Scoping Australian macroalgae for health and nutritional applications

Janice Irene McCauley
University of Wollongong

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Scoping Australian Macroalgae for Health and Nutritional Applications

A thesis submitted in partial fulfilment of the requirements of the award for the degree of

Doctor of Philosophy

From

The University of Wollongong

Janice Irene McCauley

School of Chemistry

(August 2016)
This thesis is dedicated to my partner Guillaume who encouraged me to pursue this goal, my parents who supported me every step of the way and for my beautiful daughter Blandine.
THESIS DECLARATION

I, Janice Irene McCauley, declare that this thesis is submitted in accordance with the regulations of the University of Wollongong in partial fulfilment of the requirements for the degree of Doctor of Philosophy. The written and experimental work described in this thesis is original and contains no material that has been accepted for the award of any other degree or diploma at any University and to the best of my knowledge contains no material previously published or written by another person, except where due reference is made in the text.

Janice Irene McCauley
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This has been a rewarding journey and I have met many interesting people along the way. Firstly, I would like to thank my supervisor A/Prof. Danielle Skropeta for your continued guidance and support. I can only hope that your meticulous eye for detail has rubbed off, just a little. A big thank you to my co-supervisor Dr. Pia Winberg whose enthusiasm and ideas are inspirational. After our meetings I felt like I could take on the world. Thank you to A/Prof. Barbara Meyer, my ‘Honorary Supervisor’. I truly appreciate all that you have done for me and for making me feel so welcome in your laboratory. Thank you to Prof. Marie Ranson for introducing and teaching me tissue culture, it has been a privilege and I have learnt from the best. Also, thank you Dr Lezanne Ooi and A/Prof. Ronald Sluyter for always being on-hand to pass on your knowledge of molecular and cell biology. Thank you to my seaweed colleagues, Tobias, Lauren and Drew. I started this journey feeling so alone and now look at us, what a team! A special shout-out is given to Nicole. I have thoroughly enjoyed your company, friendship and positivity. Also I must not forget Craig and Andrew at the farm! Thank you Craig for the numerous occasions you hand-delivered samples to me in Wollongong from Nowra! Thank you to my colleagues and friends in chemistry, Remi, Andrew and Chris. We have had many laughs along the way to keep us sane! Lastly a big ‘thank you’ to all the technical staff, both within Chemistry and Biology that have assisted me throughout the course of this project. They really are the true heroes working behind the scenes!
ABSTRACT

Macroalgae are an abundant and diverse group of organisms. Their polysaccharides are successfully utilised in industry due to their rheological properties, but the diverse and unique functional metabolites in macroalgae are still poorly exploited. This holds a lot of promise for health and nutritional applications. However, the variability across taxa and shifting metabolites in response to environmental conditions has resulted in an inconsistent or complex story of nutritional and health opportunities from macroalgae. In addition, the methods used to analyse metabolites from sources other than macroalgae may not be directly transferable, due to peculiarities of the algal organic tissue matrix. Therefore standardising the methods for characterisation of important macroalgal metabolite profiles and the cultivation effects on metabolites of target taxa are two of the aims of this work. Consistent and validated metabolic and compositional metabolite profiles will be required to deliver traceable, quality-controlled biomass with optimal chemistries for applications in health and nutrition.

South Eastern Australia is a diversity hot spot for algal flora and species endemism, yet it is poorly understood which species should be targeted for cultivation towards food and health markets. Here for the first time, typical
Abstract

Macroalgae of the Eastern temperate coastal zone of New South Wales (NSW), Australia were explored including the taxa *Hormosira banksii, Phyllopora comosa, Myriogloea sciurus, Ecklonia radiata, Solieria robusta* and the green macroalgae *Ulva.* Particular attention was directed towards their fatty acid (FA) profiles, anti-inflammatory, anti-oxidant and cell toxicity or functionality. In doing so, robust methods were tested and developed to establish optimal protocols for effective metabolite extraction and characterisation.

All taxa showed variable FA profiles with *Ulva* sp. representing the most desirable (n-6/n-3 ratio of 0.4) and the only species to contain the FA C22:6n-3 in this research. All non-polar extracts strongly inhibited the production of the inflammatory-mediator nitric oxide (NO). Furthermore, it was demonstrated for the first time that *Ulva* extracts were non-toxic on a primary cell line whilst selectively exhibiting toxicity against pancreatic cancer cells *in vitro.*

Application of an optimised extraction protocol revealed nutrients to be the key growth factor for the quality and quantity of *Ulva* FA. Neutral lipids can be increased by nutrient starvation, however at a detriment to FA quality. Under optimal growth (high nutrients) *Ulva* yields 56 mg of lipids per gram of dried biomass (mg.g⁻¹ d.w) that is rich in polyunsaturated fatty acids (PUFA, 54%) with a low n-6/n-3 ratio (0.2) and high levels of C18:4n-3,

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1 Species name withheld for proprietary reasons
C20:5n-3 and C22:5n-3. The standardised preparation of samples was a vital consideration as analysis of dried biomass, when compared to a fresh extraction protocol, resulted in a loss of 84% in yield and therefore a substantial underestimation of lipid content.

Under optimal growth conditions, *Ulva* can further yield 1.5 mg.g\(^{-1}\) d.w total chlorophyll, 0.1 mg.g\(^{-1}\) d.w carotenoids, 1.6 mg.g\(^{-1}\) d.w phenolics and 0.2 mg.g\(^{-1}\) d.w flavonoids. Low nutrient growth conditions reduced the presence of pigments by 98%, phenolics by 34% and anti-oxidant activity by 87%, whilst substantially increasing flavonoid content (5-fold). All extracts from cultivated *Ulva* samples inhibited NO (≥76%) although acetone extracts demonstrated higher inhibition (94 - 97%) than ethanol extracts (76 - 91%). Furthermore all pigment and phenolic yields were strongly influenced by extraction solvent (e.g. acetone versus ethanol).

These results together present quantitative yields for two major and bio-functionally important metabolite classes from macroalgae (FA and phenolics). The highest yield of FA with a nutritionally favourable profile was 5.6% d.w. in *Ulva* sp. when extracted using chloroform and methanol (2/1 v/v). Furthermore, when extracted with ethanol (100%) a yield of 4.1% d.w. total FA can be achieved along with the highest yielding phenolic content (0.2% d.w.) under optimised culture. This FA yield (% d.w.) for *Ulva* is one of the highest yields reported in the literature. Thus the FA content of this Australian cultivated macroalgal biomass together with its
high phenolic content and anti-inflammatory activities has the potential for applications in food and health markets. This is an essential step forward in delivering robust and reliable methods of production and analysis that can be applied to deliver regulatory standards and nutritional or health claims from macroalgal biomass.
PUBLICATIONS

Publications arising from the work undertaken during the course of this thesis to date are listed below.


Conference presentations where the work undertaken in this thesis has been presented are listed below.


8. **2013**, University of New South Wales 4th October: Royal Australian Chemical Institute Natural Products One-Day Symposium - Poster Presentation: Janice McCauley, Pia Winberg, Marie Ranson & Danielle Skropeta, “Anti-inflammatory and anti-cancer potential of Australian marine macroalgae - role in gut health as dietary therapeutics”

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2 **Presenting author**
PREFACE

The work presented in this thesis is part of a collaboration with industry partner Venus Shell Systems Pty. Ltd. This is a local company based in Nowra (NSW, Australia) that grows quality controlled seaweed biomass targeted for use in high value products. Venus Shell Systems collaborates with Manildra Group Pty. Ltd (Bomaderry, NSW, Australia), which is home to the largest flour mill in Australia. Manildra Group strives for constant innovation to supply industries with a diverse product range from food, health ingredients, fuel and energy. As a result of this innovation carbon dioxide (CO₂) waste product from the Manildra plant is piped and pumped directly into the algal cultivations, promoting a clean and sustainable industry practice. Venus Shell Systems is also a partner of Blue Biotech Shoalhaven, an innovative industry group that has been established to promote local innovation within the community (Shoalhaven, NSW, Australia) and member businesses in aquatic biotechnology. All research presented within this thesis contributes to the future product development opportunities that are currently being scoped by Venus Shell Systems.

The macroalgae investigated within this thesis include species collected from the South East coast of Australia, along with a cultivated Australian Ulva sp. (Chlorophyta).

3 http://www.venusshellsystems.com.au
4 http://www.manildra.com.au
5 http://www.bluebiotech.com.au
The cultivated *Ulva* is provided by Venus Shell Systems and is cultured using proprietary methods. Therefore the specific cultivation details and species information are withheld.

This thesis consists of seven chapters with the work presented in journal article style. **Chapter 1** offers a general introduction to macroalgae and macroalgal chemistries, highlighting the reported bioactivities of algal metabolites and extracts. It presents the current state of the seaweed industry and the areas for future growth and expansion and presents the aims of this thesis.

Following on from the introduction, **Chapter 2** is a published book chapter that covers the rationale and methods of two anti-cancer screening assays, one of which is routinely used and referred to throughout this thesis, presented in thesis format. Following on, each subsequent chapter (**Chapters 3-6**) is presented as a complete manuscript and is a combination of both published (final accepted versions) and prepared manuscripts in thesis format. The status of each manuscript will be defined in the foreword to each chapter.

The foreword to each chapter will link the separate works together. Where necessary, further unpublished findings that resulted from that body of work will be incorporated as part of the relevant chapter and presented immediately after the manuscript titled “Further Investigations”.

After the presentation of each manuscript (**Chapter 3-6**) a general discussion with future recommendations is provided (**Chapter 7**). This is followed by a brief
Conclusion and the Appendices. Due to the journal article style of this thesis, references are provided at the end of each chapter.
ABBREVIATIONS

AA  Arachidonic acid
ALA  Alpha linolenic acid
BHT  Butylated hydroxytoluene
d.w. equiv.  Dry weight equivalent(s)
DCM  Dichloromethane
DGDG  digalactosyldiacylglycerol
DHA  Docosahexaenoic acid
DMSO  Dimethyl sulfoxide
DPPH  2,2-diphenyl-2-picrylhydrazyl
d.w.  Dry weight
eNOS  Endothelial nitric oxide synthase
EPA  Eicosapentaenoic acid
Equiv.  Equivalent(s)
EtOAc  Ethyl acetate
EtOH  Ethanol
FA  Fatty acid(s)
GC-FID  Gas chromatography flame ionisation detector
GC-MS  Gas chromatography mass spectrometry
GLA  Gamma-linolenic acid
HPLC  High pressure liquid chromatography
HSV-1  Herpes Simplex Virus Type 1
HSV-2  Herpes Simplex Virus Type 2
IC_{50}  Inhibitory concentration at 50%
IHMRI  Illawarra Health and Medical Research Institute
IL-23  Interleukin-23
IL-6  Interleukin-6
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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</thead>
<tbody>
<tr>
<td>LA</td>
<td>Linoleic acid</td>
</tr>
<tr>
<td>lc-PUFA</td>
<td>Long chain polyunsaturated fatty acid(s)</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MGDG</td>
<td>monogalactosyldiacylglycerol</td>
</tr>
<tr>
<td>min.</td>
<td>Minutes</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(sulphophenyl)-2H-tetrazolium inner salt</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MUFA</td>
<td>Monounsaturated fatty acid(s)</td>
</tr>
<tr>
<td>n-3</td>
<td>Omega-3</td>
</tr>
<tr>
<td>n-6</td>
<td>Omega-6</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid(s)</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Revere phase high pressure liquid chromatography</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>rt</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SDA</td>
<td>Stearidonic acid</td>
</tr>
<tr>
<td>SFA</td>
<td>Saturated fatty acid(s)</td>
</tr>
<tr>
<td>SMFC</td>
<td>Shoalhaven Marine and Freshwater Centre</td>
</tr>
<tr>
<td>TFA</td>
<td>Total fatty acids</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TPC</td>
<td>Total phenolic contents</td>
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CHAPTER 1 GENERAL INTRODUCTION
Seaweed

Seaweeds inhabit the rocky intertidal and subtidal coastal zones all over the world (Figure 1). Seaweed refers to all large macroalgal species that are either classified as belonging to the class Ochrophyta or Phaeophyta (brown macroalgae), Rhodophyta (red macroalgae) or Chlorophyta (green macroalgae). The aquatic environment of marine macroalgae is competitive and constantly changing with many abiotic stressors. Combined with complex reproduction cycles marine macroalgal species are highly diversified with an estimated 72,500 species described [1]. This has resulted in macroalgae exhibiting a large array of chemical constituents known as secondary metabolites [2, 3] and includes terpenes, phenols, fatty acids (FA), lipopetides, amides, alkaloids, terpenoids, lactones, pyrroles and sterols [4, 5].

Figure 1 (i) Diversity hot spot for macroalgae at Seven Mile Beach, Gerringong, South East Coast of NSW, Australia (04/04/2011); (ii) freshly washed ashore samples of Ecklonia Radiata and Phyllospora comosa being collected from a drained intertidal rock swimming pool (iii) Hormosira banksii inhabiting the intertidal rock pools (iv) Ecklonia radiata (bottom left) and Phyllospora comosa (top).
Metabolites are classified as either secondary or primary. Primary metabolites include carbohydrates, proteins and nucleic acids. These compounds occur via fundamental biosynthetic pathways and are essential for survival. In contrast, secondary metabolites are derived from these primary metabolites and occur via highly variable biosynthetic pathways and shift more readily in response to environmental pressures [6-8].

Secondary metabolites have many roles in chemical signalling and can exhibit varying degrees of bioactivity. Bioactivity has been defined as metabolic or physiological response as a result of a molecule or ion binding to a ligand [9]. As macroalgae are abundant and very easy to collect, they have been extensively explored in natural product research. Their isolated secondary metabolites and/or extracts exhibit an array of beneficial in vitro and in vivo biological activities. This is often accompanied with minimal toxicities [10-12] when compared to other marine organisms, such as dinoflagellates (harmful algae blooms) [13] or deep-sea marine organisms [14]. However, some macroalgal species do also produce toxins, such as domoic acid in Chondria armata (Rhodophyceae) [15] and caulerpin in Caulerpa taxifolia (Chlorophyceae) [16]. Thus as the opportunities for macroalgal cultivation increase, improved knowledge of the diverse metabolites is of importance for both nutritional safety, as well as natural product opportunities.

Considering the diversity of macroalgae only a handful of species have been thoroughly investigated for isolation of their metabolites and determination of bioactivity (Table 1). Such species include Laurencia sp. and other species from the order Ceramiales (Rhodophyta); Sargassum and Dictyota sp. from the order Fucales and Dictyotales,
respectively (Phaeophyta); and *Ulva* and *Caulerpa* sp. from the order Ulvales and Bryopsidales, respectively (Chlorophyta). As chlorophyta are less explored, this presents opportunities for further investigations (**Figure 2**).
The distribution of reported bioactive metabolites from macroalgal species and their respective orders across each of the three phyla of marine macroalgae (*), as summarised from seven extensive review publications [10-12, 17-20].

<table>
<thead>
<tr>
<th>Rhodophyta Order</th>
<th>Rhodophyta Genus</th>
<th>%</th>
<th>Phaeophyta Order</th>
<th>Phaeophyta Genus</th>
<th>%</th>
<th>Chlorophyta Order</th>
<th>Chlorophyta Genus</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceramiales</td>
<td>Laurencia</td>
<td>45</td>
<td>Fucales</td>
<td>Sargassum</td>
<td>27</td>
<td>Ulvales</td>
<td>Ulva</td>
<td>19</td>
</tr>
<tr>
<td>Symphyocladia</td>
<td>Cystoseira</td>
<td>6</td>
<td>Bryopsidales</td>
<td>Caulerpa</td>
<td>19</td>
<td>Bryopsidales</td>
<td>Codium</td>
<td>8</td>
</tr>
<tr>
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<td>Rhodomela</td>
<td>3</td>
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<td>Dictyota</td>
<td>14</td>
<td>Dictyotales</td>
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<tr>
<td>Ceramiales</td>
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<td>Dictyotales</td>
<td>Canistrocarpus</td>
<td>2</td>
<td>Cladophorales</td>
<td>Cladophora</td>
<td>8</td>
</tr>
<tr>
<td>Ceramiales</td>
<td>Odonthalia</td>
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<td>Dictyotales</td>
<td>Zonaria</td>
<td>4</td>
<td>Dasycladales</td>
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<td>Ceramiales</td>
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<td>Dictyotales</td>
<td>Lobophora</td>
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<td>Desmarestiales</td>
<td>Chondria</td>
<td>1</td>
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<tr>
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<td>Desmarestiales</td>
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<td>Corallinales</td>
<td>Hydrolithon</td>
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<td>Laminariales</td>
<td>Ecklonia</td>
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<td>Laminariales</td>
<td>Ecklonia</td>
<td>8</td>
</tr>
<tr>
<td>Gracilariales</td>
<td>Gracilaria</td>
<td>6</td>
<td>Ishigeales</td>
<td>Ishige</td>
<td>4</td>
<td>Ectocarpales</td>
<td>Leathsia</td>
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<tr>
<td>Plocamiales</td>
<td>Plocamium</td>
<td>4</td>
<td>Ectocarpales</td>
<td>Dictyopteris</td>
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<td>Ectocarpales</td>
<td>Stypopodium</td>
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<td>Gigartinales</td>
<td>Sphaerococcus</td>
<td>4</td>
<td>Ectocarpales</td>
<td>Dictyopteris</td>
<td>2</td>
<td>Ectocarpales</td>
<td>Canistrocarpus</td>
<td>2</td>
</tr>
<tr>
<td>Incertae sedis</td>
<td>Callophycus</td>
<td>4</td>
<td>Ectocarpales</td>
<td>Taonia</td>
<td>2</td>
<td>Ectocarpales</td>
<td>Canistrocarpus</td>
<td>2</td>
</tr>
<tr>
<td>Peyssonneliales</td>
<td>Peyssonnella</td>
<td>2</td>
<td>Ectocarpales</td>
<td>Monostroma</td>
<td>2</td>
<td>Ectocarpales</td>
<td>Canistrocarpus</td>
<td>2</td>
</tr>
<tr>
<td>Rhodymeniales</td>
<td>Ceratodictyon</td>
<td>1</td>
<td>Ectocarpales</td>
<td>Canistrocarpus</td>
<td>2</td>
<td>Ectocarpales</td>
<td>Canistrocarpus</td>
<td>2</td>
</tr>
<tr>
<td>Halymeniales</td>
<td>Grateloupia</td>
<td>1</td>
<td>Ectocarpales</td>
<td>Canistrocarpus</td>
<td>2</td>
<td>Ectocarpales</td>
<td>Canistrocarpus</td>
<td>2</td>
</tr>
<tr>
<td>Hapalidiales</td>
<td>Lithothamnion</td>
<td>1</td>
<td>Ectocarpales</td>
<td>Canistrocarpus</td>
<td>2</td>
<td>Ectocarpales</td>
<td>Canistrocarpus</td>
<td>2</td>
</tr>
<tr>
<td>Nemaliales</td>
<td>Galaxaura</td>
<td>1</td>
<td>Ectocarpales</td>
<td>Canistrocarpus</td>
<td>2</td>
<td>Ectocarpales</td>
<td>Canistrocarpus</td>
<td>2</td>
</tr>
<tr>
<td>Bonnemaisoniales</td>
<td>Asparagopsis</td>
<td>1</td>
<td>Ectocarpales</td>
<td>Canistrocarpus</td>
<td>2</td>
<td>Ectocarpales</td>
<td>Canistrocarpus</td>
<td>2</td>
</tr>
</tbody>
</table>

*Classification information obtained through Guiry and Guiry 2016 [21].

**Dominant Orders highlighted in bold.**
Reported biological activities from macroalgae include anti-hypertensive, anti-oxidant, anti-inflammatory, anti-allergic, anti-viral, anti-bacterial, cytotoxic, anti-mitogenic, anti-cancer, anti-tumour, anti-thrombic and anti-coagulant activities [22-24]. As such, it is frequently proposed that macroalgae either as a whole, a concentrated extract or an individual macroalgal compound, have the potential for commercial applications as bioactive ingredients in a range of areas including food products [25-27].

Figure 2 The % distribution of reported bioactives across each of the three phyla of marine macroalgae as summarised from seven extensive review publications [10-12, 17-20].

**Seaweed industry**

Currently the demand for seaweed product exceeds the supply of wild harvest. In 2014, farmed algae produced 23.8 million tonnes with 1.1 million tonnes collected from wild harvest [28]. To date, algal cultivation has an estimated total output of 27 million tonnes annually with an estimated global net worth of US$6 billion [28, 29]. Thirty-one countries are officially recognised as producers from algae aquaculture, yet it is
estimated up to 50 countries actively participate in algae farming [29, 30]. However, the majority of this cultivated algae production comes from just eight countries, with China dominating (Figure 3) [30].

The production in these countries is now well established and is scaling to meet the fluctuating supply and demand, with the total world production of farmed seaweeds doubling from 2000 to 2012 [28]. Considering the diversity of defined seaweed species [1], the global seaweed demand is met with the cultivation of just a few select species (Table 2). In particular, *Kappaphycus alvarezii* and *Eucheuma* aquaculture in Indonesia was a major contributor to a recent growth in statistics of world aquatic plant production and are a major source of carrageenan [29]. These species dominate international trade and are primarily concerned with raw materials for phycocolloid extraction [31].
Phycocolloids are polysaccharides and include alginates (Phaeophyta) and agars and carrageenans (Rhodophyta), able to form highly viscous gels for use as gelling, thickening and stabilizing agents in the food and pharmaceutical industries [32, 33].

Therefore, in considering current markets alongside the large number of macroalgal species that have been defined and their reported bioactives, there exists a unique opportunity to expand the current product range, particularly from cultivated green seaweed as there are no major chlorophyta productions on the market.

<table>
<thead>
<tr>
<th>Seaweed species</th>
<th>Group</th>
<th>Common Name</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Saccharina</em> spp.</td>
<td>Phaeophyta</td>
<td></td>
<td>Alginate</td>
</tr>
<tr>
<td><em>Laminaria</em> spp.</td>
<td>Phaeophyta</td>
<td>Kombu, Japanese kelp</td>
<td>Wholefood&lt;sup&gt;6&lt;/sup&gt;, Alginate</td>
</tr>
<tr>
<td><em>Undaria pinnatifida</em></td>
<td>Phaeophyta</td>
<td>Wakame</td>
<td>Wholefood, fucoidan</td>
</tr>
<tr>
<td><em>Ascophyllum</em> spp.</td>
<td>Phaeophyta</td>
<td></td>
<td>Alginate, phenolics</td>
</tr>
<tr>
<td><em>Lessonia</em> spp.</td>
<td>Phaeophyta</td>
<td></td>
<td>Alginate</td>
</tr>
<tr>
<td><em>Porphyra</em> spp.</td>
<td>Rhodophyta</td>
<td>Nori or laver</td>
<td>Wholefood</td>
</tr>
<tr>
<td><em>Eucheuma</em> spp.</td>
<td>Rhodophyta</td>
<td></td>
<td>Carrageenan</td>
</tr>
<tr>
<td><em>Gracilaria</em> spp.</td>
<td>Rhodophyta</td>
<td></td>
<td>Wholefood, Agar</td>
</tr>
<tr>
<td><em>Chondrus</em> spp.</td>
<td>Rhodophyta</td>
<td></td>
<td>Wholefood, Carrageenan</td>
</tr>
<tr>
<td><em>Kappaphycus</em> spp.</td>
<td>Rhodophyta</td>
<td></td>
<td>Carrageenan</td>
</tr>
<tr>
<td><em>Gelidium</em> spp.</td>
<td>Rhodophyta</td>
<td></td>
<td>Agar</td>
</tr>
</tbody>
</table>

<sup>6</sup> Wholefoods are foods that are refined as little as possible. Seaweed as a wholefood is often offered to consumers as either a fresh, frozen or dried product.
Biological Activities of Macroalgae (Inflammation)

Dietary Bioactives

One niche market area is functional food products and/or ingredients for optimisation of health and disease prevention. As opposed to simply supplying nutrition, a functional food has the ability to modulate *in vivo* physiological systems, for example immune, endocrine, nervous, circulatory and digestive systems [35]. Dietary incorporation of these foods and their metabolites has the potential to contribute to disease prevention by interacting at a cellular level to modify physiological responses [35]. One particular area for potential development is the application of algal-derived bioactives to target inflammation.

In Western societies there is a rise in the number of disorders, collectively referred to as chronic, inflammatory, noncommunicable diseases [36, 37], typically characterised by slow progression and long duration [38]. Examples include allergies, asthma and inflammatory bowel, cardiovascular, metabolic and neurological diseases [36]. These afflictions have been linked to underlying low-grade inflammation and oxidative stress [36-38]. The immune response of inflammation is a complex and multifactorial network of chemical signals [39], wherein an important mediator of inflammation is the enzyme nitric oxide synthase (NOS), which regulates nitric oxide (NO) production.

The enzyme exists in both inducible (iNOS) and constitutive isoforms, which includes both endothelial NOS (eNOS) and neuronal NOS (nNOS) and it is important to differentiate between the two isoforms. The two constitutive forms of this enzyme
(eNOS and nNOS) are under enzymatic control as a result of calcium and calmodulin binding and result in small amounts of NO with important physiological roles such as acting as a vascular relaxing agent or neurotransmitter [40]. A third iNOS enzyme however generates NO as a response to microbes, cytokines, and other activating stimuli and can generate very high and toxic levels of NO [40, 41]. It is this elevated and sustained NO production from iNOS that is characteristic of prolonged inflammatory disorders resulting in tissue injury and subsequently cancer [39-41]. Experimental studies have suggested inflammatory cells and their associated mediators such as interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α) and IL-23 combined with reactive oxygen species (ROS) form a microenvironment that favours cancer development [42-46]. As a result, compounds that exhibit anti-oxidant and anti-inflammatory biological activities are said to have the ability to confer protection and reduce the risk of or aid the healing of disease [47, 48].

Macroalgae are readily consumed as a whole food, particularly in many coastal communities of Asia and have a history of being used in traditional medicines [49]. Evidence has shown that a number of bioactive metabolites isolated from macroalgae can influence a number of key molecular events that are involved in the inflammation pathway [22-24, 50]. For example polysaccharides, phenolics, pigments and terpenoid derivatives have all shown the potential to counter-act the immune response to inflammation. For example either via the reduction of NO and other pro-inflammatory immune mediators [23], reduction of oxidative stress by scavenging of free radicals [22], and/or inhibiting cancer via selectively inducing cancer cell death or cell proliferation (demonstrated both in vivo and in vitro) [24].
Fatty Acids

Another important dietary class that has been implicated as regulators and mediators of inflammation are fatty acids (FA), specifically the omega-6 arachidonic acid (AA; 20:4n-6) and eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) omega-3 polyunsaturated fatty acids (PUFA) [51-54]. Inflammatory cells contain both n-6 and n-3 PUFA, which serve as substrates for either pro- or anti-inflammatory eicosanoids, respectively. In relation to the other PUFA present, AA is far more abundant and is the main substrate for the enzymatic synthesis of the pro-inflammatory eicosanoids [51]. Interestingly, it has been shown that increased dietary consumption of either n-3 or n-6 long-chain PUFA (lc-PUFA) can directly modify the proportion of their respective FA in these inflammatory cells. This indicates a correlation between dietary intake and effect on immune modulation of inflammation [51, 55]. This is due to n-3 EPA competing with the n-6 AA as a substrate for cyclooxygenase and 5-lipoxygenase enzymes [56].

Western diets are characterised by excessively high n-6 PUFA due to the increased consumption of meat, cereals (wheat, maize, rice) and seed based oils, which are high in n-6 PUFA and low in both n-3 PUFA and anti-oxidants [57, 58]. This change in diet has been linked to the increased prevalence of inflammatory and autoimmune diseases, along with cardiovascular disease and cancer [57]. Fortunately, patients with pre-existing inflammatory disorders can reduce their levels of inflammatory cytokines with dietary incorporation of EPA and DHA [56].

The main dietary source of EPA and DHA is fish and seaweed and the current dietary
recommendation for fish is 2-3 serves per week to meet their minimum dietary requirements [58]. EPA and DHA can also be obtained by encapsulated fish oil supplements. However, to date this demand for fish and fish oil is currently met with limited and unsustainable captive based fisheries with low scope for growth to meet future demands [29]. Marine algae are primary producers of EPA and DHA that fish obtain through trophic bioaccumulation and therefore have the potential to offer an alternative dietary source of these bioactive anti-inflammatory n-3 PUFA [59-61].

**Target Macroalgal Species**

For the utilisation of algal-derived ingredients as dietary bioactives to counteract inflammation, target algal species with desirable and suitable chemistries need to be selected. Numerous studies have identified the presence of biological activities across the three phyla with the potential to enhance human and animal health by positively influencing, modulating and/or eliminating certain risk factors of defined diseases (Example Table 4).

As part of a preliminary screening assessment of algal bioactives and chemistries specifically linked to targeting inflammatory disorders, a number or macroalgal species were investigated in this thesis. These species consisted of unique native and endemic Australian species of marine macroalgae, for which little biochemical and bioactivity studies have been reported. These species were from the order Fucales (*Hormosira banksii* and *Phyllospora comosa*), order Laminariales (*Ecklonia radiata*), order Ectocarpales (*Myriogloea sciurus*), order Gigartinales (*Solieria robusta*) and order Ulvales (*Ulva sp.*) (Table 3; Figure 4).
Literature reviews of similar non-Australian species belonging to these macroalgal orders show potential for these chosen Australian species to benefit health (Table 4 - Table 8).
Table 3: Australian algae species identified as having potential to positively benefit both animal and human health.

