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**Is there lignin in the *Ulva* cell wall? A
multidisciplinary structural investigation to
provide new insights into cell wall evolution
and macroscopic complexity in the
chlorophyte green seaweeds**

A thesis submitted for the degree of Doctor of Philosophy



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Abstract

Seaweeds are not only an integral component of the marine ecosystem via their role in global carbon and sulfur cycles, but also have significant economic value as crops for food and fuel, with certain intertidal seaweeds like the green 'sea lettuce' *Ulva* (Chlorophyta) appearing as attractive bioenergy feedstocks due to their rapid growth rates and polysaccharide-rich cell wall that can comprise half its total biomass.

Seaweeds have a distinct biochemistry to traditional land plant crops. For example, they lack the phenylpropanoid pathway, a key milestone in land plant evolution that enabled the biosynthesis of the secondary cell wall biopolymer lignin which confers structural support and facilitates water transport in vascular plants.

Despite this, 'lignin-like' fractions are reported in *Ulva*, and lignin has been found in a coralline red seaweed (Rhodophyta). No alternative pathway for lignin biosynthesis is provided by our current metabolic knowledge, meaning we have limited understanding of how 'lignin' arose in seaweeds. Furthermore, the only comprehensive structural investigation into seaweed 'phycolignin' to date has been performed in the coralline reds. Consequently, the presence of an equivalent component in green seaweeds like *Ulva* is still debatable.

As the primary aim of this thesis, I investigate the identity of the proposed lignin-like fraction of *Ulva* using confocal microscopy, biochemical assays, and biophysical analysis. To accomplish this, I evaluate the use of a sequential extraction protocol described for charophyte green algae (Streptophyta) to determine which cell wall polysaccharides the proposed structure associates with.

No evidence for lignin-like structures in the *Ulva* cell wall was identified during this research. Instead, I propose that the previous attributions to lignin in *Ulva* were misidentifications on account of the limitations of quantification protocols used. Interestingly, a major structural protein component is identified with possible implications for how *Ulva* mitigates osmotic stress at low tide. The absence of 'phycolignin' in the *Ulva* cell wall contrary to the lignified coralline seaweeds demonstrates that seaweeds display diverse adaptations to intertidal habitats, and provides support to the current hypothesis that lignin arose convergently in the red lineage, with green seaweeds and land plants sharing a more recent evolutionary history.

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List of Abbreviations

Common scientific abbreviations are used for mass, volume, and molarity. Standard IUPAC nomenclature is used for amino acids and glycans (Gold, 2019).

A230	Absorbance reading at 230 nm
A260	Absorbance reading at 260 nm
A280	Absorbance reading at 280 nm
Abs.	Absorbance
ABSL	Acetyl bromide soluble lignin
ACT	Actin
AGP	Arabinogalactan protein
AIC	Akaike information criterion
AICc	Corrected Akaike information criterion
AIR	Alcohol insoluble residue
ANOVA	Analysis of Variance
ASW	Artificial seawater
ATR	Attenuated total reflectance
ATR-FTIR	Attenuated total reflectance Fourier transform infrared spectroscopy
<i>BiP</i>	Binding protein
BLAST	Basic Local Alignment Search Tool
BUSCA	Bologna Unified Subcellular Component Annotator
cDNA	Complementary DNA
CI	Confidence Interval
CIR	Cellulase insoluble residue
CLSM	Confocal laser scanning microscopy
cm ⁻¹	Reciprocal wavelength
COSY	Correlation spectroscopy
CP-MAS	Cross polarisation magic angle spinning
CWP	Cell wall protein
DFRC	Derivatisation followed by reductive cleavage
DMSO	Dimethyl sulfoxide
DMSO-d ₆	Deuterated DMSO
DNA	Deoxyribonucleic acid
DOSY	Diffusion ordered spectroscopy
dPCR	Direct PCR
DTT	Dithiothreitol
ECM	Extracellular matrix

FTIR	Fourier transform infrared spectroscopy
<i>GAPDH</i>	Glyceraldehyde 3-phosphate dehydrogenase
GC	Gas chromatography
gDNA	Genomic DNA
<i>H3</i>	Histone 3
Ha	Alkali soluble cell wall fraction (acid precipitated material)
Hb	Alkali soluble cell wall fraction (non-precipitated material)
HGT	Horizontal gene transfer
HMBC	Heteronuclear Multiple Bond Correlation
HMMER	Biosequence analysis using profile hidden Markov models
HRGP	Hydroxyproline-rich glycoprotein
HSP	Heat shock protein
<i>HSP20</i>	Heat shock protein 20 family
<i>HSP70</i>	Heat shock protein 70 family
HSQC	Heteronuclear Single Quantum Coherence
LC	Liquid chromatography
MS	Mass spectrometry
Mw	Molecular weight
NCBI	National Centre for Biotechnology Information
nm	Nanometres
NMR	Nuclear magnetic resonance
No RT	No reverse transcriptase control
NTC	No template control
ORCAE	Online resource for community annotation of eukaryotes
P1	Rapidly oxalate-soluble cell wall fraction
P2	Slowly oxalate-soluble cell wall fraction
<i>PAL</i>	Phenylalanine ammonia lyase
PCR	Polymerase chain reaction
ppm	Parts per million
ppt	Parts per thousand
qPCR	Quantitative polymerase chain reaction
<i>rbcl</i>	Large subunit of ribulose biphosphate carboxylase
RFU	Relative fluorescence units
RNA	Ribonucleic acid
RSS	Residual sum of squares
RT	Reverse transcriptase
SHSP	Small heat shock protein family
TAIR	The Arabidopsis Information Resource
TLC	Thin layer chromatography
TOCSY	Total correlation spectroscopy

<i>TUA</i>	α -tubulin
<i>TUB</i>	β -tubulin
<i>tufA</i>	Elongation factor Tu
UV-Vis	Ultraviolet-visible spectroscopy
v/v	volume/volume
w/v	weight/volume
w/w	weight/weight
‰	Parts per thousand (salinity)

Declaration

I declare that all the work in this thesis is based on research carried out at the Department of Biosciences, Durham University, United Kingdom. No part of this thesis has been submitted elsewhere for any other degree qualification and all represents my own work unless specified otherwise in the text.

Statement of Copyright

The copyright of this thesis rests with the author. No quotation from it should be published without the author's prior written consent and information derived from it should be acknowledged.

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1. Introduction

1.1 Introduction to Cell Walls

Cell walls have arisen repeatedly across natural history, found in both the prokaryote and eukaryote kingdoms. In the prokaryotes, we can observe peptidoglycan cell walls in bacteria and various proteinaceous and polysaccharide-containing cell walls in the Archaea (Dörr, Moynihan and Mayer, 2019; Klingl, Pickl and Flechsler, 2019). In the eukaryotes, we observe chitinous cell walls in fungi and a diverse range of cell walls in the photosynthetic eukaryotes – typically constructed of protein and structural polysaccharides, and polyphenols such as lignin are observed in plants (Percival, 1979; Xie and Lipke, 2010; Domozych *et al.*, 2012). Perhaps the most unique cell walls are observed in the diatoms, which are enclosed in a silica-rich skeleton (Ellegaard *et al.*, 2016; Mayzel *et al.*, 2021).

Developing a structural cell wall (perhaps more accurately described as an extracellular matrix) was a key innovation in protecting cells against a wide array of biotic and abiotic factors. Cell walls protect against pathogens such as viruses and toxic compounds, while also maintaining the cell during mechano-osmotic stress (Buchmann and Holmes, 2015; Holzinger *et al.*, 2015; Vojvodic *et al.*, 2020; Zeroual *et al.*, 2020). However, cell walls do not just have a steric role; they also have an active function in cell-cell communication, environmental sensing, and signalling (Shiu and Bleecker, 2001; Galindo-Trigo, Gray and Smith, 2016; Wolf, 2017; Cronmiller *et al.*, 2019). Nor, finally, are they static – they remodel dynamically during cell growth, cell division, and cellular stress responses (Anderson *et al.*, 2010; Holzinger *et al.*, 2015; Castilleux *et al.*, 2018; Wu, Bulgakov and Jinn, 2018).

The study of cell walls also has many ‘real-world’ applications. In biomedicine, understanding the bacterial cell wall is essential for determining the molecular basis of disease in pathogens, and to identify targets for antibiotics (Bush, 2012; Felgueiras *et al.*, 2021). From an economic perspective, the cellulosic cell walls of plants are essential in many industries, including paper manufacture, textile production, and bioenergy production (John *et al.*, 2011; Moral *et al.*, 2019; Mukherjee and Keshri, 2019; Qiao *et al.*, 2019; Felgueiras *et al.*, 2021). Lignin, often a by-product of these applications, may be useful as a feedstock for bioenergy production and can be broken down into many industrially valuable chemicals (H. Wang *et al.*, 2019; Vavilala, Ghag and D’Souza, 2019).

Also, the water-soluble pectins from the plant cell wall are valued for their gelling properties in the food industry (Thakur *et al.*, 1997; Waldron, Parker and Smith, 2003)

As stated, the photosynthetic eukaryotes, comprising modern-day algae and plants, show remarkable diversity in their cell walls – with some marine algae (plankton) lacking a functional cell wall, other algae having cell walls constructed almost entirely out of proteins, and macroscopic seaweeds and land plants ‘polysaccharide rich’ having fibrillar cell walls. Furthermore, plants appear to be the only group of organisms with distinct primary and secondary cell walls – although secondary cell walls may be present in calcified red seaweeds (Martone *et al.*, 2009).

Interestingly, cell wall formation appears to correlate with multicellularity in these organisms (Domozych and Domozych, 2014). Understanding the nature of how organisms have repeatedly undergone unicellular-multicellular transitions is of great interest in evolutionary biology, with algae appearing as ideal model organisms to study why this occurs due to the array of algal morphologies we can observe (Umen, 2014; Coates, Umm-E-Aiman and Charrier, 2015). For example, algae range from the smallest known eukaryote *Ostreococcus tauri* with a diameter of less than 1 μm , to the giant kelps (*Macrocystis pyrifera*) that can grow over 50 m in size (Raymont, 1980; Palenik *et al.*, 2007). The machinery to synthesise some of the cell wall components characterised in land plants, such as arabinogalactan proteins and xylans, appears to have arisen quite early on in the evolutionary history of algae, even in lineages that lack cell walls (Popper and Tuohy, 2010). Also, whilst not all cell wall-producing algae synthesise cellulose, the most abundant natural polymer on Earth, ‘non-cellulose producing’ green algae are reported to encode a cellulose synthase gene, which has since undergone gene family expansion in land plants (Yin, Huang and Xu, 2009). However, there are many examples of convergent evolution of structural features in algal cell walls (Martone *et al.*, 2009), in addition to novel ‘lineage-specific’ cell wall components (Lerouge *et al.*, 2021). For this reason, algal cell wall evolution appears as a topic that warrants much more attention (Domozych *et al.*, 2012; Mikkelsen *et al.*, 2014; Baudet *et al.*, 2017).

Whilst the cell wall was first identified and studied in plants and is studied extensively in modern plant biology, from an evolutionary perspective, plants are an anomaly amongst photosynthetic eukaryotes, as a lot of the biochemical and morphological complexity we observe in plants likely resulted from the transition of the ancestor of plants from an aquatic to terrestrial habitat, which exposed it to a range of new environmental challenges (Fürst-Jansen, De Vries and De Vries, 2020). However,

research of the closest algal relatives to plants indicates that the aquatic ancestor of plants may have already had a cell wall with a polysaccharide composition similar to those seen in land plants today (albeit more straightforward), which is indicative of an aquatic origin (Sørensen *et al.*, 2011; Mikkelsen *et al.*, 2014; O'Rourke *et al.*, 2015). By contrast, seaweeds (marine macroalgae) evolved unique cell walls and acquired multicellularity independently in an aquatic environment several times across three algal lineages – the red (Rhodophyta), brown (Phaeophyta), and green (Chlorophyta).

As a result, algae are key to describing cell wall evolution, investigating why microscopic-macroscopic transitions have reoccurred throughout natural history, and understanding the ecological reasons for cell wall evolution (Umen, 2014). Contrasting seaweeds and plants will help us identify conserved or convergent features in these organisms that may have contributed to multicellularity in distinct environments.

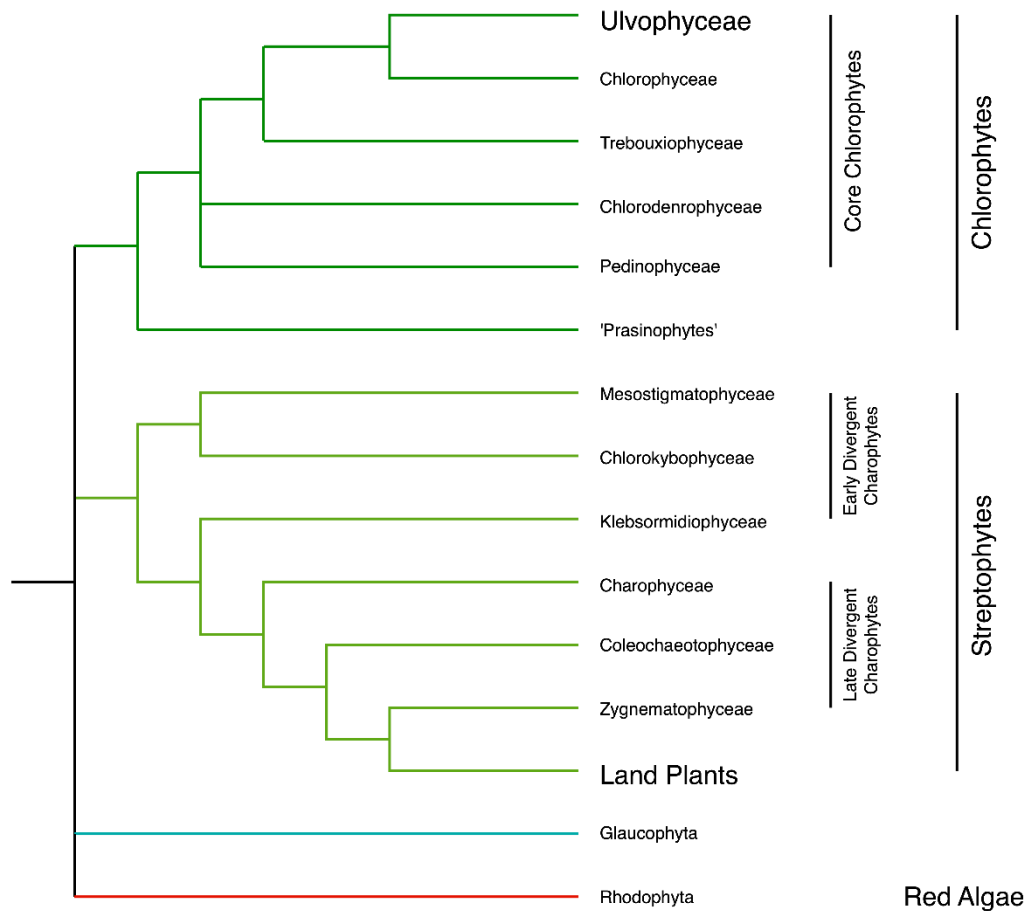


Figure 1.1. Predicted evolutionary history of the Archaeplastida, which acquired a plastid via primary endosymbiosis. The 'green lineage' consists of the streptophytes – the charophyte green algae and land plants, and the chlorophyte green algae. Glaucophytes are a relatively small group of freshwater microalgae, whilst rhodophytes are the red algae. Phaeophytes (brown algae) acquired plastids by secondary endosymbiosis and are not part of this lineage.

1.2 The World of Algae

The story of how distinct algal groups arose is a complex one, and the precise evolutionary relationships between the different groups are difficult to determine definitively, and are much debated (Cavalier-Smith, 1981; Fang *et al.*, 2017; Lee, 2018). However, based on our current molecular understanding, we can group algae by when and how they acquired their plastids, hence the ability to photosynthesise (Burki *et al.*, 2020).

The Archaeplastida – including the Rhodophyta (red algae) and the Chloroplastida (informally referred to as the ‘green lineage’), and the glaucophyte algae have a primary plastid (De Clerck, Bogaert and Leliaert, 2012; Price *et al.*, 2016). Whilst the nature of endosymbiosis in algae is still a subject of much debate, the commonly accepted view is the plastid in these algae was thought to be acquired directly by endosymbiosis of a photosynthetic prokaryote (i.e., a cyanobacterium) probably approximately 1.5 billion years ago (Yoon *et al.*, 2004). The brown algae, the Phaeophyta, have secondary plastids, which means the ancestor of these algae acquired their plastids by endosymbiosis of a cell that had already undergone endosymbiosis (hence, secondary endosymbiosis). Unlike the red and green lineages, all the Phaeophyta are multicellular seaweeds (e.g., kelps and fucoid algae), whereas the red and green algae can have unicellular, multicellular, or macroscopic forms (Yoon, Zuccarello and Bhattacharya, 2010; De Clerck, Bogaert and Leliaert, 2012; Hurd *et al.*, 2014; Fang *et al.*, 2017). In the case of the Stramenopile eukaryotes (which includes the brown algae, unicellular diatoms, and several other diverse protists), secondary endosymbiosis is hypothesised to have occurred via the acquisition of at least one, possibly more red algae. Other groups of algae (e.g., euglenids, chlorarachniophytes, and haptophytes) likely acquired plastids from similar secondary endosymbiosis events, and in the case of the dinoflagellates, tertiary endosymbiosis may have occurred (Rockwell, Lagarias and Bhattacharya, 2014; Lee, 2018; Burki *et al.*, 2020; Stephens *et al.*, 2021).

Of all these groups, the green lineage appears to have been the most extensively studied, with this monophyletic group containing all green algae and the land plants. The green lineage is estimated to have diverged over 700 million years ago into two clades; streptophytes and chlorophytes (Leliaert *et al.*, 2012; Becker, 2013; Kumar *et al.*, 2017). The former clade, the streptophytes, consists of the charophytes – a group of freshwater algae, and the embryophytes – which we know better as the land plants. On the other half of the split lie the chlorophyte green algae. Whilst the chlorophytes are

primarily aquatic; there are a few terrestrial representatives and lichen-forming species (Chapman *et al.*, 2001; Pichler *et al.*, 2020). This clade is highly diverse, consisting of unicellular plankton, multicellular seaweeds, and a range of colonial, filamentous, and siphonous representatives (Brodie, Maggs and John, 2008; Cocquyt *et al.*, 2010; Fang *et al.*, 2017).

Whilst the study of land plants has an immediately apparent economic value (i.e., for improving the output of land crops) and the charophyte algae (particularly the 'late divergent' taxa) are of interest in studies of land plant evolution (Domozych, Popper and Sørensen, 2016), many of the chlorophyte green algae have untapped potential for various biotechnology applications (Bolton *et al.*, 2016; Trivedi *et al.*, 2016; Zhang *et al.*, 2016; Moreira *et al.*, 2022). As marine organisms, the chlorophytes play a critical ecological role in sulfur cycles and primary production, with the planktonic chlorophytes estimated to contribute ~20% of global primary production (Lyons, Scheibling and van Alstyne, 2010; Rousseaux and Gregg, 2013; van Alstyne *et al.*, 2016). The efficiency of carbon fixation in both the microalgal and macroalgal chlorophytes has also made them attractive targets for carbon capture processes (Moreira and Pires, 2016; Duarte *et al.*, 2017; G. Gao *et al.*, 2018; Onyeaka *et al.*, 2021). Due to their diverse forms and the observation that multicellularity arose independently many times within this lineage, the chlorophyte algae provide excellent evolutionary models for studying why transitions to multicellularity have occurred recurrently throughout natural history (Umen, 2014). However, our understanding of the biology of chlorophytes, particularly green seaweeds, is limited.

1.3 Chlorophytes: A Diverse Group of Green Algae

1.3.1 What are Chlorophytes?

The most numerous group of chlorophytes are the prasinophytes, a paraphyletic group of marine phytoplankton (Fang *et al.*, 2017). Despite being a unicellular clade, the prasinophytes are diverse – with some coccoid and flagellate representatives and some species lacking a functional cell wall (Becker, Marin and Melkonian, 1994). The position of these algae in the taxonomic tree has not been resolved, with ‘prasinophyte’ algae being found in a series of often debated clades. However, these remain an ecologically important group of algae, with two of the better-known studied species including *Ostreococcus* and *Micromonas* (Mameiellophyceae) (Palenik *et al.*, 2007; van Baren *et al.*, 2016; Tragin and Vaulot, 2019). Microplankton are ecologically important due to their primary production and role in marine ecosystems (Worden *et al.*, 2015; Onyeaka *et al.*, 2021). Furthermore, their relatively simple genomics, cellular organisation, and ease of manipulation in the lab provide ideal model organisms for biological research. For example, *Ostreococcus tauri* has been used extensively for research into the molecular basis of stress signalling and the circadian clock (Eckardt, 2009; Morant *et al.*, 2010; Thommen *et al.*, 2010, 2012; Djouani-Tahri *et al.*, 2011; Hindle *et al.*, 2014)

The second group of chlorophytes, the ‘core chlorophytes’ represent a highly diverse group of green algae thought to have evolved from one of the planktonic prasinophyte clades (Fučíková *et al.*, 2014; Fang *et al.*, 2018). The main representatives of the core chlorophytes include the Trebouxiophyceae (including the unicellular freshwater alga *Chlorella* and the lichen-forming *Trebouxia*), the Chlorophyceae (including the ‘volvocine’ algae - unicellular *Chlamydomonas* and the ‘multicellular’ *Volvox*), and also the Ulvophyceae (containing green macroalgae such as the seaweeds *Ulva*, *Cladophora*, and *Caulerpa*) (John, 2003; Cocquyt *et al.*, 2010; Umen, 2014; Fang *et al.*, 2017; Hou *et al.*, 2022). In addition to these groups, two unresolved smaller clades of unicellular algae, Pedinophyceae and Chlorodendrophyceae, are often included in the core chlorophyte classification (Fang *et al.*, 2017).

The microalgal core chlorophytes (e.g., *Chlamydomonas reinhardtii*) have often been exploited for economic use and used by plant biologists as a model for understanding physiological processes in higher plants (Harris, 2001; Fu *et al.*, 2019). In the case of *Chlamydomonas*, the simple growth requirements and the establishment of a series of genetic tools to facilitate molecular biology in these organisms have established them as important model species in plant science (Zhang, Wang and Wang, 2021). Both land

plants and chlorophytes are united by their ability to synthesise chlorophyll a and chlorophyll b, as well as other photosynthetic pigments such as carotenoids and xanthophylls (Couso *et al.*, 2012; Christa *et al.*, 2017; He *et al.*, 2018). Furthermore, both algae and land plants store starch granules in the chloroplast (Malinova *et al.*, 2018; Prabhu *et al.*, 2019), and cellulose fibres can be synthesised by some chlorophytes (but not all) for the cell wall (Wahlström, Edlund, *et al.*, 2020; Goma *et al.*, 2022). As a result, microalgae can often be used as a fast-growing proxy for plants in physiological experiments— as plants take a while to grow. However, there are many fundamental biochemical differences between plants and algae that are important to note from an evolutionary perspective (see this section cf. 1.3.2.1).

From a commercial perspective, certain microalgae such as *Haematococcus* (Chlorophyceae) produce economically valuable pigments, whilst others such as *Chlorella* (Trebouxiophyceae) are grown for their nutritional value (Guerin, Huntley and Olaizola, 2003; Ru *et al.*, 2020). Some microalgae such as *Botryococcus braunii* (Trebouxiophyceae) and *Scenedesmus obliquus* (Chlorophyceae) can be cultivated for bioenergy purposes, particularly for biodiesel production due to the remarkable concentrations of hydrocarbons they can produce (Wijffels and Barbosa, 2010; Abomohra, El-Sheekh and Dieter, 2014; Hirano *et al.*, 2019). Filamentous chlorophytes such as *Oedogonium* also show promise for similar biodiesel production and starch biosynthesis (Zhang *et al.*, 2016). However, the macroalgal chlorophytes (i.e., the ‘green seaweeds’) are increasingly appearing to be attractive as bioenergy crops, with the recovery of valuable products from microalgae often being an inefficient process on an industrial scale (Barros *et al.*, 2015).

Green seaweeds such as *Ulva* and *Monostroma* are cultivated extensively in the Pacific and Southeast Asia as a food crop (Lindsey Zemke-White and Ohno, 1999; Radulovich *et al.*, 2015; Buschmann *et al.*, 2017; Cui *et al.*, 2021). Furthermore, the high protein content and nutritional composition of green seaweeds such as *Ulva* suit them for various aquaculture applications, such as feed and biofiltration, because of their ability to sequester heavy metals and metabolise ammonia, nitrates, and phosphates that may be toxic to other species (Bolton *et al.*, 2009, 2016; Kang, Park and Chung, 2011; Charrier *et al.*, 2017; Moreira *et al.*, 2022). Green seaweeds are also characterised by high levels of antioxidant production and may also have pharmaceutical and nutritional value for humans (Mezghani *et al.*, 2016; Gomez-Zavaglia *et al.*, 2019; Jacobsen *et al.*, 2019).

The polysaccharide-rich biomass of seaweeds makes them ideal feedstocks for hydrolysis into bioethanol and biogas (Kraan, 2013; Bikker *et al.*, 2016; Trivedi *et al.*, 2016). For example, cell wall polysaccharides comprise ~50% (w/w) of the dry biomass in *Ulva* (Lahaye and Robic, 2007), with the majority of the *Ulva* extracellular matrix composed of a group of sulfated rhamnoglucuronans called ulvans, which may also have utility in various biomaterials and biomedical applications due to their unique biochemical properties (Chiellini and Morelli, 2011; Alves *et al.*, 2012; Kidgell *et al.*, 2019; de Carvalho *et al.*, 2020). Green seaweeds also exhibit rapid growth rates with simple growth requirements and can be grown perhaps even competitively with microalgae (Bolton *et al.*, 2009). These growth rates are a clear advantage over typical land plant crops used for bioenergy, and as aquatic organisms, seaweeds do not compete for land resources. For example, the fastest growing multicellular alga reported currently, *Ulva meridionalis*, is reported to quadruple itself daily under optimum growth conditions in the lab (Hiraoka *et al.*, 2020; Tsubaki *et al.*, 2020).

However, the seaweeds are understudied relative to the microalgae, and the economic viability of cultivating green seaweeds as a 'single use' bioenergy crop in the Western hemisphere is debated (van den Burg *et al.*, 2016). To exploit *Ulva* commercially, a biorefinery approach has been proposed as a more economically viable way of making the best use of *Ulva* biomass (Bikker *et al.*, 2016; Magnusson *et al.*, 2016; Trivedi *et al.*, 2016; Prabhu *et al.*, 2020). Here, the valuable components of algal biomass are extracted sequentially – so that the biomass is used for multiple purposes instead of just one, with a multi-use crop being more valuable than a single-use crop (van den Burg *et al.*, 2016). Nonetheless, this approach appears necessary for helping seaweeds reach their full economic potential. Whilst there has been a wealth of molecular and genomic data available for several microalgae to facilitate 'molecular phycology' until recently, similar studies have not been possible in seaweeds.

1.3.2 The Green Lineage: Chlorophytes vs Plants

1.3.2.1 General Physiological and Morphological Differences

Despite various chlorophyte algae providing simple models for many biological processes in land plants, there are some differences between the streptophytes (land plants and charophytes) and chlorophyte algae due to the distinct habitats they have adapted to, which has led to divergence in morphology, physiological, biochemical, and metabolic processes. For example, in addition to the obvious differences in morphology between the macroscopic plants and planktonic microalgae, the land plants, or ‘embryophytes’ are characterised based on their unique life cycle amongst photosynthetic eukaryotes (i.e., they form embryos) (De Vries and Weijers, 2017).

Additionally, whilst green macroalgae exhibit a diverse range of macroscopic structures, they are generally less structurally complex than plants. Unlike plants, they do not exhibit major cellular differentiation patterns or discrete organs (Brodie, Maggs and John, 2008). As marine organisms, the macroscopic chlorophytes do not require the ‘root-to-shoot’ system of land plants, because water, nutrients, and carbon are all absorbed through the water column, consistent with the generally limited cellular differentiation observed for these species. The mechanism of reproduction for the model chlorophyte macroalga used in this thesis (*Ulva*) is outlined subsequently (in Figure 1.5), as are the morphologies of several of the diverse chlorophyte macroalgae (1.4).

From a biochemical perspective, the colonisation of land by embryophytes is thought to have contributed to a range of land plant-specific pathways (mainly to do with secondary metabolism) that were assumed not to occur in the green algae (C. Wang *et al.*, 2015; De Vries *et al.*, 2017; De Vries and Archibald, 2018; Berens *et al.*, 2019; Fürst-Jansen, De Vries and De Vries, 2020). However, with renewed research interest in green algae (both chlorophytes and charophytes) and the availability of more genomic information for these species, some of these observations appear to have originated earlier in evolutionary history than initially anticipated (De Vries *et al.*, 2018). I will now look at several of these in more detail.

1.3.2.2 Pyrenoids

As primarily aquatic organisms, chlorophyte algae must assimilate dissolved CO₂ (primarily as HCO₃⁻), which has a notably lower diffusion rate in water than in air (Zou and Gao, 2002; Meyer and Griffiths, 2013). Furthermore, Rubisco (Ribulose-1,5-bisphosphate carboxylase-oxygenase), the enzyme that catalyses carbon fixation in photosynthetic organisms, is not completely efficient at this process, as O₂ competes with CO₂ at the active site (Meyer, Whittaker and Griffiths, 2017; Barrett, Girr and Mackinder, 2021). Despite this, algae exhibit extremely efficient carbon fixation properties (Huan et al., 2016; Moreira and Pires, 2016; G. Gao et al., 2018; Tsubaki et al., 2020; Onyeaka et al., 2021; Yitao Wang et al., 2021).

Chlorophyte photosynthesis is therefore facilitated by CO₂ concentrating mechanisms (CCM), which selectively increases the concentration of CO₂ at Rubisco. The site of this process is the pyrenoid, a proteinaceous subcellular compartment found in the chloroplast of algae, consisting of Rubisco and the associated pyrenoid proteome (e.g., linker proteins) that is required to facilitate pyrenoid assembly and CCM (Wunder et al., 2018; Barrett, Girr and Mackinder, 2021).

Whilst pyrenoids are present in all algal lineages, plants appear to have lost pyrenoids since their transition to land, because they no longer use dissolved inorganic carbon as their carbon source, although CO₂ diffusion in the gas phase is still a limiting factor (Morison et al., 2005; Théroux-Rancourt et al., 2021). Land plants also may compensate for the inefficiencies of Rubisco by synthesising more of it (Raven, 2013), and they have evolved a range of carbon fixation methods (i.e., C₃, C₄, and Crassulacean acid metabolism – CAM) which have differing efficiencies (Armarego-Marriott, 2017). However, pyrenoids are found in the streptophyte algae (the freshwater charophytes) and in one group of land plants – the hornworts (Villarreal and Renner, 2012). It is unclear whether the common ancestor of land plants had already lost pyrenoids, and hornworts regained it specifically, or whether the land plant ancestor had a pyrenoid and only the hornworts retained it, as it appears that pyrenoids evolved independently in the various algal lineages (Barrett, Girr and Mackinder, 2021).

The pyrenoid is a crucial puzzle in algal physiology, because its efficiency has made various chlorophyte microalgae and macroalgae candidates for ‘carbon-capture’ applications to combat anthropogenic CO₂ emissions. Indeed, pyrenoids are estimated to contribute to 44% of all global carbon fixation activities across all organisms that have them (Mackinder et al., 2016; Moreira and Pires, 2016; Fernand et al., 2017; G. Gao et al.,

2018). The efficiency of CCM in algae is also of interest in crop science for improving the photosynthetic output of land plants (Fei *et al.*, 2022).

CCM and pyrenoid assembly has been studied extensively in the model alga *Chlamydomonas* (Wang, Duanmu and Spalding, 2011; Wunder *et al.*, 2018; Barrett, Girr and Mackinder, 2021), but less so in the macroalgal chlorophytes, although more recent investigations into the CCM of the intertidal seaweed *Ulva* have been reported (Suzuki *et al.*, 2014; Liu *et al.*, 2020; Yu Wang *et al.*, 2021). Investigations of CCM in seaweeds will be particularly interesting from an evolutionary perspective as, unlike microalgae, intertidal seaweeds must assimilate dissolved and atmospheric CO₂ when immersed and emersed, respectively (Zou and Gao, 2002; Xu *et al.*, 2012; Huan *et al.*, 2016).

1.3.2.3 Terrestrialisation: New Stressors, New Metabolites

Stress Signalling

In general, the terrestrialisation of land plants led to expansions of gene families associated with the cellular stress response and stress signalling (Fujita *et al.*, 2006; Holzinger and Pichrtová, 2016; Fürst-Jansen, De Vries and De Vries, 2020; Lamers, van der Meer and Testerink, 2020). One key innovation in the evolution of plants has been the development of ‘phytohormones’, which allowed more complex plants to regulate physiology in response to environmental changes, having an essential role in growth and development, facilitating most (if not all) phases of the plant life cycle (C. Wang *et al.*, 2015). Phytohormones are also key components of plant stress tolerance, controlling the expression of important stress response pathways and cross-talking with other stress signalling pathways via reactive oxygen species (i.e., regulating NADPH oxidase) and the mitogen-activated protein kinase pathway (Cramer *et al.*, 2011; Berens *et al.*, 2019).

Chlorophytes can make some, but not all, ‘phytohormones’. There appears to be no evidence of strigolactones, brassinosteroids, or jasmonates in chlorophyte algae, but these algae do appear to produce auxin, ethylene, abscisic acid, cytokinins, and gibberellins (Delaux *et al.*, 2012; C. Wang *et al.*, 2015; Lu and Xu, 2015). In the macroalga, *Ulva*, homologs for genes involved in the biosynthetic pathway for abscisic acid, ethylene, and salicylic acid were identified in the *U. compressa* genome. In addition to auxin, gibberellin and cytokinins, the production of these hormones has also been demonstrated experimentally in *U. compressa*, *U. rigida* and *U. fasciata* (Stirk *et al.*, 2009; El Shoubaky and Salem, 2016; De Clerck *et al.*, 2018).

There are relatively few reports documenting the function of these chemicals in seaweed which makes their exact roles unclear. An early report noted that different combinations of plant hormones stimulate the development of axenic *U. lactuca* cultures (Provasoli, 1958); however, in more recent work, the early development of *U. compressa* gametes was not influenced by hormone treatment (De Clerck *et al.*, 2018). It is likely that these compounds function as general ‘stress metabolites’ in algae, as observed in cyanobacteria and fungi, as various microalgae (*Chlorella vulgaris*, *Chlamydomonas reinhardtii* and *Haematococcus pluvialis*) treated with exogenous abscisic acid, auxin, and cytokinin had increased resistance to osmotic and oxidative stress (Maršálek, Zahradníčková and Hronková, 1992; Kobayashi *et al.*, 1997; Piotrowska-Niczyporuk *et al.*, 2012).

Although chlorophytes have some of the same biosynthetic pathways for ‘phytohormones’ in plants, they appear to lack the complex regulatory pathways observed in plants for these metabolites, which enables them to regulate plant physiology. These signalling pathways appear to be streptophyte specific, with evidence suggesting the expanded signalling pathways for auxins, cytokinins, and strigolactones found in plants first arose in charophytes, and signalling pathways for abscisic acid, jasmonate and salicylic acid being found only in land plants (C. Wang *et al.*, 2015; Fürst-Jansen, De Vries and De Vries, 2020). By comparison, chlorophytes and basal charophytes lack a range of the hormone receptors characterised in plants. For the ones they have (e.g., ethylene response factors), the corresponding family is highly expanded in plants (C. Wang *et al.*, 2015). In addition to phytohormones, the land plants appear to have major expansions in other stress signalling pathways compared to the chlorophyte algae – including in reactive oxygen species signalling with NOX (NADPH oxidase), in the kinome with receptor-like kinases (RLK), and also some functional differences in calcium channels and calcium sensors are observed (Lehti-Shiu *et al.*, 2009; Mittler *et al.*, 2011; Bickerton *et al.*, 2016; Galindo-Trigo, Gray and Smith, 2016; Pittman and Hirschi, 2016; Wolf, 2017). However, as with the ‘phytohormones’, these pathways are all present in algae (Hervé *et al.*, 2006; Anderson *et al.*, 2011; van Hees and van Alstyne, 2013; Diaz and Plummer, 2018), but it appears that the expansion of these processes in plants is directly linked to an adaptation to life outside water – leading to the increased complexity we observe for plants today (De Vries and Archibald, 2018; Berens *et al.*, 2019; Fürst-Jansen, De Vries and De Vries, 2020).

Secondary Metabolites - Phenolics

In addition to stress signalling, land plants have developed the ability to synthesise a diverse range of secondary metabolites that protect them against biotic and abiotic stress (Maeda, 2019; Dong and Lin, 2021). For instance, the development of polyphenol biosynthesis is hypothesised to have initially functioned as a protection mechanism against the increased UV irradiance experienced on land, consistent with the observation that the more basal plants (i.e., bryophytes) synthesise an essential group of polyphenols – the flavonoids (Wang *et al.*, 2017; Davies *et al.*, 2020). In vascular plants, polyphenols also perform an important structural function, with the structural biopolymer lignin comprising the second most abundant polymer in plant biomass after cellulose (Dixon and Barros, 2019).

Polyphenol biosynthesis in plants (including lignin biosynthesis) is facilitated by the development of the phenylpropanoid pathway (Figure 1.2), which is initiated by the conversion of the aromatic amino acid products of the shikimate pathway (typically phenylalanine, but also tyrosine) to *p*-coumaric acid, which can then be ligated with coenzyme A and directed to various biosynthetic pathways to synthesise a diverse range of polyphenols (Falcone Ferreyra, Rius and Casati, 2012; Tohge *et al.*, 2013; Dixon and Barros, 2019; Wen, Alseekh and Fernie, 2020; De Vries *et al.*, 2021). The ability to convert aromatic amino acids to phenylpropanoids was hypothesised to have occurred early in plant development with the acquisition of the PAL gene (phenylalanine ammonia lyase) from a soil bacterium (Emiliani *et al.*, 2009; Levy, Sarkissian and Sriver, 2018). This family has since been expanded in land plant genomes (De Vries *et al.*, 2021). Interestingly, there is evidence that some of the later divergent charophytes synthesise lignin and other phenolics such as flavonoids, meaning the mechanism behind the acquisition of this pathway may have occurred earlier on than initially anticipated (Sørensen *et al.*, 2011; Fernie, 2019; de Luca and Lauritano, 2020).

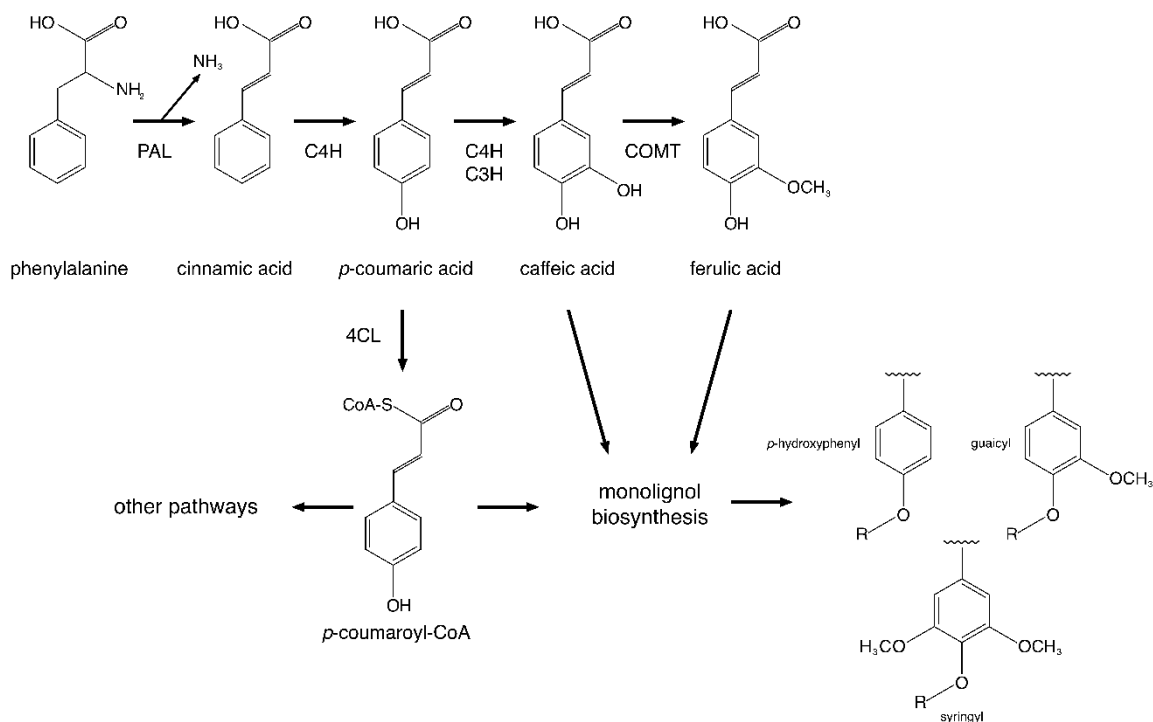


Figure 1.2. Phenylpropanoid pathway. Enzymes abbreviations refer to phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H), *p*-coumaroyl shikimate 3'-hydroxylase (C3'H), caffeic acid O-methyltransferase (COMT), and 4-hydroxycinnamoyl-CoA ligase (4CL). The monolignol pathway is summarised in Figure 1.10.

Regardless, the absence of ammonia-lyase genes and a functional phenylpropanoid pathway outside the streptophyte lineage means the metabolome of chlorophytes is different to that observed in plants, despite primary metabolism (post carbon fixation) mainly being comparable between the two lineages (Maeda, 2019). Furthermore, algae are reported to lack the biosynthetic machinery to synthesise many other phytochemicals in plants, such as alkaloids and isoprenoids (Wink, 2008). In the case of the isoprenoids, the absence of the mevalonate pathway in chlorophytes may represent a lineage-specific loss rather than a specific adaptation by land plants (Lohr, Schwender and Polle, 2012), as some 'phyco'-isoprenoids can be synthesised by an alternate pathway (Ishimatsu *et al.*, 2012; Volkman, 2014). Nonetheless, this difference in metabolic machinery appears as a major distinction between the needs of a marine and a terrestrial ecosystem.

Despite this, we know a diverse range of polyphenols are present in chlorophyte microalgae and seaweeds, with a primary role in stress tolerance. Their origin remains uncertain as the known biosynthetic pathway for these compounds is absent (Nwosu *et al.*, 2011; Martins *et al.*, 2013; Trigui *et al.*, 2013; Mezghani *et al.*, 2016; Klejdus *et al.*, 2017; Kumar *et al.*, 2020). Interestingly, flavonoid biosynthesis has been characterised in a diverse range of algae, in addition to chlorophytes (Goiris *et al.*, 2014; Al-Malki *et al.*, 2018). Phenolics are also produced by eukaryotic algae outside the Archaeplastida, with the brown seaweeds (mainly the furoid algae) known for their high polyphenol content, particularly for the synthesis of ‘phlorotannins’ and the corresponding phloroglucinol subunits (Bertoni, 2013; Kim *et al.*, 2013; Casas *et al.*, 2016; Emeline *et al.*, 2021; Shrestha, Zhang and Smid, 2021).

Notably, phlorotannins are associated with the brown seaweed cell wall in addition to their primary role in the abiotic and biotic stress defence (Birkemeyer *et al.*, 2020). Phlorotannin subunits are thought to be synthesised as a by-product of the acetate-malonate pathway (typically associated with fatty acid biosynthesis), where malonyl-CoA may be condensed to phloroglucinol. Still, this has not been proven experimentally in these species (Shrestha, Zhang and Smid, 2021). This process is possibly catalysed by a type III polyketide synthase gene (PKSIII) which is also known in land plants, such as chalcone synthases which catalyse the initial step in flavonoid production (Meslet-Cladière *et al.*, 2013). However, flavonoid biosynthesis requires input from the phenylpropanoid pathway (i.e., *p*-coumaryl CoA), although the other representatives of this family can synthesise a diverse range of aromatic polyketides without this component (Moore *et al.*, 2002; Jiang *et al.*, 2006; J. Wang *et al.*, 2020). Phlorotannins are not characterised in green seaweeds, although some evidence for phloroglucinol in the *Ulva* cell wall is reported (Briand and Morand, 1997). Furthermore, the presence of chalcone synthase and polyketide synthase genes in the main chlorophytes group is uncertain (Jiang *et al.*, 2006; Naake *et al.*, 2021), despite homologs being present in diverse marine plankton (de Luca and Lauritano, 2020). As a result, the relevance of this observation for chlorophytes is inconclusive.

1.3.3 Microalgae vs Macroalgae – A New Perspective

As discussed, whilst we can generally identify the fundamental biochemical differences between plants and algae, there are exceptions, conserved mechanisms, and points of convergence that are still poorly understood. More insight may be provided by an alternative comparison between the microalgal and macroalgal chlorophytes. Here, the seaweeds appear ideal models for understanding why unicellular to multicellular transitions occur and why increasing complexity is observed in some organisms. This is a reoccurring observation across the eukaryotic lineages. The chlorophytes appear as an ideal group of species for this purpose due to the diversity in forms observed (discussed previously §1.3.1) and because the chlorophytes have been studied more extensively than the red and brown algae due to their more recent common ancestry with land plants.

Furthermore, we have genomic information from many of the microalgal representatives. Analogous investigations are of interest in the streptophyte lineage for understanding what expansions in the aquatic ancestor of land plants facilitated their eventual colonisation of land and then the resulting increases in complexity observed on land (Mikkelsen *et al.*, 2014; Domozych, Popper and Sørensen, 2016; Nishiyama *et al.*, 2018; Jiao *et al.*, 2020). As macroalgal chlorophytes developed increased complexity in a saltwater environment, they provide a unique model for comparison to streptophytes which evolved in freshwater or terrestrial environments.

Whilst comparisons between the unicellular and ‘multicellular’ volvocine chlorophytes have been performed (Chlamydomonadales or Volvocales), the multicellular representatives of this group (e.g., *Volvox*) do not form the same complexity as the macroalgal seaweeds. They are better described as colonial organisms, formed of individual cells that associate in colonies of varying sizes that can contain more than 500 cells (Herron *et al.*, 2009; Prochnik *et al.*, 2010; Herron and Nedelcu, 2015). Interestingly, genomic investigations into *Volvox* and the unicellular *Chlamydomonas* have identified very few fundamental differences between these algae (Prochnik *et al.*, 2010; Umen, 2014). As a result, a diverse range of algae needs to be studied to better understand the reasons behind multicellular transitions, with the macroscopic green seaweeds appearing as ideal models for this purpose, with many of these seaweeds having both a unicellular (swarmer) and multicellular phase (Wichard and Oertel, 2010; Hurd *et al.*, 2014).

Unfortunately, we have limited knowledge about green seaweed biology compared to land plants and chlorophyte microalgae. Due to their complexity and unique biochemical composition, seaweeds are more challenging to work with in the lab than microalgae (Charrier, Wichard and Reddy, 2017), and whilst seaweed farming is a major industry in the Pacific and South East Asia (Lindsey Zemke-White and Ohno, 1999; Radulovich *et al.*, 2015; Buschmann *et al.*, 2017), plant science is a substantially more advanced field than ‘macro-phyecology’ as land crops are an indispensable part of our agricultural economy. However, the current interest in macroalgal biomass for various industrial applications and recent research developments now makes certain groups of seaweeds much more viable model organisms, facilitating both applied and fundamental biological investigations (De Clerck *et al.*, 2018; Arimoto *et al.*, 2019; Blomme *et al.*, 2021; He *et al.*, 2021).

The recent publication of the *Ulva compressa* genome has indicated that there are some genomic differences between seaweeds and the available sequenced microalgae, including the loss and gain of specific transcription factor and transcription regulator families and expansions in genes encoding for proteins with an extracellular domain (De Clerck *et al.*, 2018). This may mirror the expansions in extracellular domain proteins observed in the colonial volvocine algae relative to the unicellular *Chlamydomonas* (Prochnik *et al.*, 2010). However, it is unclear to what extent these apparent changes in green seaweeds have contributed to multicellularity and macroscopic forms without further investigation. For example, not all seaweeds are multicellular (Brodie, Maggs and John, 2008; Cocquyt *et al.*, 2010). As ‘molecular phyecology’ is a relatively new discipline, there remains a lot we do not know about seaweed biology. These diverse algae are now discussed.

1.4 The Ulvophyceae: Green Seaweeds

1.4.1 General Biology

Whilst chlorophyte macroalgae can be found in one order of the typically unicellular Trebouxiophyceae (i.e., the Prasiolales) and various colonial and filamentous algae can be identified in the Chlorophyceae (e.g., *Volvox* and *Oedogonium*), all other chlorophyte seaweeds are typically classified as representatives of the Ulvophyceae (Kornmann and Sahling, 1974; Cocquyt *et al.*, 2010). However, the relationship between some of the constituent clades is uncertain, and the Ulvophyceae may not necessarily be a monophyletic group (Hou *et al.*, 2022). Despite sharing a common unicellular ancestor, multicellularity is hypothesised to have evolved independently multiple times across this lineage (Cocquyt *et al.*, 2010), resulting in the diverse macroscopic forms we observe across the various ulvophyte orders today, with some representatives also retaining a unicellular form. For example, the Dasycladales and Bryopsidales seaweeds represent macroscopic unicellular organisms with a 'siphonous' morphology, where the whole body of the algae is formed from a single cell which can be uninucleate (e.g., *Acetabularia*) or multinucleate (e.g., *Caulerpa* and *Codium*), and can form remarkably complex structures and large sizes. Some of these seaweeds (i.e., *Caulerpa* and *Codium*) are notable invasive species (Belton *et al.*, 2014; Drouin, McKindsey and Johnson, 2016; Rothäusler *et al.*, 2022). Another group, the Cladophorales, are multicellular, but the individual cells are multinucleate and siphonous, forming long filamentous alga. As such, these species are known as 'siphonocladous', hence 'Cladophorales' (Choo, Snoeijs and Pedersén, 2004; Brodie, Maggs and John, 2008; Hurd *et al.*, 2014; Umen, 2014; Confalonieri *et al.*, 2017; Cremen *et al.*, 2019).

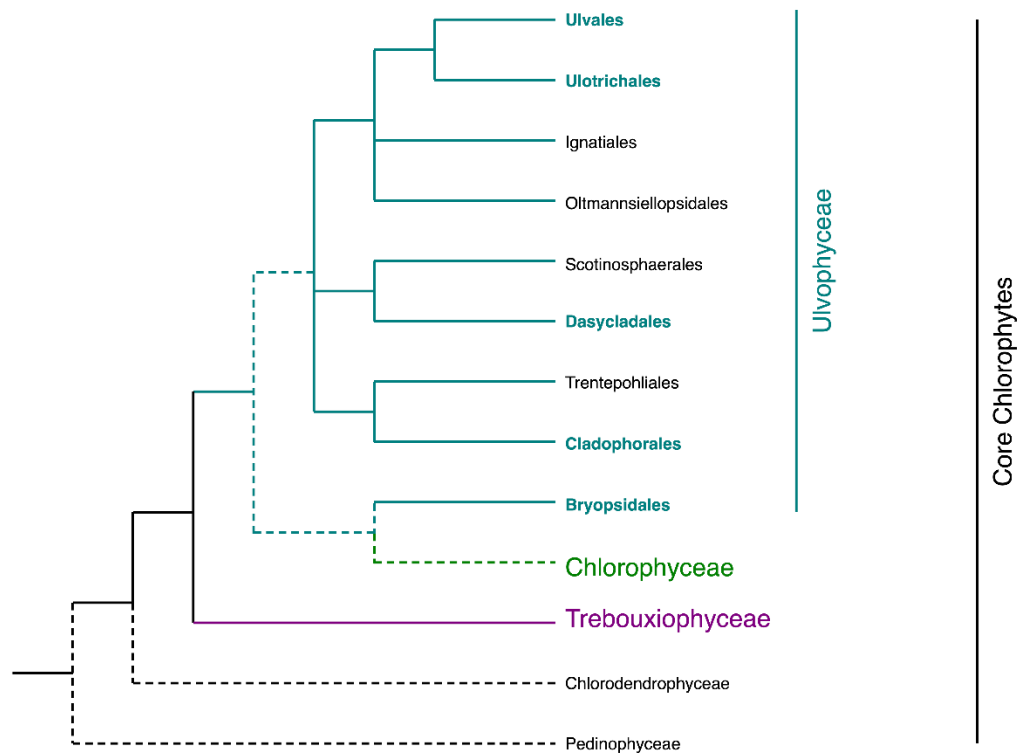


Figure 1.3. The current hypothesis of phylogenetic relationships in the Ulvophyceae and core chlorophytes. Dotted lines indicate uncertain relationships. Clades in bold correspond to the orders representing the main green seaweeds, including Ulvales, Ulotrichales, Dasycladales, Cladophorales, and Bryopsidales. Image adapted from Hou, Z. *et al.* (2022). Phylotranscriptomic insights into a Mesoproterozoic–Neoproterozoic origin and early radiation of green seaweeds (Ulvophyceae). *Nature Communications*, 13(1).

However, the best-characterised representatives of the Ulvophytes are the Ulvales and Ulotrichales, which includes blade-like and filamentous uninucleate seaweeds such as *Ulva* (Ulvales) and *Monostroma* (Ulotrichales), both of which are economically important crops, particularly in Southeast Asia (Wichard *et al.*, 2015; Cui *et al.*, 2021). *Ulva* (commonly referred to as ‘sea lettuce’) is a morphologically plastic alga that is typically characterised by either a leaf-like blade formed from a bilayer of cells (Figure 1.4, c-d) or a filamentous morphology constructed as a cylinder of cells (Figure 1.4, a-b), which may or may not include branching depending on the species. The body of the seaweed is then attached to a substrate via a small structure called a holdfast, which anchors the alga in flowing water (Brodie, Maggs and John, 2008). However, ‘free floating’ seaweeds are common (Yabe *et al.*, 2009; S. Wang *et al.*, 2018). Seaweed from the latter group was classified as a separate genus (*Enteromorpha*) until genetic evidence via species barcoding demonstrated otherwise (Hayden *et al.*, 2003).

In fact, due to the morphological plasticity of *Ulva*, intermediate morphologies are often observed, and many species of *Ulva* display both morphotypes, sometimes in the same populations (Løvlie, 1978; Loughnane *et al.*, 2008; Steinhagen, Weinberger and Karez, 2019). Despite this morphological plasticity being widely reported in the literature, the basis for this is still poorly understood and remains an area of research interest. Due to this, it is often difficult to differentiate species of green seaweed in the field without species barcoding. However, species identification based upon macroscopic and microscopic features is possible with some experience. For example, *Ulva* species that typically form filamentous thalli can often be distinguished by the degree of branching observed or by cell orientation, chloroplast morphology, and the number of pyrenoids present.

Additionally, whilst *Monostroma* appears morphologically similar to ‘sea lettuce’ type *Ulva*, it can be differentiated from microscopic investigations as the blade is only one cell thick (Brodie, Maggs and John, 2008). Despite this, until recently, the model seaweed *Ulva mutabilis* (Føyn) has frequently been referred to as a separate species to the widespread *U. compressa* in the literature due to the observed morphological plasticity of *U. mutabilis* compared to the typical ‘filamentous’ morphology of *U. compressa*, until recent genetic and cross-breeding studies confirmed that they were conspecific (Steinhagen *et al.*, 2019). As such, species barcoding (typically by sequencing the *tufA* or *rbcL* genes) remains an essential tool in seaweed ecology (Fama *et al.*, 2002;

Saunders and Kucera, 2010; Steinhagen *et al.*, 2019; Steinhagen, Düsedau and Weinberger, 2021).

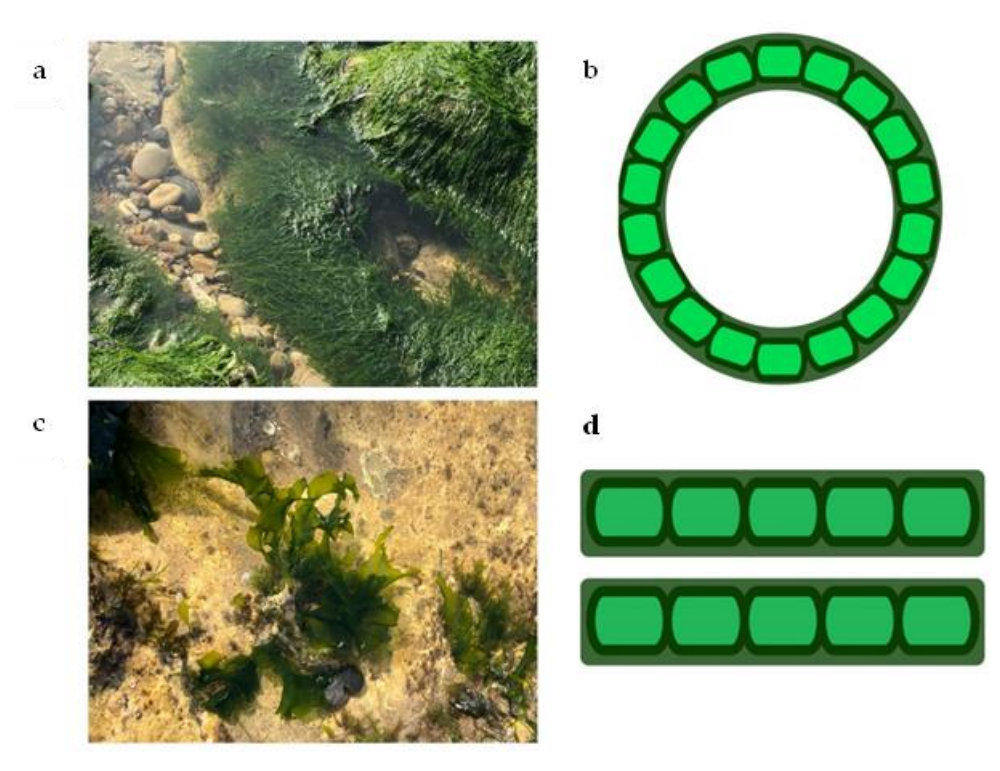


Figure 1.4. Morphological characteristics of *Ulva*. *U. compressa* displays a filamentous morphology (a), with thalli constructed from a 'cylinder' of cells (b). 'Blade'-type *Ulva* of 'sea lettuce' morphology is constructed from a bilayer of cells (d). Images collected at low tide from Harbour Beach, Seaham, Durham, UK.

The methods of reproduction of the Ulvophyceae vary; for example, Bryopsidales can propagate sexually and asexually – i.e., by fragmentation (Ceccherelli and Cinelli, 1999; Phillips, 2009). Furthermore, many green seaweeds can reproduce by more than one mechanism (Løvlie and Bryhni, 1978; Lin *et al.*, 2008; Alström-Rapaport, Leskinen and Pamilo, 2010; Carl *et al.*, 2014; Gao *et al.*, 2017b). In the case of *Ulva* (Figure 1.5), the traditional life cycle model is characterised by generation-to-generation alternation between a haploid gametophyte and a diploid sporophyte phase, both of which are morphologically identical, or 'isomorphic' (Wichard, 2015). However, this process can be more complex.

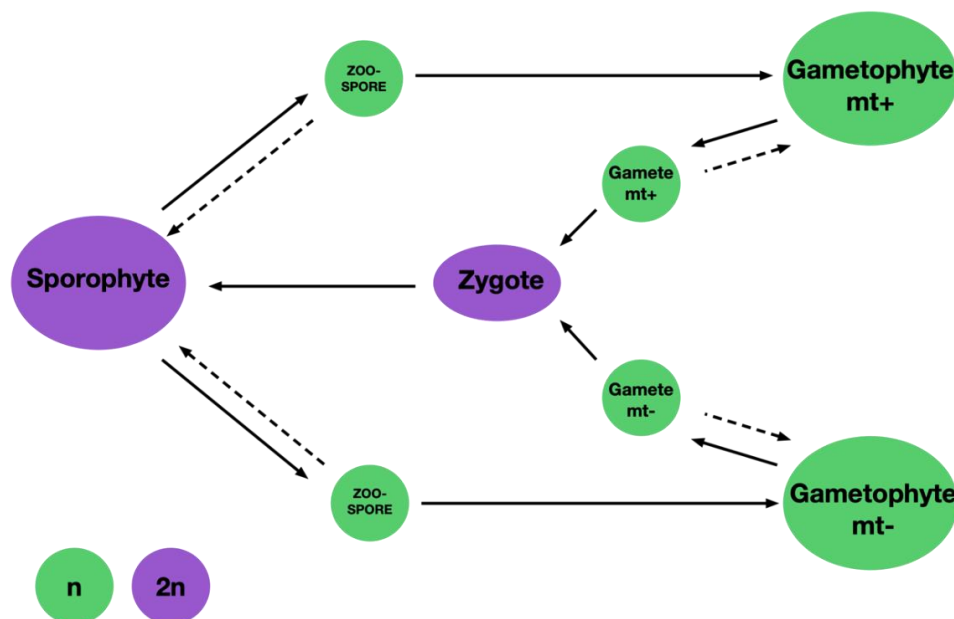


Figure 1.5. The life cycle of *Ulva* is characterised by alternating between a haploid gametophyte phase and a diploid sporophyte phase. Solid lines represent expected transitions in the classic alternation of generations model, and dotted lines represent possible transitions that can occur *in vitro* or in certain seaweed populations.

Typically, biflagellate gametes (of either mt⁺ or mt⁻ mating type) are released by differentiated gametophytes (via gametogenesis) and fuse to create a zygote that develops into a diploid sporophyte. However, if unmated, they may parthenogenetically revert to the gametophyte phase. This allows clonal cultures of *Ulva* under to be maintained under laboratory conditions indefinitely with suitable protocols (Spoerner *et al.*, 2012; Wichard, 2022). In the diploid phase, sporophytes divide by meiosis to release quadriflagellate zoospores, which develop asexually into gametophytes of the respective mating type. However, both swarmer phases (gametes and zoospores) can undergo spontaneous diploidisation into sporophytes (Liu *et al.*, 2017). Obligate asexual strains are reported that reproduce exclusively by this process, where a diploid adult develops asexually from ‘zoosporoids’ that are either biflagellate (like gametes) or quadriflagellate like gametes (Hiraoka *et al.*, 2003; Kostamo *et al.*, 2008). The nature of the reproductive cycle is not species-specific; both sexual and asexual strains of certain *Ulva* species, such as *U. prolifera*, are reported (Hiraoka *et al.*, 2003; Ichihara *et al.*, 2019). This reproductive plasticity has likely contributed to their

ecological success, with *Ulva* displaying widespread distribution on coastlines in both hemispheres and demonstrating how certain *Ulva* species can show extremely rapid growth rates under certain conditions (Tsubaki *et al.*, 2020).

Understanding the dynamics of the *Ulva* life cycle is particularly important from an ecological perspective, with certain species associated with 'green tide' events (

Figure 1.6), where certain populations of *Ulva* proliferate rapidly in an algal bloom, causing extensive ecological and environmental damage (van Alstyne *et al.*, 2016; Gao *et al.*, 2017b, 2017a; Yongyu Zhang *et al.*, 2019). Reproductive transitions can occur due to various environmental conditions (Gao *et al.*, 2010, 2017b; Kim *et al.*, 2011; van Alstyne *et al.*, 2016; Y. Wang *et al.*, 2019; Liu *et al.*, 2020; He *et al.*, 2021). Such re-occurring events are increasingly associated with human activities, where the leaching of nutrients into water bodies (e.g., via agricultural run-off or pollution) stimulates rapid algal growth. The eventual decomposition of seaweed releases toxic compounds and gases (e.g., hydrogen sulfide), causing the death of local flora and fauna, posing health risks to humans, and disrupting economic activities. Conversely, the ability of *Ulva* to proliferate rapidly is of interest from a biotechnology perspective because high amounts of biomass can be generated under the right conditions. Such green tide events are particularly common in the Yellow Sea, around of the coast of Brittany, and along the shoreline of Ireland (Charlier, Morand and Finkl, 2008; Yabe *et al.*, 2009; Liu *et al.*, 2013; Wan *et al.*, 2017; Yongyu Zhang *et al.*, 2019).



Figure 1.6. *Ulva prolifera* growth during a green tide event in the Yellow Sea (2008). Image reprinted from Gao et al. (2010). A Strategy for the Proliferation of *Ulva prolifera*, Main Causative Species of Green Tides, with Formation of Sporangia by Fragmentation. PloS one. 5. e8571.

Since the original isolation of the chlorophyte seaweed *Ulva compressa* (syn. *U. mutabilis*) by Bjørn Føyn (1958), *Ulva* representatives have been utilised in a range of developmental biology studies, characterising the *in vitro* life cycle, the nature of early *Ulva* development and morphogenesis, and the dynamics of cellular differentiation during gametogenesis and sporulation (Nordby and Hoxmark, 1972; Hoxmark, 1975; Løvlie, 1978; Løvlie and Bryhni, 1978; Stratmann, Paputsoglu and Oertel, 1996; Spoerner et al., 2012). Interestingly, more recent studies have shown that *Ulva* must form symbiotic relationships with at least two specific groups of marine bacteria (*Roseovarius* and *Marinobacteria*) to ensure that correct morphogenesis occurs during early development, with *Ulva* seedlings grown under axenic conditions failing to differentiate properly (Spoerner et al., 2012; Vesty et al., 2015; Wichard, 2015; Ghaderiardakani, Coates and Wichard, 2017). Correct morphogenesis is only induced in the presence of morphogenetic compounds produced by these bacterial species, which can be achieved experimentally either through inoculation of a culture medium (Marshall et al., 2006; Spoerner et al., 2012) or by exposure to sterile seawater containing the morphogenetic compounds (Grueneberg et al., 2016).

Moreover, as an outcome from this work, a series of methods have been established that enable *Ulva* to be cultivated in the lab, including the determination of appropriate culture conditions, protocols to induce and purify swarmers, and methods to maintain clonal cultures *in vitro* indefinitely (Stratmann, Paputsoglu and Oertel, 1996; Spoerner *et al.*, 2012). More recently, methods to genetically transform and manipulate *Ulva* have been reported (Oertel, Wichard and Weissgerber, 2015; Wu, Jiang, Guo, *et al.*, 2018; Wu, Jiang, Zhao, *et al.*, 2018; Blomme *et al.*, 2021), and accompanied by the publication of genomes for two ecologically relevant species *U. compressa* and *U. prolifera* in 2018 and 2021 respectively (De Clerck *et al.*, 2018; He *et al.*, 2021). As a result, *Ulva* appears to be an ideal ‘model seaweed’ for macroalgal research, with similar protocols for the other green seaweeds generally absent in the literature. However, a Bryopsidales genome (*Caulerpa lentillifera*) has also been published (Arimoto *et al.*, 2019).

1.4.2 Intertidal Ecology: Why are Seaweeds Unique?

From an ecological perspective, whilst chlorophyte microalgae and green seaweeds are both ‘marine’ species, they occupy different ecological niches that face distinct challenges. Whilst some seaweeds (e.g., kelps) are submerged constantly (in the subtidal zone), others (including the green seaweeds, furoid algae, and reds) survive in the intertidal and the ‘splash’ zone (Einav and Israel, 2007; Gómez and Huovinen, 2011; Kokabi *et al.*, 2016). Here, due to the tide cycle, seaweeds are periodically emersed and immersed, causing their periodic dehydration and rehydration. Planktonic algae do not face these stressors because they continuously float with the water column, whilst seaweeds are anchored to a substrate. Like other intertidal species, such as corals and invertebrates (Levy *et al.*, 2007; Goto and Takekata, 2015), seaweed physiology appears to be synchronised with the tide cycle. For example, swarmer release coincides with low tide and spring tide specifically – possibly indicative of semi-lunar rhythmicity (Titlyanov, Titlyanova and Lüning, 1996; Lüning, Kadel and Pang, 2008). Semi-lunar fluctuations in metabolite production are also reported in *Ulva*, as are tidal oscillations in protein phosphorylation (Tal *et al.*, 2011; Parages, Capasso, *et al.*, 2014; Parages, Figueroa, *et al.*, 2014).

Green seaweeds are frequently located in tide pools with highly variable and rapidly changing conditions (Brodie, Maggs and John, 2008; Hurd *et al.*, 2014). Environmental factors such as wind, geology, and zonation dictate the frequency and duration of seaweed emersion (Ganning, 1971; Einav and Israel, 2007). Furthermore,

they must withstand biotic stresses, being a food source for many aquatic and terrestrial species in the intertidal zone (Davies and Falconer, 2001; Leidenberger, Harding and Jonsson, 2012). Zonation is an important concept in intertidal ecology, as intertidal species populating the 'upper' intertidal zone are submerged less frequently and exposed longer to variable environmental conditions (Figure 1.7). These species, therefore, must demonstrate a higher degree of stress tolerance. For example, studies have demonstrated that greater antioxidant production following dehydration is greater in seaweeds collected from the upper intertidal zone than in those collected from the lower zone (Collen and Davison, 1999; Flores-Molina et al., 2014).

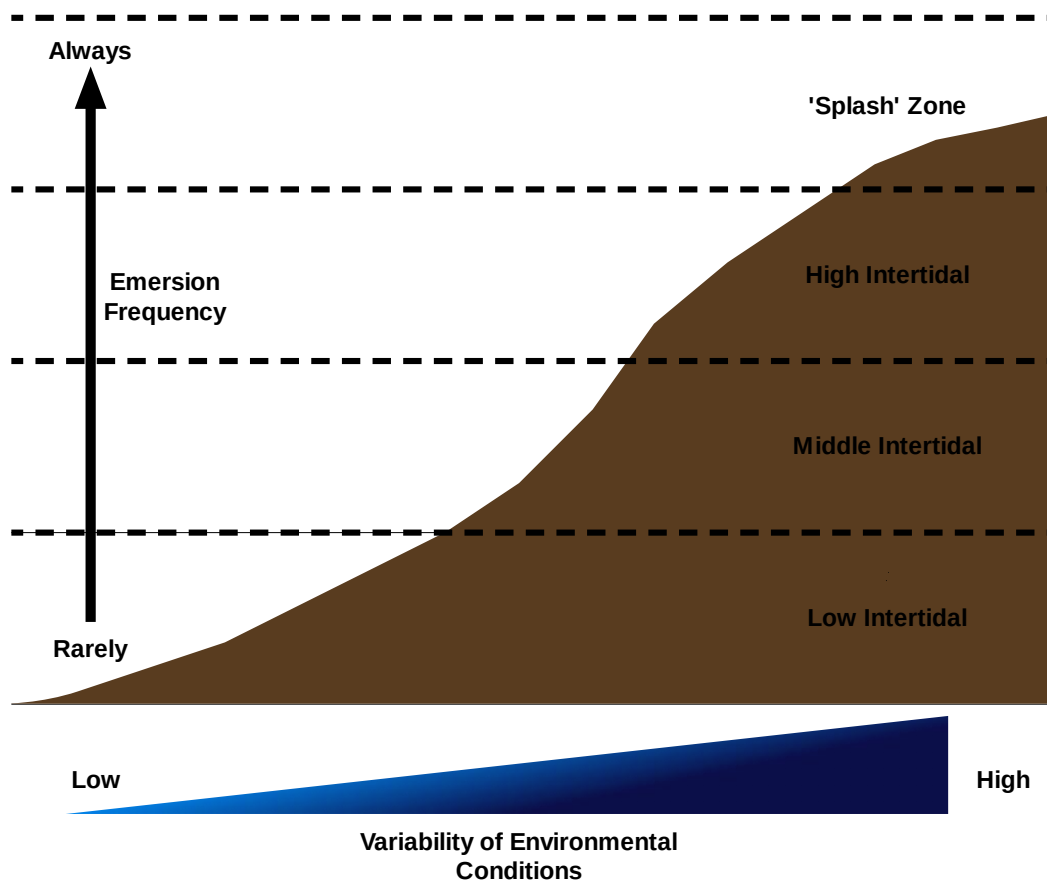


Figure 1.7. The relationship between intertidal zonation and environmental variability

Interestingly, whilst ‘sea-lettuce’ species such as *U. lactuca* are more commonly found in the mid-intertidal zone, typically filamentous species (e.g., *U. compressa*, *U. intestinalis*) tend to persist in the upper intertidal zone and the splash zone, and can also be distributed in brackish and freshwater environments (Einav and Israel, 2007; Gómez and Huovinen, 2011; Kokabi *et al.*, 2016). The widespread gutweed *U. compressa* thrives in a range of habitats where it is subjected to more variable environmental conditions (Ganning, 1971; Bischof *et al.*, 2006; Einav and Israel, 2007; Flores-Molina *et al.*, 2014). Several reports document remarkable salinity, heat, and desiccation tolerance in filamentous *Ulva*, indicating that stress tolerance may be a factor in its previously described morphological plasticity (Choo, Snoeijs and Pedersén, 2004; Luo and Liu, 2011; Carl, de Nys and Paul, 2014; Holzinger *et al.*, 2015).

The primary forms of environmental stress seaweeds face at low tide include dehydration, increased irradiance, variable salinity, and variable temperature (Ganning, 1971; Morris and Taylor, 1983; Fierro *et al.*, 2017; Legrand, Riera, *et al.*, 2018). Many of these stress factors interact – for example, in summer months, the rates of dehydration are increased, and hypersaline stress may be induced by evaporating water in tide pools or local geological factors impacting the ionic composition of water. Coastal geology also affects stress intensity; shallow tide pools are heated and cooled much faster than larger pools (Ganning, 1971; Morris and Taylor, 1983; Smit and Glassom, 2017; Legrand, Riera, *et al.*, 2018). Hyposaline stress may be induced by rainfall diluting tide pool seawater at low tide, or from run-off from freshwater sources, particularly further up the coastline (Morris and Taylor, 1983; Jocque, Vanschoenwinkel and Brendonck, 2010).

Seaweeds are exposed to unimpeded sunlight upon emersion, whilst when immersed they are exposed to low light conditions. Generally, seaweeds must tolerate low and variable light conditions, with the depth of the water column (and hence light availability) changing with the tide cycle (Fortes and Lüning, 1980). For example, changing water depth and UV penetration were demonstrated to impact the photosynthetic rates of the red seaweed *Gracilaria* spp. *in vitro* (Xu *et al.*, 2018). Interestingly, the light requirement of seaweed populations appears to correspond with zonation – i.e., seaweeds from deeper water are saturated at lower irradiance than seaweeds that populate shallower water (Markager and Sand-Jensen, 1992; Leukart and Lüning, 1994; Sagert *et al.*, 1997)

However, based on their ecological success in this environment, green seaweeds appear as a highly robust set of organisms, demonstrating remarkable stress tolerance, particularly to desiccation. A primary defence against dehydration seems to be morphology and the ability to retain water (Norton, 1991; Schagerl and Möstl, 2011). Based upon a series of studies into the dynamics of desiccation stress in *Ulva*, it appears that the cellular stress responses are not activated by emersion itself but rather when water content drops by a certain level or below a 'critical water content' (Contreras-Porcia et al., 2011; Kumar et al., 2011; Flores-Molina et al., 2014; Gupta and Kushwaha, 2017). This appears the most practical form of protection as stress responses are energetically costly. As emersion is a temporary and predictable event (as seaweeds will be re-immersed at low tide), the longer water can be retained, the less the metabolic impact on the cell is (Lichtenthaler, 1998). Water retention is influenced by thallus morphology, size and structure, with filamentous seaweeds appearing to be very resistant to desiccation (Choo, Snoeijs and Pedersén, 2004; Luo and Liu, 2011; Carl, de Nys and Paul, 2014; Holzinger et al., 2015). This has been demonstrated experimentally in the brown seaweed *Fucus spiralis*, where morphology and thallus size were shown to influence desiccation tolerance (Schagerl and Möstl, 2011). The morphological plasticity of *Ulva* likely contributes to their success, with ruffled, ribbon-like, branched, and filamentous forms described that likely assist water retention (Brodie, Maggs and John, 2008).

However, once seaweeds are dehydrated, they appear to induce physiological changes that protect the cell. Dynamic changes in cell wall structure are observed under dehydration stress in *U. compressa* due to the plasticity of the 'inner cell wall', which moves with the plasma membrane to prevent plasmolysis (Holzinger et al., 2015). Furthermore, a series of osmoprotectant metabolites are synthesised in desiccation-stressed *U. lactuca*, such as glycine and betaine (Gupta and Kushwaha, 2017). Whilst this has not been studied extensively in the literature, various transcriptional, proteomic, and biochemical reports document the expected cellular stress response in *Ulva* following multiple forms of 'intertidal stress', including the upregulation of molecular chaperones and activation of the ubiquitin-proteasome pathway to maintain protein homeostasis, the biosynthesis of antioxidants to maintain redox homeostasis, and the upregulation of various photoprotective mechanisms employed in other algae and plants to protect the photosynthetic machinery (Jia et al., 2011; Luo and Liu, 2011; H.

Zhang *et al.*, 2012; Tominaga *et al.*, 2012; Fan *et al.*, 2018; He *et al.*, 2018, 2019; Yang *et al.*, 2019; Y. Wang *et al.*, 2019; Liu *et al.*, 2020).

As stated, intertidal *Ulva* are capable of photosynthesis when emersed and immersed and perform both C₃ and C₄ mediated photosynthesis (Xu *et al.*, 2012; Liu *et al.*, 2020). Interestingly, there appear to be distinct photoprotection methods in the Ulvophyceae – with Ulvales and Cladophorales reported to perform non-photochemical quenching (i.e., the thermal dissipation of excess light) via the xanthophyll cycle as performed in land plants, whilst in seaweeds of the Bryopsidales, the xanthophyll cycle appears to be absent or decoupled from the process (Christa *et al.*, 2017). Furthermore, *Ulva* appears to have stress-responsive orthologs of the light-harvesting complex II and a chlorophyll pigment binding protein called PsbS, which was characterised initially as a novel photoprotective mechanism in plants (X. Zhang *et al.*, 2012; Croce, 2015). As the PsbS protein does not form part of the photoprotection response in *Chlamydomonas*, it may reflect an adaptation to ‘terrestrial’ light levels when emersed (X. Zhang *et al.*, 2012). Additionally, based on preliminary genomic investigations, *U. compressa* appears to encode two photoreceptor families, one that corresponds to a blue-light receptor found in planktonic marine algae (which is required as blue light penetrates furthest into the water column) and one that is a homolog to the pCRY receptor characterised in *Chlamydomonas* and land plants, possibly indicative of two independent pathways for light perception. Nonetheless, intertidal green seaweeds have developed unique adaptations to withstand variable irradiation and intertidal stress.

More algal-specific studies have been reported more recently, for example, studies of carbonic anhydrase expression to determine how the CCM is impacted by irradiance stress (Yu Wang *et al.*, 2021) and the effect of metal stress on *Ulva* gene expression – mainly focusing on the metal chelating metallothionein family (Laporte *et al.*, 2016; Zúñiga *et al.*, 2020). From an applied perspective, determining how seaweeds tolerate the dynamic conditions faced in the intertidal zone may hold value in ‘agri-tech’ for researchers aiming to develop more ‘stress tolerant’ land crops, with environmental stress events such as drought having an ever-increasing impact on our economy and agricultural outputs. These diverse yet understudied organisms likely have unique mechanisms of tolerating stress, particularly dehydration, that may provide novel targets for genetic modification studies in land crops. Furthermore, due to their remarkable ability to sequester heavy metal ions, they have been proposed for various

bioremediation activities in polluted habitats (Elizondo-González et al., 2018; G. Gao et al., 2018; Tremblay-Gratton et al., 2018).

Notably, the cell wall composition of seaweeds appears to be impacted by seasonal and environmental conditions (Holzinger *et al.*, 2015; Kidgell *et al.*, 2019; Zeroual *et al.*, 2020). However, comprehensive studies documenting their role in abiotic stress are generally absent from the literature, with most reports on seaweed cell wall composition reporting from an economic perspective (e.g., assessing the biochemical properties of cell wall polysaccharides for the proposed bioenergy and biomaterials applications), rather than from a biological perspective that will help us determine the role of the seaweed cell wall in adaptation to environmental stress.

1.4.3 The *Ulva* Cell Wall: Evolutionary and Economic Interest

1.4.3.1 Are Cell Wall Complexity and Organism Complexity Linked?

One notable difference between macroalgae and chlorophyte microalgae is the cell wall. The extracellular matrices (ECM) of chlorophytes demonstrate considerable diversity, with many of the planktonic species (e.g., prasinophytes like *Ostreococcus* and *Micromonas*) lacking a functional cell wall, whilst other microalgae have cell walls composed almost entirely out of proteins, whilst in contrast the green seaweed extracellular matrix is comparatively more 'plant-like' and consists of a fibrillar component and acidic polysaccharides (Percival, 1979; Becker, Marin and Melkonian, 1994; Lahaye and Robic, 2007; Domozych *et al.*, 2012).

Whilst on first impression, cell wall complexity in chlorophytes appears to correlate with organism complexity, distinguishing the different algal groups based on 'cell wall type' is complex. For example, filamentous *Oedogonium* (Chlorophyceae) has a cellulosic cell wall, whilst other representatives of Chlorophyceae (e.g., the colonial and unicellular Volvocales) have a proteinaceous cell wall with no significant polysaccharide fraction or cellulose (Estevez, Leonardi and Alberghina, 2008b). Here, the cell wall of the model organism *Chlamydomonas* and the closely related *Volvox* are characterised by the presence of hydroxyproline-rich glycoproteins and glycine-rich glycoproteins in the cell wall, similar to various glycoproteins identified in land plants, despite these algae lacking a significant structural polysaccharide component in the ECM (Goodenough and Heuser, 1985; Voigt, 1985; Adair *et al.*, 1987; Voigt and Frank, 2003; Voigt *et al.*, 2014). By contrast, another well-characterised freshwater microalga *Chlorella* (Trebouxiophyceae) has a polysaccharide-rich wall composed of cellulose and uronic acid-rich

polysaccharides (Kapaun, Loos and Reisser, 1992; Dunker and Wilhelm, 2018; Weber *et al.*, 2022). As a result, more extensive investigations into the cell wall evolution of chlorophytes appear necessary to determine the factors that have contributed to this diversity.

Interestingly, similar observations are noted in the streptophyte algae, with the 'higher' charophytes such as the Charales, Coleochaetales, and Zygnematales demonstrating a 'plant-like' cell wall, with pectin, hemicelluloses, cellulose, glycoproteins, and possibly lignin whilst the basal charophytes lack a functional cell wall in the same way as do the planktonic chlorophytes (Popper and Fry, 2003; Sørensen *et al.*, 2011; O'Rourke *et al.*, 2015). Importantly, for the research outlined in my thesis, identifying lignin in the various charophyte cell walls has significant evolutionary implications, with lignification traditionally considered a vascular plant-specific adaptation. Nonetheless, the progression in cell wall complexity with increased morphological complexity observed in the two branches of the green lineage is noteworthy. Further investigation into chlorophyte cell wall evolution appears necessary to ascertain the role cell wall evolution has on multicellular transitions.

Despite the development of analogous fibrillar cell walls, the structure and composition of green seaweed cell walls are considerably different from those observed in the multicellular charophytes and plants, reflecting their adaptation to a different environment. Unlike land plants, and certain red seaweeds (e.g., coralline red seaweeds), green seaweeds do not have distinct primary and secondary cell walls. The *Ulva* cell wall can generally be distinguished into an 'amorphous' pectin-like component and a structural component formed from fibrillar cellulose (Bobin-Dubigeon *et al.*, 1997). Furthermore, reflecting the distinct macroscopic morphologies of the Ulvophyceae, the cell walls of the different green seaweed orders are divergent from each other, with the siphonous and siphonocladous macroalgae (Bryopsidales, Dasycladales, and Cladophorales) having a cell wall that is distinct from the blade-like Ulvales-Ulotrichales seaweeds (Percival, 1979).

Based on the extractability of the cell wall, we can divide the green seaweed cell wall into three major fractions. These include an aqueous-soluble fraction of 'sulfated polysaccharides', an alkali-soluble fraction consisting of hemicellulose-like material, and an insoluble fraction corresponding to α -cellulose. The major components of the *Ulva* cell wall and its compositional differences to the typical plant cell wall will now be discussed.

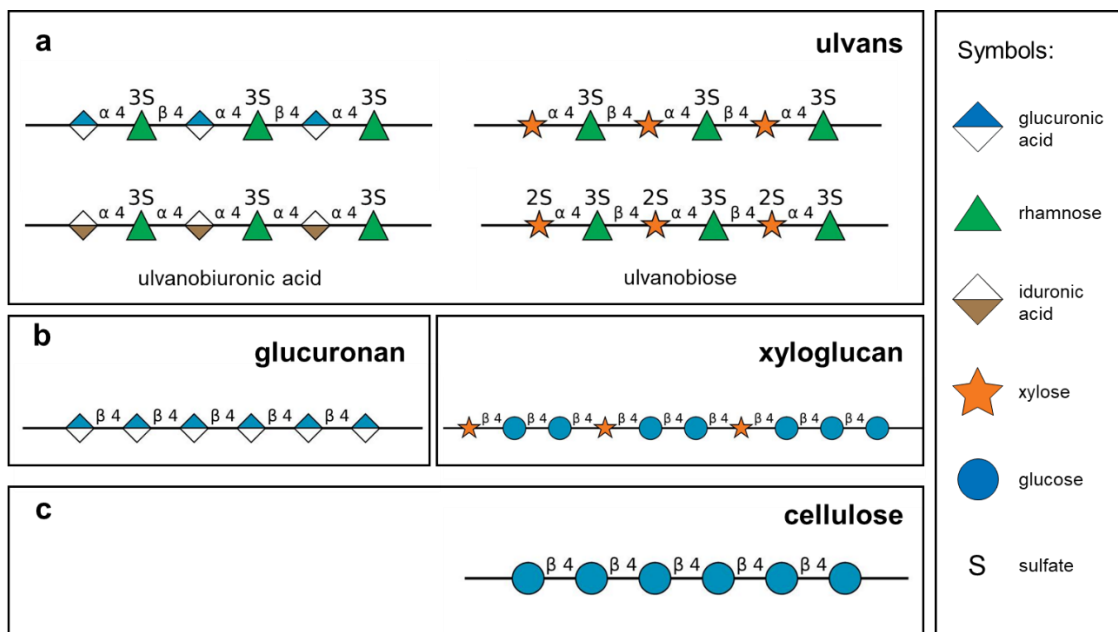


Figure 1.8. The major polysaccharides characterised in the *Ulva* cell wall include a) sulfated ulvans, b) two alkali-soluble polysaccharides – glucuronan and xyloglucan, and c) cellulose. Figures constructed with DrawGlycan-SNFG (Cheng, Zhou and Neelamegham, 2016).

1.4.3.2 The 'Soluble' Cell Wall: Sulfated Polysaccharides

The bulk of most macroalgal cell walls comprises acidic 'pectin-like' polysaccharides. These include the hydrocolloids of the phaeophyte browns (alginate, fucans) and rhodophyte red seaweeds (carrageenan and agar), and the ulvans of the chlorophyte green seaweeds (Boney, 1981; Pereira *et al.*, 2003; Chiovitti *et al.*, 2008; Jiao *et al.*, 2011; Martín-del-Campo *et al.*, 2021). For example, ulvan is reported to comprise up to 29% (w/w) of the biomass in many Ulvales (Lahaye and Robic, 2007). In the reds, carrageenophytes such as *Kappaphycus* and *Chondrus* may be composed of up to 50% (w/w) carrageenan (Rhein-Knudsen, Ale and Meyer, 2015; Syamsuddin *et al.*, 2019; Martín-del-Campo *et al.*, 2021). These 'pectin-like' polymers differ from the highly conserved land plant pectins homogalacturonan (HGA) and rhamnogalacturonan (RG-I, RG-II) through their sugar composition, with more diversity being observed in macroalgal 'pectins' (Vogel, 2008; Lerouge *et al.*, 2021). Algal representatives not only have a backbone composed can be constructed of arabinose (Ara), galactose (Gal), and rhamnose (Rha) as observed in plant pectin, but they also contain mannose (Man), xylose (Xyl), glucuronic acid (GlcA), and iduronic acid (IdoA) residues (Percival, 1979).

The most notable difference that can be observed between the cell walls of land plants and macroalgal cell walls is perhaps the presence of sulfated polysaccharides across the green, red, and brown lineages (Aquino, Grativol and Mourão, 2011; Jiao *et al.*, 2011; Patel, 2012; Confalonieri *et al.*, 2017). Sulfation of polysaccharides is hypothesised to be an osmoregulatory adaptation to saline environments and desiccation stress. Here, sulfation is thought to increase the negative charge of the cell wall, facilitating ion transport at a high salt concentration (Aquino, Grativol and Mourão, 2011). Sulfated polysaccharides are also present in planktonic microalgae and marine invertebrates (Giordano, Norici and Hell, 2005; Ganesan *et al.*, 2020). Whilst this modification is essentially absent in land plants and charophyte green algae, sulfated polysaccharides are observed in various halophytic plants and seagrasses (Aquino, Grativol and Mourão, 2011; Pfeifer and Classen, 2020), with seagrasses representing a divergent group of monocots that re-colonised the marine environment after terrestrialisation (Olsen *et al.*, 2016). In these species, sulfated galactan, arabinogalactans, and glucans are identified, supporting the hypothesis that polysaccharide sulfation is an adaptation to marine environments.

The sulfated polysaccharides found in the Ulvophyceae can be divided into three groups (Percival, 1979; Lahaye and Ray, 1996; Domozych *et al.*, 2012; Confalonieri *et al.*, 2017). The first group, found in the Bryopsidales and Cladophorales (e.g., *Codium*, *Cladophora*, *Caulerpa*, and *Chaetomorpha*) are characterised by the absence of uronic acid residues in the sulfated polysaccharide backbone. These polysaccharides are characterised as sulfated xyloarabinogalactans, with varying β -(1 $\rightarrow$ 3)-L-arabinans, β -(1 $\rightarrow$ 4)-D-galactans, and xylose backbones with variable sulfation patterns.

The second two groups are characterised by a rhamnose and 'uronic acid' containing backbone, typically glucuronic acid. These include the sulfated glucuronoxylorhamnogalactans of the Dasycladales seaweeds (e.g. *Acetabularia* spp.), and perhaps the best-characterised group of the three, the glucuronoxylorhamnans of the Ulvales and Ulotrichales, including *Ulva*, *Monsotroma*, *Gaylaria* and *Ulothrix*. The third group includes the ulvans, a group of heterogeneous, linear, polyanionic polysaccharides (Lahaye and Robic, 2007). The ulvans are perhaps the most economically valuable component of the *Ulva* ECM, being proposed for several biomaterials and biomedical applications due to ulvans gelling properties and potential bioactive properties *in vivo* (Pengzhan *et al.*, 2003; Patel, 2012; Ibrahim *et al.*, 2022).

The typical ulvan structure consists of repeating uronic acid $\rightarrow$ rhamnose disaccharide subunits joined by a series of α or β glycosidic (1 $\rightarrow$ 4) bonds, with sulfate esters located preferentially on C-3 of rhamnose. However, this is not always the case, and four typical repeating units are reported for ulvan in the literature (Lahaye and Robic, 2007; Robic, Gaillard, *et al.*, 2009; Kidgell *et al.*, 2019; Wahlström, Nylander, *et al.*, 2020). The first two, denoted ulvanobiuronic acids, have the uronic acid $\rightarrow$ rhamnose repeat structure and are the most abundant form characterised in this group of algae. These disaccharides are either $\rightarrow$ 4)- β -D-GlcA-(1 $\rightarrow$ 4)- α -L-Rha3S-(1 $\rightarrow$, or $\rightarrow$ 4 α -L-IdoA-(1 $\rightarrow$ 4)- α -L-Rha3S-(1 $\rightarrow$, where glucuronic acid (GlcA) is substituted for iduronic acid (IdoA) in the latter. A less common ulvanobiose disaccharide repeat structure is also reported, where the uronic acid is substituted for xylose or xylose-2-sulfate (i.e., sulfated at C-2), as $\rightarrow$ 4)- β -D-Xyl-(1 $\rightarrow$ 4)- α -L-Rha3S-(1 $\rightarrow$. Various branches may occur off the ulvan backbone, for example, 1 $\rightarrow$ 2 branching of glucuronic acid and xylose from the C-2 of rhamnose, in addition to sulfation of the C-2 of glucuronic acid and rhamnose subunits. However, most sulfation in ulvan still occurs on C-3 of rhamnose (Lahaye and Robic, 2007). Small amounts of other sugars such as glucose, mannose, and galactose are often characterised in ulvan extracts; however, it is often unclear how these are

incorporated into the polymer or whether they represent unavoidable contamination from the extraction process (Bobin-Dubigeon et al., 1997; Robic, Sassi, et al., 2009; Yaich et al., 2013; Glasson et al., 2017; Tabarsa et al., 2018; Zeroual et al., 2020; Ibrahim et al., 2022). For example, a repeating ulvanobiose backbone from an ulvan denoted UPP was characterised in *Ulva pertusa* that contained galactose and arabinose in addition to rhamnose-3-sulfate and xylose (Gao et al., 2020).