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Phylum</th>
<th>Class</th>
<th>Order</th>
<th>Family</th>
<th>Genus</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phaeophyta</td>
<td>Phaeophyceae</td>
<td>Fucales</td>
<td>Fucales</td>
<td>Hormosiraceae</td>
<td>Hormosira</td>
<td>H. banksii</td>
</tr>
<tr>
<td>Chlorophyta</td>
<td>Ulvophyceae</td>
<td>Ulvales</td>
<td>Laminariales</td>
<td>Ulvaceae</td>
<td>Ulva</td>
<td>Ulva species #</td>
</tr>
<tr>
<td>Rhodophyta</td>
<td>Florideophyceae</td>
<td>Florideophyceae</td>
<td>Florideophyceae</td>
<td>Florideaceae</td>
<td>Solieria</td>
<td>S. robusta</td>
</tr>
<tr>
<td>Rhodophyta</td>
<td>Florideophyceae</td>
<td>Florideophyceae</td>
<td>Florideophyceae</td>
<td>Florideaceae</td>
<td>Solieria</td>
<td>S. robusta</td>
</tr>
<tr>
<td>Rhodophyta</td>
<td>Florideophyceae</td>
<td>Florideophyceae</td>
<td>Florideophyceae</td>
<td>Florideaceae</td>
<td>Solieria</td>
<td>S. robusta</td>
</tr>
</tbody>
</table>

Kingdom: Phaeophyta, Chlorophyta, Rhodophyta
Phylum: Phaeophyceae, Florideophyceae, Rhodophyta
Class: Florideophyceae
Order: Ulvales
Family: Ulvaceae
Genus: Hormosira, Ulva, Solieria
Species: H. banksii, Ulva species #, S. robusta

Kingdom: Phaeophyta
Phylum: Phaeophyceae
Class: Florideophyceae
Order: Ulvales
Family: Ulvaceae
Genus: Hormosira, Ulva, Solieria
Species: H. banksii, Ulva species #, S. robusta

Classification information provided by Guiry and Guiry 2016 [21]

Macroalgal species investigated here: order Fucales (Hormosira banksii (also known as Neptune’s necklace) and Phyllospora comosa); order Laminariales (Ecklonia radiata); order Florideales (Myriogloea sp.); order Florideales (Solieria robusta) and order Ulvales (also known as Nereocystis nereocystis and Phyllospora comosa). The photos of H. banksii, M. sciurus and S. robusta were provided courtesy of Pia Winberg, Venus Shell Systems, Pty. Ltd., Venus Shell Systems, Pty. Ltd., Mundamia, NSW, 2540, Australia.
Phaeophyta

A range of reported biological activities from species belonging to the phylum Phaeophyta, order Laminariales are summarized in Table 4. Ecklonia is a commonly investigated genus with regards to its biological activity. Anti-oxidant, anti-inflammatory, anti-cancer, neuro-protective and acetyl (AChE) and butyryl-cholinesterase (BChE) inhibitory activities dominate this phylum and order with many of these activities attributed to isolated phlorotannins (Table 4). Acetyl- and butyryl-cholinesterase inhibitors have the potential to be used as a therapeutic strategy to treat and/or slow the progression of Alzheimer’s disease [62].
Table 4 Identified bioactive components and biological activities from species belonging to the Phylum Phaeophyta, order Laminariales.

<table>
<thead>
<tr>
<th>Bioactive Component</th>
<th>Activity</th>
<th>Species</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phloroglucinol</td>
<td>Anti-oxidant</td>
<td>Ecklonia cava, Ecklonia stolonifera</td>
<td>[63-66]</td>
</tr>
<tr>
<td></td>
<td>Neuro-protective</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AChE and BCHE inhibitory*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eckol</td>
<td>Anti-oxidant</td>
<td>Ecklonia cava, Ecklonia stolonifera</td>
<td>[63-70]</td>
</tr>
<tr>
<td></td>
<td>Neuro-protective</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AChE and BCHE inhibitory</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hepato-protective</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-hypertension, Anti-diabetic</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Radio-protective</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dieckol</td>
<td>Anti-oxidant</td>
<td>Ecklonia cava</td>
<td>[63-66, 68, 69, 71]</td>
</tr>
<tr>
<td></td>
<td>Neuro-protective</td>
<td>Ecklonia stolonifera</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AChE and BCHE inhibitory</td>
<td>Eisenia bicyclis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-hypertension</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Radio-protective</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Melanogenesis inhibitors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6,6’-Bieckol</td>
<td>Anti-HIV-1</td>
<td>Ecklonia cava</td>
<td>[65, 66, 72-75]</td>
</tr>
<tr>
<td></td>
<td>AChE and BCHE inhibitory</td>
<td>Ecklonia stolonifera</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-oxidant</td>
<td>Eisenia bicyclis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-allergic</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-inflammatory</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6,8’-Bieckol</td>
<td>Anti-allergic</td>
<td>Eisenia arborea</td>
<td>[74, 76]</td>
</tr>
<tr>
<td>8,8’-Bieckol</td>
<td>Anti-allergic</td>
<td>Eisenia arborea</td>
<td>[74, 76]</td>
</tr>
<tr>
<td>Fucodiphlorethol G</td>
<td>AChE and BCHE inhibitory</td>
<td>Ecklonia stolonifera, Ecklonia cava</td>
<td>[65, 66]</td>
</tr>
<tr>
<td>Eckstolonol</td>
<td>Neuro-protective</td>
<td>Ecklonia cava</td>
<td>[64]</td>
</tr>
<tr>
<td>Phlorofucofuroeckol A</td>
<td>AChE and BCHE inhibitory</td>
<td>Ecklonia stolonifera, Ecklonia kurome, Ecklonia cava</td>
<td>[65, 66, 68, 74, 77]</td>
</tr>
<tr>
<td></td>
<td>Algicidal</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-oxidant</td>
<td>Ecklonia cava</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-hypertension</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-allergic</td>
<td>Eisenia arborea</td>
<td></td>
</tr>
<tr>
<td>Phlorofucofuroeckol B</td>
<td>Anti-allergic</td>
<td>Eisenia arborea</td>
<td>[74, 78, 79]</td>
</tr>
<tr>
<td>7-Phloroeckol</td>
<td>AChE and BCHE inhibitory</td>
<td>Ecklonia stolonifera, Ecklonia cava</td>
<td>[65, 66, 80]</td>
</tr>
<tr>
<td></td>
<td>Anti-oxidant</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Melanogenesis inhibitors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Phloroeckol</td>
<td>Hepato-protective activity</td>
<td>Ecklonia stolonifera</td>
<td>[67]</td>
</tr>
<tr>
<td>Triphlorethol-A</td>
<td>Anti-oxidant</td>
<td>Ecklonia cava</td>
<td>[81]</td>
</tr>
<tr>
<td>Dioxinodehydroeckol</td>
<td>Anti-cancer</td>
<td>Ecklonia cava</td>
<td>[82]</td>
</tr>
<tr>
<td>2,7’-Phloroglucinol-6,6’-Bieckol</td>
<td>Anti-oxidant</td>
<td>Ecklonia cava</td>
<td>[83]</td>
</tr>
<tr>
<td>Triphlorethol A</td>
<td>Neuro-protective</td>
<td>Ecklonia cava</td>
<td>[64]</td>
</tr>
<tr>
<td>Methanol Extract</td>
<td>Anti-inflammatory</td>
<td>Undaria pinnatifida,</td>
<td>[84]</td>
</tr>
</tbody>
</table>

*Acetyl- (AChE) and butyryl-cholinesterase (BChE) inhibitory
The range of reported biologically active components from the phylum Phaeophyta, order Fucales is summarized in Table 5. Anti-inflammatory, anti-cancer, anti-oxidant and other immuno-modulating activities are commonly reported from the genus *Sargassum*. These activities are often attributed to their unique sulfated polysaccharides, known as fucoidan (Table 5).

**Table 5** Identified biological activities from the Phylum Phaeophyta, order Fucales.

<table>
<thead>
<tr>
<th>Bioactive Component</th>
<th>Activity</th>
<th>Species</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fucotriphlorethol A</td>
<td>Anti-oxidant</td>
<td><em>Fucus vesiculosus</em></td>
<td>[85]</td>
</tr>
<tr>
<td></td>
<td>Anti-inflammatory</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-cancer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trifucodiphlorethol A</td>
<td>Anti-oxidant</td>
<td><em>Fucus vesiculosus</em></td>
<td>[85]</td>
</tr>
<tr>
<td></td>
<td>Anti-inflammatory</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-cancer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fucoxanthin</td>
<td>Anti-oxidant</td>
<td><em>Sargassum siliquastrum,</em> <em>Myagropsis myagroides</em></td>
<td>[86-88]</td>
</tr>
<tr>
<td></td>
<td>Anti-inflammatory</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meroditerpenoids</td>
<td>Anti-fouling</td>
<td><em>Halidrys siliquosa</em></td>
<td>[89]</td>
</tr>
<tr>
<td>Diterpene</td>
<td>Anti-mitotic</td>
<td><em>Bifurcaria bifurcata</em></td>
<td>[90]</td>
</tr>
<tr>
<td>Fucoidan</td>
<td>Anti-cancer</td>
<td><em>Fucus evanescens</em></td>
<td>[91-93]</td>
</tr>
<tr>
<td></td>
<td>Angiogenesis</td>
<td><em>Ascophyllum nodosum</em></td>
<td></td>
</tr>
<tr>
<td>Polysaccharides (Fucoidan)</td>
<td>Anti-tumor</td>
<td><em>Sargassum fusiforme</em></td>
<td>[94, 95] [96]</td>
</tr>
<tr>
<td></td>
<td>Immuno-modulatory</td>
<td><em>Sargassum pallidum</em></td>
<td></td>
</tr>
<tr>
<td>Polysaccharide (Fucoidan)</td>
<td>Immuno-modulatory</td>
<td><em>Sargassum fusiforme</em></td>
<td>[97-99]</td>
</tr>
<tr>
<td>Methanol Extract</td>
<td>Anti-inflammatory</td>
<td><em>Sargassum hemiphyllum,</em> <em>Sargassum wightii</em></td>
<td>[100, 101]</td>
</tr>
<tr>
<td>Water Extract</td>
<td>Anti-cancer</td>
<td><em>Sargassum oligocystum</em></td>
<td>[102]</td>
</tr>
</tbody>
</table>

Species from the order Ectocarpales have been less investigated for their biological activity when compared to other Phaeophyta species. Similarly, bioactivities reported are attributed to their sulfated polysaccharides (Fucoidan), extracted either from *Cladosiphon okamuranus* or *Adenocystis utricularis* (Table 6). Fucoidan, the sulfated polysaccharide from Phaeophyta species, is a well regarded bioactive. Reported
activities include anti-cancer, anti-coagulant, anti-thrombotic, anti-viral, immunomodulatory, anti-oxidant and anti-inflammatory activities [103-106].

Table 6. Identified biological activities from the Phylum Phaeophyta, order Ectocarpales.

<table>
<thead>
<tr>
<th>Bioactive Component</th>
<th>Activity</th>
<th>Species</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fucoidan</td>
<td>Anti-viral</td>
<td><em>Cladosiphon okamuranus</em></td>
<td>[107-112]</td>
</tr>
<tr>
<td></td>
<td>Anti-HSV-1, HSV-2*</td>
<td><em>Adenocystis utricularis</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Newcastle Disease Virus</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-tumor</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-proliferative</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Rhodes Simplex Virus 1 and 2*

**Rhodophyta**

A number of biological activities from the phylum Rhodophyta, order Gigartinales are presented in Table 7. The bioactivity is reported from a variety of species and includes the commonly reported activities such as anti-oxidant, anti-cancer, anti-fungal, anti-viral and anti-inflammatory. Interestingly, hypolipidemic activity was represented within this phylum and order (Table 7). Hypolipidemic refers to the ability to modulate lipid and lipoprotein levels in blood plasma. Hyperlipidemia is characterised by high levels of low-density blood lipoproteins. This is a risk factor for atherosclerosis, an inflammatory disorder resulting in the accumulation of lipids within artery walls [113]. The identified hypolipidemic activity listed in Table 7 was attributed to an ethanol extract and a screen across a number of species indicated that *Solieria robusta* was the most effective in reducing the lipid profile in diet-induced hyperlipidemic rats [114]. Ethanol extracts will contain, to an extent, both FA and polyphenols which can influence lipid metabolism [115, 116].
Table 7 Identified biological activities from the Phylum Rhodophyta, order Gigartinales.

<table>
<thead>
<tr>
<th>Bioactive Component</th>
<th>Activity</th>
<th>Species</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol, Ethanol, Water &amp; other Organic Extracts</td>
<td>Anti-oxidant</td>
<td>Kappaphycus alvarezi</td>
<td>[114, 117-122]</td>
</tr>
<tr>
<td></td>
<td>Anti-viral (HSV-1, HSV-2*)</td>
<td>Hypnea musciformis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hypolipidemic activity</td>
<td>Solieria robusta</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-cancer</td>
<td>Hypnea musciformis,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AChE inhibitory</td>
<td>Ochtodes secundiramea</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alpha-amylase</td>
<td>Eucheuma denticulatum</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-inflammatory</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polysaccharides</td>
<td>Anti-oxidant</td>
<td>Gigartina skottsbergii</td>
<td>[123-126]</td>
</tr>
<tr>
<td></td>
<td>Anti-viral (HSV-1, HSV-2)</td>
<td>Sphaerococcus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-inflammatory</td>
<td>coronopifolius</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hypnea spinella</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Schizymenia binderi</td>
<td></td>
</tr>
<tr>
<td>Protein (Lectin)</td>
<td>Anti-bacterial</td>
<td>Kappaphycus striatum</td>
<td>[127]</td>
</tr>
</tbody>
</table>

*Herpes Simplex Virus 1 and 2

**Chlorophyta**

Chlorophyta spp. as a whole have been less represented in the literature when compared to Rhodophyta and Phaeophyta. However, the genus *Ulva* is well represented (Table 1). *Ulva* species of macroalgae can have two different morphologies. For example, *Ulva* can have a highly variable distromatic leafy thallus (Figure 5a) or a monostromatic tubular or filamentous thallus (Figure 5b). Filamentous morphologies were once assigned as belonging to the genus *Enteromorpha* [128, 129]. However, it has been defined that *Enteromorpha* and *Ulva* are not distinct genera and therefore reported bioactives for both *Ulva* and *Enteromorpha* have been included in this literature review [130, 131]. Identified biological activities from the most commonly reported *Ulva* species of macroalgae are presented in Table 8. These species include the leafy distromatic species *Ulva fasciata, U. pertusa and U. lactuca* and the monostromatic filamentous species *U. intestinalis*. Less commonly reported species are represented in Table 9. The defined bioactivities have been mainly attributed to extracts, as opposed to isolated compounds (Table 4) with anti-oxidant, anti-inflammatory and anti-bacterial activities strongly
represented (Table 8 and Table 9). The numerous reported biological activities of *Ulva* and its ability to grow rapidly in culture makes it an attractive target to pursue for investigation [132, 133].

![Ulva species of macroalgae (Chlorophyta) can exist as either a (a) distromatic, leafy thallus or (b) monostromatic tubular or filamentous thallus. Both morphologies can be found in close proximities to one another, as shown above, with leafy morphologies found habitating the exposed intertidal rock pools and filamentous morphologies on exposed intertidal rock substrates (Seven Mile Beach, Gerringong, NSW, Australia)](image)

**Figure 5** *Ulva* species of macroalgae (Chlorophyta) can exist as either a (a) distromatic, leafy thallus or (b) monostromatic tubular or filamentous thallus. Both morphologies can be found in close proximities to one another, as shown above, with leafy morphologies found habitating the exposed intertidal rock pools and filamentous morphologies on exposed intertidal rock substrates (Seven Mile Beach, Gerringong, NSW, Australia)
<table>
<thead>
<tr>
<th>Species</th>
<th>Reference</th>
<th>Component</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ulva fasciata</td>
<td></td>
<td>Guaiane S</td>
<td>Anti-bacterial</td>
</tr>
<tr>
<td>Ulva labdane Diterpenoids</td>
<td>[135]</td>
<td>Anti-bacterial</td>
<td></td>
</tr>
<tr>
<td>Ulva sesquiterpenoids</td>
<td>[136]</td>
<td>Anti-oxidant</td>
<td></td>
</tr>
<tr>
<td>Ulva sulfolipids</td>
<td>[137]</td>
<td>Anti-viral; Anti-tumor; Anti-microbial</td>
<td></td>
</tr>
<tr>
<td>Ulva glycolipids</td>
<td>[138]</td>
<td>Anti-cancer; Anti-microbial; Anti-viral</td>
<td></td>
</tr>
<tr>
<td>Ulva ethyl alcohol extracts</td>
<td>[139]</td>
<td>Anti-viral</td>
<td></td>
</tr>
<tr>
<td>Ulva methanol &amp; butanol extracts</td>
<td>[140]</td>
<td>Anti-microbial; Haemolytic activity</td>
<td></td>
</tr>
<tr>
<td>Ulva dichloromethane extracts</td>
<td>[141]</td>
<td>Anti-tumor</td>
<td></td>
</tr>
<tr>
<td>Ulva ethanol extracts</td>
<td>[142, 143]</td>
<td>Anti-inflammatory; Anti-oxidant; Anti-microbial</td>
<td></td>
</tr>
<tr>
<td>Ulva dichloromethane &amp; methanol extracts</td>
<td>[144]</td>
<td>Anti-viral</td>
<td></td>
</tr>
<tr>
<td>Ulva water, alcohol &amp; organic extracts</td>
<td>[145]</td>
<td>Anti-oxidant</td>
<td></td>
</tr>
<tr>
<td>Ulva methanol extracts</td>
<td>[146]</td>
<td>Anti-oxidant</td>
<td></td>
</tr>
<tr>
<td>Ulva chloroform &amp; alcohol extracts</td>
<td>[147, 148, 150]</td>
<td>Anti-bacterial; Anti-fungal; Anti-inflammatory</td>
<td></td>
</tr>
<tr>
<td>Ulva chloroform &amp; alcohol extracts</td>
<td>[151]</td>
<td>Anti-tumor</td>
<td></td>
</tr>
<tr>
<td>Ulva ethanol extracts</td>
<td>[152]</td>
<td>Anti-bacterial</td>
<td></td>
</tr>
<tr>
<td>Ulva mono-unsaturated fatty acid derivatives</td>
<td>[153]</td>
<td>Anti-inflammatory; Anti-oxidant; Anti-AChE</td>
<td></td>
</tr>
<tr>
<td>Ulva water, alcohol &amp; organic extracts</td>
<td>[154]</td>
<td>Anti-oxidant; Anti-AChE</td>
<td></td>
</tr>
<tr>
<td>Ulva methanol extracts</td>
<td>[155, 156]</td>
<td>Anti-bacterial, Anti-oxidant, Anti-AChE</td>
<td></td>
</tr>
<tr>
<td>Ulva chloroform &amp; alcohol extracts</td>
<td>[157, 158]</td>
<td>Anti-genotoxic; Anti-hyperglycaemic; Anti-genotoxic</td>
<td></td>
</tr>
<tr>
<td>Ulva water, alcohol &amp; organic extracts</td>
<td>[160, 161]</td>
<td>Anti-oxidant; Anti-bacterial</td>
<td></td>
</tr>
<tr>
<td>Ulva methanol extracts</td>
<td>[162]</td>
<td>Anti-bacterial</td>
<td></td>
</tr>
<tr>
<td>Ulva water, alcohol &amp; organic extracts</td>
<td>[163]</td>
<td>Anti-bacterial</td>
<td></td>
</tr>
<tr>
<td>Ulva dichloromethane extracts</td>
<td>[164]</td>
<td>Anti-oxidant; Anti-bacterial</td>
<td></td>
</tr>
<tr>
<td>Ulva ethanol extracts</td>
<td>[165]</td>
<td>Anti-oxidant; Anti-bacterial</td>
<td></td>
</tr>
<tr>
<td>Ulva chloroform &amp; alcohol extracts</td>
<td>[166]</td>
<td>Anti-oxidant; Anti-bacterial</td>
<td></td>
</tr>
<tr>
<td>Ulva water, alcohol &amp; organic extracts</td>
<td>[167]</td>
<td>Anti-oxidant; Anti-bacterial</td>
<td></td>
</tr>
<tr>
<td>Ulva methanol extracts</td>
<td>[168]</td>
<td>Anti-oxidant; Anti-bacterial</td>
<td></td>
</tr>
<tr>
<td>Ulva chloroform &amp; alcohol extracts</td>
<td>[169]</td>
<td>Anti-oxidant; Anti-bacterial</td>
<td></td>
</tr>
<tr>
<td>Ulva water, alcohol &amp; organic extracts</td>
<td>[170]</td>
<td>Anti-oxidant; Anti-bacterial</td>
<td></td>
</tr>
<tr>
<td>Ulva methanol extracts</td>
<td>[171]</td>
<td>Anti-oxidant; Anti-bacterial</td>
<td></td>
</tr>
<tr>
<td>Ulva chloroform &amp; alcohol extracts</td>
<td>[172]</td>
<td>Anti-oxidant; Anti-bacterial</td>
<td></td>
</tr>
<tr>
<td>Ulva water, alcohol &amp; organic extracts</td>
<td>[173]</td>
<td>Anti-oxidant; Anti-bacterial</td>
<td></td>
</tr>
<tr>
<td>Ulva methanol extracts</td>
<td>[174]</td>
<td>Anti-oxidant; Anti-bacterial</td>
<td></td>
</tr>
<tr>
<td>Ulva chloroform &amp; alcohol extracts</td>
<td>[175]</td>
<td>Anti-oxidant; Anti-bacterial</td>
<td></td>
</tr>
</tbody>
</table>

Table 8: Biological activities from commonly investigated species of Ulva, order Ulvales, Phylum Chlorophyta (including Phaeophyceae & Rhodophyceae associated species)
<table>
<thead>
<tr>
<th>Species</th>
<th>Component</th>
<th>Activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ulva prolifera</em></td>
<td>Pheophytin</td>
<td>Anti-oxidant</td>
<td>[163]</td>
</tr>
<tr>
<td></td>
<td>Enzyme Assisted Extracts</td>
<td>Cholinesterase; Anti-oxidant; Anti-inflammatory</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Methanol &amp; Organic Extracts</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ulva compressa</em></td>
<td>Methanol Extracts</td>
<td>Anti-inflamatory</td>
<td>[84]</td>
</tr>
<tr>
<td></td>
<td>Chloroform &amp; Alcohol Extracts</td>
<td>Anti-bacterial</td>
<td>[166]</td>
</tr>
<tr>
<td></td>
<td>Methanol &amp; Water Extracts</td>
<td>Anti-oxidant</td>
<td>[167]</td>
</tr>
<tr>
<td></td>
<td>Polyunsaturated Fatty Acids (C18:4 n-3 &amp; C18:3 n-6)</td>
<td>Anti-bacterial</td>
<td>[169]</td>
</tr>
<tr>
<td><em>Ulva linza</em></td>
<td>Methanol Extracts</td>
<td>Anti-inflamatory; Anti-proliferative; Anti-oxidant</td>
<td>[84, 168]</td>
</tr>
<tr>
<td></td>
<td>Chloroform &amp; Alcohol Extracts</td>
<td>Anti-bacterial</td>
<td>[152]</td>
</tr>
<tr>
<td></td>
<td>Methanol &amp; Organic Extracts</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ulva reticulata</em></td>
<td>Chloroform &amp; Alcohol Extracts</td>
<td>Anti-bacterial</td>
<td>[173]</td>
</tr>
<tr>
<td></td>
<td>Methanol Extracts</td>
<td>Cholinesterase inhibitory</td>
<td>[174]</td>
</tr>
<tr>
<td><em>Other species (Enteromorpha prolifera, E. tubulosa, E. flexuosa, E. antenna, U. clathrata)</em></td>
<td>Methanol &amp; Organic Extracts</td>
<td>Anti-oxidant; Larvicidal</td>
<td>[167, 175]</td>
</tr>
</tbody>
</table>

Table 9: Biological activities from less commonly investigated species of *Ulva* order *Ulvales*, phylum Chlorophyta (* including Enteromorpha prolifera assigned species,*
Thesis Aims

Currently, the utilisation of algal-derived dietary bioactives is yet to be realised at any level of significance [27]. South Eastern Australia is a diversity hot spot for algal flora and species endemism [176, 177]. Thus, there is a unique opportunity in Australia for the cultivation of selected Australian seaweed species to deliver quality controlled, traceable, high-value dietary bioactives for both human and animal nutrition, particularly in the area of inflammation.

Thus, the aims of this thesis are to:

1. Screen Australian macroalgal species for metabolites of interest relating to anti-inflammatory, anti-oxidant and anti-cancer functionalities;
2. To develop and test robust methods for effective extraction and characterisation of the metabolites of interest and;
3. To test the environmental effects of cultivation on biomass fractions that include FA and pigments, and to demonstrate that these fractions have different effects on anti-oxidant, anti-inflammatory and anti-cancer potential.

These aims were addressed across the following five chapters that have resulted in one published book chapter, two published manuscripts and two prepared manuscripts for submission. **Chapter 2** presents two commonly utilised methods in anti-cancer screening, one of which is used and referred to throughout this thesis in subsequent chapters. **Chapter 3** presents the results for the anti-inflammatory and metabolite screening of six endemic Australian seaweeds across the three phyla and tests the safety
and efficacy of these crude extracts. **Chapter 4** explores the variability of metabolic profiles for FA for *Ulva* sp. as they can vary to a large extent based on environmental stimuli and therefore cultivation conditions. This chapter also explores methods for FA profile characterisation. **Chapter 5** explores the distribution of these FA for *Ulva* sp. across neutral and phospholipids. In **Chapter 6**, the pigment, phenolic and flavonoid variability across cultivation conditions is similarly explored for *Ulva* sp. with an increased emphasis on the specific fractions to which oxidative functions could be linked. Finally a discussion is provided in **Chapter 7**, which ties together the overall assessment of Australian seaweed candidates for the extraction of metabolites and development of products that can offset the chronic inflammatory disorders that are increasingly prevalent in western societies.
Chapter 1 General Introduction

References


Chapter 1 General Introduction


110. Aguilar-Briseño, J.A., et al., Sulphated polysaccharides from Ulva clathrata and Cladosiphon okamuranus seaweeds both inhibit viral attachment/entry and cell-cell fusion, in NDV infection. 2015.


This thesis assesses a number of Australian seaweed candidates for potential bioactives that can offset chronic inflammatory disorders. Ideally, these bioactives are to be incorporated as dietary components for both animal and human nutrition, as a whole food, fraction, or concentrated extract. Therefore, the level of toxicity of the sample must be determined alongside other biological assays.

Here, Chapter 2 presents the final accepted version of a published book chapter\(^8\) (2013) that describes enzyme- and cell-based anti-cancer assays presented in thesis format. The title page of the final published version has been provided in Appendix A. The cell-based assays described are used to determine the level of toxicity as a measure of inhibition of cell proliferation and thus cell metabolism. Note, it is not a measure of absolute cell death, which must be determined by complementary Trypan blue staining (non-viable cells will stain blue) and cell counting if necessary. In addition to evaluation of end-point data, cells should be monitored during the assay for morphological changes. Photographing cells is also highly recommended and aids enormously when revisiting results for discussion at later dates.


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Secondly, it is also important to consider anti-cancer screening against both an established cell line and primary cell line. Established cell lines are immortal cancer cell lines allowing for an indefinite number of subcultures *in vitro*. In contrast, a primary cell line has finite division potential and is more representative of a normal cell. Therefore any determined cytotoxicity for potential anti-cancer agents should ideally be selective for an immortal cancer cell line. Nevertheless, it is a useful preliminary screen for extract or metabolite toxicity and to determine whether or not the more expensive and limited primary cell cultures should be included in future investigations.
CHAPTER 2 BIOASSAYS FOR ANTI-CANCER ACTIVITIES

Janice I. McCauley,¹ Ana Zivanovic¹,² and Danielle Skropeta¹,²*

¹School of Chemistry, Faculty of Science, Medicine, and Health (SMAH), University of Wollongong, NSW, 2522, Australia
²Centre for Medical and Molecular Bioscience, University of Wollongong, Wollongong, NSW, 2522, Australia

Corresponding Author*
Danielle Skropeta
Phone: +61-2-42214360
Fax: +61-2-42214287
Email: skropeta@uow.edu.au

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Contributions

40% Janice McCauley Final literature review, structure and formatting, addressing final edits and submission
40% Ana Zivanovic Initial literature review, structure and formatting
20% Danielle Skropeta Proof-reading and editing, overall structure and guidance

As agreed by supervisor A/Prof. Danielle Skropeta

Summary

The MTT/MTS *in vitro* cell proliferation assay is one of the most widely used assays for evaluating preliminary anti-cancer activity of both synthetic derivatives and natural products and natural product extracts. The highly reliable, colorimetric based assay is readily performed on a wide range of cell lines. This assay gives an indication of whole cell cytotoxicity, however to determine the exact molecular target further assays need to be performed. Of these, kinase inhibition assays are also one of the most widespread enzyme inhibition screening assays performed. Kinases are enzymes that play a key role in a number of physiological processes and their inhibitors have been found to exhibit anti-cancer activity against various human cancer cell lines. Herein, we describe the methods for performing both *in vitro* MTT/MTS cytotoxicity and kinase enzyme inhibition assays. These are two of the most useful anti-cancer screening techniques available that are relatively economical and can be easily and routinely performed in the laboratory to characterise anti-cancer activity. Both assays are highly versatile and can be modified to test against targeted disease processes by using specific kinase enzymes or cell lines.

**Key words:** MTS/MTT assays, cytotoxicity, anti-cancer activity, human cancer cell lines, enzyme inhibition, kinases
Introduction

A goal of many natural product chemistry and organic synthesis laboratories is ultimately drug discovery. An important aspect of the drug development process is testing both natural products and synthesised compounds for bioactivities that are involved in targeted diseases processes. Cancer is a general term to define a number of diseases that are characterised by the uncontrolled proliferation of cells resulting from the disruption or dysfunction of regulatory signaling pathways that are normally under tight control [1, 2]. Cancer can spread rapidly and invade other tissues and organs and different cancers are recognized to have unique characteristics or markers [3].

To assess for preliminary anti-cancer activity in terms of cell viability, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) in vitro cytotoxicity assays are considered two of the most economic, reliable and convenient methods. This is based on their ease of use, accuracy and rapid indication of toxicity [4], as well as their sensitivity and specificity [5]. Both assays are in vitro whole cell toxicity assays that employ colorimetric methods for determining the number of viable cells based on mitochondrial dehydrogenase activity measurement and differ only in the reagent employed. In the MTT assay, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide is bioreduced by dehydrogenase inside living cells to form a coloured formazan dye, while in the MTS assay, a similar bioconversion takes places utilising 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazo-lium, inner salt and an electron coupling reagent (phenazine
Chapter 2 Bioassays for Anti-cancer Activities

ethosulfate) PES (Figure 6).

The MTT assay requires the addition of solubilising agents to dissolve the insoluble formazan product formed, while the MTS assay generates a water-soluble formazan product, thus simplifying the assay. The number of viable cells is measured through colorimetry and works on the principle that the mitochondrial dehydrogenase enzymes which produces NADH or NADHP, reduces the colourless tetrazolium salt into a coloured aqueous soluble formazan product by the mitochondrial activity of viable cells at 37°C (Figure 6). The quantity of the coloured product is directly proportional to the number of live cells in the culture since the MTT/MTS reagent can only be reduced to formazan by metabolically active cells.

![MTS and Formazan Diagram](image)

**Figure 6** The reduction of the MTS tetrazolium salt to the red formazan product by viable cells.

This is a useful assay to characterise potential anti-cancer agents and can be performed routinely and easily in the laboratory without the need to forego intellectual property. The MTT and MTS assays assess for toxicity to the particular cell under investigation (not anti-cancer activity per se). Therefore, most researchers screen for cytotoxicity against either murine or human cancer cell lines, as well as against a normal cell line.
such as peripheral blood lymphocytes. A selectivity index of the compound for cancer cells over normal cells can then be determined. It is also advisable to confirm MTS/MTT results with qualitative observations under the microscope of the cell morphology both before and after the assay. This can often assist in identifying potential modes of actions and deciding which further assays should be performed such as caspase activation, assessing stage of cell cycle arrest and microtubule stabilisation or destabilisation [6]. In addition to performing MTT or MTS assays against specific cell types, many researchers worldwide also submit their compounds to screening by the National Cancer Institute (NCI, http://www.cancer.gov), which is part of the US Government National Institutes of Health (NIH). The NCI offers a rapid in vitro primary anti-cancer drug screen to support cancer researchers worldwide [7]. The screen, which is performed at no cost to the researcher other than shipping of their sample, consists of a panel of 60 different human tumour cell lines from several cancer types including leukaemia, melanoma and cancers of the lung, colon, brain, ovary, breast, prostate and kidney. The NCI60 screen tests the degree of growth, inhibition or cytotoxicity of a compound against each cell line over a range of concentrations to generate a characteristic profile or fingerprint of cellular response [8]. A computer program (COMPARE) is used to assess the pattern of response across the cell lines, which can point towards a likely mechanism of action or identify compounds with unique modes of activity and/or selectivity’s for specific cell types. Operational details are provided by the NCI Developmental Therapeutics Program (DTP) and can be found at http://dtp.nci.nih.gov and for information regarding the submission of compounds for testing in the NCI screens see
http://dtp.nci.nih.gov/docs/misc/common_files/submit Compounds.html [9]. Only pure compounds are screened in the assay and the supplier of the sample is required to provide the molecular structure of any compounds submitted, which are then reviewed and considered for testing.

The above assays are whole cell assays, however, often a researcher will want to determine activity against a specific molecular or cellular target in order to confirm the mechanism of action and to assess for selectivity towards different targets and any off-target effects. This can be achieved using in vitro inhibition assays against either purified enzymes or cell-free extracts enriched in the enzyme target of interest. One such anti-cancer screening method that can be easily and routinely performed in the laboratory in low-, medium- or high-throughput format is protein kinase inhibition [10]. After G protein-coupled receptors (GPCRs), protein kinases are the second most important anti-cancer drug target being pursued today.

Unlike MTS-type assays, which are cell based, these assays are based on detecting the degree to which the potential drug compound can inhibit an enzyme’s activity. Most drugs used today demonstrate their bioactivity by acting as either receptor antagonists or as enzyme inhibitors. Enzymes are popular drug targets as they play a significant role in a number of disease processes and are susceptible to inhibition by small drug-like molecules [11].

Protein kinases are an abundant group of enzymes in the human body with approximately 518 different protein kinases encoded in the human genome [12]. Kinases catalyse the chemical transfer of a phosphate group from a high-energy
molecule such as adenine triphosphate (ATP) to a hydroxyl-containing substrate such as serine, threonine and tyrosine, and are divided into different families based on their selectivity for these amino acids.

As one of the most abundant groups of enzymes in the human body, protein kinases are important in almost every major pathway in eukaryotic cells. They play a central role in the regulation of cellular activities and in signal transduction in signal transmission pathways. Moreover, kinases have important roles in metabolism, cell growth, apoptosis, immune response, gene expression, oncogenesis, cell differentiation and proliferation, metabolism, DNA damage repair and cell motility [2]. As a result, the deregulation of kinases has been identified to be the main cause in an increasing list of diseases [13]. An estimated one-third of pharma drug discovery programs now focus on targeting cancer-related kinases with the aim of developing potent and selective inhibitors with lower side effects than treatments that traditionally focus on DNA and chromosome regulation [14-17]. An example of a kinase inhibitor that has successfully proceeded onto the pharmaceutical market is Imitinib (Gleevec, Novartis), which is a tyrosine kinase inhibitor that has dramatically improved the prognosis for sufferers of chronic myeloid leukaemia after being the first small-molecule kinase inhibitor to be approved for human use [13].

c-AMP Dependent protein kinase A (protein kinase A, PKA) is an enzyme involved in the phosphorylation of a wide range of proteins, ion channels and transcription factors [18]. It has been demonstrated to regulate a number of physiological processes including cardiovascular function, steroid biosynthesis, reproductive function,
myogenesis, adipocyte metabolism, exocytotic processes and immune function [19, 20]. PKA was also found to play a key role in memory processes [21]. The cAMP-PKA pathway has been linked to the promotion of malignant phenotypes of head and neck squamous cell carcinoma [22] and demonstrated to be activated in a range of tumours [23]. Conversely, PKA inhibitors have been found to display both \textit{in vitro} and \textit{in vivo} anti-tumour activity against various human cancer cell lines and to enhance monocyte function in HIV-infected patients [24]. Thus, it is becoming increasingly apparent that the ability to selectively inhibit PKA provides a new way of potentially modulating cancer, immune function, and memory disorders such as Alzheimer’s disease, Parkinson’s disease and schizophrenia [19, 21, 25].

Herein, we describe methods for performing both the MTT/MTS cell proliferation cytotoxicity assay and for screening against the enzyme PKA. Both methods are widely used in our laboratory [6, 26, 27].

\textit{Materials}

Carry out all procedures at room temperature unless otherwise specified.

\textit{Protein kinase assay}

This protocol is for the analysis of 14 samples per plate – each sample is run twice at 100 µg/mL and in triplicate. This gives six data points per % inhibition reading. Alternatively, 14 samples can be run once at two different concentrations (100 µg/mL, 1 µg/mL) in triplicate. This will give three data points per % inhibition reading (adjust
protocol accordingly). The volumes provided in this protocol are intended for a 96-well plate. The actual volumes used can be adjusted as needed.

1. **Prepare sample solutions:** Prepare a 5 mg/mL stock solution of sample in 100% dimethyl sulfoxide (DMSO).

2. **Sample dilution:** Make 1 mL of a 1 mg/mL secondary stock solution (in 20% DMSO) of the sample by taking 200 µL of the above 5 mg/mL stock and adding 800 µL of Milli Q water. During the assay, this will be diluted by 1:10 to give a final concentration of 100 µg/mL in 2% DMSO [see Notes 1 & 2].

3. **Prepare kinase reaction buffer:** Dissolve 48.5 mg of tris(hydroxymethyl)aminomethane (Tris), 19.0 mg of magnesium chloride (MgCl$_2$) and 1.0 mg of bovine serum albumin (BSA) in 2 mL of ultra-pure H$_2$O and then make up to 10 mL in a falcon tube. Adjust the pH to 7.5 using aqueous hydrochloric acid (HCl) if required. This will make 10 mL of reaction buffer that is sufficient for 1 x 96-well plate (i.e. 45 µL x 96 wells = 4.3 mL, plus 5 mL for adenosine triphosphate (ATP) stock) [see Note 3].

4. **Enzyme preparation:** The PKA enzyme (Promega) used herein is supplied in buffer as 2500 units at 114 units/µL. Dissolve whole contents of tube containing enzyme in a total of 2 mL of kinase reaction buffer (see step 3 above). Aliquot out 10 x 200 µL of this solution into labeled Eppendorf tubes and store at -70 °C, to give 250 units of activity per tube. The assay described herein utilises 2.50 units per well, i.e. 2.50 units x 96 wells = 240 units in total. Thus the supplied enzyme will enable testing of 10 x 96-well plates [see Note 4].
5. Kemptide (PKA) specific substrate (Promega, Australia) (10 mg/mL). The substrate is used as supplied and stored at -20 ºC [see Note 5].

6. **Prepare ATP solution:** Label 3 tubes: ATP stock 1, ATP stock 2 and ATP stock 3 (the last one is used in the assay). Weigh 5.51 mg of ATP and make up to 500 μL with kinase reaction buffer to give a 20 mM solution of ATP (stock 1). Take 100 μL of ATP stock 1 and dilute to 500 μL in kinase reaction buffer to give a 4 mM solution. Take 15 μL of the ATP stock 2 and dilute to 3000 μL in kinase reaction buffer to give a 20 μM solution. This last solution is used in the assay to give a final concentration of 10 μM [see Note 6].

7. 96-well opaque white (non-sterile) plates (Corning #3912).

8. Micropipettes.

9. Luminometer compatible for 96-well plates.

10. **20% DMSO solution:** Add 800 μL of distilled water to 200 μL of pure DMSO.

11. A known kinase inhibitor standard reference for positive control, e.g. staurosporine, H-9 or H-89 (Sigma-Aldrich) [see Note 7].

12. **Kinase-Glo® Reagents:** Kinase-Glo® Buffer and Kinase-Glo® Substrate (Promega Corporation, Australia). Store at -20°C.

**MTS assay**

Plates containing compound dilutions should be discarded in cytotoxic waste bins. All pipette tips that came into contact with the test compounds should also be disposed of in cytotoxic waste bins

1. **Sample preparation:** Prepare a 4 mg/mL stock solution of sample in DMSO.
2. **MTS reagent:** Should be stored at -20 °C for long-term storage and protected from light. Reagent should be warmed up to room temperature before use in the assay.

3. RPMI tissue culture medium containing 5% fetal bovine serum (FBS) [see Note 8].

4. Stock cultures of cells should be maintained in RPMI medium containing 5% FBS (fetal bovine serum).

5. Clear, sterile 96-well microplates.

6. Trypan blue solution (0.4%, liquid, sterile-filtered, suitable for cell culture).

7. Dimethyl sulfoxide (DMSO).

8. Human, leukemic, monocyte-like, histolytic lymphoma (U937) and human, metastatic breast adenocarcinoma (MDA-MB-231) cancer cell lines, or other cell lines as required [see Note 9]


11. Sterile incubator.

12. UV spectrophotometer compatible for 96-well plate.

**Methods**

The kinase assay described here utilises the Kinase-Glo® Luminsecnt Kinase Assay Platform from Promega and is performed according to the manufacturer’s instructions [28] with minor modifications as used in our laboratory. The method below is relevant to screening against PKA, however a wide range of kinases can be used in this assay.
including GSK-3β, PI3K, Src and MAPK [28, 29]. In theory, potentially any kinase could be used, provided the appropriate substrate is also utilised.

**Kinase Assay**

1. To wells A/F1-12 of a 96-well plate, add 5 µL of the 1 mg/mL stock solution of the test compounds, which will give a final concentration of 100 µg/mL sample in 2% DMSO (Table 10). To the positive control wells (G1-6) and the negative control wells (H1-6), add 5 µL of the 20% DMSO solution.

2. To wells G/H7-12, add 5 µL of the internal standard (e.g. staurosporine).

3. Thaw a single tube of the enzyme (250 units per tube) and make up to 2 mL with the kinase reaction buffer to give a concentration of 125 units/mL. This will give a final concentration of 50 units/mL (or 2.50 units/well).

4. To six of the positive control wells (G1-6), add 20 µL of the reaction mixture containing 2.5 X the optimal concentration of kinase in kinase reaction buffer.

   The positive control should provide 100% luminescence. There will be 1880 µL of this reaction mixture remaining.
5. Add 20 µL of the kemptide (PKA peptide substrate, 10 mg/mL) to the remaining 1880 µL of kinase mixture. This will provide 200 µg of the kinase substrate in 1900 mL to give a 140 µM substrate/enzyme solution in buffer. This amount is sufficient for 1 x 96-well plate, giving a final concentration of 56 µM in each well per 50 µL reaction.

6. To all remaining wells, add 20 µL of the above reaction mixture (step 5) containing 2.5 X the optimal concentration of kinase and kinase substrate in 1X kinase reaction buffer. (Negative controls, 0% luminescence).

7. To all wells, add 25 µL of the ATP solution (20 µM, stock 3 ATP solution). This will give a final concentration of 10 µM in each well per 50 µL reaction. See Table 11 for a summary of the reagent volumes added to the 96-well plate.

8. Using a plate shaker, gently shake the plate and incubate at room temperature for the optimal amount of time [see Note 10].

9. **Prepare Kinase-Glo® reagent:** The kinase buffer should be stored in the freezer and thawed at room temperature. Add the Kinase-Glo® Substrate to the Kinase-Glo® Buffer and add 50 µL of this mixture to each well [see Note 11].

10. Mix the plate and incubate for 10 minutes at room temperature. Due to the long half-life of the Kinase-Glo® signal, the plates may be left longer before reading, if desired. Record luminescence, which will be directly proportional to percent inhibition of the controls [see Notes 12-14].
Table 11 Kinase assay summary of reagent concentrations and volumes added to plate.

<table>
<thead>
<tr>
<th></th>
<th>Initial Stock Conc.</th>
<th>Standard Wells Conc. (µL)</th>
<th>Positive Control Conc. (µL)</th>
<th>Negative Control Conc. (µL)</th>
<th>Final Conc. (in 50 µL)</th>
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<tr>
<td>Sample</td>
<td>1 mg/mL</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>DMSO solution</td>
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<td>-</td>
<td>5</td>
<td>5</td>
<td>2 %</td>
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<td>Enzyme/no substrate (2.5X)</td>
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<tr>
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<td>56 µM</td>
</tr>
<tr>
<td>ATP solution (2X)</td>
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<tr>
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<td>50</td>
<td>50</td>
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</tbody>
</table>

**MTS Assay**

The MTS assay described here utilises Promega’s MTS CellTiter 96® AQueous One Solution Cell Proliferation assay and is performed according to the manufacturer’s instructions [30, 31].

1. **Determination of cell number and viability:** Place 20 µL cells + 20 µL Trypan blue on parafilm and place 20 µL of this mixture under a cover-slip on the haemocytometer. Count the number of cells using the equation provided [see Note 15] to determine the volume required to get a cell concentration of 111,000 cells/mL.

2. **Day 1 - Setting Up the Plate:** Pipette 610 µL of cells (as determined in Step 1 of the procedure) into a new Falcon tube and centrifuge at 1600 rpm for five min. (For four plates, pipette 2.44 mL of cells).

3. **Drain off supernatant and resuspend the pellet into 2.5 mL of media.** This will give you 2.5 mL of cell solution at 111,000 cells/mL. (For four plates, you would resuspend in 10 mL of media).
4. Pipette 100 µL of media into wells A1-A12, H1-H12, B1-G1, B11-G11, B12-G12, B5-B7, E5-E7, F5-F7 and G5-G7 as per Figure 7. These wells are used as blanks to stop any interference that may occur when the spectrophotometer reads the absorbance values for the test wells.

![Figure 7](Image)

**Figure 7** 96-Well microplate set-up for the MTS assay.

5. Pipette 90 µL of media into wells B8-G8, B9-G9, B10-G10. These wells will form the sample background controls.

6. Pipette 90 µL of cell solution into wells B2-G2, B3-G3, B4-G4. These wells will form the sample wells.

7. Pipette 90 µL of cell solution into wells D5-D7. These wells will form the 2.5% DMSO controls.

8. Pipette 100 µL of cell solution into wells C5-C7. These wells will form the cell controls.

9. Incubate plates for 24 hours at 37 °C with 5% carbon dioxide (CO₂).
10. **Day Two - Diluting Test Compounds:** Prepare a microtitre plate for serial dilutions of the test compounds as shown in Figure 8 for each compound to be tested. Wells A3-G3 should all contain a final volume of 100 µL.

![Figure 8](image)

**Figure 8** Preparation of microtitre plate for 1:2 serial dilutions. Add 100 µL of DMSO and 100 µL of the test compound (4 mg/mL in DMSO) to well A1. Next, add 100 µL of DMSO to wells B1 to G1. Then, 100 µL is transferred from A1 to B1; B1 to C1 and so forth until F1, where 100 µL is removed and discarded. G1 should contain just 100 µL of 100% DMSO. Next, 50 µL of media is added to wells A2-G2 and A3-G3 and 50 µL transferred from A1 to A2 and then from A2 to A3 to give 100 µL of the sample at a concentration of 500 µg/mL in 25% DMSO in media. This is repeated for wells B2-B3, C2-C3, etc to give a series of 1:2 dilutions of the sample.

11. **Adding serial dilutions of samples to cells:** Using the plate of cells prepared on Day 1, pipette 10 µL of each sample dilution of the test compound in 25% DMSO (i.e. A3-F3, step 11) in triplicate into the 18 wells containing 90 µL of cell solution (i.e. 10 µL of the sample at 500 µg/mL in triplicate into wells B2-B4, 250 µg/mL sample into C2-C4 etc. (Figure 7), down to the 15.5 µg/mL sample into G2-4). Repeat this step, by adding 10 µL of the serial dilutions of the sample in triplicate to the 18 wells containing 90 µL of media (e.g. wells B8-G8, B9-G9, B10-G10). The test compounds will now have a final concentration of 2.5% DMSO in all wells.
12. Pipette 10 µL of the 0 µg/mL in 25% DMSO (i.e. G3 from step 11) into the three remaining wells containing 90 µL cells (wells D5-D7). This will be the 2.5% DMSO control.

13. Incubate plate for 24 hours at 37 ºC and 5% CO₂ [see Note 16].

14. **Day Three - Adding MTS Reagent and Plate Reading:** Thaw MTS reagent before use (~1 mL MTS reagent required per plate).

15. Pipette 20 µL of the MTS reagent into all sample wells (e.g. B2-G2, B3-G3, B4-G4, B5-D5, B6-D6, B7-G7, B8-G8, B9-G9, B10-G10) [see Note 17].

16. Incubate for three hours at 37 ºC and 5% CO₂.

17. Wrap the plates in aluminium foil and take to the spectrophotometer. Read the absorbance of the whole plate at 490 nm [see Note 18].

**Notes**

1. A final concentration of 2% DMSO was used for the sample and was found not to interfere with results at this concentration. Standards and controls used in the assay were also dissolved in a final concentration of 2% DMSO. Other solvents such as 2% ethanol can be used, provided all standards/controls are also prepared in this solvent.

2. We found that a starting stock solution of 1 mg/mL of sample (diluted to 100 µg/mL in the assay) was appropriate to identify kinase inhibitory activity. Samples can be tested at lower concentrations or over a range of different concentrations to determine IC₅₀ values.
3. We found that it is best to prepare the buffer solution fresh each time and to store the buffer at room temperature.

4. For kinases other than PKA, the optimum amount of kinase enzyme will need to be determined and the quantity of other reagents adjusted accordingly [28].

5. Kemptide (PKA Peptide Substrate) is a synthetic peptide substrate for PKA derived from the PKA phosphorylation site in liver pyruvate kinase.

6. This assay can be used to identify whether the kinase inhibitor is ATP competitive or non-competitive, by utilising various ratios of ATP (e.g. <10 µM ATP for ATP-competitive inhibitors and >100 µM for ATP non-competitive inhibitors). In general, we found it is best to prepare ATP solutions fresh, while the ATP reagent itself should be stored in the freezer.

7. We include an internal reference of a known kinase inhibitor such as staurosporine in our assays (IC₅₀ = 7 nM vs PKA; IC₅₀ = 0.7 nM vs PKC ) by adding 5 µL of a 1 mg/mL solution of the inhibitor (in 20% DMSO) to six of the wells in place of a sample set. Staurosporine is a potent inhibitor of PKC and can induce apoptosis in Jurkat cells. Other PKA inhibitors include the isoquinoline sulfonamides H-9 (IC₅₀ = 2 µM) and H-89 (IC₅₀ = 48 nM).

8. RPMI-1640 was developed by Moore et al. [32, 33] at Roswell Park Memorial Institute.

9. Human, leukemic, monocyte-like, histolytic lymphoma (U937) and human, metastatic breast adenocarcinoma (MDA-MB-231) cancer cells were obtained from American Type Culture Collection (ATCC, VA, USA) distributed by Cryosite, NSW, Australia. Cells were regularly cultured in vitro in culture
medium consisting of RPMI-1640 medium, along with 2 mM L-glutamine, 5.6% (2 g/L) NaHCO₃ and 5% foetal calf serum. The cells were maintained in a Huracell incubator (Kendro Laboratory Products, Langenselbold, Germany) at 37 °C with a humidified atmosphere containing 5% CO₂. However, a wide range of both normal and cancer cell lines can be used in this assay.

10. We found that thawing the Kinase-Glo® reagents (Kinase-Glo® substrate and Kinase-Glo® buffer) generally took one hour and we also allowed an hour for the incubation time. We strongly advise optimizing the kinase reaction conditions so that the reaction can be run at room temperature to avoid formation of temperature gradients. Information regarding optimization of kinase reaction conditions can be found in the manufacturer’s Technical Bulletin [28].

11. The choice of Kinase-Glo® reagent will depend on the desired ATP concentration to be used in the assay. This information can be found in the manufacturer’s protocol, Promega Corporation, Australia [28, 34].

12. We find that it is best to read the plate within 15 mins after the addition of the Kinase-Glo® reaction mixture.

13. Instrument settings depend on the manufacturer. An integration time of 0.25–1 second per well should serve as a guideline. We found that the best plate reading is immediately after adding the Kinase-Glo® Buffer and when the incubation is performed at room temperature.

14. The most common assay detection methods are colorimetric or fluorescence-based. However, luminescent-based detection is particularly desirable for
coloured products such as natural products, as coloured extracts and compounds can lead to false results when using absorbance as the end point. Utilization of luminescence can be more beneficial for biological screening by identifying hits with a lower number of false positives [35].

15. An example calculation of cell concentration of which the values obtained will be used in the method protocol described herein:

\[
\text{No. of Cells} = \frac{(\# \text{ of cells in 4 grids})}{4} \times 2 \times 10000
\]

For example if we count 91* cells:

\[
\text{No. of Cells} = \frac{91}{4} \times 2 \times 10000 = 455000 \text{ cells}
\]

Therefore, to get a cell concentration of 111,000 cells/mL, you will need:

\[
\frac{111000 \text{ cells/mL}}{455000 \text{ cells}} = 0.244 \text{ mL} = 244 \mu L
\]

For the preparation of one plate the cells will be resuspended in 2.5 mL media therefore the volume of cells required would be:

\[
244 \mu L \times 2.5 = 610 \mu L
\]

Adjust accordingly if more plates are required.

For example, the preparation of 4 plates will require:

\[
244 \mu L \times 10 \text{ mL (2.5 ml per plate x 4 plates)} = 2.44 \text{ mL}
\]

16. Longer incubation times of 48 – 72 hours are also routinely employed in this assay.

17. The MTS reagent is light sensitive so this step should be performed with the lights off in the cytotoxic cabinet.
18. As this is a colorimetric assay, it should be kept in mind that both coloured compounds and natural product extracts may interfere with the absorbance reading, and appropriate background controls should always be performed.

_Acknowledgements_

The School of Medicinal Chemistry and the School of Chemistry, University of Wollongong, supported this work.
References


FOREWORD TO CHAPTER 3

The previous Chapter 2 explored an anti-cancer cell based method that can be used as a preliminary assessment of a sample’s toxicity. This assay was first established in order to proceed with the following work (Chapter 3), which involved screening wild collections of macroalgae, including one cultivated species of Australian macroalgae for anti-inflammatory and anti-cancer activities and determination of their fatty acid profiles. An anti-inflammatory assay was also established in house to measure the amount of nitric oxide produced in cell supernatants in bacterial lipopolysaccharide stimulated inflammatory cells. A thorough explanation of the methods for this anti-inflammatory cell-based assay is provided in Appendix B.

This chapter presents the final accepted version (in thesis format) of a published manuscript9 (2015) within the Journal of Applied Phycology (Impact factor 2.372). The title page of the article, as it appears in the printed journal can be found in Appendix C.

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CHAPTER 3 SELECTING AUSTRALIAN MARINE MACROALGAE BASED ON THE FATTY ACID COMPOSITION AND ANTI-INFLAMMATORY ACTIVITY

Janice I. McCauley,1 Barbara J. Meyer,2 Pia C. Winberg,2,3 Marie Ranson,4,5,6 and Danielle Skropeta1,6*

1School of Chemistry, Faculty of Science, Medicine, and Health (SMAH), University of Wollongong (UOW), NSW, 2522, Australia
2School of Medicine, SMAH, UOW, Wollongong, NSW, 2522, Australia
3Venus Shell Systems, Narrawallee, NSW, 2539, Australia
4Illawarra Health and Medical Research Institute, UOW, Wollongong, NSW, 2522, Australia
5School of Biological Sciences, SMAH, UOW, NSW, 2522, Australia
6Centre for Medical and Molecular Bioscience, UOW, Wollongong, NSW, 2522, Australia

Corresponding Author*
Danielle Skropeta
Phone: +61-2-42214360
Fax: +61-2-42214287
Email: skropeta@uow.edu.au

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Contributions

60% Janice McCauley Performed the work and prepared/edited and submitted the paper
10% Barbara Meyer Provided GC access, training and supervision
10% Pia Winberg Editing and input on project design
5% Marie Ranson Guidance for molecular and cell biology techniques
15% Danielle Skropeta Oversight of project, editing and proof-reading

As agreed by supervisor A/Prof. Danielle Skropeta

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 anti-proliferative. 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 with 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 [1, 2]. Increasingly they are being recognised as a rich source of biologically active metabolites for utilisation in functional foods, nutritional supplements and/or pharmaceuticals [3]. This is due to important functional roles of these metabolites in a range of biological processes including cell mediation and immune responses [4-9]. Research in medical and health applications of seaweed metabolites now dominate the publication of phycology related scientific literature [10]. There is particular promise for chronic inflammatory diseases and cancers of the digestive system [11, 12] (Figure 9). 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) [13]. 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 [13]. Conversely, n-3 PUFA can be enzymatically converted to bioactive autacoids with inflammatory-resolving properties that compete with and offset inflammation status [14, 15]. 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 [16, 17]. The pathogenesis of diseases includes cardiovascular disease, cancer and other inflammatory disorders [17, 18]. Thus, a greater consumption of long chain n-3 FA may offer protective effects by suppressing proinflammatory activity [18, 19].

Figure 9 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 [16]. Conversion of ALA to EPA and DHA is limited and therefore dietary consumption of these two long-chain PUFA is highly recommended [20, 21]. 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 [22, 23]. 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 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 [16, 24]. 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 [25]. 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 [26]. 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 [27] and subsequently cancer [25]. Suppression of NO production has been observed with n-3 PUFA via inhibition of iNOS [28]. 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 [11]. 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 [15].

The metabolic profiles and biological activity of extracts from northern hemisphere macroalgal species have been well described including both genetic and spatial/temporal variations [29, 30]. However in Australia, which is a macroalgal biodiversity hotspot [31] with high species richness and endemism of benthic flora [32], 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 FA 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 FA profiling fresh samples were collected, cleaned and freeze-dried immediately prior to analysis. The *Ulva* sp. used herein was obtained from culture, kept and maintained for several months (*Table 12*).

<table>
<thead>
<tr>
<th>Species</th>
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<th>Order</th>
<th>Location</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Date</th>
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<td>Ochrophyta</td>
<td>Laminariales</td>
<td>Jervis Bay</td>
<td>150.7347</td>
<td>35.0653</td>
<td>10/4/14</td>
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<td>Jervis Bay</td>
<td>150.7347</td>
<td>35.0653</td>
<td>10/4/14</td>
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<td>35.0653</td>
<td>10/4/14</td>
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<td>Jervis Bay</td>
<td>150.7347</td>
<td>35.0653</td>
<td>10/4/14</td>
</tr>
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<td>Gerrinong</td>
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<td>34.7423</td>
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<td>04/4/12</td>
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<td>Ectocarpales</td>
<td>Manyana</td>
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<td>35.2500</td>
<td>01/11/12</td>
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<td>Chlorophyta</td>
<td>Ulvales</td>
<td>Cultivated</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Fresh samples collected and immediately freeze-dried for species fatty acid profiling; Freeze-dried sample prepared from frozen voucher sample; Species name withheld; species maintained in culture.*

*Extraction and Transesterification of Fatty Acids*

Lipids were extracted using the method of Folch et al. [33] with modifications by Kumari et al. [34]. 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 [35] 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. [36]. 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 µg.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) [37]. 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 ºC, 5% CO$_2$), including controls.

The quantity of nitrite (NO$_2^-$) 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. NO$_2^-$ concentrations were calculated using a standard curve. NO inhibition was determined as % of LPS induced inflammatory controls [38].

**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 [39]. 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 FA were identified across the six algal species (Table 13). 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 13 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).

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Ochrophyta</th>
<th>Rhodophyta</th>
<th>Chlorophyta</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Molar Area</td>
<td>E. radiata</td>
<td>M. sciurus.</td>
<td>H. banksii</td>
</tr>
<tr>
<td>10:0</td>
<td>8.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.3&lt;sup&gt;ad&lt;/sup&gt;</td>
<td>3.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>11:0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>12:0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>14:0</td>
<td>10.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>14:1 n-7</td>
<td>0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>15:0</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
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<td>15:1</td>
<td>0.2</td>
<td>0.3</td>
<td>0.1</td>
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<td>16:0</td>
<td>27.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>46.3&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>18:0</td>
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<td>0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.3&lt;sup&gt;abc&lt;/sup&gt;</td>
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<td>1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.5&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>0.1</td>
<td>0.1</td>
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<td>1.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.2&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>0.0</td>
<td>0.7</td>
</tr>
<tr>
<td>20:5 n-3 (EPA)</td>
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<td>0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.8&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>22:0</td>
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<td>0.3</td>
<td>1.3</td>
</tr>
<tr>
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<tr>
<td>22:2 n-6</td>
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<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>24:1 n-9</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

- **Σ SFA**: 50.7<sup>a</sup> | 65.5<sup>b</sup> | 40.6<sup>c</sup> | 42.0<sup>c</sup> | 60.8<sup>b</sup> | 47.4<sup>a</sup> |
- **Σ MUFA**: 33.0<sup>b</sup> | 26.7<sup>bce</sup> | 24.6<sup>cde</sup> | 20.8<sup>cde</sup> | 17.2<sup>c</sup> | 26.0<sup>f</sup> |
- **Σ PUFA**: 16.3<sup>d</sup> | 7.8<sup>c</sup> | 34.8<sup>a</sup> | 37.2<sup>a</sup> | 22.0<sup>b</sup> | 26.5<sup>b</sup> |
- **n-6/n-3**: 3.0<sup>a</sup> | 1.8<sup>b</sup> | 1.5<sup>c</sup> | 3.8<sup>d</sup> | 15.7<sup>e</sup> | 0.4<sup>d</sup> |
- **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
Key differences in abundance across species

Analysis of the quantified FA 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) (Figure 10). Transformation of the data to reduce the effect of abundance indicated that phyla differences are significantly distinct in terms of the composition of FA 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).

Figure 10 Multivariate Dimensional Scaling plot comparing similarities of species FA 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 (Figure 10). 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 (Figure 11). *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 (Figure 11). 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) (Figure 11). 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).
Figure 11 Key differences in FA 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 14), 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 14). 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 14. 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 14 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 µg.mL\(^{-1}\) from two independent trials done in duplicate.