Whilst functional modifications such as *O*-acetylation and methylation are widely reported for plant pectins (Figure 1.9); we are currently unsure to what extent ulvans are modified to in the same way (Marry et al., 2000; Fellah et al., 2009; Zhu et al., 2014; Kyomugasho et al., 2015; Wu, Bulgakov and Jinn, 2018). However, various iterations of the biochemical pathway for ulvan biosynthesis and modification have been proposed in the literature and are now supported with data from ulvophyte genomes, which will directly facilitate experimental investigation into cell wall biosynthesis under different environmental conditions (Ficko-Blean, Hervé and Michel, 2015; De Clerck et al., 2018; Arimoto et al., 2019; He et al., 2021). For example, a series of sulfotransferases likely involved in cell wall modification were identified in the *U. compressa* genome (De Clerck et al., 2018). As a result, we will be able to improve our understanding of the dynamics of ulvan biosynthesis and its role in the cell with further investigation.

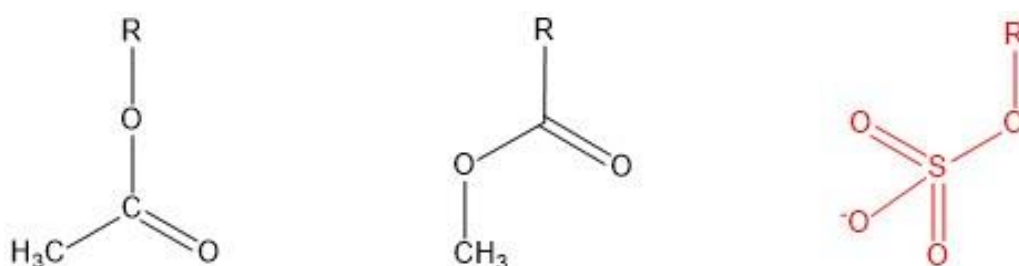


Figure 1.9. Functional modifications observed on plant cell wall polysaccharides include methyl groups (left) and *O*-acetyl groups (middle). Seaweed cell wall polysaccharides are often sulfated (right).

Whilst 'water-soluble', the ionic associations of ulvan with the cell wall limit the yield achievable during the extraction of ulvans from green seaweed biomass. As discussed by a comprehensive review on ulvan extraction that collated procedures and outcomes from different protocols (Kidgell *et al.*, 2019), chelating agents such as EDTA or oxalate are often used to scavenge cations that may encourage ulvan-cell wall interactions. Extraction yields are optimised in slightly acidic conditions (pH 4.5) and with elevated temperature, typically a little below the boiling point of water. As with most extraction protocols for biopolymers, there is a trade-off between yield and polymer quality, with excessive heat, acidic conditions, and extraction time increasing the likelihood of ulvan degradation, depolymerisation, and desulfation. As a result, the yield, sulfate content, and molecular weight of ulvans reported in the literature varies depending on the extraction protocol used and the source of biomass (Yaich *et al.*, 2013, 2017; Kidgell *et al.*, 2019; Guidara *et al.*, 2021). Ulvan content and composition is also affected by biological factors, including environmental stress, alga maturity, and season (Robic, Sassi, et al., 2009; Kidgell et al., 2019).

Although the biochemistry of ulvans has been assessed extensively, very few reports consider the *in vivo* function of the polymer in the *Ulva* cell wall. In our current understanding of the *Ulva* cell wall, ulvan is thought to carry out an analogous function to pectin in the plant cell wall by embedding the structural component of the cell wall (cellulose) and 'glueing' cells together (Bobin-Dubigeon *et al.*, 1997). Holzinger and colleagues (2015) reported that the ulvan layers are highly flexible under osmotic stress. The ulvan matrix also protects the cell against toxins, providing a negatively charged matrix for metal ions and exogenous compounds to adhere to, limiting entry into the cell (Chi *et al.*, 2021).

1.4.3.3 The 'Alkali' Soluble Cell Wall: Hemicelluloses

Two alkali-soluble polysaccharides have been characterised in the *Ulva* cell wall, a neutral xyloglucan and an acidic glucuronan (Lahaye, Jegou and Buleon, 1994; Ray and Lahaye, 1995; Lahaye *et al.*, 1996; Bobin-Dubigeon *et al.*, 1997; Ray, 2006; Redouan *et al.*, 2009). In these reports, alkali-soluble polysaccharides are separated from the sulfated polysaccharide fraction by extraction under alkaline conditions (e.g., with 6 M NaOH or 4 M KOH) and typically at lower temperatures. Both these polysaccharides represent a relatively minor component of the *Ulva* cell wall compared to the major fractions of ulvan and α -cellulose (Lahaye and Robic, 2007). As a result, few references are available documenting the quantification of xyloglucan and glucuronan content of the cell wall. However, yields of ~2.5% (w/w) have been reported for glucuronan (Redouan *et al.*, 2009).

Xyloglucan represents an important hemicellulose of the land plant cell wall, which is also found in charophytes (Del-Bem, 2018; Kim *et al.*, 2020; Mikkelsen *et al.*, 2021). The predominant role of xyloglucan is to structurally support the fibrillar cellulose network of the primary cell wall by cross-linking cellulose microfibrils in both a non-covalent and covalent manner (Zhou *et al.*, 2007; Eklöf and Brumer, 2010; Terrett and Dupree, 2019). Modification and regulation of the hemicellulose network, therefore, is necessary for facilitating cell division and growth (Xiao *et al.*, 2016).

Ulva xyloglucan differs in structure to the xyloglucan hemicellulose characterised in plants, which is formed from a β -(1 $\rightarrow$ 4) linked glucose backbone with branching α -(1 $\rightarrow$ 6) linked xylose residues. The xyloglucan branch may also be completed with a β -(1 $\rightarrow$ 2) linked galactose to the xylose and an α -(1 $\rightarrow$ 2) linked fucose to the galactose (Fry *et al.*, 1993; Schultink *et al.*, 2014; Del-Bem, 2018). In *Ulva*, a β -(1 $\rightarrow$ 4) linked trisaccharide of $\rightarrow$ 4)- β -D-Xyl-(1 $\rightarrow$ 4)- β -D-Glu -(1 $\rightarrow$ 4)- β -D-Glu-(1 $\rightarrow$ is proposed as the repeating structure (Lahaye, Jegou and Buleon, 1994). However, alternative xylose $\rightarrow$ glucose backbone structures in *Ulva* are likely, with possible branching and some degree of xylose sulfation (Ray, 2006). Despite the structural differences, xyloglucan endotransglucosylase (XET) activity required for reorganisation of xyloglucan-xyloglucan and xyloglucan-cellulose interactions in the vascular plant primary cell wall was verified experimentally in growing *Ulva* cells, which likely indicates that this hemicellulose performs a similar function in seaweed to plants (van Sandt *et al.*, 2007). A possible homolog for this gene was identified in the *U. compressa* genome (De Clerck *et al.*, 2018).

The second alkali-soluble polysaccharide is glucuronan (β -(1 $\rightarrow$ 4)-glucuronic acids). Glucuronans are perhaps the least well-characterised polysaccharide of the *Ulva* cell wall, despite being synthesised in various prokaryotic and green algal species (Pau-Roblot *et al.*, 2002). In particular, isolated bacterial glucuronans are of interest for their 'pectin-like' gelling and stabilising properties for biomaterials. In addition, algal glucuronans have been screened for bioactive effects on plant growth and defence (Delattre *et al.*, 2005; Abouraïcha *et al.*, 2017; Dubessay *et al.*, 2021). Due to the properties of this uronic acid-rich polymer, this polymer is likely associated with ulvan in the cell wall in the amorphous 'embedding matrix' around the cell. This is supported by staining, histochemical and electron microscopy analysis, which suggest the majority of glucuronan is likely located in the intercellular extracellular matrix, with some also associated with the fibrillar α -cellulose-xyloglucan skeleton (Bobin-Dubigeon *et al.*, 1997; Holzinger *et al.*, 2015). It is likely that they are present as minor components and commercially they are probably less valuable than ulvan, so have been less well studied.

Interestingly, the *Ulva* cell has a notably low arabinose and mannose content and does not appear to synthesise β -(1 $\rightarrow$ 3) or β -(1 $\rightarrow$ 4) xylans, with the former characterised in other ulvophytes (Percival, 1979; Pengzhan *et al.*, 2003; Domozych *et al.*, 2012; Yaich *et al.*, 2013, 2017; Tabarsa *et al.*, 2018; Přerovská *et al.*, 2021). As a result, we do not have any evidence for any other hemicelluloses typically observed in plants in the *Ulva* cell wall, such as arabinoxylans, mannans, and mixed linkage β -glucans. These structures in plants (particularly arabinoxylans in monocots) are known to be esterified to phenolics such as ferulic acid and coumaric acid and also associated with lignin, a large heterogenous polyphenol (Lichtenthaler and Schweiger, 1998; Ram, Dowell and Seitz, 2003; De Oliveira *et al.*, 2015; Terrett and Dupree, 2019). However, no equivalent phenolic association has been identified for algal hemicellulose.

1.4.3.4 The Insoluble Cell Wall

The second most abundant component of the *Ulva* cell wall comprises the insoluble fraction, which corresponds to cellulose microfibrils that form the skeletal structure of the cell wall. The fibrillar structure of cellulose offers rigidity to the cell, in contrast to the plasticity of the amorphous ulvan component (§1.4.3.2). The cellulose content of *Ulva* has been characterised in several different species, with highly variable yields observed, likely due to the use of varying extraction conditions and varied environmental conditions from the collected material (Yaich *et al.*, 2011; Moral *et al.*, 2019; Mukherjee and Keshri, 2019; Prabhu *et al.*, 2020; Wahlström, Edlund, *et al.*, 2020; Thygesen *et al.*, 2021; Goma *et al.*, 2022).

Some reports document the presence of xylose sugars within the *Ulva* cellulose extracts, with some proposing that xylose may be incorporated into the cellulose polysaccharide chain (Lahaye, Jegou and Buleon, 1994; Wahlström, Edlund, *et al.*, 2020). However, more recently, it has been suggested that the xylose identified in extracts is due to co-extraction of the previously described algal xyloglucan that persists in the insoluble fraction, supporting the view that the ‘hemicelluloses’ of *Ulva* cross-link the cell wall in an analogous manner to that in the classic plant cell wall model (Wahlström *et al.*, 2020).

Interestingly, cellulose may be associated with proteins in the cell wall (Lahaye, Jegou and Buleon, 1994). However, most reports of cellulose isolation in *Ulva* document the use of harsh extraction conditions with a ‘bleaching’ step or strong acid treatment, which removes this component to acquire ‘pure cellulose’ (Wahlström, Edlund, *et al.*, 2020; Thygesen *et al.*, 2021; Chopra and Manikanika, 2022). Cellulose from seaweed may have utility as pulp in the paper industry (Moral *et al.*, 2019), or as a replacement for ‘lignocellulosic biomass’ in bioenergy (Michalak, 2018), due to the low or non-existent ‘lignin’ content of algal biomass, it can be hydrolysed a lot more efficiently (see 1.4.3.5 for discussion on lignin). Furthermore, the rapid growth rates exhibited by green seaweeds provide a competitive advantage over land crops, as more cellulose can be generated more quickly.

Whilst cellulose is found across prokaryotic and eukaryotic lineages, as discussed previously, it is not a universal component in the chlorophyte cell wall, despite there being evidence for cellulose synthases within diverse chlorophyte genomes. For example, cellulose synthases have been identified in the genome for the volvocine algae, including *Chlamydomonas*, which we know lack it as a structural component (Yin, Huang

and Xu, 2009). The absence of cellulose is also observed in the siphonous and siphonocladous ulvophytes, which instead synthesise β -(1 $\rightarrow$ 4) mannans or β -(1 $\rightarrow$ 3) xylans (Percival, 1979; Fernández *et al.*, 2015). There is little biochemical evidence for either of these structures in the *Ulva* cell wall, despite the identification of homologs for several genes that could be involved in their biosynthesis in the genome (De Clerck *et al.*, 2018).

1.4.3.5 What Else?

Glycoproteins

Apart from the composition of the major polysaccharide fractions, we know relatively little else about the *Ulva* cell wall. The proteinaceous cell walls of the volvocine algae are known to have comparable glycoproteins to those observed in land plants, but the glycoprotein component of the *Ulva* ECM has not been characterised to any great extent in the literature. However, a series of orthologs for genes associated with hydroxyproline-rich glycoproteins have been identified in the *U. compressa* genome, including extensins, which have a crucial role in cross-linking and reinforcing the plant cell wall (Lamport *et al.*, 2011; De Clerck *et al.*, 2018). The presence of these proteins has also recently been verified experimentally, where arabinogalactan-like proteins with unique glycosylation profiles were purified from *U. lactuca* biomass (Přerovská *et al.*, 2021). Similar proteins have been identified in the siphonous seaweed *Codium* (Estevez *et al.*, 2009; Fernández *et al.*, 2010). Nonetheless, more work will be required to determine the role and function of these proteins in the intertidal ecosystem. Notably, in plants, these proteins are associated with osmotic and saline stress tolerance, which may be indicative of a role in 'intertidal stress' tolerance in green seaweeds (Derek T.A. Lamport, Kieliszewski and Showalter, 2006; Yang *et al.*, 2010, 2011; Zagorchev and Odjakova, 2011; Yang, Rao and Horst, 2013; Chen *et al.*, 2014).

Is There 'Phycolignin'?

Whilst lignin comprises a significant component of the vascular plant cell wall; algal cell walls lack lignin in a way that reflects their previously described differences in metabolic capabilities (1.3.2.3). This makes seaweed biomass a particular attractive biomass feedstock for biofuel production and paper making, because the presence of lignin in plant-derived feedstocks (i.e., lignocellulosic biomass) reduces the hydrolysability of polysaccharides, which means that time-consuming and costly 'delignification' protocols are often required (Himmel *et al.*, 2007; Grabber *et al.*, 2019). Whilst methods to modify lignin in proposed bioenergy crops by biochemical pre-treatment and genetic manipulation have been proposed (Welker *et al.*, 2015; Grabber *et al.*, 2019; da Costa *et al.*, 2021; Danila *et al.*, 2021), the use of seaweeds provides a ready 'lignin free' biomass source that can be processed much more efficiently (Michalak, 2018), which as previously discussed demonstrate far higher growth rates than land plants (1.3.1). This improved 'digestibility' of seaweed may also have value for agricultural purposes – i.e., in animal feed (Ventura and Castañón, 1998; Kammoun *et al.*, 2020).

The evolution of the lignin within the secondary cell walls of vascular plants is considered a key contribution to the increased complexity and tolerance to terrestrial environmental stresses observed within the streptophyte lineage (Boerjan, Ralph and Baucher, 2003; Lei, 2017; Renault *et al.*, 2017; Renault, Werck-Reichhart and Weng, 2019). Lignin is a complex polyphenol that stiffens the plant cell wall, provides structural support to vascular structures, and forming a structural barrier that protects against pathogen infection and herbivores (Liu, Luo and Zheng, 2018). Lignin confers structural support by cross-linking the cell wall fibres, forming a series of covalent and non-covalent interactions with hemicelluloses (Kang *et al.*, 2019; Terrett and Dupree, 2019). The development of lignified vascular tissue facilitated the increased complexity we can observe in plants, enabling more efficient water and nutrient transport in a terrestrial environment where water is a limiting factor (De Vries and Archibald, 2018). By contrast, aquatic organisms such as seaweeds do not require vascular transport, as water and nutrients are absorbed continuously from the water column, hence there appears to be no obvious evolutionary pressure that appears to require lignification (Hurd *et al.*, 2014). Lignification also comprises part of the land plant stress response, with multiple studies documenting lignin-associated responses to heat shock, drought, hypo-osmotic,

and oxidative stressors (van de Mortel *et al.*, 2006; Moura *et al.*, 2010; Bhardwaj *et al.*, 2014; Cesarino, 2019; Chun *et al.*, 2019; Coleman, Brunner and Tsai, 2021; Li *et al.*, 2022).

As discussed (§1.3.2.3), in land plants the principal subunits of the lignin polymer, the monolignols, are synthesised as products of the shikimate-phenylpropanoid pathway with the phenylpropanoid component initiated by the deamination of phenylalanine to *trans*-cinnamic acid by the phenylalanine ammonia-lyase (PAL) enzyme, which is subsequently converted to *p*-coumaric acid via cinnamic acid 4-hydroxylase, or C4H (Dixon and Barros, 2019; De Vries *et al.*, 2021; Xue *et al.*, 2022). In certain plants (e.g., monocots), *p*-coumaric acid can be synthesised directly by enzymes with tyrosine ammonia lyase activity such as TAL or PTAL (Barros *et al.*, 2016). A cascade of enzymes then synthesises the respective monolignols *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol from *p*-coumaric acid. During lignification, the free radicals of these monolignols are polymerised within the apoplast, with this process thought to be catalysed by oxidase enzymes called laccases and class III peroxidases (Marjamaa, Kukkola and Fagerstedt, 2009; J. Wang *et al.*, 2015; Ganewatta, Lokupitiya and Tang, 2019; Perna *et al.*, 2020). Once polymerised, the respective monomers are known as 4-hydroxyphenyl (H), guaicyl (G), and syringyl (S) subunits which are derived from *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol respectively (Ralph and Landucci, 2010; Lu *et al.*, 2017).

The degree of lignification and its composition within plant cell walls is highly variable, influenced by species, tissue type, developmental phase, and environmental conditions. For example, the lignin in dicots is typically G and S type lignin, whereas monocot lignin usually has all 3 (Welker *et al.*, 2015; Dixon and Barros, 2019). Furthermore, due to the relative non-specificity of lignin polymerisation, many phenolic structures can be incorporated into a lignin complex, including hydroxycinnamates (ferulate and *p*-coumarate) and hydroxystilbenes, with the latter two typically observed in monocot plants (Yelle, Ralph and Frihart, 2008; Ralph, 2010; Mansfield *et al.*, 2012; del Río *et al.*, 2017).

The acquisition of PAL, the development of the phenylpropanoid pathway, and the emergence of lignin in vascular plants are considered to be key events that contributed to the success of plants on land (De Vries *et al.*, 2017). In the case of the PAL gene, it is hypothesised that this was acquired in the common ancestor of land plants by a horizontal gene transfer event from a soil bacterium (Emiliani *et al.*, 2009). This progression in complexity is reflected by the observation that the more basal land plants

(i.e., the bryophytes) synthesise phenylpropanoids such as flavonoids and have 'lignin-like' structures in their cell walls (Renault *et al.*, 2017; Davies *et al.*, 2020). However, as stated previously, lignin has been positively identified in charophyte green algae in various *Coleochaete* species, indicating that the biosynthetic machinery to synthesise lignin in plants may predate the divergence of plants (Sørensen *et al.*, 2011).

Whilst these genomic observations provide evidence to indicate there is a 'proto'-phenylpropanoid pathway in some of the late divergent charophytes with phenylalanine ammonia lyase identified in some (but not all) of the later divergent charophyte genomes (Hori *et al.*, 2014; Nishiyama *et al.*, 2018; Jiao *et al.*, 2020), the positive identification of lignin in a divergent rhodophyte seaweed *Calliarthron cheilosporioides* (Corallinales) is evidence that lignin structures have evolved independently outside the streptophyte lineage (Martone *et al.*, 2009). In this species, using immunolabelling and diagnostic gas chromatography-mass spectrometry, lignified cell walls containing β -O-4 linked syringyl and guaicyl subunits were positively identified as observed in plant lignin (Lu and Ralph, 1997).

Guaicyl and syringyl monolignols are also proposed to be present in *Ulva* from metabolomic analyses (Gupta *et al.*, 2013). In addition, there are several biochemical reports that indicate 'lignin-like' fractions in *Ulva*, with lignin content estimated by various methods (Espiñeira *et al.*, 2011; Yoza and Masutani, 2013; Yaich *et al.*, 2015; Bikker *et al.*, 2016; Fernandes *et al.*, 2019; Moral *et al.*, 2019; Jonjaroen, Ummartyotin and Chittapun, 2020). Additionally, *Ulva* cell walls appear to demonstrate UV-induced cell wall autofluorescence comparable to lignified and phenolic-associated cell walls in plants, indicating that there may be an equivalent component associated (Pegg, Gladish and Baker, 2021). Finally, a lignin-like structure is hypothesised to be present in fucoid algae (Ford *et al.*, 2020; Alzate-Gaviria *et al.*, 2021). However, to date, no report documents the definitive presence of 'phycolignin' in *Ulva* or any other seaweed, as performed for *Calliarthron* by Martone (2009).

Whilst algae have a few of the genes that make up the core phenylpropanoid pathway in plants (Figure 1.10), the absence of ammonia lyases in algal genomes (including *Calliarthron*) indicates that 'phycolignin' must be synthesised by an alternative yet unknown pathway (De Vries *et al.*, 2017, 2021; Xue *et al.*, 2022). Without the conversion of phenylalanine to *trans*-cinnamic acid or the conversion of tyrosine to *p*-coumaric acid, which forms the fundamental step in this pathway, our current metabolic understanding does not provide any explanation for how the classic phenolics

including monolignols and flavonoids (both of which have been identified in algae) can be produced. In prokaryotes, the ability to produce phenolics is associated with the presence of ammonia-lyase genes, and PAL is identified in various cyanobacteria, which also populate marine environments (Moore *et al.*, 2002; Hill *et al.*, 2003; Moffitt *et al.*, 2007; Kukil and Lindberg, 2022). However, no equivalent mechanism appears to be present in any non-streptophyte alga.

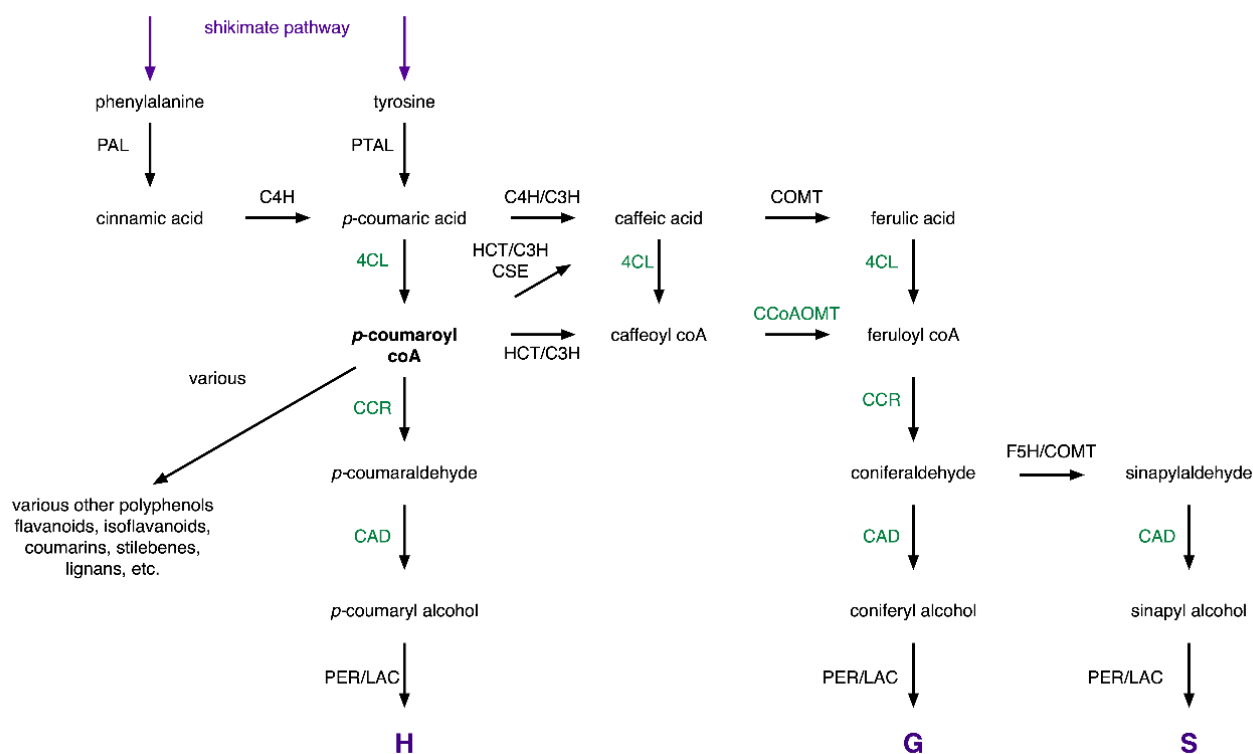


Figure 1.10. Phenylpropanoid pathway and monolignol biosynthesis. H, G, and S correspond to hydroxyphenyl, guaiacyl, and syringyl monolignols. Enzymes in green have been positively identified in chlorophyte genomes (Labeeuw *et al.*, 2015). Image adapted from Dixon, R. A., & Barros, J. (2019). Lignin biosynthesis: Old roads revisited and new roads explored. *Open Biology*, 9(12) and Xue, J. Y., *et al.* (2022). Transcriptome of the coralline alga *Calliarthron tuberculosum* (Corallinales, Rhodophyta) reveals convergent evolution of a partial lignin biosynthesis pathway. *PLOS ONE*, 17(7). Enzymes abbreviations refer to phenylalanine ammonia-lyase (PAL), phenylalanine-tyrosine ammonia lyase (PTAL), cinnamic acid 4-hydroxylase (C4H), p-coumaroyl shikimate 3'-hydroxylase (C3'H), caffeic acid O-methyltransferase (COMT), 4-hydroxycinnamoyl-CoA ligase (4CL), caffeoyl-CoA O-methyltransferase (CCoAOMT), cinnamyl alcohol dehydrogenase (CAD), cinnamoyl CoA reductase (CCR), hydroxycinnamoyl transferase (HCT), caffeoyl shikimate esterase, CSE, ferulic acid 5-hydroxylase (F5H), class III peroxidase (PER), and laccase (LAC).

The presence of 4CL, CCR, and CAD in algal genomes could theoretically catalyse the *p*-coumaric acid → *p*-coumaraldehyde → *p*-coumaryl alcohol cascade if an alternate method of generating *p*-coumaric were present (Figure 1.10). However, the enzymes required to produce the G and S monolignols that are hypothesised to be the components of ‘phycolignin’, are absent. Furthermore, the ‘phenylpropanoid’ associated genes identified in green and red algal genomes appear to be conserved across photosynthetic eukaryotes, including brown algae, haptophytes, dinoflagellates, and diatoms, indicating that these gene families likely originated in an ancestral eukaryote, long predating the phenylpropanoid pathway and emergence of lignin (Labeeuw *et al.*, 2015; De Vries *et al.*, 2021). These enzyme families also are likely to have multiple functions in the cell, not necessarily linked with phenylpropanoid synthesis, with the plant CAD family being divergent from those observed in other eukaryotes (Guo, Ran and Wang, 2010). This divergence is also observed for other gene families involved in the pathway (De Vries *et al.*, 2021). In addition, several algal species, including *Ulva*, were experimentally demonstrated to lack significant peroxidase activity against lignin monomers, and the sequenced macroalgal genomes appear to lack the laccase and class III peroxidase families associated with lignin polymerisation in land plants (Espiñeira *et al.*, 2011). However, a series of extracellular peroxidases with unknown functions were identified in the *U. compressa* genome (De Clerck *et al.*, 2018). Regardless, it is hypothesised that the ‘phycolignin’ identified in *Calliarthron* arose from convergent evolution rather than from a conserved ancestral pathway (Martone *et al.*, 2009), as would ‘phycolignin’ identified in any other seaweed.

Despite the contention about the biosynthetic pathway, as stated previously for chlorophytes (1.3.2), we know that seaweeds across the green, red, and brown lineages produce a diverse range of polyphenols (García-Casal *et al.*, 2008; Nwosu *et al.*, 2011; Martins *et al.*, 2013; Trigui *et al.*, 2013; Mezghani *et al.*, 2016; Klejdus *et al.*, 2017; Al-Malki *et al.*, 2018; Mekinić *et al.*, 2019; Cotas *et al.*, 2020; Ford *et al.*, 2020; Kumar *et al.*, 2020; Alzate-Gaviria *et al.*, 2021; Lomartire *et al.*, 2021). Furthermore, as discussed previously (1.3.2.3), the phlorotannins in fucoid algae are known to associate with the cell wall and are possibly synthesised by a ‘PAL-independent’ mechanism. Identification of whether a comparable ‘lignin-like’ structure associates with the green seaweed cell wall will offer a new perspective on the debate on about how lignin evolved, and how it contributes to multicellularity, as it would suggest that convergent evolution of lignin occurred three

times across lineages that developed macroscopic forms independently (plants, red seaweed, and green seaweed).

The reasons why marine organisms such as seaweed would need to evolve lignin must also be considered as part of this discussion, with lignin typically considered to be an adaptation to the unique challenges faced by plants on land – they need to conduct water in a water-depleted environment, and the need for increased structural support (Liu, Luo and Zheng, 2018). Seaweeds do not require vascular tissue, so perhaps ‘phycolignin’ is primarily needed to protect alga against damage from tidal forces (in ‘rigid’ algae such as *Calliarthron*) or for the maintenance of cell integrity during the highly variable osmotic conditions faced during the tide cycle (Vogel, 1984; Boller and Carrington, 2006; Martone and Denny, 2008). Lignin in plants also acts as a barrier to pathogens and helps to prevent the accumulation of toxins (e.g., heavy metals), which are also relevant environmental stressors for seaweeds (Zeroual *et al.*, 2020; Zúñiga *et al.*, 2020). Notably, lignin is retained in the seagrasses, albeit at a lower level than in terrestrial plants, indicating that it may have some function in marine adaptation (Klap, Hemminga and Boon, 2000; Pfeifer and Classen, 2020). The presence of this structure in *Ulva* and its putative role in the intertidal zone will be investigated in this thesis.

1.5 Thesis Outline

In this thesis, I outline a comprehensive investigation into the hypothesised ‘lignin-like’ component of the *Ulva* cell wall, which has not been performed previously for *Ulva*, nor for any other chlorophyte seaweed. This research aims first to identify whether a structural polyphenol is present in the *Ulva* cell wall, then to characterise the structure of this component, and to contrast it with lignin structures in representative plant biomass to infer how this structure has evolved in the absence of the phenylpropanoid pathway. In this thesis, I will refer to *Ulva*’s proposed lignin-like fraction as ‘phycolignin’ to distinguish it from true lignin in vascular plants.

To achieve this, I describe the use of confocal laser scanning microscopy to screen for ‘phycolignin’ *in vivo* and describe a cell wall fractionation protocol recently characterised for charophyte macroalgae to isolate the constituent components of the *Ulva* cell wall (Chapter 3). Here, I outline the use of various biochemical tests to identify whether the proposed ‘phycolignin’ associates with any specific cell wall polysaccharides. Then, I interpret data generated from biophysical analysis of fractions of interest for structural determination (Chapters 4 and 5). This was performed using

Fourier transform infrared spectroscopy and multidimensional nuclear magnetic resonance experiments. Finally, in the discussion chapter (Chapter 7), I discuss the outcomes of this investigation and how this work provides insight into how green seaweeds and plants have adapted to marine and terrestrial habitats, having developed multicellularity independently in both.

Lignin composition and structure is highly diverse. Accordingly, I compare *Ulva* biomass with barley straw which represents an alternate 'lignin-positive' biomass feedstock which has found utility in many of the industrial processes for which *Ulva* has been proposed for, such as bioethanol and biogas production, paper making, and biorefineries (Agu *et al.*, 2021; Raud *et al.*, 2021). In this thesis, I also perform an initial investigation into the effect of various abiotic factors discussed previously on cell wall structure (§1.4.2) to provide a platform for more comprehensive research in the future. The comparison with barley straw biomass will provide some industrial context to the thesis. With an improved understanding of the biochemical composition of the *Ulva* cell wall, we can better exploit it as a bioenergy crop via process development and biological manipulation.

Furthermore, in a supplementary research chapter (Chapter 6), I investigate the potential of the model seaweed *U. compressa* for 'molecular phycology' as a follow-up to the publication of the *U. compressa* genome, which immediately preceded the outset of my doctoral research project. Here, I report appropriate reference genes for quantitative PCR of *U. compressa* across various environmental stress conditions and perform a concise gene expression study to assess how a conserved group of *Ulva* 'stress proteins' respond to different stress combinations. As an outcome of this work, I also present some bioinformatic characterisation of the family of genes assessed, which may supplement more comprehensive studies in the future. The application of a molecular genetics approach to seaweed research has only become feasible in recent years; as such, there are a series of outstanding experiments for fundamental biological processes that need to be performed in seaweeds before we can carry out the more comprehensive transcriptomic and proteomic studies that are commonplace in plant and microalgal models. The research discussed in this chapter will demonstrate that molecular biology studies can be performed in a traditionally challenging organism to work with in vitro and will provide insight into how *Ulva* regulates the cellular stress response under dynamic intertidal conditions. Understanding the molecular basis of stress tolerance in an ecologically important alga such as *Ulva* is not only of ecological value but also will

be of benefit in 'agri-tech' for researchers looking to develop more stress tolerant land crops.

2. Materials and Methods

2.1 General Methods

2.1.1 Sample Collection

All macroalgal samples were collected at low tide from tide pools at Harbour Beach, Seaham (54°50'25.1"N 1°19'50.9"W, County Durham, UK). Collections were performed throughout the year, with greater algal coverage observed at the sampling site during the spring and summer months. Seaweed samples identified at this site included representatives of the Chlorophytes (*Ulva linza*, *Ulva intestinalis*, *Ulva intestinaloides* and *Ulva compressa*) and the Rhodophyceae (*Mastocarpus stellatus*). *Ulva* species were identified via microscopic and macroscopic morphological characteristics and species barcoding (§2.1.6). *Ulva compressa* (syn. *Ulva mutabilis*) was used as the model species in this project due to the availability of the fully sequenced genome and its widespread use as a model organism within phycological research (Wichard et al., 2015; De Clerck et al., 2018; Steinhagen et al., 2019). Various sources of plant biomass for the described staining experiments (§2.1.7.1) were collected from multiple sites around the Durham University campus, whilst barley straw (*Hordeum vulgare*) was purchased from a local garden centre.

All wild collected macroalgae were transported back to the laboratory in native seawater within plastic containers. Before incubation, macroalgal tissue was washed thoroughly with water and sorted by hand to remove debris and epiphytic organisms.



Figure 2.1. Photograph of the sampling site at Seaham, County Durham, UK. Macroalgal samples were collected from tide pools at low tide.

2.1.2 Culture Conditions

Washed and sorted macroalgae were maintained in artificial seawater solution (Table 2.12) within 3 L plastic tubs. Samples were incubated in a shaking incubator (MaxQ 6000, Thermo Fisher) at a standard temperature of 8°C and a 16 L: 8 D photoperiod, in accordance with expected North Sea conditions at the sampling site. Samples were agitated continuously with shaking at 80 rpm, and the light was provided with 8-Watt Natural White Fluorescent Tubes (Phillips), which achieved an approximate photosynthetic active radiation (PAR) of 70 $\mu\text{mol m}^{-2} \text{sec}^{-1}$.

For long-term maintenance of seaweed cultures, artificial seawater was replenished weekly and supplemented with Provasoli's Solution (Table 2.15). All collected seaweed was allowed to acclimate to incubator conditions for at least two weeks before any growth or stress experiments described in this thesis were performed.

2.1.2.1 Abiotic Stress Experiments

A series of experiments were carried out in *U. compressa* to mimic various intertidal environmental conditions in the laboratory. Thalli were transferred to 250 mL Erlenmeyer flasks for abiotic stress experiments. Samples were observed routinely under a microscope to assess the effect of experimental conditions on cell morphology and viability.

Saline

Thalli were exposed to hyposaline stress at salinities of 0 ‰ (parts per thousand) or 20 ‰ and hypersaline stress at salinities 50 ‰ or 90 ‰. Thalli were incubated in standard artificial seawater (35 ‰) as a control. For 'saline shock' experiments (0 ‰ and 90 ‰), *U. compressa* thalli were incubated in media for 3 hours under standard incubator conditions (8°C, 80 rpm agitation). For salinity acclimation experiments (20 ‰ and 50 ‰), thalli were incubated for two weeks under standard incubator conditions under the 16 L: 8 D photoperiod.

Hypersaline media was prepared by blending concentrated NaCl stock (5 M) with artificial seawater to the desired salinity due to the insolubility of the sea salt mix (Sigma) at concentrations greater than 38 ‰ (Sea salts Product Specification, 2022). Hyposaline media was prepared by diluting artificial seawater with Milli-Q or using Milli-Q to simulate freshwater (0 ‰). Nutrients (sodium nitrate and β -glycerophosphate

disodium hydrate) were added to all media at the standard concentrations used for routine culture work (see Table 2.12).

CuCl₂

Metal stress experiments were performed by incubating *Ulva* thalli in artificial seawater containing 100 μ M CuCl₂ for 24 hours, which was added to seawater from a 10 mM stock solution. Following this, samples were transferred back to control conditions (artificial seawater with no CuCl₂) for 72 hours to assess how seaweed recovered following metal exposure. Control samples were collected before exposure to stress as a comparison.

Heat Shock and Dual Stress

Samples were incubated in constitutive darkness the day before the combined stress experiments in artificial seawater. *U. compressa* thalli were then exposed to stress for 3 hours, at control temperature (10°C) or under heat shock (37°C), in artificial seawater pre-cooled or pre-heated to the set temperature of the respective incubator. At both temperatures, samples were either kept in the dark or exposed to standard light conditions (70 μ mol m⁻² sec⁻¹). A set of thalli were also removed from the media and were distributed evenly across multiple Petri dishes (Thermo Scientific) at both temperatures to simulate emersion at low tide. The emersion experiment was also performed in the presence and absence of light at the control temperature. Stressed samples were returned to control conditions (10°C, artificial seawater, dark) after the 3-hour stress experiment to assess whether they remained viable after testing. All experiments were set up in a dark room to prevent premature exposure to light. Artificial seawater was pre-heated to 37°C for heat shock experiments.

2.1.2.2 Sporulation Induction and Settlement

Gametogenesis and sporogenesis were induced in *U. compressa* samples by collecting individual samples from the storage incubator in the morning. Small fragments were cut from the apical regions of thalli and gently washed in artificial seawater. Fragments were then placed in 6 well plates (Sarstedt), 90 mm Petri dishes (Thermo Scientific) or culture flasks (Nunc) containing Provasoli's Enriched Seawater and incubated at 21°C under a 16 L: 8 D photoperiod. Induction was also trialled without seawater enrichment and under standard growth conditions (8°C). Fragments were inspected daily for evidence of differentiation using a compound microscope. Once there was evidence of swarmer release and settlement, culture flasks were returned to standard growth conditions (8°C, 16 L: 8 D photoperiod), and media was replenished weekly. *U. compressa* thalli were transferred to larger culture flasks once they grew too large for wells. Cultures were maintained as clonal populations during this project. For imaging experiments, glass coverslips were placed at the bottom of culture flasks to facilitate spore attachment.

An alternative method to induce gametogenesis was also trialled, following the protocol first reported for *U. mutabilis* by Strattman and colleagues (1996). This protocol was carried out with some slight modifications as follows:

1. Individual seaweed thalli were collected from the incubator in the morning.
2. Thalli were diced into small fragments (< 5 mm²)
3. Fragments were washed three times for 15 minutes with seawater to remove *Ulva* sporulation inhibitors from the fragmented sample. Fragments were recovered between each wash using a 100 µM mesh.
4. Seaweed fragments were placed in 50 mL enriched seawater in a petri dish.
5. Petri dishes were incubated at 21°C under a 16 L: 8 D photoperiod for 3 days. Fragments were inspected under the microscope daily for evidence of differentiation
6. After three days, biflagellate gametes from gametophytes or quadriflagellate spores from sporophytes are identified under the microscope if induction was successful.
7. Media was replaced through a 100 µM mesh one more time to induce swarmer release. This step dilutes swarming inhibitor from the media.
8. Swarmer can be harvested from the media by exposing the dish to light, with gametes demonstrating positive phototaxis and spores demonstrating negative phototaxis.

2.1.3 Imaging

2.1.3.1 Fluorescence Microscopy

Sample Preparation

All fluorescence microscopy was performed with live macroalgal or plant tissue. As a result, sample preparation requirements were minimal. For imaging, *Ulva* tissue was sliced carefully into small fragments (approximately 1-2 cm²) and then mounted in a drop of artificial seawater solution on a glass slide with a number 1.5 cover slip.

Most imaging experiments discussed in this thesis concerned macroalgal autofluorescence; as such extensive staining protocols were not required for the most part. However, protocols for the staining experiments that were required for this project are outlined in the biochemical tests section (2.1.7). All imaging for staining experiments was performed in parallel with a non-stained control.

Epifluorescence Microscopy

Epifluorescence microscopy was performed using a Zeiss Axiovert 200M Apotome and a Zeiss 63x Plan-APOCHROMAT Oil PH3 lens (1.4 NA). Images were acquired with the available filter cubes listed in Table 2.1.

Filter Cube	Excitation	Emission
DAPI	335 - 383 nm	420 LP
Alexa Fluor 488	450 - 490 nm	515 LP
HE YFP	482 - 512 nm	520 - 550
Alexa Fluor 546	540 - 552 nm	590 LP

Table 2.1. Filter cubes of the Zeiss Axiovert 200M Apotome

Confocal Laser Scanning Microscopy (CLSM)

All confocal microscopy was performed using a Leica SP5 CLSM using a Leica 63X HCX PL APO Oil UV lens (1.4 NA), with excitation with either the 405 nm or the tuneable Argon laser line (458nm, 478nm, 488nm, 494nm, and 514nm). Emission filter (PMT) settings for image acquisition were modified based upon the fluorophore of interest and are stated in the figure legends for the presented images. Emission spectra (or λ scans) were generated using the CLSM xy λ function to establish appropriate detection windows for each fluorophore of interest.

Image Analysis

All image analysis, including annotations with scale bars and calibration bars, LUT selection, background subtraction, region of interest selection (ROI), and calculation of fluorescence intensity was performed with Fiji/ ImageJ. Ratiometric analysis for dual excitation experiments was performed using the Image Calculator function ($channel\ 1 / channel\ 2$).

2.1.4 Extraction Protocols

2.1.4.1 Tissue Homogenisation

For all extraction experiments, fresh macroalgal tissue was rinsed briefly with Milli-Q water to remove excess salt and debris that would interfere with extraction protocols. Samples were blotted dry, then flash frozen with liquid nitrogen. Frozen tissue was either wrapped in aluminium foil and transported in liquid nitrogen to a -80°C freezer for storage until required or was ground to a fine powder with more liquid nitrogen with a pre-chilled pestle and mortar for immediate extraction work. Pestles and mortars were cleaned thoroughly before tissue homogenisation by soaking in a dilute sodium hypochlorite solution overnight, before being rinsed in deionised water, and then dried in a drying oven. The amount of fresh biomass processed during homogenisation was dependent on the experiment.

2.1.4.2 RNA

RNA Isolation

All RNA extractions were performed using the PowerPlant RNA Extraction Kit (Qiagen), following the manufacturer's instructions to bind, wash and elute RNA from a silica membrane spin column. The kit protocol is summarised in Figure 2.2. The protocol was modified slightly at the sample preparation and cell lysis stage, as tissue was homogenised manually under liquid nitrogen as previously described in 2.1.4.1 rather than using a bead beater as recommended by the manufacturer. Due to the presence of a robust cell wall in seaweeds that makes nucleic acid isolation difficult, manual homogenisation using liquid nitrogen was preferred to automated homogenisation methods to ensure that cells were effectively lysed during the RNA extraction process, with insufficient nucleic acid yields being achieved in preliminary trials where a bead beater was used (Bullet Blender, Next Advance). An on-column DNase digestion step

was carried out for all extraction trials to remove genomic DNA, using the RNase-Free DNase Kit (Qiagen). Samples were eluted with 50 μ L RNase-free water. All RNA samples were stored at -80°C until required.

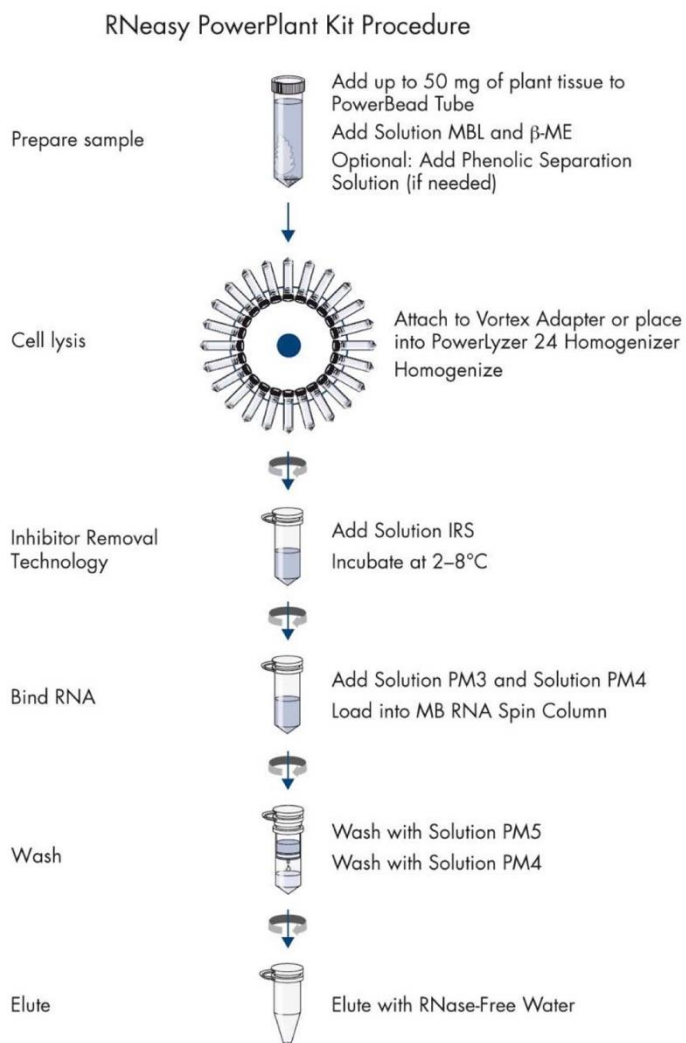


Figure 2.2. RNA extraction protocol from RNeasy PowerPlant Kit.
Protocol reprinted from the RNeasy Power Plant Kit Handbook (QIAGEN, 2017).

RNA Assessment

RNA was quantified using a NanoDrop spectrophotometer using 1 μ L sample (Thermo Fisher), and RNA integrity was assessed by denaturing gel electrophoresis (2.1.5.1). Degraded RNA samples were not used for cDNA synthesis.

cDNA Synthesis

cDNA synthesis was performed using the ReadyScript cDNA Synthesis Mix (Sigma Aldrich) using 1 μ g of RNA template. This kit is supplied as a 5X master mix consisting of all the components required for cDNA synthesis. All reactions were prepared in 0.2 mL PCR tubes with components on ice as per Table 2.2.

Component	Amount	Final concentration
5X Master Mix	4 μ L	1X
RNA	1 μ g	
Water	Add to 20 μ L	

Table 2.2. Protocol for cDNA synthesis with the ReadyScript cDNA Synthesis Mix (Sigma Aldrich)

The thermal cycler (G-Storm GS1) parameters shown in Table 2.3 were used for the reverse transcription reaction.

Step	Temperature	Time
1	25°C	5 minutes
2	42°C	30 minutes
3	85°C	5 minutes
Hold	4°C	

Table 2.3. Thermal cycler conditions for reverse transcription reactions.

As this cDNA synthesis kit is supplied as a master mix (which means that reverse transcriptase cannot be omitted from the reaction), corresponding 'no RT' (reverse transcriptase) controls for each RNA sample were manufactured by adding nuclease-free water to 1 μ g RNA to a final volume of 20 μ L.

Completed reactions were diluted with nuclease-free water (1/5, 1/10, or 1/100) and stored at -20°C until required for quantitative PCR (qPCR). The success of the cDNA synthesis reaction was assessed by amplification of reference genes by the methods discussed in 2.1.5.5.

2.1.4.3 Protein

Grinding buffer containing 10 mM TRIS, 1 mM EDTA, 1% (v/v) Triton X-100, and 1% (v/v) 2-mercaptoethanol was added to homogenised tissue at a ratio of 10 ml buffer per 1 g biomass. Suspended biomass was then transferred to 15 ml falcon tubes. Tubes were vortexed thoroughly, and then centrifuged at 3000 g for 10 minutes. The supernatant was recovered from each sample and transferred to a larger falcon tube (50 mL). Protein was then precipitated from the recovered supernatant using acetone, which was added to each tube at a 4:1 ratio (4 parts acetone, 1 part supernatant). Samples were incubated overnight at room temperature before pelleting the precipitated protein by centrifugation. Once the residual acetone had evaporated from the pellet, protein samples were suspended in 600 μ L lysis buffer containing 6 M urea, 2 M thiourea, and 4% (w/v) CHAPS and transferred to 2 mL Eppendorf tubes. Protein concentration was estimated using 2 μ L sample with the Bradford Protein Assay protocol (§2.1.5.2) using BSA in lysis buffer as a standard.

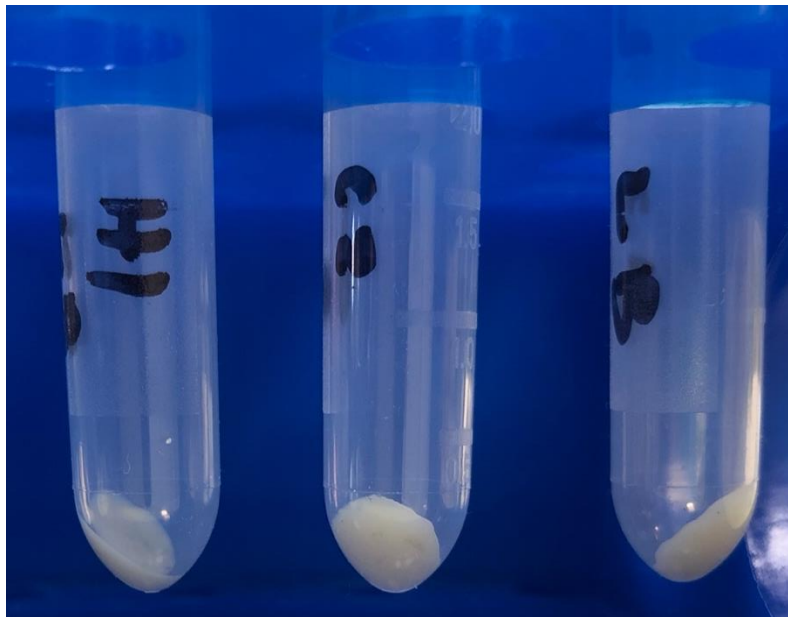


Figure 2.3. Extracted protein from *Ulva* spp. biomass forms a soft white pellet following acetone precipitation and centrifugation

Cell Wall Protein

Cell wall proteins were extracted using 0.5 g cell wall extract prepared as described in (2.1.4.4). Samples were incubated with 50 mL 1 M LiCl, 1 M NaCl, or 0.2 M CaCl₂ on a roller mixer at room temperature for 2 hours, with salt extraction being reported in the literature as a successful method for liberating ionically bound cell wall proteins in both algal and plant biomass (Voigt, 1985; Chivasa et al., 2002; Boudart et al., 2005; Voigt et al., 2014). Insoluble material was pelleted by centrifugation (3000 g, 10 minutes), and the supernatant was recovered for acetone precipitation at a 4:1 ratio as previously described (4 parts acetone, 1 part supernatant). Precipitated material was collected by centrifugation, then washed 4 times with 80% acetone with further centrifugation steps performed to recover material. Following the final wash, residual acetone was allowed to evaporate from the pellet at room temperature before resuspension of the pellet in lysis buffer (Table 2.21).

2.1.4.4 Cell Wall Fractionation

Cell walls were isolated from homogenised macroalgal and plant tissue as the alcohol insoluble residue (AIR) from four sequential ethanol washes (70% w/w) and a final wash in acetone. Biomass was recovered between each wash via centrifugation (4000 rpm, 10 minutes) and filtration through Miracloth, and the ethanol or acetone was discarded. Each wash step was performed for at least 24 h with constant mixing using a magnetic stirrer. Following removal of acetone in the final wash step, residual acetone was allowed to evaporate from the residue at room temperature in a fume cupboard. 2 g of AIR was then fractionated into pectin-like, hemicellulose-like, and cellulose-like fractions by a sequential extraction protocol for macroalgal tissue most recently described by O'Rourke and colleagues (2015). This protocol is summarised in Figure 2.4.

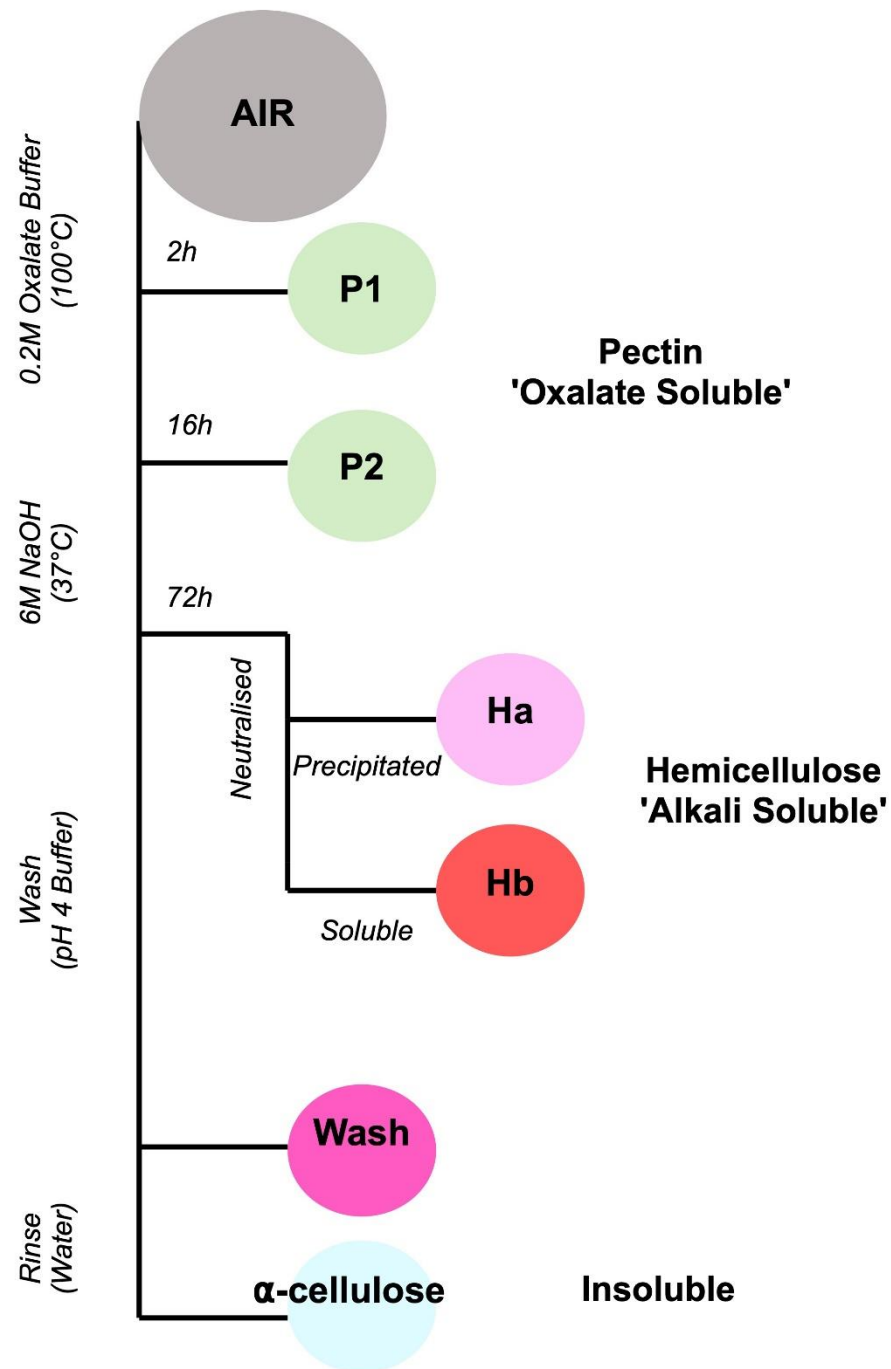


Figure 2.4. Flow chart of cell wall fractionation protocol for macroalgal biomass, most recently described by O'Rourke and colleague (2015).

Firstly, two successive extractions of 'pectin-like' polysaccharides were performed with pH 4.2 ammonium oxalate solution (Table 2.22) at 100°C. The first extraction step was performed for 2 hours, and the rapidly oxalate-soluble fraction (P1) was retrieved as the supernatant by centrifugation (3000 g, 10 mins) and filtration with Whatman filter paper (if required). The insoluble residue from this process was then extracted for a further 16 h with fresh oxalate buffer, yielding the slowly oxalate-soluble fraction (P2). The oxalate-insoluble material was then incubated for 72 hours at 37°C in 6 M NaOH to extract hemicelluloses (Ha and Hb). Alkali-soluble material, recovered by centrifugation and filtration (if required), was then neutralised with glacial acetic acid and stored as the temporary 'hemicellulose' fraction. The insoluble material that remained after NaOH extraction was then washed several times with oxalate buffer at pH 4.0, and the pooled washings were stored as the 'wash' fraction. The remaining solid was then rinsed several times with Milli-Q to be retained as the insoluble α -cellulose fraction.

Following extraction, the extracts in solution – including the oxalate-soluble P1 and P2 fractions, the alkali-soluble hemicellulose extracts, and the pooled wash samples were dialysed against water 3 times for 16-hour intervals using cellulose membrane dialysis tubing with a molecular weight cut-off of 12 KDa (Sigma-Aldrich). Material that precipitated post-neutralisation in the hemicellulose extract was recovered after dialysis via centrifugation as the Ha fraction, with the material that remained in solution corresponding to Hb. The Ha sample was rinsed several times with water as performed for the α -cellulose fraction. Both the solid (Ha and α -cellulose) and solubilised extracts (P1, P2, Hb, and wash) were then lyophilised for biochemical analysis and assessment of yield.

A separate experiment was performed where the oxalate insoluble fraction was retrieved before 6 M NaOH treatment. This sample was rinsed with water and lyophilised as performed for the solid Ha and α -cellulose extracts. All extraction steps were performed at a ratio of 50 mL extraction buffer per 1 g biomass being processed.

Enzymatic Digestion Protocol

A supplementary digestion of the cell wall fractions obtained during the sequential extraction protocol was performed to enrich the hypothesised 'phycolignin' associated with the *Ulva* cell wall that would remain in the insoluble residue following successful hydrolysis of the associated polysaccharides. The enrichment protocol used was adapted from a method initially described by Hortling and colleagues (1992) for purifying residual lignin from wood powder and kraft pulps using cellulase.

Powdered cell wall fractions (100 mg) were hydrolysed in 50 mL digestion buffer containing 1% (w/v) Cellulase Onozuka R-10 (Duchefa) or 1% (w/v) Driselase in 0.05 M acetate buffer within 50 mL falcon tubes. Samples were incubated at 40°C for 3 days with continual rotation. The insoluble residue from enzymatic hydrolysis was then pelleted by centrifugation (4400 rpm, 10 minutes). For the α -cellulose fraction, a supplementary extraction in sodium hydroxide was then performed to solubilise lignin, lignin-like compounds, and other alkali-soluble compounds from the residue. As stated previously, the α -cellulose fraction from the sequential extraction corresponds to the insoluble component of the cell wall, with soluble ulvans and hemicelluloses extractable with oxalate buffer and sodium hydroxide, respectively. Therefore, it was hypothesised that 'lignin' could be separated from residual cellulose in the insoluble pellet left following hydrolysis by solubilisation in sodium hydroxide.

For this purpose, the hydrolysate (supernatant) from the previous centrifugation step was discarded, and the pellet was solubilised in 25 mL 0.5 M NaOH. The residue that did not solubilise in 0.5 M NaOH was then removed from the solution by filtration through a Grade 601 Filter Paper (Fisher Scientific). The NaOH soluble fraction was then precipitated from the filtrate by slowly dropping 0.5 M HCl into the solution to a final pH of 2.5. The precipitate from this step was pelleted by centrifugation, then washed 6 times with Milli-Q water by repeated resuspension in water, vortexing, and centrifugation. The digested, purified, and washed sample was then lyophilised for analysis. Two samples were prepared and obtained by this method. The first, 'cellulase insoluble residue 1' (CIR-1), was washed and lyophilised immediately after enzymatic hydrolysis, and the second sample, 'cellulase insoluble residue 2' (CIR-2), was subjected to the described purification protocol.

2.1.5 General Molecular Biology Protocols

2.1.5.1 Gel Electrophoresis

DNA

The agarose concentration prepared was modified based on the anticipated amplicon size. Genomic DNA (gDNA) and PCR products > 250 base pairs were mixed with 6X DNA Loading Dye (Thermo Scientific) and assessed alongside the GeneRuler 1 kb DNA Ladder (Thermo Scientific) on 0.8% (w/v) agarose-TAE gels which were run for 1 hour at 80 V. Small amplicons (< 250 base pairs) were assessed on 3.5% (w/v) agarose-TAE (1X) gels alongside HyperLadder 25 bp (Bioline). Products for sequencing were run on 1% (w/v) low melting point agarose-TAE (1X) gels with the appropriate DNA ladder. All agarose gels were prepared with ethidium bromide and were imaged under UV light using a transilluminator.

RNA

The integrity of isolated RNA (2.1.4.2) was assessed on denaturing RNA gels. Here, 5 µL RNA loading buffer (Table 2.16) was added to 300 ng RNA sample. Samples were incubated at 65°C for 10 minutes, then were loaded onto a 1.2% (w/v) agarose-MOPS (1X) gel. Gels were run in 1X MOPS running buffer for 30 minutes at 75 V before imaging. Gels were inspected for evidence of RNA degradation under UV illumination, indicated by a low molecular weight smear on the gel and the absence of visible ribosomal RNA (rRNA) bands (28S and 18S).

2.1.5.2 Bradford Protein Assay

A Bradford Protein Assay (Bio-Rad) was performed to quantify protein samples according to the kit instructions with modification. Concentrated Bradford reagent was diluted 1/5 in Milli-Q, then filtered through Whatman filter paper to remove any precipitates. A 1 mg/ml bovine serum albumin (BSA) standard was prepared in lysis buffer to generate a 5-point standard curve. Samples were mixed with 0.9 mL Bradford reagent, 80 μ L Milli-Q, and 10 μ L 0.1 N HCl, according to Table 2.4. Absorbance was measured at 595 nm after a 5-minute incubation period.

Sample	Bradford Reagent (μ L)	Milli-Q (μ L)	0.1 N HCl (μ L)	Lysis Buffer (μ L)	Sample (μ L)
Blank	900	80	10	10	0
Standard (BSA in Lysis Buffer)	900	80	10	Varied (9, 8, 6, 2, or 0)	Varied (1, 2, 4, 8, or 10)
Protein Extract	900	80	10	8	2

Table 2.4. Sample volumes for Bradford Assay.

2.1.5.3 Primers

All primers were purchased from Integrated DNA Technologies (IDT) as 25 nmol DNA oligonucleotides unless otherwise stated. Upon delivery, lyophilised primers were reconstituted in 1X IDTE buffer (10 mM TRIS, 0.1 mM EDTA, pH 8.0) to a concentration of 100 μ M. All primers were stored at -20°C.

For conventional PCR, primer stocks were manufactured by diluting forward and reverse primers 1 in 10 in nuclease-free water to a concentration of 10 μ M. For quantitative PCR (qPCR), primer stocks were prepared by a 1 in 20 dilution to 5 μ M. Sequencing primers were diluted to 3.2 μ M (see §2.1.6).

2.1.5.4 Direct PCR

The Phire Plant Direct PCR Master Mix (Thermo Scientific) was used for PCR in *U. compressa*. Direct PCR enables PCR amplification with plant material without the need for DNA extraction (Li *et al.*, 2017). The kit protocol was slightly modified for use in *Ulva*, with the polysaccharide-rich biomass of macroalgae known to be inhibitory for molecular assays (Jin *et al.*, 1997; Wattier, Prodöhl and Maggs, 2000; Varela-Alvarez *et al.*, 2006; Greco *et al.*, 2014).

Samples were firstly prepared by homogenising a small fragment (1 mm²) of fresh seaweed tissue in 50 µL Phire dilution buffer using a bead beater (Bullet Blender, Next Advance). Homogenate was briefly centrifuged to pellet debris, and the supernatant was diluted 1 in 10 with additional dilution buffer for use as template. 20 µL reactions were set up on ice, as stated in Table 2.5.

Component	Amount	Final concentration
2X Master Mix	10 µL	1X
PEG(400)	1 µl	5%
Primers	1 µL	0.5 µM
Template	1 µL	
Water	Add to 20 µL	

Table 2.5. Reaction volumes for direct PCR using the Phire Plant Direct PCR Master Mix.

Due to the suspected presence of Taq inhibitors in *U. compressa* extracts, dPCR was trialled with and without poly(ethylene) glycol 400 (5% v/v). PEG has been shown experimentally to reverse the inhibitory effects of acidic plant polysaccharides on Taq Polymerase (Pandey, Adams and Flournoy, 1996). This was demonstrated to be the case here, where adding PEG removed inhibition of PCR reactions. PCR was performed using the parameters in Table 2.6.

Step	Temperature	Holding Time	Number of Cycles
Denaturation	98°C	5 min	1
Denaturation	98°C	5 sec	30
Annealing	Varied	5 sec	
Extension	72°C	20 min	
Extension	72°C	1 min	1
Hold	4°C	Indefinite	

Table 2.6. Thermal cycler parameters for PCR reactions set up with the Phire Plant Direct PCR Master Mix.

Gradient PCR was carried out to determine an appropriate annealing temperature for new targets. This was performed by programming a thermal gradient on the G-Storm GS1 thermal cycler for the annealing temperature parameter; as such multiple annealing temperatures could be assessed simultaneously. No template controls were included in each run to check for contamination.

2.1.5.5 Quantitative PCR

Parameters

Quantitative PCR (qPCR) was performed using the Biotium 'Forget-Me-Not' qPCR kit. This kit uses the 'EvaGreen' dye, a less toxic but spectrally similar fluorescent dye to SYBR Green (Mao, Leung and Xin, 2007). 20 µL qPCR reactions were prepared as per Table 2.7. qPCR was performed using a Rotor-Gene Q thermal cycler (Qiagen) using the parameters in Table 2.8.

Component	Amount	Final concentration
2X Master Mix	10 µL	1X
Primers	1 µL	0.25 µM
cDNA	1-3 µL	
Water	Add to 20 µL	

Table 2.7. Reaction volumes for qPCR with the Biotium 'Forget-Me-Not' qPCR kit.

Step	Temperature	Holding Time	Number of Cycles
Enzyme activation	95°C	2 min	1
Denaturation	95°C	5 sec	50
Annealing	60°C	10 sec	
Extension	72°C	10 sec	
Dissociation/ melt curve	65-95°C		

Table 2.8. Parameters for qPCR performed using the Rotor-Gene Q thermal cycler

qPCR experiments were performed using at least 3 biological replicates for each test condition and 3 technical replicates for each sample. A no template control was included for each primer set analysed. Respective no reverse transcriptase controls for each cDNA sample used were assayed with the primers for reference genes (Table 2.9) to detect if residual genomic DNA not removed during the extraction process interfered with the qPCR reaction.

Primer Design

Primers for the qPCR test and reference genes were designed using the *U. mutabilis* genome (De Clerck *et al.*, 2018) available from the ORCAE online resources (Sterck *et al.*, 2012). Potential reference gene targets were selected following a literature review for commonly used qPCR control genes used for development and stress response experiments in plants and algae.

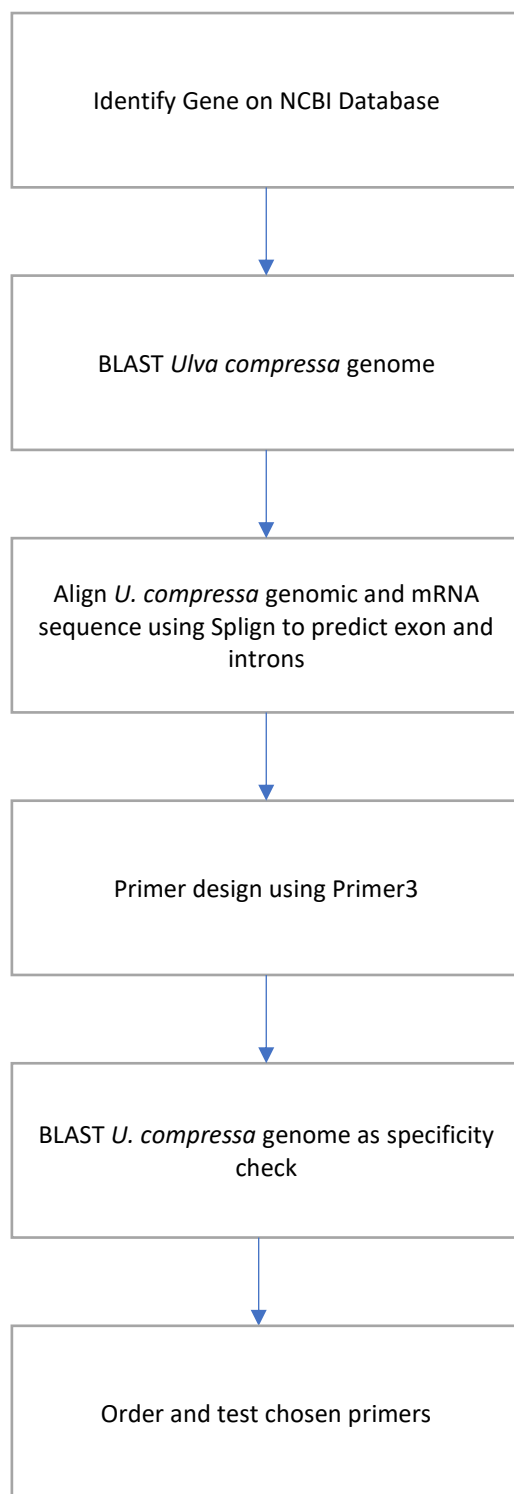


Figure 2.5. Flow chart of the primer design process

The protocol for primer design is summarised in Figure 2.5. Briefly, target genes in the *U. mutabilis* genome were identified by the BLAST (Basic Local Alignment Search Tool) algorithm using plant or algal homologs (Altschul *et al.*, 1990), and reciprocal BLAST searches were performed. Primers were designed to span exon-exon junctions where possible to limit the risk of contaminating genomic DNA in samples being amplified. If a gene model was unavailable, exon and intron sequences were predicted by aligning *U. mutabilis* mRNA and genomic sequences using the Splign alignment tool (Kapustin *et al.*, 2008).

The Primer 3 bioinformatic tool (Untergasser *et al.*, 2012) was then used to assist primer design from the desired sequence regions, with parameters set for an optimum amplicon size of 100-200 base pairs and a T_m of 60°C. The specificity of returned primers was then assessed by performing a BLAST of individual sequences against the *U. mutabilis* genome. Suitable primers were ordered from IDT as 25 nm oligonucleotides. All primer sequences designed for qPCR are shown in Table 2.9.

Gene	Orcae ID	Cellular function	Primers (5'-3')
Actin	UM112_0003	Cytoskeleton	TCTTGGCTCTGAAGTATCCCA GGGCGTTAAAAGTCTCGAAC
α -tubulin	UM021_0090		CACTTCTTCGTCACAATGCGT TGTTGAAAGCGTCGTCACCT
β -tubulin	UM182_0008		GACTCGACCGGCACATACC CCAGTCTGACCGAACACGAA
Glyceraldehyde 3-phosphate dehydrogenase	UM040_0048	Glycolysis	AGGTTGTCATCACTGCTCCC GAGTGGGTGGTGGTCATTGT
Histone 3	UM003_0201	Nucleosome structure	TTTCCAGACCGCTTCACCAG GACTTACGAGCTGCCTTGGT
Heat shock protein 20 like family	UM038_0098	Molecular chaperone, stress response	TGGCTTTAATCCTGACGGCA CTGACTGCGCCATCATCTCT
Heat shock protein 20 family (chloroplast localised, putative)	UM003_0083		CTGGCGGATAGGACACGG AGACAGCGAAACGTGATCCC
Heat shock protein 70 family	UM041_0091		GCCAAGCCCAGTGTGAAGAA GGAGCCTGTGTTTCGTCTCT
Heat shock protein 70 family (chloroplast localised, putative)	UM002_0182		GATGGGGATAACAGCCTGGG CGATTTTACAACGCTCGGCC
Heat shock protein 70 family (mitochondria localised, putative)	UM039_0067		TGAAATGCTTGTGGCCTGC CGAGTACTTCTTGCTCCCG
Heat shock protein 70 family (endoplasmic reticulum localised, putative)	UM012_0036		GAGGTCGAGAGGATGGTGC CGATTTTGTCCCTCAGCCGA

Table 2.9. Primers used for qPCR.

Primer QC

All new primers were assessed with the qPCR kit using a control sample on a conventional thermal cycler (G-Storm GS1) as an initial assessment of design success and specificity. PCR products were assessed alongside HyperLadder 25 bp (Bioline) on a 3.5% (w/v) agarose-TAE (1X) gel run at 80V for 1 hour. The control sample for primer assessment was manufactured by pooling multiple *U. compressa* cDNA samples together.

Following this, primer linearity was assessed by qPCR. Serial dilutions of the control sample were manufactured in nuclease-free water, from undiluted cDNA ('neat') to 1/10,000. Samples from this dilution series were assayed in triplicate using each new primer set, and linearity performance was assessed from the linear regression generated by plotting log dilution against the mean quantification cycle (C_q) value obtained at each dilution.

Data Analysis

The analysis of raw fluorescence data from qPCR experiments was carried out in RStudio using the 'qpcR' package (version 1.4-1 Spiess, 2018). Firstly, background subtraction was performed for all fluorescence data by subtracting the mean fluorescence value obtained from qPCR cycles 1 to 5 from all data points. Using a preliminary data set (i.e., data generated during the previously described standard curves for primers), the 'goodness of fit' of various sigmoidal curve fit models were compared to identify the best-suited model to fit the qPCR data generated in this thesis. The goodness of fit was determined by bias-corrected Akaike Information Criterion (AICc), maximum log-likelihood, and residual variance. Four, five, and six-parameter logistic regression models were tested (see equation a, b, and c). Based on this analysis, the best-suited model was the 5-parameter logistic model, therefore it was used to fit all qPCR fluorescence data generated in this thesis.

a. Four-parameter logistic

$$f(x) = c + \frac{d - c}{1 + \exp(b(x - e))^f}$$

b. Five-parameter logistic

$$f(x) = c + \frac{d - c}{(1 + \exp(b(x - e)))^f}$$

c. Six-parameter logistic

$$f(x) = c + k \cdot x + \frac{d - c}{(1 + \exp(b(x - e)))^f}$$

The C_q (quantification cycle) of fitted data was determined by the Cy_0 method (Guescini *et al.*, 2008). The C_q value is a measure of how much template is present in a sample, with lower C_q values indicative of higher amounts of template. Briefly, a tangent is fit to the inflection point of the curve model, and C_q is determined as the cross point at the x-axis. This is performed by the equation $Cy_0 = cpD1 - \frac{F_{cpD2}}{slope}$, where $cpD1$ represents the cycle at the maximum of the first derivative of the curve model, and F_{cpD2} represents the maximum fluorescence of the second derivative of the model.

Determination of Relative Gene Expression

Obtained C_q data was analysed by the method described by Vandesompele and colleagues (2002) for normalisation of data using the geometric average of multiple reference genes. Here, relative gene expression compared to an untreated control sample is calculated in samples exposed to different experimental conditions. This analysis method is summarised in Figure 2.6. Amplification efficiency (E) was calculated from the efficiency curve derived from fitted curves by the equation $E_n = \frac{F_n}{F_{n-1}}$, with F representing fluorescence. The E required for relative gene expression analysis was taken from where $n = C_q$ (as determined by the Cy_0 method). Relative gene expression, or fold change was expressed as log fold change (log2) for statistical analysis. Data was tested with either one-way or two-way analysis of variance (depending upon the number of independent variables in the experiment), followed by post hoc analysis using Tukey honest significance test to determine statistical differences between mean values. Statistical significance was determined when $p < 0.05$.

Appropriate reference genes for *Ulva* were determined by assessing the stability of gene expression for putative ‘housekeeping’ genes across a range of experimental conditions. Potential reference gene targets were selected following a literature review for commonly used qPCR control genes used for development and stress response experiments in plants and algae. The stability of candidate reference genes was ranked using the geNorm and Normfinder algorithms (Vandesompele *et al.*, 2002; Andersen, Jensen and Ørntoft, 2004). The most stable genes determined by this process were selected as the internal controls for all qPCR experiments discussed in this thesis.

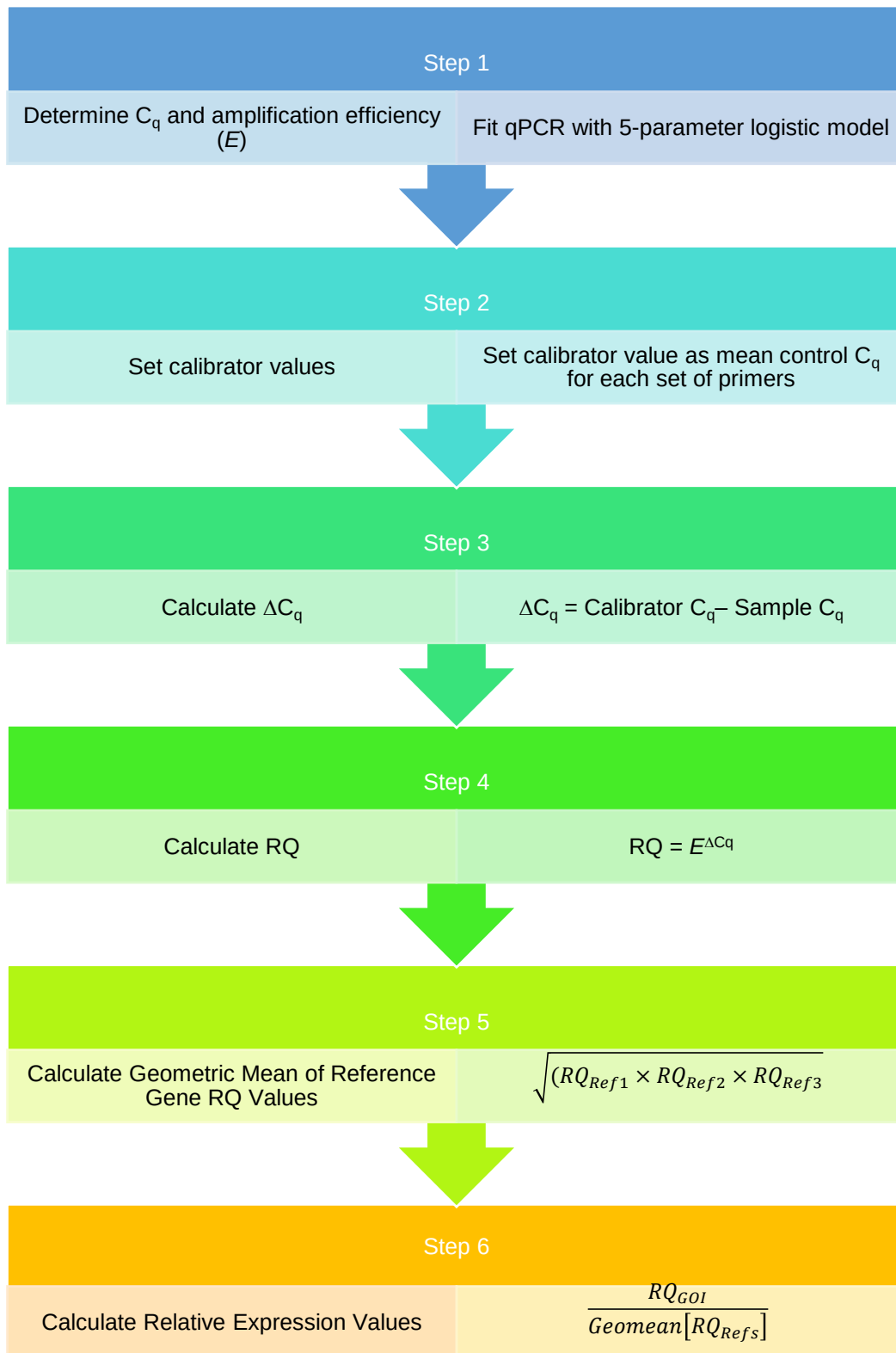


Figure 2.6. Protocol for qPCR data analysis by the method reported by Vandesompele and colleagues (2002)

2.1.6 Species Identification

2.1.6.1 Morphology and Microscopy

Green macroalgae collected from the sampling site were identified based on morphological characteristics as described in the literature (Brodie, Maggs and John, 2008). At the sampling site, *U. compressa* was identified firstly from macroscopic observations, with the simplest to identify characteristic of *U. compressa* being the ‘filamentous’ morphology of tubular-type *Ulva* it typically exhibits (Figure 2.7, a). Despite this, *U. compressa* is known to be morphologically plastic, with some populations of *U. compressa* known to exhibit a ‘blade’ type morphology (Steinhagen, Weinberger and Karez, 2019). *U. compressa* can typically be distinguished from other tubular species such as *U. intestinalis* via the presence or absence of branching, with branching observed less frequently on *U. intestinalis* (Figure 2.7, f). Notably, we know that *U. compressa* is conspecific with the model seaweed *U. mutabilis*, for which a genome is available (De Clerck et al., 2018; Steinhagen et al., 2019).

Both *U. compressa* and *U. linza* appeared to be the most abundant types of Ulvophytes present at the sampling site. *U. linza* also demonstrates morphological plasticity; however, as shown in Figure 2.7, both species can be distinguished by the typical ‘ribbon morphology’ of *U. linza* thalli (Figure 2.7, b), which can grow much wider than *U. compressa*.

The two can be distinguished more conclusively via microscopic investigations. *U. compressa* is formed as a hollow cylinder of a single layer of cells (Figure 2.7, f). *U. linza* is formed by a bilayer of cells, consistent with the ‘sea lettuce’ morphology (Figure 2.7, c). Whilst both species have one pyrenoid, the chloroplast of *U. compressa* has a characteristic ‘hood’ shape (Figure 2.7, d) and is localised to one end of the cell. In *U. linza*, the chloroplast appears to fill the cell (Figure 2.7, e).

Whilst morphological characteristics are used routinely in phycology for species identification; this typically requires extensive expertise in algal ecology and taxonomy. As stated, identifying *Ulva* species is a particularly challenging task due to the morphological plasticity observed in this group. The reasons for this are still poorly understood, with the morphological diversity observed in some *Ulva* populations not necessarily showing an obvious relationship to environmental conditions or developmental phase (Niesenbaum, 1988). As a result, the morphological species identification described here was supported by species barcoding.

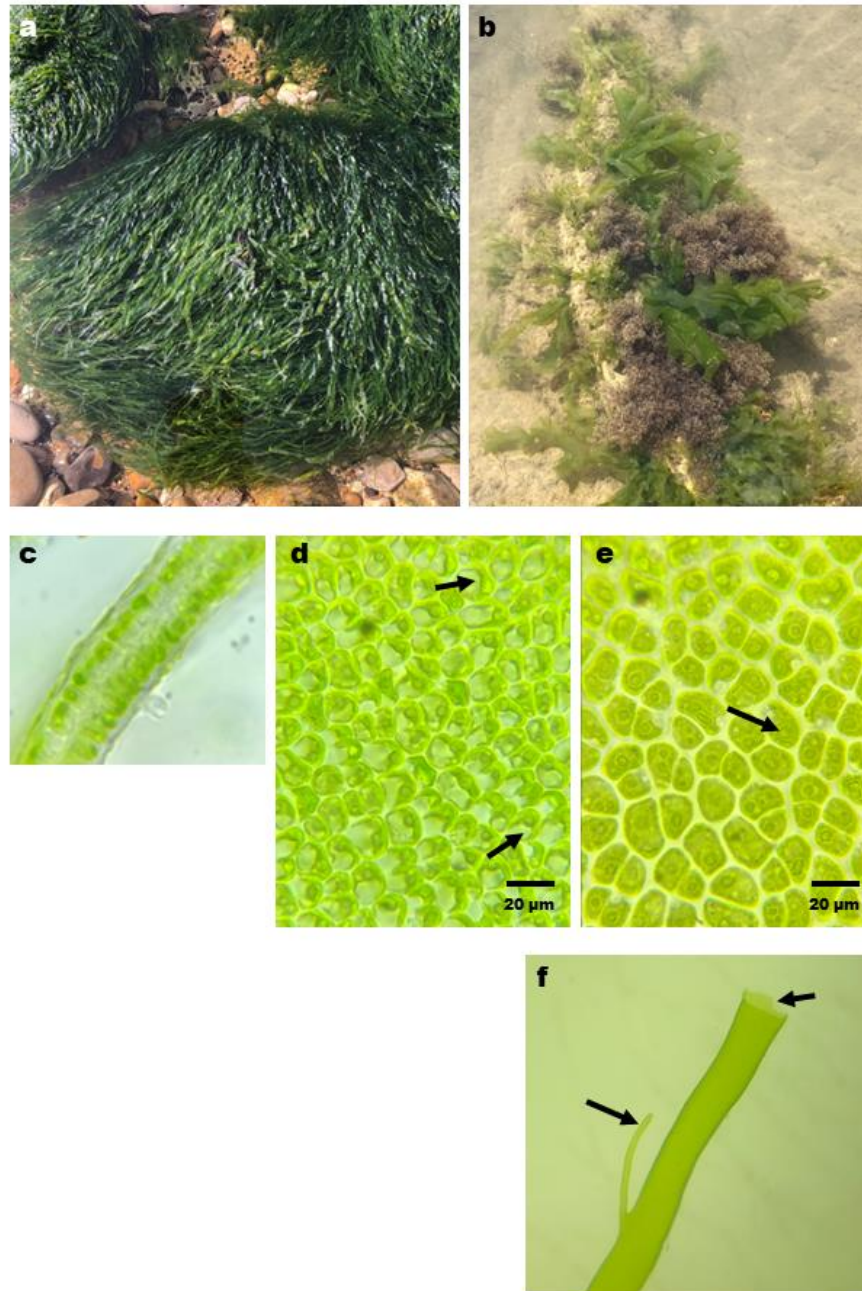


Figure 2.7. Identification of *Ulva* via macroscopic and microscopic features. *U. compressa* (a) and *U. linza* (b) display morphological differences at the sampling site. *U. linza* fronds are formed from a bilayer of cells, as shown by the cross-section in (c). Both *U. compressa* (d) and *U. linza* (e) typically have one pyrenoid (labelled by arrows), but *U. compressa* can typically be distinguished from other species by the characteristic 'hood' shaped chloroplast. *U. compressa* has a 'cylindrical' morphology with frequent branching (both labelled by arrows, f).

2.1.6.2 Sequencing

All sequencing was performed by DBS Genomics (Durham University) using an Applied Biosystems 3730 DNA Analyser. PCR products required for sequencing (amplified via direct PCR, 2.1.5.4) were run on 1% (w/v) low melting agarose-TAE (1X) gels and visualised under UV light (2.1.5.1). The desired bands were carefully cut from the gel, and DNA was then purified using the QIAquick Gel Extraction Kit (QIAGEN), following the manufacturer's instructions. DNA purified using this kit was eluted with nuclease-free water instead of the kit-provided elution buffer.

A supplementary ethanol precipitation was performed on gel extracts of insufficient concentration for sequencing as follows:

1. 3 M sodium acetate (pH 5.2) was added to DNA samples at a 1/10 volume
2. A 3X volume of ice-cold ethanol was added to samples
3. Samples were briefly mixed, then incubated at -20°C for 1 hour
4. Samples were then centrifuged for 30 minutes at 14,000 G
5. The supernatant was discarded, and the pellet washed with 70% ethanol
6. Samples were centrifuged for a further 15 minutes at 14,000 G
7. The supernatant was discarded, and residual ethanol was allowed to evaporate at room temperature by leaving sample tube caps open
8. The DNA pellet was resuspended in the desired volume of nuclease-free water

2.1.6.3 Species Barcoding

The chloroplastic *tufA* gene was amplified from a sample predicted to be *U. compressa* at the sampling site and sequenced from both ends using 5'-GGNGCNGCNCAAATGGAYGG-3' and 5'-CCTTCNCGAATMGCRAAWCGC-3' as forward and reverse primers (Fama et al., 2002; Ghaderiardakani, Coates and Wichard, 2017; Steinhagen et al., 2019). The use of the chloroplast *tufA* gene is widely reported for chlorophyte species identification and is typically used in preference to *rbcl* and ITS as a biomarker in phylogenetic studies (Saunders and Kucera, 2010; Wolf et al., 2012; Du et al., 2014; Steinhagen et al., 2019; Steinhagen, Düsedau and Weinberger, 2021).

Both the forward and reverse sequences were aligned using CLUSTALW to obtain a consensus sequence of 765 bp (Thompson, Higgins and Gibson, 1994). This consensus was aligned with the GenBank database using BLASTN to identify organisms with a similar sequence, where the identity of *U. compressa* was verified, with the top hits corresponding to the *U. compressa* *tufA* gene (percentage identity = 99.87%, e = 0.0).

Description	Scientific Name	Max Score	Total Score	Query Cover	E	Per. ident	Acc. Len	Accession
<i>Ulva compressa</i> voucher S 14 B elongation factor Tu (tufA) gene, partial cds; chloroplast	<i>Ulva compressa</i>	1404	1404	99%	0	99.87	773	MF979645.1
<i>Ulva compressa</i> chloroplast	<i>Ulva compressa</i>	1404	1404	100%	0	99.74	89164	MK069585.1
<i>Ulva compressa</i> voucher SV_804 elongation factor Tu (tufA) gene, partial cds; chloroplast	<i>Ulva compressa</i>	1404	1404	100%	0	99.74	798	OL421446.1
<i>Ulva compressa</i> voucher SV_803 elongation factor Tu (tufA) gene, partial cds; chloroplast	<i>Ulva compressa</i>	1404	1404	100%	0	99.74	798	OL421445.1
<i>Ulva compressa</i> voucher SV_802 elongation factor Tu (tufA) gene, partial cds; chloroplast	<i>Ulva compressa</i>	1404	1404	100%	0	99.74	798	OL421444.1
Consensus Sequence	ACGAAGAGCATTTATTATTAGCTAAACAAGTAGGTGTTCTAATATGTTGTTTTTTAAATAAGAAGATCAAGTAGATGATGCTGAATTATTAGAGTTAGTTCAATTAGAAGTCAAGAAACACTTGATGCTTATGATTTCCCGGTGAAGATATACCTATTGTAACAGGTTTCGGCTTTACTAGCACTAGAGGCGNTAATTGAAGGTACTGATGTTCTGATAACAAATGGGTTAATAAAATCTATGATTTAATGAAAGAAGTTGATAATTATATTCCAACCTCCTGAGCGTGAAACAGATAAAACATTTCTTAATGGCAATAGAAGATGTAATCTCTATTACTGGTCTGGAACTGTTGCAACTGGACGTGTTGAACGTGGTGTITTTAAAAACAGGTGAAACAGTAGATCTTGTTGGATTAGGGGATACAAAAATGTAACAGTTACTGGATTAGAAATGTTCCAGAAAACCTTTAGATGAAACAGTTGCGGGAGATAATGTAGGTGTATTACTTCGAGGTGTTCAAAAAGATGAAATTCACGAGGTATGTTAATTGCTGCTCCAAATTCAATTGAACCTCATACAAAATTGAAGCACAAAGTTATGTTTTAACAAAAGACGAAGGTGGTCGCCACACTCCATTTTCCAGGTTACCGACCTCAATTTTATGTTAGAACAACTGATGTACAGGTAAAATTGAAAACTTTACAGCAGACGATGGATCTGAAACAAAAATGGTAATTCAGGAGATCAGTAA							

Table 2.10. Summary of best matches from BLASTN analysis for *tufA* consensus sequence.