<table>
<thead>
<tr>
<th>Species</th>
<th>Extract</th>
<th>RAW 264.7</th>
<th>WT MEF</th>
<th>MIA PaCa-2</th>
<th>% ↓ NO</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. radiata</em></td>
<td>DCM</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>EtOAc</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>BuOH</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>M. sciurus</em></td>
<td>DCM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>EtOAc</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>BuOH</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td><em>H. banksii</em></td>
<td>DCM</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>EtOAc</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>BuOH</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>P. comosa</em></td>
<td>DCM</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>EtOAc</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>BuOH</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>S. robusta</em></td>
<td>DCM</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>EtOAc</td>
<td>nt</td>
<td>nt</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>BuOH</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Ulva sp.</em></td>
<td>DCM</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td></td>
<td>EtOAc</td>
<td>+</td>
<td>-</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>BuOH</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**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)

**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 15).

Table 15 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.

<table>
<thead>
<tr>
<th>Species Name</th>
<th>PUFA Content</th>
<th>Abundance</th>
<th>Low n-6/n-3 Ratios</th>
<th>LA/ALA &lt; 1</th>
<th>Selective Cancer Cell Toxicity</th>
<th>iNOS Inflammatory Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ecklonia radiata</td>
<td>YES</td>
<td>YES</td>
<td></td>
<td></td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>Myriogloea sciurus</td>
<td>YES</td>
<td>YES</td>
<td></td>
<td></td>
<td>YES</td>
<td></td>
</tr>
<tr>
<td>Hormosira banksii</td>
<td>HIGH</td>
<td>YES</td>
<td></td>
<td></td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>Phyllospora comosa</td>
<td>MED</td>
<td>YES</td>
<td></td>
<td></td>
<td>YES</td>
<td></td>
</tr>
<tr>
<td>Solieria robusta</td>
<td>YES</td>
<td>YES</td>
<td></td>
<td></td>
<td>YES</td>
<td></td>
</tr>
<tr>
<td>Ulva sp.</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
</tr>
</tbody>
</table>

Species name withheld.

With the exception of the well-studied green macroalgal genus Ulva [40-42] the five other macroalgal species described herein have been studied in other contexts such as their protein, trace element and carbohydrate content [43], 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 [44]. 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 [45].

Apart from palmitic acid (C16:0), which was the dominant FA in all six species and is consistent with other macroalgae [29, 30, 44] 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 [46, 47].

From the four Ochrophyta species, the two fucales species showed comparable total SFA, MUFA and PUFA levels [47, 48]. *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 [47, 48]. 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-chain 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 [49].

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 [49, 50]. 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 Aknin et al [51]. However, EPA has been reported in higher amounts in other red algae (order Gigartinales) from the Pacific coast of North California [48]. In terms of
total SFA, MUFA, PUFA, the results obtained were comparable to reported values for Rhodophyta species [47]. 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 [49].

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 [30, 52, 53]. As a desirable ratio for human health is around 1 [16, 17], 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 [3, 54, 55]. 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 [15, 56]. Interestingly, n-3 PUFA have been reported as modulators of the activity of nitric oxide synthase (NOS) [57], 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 [58], linked to suppression of inflammatory responses [59]. 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 [60]. 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) [61].

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) [62]; halogenated compounds from the Malaysian red alga Laurencia snackeyi [63] and the porphyrin derivatives, pheophorbide a and pheophytin a, along with the xanthophyll fucoxanthin, from the edible brown alga Saccharina japonica (order Laminariales) [64]. Other bioactive algal metabolites isolated from non-polar extracts include cytotoxic, antibacterial linear sesquiterpenoids from the green alga Penicillus capitatus (class Ulvophyceae, order Bryopsidales) [65]; antibacterial labdane diterpenoids from Ulva
fasciata [66]; and antiatherosclerotic phytosterols from the brown alga *Sargassum fusiforme* (order Fucules) [67].

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 anti-cancer, anti-viral, anti-bacterial, anti-fungal and immunomodulating activities [8, 68, 69]. In particular, a number of promising anti-cancer 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 [70], 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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establish the biological screening

Conflicts of Interest

The authors declare no conflict of interest
References


Chapter 3 Metabolite and Bioactivity Screening


42. Ortiz, J., et al., Dietary Fiber, amino acid, fatty acid and tocopherol contents of the edible seaweeds *Ulva lactuca* and *Durvillaea antarctica*. *Food Chemistry*, 2006. 99: p. 89-104.


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61. Yoo, M.-S., et al., Fucosterol isolated from *Undaria pinnatifida* inhibits lipopolysaccharid-induced-production of nitric-oxide and pro-inflammatory cytokines via the inactivation of


FOREWORD TO CHAPTER 4

The metabolite and bioactivity screening of wild and cultivated macroalgal species presented in the previous Chapter 3 established a number of key species of interest for future investigations (Figure 12).

Figure 12 A summary of the major findings and most interesting macroalgal candidates presented in Chapter 2, as a result of preliminary metabolite and bioactivity screening of five macroalgal species obtained from wild collection and one cultivated Ulva sp. (provided by Venus Shell Systems Pty. Ltd.)

**Myriogloea sciurus and Hormosira banksii**

*Myriogloea sciurus* showed anti-inflammatory activity at a consistently high level across all extracts with little toxicity. Interestingly, *Hormosira banksii* showed selective

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10 Venus Shell Systems, Narrawallee, NSW, 2539, Australia
anti-cancer activity against pancreatic cancer. Whilst cancer and natural product isolation for potential anti-cancer drug compounds is beyond the scope of this thesis, this finding was subsequently explored and the active extract was purified into 24 fractions using column chromatography (Supplementary Methods Appendix D) (Figure 13).

![Figure 13](image)

**Figure 13** The % cytotoxicity on pancreatic cancer (MIA PaCa-2) cells (as a measure of inhibition of cell proliferation) of 24 fractions generated by silica-gel chromatography purification of the active *Hormosira banksia* extract which was identified by bioactivity screening work presented in Chapter 2. High anti-cancer activity was retained in 4 fractions (F9, F10, F11 and F22).

As fraction 10 had one of the highest yields (mg) of product it was chosen for further analysis by analytical high-pressure liquid chromatography (HPLC), which revealed 9 minor and 2 major peaks (Supplementary Results Appendix E). This indicates the
presence of 12 compounds. Further preparative HPLC revealed the most dominant peak to be two compounds indicating the presence of 13 compounds in fraction 10, in which one or all may be responsible for selectively inhibiting pancreatic cancer (Supplementary Results Appendix E) However, poor yields (< 1 mg) of the final isolated compound(s) limited further structural characterisation via mass spectrometry and nuclear magnetic resonance spectroscopy (NMR). However, these preliminary investigations of Hormosira banksia highlight this macroalgae as a novel and unique Australian species to pursue, particular as part of a future natural product focused project. Myriogloea sciurus also warrants further investigations as species from the order Ectocarpales have mainly been investigated with regards to their water-soluble polysaccharides or Fucoidan (as outlined in Chapter 1) and herein we demonstrate high anti-inflammatory potential across three organic extracts of differing polarities with limited to zero toxicity against both established and primary cells lines.

Ulva sp.

Of greater interest herein were the positive results obtained for the cultivated Ulva sample, which not only showed high anti-inflammatory functionalities across all three organic extracts, but also a diverse fatty acid profile. The nonpolar dichloromethane extract and the more polar ethyl acetate extract showed the strongest anti-inflammatory activities and it was hypothesised that that this could be due to the presence of anti-inflammatory fatty acids and pigments. As the metabolism of macroalgae responds readily to their environment it is important to determine the cultivations conditions that enhance that metabolite and bioactivity profile of interest, particular in regard to their
fatty acids. Fatty acids are one of the most variable compositional categories that differ across taxa but they also readily change within taxa in response to the environment. The analysis of fatty acids and pigments were undertaken to determine if variability due to cultivation of different biomass fractions was significant and quantifiable.

Furthermore, due to the variability found in the reporting of fatty acids for *Ulva* sp. in the literature, along with first-hand experience with fatty acid variability across different protocols in-house, it was considered necessary to consider the extraction protocols and analytical methods and their affect on the fatty acid profile.

Therefore work was undertaken to determine the effect of cultivation conditions and extraction method on the fatty acid profiles of a cultivated *Ulva* sp. This work presented in Chapter 4, is the final accepted version in thesis format of a published manuscript\(^\text{11}\) (2016) within the journal *Food Chemistry* (Impact factor 3.391). The title page of the article as it appears in the printed journal can be found in Appendix F.


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CHAPTER 4 PARAMETERS AFFECTING THE ANALYTICAL PROFILE OF FATTY ACIDS IN THE MACROALGAL GENUS ULVA

Janice I. McCauley, a Barbara J. Meyer, b Pia C. Winberg, h,c and Danielle Skropeta a,d

aSchool of Chemistry, Faculty of Science, Medicine, and Health (SMAH), University of Wollongong, NSW, 2522, Australia
bSchool of Medicine, SMAH, University of Wollongong, Wollongong, NSW, 2522, Australia
cVenus Shell Systems Pty. Ltd., Narrawallee, NSW, 2539, Australia
dCentre for Medical and Molecular Bioscience, University of Wollongong, Wollongong, NSW, 2522, Australia

*Corresponding Author
Janice McCauley
Phone: +61-2-42214001
Fax: +61-2-42214287
Email: jim479@uowmail.edu.au

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Contributions

65% Janice McCauley Performed the work and prepared/edited and submitted the paper
10% Barbara Meyer GC access, training and supervision
10% Pia Winberg Editing and input on project design
15% Danielle Skropeta Oversight of project, guidance with paper structure and editing and proof-reading

As agreed by supervisor A/Prof. Danielle Skropeta

Abstract

The fatty acids (FA) of *Ulva* have potential to contribute to nutrition. However, the large variability of FA profiles of *Ulva* species; thus the quality and quantity of FA in relation to nutrition is poorly defined. Herein we investigate the FA profile of 74 cultured *Ulva* samples crossing five culture regimes, six extraction regimes and four post-harvesting processes. This is compared alongside a comprehensive review of FA profiles of *Ulva* spp. With regard to the literature, *Ulva* is characterised by C16:0 (30.5 ± 11.5 %), C18:3 n-3 (14.5 ± 6.3 %), C18:4 n-3 (12.5 ± 5.4 %), C16:4 n-3 (8.9 ± 4.8 %) and C18:1 n-7 (10.1 ± 4.0 %). The investigated *Ulva* fell within the reported range of specific FA. High nutrient conditions showed the most desirable FA profile for health, along with the highest total FA content (56 mg.g⁻¹ dry weight equivalent) when extracted with an optimised protocol.

**Key words:** PUFA, desiccation, extraction, cultivation, environment, nutrition, health
Introduction

The marine macroalgal genus *Ulva* (Chlorophyta) is a source of valuable long-chain polyunsaturated fatty acid (LC-PUFA) profiles, in particular the biologically important eicosapentaenoic acid (C20:5 n-3, EPA) [1]. *Ulva* spp. also show the presence of trace amounts of docosahexaenoic acid (C22:6 n-3, DHA), however more abundant LC-PUFA include the DHA precursor, docosapentaenoic acid (C22:5 n-3, DPA), along with the more unique C18:4 n-3 and C16:4 n-3 PUFA [1]. *Ulva* grows abundantly in waters enriched by nutrients and is frequently involved in green tides in eutrophicated coastal areas producing a high quantity of biomass [2]. This characteristic of *Ulva* means that it is well suited for cultivation. Therefore as whole biomass or as part of a sequential biorefinery system of production, cultivated *Ulva* biomass has the potential to offer a sustainable source of essential FA for human and animal consumption and nutrition.

The feasibility of cultivating marine macroalgae, as a sustainable source of high value products is largely dependent on the cost-effectiveness and scalability of cultivation to deliver high yields of quality controlled biomass. *Ulva* is a cosmopolitan genus (Figure 14) and can readily adjust its metabolism under specific environmental conditions, wherein the lipids and FA play an important role in this adaptation [3]. As such, the profiles of reported FA for *Ulva* show a wide degree of natural variation. For example *U. intestinalis* has demonstrated an increase in unsaturation with increasing mineralisation in a study of a number of abiotic factors [3].
Seasonal comparison of *U. lactuca* samples collected in Western Ireland showed a notable decrease in the n-6/n-3 ratio when collected in November (cf. June) [4]. Furthermore, the range of reported n-6/n-3 values were also more favourable when compared to an *U. lactuca* specimen collected from Northern Chile [5]. However, one of the appeals of *Ulva* in particular as a target genus, is its ability to be grown effectively and rapidly in culture, while tailoring PUFA profiles through nutrient replete conditions, which have been demonstrated on the laboratory scale [6]. It has also been shown that culture conditions including temperature [7], pH [8] and nutrient availability along with the presence of organic carbon [6], will also influence the lipid profile of
many algae including *Ulva* spp. Despite this, there are species-specific patterns within that variability that can be determined with experimental means and comprehensive review of the data. Yet little has been done beyond the laboratory scale in developing controlled commercial production systems. Therefore it is important to demonstrate the degree to which scaled cultivation conditions can be controlled to deliver the desired compositional profiles. A second consideration that could drive the variability of reported FA content and profiles is whether or not there are effects on the FA composition due to post-harvest processing, and in particular the drying process, along with effects due to variations in analysis methods on the quantification of these desired traits. This is important in delivering, from the farm to the consumer, a high quality health food product and/or ingredient with well-established processing and/or quality controlled testing criteria.

For example a number of studies have demonstrated that the choice of analytical method and any deviations of the method can significantly influence the FA yield and profile. For example, comparisons across standard extraction and transesterification techniques such as the International Organization for Standardization (ISO) method, the Association of Official Analytical Chemists (AOAC) method and the Folch method [9] found no significant differences in regards to total FA recoveries of flaxseed oil [10]. However, monounsaturated fatty acids (MUFA) and PUFA were found to be underestimated after extraction with the ISO method when compared with AOAC and Folch methods [10].
In terms of the effects from extraction methods on FA yields, the comparison of extraction-transesterification techniques versus direct transesterification showed significantly increased FA yields in lipid-producing microheterotrophs for direct transesterification methods [11]. Various solvents and solvent combinations have also been explored with higher FA recoveries found with either the Folch or Bligh and Dyer extractions protocols [12]. These protocols both use a ratio of the nonpolar solvent chloroform with the more polar methanol [12]. In macroalgae specifically, Kumari et al (2011) did a comparative evaluation of Bligh and Dyer, Folch and Cequier-Sánchez methods for the quantitative determination of total lipids and FA, along with investigating the effect of sonication and addition of a buffer in the solvent system. For *U. fasciata* no added benefit was observed for a buffered solvent system with high lipid and FA yields obtained for the traditional Folch extraction. Therefore, for the development of a nutritionally valuable, high yielding algal-derived oil, it is important to investigate both environmental effects of cultivation and effects due to post-harvest processing and analysis methods.

Herein we present the FA profile of a medium-scaled production of a genetically identified strain of one sp. of *Ulva* as a model to test the effects of environment and processing. The FA profile is presented and compared against a comprehensive literature review for the FA profile of the genus *Ulva*. The effects of environmental conditions were compared across five different medium-scale (5 m³) culture conditions; low nutrients, high nutrients, shaded, added glucose and acetic acid boost. Thirdly, this research explores the effects of post-harvesting processes and analysis methods on the FA composition and total content of the cultured algae. The findings presented have
important implications for the application of *Ulva* spp. in nutrition. This includes the development of diverse techniques to establish the best practices for analysis of FA in *Ulva* spp. as well as understanding the scale and type of effects from the environment.

*Materials and Methods*

*Analysis of the literature*

A literature review of the reported FA profiles for the genus *Ulva* was conducted. The search was conducted using Web of Science with “*Ulva*” and “fatty acids” as key search terms. The review was comprised of 40 FA profiles from 23 references. Only papers reporting profiles as a percentage of total FA were considered. Due to large variations in the level of reporting of *Ulva* FA profiles, data was modified for analysis. All structural variations of identified C14, C16, C17 and C20 MUFA were grouped and identified at the level of C14:1, C16:1, C17:1 and C20:1 respectively. Similarly, this was applied to all identified C16:2 and C20:2 PUFA. Identified *cis* and *trans* isomers of the C18:1 n-9 were also analysed collectively as C18:1 n-9. Furthermore, a number of identified FA were excluded due to (1) the bond position not being specified for a commonly reported FA and/or (2) FA is uncommon and/or appears less than 3 times out of the 40 profiles reviewed. Data was aligned and visualised using GraphPad Prism version 6.00, GraphPad Software.

*Experimental Samples*

A total of 74 *Ulva* samples were analysed over the course of two yearly cultivation periods. The *Ulva* samples were from scaled, cultivated stock, provided by Venus Shell
Systems Pty. Ltd. Samples were from controlled cultivation conditions of high nutrient (natural sunlight with nutrient-rich media); low nutrient (natural sunlight with reduced nutrients); low light (reduced natural sunlight with nutrient-rich media), added acetic acid (natural sunlight with nutrient-rich media supplemented with acetic acid) and added glucose (natural sunlight with nutrient-rich media supplemented with glucose). For investigations into desiccation effects, samples were oven-dried (70 °C, 48 hrs), freeze-dried (liquid nitrogen followed by lyophilisation for 48 hrs), fresh (stored -4 °C, 48 hrs) or frozen (-18 °C). Samples were extracted directly, or milled using a mortar and pestle (5 mins, continuously), sonicated (15 mins, rt) or co-incubated with enzyme (Onozuka R10, 2% w/v, rt, 15 mins) to test for effects of processing techniques.

**Extraction and Transesterification**

Lipids were extracted based on a standard method (referred to as extraction regime E1) of Folch et al. [9], with modifications based on Kumari, Reddy [13]. In brief, two grams of non-homogenized fresh sample was extracted with 3 x 6 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), with two extracts left overnight. The extract was evaporated to a consistent weight (dryness) in pre-weighed 9 mL Teflon lined screw top glass tubes under a stream of nitrogen. Crude lipid extracts were transesterified according to the method of Lepage and Roy [14] with added BHT (0.01% w/v).
Extraction Regimes

Further modifications to the standard method (E1) were also investigated and involved alterations to the solvent and mass ratio, extraction time and number of extractions, as well as the inclusion of a pre-treatment step. Specific modifications to the method (E1) involved pre-homogenization of the biomass with a mortar and pestle (5 mins, continuously) and the removal of one overnight incubation (E2); pre-treatment of the biomass with an enzyme (Onozuka R10, 2% w/v, rt, 15 mins) before extraction (E3); pre-treatment of the biomass with sonication (15 mins, rt) before extraction (E4); pre-homogenization of the biomass with a mortar and pestle (5 mins, continuously) and an increase in the number of consecutive reactions from 3 to 6 (limit observed where no more colour could be extracted) (E5) and; increase in the solvent to mass ratio from 3:1 to 6:1 with the removal of two overnight incubations (E6).

FA Analysis

FA were analysed by flame-ionization gas chromatography (GC-FID) (model GC-17A; Shimadzu, Rydalmere, NSW, Australia) using a 50 m x 0.25 mm internal diameter capillary column. One microlitre 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). Samples were also analysed by GC mass spectrometry (GC-MS), performed on a Shimadzu QP-5050A GC-MS system equipped with a BP-5 fused silica Rxi-5ms capillary column (5% phenyl/95% polysiloxane, 30 m x 0.25 mm, 0.25 um film thickness, Restek), using helium carrier gas (1.0 mL.min⁻¹) and samples of one microlitre over the temperature range of 80 - 300 °C. FA were identified by comparison
with known standards (Nu-chek or Sigma, Sydney, Australia) and comparison with
mass spectral database library. *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 equivalent (d.w. equiv.).

**Statistical Analysis**

The relative compositional profiles and content of FA across the five cultivation
conditions, six extraction regimes and four post-harvesting processes were assessed
using Two-way ANOVA and Tukey’s multiple comparisons to determine significantly
different means using GraphPad Prism version 6.00, GraphPad Software.
Results and Discussion

FA Profile of the genus Ulva

A review of 40 reported FA profiles from 23 references of the genus Ulva was used to establish a generic FA profile for the genus Ulva and to determine the degree of variability that can occur if spp., environment and/or processing variables are viewed collectively. Furthermore, it provides a reference upon which to base our comparisons. Figure 14 shows the diverse locations of the collected Ulva samples that were reviewed and analysed. The number of FA reported varied between 7 and 31, with an average of 16. From the 12 spp. of Ulva that were reported, U. lactuca and U. pertusa were heavily represented (33 % and 25 % respectively). Furthermore, the most commonly used extraction method was the traditional extraction protocol of Bligh and Dyer (55 %) followed by the Folch method (23 %). Analyses were more commonly conducted on fresh samples (30 %), followed by dried and frozen samples (both 23 %), with extraction on freeze-dried biomass representing 20 %. A number of publications reported a pre-treatment step of boiling in water or isopropyl alcohol to inactivate enzymes or for de-proteinisation of the sample (Table 16).
Location
Nagasaki Prefecture, Japan
Brittany, France
Nagasaki Prefecture, Japan
Alert Bay, Cormorani Island, Canada
Nagasaki Prefecture, Japan
Amur Bay, Sea of Japan
Stark Strait, Japan
Stark Strait, Japan
Elton Lake Basin, Russia
Eastern Gulf of Finland
Caspian basin, Russia
Caspian basin, Russia
Romanian Black sea coast
Monastir-Tunisia
Baja California Peninsula, Mexico
Baja California Peninsula, Mexico
Baja California Peninsula, Mexico
Baja California Peninsula, Mexico
Baja California Peninsula, Mexico
Baja California Peninsula, Mexico
Bohai Sea, China
Persian Gulf of Iran
Galway Bay, Western Ireland
Galway Bay, Western Ireland
Bodega Bay, California
Coastal area, Northern Chile
Egyptian Mediterranean
Mission Bay, San Diego, California
Nagasaki Prefecture, Japan
Algal Culture
Algal Culture
Algal Culture
Algal Culture
Algal Culture
Algal Culture
Bohai Sea, China
Fukuyama Cho, Southern Japan
Fukuyama Cho, Southern Japan
Algal Culture Filamentous. sp.
Algarve coast, Portugal

Extraction
Methanol
Bligh & Dyer
Methanol
Folch
Methanol
Bligh & Dyer
Bligh & Dyer
Bligh & Dyer
Bligh & Dyer
Folch
Bligh & Dyer
Bligh & Dyer
Hexane
AFNOR, 1984
Folch
Folch
Folch
Folch
Folch
Folch
Bligh & Dyer
Bligh & Dyer
Direct transesterification
Direct transesterification
Bligh & Dyer
Folch
Bligh & Dyer
Bligh & Dyer
Methanol
Bligh & Dyer
Bligh & Dyer
Bligh & Dyer
Bligh & Dyer
Bligh & Dyer
Bligh & Dyer
Bligh & Dyer
Bligh & Dyer
Bligh & Dyer
Bligh & Dyer
Direct transesterification

Biomass Extracted
Dried powder
Frozen
Dried powder
Lyophilised
Dried powder
Fresh
Not specified
Not specified
Inactivated fresh (boiled 10 mins i-PrOH)
Fresh
De-proteinisation in i-PrOH 15 mins 80 degrees
De-proteinisation in i-PrOH 15 mins 80 degrees
Freeze-dried powder
Dried powder
Fresh
Fresh
Fresh
Fresh
Fresh
Fresh
Boiled water then dried and ground
Freeze-dried powder
Freeze-dried
Freeze-dried
Boiling water (2 mins) to inactivate enzymes
Dried powder
Dried powder
Rehydrated lyophilised
Dried powder
Inactivated (boiling water) then frozen -20 under N
Inactivated (boiling water) then frozen -20 under N
Inactivated (boiling water) then frozen -20 under N
Inactivated (boiling water) then frozen -20 under N
Inactivated (boiling water) then frozen -20 under N
Inactivated (boiling water) then frozen -20 under N
Boiled in water then dried and ground
Inactivated (boiling water) then frozen -20 under N
Inactivated (boiling water) then frozen -20 under N
Freeze-dried
Freeze-dried powder

Table 16 Summary of the literature reviewed for the FA profile of the genus Ulva.
Spp.
U. arasakii
U. armoricana
U. congloboto
U. fenestrata
U. fasciata
U. fenestrata
U. fenestrata
U. fenestrata
U. intestinalis
U. intestinalis
U. intestinalis
U. intestinalis
U. intestinalis
U. lactuca
U. lactuca
U. lactuca
U. lactuca
U. lactuca
U. lactuca
U. lactuca
U. lactuca
U. lactuca
U. lactuca
U. lactuca
U. lactuca
U. lactuca
U. linza
U. lobata
U. pertusa
U. pertusa
U. pertusa
U. pertusa
U. pertusa
U. pertusa
U. pertusa
U. pertusa
U. pertusa
U. pertusa
Ulva. sp.
Ulva. sp.

Ref
Alamsjah, Hirao [15]
Kendel, Wielgosz-Collin [16]
Alamsjah, Hirao [15]
Colombo, Risé [17]
Alamsjah, Hirao [15]
Khotimchenko [18]
Hotimchenko [19]
Hotimchenko [19]
Nesterov, Rozentsvet [3]
Gubelit, Makhutova [20]
Rozentsvet and Nesterov [21]
Rozentsvet and Nesterov [21]
Horincar, Parfene [22]
Yaich, Garna [23]
Serviere-Zaragoza, Hurtado [24]
Serviere-Zaragoza, Hurtado [24]
Serviere-Zaragoza, Hurtado [24]
Serviere-Zaragoza, Hurtado [24]
Serviere-Zaragoza, Hurtado [24]
Serviere-Zaragoza, Hurtado [24]
Li, Fan [25]
Rohani-Ghadikolaei and Abdulalian [26]
Schmid, Guihéneuf [4]
Schmid, Guihéneuf [4]
Khotimchenko, Vaskovsky [27]
Ortiz, Romero [5]
El Maghraby and Fakhry [28]
Nelson, Phleger [29]
Alamsjah, Hirao [15]
Floreto, Teshima [30]
Floreto, Teshima [30]
Floreto, Teshima [30]
Floreto, Teshima [30]
Floreto, Hirata [31]
Floreto, Hirata [31]
Li, Fan [25]
Floreto, Hirata [32]
Floreto, Hirata [32]
McCauley, Meyer [33]
Pereira, Barreira [34]

Other Notes

Collected shade
Collected Light

10.9 g/L salinity
31.2 g/L salinity

May 2002
May 2003
Aug 2003
Nov 2003
Feb 2004
May 2004

Temp 14 °C (June)
Temp 12 °C (Nov)

N = 0 mM
N = 0.5 mM
P = 0 µM
P = 75 µM
Light Int. 195 µE/m2/s
Light Int. 15 µE/m2/s

10th April 17.8 °C
22nd July 28.4 °C
Cultured

113


Consolidation of the literature, shown in Figure 15, shows the FA profile of *Ulva* macroalgae being comprised of $35.3 \pm 13.7\%$ saturated fatty acids (average ± standard deviation, SFA), $15.9 \pm 6.6\%$ MUFA and $41.8 \pm 15.6\%$ PUFA. The lowest degree of variation occurs within the MUFA. Notably larger increases in total SFA with reduced PUFA content has been reported. For example samples collected from Tunisia [23], the Egyptian Mediterranean [28], Persian gulf of Iran [26] and the Algarve coast, Portugal [34] report SFA between 59 - 72 % of total FA with PUFA between 6 - 24%. In contrast, FA profiles of *Ulva* collected from the sea of Japan [18, 19] and the Chinese Bohai sea [25] showed a reported range of SFA between 21 - 33 % and PUFA between 49 - 61 %. Herein, analysis of the FA composition of 74 samples of one cultured *Ulva* sp. over the course of two years fell well within the ranges of the reported literature (SFA $32.8 \pm 5.8\%$, MUFA $16.5 \pm 4.3\%$ and PUFA $51.0 \pm 7.3\%$) (Figure 15).

The most commonly reported SFA for *Ulva* spp. were found to be C14:0 (1.8 % ± 2.1), C16:0 (30.5 % ± 11.5), C18:0 (1.5 % ± 1.8) and C22:0 (1.7 % ± 1.5) with C16:0 being the most dominant FA, a main characteristic of all macroalga in general [34]. The cultured *Ulva* sample reported similar values: C14:0 (1.1 ± 0.7 %), C16:0 (26.5 ± 5.2 %), C18:0 (0.8 ± 0.7 %) and C22:0 (0.5 ± 0.2 %) (Figure 15). In general, C16:0 was also the most variable FA, with the lowest content (14 %) reported in an *Ulva* sample from Chile [5] and the highest content (59.4 %) reported from a Tunisian sample [23].
Figure 15 summates the characteristic FAs and their representative abundance (as a percentage of total FA) for the genus Ulva as reported in the literature (review of 40 profiles from 23 references as outlined in Table 1). Graph represents reported values alongside literature average ± standard deviation. This is further compared with a cultured filamentous Ulva sp. (as reported herein) and represents the average of 74 analysed samples which included five different culture regimes, six extraction regimes, and four post-harvesting processes.
Commonly reported MUFA from least abundant to most abundant were C14:1 (1.5 ± 1.2 %), C18:1 n-9 (4.1 ± 4.1 %), C16:1 (4.4 ± 3.2 %) and C18:1 n-7 (10.1 ± 4.0 %), with comparable findings for our cultured Ulva sp. (Figure 15). There was little variability in the literature in regards to MUFA content, although some discrepancy in regards to the reporting and level of identification of C18:1 was apparent. For example, in some cases bond position is not specified [3, 23]. In other cases, only C18:1 n-7 [30] or C18:1 n-9 is reported [26]. In these cases the abundance of the respective C18:1 showed variation between 4.9 and 15.9 % of total FA. In instances where both FA were identified [19, 20, 25], the abundance of C18:1 n-7 to C18:1 n-9 was often greater than 1-fold and reflects the literature trend as shown in Figure 15. Notably, papers identifying only C18:1 n-9, show it to be more abundant than the literature average. For example Rohani-Ghadikolaei and Abdulalian [26] reported an abundance of C18:1 n-9 of 11.5 % representing a 2-fold increase when compared to the literature average. This raises the question as to whether or not the bond position of the C18:1 MUFA is being correctly identified or is simply a matter of intra-species variation that is known to occur [4, 24, 30]. Indeed we found that comparison of our sample with a standard FA methyl ester mixture lacking C18:1 n-7, C18:1 n-9 can be easily assigned to the more dominant C18 MUFA due to similar retention times. Only upon running an additional C18:1 n-7 standard were we able to assign both C18:1 n-9 and C18:1 n-7.

*Ulva* was further characterised by abundant C16 and C18 PUFA comprising about 45 % and 40 % of total FA respectively. Of note C18:4 n-3, a characteristic FA of marine algae and C16:4 n-3 a distinguishing characteristic FA for green algae, along with high
levels of C18:2 n-3 and C18:3 n-3 [1]. The FA in order of increasing abundance were C18:2 n-6 (5.3 ± 3.6 %), C16:4 n-3 (8.9 ± 4.8 %), C18:4 n-3 (12.5 ± 5.4 %) and C18:3 n-3 (14.5 ± 6.3 %). The FA C18:2 n-6 (8.3 ± 3.5 %) and C18:3 n-3 (19.5 ± 3.4 %) for the investigated cultured Ulva were marginally above the literature average. However, C18:2 n-6 and C18:3 n-3 have been reported to be as high as 21 % of total FA for C18:2 n-6 [15] and 28.8 % for C18:3 n-3 [21].

LC-PUFA combined, as reported in the literature for Ulva comprised approximately 6.0 % of total FA. Most commonly reported LC-PUFA from least abundant to most abundant are C20:4 n-6 (0.9 ± 0.6), C22:6 n-3 (1.1 ± 2.3), C20:5 n-3 (1.6 ± 1.2) and C22:5 n-3 (2.2 ± 1.0). The cultivated Ulva sample fell well within the reported range of the literature (Figure 15). It was found that DHA content (C22:6 n-3) is often reported to be present in trace amounts, on average < 2 %, however DHA content has been reported to be as high as 9 % [28] from the only reported FA profile for U. linza.

**Effect of environmental conditions on FA profile (% total FA)**

Over the course of two years the FA profile of a cultured filamentous sp. of Ulva was analysed to investigate the effect of different medium-scale open pool outdoor cultivation conditions. The 74 samples investigated included five different culture regimes; high nutrients, low nutrients, low light, added acetic acid and added glucose and also included samples prepared by six different extraction regimes and four different post-harvesting processes. FA profiles from the respective culture conditions are presented in Table 17. The most notable shifts occurred between the low nutrient and high nutrient treatments. Multiple comparisons indicated a significant increase in the
proportion of total SFA (41%) and decrease in PUFA (25%) for the low nutrient group when compared to the high nutrient group (P < 0.005). This is due to changes in C16:0 content (Table 17).

Table 17 The FA profile of a filamentous Ulva sp. from five different culture treatments. Data is expressed as the % of total FA. Data represents the average ±se of 74 Ulva samples investigated over a two-year period. Means with different letters are significantly different (Tukey’s multiple comparisons, p < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>High Nutrients (n = 35)</th>
<th>Low Nutrients (n = 9)</th>
<th>Low Light (n = 12)</th>
<th>+Acetic Acid (n = 12)</th>
<th>+Glucose (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C8:0</td>
<td>0.11 ± 0.05</td>
<td>0.15 ± 0.12</td>
<td>- -</td>
<td>0.72 ± 0.12</td>
<td>- -</td>
</tr>
<tr>
<td>C10:0</td>
<td>1.41 ± 0.31</td>
<td>0.79 ± 0.35</td>
<td>1.76 ± 0.33</td>
<td>0.46 ± 0.09</td>
<td>1.68 ± 0.37</td>
</tr>
<tr>
<td>C11:0</td>
<td>0.27 ± 0.04</td>
<td>2.20 ± 0.03</td>
<td>0.44 ± 0.13</td>
<td>0.97 ± 0.32</td>
<td>0.29 ± 0.05</td>
</tr>
<tr>
<td>C12:0</td>
<td>0.74 ± 0.06</td>
<td>0.45 ± 0.09</td>
<td>0.75 ± 0.07</td>
<td>0.55 ± 0.09</td>
<td>0.88 ± 0.12</td>
</tr>
<tr>
<td>C14:0</td>
<td>1.19 ± 0.14</td>
<td>1.27 ± 0.13</td>
<td>1.15 ± 0.18</td>
<td>0.81 ± 0.02</td>
<td>1.05 ± 0.17</td>
</tr>
<tr>
<td>C14:1 n-7</td>
<td>0.41 ± 0.12</td>
<td>0.27 ± 0.24</td>
<td>0.46 ± 0.09</td>
<td>0.03 ± 0.02</td>
<td>0.76 ± 0.19</td>
</tr>
<tr>
<td>C15:0</td>
<td>0.26 ± 0.03</td>
<td>0.25 ± 0.07</td>
<td>0.34 ± 0.02</td>
<td>0.20 ± 0.03</td>
<td>0.38 ± 0.02</td>
</tr>
<tr>
<td>C15:1</td>
<td>0.47 ± 0.06</td>
<td>0.47 ± 0.09</td>
<td>0.50 ± 0.09</td>
<td>0.42 ± 0.07</td>
<td>0.26 ± 0.06</td>
</tr>
<tr>
<td>C16:0</td>
<td>24.46 ± 0.67a</td>
<td>35.22 ± 0.11b</td>
<td>25.33 ± 0.83a</td>
<td>28.22 ± 1.01c</td>
<td>24.75 ± 1.27a</td>
</tr>
<tr>
<td>C16:1 n-7</td>
<td>5.25 ± 0.53</td>
<td>4.30 ± 0.26</td>
<td>5.22 ± 0.64</td>
<td>4.22 ± 0.29</td>
<td>4.56 ± 0.79</td>
</tr>
<tr>
<td>C17:0</td>
<td>0.70 ± 0.09</td>
<td>0.63 ± 0.12</td>
<td>1.56 ± 0.12</td>
<td>0.25 ± 0.05</td>
<td>1.25 ± 0.23</td>
</tr>
<tr>
<td>C17:1 n-7</td>
<td>0.35 ± 0.05</td>
<td>0.34 ± 0.08</td>
<td>0.69 ± 0.08</td>
<td>0.18 ± 0.04</td>
<td>0.41 ± 0.14</td>
</tr>
<tr>
<td>C16:4 n-3</td>
<td>12.11 ± 0.40a</td>
<td>4.79 ± 0.19b</td>
<td>10.92 ± 0.57ac</td>
<td>10.37 ± 0.44ac</td>
<td>8.49 ± 0.66c</td>
</tr>
<tr>
<td>C18:0</td>
<td>0.61 ± 0.07</td>
<td>1.36 ± 0.06</td>
<td>0.99 ± 0.21</td>
<td>0.34 ± 0.01</td>
<td>1.71 ± 0.27</td>
</tr>
<tr>
<td>C18:1 n-9</td>
<td>0.83 ± 0.11</td>
<td>2.09 ± 0.08</td>
<td>0.78 ± 0.09</td>
<td>0.42 ± 0.04</td>
<td>1.23 ± 0.08</td>
</tr>
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<td>C18:1 n-7</td>
<td>8.62 ± 0.76a</td>
<td>9.70 ± 0.24ab</td>
<td>7.16 ± 0.75a</td>
<td>12.30 ± 0.08a</td>
<td>10.79 ± 1.20b</td>
</tr>
<tr>
<td>C18:2 n-6</td>
<td>6.61 ± 0.30a</td>
<td>12.90 ± 0.08b</td>
<td>9.51 ± 0.86c</td>
<td>6.90 ± 0.15c</td>
<td>11.62 ± 1.71b</td>
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<td>0.42 ± 0.02</td>
<td>1.32 ± 0.11</td>
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<tr>
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<td>14.17 ± 0.16b</td>
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<td>20.09 ± 0.31a</td>
<td>17.81 ± 0.62c</td>
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<tr>
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<td>7.80 ± 0.35c</td>
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<td>0.08 ± 0.02</td>
<td>0.16 ± 0.03</td>
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<tr>
<td>C20:4 n-6</td>
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<td>1.53 ± 0.15</td>
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<td>C20:5 n-3</td>
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<td>1.44 ± 0.23</td>
<td>1.30 ± 0.06</td>
<td>1.10 ± 0.06</td>
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<td>0.31 ± 0.05</td>
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<td>0.06 ± 0.01</td>
<td>0.17 ± 0.10</td>
</tr>
<tr>
<td>C22:5 n-3</td>
<td>1.81 ± 0.13a</td>
<td>1.03 ± 0.13b</td>
<td>1.09 ± 0.06b</td>
<td>2.20 ± 0.22c</td>
<td>1.01 ± 0.10b</td>
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<td>C22:6 n-3</td>
<td>0.09 ± 0.03</td>
<td>0.01 ± 0.27</td>
<td>0.03 ± 0.02</td>
<td>0.15 ± 0.15</td>
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</tr>
</tbody>
</table>

% SFA    | 30.30 ± 0.75a           | 42.83 ± 0.11b         | 32.74 ± 0.56c     | 33.08 ± 1.43c         | 32.36 ± 0.96c   |
% MUFA   | 16.03 ± 0.89             | 17.61 ± 0.21          | 15.14 ± 0.84      | 17.73 ± 0.29          | 18.33 ± 2.13    |
% PUFA   | 53.90 ± 1.22a            | 40.63 ± 0.19b         | 52.66 ± 1.42a     | 49.72 ± 1.33c         | 49.32 ± 0.94c   |
n-6/n-3  | 0.23 ± 0.01a             | 0.74 ± 0.01b          | 0.37 ± 0.05c      | 0.23 ± 0.01a          | 0.55 ± 0.08bc   |
It is regarded that nitrogen is an important factor in algal lipid metabolism and that nitrogen starvation can contribute to the formation and accumulation of triacylglycerides, comprising of predominantly SFA [35]. This is often considered in a positive context with applications such as biodiesel production. Results here suggest that for the development of an algal-derived oil for human and animal nutrition, nitrogen-deplete conditions are unfavourable due to this shift of PUFA to SFA. Also unfavourable was a significant shift in C18:3 n-3 to C18:2 n-6, with the low nutrient group showing a reduction in C18:3 n-3 of 32% and an increase in C18:2 n-6 of 95% when compared to the high nutrient group. The relative abundance of C16:4 n-3 and C18:4 n-3 PUFA were also shown to be affected by environmental conditions. Stearidonic acid (C18:4 n-3) showed a lower relative abundance for the added glucose and low nutrient groups. For example, low nutrients showed a reduction in C18:4 n-3 of 54% compared to the high nutrient treatment. Similarly C16:4 n-3 showed a decrease of 60% for the low nutrient group when compared to the high nutrient group. These results suggest that the high nutrient treatment has the most desirable FA profile and importantly, a lower n-6/n-3 ratio, for human and animal health applications. We found no added benefit with regard to the FA profile of *Ulva* with either glucose supplementation or acetic acid supplementation, except for a slight increase in n-3 DPA content with acetic acid supplementation when compared to the high nutrient group.

*Effect of freezing, drying and homogenisation on the FA profile (% total FA) and total FA content (mg.g⁻¹ d.w. equiv.)*
Freeze-drying is a drying process in which the solvent (usually water) is crystallized at low temperature and thereafter sublimated from the solid state directly into the vapor phase [36]. Freeze-drying is a common technique to preserve diverse sources of fresh material before analysis and extraction of bioactives. Reviews of the effects of freeze-drying on extraction efficiencies of plant phytochemicals reveal highly variable results [37].

From the analysis of 74 samples, including five cultivation regimes; four different extraction regimes and four different post-harvesting processes, no significant effects of drying (freeze-dried or oven-dried) were found on the relative FA composition, (F (3, 210) = 0.041, P = 0.99). There is an underlying assumption that freeze-drying, as opposed to oven or air-drying, best preserves the bioactive chemical constituents of plant biomass [37]. Our results suggest that both oven and freeze-drying are effective for FA preservation and do not alter the relative FA composition of the Ulva macroalgae. This is in agreement with Molina Grima et al. [38] who showed that freeze-drying did not affect the FA profiles of microalgal samples.

Effects were seen with homogenisation in regards to FA total content (mg.g⁻¹ d.w. equiv.). As expected, the FA yields increased by 2- and 19-fold for both freeze-dried and oven-dried homogenised samples respectively when compared to non-homogenised samples (Figure 16a). However, no benefit from homogenisation was observed for extracted fresh biomass and even showed a slight decrease (Figure 16a). The highest yield was obtained from the extraction of non-homogenised fresh biomass, which achieved a total FA content of 51 mg.g⁻¹ d.w. equiv. Our results suggest that non-
homogenised fresh biomass for the filamentous *Ulva* sp. investigated herein is the most efficient method for determination of total FA content, as it eliminates both a drying and processing step. We found that by using fresh non-homogenised *Ulva*, we were able to significantly double the extraction yields from 3.1 to 6.6 mg·g\(^{-1}\) fresh weight when compared to the extraction of homogenised pre-frozen biomass, Figure 16b. Furthermore it is more convenient, particularly for high throughput analysis, as it removes a processing step. If the biomass is to be dried and stored, homogenisation before analysis is necessary to maximise yields and avoid underestimation of total content. We found milled oven-dried biomass preferable, as it showed an increase in yield of 49.9 % when compared to milled lyophilised biomass (Figure 16a).

Figure 16 (a) Comparison of total FA content of non-homogenised freeze-dried, fresh and oven-dried samples versus homogenised samples and (b) comparison of the total FA content of non-homogenised fresh samples compared to homogenised frozen samples when extracted with the same protocol. Dotted line represents reported literature value for frozen, homogenised equivalent extracted under a similar protocol [13].
In contrast, Ryckeboch et al. [39] reported no significant difference in FA yields for freeze-dried algae when compared to fresh. However it is important to consider that different extraction protocols were employed and extractions were performed on microalgae, as opposed to macroalgae with an extensive extracellular matrix [13]. Martins et al (2012) also noted the presence of significant matrix effects between macroalgal species and suggest that the analysis method for different organisms should be selected carefully. This highlights that the pre-treatment of Ulva biomass, or other samples of interest, is an important consideration when quantifying algal FA from the macroalgal genus Ulva.

*Effect of extraction regimes and environmental conditioning on total FA content (mg g⁻¹ d.w. equiv.)*

From the analysis of 36 fresh-only samples significant differences in the extraction efficiency of different protocols were observed. As shown in Figure 17a, extraction protocols E1, E3 and E6 exhibited significantly higher yields than E2, E4 and E5 (F(5,120) = 37.17, P < 0.0001). Compared to a standard regime (E1), which involved three consecutive extractions of a 3:1 solvent to mass ratio with two over-night incubations, the removal of one over-night incubation (E2) reduced the yield by 59%. The addition of a membrane disruption step using either an enzyme (E3) or sonication (E4) did not have any benefit when compared to the control (E1). Similarly, Lewis, Nichols [11] found no significant effect of sonication on extracted FA. Furthermore, increasing the number of consecutive extracts per sample from three (E1) to six (E5) did not significantly increase yields. The most efficient extraction method was found to be
E6, which involved extraction of three immediate consecutive extractions with a higher solvent to mass ratio (6:1), providing yields comparable to both the standard (E1) and extended extraction (E5) regimes. As a result, E6 is the method of choice for high throughput analysis as extraction can be complete in under an hour. The variations in total FA contents observed highlight the importance of method optimisation for the sample of interest. This is potentially due to cellular matrix effects [11, 40]. Extraction methods have also been highlighted as another key element in the quantification of lipids and FA [13].

![Figure 17](image)

**Figure 17** (a) The total FA yields (mg.g⁻¹ d.w. equiv) from fresh *Ulva* samples from six different extraction protocols. Extraction 1 (E1) represents the standard extraction involving 3 extractions with a 3:1 solvent to mass ratio with 2 over-night incubations; E2 involves the removal of one over-night incubation; E3 involves a pre-treatment step with an enzyme; E4 involves a pre-treatment step with sonication; E5 shows an extended extraction involving 6 extractions with a 3:1 solvent to mass ration with 2 over-night incubations and E6 represents three immediate consecutive extractions with a 6:1 solvent to mass ratio; (b) total FA yields (mg.g⁻¹ d.w. equiv.) of samples from four different culture treatments extracted with E6 protocol. Means with different letters are significantly different (Tukey’s multiple comparisons, p < 0.05).

As a result, cultured samples that were extracted using the E6 protocol were compared to determine the effect of culture on total content (**Figure 17b**). High nutrients showed
significantly higher total FA content with an increase of 23 mg.g\(^{-1}\) d.w. equiv. when compared to the low nutrient treatment \((F (3, 32) = 78.74, P = < 0.0001)\). High nutrient levels have been shown to increase crude lipid content [30]. Furthermore a study by Ak, Öztaşkent [6] showed no effect of low sodium nitrate (NaNO\(_3\)) concentrations (0.02 - 0.11 g/L) on lipid content with increased lipid content observed for control regimes which had a much higher concentration of NaNO\(_3\) (1.5 g/L). Our results indicate that the high nutrient regime, at this scale of cultivation, in terms of total FA content, desirable PUFA profile and n-6/n-3 ratios, is the optimal growth environment for potential biomass biorefinery of an algal-oil for human and/or animal nutrition.

*Monitoring of FA profile consistency of treatments and time.*

The potential utilisation of cultured algal biomass for higher value products, such as an algal-derived oil for human and animal nutrition, is ensuring batch-to-batch reproducibility between harvests. The tests for reproducibility of results using two variables with similar SFA and PUFA ratios showed no significant change in the overall FA profile in terms of total SFA, MUFA and PUFA as a percentage of total extract composition \((F (3,33)= 0.05 P = 0.98)\) (Figure 18).
Conclusion

Production controlled Ulva biomass through cultivation has future potential to generate an algal-based oil for human and/or animal nutrition, as part of a biorefinery process for high value products. Here we demonstrate that despite inconsistencies in the analysis of FA of Ulva in the literature, there is a key profile of abundant and some unique FA for the genus, which is high in unsaturated FA (about 60%) compared to SFA. These include C16:4 n-3, C18:4 n-3, C18:2 n-6 and C18:3 n-3 FA which are thought to contribute to the algicidal and competitive strategy of Ulva spp. [15]. We found that the literature was not consistent in reporting all potential FA in Ulva spp. and that it is important to include standards, or other identification techniques such as GC-MS, for feature FA such as C16:4 n-3 and C18:4 n-3 as well as C18:1 n-7, the latter likely to have been under reported in the literature. Despite some clear characteristics of the FA profiles of Ulva spp. there is a range of variation in the relative amounts of different FA.
One reason for this could be the environment as our work here demonstrated. We found C16:0, C16:4 n-3, C18:1 n-7, C18:2 n-6, C18:3 n-3 and C18:4 n-3 FA to be most affected by the environment. Notably, Ulva cultivated under low nutrient conditions showed accumulation of specific fatty acids, in particular C16:0 and C18:2 n-6 at the detriment of n-3 PUFA. However, this did not translate into greater total FA content. Future research may consider conditioning the low nutrient treated algae to the point of suspended growth, often accompanied with significant loss of pigments to see if a boost in content can be observed. However, from a commercial production point of view, any stressed condition is unfavourable as it dramatically reduces cell division. High nutrient conditions on the other hand showed the most desirable FA profile for health applications. This environmental condition also displayed the highest total FA content achieving 56 mg per gram of dry weight, 30 mg of which is comprised of PUFA. From a nutritional perspective, the appeal of this FA profile from the high nutrient condition lies in the low n-6/n-3 ratio. It is rich in C18:3 n-3, in contrast to plant derived oils which are high in C18:2 n-6. Ulva is also unique from plant and fish derived oils due to high levels of C18:4 n-3, as well as offering essential dietary EPA and DHA, which are generally absent in land plants. From a commercial production point of view, high nutrient conditions are more favourable for achieving a higher turnover and such biomass could have considerable future value for FA supplementation in the diet of animals or humans.

We have demonstrated that post-harvest processing and extraction methods are important considerations in FA quantification and should be selected carefully to avoid underestimation of total content. Future work should consider the scalability and
logistics of post-harvest treatments and processing, along with exploration of safer and less toxic extraction alternatives, such as supercritical CO₂ extraction.

**Acknowledgments**

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**Conflicts of Interest**

The authors declare no conflict of interest
Chapter 4 Ulva Fatty Acids

References


FOREWORD TO CHAPTER 5

The previous Chapter 4 explored the fatty acid profiles of cultivated *Ulva* to determine the optimal growth conditions to achieve high quality fatty acids that are optimal for human and animal nutrition. In particular, emphasis was placed on the effect of the methods on the fatty acid profiles and yields as a dominant theme of the previous chapter and manuscript.

The fresh cultivation experiments that were investigated as part of Chapter 4 were harvested and the biomass stored as a finely milled powder. These dried samples were subsequently analysed for all the following investigations herein. Interestingly, at the time of harvesting, the biomass from nutrient starved treatment showed a dramatic loss of pigments, differing from the nutrient starved biomass analysed previously in Chapter 4. This had a dramatic affect on total lipid yields and this is explored in the following Chapter 5, along with investigating the effect of cultivation on the distribution of fatty acids across both neutral and phospholipid fractions. In particular, emphasis is placed on the potential health benefits an algal-derived oil may offer human and animal nutrition. The chapter is presented as a prepared short communication for submission.\(^\text{12}\) In addition, results of further investigations that were not incorporated

\(^{12}\) McCauley, J.I., et al., Fatty acid composition of neutral and phospholipids of an emerging algal-based and sustainable omega-3 oil alternative, *Prepared Short Communication for Submission*
in the short communication are presented at the end of the chapter
CHAPTER 5 FATTY ACID COMPOSITION OF NEUTRAL AND PHOSPHOLIPIDS OF AN EMERGING ALGAL-BASED AND SUSTAINABLE OMEGA-3 OIL ALTERNATIVE

Janice I. McCauley, a Barbara J. Meyer, b Pia C. Winberg, b,c and Danielle Skropeta a,d

aSchool of Chemistry, Faculty of Science, Medicine, and Health (SMAH), University of Wollongong, NSW, 2522, Australia
bSchool of Medicine, SMAH, University of Wollongong, Wollongong, NSW, 2522, Australia
cVenus Shell Systems Pty. Ltd., Mundamia, NSW, 2540, Australia
dCentre for Medical and Molecular Bioscience, SMAH, University of Wollongong, NSW, 2522, Australia

Corresponding Author*
Janice McCauley*
Phone: +61-2-42214001
Fax: +61-2-42214287
Email: jim479@uowmail.edu.au

Contributions

65% Janice McCauley  Performed the work and prepared the paper
10% Barbara Meyer  GC access, training and supervision
10% Pia Winberg  Major editing and input on project design
15% Danielle Skropeta  Oversight of project, editing and proof-reading

As agreed by supervisor A/Prof. Danielle Skropeta
Abstract

*Ulva* has a high quality omega-3 fatty acid (n-3 FA) profile that can respond to the environment yet it is poorly understood which lipids are affected. This has implications for FA bioavailability and processing. Here nutrient-rich conditions resulted in 61-69% of total FA (2.6-3.4 mg.g⁻¹) bound within neutral lipids, characterised by 16:0 (18-26%), 18:3n-3 (24-25%), 16:4n-3 (13-17%), 18:2n-6 (11-15%), 18:1n-7 (6-9%) and 18:4n-3 (4-6%). Low-pigment (nutrient-starved) conditions increased neutral lipids (10 mg.g⁻¹) with increased 16:0, 18:1n-7, 18:2n-6, 18:3n-3 and reduced 16:4n-3 and 18:4n-3 (p<0.0001). Despite the increased yield of 18:3n-3, it represented a lower percentage (10%) of total extract leading to an unfavourable n-6/n-3 ratio (1.6 c.f. 0.3 for the nutrient-rich, high-pigment culture). All phospholipid fractions were characterised by the presence of 16:0, 16:1n-7, 18:1n-7, 18:2n-6 and 18:3n-3. However, the low-pigment (nutrient-starved) biomass showed reduced phospholipids (12%) as a percentage of total lipids (c.f. nutrient-rich conditions 31-38%). In summary, the n-6/n-3 ratio, phospholipid fraction and relative amounts of unique n-3 FA (18:4n-3) are significantly higher in nutrient-rich conditioned biomass and are potentially more optimal for health. Thus it may be important to optimise the high quality rather than the quantity of fatty acids for nutritional applications through the cultivation of *Ulva*.

**Key words:** *Ulva*, chlorophyta, PUFA, health, nutrition, cultivation
Introduction

Fish oil is a highly priced commodity. The recommendation for the consumption of 2-3 serves of fish per week has led to increased awareness of the health benefits of omega-3 fatty acids (n-3 FA) [1]. These recommendations can be obtained through diet, however this is not always practical and has created the demand for encapsulated fish oil supplements [2]. Fish oils are also sought for aquaculture, along with high protein fishmeal. To date, this demand is currently met with limited captive based fisheries [3] but has low scope for growth to meet future demands. Typical fish oil preparations are high in n-3 long chain (≥ 20 carbons) polyunsaturated fatty acids (LC n-3 PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Table 18).

Whilst EPA and DHA are high in certain microalgae, it is typically low in the green macroalgae Ulva (Table 18). However, Ulva spp. have a unique fatty acid profile characterised by high levels of alpha-linolenic acid (18:3n-3, ALA) and stearidonic acid (18:4n-3, SDA) [4]. SDA is the first metabolite of ALA in the metabolic pathway leading to EPA and DHA and has been identified as an efficient precursor of EPA synthesis (Figure 19) [3]. The metabolic conversion of ALA represents approximately 6% for EPA and 3.8% for DHA with a further loss in efficiency up to 50% with a high n-6 diet [5]. Adequate dietary provision of EPA and DHA can be obtained through direct supplementation [5]. However, studies have indicated that dietary supplementation with SDA can increase human tissue EPA levels much more effectively than ALA [2]. As a result, it has been proposed that SDA oils could offer an attractive alternative to EPA-containing fish oils [6].
### Table 18

<table>
<thead>
<tr>
<th>Source</th>
<th>Microalgae</th>
<th>Seed</th>
<th>Fish</th>
<th>Vegetables</th>
<th>Fruits</th>
<th>Seafood, Meat</th>
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Ref. De Silva, Francis [7]
Özcan [8]
Custódio, Soares [9]
Vidyashankar, Sireesha [10]

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<th>22:5n-3</th>
<th>22:6n-3</th>
<th>18:1n-7</th>
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AA: arachidonic acid; ALA: alpha-linolenic acid; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; la: linolenic acid; SDA: stearidonic acid.

and other-oxidized oils.

and other-fatty-acids.
Ulva is readily cultivated at scale for commercialisation and together with its unique and relatively abundant n-3 content offers an attractive source of quality controlled and traceable biomass for nutritional applications [11]. Furthermore, green algae are

**Figure 19** Biosynthesis pathways of n-6 and n-3 polyunsaturated fatty acids. Adapted from Pereira, Barreira [12]. AA: arachidonic acid; ALA: alpha-linolenic acid; DHA: docosahexaenoic acid; DPA: docosapentaenoic acid; EPA: eicosapentaenoic acid; GLA: gamma-linolenic acid; LA: linoleic acid; SDA: stearidonic acid
naturally rich in neutral polar glycolipids (up to 50%) with various levels of charged polar phospholipids (typically between 10-20%) [13] (Figure 20) differing from traditional fish oils.

![Figure 20](image)

**Figure 20** Typical lipid composition (%) of neutral polar glycolipids and highly polar charged phospholipids of green algae of whole cells, chloroplast envelopes and thylakoids. Representative glycolipids: monogalactosyldiacylglycerol (MGDG); digalactosyldiacylglycerol (DGDG). Adapted from Harwood (1998).

This has implications for FA bioavailability as marine derived PUFA from fish oil are commonly bound within triacylglycerides, which are bioavailable, however they may not be as efficient as other lipids classes [14]. For example, Krill oil is unique in that as it has a substantial proportion of EPA and DHA in abundant phospholipids and there is evidence to suggest that polar lipids have a higher bioavailability than nonpolar triacylglycerides [15]. More recently it was shown that tissue uptake of LC n-3 PUFA from an algal oil containing EPA partly conjugated to 15% phospholipids, as well as glycolipids was found to be equivalent to krill oil containing EPA partly conjugated to 40% phospholipids, demonstrating a promising bioavailability for algal derived oils [14].
Limited studies have explored the fatty acid composition of *Ulva* phospholipids [16], as the characterisation of major glycolipids is more common [17-19]. Previously we demonstrated that PUFA and n-6/n-3 ratios can be optimised through cultivation, influencing these profiles by up to 2-fold [11]. However it is unclear what lipid classes comprise these fatty acid shifts and this has implications for the bioavailability and extraction optimisation of these lipid classes. Therefore in scoping the potential of an *Ulva*-derived n-3 alternative, this communication further develops the understanding of the composition of fatty acids across the phospholipid and neutral fractions from a cultivated Australian *Ulva* species.

*Materials and Methods*

*Samples*

The samples (dried, milled) were provided by Venus Shell Systems Pty. Ltd and represented four culture regimes (5m$^2$): (1) control (natural sunlight with nutrient-rich media); (2) nitrogen starved, low pigment (natural sunlight with reduced nutrients); (3) shaded (reduced natural sunlight with nutrient-rich media) and; (4) acetic acid nutrient boost (natural sunlight with nutrient-rich media supplemented with acetic acid).

*Extractions and Transesterification*

The lipids were extracted twice in triplicate as described by Kumari et al. 2011 with modifications [20]. In brief, samples (250 mg) were extracted with chloroform/methanol (3 x 3 mL; 2/1, v/v) with butylated hydroxytoluene (0.001%) by brief vortexing and centrifugation (3000 rpm, rt, 15 mins). The phospholipids were
isolated from the crude extract by the addition of cold acetone (2 x 3 mL) with added magnesium chloride (4 % in ethanol) and centrifugation (3000 rpm, 4 ºC, 15 mins). The decanted acetone soluble (neutral) lipids and the insoluble residue (phospholipids) were dried under nitrogen and transesterified as per Lepage and Roy [21]. Extract compositions were qualitatively assessed by thin layer chromatography to confirm isolation of phospholipids.

**Pigments**

The pigments (250mg) were extracted with methanol (3 x 3 mL) by brief vortexing and centrifugation (3000 rpm, rt, 15 mins) and quantified as per Lichtenthaler [22].

**Fatty acid analysis**

The samples (1µL) were analysed by flame-ionization gas chromatography (GC-FID) (GC-17A; Shimadzu) using a 50m x 0.25mm internal diameter capillary column and analysis performed using Shimadzu software (Class-VP 7.2.1 SP1). Fatty acids were identified with known standards (Sigma) and an internal standard (heneicosanoic acid) was used for quantification. The data are represented as molar % extract composition ± standard deviation Quantified fatty acid data are represented as mg.g⁻¹ dry weight (d.w.) ± standard error.

**Statistical Analysis**

The data were assessed using Two-way ANOVA and Tukey’s multiple comparisons using GraphPad Prism version 6.00.
Results and Discussion

Previously we have shown that *Ulva* is characterised by abundant C16:0, C18:3n-3, C18:4n-3, C16:4n-3 and C18:1n-7 fatty acids with high nutrient conditions delivering biomass with the most desirable fatty acid profile for health and nutritional applications [11]. Here we determine the effect of these cultivation conditions on the distribution of fatty acids across the neutral and phospholipid fractions (Table 19). The low nutrient treated algae showed a significant loss of pigments both visually and spectrophotometrically (Figure 21).