2.1.7 Biochemical Tests

2.1.7.1 Histochemical Staining

Non-Fluorescent Staining

The Wiesner (or phloroglucinol-HCl) and toluidine blue O methods were used to identify lignin-like structures in plant and algal samples. A drop of phloroglucinol-HCl (3% w/w) or toluidine blue O solution (0.02 % w/w) was added to a cover slip mounted with a small fragment of seaweed or plant tissue or a cell wall extract. Evidence for lignin was then assessed under a light microscope based upon the colourimetric changes observed. For phloroglucinol-HCl staining, lignified tissues stain pink, red, or purple depending on lignin composition (Pomar, Merino and Barceló, 2002). A colour change from blue to green is observed when toluidine blue O binds lignin and other phenolics (Pradhan Mitra and Loqué, 2014).

Direct Yellow 96

Ulva cell walls were stained with Direct Yellow 96 (Solophenyl Flavine 7GFE), which is a fluorescent dye hypothesised to stain cellulose and xylan structures *in vivo*, having been used successfully in both fungal and plant models in the literature (Hoch et al., 2005; Ursache et al., 2018; Hubbe et al., 2019). Successful staining of *Ulva* cell walls with Direct Yellow 96 has previously been reported in our lab, meaning that an appropriate staining protocol had already been established at the outset of this project, as well as appropriate acquisition windows for confocal microscopy (excitation – 405 nm, emission – 490 to 580 nm).

Small sections of *Ulva* thalli (~1-2 cm²) were incubated overnight in the dark at 8°C in a 0.1 mg/ml Direct Yellow 96 solution prepared in artificial seawater. Thalli were rinsed several times with seawater and were kept in the dark before imaging.

Basic Fuchsin

Basic Fuchsin (CAS: 569-61-9) is a fluorescent magenta dye composed of a commercial mixture of pararosaniline, rosaniline, magenta II, and new fuchsine.

Basic Fuchsin staining is frequently cited in the literature as a method of staining lignified secondary cell walls in plants (Mähönen et al., 2000, 2006; Yokoyama et al., 2007; Carlsbecker et al., 2010; Kapp et al., 2015; Taylor-Teeples et al., 2015; Kalmbach et al., 2017; Ursache et al., 2018). As a result, it was trialled here to stain the hypothesised 'phycolignin' in the macroalgal cell wall.

As the use of this stain has not previously been described for macroalgae, various concentrations and incubation timings were trialled. Initially, a 1 mg/ml stock was prepared, and different working concentrations from 0.0001% to 0.1% (v/v) (were prepared in artificial seawater, with stain concentrations of 0.001% and 0.01% reported in various plant staining protocols (Mähönen et al., 2000; Yokoyama et al., 2007; Kapp et al., 2015). Thalli were incubated in solution in the dark for 5 minutes, 3 hours, and overnight as performed for Direct Yellow 96 staining before imaging. Staining trials were also conducted where a drop of concentrated dye (0.1% to 1%) was placed on plant or algal tissue mounted on a slide and incubated at room temperature for 5 minutes before washing with seawater. Confocal imaging for Basic Fuchsin staining trials was performed with a 543 nm laser line, and emission was acquired from 600-650 nm.

Nile Red

The lipophilic Nile Red stain has been reported to stain suberin and cutin structures in the plant cell wall successfully (Li et al., 2007; Danila et al., 2021). *Ulva* thalli were incubated in artificial seawater containing 0.01% or 0.05% Nile Red for the same time intervals as performed for Basic Fuchsin, with the same excitation and emission parameters for imaging (excitation – 543 nm, emission – 600-650 nm).

2.1.7.2 Acetyl Bromide Soluble Lignin Assay

The acetyl bromide soluble lignin (ABSL) method was used to quantify the lignin content of cell wall extracts and fractions according to methods previously described for use with herbaceous plants, with a few modifications intended to limit interference from polysaccharide degradation (Hatfield et al., 1999; Barnes and Anderson, 2017). Powdered sample (20 mg) was distributed into 10 mL glass test tubes with screwcaps before the addition of 1 ml 25% acetyl bromide (in glacial acetic acid). The tubes were then vortexed and transferred to a 50°C water bath for incubation for 2 hours. Incubating samples were gently mixed by hand every 15 minutes. The assay was stopped immediately following incubation by transferring samples to an ice bath and adding glacial acetic acid (5 mL) to samples on ice. Completed reactions (300 µL) were then mixed with 1.5 N NaOH (400 µL) and 0.5 M hydroxylamine hydrochloride (300 µL), and absorbance was measured at 280 nm. The lignin content of each sample was estimated as per the Beer-Lambert Law using standard curves generated from kraft lignin (Sigma-Aldrich).

2.1.8 Analytical Chemistry

A series of analytical techniques were performed on the isolated cell wall samples (2.1.4.4) for structural characterisation.

2.1.8.1 Elemental Analysis

Elemental analysis was performed at the Stable Isotope Biogeochemistry Laboratory (Department of Earth Sciences, Durham University) using an ECS 4010 CHNSO elemental analyser (Costech) for carbon, sulfur, and nitrogen detection. Here, samples are thermally combusted in the presence of oxygen and are released as N₂, CO₂, H₂O, and SO₂ gases. Gases are separated by gas chromatography and then analysed by a thermal conductivity detector to determine elemental composition.

2.1.8.2 ATR-FTIR

Powdered biomass from cell wall isolation protocols (2.1.4.4) was assessed by attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR). Scans were performed from 4000 to 500 cm^{-1} at a 1 cm^{-1} resolution using a Perkin Elmer Frontier FTIR spectrometer with a Specac Quest ATR accessory attached. All scans were performed in transmittance mode, and data was converted to absorbance for analysis.

Spectra were normalised for comparison by the min-max normalisation method (Baker et al., 2014; Gautam et al., 2015; Zanca et al., 2020; Barboux, Bousta and di Martino, 2021; Javier-Astete, Jimenez-Davalos and Zolla, 2021), with the maximum peak of the obtained cell wall fractions corresponding to the maximum band attributed to glycosidic linkages of the carbohydrate backbone at $\sim 1035 \text{ cm}^{-1}$. Normalisation via this peak enabled ratiometric comparison of different samples, meaning that changes in peaks attributed to other functional groups in the infrared spectra could be assessed relative to the vibrational contribution from sugars in a sample. Differential spectra were generated by numerical subtraction of infrared spectra from each other to better resolve functional differences between samples (McCann et al., 1992; Gezici et al., 2012).

2.1.8.3 Solution State NMR

Sample Preparation and ^1H NMR

Solution state ^1H NMR was performed on cell wall extracts (20 mg) solubilised in 750 μL DMSO- d_6 . Due to the general insolubility of cellulose in NMR solvents, samples were suspended in DMSO- d_6 and allowed to mix for several hours with regular vortexing until a homogenous solution was achieved. Following this, the DMSO- d_6 soluble component was retrieved by a brief centrifugation step to pellet insoluble material, and the supernatant was extracted for analysis. Initial ^1H spectra were acquired using a Bruker Avance III HD spectrometer (500 MHz) with a BBO 400S1 probe (Bruker) with 750 μL DMSO- d_6 solubilised sample. Subsequent ^1H spectra for samples of interest and multi-dimensional analysis were acquired using a 700 MHz instrument using a CPP TCI 700S3 probe (Bruker). All chemical shifts were reported relative to tetramethylsilane (TMS).

Initial scans were performed with the standard Bruker program for a 90° pulse ('zg') with an acquisition time of 4 s, relaxation delay of 0.1 ms, a pulse width of 12 μs , and a receiver gain of 20.2. ^1H was acquired at 400.13 MHz. Analysis was performed at 293.9 K. For analysis on the 700 MHz instrument, an acquisition time of 4.7 s was used,

with a relaxation delay of 1 s, a pulse width of 8 μ s, and a receiver gain of 36. ^1H NMR was acquired at 688.74 MHz. Whilst a single scan was generated during the initial analysis, multiple scans were performed subsequently (reported with data).

^{13}C - ^1H HSQC

A series of multiplicity-edited ^{13}C - ^1H HSQC experiments were performed using a modified version of the Bruker standard pulse sequence for adiabatic-pulse HSQC ('hsqcedetgpsisp2.3') using an acquisition time of 150 ms, relaxation delay of 1 s, a pulse width of 8 μ s, and a receiver gain of 101. Spectra were acquired in F2 (^1H) from 13 ppm to -3 ppm, and in F1 (^{13}C) from 150 ppm to ~0 ppm. A total of 64 scans were performed per experiment. Resonances from ^1H and ^{13}C nuclei were acquired at 699.74 MHz and 175.96 MHz respectively. Analysis was performed at 298.2 K. All data analysis was performed in MestReNova.

2.1.8.4 Solid-State State NMR

Whilst the oxalate-soluble (pectin) and alkali-soluble (hemicellulose) cell wall fractions obtained via the sequential extraction protocol (2.1.4.4) readily solubilised in DMSO- d_6 , the solubility of the α -cellulose fraction in NMR solvents was poor. As a result, the α -cellulose fractions were also assessed by solid-state NMR spectroscopy.

^{13}C CP-MAS

A total of 80 mg α -cellulose fraction was submitted for ^{13}C cross-polarisation magic-angle spinning analysis using the 500 MHz Bruker Avance III HD spectrometer operating at 100.63 MHz for ^{13}C , and 400.117 for ^1H with a 4 mm double-resonance magic-angle spinning (MAS) probe spun at 10 KHz. Cross-polarisation was recorded with a 1 ms contact time and a recycle delay of 4 s, using total suppression of sideband (TOSS) suppression. Cross polarisation was acquired using the pulse program 'cptoss'. Supplementary phase-edited experiments were performed using the 'cppti' program to determine multiplicity. Chemical shifts (ppm) were determined relative to TMS. Spectra were acquired at 298 K.

All ATR-FTIR and NMR analysis was performed at the Department of Chemistry (Durham University) at the solution-state and solid-state NMR services respectively.

2.2 Materials

Enzymes, Kits, and Reagents

Supplier	Kit
Bioline	HyperLadder 25 bp
Biotium	'Forget-Me-Not' qPCR kit
Duchefa Biochemie	Cellulase Onozuka R-10
Goss Scientific Instruments	Dimethyl sulfoxide-d ₆ (deuterated)
New England Biolabs	Proteinase K, Molecular Biology Grade
Qiagen	DNeasy Plant Mini Kit
	QIAquick Gel Extraction Kit
	PowerPlant RNA Extraction Kit
Sigma-Aldrich	Basic fuchsin
	Cellulose fibres (medium)
	Driselase from Basidiomycetes sp
	Lignin, alkali
	ReadyScript cDNA Synthesis Mix
	Sea salts
Thermo Scientific	6X DNA Loading Dye
	GeneRuler 1 kb DNA Ladder
	GeneJET Plasmid Miniprep Kit
	Phire Plant Direct PCR Master Mix
	RNAse A, DNase and protease-free

Table 2.11. Enzymes, kits, and reagents used for research in this thesis.

Media

Artificial Seawater (ASW)	
Component	Amount
Sea salts	35 g
Sodium nitrate	70 mg
β-glycerophosphate disodium hydrate	10 mg
Milli-Q	to 1 L

Table 2.12. Composition of artificial seawater (35 ‰) for culturing *Ulva*

Provasoli's Enrichment Solution (Stocks)		
Primary Stocks		per litre
1	Sodium nitrate	35 g
2	β -glycerophosphate disodium hydrate	50 g
3	<u>Iron-EDTA:</u>	
	Ferrous Ammonium Sulfate Hexahydrate	0.7 g
	Na ₂ EDTA	0.6 g
4	Vitamin B ₁₂	0.025 g
5	Thiamine	0.05 g
6	Biotin	0.05 g
7	<u>Trace Metals:</u>	
	Na ₂ EDTA	1.0 g
	H ₃ BO ₃	1.12 g
	MnSO ₄ ·4H ₂ O	0.12 g
	ZnSO ₄ ·7H ₂ O	0.022 g
	CoSO ₄ ·7H ₂ O	0.005 g

Table 2.13. Stocks for preparation of Provasoli's Enrichment Media

Provasoli's Enrichment Media	
Stock (see Table 2.13)	Amount
1 (Sodium nitrate)	8 mL
2 (β -glycerophosphate disodium hydrate)	110 mL
3 (Iron-EDTA)	100 mL
4 (Vitamin B ₁₂)	3.5 mL
5 (Thiamine)	8 mL
6 (Biotin)	8 mL
7 (Trace Metals)	200 mL
Milli-Q	to 1 L

Table 2.14. Provasoli's Enrichment Media. Stocks 4-6 were filter sterilised. Filter sterilised stocks were added after the other stocks (1-3, 7, and Milli-Q) were mixed and autoclaved. Stocks were prepared as stated in Table 2.13. All stocks and completed media were stored in the dark at 4°C.

Provasoli's Enriched Seawater	
Component	Amount
Provasoli Enrichment Media	20 mL
Artificial Seawater	to 1 L

Table 2.15. Provasoli's Enriched Seawater.

Buffers

RNA Loading Buffer	
Component	Amount
Formamide	500 μ L
Formaldehyde	175 μ L
10X MOPS	50 μ L
Milli Q	50 μ L
Ethidium Bromide (10 mg/ml)	10 μ L
Orange G	

Table 2.16. Composition of RNA Loading Buffer. Stored at -20°C.

50X TAE Buffer	
Component	Amount
TRIS	2 M
EDTA	0.05 M
Acetic Acid (Glacial)	57.1 mL
Milli-Q	to 1 L

Table 2.17. Composition of 50X TAE (Tris-Acetate-EDTA) Buffer

10X MOPS	
Component	Amount
MOPS	41.8 g
EDTA	0.01 M
Sodium Acetate (pH 7.0)	0.02 M
Milli-Q	to 1 L

Table 2.18. Composition of 10X MOPS Buffer. Stored in the dark.

1X TE Buffer (pH 8.0)	
Component	Amount
TRIS	10 mM
EDTA	1 mM / 0.1 mM (IDTE)

Table 2.19. 1X TE Buffer. Prepared with 1 mM EDTA or 0.1 mM EDTA for primer storage.

Grinding Buffer (Protein Extraction)	
Component	Amount
TRIS	10 mM
EDTA	1 mM
Triton-X-100	1% (v/v)
2-mercaptoethanol	1% (v/v)

Table 2.20. Composition of grinding buffer for protein extraction. 2-mercaptoethanol added to aliquots of solution on the day of use.

Lysis Buffer	
Component	Amount
Urea	6 M
Thiourea	2 M
CHAPS	4% (w/v)

Table 2.21. Composition of protein lysis buffer. Stored at -20°C in 1 mL aliquots.

Ammonium Oxalate Solution (pH 4.2)	
Stock	Amount
Ammonium oxalate monohydrate (0.2 M)	250 mL
Oxalic acid (0.2 M)	50 mL

Table 2.22. Ammonium oxalate solution (pH 4.2) for cell wall fractionation protocol

3. Imaging and Fractionation of *Ulva* Cell Walls

3.1 Introduction

3.1.1 General Introduction

As discussed in chapter 1, the initial identification of lignin outside the streptophyte lineage in the red seaweed *Calliarthron cheilosporioides* (Rhodophyceae) by Martone and colleagues (2009) has not been replicated to date in any other algal species. Strong evidence of a 'lignin-like' structure is reported in the charophyte green algae (e.g., *Coleochaete*), which are representatives of the streptophyte clade alongside land plants. However, this is consistent with the presence of a 'proto'-phenylpropanoid pathway within this group to facilitate monolignol biosynthesis (Sørensen et al., 2011; De Vries et al., 2017). Despite there being a handful of publications in the literature that provide preliminary evidence that a similar component is present in the cell walls of other seaweeds, including the furoid browns (*Sargassum* spp.) and chlorophyte green seaweeds (Ulvophyceae) such as *Ulva*, no comprehensive investigation has been performed to date that conclusively verifies the presence of 'phycolignin' outside the coralline *C. cheilosporioides* species (Gupta et al., 2013; Yaich et al., 2015; Alzate-Gaviria et al., 2021).

The current evidence for phycolignin in these alternative algal models is also contentious for two reasons. Firstly, the limitations of the analytical methods documented in these reports may have led to misdiagnosis of lignin structures, which is particularly an issue in species with polysaccharide-rich biomass (i.e., algae) and will be discussed in more detail subsequently in this thesis in section 3.1.2 (Hatfield et al., 1999; Bunzel, Seiler and Steinhart, 2005; Schäfer et al., 2015). Secondly, there is an absence of supporting genomic information to support the biosynthesis of a 'lignin-like' structure within the major algal lineages, with the evolution of the phenylpropanoid pathway linked to the terrestrialisation of plants within the streptophyte lineage, and 'true' lignin only being present in vascular plants (Labeeuw et al., 2015; De Vries et al., 2017, 2021; Xue et al., 2022). Whilst the evolutionary basis for how and when the constituent enzymes of this pathway appeared within the streptophyte lineage is debated, the absence of the fundamental enzyme phenylalanine ammonia-lyase (PAL) in the currently available rhodophyte and chlorophyte genomes raises questions about any phycolignin hypothesis, as the PAL enzyme is required for the first step of phenylpropanoid biosynthesis, the conversion of the amino acid phenylalanine to

cinnamic acid (Barros et al., 2016; De Vries et al., 2017, 2021). Despite this, cinnamic acid derivatives and phenylpropanoids such as flavonoids have been positively identified in both prokaryotic and eukaryotic algae, including chlorophytes (Trigui et al., 2013; Goiris et al., 2014; Al-Malki et al., 2018), providing further confirmation, were any needed, that our current understanding of algal secondary metabolism is limited.

In this chapter, I aim to characterise the proposed phycolignin fraction in the model seaweed *Ulva compressa*, by using a range of lignin detection techniques reported frequently in the literature for *in vivo* and *in vitro* analysis of lignocellulosic biomass. As discussed in the general introduction (see Chapter 1, section 1.4.3.5), it is somewhat surprising that the current evidence for phycolignin is more convincing in rhodophytes than the chlorophytes, with the chlorophytes sharing a more recent evolutionary history with plants than red algae; as such, the model green seaweed *U. compressa* provides an ideal model for comparison. Some of these lignin detection methods have previously been used with algal biomass with variable success; others have not been applied to *Ulva* before, representing a novel approach. The utility of these methods is now discussed.

3.1.2 Methods for Lignin Analysis

3.1.2.1 Non-Invasive

Autofluorescence

One simple and non-invasive method of monitoring the physiological state of a cell is autofluorescence imaging. Differences in spectral properties between biological samples have been used as biomarkers for cell identification, development, stress, and viability in multiple model systems (Elbrächter, 1994; Ying and Dobbs, 2007; Schulze *et al.*, 2011). Plants and algae provide great models for autofluorescence imaging due to the abundance of fluorescent metabolites in cells (Ying and Dobbs, 2007; Terryn, Paës and Spriet, 2018; Donaldson, 2020; Pegg, Gladish and Baker, 2021). Whilst autofluorescence is typically considered a source of interference for fluorescence microscopy when imaging experimentally labelled structures in plants, advances in imaging techniques, including sequential excitation and spectral unmixing facilitated by confocal laser scanning microscopy (CLSM), fluorescence lifetime imaging (FLIM), and Förster resonance energy transfer (FRET) allow resolution of multiple fluorescent compounds *in vivo*, enabling analysis of different structures under different conditions (Waters, 2009;

Donaldson and Radotic, 2013; Jonkman and Brown, 2015; Terryn, Paës and Spriet, 2018; Chacko and Eliceiri, 2019).

The development of the confocal microscope (Minsky, 1988), followed by the expansion of this technique by confocal laser scanning microscopy (Davidovits and Egger, 1969), were two essential steps that enabled biological imaging to become an essential tool in the modern biological sciences, providing direct advantages over traditional epifluorescence microscopy by removing ‘out of focus’ light, improving resolution, and facilitating 3-dimensional imaging (i.e., the generation of Z-stacks) via sequential optical sectioning (Elsayed *et al.*, 2017; Langenaeken *et al.*, 2020; von der Heyde and Hallmann, 2022). This is facilitated by using two ‘pinholes’ in the imaging set-up, the first of which directs the illuminating light beam to a specific point on the specimen, and the second of which prevents the acquisition of ‘out of focus’ light emitted from the sample by the detector. By selectively exciting and acquiring the emission from one point of a sample at a time, high-resolution images can be generated. This approach enables the imaging of thick biological samples, meaning that live tissue can be imaged without extensive sample preparation, depending on organism complexity (Waters, 2009; Jonkman and Brown, 2015; Justyna, 2017). Macroalgae such as *Ulva* are therefore well-suited for non-invasive imaging experiments due to their low complexity (with *Ulva* being two cells thick) and simple media requirements. As a result, confocal microscopy of macroalgae is widely reported in the literature (Elsayed *et al.*, 2017; Zhang *et al.*, 2017; Prabhu *et al.*, 2019; Blomme *et al.*, 2021; Pegg, Gladish and Baker, 2021; von der Heyde and Hallmann, 2022).

The main contributor to autofluorescence in plants and algae is the chloroplast, predominately from chlorophyll pigments, which display strong red fluorescence upon excitation with ultraviolet, blue, and green light (Gonzalez *et al.*, 2010; Schulze *et al.*, 2011; Donaldson, 2020). The second major source of plant autofluorescence is the broad blue-green fluorescence observed upon excitation with ultraviolet light, typically attributed to phenolic structures, with lignified cell walls (e.g., in xylem or sclerenchyma tissue) being the major structure observed. The use of autofluorescence to monitor lignin structures *in vivo* is widely reported in the literature, particularly in wood or paper biomass, but also in herbaceous plants (Pomar, Merino and Barceló, 2002; Barsberg and Nielsen, 2003; Radotić *et al.*, 2006; Donaldson and Radotic, 2013; Pradhan Mitra and Loqué, 2014; Donaldson and Williams, 2018; Baldacci-Cresp *et al.*, 2020). Imaging facilitates non-invasive studies of lignin content, distribution, and composition. The

spectral properties of lignin are generally attributed to phenolic stilbene and phenylcoumaran structures that can be incorporated into the lignin complex (see Figure 3.1). However, due to the heterogeneity and complexity of lignin, there are a range of constituent fluorophores that contribute to the broad spectral properties observed (Albinsson *et al.*, 1999; Ralph *et al.*, 1999; Radotić *et al.*, 2006; Martínez *et al.*, 2008; del Río *et al.*, 2017). Recently, 'blue-green' autofluorescence was reported in the cell walls of various algal species, including *Ulva*, but not attributed to a specific structure (Pegg, Gladish and Baker, 2021).

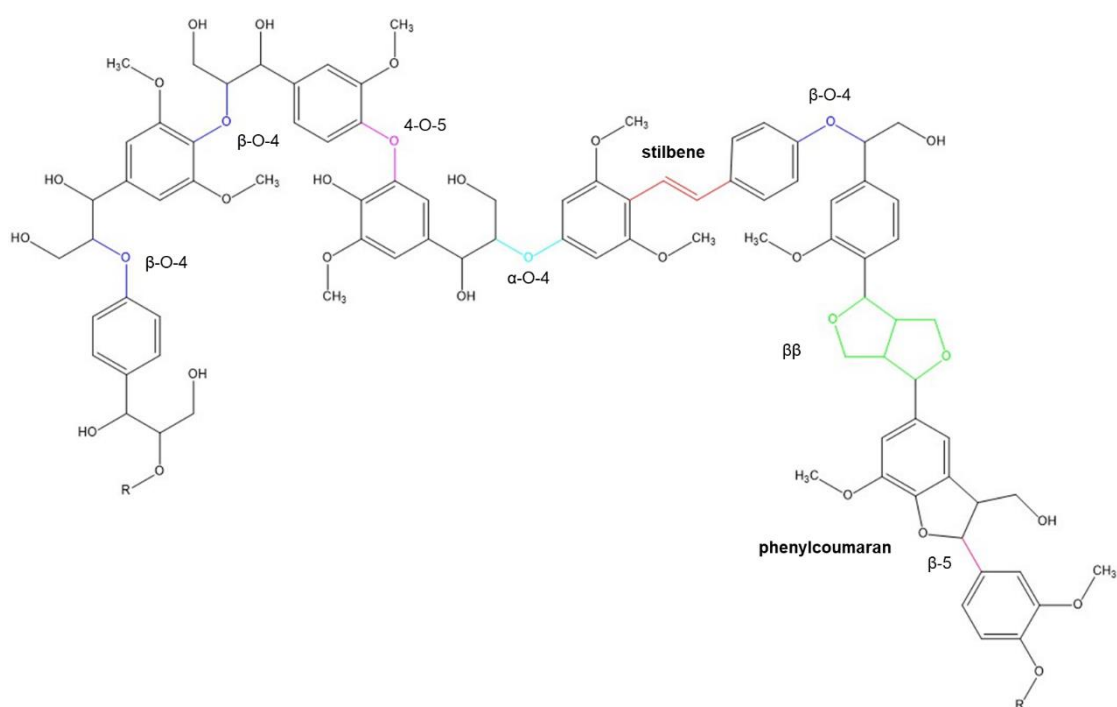


Figure 3.1. A model of a lignin polymer with frequently observed linkages labelled.

Histochemical Staining

Despite the simplicity of autofluorescence imaging, the amount of structural information that can be determined using this method alone is limited. Many aromatic compounds are present in biological specimens that display blue-green emission, including a range of secondary metabolites such as aromatic amino acids, phenolics, terpenoids, and alkaloids which are abundant in the plant metabolome (Donaldson, 2020). In addition to lignin, phenolics such as ferulate, coumarate, and flavonoids associate with the plant cell wall, as do the waxy biopolymers suberin and cutin that display similar fluorescence properties to phenolics, with suberin containing an aromatic domain (Hutzler et al., 1998; Lichtenthaler and Schweiger, 1998; Vogel, 2008; Kapp et al., 2015; Donaldson and Williams, 2018). Whilst it is reported that the fluorescence properties of ferulates can be modulated with varied pH as a diagnostic method (Harris and Hartley, 1976), in general, these compounds cannot be distinguished easily from each other *in vivo* without prior information on cellular structure (Hutzler et al., 1998; Donaldson and Williams, 2018).

A straightforward way of distinguishing lignin autofluorescence from other aromatic structures in cell wall imaging studies is the use of 'lignin-specific' stains that target structures associated with the lignin complex. Multiple methods for lignin detection are cited in the literature, with the most frequently referenced approach being the Wiesner test, also known as phloroglucinol-HCl staining (Espiñeira *et al.*, 2011; Pradhan Mitra and Loqué, 2014; Taylor-Teeples *et al.*, 2015; Gallego-Giraldo *et al.*, 2016; Hubbe *et al.*, 2019). In this method, lignified structures are stained pink or red and can be observed simply by light microscopy. Whilst the exact basis for this reaction is not fully understood; it is hypothesised that phloroglucinol-HCl selectively targets the fraction of β -O-4 linked aromatic aldehyde structures associated with the lignin complex including coniferyl aldehyde, cinnamyl aldehyde and sinapyl aldehyde, with the intensity of the colour change from pink to red being influenced by lignin chemical composition and tissue type (Kutscha and Gray, 1972; Pomar, Merino and Barceló, 2002). Attempts to stain macroalgae with phloroglucinol-HCl were reported for the freshwater chlorophyte *Oedogonium bharuchae*, which has a cellulosic cell wall, but proved unsuccessful (Estevez, Leonardi and Alberghina, 2008).

Other available methods for lignin detection include the colourimetric toluidine blue O dye, which differentially stains pectic and lignin-rich cell walls pink and green, respectively, and the Mäule test in which potassium permanganate is used to oxidise

syringyl subunits in lignin forming a characteristic purple-red Mäule stain (Pradhan Mitra and Loqué, 2014; Yamashita et al., 2016). Fluorescent dyes for lignin with variable spectral properties and sensitivity that have utility in confocal microscopy are also available. Such dyes include acriflavine, auramine, safranine, and basic fuchsin, which have been used to stain lignin successfully in a range of species (Mähönen et al., 2000; Bond et al., 2008; Kapp et al., 2015; Ursache et al., 2018; Hubbe et al., 2019; Baldacci-Cresp, Spriet, et al., 2020). To my knowledge, no reports of 'lignin-specific' fluorescent dyes being applied to seaweeds have been documented in the literature.

3.1.2.2 Invasive Methods

Two main approaches are reported in the literature for quantifying lignin content in lignocellulosic biomass, not counting biophysical methods, which are discussed and utilised in the subsequent chapters. As with the histochemical staining methods, most of these methods have only been modified slightly since their original publication as the biochemical principles behind lignin analysis are unchanged and as non-invasive biophysical techniques become more accessible and favourable in routine research (Ralph and Landucci, 2010; Lu *et al.*, 2017).

Firstly, gravimetric methods such as the acid-detergent and Klason lignin protocols provide estimates of lignin content from the mass of the insoluble hydrolysis remaining after sulfuric acid hydrolysis of cell wall polysaccharides (Rodrigues, Faix and Pereira, 1999; Fukushima and Hatfield, 2004; Hatfield and Fukushima, 2005; Gómez-Ordóñez, Jiménez-Escrig and Rupérez, 2010; H. Wang et al., 2019). Whilst crude, these methods are simple, do not require extensive sample preparation, and do not require extensive analytical expertise. More recently, acidic lithium bromide has been proposed as a safer and less cumbersome alternative to hydrolysis with concentrated sulfuric acid. However, the sulfuric acid method appears to remain more popular likely due to cost (Li, Pan and Alexander, 2016).

The second approach involves the use of the derivatisation-based thioglycolic acid or acetyl bromide assays, where lignin from biomass is selectively solubilised in an acidic solvent, and lignin concentration is estimated by absorbance readings against a standard curve generated from a sample of known lignin content (Fukushima and Hatfield, 2004; Hatfield and Fukushima, 2005; Suzuki *et al.*, 2009; Moreira-Vilar *et al.*, 2014). These derivatisation-based methods facilitate a more in-depth structural assessment of lignin; for example, acetyl bromide solubilised samples can be further

processed for gas chromatography-mass spectrometry (GC-MS) by the derivatisation followed by the reductive cleavage method (DFRC) which can be used to determine monolignol composition and structure (Lu and Ralph, 1997; Reale *et al.*, 2004; Letourneau and Volmer, 2021). This method has been used successfully to study lignin composition in various biomass sources, including algae, herbaceous plants, and wood biomass (Bunzel, Seiler and Steinhart, 2005; Guerra *et al.*, 2006; Martone *et al.*, 2009; Schäfer, Trierweiler and Bunzel, 2018). The presence of lignin-like structures in the Coleochaetales was also verified by thioacidolysis (Sørensen *et al.*, 2011), another derivatisation-based method that has been used universally in lignin analysis (Pomar, Merino and Barceló, 2002; Cass *et al.*, 2015; Gallego-Giraldo *et al.*, 2016; Yu Zhang *et al.*, 2019). The workflow behind these methods is summarised in Figure 3.2.

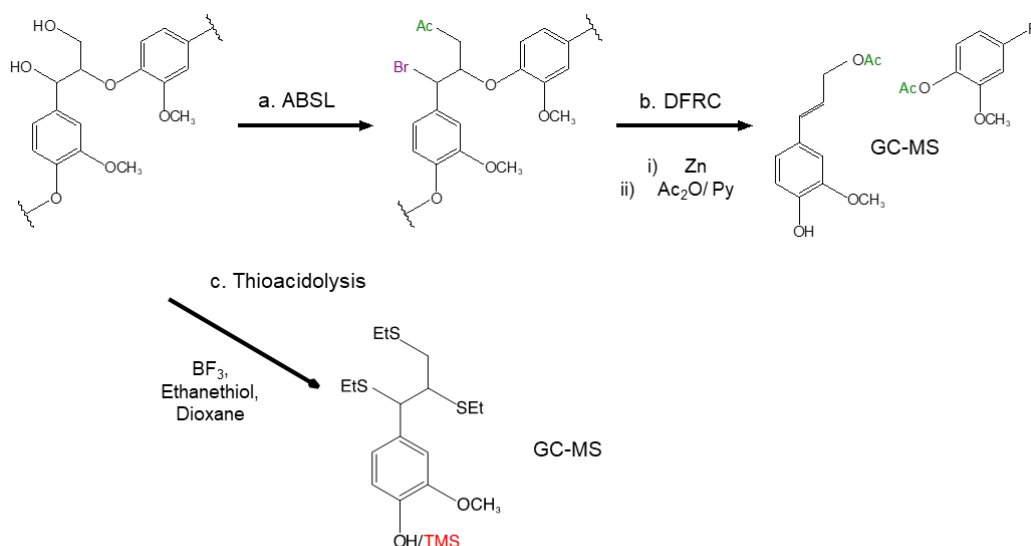


Figure 3.2. Summary of the derivatisation-based methods of lignin analysis. Lignin is selectively solubilised by the acetyl bromide soluble lignin method, with 25% (v/v) acetyl bromide in glacial acetic acid (a). Acetyl bromide derivatised lignin is then processed via the derivatisation by reductive cleavage (DFRC) method, which releases acetylated monomers that can be analysed by gas chromatography-mass spectrometry (b). Alternatively, the thioacidolysis method releases lignin monomers that can also be analysed by mass spectrometry (c).

Despite several of these lignin quantification methods being applied to *Ulva* biomass, the reliability of the findings in these reports is uncertain due to the limitations of these methods for samples that are expected to have a low lignin content (Hatfield *et al.*, 1999; Schäfer *et al.*, 2015). The evidence for lignin in green seaweeds has most frequently been provided by the gravimetric methods (Ventura and Castañón, 1998; Yaich *et al.*, 2011, 2015; Yoza and Masutani, 2013; Moral *et al.*, 2019; Mukherjee and Keshri, 2019), but have not been accompanied with methods of structural analysis to verify the presence of lignin in the insoluble residue, and these methods are generally assumed to overestimate lignin content either due to artefacts from degraded polysaccharides that form under the acidic conditions used, or other acid-insoluble polymers that persist in the resulting insoluble residue (Hatfield *et al.*, 1999; Bunzel, Schüßler and Tchetseubu Saha, 2011). For example, the presence of protein in samples is reported to cause overestimations of lignin content by these methods (Rencoret *et al.*, 2015; Kim *et al.*, 2017), and Klason lignin content in leaves is often overestimated relative to stem tissue due to differences in biochemical composition (Toda *et al.*, 2015). Notably, *Ulva* biomass can have relatively high protein content under certain growth conditions (Bolton *et al.*, 2016). Attempts to use the derivatisation-based thioacidolysis method on macroalgal biomass (*Ulva rigida* and *Mastocarpus stellatus*) were also reported to be unsuccessful, suggesting the ‘lignin-like’ content is below the sensitivity limit for these assays or is absent (Espiñeira *et al.*, 2011). This is supported by more recent reports where cellulose is successfully isolated from *Ulva*, where extracts are reported as being ‘lignin-free’ (Wahlström, Edlund, et al., 2020; Thygesen et al., 2021).

The most convincing evidence for phycolignin in the Ulvophyceae is provided by the DFRC method, where guaicyl and syringyl monolignols appeared to be identifiable in the resulting GC-MS analysis from *U. lactuca* biomass (Gupta *et al.*, 2013). Nonetheless, further critique of the approach used in this study suggests that these observations may not be conclusive. Firstly, it appears that unextracted *Ulva* biomass analysed, rather than specific cell wall extracts. As such, the observations cannot be directly linked to a cell wall structure, nor can an alternative extractive from the relatively understudied *Ulva* metabolome be ruled out (Martone *et al.*, 2009). ‘Noise’ was reported to be an issue in the DFRC analysis of various sources of plant biomass with low lignin content – which required method optimisation (e.g., sample pre-treatment to remove interferents) to acquire ‘analysable’ data (Bunzel, Seiler and Steinhart, 2005). The solubilisation of co-extractives in acetyl bromide is particularly an issue for the

corresponding quantitative acetyl bromide lignin assay, with aromatic amino acids, phenolics, and various other metabolites absorbing in the same spectral region used to quantify lignin (Desjardins and Conklin, 2010; Page, 2010; Kielkopf, Bauer and Urbatsch, 2020).

Secondly, as with the gravimetric methods, the acidic conditions from the acetyl bromide: acetic acid solution required to derivatise lignin also induce the formation of aromatic groups from degraded polysaccharides; for example xylose residues are known to degrade to furfurals (Hatfield *et al.*, 1999; Binder *et al.*, 2010; Moreira-Vilar *et al.*, 2014). This, in addition to co-extractives, has been demonstrated experimentally to interfere with the analysis of lignin in acetyl bromide-derivatised samples by spectrophotometric methods or GC-MS, particularly in samples expected to have a low lignin content (Hatfield *et al.*, 1999; Bunzel, Seiler and Steinhart, 2005; Schäfer *et al.*, 2015). The compositional differences between seaweed and plants may exacerbate this interference, with acetyl bromide-based methods for lignin designed initially to quantify lignin in 'lignin-rich' wood biomass where polysaccharide and co-extractive interference is less significant (Sluiter *et al.*, 2008). Since the original publication of the method (Johnson, Moore and Zank, 1961; Iiyama and Wallis, 1988), these methods have been optimised to limit the known interference that occurs to facilitate use with herbaceous plants (Hatfield, Brei and Grabber, 1996; Xue *et al.*, 2008; Barnes and Anderson, 2017). Nonetheless, some degree of overestimation is still expected when using the acetyl bromide soluble lignin assay as, practically, degradation cannot be eliminated entirely. As discussed, there appears to be strong evidence of similar interference in the reported chromatogram for *U. lactuca* as reported by Bunzel and colleagues (2005) and Schäfer and colleagues (2015) for plants with low lignin content (*Raphanus sativus* and *Asparagus officinalis*). Furthermore, no monolignols were identified by GC-MS with the analogous derivatisation-based thioacidolysis protocol in *U. rigida* (Espiñeira *et al.*, 2011). As a result, the possibility that lignin has been misidentified in *Ulva* due to artefacts must be considered.

By contrast, the analysis performed for the Rhodophyte seaweed *C. cheilosporioides* by the DFRC method appears to be conclusive evidence for lignin being present outside the Streptophyta, being performed on various cell wall extracts, and the resulting GC-MS analysis being supported by parallel analysis of synthetic lignin structures and a 'lignin positive' *Arabidopsis thaliana* cell wall extract (Martone *et al.*, 2009). Lignin was also positively identified by other methods in this report, including transmission

electron microscopy and immunolabelling. Additionally, guaicyl and syringyl monolignols were also positively identified in various representatives from the charophyte Coleochaetales genera by the thioacidolysis method and immunolabelling (Sørensen *et al.*, 2011). Due to the positive identification of ‘phycolignin’ in both charophyte and rhodophyte macroalgae, it is evident that these methods need to be reassessed for the chlorophytes.

3.2 Chapter Aims

In this chapter, I aim to:

- Image the hypothesised ‘phycolignin’ in the *Ulva* cell wall *in vivo* using confocal laser scanning microscopy, using a combination of autofluorescence imaging and lignin staining methods (section 3.3)
- Isolate and fractionate the *Ulva* cell wall into ulvan, ‘hemicellulose’, and cellulose using a sequential extraction protocol for macroalgae, and screen the extracted cell wall fractions with various conventional lignin detection methods to identify which components of the cell wall associate with a lignin-like structure (section 3.4)
- Verify the suitability of conventional lignin quantification methods (i.e., acetyl bromide solubilisation) for analysing *Ulva* biomass by assessing the degree of interference observed with various other organic compounds
- If ‘phycolignin’ is successfully identified in cell wall extracts, perform structural characterisation of this component using the derivatisation followed by reductive cleavage protocol (DFRC) as demonstrated in the red seaweed *C. cheilosporioides* (Martone *et al.*, 2009)

3.3 Imaging

3.3.1 Screening of *Ulva* Cellular Autofluorescence Properties Using Confocal Laser Scanning Microscopy

A series of emission scans (λ scans) were performed using the xy λ function of the confocal microscope (Leica SP5) to characterise the autofluorescence properties of *Ulva* cells and to determine appropriate acquisition windows for imaging fluorophores of interest (Figure 3.3). Here, two sources of fluorescence were identified in *Ulva* tissue following excitation with a tuneable Argon laser (488 nm), 'non-specific' green autofluorescence ($\lambda_{\text{max}} = 525$ nm), and red autofluorescence corresponding with chloroplasts ($\lambda_{\text{max}} = 660$ nm). Both chloroplast and 'non-specific green autofluorescence' have been used by plant biologists and phycologists as biomarkers for assessing the degree of physiological stress a cell is under (Gonzalez *et al.*, 2010; Schulze *et al.*, 2011; Sathasivam and Ki, 2019; Takahashi, 2019). A reduction in chloroplast autofluorescence is typically associated with a decline in photosynthetic activity and pigment breakdown (Dorsey *et al.*, 1989; Jiang, Howell and Flore, 1999; Schulze *et al.*, 2011; Winnicki *et al.*, 2021). Conversely, an increase in 'non-specific' green autofluorescence in cells correlates with increased stress and a loss of cell viability. This is not just observed in photosynthetic organisms; increases in green autofluorescence in the cytoplasm, possibly attributed to flavins and nicotinamide adenine dinucleotide (NAD), can be observed during the stress response of both prokaryotic and eukaryotic cell lines (Surre *et al.*, 2018; Chacko and Eliceiri, 2019; Müllerová *et al.*, 2022). However, in photosynthetic organisms, the most obvious increases in green autofluorescence are co-localised with chloroplast autofluorescence, indicating that various chloroplast-associated pigments may contribute to this signal (Beck and Scheibe, 2003; Hörtensteiner, 2006; Chung *et al.*, 2015). However, to date, the identity of the contributing fluorophores has not been confirmed in algae.

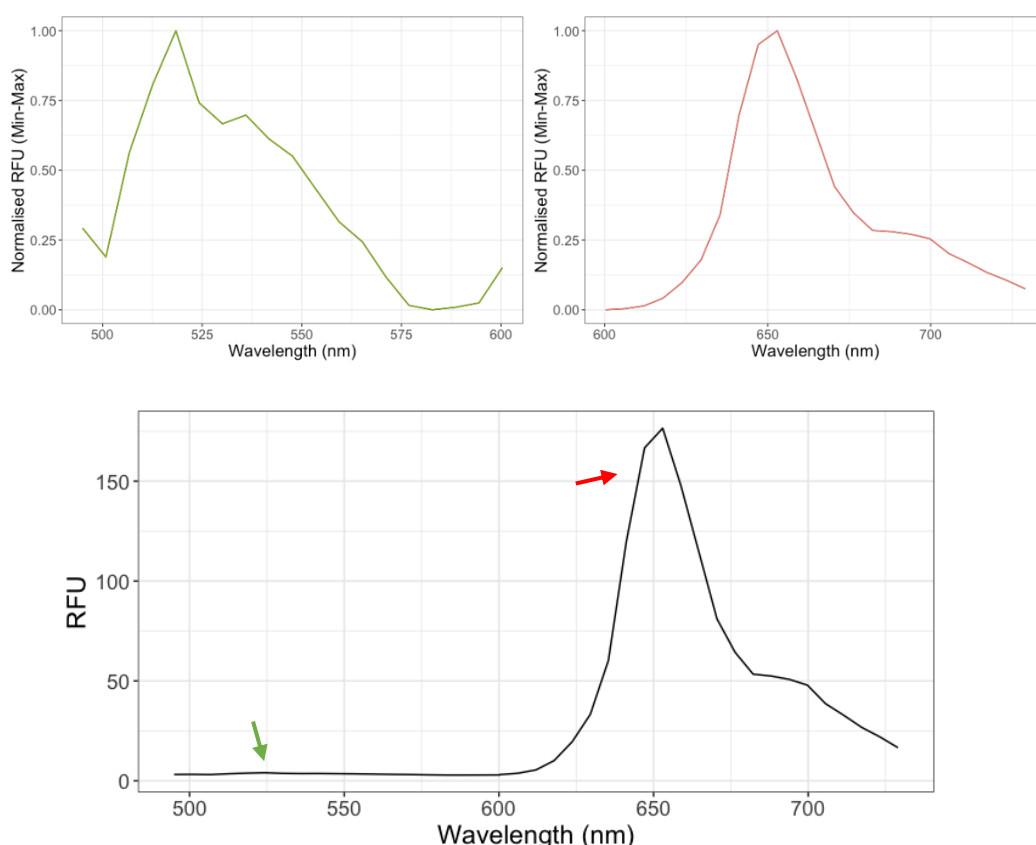


Figure 3.3. Spectral properties of *Ulva* cells. λ scans were performed on *Ulva* tissue across the green (500-600 nm, a) and red (> 600 nm, b) spectral regions at 5 nm step size with a 488 nm laser line to determine appropriate acquisition windows for collection of 'non-specific' green autofluorescence and red chloroplast autofluorescence. *Ulva* was exposed to 'heat shock' (37°C, 3 hours) to induce non-specific green autofluorescence. Emission spectra presented are min-max normalised means from duplicate scans (a, b), and non-normalised spectra are also presented (c).

The observed green autofluorescence only gave a faint signal under the 488 nm laser relative to the red chloroplast autofluorescence (Figure 3.4), which appeared as the major fluorophore in *Ulva* cells. Green autofluorescence was mostly undetectable in unstressed *Ulva* (Figure 3.4, b) and was only visible following heat shock stress (Figure 3.4,e), consistent with observations from other model systems that green autofluorescence is stress induced. Green autofluorescence was predominantly intracellular and appeared to be co-localised with chloroplast autofluorescence, most likely indicative of a stress response to heat shock that induces a change in chloroplast metabolism or of the breakdown of cellular components and a reduction of cell viability. However, as the source of non-specific green autofluorescence in algae is poorly understood, the exact dynamics of this observation could not be determined

conclusively. As this was beyond the scope of this research, this observation was not investigated further.

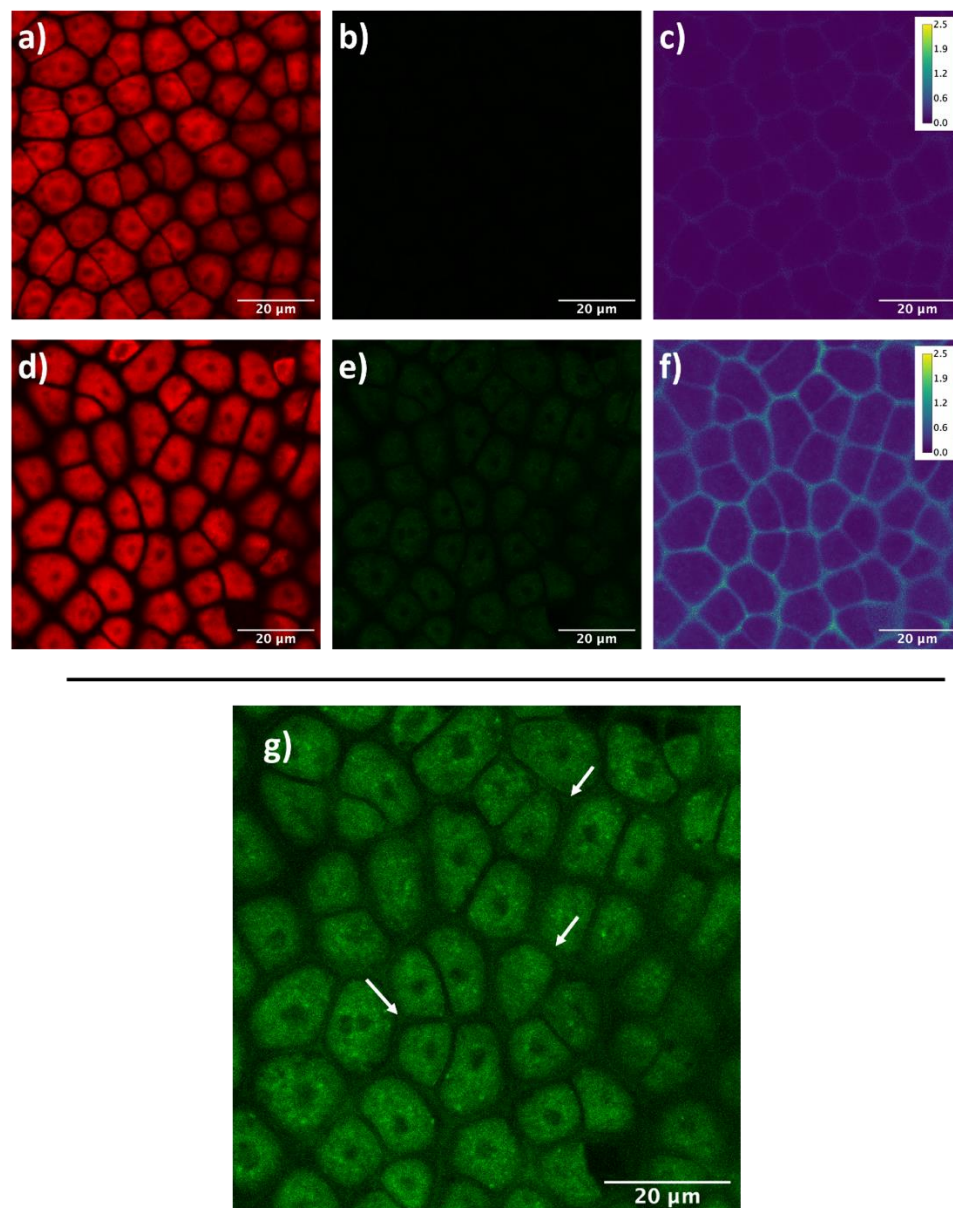


Figure 3.4. Autofluorescence of *Ulva* cells under normal growth conditions (a-c) and following heat shock (d-f). (a) Chloroplast autofluorescence of unstressed *Ulva* (excitation: 488 nm, emission windows: 650-700 nm). (b) Non-specific green autofluorescence of unstressed *Ulva* (excitation: 488 nm, emission windows: 510-580 nm). (c) Initial evidence for a cell wall fluorophore in *Ulva* was provided by ratiometric analysis of the red (a) and green channels (b), performed by the calculation $\frac{green}{red}$ using the Image Calculator function of ImageJ/Fiji. The LUT indicated by the calibration bar represents the calculated ratio at each pixel. (d-f) Respective channels for heat shock stressed samples, which were incubated at 50°C for 30 minutes in artificial seawater. (g) The possible cell wall fluorophore is identifiable by the white arrows in the brightness and contrast adjusted image from the green channel for heat shock stressed *Ulva* (e), where a distinct fluorescent structure is observed at cell-cell boundaries.

Preliminary evidence for a cell wall fluorophore in *Ulva* was identified in the green channel following heat shock stress (Figure 3.4, g), where a distinct structure at cell-cell boundaries was observed (labelled by the white arrows) that appeared independent of the intracellular autofluorescence signal. Ratiometric analysis of confocal images (Figure 3.4, c & f), calculated by $\frac{\text{green}}{\text{red}}$ (G/R) for images acquired from the two channels at the same *xy* coordinates was performed to support this observation, as the signal observed from the green channel was relatively faint and difficult to discern. Following this analysis, a distinct fluorophore correlating with the location of the *Ulva* cell wall was identified more clearly. The higher ratio observed at the cell wall following heat shock (Figure 3.4, f) indicated that this structure could be stress-responsive. It was predicted at this stage that this observation might correspond to the hypothesised 'lignin-like' fraction of *Ulva*, with lignin structures in plants known to fluoresce under blue-green excitation (488 nm) in addition to UV excitation (Donaldson, 2013; Donaldson and Radotic, 2013; Donaldson and Williams, 2018).

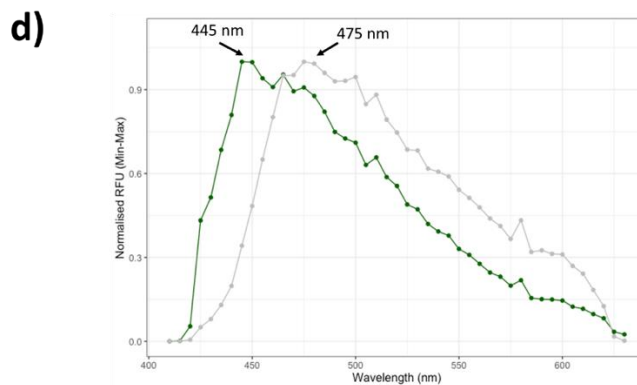
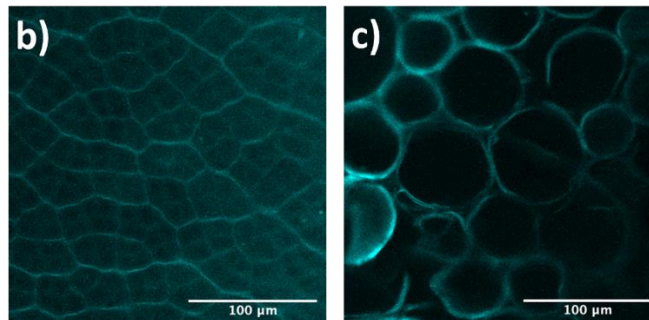
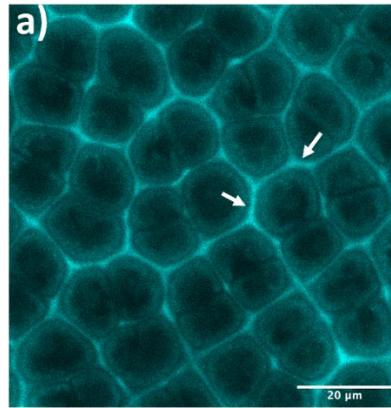


Figure 3.5. Spectral properties of cell wall fluorescence. (a) Blue-green autofluorescence (emission windows: 415-580 nm) was observed in *Ulva* using a 405 nm laser line following the determination of appropriate acquisition windows via λ scan (b-d). *Ulva* cell walls (b) and lignified tissue from plant stem (*Arabidopsis*) cross-sections (c) were subjected to a series of λ scans with the 405 nm laser line from 410-630 nm at a 5 nm step size. Spectral properties and $\lambda^{\max}$ are reported (d). The emission spectra presented are the min-max normalised mean calculated from a series of emission spectra generated from regions of interest selected from images acquired from 3 biological replicates of *Ulva* thalli and plant stem cross-sections. ROI were selected manually using the ImageJ/Fiji line tool ($n = 180$).

However, imaging of the observed cell wall autofluorescence in the green channel (Figure 3.4, g) could not be reproduced or optimised further by modification of CLSM parameters under 488 nm excitation or with any of the other available blue laser lines (458, 478, and 494 nm). Using these laser lines, the fluorescence of the cell wall remained undetectable or faint, depending on culture conditions. As a result, a useable λ scan for the observed cell wall structure could not be generated with blue-green excitation. However, cell wall autofluorescence was consistently observed in *Ulva* tissue following excitation with a 'UV' diode laser (405 nm), including in *Ulva* cultured under standard growth conditions (Figure 3.5). Unlike the images of cell wall autofluorescence generated under 488 nm excitation, no apparent intracellular fluorescence was observed following the UV excitation of control tissue. Instead, autofluorescence corresponding to cell-cell boundaries was observed specifically.

The λ scans performed using these parameters (Figure 3.5, d) indicated that the cell wall fluorophore in *Ulva* ($\lambda^{\text{max}} = 445$ nm, Figure 3.5, a & b) had a similar emission profile *in vivo* to that of lignified plant tissue imaged under the same settings ($\lambda^{\text{max}} = 475$ nm, Figure 3.5, c), possibly indicating that the *Ulva* cell wall contains a 'plant-like' phenolic fraction. However, the blue shift of the emission maxima of the *Ulva* cell wall relative to plant lignin and the lower signal observed in the *Ulva* cell wall upon excitation with the 488 nm laser suggested that structural differences to 'true' lignin were likely. Furthermore, autofluorescence intensity was greater in the plant stem (Figure 3.6), although it is difficult to compare different tissue types. Following analysis of λ scan data, parameters for imaging the cell wall fluorophore were defined for further investigations (excitation: 405 nm, emission: 415-580 nm).

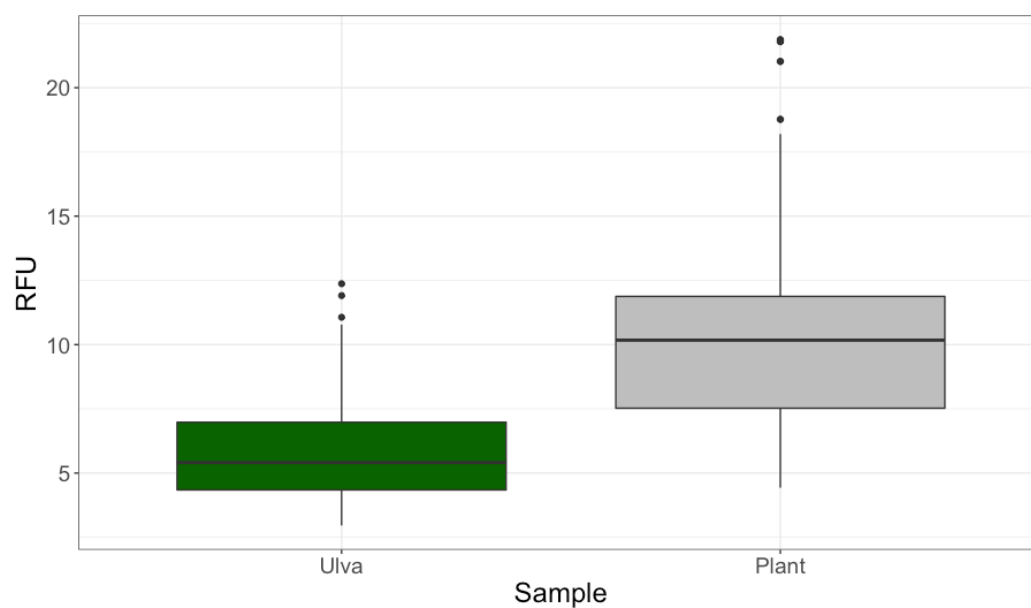


Figure 3.6. The emission intensity of cell wall fluorescence at $\lambda_{\max}$ under 405 nm excitation for spectra is presented in Figure 3.5. Boxplots represent minimum and maximum values (whiskers), interquartile range (box, from 25th to 75th percentile), median value (line), and outliers (dots). $\lambda_{\max}$ was calculated from emission spectra generated from multiple regions of interest selected from images acquired of 3 biological replicates of *Ulva* thalli and plant stem cross-sections. ROI were selected manually using the ImageJ/Fiji line tool (n = 180).

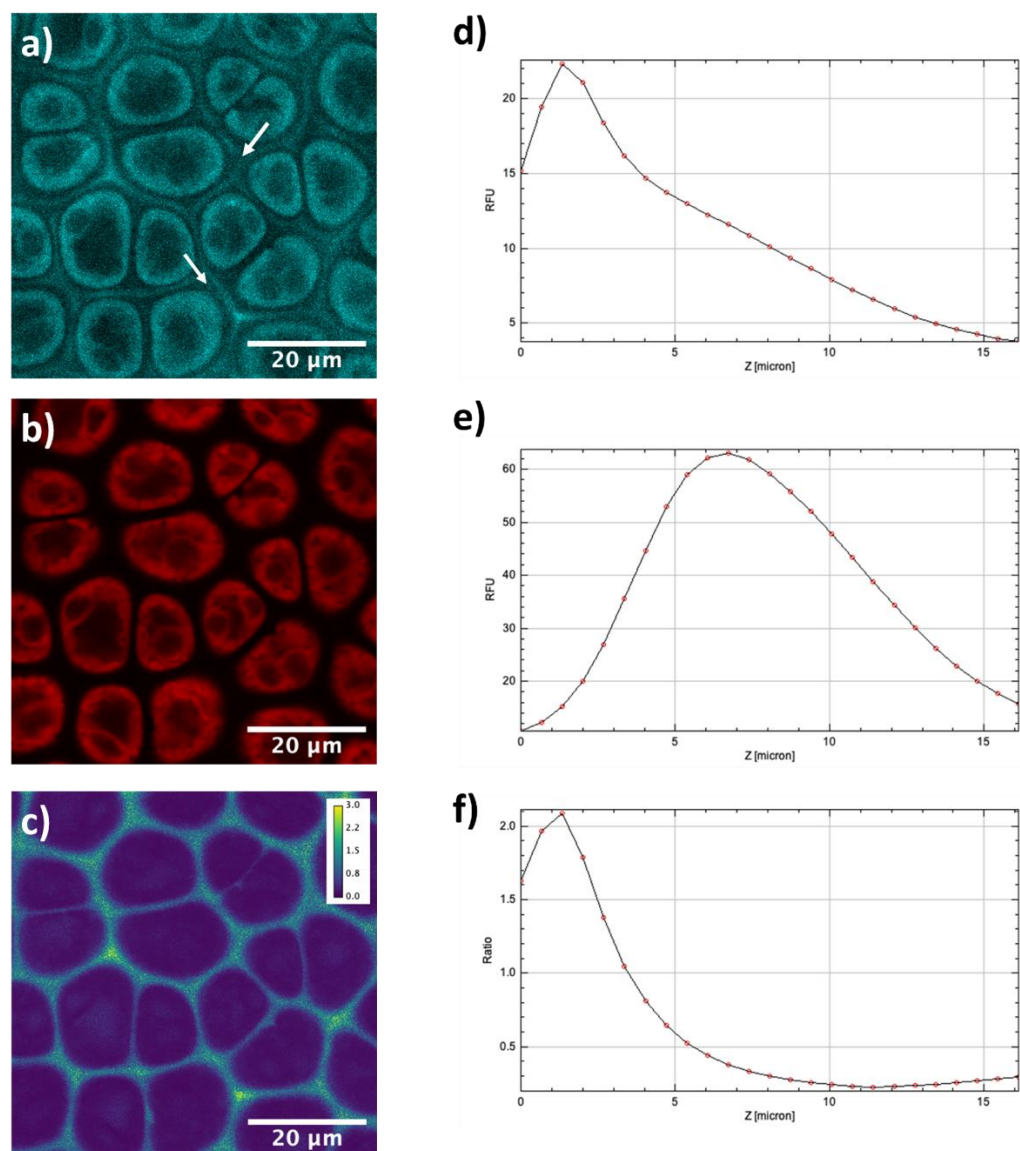


Figure 3.7. Profile (z-axis) of *Ulva* cell wall autofluorescence (a), relative to chlorophyll (b). (a) Maximum intensity plot of blue-green *Ulva* cell wall autofluorescence (excitation: 405 nm, emission: 415-580 nm) from z-stack. (b) Maximum intensity plot of chlorophyll autofluorescence from z-stack (excitation: 405 nm, emission: 650-700 nm). (c) Maximum intensity plot of z-stack generated from ratiometric analysis (blue/red). Corresponding profile plot for each stack where the x-axis corresponds to depth (z, micron, μm) and the y-axis corresponds to signal intensity expressed as RFU (d and e) or ratio (f). z-stacks were collected from sequential scans for the blue-green and red channels at a step size of 0.67 μm . All image processing was performed in ImageJ/Fiji. White arrows mark fluorescent cell wall structures (a).

Confocal microscopy enables the acquisition of z-stacks or 3-dimensional images of tissue. A z-stack, with the z-axis referring to sample depth, is collated from multiple images collected from different focal planes of the same sample (z-depths), which the operator can define. Following the acquisition of images at an appropriate number of intervals, a 3-dimensional image can be generated.

Accordingly, attempts were made to generate a 3-dimensional image of the cell wall fluorophore in live *Ulva* tissue using the *xyz* function of the confocal microscope to better characterise how this structure was associated with the cell wall (Figure 3.7). However, it was difficult to obtain a 'clean' z-stack image of the cell wall fluorophore in *Ulva* without photobleaching occurring, as due to the generally weak autofluorescence observed from the cell wall, the required laser intensity to acquire images was high (~20%). Therefore, high-quality 3-dimensional images could not be generated. Additionally, the extended exposure to the 405 nm laser throughout the z-scan appeared to induce changes in live cells, as demonstrated by the intracellular autofluorescence observed (Figure 3.7, a, white arrows), which was not observed when single *xy* images were acquired previously. Nonetheless, profile plots of *z* vs fluorescence intensity (RFU) were generated from z-scans of chloroplast autofluorescence (excitation: 405 nm, emission: 650-700 nm) and cell wall autofluorescence (excitation: 405 nm, emission: 415-580 nm).

Here, the z-depth of maximum cell wall fluorescence did not correspond with the maximum fluorescence of the chloroplast channel. Cell wall signal was most intense at a shallower z-depth before chloroplast autofluorescence could be observed and rapidly declined as the z-stack progressed deeper into tissue and chloroplast signal increased (Figure 3.7, d & e). This observation was consistent with the ratiometric analysis (Figure 3.7, f), performed by *blue/red*. This analysis may indicate that the fluorophore is present 'above' the chloroplast and, therefore, the cell, with the chloroplast comprising most of the cell volume in *Ulva*. This may suggest that the observed fluorescence corresponds to the 'external cell wall' rather than the 'lateral' cell wall, which is coplanar with cells in the *Ulva* bilayer (Figure 3.8). However, without higher-quality images, interpretations of this analysis must necessarily be limited. Due to the observed limitations, further optimisation was abandoned, with this approach likely having more value if the structure could be stained with a fluorescent dye. Staining attempts are documented in 3.3.3.

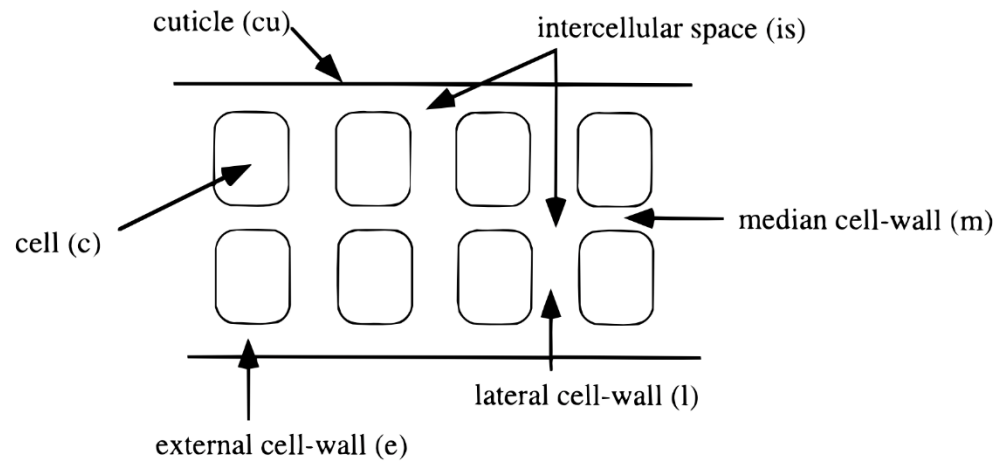


Figure 3.8. Model of the *Ulva* cell wall in a distromatic thallus. Image reprinted from Bobin-Dubigeon *et al.* (1997). Factors limiting the biodegradation of *Ulva* sp cell-wall polysaccharides. *Journal of the Science of Food and Agriculture*, 75(3).

3.3.2 Dynamics of ‘Blue-Green’ Cell Wall Autofluorescence in *Ulva* in Response to Abiotic Stress

3.3.2.1 Salinity

The previous imaging data (§1.3.1) provided strong evidence of an aromatic compound in the *Ulva* cell wall, which was consistent with the hypothesis that there is a phycolignin fraction present in seaweed that is structurally distinct from true lignin in plants. Whilst the function of lignin in the plant secondary cell wall is understood, it is unclear what role any analogous structure would have in an intertidal seaweed, with the evolution of lignin attributed to being an adaption to life on land. However, dynamic changes in lignin biosynthesis have been observed in response to various types of stress, including osmotic, drought, and oxidative stress, which are also environmental conditions that seaweeds must withstand in coastal habitats (van de Mortel *et al.*, 2006; Moura *et al.*, 2010; Cesarino, 2019; Chun *et al.*, 2019; Coleman, Brunner and Tsai, 2021; Li *et al.*, 2022). Therefore, it appeared appropriate to assess whether this fraction in *Ulva* was also influenced by variable environmental conditions, with the phenylpropanoid pathway itself hypothesised to have originated as a means of stress tolerance to life on land (Renault *et al.*, 2017; Davies *et al.*, 2020). In the first instance, cell wall autofluorescence appeared as a simple method to assess whether the proposed phycolignin structure in *Ulva* responded dynamically to stress, with the fluorescence properties of plant lignin known to be influenced by its structure, abundance, and interactions with carbohydrates (Donaldson, 2013; Donaldson and Radotic, 2013).

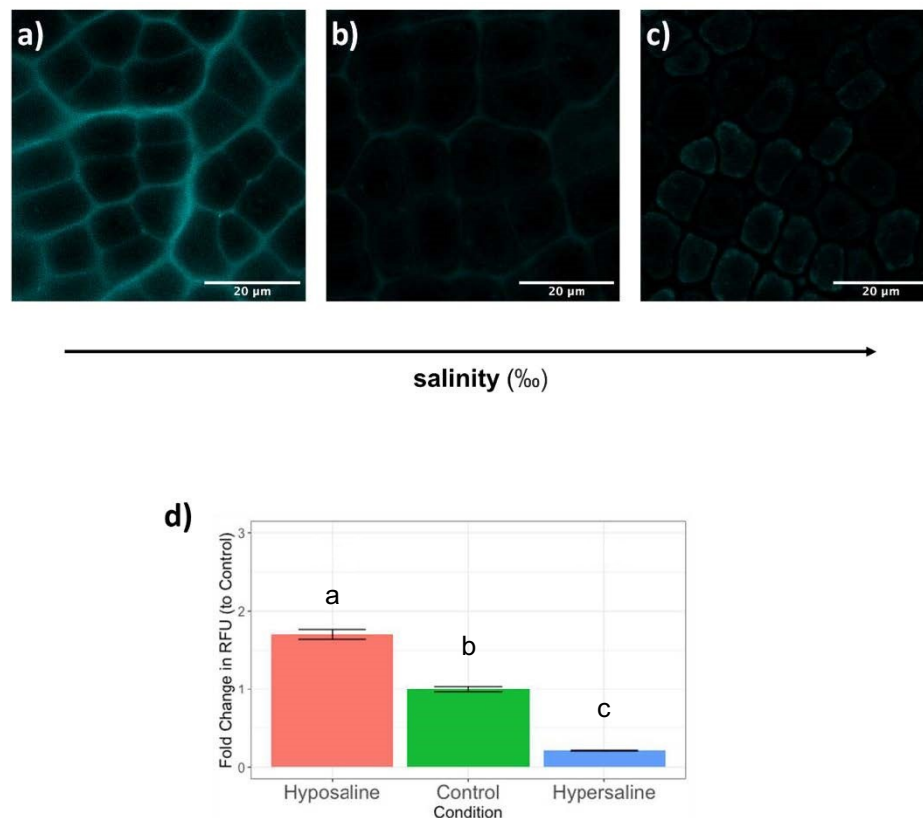


Figure 3.9. *Ulva* cell wall autofluorescence responds dynamically to short-term osmotic stress. *Ulva* fronds were cultured at 0 ‰ (a), 35 ‰ (b), or 90 ‰ salinity (c) for 3 hours prior to imaging (excitation: 405 nm, emission: 415-580 nm). The fold change in fluorescence intensity at cell-cell boundaries following stress (relative to the 35 ‰ cultured control) is reported as a bar plot with the error bars denoting 95% confidence intervals (d). Fluorescence intensity was calculated from ROI manually selected with the ImageJ/Fiji line tool from images acquired from 3 biological replicates (with 3 regions of tissue imaged per biological replicate). A total of 400 regions of interest (ROI) were analysed for each stress condition. Data was analysed by one-way ANOVA, followed by Tukey's HSD post hoc test. Letters represent outcomes of post hoc analysis, here treatments that share a common letter are not statistically different ($p > 0.05$)

As shown in Figure 3.9, signal from the cell wall fluorophore appeared to be significantly influenced by osmotic stress (one way ANOVA; $F = 533.49$, $p < 0.001$). Following 3 hours of hyposaline shock (0 ‰), the intensity of the cell wall fluorophore increased significantly (1.7x fold change, $p < 0.001$) relative to *Ulva* cultured under standard seawater salinity (35 ‰). Under hypersaline stress (90‰), cell wall fluorescence disappeared (0.21x fold change, $p < 0.001$), instead, an increase in intracellular fluorescence was observed under imaging parameters that was absent in unstressed and hyposaline treated samples.

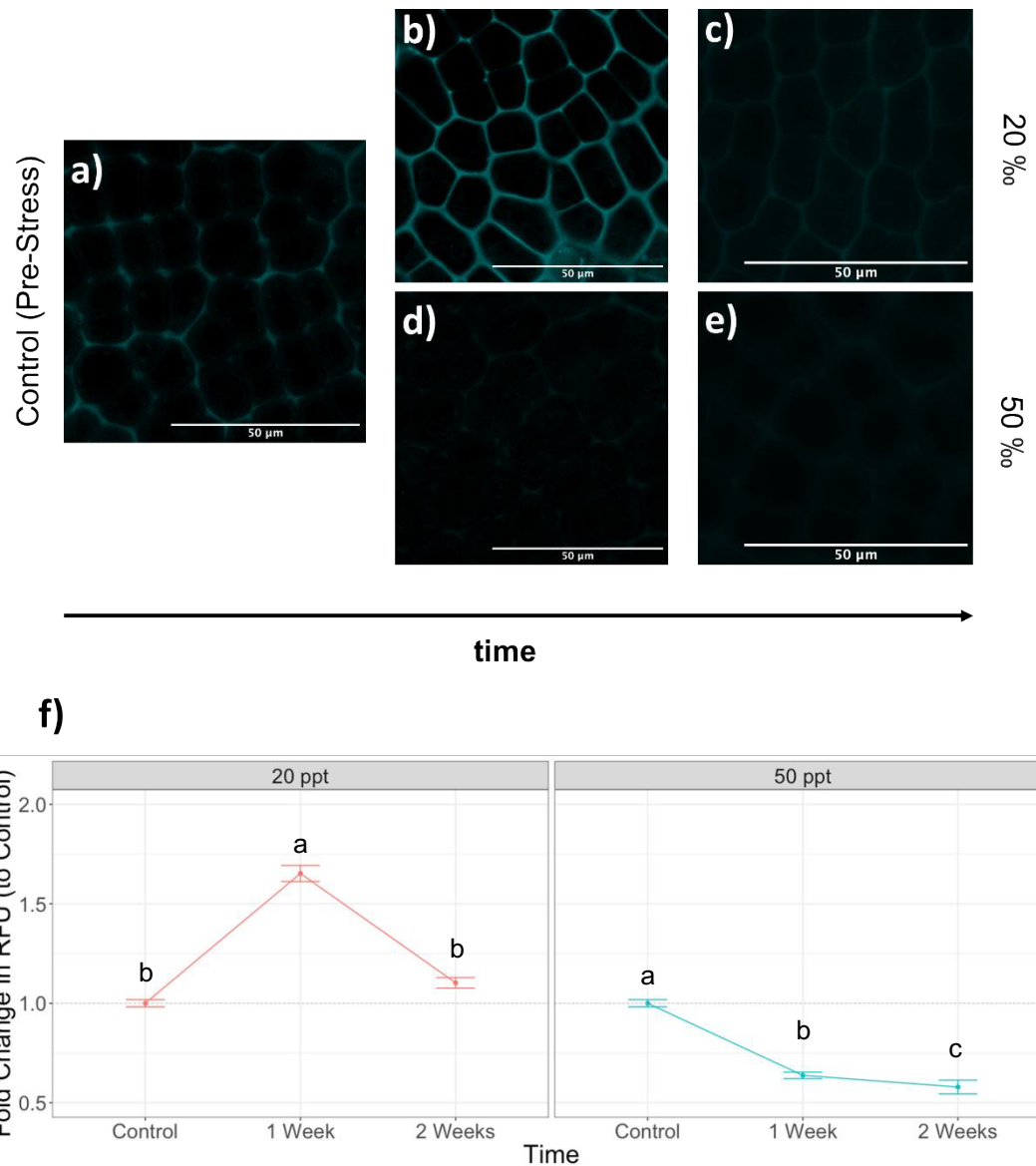


Figure 3.10. *Ulva* cell wall autofluorescence responds dynamically to longer-term osmotic stress. *Ulva* fronds were incubated under control conditions (35 ‰, a) or under hyposaline (20 ‰, b and c) or hypersaline (50 ‰, d and e) conditions for two weeks. Samples were imaged after one (b, d) and two weeks (c, e) (excitation: 405 nm, emission: 415-580 nm). The fold change in fluorescence intensity at cell-cell boundaries following stress (relative to the 35 ‰ cultured control imaged at each time interval) is reported as a line with the error bars denoting 95% confidence intervals (f). Fluorescence intensity was calculated from ROI manually selected with the ImageJ/Fiji line tool across 3 biological replicates (with 3 regions of tissue imaged per replicate). A total of 200 regions of interest (ROI) were analysed per interval. Data was analysed by one-way ANOVA, followed by Tukey's HSD post hoc test. Letters represent outcomes of post hoc analysis, here treatments that share a common letter are not statistically different ($p > 0.05$)

Additionally, *Ulva* was cultured in 20 ‰, 35 ‰, and 50 ‰ seawater for two weeks to assess whether similar changes occurred following long-term acclimation to moderate saline stress (Figure 3.10). Changes in cell wall autofluorescence following hyposaline (20 ‰) and hypersaline stress (50 ‰) relative to control tissue (35 ‰) were consistent with those observed in the initial saline shock experiment, with one-way ANOVA identifying that significant changes in relative autofluorescence occurred across the time course at both 20 ‰ ($F = 97.71$, $p < 0.001$) and 50 ‰ ($F = 158.4$, $p < 0.001$). *Post hoc* analysis demonstrated significant changes occurred after 1 week, with an estimated 1.7x fold change in autofluorescence observed relative to control tissue under hyposaline stress ($p < 0.001$), and a 0.6x fold change observed under hypersaline stress ($p < 0.001$). After two weeks, whilst the relative differences in signal to *Ulva* cultured in control (35 ‰) seawater were still apparent at 50 ‰ salinity (0.6x fold change, $p < 0.001$), the signal in the cell walls of 20‰ seawater cultured samples declined back to control levels (1.1x fold change, no significant difference to the control). This data provided strong evidence that the observed aromatic in the cell wall was responsive to osmotic and saline stress, possibly due to changes in structural composition, the interaction with the cell wall, or abundance.

3.3.2.2 Other (Heat Shock, CuCl_2)

As illustrated in Figure 3.11, significant changes in cell wall autofluorescence were also observed in *Ulva* exposed to alternative stressors, including CuCl_2 treatment (ANOVA: $F = 20.32$, $p < 0.001$) and heat shock ($F = 42.68$, $p < 0.001$).

The observed changes in cell wall autofluorescence were inconsistent under heat shock. Whilst the initial observation of cell wall autofluorescence was made in *Ulva* samples exposed to heat shock, this observation was performed before the parameters for imaging were optimised. The increase in cell wall autofluorescence following heat shock could not be replicated with UV excitation (405 nm), and instead, a decline was observed relative to the control (0.54x fold change, $p < 0.001$). However, the intensity of cell wall autofluorescence did appear to increase in heat-shocked samples following a 3-day recovery period under control conditions relative to unstressed samples (1.28x fold change, $p < 0.001$), in contrast to the hyposaline shock experiment (0‰), where the previously observed induction of cell wall signal was demonstrated to be reversible after the same recovery period (Figure 3.11). This reversibility perhaps indicated that the observed autofluorescence represented a transient change in cell wall properties rather than ‘lignification’ and secondary cell wall biosynthesis.

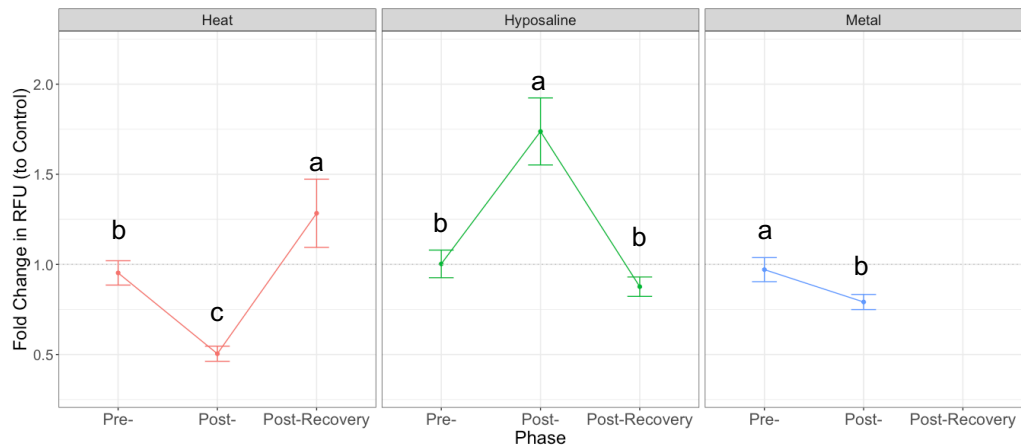


Figure 3.11. Response and recovery dynamics of *Ulva* cell wall autofluorescence following CuCl_2 , hyposaline, and heat shock treatment. *Ulva* fronds were incubated under control conditions and various abiotic stress conditions. Following exposure to abiotic stress, *Ulva* was returned to control conditions for 3 days as a 'recovery' experiment, after which autofluorescence was re-imaged. Respective abiotic stresses and recovery samples include *Ulva* exposed to heat shock for 3 hours at 35°C , a corresponding heat shock recovery sample, *Ulva* exposed to hyposaline stress (0 ‰) for 3 hours, a corresponding hyposaline recovery sample, and *Ulva* exposed to 24 hours CuCl_2 stress (100 μM). Images were not acquired for the CuCl_2 recovery sample. Unstressed *Ulva* was imaged at each time interval. Fluorescence was acquired under standard parameters (excitation: 405 nm, emission: 415-580 nm). The fold change in fluorescence intensity at cell-cell boundaries following stress and recovery (relative to the control sample imaged at each time interval) is reported as a line with the error bars denoting 95% confidence intervals (g). Fluorescence intensity was calculated from ROI manually selected with the ImageJ/Fiji line tool across 3 biological replicates (with 3 regions of tissue imaged per replicate). A total of 120 regions of interest (ROI) were analysed per interval. Data was analysed by one-way ANOVA, followed by Tukey's HSD post hoc test. Letters represent outcomes of post hoc analysis, here treatments that share a common letter are not statistically different ($p > 0.05$)

3.3.2.3 Screening for Evidence of ‘Free Radical Polymerisation’ using Exogenous ROS and ROS Scavengers

In the most accepted model, lignification in plants occurs via free radical polymerisation of phenolics in the apoplast, where the oxidative activity of laccase enzymes and class III peroxidase catalyses the conversion of monolignols into free radicals that then assemble randomly into the heterogeneous lignin complex. It was hypothesised that if the *Ulva* cell wall fluorophore was ‘lignin-like’ and assembled by an analogous mechanism to true lignin in plants, the stress-induced changes in autofluorescence observed here could be prevented with ROS inhibitors or stimulated by exogenous ROS treatment.

No laccase homologs are present in the *U. mutabilis* genome, nor are representatives from the class III peroxidase family, both of which are associated with lignin polymerisation in plants (Hiraga et al., 2001; Passardi, Penel and Dunand, 2004; Marjamaa, Kukkola and Fagerstedt, 2009; J. Wang et al., 2015; Perna et al., 2020). However, extracellular haem peroxidases (n = 21) of unknown function have been identified in the *U. mutabilis* genome, and extracted peroxidases from *U. rigida* have been shown experimentally to oxidise syringyl monolignols *in vitro* (Espíñeira et al., 2011; De Clerck et al., 2018). Furthermore, laccases represent a broad class of enzymes, with laccases identified in fungi and bacteria (Chauhan, Goradia and Saxena, 2017; Mehra et al., 2018). As a result, the presence of enzymes with ‘laccase activity’ in *Ulva* cannot be ruled out definitively from genomic data.

U. compressa thalli were treated with the laccase inhibitor dithiothreitol during incubation under hyposaline conditions (0 ‰, 3 h) to assess whether the observed increase in cell wall autofluorescence could be inhibited. Dithiothreitol (DTT) is used as a reducing agent in various cell culture protocols and has been shown to inhibit laccase activity *in vitro* (Johannes and Majcherczyk, 2000). Treatment of gerbera flowers (*Gerbera jamesonii*) with various concentrations of DTT was also demonstrated to inhibit lignification *in vivo*, with significantly lower lignin contents and reduced laccase activity observed in treated plants relative to control plants (Gerabeygi, Roein and Rezvanipour, 2021). As a result, this approach was trialled with *Ulva*. However, adding DTT (0.1-50 mM) to hyposaline media did not appear to affect the fluorescence properties of samples relative to the control, indicating that the fluorescence source was unrelated to laccase activity.

3.3.3 Screening for 'Lignin-Like' Structures in the *Ulva* Cell Wall Using Conventional Histochemical Stains

As a control, the tissue of the plant stem (*Arabidopsis*) was successfully stained and labelled with the fluorescent dye basic fuchsin. Lignified plant tissue (the plant stem and barley straw) was also identified successfully with the non-fluorescent toluidine blue O and phloroglucinol-HCl methods, with a colourimetric change of toluidine blue O from blue to green observed in secondary cell walls, and a pink or red colouration observed in secondary cell walls following phloroglucinol-HCl staining.

However, attempts to stain *Ulva* cell walls *in vivo* by these methods were unsuccessful. No colourimetric change was observed following phloroglucinol-HCl staining, and the colour change from blue to pink observed following toluidine blue O staining was indicative of the abundance of acidic pectin-like polysaccharides in the seaweed cell wall, but not of any significant polyphenol content. Modification of the basic fuchsin staining protocol in *Ulva* by varying dye concentration (0.01 to 1% v/v), buffer (seawater or phosphate-buffered saline), and sample incubation time (various times from 5 mins to 24 h) did not affect the uptake of the stain, nor did exposing *Ulva* to hyposaline stress (0 ‰) preceding or during staining. By contrast, the *Ulva* cell wall was successfully stained with Direct Yellow 96; a general cell wall dye hypothesised to associate strongly with cellulose, hemicellulose, and xyloglucan (Figure 3.12).

Reproducible staining protocols that distinguish UV-excited fluorophores other than lignin in the plant cell wall are mostly unavailable, and as a result, these structures could not be screened for by a histochemical approach. One exception was suberin, with several studies documenting successful staining of suberin, cutin, and non-fluorescent waxes in the plant cell wall with lipophilic dyes such as Nile Red, BODIPY or Fluorol Yellow 088 that target the extended polyaliphatic regions of these polymers rather than the aromatic groups (Simpson and Ohlrogge, 2016; Ursache *et al.*, 2018; Hubbe *et al.*, 2019; Danila *et al.*, 2021). Despite Nile Red appearing to stain intracellular lipid bodies *in vivo* successfully, there was no evidence of adherence of this dye to the *Ulva* cell wall. As per the attempts at basic fuchsin staining of lignin, modification of the protocol did not affect outcomes.

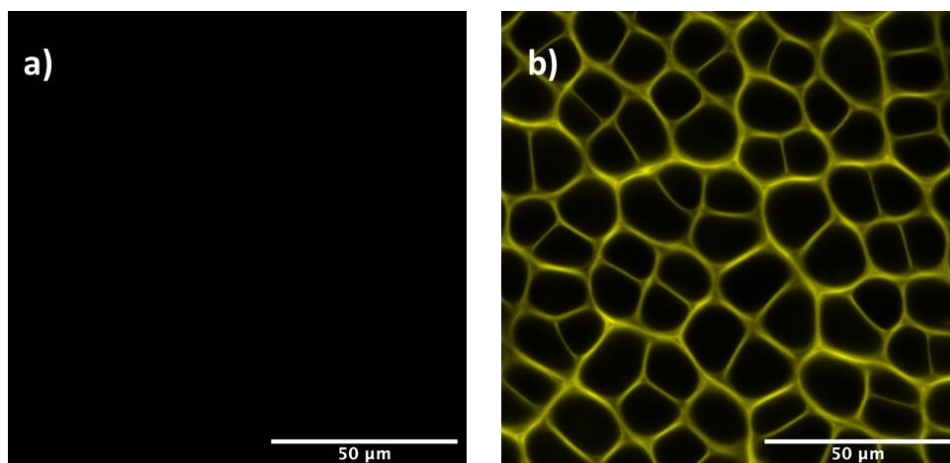


Figure 3.12. *U. compressa* cell walls did not stain with the lignin dye Basic Fuchsin; however, cellulosic cell walls were successfully stained with Direct Yellow 96.

3.4 Cell Wall Fractionation

Whilst the observed fluorescence properties of the *U. compressa* cell wall appeared to be consistent with a plant-like phenolic, the failure to positively identify phycolignin in the cell wall with lignin dyes indicated that no more structural information could be obtained by this approach, as other aromatic structures cannot be distinguished easily *in vivo*. Therefore, at this stage I performed cell wall extractions to facilitate more extensive analysis using the lignin detection methods described previously (3.1.2.2) to assess whether any phycolignin component was present or whether the fluorescence was attributable to another structure.

3.4.1 Sequential Extraction Protocol

The sequential extraction protocol for macroalgal cell walls isolates and fractionates the cell wall into two oxalate-soluble fractions (P1, P2), alkali-soluble fractions (Ha, Hb), and one insoluble fraction, corresponding to pectic, hemicellulose-like, and α -cellulose polysaccharides respectively (see Materials and Methods, section 2.1.4.4). In *Ulva*, this was hypothesised to correlate to the sulfated ulvans, the minor hemicellulose fractions (glucuronan and xyloglucan), and cellulose, respectively, based on the known cell wall composition of these algae (Lahaye and Robic, 2007). A wash fraction is also obtained from the insoluble residue following extraction of hemicelluloses with 6 M NaOH. This method has been used successfully for various charophyte green algae, including representatives from the Charales, Coleochaetales, and Klebsormidiales (O'Rourke *et al.*, 2015).

Parallel extractions were performed with plant biomass (barley straw – *Hordeum vulgare*) as a ‘lignin-positive’ control to verify whether lignin, other cell wall-associated phenylpropanoids, and any other structural components of interest were co-extracted with cell wall polysaccharides during the fractionation protocol. With the lignin content of barley and other monocots cited at approximately 20%, and other phenolics such as coumarates and ferulates known to be esterified to hemicelluloses in these species (e.g., arabinoxylans), barley straw appeared as a suitable source of biomass for comparison (Love, Snape and Jarvis, 1998; Krawczyk et al., 2008; Vogel, 2008; Sun et al., 2011; Yu Zhang et al., 2019). Furthermore, barley straw represented an alternate biomass feedstock used for many of the processes for which *Ulva* has been proposed (e.g., bioethanol production) (Agu et al., 2021; Raud et al., 2021). Extractions were also performed with the carrageenophyte seaweed *Mastocarpus stellatus* as a representative of the Rhodophyceae in which phycolignin was first identified, with *M. stellatus* used frequently in phycology as a model red seaweed for biochemical, molecular, and ecological research (Collén and Davison, 1999; Pereira et al., 2003; Robba et al., 2006; Gómez-Ordóñez, Jiménez-Escrig and Rupérez, 2010; Gómez-Ordóñez and Rupérez, 2011; Flores-Molina et al., 2014; McCoy et al., 2020).