![Chlorophyll a/b and total carotenoid content](image)

**Figure 21** Chlorophyll a/b and total carotenoid content (ug.mL\(^{-1}\)) of methanol extracts for control (high-nutrient) versus nutrient-starved cultivated *Ulva* samples (F (1,12) = 625.0, P < 0.0001).

**Total Content (mg.g\(^{-1}\) d.w.)**

The combined neutral and phospholipids in this experiment yielded an average of 3.03 mg.g\(^{-1}\) d.w total fatty acids across the nutrient-rich conditions which included the control, shaded and acetic acid cultures (Figure 22), although we have previously reported that we can obtain 56 mg.g\(^{-1}\) d.w. equivalent using an optimised extraction for fresh biomass cultivated under nutrient-rich conditions [11]. The low pigment (nutrient-starved) samples showed a significant 3-fold increase in total content (12.01 mg.g\(^{-1}\)
d.w.) when compared to the other samples (Figure 22) \((p < 0.0001)\). This increase in content occurred within the neutral fraction, which represented 88\% of the total lipids, suggesting accumulation of triacylglycerols. The major contributing fatty acids to this increase in total content within the neutral fraction of low-pigment (nutrient-starved) biomass were 16:0 \((4.02 \pm 0.15 \text{ mg.g}^{-1}\text{ d.w.})\), 18:1\text{n}-7 \((1.79 \pm 0.06 \text{ mg.g}^{-1}\text{ d.w.})\), 18:2\text{n}-6 \((2.30 \pm 0.08 \text{ mg.g}^{-1}\text{ d.w.})\) and 18:3\text{n}-3 \((1.09 \pm 0.03 \text{ mg.g}^{-1}\text{ d.w.})\). Although 18:3\text{n}-3 increased in yield it represented a lower percentage of total extract composition (10.0\%) when compared to the nutrient-rich conditions (24.3 - 24.7\%) (Table 2) resulting in a higher and unfavourable n6/n3 ratio (Figure 22). Due to the increase in neutral lipids in the low-pigment (nutrient-starved) biomass, phospholipids represented only 12\% of total lipids, a 65\% reduction in phospholipids when compared to remaining nutrient-rich samples (31 - 38\%), which contained between 61-69\% neutral lipids. Elsewhere \textit{Ulva} neutral lipids have been shown to represent up to 76\% with phospholipids constituting 24\% [16], however phospholipid content in \textit{Ulva} sp. has been reported to be as high as 50\% [23].
Figure 22 Total content (mg g\(^{-1}\) d.w.) of saturated fatty acid (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) of neutral and phospholipid fractions for the average yield (± standard error) of nutrient-rich cultivation conditions (control, shaded, acetic acid cultivations) versus the yield for low pigment (nutrient-starved) conditions. *Significantly different (p < 0.0001)

Phospholipid Fractions

The acetone insoluble *Ulva* phospholipid extract (highly charged polar lipids) was characterised by high levels of saturated FA (SFA) (51.2 - 59.5%) with lower PUFA (21.3 - 29.7%) when compared with the corresponding neutral fractions (Table 19). Samples showed abundant 16:0 (49.6 - 58.8%), 16:1n-7 (1.4 - 5.8%), 18:1n-7 (11.8 - 17.5%), 18:2n-6 (8.6 - 13.1%) and 18:3n-3 (7.9 - 13.9%), in agreement with similar findings for *Ulva* [16]. The n-3 PUFA 16:4n-3 and 18:4n-3 were present in trace amounts (< 2.2%) but have been reported as high as 2.2% and 3.3% respectively [16]. The low pigment (nutrient-starved) samples showed reduced 16:1n-7 (1.4%) when compared to the other samples (3.3 - 5.8%). Low pigment and acetic acid samples also showed higher 16:0 (56.9% and 58.8 respectively) compared to the control (nutrient-rich) and shaded cultures (49.6 and 52.8% respectively). Low pigment samples were
significantly higher in 18:1n-7 (17.5%) with a significant reduction in 18:3n-3 (7.9%) when compared to the other nutrient-rich samples (13.3 - 13.9%). No significant differences were observed in the LC PUFA across the different cultures for the phospholipid fractions (F (3,102) = 1.841, P = 0.1444). The PUFA 20:4n-6 was present in trace amounts (≤ 0.6%) across control, low pigment and shaded culture regimes whilst 20:5n-3 was slightly more abundant in the control and low pigment cultures (1.1% and 1.5% respectively) (Table 19).

Neutral Lipid Fractions

The neutral lipid fraction (acetone soluble), which represented between 61-88% of total lipid yield comprised between 37.3 - 66.1% of total PUFA across samples, indicating that most PUFA reside within the neutral lipid fraction (Table 19). Ulva commonly exhibits high PUFA due to abundant 18:3n-3 [24] and herein represents approximately 25% of the neutral fraction for nutrient-rich conditions which include the control, shaded and acetic acid cultivations. An increase in total content in the neutral fraction under starved conditions (Figure 22) suggests an accumulation of triacylglycerols for energy storage, although neutral glycolipids are also present in this fraction. In low-pigment (nutrient-starved) conditions the relative amount of 18:3n-3 is significantly lower (10%), although the absolute amount in dried biomass is higher, contributing to an increase in PUFA content (Figure 22). Higher relative abundance of 18:4n-3 (4.4 - 5.8%) and 16:4n-3 (13.1 - 16.6%) were evident across the remaining samples and represent two characteristic fatty acids of Ulva [4]. High 18:4n-3 in neutral glycolipids has also been reported by Sanina et al. [17].
| Phospholipid Fraction | Ulva | 22.4±3.3 | 22.4±3.3 | 22.4±3.3 | 22.4±3.3 | 22.4±3.3 | 22.4±3.3 | 22.4±3.3 |
|-----------------------|------|----------|----------|----------|----------|----------|----------|
| PUFA                  |      | 16.8±0.4 | 15.5±0.7 | 19.9±1.6 | 20.4±0.9 | 11.5±0.4 | 9.0±0.4  | 20.6±0.5 |
| MUFA                  |      | 59.5±1.6 | 54.8±1.8 | 58.8±3.7 | 51.2±1.3 | 28.9±1.8 | 24.9±0.6 | 42.1±0.5 |
| SFA                   |      | 0.3±0.1  | 0.1±0.3  | 0.3±0.1  | 0.1±0.3  | 0.3±0.1  | 0.1±0.3  | 0.3±0.1  |

**Table 19**

Fatty acid composition of the neutral and phospholipids of Ulva from different cultivations. Means with different letters are significantly different (Tukey's multiple comparisons, p < 0.001). Values: average ± standard deviation. SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.
The marked relative and absolute reduction of 16:4n-3 (2.1%) and 18:4n-3 (1.1%) in the low-pigment (nutrient-starved) samples indicate that PUFA can be shifted by the environment, consistent with our previous report [11]. Herein we show that this occurs primarily in the neutral lipid fraction and only for these specific PUFA. Indeed most of the other PUFA increased in content per gram of dry weight in nutrient-starved material. Green macroalgae do not naturally accumulate significant amounts of triacylglycerols [25]. *Ulva* is one of the few species that can be pushed to accumulate triacylglycerols with a subsequent relative increase in SFA and relatively lower PUFA content as a percentage of total extract composition [26]. This is evident in Table 19, although we also show that there is an absolute increase in PUFA content per gram of seaweed dry weight (Figure 22). The low pigment nutrient-starved samples showed significantly higher 16:0 (40.1%) and 18:1n-7 (16.2%) when compared to the other samples along with a 7-fold increase in the absolute amount of 18:2n-6 subsequently increasing the n-6/n3 ratio (Table 19; Figure 22). This is consistent with nitrogen starvation effects reported for *Ulva* [23, 27]. Significant effects of culture were observed on the extract composition of LC PUFA within the neutral fractions (F (3,114) = 46.61, P < 0.0001) with the control (nutrient-rich) culture having significantly higher relative abundance of 20:4n-6, 20:5n-3 and 22:5n-3 when compared to the low pigment (nutrient-starved) biomass.

**Conclusion**

High nutrient cultivation (control regime) provided biomass with desirable n-6/n-3 (0.3) and 18:2n-6/18:3n-3 (0.5) ratios, along with higher relative levels of 18:4n-3, 20:5n-3,
22:5n-3 (Table 20). This fatty acid profile was predominant in the more abundant neutral lipid fraction, which comprised 62% of the total lipid extracted (3.4 mg.g\(^{-1}\) d.w.). In contrast the phospholipid fraction had a higher ratio of SFA, although the n-6/n-3 ratio was still reasonable (0.7), and comprised 38% of the biomass for the high nutrient cultivation. The low pigment (nutrient-starved) Ulva provided for a 3-fold increase in overall lipid yields (12.0 mg.g\(^{-1}\) d.w.) and included both increased SFA, monounsaturated FA (MUFA) and PUFA per gram of seaweed dry weight, as has been found for other algae and we demonstrate that this increase is primarily in the neutral lipid fraction. This increase however was at a detriment to the oil’s profile with increases in SFA content being far greater than increases in MUFA and PUFA. This also resulted in less desirable n-6/n-3 ratios, as well as a lower ratio of phospholipids (12%).
Table 20 A qualitative summary of the neutral and phospholipid fractions of Ulva from four cultivation regimes

<table>
<thead>
<tr>
<th>Neutral Fraction</th>
<th>Control</th>
<th>Low pigment</th>
<th>Shaded</th>
<th>Acetic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low n-6/n-3</td>
<td>Yes</td>
<td></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Low 18:2n-6/18:3n-3</td>
<td>Yes</td>
<td></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>LC-PUFA Content</td>
<td>++++</td>
<td>+</td>
<td>++++</td>
<td>+++</td>
</tr>
<tr>
<td>n-3 Content</td>
<td>++++</td>
<td>+</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>18:4n-3 Content</td>
<td>++++</td>
<td>++</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>Total Content</td>
<td>+++</td>
<td>++++</td>
<td>++</td>
<td>+</td>
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</table>

<table>
<thead>
<tr>
<th>Phospholipid Fraction</th>
<th>Control</th>
<th>Low pigment</th>
<th>Shaded</th>
<th>Acetic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low n-6/n-3</td>
<td>Yes</td>
<td></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Low 18:2n-6/18:3n-3</td>
<td>Yes</td>
<td></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>LC-PUFA Content</td>
<td>++++</td>
<td>++++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>n-3 Content</td>
<td>+++</td>
<td>+</td>
<td>++++</td>
<td>++</td>
</tr>
<tr>
<td>18:4n-3 Content</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Total Content</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

**Key Neutral:** LC-PUFA (++++) > 5% total extract; (+++) 4 - 5%; (++) 2 - 3%; (+) < 2% and n-3 content: (++++) > 40% total extract; (+++) 30-40%; (++) 20 - 29%; (+) < 20%

**Key Phospholipid:** LC-PUFA: (++++) 1.5 - 2% total extract; (+++) 1 - 1.5%; (++) 0.5 - 1%; (+) < 0.5%

**Key 18:4n-3:** (++++) > 15%; (+++) 14-15%; (++) 12 - 13%; (+) < 12%

**Key Total Content:** (++++) > 5 mg.g⁻¹ d.w.; (+++) > 2 mg.g⁻¹ d.w.; (++) 1 - 2 mg.g⁻¹ d.w.; (+) < 1 mg.g⁻¹ d.w.

These results highlight that Ulva cultivated under high nutrient conditions offers a unique and sustainable source of biomass rich in n-3 PUFA (Table 20). The neutral lipid fraction is highlighted as the richest fraction and target for a nutritionally potent PUFA extract and could direct the future choice of extraction protocol, although it is not yet understood how these are distributed across the different neutral lipid classes such as glycolipids and triacylglycerols. For example a one-step neat super-critical carbon dioxide (SC-CO₂) extraction without ethanol supplementation could optimise a sequential biorefinery process that concentrates the neutral lipids fractions, such as glycolipids, to deliver a nutritionally valuable high-end product [28]. However increased content of PUFA can also be achieved with nutrient starvation but with a
consequence of less desirable n-6/n-3 ratios and these would have to be altered through a selective biorefinery process.

**Acknowledgements**

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**Conflict of Interest**

The authors declare no conflict of interest
References


FURTHER INVESTIGATIONS
**Method Optimisation**

The optimisation of the fatty acid extraction of fresh filamentous *Ulva* was a key finding presented in Chapter 4 (p. 101-130). Key aspects of this optimisation for fresh biomass involved:

1. Removal of the homogenisation step
2. Increasing the solvent to mass ratio from 3:1 (2 g fresh in 6 ml solvent) to 6:1 (0.5 g fresh in 3 mL solvent)
3. Removing two overnight incubations from three sequential extractions thereby reducing extraction time from 3 days to 1 day.

Chapter 5 then employed this optimised extraction protocol for the extraction of the harvested, and subsequently oven-dried and milled biomass from these same four different cultivation regimes:

1. High nutrients with natural sunlight;
2. Low nutrients with natural sunlight;
3. High nutrients with reduced natural sunlight (low light) and;
4. High nutrients plus acetic acid supplementation with natural sunlight

The extraction is quick and convenient and involved extracting 250 mg of oven-dried, milled biomass in 3 mL of solvent, thus differing only in the solvent to mass ratio (12:1 as opposed to 6:1 for fresh). As the solvent ratio was shown to be an influential variable in the extraction of fresh biomass, this increase in solvent ratio for the extraction of...
dried biomass was determined to be appropriate at the time. Whilst a variation in yield was expected, it was not anticipated that this optimized extraction method for fresh would result in such dramatic reduction in yield for dried biomass (from 56 mg·g⁻¹ dry weight equivalent to 3 mg·g⁻¹ dry weight) (Figure 23). Therefore, further investigations were carried out to see if this loss in yield could be recovered. It was found that the optimised method for the extraction of fatty acids from dried biomass was the 3-Day extraction protocol that involved 2 over-night incubations and a solvent to mass ratio of 24:1 (250 mg in 6 mL extraction solvent) (Figure 23)

**Figure 23** Comparison of the 3-Day extraction protocol for fresh (non-homogenised) and dried (milled) biomass which involved either 2 g fresh or 250 mg dried biomass in 3 x 6 mL extraction solvent with 2 overnight incubations versus the 1-Day extraction protocol involving either 500 mg fresh or 250 mg dried biomass in 3 x 3 mL consecutive extractions.

**3-Day Protocol** - Lipids extracted based on a standard method of Folch et al. 1957 [1] with modifications based on Kumari et al. 2011 [2] In brief, either two grams of non-homogenized fresh sample or 250 mg of dried biomass was extracted with 3 x 6 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), with two extracts left overnight. The extract was evaporated to a consistent weight (dryness) in pre-weighed 9 mL Teflon lined screw top glass tubes under a stream of nitrogen. Crude lipid extracts were transesterified according to the method of Lepage and Roy 1986 [3] with added BHT (0.01% w/v).

**1-Day Protocol** Involved either 500 mg of non-homogenized fresh sample or 250 mg of dried biomass was extracted with 3 x 3 mL consecutive extractions of chloroform/methanol (2/1, v/v) with 0.001% butylated hydroxytoluene (BHT) by vortexing (~1 min) and centrifugation (3000 rpm, rt, 15 mins).
Whilst the there were no significant effects of the two extraction protocols on the total fatty acid content recovery of fresh biomass, a significant increase and complete recovery of total fatty acid yields is observed with the 3-Day extraction protocol ($P < 0.0001$) for dried biomass. This indicates that the extraction of dried milled powder benefits positively from longer extraction times. It is possible that the solvent requires more time to penetrate the matrix of dried biomass and dissolve the lipids compared to fresh biomass. Future investigations of dried biomass should consider the addition of a magnetic stirring bar during the extraction process and/or further increase the solvent ratio to try and reduce the extraction time and thus increasing sample through-put.

**Non-toxic Solvent Extraction**

The results presented in Chapters 4 and 5 propose the idea that cultivated *Ulva* has the potential to be formulated as an algal-derived oil supplement to complement human and animal nutrition. However, the traditionally utilised lipid extraction protocol, which uses a fixed ratio of chloroform to methanol is toxic and unsuitable for up-scaling at any significant level beyond the laboratory due to its toxicity and lack of food grade compatibility. Thus whilst it is an effective solvent extraction for initial characterisation, safer non-toxic extraction techniques need to be considered and different solvents have been found to effect both yield and composition of lipid extracts [4]. Therefore, additional fresh samples cultured under optimal conditions for fatty acid output (provided by Venus Shell Systems\footnote{Venus Shell Systems, Narrawallee, NSW, 2539, Australia}) were analysed on a separate occasion to determine
if extraction with 100% ethanol can achieve comparable fatty acid yields and composition as previously demonstrated in Chapter 4 and 5 (Figure 24).

**Figure 24** The total fatty acid yields (TFA), saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) (mg g⁻¹ d.w. equivalent) for fresh *Ulva* biomass, which was conditioned under optimal conditions (high nutrients) for desired fatty acid output and extracted with two different solvents (ethanol and chloroform: methanol 2/1 v/v) as per the optimised protocol outlined in Chapter 4 (P < 0.0001).

In terms of yields, ethanol compared very favourable to the traditional chloroform and methanol extraction. Performing the lipid extraction with 100% ethanol yielded significantly higher total fatty acids and total polyunsaturated fatty acids when compared to the traditional chloroform and methanol solvent mixture. Furthermore there was no significant effect of solvent on the fatty acid composition (% extract) $F(1,100) = 3.898$, $P = 0.0511$. This result is in contrast to the findings reported by Schmid et al. 2016 [4] in which ethanol was found to be the second most efficient solvent after chloroform and methanol. However, their analyses were conducted on freeze-dried powders of *Laminaria digitata* (Phaeophyta) and *Palmaria palmata* (Rhodophyta) differing in our analysis of fresh filamentous *Ulva* (Chlorophyta). However, in support
of our finding Schmid et al. 2016 [4] did find that ethanol delivered the highest levels of PUFA.

Thus ethanol is deemed as a suitable alternative to the traditional chloroform and methanol solvent system for the extraction of lipids from fresh filamentous *Ulva* with total fatty acids representing 4.1 % dry weight (d.w.). This is one of the higher yields reported in the literature. For example Kumar et al. 2011 [5] reported total lipid contents of 1.83, 2.03 and 2.00 % d.w. for wild collections of *Ulva fasciata*, *U. reticulata* and *U. rigida* respectively. Recently, Trivedi et al. 2016 [6] further reported up to 2.7 % d.w. for wild collections of *Ulva fasciata* using a sequential extraction process of multiple algal components. With regards to cultivated *Ulva*, Mata et al. 2016 [7] reported a total fatty acid yield of 2.0 % d.w. for cultivated *U. ohnoi* whilst Carl et al. 2016 [8] reported a lipid yield of 3 % d.w. for cultivated *U. tepida*. 
References


FOREWORD TO CHAPTER 6

After the biological screening of a number of Australian macroalgal taxa (Chapter 3), attention was directed towards the cultivation candidate *Ulva*. Due to the strong anti-inflammatory activity identified in *Ulva* extracts in Chapter 3, it was hypothesised that two major metabolite classes may be responsible for this activity. These two metabolite classes were fatty acids and phytochemicals (pigments and phenolics). Thus Chapter 4 was dedicated to exploring the fatty acid profiles of *Ulva*. It offered a comprehensive review of the literature and presented the fatty acid profiles for cultivated *Ulva* from five different treatments conditions, along with method optimisation. Chapter 5 then explored the distribution of these fatty acids across the neutral and phospholipids, along with further highlighting considerations for the extraction method. As a result of the work presented in Chapters 4 and 5, the nutritionally value of the proposed algal-derived oil from cultivated *Ulva* from Venus Shell Systems15 was clearly defined. The four samples, from four different treatment regimes, that were analysed in Chapter 5 were a dried commercially milled powder, provided by Venus Shell Systems. The following Chapter 6 now presents the phytochemical analysis of these same four samples to offer characterisation and quantification of pigments, phenolics and flavonoid content and correlation of this content to anti-oxidant and anti-inflammatory activity. It is presented as a prepared full research manuscript for submission.16.

15 Venus Shell Systems, Narrawallee, NSW, 2539, Australia
CHAPTER 6 ANTI-OXIDANT AND ANTI-INFLAMMATORY ACTIVITIES OF PIGMENT EXTRACTS OF CULTIVATED ULVA

Janice I. McCauley, a Pia C. Winberg, b,c Barbara J. Meyer, b and Danielle Skropeta a,d

aSchool of Chemistry, Faculty of Science, Medicine, and Health (SMAH), University of Wollongong, NSW, 2522, Australia
bSchool of Medicine, SMAH, University of Wollongong, Wollongong, NSW, 2522, Australia
cVenus Shell Systems Pty. Ltd., Mundamia, NSW, 2540, Australia
daCentre for Medical and Molecular Bioscience, SMAH, University of Wollongong, NSW, 2522, Australia

Corresponding Author*
Janice McCauley*
Phone: +61-2-42214001
Fax: +61-2-42214287
Email: jim479@uowmail.edu.au

Contributions

70% Janice McCauley Performed the work and prepared the paper
10% Pia Winberg Editing and input on project design, guidance with data interpretation/analysis
5% Barbara Meyer Laboratory access, equipment and training
15% Danielle Skropeta Oversight of project, paper structure and guidance, editing and proof-reading

As agreed by supervisor A/Prof. Danielle Skropeta
Abstract

Macroalgae are rich in photosynthetic pigments and phenolics. The biomass from cultivated *Ulva* sp. (Chlorophyta) is a potentially sustainable and alternative source of these nutritionally important metabolites. Algae can readily adjust their metabolism and thus the production of these pigments and anti-oxidant molecules as a response to their environment. Therefore, cultivation conditions can be engineered to optimise these metabolite yields. However, in contrast to microalgal research, this has not been explored as extensively for cultivated macroalgae. Thus, herein we determine the effect of both cultivation and extraction processes on the yield and bioactivity, including both anti-inflammatory and anti-oxidant functionalities, of pigments, phenolics and flavonoids of a cultivated *Ulva* sp. It was found that ethanol (95%) extracts of high nutrient cultures with natural sunlight were optimal to maximise levels of total chlorophyll (1.48 mg.g\(^{-1}\) dry weight (d.w)), phenolics (1.58 mg.g\(^{-1}\) d.w.) and corresponding anti-oxidant activity (18.8 % inhibition 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical). In contrast, acetone extracts of high nutrient cultures under natural sunlight showed the highest carotenoid content (0.1 mg.g\(^{-1}\) d.w.) and anti-inflammatory activity (97% inhibition of nitric oxide (NO) in lipopolysaccharide (LPS) stimulated macrophages). Low nutrient conditions under natural sunlight however was found to substantially increase flavonoid content (1.27 mg.g\(^{-1}\) d.w). No cell toxicity (100 µg.mL\(^{-1}\)) was observed in any extracts. In contrast, results indicate that ethanol (95%) extracts, which contain the highest level of pigments and phenolics, promoted cell proliferation at twice the rate when compared to controls. These results establish a
foundation upon which to further optimise macroalgal culture for the production of desired metabolites, such as pigments and phenolics, for functional food products.
Introduction

Phytochemicals from plants have been increasingly highlighted as metabolites of importance with a range of health benefits [1]. These phytochemicals are abundant within fruits and vegetables and include polyphenol compounds such as phenolic acids and flavonoids, terpenoid derived compounds such as carotenoids, lutein, lycopene and β-carotene, and substituted tetrapyrrole chlorophylls [2, 3]. Specific phytochemicals have strong anti-oxidant and anti-inflammatory activities that have the ability to confer protection and reduce the risk or aid the healing of disease [4, 5].

For example, the phenolic oleuropein has been shown to reduce damaging lipid peroxidation caused by free radicals as well as suppress nitric oxide (NO) production via inducible nitric oxide synthase (iNOS) [6]. Whilst NO is an important immune modulator, high and persistent levels can lead to cellular and DNA mutagenesis and damage [7, 8]. The ability of phytochemicals to confer health benefits is evident in studies that correlate diets rich in fruit and vegetables with a lower risk of hypertension, coronary heart disease, stroke [9] and reduced risk for cancer [10, 11]. The therapeutic potential of phytochemicals has also been highlighted in the treatment of chronic inflammatory disorders such as inflammatory bowel disease [12] and protection of the liver due to their hepatoprotective effects [13]. These phytochemical classes are also abundant in algae, and cultivated macroalgae has been highlighted as a potential source for these high value anti-oxidant and anti-inflammatory phenolics and pigments [14-18].
Due to the high natural chemical variability of wild seaweeds, controlled cultivation is essential to deliver consistent biomass [19, 20]. Therefore the cultivation conditions that optimise the desired bioactive profile must be defined. It is known that microalgae can shift the type and contents of pigments and or phenolic compounds in culture [21], and this process has been commercialised for the production of pigments such as β-carotene and astaxanthin production by stressing cultures of Dunaliella salina or Haematococcus pluvialis, respectively [22]. In contrast, other studies have shown that optimal growth conditions of high light and high nutrients favour pigment profiles [23]. Whilst the effect of cultivation on pigment profiles has not been explored as comprehensively for macroalgae, such as Ulva spp. [24, 25], there are early findings consistent with improved pigment profiles with optimal nutrient and growth conditions. For example, improved pigment and anti-oxidant profiles for Ulva spp. has been shown for high light and high nutrient cultivation [26]. Specific responses to different culture media with subsequent shifts in pigments were also correlated to changes in anti-oxidant activities for an Ulva lactuca sample [27].

Ulva spp. are a source of both oxidant-scavenging phenolics (Table 21) and chlorophyll and carotenoid pigments [27, 28]. Evidence also shows that these profiles can be shifted in culture. For example regarding chlorophyll content, Ulva lactuca cultured in artificial seawater and low nutrients showed a loss of pigments, however the pigment concentration can be recovered with complete nutrient provision by nitrate and phosphate supplementation [29]. Similarly, Gómez Pinchetti et al. 1998 [30] found maximum chlorophyll content in nitrogen enriched cultivation conditions for Ulva spp. Variation in pigments have also been shown across species from different habitats. For
example a subtidal *Ulva rotundata* sample showed a three-fold increase in pigments (chlorophyll a, chlorophyll b and carotenoids) when compared to an intertidal *Ulva olivascens* sample [31]. Figueroa et al. 2003 [31] further showed that these two species had different phytochemical adaptive responses to ultraviolet light treatments.

Whilst a number of studies have reported on the total phenolic content of *Ulva* (Table 21), the effect of cultivation on total phenolics has been less investigated. Cabello-Pasini et al. 2011 [32] however did show a 60% increase in phenolics for *Ulva rigida* when cultured with high nitrate levels. Studied with other species of macroalgae have also shown that low-stocking densities of land-based cultivated *Derbesia tenuissima* (class Ulvophyceae) can boost anti-oxidant capacity by up to 88% with 22% higher phenolic content when compared to high-stocking densities [33]. This indicates a potential effect of internal fluctuating light intensities resulting from the different stocking densities [33].

Other important considerations in the determination of total pigments and phenolics are effects of the extraction process. Thus, it is important to select solvents and maintain consistent condition of the biomass prior to extraction (Table 21) as both variables have been shown to influence phenolic yields and anti-oxidant capacities [1]. In particular, freeze-dried biomass has been shown to better preserve phenolic content as opposed to oven-drying, and may explain the higher overall phenolic content observed for freeze-dried *Ulva lactuca* as opposed to the air-dried *Ulva rigida* as evident in Table 21 [34]. Thus it is hard to compare across research findings when the methods of extraction are inconsistent. With regards to solvents, aqueous methanol, ethanol and acetone solutions
are commonly employed (Table 21). However, when compared to methanol, both acetone and ethanol have been shown to yield higher amounts of phenolics and flavonoids, with aqueous ethanol extracts showing the highest anti-oxidant activity [35]. Therefore, herein we have investigated the effects of cultivation and extraction solvents on the yields of broad pigment categories (chlorophyll a; chlorophyll b; carotenoids; phenolics and flavonoids) in one Australian species of Ulva. Two extraction solvents (acetone and 95% ethanol) were used on algal biomass from four cultivation conditions. The extracts were then tested across three basic assays for anti-oxidant, anti-inflammatory and cell toxicity. These results have the potential to aid in the implementation of future protocol systems of cultivation and production. This will help to deliver improved consistency in the functional aspects of algal-derived novel foods. This is the first screening study to explore the effect of cultivation conditions and the effect of extraction solvent on the pigments profiles and anti-oxidant and anti-inflammatory bioactivities of a cultivated Ulva sp.
Table 21  Reported phenolic contents of various species of *Ulva* macroalgae (Chlorophyta) expressed as mg phloroglucinol (PHGE), mg gallic acid (GAE) or µg pyrocatechol (PE) equivalents per gram of extract unless otherwise specified.

<table>
<thead>
<tr>
<th>Species</th>
<th>Location</th>
<th>Biomass</th>
<th>Extract</th>
<th>mg PHGE·g⁻¹</th>
<th>mg GAE·g⁻¹</th>
<th>µg PE·g⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ulva rigida</em></td>
<td>Tunisia Air-Dried</td>
<td>Chloroform</td>
<td>13 ± 1.1</td>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tunisia Air-Dried</td>
<td>Ethyl acetate</td>
<td>19 ± 0.8</td>
<td>a</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tunisia Air-Dried</td>
<td>Butanol</td>
<td>6 ± 0.3</td>
<td>a</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tunisia Air-Dried</td>
<td>Water</td>
<td>5 ± 0.6</td>
<td>a</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Marmara sea</td>
<td>Methanol</td>
<td>0.73 ± 0.1</td>
<td>b*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Marmara sea</td>
<td>80% Methanol</td>
<td>2 ± 0.3</td>
<td>c#</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ulva lactuca</em></td>
<td>Adriatic sea</td>
<td>Acetone</td>
<td>58 ± 1.1</td>
<td>d</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Commercial</td>
<td>Ethanol</td>
<td>6.0 ± 0.01</td>
<td>e</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ulva intestinalis</em></td>
<td>Persian Gulf Air-Dried</td>
<td>80% Methanol</td>
<td>1 ± 0.1</td>
<td>g</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ulva clathrata</em></td>
<td>Persian Gulf</td>
<td>80% Methanol</td>
<td>5 ± 0.7</td>
<td>g</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ulva linza</em></td>
<td>Persian Gulf</td>
<td>80% Methanol</td>
<td>2 ± 0.3</td>
<td>g</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Ulva flexuosa</em></td>
<td>Persian Gulf</td>
<td>80% Methanol</td>
<td>3 ± 0.2</td>
<td>g</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*mg gallic acid equivalents per 100-gram fresh tissue; µg pyrocatechol equivalents per gram dry weight powder*

*References:* a) Trigui et al. 2013 [36]; b) Yildiz et al. 2012 [37]; c) Yildiz et al. 2014 [38]; d) Kosanić et al. 2015 [39]; e) Boisvert et al. 2015 [40]; f) Kellogg and Lila 2013 [41]; g) Farasat et al. 2014 [42].
Materials and Methods

Algal Samples and Cultivation

The *Ulva* samples were provided as a dried commercially milled powder from Venus Shell Systems Pty. Ltd., Mundamia, New South Wales, 2540, Australia. Samples were from controlled scaled, cultivated stock and conditioned either in the presence of high nutrients (natural sunlight with ammonia enriched media, control regime), low nutrients (natural sunlight with reduced nutrients), low light (reduced natural sunlight with ammonia enriched media) or added acetic acid (natural sunlight with ammonia enriched media and added acetic acid supplementation).

Extraction

All samples (250 mg) were extracted in triplicate in 95% ethanol or 100% acetone (3 x 3 ml; total volume 9 ml) with brief vortexing followed by centrifugation (3000 rpm, 10 mins, rt). The extracts were immediately analysed for pigments and a portion of the extract set aside for determination of phenolic and flavonoid contents and testing of anti-oxidant activity.

Total Phenolic Content

Total phenolic content was determined as per the 96-plate method described by Herald et al. 2012 [43]. Extracts or standards were diluted 1:4 with Milli-Q (75 µl Milli-Q; 25 µl) and mixed thoroughly with 25 µL of diluted Folin-Ciocalteu (1:1 with Milli-Q) reagent using a multi-channel pipette. After 6 minutes, 100 µL of Na₂CO₃ was added to
each well and the plate incubated for 90 minutes in the dark, after which absorbance was read at 765 nm (SpectraMax). The values were determined from a calibration curve prepared with gallic acid (0 - 500 µg.mL\(^{-1}\)) and results expressed µg gallic equivalents per mL extract (µg quercetin.mL\(^{-1}\)) and µg gallic equivalents per g dry weight (µg quercetin.g\(^{-1}\) d.w.). Each extracted sample was analysed in triplicate with each set of cultivation data having a sample number (n) of 9.

**Total Flavonoid Content**

Total flavonoid content was determined as described by Santas et al. 2008 [44]. In brief, 100 µL of extract was diluted (1:1) with Milli-Q water and 200 µL of a 2% AlCl\(_3\) in methanol solution was added (5% acetic acid in methanol). The plate was left to incubate for 10 mins at room temperature before absorbance was read at 430 nm (SpectraMax). The values were determined from a calibration curve prepared with quercetin (0 - 500 µg.mL\(^{-1}\)) and results expressed as µg quercetin equivalents per mL extract (µg quercetin.mL\(^{-1}\)) and µg quercetin equivalents per g dry weight (µg quercetin.g\(^{-1}\) d.w.). Each extracted sample analysed 6 individual times with each set of cultivation data having a sample number (n) of 18.

**Total Chlorophyll and Carotenoid Content**

Total chlorophyll and carotenoid content were determined by ultraviolet–visible spectroscopy as per Foo et al. 2015 [45] and Kumar et al. 2010 [46] utilising the equations described by Lichtenthaler 1987 [47]. If necessary the samples were diluted to fall within the absorbance range of 0.3 and 0.85 [48]. Total content of chlorophyll a,
chlorophyll b and carotenoids (xanthophylls and carotenes) determined for both ethanol 95% (v/v) and acetone 100% (v/v) using the following equations and then results expressed as µg·g⁻¹ dry weight (d.w.).

Equations for determination of pigments in 95% ethanol:

\[
\begin{align*}
\text{µg·mL}^{-1}
\text{Chlorophyll a} &= 13.36A_{664.2} - 5.19A_{648.6} \\
\text{µg·mL}^{-1}
\text{Chlorophyll b} &= 27.43A_{648.6} - 8.12A_{664.2} \\
\text{µg·mL}^{-1}
\text{Total chlorophylls} &= 5.24A_{664.2} + 22.24A_{648.6} \\
\text{µg·mL}^{-1}
\text{Total carotenoids} &= 1000A_{470} - 2.13Ca - 97.64Cb/209.
\end{align*}
\]

Equation for the determination of pigments in 100% acetone:

\[
\begin{align*}
\text{µg·mL}^{-1}
\text{Chlorophyll a} &= 11.24A_{661.6} - 2.04A_{644.8} \\
\text{µg·mL}^{-1}
\text{Chlorophyll b} &= 20.13A_{644.8} - 4.19A_{661.1} \\
\text{µg·mL}^{-1}
\text{Total chlorophylls} &= 7.05A_{661.6} + 18.09A_{644.8} \\
\text{µg·mL}^{-1}
\text{Total carotenoids} &= 1000A_{470} - 1.90Ca - 63.14Cb/214.
\end{align*}
\]

Assessment of Antioxidant Activity via 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) Assay

After extraction, the extracts were directly assessed for their free radical scavenging activity for correlation against phenolic and flavonoid content. The anti-oxidant activity was assessed by the 96-plate DPPH method as described by Herald et al. 2012 [43] with some modifications [49]. In brief, 200 µl of 150 µM DPPH solution in ethanol is added to 25 µL of extract or blank. The blank is left to incubate in the dark for up to 6 hours and absorbance read at 517 nm (SpectraMax). The percentage (%) of DPPH scavenged is calculated as the following equation:

\[
\text{Absorbance of the Control - Absorbance Sample / Absorbance Control} \times 100
\]
Each extracted sample was analysed in triplicate with each set of cultivation data having a sample number (n) of 9.

Assessment of Cytotoxicity

After phytochemical analysis the triplicate extractions for each sample were combined and dried under nitrogen and 10 mg.g\(^{-1}\) stocks in 100% dimethyl sulfoxide were prepared for biological evaluation. The extracts were assessed for cytotoxicity (100 µg.mL\(^{-1}\)) on Abelson murine leukaemia virus-induced tumour macrophage (RAW 264.7) cell lines as described previously [50, 51]. The cytotoxicity of the extracts was calculated as the % inhibition of cell proliferation against controls (24 hour incubation) using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) reagent (Promega). Data was normalised against a control for analysis.

Assessment of Anti-inflammatory Activity

The anti-inflammatory activity was assessed as the percent (%) reduction of nitrite (NO\(_2^–\)) in the cell supernatants determined spectrophotometrically using Griess reagent (1 % sulphanilamide and 1 % N-(1-naphthyl)ethylenediamine dihydrochloride solution) as previously described [51]. In brief, RAW 264.7 macrophage cells were co-incubated with 1 µg.mL\(^{-1}\) lipopolysaccharides (LPS) and algal extract (100 µg mL\(^{-1}\)) for 24 h (37 °C, 5 % CO\(_2\)), including controls in 24-well plates. After incubation an equal volume of Griess reagent was added to 50 µL of cell supernatant or standard (0 - 1000 µg.mL\(^{-1}\) sodium nitrite) and mixed thoroughly. After 10 minutes the absorbance was measured at
550 nm with a baseline correction at 650 nm [52] and the amount of nitrite determined using a standard curve (0 - 100 µM sodium nitrite). Data expressed as a % reduction of nitrite compared to controls.

**Statistical and Data Analysis**

Two-way ANOVA and Tukey’s multiple comparisons were used to determine significantly different means using GraphPad Prism version 6.00.
Results and Discussion

Pigments

The pigments of chlorophyta species consist mainly of chlorophyll a and b, β-carotenes, lutein and several xanthophylls [53]. A significant effect of the Ulva cultivation treatment was observed on the pigment profiles when extracted with acetone (F (3, 32) = 2486, P < 0.0001) (Figure 25). Low nutrient conditions (-N) showed the lowest total chlorophyll content (17.1 µg.g\(^{-1}\) d.w.) and total carotenoids (2.6 µg.g\(^{-1}\) d.w.) in both extraction solvents, which is consistent with reported environmental stress effects such as nitrogen limitation [54-56]. In contrast, the high nutrient regime (+N) showed a dramatic 57-fold increase in total chlorophyll content (990.9 µg.g\(^{-1}\) d.w.) and 37-fold increase in total carotenoid content (102.8 µg.g\(^{-1}\) d.w.) (Figure 25).

Cultivation with high nutrients under shaded conditions (-L) significantly reduced the level of total chlorophyll by 49% and carotenoid content by 73% when compared to the high nutrients with natural sunlight. Acetic acid (+AA) cultivation reduced the acetone yields for total chlorophyll and carotenoids by 66% and 78% to 336.7 µg.g\(^{-1}\) and 22.3 µg.g\(^{-1}\) d.w., respectively. Whilst supplementation with an organic carbon source was investigated herein, supplementation with an inorganic carbon source (elevated carbon dioxide (CO\(_2\)) has also been shown to decrease total chlorophyll and carotenoid content in cultures of Ulva rigida relative to a normal CO\(_2\) control [55]. Ethanol extracts (95% v/v), showed similar trends as for the acetone extracts across the different cultures with significant differences across culture treatments (F (3, 32) = 1101, P < 0.0001) (Figure
For both acetone and 95% ethanol extracts, high nutrients delivered the highest combined yield for pigments (2085 µg.g\(^{-1}\) d.w. acetone; 3045 µg.g\(^{-1}\) d.w. 95% ethanol).

Significantly higher yields of total chlorophylls (1480.5 µg.g\(^{-1}\) d.w.) across all cultures were obtained with ethanol extracts while the highest yields of carotenoids across all cultures were obtained for the acetone extracts (chlorophyll a F (1, 16) = 150.7, p < 0.0001; chlorophyll b F (1, 16) = 226.8, p < 0.0001; total chlorophyll F (1, 16) = 211.0, p < 0.0001 and; total carotenoids F (1, 16) = 12.33, p = 0.0029). This is likely due to major carotenoids such as β-carotene and lutein having a higher solubility in acetone compared to ethanol [57].

The increase in carotenoid levels for the high nutrients with natural sunlight may be an acclimation response for high sun exposure as carotenoids prevent chlorophyll photoxidation [54, 58]. The resulting higher a/b ratio is also indicative of the higher chlorophyll a content responsible for light processing, in contrast to the accessory chlorophyll b pigment responsible for light harvesting. Continual low light conditions did not accumulate a pigment concentration beyond that achieved with full sunlight with natural fluctuations. Accumulation of light harvesting pigments is one adaption of low light plants in an attempt to maintain a high efficiency of light absorbance [59] and Ulva has been shown to exhibit adaptive responses to ultraviolet light treatments [31]. However, limited studies have compared the effect of natural sunlight versus continual shaded (low light) conditions for large-scale open pool cultivations of Ulva.
Figure 25. Total chlorophyll, carotenoid content (µg g⁻¹ d.w.) and chlorophyll a/chlorophyll b ratios for 100% acetone and 95% ethanol extracts of *Ulva* from four different cultivation treatments: + N: high nutrients with natural sunlight; - N: low nutrients with natural sunlight; - L: high nutrients under shaded sunlight; + AA: high nutrients with natural sunlight and acetic acid supplementation.
Light quality on the other hand, has been investigated for *Ulva lactuca*, but with no significant effects on pigment concentration [56] while high nutrients significantly increased levels of pigments [56]. Exposed wet thalli of *Ulva fasciata*, *U. lactuca* and *U. reticulata* have shown a decrease in total pigments in response to ultraviolet B radiation exposure over time [60]. However, this is not representative of true cultivation conditions where algal cells can divide and acclimate and is likely the result of pigment photoxidation. Sand-Jensen 1988 [59] suggested that *Ulva* found naturally in high light, intertidal zones utilises other light adaptive mechanisms than a shade adaptive mechanism of increased pigments in low light; a selective advantage to natural fluctuating light conditions. Similarly, increasing chlorophyll content with increasing light intensity has been shown in microalgae [61, 62]. Results herein, along with the reported literature suggest that nutrients are the strongest determinant of pigment concentration in natural light conditions.

**Phenolics and Antioxidant Activity**

**Acetone extracts**

For acetone extracts there was a significant effect of increased flavonoid content and DPPH activity for low nutrient cultures (*F* (3, 132) = 100.5, *P* < 0.0001) (Figure 26), while there were no significant differences in acetone-extracted phenolics across the different cultivation regimes (6.15 - 9.08 µg.mL⁻¹ GAE). Low nutrients showed a 5-fold increased in flavonoid concentration (35.89 µg.mL⁻¹ QE) when compared to the other treatment groups (3.95 - 6.96 µg.mL⁻¹ QE). Low light (6.95 µg.mL⁻¹ QE) and samples
supplemented with acetic acid (6.76 μg.mL\(^{-1}\) QE) also showed slightly elevated flavonoid content compared to natural sunlight (3.95 μg.mL\(^{-1}\) QE) but an interaction of this effect with nutrients was not tested and the scale was much smaller than that of nutrient depletion.

As expected, acetone-extracted flavonoid content did not exceed phenolic content for the high nutrient regimes (+ N, - L and + AA) as the phenolic assay measures all compounds capable of reducing the Folin-Ciocalteu reagent, including flavonoids (Figure 26). However the flavonoid content of the nutrient-starved regimes was unusually higher than the phenolic content and requires further investigation. It may be possible that the low nutrient cultures increase the synthesis of flavonoids, including flavonols as a defence mechanism in response to environmental stress [63, 64]. The flavonoids may also be highly substituted thus limiting their ability to react with the Folin-Ciocalteu reagent. However the higher flavonoid content of the low nutrient conditioned biomass did not equate to higher anti-oxidant activity (7.2 % inhibition) compared to the high nutrients with natural sunlight (12.4 %) (P < 0.001).
Figure 26 (a) The phenolic (µg.mL⁻¹ gallic acid equivalent), (b) flavonoid content (µg.mL⁻¹ quercetin equivalents) and (c) relative DPPH free radical scavenging activity (% inhibition of DPPH free radical) of acetone and ethanol (95% v/v) extracts for Ulva biomass conditioned using four different cultivations treatments; nutrient-rich (+ N); nutrient-starved (- N), nutrient-rich; low-light (- L) and; nutrient-rich with added acetic acid supplementation (+ AA).
Ethanol Extracts

A significant effect of cultivation on the phenolic content, flavonoid content and DPPH activity was observed for extracts from 95% ethanol compared to acetone extracts (F (3, 132) = 36.23, P < 0.0001). Furthermore, when extracted with 95% ethanol there is also a significant effect of both solvent (F (1, 64) = 98.23, P < 0.0001) and cultivation (F (3, 64) = 23.11, P < 0.0001) on phenolic yields, which was not observed for acetone (Figure 26). Extraction with 95% ethanol for the high nutrient culture with natural sunlight resulted in a 4-fold increase in total phenolics when compared to acetone extracts (44.58 c.f. 9.08 µg.mL\(^{-1}\) GAE respectively). Apart from phenolics, all other trends observed for the acetone extracts were maintained with the 95% ethanol extraction with the low nutrient regime showing a marked increase in flavonoid content (34.26 µg.mL\(^{-1}\) QE) when compared to the other regimes (6.23 - 9.66 µg.mL\(^{-1}\) QE) (Figure 26). Anti-oxidant activity was again highest in the high nutrient culture with natural sunlight and with ethanol extract (18.8% inhibition), which is potentially due to the significantly higher levels of phenolics (44.58 µg.mL\(^{-1}\) GAE).

Comparison of the yields and activity of Ulva phenolics found here with reported literature is difficult due to the different standards (phenolic equivalents) used and different methods of reporting yields (Table 21). Table 22 shows the dry weight equivalents (mg.g\(^{-1}\) d.w.) for phenolic and flavonoid contents across the four cultivation regimes when extracted with either acetone or 95% ethanol. Yildiz et al. 2014 [38] reported a value of 1.7 ± 0.3 mg GAE per g of dry weight for a dried Ulva rigida sample extracted with 80% methanol, supporting our reported value herein of 1.58 ±
0.21 mg GAE per g of dry weight for the high nutrient culture with natural sunlight. However other studies with freeze dried material showed much higher levels of PHGE equivalents and these equivalents and pre-processing effects were not explored here.

Herein our results indicate that high nutrients with natural sunlight is optimal for phenolics and antioxidant activity, relative to the other treatment regimes, however for more definitive, absolute amounts regarding total content, further investigations should be carried out to optimise the extraction for algal samples. It is possible that higher yields may be obtained by extraction of fresh or freeze-dried material as opposed to oven-dried, as investigated herein [34, 65].

### Table 22

The phenolic total content (gallic acid equivalents, GAE) and flavonoid (quercetin equivalents, QE) content per gram of dried *Ulva* powder for acetone and 95% ethanol extracts from four different cultivations treatments (nutrient-rich, nutrient-starved, nutrient-rich low-light and nutrient-rich with added acetic acid supplementation).

<table>
<thead>
<tr>
<th>Acetone</th>
<th>Phenolics (mg.g⁻¹ d.w. GAE)</th>
<th>Flavonoids (mg.g⁻¹ d.w. QE equiv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrient-starved (- N)</td>
<td>0.25 ± 0.01</td>
<td>1.27 ± 0.02</td>
</tr>
<tr>
<td>Nutrient-rich (+ N)</td>
<td>0.32 ± 0.01</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>Low light (- L)</td>
<td>0.28 ± 0.01</td>
<td>0.25 ± 0.03</td>
</tr>
<tr>
<td>+ Acetic Acid (+ AA)</td>
<td>0.22 ± 0.01</td>
<td>0.24 ± 0.02</td>
</tr>
<tr>
<td><strong>Ethanol (95% v/v)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nutrient-starved (- N)</td>
<td>1.04 ± 0.10</td>
<td>1.21 ± 0.07</td>
</tr>
<tr>
<td>Nutrient-rich (+ N)</td>
<td>1.58 ± 0.21</td>
<td>0.22 ± 0.03</td>
</tr>
<tr>
<td>Low light (- L)</td>
<td>0.39 ± 0.03</td>
<td>0.34 ± 0.02</td>
</tr>
<tr>
<td>+ Acetic Acid (+ AA)</td>
<td>0.47 ± 0.07</td>
<td>0.33 ± 0.02</td>
</tr>
</tbody>
</table>

The chemical nature of the algal phenolics themselves will also direct the choice of solvent-system so further solvents/solvent combinations should be investigated [1, 66]. For example Vijayavel and Martinez 2010 [67] reported appreciable NO, hydroxyl and superoxide radical scavenging dose responses for *Ulva fasciata* in 100% ethanol
extracts with corresponding total phenolic content of 0.21 mg GAE per g algal weight, an 87% decrease compared to the total content reported herein. Aqueous organic solvents, such as 80% methanol or 95% ethanol have also been shown to extract higher quantities of phenolic compounds than pure organic solvents [68] suggesting that the acetone extraction herein may benefit by addition of a proportion of water.

Anti-inflammatory and Cytotoxicity

With regards to the inhibition of LPS-induced NO, no significant effects of cultivation were observed for either the acetone or 95% ethanol extracts (F (3, 24) = 0.3892, P = 0.7618). All samples exhibited strong anti-inflammatory activity ≥ 76 % with no evidence of cell cytotoxicity (% inhibition on cell proliferation) (Figure 27). However, the extraction solvent did have a significant effect on the inhibition of NO (F (1, 24) = 16.49, P = 0.0005) with acetone extracts exhibiting significantly higher inhibition of NO (94 - 97 %) than the 95% ethanol extracts (76 - 91 %) (Figure 27). It is unclear which phytochemical is responsible for the increased NO activity as there are no obvious differences in the profiles of the solvents that might explain this, other than that carotenoids were generally higher in the acetone extracts but mostly in the nutrient rich conditions (above).
The increase in cell proliferation (normalised to control) and corresponding % inhibition of nitric oxide (NO) for acetone and 95% ethanol extracts of *Ulva* from four different cultivations treatments (nutrient-rich, nutrient-starved, nutrient-rich low-light and nutrient-rich with added acetic acid supplementation).

Kellogg and Lila 2013 [41] reported similar levels of NO inhibition, up to 92 % for hexane, ethyl acetate and water extracts from an *Ulva lactuca*. We have also previously reported strong NO inhibition (>76 %) for *Ulva* dichloromethane and ethyl acetate extracts [51]. This high NO inhibition across a variety of extracts with differing polarities suggests that the anti-inflammatory functionality of *Ulva* is likely due to a number of different structurally classed molecules such as fatty acids, phenolics, terpenoids with possible synergistic effects. This however would need to be defined through further purification and a bioassay guided fractionation approach.
With regards to cell toxicities of both extracts, there was no observed inhibition of cell proliferation. In contrast, cells incubated with *Ulva* acetone and 95% ethanol extracts (100 µg.mL\(^{-1}\)) showed enhanced cell growth. When normalised against a control, both extracts indicated enhanced proliferation up to 1-fold. When normalised, significant differences between 95% ethanol and acetone extracts were observed (F (1, 56) = 395.9, P < 0.0001) (Figure 27). *Ulva* extracted with 95% ethanol showed a higher rate of cell proliferation. This may correlate with the increased phenolic and total chlorophyll content observed. Furthermore the increased NO inhibition observed for the acetone extracts may be indicative that there was less LPS induced cells producing NO. Future investigations should correct NO findings against a cell number or mg of cell protein [52]. With regards to cultivation, no significant differences were observed across the treatments for either the acetone or 95% ethanol extracts (F (3, 56) = 1.981, P = 0.1272). It is possible that the extracts are conferring a significant protective effect that increases cell viability. Indeed *Ulva rigida* ethanol extracts have been reported to protect human carcinoma cells from death induced by oxidative stress due to H\(_2\)O\(_2\) exposure [69].

**Conclusion**

In summary, across all samples investigated here, acetone extracts of high nutrient natural sunlight biomass showed the highest level of carotenoids and inhibition of NO (Table 23). Important carotenoids present in *Ulva* include β-carotene, lutein [70] and have been shown to influence important inflammatory mediators [71]. In contrast, the low pigment, low nutrient samples when extracted with acetone showed a higher
concentration of flavonoids. Flavonoids are also anti-oxidants, but they also exhibit a vast range of pharmacological activities including anti-inflammatory, anti-diabetic, hepato- and gastro-protective [72]. It has also been implied that the larger molecular weight flavonoids exert their beneficial effects through their interactions with gut microbiota [73]. However these biological activities were not measured or evident here. The high flavonoid content of low nutrient culture is highlighted as a key area to investigate using a bioassay guided fractionation approach to identify the metabolite that is being enhanced through nutrient-starved culture conditions and for considering further biological assays. However, the optimal cultivation condition of high nutrients and ethanol extractions provided for the highest chlorophyll and phenolic content with associated high anti-oxidant activity (Table 23). It will be important to build on these findings by increasing the identification of phytochemicals present in the diverse extracts and expanding the types of biological assays along with establishing concentrations at 50% inhibition (IC$_{50}$) for purified fractions.

**Acknowledgments**

This work was supported financially by the Centre for Medical and Molecular Bioscience, University of Wollongong (UOW) and an Australian Postgraduate Award (JM). We further thank the Rural Industries Research and Development Corporation (RIRDC) who funded the FA standards.

**Conflicts of Interest**

The authors declare no conflict of interest
### Table 23

Summary of the highest detected concentrations of chlorophyll, carotenoids, flavonoids, and the highest detected anti-inflammatory, antioxidant, and cell proliferative activity for four different cultivations of Ulva macroalga, extracted with two different solvents.

<table>
<thead>
<tr>
<th>Extraction Solvent</th>
<th>Culture Conditions</th>
<th>Chlorophyll</th>
<th>Carotenoids</th>
<th>Flavonoids</th>
<th>Phenolics</th>
<th>DPPH</th>
<th>Biological Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>95% Ethanol (control)</td>
<td>Low light, nutrient-rich (control)</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>NO</td>
<td>Anti-inflammatory</td>
</tr>
<tr>
<td>95% Ethanol (control)</td>
<td>Low light, nutrient-starved</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>Anti-inflammatory</td>
</tr>
<tr>
<td>Acetic acid + Acetic acid</td>
<td>Nutrient-rich (control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetic acid</td>
<td>Nutrient-starved</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 23: Summary of the highest detected concentrations of chlorophyll, carotenoids, flavonoids, along with the highest detected anti-inflammatory, anti-oxidant, and cell proliferative activity.
References


CHAPTER 7 GENERAL DISCUSSION & FUTURE DIRECTIONS
Increasingly consumers are being exposed to information regarding the link between diet and health. However, a proportion of the population will find it challenging to meet the simple dietary intake requirements for fresh fruit and vegetables [1]. Specifically, diets that are high in omega-6 and trans-unsaturated fatty acids have led to an increasing prevalence of non-transmissible diseases [2]. For example, allergies, asthma and inflammatory bowel, cardiovascular, metabolic and neurological diseases, all of which are characterised by a persistent underlying low-grade inflammation [3-5]. However, diets that are rich in plant phytochemicals and anti-inflammatory omega-3 fatty acids confer protective benefits against these inflammatory diseases [6-11]. These metabolite classes are abundant in algae and cultivated macroalgal crops have been highlighted as a potential source for these high value anti-oxidant and anti-inflammatory compounds [12-16].

A review of bioactive compounds in seaweed by Holdt and Kraan 2011 [17] demonstrates that, collectively, seaweeds are a valuable source of nutrition including essential dietary vitamins, minerals, fatty acids and amino acids. In addition, the functionality of seaweeds also extends beyond that of simply providing nutrition, as they are also a source of other functional compounds (Chapter 1). Northern hemisphere seaweeds dominate this field of research, and yet Australia has a unique flora of macroalgae in which the chemistry and biological activity is still poorly understood. Therefore a number of Australian seaweeds were investigated herein to establish their potential for cultivation for food and health markets (Chapter 3). These taxa included Hormosira banksii, Phyllospora comosa, Ecklonia radiata, Myriogloea sciurus, Solieria robusta and the green macroalgae Ulva spp. In particular, the study focused on
dominant seaweed traits of interest, such as metabolites that function as anti-inflammatories that could confer protection against underlying low-grade inflammation that characterises many lifestyle diseases.

One anti-inflammatory class of molecules are polyunsaturated fatty acids (PUFA), in particular omega-6 (n-6) fatty acids, which are substrates for pro-inflammatory mediators and omega-3 (n-3) fatty acids, which are substrates for anti-inflammatory mediators [18]. This is why a reduced ratio of dietary inflammatory n-6 fatty acids to anti-inflammatory n-3 fatty acids (n-6/n-3) is considered important to suppress chronic inflammatory disease [9, 19]. Therefore characteristics of the fatty acid profiles for these Australian taxa were investigated. All six taxa had significantly different omega-6 to omega-3 ratios, although the environmental conditions had a big influence on this too. However it was the green seaweed Ulva that was most nutritionally desirable, exhibiting a low n-6/n-3 ratio of 0.4 and the only species to contain the important omega-3 docosahexaenoic acid (DHA), albeit at low concentrations. The consumption of n-6 to n-3 in a typical Western diet is approximately 15:1, while ratios of between 2.5-5 to 1 are desirable to reduce the risk of disease [9]. Furthermore, the non-polar (lipid rich) extracts of all six algal species strongly inhibited production of the inflammatory-mediator nitric oxide (Chapter 3). Investigations into cell toxicity also revealed that extracts of both the Ulva sp. and H. banksii showed selective cytotoxicity towards a human pancreatic cancer cell line, an interesting finding requiring further exploration in the future.
As a result, *Ulva* sp. and *H. banksii* and their extracts and fractions were identified as key candidates for further investigation. There is also a potential opportunity to pursue characterisation of the active component(s) as a new future research project emerging from the work described herein. Identification of a novel bioactive may further direct future anti-inflammatory drug design through a synthetic chemistry approach. However, the attribution of a biological activity to an extract is an important first step in characterising the functionalities of the biomass as a whole food matrix. Such information contributes to more immediately attainable goals in which seaweeds, as either a whole food, concentrated extracted or isolated class of metabolites have the potential to contribute to human and animal nutrition as dietary functional foods and/or ingredients with anti-inflammatory functionalities.

Collaborations with industry partner Venus Shell Systems Pty. Ltd.\textsuperscript{18} enabled the analysis of biomass from large-scale cultivation experiments to further explore the fatty acid profiles and anti-inflammatory activity of the identified key candidate taxon *Ulva* sp. There have been a number of reports quite recently of promising results regarding large-scale cultivation of *Ulva* [20-23] and similar chlorophyta species such as *Derbesia tenuissima* [24, 25]. These studies contribute important knowledge of various aspects of cultivation (stocking density, seasonal productivities, harvesting and post-processing) for chlorophyta species to maximize biomass yields and quality. With regard to quality, they have focused on key compounds for biorefinery and commercial utilisation, such as fatty acids and lipids [24], carbohydrates, amino acids [22], protein [23], mineral salt

\textsuperscript{18}Venus Shell Systems, Narrawallee, NSW, 2539, Australia
[20] and even phenolics and antioxidant activity [25]. On the basis of the results presented in Chapter 3, it was hypothesised that the presence of two metabolites classes are likely candidates for the strong anti-inflammatory activity of the extracts. These include lipids rich in polyunsaturated fatty acids and pigments (including phenolics).

*Ulva* can readily adjust its metabolism under specific environmental conditions, wherein the lipids and fatty acids play an important role in this adaptation [26]. As such, the profiles of reported FA for *Ulva* can show a wide degree of natural variation (comprehensive review presented Chapter 4). Therefore, in complement to the reported cultivation studies of *Ulva* described above, Chapter 4 demonstrates that growth conditions are also an important consideration for biomass yield and quality. It was demonstrated that high nutrient growth conditions deliver the most desirable fatty acid profile for health with PUFA constituting 54% of the lipid extract. The n-6/n-3 was a very favourable low 0.2, and important omega-3 PUFAs comprised up to 9%, 2% and 1% of stearidonic acid (SDA; C18:4n-3), eicosapentaenoic acid (EPA; C20:5n-3) and docosahexaenoic acid (DHA; C22:5n-6), respectively. A number of studies have shown potential for the rare stearidonic acid (C18:4 n-3) to boost tissues levels of the anti-inflammatory eicosapentaenoic acid (EPA, C20:5 n-3) [27-29]. Furthermore, via a thorough a comparison of lipid extraction procedures and subsequent development of an optimised protocol for the extraction of fresh filamentous *Ulva*, a yield of 56 mg.g⁻¹ dry weight equivalent could be achieved.
Chapter 5 explored the effect of environment on the distribution of these important polyunsaturated fatty acids across the phospholipid and neutral lipid fractions. These fractions perform important physiological roles in different cellular structures. In particularly the fatty acid composition of Ulva phospholipids, which constitute between 10-50% of cell membranes and chloroplasts [30, 31] has been poorly explored [32], compared with the typically more abundant glycolipids [33-35]

It is important to consider in which lipids the important omega-3 fatty acids are bound as this may have implications regarding bioavailability of the algal-derived oil. Fish derived PUFA from fish oil are commonly bound within triacylglycerides, however it has been postulated that they may not be as efficient as other lipids classes, such as triacylglycerides [14]. For example, Krill oil is unique in that as it has a substantial proportion of EPA and DHA in abundant phospholipids and this has been used in marketing to differentiate itself from the other typical fish oils. Whilst there is evidence to suggest that polar lipids have a higher bioavailability than nonpolar triacylglycerides further studies are required [15]. Nethertherless, for a marketable algal-derived oil this is an important consideration. Secondly, such information can direct future extraction protocols as different lipids have differing solubilities.