3.4.1.1 Extraction Yields

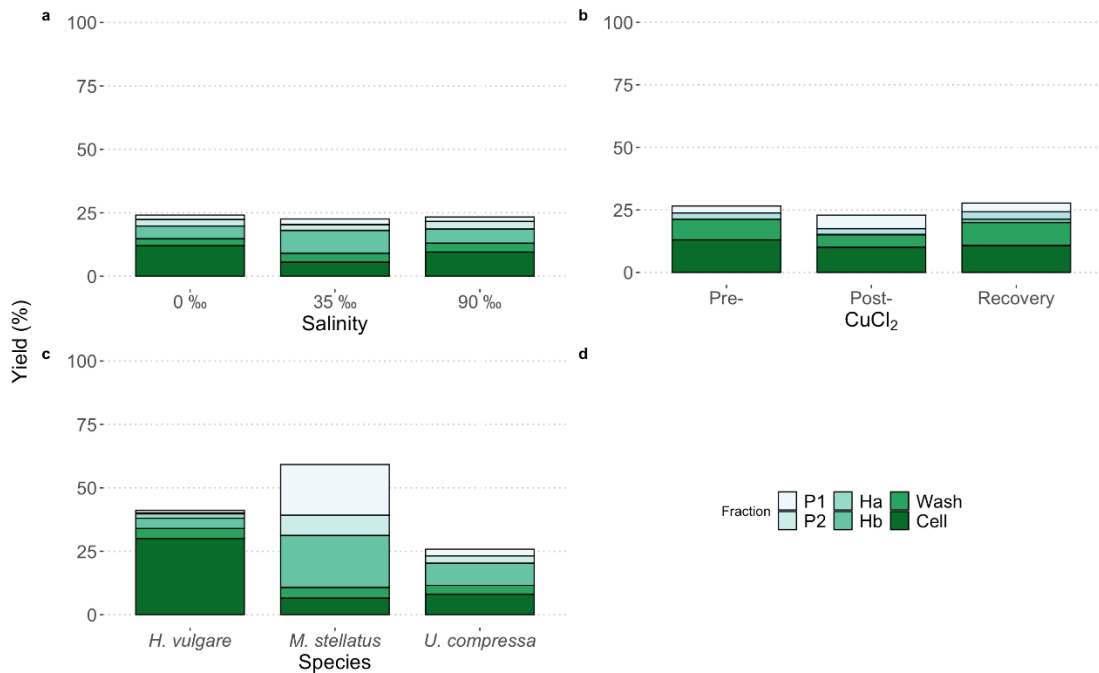


Figure 3.13. Obtained yields (% w/w) from cell wall fractionation protocol. The recovered yield for each cell wall fraction is expressed as a percentage of the starting mass of the AIR used. For the salinity experiment, extractions were performed on *U. compressa* biomass subjected to variable saline conditions for 3 hours (0 ‰, 35 ‰, and 90 ‰). For the CuCl₂ experiment, *U. compressa* was subjected to 100 µM CuCl₂ in artificial seawater for 24 hours (post-), with the pre- sample representing the unstressed control. The recovery sample was generated by returning stressed *Ulva* to control growth conditions for 72 hours before extraction. Extractions were also performed on barley straw (barley straw) and the red seaweed *M. stellatus* for comparison with *U. compressa*. The wash fraction was not recovered from the *U. compressa* CuCl₂ experiment.

As shown in Figure 3.13, the majority of starting biomass was lost throughout the extraction process, with the *M. stellatus* extract having the highest recovery (59% w/w) and the *U. compressa* samples having the lowest (24% w/w). Two hypotheses were proposed to explain this observation. First, that material was lost throughout the fractionation process through dialysis. The second explanation proposed was that the starting biomass (AIR) might contain residual water that was not removed after acetone drying. This residual water would inflate the mass estimations of the starting biomass relative to the isolated cell wall fractions, which were prepared by lyophilisation. The latter theory was tested experimentally, where 1 g of *U. compressa* AIR prepared by the standard ethanol washing method (see Materials and Methods, section 2.1.4.4) was subjected to lyophilisation for 72 hours. A slight loss in mass was observed following

this process (from 1.04 g to 0.93 g), indicating that ~10% (w/w) residual water persisted in this AIR sample after acetone drying. However, this change only accounted for a small proportion of the loss observed during extraction, suggesting that material loss during dialysis was more likely the bigger factor. In particular, acidic polysaccharides such as pectins and ulvans are likely to adhere to dialysis tubing. This could be mitigated by dialysing against a slightly acidic buffer instead of water, as demonstrated for hyaluronic acid extractions (Kohsaka *et al.*, no date). It is hypothesised that the 6 M NaOH treatment in this protocol causes hydrolysis of oxalate-insoluble polysaccharides, leading to loss during dialysis (unpublished).

Preparation of 'AIR' represents a crude method of cell wall isolation, as whilst it removes pigments, lipids, and small molecules, other alcohol insoluble biopolymers such as proteins will persist and may be removed and degraded as polysaccharide fractions are extracted (Foster, Martin and Pauly, 2010; Fangel *et al.*, 2021). For example, in *Arabidopsis thaliana*, the composition of 'AIR' content has been reported as ~15-30% protein and ~30-50% cell wall sugars, with variable starch and a substantial 'uncharacterised' component (Leboeuf, Thoirion and Lahaye, 2004). The estimated dry cell wall polysaccharide content in total *Ulva* biomass is ~30-55%, and protein ~25%, but both are variable and depend on developmental and environmental factors (Lahaye and Robic, 2007; Shuuluka, Bolton and Anderson, 2013; Bolton *et al.*, 2016). Additionally, a high mineral content is often reported in *Ulva* (Magnusson *et al.*, 2016). As a result, it is incorrect to assume that the ~25% (w/w) recovery from starting AIR observed here for *U. compressa* represents ~25% (w/w) recovery of all cell wall polysaccharides. During this work, ethanol washing only removed a mean of ~20% of dry mass from *Ulva*, which, based upon the expected range for cell wall polysaccharide content reported by Lahaye and Robic (2007), would mean that the AIR would theoretically contain 36-60% (w/w) cell wall polysaccharides that could be extracted. Notably, the polysaccharide content of *U. compressa* may be lower than other species (Kidgell *et al.*, 2019). However, again this will be dependent on many biological variables. Methods to purify alcohol insoluble residue are reported (e.g., chloroform and dimethyl sulfoxide), however, this was not deemed appropriate for the current experimental design as lignin is soluble in these solvents, and the removal of proteins was not desired as cell wall-associated proteins possibly represented structures of interest in this research, as aromatic amino acids display fluorescence (Arnon *et al.*, 2021). The root causes of this loss are investigated in the next chapter, using biophysical analysis to determine the structural composition and

supplementary treatments on cell wall polymers (e.g., NaOH) to assess the effect on yield (see chapter 4, section 4.5.3).

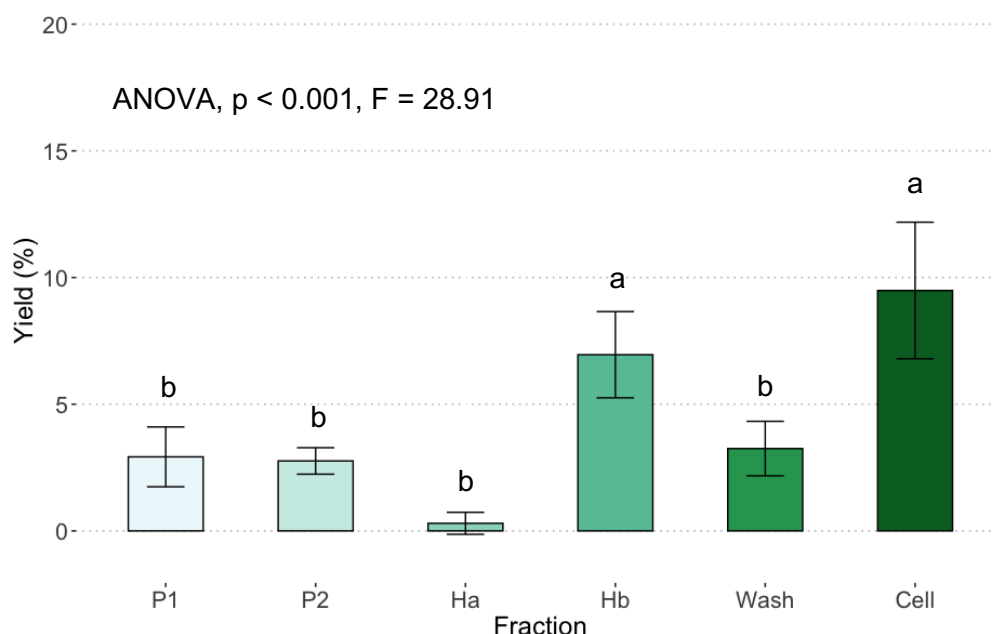


Figure 3.14. Obtained yields (% w/w) from cell wall fractionation protocol across all extractions performed with *U. compressa* biomass (n=7). Bar plots and error bars represent the mean percentage yield and 95 confidence intervals. Yields were analysed by one-way ANOVA, followed by Tukey's HSD post hoc test. Letters represent outcomes of post hoc analysis, here fractions that share a common letter did not have statistically different yields ($p > 0.05$)

The largest fraction in *U. compressa* was consistently the α -cellulose fraction, with a mean yield of 9.5% (w/w) obtained across all extractions (Figure 3.14), consistent with the absence of a dialysis step in acquiring the 'insoluble' fraction. The mean yield for P1 (2.9% w/w) and P2 (2.8% w/w) extracts was surprisingly lower than expected, with these fractions predicted to correspond to the water-soluble sulfated ulvans that are the main component of the *Ulva* extracellular matrix. However, reported ulvan extraction yields do vary significantly in the literature and are affected by method, environmental conditions, and the physiology of the alga. In a literature review on the subject performed by Kidgell and colleagues (2019), median yields for ulvans by various extractions methods ranging from ~3-40% are reported, with the mean combined ulvan yield reported in the present work falling within this range (5.7% w/w). Also, higher total ulvan yields were observed in individual extracts, for example, in the CuCl_2 stressed sample (15.6% w/w) and the corresponding recovery sample (10.9% w/w).

Mean hemicellulose yields of 0.3% (w/w) and 7.0% (w/w) were obtained in *U. compressa* for Ha and Hb, respectively, with a corresponding wash yield of 3.3% (w/w).

The alkali-soluble fraction of the *Ulva* cell wall corresponds with xyloglucans and glucuronans. However, both represent minor fractions in the *Ulva* cell wall (Lahaye and Robic, 2007); as a result, the relatively high Hb yield was somewhat unexpected.

By contrast, the yields obtained for barley straw and *M. stellatus* largely correlated with what is known about the biochemical composition of these species (Figure 3.13), with barley having a low pectin content (1.3% w/w) and being primarily composed of lignocellulose (Vogel, 2008), with hemicellulose and cellulose yields of 1.8% (w/w), 40% (w/w), and 30% (w/w) observed respectively for the Ha, Hb and α -cellulose fractions. In barley straw, most biomass is expected to be lignocellulose (~70% w/w). Therefore, the total recovery of ~42% (w/w) may indicate some depolymerisation and loss through dialysis (Krawczyk *et al.*, 2008; Lara-Serrano *et al.*, 2019; Serna-Díaz *et al.*, 2020; Serran-Parareda *et al.*, 2020). For *M. stellatus*, the major fraction was oxalate soluble material (28% w/w), with the majority segregating in the rapidly soluble P1 fractions (20% w/w). The pectic fraction of *M. stellatus* is known to correspond with sulfated carrageenan (α 1-3 and β 1-4 linked galactopyranoses), for which various extraction methods and structural analysis methods have been widely characterised in the literature (Pereira *et al.*, 2003; Gómez-Ordóñez and Rupérez, 2011). However, whilst it is known that *M. stellatus* synthesises cellulose (Gómez-Ordóñez, Jiménez-Escrig and Rupérez, 2010), as shown by the yield of 6.5% (w/w) obtained for the α -cellulose fraction, the identity of other 'non-carrageenan' polysaccharides in carrageenophytes is not widely reported in the literature. Here, the higher total recovery (59% w/w) suggests that more material was extracted from *M. stellatus* before the sodium hydroxide extraction step. In contrast, little oxalate-soluble material was acquired from barley straw. The composition of cell wall extracts and the causes of the low yields are assessed more extensively in the subsequent research chapters.

3.4.2 Screening of Cell Wall Fraction Fluorescence Properties Using Confocal Laser Scanning Microscopy

Following extraction, a series of emission scans (λ) using the confocal laser scanning microscope (Leica SP5) were performed on the powdered cell wall fractions using the same parameters as those used to observe the cell wall fluorophore *in vivo* previously (Figure 3.5) to assess whether the hypothesised fluorophore was co-extracted during the sequential extraction protocol (excitation: 405 nm, emission: 415 nm $\rightarrow$).

The polysaccharides of the cell wall are usually considered non-fluorescent; as a result, any UV-excited fluorophore was hypothesised to correspond to a structure of interest, including phycolignin. However, there is evidence that cellulosic fibres display fluorescence properties in the absence of lignin (Grönroos et al., 2018; Ding et al., 2020). As a result, pure cellulose fibres (Sigma) were also tested as a control. Spectra from *U. compressa* fraction were contrasted with extracts from the ‘lignin positive’ control barley straw; however, due to the negligible pectin and Ha yields for barley straw, these fractions could not be compared.

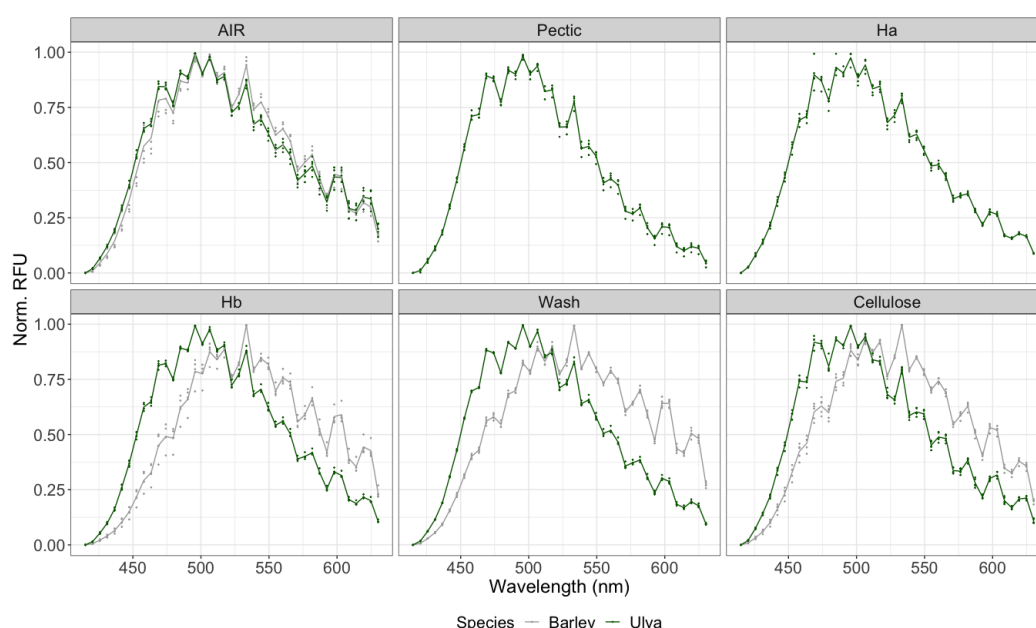


Figure 3.15. Autofluorescence properties of extracted cell wall fractions in *U. compressa* and barley straw. Powdered cell wall fractions were subjected to a series of λ scans with the 405 nm laser line from 410-630 nm at a 5 nm step size. Spectra were not generated for barley straw ‘pectic’ (oxalate-soluble) and Ha fractions. The emission spectra presented are the min-max normalised mean calculated from a series of emission spectra generated from 5 sets of powdered samples on glass slides.

As shown in Figure 3.15, all cell wall fractions in *U. compressa* displayed fluorescence properties under 405 nm excitation. The λ_{max} of the total cell wall extract (AIR) in *U. compressa* was comparable to that of barley straw. However, in the individual cell wall extracts (Hb, wash, and α -cellulose), the barley straw λ_{max} was redshifted relative to the *U. compressa*, with λ_{max} of 533 nm and 496 nm observed, respectively (Figure 3.16 and Table 3.1). A similar redshift of cell wall autofluorescence was observed previously *in vivo* in lignified plant tissue relative to *Ulva* (Figure 3.5). Overall, the intensity of extract fluorescence did not vary significantly between *U.*

compressa and barley straw or between any of the individual fractions except for the Hb sample ($p < 0.05$) and wash sample ($p < 0.01$), where significant increases in RFU was observed in *U. compressa* relative to the barley straw fractions (Figure 3.17).

Emission Properties of Extracted Cell Wall Fractions (CLSM)		
$\lambda_{\max}$ (nm)		
<u>Fraction</u>	<u>barley straw</u>	<u><i>U. compressa</i></u>
AIR	502	496
P1	-	-
P2	-	496
Ha	-	488
Hb	533	496
Wash	533	496
Cell	533	496

Table 3.1. Emission maxima ($\lambda_{\max}$) of extracted cell wall samples from emission spectra are presented in Figure 3.15.

Excitation of a pure lignin control (alkali lignin, Sigma) induced weak autofluorescence with a $\lambda_{\max}$ of 533 nm observed (Figure 3.16). Interestingly, pure cellulose fibres (Sigma) also displayed weak fluorescent properties under 405 nm excitation, with a $\lambda_{\max}$ of 523 nm. The shift in $\lambda_{\max}$ between pure lignin and cellulose (46 nm, $p < 0.01$) was comparable to the shift in wavelength observed between the emission spectra for barley straw and *U. compressa* extracts (31 nm, $p < 0.0001$), possibly reflecting the different biochemical compositions of the extracts, with barley straw biomass being known to be composed predominantly of lignocellulose.

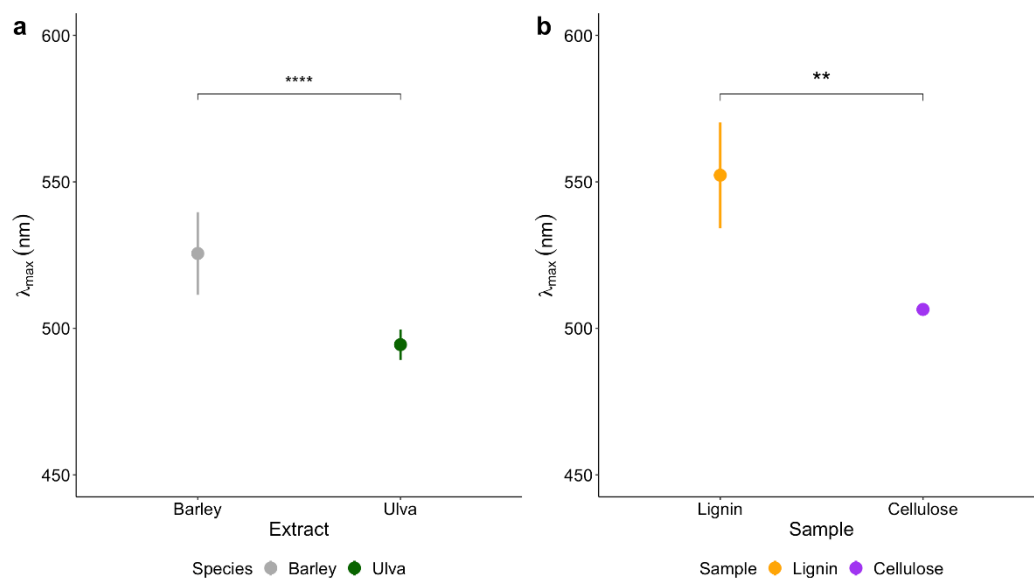


Figure 3.16. Comparison of $\lambda_{\max}$ in (a) *U. compressa* and barley straw cell wall fractions (b) pure lignin (kraft lignin) and pure cellulose standard (Sigma). Points represent mean value; the line represents the range from minimum to maximum value. Powdered samples were subjected to a series of λ scans with the 405 nm laser line from 410-630 nm at a 5 nm step size ($n = 5$). $\lambda_{\max}$ was compared by the Wilcoxon test, with statistical significance indicated by $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), or $p < 0.0001$ (****).

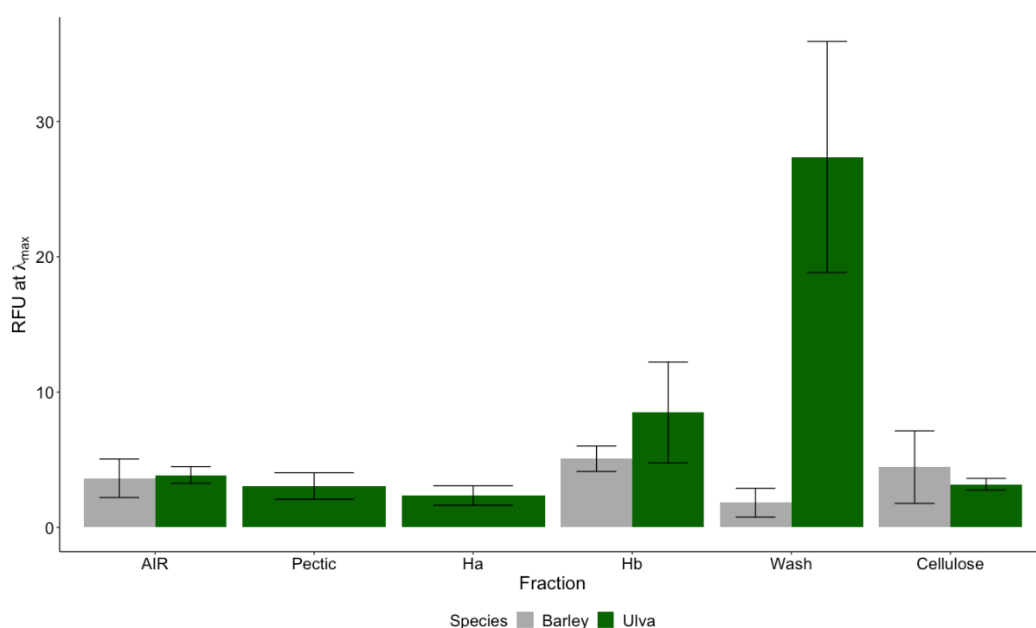


Figure 3.17. The emission intensity of cell wall fractions at λ_{max} for *U. compressa* and barley straw. The bar plot represents mean values calculated from λ_{max} from the 5 emission scans performed per sample, with error bars representing standard deviation.

The autofluorescence of extracted cell walls was also red-shifted compared to the *in vivo* measurements acquired previously for barley straw and *U. compressa* (Figure 3.18). This shift could be because of functional changes in samples induced by the extraction process and the different interactions that cell wall polysaccharides will exhibit *in vivo*.

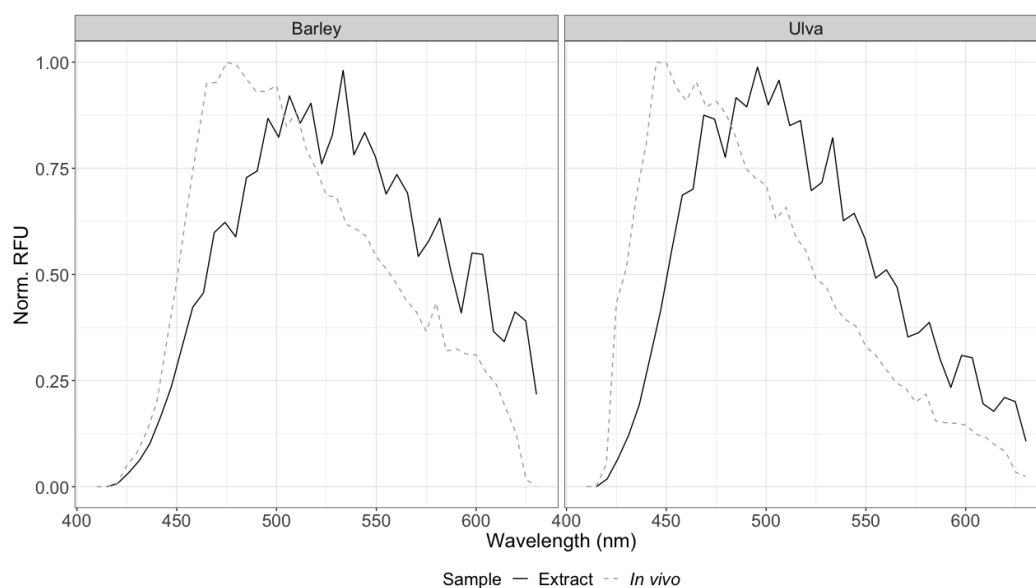


Figure 3.18. Comparison of cell wall autofluorescence *in vivo* and extracted cell walls (i.e., ‘alcohol insoluble residue’). Emission spectra presented are the min-max normalised means.

3.4.3 Screening for ‘Lignin-Like’ Structures in Cell Wall Fractions Using Conventional Histochemical Stains

Following the assessment of fluorescence properties, which were consistent with the successful extraction of the observed cell wall fluorophore, histochemical staining with phloroglucinol-HCl and toluidine blue O reagent was repeated with extracted cell wall fractions to screen for lignin-like compounds. One possible explanation for the previous failure to stain macroalgal cell walls *in vivo* (3.3.3) might have been due to a low content of any ‘lignin-like’ structure in the cell wall, taking it below the limit of sensitivity for successful staining in live tissue. Therefore, it was hypothesised that if the proposed phicolignin fraction from either *U. compressa* or *M. stellatus* was similar structurally to true lignin and was extracted during the cell wall fractionation protocol, staining attempts by these methods might now be successful for the isolated cell wall fractions.

Staining was performed by placing a drop of reagent on powdered cell wall fraction on a glass slide and noting the colour change. As shown in Table 3.2, the AIR, Hb and α -cellulose fractions in barley straw were successfully stained positive with phloroglucinol-HCl, indicated by a colour change of powder to pink. However, no cell wall fraction from *U. compressa* was successfully stained with phloroglucinol-HCl reagent, and the colourimetric changes observed upon reaction with TBO stain provided

no evidence of phenolics. The same negative results were observed in the *M. stellatus* cell wall extracts.

	Qualitative Identification of Lignin (Phloroglucinol-HCl Staining)			Estimated Lignin Content (Acetyl Bromide Soluble Lignin Method)		
	yes/no			% (w/w)		
Fraction	barley straw	<i>U. compressa</i>	<i>M. stellatus</i>	barley straw	<i>U. compressa</i>	<i>M. stellatus</i>
AIR	yes	no	no	53.04	1.03	38.1
P1	no	no	no	-	2.16	3.83
P2	-	no	no	-	3.72	4.23
Ha	no	no	no	7.24	7.62	-
Hb	yes	no	no	18.94	7.15	4.23
Wash	no	no	no	14.1	4.53	
Cell	yes	no	no	10.77	7.78	2.79

Table 3.2. Biochemical screening for lignin in cell wall extractions with a qualitative (phloroglucinol-HCl) and quantitative method (acetyl bromide soluble lignin assay).

3.4.4 Screening for ‘Lignin’ in Cell Wall Fractions Using Quantitative Lignin Detection Methods

3.4.4.1 Derivatisation-Based (Acetyl Bromide)

The acetyl bromide soluble lignin (ABSL) content of *U. compressa* pre-fractionation cell wall extracts (AIR) exposed to the variable saline conditions imaged previously was assessed (Figure 3.19). This method was selected as the selective solubilisation of polyphenols in acetyl bromide: acetic acid reagent facilitates further structural analysis of lignin composition post-quantification by GC-MS, using the derivatisation by reductive cleavage (DFRC) protocol (Lu and Ralph, 1997; Martone *et al.*, 2009), which would offer insight into how the proposed phycolignin is composed relative to true lignin in plants. Here, the ABSL assay was carried out according to Barnes and Anderson (2017), with modifications described initially by Hatfield and colleagues (1999) to limit interference from certain polysaccharides (e.g., xylans), which break down to furfurals in acidic conditions (Binder *et al.*, 2010), contributing to overestimation of lignin content as furfurals absorb in the same region as lignin at ~284 nm.

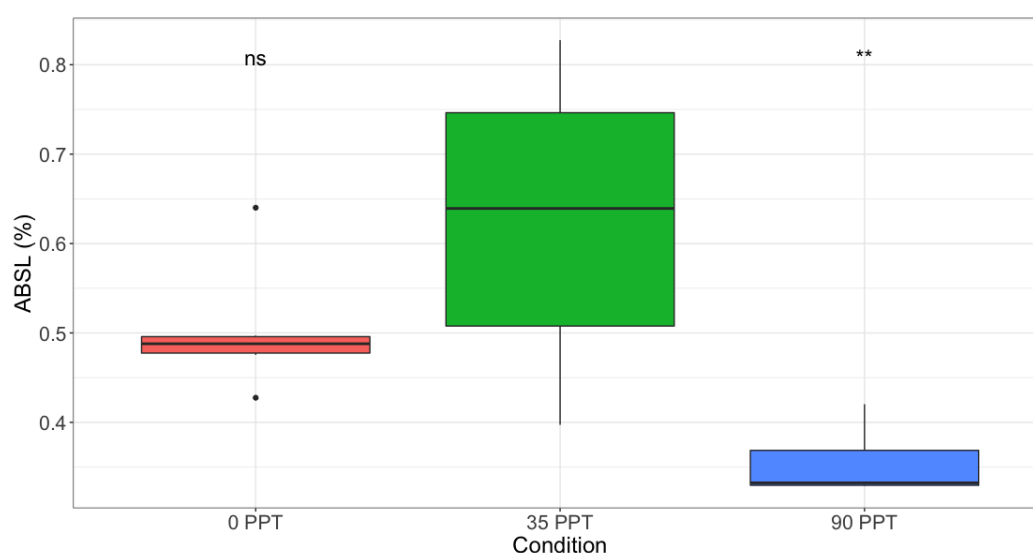


Figure 3.19. Acetyl bromide soluble lignin content (%) of total cell wall extracts (AIR) from *U. compressa* exposed to variable salinity. The data is presented as boxplots from n=6 biological replicates. Boxplots represent minimum and maximum values (whiskers), interquartile range (box, from 25th to 75th percentile), median value (line), and outliers (dots).

In contrast to the measured autofluorescence *in vivo* (Figure 3.9), no significant increase in ABSL content was observed following 3 hours of hyposaline stress (0 ‰). Instead, a slight decline was observed relative to the control (35 ‰). However, the change in ABSL content under hypersaline stress (90 ‰) mirrored the decrease in RFU observed from *in vivo* imaging of *U. compressa* tissue, with a significant decline observed ($p < 0.01$). Overall, the estimated ABSL content for *U. compressa* AIR was low, with unstressed *U. compressa* having a predicted ABSL content of 0.63% (w/w) in this experiment.

The estimated ABSL content of individual cell wall fractions was then assessed to determine whether the seemingly solubilised 'lignin-like' structure was preferentially associated with any specific cell wall fraction (Table 3.2). The measured ABSL content of AIR for the subsequent extractions performed for cell wall fractionation was slightly higher than in the previous experiment (1.03% w/w). However, this was likely a reflection of biological variability or due to differential phycolignin yields between the different experiments as a result of the larger amount of biomass processed for the cell wall fractionation process, with bulk extractions (2 g) required to obtain sufficient yields of cell wall fractions (Figure 3.13). By contrast, only small amounts of AIR (~50 mg)

were required for the former saline stress experiment. However, the ABSL of *U. compressa* AIR was still low relative to that of *M. stellatus* (38.1%) and barley straw (53.1%). As stated previously (§3.4.1), contents for barley, wheat and other grasses are cited at approximately 20% (w/w) in the literature, indicating that the obtained ABSL value here was likely an overestimation, consistent with the expected limitations of the assay (3.1.2.2). However, the large signal observed for barley straw AIR relative to *U. compressa*, in addition to the positive identification of lignin by the phloroglucinol-HCl method, indicated that solubilised lignin and cell wall-associated polyphenols were likely the main contributors to the calculated ABSL content. Monocots such as barley are also known for their high ferulic acid content, which typically associates with the cell wall as esters off the hemicellulose arabinoxylan and are also likely to be incorporated into the lignin complex (Lichtenthaler and Schweiger, 1998; Ram, Dowell and Seitz, 2003; Salleh *et al.*, 2011; De Oliveira *et al.*, 2015; Dixon and Barros, 2019). Therefore, it appears possible that ferulates and other phenylpropanoids present in the barley cell wall are solubilised by acetyl bromide and contribute to the observed signal.

Whilst insufficient pectin yields were acquired from barley straw for the ABSL assay (Figure 3.13), estimated lignin contents of 7.24%, 18.94%, 14.10%, and 10.77% were acquired for the Ha, Hb, wash, and α -cellulose fractions respectively, demonstrating that lignin was likely associated with hemicellulose and cellulose fibres consistent with the known composition of 'lignocellulose' in plants. In *U. compressa*, estimated ABSL was highest in the hemicellulose (Ha and Hb) and α -cellulose fractions (7.62%, 7.15%, and 7.72%, respectively), possibly indicating that the hypothetical phycolignin was associated with the fibrillar skeleton of the cell wall in *Ulva* by a similar mechanism to lignin in the plant secondary cell wall (Liu, Luo and Zheng, 2018; Kang *et al.*, 2019; Terrett and Dupree, 2019). By comparison, ABSL content was lower in the pectic fractions corresponding to ulvans (2.16% and 3.72%), which comprise the amorphous component of the *Ulva* extracellular matrix.

Surprisingly, the ABSL content of all obtained *M. stellatus* fractions was low (2.79-4.23%), indicating that the high ABSL content in the *M. stellatus* AIR may have been attributable to residual extractives in the sample following ethanol washing rather than a phycolignin, with the sample retaining a slight red colour that could neither be removed with successive ethanol nor acetone washes. This hypothesis was supported by the failure of the phloroglucinol-HCl method in staining this sample. The red colour of Rhodophyte algae is attributable to water-soluble phycobiliproteins, which are

essentially insoluble in ethanol, and display unique fluorescence properties as chromophores (Saluri, Kaldmäe and Tuvikene, 2019). Nonetheless, it appears that the contributing component to the ABSL absorbance in *M. stellatus* was removed following the initial oxalate treatment at 100°C and was likely removed from fractions during dialysis.

3.4.4.2 Interference

As discussed previously (3.1.2.2), one limitation of derivatisation-based methods for lignin analysis (e.g., the acetyl bromide protocol) in polysaccharide-rich biomass is interference from aromatic polysaccharide breakdown products that form under acidic conditions, which also absorb at ~280 nm. This is most likely an issue in biomass where a low lignin content is expected in a sample relative to polysaccharide content, for example, in macroalgae. Therefore, the extent to which this skewed the previously described results were assessed experimentally.

Firstly, UV-Vis absorbance spectra (250-320 nm) for a selection of the completed acetyl bromide reactions outlined in Table 3.2 were acquired and compared to pure lignin as an initial assessment of the reliability of the method, with the presence of interferents in the acetyl bromide reaction reported to cause distinctive changes in the absorbance spectra in this region (Hatfield and Fukushima, 2005). Figure 3.20 shows a clear absorbance shoulder in the pure lignin standard (alkali lignin, Sigma) at 280 nm. However, with the exception of the *U. compressa* wash fraction, none of the cell wall extracts displayed a similar profile. Instead, broad descending spectra were observed over the region of interest with no readily discernible peaks. In particular, the *U. compressa* and *M. stellatus* AIR displayed no apparent shoulder in this spectral region, nor did the *U. compressa* α -cellulose. The spectra for the *U. compressa* pectic and hemicellulose fraction appeared to share a shoulder shifted towards ~275 nm. However, this was subtle. The spectra acquired for barley samples also displayed a subtle shoulder in this region, with the most obvious peak being observed in the α -cellulose. Whilst a clear peak was observed in the wash sample, this fraction could not be attributed to a particular structure at this stage of the project, with one hypothesis being that this fraction corresponded to degraded polymers and various small molecules released by the 6 M NaOH treatment.

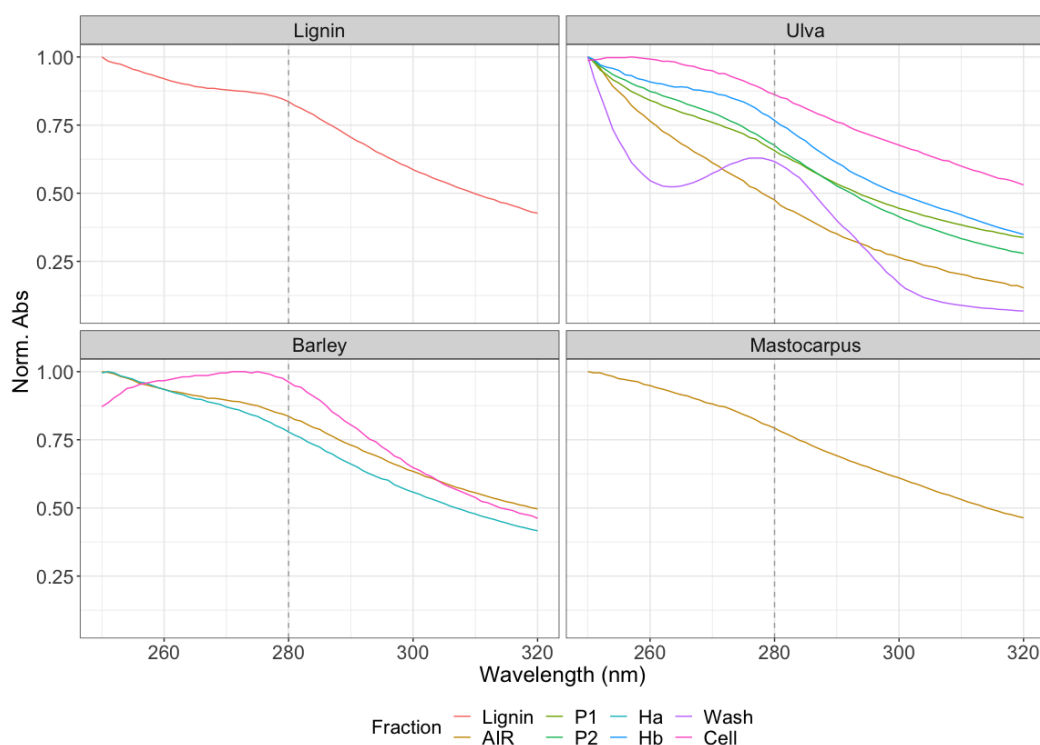


Figure 3.20. Absorbance spectra of completed acetyl bromide soluble lignin reactions for cell wall extracts and pure lignin. Absorbance spectra are normalised for comparison using maximum absorbance values. The dotted line represents the absorbance reading at 280 nm for lignin quantification.

As a result, the absence of clear absorbance peaks at 280 nm following the acetyl bromide reaction in cell wall samples provided some initial doubts about the reliability of this assay, with it being unclear how much of the observed signal could be attributed to the background from degraded polysaccharides and other co-extractives rather than a 'lignin-like' structure specifically. As a result, an interference experiment was performed with the monosaccharides glucuronic acid (GlcA) and xylose (Xyl), pure cellulose (Sigma), and the aromatic amino acid L-tryptophan to assess whether the absorbance signal observed in *U. compressa* cell wall fractions could be explained by degradation of cell wall sugars or aromatic amino acids from proteins that also absorb at 280 nm. Both GlcA and Xyl were tested as they represent residues that are present in the *Ulva* cell wall polysaccharides ulvan (GlcA and Xyl), glucuronic acid (GlcA), and xyloglucans (Xyl).

As shown in Figure 3.21, the candidate interferents readily solubilised in acetyl bromide: acetic acid solution even before the required incubation step (50°C) was performed (see Materials and Methods, section 2.1.7.2). Here, colourimetric changes were observed in the reagent for the lignin control (to light brown), xylose (to green),

and tryptophan (dark brown), which provided initial evidence that an absorbance signal would be acquired from these samples.

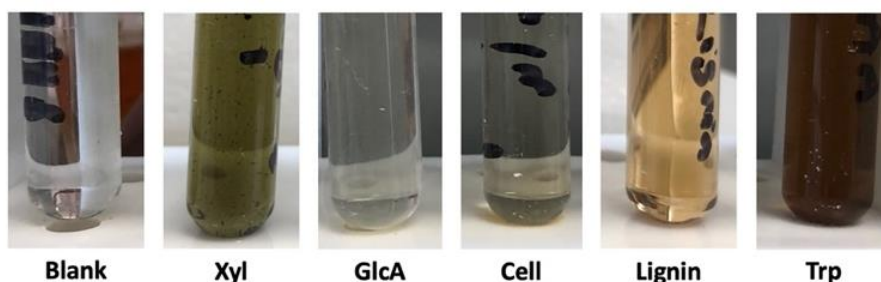


Figure 3.21. Pictures of candidate interferents and lignin solubilised in acetyl bromide: acetic acid reagent. These images were acquired before the 50°C incubation step indicating that interferents readily solubilised in acetyl bromide solution.

This hypothesis was confirmed by the post-reaction absorbance measurements (Figure 3.22), where each interferent tested displayed absorbance at 280 nm, which fell within the linear range of a standard curve prepared with the pure lignin sample, demonstrating that all likely interfered with ABSL measurements. Interference was tested at various sample concentrations, and a ‘relative percentage’ was calculated to correlate the tested concentrations to their corresponding abundance in a hypothetical test sample. For example, the spike with 10 mg cellulose represented an acetyl bromide sample with a cellulose content of 50% (w/w), with 20 mg biomass used per acetyl bromide reaction in the cited protocol (Barnes and Anderson, 2017).

The 10 mg cellulose sample gave an absorbance reading above the limits of the standard curve, indicating that cellulosic fibres were a major source of interference with the assay, suggesting that the data obtained previously from the α -cellulose extracts was likely attributable to noise (Table 3.2). The increase in absorbance signal seen with cellulose appeared to occur in a somewhat dose-dependent manner, which increased the confidence that cellulose (or a breakdown product of cellulose) was responsible for the observed signal. This observation contradicted findings made by Hatfield and colleagues (1999), that whilst xylans (β 1 $\rightarrow$ 4 Xyl) induced significant interference with the acetyl bromide assay, cellulose did not. However, the former observation regarding xylan interference was consistent with the interference observed from the xylose spike experiment here, with xylose residues known to degrade under acidic conditions into furfurals that absorb at 284 nm (Hatfield *et al.*, 1999; Binder *et al.*, 2010).

Interestingly, the uronic acid GlcA did not appear to induce as much interference as cellulose fibres or Xyl. However, the measured absorbance using a 5 mg or 10 mg sample fell within the linear range of the assay. However, using more GlcA in the reaction (> 20 mg) induced absorbance readings that resulted in an estimated 'ABSL content' above the range of the curve. The lower interference observed overall for GlcA correlated with, the lower ABSL content observed for the ulvan fractions in *U. compressa*, whereas the higher ABSL observed in the hemicellulose and α -cellulose fractions appeared to reflect the higher absorbance values observed from the xylose and pure cellulose interference experiments respectively, with 'xyloglucan' likely being the predominant hemicellulose in the *Ulva* cell wall (Lahaye, Jegou and Buleon, 1994). Finally, this experiment also demonstrated that proteins were a likely source of overestimated lignin content, with the highest absorbance readings from all samples tested obtained from tryptophan.

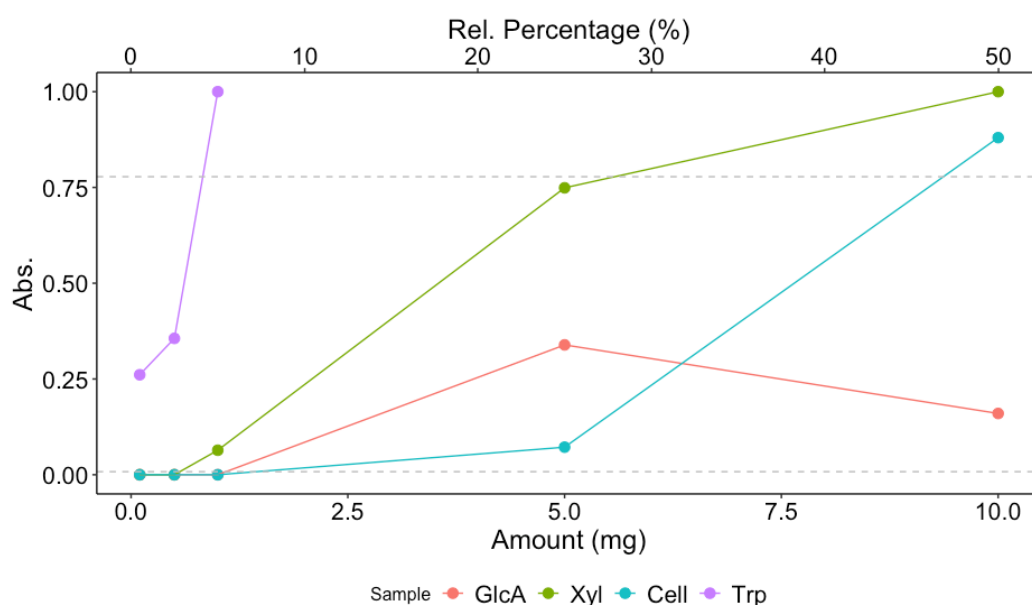


Figure 3.22. Measured absorbance from candidate interferents subjected to the acetyl bromide soluble lignin protocol. Dotted lines represent the linear range of a standard curve prepared with kraft lignin (Sigma). The data presented is the mean from duplicate absorbance readings.

In addition to the interference experiment, there did not appear to be any significant correlation between the ABSL content (% w/w) of samples and the corresponding cell wall autofluorescence properties, either for λ_{max} (not shown) or signal intensity (Figure 3.23), with a slight negative trend observed for the latter. The lack of a clear link between the fluorescence properties of cell wall fraction and the estimated lignin (or polyphenol) content indicated that the observed autofluorescence properties were not necessarily attributable to the presence of lignin-like structures or that the acetyl bromide assay was a poor predictor of the observed fluorophore. The evidence here indicated that a combination of factors was likely.

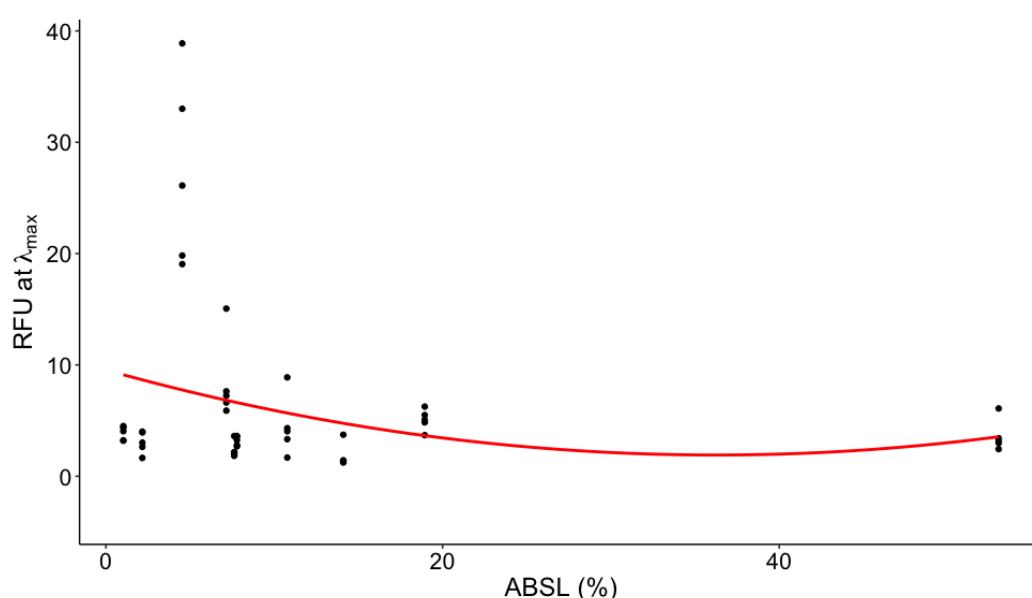


Figure 3.23. Acetyl bromide soluble lignin content (%) plotted against signal intensity (RFU at λ_{max}) for extracted cell wall samples. No relationship between the two variables could be observed.

3.5 Discussion

3.5.1 Autofluorescence Imaging Provides Evidence of Osmotic Stress Induced Changes Associated with the *Ulva* Cell Wall

The *in vivo* imaging data reported in this chapter correlated with an aromatic structure in the *Ulva* cell wall, which during my research, I tentatively attributed to phycolignin due to the similar fluorescence properties it had compared to the lignified plant tissue. The images acquired for *Ulva* cell wall autofluorescence from live tissue in this chapter are comparable to those acquired by Pegg and colleagues (2021) in fixed tissue, which to my knowledge, is the only other report in the literature that documents the observation of this fluorophore in macroalgae. Here, the authors hypothesise that this autofluorescence is due to phenolics and note that the observed signal was significantly less intense than in embryophyte cell walls which contain lignin. However, they do not provide a direct comparison of the spectral properties of the two. The comparative λ scans on *Ulva* and plant tissue performed in this chapter (Figure 3.5) supported the conclusions from this paper that this cell wall aromatic may resemble a plant phenolic. However, the shift in $\lambda^{\max}$ indicates that there may be structural or compositional differences.

Another advance on the report by Pegg and colleagues provided by my investigations in this chapter is the observation that the intensity of the observed cell wall autofluorescence in *Ulva* changes dynamically with osmotic stress. The ability to monitor metabolic changes in real-time remains an advantage for imaging live tissue over fixed tissue, despite the sacrifice in image quality, as laser strength must be given more consideration in live tissue to prevent photodamage (Justyna, 2017).

Notably, the contrasting effects that hypersaline and hyposaline stress had on cell wall fluorescence may offer insight into the biological function of the fluorophore, with intertidal *Ulva* exposed to highly variable osmotic conditions throughout the tide cycle from variable salinity and desiccation at low tide, indicating that this response may be related to osmoprotection. The observation further supported this theory that the hyposaline stress-induced increase in fluorescence signal was reversible when tissue was returned to standard seawater conditions. Changes in lignin content in response to osmotic and salt stress are reported in various plants (van de Mortel *et al.*, 2006; Moura *et al.*, 2010; Cesarino, 2019; Chun *et al.*, 2019; Coleman, Brunner and Tsai, 2021; Li *et al.*, 2022). Here, lignin stiffens the plant cell wall, maintaining cell integrity under hypo-osmotic conditions. This data may indicate that the hypothesised phycolignin might

have an equivalent role in *Ulva*, which is exposed to highly variable osmotic conditions throughout the tide cycle. The increased autofluorescence under hyposaline stress could represent an increase in cell wall rigidity, preventing cells from bursting under the increased turgor pressure.

Conversely, a reduction in phycolignin under hyper-osmotic conditions, as indicated by the apparent loss in autofluorescence signal observed after hypersaline stress, may facilitate increased flexibility of the cell wall and prevent plasmolysis. Alternatively, the dynamic changes in cell wall autofluorescence could reflect changes in total cell wall composition under osmotic stress rather than absolute changes in aromatic content, with lignin autofluorescence strongly impacted by how it interacts with other polymers, with the hemicellulose-lignin interaction being one association reported to quench signal (Donaldson, 2020). Dynamic cell wall stiffening and loosening in response to dehydration-rehydration cycles has been described previously in *U. compressa*, where the inner ulvan-rich layers of the *Ulva* cell wall move with the plasma membrane during dehydration stress preventing plasmolysis (Holzinger *et al.*, 2015). It is possible that the proposed phycolignin fraction also has a role in this remodelling.

3.5.2 Evidence Indicates Source of Fluorescence is Structurally Different to Lignin and other Phenolics in Plants

The recalcitrance of *Ulva* to the histochemical tests for lignin detection and the general lack of cell wall signal observed under blue-green excitation (488 nm) provided early evidence that the observed fluorophore was not necessarily 'lignin-like'. The failure of histochemical tests to detect lignin in *Ulva* both *in vivo* and in cell wall extracts is consistent with reports of unsuccessful phloroglucinol-HCl and TBO staining of various macroalgae (including charophytes and chlorophytes) in the literature, indicating that lignin-like material is either below the limit of detection for these methods, or is absent in these species. Interestingly, despite the failure of phloroglucinol-HCl staining in *Ulva*, coniferaldehyde was positively identified in the metabolome of *U. lactuca* (Gupta *et al.*, 2013), with the phloroglucinol-HCl reagent thought to target β -O-4 linked aromatic aldehydes (Pomar, Merino and Barceló, 2002). To my knowledge, basic fuchsin staining of macroalgae has not been reported in the literature. However, the failure of uptake observed here is consistent with the negative results of phloroglucinol-HCl testing, with the basic fuchsin-lignin reaction being analogous to the Schiff test for aldehydes (Tobie, 1942). This may suggest that if

building blocks for lignin are present in *Ulva*, they may not be polymerised as a lignin complex (β -O-4 linkages) and may associate with the cell wall as monomers or oligomers.

The apparent lack of response of cell wall signal observed *in vivo* following exposure to oxidative stress via CuCl_2 treatment (Figure 3.11) and the inability to inhibit fluorescence with a ROS scavenger were consistent with this hypothesis. Dithiothreitol treatment of land plants (*Gerbera jamesonii*) has been shown to inhibit lignin biosynthesis (Gerabeygi, Roein and Rezvanipour, 2021). However, dithiothreitol treatment in the present work did not inhibit stress-induced increases in autofluorescence following hyposaline stress, indicating that the observed fluorophore is not assembled by free-radical coupling. However, as stated in 3.5.1, the autofluorescence signal is not necessarily correlated with abundance or the degree of polymerisation but could be impacted by the association of the fluorophore with another. More conclusively, no laccases or class III peroxidases can be identified in chlorophyte genomes, both of which are required for lignin polymerisation. This is contrary to the observations by Martone and colleagues (2009) with *C. cheilosporioides*, where β -O-4 linked monolignols were identified following a GC-MS workflow, demonstrating that phycolignin in red seaweed polymerises analogously to true lignin. It is unclear why 'phycolignin' in a rhodophyte seaweed would polymerise and be more structurally like plant lignin than the equivalent component in the chlorophyte seaweeds, which are more closely related to plants, especially as the phenylpropanoid pathway is absent in both algal lineages. From the available genomic information, there does not appear to be a functional phenylpropanoid pathway in *Calliarthron* (Xue *et al.*, 2022). The lack of the PAL enzyme or equivalent ammonia lyases in both lineages that can catalyse the fundamental step in the phenylpropanoid pathway (L-phenylalanine $\rightarrow$ cinnamic acid) raises questions as to whether seaweeds can synthesise true monolignols. Nonetheless, the comprehensive information on phycolignin structure in *C. cheilosporioides* provided by the DFRC protocol and GC-MS demonstrates that imaging experiments performed in isolation are a limited tool for structural analysis.

Despite the evidence for phycolignin in red seaweed, attempts to observe cell wall autofluorescence in the same spectral region (excitation: 405 nm) as *U. compressa* and true lignin *in vivo* in various red seaweeds including *Palmaria* spp., *Pyropia* spp., and *M. stellatus* were unsuccessful (not shown). No *Calliarthron* spp. could be located in this project for comparison. However, the absence of an obvious cell wall fluorophore in

various rhodophytes may indicate that the evolution of phycolignin within these lineages may be more complicated than anticipated. Interestingly, Pegg and colleagues (2021) observe comparable cell wall autofluorescence in various bryophytes and charophyte green algae to cell wall autofluorescence they observe in *Ulva*. However, some charophytes and the bryophytes are known to have a rudimentary phenylpropanoid pathway, which is absent outside Streptophyta (Labeeuw *et al.*, 2015; De Vries *et al.*, 2017, 2021).

These hypothesised structural differences may also be supported by the ‘blue-shift’ in the λ_{max} of the *Ulva* cell wall relative to lignified plant tissue, which may indicate that the algal fluorophore is smaller and less complex than lignin, with polymer size reported to impact the fluorescence properties of lignin (Shen *et al.*, 2022). However, as previously stated, it is difficult to determine this conclusively as many factors impact emission properties.

Additionally, the failure of Nile Red to adhere to the *Ulva* cell wall likely ruled out the fluorophore being a suberin-like polymer. Successful staining of waxy cuticles using lipophilic dyes such as Nile Red and Fluorol Yellow 088 is reported in various plant species (Li *et al.*, 2007; Kalmbach *et al.*, 2017; Ursache *et al.*, 2018; Danila *et al.*, 2021). This staining was performed as suberin and cutin represent some of the only non-lignin UV-excited fluorophores in the plant cell wall that can be reproducibly stained by histochemical methods, with the presence of these structures in the *Ulva* cell wall not previously considered (Pradhan Mitra and Loqué, 2014; Hubbe *et al.*, 2019). As stated in 3.1.2.1, methods for staining other aromatic-associated compounds *in vivo* are largely unavailable; therefore, cell wall-associated compounds such as aromatic amino acids, hydroxycinnamates, and flavonoids could not be easily identified *in vivo*.

3.5.3 The Acetyl Bromide Method is Unsuitable for Use with Macroalgal Biomass

The interference experiments in this chapter indicated that the estimated ‘lignin-like’ content of seaweed cell wall fractions and the apparent stress-induced changes in lignin-like content reported were unreliable and thus provided insufficient evidence for ‘phycolignin’ being present in *U. compressa* biomass. This was supported by the repeated failure to stain samples by the phloroglucinol-HCl method, which is considered a diagnostic test for lignin. Instead, the statistically significant changes observed in estimated ABSL content following hypersaline stress may have reflected possible

compositional changes in the cell wall, with the different sugars tested in the interference experiment demonstrating different absorbance properties in acetyl bromide: acetic acid reagent (Figure 3.22). However, the absolute compositional changes under hypersaline and hyposaline stress could not be verified conclusively from the limited yield data available (Figure 3.13).

With insufficient evidence available to support the finding that a 'lignin-like structure' in *U. compressa* was solubilised in acetyl bromide, and with no available reference for a phycolignin structure, the intended derivatisation followed by reductive cleavage (DFRC) method for lignin identification was not carried out as initially planned, due to the possibility of artefacts being misidentified as structures of interest (Bunzel, Seiler and Steinhart, 2005; Schäfer et al., 2015). The observations here are consistent with the critique of the prior literature discussed in 3.1.2.2 that conventional biochemical tests for lignin quantification are most likely unsuitable for macroalgal biomass due to the compositional differences between plants and algae. The acetyl bromide interference experiment performed here may indicate that some of the previously identified phycolignin structures in the literature may represent misidentified artefacts produced by the acidic conditions used in these methods or other interferents. This analysis supported the hypothesis that a 'lignin-like' structure was not present in *Ulva*, and the autofluorescence observed must be attributable to something else. Nonetheless, further structural analysis appeared necessary to resolve this question.

3.5.4 Extracted Polysaccharides Display Fluorescence Properties

The λ scans of the isolated cell wall fractions indicated that a fluorophore with similar properties to that observed *in vivo* was successfully extracted by the sequential extraction protocol, despite the low recovery (Figure 3.13), and as observed *in vivo* displayed a similar blue-shift in (λ_{max}) relative to the equivalent barley straw extracts. Overall, the emission spectra of the extracted cell wall fractions generally resembled cited fluorescence properties of lignocellulose samples excited in the UV (Donaldson, 2013; Auxenfans, Terryn and Paës, 2017; Grönroos *et al.*, 2018). However, the observed emission from pure cellulose fibres, the observation that fluorescence properties were not attributable to specific cell wall fractions, and the negative results obtained from biochemical screening for lignin indicated that the observed signal in *U. compressa* was

not necessarily attributable to a phenolic or 'lignin-like' structure as initially concluded from *in vivo* imaging (Figure 3.5).

Whilst the fluorescence properties of lignocellulose are typically attributed to lignin specifically, with polysaccharides traditionally considered as 'non-fluorescent', various cellulosic materials, bacterial celluloses, and algal celluloses are known to exhibit fluorescence properties in the absence of lignin (Olmstead and Gray, 1993; Grönroos *et al.*, 2018; Khalid *et al.*, 2019; Ding *et al.*, 2020; Qin *et al.*, 2022). The basis of these properties in cellulose is poorly understood, with an unsaturated structure such as an aromatic ring required for fluorescence (Sauer, Hofkens and Enderlein, 2011). As a result, these properties are typically attributed to artefacts produced by standard cellulose extraction protocols, such as the formation of aromatic quinones (Bikova and Treimanis, 2004; Grönroos *et al.*, 2018). However, this does not explain the correlation with the *in vivo* fluorescence properties. Additionally, the autofluorescence of pectin and hemicellulose-based biomaterials is reported in the literature (Liu *et al.*, 2008), consistent with the fluorescence observed for the ulvan and hemicellulose fractions extracted here. Other explanations for 'lignin independent' polysaccharide fluorescence include carboxyl and carbonyl groups and glycosidic linkages (Grönroos *et al.*, 2018; Ding *et al.*, 2020). As hypothesised for the acetyl bromide data, stress-induced changes in cell wall composition could also explain the dynamic signal changes observed *in vivo* if the source of cell wall fluorescence was the polysaccharide fibres.

Broad emission properties are reported for cellulosic materials under UV excitation, with emission maxima (λ_{max}) ranging from 422 nm to 495 nm reported for various cellulosic materials (Grönroos *et al.*, 2018). In addition, broad red-shifted emission properties (570-585 nm) were also observed with cellulose isolates excited with green wavelengths (510-530 nm), where the absence of contaminating lignin was verified experimentally (Ding *et al.*, 2020).

In this chapter, using 405 nm excitation, a λ_{max} of 496 nm is observed for the *U. compressa* α -cellulose extract, and a λ_{max} of 523 nm was acquired from the cellulose standard (Figure 3.16), with the fluorescence properties of cellulosic materials known to vary with source and extraction method (Donaldson, 2013; Auxenfans, Terryn and Paës, 2017; Chabbert *et al.*, 2018). As stated previously, common extraction steps for cellulosic material, such as hot alkaline treatment, may induce the formation of aromatic groups on sugar residues, which has been hypothesised to contribute to the observed fluorescence properties. For example, the intensity of the observed emission from *U.*

compressa cell wall fractions increased significantly relative to the others in the sodium hydroxide soluble hemicellulose fraction (Hb) and the resulting wash sample from this process. However, this was not reflected in the spectra for the corresponding barley straw samples, and fluorescence was observed in all *U. compressa* fractions before and after sodium hydroxide treatment, indicating that the alkali treatment was not the source of the emission properties. Additionally, the fluorescence in the pre-fractionation sample (alcohol-insoluble residue) showed that neither the extraction temperature nor the buffers used could explain the observed emission.

Functional modification of cellulose fibres is also known to modify the observed fluorescence properties. For example, increased intensity and broader emission bands are observed in amino-functionalised cellulose, likely due to the formation of imine groups between carbonyl groups on glucose residues and amino groups (Martínez Urreaga and de la Orden, 2007; Shen *et al.*, 2020). Interestingly, the natural biopolymer chitin, composed of repeating subunits of the amide sugar *N*-acetylglucosamine, displays similar fluorescence properties to those observed here (Rabasović *et al.*, 2015). However, to my knowledge, there is no evidence of amino sugars being present in the *Ulva* cell wall. However, some microalgae and red coralline algae may synthesise chitin (Kapaun and Reisser, 1995; Rahman and Halfar, 2014).

The presence of proteins in cell wall extracts containing aromatic acid residues (tryptophan, phenylalanine, and tyrosine) would also induce signal over the same spectral region (Ghisaidoobe and Chung, 2014; Arnon *et al.*, 2021). Unicellular chlorophytes from the Chlorophyceae (e.g., Volvocales) are known to have hydroxyproline-rich cell walls, consisting of extensins, arabinogalactan-like proteins, and other hydroxyproline-rich glycoproteins (Goodenough and Heuser, 1985; Adair *et al.*, 1987; Voigt and Frank, 2003; Voigt *et al.*, 2014), with these proteins, reported to have a variable aromatic amino content (Přerovská *et al.*, 2021). Aromatic amino acid-containing proteins have generally been characterised as components of plant and algal cell walls (Burczyk *et al.*, 1999; Kim *et al.*, 2017). Similar cell wall proteins have recently been identified in the cell wall of *U. lactuca* (Přerovská *et al.*, 2021).

3.5.5 More Comprehensive Analysis is Required to Characterise the Observed Cell Wall Dynamics

The use of imaging to study lignin, as performed in this chapter here, is not without its limitations. As per most modern confocal microscopes, the confocal laser scanning microscope (CLSM, Leica SP5) used in this project was equipped with a 405

nm diode for UV imaging rather than an actual UV laser (< 400 nm). The use of a 405 nm diode is typically preferred to shorter wavelength lasers both for cost and safety reasons and also due to the widespread use of DAPI as a stain in biological research, which is strongly excited at this wavelength (Kapuscinski, 1995; Żurek-Biesiada, Kędracka-Krok and Dobrucki, 2013). As a result, the imaging and discrimination of different aromatics *in vivo* is limited using the CLSM set-up. Additionally, an appropriate laser line was unavailable to enable the use of more advanced techniques available with the CLSM, which may have provided more substantial information on autofluorescence dynamics, including fluorescence lifetime imaging microscopy (FLIM) and Förster resonance energy transfer (FRET) which have both been used successfully for the analysis of lignin *in vivo* (Donaldson and Radotic, 2013; Terryn, Paës and Spriet, 2018). However, cell wall autofluorescence would not be observed with the available FLIM laser lines of the Leica SP5 used in this project (470 nm and 640 nm), with the 405 nm laser required to observe the fluorescence *in vivo*. Time-resolved fluorescence microscopy via FLIM would enable better resolution of the observed cell wall fluorescence properties, as whilst different fluorophores may have overlapping emission profiles, the lifetime (i.e., the time between excitation and emission of a photon by a fluorophore) may differ. This would facilitate better analysis of the structural changes of the cell wall under the abiotic stress conditions tested and facilitate a more informative structural comparison with true lignin.

Imaging of the fluorophore also required extensive optimisation of parameters. Issues encountered included photobleaching during the acquisition of 3-dimensional images, which limited the reliability of the quantitative analysis performed across the different stress conditions tested. Overall, phycolignin autofluorescence was relatively weak compared to chloroplast autofluorescence and the lignin structures in plant tissue, meaning extensive optimisation of laser intensity, pinhole width, and acquisition settings was required to acquire images of a high enough quality for analysis. The application of ratiometric analysis somewhat mitigated these issues; however, autofluorescence imaging remained a limited tool for acquiring substantial structural information about the *Ulva* cell wall. In a couple of reports, immunolabelling and TEM have been used to verify the presence of 'monolignols' in charophyte green algae (*Coleochaete scutate*) and the red seaweed *C. cheilosporioides* (Martone *et al.*, 2009; Sørensen *et al.*, 2011). To date, this has not been performed for a chlorophyte species, meaning this approach may have value for future investigations into the proposed phycolignin

fraction of *Ulva*. Nonetheless, combined with the inconclusive biochemical analyses, these limitations indicated that a new approach was necessary.

3.5.6 Conclusions

The post-extraction observations did not support the hypothesis that the observed cell wall autofluorescence *in vivo* was attributable to a phycolignin structure. Instead, these findings appeared to support an alternate view that the cell wall autofluorescence was due to reorientating cell wall fibres or a proteinaceous cell wall rather than a 'plant-like' phenolic specifically.

Furthermore, the work in this chapter demonstrates that the frequently cited derivatisation-based methods for lignin analysis were unsuitable for use with *Ulva* biomass due to the extensive interference observed. This raised uncertainty about the reliability of the prior reports in the literature that used these methods, which provided the initial evidence that *Ulva* may have a phycolignin structure.

Lower than expected yields were also obtained from *Ulva* cell wall fractions with the described cell wall fractionation protocol, suggesting a proportion of cell wall polysaccharides were lost through dialysis, likely because of alkaline hydrolysis. However, the successful extraction of fluorescent cell wall fractions for *Ulva* and the positive identification of lignin in the barley straw extract indicated that the structures of interest were successfully co-extracted during this process for the research discussed in this thesis. Nonetheless, this observation required further investigation to critique the suitability of this method for obtaining seaweed cell wall polymers in future investigations and determine if modifications were required.

As a result, a non-destructive analytical approach was required to establish whether a 'lignin-like' structure was associated with the *Ulva* cell wall and to determine the structural composition of the obtained cell wall fractions. This work is outlined in the next two research chapters with biophysical analysis (ATR-FTIR and NMR).

4. ATR-FTIR Spectroscopy of *Ulva* Cell Wall Extracts

4.1 Introduction

4.1.1 General Introduction

In the previous chapter, a sequential extraction protocol was performed to isolate different cell wall polysaccharides from *Ulva compressa* biomass. These polysaccharide fractions were screened for with various biochemical tests frequently used to detect lignin in lignocellulosic biomass, including the Wiesner test (phloroglucinol-HCl method) and the acetyl bromide soluble lignin assay. However, whilst lignin was positively identified in barley straw extracts that were processed identically to *U. compressa* by the same fractionation protocol, there was no evidence for an equivalent structure being associated with macroalgal cell wall polysaccharides (*U. compressa* and *Mastocarpus stellatus*). Upon review of the initial evidence provided in the literature that suggested an equivalent structure (or ‘phycolignin’) was present in seaweeds (Yaich *et al.*, 2011, 2015; Gupta *et al.*, 2013; Yoza and Masutani, 2013; Li *et al.*, 2016; Moral *et al.*, 2019), and experimental investigation into some of the protocols used in these reports, it was concluded that many of the ‘destructive’ methods typically used for studying lignocellulosic biomass in bioenergy and biomaterials research were not suitable for investigating this hypothesised structure in the present work, due to the extensive interference observed with macroalgal sugars under the acidic conditions required by these methods (Hatfield *et al.*, 1999). As a result, I suspected that the identified ‘phycolignin’ in *Ulva* in the prior literature may have been attributable to artefacts; consequently, a ‘non-destructive’ analytical approach appeared necessary to resolve the research aims of this thesis.

One non-destructive and routine method for the structural analysis of biopolymers is attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR). FTIR is an effective tool for the structural characterisation of biopolymers, but it is often difficult to interpret, particularly for complex biopolymers in understudied organisms where limited reference spectra are available in the literature (Coates, 2006; Barth, 2007; Liu *et al.*, 2021). However, FTIR methods have been used successfully in the literature for studying biopolymers in various macroalgal species (including *Ulva*) and for characterising the lignocellulosic biomass of both wood and herbaceous plants that contain lignin, hemicelluloses, and cellulose (Pereira *et al.*, 2003; Popescu *et al.*, 2007; Robic *et al.*, 2009; Hernández-Garibay, Zertuche-González and

Pacheco-Ruíz, 2011; Trivedi *et al.*, 2016; Traoré, Kaal and Martínez Cortizas, 2018; Deng *et al.*, 2019; Prabhu *et al.*, 2019; Wahlström, Nylander, *et al.*, 2020; da Costa *et al.*, 2021; Md Salim, Asik and Sarjadi, 2021).

In addition to the ‘phycolignin’ investigations, the generation of ATR-FTIR data from *Ulva* cell wall polymers in this chapter will also help assess if this approach is appropriate for further ‘routine screening’ of macroalgal biopolymers by evaluating how much biologically relevant structural information can be determined by this method. For example, identifying whether functional modifications that are known to occur on plant polysaccharides (e.g., methylation and *O*-acetylation of pectin and hemicelluloses) are observable in macroalgae will offer evolutionary insight into how cell walls are structurally adapted to diverse environments (Gnanasambandam, R., Proctor, 2000; Marry *et al.*, 2000; Synytsya *et al.*, 2003; Fella *et al.*, 2009; Kyomugasho *et al.*, 2015; Wu, Bulgakov and Jinn, 2018). If distinct changes can be observed by this approach, FTIR may be a suitable tool for studying cell wall remodelling in subsequent stress and development studies to assess how the *Ulva* cell wall changes functionally under different environmental conditions.

4.1.2 FTIR of Biopolymers

4.1.2.1 Infrared Spectroscopy: Theory

In all spectroscopy techniques, such as fluorescence spectroscopy and imaging (discussed in the previous chapter), a transition between energy states must be observed and measurable following the absorbance of energy by a structure of interest (Banwell, 1983). In infrared spectroscopy, this is facilitated by the observation that the bonds between atoms in a molecule are not static. Instead, they are in constant motion relative to each other, defined as a molecular vibration or ‘vibrational mode’, which depends on the bond type and local chemical environment. These vibrations occur even at ‘ground state’ (i.e., when no energy is applied). However, a transition in vibrational energy is observed upon absorption of mid-infrared energy, and the bond vibrates faster. For a bond to be excited, the frequency of the IR energy must match up with the frequency of the bond vibration, meaning that different bonds can be distinguished on an IR spectrum (Harris, 1977; Banwell, 1983; Urban, 1993; Engel, 2006).

As described in Figure 4.1, vibrational modes are grouped generally as either stretching motions (ν) or bending motions (δ), with stretching being the most prominent vibration that is observed during an infrared spectroscopy experiment (Nasdala *et al.*,

2004). Notably, stretching can be coupled as discrete asymmetric and symmetric stretches (Coates, 2006; Griffiths and de Haseth, 2007; Berthomieu and Hienerwadel, 2009). For example, a hypothetical isolated bond X-Y (with X and Y representing different atoms) will have one stretching motion, whilst a corresponding X-Y₂ group will have a symmetric vibration where both X-Y stretches are synchronised and an asymmetric vibration where whilst one X-Y stretch constricts the other stretches. Coupled vibrations will vibrate at a different frequency to single bonds and at a different frequency to bonds with more constituent bonds. As a result, they can be distinguished by FTIR. Notably, asymmetric stretches are always observed at a higher frequency than the corresponding symmetric stretch (Coates, 2006). Coupling is also observed for the bending vibrations, which will either move 'in-plane' (scissoring and rocking) or 'out of plane' via wagging or twisting (Nasdala *et al.*, 2004; Coates, 2006; Berthomieu and Hienerwadel, 2009).

However, some bonds are 'IR inactive'. For a bond to be 'IR active', a change in 'dipole moment' must be observed for the functional group to facilitate the absorbance of infrared energy (Banwell, 1983; Coates, 2006; Baker *et al.*, 2014). Using a typical textbook explanation of this concept (Engel, 2006), the CO₂ molecule has a coupled C=O stretch (O=C=O) that may be asymmetric or symmetric (as shown in Figure 4.2). In an asymmetric stretch, there is a net change in dipole, with oxygen (O^{δ-}) being more electronegative than carbon. As the stretches occur asynchronously, the dipole oscillates as the two oxygen atoms constrict and stretch away from the carbon sequentially. Both bonds move in tandem in a symmetric stretch, meaning the dipole is not changed with the vibrational movement. If the vibrational mode induces a change in dipole moment for a bond (i.e., the local charge), the dipole will be able to interact with the applied electromagnetic energy (infrared radiation) and absorbs energy which oscillates at the same frequency as the dipole (Harris, 1977; Banwell, 1983; Engel, 2006; Griffiths and de Haseth, 2007). Therefore, using the CO₂ model, the asymmetric stretch will absorb IR, but the symmetric stretch cannot. With larger molecules (e.g., polymers) and more complicated vibration patterns, the dynamics of how dipole moments form are more complex. Nonetheless, the same principle applies.

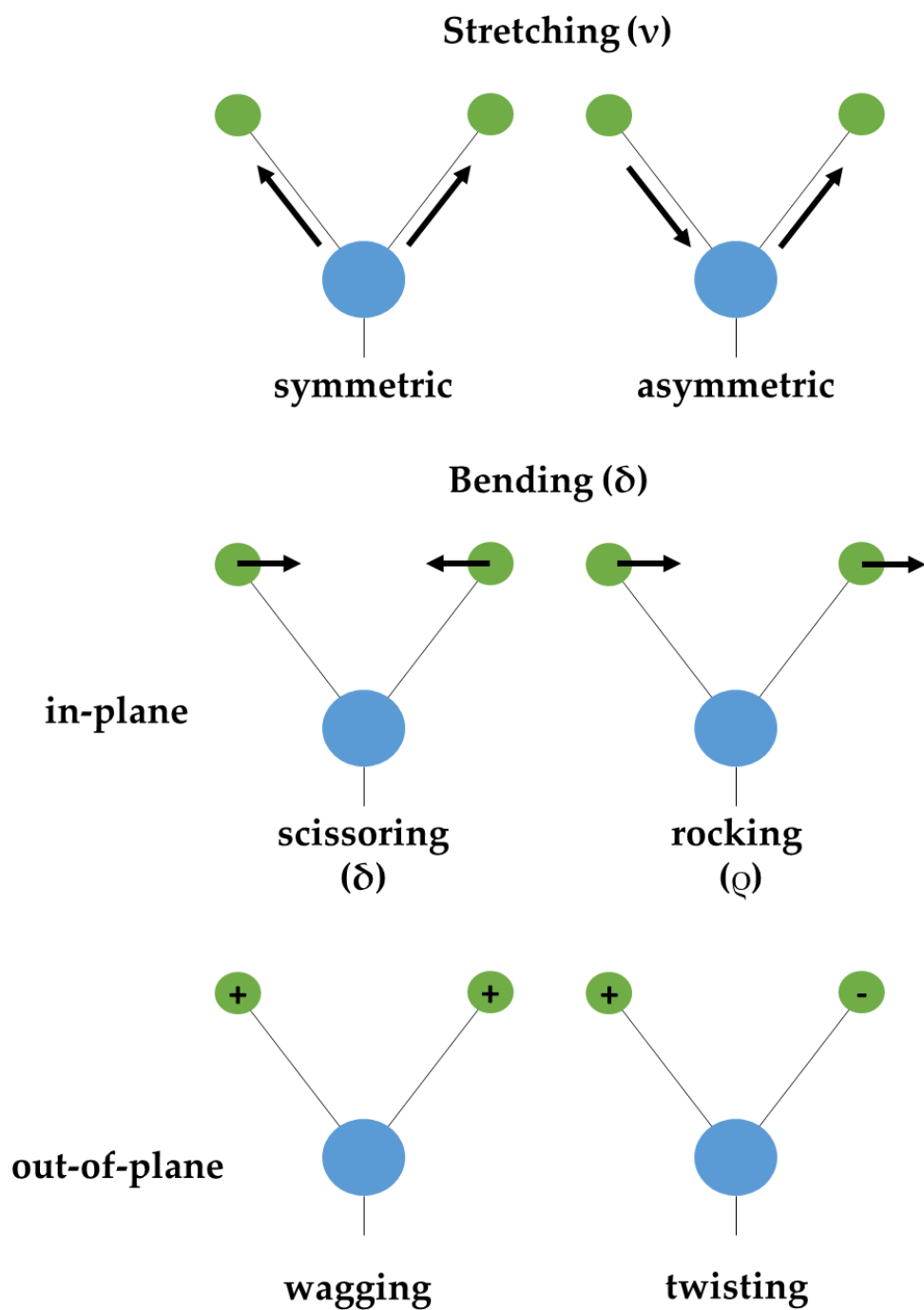


Figure 4.1. A summary of the vibration modes observed in infrared spectroscopy.

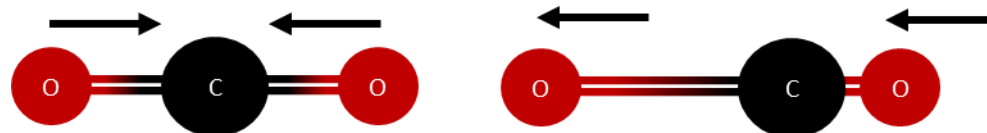


Figure 4.2. Infrared spectroscopy requires a change in dipole moment, demonstrated by symmetric (left) and asymmetric (right) stretches for CO₂. No oscillating dipole moment is observed for the symmetric stretch, unlike the asymmetric stretch (with red signifying negative charge).

Originally, infrared spectroscopy was performed by a 'dispersive' method analogous to the UV-Vis approach, where a single wavelength of light irradiates the sample, and the resulting absorbance is measured. The wavelength is then sequentially increased, and the process is repeated in a 'scan' until the desired spectral region has been assessed (Urban, 1993). This method has now been superseded by Fourier transform infrared spectroscopy (FTIR), which facilitates much faster data acquisition in seconds rather than minutes and improves the signal-to-noise ratio compared to the dispersive method (Baker *et al.*, 2014). The key component of the FTIR spectrometer is the Michelson interferometer (see Figure 4.3), which contains a stationary mirror, a beam splitter, and a moving mirror (Griffiths, 1980). Simply, polychromatic light (i.e., from a range of frequencies) is passed through the interferometer, and the adjustment of the moving mirror modulates what frequencies of light pass through to the sample. An interferogram is generated from this process, which plots mirror position against transmittance from the sample. This interferogram is subsequently converted to the FTIR spectrum computationally by a Fourier transform algorithm, which transforms functions of space or time into functions of frequency (Banwell, 1983; Urban, 1993; Barth, 2007; Griffiths and de Haseth, 2007; Baker *et al.*, 2014). Here, the mirror position is converted to wavelength (cm⁻¹) on the x-axis, plotted against transmittance (i.e., the ratio of light measured by the detector relative to the input). Transmittance can be further converted into absorbance for data analysis (Coates, 2006; Lee *et al.*, 2018).

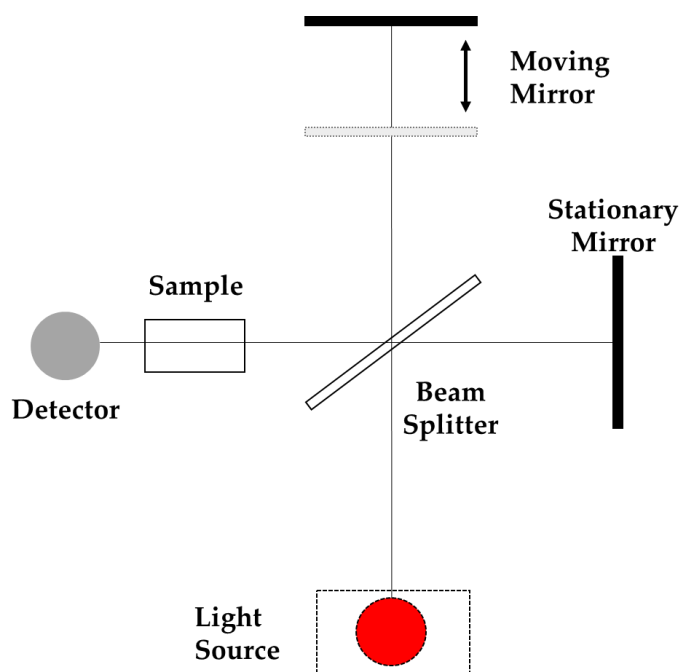


Figure 4.3. A diagram of the Michelson interferometer

Whilst FTIR can be performed in the solid, liquid, or gas phase, the requirement for a sample preparation workflow is one limitation of the standard method (Coates, 2006). An IR ‘invisible’ solvent is required to dissolve a sample in the solution state. In the solid-state, samples must be prepared in a potassium bromide (KBr) pellet as the absorbances of solids in the mid-IR spectrum are too high to quantify (Stehfest, Toepel and Wilhelm, 2005; Coates, 2006; Baker *et al.*, 2014). KBr represents an IR invisible compound that is typically used to ‘dilute’ the absorbance of solid samples that will not dissolve in solution for FTIR (Coimbra *et al.*, 1999; Pandey and Pitman, 2003; Zimmermann, 2010; Fan, Dai and Huang, 2012; Gao *et al.*, 2020).

A further enhancement to the FTIR method was provided by the development of attenuated total reflectance-FTIR or ATR-FTIR, which enabled FTIR to be performed without any sample preparation being required (Gómez-Ordóñez and Rupérez, 2011; Baker *et al.*, 2014; Hofko *et al.*, 2017; Liu *et al.*, 2021). Here, the ATR accessory directs the applied infrared light to a crystal (e.g., diamond or germanium) in contact with the sample at a defined angle (Urban, 1993; Grdadolnik, 2002; Guiliano *et al.*, 2007). Application of the light to the crystal creates an evanescent wave that radiates outside the crystal into the sample, where it is absorbed (Figure 4.4). The depth to which this wave penetrates the sample is dependent on the wavelength frequency, the angle of the

applied IR beam, and the refractive index of the crystal used; however, it is typically on the scale of a few microns depending on the experimental set up (Averett, Griffiths and Nishikida, 2008). Following absorbance, the attenuated light passes back through the crystal for detection.

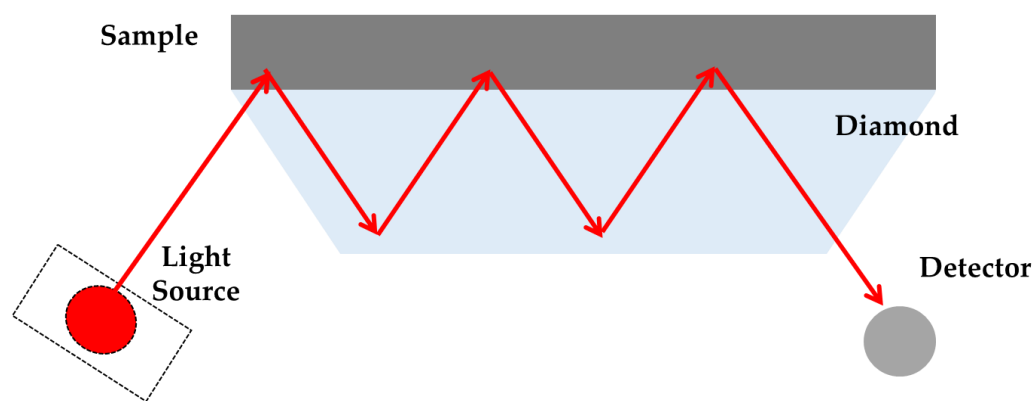


Figure 4.4. Schematic of ATR-FTIR theory.

As a result, ATR-FTIR can be directly performed on powder, biological tissue, and biomaterials without the prior requirement of solubilising a sample in solvent (for solution-state FTIR) or a KBr pellet for powdered analysis (Baker *et al.*, 2014; Canteri *et al.*, 2019; Liu *et al.*, 2021). ATR-FTIR can also be performed directly on liquids. This sampling flexibility makes modern FTIR analysis an extremely fast and versatile approach, where significant amounts of structural information can be obtained in a matter of seconds, finding utility in both plant research and algal biotechnology as a non-invasive tool for structural analysis. By comparison, other analytical techniques, such as mass spectrometry, require extensive sample preparation and are destructive (Galen and Feiters, 2016; Segers *et al.*, 2019; Letourneau and Volmer, 2021). NMR scans can take a few minutes, but more extended experiments are typically preferred to improve the signal to noise, and whilst no sample preparation is necessarily required for NMR (e.g., solid-state), better spectra are generally observed via solution state analysis (Lesage, 2009; Mansfield *et al.*, 2012; Carbajo and Neira, 2013).

However, discrimination of FTIR peaks is challenging in macromolecules relative to smaller compounds due to the presence of many functional groups in biopolymers that will have overlapping vibrational frequencies (Barth, 2007; Traoré, Kaal and Martínez Cortizas, 2018; Liu *et al.*, 2021). As the composition of biopolymers is predominantly CHO (carbon, hydrogen, and oxygen), the formation of hydrogen bonds between electronegative functional groups containing oxygen causes significant

increases in peak intensity, peak width, and absorption shifts due to the modulation of vibrational modes from this interaction (Kačuráková *et al.*, 1999; Coates, 2006; Berthomieu and Hienerwadel, 2009; Vasylieva *et al.*, 2018). This makes regions of the FTIR spectrum which correspond to electronegative functional groups that can form hydrogen bonds (e.g., carbonyl and hydroxyl groups) challenging to interpret, including the 'carbohydrate' backbone region of polysaccharides, which corresponds to multiple C-O and C-O-C peaks of sugars and glycosidic linkages (Schwanninger *et al.*, 2004; Popescu *et al.*, 2007; Liu *et al.*, 2021).

Due to this, it is also important to remove water from samples (e.g., via lyophilisation) before analysis, with the -OH group from water forming a prominent, broad peak around in the fingerprint region of biopolymers at $\sim 1600\text{ cm}^{-1}$ and the observed -OH stretch of all biopolymers at $\sim 3200\text{-}3600\text{ cm}^{-1}$ in the functional region (Vasylieva *et al.*, 2018). However, some polymers (including proteins and polysaccharides) may retain residual water after lyophilisation due to water adsorbed to hydrophilic structure, with the corresponding -OH resonances readily observable in the resulting FTIR spectra (Townes, 1995; Popescu *et al.*, 2007; Mishra and Malhotra, 2012; Md Salim, Asik and Sarjadi, 2021).

As a result, FTIR is useful for fast, initial analysis of polymer structure (Barth, 2007; Baker *et al.*, 2014; Canteri *et al.*, 2019), but for more specific structural analysis, it is often performed in combination with other techniques such as nuclear magnetic resonance (Gao *et al.*, 2015; Jiang *et al.*, 2017; Besser *et al.*, 2018; Wahlström, Edlund, *et al.*, 2020; Ibrahim *et al.*, 2022). Complementary NMR analysis is discussed in the next chapter.

4.1.2.2 Identification of Functional Groups

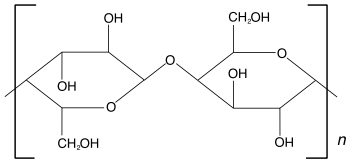
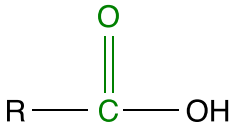
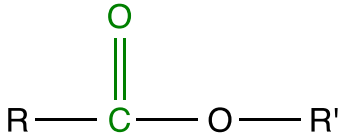
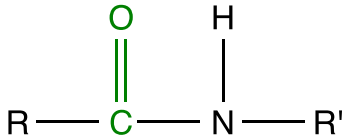
Plant and macroalgal cell wall polysaccharides are associated with various functional groups. The most predominant functional modification related to the 'soluble' component of the seaweed cell wall is sulfation (Lahaye and Robic, 2007; Aquino, Grativol and Mourão, 2011). Furthermore, the characteristic vibrations associated with different polysaccharide structures from various sources have been widely characterised in the literature, enabling the cell wall fractions obtained previously to be identified by FTIR analysis in this chapter (McCann *et al.*, 1992; Marry *et al.*, 2000; Pereira *et al.*, 2003; Schwanninger *et al.*, 2004; Popescu *et al.*, 2007; Fan, Dai and Huang, 2012; Liu *et al.*, 2021).

As the analysis discussed in this chapter required the characterisation of multiple functional groups from the extracted cell wall fractions, the structure of the relevant functional groups, corresponding nomenclature, and expected resonances associated with these groups are summarised in Table 4.1 for reference, which indicates where different functional groups can be identified.

Functional Region: Single-Bonds and Triple-Bonds

Researchers often divide the mid-infrared spectrum into discrete regions to facilitate a more straightforward analysis of biochemical structures. It is often not practical or necessary to assign every peak in the FTIR spectrum, particularly in samples with overlapping resonances (Nandiyanto, Oktiani and Ragadhita, 2019). Here, a sequential approach to analysis simplifies the peak assignment process.

The first region that is assessed is the general 'functional region' of the IR spectrum, observed from 4000 cm^{-1} to $\sim 2500\text{ cm}^{-1}$, which corresponds to single bond stretches that in biopolymers can be used in the first instance to identify what the compound is (Coates, 2006; Türker-Kaya and Huck, 2017). These stretches include the characteristic broad O-H and C-H stretches of all biopolymers and the N-H stretches of proteins and ribonucleic acids (Barth, 2007; Fan, Dai and Huang, 2012; Elmarzugi *et al.*, 2014; Fuller, Andaya and McClay, 2018). Following this, peaks attributed to triple bonds are observed from $\sim 2500\text{ cm}^{-1}$ to $\sim 2000\text{ cm}^{-1}$. However, alkyne ($\text{C}\equiv\text{C}$) and cyano groups ($\text{C}\equiv\text{N}$) are observed infrequently in natural compounds; as such, this region is usually (but not always) unimportant in biological studies (Amsler, 2008). Furthermore, the 'triple bond' region is notably noisy in ATR-FTIR spectra as the diamond from the ATR accessory displays an absorbance band in this region (Nechaev and Khokhryakov, 2013; Lai *et al.*, 2020).

Group	Structure	Associated Vibrations
Hydroxy	— OH	$\nu(\text{OH})$ $\sim 3650\text{--}3200\text{ cm}^{-1}$ $\delta(\text{OH})$ $\sim 1640\text{ cm}^{-1}$ (water) $\sim 1400\text{ cm}^{-1}$ (alcohol and phenol)
Aliphatic	CH, CH ₂ , CH ₃	$\nu(\text{CH})$ $\sim 3000\text{--}2850\text{ cm}^{-1}$ $\delta(\text{CH})$ $\sim 1480\text{--}1350\text{ cm}^{-1}$ (in-plane) $< 900\text{ cm}^{-1}$ (out-of-plane)
'Sugar Backbone'		$\nu(\text{CO}), \nu(\text{COC}), \nu(\text{COH}),$ and various $\delta(\text{CH})$ $\sim 1200\text{--}900\text{ cm}^{-1}$
Carbonyl (Carboxyl)		$\nu(\text{C=O})$ $\sim 1600\text{ cm}^{-1}$
Carbonyl (Ester)		$\nu(\text{C=O})$ $\sim 1750\text{ cm}^{-1}$
Carbonyl (Amide)		$\nu(\text{C=O})$ $\sim 1680\text{ cm}^{-1}$

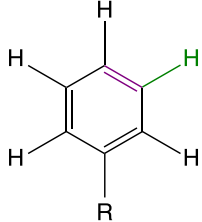
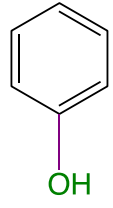
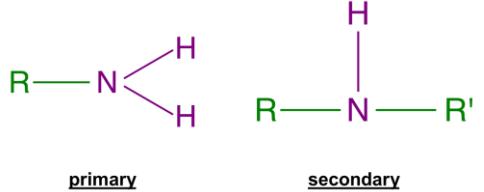
Aromatic		$\nu(\text{C}=\text{C})$ $\sim 1600\text{ cm}^{-1}$ and $\sim 1500\text{ cm}^{-1}$ $\delta(\text{CH})$ $\sim 1225\text{--}950\text{ cm}^{-1}$ (in-plane) $< 900\text{ cm}^{-1}$ (out-of-plane)
Phenolic		$\nu(\text{OH})$ $\sim 3650\text{--}3500\text{ cm}^{-1}$ $\delta(\text{OH})$ $\sim 1410\text{--}1310\text{ cm}^{-1}$ $\nu(\text{CO})$ $\sim 1200\text{ cm}^{-1}$
Amine		$\nu(\text{NH})$ $\sim 3500\text{--}3300\text{ cm}^{-1}$ $\delta(\text{NH})$ $\sim 1650\text{--}1550\text{ cm}^{-1}$ (primary) $\sim 1600\text{--}1500\text{ cm}^{-1}$ (secondary) $\nu(\text{CN})$ $\sim 1350\text{--}1200\text{ cm}^{-1}$

Table 4.1. The main functional groups observed in biopolymers by infrared spectroscopy and the associated structures and vibrations

Functional Region: Double-Bonds

The double bond region is then observed from $\sim 2000\text{ cm}^{-1}$ to $\sim 1500\text{ cm}^{-1}$, consistent with carbonyl groups ($\text{C}=\text{O}$), aromatic structures and alkenes ($\text{C}=\text{C}$), and less frequently imine ($\text{C}=\text{N}$) and azo groups ($\text{N}=\text{N}$), although imine and azo groups are present in various prokaryote, fungal, algal and plant synthesised metabolites (Ju and Parales, 2010; Dembitsky, Glorizova and Poroikov, 2017). In biopolymers, this region is more accurately represented by resonances $< 1800\text{ cm}^{-1}$, with carbonyl resonances $> 1800\text{ cm}^{-1}$ attributed to transition metal carbonyl groups, which are often not relevant to biological investigations (Coates, 2006).

As demonstrated in Table 4.1, the vibration of the carbonyl group is shifted depending on the associations of the functional group. Ester groups are observed at a higher wavelength ($\sim 1740\text{ cm}^{-1}$) than the corresponding carboxylate group ($\sim 1600\text{ cm}^{-1}$), with ester peaks in plant polysaccharides associated with *O*-acetylation and methylation, in addition to esterified phenolics (such as the hydroxycinnamates ferulic acid and coumaric acid) and polyphenols such as lignin (McCann *et al.*, 1994; Gnanasambandam, R., Proctor, 2000; Marry *et al.*, 2000; Synytsya *et al.*, 2003; Baum *et al.*, 2017; Canteri *et al.*, 2019).

Peaks corresponding with aromatic esters (i.e., corresponding with lignin) are typically observed at a slightly lower resonance than saturated esters, as are amide groups. However, this is often difficult to distinguish in a complex biopolymer spectrum (McCann *et al.*, 1994; Marry *et al.*, 2000; Sun *et al.*, 2011). For polysaccharides containing carboxylate groups (pectin and hemicelluloses), the prominent asymmetric carboxyl stretch is typically accompanied by a smaller symmetric carboxyl stretch at $\sim 1400\text{ cm}^{-1}$ in the fingerprint region (Gnanasambandam, R., Proctor, 2000; Gómez-Ordóñez and Rupérez, 2011; Hofko *et al.*, 2017). For these polysaccharides, the prominence of the carboxyl peak is inversely proportional to the prominence of the ester peak, meaning FTIR can be used as a simple tool for determining the relative degree of esterification of pectin and hemicelluloses (Gnanasambandam, R., Proctor, 2000; Fellah *et al.*, 2009; Canteri *et al.*, 2019).

However, the observed carbonyl vibrations are sensitive to various factors, including the effect of hydrogen bonding (discussed previously, 4.1.2.1) and the effect of the extraction protocol. Vibrations in the solid state may be shifted relative to solution-state FTIR analysis (Sachdeva and Cai, 2009). Notable, alkaline treatment, such as the

sodium hydroxide extraction step performed in the present protocol, is known to impact the carbonyl spectrum in pectin and induce ester hydrolysis, causing a reduction of the ester peak (Gnanasambandam, R., Proctor, 2000; Liang *et al.*, 2022). This may impact the biological conclusions from the spectra; as a result, the effect of this on the extracted *Ulva* fractions will be assessed during this chapter (§4.5).

Aromatic ring vibrations (C=C-C) are observed typically as a pair of peaks at $\sim 1600\text{ cm}^{-1}$ and $\sim 1500\text{ cm}^{-1}$. However, these often can be shifted in certain aromatics, depending on the type of substitution on the ring (Schwanninger *et al.*, 2004; Popescu *et al.*, 2007; Lu *et al.*, 2017). One challenge for identifying aromatic structures with FTIR in samples of mixed composition is that broad peaks corresponding with NH scissoring from amine groups are also observed in this region, and the NH stretch observed in the single bond region typically overlaps with the broad OH stretch (Coates, 2006; Barth, 2007; Mayers, Flynn and Shields, 2013; Guerrero, Kerry and de La Caba, 2014; Nunes *et al.*, 2020). However, amine structures are typically accompanied by accompanying C-N stretches in the fingerprint regions (discussed subsequently). In the presence of protein, two bands attributed to NH scissoring are typically observed as amide I and amide II, corresponding to primary and secondary amines, respectively (Coates, 2006; Barth, 2007). Nonetheless, supplementary biochemical information such as the performance of biochemical assays to detect aromatics (discussed in the previous chapter) and elemental analysis to detect amines (§4.2) provides significant support to assignments in this region.

Fingerprint Region

The fingerprint region ($< 1500\text{ cm}^{-1}$) is the most complex region of the FTIR spectrum to interpret, consisting of multiple overlapping resonances (Türker-Kaya and Huck, 2017; Nandiyanto, Oktiani and Ragadhita, 2019). However, due to this complexity, the peaks observed in this region can be used to distinguish between different polymers that contain the same functional groups, for example, for distinguishing between different types of polysaccharides (Traoré, Kaal and Martínez Cortizas, 2018; Javier-Astete, Jimenez-Davalos and Zolla, 2021; Liu *et al.*, 2021). The focus of this chapter is the analysis of this region in the obtained cell wall fractions for the identification of 'phycolignin'.

As discussed previously, this region can be used to support the assignments made in the various functional region; for example, an aromatic ring stretch (C=C-C) will be

accompanied by multiple C-H resonances in the fingerprint region (Coates, 2006; Lu *et al.*, 2017; Javier-Astete, Jimenez-Davalos and Zolla, 2021). An amine bend will be accompanied by C-N stretches between 1350 cm⁻¹ and 1200 cm⁻¹, which will be shifted depending on the structure of the compound (i.e., aromatic or aliphatic) (Coates, 2006; Barth, 2007).

This region also contains the 'sugar backbone' region, which is observed in all polysaccharides from ~1200 cm⁻¹ to 900 cm⁻¹, consisting of various resonances associated with C-O, C-OH, and C-O-C bonds associated with sugar structures and glycosidic linkages from 1200 to 1000 cm⁻¹, with other CH bending vibrations associated with sugars observed below from 1000 cm⁻¹ to 900 cm⁻¹ (McCann *et al.*, 1992; Kačuráková *et al.*, 1999; Marry *et al.*, 2000; Schwanninger *et al.*, 2004; Popescu *et al.*, 2007). Typically, the spectral maximum is observed in this region for polysaccharides at ~1035 cm⁻¹, corresponding with a C-O stretch, which can be used to normalise data for comparative analysis of peak intensity across samples (Guerrero, Kerry and de La Caba, 2014; Baum *et al.*, 2017; Hofko *et al.*, 2017; Zanca *et al.*, 2020; Barboux, Bousta and di Martino, 2021).

Due to the extensive overlap, the sugar composition of polysaccharides is better characterised by chromatographic methods such as thin layer, high-performance liquid, and gas chromatography (Virkki *et al.*, 2005; O'Rourke *et al.*, 2015). However, this is beyond the scope of this research project, with the sugar composition of the extractable polysaccharides of *Ulva* characterised within the literature (Lahaye, Jegou and Buleon, 1994; Lahaye and Robic, 2007; Kiggell *et al.*, 2019; Wahlström, Edlund, *et al.*, 2020; Wahlström, Nylander, *et al.*, 2020). Nonetheless, some resonances that can be observed in the FTIR spectra can be attributed to specific sugars; for example, xylose-rich polysaccharides display a unique fingerprint and are used in this chapter to support the identification of the acquired fractions where relevant (Marry *et al.*, 2000; Mishra and Malhotra, 2012; Sonawane, Bhalekar and Shimpi, 2014; Liu *et al.*, 2021). This will be supported by NMR analysis in the next chapter

The carbohydrate backbone region overlaps with the C-O stretch of phenolics (observed at ~1200 cm⁻¹). As a result, lignin cannot be distinguished here. However, the O-H bending of phenols and alcohols can be observed elsewhere in the fingerprint region, as can various aliphatic CH bends that are distinguishable from aromatic CH bends observed at lower wavelengths (Coates, 2006). As a result, an extensive amount of structural information can be generated from FTIR spectra both non-invasively and rapidly (Baker *et al.*, 2014).

4.1.3 Chapter Aims and Hypotheses

In this and the subsequent chapter, I aim to characterise the structure of the cell wall fractions isolated from *U. compressa* using a biophysical approach. Here, ATR-FTIR is used as the primary analytical method, with carbon nitrogen sulfur elemental analysis used to support assignments where necessary.

- Firstly, I attempt to verify the identity of the individual cell wall fractions by identifying functional groups in FTIR spectra corresponding to cell wall polysaccharides.
- Secondly, I screen for evidence of ‘phycolignin’ and other structural components associated with polysaccharides that may correspond with the observed cell wall fluorophore identified in the previous chapter
- Finally, I assess whether changes in either can be observed in extracts from the different abiotic stress conditions tested (metal, hyposaline, and hypersaline stress).

This analysis will be expanded upon in the following research chapter (Chapter 5), where I will employ various nuclear magnetic resonance (NMR) experiments to verify and supplement the observations made in this chapter.

4.2 Elemental Analysis

Two functional groups predicted to be present in macroalgal cell wall extracts in addition to the expected carbohydrate fingerprint were sulfate esters from sulfated polysaccharides (ulvans and carrageenan) and amine and amide resonances from proteins can be co-extracted during the isolation of cell wall polysaccharides. Various chlorophytes are known to have protein-rich cell walls (e.g., *Chlamydomonas*) consisting primarily of hydroxyproline-rich proteins and extensins and are also known to be associated with various polyamine structures (Voigt, 1985; Domozych *et al.*, 2012; Burczyk *et al.*, 2014; Mahdy *et al.*, 2014; Schweikert and Burritt, 2015; Mócsai *et al.*, 2019). By comparison, the *Ulva* cell wall's protein content is understudied. However, similar hydroxyproline-rich proteins have been identified (De Clerck *et al.*, 2018; Přerovská *et al.*, 2021).

Whilst peaks corresponding to these functional groups have been reported previously in cell wall polysaccharides in the literature (Table 4.1), as stated, the spectral region where these structures vibrate does overlap with others. Therefore, elemental analysis was used to determine cell wall extracts' sulfur and nitrogen content to increase the confidence in the peak assignments made in this chapter for these functional groups. Here, sulfur content was used to determine where the sulfated polysaccharides from macroalgae segregated during the fractionation protocol, enabling peaks corresponding with sulfate esters to be identified and distinguished from other functional groups. Nitrogen content was used as an estimate of protein content, with samples predicted to have high levels of co-extracted protein expected to have bands corresponding to amine (δNH) and amide ($\nu\text{C=O}$) functional groups in the corresponding infrared spectra.

4.2.1 Sulfur

An estimated sulfur content of 1.54% and 2.01% was observed in the rapidly oxalate soluble and slowly oxalate soluble fractions for *Ulva*, consistent with the expected ulvans, which are typically sulfated on the C-3 of rhamnose residues or less frequently on C-2 of xylose residues (Lahaye and Robic, 2007). As with extraction yields, sulfate contents of ulvan from *Ulva* are highly variable in the literature (Kidgell *et al.*, 2019). However, the sulfur content reported here was somewhat lower than those reported recently in the literature for ulvans, which range from ~5-10% (Wahlström, Nylander, *et al.*, 2020; Ibrahim *et al.*, 2022). This may indicate that the extraction protocol

may remove some of the sulfation. However, populations of *U. compressa* may exhibit a lower degree of sulfation than other *Ulva* species (Kidgell *et al.*, 2019).

Lower sulfur contents were estimated for all other cell wall fractions in *Ulva* (0.41-0.71%). However, an estimated sulfur content of 0.94% was observed in the wash, indicating that sulfation predominantly occurred on the oxalate-soluble fractions. This observation was consistent with the previous literature, with the alkali-soluble and insoluble fractions of the *Ulva* cell wall known to lack a major sulfation pattern. Similarly, a low sulfur content was observed in barley straw samples (< 0.81%), with sulfated polysaccharides being an adaptation specific to marine algae in photosynthetic eukaryotes (Aquino, Grativol and Mourão, 2011). However, it must be noted that due to sampling limitations the elemental percentages described in this chapter represent single replicate values from a cell wall sample combusted during elemental analysis, with samples preferentially retained for lignin screening (for example, the acetyl bromide soluble lignin assay described in the previous chapter). As such, this analysis can only be considered an initial assessment of the elemental composition of these fractions, with more comprehensive analysis required to more accurately determine the factors that impact sulfur content. For the purposes of this study this data is sufficient to assist with FTIR assignments, and more in-depth analysis of cell wall sulfation patterns are largely beyond the scope of this study and have previously been reported in the literature for *Ulva* (Kidgell *et al.*, 2019).

4.2.2 Nitrogen

Calculations of protein content from elemental analysis make two assumptions: firstly, that all nitrogen is from protein, and secondly, the average nitrogen content of all protein in the sample is 16% (Krul, 2019; Hayes, 2020). Whilst these limitations cause protein content to be overestimated, for this research, this method appeared as a simple tool for identifying which cell wall fractions were likely associated with protein. These predictions would then be verified or disproved by the subsequent biophysical analysis discussed in this chapter (in section 4.3). This approach has been used extensively in macroalgal research (Yaich *et al.*, 2011; Mayers, Flynn and Shields, 2013; Shuuluka, Bolton and Anderson, 2013; Gao *et al.*, 2017c, 2018).

Negligible nitrogen content was observed in barley straw biomass (alcohol insoluble residue) in contrast to *U. compressa* (shown in Table 4.2), consistent with the anticipated differences in protein content between the two sources of biomass (Vogel, 2008). Estimated protein contents were 25.08%, 26.47%, and 30.99% for *U. compressa* extracted under control, hypersaline, and hyposaline stressed conditions, with typical protein contents of 10% to 26% reported for *Ulva* in the literature (Shuuluka, Bolton and Anderson, 2013). As discussed in the previous chapter (see chapter 3, section 3.4.1.1), the presence of ~25% protein in the AIR is consistent with low post-fractionation recovery observed for the *U. compressa* extractions, indicating a significant proportion of the AIR was not extractable cell wall polysaccharide.

An estimated 2.81% and 4.35% nitrogen content was obtained in the rapidly oxalate soluble and slowly oxalate soluble fractions (ulvan 1 and ulvan 2), corresponding with an estimated protein content of 17.9% and 27.1%, respectively. Similar estimated protein contents were observed from the hypersaline (27.89%) and hyposaline-stressed slowly soluble extractions (24.26%). Co-extraction of ulvans with protein is reported in the literature due to the ionic associations these acidic polysaccharides can form with proteins in the cell wall (Robic *et al.*, 2009; Costa *et al.*, 2012; Yaich *et al.*, 2013; Wahlström, Nylander, *et al.*, 2020)

It was hypothesised that protein would be solubilised during the 6 M sodium hydroxide treatment and thus would segregate with the hemicellulose fractions (Ha, Hb), based on the known properties of proteins in concentrated alkaline solution (Deleu *et al.*, 2019). However, the Ha fraction had a non-measurable nitrogen content, and the Hb fraction had the second lowest estimated protein content (3.03%) of all fractions. This

observation may indicate that proteins are not tightly bound to hemicelluloses in *U. compressa*, and most weakly bound protein is removed during the oxalate extraction.

Interestingly, higher nitrogen contents were observed in the insoluble α -cellulose fraction in *Ulva* (3.10% in the control, increasing to 4.61% under hyposaline stress), suggesting that the sodium hydroxide treatment had not hydrolysed protein or that cellulose was associated with another nitrogenous metabolite. The observation that the washing step did not remove this structure, nor the other extraction buffers (oxalate and sodium hydroxide), may indicate that it is strongly associated with *Ulva* α -cellulose. Assuming this is protein, this corresponds to a predicted protein content of 19.7% in the control and 21.24 and 28.74% for the hypersaline and hyposaline samples, possibly indicative of some stress-induced change. However, as stated previously, generating more replicates in follow up studies will be required to confirm this. By contrast, the barley straw cellulose at an estimated protein content of 4.1%.

Interestingly, whilst the nitrogen content was low in the control wash sample, this increased in hyposaline and hypersaline stress (to 2.63% and 9.64%), consistent with the increases observed in the α -cellulose fraction. This was accompanied by a decline in the estimated carbon content relative to the other 'polysaccharide-rich' fractions, suggesting that the wash was not primarily composed of CHO as sugars or polysaccharides would be. As a result, it is hard to diagnose what this fraction corresponds to without further analysis.

Fraction	%N	%C	Estimated Protein (%)
AIR	4.01	34.52	25.08
<i>Hypersaline</i>	4.24	31.55	26.47
<i>Hyposaline</i>	4.96	34.69	30.99
Oxalate Soluble 1	2.86	32.88	17.85
Oxalate Soluble 2	4.34	34.42	27.13
<i>Hypersaline</i>	4.46	31.31	27.89
<i>Hyposaline</i>	3.88	32.56	24.26
Alkali Soluble (Ha)	Neg	39.66	NA
Alkali Soluble (Hb)	0.49	34.17	3.03
Insoluble Cellulose	3.10	36.07	19.37
<i>Hypersaline</i>	3.40	36.64	21.24
<i>Hyposaline</i>	4.61	33.20	28.78
Wash	1.31	20.83	8.21
<i>Hypersaline</i>	9.64	19.27	60.25
<i>Hyposaline</i>	2.63	27.56	16.43

Table 4.2. Elemental analysis of carbon and nitrogen in *U. compressa* cell wall fractions. The protein content is estimated by the conversion factor of 6.25 (Shuuluka, Bolton and Anderson, 2013; Krul, 2019). Elemental percentages represent the single result obtained when a sample of cell wall fraction was combusted during elemental analysis.

4.3 Characterisation of Cell Wall Fractions using ATR-FTIR

With the aim of this chapter to identify a ‘phycolignin’ structure via biophysical analysis, the first step was to characterise the spectra of lignin as a reference. Firstly, this was performed via literature review – with numerous reports of lignin analyses via FTIR reported in the literature, both for lignin purified by a range of extraction methods, and in lignocellulosic biomass obtained from a range of species, including herbaceous plants and woody biomass (Pandey and Pitman, 2004; Schwanninger *et al.*, 2004; Popescu *et al.*, 2007; Kline *et al.*, 2010; Baicioğlu, Zimmermann and Kohler, 2015; Watkins *et al.*, 2015; Javier-Astete, Jimenez-Davalos and Zolla, 2021; Md Salim, Asik and Sarjadi, 2021). The analysis of lignin is widely reported due to the industrial interest in lignocellulosic biomass for bioenergy production and producing pulp for paper-making applications (Ibarra *et al.*, 2004; Ajao *et al.*, 2018; H. Wang *et al.*, 2019; Jiang *et al.*, 2020; Liao *et al.*, 2020). Reports documenting the analysis of lignocellulose are particularly relevant for the research aims of this chapter, with how lignin interacts with the polysaccharides of the secondary cell wall (hemicellulose and cellulose) influencing the observed spectroscopic properties (Hafrén, 2007).

For this purpose, I also assessed a pure lignin standard (alkali lignin, Sigma) by FTIR (Figure 4.5) and cross-referenced the identified peaks with those determined from my literature review. Assignations for lignin used in this chapter are documented and referenced in Table 4.3.

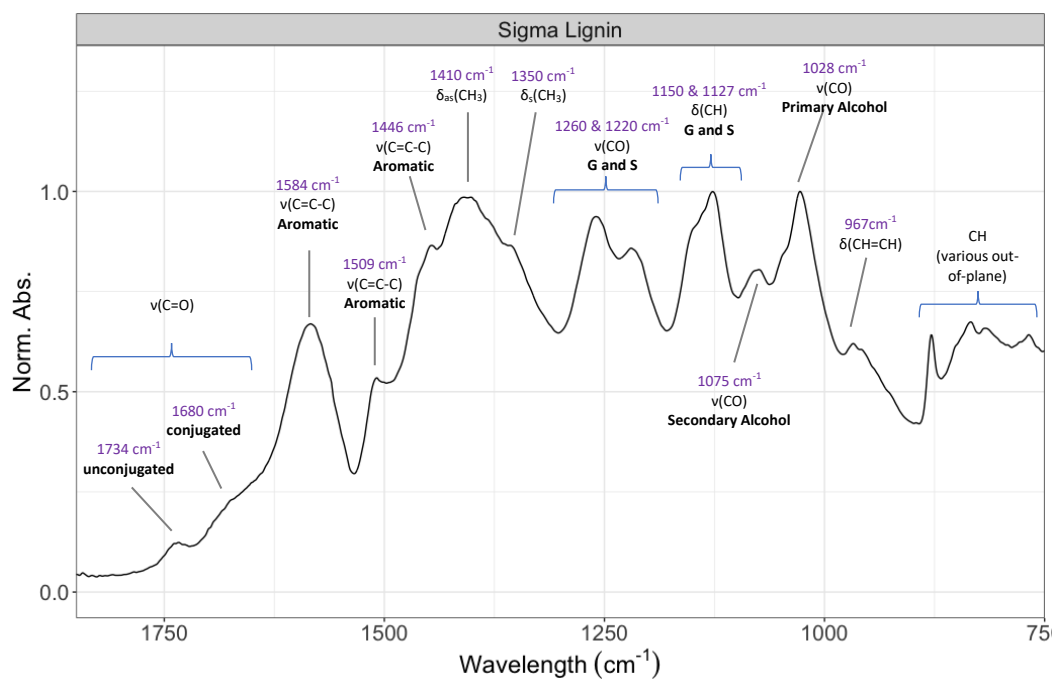


Figure 4.5. Kraft lignin (Sigma) as a reference for analysing extracted cell wall fractions. The 'double bond' region and fingerprint region of the mid-infrared spectrum are plotted and annotated in this figure. Full assignments are reported in Table 4.3.

Assignment	Functional Group	Peak (cm ⁻¹)
Hydroxy (Phenolic)	ν O-H	3245
Methyl	ν C-H	2980
Unconjugated Ester	ν C=O	1734
Conjugated Ester	ν C=O	1680
Aromatic Ring	ν (C=C-C)	1584
Aromatic Ring	ν (C=C-C)	1509
Aromatic Ring	ν (C=C-C)	1446
Methyl from OMe (CH ₃)	δ_{as} CH	1410
Methyl from OMe (CH ₃)	δ_s CH	1350
OMe (Guiacyl)	ν CO	1260
OMe (Syringyl)	ν CO	1220
Aromatic CH	δ CH	1150
Aromatic CH	δ CH	1127
Phenolic	ν C-O	1075
Phenolic	ν C-O	1028
Aromatic Ring	δ CH=CH	967
Aromatic CH	δ CH	879
		834
		768

Table 4.3. Assignations for the kraft lignin sample plotted in Figure 4.5. Assignations were determined from a literature review of FTIR studies performed on lignin and lignocellulosic biomass (Pandey and Pitman, 2003, 2004; Schwanninger et al., 2004; Popescu et al., 2007; Fan, Dai and Huang, 2012; Liu et al., 2014; Watkins et al., 2015; Fahey, Nieuwoudt and Harris, 2017; Lu et al., 2017; Traoré, Kaal and Martínez Cortizas, 2018; Fodil Cherif et al., 2020; Md Salim, Asik and Sarjadi, 2021).

4.3.1 Oxalate-Soluble Fraction

Two ‘pectic’ fractions were acquired during the sequential extraction protocol, with the rapidly oxalate-soluble material segregating into P1 after two hours of extraction at 100°C and more recalcitrant material being solubilised in the slowly oxalate-soluble fraction (P2) after a further 16 hours (see Methods and Materials, section 2.1.4.4). Differences in polymer solubility were hypothesised to occur from variability in polysaccharide composition and the associated functional modifications (e.g., sulfation, acetylation). Here, the oxalate-soluble fraction was expected to correspond to ulvans in *U. compressa* (Figure 4.6), carrageenan (α (1-3) and β (1-4) linked sulfated galactopyranoses) in *M. stellatus*, and the pectins homogalacturonan and rhamnogalacturonan I in barley straw, based upon the known cell wall composition of these species (Lahaye and Robic, 2007; Vogel, 2008; Hilliou *et al.*, 2012). As a result, the rapidly oxalate-soluble and slowly oxalate-soluble fractions in *U. compressa* were tentatively named ‘ulvan 1’ and ‘ulvan 2’ and named ‘carrageenan 1’ and ‘carrageenan 2’ in *M. stellatus*.

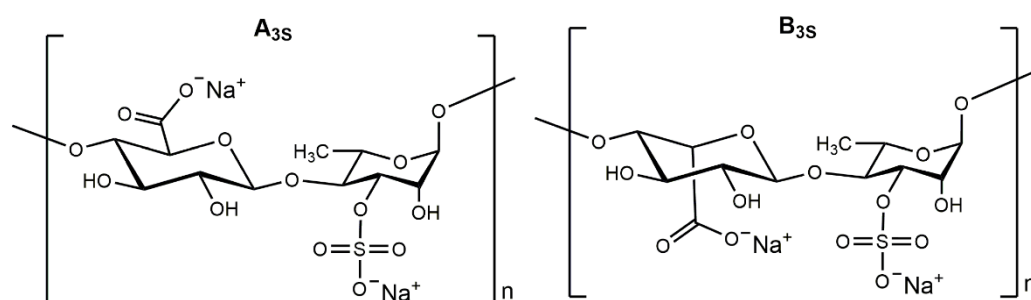


Figure 4.6. Repeating structure of ulvanobiuronic acids (ulvans).

Image reprinted from Cindana Mo'o, F. R., *et al.* (2020). Ulvan, a Polysaccharide from Macroalga *Ulva* sp.: A Review of Chemistry, Biological Activities and Potential for Food and Biomedical Applications. *Applied Sciences*, 10(16), 5488. <https://doi.org/10.3390/app10165488>

4.3.1.1 Identification of Ulvans in *U. compressa*

The aqueous-soluble fraction of plant cell walls has been characterised extensively in the literature using FTIR. As a result, assigning the main peaks for the equivalent fractions in *U. compressa* was possible following an extensive literature review (Table 4.4).

Assignment	Functional Group	Peak (cm ⁻¹)	References
Hydroxyl	vOH	3217	(Coates, 2006)
Methyl	vCH	2981	
Ester	vC=O (RCOOR')	1742	(McCann <i>et al.</i> , 1994; Gnanasambandam, R., Proctor, 2000; Synytsya <i>et al.</i> , 2003; Coates, 2006; Fellah <i>et al.</i> , 2009; Baum <i>et al.</i> , 2017)
Primary Amine	δNH (NH ₂)	1644	(Mayers, Flynn and Shields, 2013; Nunes <i>et al.</i> , 2020; Wahlström, Nylander, <i>et al.</i> , 2020)
Carboxyl	v _{as} C=O (RCOO ⁻)	1596	(Robic <i>et al.</i> , 2009; Yaich <i>et al.</i> , 2017)
Secondary Amine	δNH (NH)	1530	(Barth, 2007; Mayers, Flynn and Shields, 2013; Guerrero, Kerry and de La Caba, 2014; Nunes <i>et al.</i> , 2020)
Carboxyl	v _s C=O (RCOO ⁻)	1428	(Robic <i>et al.</i> , 2009; Yaich <i>et al.</i> , 2017)
Methyl	δCH ₂	1394	(Schwanninger <i>et al.</i> , 2004; Popescu <i>et al.</i> , 2007; Szymanska-Chargot <i>et al.</i> , 2015; Liu <i>et al.</i> , 2021)
Sulfate	vS=O	1250	(Ray, 2006; Zhang <i>et al.</i> , 2010; Hernández-Garibay, Zertuche-González and Pacheco-Ruíz, 2011; Guidara <i>et al.</i> , 2021)
	vS=O	1216	

Sugar Backbone	vCOC	1126	(McCann <i>et al.</i> , 1994; Marry <i>et al.</i> , 2000; Synytsya <i>et al.</i> , 2003; Baum <i>et al.</i> , 2017; ben Amor <i>et al.</i> , 2021; Liu <i>et al.</i> , 2021; Gomaa <i>et al.</i> , 2022)
	vCOC	1036	
	qCH ₃ (Rha)	987	(Marry <i>et al.</i> , 2000)
	vCOC (α 1→4 glycosidic bond)	926	(Prabhu <i>et al.</i> , 2019)
Sulfate	C-O-S (axial)	844	(Castro <i>et al.</i> , 2006; Ray, 2006; Zhang <i>et al.</i> , 2010;
	C-O-S (equatorial)	787	

Table 4.4. Assignations for the oxalate-soluble fractions (ulvan 1 and ulvan 2) of *U. compressa*.

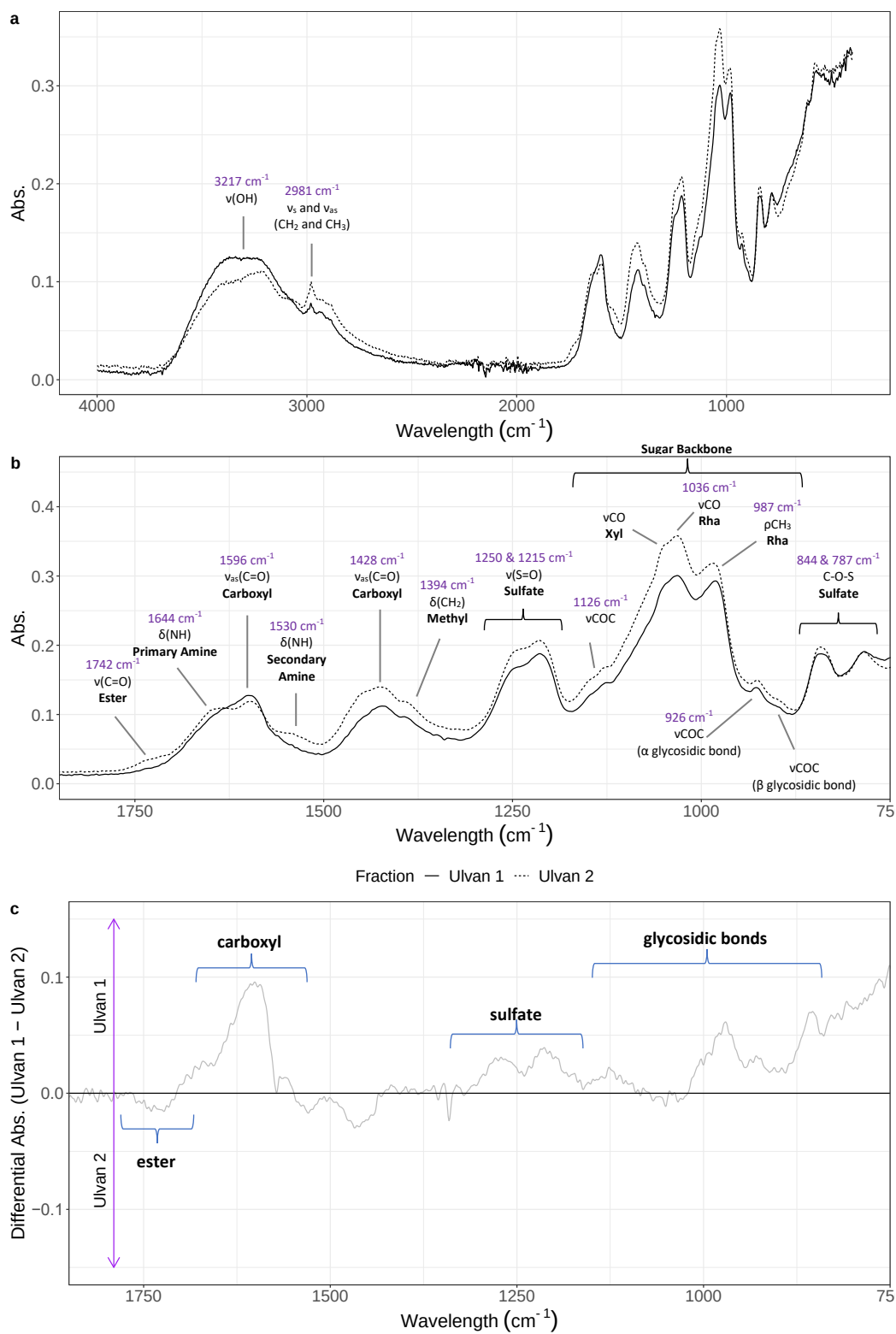


Figure 4.7. Rapidly oxalate-soluble and slowly-oxalate soluble fractions of *U. compressa* (ulvan 1 and ulvan 2). The full mid-infrared spectrum is presented in (a), with the ‘double bond’ and fingerprint region of the spectrum presented and annotated in (b). A differential plot (c) is presented, which emphasises the functional differences between the two oxalate soluble fractions, calculated by *ulvan 1* – *ulvan 2*. For (c), spectra were normalised based upon the min-max method as discussed in the Materials and Methods (section 2.1.8.2) to facilitate ratiometric comparison of the two fractions, with the νCO peak at 1036 cm^{-1} attributed to rhamnose representing the maxima in both *ulvan 1* and *ulvan 2*. Peak attributions are referenced in Table 4.4. No evidence of lignin or other aromatic groups is evident in either fraction.

In *U. compressa*, whilst no aromatic or phenolic-associated peaks consistent with a ‘lignin-like’ structure were identified, the characteristic hydroxyl (νOH) and methyl stretch (νCH) of all polysaccharides were identified at 3217 cm^{-1} and 2981 cm^{-1} . In the fingerprint region, major peaks corresponding with ulvan and other pectin-like polymers were observed in both *ulvan 1* and *ulvan 2*, including the asymmetric and symmetric carboxyl stretches at 1596 cm^{-1} and 1428 cm^{-1} ($\nu\text{C=O}$), two stretches corresponding to sulfate esters at 1250 cm^{-1} and 1216 cm^{-1} ($\nu\text{S=O}$), and a series of overlapping bands corresponding to the ulvan carbohydrate backbone (C-O, C-O-C, and C-H) identified at 1126 cm^{-1} , 1036 cm^{-1} , and 987 cm^{-1} , with the maxima at 1036 cm^{-1} likely representing the 1→4 glycosidic bonds (C-O-C) between glucuronic acid (GlcA) and rhamnose (Rha) residues (Table 4.4). The peak at 987 cm^{-1} was attributed to in-plane rocking from methyl groups on rhamnose residues (ρCH_3), based upon the identification of this peak in the literature in ‘rhamnose-rich’ pulp obtained from sugar beet cell walls (Marry *et al.*, 2000). The small peak at 927 cm^{-1} may be attributable to C-O-C stretching from α (1→4) linkages, likely corresponding to iduronic acid (IdoA) and Rha. However, this peak was not observed in any specific reference spectra for ulvan from the literature (Lahaye and Robic, 2007; Gómez-Ordóñez and Rupérez, 2011; Prabhu *et al.*, 2019). Two additional peaks indicative of polysaccharide sulfation were also identified at 844 cm^{-1} and 787 cm^{-1} (Castro *et al.*, 2006; Ray, 2006; Zhang *et al.*, 2010; Yaich *et al.*, 2017; Nunes *et al.*, 2020). Whilst these peaks are sometimes attributed to CH bending of sugars by some authors (Robic *et al.*, 2009; Hernández-Garibay, Zertuche-González and Pacheco-Ruíz, 2011), these peaks were verified to be linked to the sulfation modification by Castro and colleagues (2006) by experimental de-sulfation and re-sulfation of ulvan extracts.

4.3.1.2 Comparison of the Rapidly Soluble and Slowly Soluble Ulvan Fractions

As shown by the comparative and differential plots for ulvan 1 and ulvan 2 (Figure 4.7), the differences between the two ulvan fractions were subtle. A small shoulder at 1742 cm^{-1} attributable to saturated ester groups ($\nu\text{C=O}$) was observable in the ulvan 2 fraction but not the ulvan 1 fraction, possibly indicative of *O*-acetyl or methyl esters that are widely observed in pectic polysaccharides (McCann *et al.*, 1994; Gnanasambandam, R., Proctor, 2000; Marry *et al.*, 2000; Canteri *et al.*, 2019). Interestingly, the shoulder observed at 1644 cm^{-1} appears more evident in ulvan 2 than in ulvan 1. This observation might reflect the differential esterification patterns of the two fractions; however, this shoulder remains within the expected region cited for the asymmetric stretch of carboxyl groups (Gnanasambandam, R., Proctor, 2000). Alternatively, this band may have some contribution from a primary amine (δNH) scissoring vibration (Coates, 2006; Guerrero, Kerry and de La Caba, 2014; Fuller, Andaya and McClay, 2018), consistent with the proteins identified with this fraction via elemental analysis (Table 4.2), with an additional peak consistent with secondary amine (δNH) scissoring identified at 1530 cm^{-1} . Most extraction protocols for ulvans typically co-extract proteins, with sulfated negatively charged polysaccharides likely forming strong associations with proteins in algal cell walls (Costa *et al.*, 2012; Glasson *et al.*, 2017; Sari-Chmaysssem *et al.*, 2019; Wahlström *et al.*, 2020).

In contrast, the asymmetric carboxyl peak (1596 cm^{-1}) was more prominent in ulvan 1 than ulvan 2, consistent with the expected inverse relationship in peak intensity that is observed in the FTIR spectra of pectins between the ester bands at 1740 cm^{-1} and the carboxylate associated bands at 1600 cm^{-1} (Gnanasambandam, R., Proctor, 2000; Marry *et al.*, 2000; Szymanska-Chargot *et al.*, 2015; Liu *et al.*, 2021). Here, more esterified pectins have a more prominent ester band, with the carboxyl band less noticeable as more C=O groups are functionalised as esters. Differential peaks associated with sulfate esters at 1250 cm^{-1} and 1216 cm^{-1} may indicate that ulvan 1 has a different sulfation pattern from ulvan 2. However, this was not reflected by the elemental analysis reported previously (§4.2.1).

The differential in the carbohydrate backbone region corresponding to various overlapping C-O, C-OH, and C-O-C indicates that there are likely compositional differences between the two fractions. However, it is hard to resolve the exact changes due to the overlapping resonances. Compositional analysis of sugars via the

chromatographic methods discussed in section 4.1.2.2 would be a more appropriate tool to investigate these changes. However, as stated, this is beyond the scope of this thesis.

4.3.1.3 Cross-Species Comparison of Oxalate-Soluble Fraction

Structurally, the FTIR data appeared to support what is known about the respective pectic fractions of the different species extracted from. As the differences between the rapidly and slowly oxalate-soluble ulvan and carrageenan fractions were subtle in *U. compressa* and *M. stellatus*, and as no significant rapidly soluble pectin fraction was obtained in barley straw for ATR-FTIR analysis, spectra from the respective slowly oxalate-soluble fractions (P2) were used for the cross-species comparison.

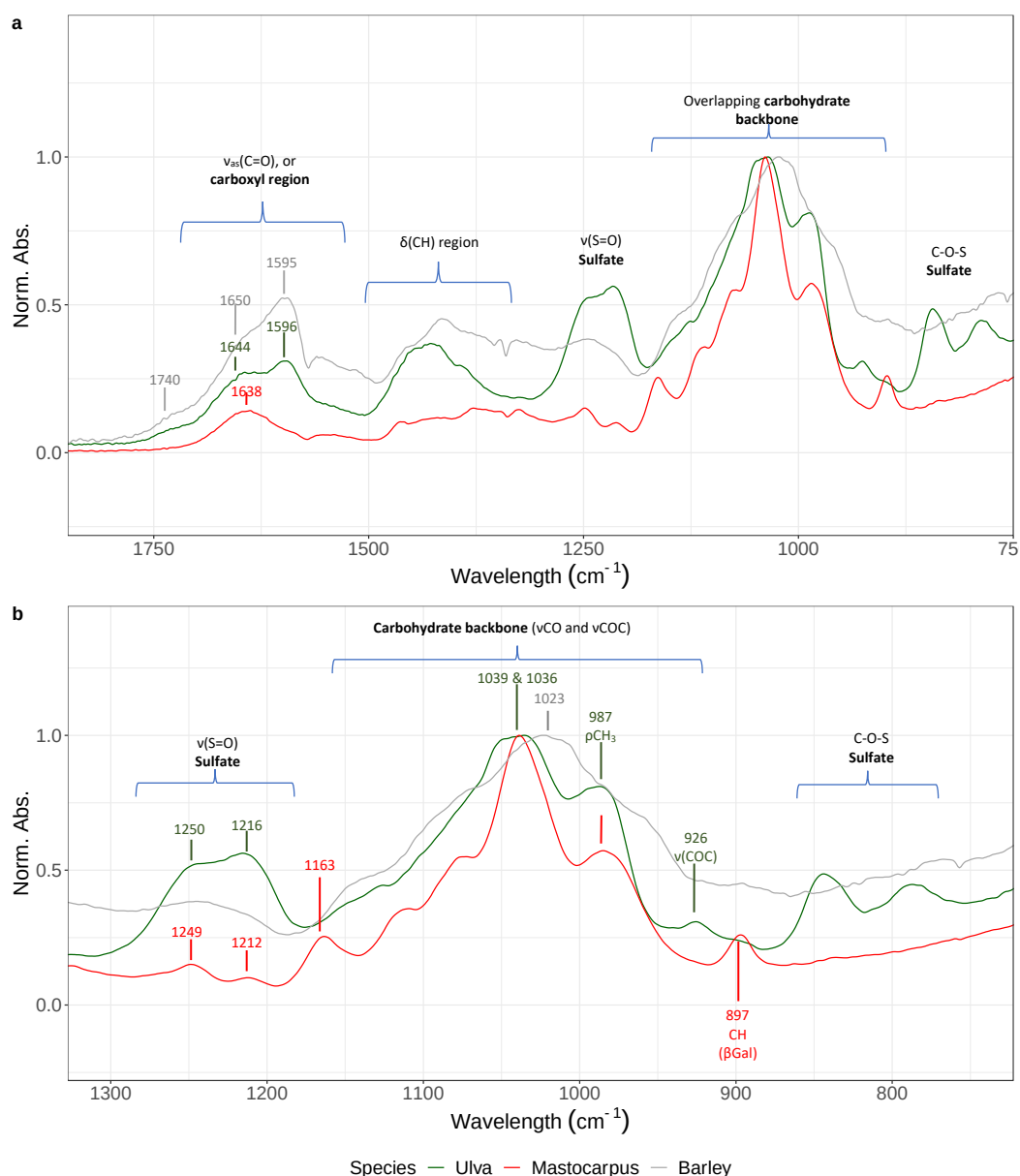


Figure 4.8. Comparison of slowly-soluble oxalate fraction (P2) from *U. compressa* (green), *M. stellatus*, and barley straw (grey). The double bond and fingerprint region (1800 cm^{-1} to 750 cm^{-1}) is presented in (a), where differences in $\nu(\text{C}=\text{O})$ associated resonances are observed, notably the lack of an ester shoulder at $\sim 1740\text{ cm}^{-1}$ and the shifted asymmetric carboxyl peak (to 1638 cm^{-1}) in the *M. stellatus* fraction. The spectral region from 1300 cm^{-1} to 750 cm^{-1} is magnified in (b) to facilitate the annotation of sulfate and carbohydrate backbone-associated peaks. Sulfate-associated peaks are identifiable on both the *U. compressa* (1250 , 1216 , 844 and 787 cm^{-1}) and *M. stellatus* (1249 and 1212 cm^{-1}) spectra, but not the barley straw extract. Functional differences are observed in the ‘carbohydrate backbone’ region (related to glycosidic linkages), indicative of compositional differences.

There appears to be no ester contribution at $\sim 1740\text{ cm}^{-1}$ in carrageenan. However, this shoulder is clearly visible in the barley straw pectin fraction (Figure 4.8, a). Interestingly, the characteristic carboxyl peak defined at 1596 cm^{-1} in ulvan is shifted to 1638 cm^{-1} in carrageenan. However, this corresponds with the previously identified 'shoulder' in ulvan at 1644 cm^{-1} that may also be contributed to by this functional group. The peak for the corresponding symmetric stretch (likely at 1463 cm^{-1} or 1420 cm^{-1}) is present, albeit small. In barley straw pectin, the carboxyl asymmetric stretch is observed at 1595 cm^{-1} , and the corresponding shoulder is observed at 1650 cm^{-1} .

A series of minor peaks that were difficult to assign based on the existing literature were observed in the carrageenan spectra, including bands at 1552 cm^{-1} , 1463 cm^{-1} , 1420 cm^{-1} , 1378 cm^{-1} , and 1325 cm^{-1} . Possible assignments for the 1552 cm^{-1} peak include NH scissoring from a secondary amine group, with the 1378 cm^{-1} and 1325 cm^{-1} peaks possibly corresponding with a C-N stretch, perhaps indicative of carrageenan-associated proteins analogous to those identified in the *U. compressa* ulvan fractions (Figure 4.7).

As expected, whilst the sulfate ester-associated peaks are present in the seaweed extracts (carrageenan - 1249 cm^{-1} and 1212 cm^{-1} , ulvan - 1250 cm^{-1} and 1216 cm^{-1}), this profile is absent in pectin, as polysaccharide sulfation is mostly absent within the land plant lineage (Aquino, Grativol and Mourão, 2011; Jiao *et al.*, 2011). Interestingly, carrageenan lacks the additional sulfate peaks (C-O-S) present in ulvan at 844 cm^{-1} and 787 cm^{-1} , most likely due to the known differences in sulfation pattern between the two polymers, with ulvan predominantly sulfated on carbon-3 (C-3) of rhamnose residues, and carrageenan having a varied sulfation pattern on C-2, C-4, and C-6 (Lahaye and Robic, 2007; Rhein-Knudsen, Ale and Meyer, 2015). Notably, Mainreck (2011) demonstrates that in sulfated glycosaminoglycans, peaks at resonances below 1015 cm^{-1} shift with the carbon group that the sulfate group is esterified to. However, none of the C-4-S or C-6-S bands identified at 853 cm^{-1} , 822 cm^{-1} , or 730 cm^{-1} in this paper were evident in the present carrageenan spectra.

A different spectral profile was observed in the carbohydrate backbone region for the three species, likely due to the differences in sugar composition, with ulvans predominantly being rhamnogalacturonan (ulvan), carrageenan consisting of glucopyranose, and the pectic fraction from barley straw likely containing homogalacturonan (HGA) and rhamnogalacturonan I or II, which are present albeit at a low level in barley straw (Langenaeken *et al.*, 2020). The glycosidic bond maxima at

band I (C-O-C) was comparable in the seaweed extracts (carrageenan - 1039 cm⁻¹, ulvan - 1036 cm⁻¹), despite the broader spectra observed in this region for ulvans. This was shifted to 1023 cm⁻¹ in barley straw pectin. The absence of the rhamnose attributed peak at 987 cm⁻¹ in barley straw may indicate that this fraction was predominantly homogalacturonan type rather than rhamnogalacturonan. However, the presence of a band in this region for the carrageenan fraction which should not contain rhamnose may indicate that this band may also be attributable to other sugar-associated vibrations (McCandless, Craigie and Walter, 1973; Hilliou *et al.*, 2012; Martín-del-Campo *et al.*, 2021). The presence of a band at 897 cm⁻¹ in carrageenan may be due to the abundance of galactose residues in the carrageenan backbone, with a major band at ~895 cm⁻¹ attributed to CH₂ rocking in β-galactose documented in spectra for various carrageenophytes including *M. stellatus* (Pereira *et al.*, 2003; Gómez-Ordóñez and Rupérez, 2011). The presence of an equivalent peak in barley straw P2 at 896 cm⁻¹ provides further support for this assignment, possibly corresponding to homogalacturonan. Again, no aromatic associated bands were identified in the pectin or carrageenan spectra as per the ulvan fraction.

4.3.1.4 Stress-Induced Changes in Ulvan Spectra

The differences in the spectra observed under metal stress and osmotic in the ulvan fractions were subtle. In the case of metal stress, it appeared to be primarily associated with the C=O associated ester and carboxyl peaks. In ulvan 1, whilst no ester peak was identifiable in any stress condition, a decline in the peak attributed to the asymmetric carboxyl peak at ~1596 cm⁻¹ was observed in the extract obtained following 24 h metal stress (CuCl₂) relative to the control, and this decrease persisted in the metal recovery sample, which was returned to control conditions for three days after metal stress before extraction (Figure 4.9). For ulvan 2, there appeared to be a reversible shift in both the carboxyl and the ester peak identified specifically in this fraction (~1742 cm⁻¹), with a decrease in both observed following exposure to CuCl₂ stress, which was reversed upon return to control conditions in the recovery sample (Figure 4.10). A similar post-stress decline was observed in carbonyl resonance following 3 hours of hyposaline or hypersaline stress (0 and 90 ‰, not plotted).

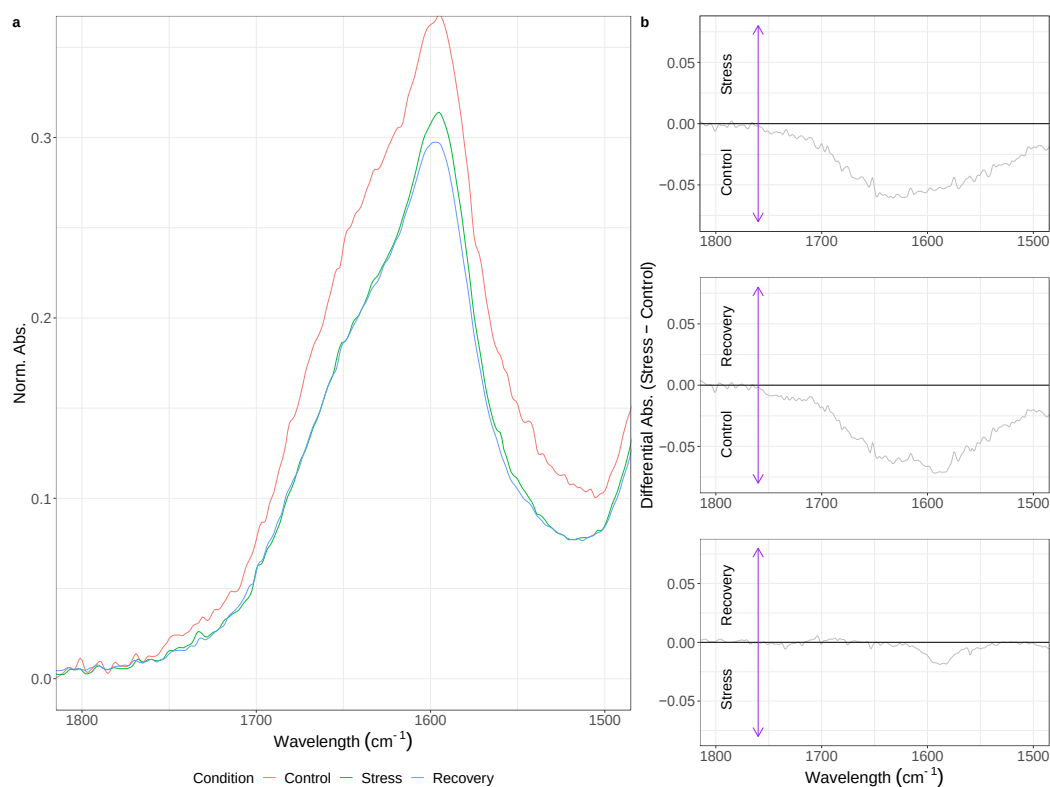


Figure 4.9. Comparison of the carboxyl region in the rapidly oxalate soluble fraction of *U. compressa* (P1) in samples exposed to CuCl_2 stress. Cell wall fractionation was performed on a sample of unstressed *U. compressa* thalli before CuCl_2 treatment (control, red), following 24 h incubation in artificial seawater (35 ‰) containing 100 μM CuCl_2 (stress, green), and following a 72 h recovery period where stressed thalli were returned to control growth conditions before extraction (recovery, blue). The spectral region from 1800 to 1500 cm^{-1} where $\nu(\text{C}=\text{O})$ associated resonances are observed is presented in (a), and corresponding differential plots calculated by *stress* – *control*, *recovery* – *control*, and *recovery* – *stress* are presented in (b).

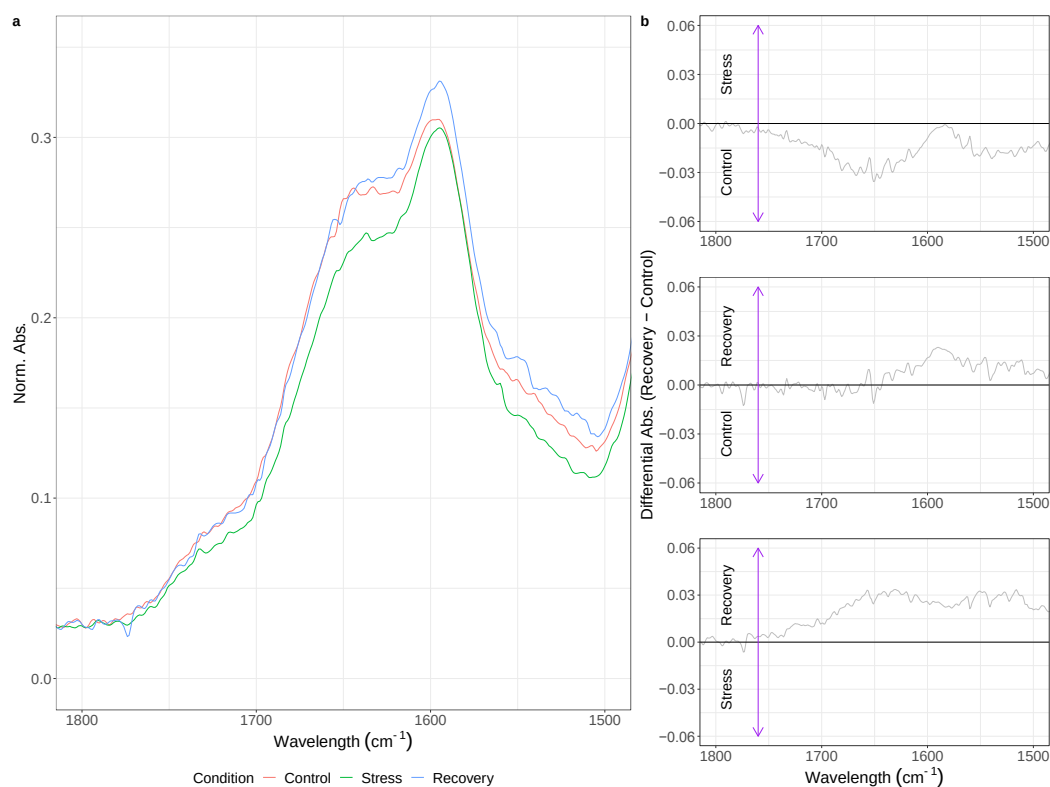


Figure 4.10. Comparison of the carboxyl region in the slowly oxalate soluble fraction of *U. compressa* (P2) in samples exposed to CuCl_2 stress. The spectral region from 1800 to 1500 cm^{-1} where $\nu(\text{C}=\text{O})$ associated resonances are observed is presented in (a), and corresponding differential plots calculated are shown in (b), where reversible changes in ester and carboxyl associated bands are observed after stress and recovery.

4.3.2 Alkali-Soluble Fraction

After extraction of the oxalate soluble pectic fraction, two sodium hydroxide soluble fractions were obtained, corresponding to the hemicellulose fractions Ha and Hb. The first fraction (Ha) was recovered from the material precipitated after neutralising the sodium hydroxide extract with glacial acetic acid, whilst the second fraction (Hb) corresponds to the material that remained in solution during this process. Here, the alkali-soluble fraction in *U. compressa* was expected to correspond to the minor glucuronan and xyloglucan components of the *Ulva* cell wall (Lahaye, Jegou and Buleon, 1994; Redouan *et al.*, 2009). A similar extract was expected in barley straw, where arabinoxylans comprise the most abundant hemicellulose (Krawczyk *et al.*, 2008; Sun *et al.*, 2011; Ma, Dang and Xu, 2016; Langenaeken *et al.*, 2020). The barley straw extract was also hypothesised to have functional groups corresponding to ferulate, coumarate, and lignin which comprise a significant component of monocot biomass and are esterified to hemicelluloses (Lichtenthaler and Schweiger, 1998). These phenolic structures may be esterified or etherified to sugar residues (Maryana *et al.*, 2014; Aminzadeh, Zhang and Henriksson, 2017; Nishimura *et al.*, 2018; Terrett and Dupree, 2019; Antonopoulou *et al.*, 2022). By contrast, few scientific reports document the structure and composition of the alkaline soluble polysaccharides of red macroalgae, including *Mastocarpus* spp. and the closely related model seaweed *Chondrus crispus*, as due to the economic value of carrageenan, there is limited interest in the other components of the red seaweed cell wall. However, alkali-soluble glucomannans and glucans have been characterised in another red carrageenophyte, *Kappaphycus alvarezii* (Lechat *et al.*, 2000).

4.3.2.1 Characterisation of Precipitated Hemicelluloses

Whilst no substantial Ha fraction was obtained from *M. stellatus* biomass, the resulting spectra from *U. compressa* and barley straw were near identical in profile (Figure 4.11), with the assignments for both spectra presented in Table 4.5.

Assigination	Functional Group	Peak (cm ⁻¹)	References
Hydroxyl	vOH	3340 (3361)	(Coates, 2006)
Methyl	vCH	2981 (2980)	
	vCH	2888 (2895)	
Adsorbed Water	δ(OH)	1638 (1660)	(Fan, Dai and Huang, 2012; Mishra and Malhotra, 2012; Vasylieva et al., 2018; Gomaa et al., 2022)
Aromatic Ring	v(C=C-C)	- (1596)	See Table 4.3
	v(C=C-C)	- (1555)	
	v(C=C-C)	- (1507)	
CH bending from various alkyl groups from pyranose sugars and side groups	δCH	1462 (1458)	(Popescu <i>et al.</i> , 2007; Sun <i>et al.</i> , 2011; Traoré, Kaal and Martínez Cortizas, 2018; Javier-Astete, Jimenez-Davalos and Zolla, 2021; Liu <i>et al.</i> , 2021).
	δCH	1416	
	δCH	1382 (1380)	
	δCH	1312 (1319)	
	δCH	1312 (1319)	
Sugar Backbone	v(CO) or v(S=O)	1248 (1250)	(Szymanska-Chargot <i>et al.</i> , 2015; Acquah <i>et al.</i> , 2016; Javier-Astete, Jimenez-Davalos and Zolla, 2021)
	v(CO) or v(S=O)	1214 (1213)	

Xylose	v(CO)	1163 (1162)	(McCann <i>et al.</i> , 1992; Liu <i>et al.</i> , 2021)
	v(CO)	1034 (1031)	
	ρCH ₃	973 (971)	(Marry <i>et al.</i> , 2000)
Xylose	δCH	895 (896)	(Szymanska-Chargot and Zdunek, 2013)

Table 4.5. Assignations for the first hemicellulose (Ha) fraction in *U. compressa* and barley straw. Assignations for barley straw are reported in parentheses. No peaks at 1596 cm⁻¹, 1555 cm⁻¹, or 1507 cm⁻¹ were observed in the *U. compressa* spectrum, consistent with the absence of aromatics.

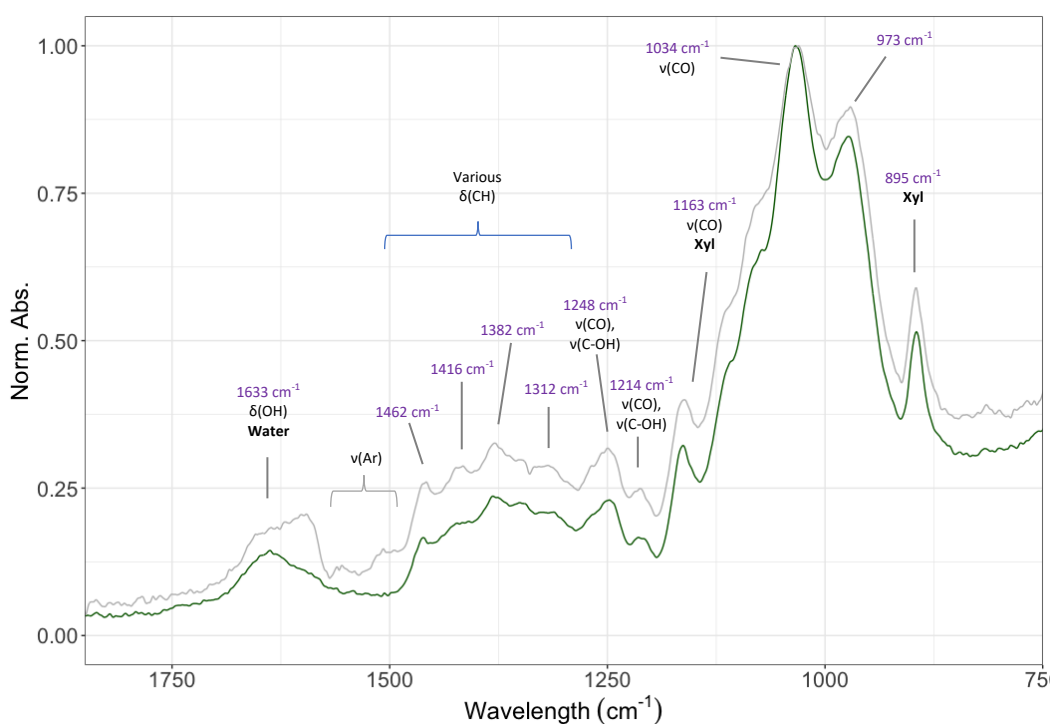


Figure 4.11. FTIR spectra for the precipitated hemicellulose fraction (Ha) in *U. compressa* (green) and barley straw (grey). Only a negligible Ha fraction was obtained from *M. stellatus*, which was insufficient for ATR-FTIR analysis. Aromatic stretches were observed in barley straw at 1595, 1555, and 1507 cm⁻¹ but not in *U. compressa*.

As with the pectic fractions, the characteristic $\nu(\text{OH})$ and $\nu(\text{CH})$ bands of polysaccharides were identified in the functional region with peaks at 3340 cm^{-1} , 2981 cm^{-1} , and 2888 cm^{-1} . However, no peak attributable to an ester group was observed in the *U. compressa* fraction, nor were carboxyl bands ($\nu_{\text{as}}\text{C}=\text{O}$ and $\nu_{\text{s}}\text{C}=\text{O}$) observed at approximately 1598 cm^{-1} and 1428 cm^{-1} as observed in the pectic fractions. Rather, the band at 1638 cm^{-1} was consistent with the adsorbed water peak typically observed in cellulose and xyloglucans (Mishra and Malhotra, 2012), which was also observed in the spectra for the α -cellulose extracts and the pure cellulose control (see Figure 4.17). This band was followed by a series of CH deformations, consistent with CH bending from $-\text{OCH}_3$ groups in pyranose structures of sugars (1462 cm^{-1} and 1416 cm^{-1}), a CH bending peak typically observed in hemicelluloses (1382 cm^{-1}), and a CH_2 wagging resonance (1312 cm^{-1}) respectively (Popescu *et al.*, 2007; Sun *et al.*, 2011; Traoré, Kaal and Martínez Cortizas, 2018; Javier-Astete, Jimenez-Davalos and Zolla, 2021; Liu *et al.*, 2021).

Consistent with the elemental analysis, the *U. compressa* Ha fraction did not appear to be sulfated, with the bands attributed to C-O-S bending in ulvans at 844 cm^{-1} and 787 cm^{-1} absent in the Ha spectra. However, weak bands in the region where the corresponding S=O stretches were characterised in ulvans were identified at 1248 cm^{-1} and 1214 cm^{-1} . However, as these bands were also present in the equivalent barley straw Ha fraction at a similar peak intensity (1250 cm^{-1} and 1213 cm^{-1}), it indicated that they were likely attributable to an alternative functional group, or if sulfate-associated, the degree of sulfation was reduced in the fraction relative to ulvans, which again was consistent with the findings from elemental analysis. Furthermore, the profile of the peaks in this region was also different in Ha, with the S=O stretches appearing as broad overlapping bands in ulvan (Figure 4.8). In contrast, these resonances formed two distinct peaks (Figure 4.11), indicating again that they were likely from a different functional group. This fingerprint is also consistent with C-O stretches and O-H bends from pyranose rings observed in polysaccharide extracts from holocellulose, starch, and pectin (Szymanska-Chargot *et al.*, 2015; Acquah *et al.*, 2016; Javier-Astete, Jimenez-Davalos and Zolla, 2021). Also, bands in this region may also correspond to C-O stretches phenolic structures in lignocellulose (e.g., guaicyl monolignols). However, no corresponding bands were identified between 1500 cm^{-1} and 1600 cm^{-1} in *U. compressa*, as expected for an aromatic compound (Lu *et al.*, 2017).

Several C-O and C-O-C peaks were identified in the sugar backbone region that were consistent with a xyloglucan polysaccharide, including those observed at 1163 cm^{-1}

and 1034 cm^{-1} (McCann *et al.*, 1992; Liu *et al.*, 2021). Additionally, the peak at 895 cm^{-1} correlates with CH rocking from C-1 of xylose (Szymanska-Chargot and Zdunek, 2013).

Overall, these observations were consistent with a linear xylose-containing polysaccharide such as algal xyloglucan (Lahaye, Jegou and Buleon, 1994), consistent with the lack of evidence for methyl, *O*-acetyl, or sulfate esters in the *U. compressa* spectra compared to ulvans. There appeared to be limited evidence of amine or amide resonances in the spectra, correlating with the low nitrogen content estimated in this fraction by elemental analysis.

Only three differences in peak profile were observable between the barley straw and *U. compressa* spectra, indicative of structural differences. Xyloglucans are a major hemicellulose fraction in plants; however, the structure and sugar composition of plant xyloglucan is known to differ from the 'xyloglucans' isolated from *Ulva* (Lahaye, Jegou and Buleon, 1994).

Firstly, the peak at the adsorbed water region ($\sim 1638\text{ cm}^{-1}$) was broader in barley straw Ha than *U. compressa*, with two shoulders observed at 1660 cm^{-1} and 1596 cm^{-1} , with the former band possibly indicative of carboxyl groups or a primary amine stretch. (Popescu *et al.*, 2007; Zhou *et al.*, 2015; Javier-Astete, Jimenez-Davalos and Zolla, 2021). Secondly, two additional peaks were observed within the aromatic region at 1555 cm^{-1} and 1507 cm^{-1} of the barley sample, likely representing aromatic skeletal vibrations of lignin or other aromatics, consistent with the spectra generated for kraft lignin previously (Figure 4.5). Peaks attributed to aromatic ring vibrations can also be identified in lignocellulose at $\sim 1600\text{ cm}^{-1}$ (Lu *et al.*, 2017), offering a possible explanation for the shoulder at 1596 cm^{-1} . Whilst the changes in this region were subtle as these peaks were small, they are more evident in the differential spectrum plotted for this region (Figure 4.13). It was concluded that the barley straw Ha extract was likely associated with lignin or another phenolic. In contrast, there was no evidence of a 'non-sugar' structure in the *U. compressa* spectra.

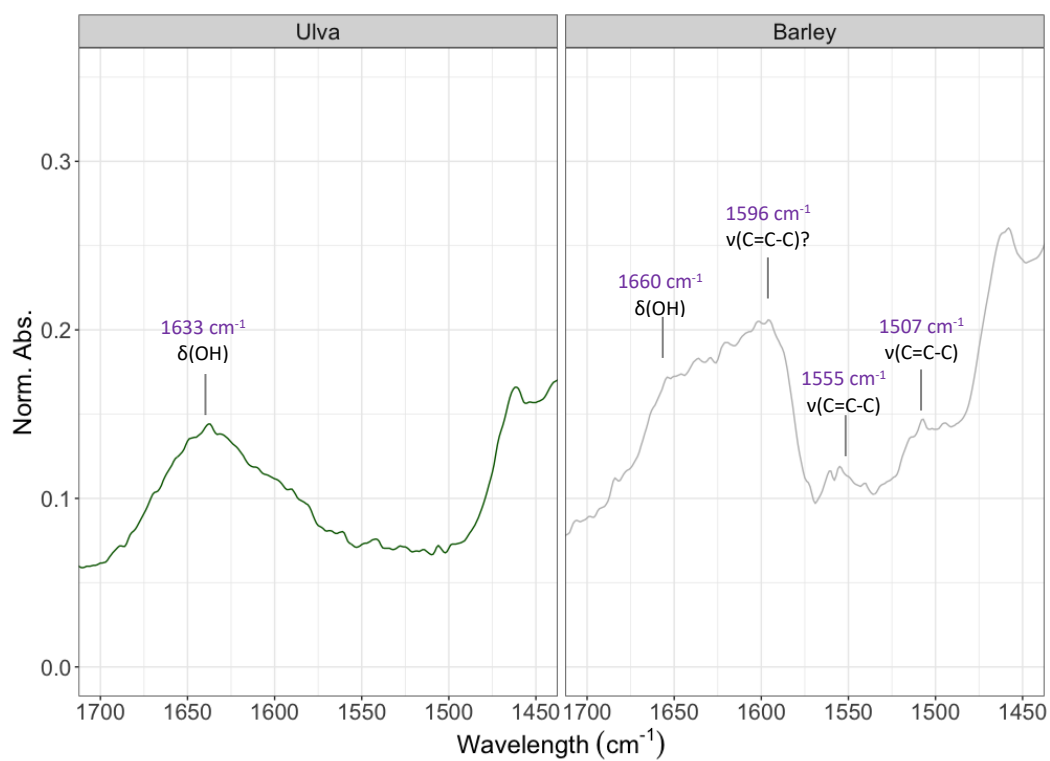


Figure 4.12. Small peaks consistent with aromatic stretches ($\nu\text{C}=\text{C}-\text{C}$) were observed in the Ha fraction from barley straw (grey) but not *U. compressa* (green).

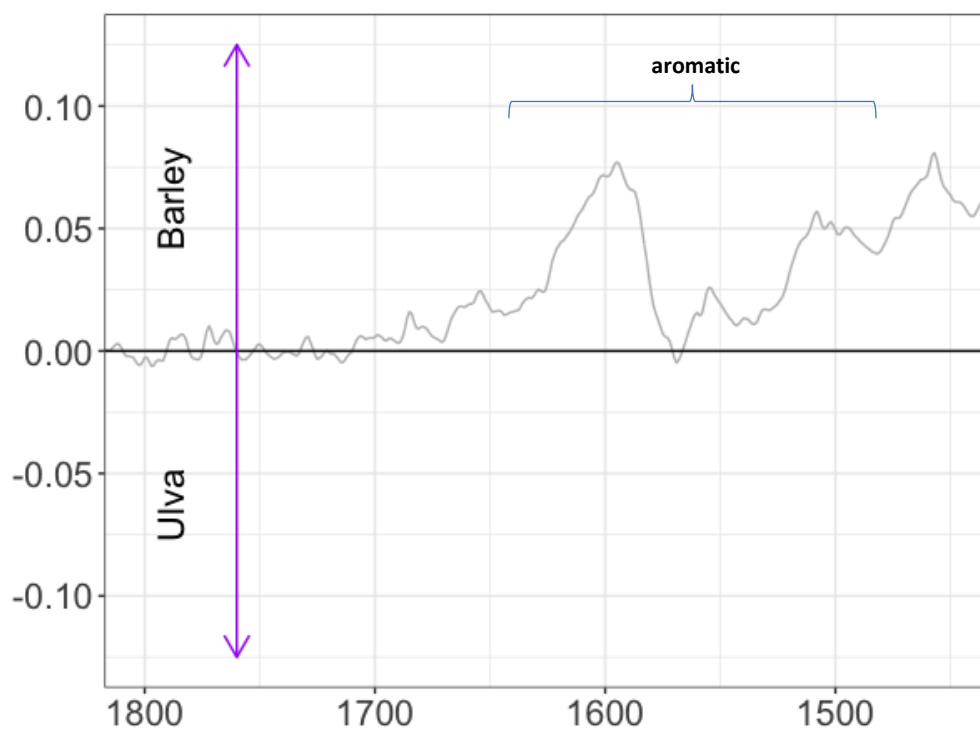


Figure 4.13. Differential spectra between the Ha fraction in barley straw and *U. compressa* illustrate the presence and absence of aromatic associated bands in both extracts, respectively. The differential plot was calculated by *H. vulgare* – *U. compressa*.

4.3.2.2 Characterisation of Non-Precipitated Hemicelluloses

The second hemicellulose fraction (Hb) appeared structurally distinct from the Ha fraction and, unlike Ha, appeared structurally distinct in *U. compressa* relative to the corresponding barley straw and *M. stellatus* fractions (Figure 4.14).

Assignment	Functional Group	Peak (cm ⁻¹)	References
Hydroxyl	vOH	3348	(Coates, 2006)
Methyl	vCH	2981	
	vCH	2889	
Carboxyl	v _{as} COOH	1608	See Table 4.4 and Table 4.5 for assignments of the peaks in oxalate-soluble and precipitated hemicellulose fractions
CH bending of hemicellulose	δCH	1460	
Carboxyl	v _s COOH	1420	
Methyl	δCH	1384	
Sulfate	vS=O	1252	
	vS=O	1220	
Sugar Backbone	vCOC	1125	
	vCOC	1037	
	vCO	996	(Schwanninger <i>et al.</i> , 2004)
	vCOC (α 1→4 glycosidic bond)	927	(Prabhu <i>et al.</i> , 2019)
Sulfate	C-O-S	845	(Castro <i>et al.</i> , 2006; Ray, 2006; Zhang <i>et al.</i> , 2010; Yaich <i>et al.</i> , 2017; Nunes <i>et al.</i> , 2020)
	C-O-S	785	

Table 4.6. Assignations for the second hemicellulose (Hb) fraction in *U. compressa*.

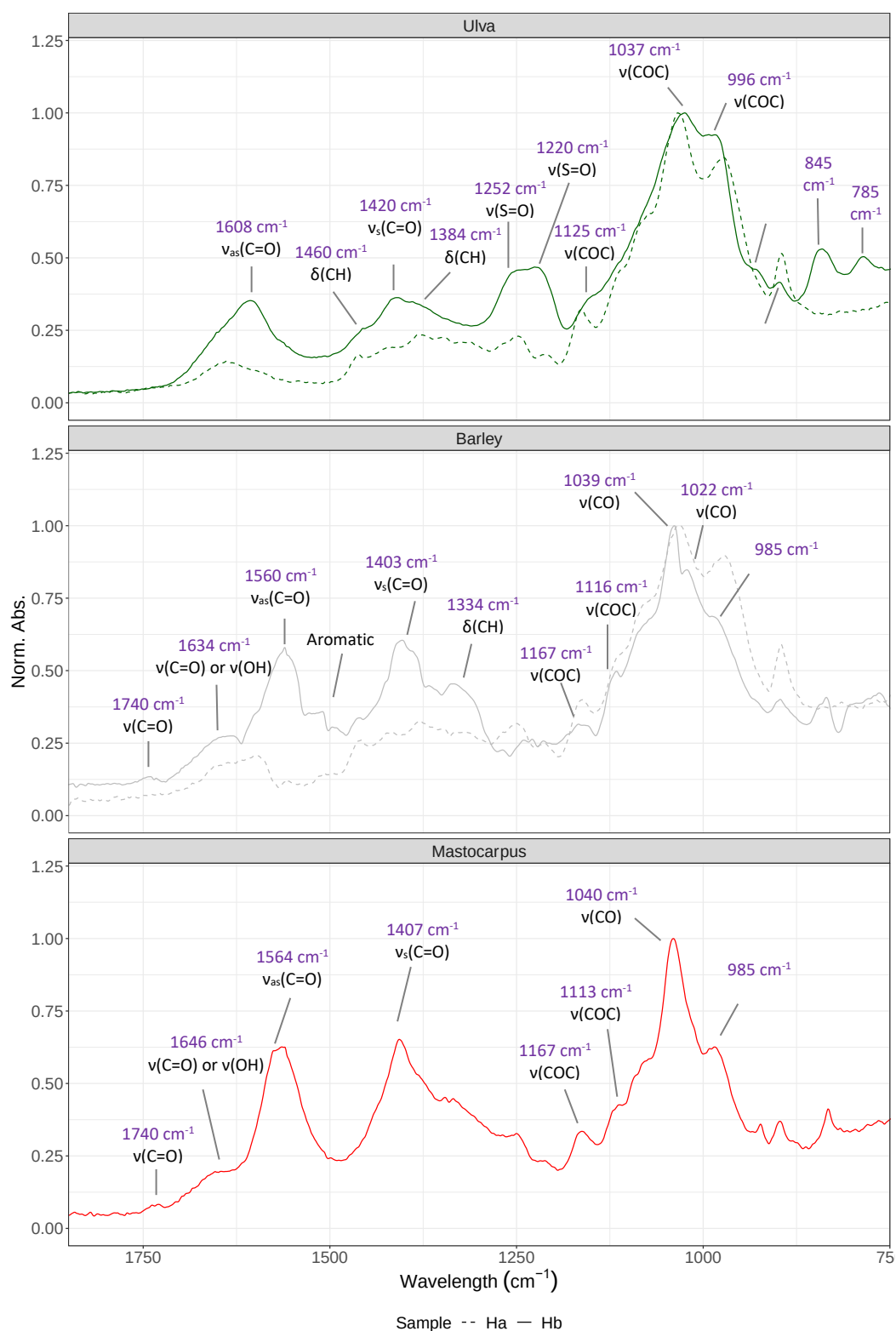


Figure 4.14. Comparison of precipitated (Ha) and non-precipitated (Hb) hemicellulose fractions in *U. compressa* (green), barley straw (grey), and *M. stellatus* (red). No Ha fraction was obtained from *M. stellatus*; only the Hb fraction is presented.

In the functional region, the νOH (3348 cm^{-1}) and νCH (2981 cm^{-1} and 2889 cm^{-1}) peaks did not appear to be shifted relative to Ha (Table 4.5), indicating that the returned fraction was predominantly polysaccharide. However, more distinctive differences in the precipitated material were observed in the fingerprint region. Whilst most of the identified peaks were present in both hemicellulose fractions, for example, the CH bending at 1460 cm^{-1} and 1384 cm^{-1} , the intensities of the observed peaks were considerably greater in Hb. Also, no contribution from adsorbed water was observed in the Hb fraction at $\sim 1650\text{ cm}^{-1}$. Most notably, a carboxylate peak was observed at 1608 cm^{-1} in Hb, which was not present in Ha, indicating that acidic polysaccharides (such as ulvans or glucuronan) likely segregated into this fraction, as observed in the oxalate-soluble fractions (Figure 4.7). In contrast to these fractions, the corresponding carboxylate symmetric stretch only appeared as a shoulder at 1420 cm^{-1} in Hb, whereas it formed a more pronounced peak in ulvan. This difference was accompanied by an increase in the observed methyl stretch at 1384 cm^{-1} (1392 cm^{-1} in ulvans, 1382 cm^{-1} in Ha).

Interestingly, whilst no ester contribution was observed in the *U. compressa* Hb like the Ha, a small peak was visible in both the barley straw and *M. stellatus* fractions at $\sim 1740\text{ cm}^{-1}$, consistent with phenolic or carboxylate esters (McCann *et al.*, 1994; Marry *et al.*, 2000; Coates, 2006). Furthermore, a shoulder was also observed in these spectra at 1634 cm^{-1} and 1646 cm^{-1} (barley and *Mastocarpus*, respectively), consistent with adsorbed water (Vasylieva *et al.*, 2018), or reflecting increased contribution from an ester group as observed in the corresponding pectic fractions (Figure 4.7). Furthermore, the asymmetric and symmetric stretches were shifted relative to *U. compressa* ($1560/1564\text{ cm}^{-1}$ and $1403/1407\text{ cm}^{-1}$), consistent with a differential functionalisation profile.

Unlike in the Ha fraction, all four peaks attributed to sulfate groups in the ulvan samples appeared to persist in Hb. Whilst bands at 1250 cm^{-1} and 1213 cm^{-1} were observed in Ha, more prominent bands were observed at 1252 cm^{-1} and 1220 cm^{-1} in Hb. Furthermore, the C-O-S resonances absent in the Ha were present in Hb at 845 cm^{-1} and 785 cm^{-1} . Whilst peaks at 1248 cm^{-1} have been shown to correlate with O-acetylation in glucuronan isolated from soil bacteria, the absence of a peak attributable to an ester group indicated that this contribution was unlikely (Pau-Roblot *et al.*, 2002). Regardless, the prominence of these bands was reduced in Hb relative to the ulvan fractions, consistent with the decrease in estimated sulfur content observed as determined by

elemental analysis (4.2.1). By contrast, the sulfation pattern observed in the carrageenan fractions was no longer present in *M. stellatus* Hb (Figure 4.14).

The profile of peaks in the sugar backbone region in Hb differed in *U. compressa* to Ha and the corresponding barley straw and *M. stellatus* fractions, likely representing the different saccharide compositions of extracts. For example, the shoulder at 985 cm⁻¹ in barley straw and *M. stellatus* Hb that was shifted to 996 cm⁻¹ in *U. compressa* may be consistent with xylans and arabinoxylans, both of which are absent in the *Ulva* cell wall (Liu *et al.*, 2021). A peak at ~985 cm⁻¹ was initially attributed to rhamnose in the oxalate-soluble fraction of *Ulva*, and the absence of this peak in the corresponding Hb sample may reflect that ulvans do not segregate into this fraction, despite the persistence of the carboxyl peak discussed previously. Additionally, whilst both barley straw and *M. stellatus* had a shoulder at 1116 cm⁻¹ (1113 cm⁻¹ in *M. stellatus*), the corresponding COC ether was shifted to 1125 cm⁻¹ in *U. compressa*. Interestingly, the 1125 cm⁻¹ peak and the 927 cm⁻¹ peak attributed to the C-O-C stretching of glycosidic bonds were also evident in the ulvan fractions, but not Ha. As stated previously (4.1.2), it is difficult to predict sugar composition from ATR-FTIR spectra.

As shown in Figure 4.15, a fingerprint consistent with lignin and hydroxycinnamic acids was identified in the barley straw Hb fraction, exclusively providing more substantial evidence of this association than the previous Ha fraction (Baicioğlu, Zimmermann and Kohler, 2015; Lu *et al.*, 2017; Md Salim, Asik and Sarjadi, 2021). This included the previously mentioned ester at 1740 cm⁻¹, in addition to various resonances at 1600 cm⁻¹ and 1510 cm⁻¹ (aromatic ring stretches), 1367 cm⁻¹ and 1334 cm⁻¹ (C-H bending from lignin side groups), 1269 cm⁻¹, 1229 cm⁻¹ (C-O stretches from guaicyl and syringyl monolignols), and aromatic C-H bending at 835 cm⁻¹ (see Table 4.3 for lignin assignments). Most of these peaks were unique to the barley straw spectra, but, in addition to the ester peak, two very small peaks corresponding to C-H bending and C-O stretching were also observed in the *M. stellatus* spectra at 1335 cm⁻¹ and 1250 cm⁻¹. However, as these peaks were small, not necessarily attributable to aromatics, and as the corresponding aromatic ring stretch was absent, these observations were inconclusive. The identification of these peaks in barley straw was supported by the positive phloroglucinol-HCl staining reported for this sample in the previous chapter (see Chapter 3, Table 3.2).

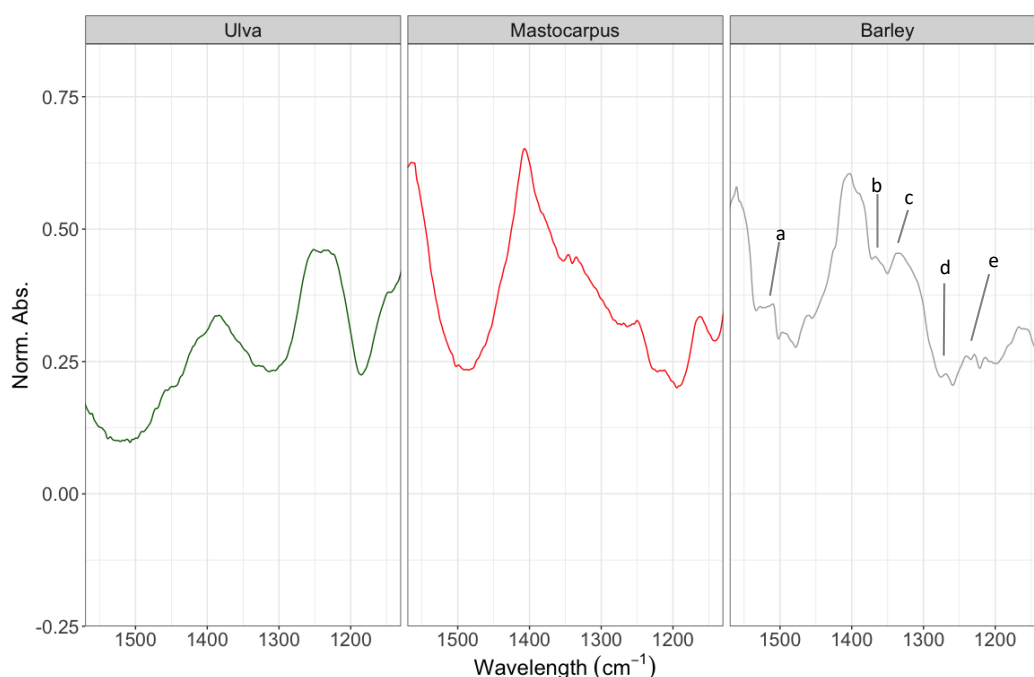


Figure 4.15. Comparison of the aromatic region of the spectra for the non-precipitated hemicellulose fractions (Hb) in *U. compressa* (green), barley straw (grey), and *M. stellatus* (red). Aromatic-associated bands are observed in barley straw specifically at 1510 cm⁻¹ (a), 1367 cm⁻¹ (b), 1334 cm⁻¹ (c), 1269 cm⁻¹ (d), and 1229 cm⁻¹ (e).

4.3.2.3 Stress-Induced Changes in Alkali-Soluble Fractions

The most notable change in the Hb fraction obtained from stressed samples was observed in the CuCl₂ stress experiment (Figure 4.16), where the ester peak at ~1740 cm⁻¹ that was absent in the Hb fraction of unstressed tissue and also immediately following 24 h stress, appeared on the spectra following the 3-day recovery period. This was accompanied by a general increase in signal across the fingerprint region, with shifts in the carbohydrate region indicative of carbohydrate composition observed relative to the stressed and unstressed sample. In particular, the peak attributed previously to CH rocking of rhamnose in the *U. compressa* oxalate soluble fraction (Figure 4.7) increased in prominence, possibly indicating that more ulvan material had segregated into this fraction, possibly consistent with the increased ulvan yields observed in this condition observed previously (see Chapter 3, Figure 3.13). These changes were not observed under osmotic stress.

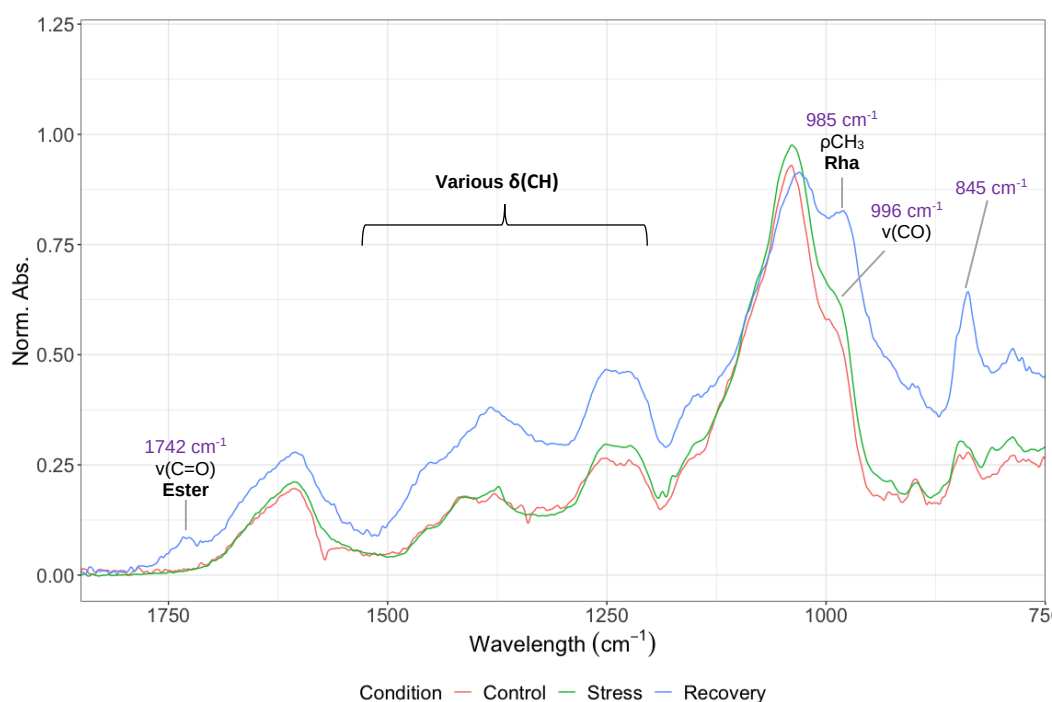


Figure 4.16. Comparison of the precipitated hemicellulose fraction of *U. compressa* (Hb) in samples exposed to CuCl_2 stress. The main functional differences are observed in the ‘recovery’ sample (blue), where samples were returned to control conditions for 72 h following 24 h of stress (green) in artificial seawater containing $100 \mu\text{M}$ CuCl_2 . Differences observed include the re-appearance of the ester peak at 1742 cm^{-1} observed in the oxalate soluble fractions, which is absent under control conditions and in the sample extracted immediately following stress. General increases are observed across the fingerprint region under sample recovery, associated with CH bending, with shifts in the carbohydrate backbone consistent with compositional changes.