If was found herein that phospholipid fractions were characterised by the presence of five major fatty acids including 16:0, 16:1n-7, 18:1n-7, 18:2n-6 and 18:3n-3 and that the majority of important omega-3’s reside in the remaining neutral fractions (triacylglycerides and glycolipids).
Thus the neutral lipid fraction was highlighted as the richest fraction and target for a nutritionally potent PUFA extract and could direct the future choice of extraction protocol. For example, two feasible options for scaling the oil extraction include either a non-toxic solvent approach such as ethanol [36] or super-critical carbon dioxide, which offers a number of benefits over traditional solvent extractions including:19

- Non-flammable, non-corrosive and inexpensive with no chemical residue;
- Low critical temperature and decreased degradation of food components;
- Higher diffusion coefficients and lower viscosity allowing for rapid matrix penetration and enhanced extraction efficiencies; and
- Selective for compounds of interest and can be recycled.

Phospholipids have a limited solubility in super-critical carbon dioxide thus requiring a co-solvent such as ethanol [38]. For example, a one-step neat super-critical carbon dioxide extraction without ethanol supplementation could optimise a sequential biorefinery process that concentrates the neutral lipids fractions, such as glycolipids, to deliver a nutritionally valuable high-end product with phospholipid by-product rich in alpha linolenic acid (18:3n-3) and linoleic acid 18:2n-6 [38]. It was further found herein that nitrogen-starvation could increase the yield for this neutral lipid compared to the high nutrient conditions, however this was at a detriment to the overall quality of the oil. Thus for products aimed at food and health markets it may be more important to optimise the high quality rather than the quantity of fatty acids through the optimised cultivation of Ulva. This understanding of the fatty acid distribution across different

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19 Rozzi, N.L. and Singh, R., K., Supercritical fluids and the food industry. *Comprehensive reviews in food science and food safety*, 2002. 1: p. 33-44 [37]
lipids can define future selective biorefinery processes. Future investigations of *Ulva* derived oils should further consider the fatty acid distributed across the different neutral lipid classes such as glycolipids and triacylglycerols. In particular, more chemistry-oriented approaches should be employed to identify and quantify lipids, for example identification of phospholipid composition by electrospray mass spectrometry.

The results presented across both Chapters 4 and 5 present the idea that an algal-derived oil from *Ulva* spp. could offset a proportion of the demand for fish derived oils. Encapsulated omega-3 fish oils are highly promoted as part of a balanced diet, and rightly so due to their health benefits associated with foetal development, cardiovascular function, and Alzheimer’s disease [39]. Fish oil is also a valuable additive in feed for aquaculture to boost tissues levels of omega-3 and replacement oils have been sought due to declining wild fish stocks [40]. There is also particular promise for oils high in stearidonic acid (C18:4 n-3) [41], such as is the case with an *Ulva* derived oil. High levels of stearidonic fatty acids are found in seed based oils from *Echium* species, however these oils are deficient in other important omega-3 such as EPA and DHA [42]. Whether the omega-3 PUFA are delivered via the whole food matrix or as an extracted oil supplement, there is additional evidence to suggest the benefits of incorporating algae in aquaculture feeds. For example, the flavour of farmed barramundi was shown to be enhanced, with other organoleptic attributes with an algal diet (*Ulva ohnoi*) [43]. Macroalgal diets have also achieved high levels of important omega-3 fatty acids in abalone [44], along with higher growth rates and overall improved condition of the abalone [45]. Indeed, the latest report from the Food and Agriculture Organisation of the United Nations [46] identified a surging demand in fish
and fish products that can only be met by aquaculture, indicating a pressing need for sustainable sources of beneficial omega-3 PUFA.

One particular limitation to our proposed algal-derived omega-3 oil from *Ulva* is the level of docosahexaenoic fatty acid (DHA), which is only present in trace amounts. In contrast to EPA, tissue levels of DHA can only be boosted with direct supplementation [47]. An interesting future project could consider options to boost the DHA content of the proposed algal-oil. Herein biomass was analysed from cultivations that differed in two growth variables (light and nutrients). Future considerations could consider exploring effects of salinity, pH, and temperature, however natural seasonal fluctuations of these variables were found not to significantly effect total lipids and fatty acid contents of a number of wild species including *Ulva linza* [48]. A more interesting and challenging project might be to explore co-culturing techniques with other algal species or even symbiotic bacteria [49]. A more simple option however may be to consider supplementation of the *Ulva* derived oil with cultured microalgal oils rich in EPA and DHA such as *Synechocystis* [50] and *Isochrysis galbana* [51], respectively. Regardless of the approach, if the both the EPA and DHA content can be successfully boosted beyond levels that are presented herein, or if supplementation to animal feeds becomes a more standard practice to boost tropic bioaccumulation, there is real possibility that an algal oil could offset a proportion of this demand for fish-derived oils in the future.

With regards to human nutrition, the human consumption of seaweed, and thus its beneficial omega-3’s and other bioactive phytochemicals faces a bigger challenge for acceptance, than it does for simple supplementing fish feed. In Western cultures
seaweed as a recognisable whole food has struggled to expand significantly beyond Asian Markets [52]. In cultures that have a more limited tradition in the utilisation of seaweed, the consumption of algal-derived functional food ingredients and/or supplements might be a more feasible option to pursue for consumer acceptance and commercial success [14] (Figure 28).

As mentioned earlier, a proportion of society’s population will struggle to meet dietary requirements for fresh fruit and vegetables. Fruit and vegetables are rich in phytochemicals [53] and include polyphenol compounds such as phenolic acids and flavonoids and terpenoid derived compounds such as carotenoids, lutein, lycopene and β-carotene [54]. Seaweeds are also a source of these beneficial and abundant bioactive phytochemicals [12, 13] and it was hypothesised based on the results of Chapter 3 that the presence of pigments and phenolics may have attributed to the high anti-inflammatory activity observed. Therefore, upon completion of the cultivation investigation (Chapter 4 and 5), the harvested biomass was dried and milled and subsequently analysed for phenolics and pigments as a potential class of metabolites for biorefinery and utilisation.

Whilst phenolics have been reported for Ulva within the literature (Chapter 6) little has been done to explore the effect of cultivation on the phytochemicals of Ulva. High nutrient conditions were again deemed optimal to deliver the highest yields of phenolic content, as well as anti-oxidant activity. Furthermore, a 4-fold increase in phenolic yield is obtained with ethanol extraction as opposed to using acetone as the extraction solvent. In contrast, acetone extracts of high nutrient cultures exhibited the highest
carotenoid content and anti-inflammatory activity. Low nutrients cultures were also correlated to substantially increased flavonoid content. These results again have important implications that can direct future extraction protocols. Furthermore, whilst this thesis presents the characterisation of the anti-inflammatory and anti-oxidant functionalities at the level of extract there are further avenues to pursue and build upon these results through further purification via a bioassay guided fractionation approach combining both molecular and chemistry techniques.

Although a direct cause and effect relationship was not pursued to the level of molecular fractions herein, the clear indications of in vitro anti-oxidative and anti-inflammatory activities of phenolic/pigment rich macroalgae is an adequate position from which to consider applying these seaweeds in nutritional studies. However, how these activities should be applied in a nutritional sense to reduce the deficiencies in the western diet still needs to be considered thoroughly. If direct consumption of these seaweeds or their extracts is required, then the effects of any processing needs to be considered. Here the optimisation of protocols for quantifying bioactive metabolites will be of great use in pursuing this direction of research to quantify effects, as was done in Chapter 5 (further investigations) based on the drying process.

In addition, any toxicity testing (Chapter 2) of the processed products will be of importance as commercially processed and whole biomass effects were not undertaken here. This area should be expanded upon to consider the content of trace elements and metals, which were not investigated here. A full investigation of these attributes should be used to characterise the seaweed biomass from a nutritional sense as well as for
safety. Following this, clinical testing of these seaweeds and their extracts as supplements or food needs to be undertaken to confer that the metabolite attributes and *in vitro* assays can be translated directly into benefits for humans and fortification of inflammatory western diets.
The development of robust analysis methods to determine the quantity and quality of cultivated seaweed is an important first step for the successful commercialisation of algal-derived products to contribute to human and animal nutrition. This could be via consumption as either a whole food, an algae-derived oil or a pigment-rich supplement.
Conclusion

In conclusion, this thesis presents, in journal article style, a combination of published and prepared manuscripts that screens the metabolite profiles and anti-inflammatory activities of a number of South Eastern Australian, macroalgal species. These included endemic and native species of Phaeophyta, Rhodophyta and Chlorophyta. All species screened here exhibited anti-inflammatory activity associated with at least one extract fraction, although there were clear characteristics in the anti-inflammatory activity and FA profiles across the level of phyla, as well as species. In terms of FA profiles, Hormosira banksii (Phaeophyta) is a promising candidate with high levels of PUFA, however Ulva sp. exhibited more favourable Omega-3 ratios, which is important for anti-inflammatory health applications. In addition, Ulva sp. exhibited selective cell toxicity against pancreatic cancer cells \textit{in vitro} and was investigated further for pigment traits that can also explain anti-inflammatory activity. Therefore a range of cultivation conditions and analytical methods were optimised to test the variability and actual content of metabolites with anti-inflammatory potential (Figure 29).

An important requisite in the screening of new natural products is the toxicity for safety concerns. In addition, targeted toxicity assays towards cancerous cell lines is of interest and these aspects were explored in Chapter 2, published as “Bioassays for Anticancer Activities” in the book Metabolomics Tools for Natural Product Discovery Methods and Protocols. Using this screen for toxicity, as well as the FA profiles and anti-inflammatory activities, Chapter 3, published in the \textit{Journal of Applied Phycology}. 

Chapter 7 Discussion & Future Directions

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(impact factor 2.372), identified the important candidates of anti-inflammatory compounds for further investigation aimed towards food and health markets.

Figure 29 Graphical summary of the chapters and corresponding publications presented in this thesis.

A challenge to the adoption of natural products is the establishment of quality-controlled procedures that can provide a comprehensive comparative assessment of the FA of cultivated *Ulva* sp. via a consistent methodological approach. This was developed in Chapter 4, also presented as a published manuscript, which appeared in the journal *Food Chemistry* (impact factor 3.391). A thorough review of FA from the genus *Ulva* identified that there were inconsistencies in the published literature, and a focus on method optimisation targeting these inconsistencies delivered a validated and comprehensive approach to the analysis of FA in *Ulva* sp., taking into account the effects of biomass preparation and extraction protocols. This was essential for the
rigorous comparative work of *Ulva* biomass, which was cultivated in different conditions to determine the effects on FA profiles presented in Chapter 5.

As a result of the findings presented in Chapters 4 & 5, a food safe solvent extraction for FA was explored and it was found that *Ulva* sp. cultivated under optimal growth conditions (high nutrients) can yield 4.1% dry weight (d.w.) of total FA. This is an important finding and represents a doubling of the average reported lipid yield for *Ulva* spp. in the literature (~2% d.w.). Due to this finding and the fact that ethanol as a solvent extracts are also rich in pigments and phenolics, the characterisation of these metabolite classes were explored. This investigation of the phenolics and pigments of cultivated *Ulva* sp., along with associated anti-oxidant and anti-inflammatory activities, is presented as a prepared full research manuscript in Chapter 6. The results presented in Chapter 6 establish that *Ulva* sp., when cultivated under optimal conditions, delivers a high yield of Omega-3 rich FA, pigments and phenolics, with associated anti-inflammatory and anti-oxidant activity.

*Thus, as Aristotle once said “in all things of nature there is something of the marvelous” and indeed Australia’s unexplored bounty of endemic macroalgae has a promising future of marvellous discovery.*
References


APPENDICES
Appendix A

Title Page Published book chapter (Chapter 2)
Appendix B

Protocols for Abelson murine leukaemia virus-induced tumor macrophage (RAW 267.4 cells)\textsuperscript{20}

Protocol for Thawing Raw 264.7 cells

1. Cells are stored in media with 20% DMSO (which helps with freezing down). Therefore there is a need to work quickly. Prepare your biological safety cabinet (BSC) and equipment.

2. Prepare tissue culture media RPMI-1640 with 10% fetal calf serum and 1% GlutaMAX with penicillin (100 U/mL) and streptomycin sulphate (100 µg/mL).
   
   a. \textbf{Note:} Have found that High Glucose DMEM media with 10% FBS (without added Glutamax) works just as well or even better I also found that you so do not need antibiotics

3. Pre-warm media and thaw cryovial of cells in water bath (37 °C)

4. Add 40 ml of culture media to a 50 mL falcon tube. Add whole contents of cryovial (1 mL) to the 40 ml of culture media to dilute DMSO (syringe works well). Spin at 1500 rpm for 5 mins. Pour off supernatant. Using a syringe, resuspend in 2 mL of culture media

\textsuperscript{20}Modifications of methods and feedback originally provided by Associate Professor Ronald Sluyter, School of Biological Sciences, Faculty of Science, Medicine and Health, University of Wollongong, Wollongong, NSW, 2522, Australia
5. Add approximately 23 mL media to 75 cm² tissue culture flask (total volume 25 mL) than add the 2 mL of resuspended cells.

6. Place in incubator with 5% CO₂ at 37 °C.

Protocol for Passaging Raw 264.7 cells

1. Prepare BSC and equipment.

2. Pour off supernatant in tissue culture flask and add ~10 mL fresh media.

3. Scape cells off bottom of flask with tissue culture scraper.
   
   a. **Note**: Contamination can easily occur with this step. Clean scraper packet with ethanol thoroughly before putting in BSC. Be careful when opening to ensure aseptic technique as plastic packet rips off-centre very easily and tears).

4. Swirl flask to make homogenous.

5. Add 2ml of scraped cells suspension to new 75 cm² flask with ~ 23 ml fresh media.

Protocol for Freezing down Raw 264.7 cells

1. The day before put a cryovial container in the -80 °C freezer to cool.

2. Prepare 10 ml of a 20% DMSO solution in culture media in a 50 ml falcon tube (2 ml 100% DMSO in 8 ml culture media). Put on ice.

3. Harvest flasks (5 x 75 cm² flasks by first pouring off supernatant. Add fresh culture media (~8 ml), scrape and pool into a 50 ml falcon tube. Spin for 5 mins at 1500 rpm.

4. Pour off supernatant, resuspend in 10 ml culture media. Put on ice.
5. Label 10 cryovials (2 cryovials per 75 cm$^2$ flask) and removed the cryovial container from the -80 °C freezer.

6. Add the cells slowly (drop wise), whilst continually stirring, to the 20% DMSO solution in CM. This will now be a cell suspension in 10% DMSO in culture media.

7. Aliquot ~1.8 ml of cell suspension in 10 % DMSO in culture media to each cryovial (do not completely fill to the 2 ml mark) to

8. Put into the cryovial container and place container back in --80 °C freezer for 24 hours before storing in liquid nitrogen.

Preparation Phosphate Buffered Saline$^{21}$

Table 24 Preparation for 1X and 10X phosphate buffered saline stock solutions.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount to add (for 1X solution)</th>
<th>Final concentration (1X)</th>
<th>Amount to add (for 10X stock)</th>
<th>Final concentration (10X)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8 g</td>
<td>137 mM</td>
<td>80 g</td>
<td>1.37 M</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2 g</td>
<td>2.7 mM</td>
<td>2 g</td>
<td>27 mM</td>
</tr>
<tr>
<td>$\text{Na}_2\text{HPO}_4$</td>
<td>1.44 g</td>
<td>10 mM</td>
<td>14.4 g</td>
<td>100 mM</td>
</tr>
<tr>
<td>$\text{KH}_2\text{PO}_4$</td>
<td>0.24 g</td>
<td>1.8 mM</td>
<td>2.4 g</td>
<td>18 mM</td>
</tr>
</tbody>
</table>

If necessary, PBS may be supplemented with the following:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount to add (for 1X solution)</th>
<th>Final concentration (1X)</th>
<th>Amount to add (for 10X stock)</th>
<th>Final concentration (10X)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$</td>
<td>0.133 g</td>
<td>1 mM</td>
<td>1.33 g</td>
<td>10 mM</td>
</tr>
<tr>
<td>$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$</td>
<td>0.10 g</td>
<td>0.5 mM</td>
<td>1.0 g</td>
<td>5 mM</td>
</tr>
</tbody>
</table>

1. Dissolve the reagents listed above in 800 mL of H$_2$O. Adjust the pH to 7.4 with HCl, and then add H$_2$O to 1 L.

2. Put the lid on the bottle loosely with autoclave tape and then autoclave it.

Lipopolysaccharide (LPS) Preparation

$^{21}$Provided courtesy of Nathanial Harris, PhD Candidate, Supervisor: Prof. Marie Ranson, School of Biological Sciences, Faculty of Science, Medicine and Health, University of Wollongong, Wollongong, NSW, 2522, Australia
1. Sample supplied as 1 mg lyophilised powder. Resuspend in 1 ml phosphate buffered saline or tissue culture media (1 mg.mL\(^{-1}\) stock solution).

   a. **Note:** a significant amount of LPS may be on the underside of the rubber lid so add 1 ml of PBS, recap and invert to mix. LPS dissolves rapidly.

2. Prepare 25 diluted aliquots in eppendorf tubes by taking 40 µL of stock solution and make up to 1 mL (0.04 mg.mL\(^{-1}\)). Store in -20 freezer. Avoid thaw and freeze cycles.

3. For testing, one 1mL eppendorf is thawed and 25 µL is then added to each well on a 24 well plate for a total volume of 1 mL. Volume required per plate 25 µL. This gives a testing concentration of 1 µg.mL\(^{-1}\).

   a. **Note:** if using a 96 well plate add 3 mL of sterile PBS to the 1 mL aliquot and add 10 µL of diluted LPS solution per well to give a final testing concentration of 1 µg.mL\(^{-1}\)

   b. **Notes 2:** I have found that for the determination of nitric oxide in LPS stimulated RAW264.7 cells that 24 well plate method give much more consistent results across replicate plates and replicate trials

1. Prepare 10 mg.mL\(^{-1}\) stocks of your test compound in 100% DMSO.

   a. **Note:** Weigh your pre-labelled vial (with lid), add portion of extract and evaporate down. Re-weigh you vial (with lid) and prepare your 10 mg.mL\(^{-1}\) using this extract weight and avoid trying to prepare a set mg amount of extract.
b. **Note 2:** Sample vials will be sprayed with ethanol before going into the BSC so use proper labelling and not just marker for labelling your vials. Also make up your stock solutions in the BSC.

c. **Notes 3:** I have found that using brand new vials and preparing stock solution in 100% DMSO in the BSC suffice for sterility. Water soluble samples will need further preparation to ensure they are sterile (sterile filtering or autoclave (solution))

2. Prepare a dilution plate using a standard 96 well plate. Pipette 10 µL sample into 90 µL media (1/10 dilution to give a 1 mg.mL\(^{-1}\) solution in 10% DMSO).

3. This 100 µL of 1 mg.mL\(^{-1}\) solution in 10% DMSO will then further be diluted 1/10 when added to the cells to give a final testing concentration of 100 µg.mL\(^{-1}\) in 1% DMSO. This achieved by added 100 µL of 1 mg.mL\(^{-1}\) solution to 875 µL cells (1.5 x 105 cell.mL\(^{-1}\)) and 25 µL of LPS (24 well plate method, adjust accordingly if needed for 96 well plate assay)

4. Test each sample in duplicate (minimum) which allow for four text samples per 24 well plate (**Figure 30**)  

   a. **Note:** With microplate (96-well plate) preparation it is standard practice to avoid use of the outside well due to the “edge effect”. Therefore avoiding the outside perimeter wells should be considered when modifying this example set up for a 96 well plate assays.
Figure 30 Example preparation for a 24 well plate for the testing of four samples at one concentration.
- S1-S4 Test sample with LPS;
- S1-S4 Test sample with PBS (Blank);
- Positive control (100% LPS-stimulated nitric oxide production)
- Negative control (Blank, 100% normal nitric oxide production in non LPS-stimulated cells.)

<table>
<thead>
<tr>
<th></th>
<th>S1 + LPS</th>
<th>S1 + LPS</th>
<th>S1 + PBS</th>
<th>S1 + PBS</th>
<th>1% DMSO + LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>S2</td>
<td>S2 + LPS</td>
<td>S2 + LPS</td>
<td>S2 + PBS</td>
<td>S2 + PBS</td>
<td>1% DMSO + LPS</td>
</tr>
<tr>
<td>S3</td>
<td>S3 + LPS</td>
<td>S3 + LPS</td>
<td>S3 + PBS</td>
<td>S3 + PBS</td>
<td>1% DMSO + PBS</td>
</tr>
<tr>
<td>S4</td>
<td>S4 + LPS</td>
<td>S4 + LPS</td>
<td>S4 + PBS</td>
<td>S4 + PBS</td>
<td>1% DMSO + PBS</td>
</tr>
</tbody>
</table>
Appendix C

Title Page - Published Manuscript (Chapter 3)

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

Janice I. McCauley · Barbara J. Meyer · Pia C. Winberg · Marie Ranson · Danielle Skropeta

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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.

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

Introduction

Macroalgae are a large and growing multibillion dollar food and biotechnology industry (FAO 2012; Bixler and Porse 2011; 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 (Wanade 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 (No 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.
Appendix D

Supplementary Methods

Thin Layer Chromatography (TLC)

For the preliminary assessment of extracts and to determine the solvent system for purification by flash chromatography, thin layer chromatography was used. TLC plates (3.5 cm x 9.0 cm) were cut from commercially available aluminum backed sheets of Silica Gel 60 F_{254} plates. The crude extracts (approx. 25 mg/ml) were transferred onto the plates by capillary action and the plates were eluted in a closed chamber with various mobile phases. After drying, the retention factors (rf) for any visible or UV active components were recorded. The plates were then further stained using iodine vapor, Hanessian’s stain (comprising 90 mL water, 5 g ammonium molybdate, 1 g cerium sulfate and 10 mL concentrated sulfuric acid) and an anti-oxidant stain (comprising of 0.2% w/v 2,2-diphenyl-2-picrylhydrazyl (DPPH) in methanol).

Flash Column Chromatography

Flash column chromatography using silica gel was used to purify extracts. Solvent system chosen based on TLC (Table 25). Column fractions combined on the basis of TLC and dried on the rotatory evaporator (Table 26). Weight recorded for each dried fraction to determine mass recovery (Table 27). Combined dried fractions were tested for biological activity and only the high yielding fractions with strong activity where analysed by reverse-phase high-pressure liquid chromatography (RP-HPLC).
Table 25 Solvent system chosen for flash column chromatography for *Hormosira banksii* ethyl acetate extract based on thin layer chromatography (TLC)

<table>
<thead>
<tr>
<th>Species</th>
<th>Extract</th>
<th>Solvent System</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hormosira banksii</em></td>
<td>Ethyl Acetate</td>
<td>Chloroform (100 %): Methanol (50 %): Chloroform/Methanol/Water (6/4/1): Methanol (100 %)</td>
</tr>
</tbody>
</table>

Table 26 Summary of combined fractions after flash column chromatography of *Hormosira banksii* ethyl acetate extract. Fractions combined based on TLC.

<table>
<thead>
<tr>
<th>Species</th>
<th>Extract</th>
<th>Fraction #</th>
<th>Combined Column Fraction</th>
<th>Eluent</th>
<th># Spot TLC</th>
<th>Mass (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hormosira banksii</em></td>
<td>Ethyl Acetate</td>
<td>1</td>
<td>#1-10</td>
<td>95% CHCl3</td>
<td>2</td>
<td>37.2</td>
</tr>
<tr>
<td>2</td>
<td>#11-27</td>
<td>92.5% CHCl3</td>
<td>2</td>
<td>27.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>#28-40</td>
<td>90% CHCl3:</td>
<td>1</td>
<td>36.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>#41-51</td>
<td>80% CHCl3</td>
<td>1</td>
<td>22.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>#52-54</td>
<td>70% CHCl3</td>
<td>2</td>
<td>15.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>#55-60</td>
<td>50% CHCl3</td>
<td>1</td>
<td>16.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>#61</td>
<td>Magic</td>
<td>1</td>
<td>1.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>#62-64</td>
<td>Magic</td>
<td>2</td>
<td>23.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>#65</td>
<td>Magic</td>
<td>2</td>
<td>36.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>#66-67</td>
<td>Magic</td>
<td>4</td>
<td>7.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>#68</td>
<td>Magic</td>
<td>3</td>
<td>12.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>#69-71</td>
<td>Magic</td>
<td>1</td>
<td>14.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>#72</td>
<td>Magic</td>
<td>2</td>
<td>31.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>#73</td>
<td>Magic</td>
<td>3</td>
<td>26.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>#74-75</td>
<td>Magic</td>
<td>1</td>
<td>5.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>#76-77</td>
<td>Magic</td>
<td>1</td>
<td>3.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>#78-79</td>
<td>Magic</td>
<td>1</td>
<td>4.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>#80-81</td>
<td>Magic</td>
<td>1</td>
<td>2.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>#82-83</td>
<td>Magic</td>
<td>1</td>
<td>5.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>#84-86</td>
<td>Magic</td>
<td>1</td>
<td>1.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>#87-88</td>
<td>Magic</td>
<td>1</td>
<td>1.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>#89</td>
<td>Magic</td>
<td>2</td>
<td>1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>#90</td>
<td>Magic</td>
<td>2</td>
<td>0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>#91-93</td>
<td>Magic</td>
<td>1</td>
<td>9.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>#94</td>
<td>Magic</td>
<td>1</td>
<td>3.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>#95-97</td>
<td>Magic</td>
<td>1</td>
<td>25.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>#98</td>
<td>Magic</td>
<td>1</td>
<td>12.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>#99</td>
<td>Magic</td>
<td>2</td>
<td>2.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>#100-134</td>
<td>Magic</td>
<td>1</td>
<td>4.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>#135-147</td>
<td>Magic</td>
<td>1</td>
<td>3.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>#148</td>
<td>Magic</td>
<td>1</td>
<td>12.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>#149</td>
<td>Magic</td>
<td>1</td>
<td>2.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>#150</td>
<td>Magic</td>
<td>1</td>
<td>3.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>#151-159</td>
<td>Magic</td>
<td>1</td>
<td>25.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>35</td>
<td>#160-161</td>
<td>Magic</td>
<td>1</td>
<td>12.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>MeOH wash</td>
<td>0% MeOH</td>
<td>1</td>
<td>2.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 27 The mass recovery for Hormosira banksii ethyl acetate extract for the combined 36 fractions after flash column chromatography on silica gel.

<table>
<thead>
<tr>
<th>Species</th>
<th>Extract</th>
<th>Initial Weight (mg)</th>
<th># Combined Fractions</th>
<th>Mass Combined Fraction (mg)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hormosira banksii</td>
<td>Ethyl Acetate</td>
<td>610 mg</td>
<td>36</td>
<td>577.7</td>
<td>95</td>
</tr>
</tbody>
</table>

Reverse-Phase - High Pressure Liquid Chromatography (RP-HPLC)

Analytical RP-HPLC was performed on a Shimadzu system with either a dual UV detector or PDA detector and Phenomenex Luna C\textsubscript{18} column (4.6 mm x 250 mm). Preparative RP-HPLC was performed on a Waters system with dual UV detector using a Waters C\textsubscript{18} column (19 x 150 mm). Mobile phase for both analytical and preparative RP-HPLC outlined in Table 28.

Table 28 Solvent system used for both preparative and analytical -HPLC

<table>
<thead>
<tr>
<th>Species</th>
<th>Extract</th>
<th>Solvent System</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hormosira banksii</td>
<td>Ethyl Acetate</td>
<td>Water (100%): Methanol (100%) (Increasing gradient over 40 mins run time)</td>
</tr>
</tbody>
</table>
Appendix E

Supplementary results

Figure 31 Analytical RP-HPLC trace for *Hormosira banksii* ethyl acetate extract, fraction # 10

Figure 32 Analytical RP-HPLC trace for *Hormosira banksii* ethyl acetate extract, isolated peak 8 (via preparative RP-HPLC) from fraction # 1
Parameters affecting the analytical profile of fatty acids in the macroagal genus Ulva

Janice I. McCauley, a,⁎ Barbara J. Meyer, b Pia C. Winberg, b,c Danielle Skropeta a,d

a School of Chemistry, Faculty of Science, Medicine, and Health (SMAH), University of Wollongong, NSW 2522, Australia
b Centre for Medical and Molecular Bioscience, University of Wollongong, Wollongong, NSW 2522, Australia
 c Venus Shell Systems Pty Ltd, Narrawallee, NSW 2539, Australia
 d Corresponding author. E-mail address: jim479@uowmail.edu.au

The feasibility of cultivating marine macroalgae, as a sustainable source of essential FA for human and animal nutrition, to offer a sustainable source of essential FA for human and animal nutrition, means that it is well suited for cultivation. Therefore as whole biomass or as part of a sequential bioenergy system of production, cultivated Ulva biomass has the potential to offer a sustainable source of essential FA for human and animal consumption and nutrition.

The feasibility of cultivating marine macroalgae, as a sustainable source of high value products is largely dependent on the cost-effectiveness and scalability of cultivation to deliver high yields of quality controlled biomass. Ulva is a cosmopolitan genus (Fig. 1) and can readily adjust its metabolism under specific environmental conditions, whereas the lipids and FA play an important role in this adaptation (Nesterov, Bugstream, & Bugstream, 2013). As such, the profiles of reported FA for Ulva show a wide degree of natural variation. For example Ulva intestinalis has demonstrated an increase in unsaturation with increasing mineralisation in a study of a number of abiotic factors (Nesterov et al., 2013). Seasonal comparison of Ulva lactuca samples collected in Western Ireland showed a notable decrease in the n-6/n-3 ratio when collected in November (cf. June) (Schmid, Guibert, & Stengel, 2014). Furthermore, the range of reported n-6/n-3 values were also more favourable when compared to an U. lactuca specimen collected from Northern Chile (Ortiz et al., 2006). However, one of the appeals of Ulva in particular as a target genus, is its ability to be grown effectively and rapidly in culture, while tailoring PUFA profiles through nutrient replete conditions, which have been demonstrated on the laboratory scale (Ak, Öztürk, Özüg, & Göksan, 2015). It has also been shown that culture conditions including temperature (An et al., 2013), pH (Spilling, Bystrøm, Enns, Røcher, & Sveavågen, 2013) and nutrient availability along with the presence of organic carbon (Ak et al., 2015), will also influence the lipid profile of many algae including Ulva spp. Despite this, there are species-specific patterns within that variability that can be determined with experimental means and

⁎ Corresponding author.
E-mail address: jim479@uowmail.edu.au (J.I. McCauley).

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