4.3.3 Insoluble Fraction

As in plants, the cell walls of *Ulva* and *Mastocarpus* all contain cellulose as the major fibrillar component (Lahaye and Robic, 2007; Gómez-Ordóñez, Jiménez-Escrig and Rupérez, 2010; Wahlström, Edlund, *et al.*, 2020). As a result, it was expected that this would comprise the bulk of the insoluble fraction (α -cellulose) rather than the insoluble mannan or xylan structures found in other macroalgal species such as *Caulerpa* and *Codium* (Domozych *et al.*, 2012). In addition, lignin, lignin-like material and structural components associated with the fibrillar components of the cell wall were also hypothesised to be present in this fraction.

The characteristic fingerprint of α -cellulose was observed in all extracts, and a pure cellulose control was analysed as a reference (Table 4.7, Figure 4.17). Interestingly, the previously described precipitated hemicellulose fraction (Ha) had a more comparable spectrum to cellulose than the respective non-precipitated hemicellulose fraction (Hb), particularly for barley straw, providing further evidence that it likely resembled a xyloglucan type polysaccharide (Figure 4.18). Surprisingly, there was no immediate evidence of an aromatic structure in any of the cellulose fractions.

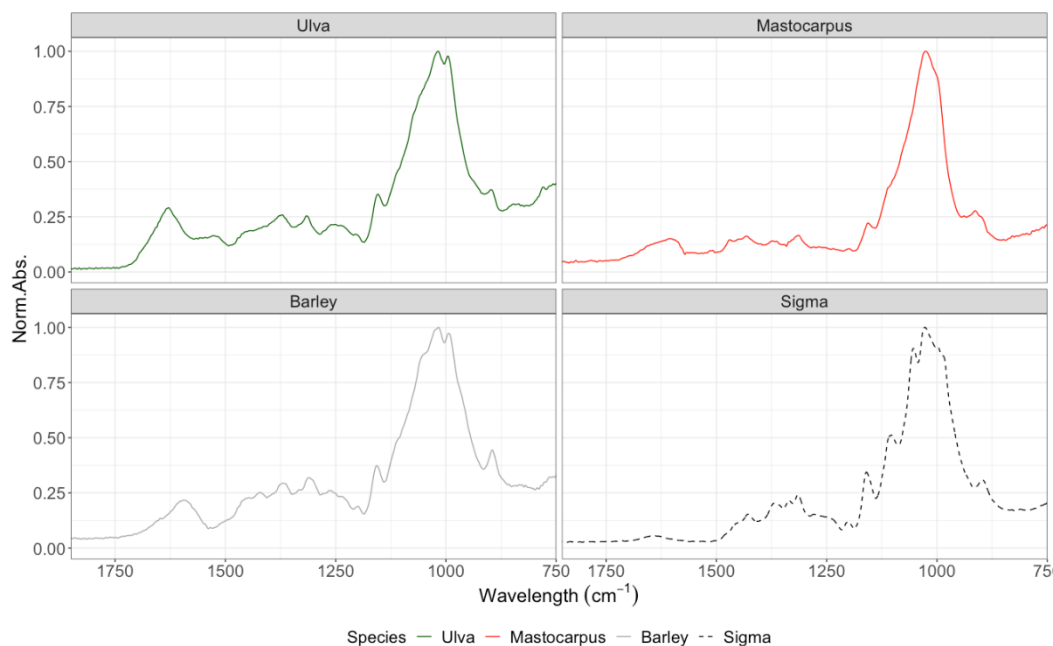


Figure 4.17. Comparison of the α -cellulose fraction in *U. compressa* (green), *M. stellatus* (red), and barley straw (grey) with a cellulose standard (dotted). The cellulose standard was obtained from Sigma (cellulose fibres, medium: C6288).

Assignment	Functional Group	Peak (cm ⁻¹)	References
Hydroxyl	vOH	3284-3297	(Coates, 2006)
Methyl	vCH	2980	
	vCH	2881-2889	
Amide ^a	vC=O	1689	(Coates, 2006; Barth, 2007)
Adsorbed Water ^b	δ(OH)	1645	(Fan, Dai and Huang, 2012; Mishra and Malhotra, 2012; Vasylieva et al., 2018; Gomaa et al., 2022)
Primary Amine ^a	δ(NH)	1627	(Barth, 2007; Mayers, Flynn and Shields, 2013; Mazurek <i>et al.</i> , 2013; Guerrero, Kerry and de La Caba, 2014; Fuller, Andaya and McClay, 2018)
Carbonyl ^c	vC=O	1600	(Oh <i>et al.</i> , 2005)
Secondary Amine	δ(NH)	1523	(Barth, 2007; Mayers, Flynn and Shields, 2013; Mazurek <i>et al.</i> , 2013; Guerrero, Kerry and de La Caba, 2014; Fuller, Andaya and McClay, 2018)
Alkyl (CH)	δ(CH)	1420-1470	(Schwanninger <i>et al.</i> , 2004; Md Salim, Asik and Sarjadi, 2021)
Alkyl (Cellulose, CH)	δ(CH)	1369-1373	(Gastaldi <i>et al.</i> , 1998; Schwanninger <i>et al.</i> , 2004; Sun <i>et al.</i> , 2005; Popescu <i>et al.</i> , 2007; Fan, Dai and Huang, 2012)

	$\delta(\text{CH})$	1310-1315	
	$\delta(\text{CH})$	1252-1262	
	$\delta(\text{CH})/\nu(\text{CO})$	1200-1206	(Fan, Dai and Huang, 2012)
Sugar Backbone	$\nu(\text{CO})$	1155-1160	(Gastaldi <i>et al.</i> , 1998; Schwanninger <i>et al.</i> , 2004; Sun <i>et al.</i> , 2005; Popescu <i>et al.</i> , 2007; Fan, Dai and Huang, 2012; Liu <i>et al.</i> , 2021)
	$\nu(\text{CO})$	1017-1028	
	$\nu(\text{CO})$	994-1000	
Anomeric CH (Cellulose) ^d	$\delta(\text{CH})$	~895	(Kačuráková <i>et al.</i> , 1999; Schwanninger <i>et al.</i> , 2004; Liu <i>et al.</i> , 2021)

Table 4.7. Assignations for α -cellulose fractions and pure cellulose control (Sigma). All samples displayed typical cellulose fingerprint, with some exceptions: (a) amide and amine-associated peaks were only observed in *Ulva* extracts, (b) adsorbed water was only observed in the 'pure cellulose' sample, (c) this peak was only observed in *M. stellatus* and barley straw cellulose, and (d) a $\delta(\text{CH})$ peak was observed at 914 cm^{-1} in *M. stellatus*, but not 895 cm^{-1} as observed in the other extracts and reference.

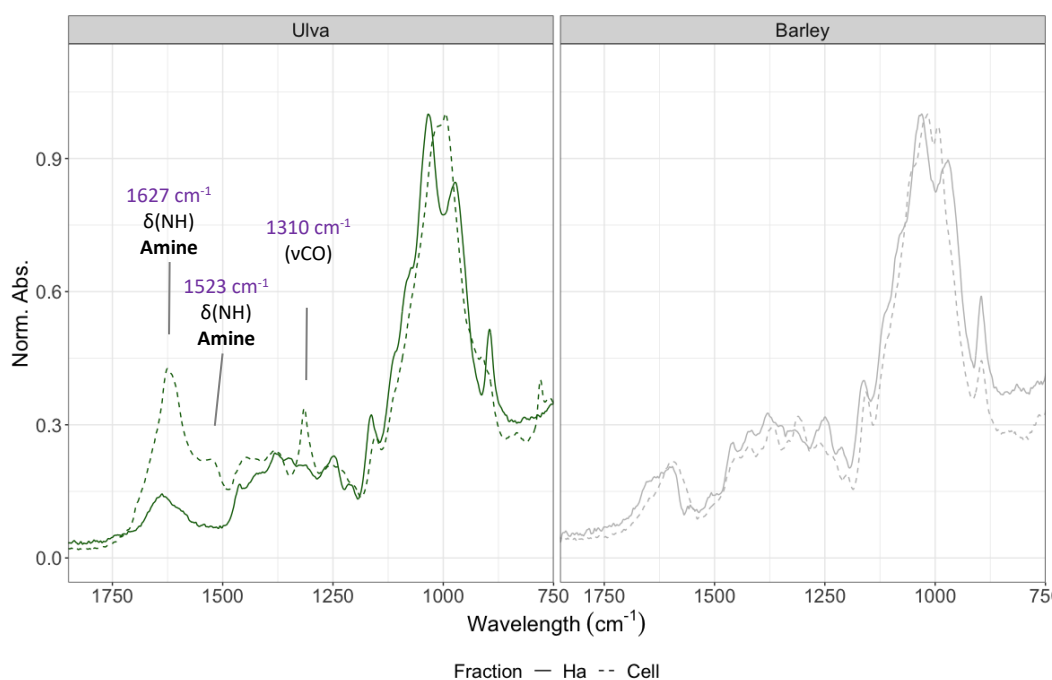


Figure 4.18. Comparison of α -cellulose fraction and precipitated hemicellulose fraction (Ha) in ‘hypersaline-stressed’ *U. compressa* (green) and the barley straw extracts for comparison (grey). The Ha fraction is represented by a solid line, and a dashed line represents a representative spectrum from an α -cellulose fraction. Annotated peaks on the *U. compressa* spectrum include peaks attributed to amine vibrations that were identified in α -cellulose specifically.

Whilst the spectra from all three species were mostly comparable, some additional bands were identified in the *U. compressa* spectra that were not associated with the expected cellulose carbohydrate fingerprint and were absent in the pure cellulose control and the extracts from the other species (Figure 4.19). Firstly, the small peak attributed to adsorbed water at 1645 cm^{-1} in the pure cellulose control was not present in the *M. stellatus* and barley straw cellulose (Figure 4.19, b). This observation and the observations from the previously discussed elemental analysis (Table 4.2) that detected an elevated nitrogen content in this sample suggested that the peak observed at 1627 cm^{-1} in *U. compressa* was consistent with an NH_2 scissoring vibration from a primary amine rather than an adsorbed water peak, as this peak was not present in the other cellulose samples processed by the same extraction method. This was further supported by the identification of a second amine peak at 1523 cm^{-1} , consistent with NH scissoring from a secondary amine. The *Ulva* α -cellulose was also unique due to the presence of a small shoulder in the spectra at 1689 cm^{-1} , which was absent in the other species and the pure control. This, with the presence of the other NH bands, was

indicative of an amide-associated carboxyl group. The presence of these three bands is consistent with spectra from the literature generated from amine-functionalised polymers or from co-extracted cellulose and protein samples (Mayers, Flynn and Shields, 2013; Guerrero, Kerry and de La Caba, 2014; Fuller, Andaya and McClay, 2018; Kouadri and Satha, 2018), indicating that the α -cellulose fraction in *Ulva* might be associated with cell wall proteins or an amine-containing metabolite such as a polyamine (McCann *et al.*, 1992; Martínez Urreaga and de la Orden, 2007; Kadimaliev *et al.*, 2015; Koshani *et al.*, 2022), both of which have been characterised in the cell walls of chlorophyte microalgae and plants (Voigt, 1985; Berta *et al.*, 1997; Lenucci *et al.*, 2005; Domozych *et al.*, 2012; Burczyk *et al.*, 2014; Voigt *et al.*, 2014; Přerovská *et al.*, 2021).

Despite the lack of a peak attributed to adsorbed water at $\sim 1645\text{ cm}^{-1}$ in barley straw cellulose, a prominent peak at $\sim 1600\text{ cm}^{-1}$ was observed in both samples (Figure 4.19, b). This peak is not typically associated with cellulose. However, this may be attributable to the extraction process, with sodium hydroxide treatment of cellulose fibres reported to induce the formation of 'sodium cellulose', where the hydroxy groups ($-\text{OH}$) from the hydroxymethyl groups of glucopyranose residues are converted to a sodium salt ($-\text{O}^-\text{Na}^+$), or carbonyl groups are modified to carbonate ions (Oh *et al.*, 2005; Williams *et al.*, 2011; Maślowski, Miedzianowska and Strzelec, 2018). The formation of these groups has been attributed to a peak at 1596 cm^{-1} in cellulose previously in the literature, which may correspond to the peak observed in the extracts here (Oh *et al.*, 2005; Ramli *et al.*, 2015). This peak was not present in *Ulva*, perhaps due to the overlap with the two amine resonances.

Interestingly, the sugar backbone of the pure cellulose fibres was slightly more complex than the extracted samples, with additional peaks attributed to the expected CO stretching of sugars in this region observed at 1103 cm^{-1} and 1053 cm^{-1} , in addition to a CH rocking resonance at 982 cm^{-1} (Figure 4.19, c). However, this was attributed to the extraction protocol, with 6 M NaOH treatment of the pure cellulose control as performed in the sequential extraction protocol (§2.1.4.4) shown to alter the profile of the peaks in the region in a subsequent experiment (see Figure 4.28).

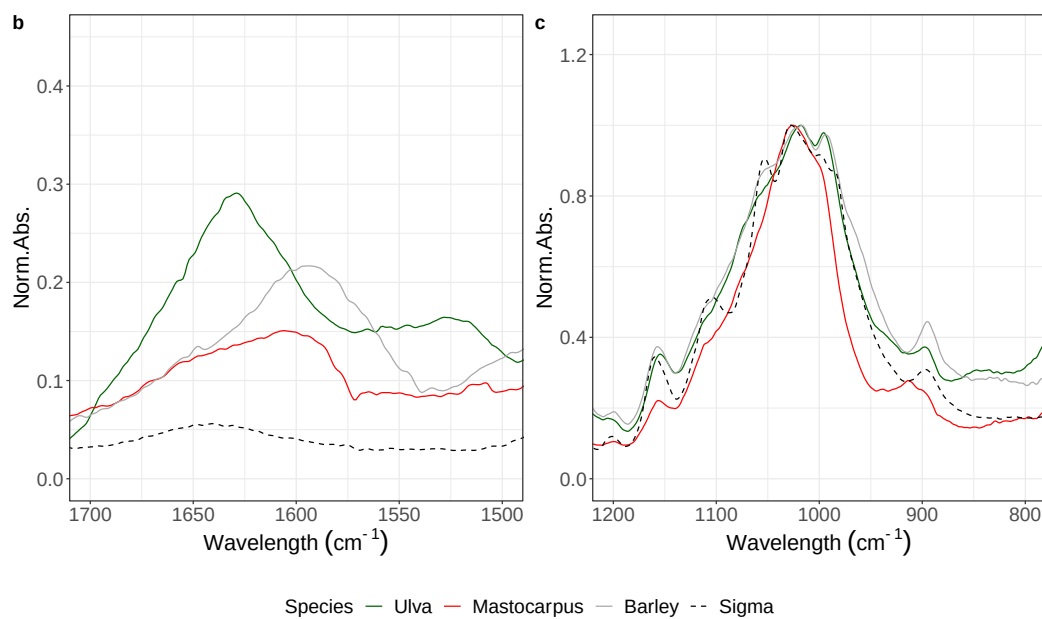
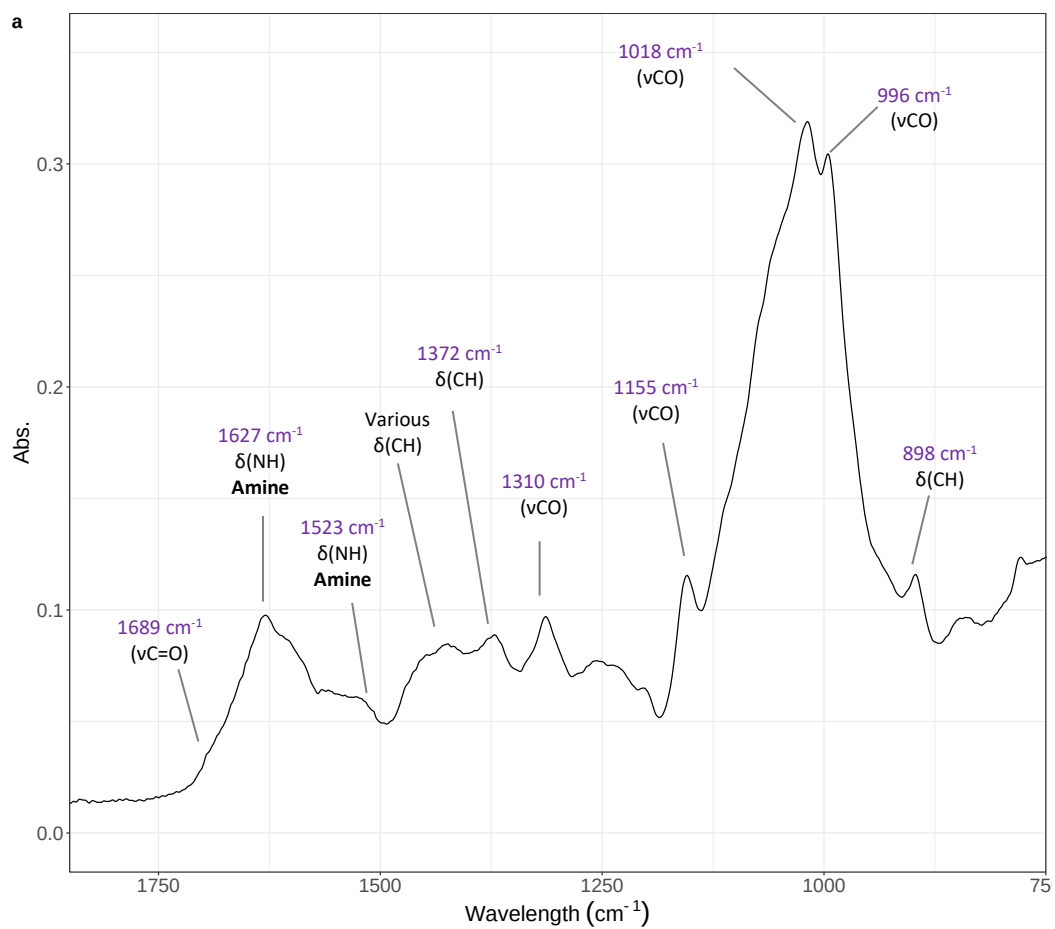


Figure 4.19. FTIR spectra of the α -cellulose fraction from *U. compressa*. The spectrum for the double bond and fingerprint region is presented in (a) and magnified in (b) and (c) for comparison with *M. stellatus* (red), barley straw (grey), and the pure cellulose standard (dotted black line). These magnified plots display improved resolution of the putative amide bands at 1627 cm^{-1} and 1523 cm^{-1} in *U. compressa* relative to the other species (a) and improved resolution of the 'carbohydrate backbone' region (b). Presented *U. compressa* spectra is a mean of spectra from extracts from 'unstressed' samples ($n=3$).

The most notable differences observed in the saline stress experiment were associated with resonances attributed to the proposed amide structure (Figure 4.20). Firstly, the shoulder at 1689 cm^{-1} attributed to an amide group ($\nu\text{C=O}$) was more prominent in the hyposaline stressed fraction (0 ‰) than in the corresponding control or hypersaline fraction. Secondly, unlike the control and hypersaline samples (90 ‰), where two discrete bands corresponding to the primary and secondary amine (NH) bands were observed at 1623 cm^{-1} and 1523 cm^{-1} , the hyposaline stressed α -cellulose had a broad peak spanning this region, with the secondary amine band observed as a shoulder at $\sim 1523\text{ cm}^{-1}$. Whilst the amine peaks appeared to increase in α -cellulose from both saline extremes relative to the control, the amide peak at 1689 cm^{-1} only appeared to be more prominent following hyposaline stress.

A broad signal was also observed from $\sim 1470\text{ cm}^{-1}$ to $\sim 1350\text{ cm}^{-1}$ in the hyposaline stressed sample specifically. Here, hyposaline-specific peaks were observed at 1463 cm^{-1} , 1437 cm^{-1} , 1411 cm^{-1} , and 1397 cm^{-1} that was not observed previously in the α -cellulose spectrum, consistent with various CH bending vibrations. A further peak at 1315 cm^{-1} was elevated in both hyposaline and hypersaline stress, corresponding with a CH_2 bending resonance identified from the cellulose fingerprint (Table 4.7). This was accompanied by a broad shoulder from $\sim 1260\text{ cm}^{-1}$ to $\sim 1205\text{ cm}^{-1}$ in the hyposaline stress sample, attributable to various C-O and C-OH stretches. Hypersaline stress appeared to induce increases in amine and C-O stretching specifically, but not the observed amide group or C-H bending (Figure 4.20, c). These changes are consistent with a stress-induced change in cellulose structure, and a possible increase in the hypothesised protein content, reflecting the observations from elemental analysis (Table 4.2). However, these structural changes are difficult to resolve with FTIR alone.

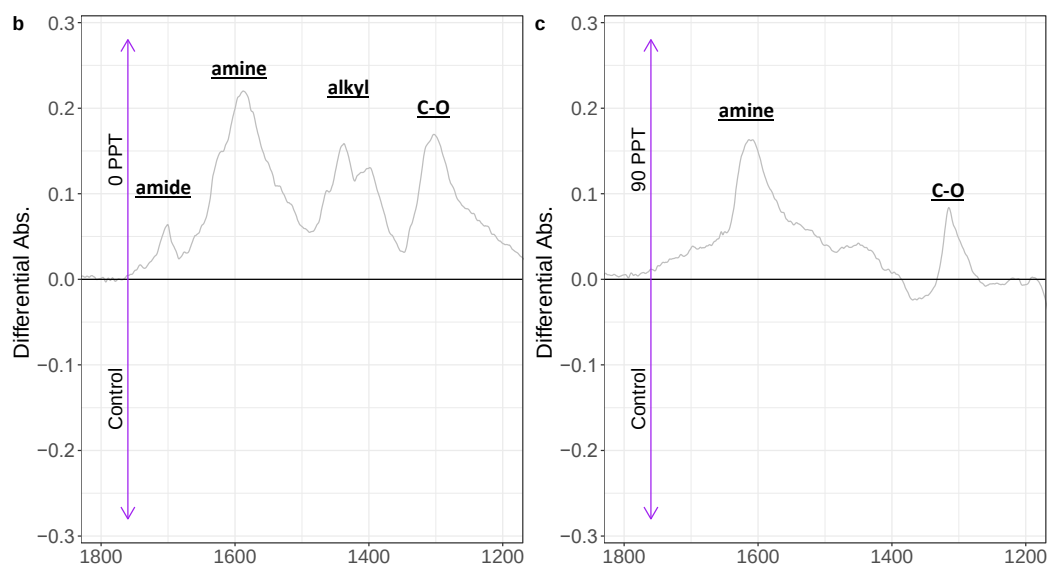
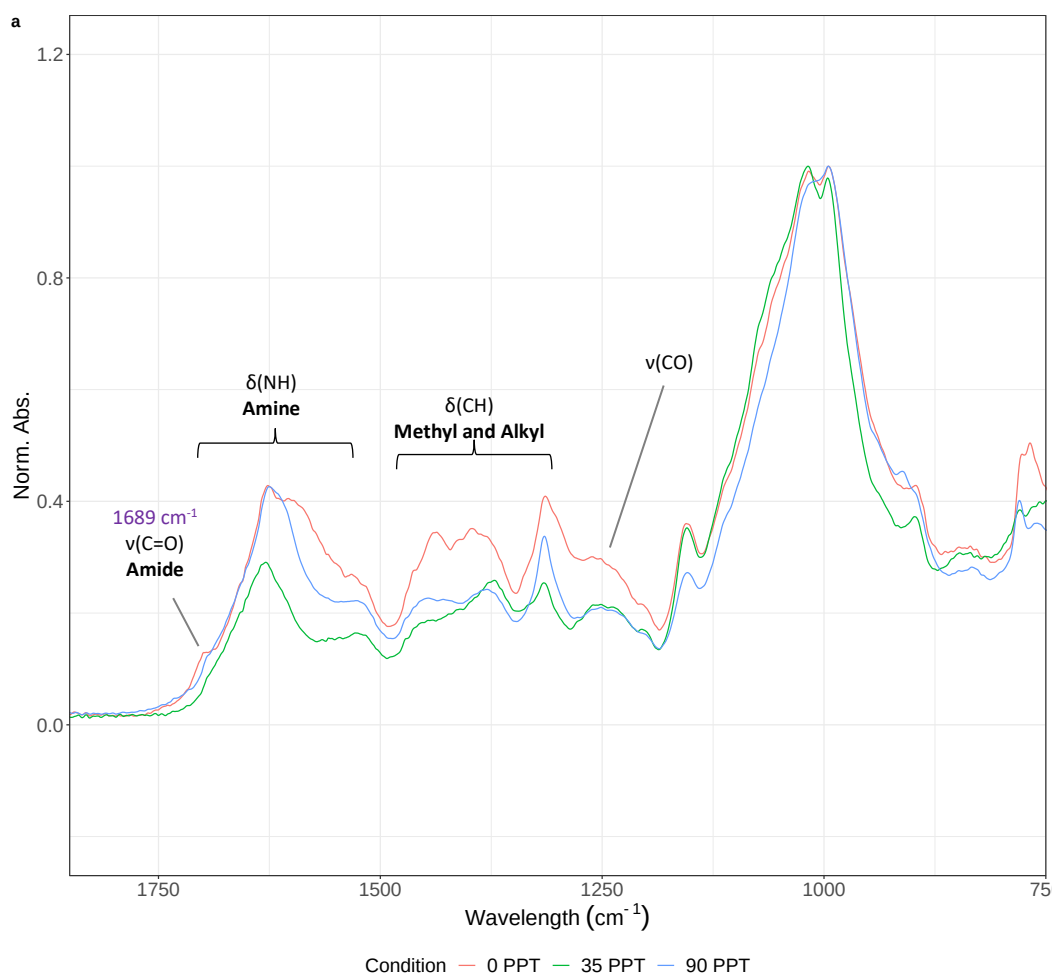


Figure 4.20. Comparison of the α -cellulose fraction of *U. compressa* in samples exposed to variability salinity. Cell wall fractionation was performed on a sample of *U. compressa* thalli exposed to hyposaline stress (0 ‰, red), control conditions (35 ‰, green), and hypersaline stress (90 ‰, blue). Saline solutions were prepared from artificial seawater solution (35 ‰), with hypersaline seawater prepared by blending artificial seawater with concentrated NaCl due to the limited solubility of sea salt mix (Sigma). Samples were incubated for 3 h in the respective media before extraction. The corresponding differential plots for the comparative plot in (a) are presented in (b) and (c) from 1800 cm^{-1} to 1200 cm^{-1} , calculated from *hyposaline* – *control* and *hypersaline* – *control* respectively.

4.4 Enzymatic Digest of Cell Wall Fractions

4.4.1 Cell Wall Enzymes

A series of enzymatic digestion experiments were performed on the polysaccharide-rich cell wall fractions of *U. compressa* (ulvan 1, ulvan 2, Ha, Hb, and α -cellulose) as it was hypothesised that if present, resonances from aromatic or lignin-like structures be masked by the signal from the ‘sugar backbone’ if they were present at relatively low levels. Previously, only the Hb fraction of barley straw displayed clear evidence of a lignin-like structure in the FTIR spectra (Figure 4.14), despite the positive identification of lignin in the corresponding α -cellulose fraction (see Chapter 3, Table 3.2). Cell wall fractions were hydrolysed with commercially available cell wall enzymes to perform this, and the insoluble residue was recovered if present. It was hypothesised that previously undetected structures might be identifiable after removing a significant amount of carbohydrate from the fraction.

No extractable insoluble residue was obtained following hydrolysis of the ulvan or alkali-soluble fraction (Hb) in *U. compressa*, indicating near full solubility of these fractions in acetate buffer and near full hydrolysis with Driselase (see Materials and Methods, section 2.1.4.4 for protocol). In the case of the Ha fraction, insufficient material was obtained from the extraction protocol to detect whether a residue could be obtained. The absence of an extractable insoluble residue for analysis in these samples likely indicated that the amount of ‘non-carbohydrate’ material in these fractions was negligible.

For the α -cellulose fraction, a small amount of ‘cellulase insoluble residue’ (CIR-1) was obtained following hydrolysis with Cellulase Onozuka R-10. This sample consisted of approximately 30 mg of a dark brown residue extracted following hydrolysis of 100 mg of starting material. A supplementary ‘lignin purification’ protocol was performed

on the CIR-1 sample (Hortling, Turunen and Sundquist, 1992), which involved extraction with 0.5 M NaOH, precipitation of solubilised material at pH 2.5 with HCl, washing, and lyophilisation. As this experiment was successful, a corresponding CIR-1 and CIR-2 (NaOH extracted) sample from barley straw α -cellulose was acquired for comparison.

As shown in Figure 4.21, where the spectra from the digested samples are compared to the pre-digest sample, the barley straw CIR-2 sample displayed strong evidence of lignin spectrum, where various peaks attributable to lignin and hydroxycinnamic acids were identified, including a carbonyl group (1640 cm^{-1}), aromatic ring bands (1595 cm^{-1} and 1505 cm^{-1}), and various C-O stretches and C-H bending corresponding to guaiacyl and syringyl monolignols from $\sim 1260\text{--}1120\text{ cm}^{-1}$ (Popescu *et al.*, 2007; Balciolu, Zimmermann and Kohler, 2015; Lu *et al.*, 2017; Md Salim, Asik and Sarjadi, 2021). These peaks are summarised in Table 4.8. Here, the supplementary sodium hydroxide extraction step also appeared to successfully remove noise from residual sugar signals still observable in the CIR-1 sample that impacted interpretation (not shown). Whilst direct covalent linkages between cellulose and lignin are reported (Zhou *et al.*, 2010), it cannot be determined from the present work whether this is the case here.

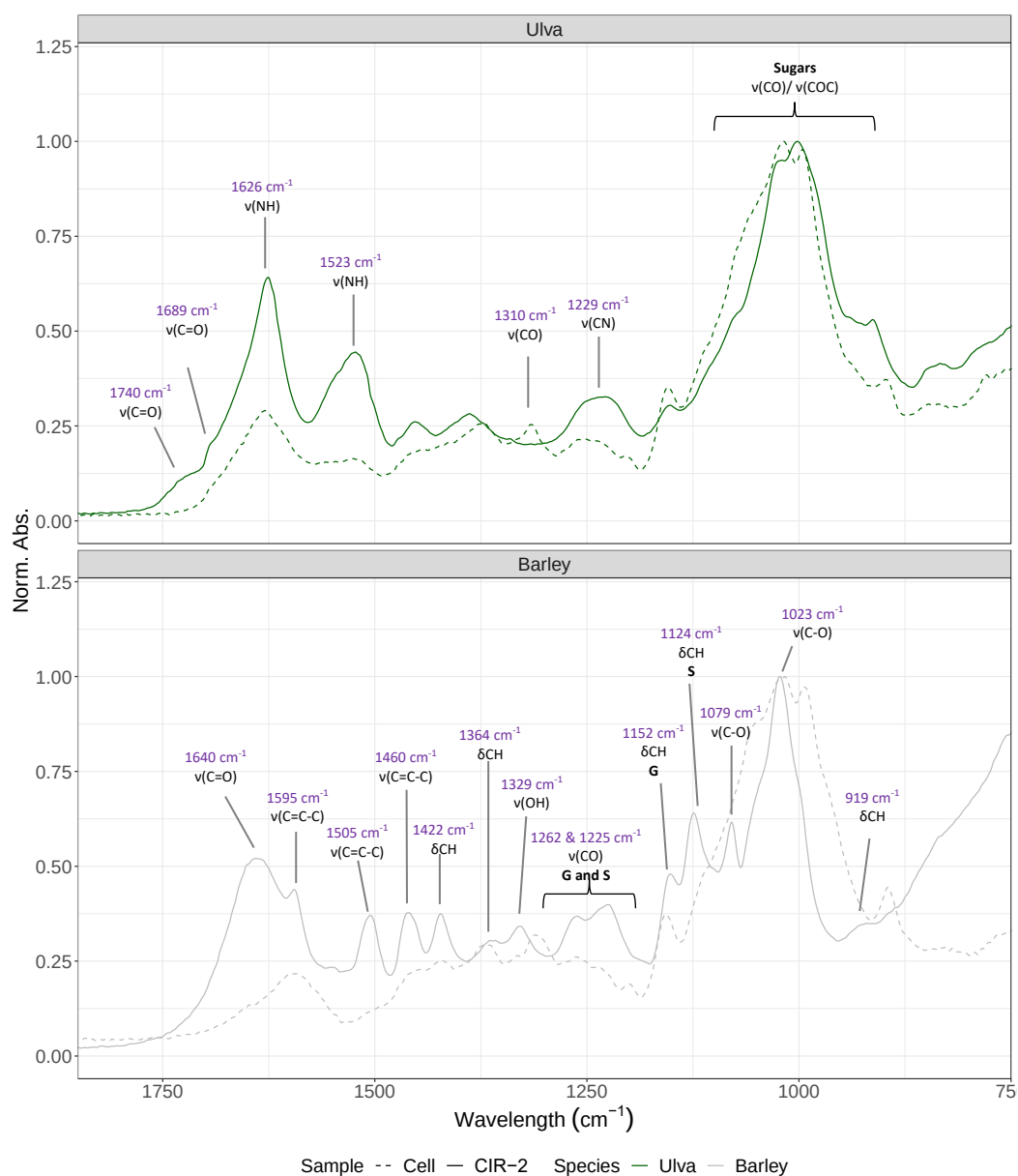


Figure 4.21. Comparison of the cellulase insoluble residue (CIR-2) acquired following cellulase digestion and purification of α -cellulose (solid line) and the 'pre-digest' α -cellulose fraction (dashed line) from *U. compressa* (green) and barley straw (grey). The CIR-2 sample corresponded to the 'cellulase insoluble' residue obtained after cellulase digestion of α -cellulose, followed by sodium hydroxide extraction, acid precipitation, washing, and lyophilisation.

Assignment	Functional Group	Peak (cm ⁻¹)
Hydroxy	vOH	3311
Methyl	vCH	2943
Ester	vC=O	1640
Aromatic Ring	v(C=C-C)	1595
Aromatic Ring	v(C=C-C)	1505
Aromatic Ring	v(C=C-C)	1460
Methyl from OMe (CH ₃)	δ _{as} CH	1422
Methyl from OMe (CH ₃) or CH from Cellulose	δ _s CH	1364
CH or OH from Phenolics ^a	δ(CH) or v(OH)	1329
OMe (Guaiacyl)	v(CO)	1262
OMe (Syringyl)	v(CO)	1225
Aromatic CH (Guaiacyl)	δ(CH)	1152
Aromatic CH (Syringyl)	δ(CH)	1124
Phenolic (-OH)	v(CO)	1079
Phenolic (-OH)	v(CO)	1023
Aromatic CH	δCH	919

Table 4.8. Assignations for the CIR-2 fraction in barley straw. Peaks were determined from previously generated reference spectra for kraft lignin (Table 4.3). (a) This peak was not identified in the reference spectra; likely assignations are a further δ(CH) or a v(OH) from a phenolic group (Schwanninger *et al.*, 2004).

None of the observed aromatic associated peaks could be identified in the corresponding *U. compressa* samples. By contrast, the increase in prominence of the two peaks attributed to NH scissoring at 1626 cm⁻¹ and 1523 cm⁻¹ in *U. compressa* CIR samples relative to undigested α -cellulose provided strong evidence that the hypothesised amine-containing compound in this fraction had been enriched successfully. The absence of these peaks in the corresponding barley straw samples indicated that the increase in these peaks was most likely due to the non-hydrolysable structure associated with this fraction and not due to contamination with residual enzyme, which appeared to be removed successfully after washing.

In addition to these differential peaks, the shoulder attributed to an amide functional group in the hyposaline-stressed *U. compressa* α -cellulose extract at 1689 cm⁻¹ was present in both the CIR samples (Figure 4.22), and an additional shoulder at 1740 cm⁻¹ was observed, consistent with an increased signal from carbonyl (ν C=O) groups. A broad peak was also apparent at 1229 cm⁻¹, which was assigned as a C-N stretch, possibly from an aromatic amine structure (Coates, 2006). Interestingly, the purification step appeared to change the profile of the observed sugar backbone somewhat from CIR-1 to CIR-2 (~1200-900 cm⁻¹), and the peak at 1310 cm⁻¹ attributed to C-O stretching of cellulose (Table 4.7) which increased in prominence following digestion in CIR-1 disappeared in the CIR-2 spectra. The persistence of the sugar backbone in the spectra from the post-digestion residue may be indicative of residual cellulose or enrichment of glycoproteins.

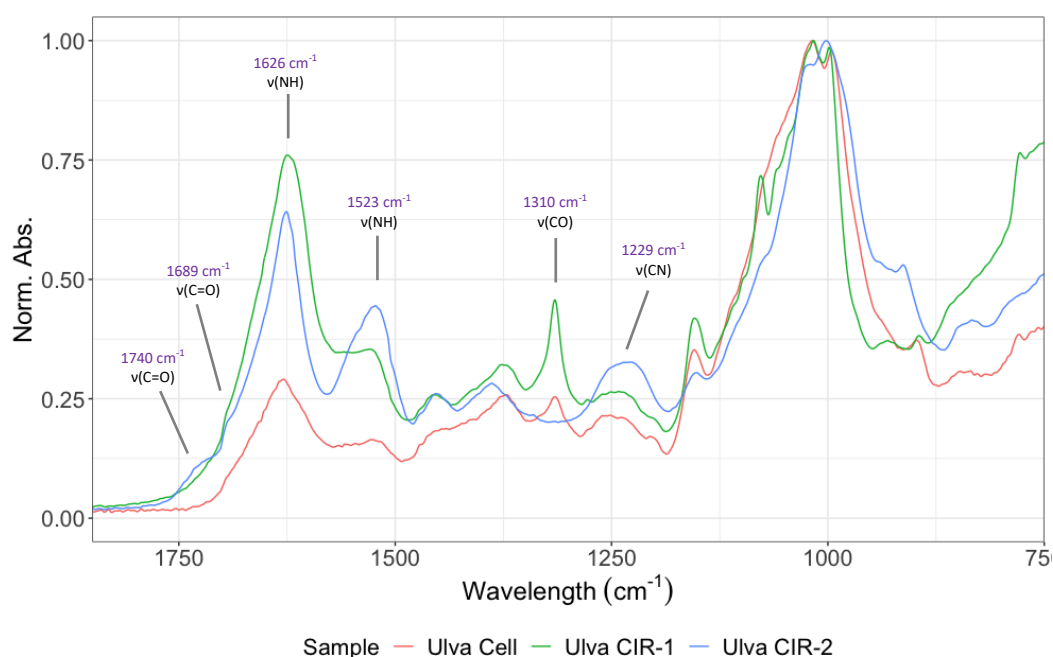


Figure 4.22. Comparison of pre-digest α -cellulose (red) and post-digest α -cellulose corresponding to cellulase insoluble residue-1 (CIR-1, green) and cellulase insoluble residue-2 (CIR-2, blue) in *U. compressa*. The CIR-1 sample corresponded to the ‘cellulase insoluble’ residue obtained after cellulase digestion of α -cellulose, which was washed several times with Milli-Q and lyophilised for analysis. The CIR-2 sample corresponded to the insoluble residue prepared by the same enzymatic hydrolysis, which was further processed by sodium hydroxide extraction and acid precipitation before washing and lyophilisation (Hortling, Turunen and Sundquist, 1992). Functional differences between the 3 samples were observed at 1740 cm^{-1} , 1689 cm^{-1} , 1626 cm^{-1} , 1523 cm^{-1} , 1310 cm^{-1} , and 1229 cm^{-1} with the 1310 cm^{-1} peak disappearing from the spectra after the supplementary purification protocol (CIR-2).

Finally, differences between barley straw and *U. compressa* CIR were observed in the single bond functional region of the spectrum (Figure 4.23). Whilst the barley straw CIR-2 sample had a broad OH stretch at 3311 cm^{-1} with the corresponding CH stretch at 2943 cm^{-1} , comparable to the kraft lignin spectrum, the corresponding *U. compressa* CIR-2 OH band appeared to have some contribution from NH stretching from a primary amine group, indicated by the sharper additional peak observed at 3276 cm^{-1} . These observations and the hyposaline stress-induced changes (Figure 4.20) provided further evidence for a protein structure in the insoluble fraction.

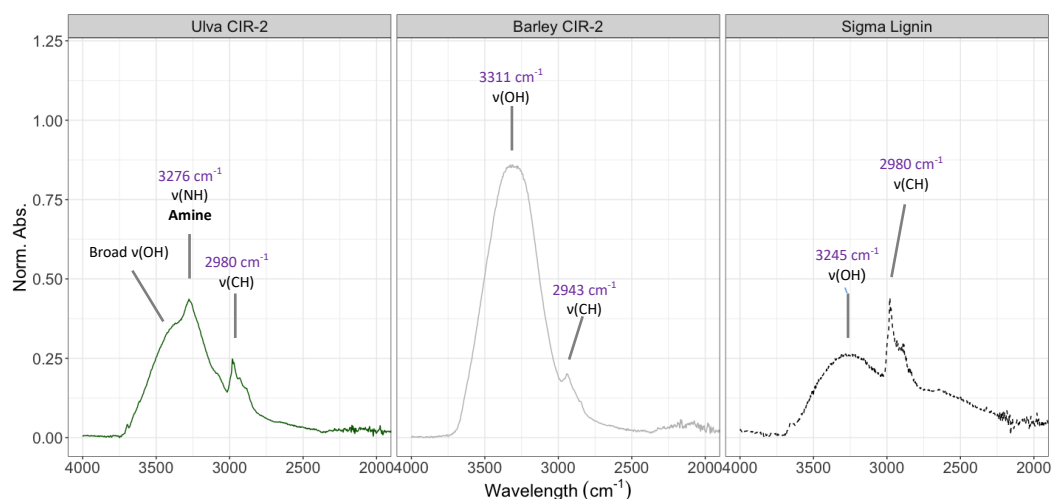


Figure 4.23. Comparison of the functional region of the FTIR spectra from the cellulase-insoluble residue (CIR-2) from *U. compressa* (green) and barley straw (grey) with the functional region from the kraft lignin standard (Figure 4.5).

4.4.2 Protease Digestion of Insoluble Fraction

Following this, a protease digest (proteinase K) was also performed on the *U. compressa* α -cellulose fraction to verify whether the identified C=O (1689 cm^{-1}) and NH stretches (1626 and 1530 cm^{-1}) were attributable to peptide bonds specifically or another amine containing metabolite. A similar approach is described by Mazurek and colleagues on petal biomass from *Arabidopsis thaliana* to verify the identity of protein-associated bands via FTIR, where a proteinase K digest was demonstrated to reduce the intensity of peaks at 1656 , 1627 , and 1540 cm^{-1} (Mazurek *et al.*, 2013). Here, it was hypothesised that these peaks would be removed from the spectra if hydrolysis of peptide bonds attributable to cellulose-associated protein occurred. As shown in Figure 4.24, the protease digest of the α -cellulose fraction appeared to induce changes that could be observed in the corresponding insoluble residue.

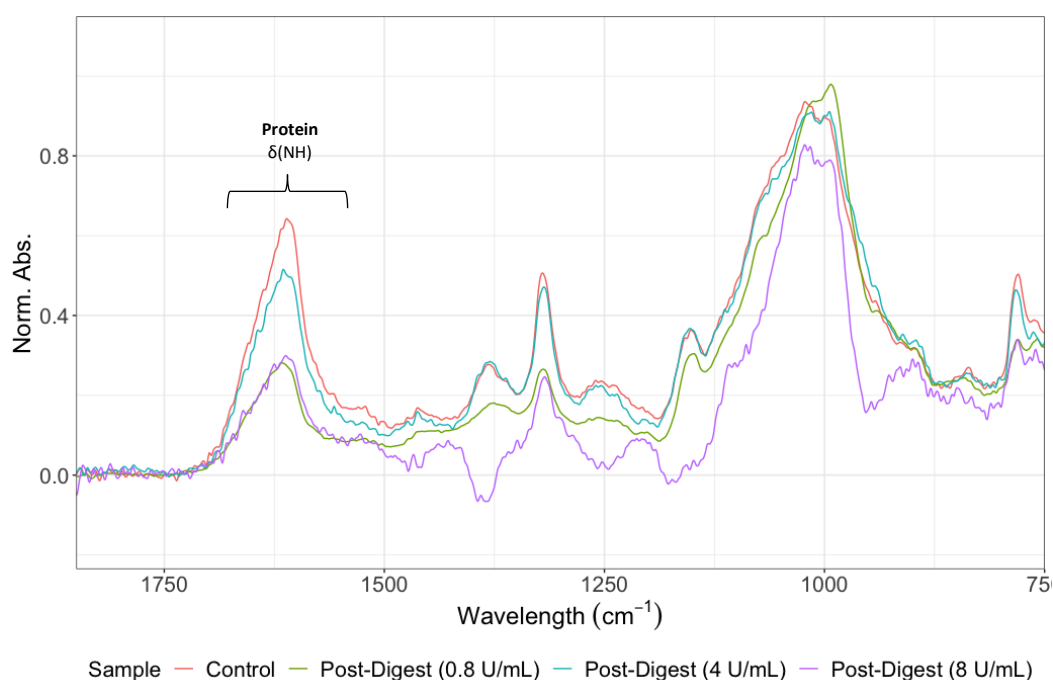


Figure 4.24. Comparison of the α -cellulose fraction before and after protease digestion. The insoluble residue was obtained following 2 h protease hydrolysis (50°C) of 20 mg α -cellulose fraction from *U. compressa* with 0.8 U/mL (green), 4 U/mL (blue), and 8 U/mL (purple) proteinase K in 50 mM Tris-HCl buffer containing 5 mM CaCl_2 . Protease was inactivated by a brief incubation step (10 mins) at 95°C. The insoluble residue was washed several times with Milli-Q and lyophilised before analysis. The control sample (red) was incubated in buffer without enzyme and was processed identically for comparison.

As shown in the resulting differential spectra (insoluble residue - control spectra, Figure 4.25), differential bands that were relatively more prominent pre-digest than post-digest included those at 1610 cm^{-1} , 1384 cm^{-1} , 1321 cm^{-1} , and 780 cm^{-1} . All differential bands were identified in the insoluble residue for the 0.8 U/mL and 20 U/mL reactions. However, only the 1610 cm^{-1} differential was observed in the 4 U/mL protease reaction, indicating that the changes induced were not necessarily dose-dependent.

Interestingly, there were no differential bands observed for the previously identified amide, secondary amine (NH), or C-N stretch bands (at 1689 cm^{-1} , 1530 cm^{-1} , and 1229 cm^{-1} , respectively), as identified in the original *U. compressa* α -cellulose, CIR-1, and CIR-2 spectra (Figure 4.25). However, the 1610 cm^{-1} differential appeared to correspond with the primary amine stretch (NH_2) observed at 1626 cm^{-1} . Whilst the differential band observed at 1321 cm^{-1} also increases in prominence in the same region in the CIR-1 sample at 1315 cm^{-1} and is within the expected region for CN stretches

(Coates, 2006), it disappeared following the sodium hydroxide solubilisation and acid precipitation required for CIR-2 preparation. Furthermore, this resonance overlaps with the known vCO fingerprint resonance for α -cellulose, and the differential is shifted relative to the CN stretch observed in CIR-2. Nonetheless, the differential spectra indicate that a compositional change is induced in the *U. compressa* α -cellulose fraction following protease treatment, most likely corresponding with the hydrolysis of cellulose-associated proteins.

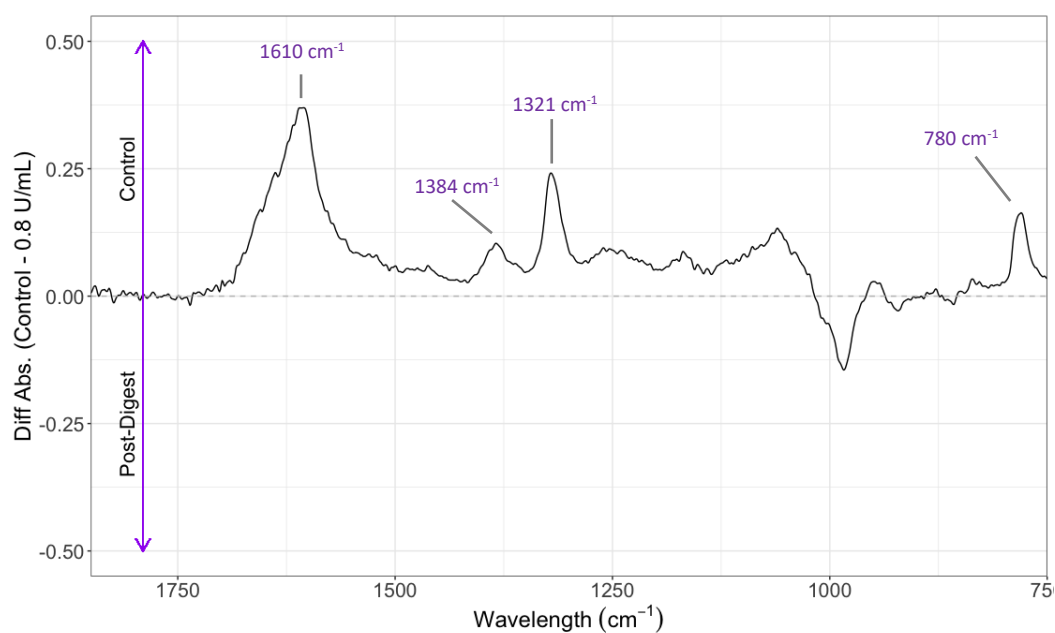


Figure 4.25. Differential plot calculated from spectral subtraction (*control – protease insoluble residue*) for the *U. compressa* α -cellulose fraction. In this plot, the ‘post-digest’ sample corresponds to the 0.8 U/mL protease digest (Figure 4.24). Differential peaks that are more prominent in the control are observed at 1610 cm^{-1} , 1384 cm^{-1} , 1321 cm^{-1} , and 780 cm^{-1} .

4.5 Effect of Extraction Protocol on Cell Wall Fractions

In addition to the described cell wall characterisation work, a few supplementary experiments were performed to test the effect of the cell wall fractionation protocol (§2.1.4.4) on the obtained *U. compressa* cell wall fractions to assess whether the protocol was suitable for future biological investigations.

For example, how algal polysaccharides are functionalised is poorly understood relative to plants, and based on my analyses, ATR-FTIR appears as a suitable tool to assess this topic further. However, to determine the suitability of the extraction protocol, it needed to be assessed whether the protocol removed functional groups of interest or induced artefacts in the spectra that would impact the resulting biological interpretations. This is now discussed.

4.5.1 Ester Groups

Two experiments were performed to assess whether the proposed esterification modification hypothesised to correspond with methylation or *O*-acetylation attributed to the 1644 cm⁻¹ and 1742 cm⁻¹ resonances in the ulvan 2 extracts were specific to ulvans (4.3.1). These ester resonances were not identified in spectra for *U. compressa* hemicellulose samples, which suggested that *Ulva* hemicelluloses were not *O*-acetylated or methylated to a notable extent, or that ester groups were removed during the 6 M NaOH extraction step by ester hydrolysis. Alkaline conditions, such as the 6 M NaOH treatment used to extract hemicelluloses, are known to remove ester groups and shift the corresponding carboxylate group in FTIR spectra (Gnanasambandam, R., Proctor, 2000; Liang *et al.*, 2022).

Esterification of plant hemicelluloses such as arabinoxylans and xyloglucan with lignin, ferulates, methyl, and *O*-acetyl groups has been characterised extensively in the literature and studied by FTIR analysis (Sun and Sun, 2002; Vogel, 2008; Sun *et al.*, 2011; Zhu *et al.*, 2014). Despite the presence of a likely ester group at ~1750 cm⁻¹ in the identically treated barley straw Hb sample spectra supporting the former explanation, and an ester peak appearing in the spectra for the 'metal recovery' Hb sample in *U. compressa* (Figure 4.16), experimental evidence was obtained to assess whether the lack of this peak in *U. compressa* Hb was a genuine biological observation.

In the first experiment, the ulvan 2 extract was subjected to the 6 M NaOH treatment before neutralisation with acetic acid, dialysis, and lyophilisation as

performed for hemicellulose extraction to assess whether the peaks of interest disappeared following processing. For the second experiment, an oxalate-insoluble residue was prepared to determine whether the assigned ester groups were still observable after the extraction of ulvans.

As shown in Figure 4.26, the ester shoulder visible at 1742 cm^{-1} disappeared in the NaOH treated ulvan 2 fraction. Furthermore, this treatment appeared to induce a shift and increase in prominence of both the asymmetric and symmetric carboxyl peaks, consistent with the previously described removal of esters in pectins. Interestingly, the peak at 1530 cm^{-1} attributed to a secondary amine group was also lost post-treatment, consistent with the removal of ulvan-associated proteins.

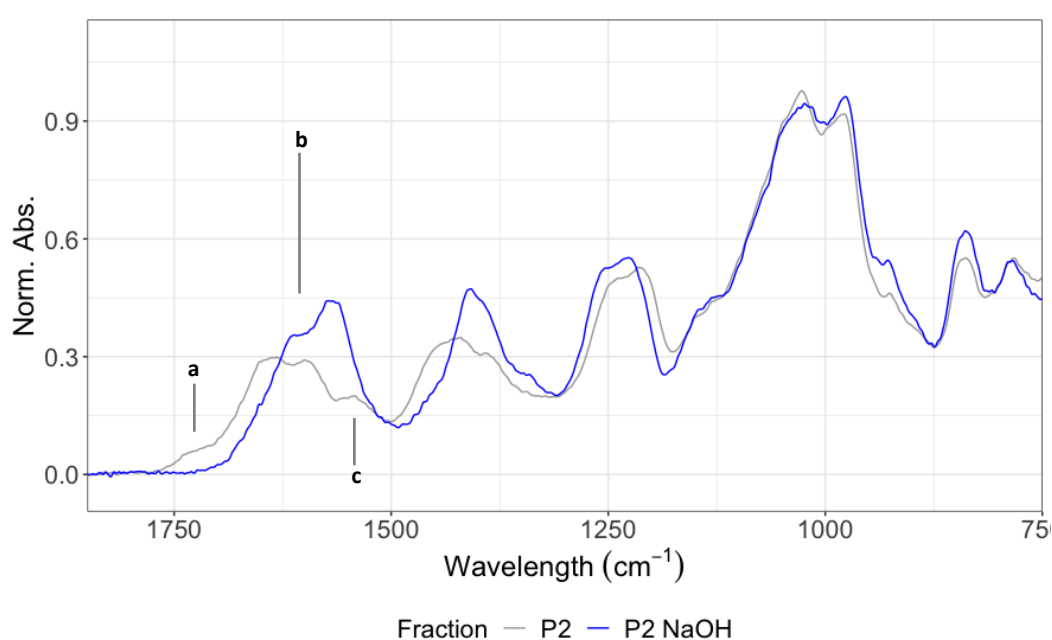


Figure 4.26. Comparison of the slowly oxalate soluble fraction of *U. compressa* before (grey) and after (blue) a supplementary treatment with 6 M NaOH as performed for hemicellulose extraction. As performed for hemicellulose extraction, P2 was incubated for 72 h at 37°C in 6 M NaOH before neutralisation with glacial acetic acid, dialysis to remove salts, and lyophilisation. Functional differences are observed following NaOH treatment, including the loss of the ester peak at 1740 cm^{-1} (a), a shift in carboxyl bands (b), and a loss of the secondary amine peak at 1530 cm^{-1} (c).

Interpreting the spectra from the oxalate-insoluble experiment was slightly more challenging, with the observed ester shoulder not readily visible in the alcohol-insoluble residue spectra (Figure 4.27). However, following removal of the ulvan 1 and ulvan 2 fractions, this shoulder becomes visible in the oxalate-insoluble residue at $\sim 1740\text{ cm}^{-1}$. Other changes observed in the oxalate-insoluble residue appear to reflect the increased contribution of α -cellulose resonances as ulvan is removed; for example, the peak at 1317 cm^{-1} attributed to CH_2 bending of cellulose I (Carrillo *et al.*, 2004; Schwanninger *et al.*, 2004; Popescu *et al.*, 2007). Interestingly, the region associated with the peaks associated with sulfation ($\nu\text{S=O}$) at 1250 cm^{-1} and 1216 cm^{-1} appeared less prominent around the 1216 cm^{-1} peak in the oxalate-insoluble residue, consistent with the removal of sulphated material.

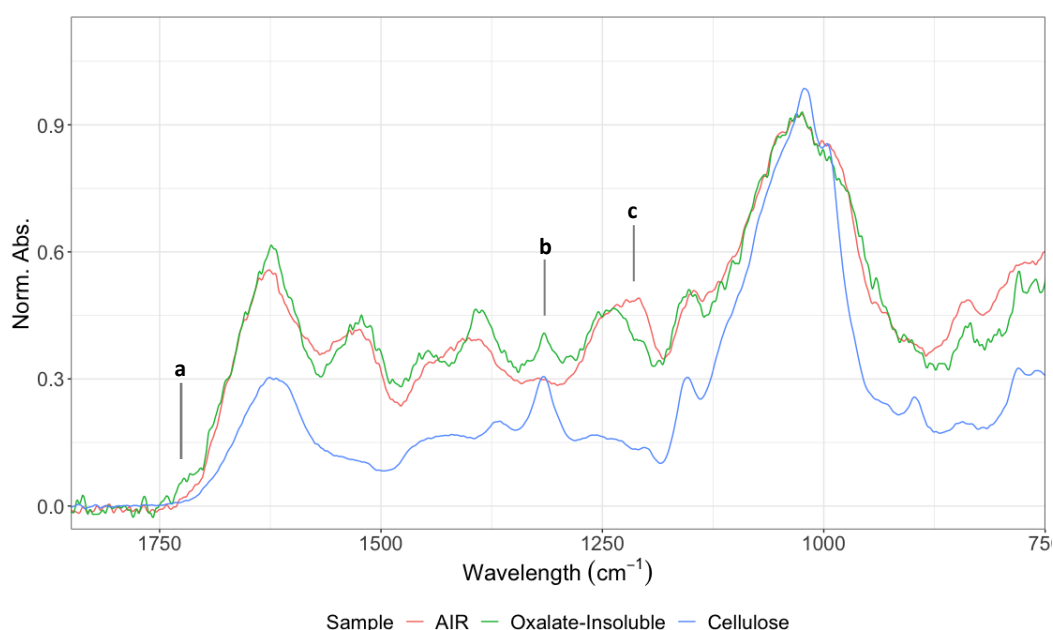


Figure 4.27. Comparison of the *U. compressa* alcohol insoluble residue (AIR, red), oxalate insoluble residue (green), and insoluble residue (α -cellulose, blue). A peak consistent with esters is present in oxalate-insoluble residue but not the AIR or α -cellulose fraction (a). Removal of oxalate soluble material results in increased contribution from α -cellulose in the infrared spectra, as shown by the appearance of a peak at 1317 cm^{-1} in the oxalate-insoluble residue (b). The relative intensity of the sulfate stretches at $1250\text{--}1216\text{ cm}^{-1}$ appears to change in the oxalate-insoluble residue, possibly consistent with a change in sulfate content.

As a result, it appears that the material that segregates into the hemicellulose fraction in *U. compressa* is esterified, but these groups are removed by the alkaline conditions required to extract them. It is unclear from this data how much ulvan segregates into the alkali-fraction fraction, but with 'pectic' peaks still present in the oxalate-insoluble fraction, it is likely that some ulvans persist after the two oxalate extractions.

4.5.2 Artefacts

Common extraction protocols for cellulosic material, including hot alkaline treatment, are known to induce the formation of aromatic structures (Bikova and Treimanis, 2004; Grönroos *et al.*, 2018). Whilst no obvious aromatic associated resonances were identified in the spectra for the extracted α -cellulose samples, and alkaline extraction was performed at a relatively moderate temperature (37°C), the effect of the 6 M NaOH treatment on the insoluble α -cellulose fraction was nonetheless tested using a pure cellulose standard (cellulose fibres, Sigma). This was performed to assess if any of the assignments reported in this chapter for α -cellulose could be attributable to artefacts. As shown in

Figure 4.28, whilst sodium hydroxide treatment appeared to induce changes in the cellulose spectra, no new peaks were identified in the sodium hydroxide treated spectra that were not observed in the untreated control, increasing the confidence that the assignments in this chapter reflected the genuine properties of *U. compressa* α -cellulose. Instead, the changes observed correlated with a loss of peaks from the cellulose fingerprint associated with CH bending at 1428 cm^{-1} , 1335 cm^{-1} , and 1315 cm^{-1} and a shift in peaks from the carbohydrate backbone, consistent with degradation.

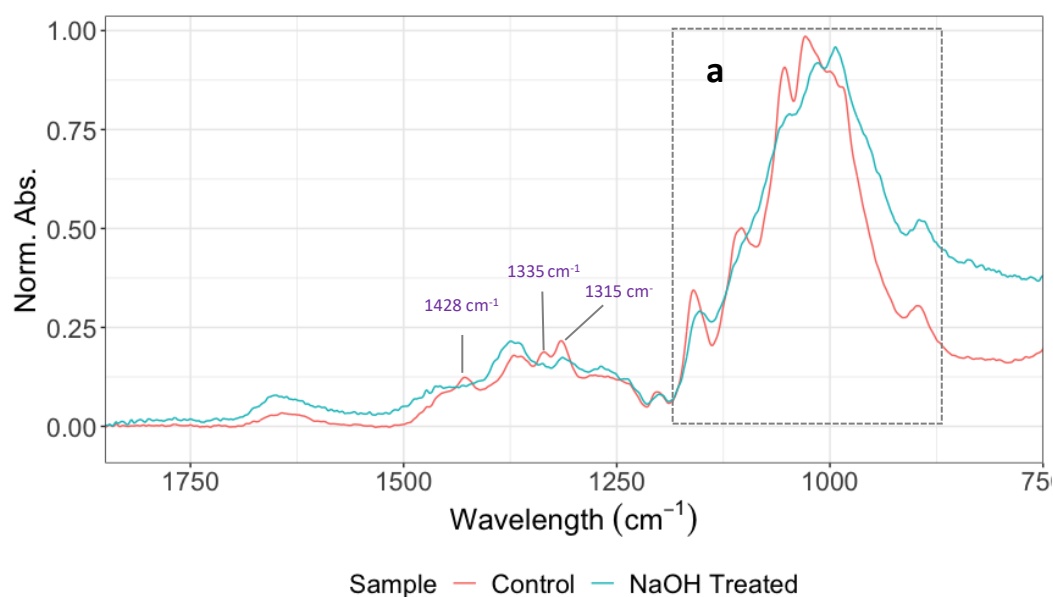


Figure 4.28. Cellulose (fibres) before and after 6 M NaOH treatment, neutralisation, and lyophilisation. Labelled peaks correspond to various δ CH resonances from the cellulose fingerprint that were lost following NaOH treatment, including the bands at 1428 cm^{-1} , 1335 cm^{-1} , and 1315 cm^{-1} . The box (a) highlights the various C-O and C-O-C shifts observed post-treatment in the ‘carbohydrate backbone’ region.

4.5.3 Yield

In the previous chapter, it was noted that poor recovery was observed during the cell wall fractionation process, particularly for extractions performed with *U. compressa* (Chapter 3, Figure 3.13). The low yields were initially attributed to loss of material through dialysis, with the hypothesis being that the 6 M NaOH treatment caused degradation of cell wall polysaccharides into smaller molecular weight structures that were not retained by dialysis tubing. Furthermore, as the ‘AIR’ represented a crude extract, the recovery of ~25% (w/w) cell wall polysaccharides for *U. compressa* may not be indicative of a considerable loss in cell wall material, with the ‘cell wall polysaccharide’ content of *Ulva* reported varying ~30-55% (w/w) of dry mass (Lahaye and Robic, 2007). For example, I have estimated that ~25% of *Ulva* AIR corresponds to protein in this chapter from elemental analysis (Table 4.2).

Whilst the presence of lignin in the ‘lignin positive’ control was verified in cell wall fractions by biochemical tests and now by FTIR, showing that this process successfully extracted lignin, this loss nonetheless required further investigation to assess the extraction protocol’s suitability for future research into the *Ulva* cell wall.

Firstly, the two supplementary extraction experiments performed in this chapter indicated that the loss occurred post- NaOH treatment as hypothesised. Firstly, in the treatment of the slowly oxalate soluble fraction with NaOH (Figure 4.26), a recovery of 25% (w/w) was observed (starting biomass: 100 mg, recovered biomass: 25 mg) following alkaline treatment, neutralisation, dialysis, and lyophilisation as described in the fractionation protocol (§2.1.4.4). This observation indicated that ulvan had been hydrolysed by NaOH treatment and lost via dialysis, as this material had already been recovered through dialysis during oxalate extraction.

Secondly, the mass of 'oxalate insoluble' residue (Figure 4.27) after both the 2 h and 16 h oxalate extractions was 83% (w/w) of the starting AIR mass (starting: 2 g, recovery: 1.66 g). Therefore, most loss appears to occur after the sodium hydroxide treatment. As stated in 4.5.1, there appears to be a contribution from 'pectic' polysaccharides in the oxalate-insoluble residue, possibly indicating that oxalate-insoluble ulvans persisted in the sample. As shown by the supplementary treatment of a ulvan-associated fraction (Figure 4.26), these polysaccharides might degrade during hemicellulose extraction, possibly explaining the limited recovery. These may alternatively represent glucuronans, but this is known to be a minor fraction in the *Ulva* cell wall (Pau-Roblot *et al.*, 2002; Lahaye and Robic, 2007; Redouan *et al.*, 2009). Modifying the protocol may be required to solubilise these hypothesised ulvans, perhaps by increasing oxalate-extraction duration or trialling a mild alkaline extraction step (Hernández-Garibay, Zertuche-González and Pacheco-Ruíz, 2011; Kidgell *et al.*, 2019).

Analysis of the Alcohol Insoluble Residue

In addition, the ATR-FTIR spectra from the pre-ethanol-washed biomass and the pre-fractionated AIR were compared to the cell wall fractions to assess how spectra changed throughout the extraction process to see if any important functional groups were lost.

Firstly, biomass acquired before ethanol washing was compared to the AIR (Figure 4.29, a). Overall, there appeared to be no major changes in the functional groups observed, with both samples appearing to have a series of broad overlapping peaks typical of a biological sample. The predominant signal appears to be from carbohydrates, resembling the 'oxalate-soluble fraction', with evidence of the sulfate peaks observed at $\sim 1250\text{ cm}^{-1}$, consistent with the observations presented in Figure 4.7.

There is also evidence of protein from an NH bend at $\sim 1530\text{ cm}^{-1}$ in both samples. The unprocessed biomass had a carbonyl peak (at $\sim 1740\text{ cm}^{-1}$) that disappeared after ethanol washing, probably attributable to pigments and other ethanol soluble metabolites, as an ester peak in this region is observed in extracted ulvans (Figure 4.7). The second difference between the spectra was observed over the carbohydrate backbone region, where a shift in peak profile was observed, also consistent with the removal of pigments, as chlorophyll and other photosynthetic pigments display C-O stretches over this region (Petrovic, Nikolic and Markovic, 2006). The similarities between the spectra appear to indicate that the AIR fraction has a similar biochemical composition to starting biomass, consistent with the observation that only $\sim 20\%$ (w/w) of biomass was removed during the ethanol washing protocol.

Interestingly, whilst there was no evidence of aromatics in the *U. compressa* AIR or pre-ethanol-washed biomass, there was convincing evidence of lignin in the pre-fractionated barley straw sample (Figure 4.29, b). This observation was consistent with the positive phloroglucinol-HCl test performed in the previous chapter (Chapter 3, Table 3.2). As performed previously for the individual cell wall fractions (§4.4), the AIR was also subjected to an enzymatic digest to facilitate FTIR analysis of the insoluble residue. Here, increases in the prominence of ester-associated, carboxy-associated, and amide-associated peaks in the normalised spectrum were observed (Figure 4.30). However, again, there was no evidence of aromatics in the resulting spectra. As no peaks of interest were present in the AIR that could not be identified in the cell wall fractions, the pooled wash sample obtained at the end of the fractionation process was analysed to assess what was removed from the AIR but not associated with the polysaccharide fractions.

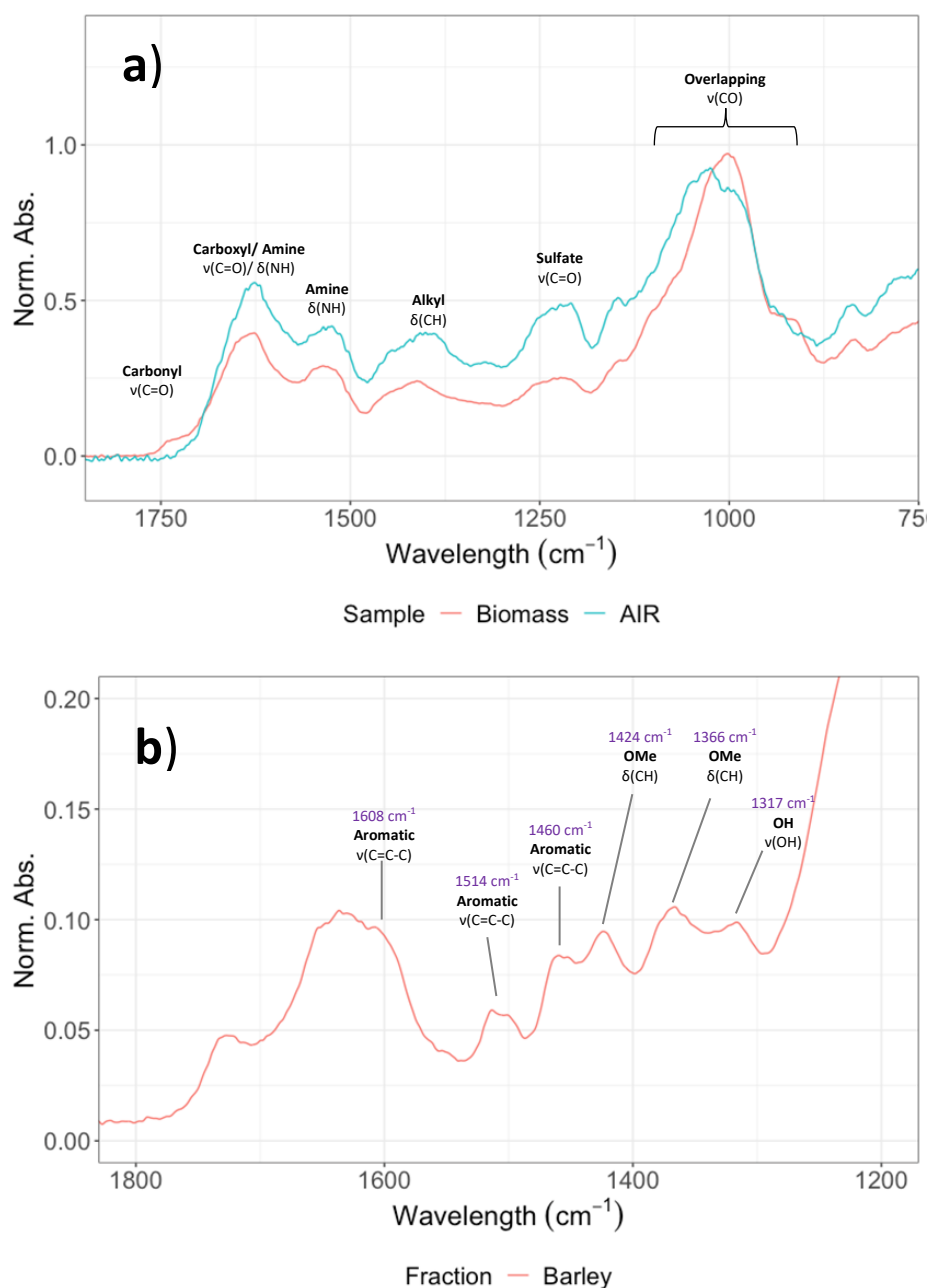


Figure 4.29. Analysis of the alcohol insoluble residue. The spectrum from *U. compressa* biomass before ethanol and acetone washing is compared to the alcohol insoluble residue acquired after this process (a). Vibrations corresponding to lignin are identified in unfractionated barley straw (b).

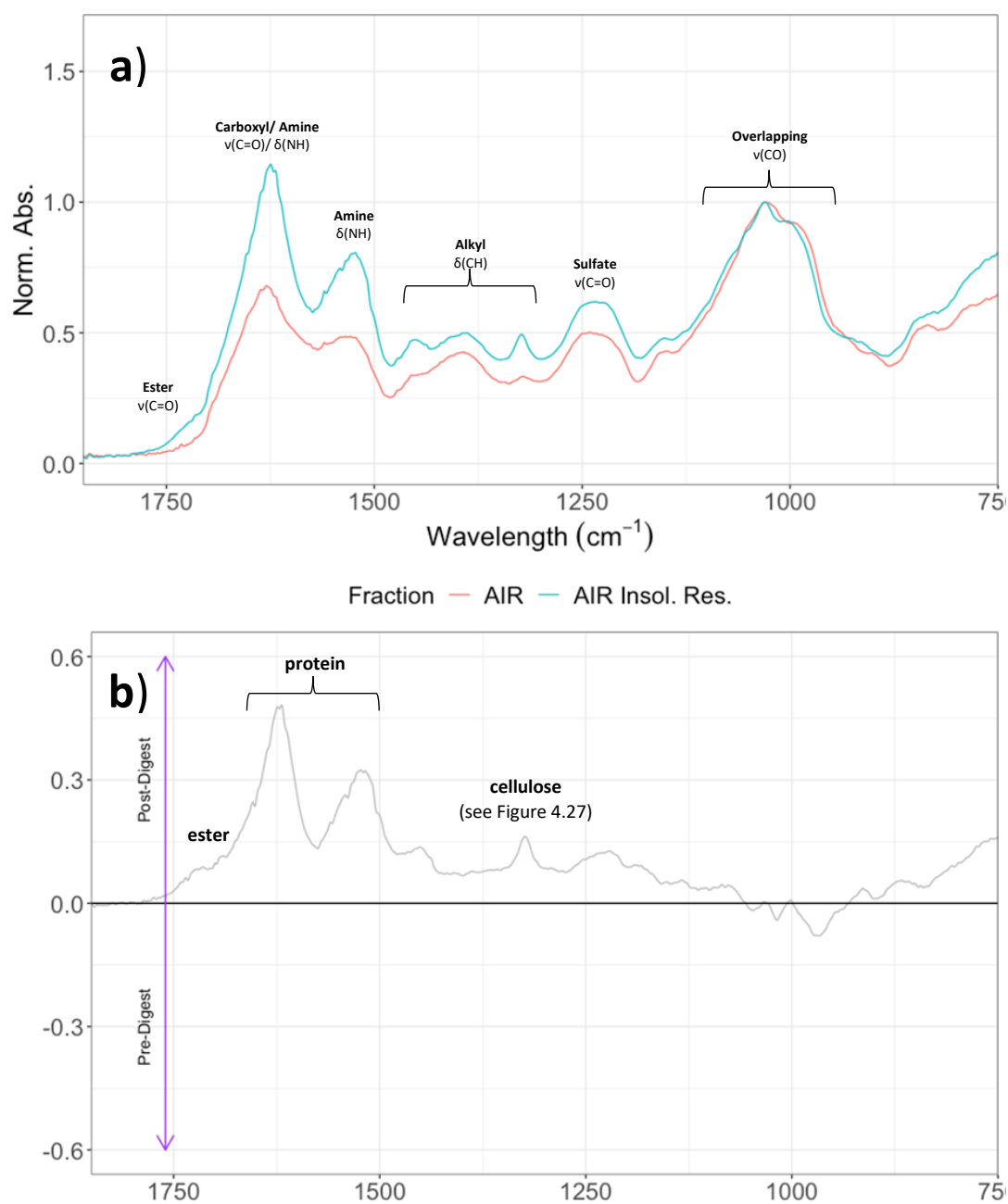


Figure 4.30. Enzymatic digestion of the *U. compressa* alcohol insoluble residue. The alcohol insoluble residue was digested with Driselase by the previously described methods (Methods and Materials, section 2.1.4.4), and the FTIR spectrum from the Driselase insoluble residue was compared to the pre-digest sample (a). The corresponding differential spectra is presented, displaying the increase in ester and protein associated peaks in the Driselase insoluble residue (b)

Analysis of the Wash Fraction

It was hypothesised that the wash fraction would provide insights into what material was degraded during the fractionation process. The wash was recovered following sodium hydroxide extraction of the hemicellulose fraction, where the resulting insoluble residue was washed several times, and the used rinse was pooled, dialysed, and lyophilised for analysis. At the outset of this work, it was uncertain what this fraction would correspond to; degraded polysaccharides, structures liberated by the sodium hydroxide treatment, and other extractives not removed by ethanol washing were hypothesised as likely candidates (O'Rourke *et al.*, 2015). Here, the low recovery for wash fractions of ~4% (w/w) (Chapter 3, Figure 3.13) is consistent with these hypotheses as 'degraded' polymers would not be retained by dialysis. The elemental analysis (Table 4.3) may also be indicative of degradation – with the lower estimated carbon content (19-28%) than the other fractions provided an initial indication that there was not necessarily a significant biopolymer component in the wash. Interestingly, the nitrogen content was variable – possibly indicative of stress-induced changes in cell wall composition and indicative of protein degradation during the sodium hydroxide treatment.

These observations were verified by FTIR analysis. Whilst identifying the peaks in the wash spectra was difficult due to the likely heterogeneity of the wash sample, clear peaks consistent with amine groups were observed in all *U. compressa* wash samples (Figure 4.31). The sharp intensity observed peaks appeared more consistent with small molecules such as amino acids rather than a large macromolecule with broad overlapping bands. Furthermore, whilst peaks ~1630 cm⁻¹ were observed for primary amine scissoring (-NH₂), there was no prominent secondary amine peak at ~1520 cm⁻¹ (-NH) in any of the samples, consistent with structures being 'free' amino acids rather than connected by peptide bonds. The primary amine band was accompanied by a peak at ~1320 cm⁻¹, attributable to a C-N stretching vibration (Coates, 2006).

The increased intensity of these peaks in the hypersaline wash compared to the control and hyposaline sample was also consistent with the elevated nitrogen estimated by elemental analysis, which increases the confidence that these peaks are amine-associated. Furthermore, the spectral region attributed to the sugar backbone (~1200 cm⁻¹ to 900 cm⁻¹) was far less prominent than the other cell wall fractions, demonstrating a different spectral profile – consistent with a general absence of a complex biopolymer in this fraction and the absence of glycosidic linkages. As shown in the functional region

(Figure 4.31, a), there did not appear to be a polysaccharide-associated OH stretch in the hypersaline stressed wash. However, the peak observed is too low to be attributable to an NH stretch. Instead, stretches in this region as typically attributed to C-H from alkenes (C=C-C), which may indicate lipids or an aromatic ring (Coates, 2006). Whilst there was a peak at $\sim 1600\text{ cm}^{-1}$, the lack of a peak at $\sim 1500\text{ cm}^{-1}$ suggested that this was more likely attributable to an unsaturated fatty acid structure than an aromatic ring (Table 4.1). This was supported by the peak at 1460 cm^{-1} identified in the hypersaline stressed wash sample exclusively, which has been attributed to CH_2 bending of fatty acids in the literature (Dreissig *et al.*, 2009; Forfang *et al.*, 2017; Shapaval *et al.*, 2019).

Furthermore, carboxyl resonances were identified in all samples ($\sim 1680\text{ cm}^{-1}$); however, it is not possible to attribute this to fatty acid specifically nor distinguish it from carboxyl groups in the predicted amino acids. Lipid accumulation is widely reported in algae in response to variable conditions (Guschina and Harwood, 2006; Elsayed *et al.*, 2017; Muzika William A. and Khan, 2017; Kostetsky *et al.*, 2018; You *et al.*, 2019; Ischebeck *et al.*, 2020). Sharp peaks were also observed at 820 cm^{-1} and 780 cm^{-1} , respectively, in all fractions (although the 820 cm^{-1} peak was absent in the hypersaline stressed sample), possibly attributed to P-O stretching of phospholipids and CH_2 rocking from fatty acids (Dreissig *et al.*, 2009).

Despite the compositional differences of the cell wall fractions, the barley straw wash spectrum was mainly comparable to that of the *U. compressa* wash, indicating that similar components were segregated into the fraction during the fractionation protocol (Figure 4.32). Whilst peaks at 1598 cm^{-1} and 1466 cm^{-1} were identified in the barley straw lignin profile (Figure 4.21), the absence of an accompanying aromatic stretch at 1500 cm^{-1} and the expected OMe and phenolic resonances in the spectrum (Table 4.8) indicated that these peaks were more likely attributable to lipids as observed in *U. compressa*. The reported lipid content of barley straw is low ($\sim 2\%$); however, the wash is only a small proportion of total biomass (Chapter 3, Figure 3.13). The absence of an aromatic signal in the wash may suggest that lignin segregates with the associated cell wall polysaccharides, specifically during the fractionation process. Finally, as with *U. compressa*, there was no strong evidence of glycosidic linkages, which indicated that polysaccharides had been degraded.

As a result, it appears that there is evidence of protein degradation and evidence of fatty acids in *Ulva* wash, which provides supporting evidence to the assumption that the pre-fractionated AIR is not just cell wall material and that the fractionation process is

selective for cell wall polysaccharides and associated structures such as polyphenols and cell wall protein, which ATR-FTIR has successfully identified. The next chapter will assess the wash more thoroughly via NMR analysis.

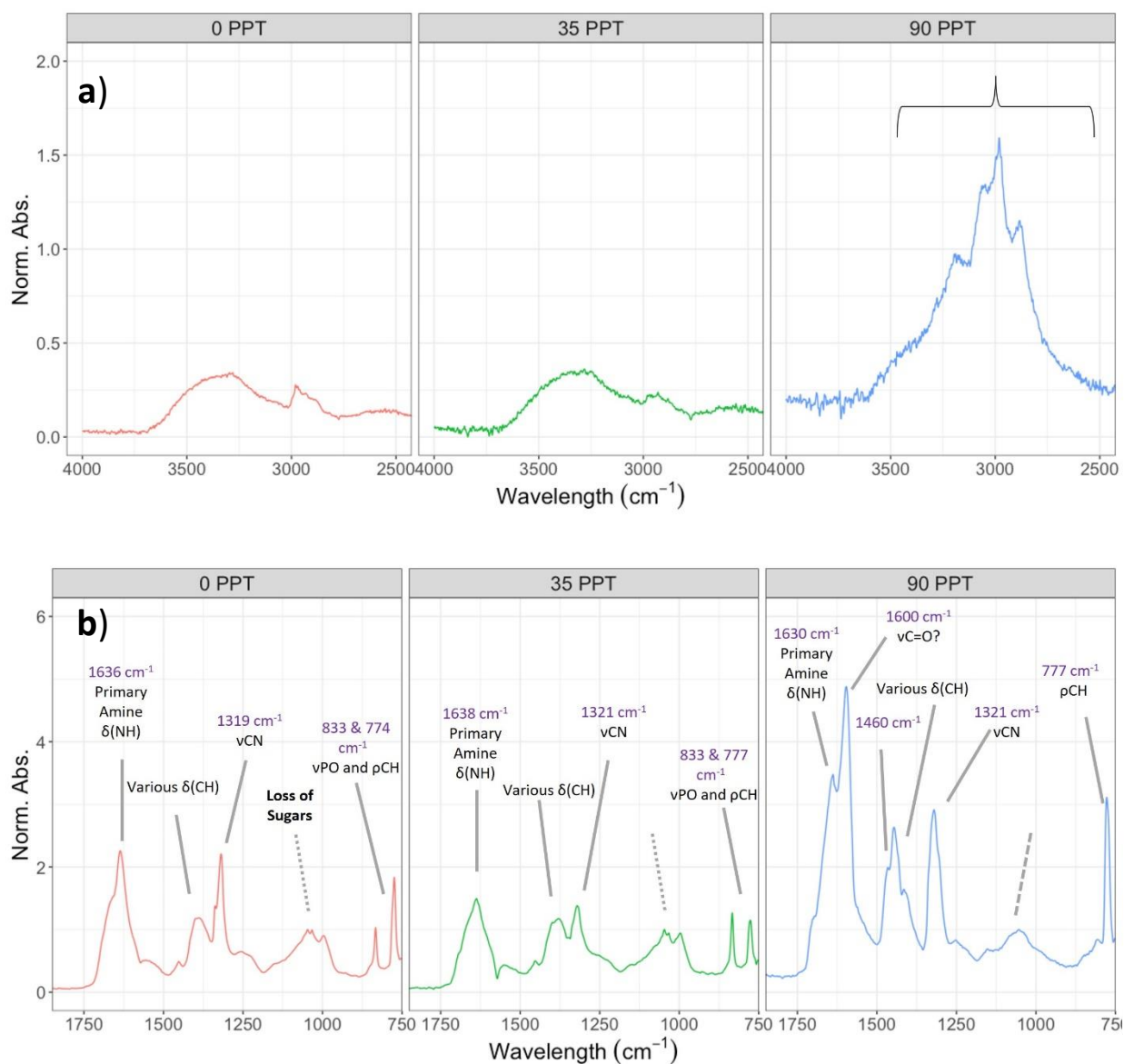


Figure 4.31. Analysis of the *U. compressa* pooled wash fractions. The functional region of the infrared spectrum is presented in a), and the corresponding fingerprint region is presented in b).

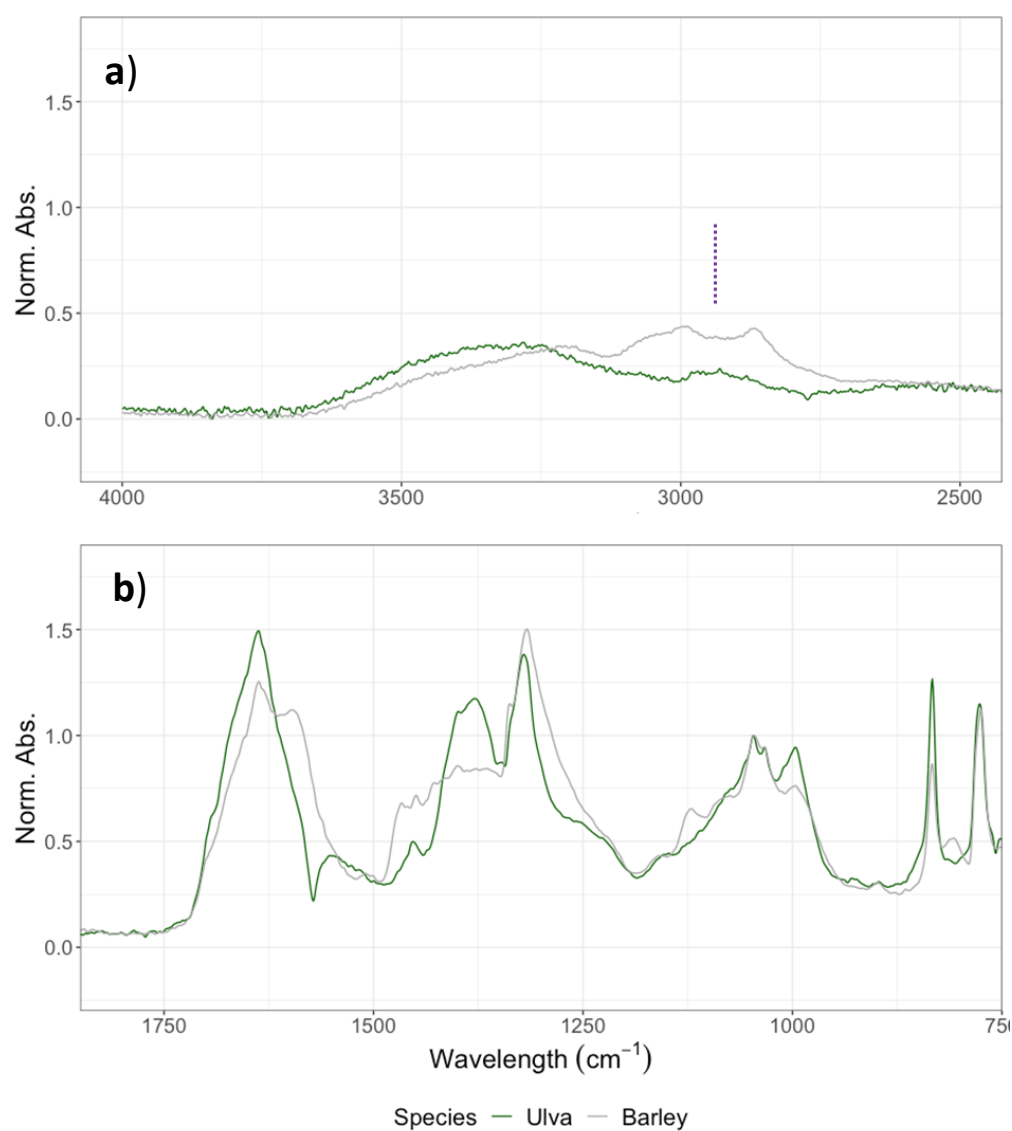


Figure 4.32. Comparison of the *U. compressa* (green) and barley straw (grey) pooled wash fractions. The functional region of the infrared spectrum is presented in a), and the corresponding fingerprint region is presented in b). The dashed purple line identifies a possible CH stretch from an alkene structure, consisted with unsaturated fatty acids (Coates, 2006)

4.6 Protein Isolation

To verify the observations from elemental analysis (Table 4.2) and subsequent FTIR analysis, a series of protein isolations were trialled on the *Ulva* cell wall fractions to assess if protein could be isolated and quantified.

However, protein extractions could not be performed directly on the ulvan and hemicellulose (Ha and Hb) due to the low yield obtained for these samples (see Chapter 3, Figure 3.13) and their likely solubility in protein extraction buffer. Therefore, an alternative extraction protocol was devised to determine at what phase in the fractionation protocol proteins were solubilised. Here, extractions were performed on the alcohol insoluble residue, the oxalate-insoluble residue and the α -cellulose extract. A comparison of protein yield before and after oxalate treatment would confirm that protein was removed during oxalate treatment, corresponding to the nitrogen content of the oxalate soluble fractions. If protein could not be isolated post-oxalate treatment, it would confirm that the lack of protein in the hemicellulose fractions (Ha or Hb) was a genuine biological observation rather than a result of alkaline hydrolysis during 6 M NaOH extraction. Finally, the post-NaOH sample (α -cellulose) was assessed in an attempt to characterise the hypothesised protein from this fraction further, as the yield from the previously described cellulase insoluble residue from this fraction was too low to quantify and could not be re-solubilised in protein buffer (see Materials and Methods, Table 2.21) or DMSO- d_6 for further analysis (as discussed in the next chapter).

Whilst no protein could be isolated from the AIR using standard detergent-based methods for protein extraction (see Materials and Methods, section 2.1.4.3 for details), protein could be liberated using various salt buffers described for extraction of cell wall proteins in algae and plants in the literature (Voigt, 1985; Chivasa *et al.*, 2002; Boudart *et al.*, 2005; Voigt *et al.*, 2014; Přerovská *et al.*, 2021). Here, salts are required to liberate proteins from negatively charged polysaccharides, such as pectin and ulvan, by chelation of carboxyl (and sulfate) groups.

No protein could be isolated from the oxalate-insoluble residue or α -cellulose fractions by detergent or salt methods, confirming the hypothesis that protein was ionically bound to the negatively charged ulvans, and the oxalate extraction removed most of the ionically bound protein before the 6 M NaOH treatment, so the lack of nitrogen in the hemicellulose fractions appeared as a genuine biological observation. The inability to isolate protein from α -cellulose may reflect covalent linkage of protein, or proteins tightly associated with the fibrillar component of the cell wall, as certain cell

wall proteins are generally ‘unextractable’ from insoluble residue (Fritz, Hood and Hood, 1991; Qi *et al.*, 1995; Voigt and Frank, 2003; Feiz *et al.*, 2006). One alternative explanation was that the identified nitrogen is not protein-associated, but based upon the analysis performed in this chapter which appears consistent with protein, the latter explanation seems unlikely.

4.7 Discussion

4.7.1 Successful Fractionation of the *Ulva* Cell Wall into Constituent Components

The ATR-FTIR analysis performed in this chapter provides a wealth of structural information for the isolated *U. compressa* cell wall fractions. Generally, the observations are consistent with the expected composition of the *Ulva* cell wall, with sulfation observed on the oxalate-soluble fractions exclusively consistent with ulvans. In contrast, the other fractions (the alkali-soluble ‘hemicellulose’ and insoluble α -cellulose) were non-sulphated (Table 4.2). This was verified by both elemental analysis, and the identification of sulfate stretches in the corresponding FTIR spectra.

Notably, two distinct populations of ‘ulvan’ were identified in the two sulphated oxalate-soluble fractions, with the slowly soluble fraction appearing to have a small peak indicative of ester groups that were not present in the rapidly soluble fraction, which may have contributed to the differential solubility of the two fractions. The solubility of ulvan and pectins under slightly acidic conditions is partially attributable to the presence of negatively charged carboxyl groups, with increased esterification of these free carboxyl groups reported to reduce pectin solubility experimentally (Monsoor and Proctor, 2001; Robic, Gaillard, *et al.*, 2009; R. S. Wang *et al.*, 2019). The esterification profile of pectin has been assessed extensively in the literature using ATR-FTIR, with the identified ester peak at $\sim 1740\text{ cm}^{-1}$ attributed to *O*-acetyl and methyl groups in pectin from various species (McCann *et al.*, 1994; Gnanasambandam, R., Proctor, 2000; Marry *et al.*, 2000; Synytsya *et al.*, 2003; Baum *et al.*, 2017; Wu, Bulgakov and Jinn, 2018). This was supported by the positive identification of an ester peak in the oxalate-soluble barley straw fraction, despite pectin comprising only a small component of barley biomass (Vogel, 2008). The identification of a comparable peak in the slowly-oxalate soluble fraction may indicate that ulvans may be modified with comparable groups.

Interestingly, carrageenan from *M. stellatus* lacked a corresponding ester peak, comparable to the FTIR spectra obtained from various carrageenan extracts in the

literature (Pereira *et al.*, 2003; Gómez-Ordóñez and Rupérez, 2011). This may explain the notably higher yields observed for carrageenan from *M. stellatus* than ulvan from *Ulva* in the previous chapter, as with the lack of esterification, carrageenan would have more comparably more 'free' carboxyl groups. However, some degree of methylation on carrageenan is reported in some species (Chiovitti *et al.*, 2008).

Here, the reversible loss and re-appearance of the ester peak in the ulvan fractions before and after metal stress appear to indicate dynamic removal and re-addition of ester groups, possibly representing a biological response to stress. A comparable reduction in the *O*-acetylation of the hemicellulose xyloglucan from *Arabidopsis thaliana* has been reported in response to metal stress, providing further support to the present observation (Zhu *et al.*, 2014). However, whilst this is a novel observation for a chlorophyte polysaccharide, this will need to be verified by appropriate biochemical assays for methylation and acetylation, as described in the literature (Wood and Siddiqui, 1971; Marry *et al.*, 2000). These groups are also identifiable via various multi-dimensional NMR techniques, which are discussed in the next chapter.

Whilst the hemicellulose fraction requires sugar analysis to confirm whether glucuronan and xyloglucan segregate into them, the spectra Ha fraction (precipitated material) is consistent with the linear xyloglucan structure found in *Ulva* (Xyl→Glu→Glu), due to the absence of peaks consistent with a carboxyl group as expected for glucuronan and other uronic acid-containing polysaccharides (Figure 4.33).

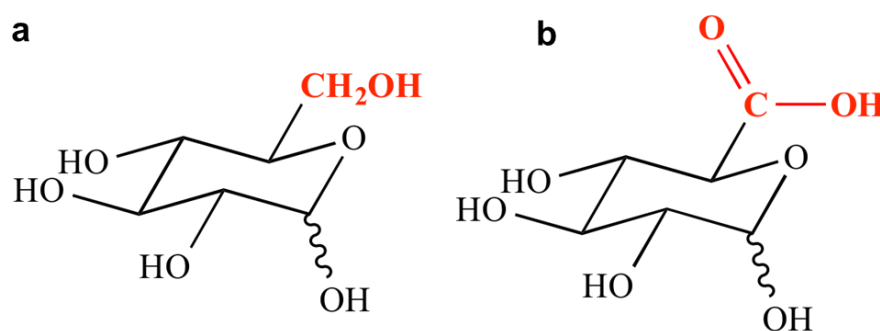


Figure 4.33. Comparison of D-glucopyranose (a) and D-glucuronic acid (b).

Furthermore, several resonances were observed consistent with xylose in the carbohydrate backbone region, distinguishing it from α -cellulose.

Due to the relatively high yield of the Hb fraction and that both the xyloglucan and glucuronan components of the *Ulva* cell wall are known to be minor fractions, in

the previous chapter, it was hypothesised that this fraction might also contain ulvan that was not solubilised in the oxalate extraction step. The observation that the oxalate insoluble residue (displayed in Figure 4.27) had ‘pectic’ peaks after removal of both oxalate soluble fractions indicated that ulvans were not fully solubilised in *U. compressa* AIR by the two oxalate extractions.

Furthermore, treatment of the slowly oxalate soluble fraction appeared to remove ester groups and shift the symmetric carboxyl peaks to a lower wavelength, as observed in the original Hb fraction, indicating that the 6 M NaOH treatment may have altered the structure of ulvans, resulting in a different spectral profile. As stated, alkaline treatment of pectin is known to induce ester hydrolysis (Gnanasambandam, R., Proctor, 2000; Chaiwarit *et al.*, 2020; Liang *et al.*, 2022). Alkaline extraction of ulvan material is reported in the literature (Ray and Lahaye, 1995; Hernández-Garibay, Zertuche-González and Pacheco-Ruíz, 2011), with similar losses of ester peaks at $\sim 1740\text{ cm}^{-1}$ reported in alkaline extracts relative to acidic extracts. Additionally, the peak attributed to rhamnose (αCH_3) in the oxalate fractions indicated that rhamnose-containing polysaccharides had segregated into that fraction (i.e., ulvans). However, this again will require sugar analysis for confirmation.

It is difficult to determine whether the ulvan that likely segregated into Hb was a ‘non-sulphated’ form of ulvan, as indicated by the elemental analysis, or whether the sulfate groups were removed artificially from these samples by NaOH treatment. The slight reduction of the peaks corresponding to S=O stretches in the oxalate-insoluble residue (not treated with NaOH) may support the former explanation, as does the observation that 6 M NaOH treatment did not remove these peaks from oxalate soluble material. Variable degrees of ulvan sulfation are reported in the literature from extracts from different populations and species (Kidgell *et al.*, 2019).

The analysis of the oxalate-insoluble fraction also indicated that the material that segregated into the Hb fraction was likely esterified, despite the lack of the corresponding peak in Hb, as the ester peak was present in oxalate-insoluble residue. This was shown to be a result of NaOH treatment, with this peak being removed successfully from the oxalate soluble fraction (Figure 4.26). However, we cannot distinguish whether this esterification profile corresponds to ‘hemicellulosic’ material or ulvans in the Hb fraction without the ability to separate the two. By contrast, the persistence of the ester group in the barley straw Hb fraction is consistent with a phenolic ester for a large polyphenol such as lignin, supported by the identification of

the lignin fingerprint in this sample and the observation that this peak persists even after alkaline extraction.

4.7.2 Limited Evidence for an Aromatic, 'Lignin-Like' or Phenolic Fraction in the *Ulva* Cell Wall

The comparison between the 'lignin positive' fractions of barley straw and *U. compressa* supports the observations from the previous chapter that a 'lignin-like' structure was not present in *U. compressa*. Consistent with the acetyl bromide soluble lignin assay and the biochemical staining, peaks corresponding to aromatic ring vibrations were positively identified in the barley straw Hb and α -cellulose fractions, alongside vibrations consistent with the phenolic and methoxy groups of monolignols and hydroxycinnamic acids. However, no evidence of these functional groups was observed in the spectra for *Ulva* or *Mastocarpus* samples, providing further evidence for the absence of a polyphenol component in algal cell walls, consistent with the lack of biosynthetic machinery to synthesise these compounds.

One point of contention from this, however, is that if aromatics were linked to the *Ulva* cell wall as small molecules rather than the hypothesised polyphenol structure, the linkage might have been hydrolysed by the NaOH treatment. For example, sodium hydroxide treatment is reported to deprotonate, cleave, and release ferulic acid from various hemicelluloses (Ram, Dowell and Seitz, 2003; Salleh *et al.*, 2011). Furthermore, some polyphenols may associate with cell wall polysaccharides via non-covalent associations (Kang *et al.*, 2019), which would be broken under the extraction conditions (Zdunek, Krysa and Szyma, 2022).

Here, FTIR analysis cannot easily distinguish between phenolics esterified to the cell wall as monomers and polyphenols. However, the presence of multiple bands associated with phenolic groups and aromatic C-H bonds was consistent with the presence of both syringyl and guaicyl monolignols of lignin, and the positive phloroglucinol-HCl staining performed previously differentiated between lignin structures and other phenolics.

However, as no aromatic peaks were observed pre-extraction in *U. compressa* biomass and alcohol insoluble residue, whilst they were readily identifiable in the barley straw, it indicated that no major aromatic component was associated with *Ulva*, covalently or non-covalently. At this stage, whilst the presence of 'phycolignin' in the *Ulva* cell wall could be ruled out conclusively, the presence of small phenolic esters could not.

Indication of sample size can be provided by NMR analyses, with more accurate estimates facilitated by methods such as diffusion-ordered spectroscopy (Groves, 2017). Here, if small phenolic esters (i.e., ferulic acid) can be positively identified in the barley extracts following alkaline treatment (i.e., in the hemicellulose and α -cellulose fractions), alongside lignin, it would indicate that these groups persist during the hemicellulose extraction process. As a result, verifying the absence of these groups in the corresponding *U. compressa* fractions would be more conclusive. This approach is discussed in the next chapter.

4.7.3 Identification of a -NH₂ Structure in the Insoluble Fraction

Despite the absence of an aromatic, the *U. compressa* oxalate-soluble and α -cellulose fractions appeared to contain protein, based upon the elemental analysis and subsequent FTIR assignments. By contrast, barley straw had a low estimated protein content, consistent with the expected differences in biochemical composition between the two biomass sources.

Co-extracted protein with *Ulva* cell wall polysaccharides has previously been reported. However, this component has not been studied significantly (Lahaye, Jegou and Buleon, 1994). Relative to microalgal chlorophytes and land plants, *Ulva*'s cell wall proteins (CWPs) are mostly uncharacterised. However, more recently, arabinogalactan-like glycoproteins were characterised in *U. lactuca* (Přerovská *et al.*, 2021), consistent with the hydroxyproline-rich cell walls observed in the various chlorophyte microalgae such as *Chlamydomonas*. This is supported by the identification of genes associated with hydroxyproline-rich proteins and extensins in the *U. mutabilis* genome (De Clerck *et al.*, 2018) and the previous identification of similar proteins in another green seaweed *Codium fragile* (Estevez *et al.*, 2009; Fernández *et al.*, 2010). Regardless, it is clear that more investigation is needed into the role, function and structure of CWP in the *Ulva* cell wall.

Here, as protein could be extracted successfully from cell wall fractions with salt buffer before oxalate extraction but not after (in the oxalate insoluble residue and α -cellulose), it indicated that the protein identified in the oxalate soluble fraction likely represented proteins that were ionically bound to the 'ulvan' matrix. Salt extraction of CWPs has been performed successfully in the previously described *U. lactuca* study to liberate ionically bound proteins (Přerovská *et al.*, 2021).

The evidence for protein in the α -cellulose fraction was somewhat surprising, as there was no corresponding evidence for segregation of proteins into the alkaline-soluble fractions, with proteins expected to be soluble in 6 M NaOH at the outset of the described experiments (Batey and Gras, 2007; Connolly, Piggott and FitzGerald, 2013; Deleu *et al.*, 2019). However, high levels of protein (~40%) have been estimated in the insoluble glucan extracts of *Ulva* previously (Lahaye, Jegou and Buleon, 1994). As protein was not solubilised or hydrolysed by the alkaline extraction step, nor was it extractable with detergent or salt buffer during the attempted protein extractions or removed during washing, it may indicate that this represents a fraction of protein that is covalently linked to *Ulva* cell wall polysaccharides.

Structural proteins are known to be covalently linked to pectin and hemicellulose in plant cell walls and chitin in fungal cell walls. Proteins such as extensins (as identified in the *U. mutabilis* genome) and other hydroxyproline-rich glycoproteins have a known function in cross-linking, reinforcing, and remodelling the plant cell wall like lignin, and are also reported to be largely insoluble, consistent with the segregation of 'insoluble' protein into the α -cellulose fraction here (Fritz, Hood and Hood, 1991; Qi *et al.*, 1995; Voigt and Frank, 2003; Feiz *et al.*, 2006). Covalent protein linkages on cellulose directly are not widely reported in plants, which may indicate that a proportion of 'hemicellulosic' material tightly associated with cellulose persists in the insoluble fraction (i.e., xyloglucan), to which protein may be linked. Detecting xylose in the α -cellulose fraction may support this hypothesis.

The dynamic changes observed in the hypothesised protein-associated peaks in saline stress relative to the control by both FTIR and elemental analysis may represent a response to osmotic stress, with the dynamic remodelling of the cell wall reported under variable osmotic conditions in *Ulva* and various land plants. Changes in cell wall protein composition may also explain the *in vivo* autofluorescence changes observed previously under different saline conditions (Chapter 3, see Figure 3.9 and Figure 3.10) as aromatic amino acids absorb at 405 nm (Ghisaidoobe and Chung, 2014; Arnon *et al.*, 2021). Extensins are known to be upregulated and modified during osmotic stress and salt stress, as are arabinogalactan proteins (Derek T.A. Lamport, Kieliszewski and Showalter, 2006; Yang *et al.*, 2010, 2011; Zagorchev and Odjakova, 2011; Yang, Rao and Horst, 2013; Chen *et al.*, 2014). In this model, protein may take the role of cross-linking the *Ulva* cell wall in the absence of lignin.

However, it must be considered that the inability to extract protein from α -cellulose may suggest that the assigned peaks are not protein associated and may be from another nitrogenous metabolite such as a polyamine. Polyamines are characterised in various chlorophyte cell walls and are known components of osmotic stress responses (Lee and Chen, 1998; Burczyk *et al.*, 2014; Schweikert and Burritt, 2015). Despite this, these metabolites are highly soluble and are reported to adhere to the cell wall rather than form covalent linkages. Therefore, the persistence of these compounds to the insoluble fraction appears unlikely (Lenucci *et al.*, 2005; Burczyk *et al.*, 2014; Díaz-Liñán *et al.*, 2021).

Furthermore, the likely presence of amide groups from the ester band at $\sim 1689\text{ cm}^{-1}$ is consistent with peptide bonds rather than a polyamine structure or a carboxyl group from a 'free' amino acid. The attribution of the amide and amine peaks to protein was supported by the removal of some of the carbohydrate components of this fraction by enzyme hydrolysis, where they increased in prominence. Furthermore, the protease digestion performed on these samples appeared to reduce these peaks and induce some form of 'degradation' in cellulose, consistent with a structural protein. With our current knowledge of the *Ulva* metabolome, there is no evidence for an alternative polyamide that could explain these observations. Regardless, this will be more extensively assessed in the next chapter for confirmation.

4.7.4 Conclusion

Whilst the analysis described in this chapter provided a considerable amount of data with a routine method for testing the hypotheses generated in the previous chapter, the limitations of FTIR (i.e., the complicated spectra) means the use of a complementary analytical tool with more resolution would greatly support the observations discussed here.

Therefore, a selection of these samples will now be analysed by NMR to support the observations made in this chapter as a final investigation of the hypothesised 'phycolignin' fraction in *U. compressa* and as an assessment of the hypothesised protein structure observed in the *U. compressa* α -cellulose fraction.

5. NMR Spectroscopy of *Ulva* Cell Wall Extracts

5.1 Introduction

5.1.1 General Introduction

In the previous chapters, I fractionated the *Ulva* cell wall and then screened for the proposed ‘phycolignin’ structure that was hypothesised. My ATR-FTIR analysis identified no obvious resonances associated with an aromatic compound in any of the *Ulva* cell wall fractions. However, aromatic structures consistent with lignin were positively identified in plant biomass extracted by the same process (barley straw). Interestingly, a series of functional groups were identified in *Ulva* specifically, particularly in the α -cellulose fraction. However, they were difficult to attribute as FTIR biopolymer spectra are often challenging to interpret. Likely candidates for these groups were amine or amide structures, possibly indicative of protein, which was supported by the identification of an elevated nitrogen content from the corresponding elemental analysis. As a result, in this chapter, I describe the use of an additional biophysical technique – nuclear magnetic resonance (NMR) to supplement these observations.

Whilst NMR spectroscopy has limitations compared to infrared spectroscopy and other analytical techniques used for analysing lignocellulosic materials such as mass spectrometry, including its lower sensitivity, higher cost and time per run, and the fact that only certain isotopes can be assessed (e.g., ^1H , ^{13}C , and ^{15}N), NMR provides significant structural information at a much higher resolution than the previously described FTIR spectroscopy approach (Keeler, 2002; Ralph and Landucci, 2010; Mansfield *et al.*, 2012; Frueh, 2014). As a result, various NMR techniques have been used successfully in biology for the non-destructive assessment of proteins, polysaccharides, total cell wall extracts, and whole cells (Pizzoferrato *et al.*, 2000; Pereira *et al.*, 2003; Li *et al.*, 2005; Mansfield *et al.*, 2012; Wang *et al.*, 2020). Furthermore, as a non-invasive method, NMR can also be used for whole-organism analysis, for example, magnetic resonance imaging (van As, Scheenen and Vergeldt, 2009).

FTIR and NMR are often used in tandem in biochemical investigations (Gilardi, Abis and Cass, 1995; Pereira *et al.*, 2003; Sun *et al.*, 2011; Qiao *et al.*, 2019; Guidara *et al.*, 2021), whilst FTIR can be used to identify what functional groups are present in a sample, NMR can be used to determine the exact structure of a compound. For example,

^1H NMR provides information on the relative number of hydrogen atoms present in a structure, which hydrogen atoms they are coupled to in a structure, and which functional group they are most likely associated with (Connor *et al.*, 2021). In FTIR, only the probable functional group can be identified. More complex interactions between nuclei can be assessed by multidimensional NMR, for example, ^{13}C - ^1H coupling analyses (Li *et al.*, 2005; Yelle, Ralph and Frihart, 2008; Mansfield *et al.*, 2012).

In this research, NMR was selected as a more reproducible ‘non-destructive’ method over mass spectrometry which is also frequently used in assessing lignocellulosic biomass (Lu *et al.*, 2017; Letourneau and Volmer, 2021). However, whilst mass spectrometry is a far more sensitive technique, being able to detect picomolar concentrations of a metabolite (C. Li *et al.*, 2021), the sample separation and derivatisation steps required for analysis risk the production of artefacts (Gu *et al.*, 2003; Bunzel, Seiler and Steinhart, 2005; Vikkula, Valkama and Vuorinen, 2006; Brondz *et al.*, 2007; Sauerschnig *et al.*, 2018; Schäfer, Trierweiler and Bunzel, 2018), which as previously discussed may result in false identification of ‘lignin-like’ structures in algae (see Chapter 3, §3.1.2). Furthermore, without an appropriate standard, mass spectrometry cannot be used quantitatively (Letourneau and Volmer, 2021), which for my research, was not possible as the presence of an ‘unknown compound’ was being investigated. Furthermore, whilst mass spectrometry can be used to identify unknown compounds (by molecular weight determination), the current knowledge of algal metabolome is limited; as a result, it appeared more appropriate to perform NMR on the isolated cell wall samples for more precise structural determination.

However, NMR spectroscopy can be a highly complicated technique, with a range of NMR publications available in the literature, across various fields of science, including mathematics, physics, chemistry, and the biological sciences. As a result, in the following sections, I introduce some fundamental concepts of NMR spectroscopy, which are essential for successfully interpreting the data generated in this chapter. For reference, my discussion of NMR theory relates to Fourier transform NMR spectroscopy (FT-NMR) rather than continuous wave NMR, which has since been superseded by FT-NMR and is now used universally in biochemistry (Carbajo and Neira, 2013).

5.1.2 Nuclear Magnetic Resonance: Theory

5.1.2.1 Basic Principles

To start, we consider some fundamentals of electromagnetism and atomic nuclei that enable us to study nuclear magnetic resonance.

A magnetic field is created by the movement of an electric charge. When the charge is rotating around an axis, this creates a magnetic moment (or dipole), the direction of which is perpendicular (i.e., at a 90° angle) to the direction of the moving charge. Certain atomic nuclei behave in similar fashion and have a property called spin (i.e., may be considered to rotate about their axis in this fashion). As a result, they exhibit a magnetic moment due to the net positive charge of the nucleus, which is assigned as the parameter μ (Keeler, 2002; Carbajo and Neira, 2013). Usually, the alignment of these 'spinning' nuclei in any given sample is random, which means that there is no net direction of the overall magnetic moment; however, in an applied magnetic field (which we define as B_0), NMR active nuclei (i.e., those with spin) become aligned with the magnetic field on what is typically defined as the longitudinal (or z) axis (Figure 5.1, b & c). Here, the net magnetic moment of the nuclei (μ) precesses (i.e., rotates like a spinning top or gyroscope) about the longitudinal axis of the magnetic field (Figure 5.1, a). The precession frequency depends on the spin properties of the nucleus (defined by the gyromagnetic ratio, γ , which is a constant that describes the relationship between spin and magnetic moment in different nuclei) and the strength of the applied magnetic field. The relationship between the precession frequency and these factors is defined by the Larmor Frequency ($\omega = \gamma B_0$), with ω representing the energy required to induce resonance in a particular nucleus (Raghothama, 2010; Bothwell and Griffin, 2011; Carbajo and Neira, 2013).

As per infrared spectroscopy (Chapter 4), for spectroscopy to work, a transition in energy level must be inducible and measurable by the method (Banwell, 1983; Engel, 2006). In NMR, this is aided by the observation that nuclei with spin have more than one orientation when aligned to the magnetic field, representing different energy levels. To understand how this occurs, we must develop my previous definition of spin using some further atomic principles. Firstly, we note that the spin properties (I) of a nucleus are defined by its proportion of neutrons: protons present. For example, nuclei with either an odd number of neutrons or protons have spin $1/2$, representing the most frequently used nuclei for NMR analyses, such as ^1H , ^{13}C , ^{15}N , and ^{31}P . The spin of these

nuclei is defined as $1/2$ because of the principle that a magnetic moment has $2I + 1$ possible orientations in a magnetic field. Nuclei with spin $1/2$ therefore have two possible orientations, either parallel ($1/2$) or anti-parallel ($-1/2$) to the applied magnetic field. NMR inactive nuclei, i.e., those with an even number of protons and neutrons (such as ^{12}C), have no spin ($I = 0$), therefore do not have a magnetic moment (μ) which, as previously mentioned, is induced as a function of nuclear spin ($\mu = I\gamma$), meaning these nuclei cannot be studied by NMR. Nuclei with spin $> 1/2$ can be assessed by NMR, and may have magnetic moments that have more than two possible orientations in a magnetic field. However, these nuclei are usually beyond the scope of biological investigations, as more complex mechanics are observed when analysing these nuclei (Keeler, 2002; Engel, 2006; Carbajo and Neira, 2013). As such, I describe NMR concerning nuclei with spin $1/2$, corresponding to the ^1H and ^{13}C analyses carried out in this chapter. Here, we observe one of the first limitations of NMR spectroscopy for studying biopolymers due to the low abundance of NMR active isotopes (with spin $1/2$) in nature, the sensitivity of NMR is limited, with ^1H being the most abundant and, therefore, the most sensitive NMR nuclei (Connor *et al.*, 2021). However, isotopic labelling protocols are reported in the literature for various organisms (Alexandre *et al.*, 2017; Andriukonis and Gorokhova, 2017; P. Wang *et al.*, 2018; Tsubaki *et al.*, 2020).

Due to the Boltzmann distribution, which states that the most stable form of an energy state will be observed more frequently at equilibrium, more nuclei precess in the parallel orientation ($1/2$) in a magnetic field at equilibrium than in the anti-parallel ($-1/2$) orientation, because the parallel orientation represents the lower energy state (Keeler, 2002). However, because the energy gap is small between the two states, only a small amount of energy is required to ‘flip’ a spin from parallel to anti-parallel orientation, which means that there is only a small population difference between the spin-states. This small energy gap contributes to the low sensitivity of NMR, as whilst the energy gap increases with magnetic strength, even with modern magnets, this population difference is still relatively small (Moser *et al.*, 2017). Nonetheless, at equilibrium, this population difference means that there is a net magnetisation parallel to the magnetic field.

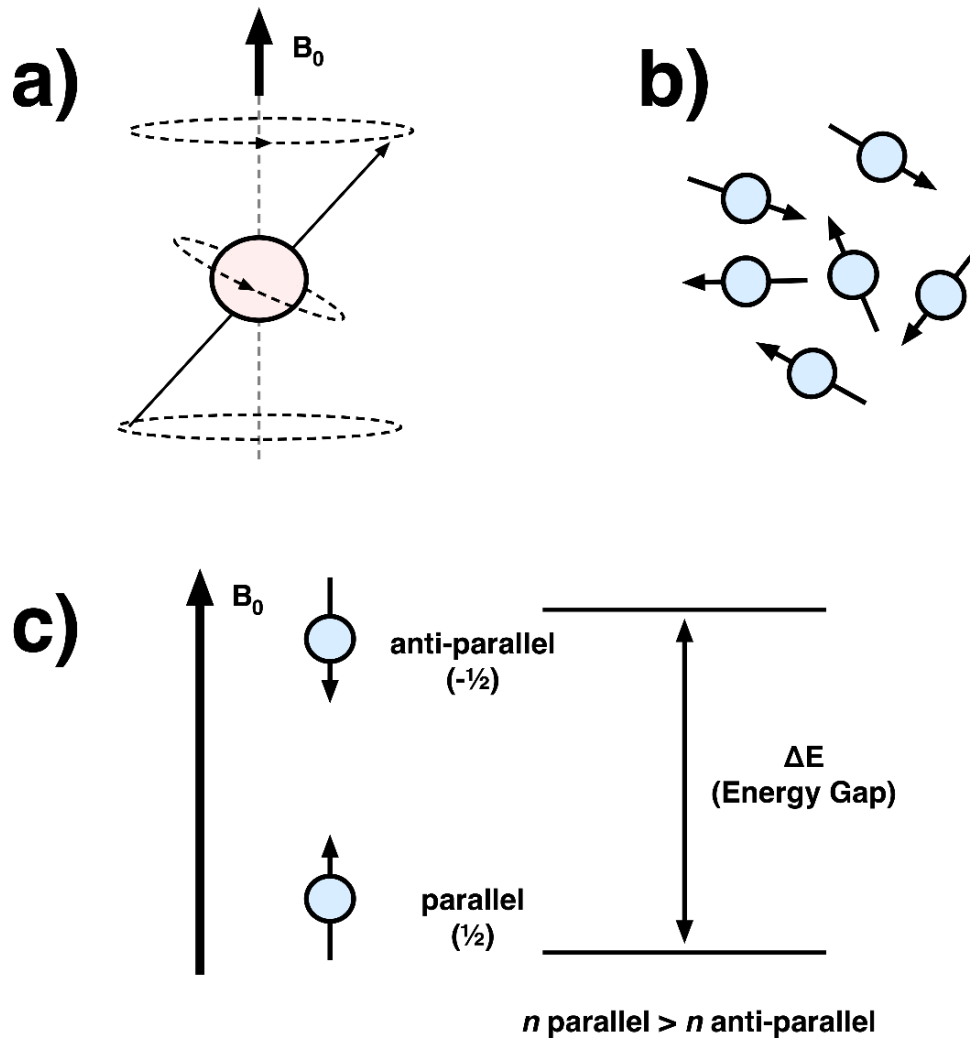


Figure 5.1. Fundamentals of nuclear magnetic resonance. A nucleus with spin precesses in alignment with a magnetic field (a). With no magnetic field, the orientation of spinning nuclei is random (b). However, in a magnetic field, spins align either parallel or anti-parallel to the applied magnetism. Here, the anti-parallel orientation represents the higher energy state, although the energy gap between the two states is small.

5.1.2.2 NMR Spectroscopy

Now that the properties of NMR active nuclei (i.e., those with spin) in a magnetic field have been defined, we consider how these conditions can be perturbed in an NMR experiment to enable structural analysis. In a standard NMR experiment, the precessing nuclei in a magnetic field are excited by a radiofrequency (RF) pulse which shifts the nuclei from equilibrium, and upon removal of the pulse, the signal released during relaxation back to equilibrium (i.e., the decay) from resonating nuclei is recorded during a fixed detection time (t_2) and analysed (Banwell, 1983; Keeler, 2002; Engel, 2006; Carbajo and Neira, 2013). The mechanism by which this occurs is now discussed.

Based upon the previously described principles (§5.1.2.1), if we consider the NMR set up in a coordinate model (xyz), at equilibrium (as shown in Figure 5.2), there is a net magnetisation of nuclei parallel to the applied magnetic field (B_0) along the longitudinal axis (z) due to there being slightly more spins orientated parallel to the axis than anti-parallel (Carbajo and Neira, 2013). However, whilst these spins precess in alignment with the magnetic field, the phase of precession from each nucleus in a sample (i.e., the angle of precession) will be random due to local magnetic effects (discussed more in 5.1.2.3), and the absence of magnetic pressure on the transverse (xy) axis. This is demonstrated in Figure 5.2 (a). As a result, applying a radio frequency pulse (B_1) to nuclei in the magnetic field perturbs equilibrium in two ways.

Firstly, the absorbance of the RF pulse induces NMR transitions of spins from the low energy parallel state to the high energy anti-parallel state (Figure 5.1, c). If pulsed for long enough, saturation occurs, meaning that the parallel and anti-parallel spin populations become equal. The effect of this is that net magnetisation is reduced on the longitudinal axis. Secondly, the pulse application causes net magnetisation to increase on the transverse axis (Figure 5.2, c). This occurs because the pulse induces coherence in the precession of nuclei, which means that the phases of spins are synchronised with the B_1 axis and are no longer random (Figure 5.2, b). This shift of magnetisation from the longitudinal to the transverse axis is referred to as 'tilting', with a pulse that tilts magnetisation completely from the longitudinal axis to the transverse axis referred to as a 90° pulse. The degree to which tilting occurs depends on pulse duration and intensity (Wider, 1998; Marusak, Doan and Cummings, 2006; Carbajo and Neira, 2013).

Upon removal of the pulse, two independent forms of relaxation occur concurrently to restore equilibrium (Figure 5.2, c). The first restores the original population difference between spin states, where slightly more parallel spins are present

than anti-parallel spins. This is called longitudinal relaxation, or T_1 relaxation, where net magnetisation oriented with the magnetic field (B_0) is restored. The second, transverse relaxation (T_2), refers to the loss of pulse (B_1) directed magnetisation, where the 'randomness' of the precession phase of the spins must be restored. Notably, T_2 relaxation is always faster than T_1 relaxation; as a result, T_1 is the rate-limiting factor determining how frequently a sample can be pulsed in NMR experiments (Keeler, 2002; Bothwell and Griffin, 2011; Carbajo and Neira, 2013).

The emission of energy during relaxation releases a decay signal which decreases exponentially (i.e., the free induction decay, or FID) that is recorded in the time-domain, with an NMR experiment typically requiring multiple replicate scans (or transients) to improve the signal to noise ratio for analysis (Hyberts, Robson and Wagner, 2013). The conversion of the FID from the time domain to the frequency-domain performed by a Fourier transform produces the resulting NMR spectrum (Figure 5.2, d). As with FTIR, the development of FT-NMR provided a significant advantage over previous attempts at the approach, which in the case of NMR was the 'continuous-wave' NMR approach, which collected spectra by sequentially exciting nuclei with an incrementally increasing wavelength of radiofrequency. By exciting all nuclei in a sample simultaneously with a radiofrequency pulse and recording the FID as a function of time, spectra can be acquired much faster (Keeler, 2002; Bothwell and Griffin, 2011; Carbajo and Neira, 2013; Frueh, 2014).

Whilst the pulse-data acquisition workflow I have described outlines the general parameters of a one-dimensional NMR experiment, this is often more complicated in modern NMR. Multi-pulse experiments (or pulse sequences) facilitate multidimensional NMR (discussed in 1.1.3.3) or can be used to selectively excite or suppress nuclei in one-dimensional NMR. Nonetheless, these experiments all follow the same basic principles.

As I have explained NMR spectroscopy so far, based upon the Larmor Frequency that defines the rate at which nuclei precess in a given magnetic field, it would be assumed that in a homogeneous magnetic field, the spin-properties of nuclei of the same species in a sample would be identical, as would be the resulting decay observed following an RF pulse. However, this is not the case, with various local magnetic effects impacting T_1 and T_2 relaxation times (and therefore the FID) and consequently allowing nuclei in different environments to be distinguished.

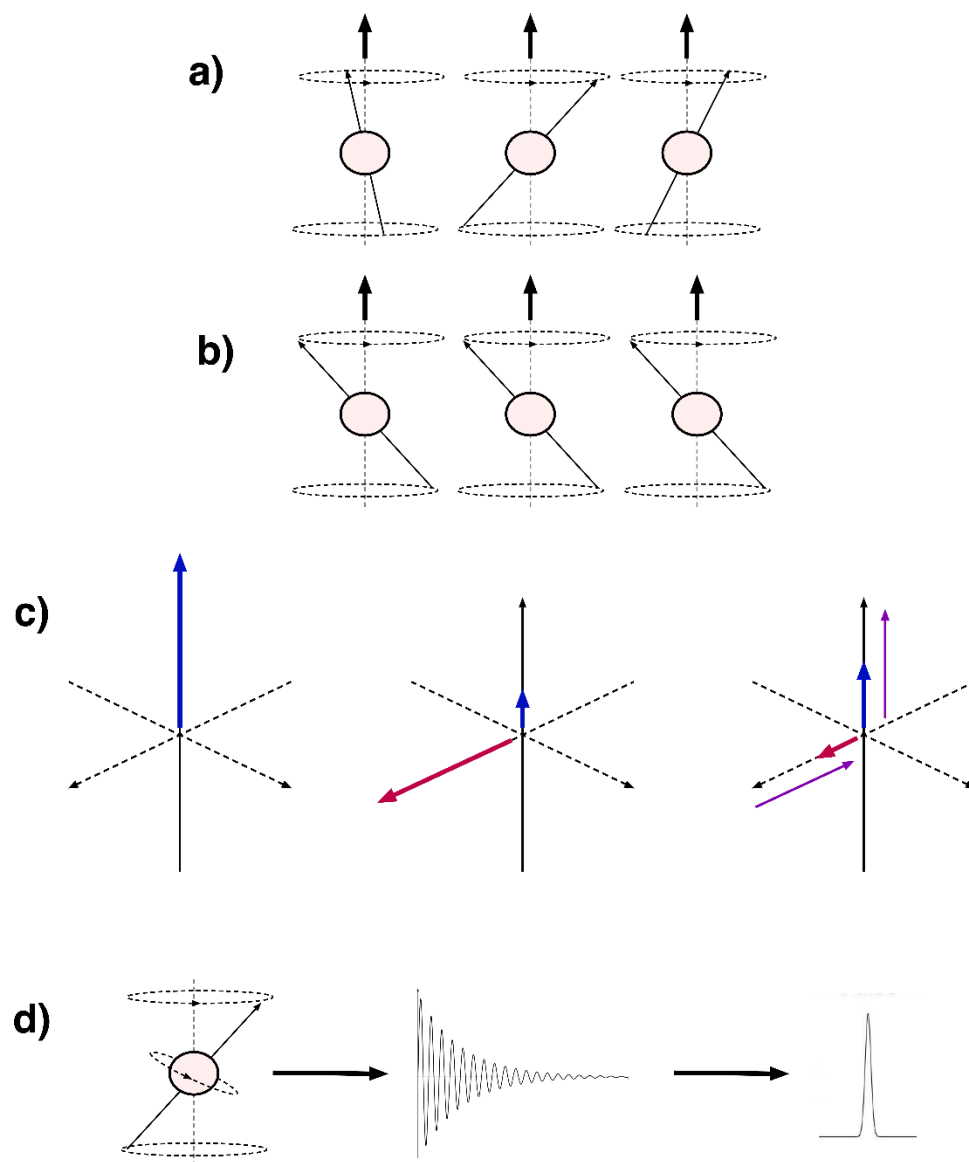


Figure 5.2. Dynamics of a NMR experiment. Precessing nuclei in a magnetic field have a random phase of precession (a), whilst coherence is observed upon application of a radiofrequency pulse (b). As shown in (c), in a magnetic field B_0 , net magnetisation is on the longitudinal axis parallel to the applied magnetic field (blue arrow). Application of a radiofrequency pulse shifts net magnetisation to the transverse axis (red arrow) from the resonance of spinning nuclei. On removal of the pulse, two forms of relaxation occur (purple arrows). The signal released from this process is measured in the time domain as a free induction decay, which is converted to the NMR spectrum by a Fourier transform.

5.1.2.3 One-Dimensional NMR: Interpretation of Chemical Shifts

As discussed previously (5.1.2.2), the resulting FID is converted via Fourier transform to the NMR spectrum, where frequency (from the RF pulse) is plotted against intensity. However, as described, this depends on the strength of the applied magnetic field, which may vary depending on the properties of the NMR instrument used (Moser *et al.*, 2017). As a result, the x-axis of the resulting spectra is reported as the chemical shift (δ) relative to a standard such as tetramethylsilane (TMS), against which the relative chemical shifts from the sample of interest are expressed. Here, chemical shift is represented (in parts per million, or ppm) via $\delta = \frac{\Delta\nu}{\nu_{\text{spectrometer}}} \times 10^6$, with $\Delta\nu$ representing the difference in resonance frequency (Hz) between the reference standard and the sample, and $\nu_{\text{spectrometer}}$ representing the applied frequency from the NMR spectrometer (MHz), enabling instrument-to-instrument differences to be accounted for (Keeler, 2002; Harris *et al.*, 2008; Carbajo and Neira, 2013). Due to this normalisation protocol, spectra across experiments can be compared.

The frequency at which a nucleus spins following a pulse depends on the local magnetic environment, which is accordingly represented in the chemical shift so that nuclei that have a higher spin (or resonance) frequency following the application of the pulse are observed downstream on the spectra, and nuclei that have a lower resonance frequency observed are upstream (Keeler, 2002; Engel, 2006; Raghothama, 2010; Carbajo and Neira, 2013). As a result, nuclei of the same type (e.g., ^1H) can be distinguished on the spectra by their local environment and thus can be used to identify the different structures present in a sample.

Electron density is the predominant factor that determines resonance frequency and hence chemical shift. For example, the standard used (i.e., TMS) represents an electron-dense compound in which nuclei (e.g., ^1H , ^{13}C) will spin at a lower frequency than in most compounds, allowing its chemical shift to be set at 0 ppm (Keeler, 2002; Harris *et al.*, 2008; Raghothama, 2010; Carbajo and Neira, 2013). Here, a nucleus in an electron-dense environment will have increased electron shielding, which reduces the effect of the applied magnetic field on the nucleus (Figure 5.3). Shielding occurs as the motion of the electrons in the electron cloud surrounding the nucleus will generate a small magnetic field, which opposes the applied magnetic field; hence the net magnetism experienced by the nucleus is reduced. Here, more energy must be applied to induce resonance in shielded nuclei (i.e., a higher radio frequency), meaning a lower chemical shift is observed.

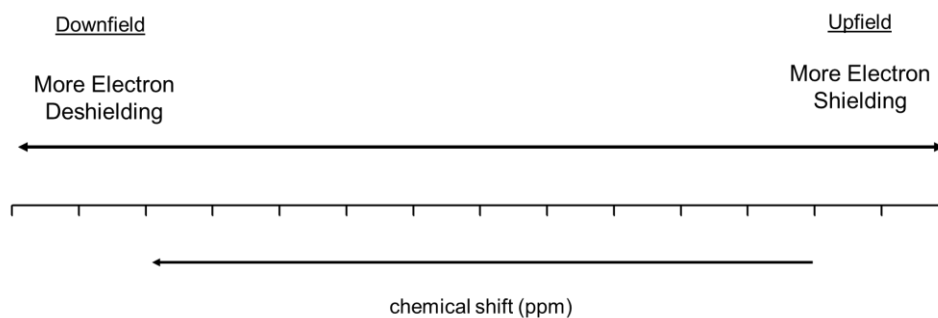


Figure 5.3. The effect of electron shielding and 'deshielding' on the chemical shifts observed in NMR spectra.

Conversely, deshielding occurs in a less dense electron environment or in environments containing 'electron withdrawing' groups (e.g., carbonyl groups) which pull the electron cloud away from the nucleus, meaning the strength of the applied pulse is experienced more intensely than for shielded nuclei. Hydrogen bonding (via -OH and -NH groups) also has a 'deshielding effect' in NMR, which as per FTIR, makes it challenging to identify these groups in macromolecules by NMR. In ^1H NMR, protons involved in -OH or -NH groups may form electrostatic interactions with electronegative groups (e.g., oxygen or nitrogen in biomolecules), which increases the effect of deshielding on the proton, with electrons pulled towards the more electronegative nuclei both at the original covalent bond and at the hydrogen bond (Raghothama, 2010; Carbajo and Neira, 2013). Due to the dynamic nature of hydrogen bonding, which is affected by multiple experimental variables (e.g., temperature, pH, solvent, and the amount of residual water in a sample), the chemical shifts for nuclei associated with -OH and -NH groups are highly variable in the resulting spectra due to the variable deshielding effects observed (Hasan, 1980; Asakawa et al., 1992; Tsuchiya et al., 1995; Abraham and Mobli, 2007). As a result, residual water in a sample heavily interferes with spectra, meaning that methods for water suppression are usually required if an NMR experiment is to be intelligible (Price, 1999; Chen *et al.*, 2020).

Interestingly, aromatic structures display unique electron shielding-deshielding properties in ^1H NMR due to diamagnetic anisotropy (Martin and Dailey, 1962; Martin et al., 1998; Wannere and Schleyer, 2003; Carbajo and Neira, 2013). In an aromatic ring, the adjacent delocalised pi electrons form a circular current upon exposure to a magnetic field (B_0), which creates an induced magnetic field that circulates in a direction opposing B_0 . Due to this, the direction of the local field inside the ring opposes the external field (B_0), while outside the ring, the local field acts in the same direction as the

external field (Figure 5.4). Therefore, the net magnetic field experienced by aromatic protons outside the ring is stronger. Because of this, aromatic resonances are characteristically shifted downstream relative to 'shielded' protons and are usually found in the chemical shift range of ~6-8 ppm (Ralph, Landucci and Ralph, 2004; Sun et al., 2011; Komatsu and Kikuchi, 2013; Grabber et al., 2019).

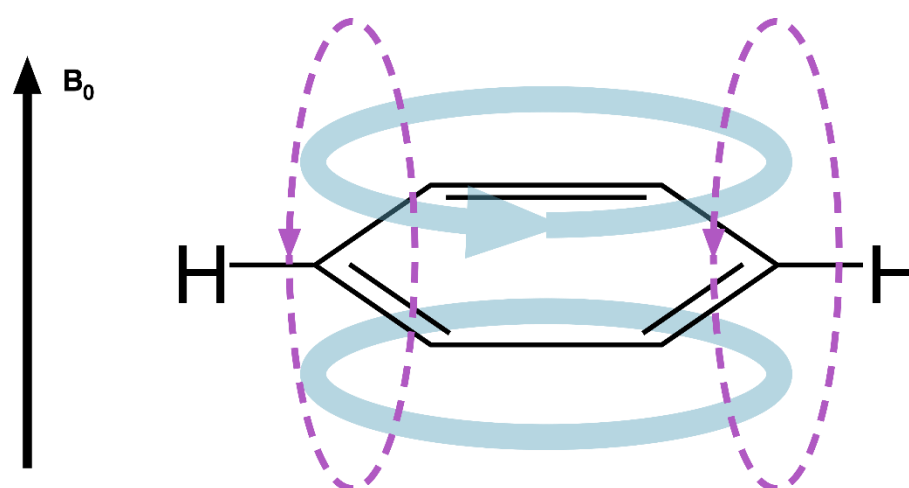


Figure 5.4. Diamagnetic anisotropy in an aromatic ring. The circular current from delocalised pi electrons (blue) induces a circulating magnetic field (dotted line, purple) that increases the effect of magnetism on aromatic protons.

Another important concept to consider is scalar coupling, also known as J coupling or spin-spin coupling. Scalar coupling occurs 'through bond' via covalent bonds that link NMR-active nuclei and is not affected by magnetic field strength (Wider, 1998; Keeler, 2002; Edison and Schroeder, 2010; Carbajo and Neira, 2013). Scalar coupling contrasts with dipole-dipole interactions (or dipolar coupling), which occur via direct 'through space' interactions between spins, thereby affecting the local environment of nuclei and influencing the resulting chemical shifts. Its properties mean that scalar coupling can be used to identify how bonds connect non-equivalent nuclei (i.e., those that experience different magnetism). In a one-dimensional NMR spectrum, this is represented by a 'splitting pattern', where a peak with multiplicity is observed for coupled nuclei. The 'n+1' rule defines the degree to which the peak is split. For example, in ^1H NMR, a proton coupled to one non-equivalent proton forms a doublet, whereas a proton coupled to two non-equivalent protons forms a triplet, and so on (Connor *et al.*, 2021). This observation is one of the main advantages of NMR over other analytical techniques, enabling precise determination of chemical structure. However, in complex macromolecules, spectra often become too crowded to interpret easily because of the presence of multiple functional groups and overlapping resonances in a sample (Fernández and Wider, 1999; Ralph and Landucci, 2010; Frueh, 2014). Scalar coupling helps to cut through this by facilitating multidimensional techniques, which are often preferred in the study of macromolecules, because couplings between nuclei can be assessed more precisely, for example, by correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY) experiments (Chen *et al.*, 2020). These techniques and their advantages are now discussed.

5.1.2.4 Multidimensional NMR

Multidimensional NMR is facilitated by 'multi-pulse' experiments in which a pulse sequence is applied to a sample to assess multiple NMR active nuclei in an experiment, rather than the single pulse used in one-dimensional NMR. These experiments are facilitated by the phenomenon of 'magnetisation transfer', where nuclei transfer polarisation to coupled nuclei of the same (homonuclear) or different (heteronuclear) species (McDermott *et al.*, 2000; Mansfield *et al.*, 2012; Confalonieri *et al.*, 2017; Schäfer, Trierweiler and Bunzel, 2018; Chen *et al.*, 2020). How magnetisation transfer occurs is complex, as are the pulse sequences designed to exploit it (Keeler, 2002; Mandal and Majumdar, 2004). However, two-dimensional NMR analysis is facilitated at the fundamental level by including an evolution time (t_1) in the pulse sequence before detection (Wider, 1998). Whilst detection time (t_2) is fixed in the NMR experiment (Figure 5.5, a), t_1 increases in increments with each replicate scan. More simply, a series of scans from one-dimensional NMR experiments are carried out with incrementally increasing evolution times (Figure 5.5, b). The addition of the t_1 variable creates a second dimension of time (hence, two-dimensional NMR), with the fixed detection time used to collect the FID representing the only time variable in the more common one-dimensional NMR experiment. As the acquired FID in t_2 varies as a function of t_1 , a two-dimensional Fourier Transform can be performed to generate a plot of f_1 (evolution frequency) against f_2 (acquisition frequency), displayed as a contour plot to represent the 2D NMR spectrum following conversion to chemical shift (Wider, 1998; Keeler, 2002; Edison and Schroeder, 2010; Carbajo and Neira, 2013). By varying pulse sequence, pulse frequency, pulse width, and the evolution time (in which heteronuclear or homonuclear couplings 'evolve'), a range of pulse sequences has been designed to facilitate a range of two-dimensional NMR experiments to study the coupling interactions between various nuclei.

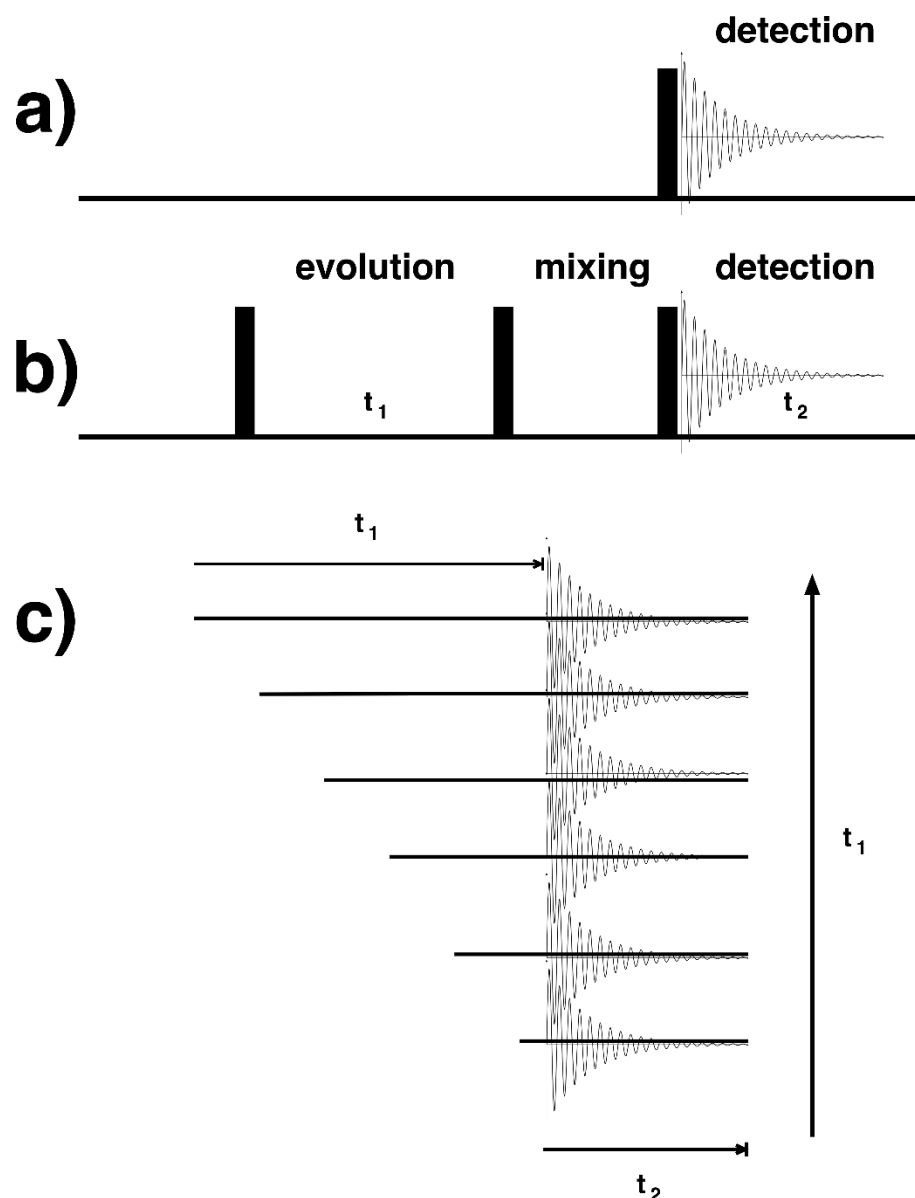


Figure 5.5. Pulse sequences for a one-dimensional NMR experiment (a) and a general two-dimensional NMR experiment (b). The duration of the evolution time is increased incrementally with each scan so that we have two time variables within the NMR experiment – t_2 (free induction decay) and t_1 (evolution time), as shown in (c). Further dimensions can be added to an NMR experiment (e.g., 3D) by adding another variable evolution time to the pulse sequence. Black bars indicate radiofrequency pulses. Variation of pulse and mixing time parameters enables a range of multidimensional experiments to be performed.

For example, two-dimensional heteronuclear correlation experiments typically use pulse sequences designed to identify ^1H coupled to another nuclear species. Pulse sequences for ^1H - ^{13}C are cited most frequently for two-dimensional analyses of biopolymer structures. However, ^1H - ^{15}N , ^1H - ^{31}P , and ^1H - ^{19}F experiments for protein, lipid, and NMR of ^{19}F -labelled biomolecules, respectively, have also been developed (Hayato Morita *et al.*, 2004; Majumdar *et al.*, 2010; Kiraly *et al.*, 2015; Arntson and Pomerantz, 2016; Furse *et al.*, 2021; Welte, Sinn and Kovermann, 2021). These correlation experiments provide considerable advantages over the one-dimensional methods, with the ‘inverse detection’ of less sensitive nuclei such as ^{13}C or ^{15}N by this method providing significant sensitivity benefits relative to one-dimensional ^{13}C or ^{15}N NMR because of the previously described ‘magnetisation transfer’ between coupled nuclei. This occurs as the typical heteronuclear pulse sequence (e.g., INEPT) uses the more sensitive nucleus (i.e., ^1H) to transfer magnetisation to the less sensitive nucleus (^{13}C or ^{15}N), which is subsequently transferred back to ^1H for detection (Fernández and Wider, 1999; McDermott *et al.*, 2000; Keeler, 2002; Mandal and Majumdar, 2004; Carbajo and Neira, 2013).

Furthermore, the identification of cross peaks for multiple nuclei via a 2D experiment provides much-improved resolution of the structure of a compound, particularly for more complex macromolecules, where extensive overlap in a one-dimensional NMR spectrum will be observed, making interpretation difficult (Edison and Schroeder, 2010; Ralph and Landucci, 2010; Frueh, 2014). This problem is a particularly thorny one when interpreting complex ^1H NMR spectra because of the low chemical shift range (~ 10 ppm) observed relative to TMS. Adding another nucleus to the analysis, which has a wider chemical shift range (e.g., ^{13}C), increases the ‘spread’ of data. Furthermore, correlative analysis can eliminate the considerable amounts of ‘noise’ and overlapping signals that complicate one-dimensional spectra. For example, ^{13}C - ^1H analysis does not show peaks for protons in -NH or -OH groups which complicate spectra via the previously described effects of hydrogen bonding (see section 5.1.2.3).

Common pulse experiments include heteronuclear single quantum coherence (HSQC), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond coherence (HMBC). Fundamentally, these methods are similar, with the output of the HSQC and HMQC pulse sequences being essentially equivalent, defining single bond correlations between the two nuclei (Mandal and Majumdar, 2004; Carbajo and Neira, 2013). However, HSQC is typically preferred for more complex

macromolecules as the pulse sequence of the HSQC experiment limits homonuclear coupling (i.e., ^1H - ^1H) during the evolution time (t_1), which appears as multiplet peaks on an HMQC spectrum, making the interpretation of an already complex spectrum (in the case of a biopolymer) even more challenging. HSQC also provides better resolution of the 'indirect' nuclei (i.e., ^{13}C). In the case of HMBC, multiple correlations can be observed from longer distances (i.e., HMBC can correlate nuclei 2 or 3 bonds away), whereas HSQC/HMQC only identifies single-bond couplings (Pau-Roblot *et al.*, 2002; Edison and Schroeder, 2010; Carbajo and Neira, 2013; Gürer *et al.*, 2021). HMBC has shown promise in the characterisation of various biological structures, including secondary metabolites, lignin, and cellulosic material (Li *et al.*, 2003; Mangoni *et al.*, 2011; Koso *et al.*, 2020). Of these methods, HSQC is perhaps the most frequently cited in the biological literature, with ^1H - ^{13}C HSQC frequently used for the analysis of lignin, lignocellulose, individual cell wall polysaccharides, and whole plant cell wall extracts (Yelle, Ralph and Frihart, 2008; Mansfield *et al.*, 2012; Gupta *et al.*, 2013; Jiang *et al.*, 2017; Guidara *et al.*, 2021).

Conversely, whilst the suppression of homonuclear coupling in heteronuclear NMR is desirable for the analysis of complex biopolymers, multidimensional homonuclear experiments such as correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY) also provide valuable structural information, with COSY reported as the first multidimensional NMR technique in the literature (Keeler, 2002; Ralph and Landucci, 2010; Carbajo and Neira, 2013; Chen *et al.*, 2020). Using the COSY pulse sequence, we can perform ^1H - ^1H correlations to identify coupled protons, which are attached to the same atom (geminal protons) or attached to neighbouring atoms and are adjacent (vicinal). Whilst we can identify ^1H - ^1H interactions in 1D NMR via multiplets (Connor *et al.*, 2021), the advantage of COSY and TOCSY is that the homonuclear correlation plot allows better discrimination of these couplings. As previously mentioned, coupling is difficult to discern in complex spectra.

5.1.2.5 Solid vs Solution State

So far, I have discussed NMR theory with the assumption that analysis of biopolymers occurs in the solution-state. Solution-state NMR is typically the first-choice method for NMR analysis, where samples are often solubilised in a deuterated solvent such as D₂O, DMSO-d₆, or CDCl₃, which is deuterated so that protons in the solvent do not distort the resulting spectra (Fulmer *et al.*, 2010). The main limitation of solid-state samples is that solid samples cannot ‘tumble’ in the way they do in solution, which means that much broader signals appear on the solid-state spectrum (Fernández and Wider, 1999; Reif *et al.*, 2021).

This occurs due to anisotropy of the magnetic field (i.e., the variation in the local environment) in the different directions surrounding the molecule, for example, due to dipolar coupling interactions between different nuclei (Carbajo and Neira, 2013). As the molecule cannot freely rotate, a series of overlapping resonances are recorded for nuclei due to this local variability, producing a broad peak on the NMR spectra. The effect of anisotropy is ‘averaged’ out in solution-state NMR with the molecule able to freely rotate in a homogeneous solution, meaning sharper discrete peaks are observed. This is analogous to how larger molecules form broader peaks on solution-state spectra, whereas sharp peaks are observed for small molecules and side groups on macromolecules that can rotate freely (e.g., methyl groups) in solvent (Fernández and Wider, 1999; Foster, McElroy and Amero, 2007; Frueh, 2014; Reif *et al.*, 2021). Further, larger amounts of sample are typically required for solid-state work to compensate for the lower sensitivity. However, with certain biological samples, solid-state analysis is more practical, particularly for polymers that are insoluble in conventional NMR solvents such as cellulose (Gilardi, Abis and Cass, 1995; Love, Snape and Jarvis, 1998; Gao *et al.*, 2015).

A few techniques have been applied to solid-state NMR analysis to overcome some of the described limitations, which have found utility in modern biological NMR (Lesage, 2009; Reif *et al.*, 2021). The first, magic-angle spinning (MAS), reduces the effect of the previous described anisotropic effects on solid-state samples by rapidly spinning samples at an angle of 54.74° to the applied magnetic field, limiting the effect of peak broadening typically observed for solid samples. Secondly, cross-polarisation is often used to compensate for the lower sensitivity of solid-state NMR, to enable less abundant nuclei such as ¹³C and ¹⁵N to be assessed in the solid-state by the induced transfer of polarisation to these nuclei via protons, by the magnetisation transfer phenomenon

described for multidimensional NMR. Both these techniques are combined in CP-MAS (i.e., cross polarisation-magic angle spinning) NMR spectroscopy, which is applied to the insoluble α -cellulose fractions in this chapter (5.2.1).

5.1.3 Chapter Aims and Hypotheses

In this chapter, I will perform various NMR analyses on the obtained cell wall fractions to:

- Support the assignation of the various functional groups identified in the isolated cell wall fractions in the previous chapter by FTIR
- Further characterise the α -cellulose fraction, where non-cellulose associated peaks were identified in the *Ulva* fraction but not barley, possibly indicative of a proteinaceous cell wall
- Identify conclusively if aromatic structures are present in the *Ulva* cell wall isolates, consistent with the hypothesised 'phycolignin' structure

A handful of multidimensional NMR experiments are reported in *Ulva* to date, which typically concerns the characterisation of ulvans and non-structural metabolism and not the presence of aromatics or other structural components of the *Ulva* cell wall (Tabarsa, Lee and You, 2012; Gupta et al., 2013; Glasson et al., 2017; Gupta and Kushwaha, 2017; Tran et al., 2018; de Carvalho et al., 2020; Gao et al., 2020; Guidara et al., 2021). As such, the work described in this chapter outlines a novel approach.

5.2 Solid-State NMR

5.2.1 ^{13}C CP-MAS

The insoluble fractions (α -cellulose) from barley straw and *U. compressa* were compared by ^{13}C cross-polarisation magic angle spinning (CP-MAS) NMR spectroscopy. Due to the previously described limitations of the solid-state analysis, this experiment was performed on α -cellulose exclusively as all the other tested fractions solubilised in DMSO- d_6 . Lignocellulosic material has been studied extensively in the literature via ^{13}C CP-MAS from various sources of plant biomass; as a result, attribution of cellulose and lignin-associated resonances could be performed following a literature review.

Both samples had the characteristic resonances reported for cellulose from 60 ppm to 108 ppm (Mc Bride, 1991; Gilardi, Abis and Cass, 1995; Gastaldi et al., 1998; Love, Snape and Jarvis, 1998; Pizzoferrato et al., 2000; Sun et al., 2005, 2011; Wang and Hong, 2016; Kouadri and Satha, 2018; Yang et al., 2018; Wahlström, Edlund, et al., 2020). Here, a peak corresponding to C-1 of cellulose was observed at 105.1 ppm in the *U. compressa* extract (Figure 5.6), followed by two peaks corresponding to the C-4 of crystalline surface and disordered cellulose structures, respectively, at 87.6 ppm and 82.4 ppm. A broad prominent peak was observed at 74.5 ppm corresponding to C-2, C-3, and C-5 resonances, with the C-6 resonance observed at 62.8 ppm. The corresponding barley resonances were comparable (Figure 5.7), with the same peaks observed at 105.6, 87.8, 84.5, 75.2, and 63.4 ppm, respectively. The resonance of C-6 in both samples (at ~63 ppm) is consistent with a disordered cellulose structure rather than crystalline, with the C-6 shift appearing at 66 ppm in crystalline samples (Mc Bride, 1991; Sun et al., 2005; Wahlström, Edlund, et al., 2020). However, more appropriate tools are available to assess the crystallinity of cellulose (e.g., X-ray diffraction), which are beyond the scope of this research (Park *et al.*, 2010).

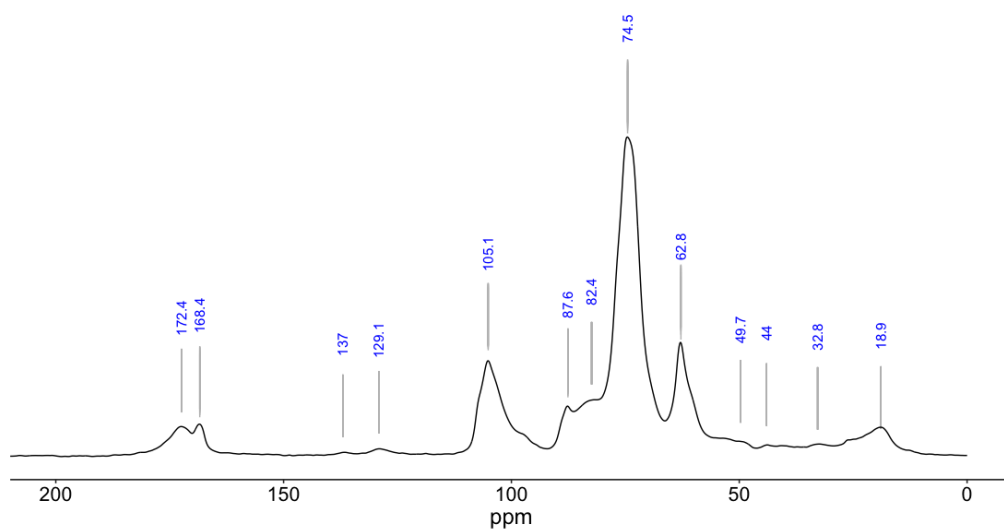


Figure 5.6. Analysis of the insoluble α -cellulose fraction of *U. compressa* by ^{13}C CP-MAS NMR

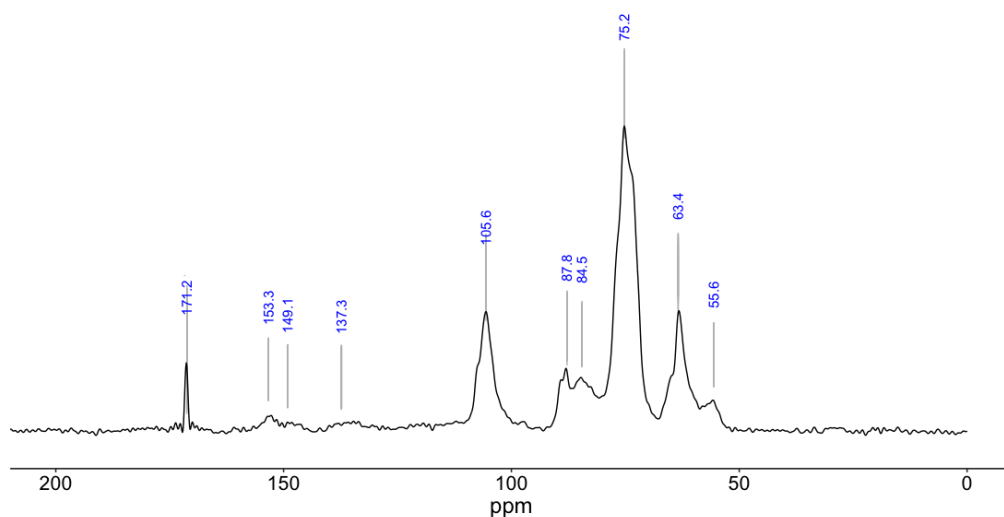


Figure 5.7. Analysis of the insoluble α -cellulose fraction of barley straw by ^{13}C CP-MAS NMR

Differences were observed in other regions of the spectra, indicating that cellulose was associated with different structures in both extracts. Firstly, whilst shifts associated with a carbonyl-associated carbon were observed in both samples at ~ 171 ppm (Figure 5.9, Figure 5.10), in barley straw, this appeared as a sharp, intense peak at 171.2 ppm (Gilardi, Abis and Cass, 1995; Love, Snape and Jarvis, 1998; Sun *et al.*, 2011; Qiao *et al.*, 2019). This could be attributable to the co-extraction of hemicelluloses (i.e., an acetyl group) or lignin which may have incorporated a range of carbonyl structures, including

cinnamaldehydes (Smith, 1955; Love, Snape and Jarvis, 1998; Kihara *et al.*, 2002; Pomar, Merino and Barceló, 2002; Sun *et al.*, 2011). In *U. compressa*, two broader peaks were observed at 172.4 ppm and 168.4 ppm, consistent with the identified carbonyl group in the corresponding FTIR spectrum for this fraction (see the peak at 1689 cm⁻¹ in Figure 4.19, Chapter 4). A series of small aromatic carbon resonances were then observed from ~150-130 ppm in both samples, the profile of which was different in both spectra. In barley straw, two peaks were observed at 153.3 ppm and 149.1 ppm that were not present in *U. compressa*, consistent with C-5 and C-4 from an aromatic ring connected to a methoxy (-OCH₃) and phenol (-OH) group, respectively (Mc Bride, 1991; Gilardi, Abis and Cass, 1995; Love, Snape and Jarvis, 1998; Lu *et al.*, 2017). Both of these structures are consistent with the guaicyl and syringyl monolignol structures in lignin and ferulic acid, which are known to be abundant in monocot cell walls (Ram, Dowell and Seitz, 2003; De Oliveira *et al.*, 2015). A further peak consistent with a carbon (C-1) from an etherified aromatic ring (i.e., C-O-R) was also identified at 137.3 ppm (Love, Snape and Jarvis, 1998; Sun *et al.*, 2011). In *U. compressa*, an equivalent peak was identified at 137 ppm, in addition to another small peak corresponding to an unsubstituted aromatic carbon at 129.1 ppm, indicating that an aromatic structure was also present in this sample (Mc Bride, 1991; Gilardi, Abis and Cass, 1995; Gao *et al.*, 2015).

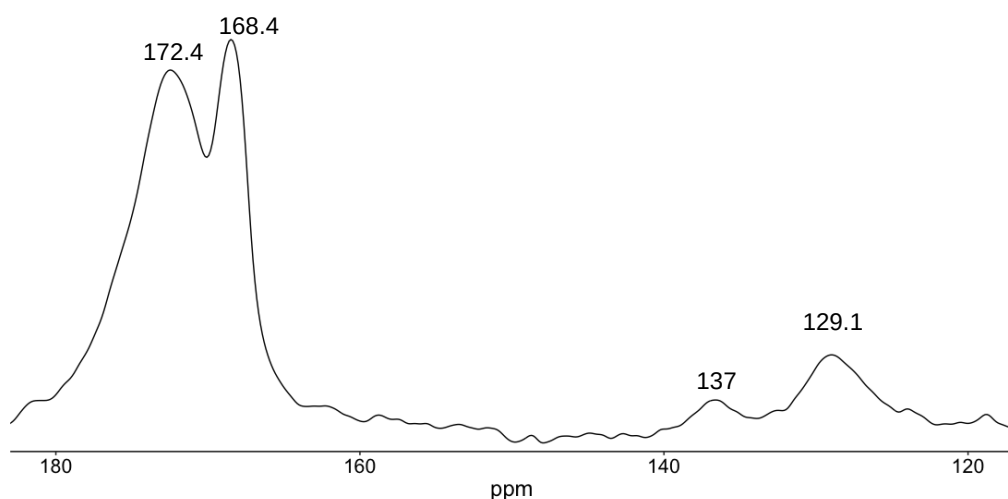


Figure 5.8. Analysis of the insoluble α -cellulose fraction of *U. compressa* by ¹³C CP-MAS NMR. *U. compressa* spectra zoomed in at putative carbonyl and aromatic region from 180 to 120 ppm. Carbonyl peaks are at 172.4 and 168.4 ppm, and aromatic peaks are identified at 137 and 129.1 ppm.

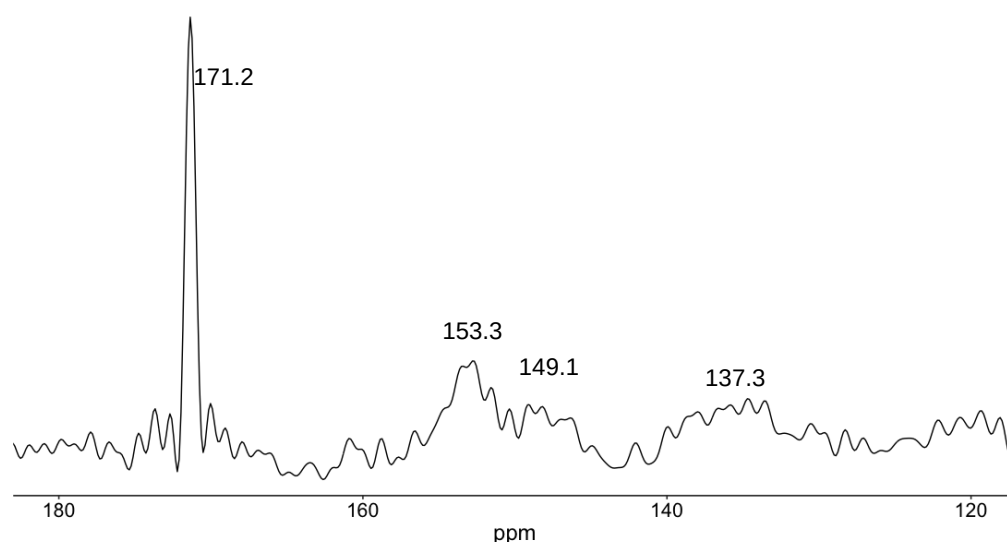


Figure 5.9. Analysis of the insoluble α -cellulose fraction of barley straw by ^{13}C CP-MAS NMR. More signal to noise is observed for the corresponding *Ulva* analysis as more scans were performed ($n = 14400$) than for the barley straw cellulose ($n = 900$). Aromatic peaks (labelled) were also observed in phase edited spectra, increasing confidence that they were not attributable to background (see Appendix III).

Further evidence of a phenolic in barley straw cellulose was identified at 55.6 ppm (Figure 5.10, a), with this peak attributed to the carbon in the previously identified methoxyl ($-\text{OCH}_3$) group, which is attached to the previously described phenolic structures in plants (Gilardi, Abis and Cass, 1995; El-Khouly et al., 2011; Sun et al., 2011; Yuan et al., 2011). This peak was not observed in *U. compressa*, indicating that the aromatic structure identified in this fraction was not one that contained this group (Figure 5.10, b). No further peaks were observed upfield of this resonance in barley straw; however, a series of small peaks associated with aliphatic ($-\text{CH}_2$, $-\text{CH}_3$) resonances were observed in *U. compressa*, with the most prominent peak observed at 18.9 ppm, consistent with a carbon from a $-\text{CH}_3$ group. These resonances were hypothesised to be consistent with protein (Mc Bride, 1991; Pizzoferrato et al., 2000; Asakura and Yao, 2009), based upon the previous elemental analysis and FTIR for this fraction, with the 18.9 ppm peak being consistent with the carbon from the $-\text{CH}_3$ group in aliphatic amino acids in various solution-state and solid-state studies (Rabenstein and Sayer, 1976; Frey and Opella, 1980; Schmidt et al., 1993; Prabhu et al., 1996; McDermott et al., 2000; Košir and Kidrič, 2001).

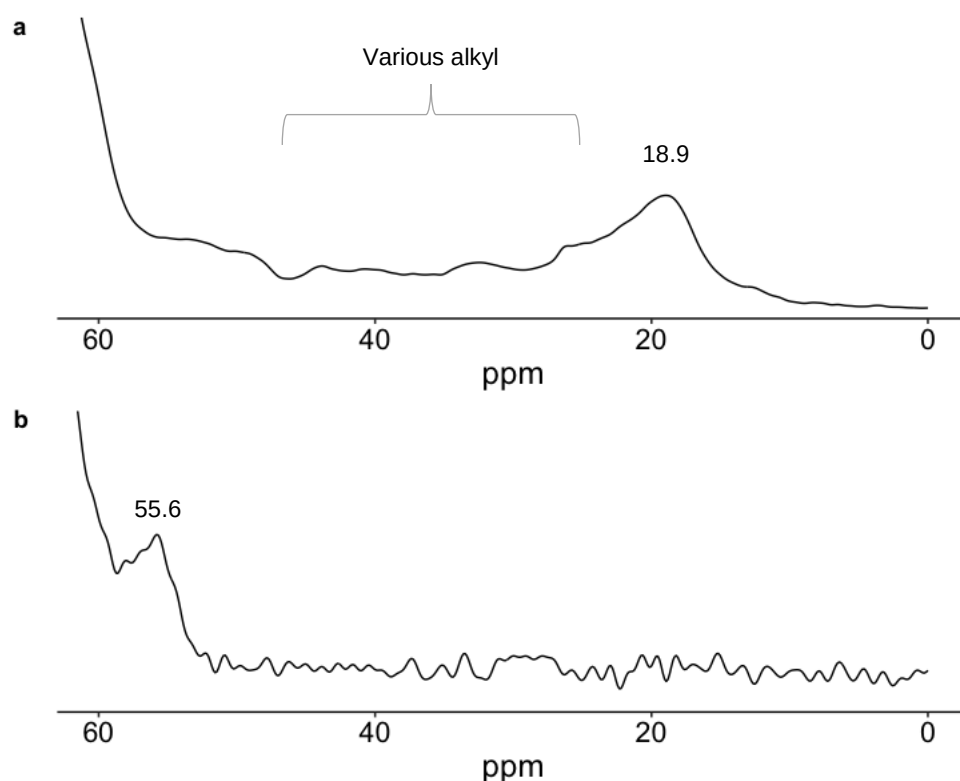


Figure 5.10. Analysis of the insoluble α -cellulose fraction by ^{13}C CP-MAS NMR from 0 to 60 ppm. (a) *U. compressa* has various small peaks corresponding with various aliphatic resonances, including a major peak at 18.9 ppm consistent with a CH_3 group from a $\text{C}\beta$ from amino acids (b). A single peak corresponding with a methoxy group ($-\text{OCH}_3$) from phenolics is observed in barley straw at 55.6 ppm.

However, it is not possible to distinguish between amino acids here because of the broadness of the observed peak. A series of further aliphatic resonances corresponding with amino acid groups were observed at 32.8, 44, and 49.7 ppm, consistent with carbons from CH_2 groups of various amino acids. However, these peaks were very subtle (Prabhu et al., 1996; Košir and Kidrič, 2001; Tian and Yin, 2004; Virkki et al., 2005). The most prominent of these resonances, the 49.7 ppm resonance, is consistent with a C_α peak from amino acids. However, the intensity of this peak was notably small relative to the carbonyl peaks observed at 172.4 ppm and 168.4 ppm, which, if protein associated, would represent carboxyl groups of the peptide (or amide) bonds (Frey and Opella, 1980; McBride, 1991; Asakura and Yao, 2009; Ma, Dang and Xu, 2016), with the C_α peak appearing more prominent in protein containing samples (Saitô and Yokoi, 1992; Asakura and Yao, 2009; Ma, Dang and Xu, 2016). In amino acids, a single carbonyl peak is observable at ~ 170 – 180 ppm (Prabhu et al., 1996; Tian and Yin, 2004). However, in a peptide, this peak will broaden due to the contribution of

overlapping resonances from the different amino acids in the sample, which has slightly different shifts in a ^{13}C spectrum, and the increased size of the molecule (Frueh, 2014). For example, the carbonyl-associated carbon in L-alanine has been shown to be shifted up-field by approximately 5 ppm when in an oligopeptide (L-Ala→L-Ala) relative to the corresponding shift observed in monomers by ^{13}C NMR (Mitra, Ostashevsky and Brewer, 2009).

The two distinct peaks observed may represent distinct populations of this functional group, with the higher resonance consistent with the carboxyl group in 'free' amino acids and the lower resonance consistent with a carbonyl in an amide group, or both peaks representing carbonyl groups from different amino acids (Saitô and Yokoi, 1992; Asakura and Yao, 2009). In silk proteins and collagen, two distinct peaks are observed on ^{13}C NMR spectra for alanine and glycine and proline and glycine, respectively (Saitô and Yokoi, 1992; Asakura and Yao, 2009). Furthermore, the effect of hydrogen bonding is known to induce shifts in the observed carbonyl resonances in this region for various peptide linkages (Asakawa *et al.*, 1992; Tsuchiya *et al.*, 1995).

Based on these interpretations, the aromatic peaks identified in *U. compressa* may reflect the presence of aromatic acids rather than a phenolic structure. For example, the aromatic carbons from phenylalanine exhibit chemical shifts at 132 ppm and 137 ppm and tyrosine peaks are reported at 128 ppm and 133 ppm (Schaefer *et al.*, 1984; Prabhu *et al.*, 1996; Košir and Kidrič, 2001). Whilst we cannot easily distinguish an aromatic amino acid from a phenolic structure in these spectra, and attempts at ^{15}N cross polarisation for these samples during my research were unsuccessful, the data generated in the previous chapter (i.e., the FTIR and elemental analysis) indicates that the aromatic peaks in *Ulva* most likely correspond to aromatic amino acids and the corresponding carboxyl peaks are attributable to amide bonds, with amine and amide peaks identified in the α -cellulose FTIR spectra (see Figure 4.19, Chapter 4). No amide peaks were identified in the corresponding FTIR spectra for barley straw cellulose, and as the nitrogen content was negligible, the peaks observed in the CP-MAS analysis of this fraction are consistent with phenolics. This is supported by the observation that no peaks were observed at ~150 ppm for *U. compressa*, indicating that the aromatic structure in *Ulva* likely did not have a phenolic group (-OH) or methoxy group (-OCH₃) attached to it, likely ruling out a 'lignin-like' structure, whereas these resonances were positively identified in barley straw.

Shift (ppm)	Assignment
172.4	C=O
168.4	C=O
137	Aromatic C (Unsubstituted)
129.1	Aromatic C (Unsubstituted)
105.1	C-1 (Cellulose)
87.6	C-4 (Cellulose, Crystalline)
82.4	C-4 (Cellulose, Disordered)
74.5	C-2, C-3, and C-5 (Cellulose)
62.8	C-6 (Cellulose)
49.7	-CH ₂ (C _α)
44	-CH ₂
32.8	-CH ₂
18.9	-CH ₃ (C _β)

Table 5.1. Identified peaks from ¹³C CPMAS NMR analysis of the α-cellulose fraction of *U. compressa*

Shift (ppm)	Assignment
171.2	C=O (Lignin)
153.3	Aromatic C-5 (Lignin, Syringyl, Connected to -OCH ₃)
149.1	Aromatic C-4 (Lignin, Guaiacyl, Connected to -OH)
137.3	Aromatic C-1 (Unsubstituted)
105.6	C-1 (Cellulose)
87.8	C-4 (Cellulose, Crystalline)
84.5	C-4 (Cellulose, Disordered)
75.2	C-2, C-3, and C-5 (Cellulose)
63.4	C-6 (Cellulose)
55.6	O-CH ₃ (Lignin)

Table 5.2. Identified peaks from ¹³C CP-MAS NMR analysis of the α-cellulose fraction of barley straw.

5.3 Solution-State NMR

5.3.1 ^1H

As stated, α -cellulose had poor solubility in NMR solvents. Nonetheless, solution-state NMR was trialled with the DMSO- d_6 soluble component of 3 *Ulva* α -cellulose extracts and the barley straw α -cellulose extract, where 20 mg of sample was mixed with 750 μL solvent (see Materials and Methods, §2.1.8.3 for details). The *Ulva* samples assessed include two from unstressed conditions and the α -cellulose fraction from hyposaline stressed *Ulva*, where an increase in the hypothesised amide-containing compound was observed via FTIR and elemental analysis (see Table 4.2 and Figure 4.20, Chapter 4).

A poor signal was observed in the ^1H NMR spectra from the α -cellulose from unstressed *Ulva* and from barley straw α -cellulose (Figure 5.11). Unlike the solid-state analysis, the cellulose backbone could not easily be defined in these samples, despite there being peaks consistent with carbohydrate ring protons in all 3 samples from 3–6 ppm, with the anomeric proton of glucose identified at ~ 5.44 ppm (Kaufmann, Mügge and Kroh, 2018; Alexandersson and Nestor, 2022). The observed peak at ~ 4.3 ppm may be attributable to xylose (Kim and Ralph, 2014; W. Wang et al., 2020). Regardless, there was a broad peak attributed to protons from water that overlapped these peaks, making interpretation of this region difficult, consistent with adsorbed water possibly identified in this fraction previously by ATR-FTIR (see Table 4.7, Chapter 4). However, as the purpose of this work was not to characterise the structure of α -cellulose, which had already been verified by ATR-FTIR and the prior solid-state analysis (Figure 5.9), interpretation of this region of the spectrum was not required.

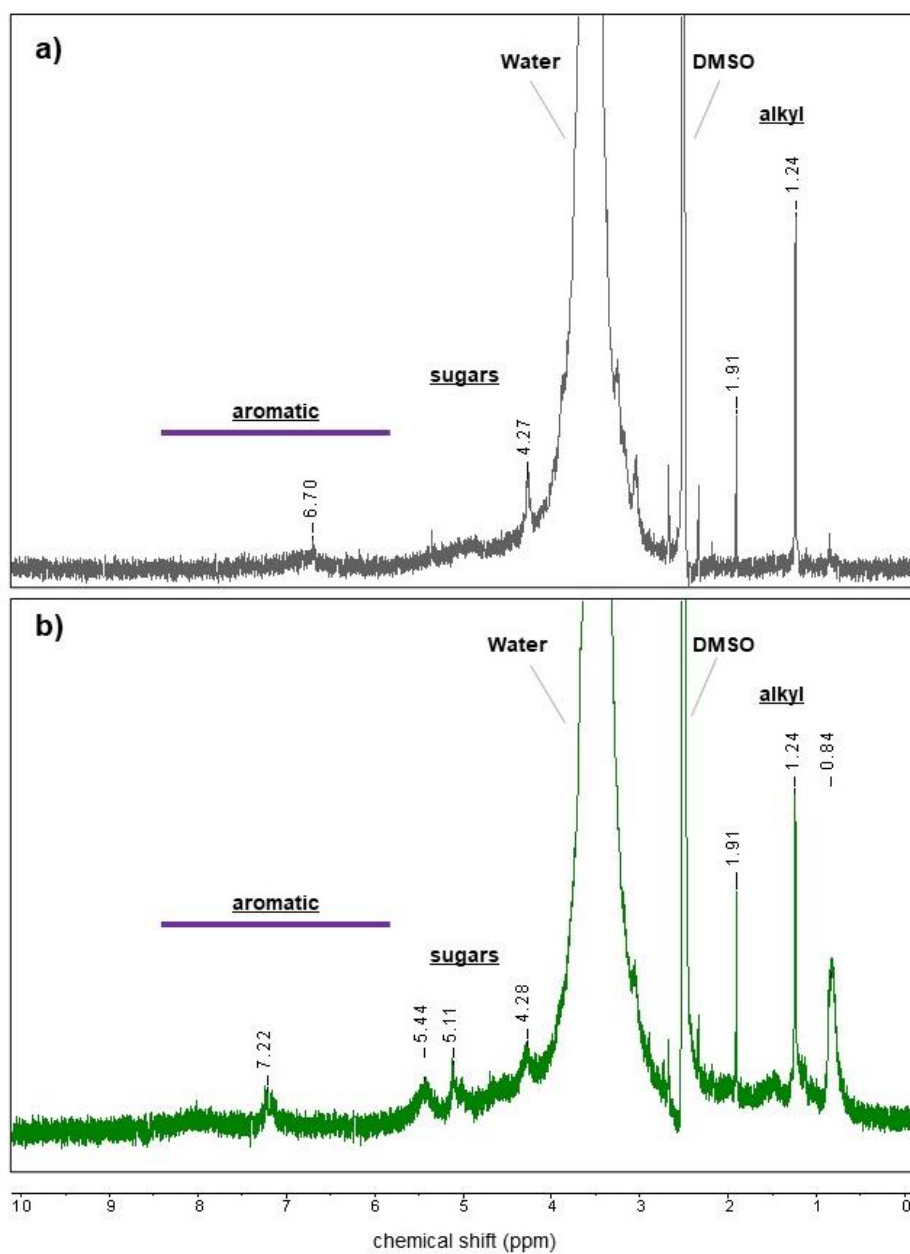


Figure 5.11. ^1H NMR spectra of DMSO-d_6 soluble material from α -cellulose fraction from barley straw (a) and *U. compressa* (b). Two *U. compressa* α -cellulose samples were assessed by this method, but only one is presented here for conciseness as both spectra show the same general features.

With respect to the ‘phycolignin’ investigation, I found only limited evidence of aromatic resonances in the *Ulva* α -cellulose spectra (Figure 5.11, b), with a slight inflection observed at ~7.25 ppm in both replicates that may indicate an aromatic structure. In barley straw, this was shifted to 6.70 ppm and slightly broader (Figure 5.11, a). The broader signal observed here may be consistent with the lignin identified by solid-state ^{13}C analysis (Figure 5.9). However, this was difficult to diagnose due to the poor resolution. Peaks at ~6.7 are typically attributed to aromatic protons from monolignol structures in lignocellulosic biomass (Sun *et al.*, 2011; Lu *et al.*, 2017). As stated previously (5.1.2.5), a large polymer such as lignin will exhibit broader peaks than a smaller molecule due to the effects of tumbling in solution (Fernández and Wider, 1999; Foster, McElroy and Amero, 2007; Frueh, 2014).

Interestingly, in both *Ulva* and barley straw, two peaks were observed at 1.19 ppm and 1.24 ppm, which are not consistent with expected glucose spectra. These peaks are consistent with alkyl protons likely from a side chain (Košir and Kidrič, 2001; Samuel *et al.*, 2011; Shi, Holland and Yarger, 2013), such as the methoxy group identified in barley straw by ^{13}C CP-MAS and the series of methyl and methylene groups observed in the corresponding *Ulva* sample (Figure 5.10, b). The observation that these peaks were present in both barley straw and *Ulva* cellulose was interesting, as a different profile of alkyl peaks was observed previously (Figure 5.10). In the prior ^{13}C analysis, *Ulva* α -cellulose did not have an obvious methoxy peak at ~55 ppm like barley straw, although there was a small shoulder in this region (Figure 5.10, a) which might be indicative of some contribution from this group. However, *Ulva* had a broader peak at 0.84 ppm, consistent with a $-\text{CH}_3$ group that was not present in barley straw, which may represent the ^{13}C peak observed at 18.9 ppm in *Ulva*, which was attributed to the C_β from an amino acid structure (Figure 5.10, a). However, due to the effects of hydrogen bonding (see §5.1.2.3), we cannot easily distinguish these resonances from protons in $-\text{NH}$ or $-\text{OH}$ groups which may be observed over a wide range of the spectra, hence the need for the subsequent ^{13}C - ^1H heteronuclear experiments.

Better solubility was observed from the α -cellulose fraction obtained from hyposaline stressed *Ulva*, which displayed a series of discernible peaks, including in the aromatic region of the ^1H spectra, with the peak at 7.22 ppm being the most prominent (Figure 5.12). This peak was consistent with the peak observed in the less-soluble *U. compressa* cellulose samples (Figure 5.11, b), which increases my confidence that the initial observation was genuine. Whilst there was still a contribution from a large water

peak at 3.39 ppm, peaks attributed to sugar protons could be identified more easily than in the 'unstressed' samples, in addition to the anomeric proton at 5.42 ppm (Kim and Ralph, 2014; Kaufmann, Mügge and Kroh, 2018; Alexandersson and Nestor, 2022). It was hypothesised that the stress-induced changes in the cellulose fraction from hyposaline stressed *Ulva* observed previously (see Figure 4.20, Chapter 4) led to the increased solubility via increased polysaccharide association with DMSO-d₆ soluble component (e.g., protein). However, there was insufficient material to perform an extensive comparison of solubility properties during this work.

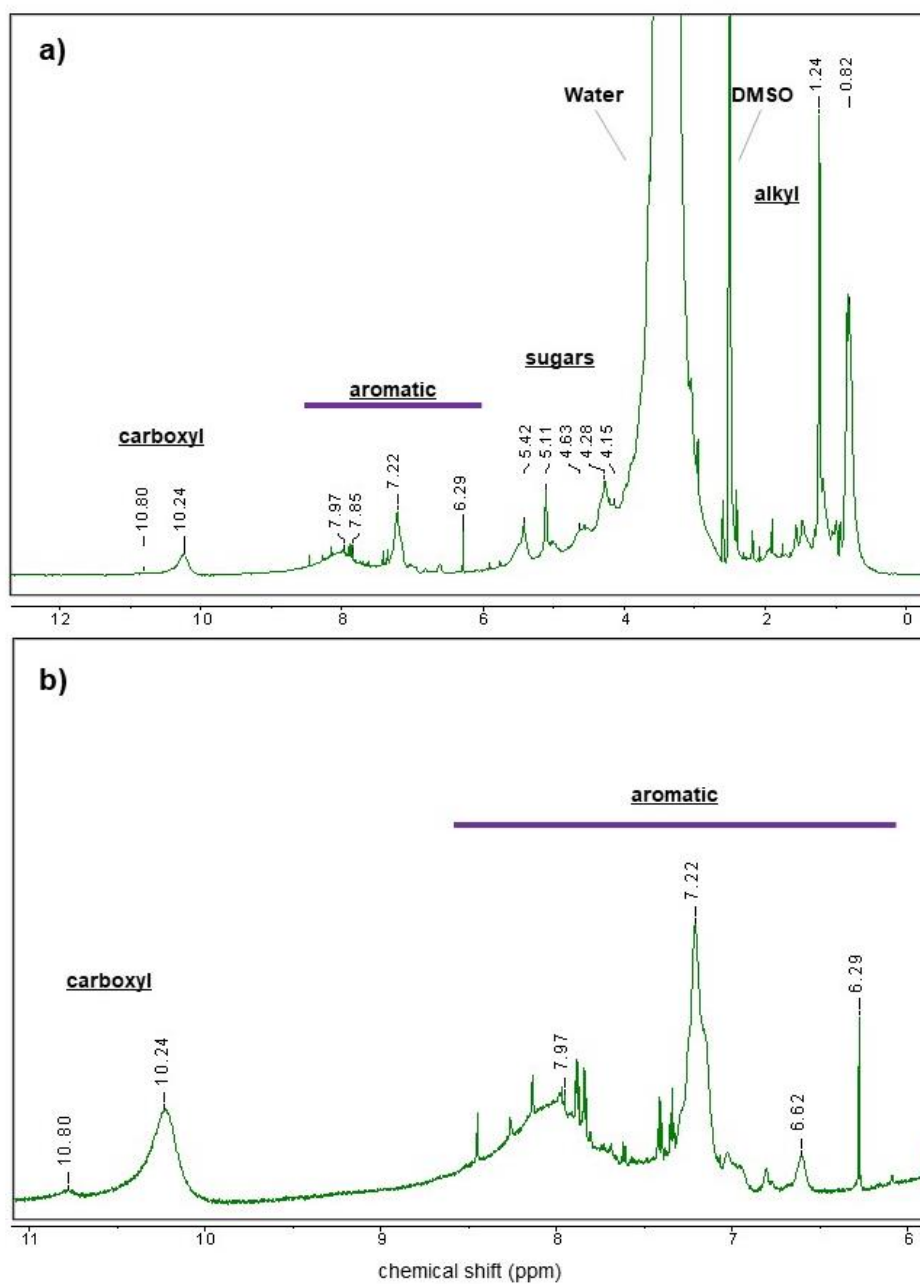


Figure 5.12. ^1H NMR spectra of DMSO- d_6 soluble material from α -cellulose fraction from *U. compressa* exposed to hyposaline stress (0 ‰, 3 h). The full spectrum for this sample is presented in (a), whilst the 'aromatic' and 'carboxyl' associated regions are presented in (b).

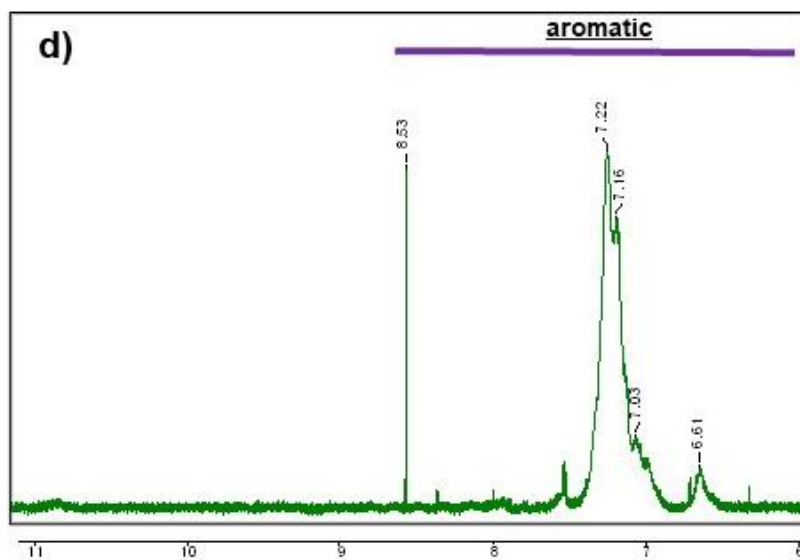
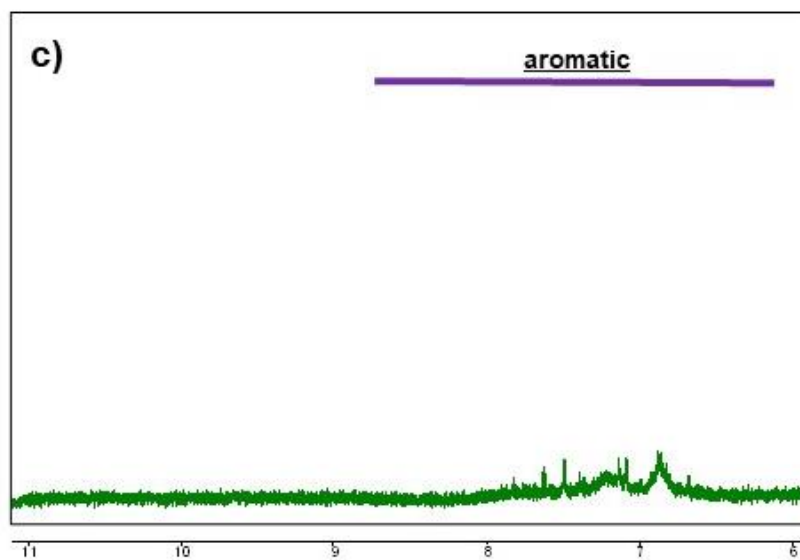
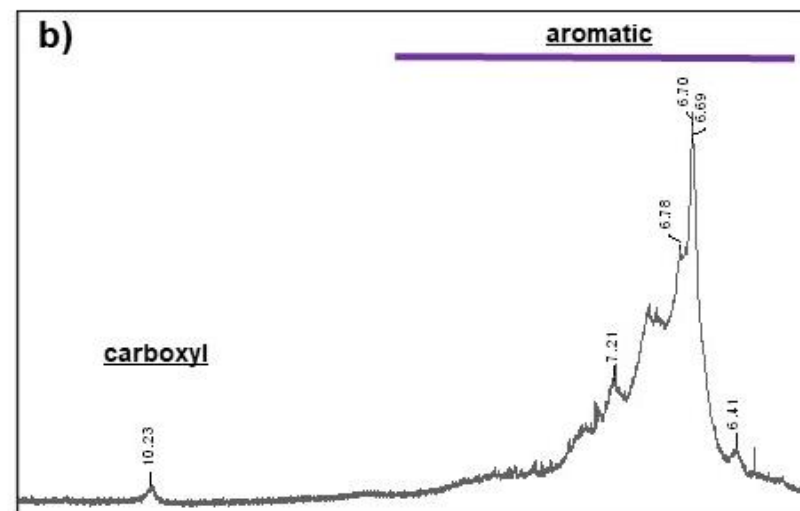
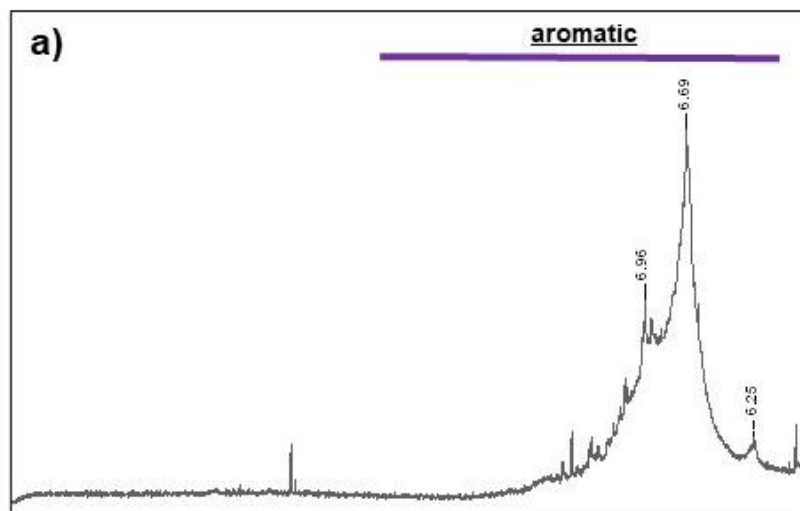
In addition to these peaks, a peak was identified at 10.24 ppm (Figure 5.12, b), consistent with a proton from a carboxylate group (Abraham, Mobli and Smith, 2003), with this group also identified in the solid-state ^{13}C spectrum. A smaller peak was also observed at 10.8 ppm, possibly consistent with the presence of two carbonyl peaks observed for *Ulva* α -cellulose by CP-MAS (Figure 5.6).

In the alkyl region, a series of smaller peaks were observed in addition to the previously identified resonances at 1.23 ppm and 0.82 ppm. Due to the complexity of these spectra, with overlapping spectra and the presence of various multiplet peaks, I performed multidimensional NMR to resolve the key structural information from this sample to fulfil the aims of this thesis. During this work, other solvents were not trialled (see 5.4.1 for discussion), as I hypothesised that the structures of interest in the cell wall fractions would be DMSO- d_6 soluble, with analysis of α -cellulose component beyond the scope of this investigation (as it was already performed by FTIR and solid-state analysis).

The other fractions (oxalate-soluble, alkaline-soluble, and wash) were all readily soluble in DMSO- d_6 , where ^1H NMR spectra with overlapping resonances and evidence of aromatic peaks were observed (see the aromatic region in Figure 5.13). These samples, in addition to the α -cellulose from hyposaline-stressed *Ulva*, were therefore processed for multidimensional NMR analysis (discussed subsequently in section 5.4.3.1). Unfortunately, an analysis could not be performed with barley straw α -cellulose for comparison due to the poor resolution observed in the previously described spectra (Figure 5.11, a). However, there appeared to be sufficient evidence from the biochemical tests (acetyl bromide soluble lignin and phloroglucinol-HCl methods), FTIR analysis, and ^{13}C CP-MAS performed here and in the previous two chapters that verified that lignin was associated with this fraction, making further analysis unnecessary. Also, based on these analyses, the barley straw hemicellulose fraction (Hb) was predicted to have a higher lignin content than the insoluble fraction (see acetyl bromide soluble lignin data, Table 3.2, Chapter 3), which made it a more suitable candidate for identifying lignin-associated peaks.

The Ha fraction had the same solubility issues as cellulose, and there was insufficient yield to perform solid-state NMR, with ~100 mg material required for sample submission. Additionally, whilst the oxalate soluble fraction was solubilised in DMSO- d_6 , it was not run. Neither of these fractions appeared to be associated with major 'aromatic resonances' in the lignin-positive control barley straw by FTIR (see

Figure 4.8 and Figure 4.11, Chapter 4), nor did they stain positive via biochemical tests (see Table 3.2, Chapter 3), suggesting that further analysis of these fractions was not required to achieve the aims of this thesis. Despite this, analysis of the oxalate-soluble ulvans would have been worth assessing for direct comparison with α -cellulose due to the hypothesised presence of protein in both fractions. However, the use of 1-dimensional and ^{13}C - ^1H HSQC for extracted ulvans has been reported before in the literature due to the economic interest in ulvans for biomaterials and bioenergy (Glasson et al., 2017; Tran et al., 2018; de Carvalho et al., 2020; Gao et al., 2020; Guidara et al., 2021).



chemical shift (ppm)

Figure 5.13. ^1H NMR spectra of DMSO- d_6 soluble material from barley straw hemicellulose (a), barley straw wash (b), *U. compressa* hemicellulose (c), and *U. compressa* wash (d). The 'aromatic' and 'carboxyl' regions of the spectra are selectively plotted here for the identification of lignin. Here, hemicellulose refers to the 'alkali-soluble' cell wall fraction that remained in solution after acid-precipitation. The wash fraction refers to the material that was solubilised as the 'alkali-insoluble' material post-hemicellulose extraction was washed to yield the insoluble α -cellulose fraction.

In the 1-dimensional spectra for the hemicellulose (Hb) and wash samples (Figure 5.13), there was clear evidence of an aromatic signal in both the barley straw samples (Figure 5.13, a-b). The same peaks were identified in both, indicating that the aromatic compound was most likely the same structure in both samples. However, a small carboxyl-associated peak was observed in the barley straw wash sample at 10.23 ppm but not in the hemicellulose sample. Interestingly, there was evidence of acetylation in hemicellulose in the ^1H NMR spectra from both barley straw and *Ulva* (Figure 5.14), despite the apparent absence of ester groups in the latter by ATR-FTIR (Lee *et al.*, 2011). This was investigated further by HSQC, as due to the complexity observed in this region in the spectra, these peaks were difficult to assign.

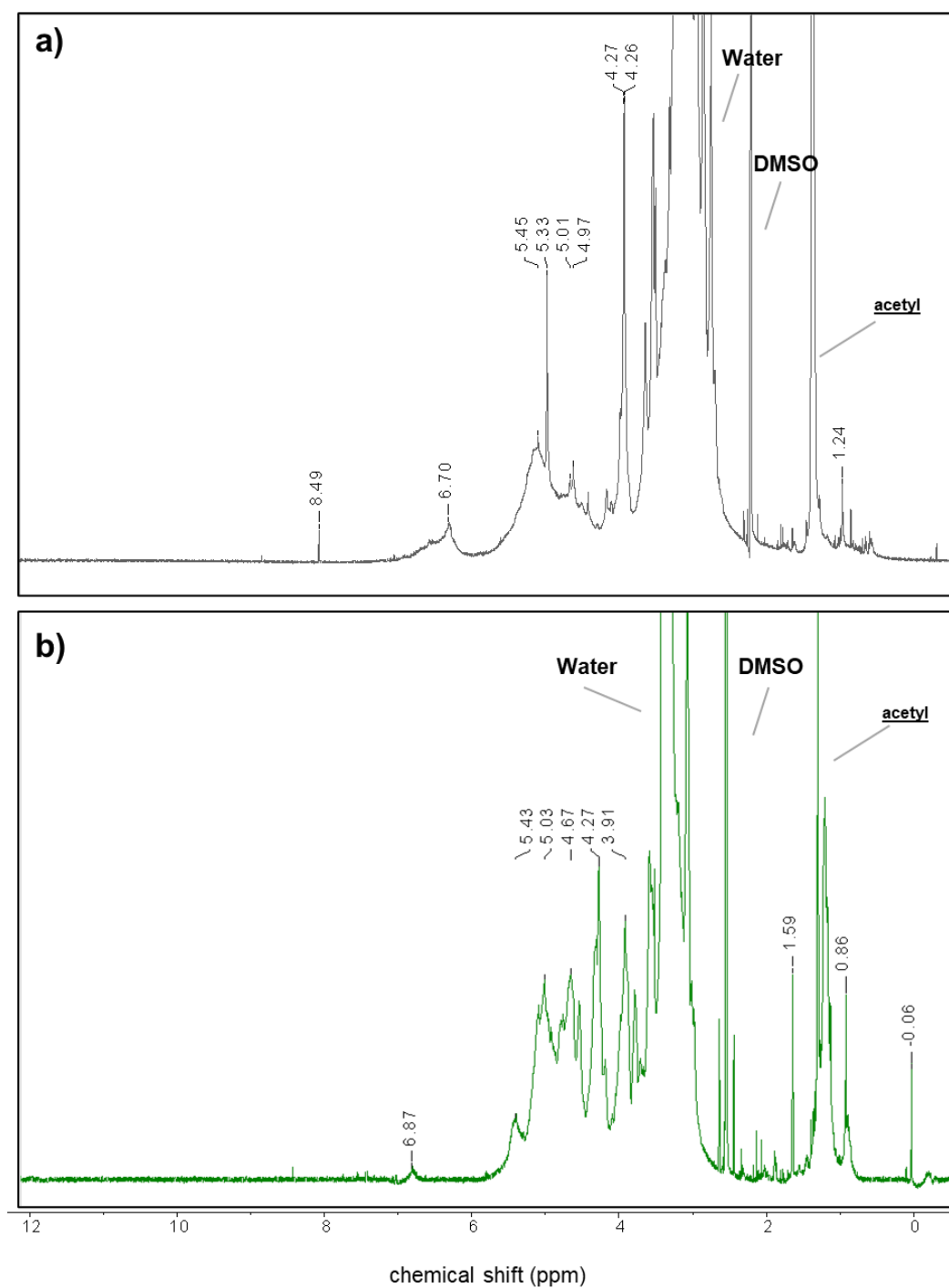


Figure 5.14. Comparison of ^1H NMR spectra from barley straw (a, grey) and *U. compressa* (b, green). Hypothesised acetyl groups are identified at 1.59 ppm and 1.22 ppm respectively.

For the aromatic region of *Ulva*, peaks were observed in the wash but not the hemicellulose fraction, indicating that the alkali-soluble fraction of *Ulva* was not associated with an aromatic or phenolic compound, as observed in plant hemicelluloses (Vogel, 2008; Kang *et al.*, 2019; Terrett and Dupree, 2019). Interestingly, the aromatic peaks in *Ulva* wash were the same as those observed in the corresponding α -cellulose sample (Figure 5.12, b), although the broad peak at ~8 ppm observed in α -cellulose was missing. The absence of the aromatic peak at ~7.22 ppm in hemicellulose suggests that this resonance was removed from the α -cellulose during washing, possibly indicative of a population of this structure that was strongly associated with cellulose (and therefore remained associated after washing), and a population that was loosely associated and removed by washing.

Both were shifted to the spectra observed in barley straw samples, where the aromatic peak was observed at ~6.7 ppm. This is consistent with the preliminary barley straw α -cellulose spectra, where a small peak at 6.7 ppm was observed (Figure 5.11, a). These differences between the two 'aromatic-containing' *Ulva* samples and the barley straw hemicellulose fraction are highlighted in Figure 5.15, where the aromatic region of all 3 samples is plotted.

Based on these observations and the conclusions from the previous chapter, we can conclude that this resonance can be attributed to monolignol structures in lignin. As such, the shifted peaks observed in the *Ulva* α -cellulose appear to reflect a different structure. Notably, the aromatic protons of the aromatic amino acids are reported to resonate in this shifted region, which is consistent with the supporting elemental analysis and FTIR (Avison *et al.*, 1990; Košir and Kidrič, 2001; Shi, Holland and Yarger, 2013; Kim *et al.*, 2017; Zompra *et al.*, 2021). However, multidimensional NMR analysis was required to verify this. Nonetheless, the absence of an equivalent structure to lignin in *U. compressa* hemicellulose is indicative of the biochemical differences between the samples, providing further evidence as to the absence of 'phycolignin' in *Ulva*.

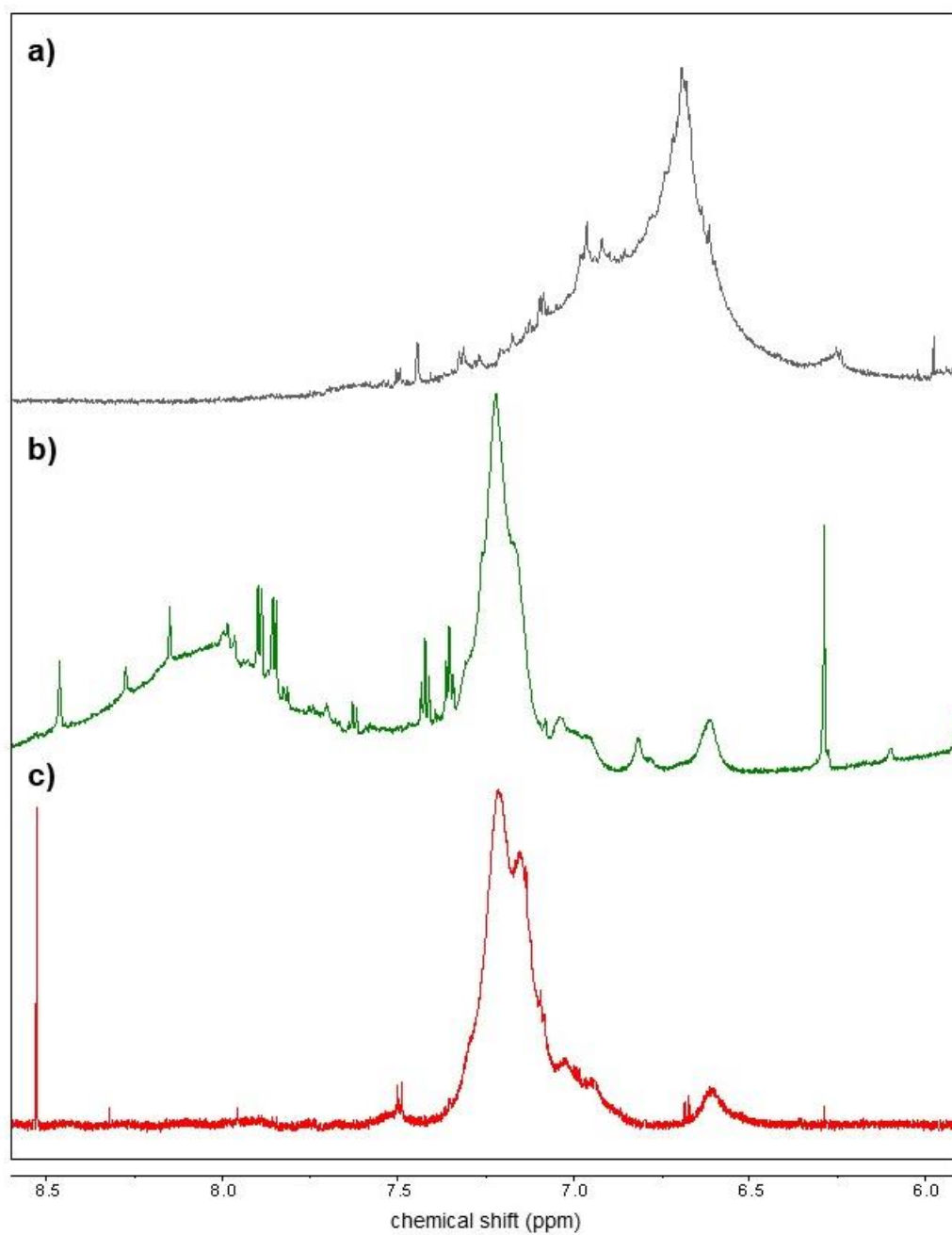


Figure 5.15. Comparison of the 'aromatic' region of the ¹H NMR spectra for barley straw hemicellulose (a grey), *U. compressa* α-cellulose (b, green), and the *U. compressa* wash fraction (c, red).

5.3.2 ^{13}C - ^1H HSQC

Whilst data was generated from the full ^{13}C - ^1H spectra, as stated previously (5.1.2.4), multidimensional NMR experiments generate a wealth of information that requires extensive analysis. As a result, the data plotted here corresponds to the aromatic region of the ^{13}C - ^1H HSQC spectra to coincide with the main aim of this thesis, the identification of 'phycolignin' in the *Ulva* cell wall. Analysis of these other regions, divided discretely into anomeric, aliphatic, and 'methyl' regions, will yield further information about the biochemical composition of *Ulva* and barley straw biomass in future investigations that are beyond the scope of this thesis as performed in the previous FTIR chapter (for example, the described investigations into ulvan esterification). Division of the complex HSQC spectrum into these key regions is widely reported in HSQC analysis of cell walls (Yelle, Ralph and Frihart, 2008; Samuel et al., 2011; Mansfield et al., 2012; Wen et al., 2013; Takeda et al., 2017; W. Wang et al., 2020). However, these regions will be discussed for the α -cellulose fraction to verify the previous observations made from ^1H and ^{13}C analysis, in addition to the anomeric region of the hemicellulose fractions as a predictor of sugar composition.

Here, the assessment of heteronuclear couplings via HSQC provided a more accurate analysis of the major structural components, which was required due to the complexity of the crowded ^1H spectra observed previously. As stated, the assessment of protons in -NH and -OH groups are challenging with 1-dimensional ^1H NMR because of the effects of hydrogen bonding (5.1.2.3). In particular, amide groups overlap the 'aromatic region' of the spectra, with the identification of these groups being particularly relevant for the *U. compressa* α -cellulose fraction (Hasan, 1980; Abraham, Griffiths and Perez, 2013). Peaks attributed to these groups could be identified by cross-referencing the shifts observed from ^1H - ^{13}C coupling with the original ^1H spectra, because these resonances are filtered out in single-bond ^{13}C - ^1H heteronuclear experiments such as HSQC.

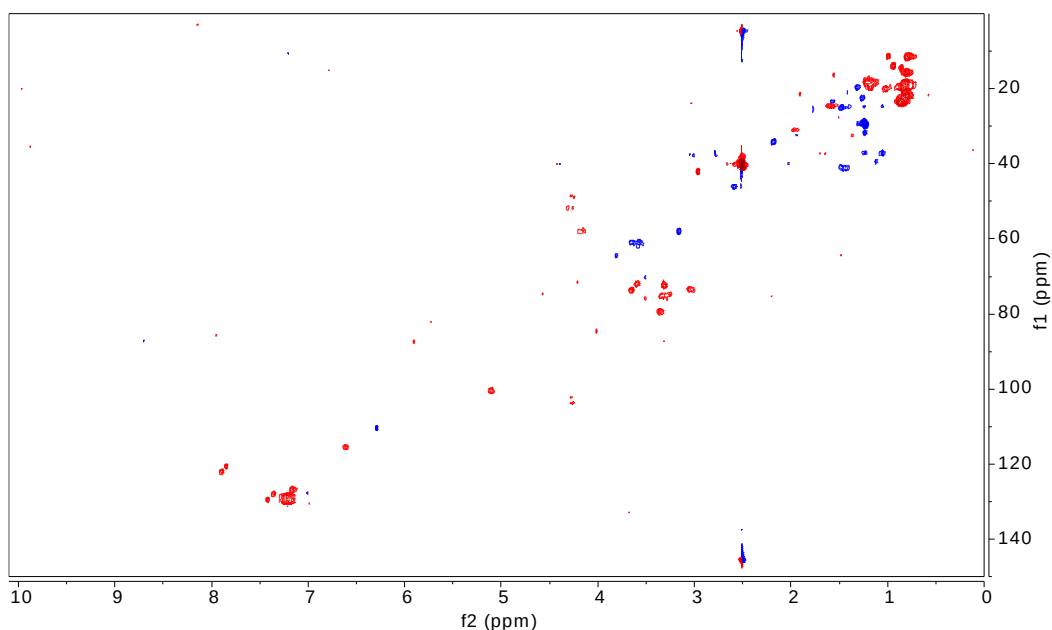


Figure 5.16. ^{13}C - ^1H HSQC of the α -cellulose fraction from hyposaline-stressed *U. compressa*. In this plot, f2 represents resonance from ^1H and f1 represents resonance from coupled ^{13}C nuclei as the chemical shift from TMS (ppm). The colour of cross-peaks represents the multiplicity of the identified couplings, with red representing the positive phase from CH or CH_3 groups and blue representing the negative phase (corresponding to CH_2 groups).

A representative ‘full spectrum’ from HSQC analysis is presented in Figure 5.16 for the *U. compressa* α -cellulose fraction, with the corresponding regions of interest magnified in Figure 5.17 for the easier presentation of heteronuclear couplings (i.e., cross-peaks). Here, the absence of cross peaks for ^{13}C for protons that resonated > 10 ppm was consistent with the peaks at 10.24 ppm from the 1-dimensional ^1H spectra being a carboxyl group, which indicated that this resonance was not attributable to a proton coupled to carbon, with carbonyl groups not visible via HSQC. Furthermore, a broad signal was not observed around > 8 ppm as observed for the 1-dimensional ^1H NMR spectra (Figure 5.12, b), indicating that the resonance observed here was not from a proton coupled to a carbon, meaning it cannot be attributed to an aromatic structure. This signal may therefore be consistent with the predicted amide group, with protons from the carboxyl group (OH) typically observed further downfield than the protons from the NH group of amides due to the increased deshielding of oxygen relative to nitrogen (Abraham, Mobli and Smith, 2003; Abraham, Griffiths and Perez, 2013). However, some cross-peaks were observed at 121.95/7.89 and 120.63/7.85 ppm ($\delta_{\text{C}}/\delta_{\text{H}}$), consistent with aromatic ring groups (Ralph and Landucci, 2010; Mansfield *et al.*, 2012),

indicating that there was some aromatic contribution from the overlapping resonances identified in this region.

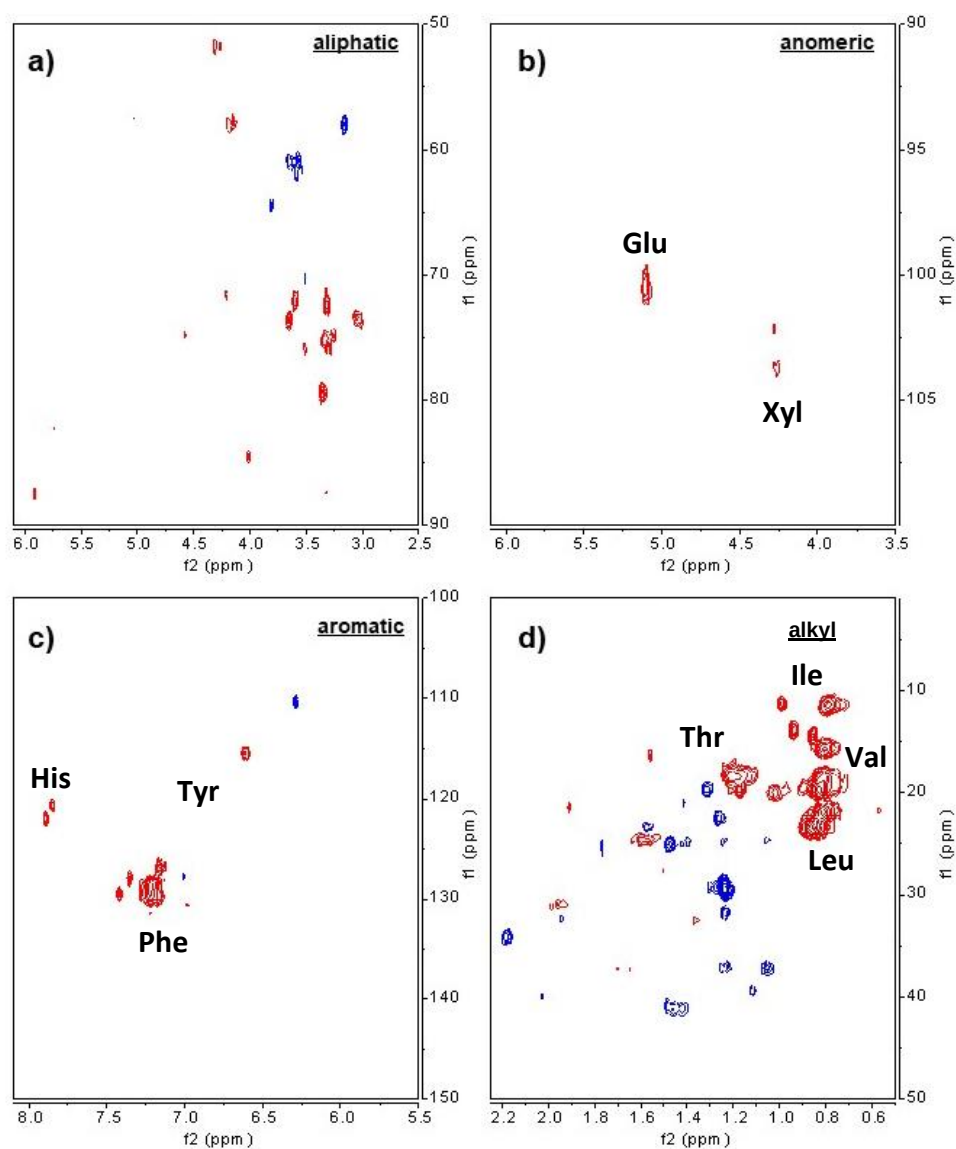


Figure 5.17. ^{13}C - ^1H HSQC of the α -cellulose fraction from hyposaline-stressed *U. compressa* magnified at the aliphatic (a), anomeric (b), aromatic (c), and alkyl (d) regions of the spectrum.

The resolution of cross peaks is improved by splitting the HSQC spectra into discrete regions of interest. In cell wall studies, the aliphatic region (δ_C – 50 to 90, δ_H – 2.5 to 6 ppm, Figure 5.17, a) displays cross peaks associated with the non-anomeric CH groups of sugars, with the anomeric CH observed in the anomeric region (δ_C – 90 to 110, δ_H – 3.5 to 6 ppm, Figure 5.17, b). The cross peak observed at 100.42/5.11 ppm (δ_C/δ_H) is consistent with the anomeric CH of $\beta(1\rightarrow4)$ linked glucose, whilst the peak at 102.18/4.28 ppm indicates that a xylose-containing carbohydrate is present in the sample (Kim and Ralph, 2014; Kaufmann, Mügge and Kroh, 2018; Dalvi *et al.*, 2020; Gao *et al.*, 2020), corresponding to the observation that cellulose extracts from *Ulva* often contain xylose, likely due to close association with algal xyloglucan (Lahaye, Jegou and Buleon, 1994; Wahlström, Edlund, et al., 2020; Gomaa et al., 2022). A series of aromatic cross-peaks were also identified (Figure 5.17, c), demonstrating the utility of this analytical method as aromatic groups were not identified in this sample in the previous chapter by ATR-FTIR (see Figure 4.20, Chapter 4).

Finally, the ‘methyl’ region (Figure 5.17, d) corresponds with alkyl groups, as discussed previously in the 1-dimensional analysis. This region is typically less important for studies of carbohydrate structure, with these resonances typically attributable to co-extracted fatty acids or amino acids that can be studied here (Alexandri et al., 2017; Asami and Reif, 2019; Hwang et al., 2021). However, modifications such as the *O*-acetylation of hemicellulose can be observed in this region (Samuel *et al.*, 2011). This is shown in Figure 5.18, where a group corresponding with an acetyl CH₃ is in the barley straw hemicellulose fraction (Figure 5.18, a) but not the corresponding *U. compressa* sample (Figure 5.18, c), consistent with the observations from the ATR-FTIR analysis in the previous chapter.

In phase-edited HSQC experiments, we can determine the multiplicity of cross peaks with a negative phase (blue) indicative of CH₂ groups and the positive phase (red) indicative of CH or CH₃ groups. The presence of a series of CH₂ resonances in the non-aromatic and non-anomeric regions of the spectra (Figure 5.17, a & d) is consistent with the observation of a major ‘non-sugar’ component of the *Ulva* α -cellulose fraction, consistent with the predicted protein association. In the methyl region specifically (Figure 5.17, d), cross peaks are identified that are consistent with methyl groups from various amino acids from immunoglobulin G protein analysed by HSQC by Hwang and colleagues (2021), consistent with the series of methyl groups observed by ¹³C CP-MAS. The cross peaks corresponded with CH₃ groups from isoleucine (δ_C/δ_H 15.74/0.81,

13.98/0.94, and 11.49/0.79 ppm), threonine (δ_C/δ_H 18.38/1.21), valine (δ_C/δ_H 19.77/0.83), and leucine (δ_C/δ_H 23.36/0.85 and 21.97 and 0.81). Notably, these were absent in the tested barley straw fractions. Whilst the other resonances (particularly the CH₂ groups) in both these regions likely correspond to other amino acids, it is difficult to identify them in a sample of mixed composition by ¹³C-¹H. As a result, we cannot be certain that these signals correspond to hydroxyproline-rich glycoproteins, with the reported cross-peaks for Hyp not easily discernible in the spectra (Tan *et al.*, 2010). Furthermore, the absence of additional cross peaks in the anomeric region (Figure 5.17, b) is not consistent with a glycoprotein HSQC, where the contribution from arabinose, galactose, and other sugars such as rhamnose would be expected (Tan *et al.*, 2010). This may be due to the inherent sensitivity limitations of NMR, and other methods of sugar analysis (i.e., chromatographic methods) may be more appropriate for assessing this.

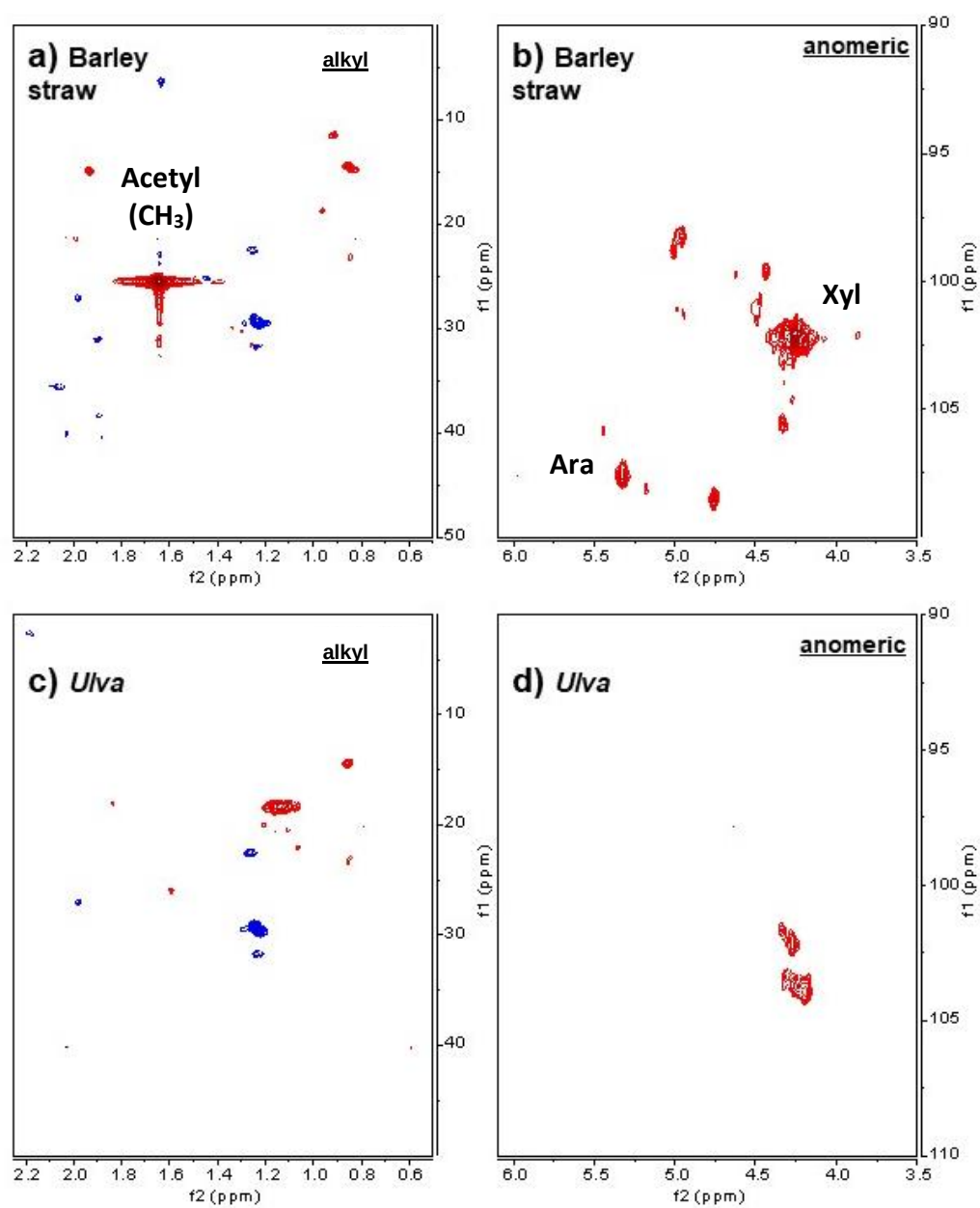


Figure 5.18 ^{13}C - ^1H HSQC spectra for hemicellulose fractions of barley straw and *Ulva*.

The anomeric regions of the hemicellulose fractions were also contrasted to support the prior ATR-FTIR analysis, as HSQC can be used to identify the constituent sugars of cell wall samples (Mansfield et al., 2012; W. Wang et al., 2020). For the *Ulva* fraction, the cross peak attributed to xylose was observed (δ_C/δ_H 103.79/4.24), with a second peak possibly consistent with glucuronic acid (δ_C/δ_H 102.25/4.27) reflecting the expected composition of the alkali-soluble component of the *Ulva* cell wall (Lahaye and Robic, 2007; Yelle, Ralph and Frihart, 2008; Tran et al., 2018; de Carvalho et al., 2020).

The more complex spectrum of the barley straw hemicellulose HSQC reflects the more diverse range of sugars reported for the alkali-soluble component of barley biomass, with arabinoxylans known to be the most abundant hemicellulose (Sun and Sun, 2002; Krawczyk et al., 2008). Cross peaks here (Figure 5.18, b) were consistent with those reported for β (1-4) linked xyloses and the corresponding 1-2 and 1-3 linked arabinans in the literature (Yelle, Ralph and Frihart, 2008; van Munster et al., 2017). These differences are consistent with the different spectral profiles observed via FTIR for these samples previously. Surprisingly, there appeared to be no contribution from rhamnose in *Ulva*, with ulvan hypothesised to segregate in this fraction based upon the FTIR spectra discussed in the previous chapter (see §4.3.1). Based on previous HSQC analyses of ulvan, the anomeric resonances from rhamnose-3-sulfate are expected at δ_C/δ_H ~100/4.9 ppm (Tran et al., 2018; de Carvalho et al., 2020; Guidara et al., 2021). Furthermore, the cross peaks of non-sulfated rhamnose are reported to be shifted even further downfield (~110/5.2 ppm), with the fraction identified as 'non-sulfated' (Gao et al., 2020). Further assessment of the other cell wall fractions that were not assessed within the scope of this work (i.e., the precipitated hemicellulose and oxalate-soluble fractions) may help resolve the presence or absence of ulvans in this fraction.

Interestingly, there were no cross-peaks observed in the anomeric region for *Ulva* wash and the 'aliphatic region' (Figure 5.19, a & b), suggesting that few sugars segregated into this fraction, consistent with the degradation hypothesised in the previous chapter (§4.5.3). Most cross-peaks were instead observed in the 'alkyl' region (Figure 5.19, c), largely corresponding with cross-peaks from the cellulose fraction (Figure 5.17, d), indicating that this fraction consisted predominantly of material that could be removed from cellulose. This is consistent with the prior elemental analysis where a low elemental carbon content (20.83 %, w/w) was verified for this sample relative to the other cell wall fractions (see Table 4.2, Chapter 4). Interestingly, most of the cross peaks identified corresponded to CH or CH₃ groups rather than CH₂ groups

(based upon the phase), which suggested that there was little contribution from lipids in this sample as hypothesised by FTIR analysis, which consists of a 'CH₂ backbone'. However, it was the hypersaline-stressed wash fraction that displayed the strongest evidence of lipids in the previous chapter (see Figure 4.31, Chapter 4).

In contrast, the HSQC for barley straw wash appeared as a 'less complex' version of the corresponding hemicellulose fraction across the plotted regions (Figure 5.19, d to f), indicating that this fraction was predominantly residual material from the alkali-extraction process. Here, there was some evidence of sugars in the anomeric region (Figure 5.19, e), a series of CH₂ peaks consistent with amino acids or lipids (Figure 5.19, d & f), and also aromatic structures (see subsequent discussion for Figure 5.20).

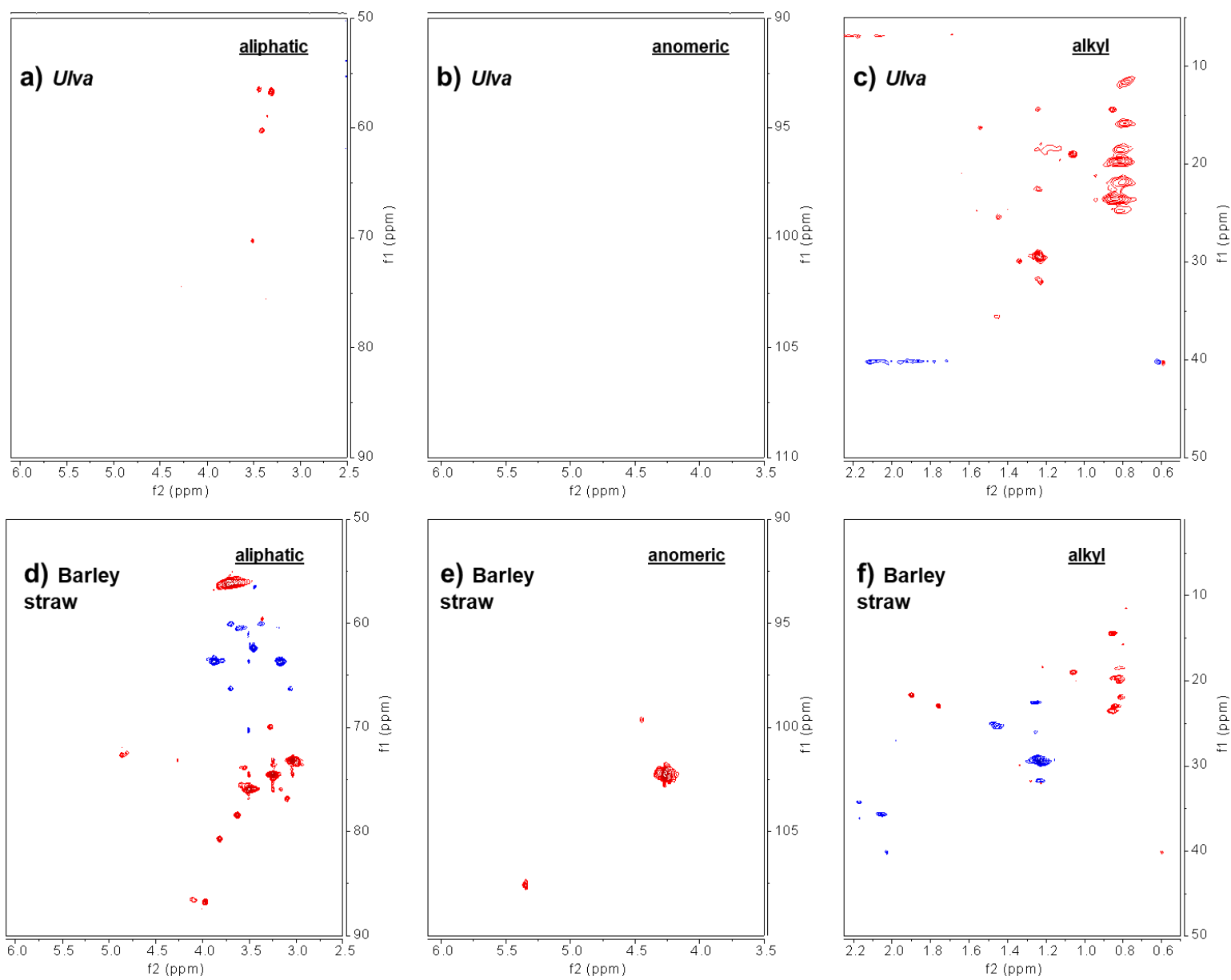


Figure 5.19. ^{13}C - ^1H HSQC spectra of the aliphatic, anomeric, and alkyl regions for the *U. compressa* (a-c) and barley straw (d-f) pooled wash fraction.

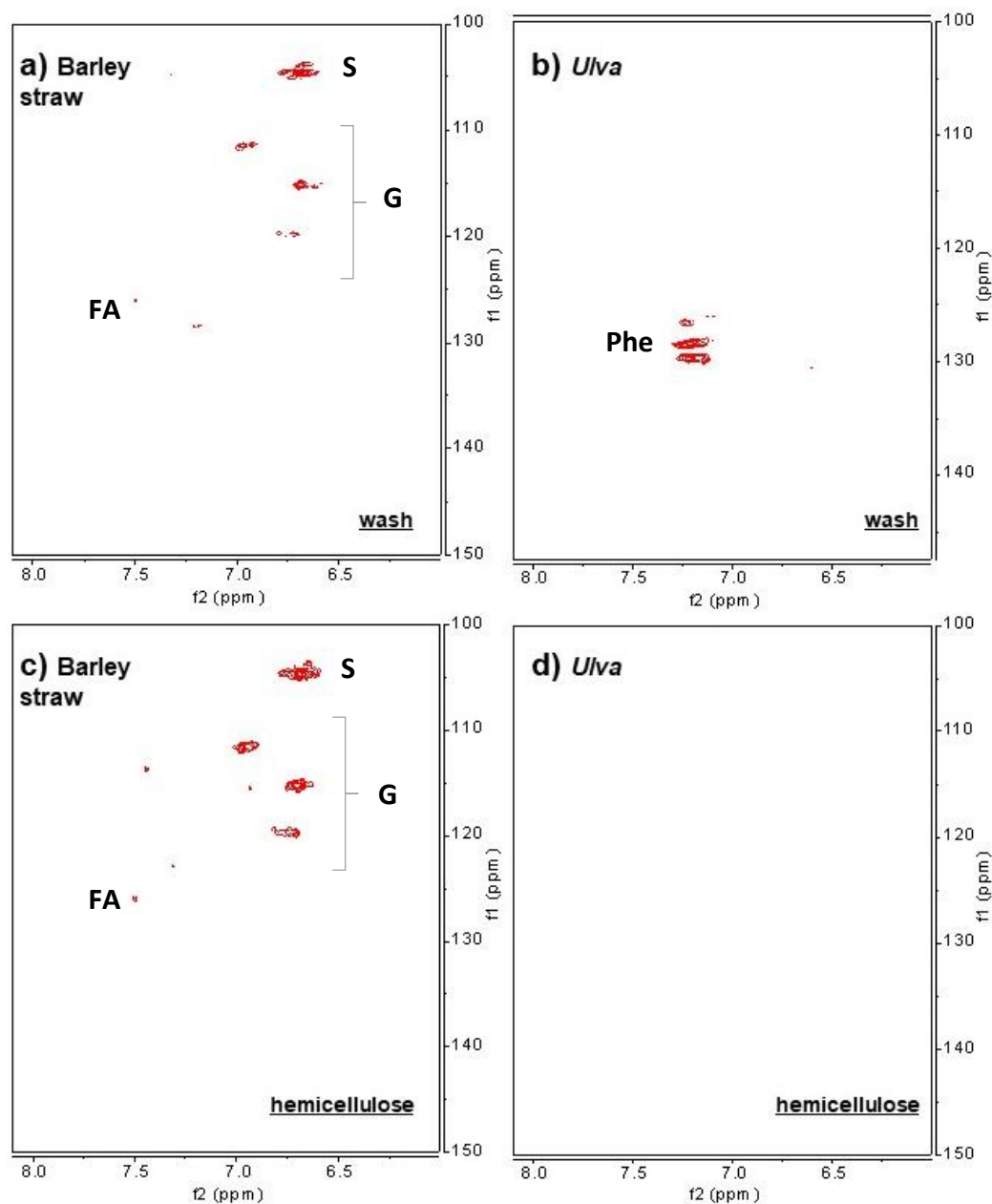


Figure 5.20. ^{13}C - ^1H HSQC spectra of the aromatic region for wash fraction for barley straw (a) and *Ulva* (b), and hemicellulose fraction for barley straw (c) and *Ulva* (d). Spectra are annotated with resonances consistent with syringyl (S), guaiacyl (G), ferulic acid (FA), and phenylalanine (Phe).

In Figure 5.20, the aromatic region from the other analysed cell wall fractions is compared to the corresponding α -cellulose sample from *Ulva*. As observed in the 1-dimensional spectrum (Figure 5.13, c), no aromatic cross-peaks were observed for *Ulva* hemicellulose (Figure 5.20, c). There were fewer cross-peaks in this region for the *Ulva* wash sample than the corresponding α -cellulose fraction, indicating that the aromatic structure that was extracted during washing was less complex than observed in the α -cellulose. However, the identified cross peaks for this sample (δ_C/δ_H 129.66/7.21 and 125.98/7.13) corresponded to those identified in α -cellulose, indicating that a proportion of this structure remained insoluble during the washing process. This is again consistent with the observations from the 1-dimensional spectrum (Figure 5.15).

The cross-peaks in the aromatic region for barley straw were different to those observed in *Ulva*, being observed at both a lower ^{13}C and ^1H resonance than the observed aromatic resonances in *Ulva*. Based on the literature, a selection of these peaks are attributable to aromatic CH groups from monolignols and hydroxycinnamic acids in cell wall samples, including guaiacyl (δ_C/δ_H 115.21/6.71), syringyl (δ_C/δ_H 104.67/6.69 ppm), and ferulate (δ_C/δ_H 125.98/7.50) (Ralph and Landucci, 2010; Wen et al., 2013; Kim et al., 2017; Takeda et al., 2017; W. Wang et al., 2020). Whilst it is difficult to distinguish between these phenolics without model compounds, the identification of these structures is consistent with lignin and phenolic association with the cell wall, while the different set of cross-peaks identified in *Ulva* indicates that equivalent structures in these algal samples are absent.

Whilst there are no HQSC studies characterising the aromatic region of *Ulva* biomass, the differences in chemical shift observed between the phenolic cross-peaks of barley straw samples and the *Ulva* cross-peaks appear to resemble the shifts observed from comparisons of phenolics with aromatic acids performed in the literature, with co-extraction of protein known to complicate studies of lignin in cell wall samples (Bunzel, Schüßler and Tchetseubu Saha, 2011; Rencoret et al., 2015; Kim et al., 2017). As shown in Figure 5.17 (c) and Figure 5.20 (b), the labelled series of cross peaks appear to correspond to aromatic CH groups from phenylalanine (δ_C/δ_H 128.69/7.22 and 127.89/7.36 ppm), histidine (δ_C/δ_H 129.5/7.42 ppm) and tyrosine (δ_C/δ_H 115.43/6.62 ppm) respectively (Košir and Kidrič, 2001; Kim et al., 2017; Zompra et al., 2021). However, there is a significant overlap between phenolic and aromatic amino acid resonances that make them difficult to resolve by ^{13}C - ^1H NMR alone (Kim et al., 2017). Furthermore, the peaks at 121.95/7.89 ppm and 120.63/7.85 ppm that were identified in *Ulva* α -cellulose

specifically cannot be attributed to any of the amino acids, nor could it be attributed to any other aromatic structure typically associated with the plant cell wall during HSQC experiments.

Due to these limitations, ^{15}N - ^1H HSQC is usually used as a diagnostic tool for amino acid identification in protein NMR (Hayato Morita *et al.*, 2004), but due to the discussed sensitivity issues, this was not possible during this research. Despite this, the supporting evidence from the prior FTIR and elemental analysis allows us to make an informed assessment of these spectra. The biochemical evidence for barley straw is consistent with lignin due to the biochemical tests and lack of nitrogen observed from elemental analysis. Conversely, the prior data (elemental analysis and FTIR) is consistent with protein, and the aromatic peaks can be attributed to aromatic amino acids rather than phenolics. This is also supported by the lack of the 'methoxy' peak associated with phenylpropanoids in the prior solid-state analysis (Figure 5.10).

5.4 Discussion

5.4.1 NMR Complements FTIR Analysis

This data, and the data obtained in the previous two chapters, has built up a comprehensive picture of the *Ulva* cell wall components obtained by the sequential extraction process applied in this thesis. The presence of lignin in barley straw biomass has been successfully verified, and as such, the absence of an equivalent, 'lignin-like' fraction in *Ulva* has also been determined.

Based upon the 3 analytical tools employed (elemental analysis, FTIR, and NMR), the hemicellulose fraction of *Ulva* appears to be predominantly sugars, with no evidence of a phenolic or protein content. This is in direct contrast to plants, where hemicelluloses are strongly associated with lignin and other phenylpropanoids which cross-link the cell wall. This was verified here in the corresponding barley straw samples.

Whilst some aromatic signal is observed in the *Ulva* wash and α -cellulose fractions, the described evidence suggests that these are associated with aromatic amino acids rather than phenolics, consistent with the conclusions from FTIR analysis, and the observation of methyl groups consistent with various aliphatic amino acids. Interestingly, xylose may be present in the insoluble fraction. With the current data, it is difficult to determine whether proteins are associated with cellulose or 'hemicelluloses' in the *Ulva* cell wall. Furthermore, the likely identification of aromatic amino acids in the

wash may indicate that some protein was hydrolysed during hemicellulose extraction or that a subset of proteins are more loosely associated with cellulose and are more easily removed. However, the amino acid composition cannot be determined definitively with the described methods. Nonetheless, the presence of a proteinaceous cell wall has likely led to inaccurate estimations of Klason lignin content previously in *Ulva*, with the presence of protein reported experimentally to interfere with lignin analyses by biochemical methods (Kim *et al.*, 2017). Due to these observations, it appears that the aims set at the beginning of this thesis have been achieved, and ‘phycolignin’ can be discounted from the model of the *Ulva* cell wall.

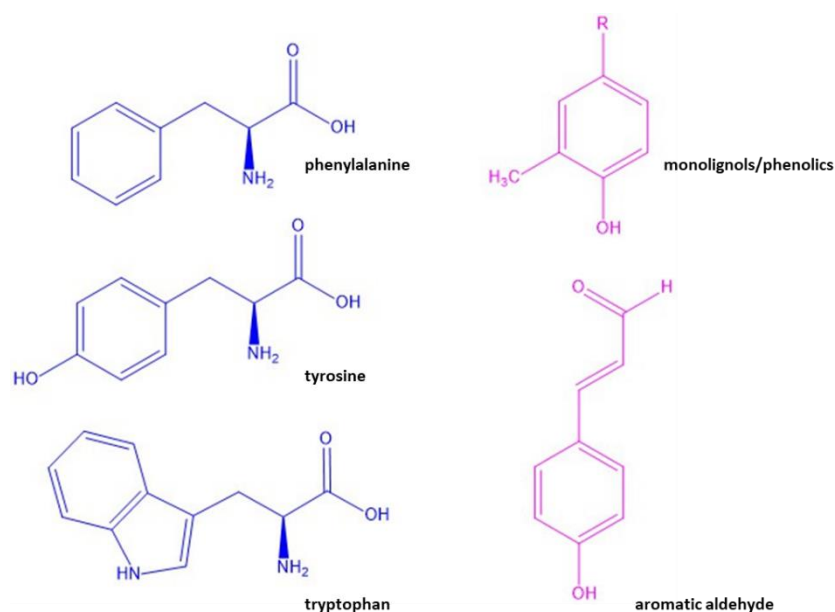


Figure 5.21. Possible candidates for aromatic structures identified in cell wall fractions

For future investigations, analysis of the oxalate-soluble fraction (corresponding to ulvans) will enable the ‘insoluble’ and soluble proteins in the *Ulva* cell wall, with the identification of ionically bound proteins verified in the previous chapters. However, one limitation to the analyses in this chapter was the insolubility of α -cellulose, which limited the NMR experiments I could perform with these samples. Whilst this generally did not affect the outcomes of analysis, based upon the supporting information from ATR-FTIR, elemental analysis, and solid-state NMR that verified the lack of ‘phycolignin’ in this fraction, alternative solvents such as ionic liquids, DMSO- d_6 with LiCl, and DMSO- d_6 with DMAC had been reported for use with cellulosic material (Shigemasa *et al.*, 1990; Wang *et al.*, 2012; Kim and Ralph, 2014; Tolbert *et al.*, 2014; Lili Zhang, Zhang, *et al.*, 2019). Due to these issues, only the ‘hyposaline stressed’ cellulose

fraction was solubilised. Contrasting cellulose fractions from *Ulva* cultured under different growth conditions will help verify whether the identified CWP's have a role in stress tolerance. Various CWP's in plants are associated with salt and osmotic stress responses, which are both particularly relevant stressors in the intertidal ecosystem (Lamport, Kieliszewski and Showalter, 2006; Yang *et al.*, 2010, 2011; Zagorchev and Odjakova, 2011; Yang, Rao and Horst, 2013; Chen *et al.*, 2014). In future investigations, I recommend that these solvents are trialled if further multidimensional analyses of these fractions are required in the solution-state

An extensive data set was generated during this chapter by the multidimensional NMR experiments performed. Some of this data may help address unanswered questions about the *Ulva* cell wall, which are beyond the main aims of this thesis. Additionally, information about the effects of the cell wall fractionation protocol on the extracted cell wall polymers may be provided by further analysis in order to improve it for future investigations. For example, HSQC was used to predict the sugar composition of the α -cellulose and hemicellulose fractions of *Ulva*. Whilst the observations were generally consistent with what was expected for these samples, further analysis of this data and the assessment of model compounds may help refine this approach for more complex samples, such as the unassessed ulvans.

Furthermore, the absence of an acetyl CH₃ group in the *Ulva* hemicellulose fraction was consistent with the lack of ester groups identified by ATR-FTIR in the previous chapter, whilst acetylation of the barley straw hemicellulose fraction was positively identified. This approach will help verify the identity of the contributing group to the ester peak observed by FTIR in this fraction by assessing the oxalate-soluble fractions from *Ulva*.

5.4.2 Isotopic Labelling May Facilitate ^{15}N NMR Analysis

Whilst the combined elemental analysis, FTIR, and described multidimensional ^1H and ^{13}C NMR analysis is consistent with the α -cellulose fraction being associated with cell wall protein, ^{15}N NMR remains the most appropriate NMR technique for analysis of nitrogenous structures (Mitri *et al.*, 2018; Shrestha *et al.*, 2022).

Here, experiments such as ^1H - ^{15}N HSQC are widely reported for biophysical analyses of protein structure and amino acid composition (Hayato Morita *et al.*, 2004; Yuwen and Skrynnikov, 2014; Kiraly *et al.*, 2015; Gyöngyösi *et al.*, 2021), and one-dimensional ^{15}N would provide definitive confirmation that the identified carboxyl group in the ^{13}C , HSQC, and FTIR spectra of *Ulva* α -cellulose samples corresponded to an amide group. Both of these experiments would have provided significant structural information about the protein associated with the *Ulva* cell wall in this thesis. This would have facilitated structural comparison with the cell wall proteins (CWPs) of the chlorophyte microalgae and various land plants, with hydroxyproline-rich glycoproteins hypothesised as the most likely candidate due to recent publications and the identification of corresponding genes in the *U. compressa* genome (De Clerck *et al.*, 2018; Přerovská *et al.*, 2021).

Here, ^{15}N CP-MAS was attempted in the solid state for the *Ulva* α -cellulose. Unfortunately, this attempt was unsuccessful, with ^{15}N NMR being an even less sensitive technique than ^{13}C NMR due to the differences in isotope abundance. Based upon the previous elemental analysis (see Table 4.2, Chapter 4), the estimated carbon content of this sample was 36.07%, and the nitrogen content was 3.1%, meaning that there were likely insufficient ^{15}N nuclei in the sample to acquire sufficient resonance for analysis. Due to this poor sensitivity and the low solubility of this fraction, an equivalent experiment (i.e., ^1H - ^{15}N HSQC) was not attempted in solution-state.

The obvious follow-up for subsequent investigations into this fraction would be to attempt to isotopically label *Ulva* cultures pre-stress and pre-extraction. Several reports of successful isotopic labelling of algae are documented in the literature for various analytical techniques. For example, the filamentous macroalga *U. meridionalis* was successfully labelled with ^{13}C using artificial seawater containing ^{13}C labelled sodium bicarbonate for NMR analysis of carbon fixation (Tsubaki *et al.*, 2020). A collaborating group has recently reported the rapid uptake of ^{15}N isotope in ^{15}N enriched seawater in the bladderwrack seaweed (*Fucus vesiculolus*), which will directly apply to the work described in this chapter for *U. compressa* (Bailes and Gröcke, 2020).

Dual labelling with ^{13}C and ^{15}N is also reported for the red seaweed *Solieria chordalis* using supplemented seawater (Legrand, Martin, et al., 2018), and ^{15}N labelling is reported for *Ulva rotundata*, *Codium marina*, and seagrasses in nitrogen acquisition studies (Alexandre *et al.*, 2017). Isotope labelling with ^{15}N has also been characterised in chlorophyte microalgae by supplementation of growth media (Sauer, Xu and Sutton, 2014; Andriukonis and Gorokhova, 2017). As a result, this appears a feasible avenue of investigation to characterise this cell wall component further, if seaweed can be propagated *in vitro* (as discussed in the next chapter), and if the dynamics of cell wall protein turnover can be determined experimentally before analysis. Furthermore, ^{13}C labelling may improve the analysis of the polysaccharide component of the *Ulva* cell wall, with this approach being successfully applied to lignocellulosic biomass (P. Wang et al., 2018). This is particularly useful for the analysis of the insoluble α -cellulose fractions, which may need to be analysed by less-sensitive solid-state analysis. This approach may also facilitate easier identification of the constituent sugars of the cell wall fractions by ^{13}C - ^1H HSQC, as attempted in this chapter.

However, alternative methods may be more practical analyses of cell wall protein, as NMR is costly and requires extensive data analysis. Furthermore, as covalently bound proteins or ‘insoluble proteins’ are difficult to isolate from the polysaccharides they cross-link (Chivasa *et al.*, 2002), as observed for the α -cellulose fraction (see §4.6, Chapter 4), enzymatic methods of protein analysis may be a more appropriate analytical approach, for example, the cellulase digest experiments performed in the previous chapter could be scaled up to acquire enough residue for analysis by conventional protein analysis. However, attempts to solubilise this sample (~10 mg) in various solvents for NMR and other analytical methods were unsuccessful. Alternatively, the amino acid composition could be assessed by direct hydrolysis of cellulose-associated protein and analysis of the lysate by chromatographic methods (Cui and Brummer, 2005; O’Rourke *et al.*, 2015; Pereira da Costa and Conte-Junior, 2015). Proteomics could also be performed by this method by performing trypsin digests on α -cellulose and analysing the released peptides by liquid chromatography-mass spectrometry (LC-MS-MS). This proteomic approach has been documented in *U. prolifera* for protein extracts labelled via the isobaric tags for relative and absolute quantitation method (iTRAQ) to assess the effect of heat stress on the *Ulva* proteome. (Fan *et al.*, 2018). However, as these enzymes are typically inhibited by residual polysaccharides, I still propose the ‘non-

invasive' NMR approach, if possible, which will also provide the most structural information.

5.4.3 Additional NMR Analysis May Resolve Additional Questions about the *Ulva* Cell Wall

Due to the inability to run ^{15}N analyses during this project, a series of other multidimensional experiments were performed to obtain supplementary information that could be used to contrast the algal and plant cell wall fractions for future investigations and remove some of the observed ambiguities encountered during peak assignment. As the previous approach had successfully identified that no lignin-like fraction was present in the *U. compressa* cell wall, these investigations were generally beyond the scope of my thesis. However, further analysis of this supplementary data (and the analysis of untested cell wall fractions) may resolve some of the additional questions about the structure of the *Ulva* cell wall raised in this thesis, including the nature of functional modifications observed on the *U. compressa* oxalate-soluble and alkali-soluble fractions, and determining the composition of these cell wall fractions. Unfortunately, due to the complex nature of these experiments, data could not be acquired for all the intended samples.

5.4.3.1 2D-TOCSY

The first of these supplementary experiments was total correlation spectroscopy (TOCSY), which is typically preferred over correlation spectroscopy (COSY) in biophysical studies for the analysis of 2D homonuclear couplings in macromolecules (§5.1.2.4). Whilst ^1H - ^1H COSY can tell us whether non-equivalent protons are geminal or vicinal to a proton, TOCSY enables the analysis of all the proton couplings in a 'spin system', meaning that we can assess how protons in a 'chain' are coupled. Homonuclear coupling enables more precise assignments of nuclei, enabling the structure of a compound to be mapped out more accurately (Keeler, 2002).

A 2D TOCSY experiment follows the same principle as COSY, and looks at homonuclear coupling. However, the standard 90° pulse of COSY (Figure 5.22, a) is replaced by an extended spin-lock pulse (Figure 5.22, b), which maintains net magnetisation in the transverse plane, extending relaxation time. The effect is that magnetisation can be transferred over more homonuclear couplings, with the number of couplings that can be observed in an experiment dependent on pulse length (Dona *et al.*, 2016). We can also perform 3-dimensional TOCSY (i.e., TOCSY-HSQC), which simplifies

complex TOCSY spectra by increasing the selectivity of the experiment (Li *et al.*, 2005; Komatsu and Kikuchi, 2013; del Río *et al.*, 2017; Brown *et al.*, 2018). For example, the addition of a ^{13}C - ^1H heteronuclear correlation pulse filters out protons not attached to a carbon.

By analysing 'spin systems' in biopolymers, we can more easily resolve which cross peaks are attributable to the backbone structure and which are attributable to side chains. 2D-TOCSY and 3D-TOCSY analysis has been shown to be particularly effective for determining the structure of side chains in carbohydrates and amino acids (Wang and Zuiderweg, 1995; Hilty *et al.*, 2002; Sandusky and Raftery, 2005; Lowman *et al.*, 2011; Duconseille *et al.*, 2015; Danmaliki and Hwang, 2022).

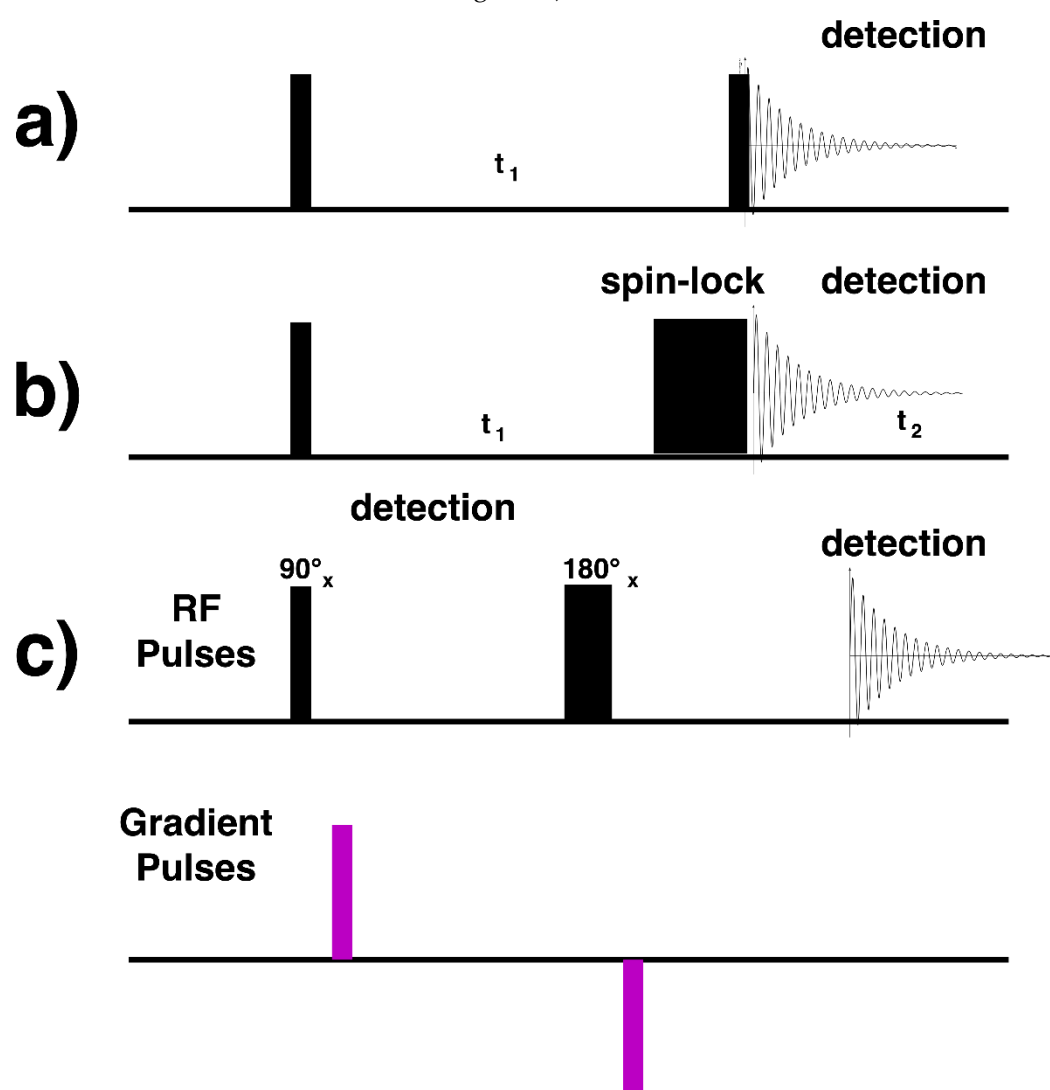


Figure 5.22. Pulse sequences for correlation spectroscopy (a), total correlation spectroscopy (b), and diffusion-ordered spectroscopy (DOSY, c).

TOCSY has also found utility in lignin analysis to assess structural changes under different chemical treatments and to analyse linkages and side chain structures in the complex lignin polymer (Ede and Ralph, 1996; Liitiä et al., 2003; Vivas et al., 2006; del Río et al., 2017; Nishimura et al., 2018). The addition of this analysis may further deconvolute some of the previous data, in which it was difficult to assess how the different alkyl resonances were associated, particularly in the *U. compressa* α -cellulose HSQC spectrum (Figure 5.17).

The use of TOCSY in combination with heteronuclear NMR analysis has been widely reported in the literature for cell wall analyses (Choudhury *et al.*, 2006; Tan *et al.*, 2010; Tabarsa, Lee and You, 2012). A combined ^{13}C - ^1H heteronuclear and ^{13}C - ^{13}C TOCSY correlation approach was demonstrated to resolve cell wall polysaccharide structure in the *A. thaliana* primary cell walls (Phyo and Hong, 2019). Here, the use of multiple-bond correlation analysis via TOCSY enabled an assessment of how different matrix polysaccharides interact with cellulose in the *A. thaliana* cell wall. In the context of the present work, this approach could be used to assess how the proposed cell wall proteins interact with *Ulva* cell wall fractions, and whilst I have verified the presence of this component in the cell wall, I have not determined the nature of its interaction. Again, the use of isotopic labelling for *Ulva* appears to be an appropriate next step (§5.4.2), as ^{13}C labelling would enable more flexibility in experiment choice, facilitating a more comprehensive analysis of cellulose in the solid state. The use of ^1H - ^1H TOCSY to supplement HSQC analysis is also reported in various metabolomic studies in *Ulva* (Gupta *et al.*, 2013; Gupta and Kushwaha, 2017), and ^1H - ^1H COSY has been used with HSQC to supplement analyses of ulvan structure and composition (Glasson *et al.*, 2017; Tran *et al.*, 2018; Guidara *et al.*, 2021). 1-dimensional TOCSY has also been used to characterise *O*-methyl groups in charophyte cell wall extracts (O'Rourke et al., 2015). The ability to precisely determine the structure of polysaccharide residues, as described by these papers, may improve the compositional analysis from the 'anomeric' region described previously.

5.4.3.2 DOSY

The second supplementary experiment that was performed was diffusion-ordered spectroscopy (DOSY). Whilst the previously described methods analyse the spin-spin interactions between nuclei, DOSY performs an analysis of the molecular diffusion properties of an NMR sample (Johnson, 1999). DOSY is facilitated by the application of two gradient pulses during a multidimensional pulse sequence (**Figure 5.22, c**). The first pulse induces a gradient in the magnetic field in the sample so that the frequency at which nuclei spin is dependent on their location along the gradient. The application of a second inverted gradient pulse refocuses the spins, but nuclei that have diffused (i.e., moved away) since the initial pulse cannot be refocused, thus giving a weak signal. By performing a series of scans with incrementally increasing gradient pulse strength (which impact the signal obtained from diffused nuclei), a 2-dimensional NMR experiment can be performed with pulse intensity and the resulting decay as the two variables (Johnson, 1999; Groves, 2017; Kandiyal *et al.*, 2019).

By coupling a DOSY experiment to chemical shift (e.g., via a 3D-DOSY HSQC), we can separate out different structures in a mixture and determine their individual diffusion properties, which are affected by both molecular weight and shape (Sandusky and Raftery, 2005; Didenko, Boelens and Rudiger, 2011; Jacquemmoz and Dumez, 2018). As a result, we can get some indication of the size of the different structures in a mixture, such as the various aromatic structures observed in the *U. compressa* and barley straw cell wall fractions. For example, DOSY has been applied to biopolymers to determine the molecular weight and size distribution of carbohydrates, proteins, lignin, and various other metabolites (Viel *et al.*, 2003; Duarte *et al.*, 2005; Yamanoi, Oda and Katsuraya, 2017; Kandiyal *et al.*, 2019; Montgomery *et al.*, 2019; Lee *et al.*, 2020).

Therefore, the application of DOSY experiments to the obtained cell wall fractions may function as a means of estimating the size of extracted cell wall polysaccharides and determining the size distribution of the various aromatic and proteinaceous structures identified within this chapter. Nonetheless, a substantial amount of data has been generated as an outcome of this work, which may form the basis for future research projects.

5.5 Conclusions

The biophysical analysis performed here and in the previous chapter has established that a 'lignin-like' structure is absent in the *Ulva* cell wall, which may provide a new perspective for how lignin structures have evolved independently outside the streptophyte lineage in a diverse red alga, but apparently not in the closer related chlorophyte seaweeds.

Furthermore, the NMR analysis is consistent with the previous elemental analysis and FTIR, which indicates that there are separate fractions of soluble and insoluble cell wall proteins associated with the amorphous ulvans and fibrillar α -cellulose components, respectively. This observation is consistent with the identification of hydroxyproline-rich glycoproteins within *Ulva* in the literature, which are well characterised in microalgal and plant models. ^{15}N NMR may facilitate further analysis of this component.

Finally, a wealth of multidimensional NMR data has been generated during this work from HSQC, TOCSY, and DOSY experiments that will require more extensive analysis, which may form the basis for future research projects into *Ulva* cell wall structure.

6. From Coast to Lab: Optimisation of Protocols for ‘Molecular Phycology’

6.1 Introduction

6.1.1 General Introduction

Macroalgal biotechnology remains an exciting field of research due to the industrial, ecological, and biomedical potential of seaweed biomass. In the case of *Ulva*, the presence of a rich extracellular matrix suits it for bioethanol and biogas production, with the main fraction of sulphated ulvans also showing promise for various biomaterials and biomedical applications (Trivedi *et al.*, 2016; Glasson *et al.*, 2017; Michalak, 2018; Gomez-Zavaglia *et al.*, 2019; ben Amor *et al.*, 2021; Guidara *et al.*, 2021). As verified in the previous research chapters, the absence of lignin in the *Ulva* cell wall also confers a direct advantage over lignocellulose for industrial applications, with the presence of lignin reducing the hydrolysability of cellulosic material, requiring costly and time-consuming ‘lignin removal’ procedures to be carried out at scale (Bolton *et al.*, 2016; Moral *et al.*, 2019; Wahlström, Edlund, *et al.*, 2020; Gomaa *et al.*, 2022). Furthermore, the fast growth rate of *Ulva* spp. relative to that of land crops and woody biomass means large amounts of biomass can be produced without competition for land resources (Kim *et al.*, 2011; Fernand *et al.*, 2017; Tsubaki *et al.*, 2020).

The high protein content of *Ulva* and the unique metabolic profile of *Ulva* may also have value for agricultural and aquaculture applications (i.e., as fertiliser and feed), as well as pharmaceutical value, with *Ulva* extracts displaying a range of bioactive properties *in vivo* (García-Casal *et al.*, 2008; Zhang *et al.*, 2010; Bikker *et al.*, 2016; Mezghani *et al.*, 2016; de Carvalho *et al.*, 2020; Moulazadeh *et al.*, 2021). Here, biorefinery (i.e., the sequential extraction of biomass into various products of different utility) is perhaps a more economically viable approach for exploiting *Ulva* biomass (Bikker *et al.*, 2016; Dong *et al.*, 2016; Balina, Romagnoli and Blumberga, 2017; Glasson *et al.*, 2017; Torres, Kraan and Domínguez, 2019; Prabhu *et al.*, 2020), with the economic feasibility of cultivating seaweed as a ‘single-use’ crop a major barrier to this industry in the Western hemisphere (van den Burg *et al.*, 2016). This unique biochemical composition is also of evolutionary interest, particular when considered in comparison to land plants, with seaweeds and plants evolving multicellularity independently in distinct environments (Umen, 2014; Coates, Umm-E-Aiman and Charrier, 2015). As seaweeds populate a

dynamic environment, with the tide cycle being the major environmental factor, it is also likely that they exhibit unique methods of stress tolerance (e.g., to desiccation) that may be of interest to researchers engineering more stress tolerant strains of crops, with tolerance to heat stress and drought of increasing concern in agriculture with the increased risk of climate change.

As demonstrated in my previous research chapters, where I performed a novel comprehensive biophysical analysis on *Ulva* cell wall structure, there are still significant gaps in the literature that would be benefited by a ‘molecular phycology’ approach, to better exploit seaweed as a crop and understanding how seaweeds have evolved multicellularity independently across multiple lineages. However, most of the current seaweed literature is still performed with ‘bulk’ material and mostly concerns the optimisation of extraction protocols (for industrial purposes) and assessments of biochemical composition under different conditions. What is now needed is a molecular approach, where defined cultures of seaweed can be manipulated *in vitro* to facilitate a better understanding of seaweed genetics, development, and physiology (Blomme *et al.*, 2021).

However, limited bespoke protocols for this type of research in seaweed are available. Researchers typically must adapt methods utilised in plant research, which often require extensive optimisation due to the different biochemical composition of seaweeds (e.g., nucleic acid extraction protocols) and are not always appropriate for diverse seaweed models (Fort, Guiry and Sulpice, 2018). Some of these issues were identified in the previous chapters, for example, during the described cell wall staining and cell wall extraction work.

Furthermore, macroalgae are highly diverse, and as a result, protocols that may be effective in one group may not be effective in another. Even within the major seaweed lineages (reds, greens, and browns), there is extensive diversity. For example, the major seaweed representatives of the Ulvophyceae, such as the Ulvales (e.g., *Ulva*), the Bryopsidales (*Caulerpa* and *Codium*), and the Cladophorales (*Cladophora*), all display distinct biochemical, physiological, and morphological characteristics, meaning appropriate model organisms for different groups must be identified, and corresponding protocols developed (Brodie, Maggs and John, 2008; Cocquyt *et al.*, 2010; Domozych *et al.*, 2012; Christa *et al.*, 2017; Arimoto *et al.*, 2019). Additionally, as intertidal conditions cannot be simulated effectively in the laboratory, establishing appropriate culturing methods is a concern – however, historically, this has been

studied quite extensively in *Ulva*, and defined conditions for growth have been established (Føyn, 1958, 1960; Nordby and Hoxmark, 1972; Løvlie, 1978; Løvlie and Bryhni, 1978; Stratmann, Paputsoglu and Oertel, 1996; Wichard and Oertel, 2010; Oertel, Wichard and Weissgerber, 2015; Wichard *et al.*, 2015).

In the case of *Ulva*, there have been a series of recent developments that have enhanced its suitability as a model seaweed, including the publication of two genomes (*U. mutabilis* and *U. prolifera*) and the development of new tools for genetic transformation and genetic editing (Oertel, Wichard and Weissgerber, 2015; De Clerck *et al.*, 2018; Blomme *et al.*, 2021; He *et al.*, 2021; Yu Wang *et al.*, 2021; Ichihara, Yamazaki and Kawano, 2022; Kwantes and Wichard, 2022). Nonetheless, there are still challenges to overcome before these approaches can be used universally, with these methods being developed for *in vitro* strains of *Ulva* rather than on a diverse range of wild-collected seaweeds. For macroalgal biotechnology to become a more practical field of science, protocols must become accessible for researchers working on local populations of seaweed as these strains are not widely accessible. These new protocols will also need to be accompanied by more robust protocols for procedures assumed to be 'routine' in molecular biology, including culturing techniques, nucleic acid isolation, protein extractions and gene expression analysis. Whilst these methods have been carried out in macroalgal research, the details of the challenges faced when applying these methods to seaweed research are typically omitted, as are the steps the researchers take to establish a working method. Again, due to the diversity of macroalgae, a 'one size fits all' approach is impractical, therefore more reports of bespoke method development for different species will greatly assist research in this field.

6.1.2 Chapter Aims and Hypotheses

In this final research chapter, I document various investigations that I carried out during my research that were intended to contribute to the development of a 'molecular toolkit' for *U. compressa* as follow-up to the publication of the corresponding genome, which coincided with the outset of my doctoral studies.

- Firstly, I attempt and optimise a protocol to induce reproduction in wild-collected *Ulva* to facilitate studies of spore settlement, early development, and genetic transformation
- Secondly, I assess the suitability of two commercial kits for isolating RNA from *Ulva* biomass, determine appropriate reference genes for a selection of abiotic stress conditions for qPCR analysis, and compare different curve fit models for fitting PCR data
- Finally, I perform a concise gene expression study in *U. compressa*, assessing the effect of combined abiotic stress on the response of various molecular chaperones hypothesised to be localised to different subcellular compartments. This work firstly demonstrates the potential of a molecular approach to macroalgal research and also provides some insights into how *Ulva* regulates stress responses in the dynamic intertidal zone

As a result of these investigations, I offer recommendations on the utility of these methods for macroalgal research, note the challenges faced when trialling these methods, and offer suggestions on how these methods could be used to supplement the research into *Ulva* cell wall structure described in the previous chapters.

6.2 Cultivation Techniques

The research performed in this thesis was carried out with wild collected *Ulva*, with collected material acclimated to defined incubator conditions prior to experimental assessment. Whilst sample collection is a simple process, and large amounts of biomass can be collected for research from rocky shores due to the wide distribution of *Ulva*, our current inability to maintain long-term cultures of seaweed in the lab limited my ability to perform molecular research in this project. Under optimum conditions, *U. compressa* theoretically can be maintained in culture indefinitely, taking approximately 3 months to cycle from generation to generation (Føyn, 1958). However, the wild-collected *Ulva* thalli in this project typically only remained viable for up to 6 weeks with weekly media replenishment and did not progress to a reproductive phase to enable propagation. By contrast, various developmental mutants of *U. mutabilis* are kept in culture by a few research groups, for example the *U. mutabilis* slender mutant which was sequenced in parallel with wild-type *U. mutabilis* in the sea lettuce genome project (Føyn, 1958; Wichard, 2015; De Clerck et al., 2018). Protocols to induce sporulation (spore release) and gametogenesis (gamete release) in *U. mutabilis* strains are also reported, for both sporophyte and gametophyte tissue respectively, demonstrating that *Ulva* can successfully be cycled in culture (Niesenbaum, 1988; Stratmann, Paputsoglu and Oertel, 1996; Gao et al., 2010; H. Wang et al., 2016).

As mature gametophyte and sporophyte *Ulva* are morphologically indistinguishable (Potter et al., 2016), having an established algal culture where the life cycle can be experimentally controlled facilitates both reproductive and molecular research, with gametophytes being preferred for the development of macroalgal gene editing tools, as gametophytes and gametes are haploid (Wichard et al., 2015). Haploids have a direct advantage over multiple ploidy counterparts for gene editing as they only have one copy of the genome, the effect of a mutated gene cannot be masked by an allele, facilitating experiments such as ‘loss-of-function’ trials (Bhowmik and Bilichak, 2021). By isolating gametophytes, clonal populations of *Ulva* can be established and maintained indefinitely in culture following induction of gametogenesis, with unfused gametes reverting to the gametophyte phase via parthenogenesis (Løvlie and Bryhni, 1978). Artificial induction of reproduction also facilitates the isolation of flagellated swimmers (spores and gametes), which lack a functional cell wall, and as such are more amenable to genetic transformation via electroporation or a polyethylene glycol-based method (Oertel, Wichard and Weissgerber, 2015; Blomme et al., 2021). Gametes can

specifically be purified and subsequently concentrated for this purpose based upon the positive phototaxis they exhibit before fusion (Stratmann, Paputsoglu and Oertel, 1996; Kuwano *et al.*, 2012; Oertel, Wichard and Weissgerber, 2015).

As a result, it appeared necessary to develop a protocol to induce reproduction in the populations of *Ulva* identified at our sampling site in this research, to facilitate more in-depth molecular phycology projects with these algal populations in the future. In the first instance, the frequently cited sporulation induction protocol first described by Stratman and colleagues (1996) for *U. compressa* was trialled on multiple *Ulva* thalli collected from Seaham beach at different times of the year. This method is hypothesised to induce sporulation or gametogenesis *in vitro* depending on tissue type by fragmentation of *Ulva* thalli, and washing away two inhibitory compounds (named sporulation inhibitors) which are hypothesised to accumulate in-between the two cell layers of *Ulva* and regulate when cells can differentiate into gametes or spores (Wichard and Oertel, 2010). Whilst there are various reports that document the successful application of this method in *U. linza*, *U. compressa* and *U. intestinalis* (Vesty *et al.*, 2015; Ghaderiardakani, Coates and Wichard, 2017), which were all identifiable at our sampling site, this method was unsuccessful in all trials performed with this material.

This protocol was carried out as follows:

1. Individual thalli were diced into small fragments (Figure 6.1)
2. Fragments were washed 3 times for 15 minutes with Provasoli's enriched seawater (see Materials and Methods, Table 2.15) using a 100 µm mesh to recover fragments each time wash media is replaced
3. Fragments were then transferred to flasks containing supplemented seawater and incubated under standard growth conditions for 3 days.
4. Three days post-induction, fragments were washed once as performed for step 2. This step is performed in the morning, consistent with the known time course of gametogenesis in the *U. mutabilis* slender mutant, where swarmers are released in the morning 3 days after induction (Katsaros *et al.*, 2017).
5. Fragments were then placed in a fresh medium, and flasks were exposed to a light source in order to purify swarmers. Gametes display positive phototaxis, whilst zoospores will move away from light. Due to this behaviour, gametes can be harvested from the media and purified (from commensal microorganisms) in order to obtain axenic cultures that are required for some developmental studies.

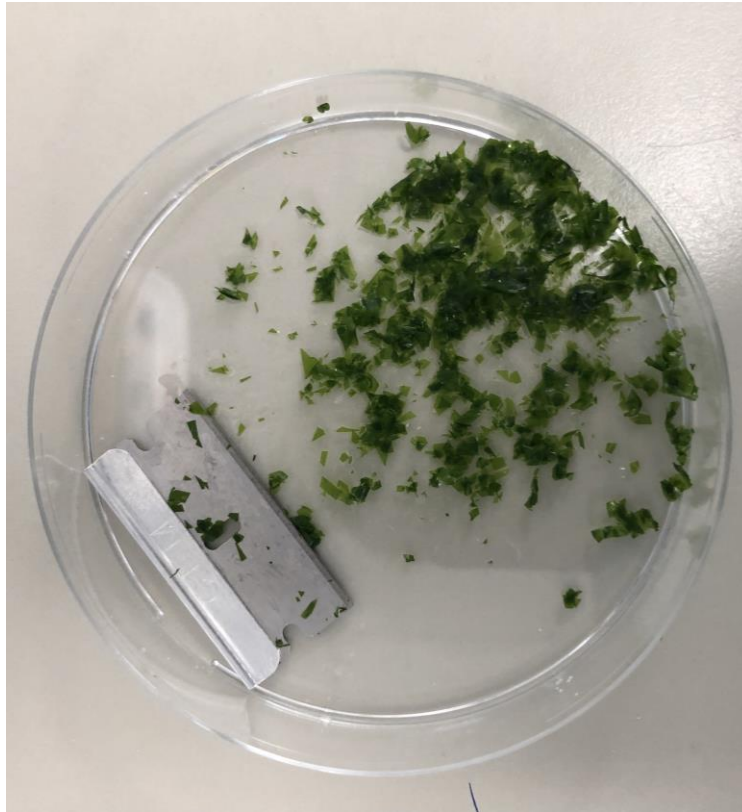


Figure 6.1. Fragmented *Ulva* tissue prepared for the induction protocol initially described by Stratmann and colleagues (1996).

Despite the life cycle of *Ulva* being typically characterised as a cycle between a sporophyte phase and a gametophyte phase from generation to generation, it is often more complex in certain populations. As stated in the introductory chapter (Chapter 1, §1.4.1), some populations are obligate sporophytes which reproduce rapidly and asexually (e.g., green tide forming species), whereas other populations are more resistant to differentiation and remain in a vegetative state for extended periods (Hiraoka et al., 2003; Lüning, Kadel and Pang, 2008; Gao et al., 2017b; Ichihara et al., 2019). These reproductive differences are not necessarily governed by species, with different populations of the same species (for example, *U. prolifera*) demonstrating different life cycles in the field (Hiraoka and Higa, 2016). Furthermore, reproductivity, and the ratio of gametophytes (see Figure 6.2 for a representative specimen) to sporophytes in a population varies with the season (Pringle, 1986; Alström-Rapaport, Leskinen and Pamilo, 2010; Potter *et al.*, 2016; Liu *et al.*, 2017). Additionally, this protocol was primarily developed for *U. mutabilis* mutants that have an established *in vitro* life cycle, which most likely exhibits developmental and physiological differences to wild-type *Ulva* at our sampling site.



Figure 6.2. Gametophyte tissue from an *U. linza* specimen collected at Seaham beach in spring 2019. Brownish discolouration at the edges of tissue (blue arrow) indicates cells have differentiated to gametangia. Translucent tissue (black arrow) is indicative of recent gamete release.

As a result, a modified protocol was devised based upon a literature search for protocols where reproduction had successfully been induced in wild-collected *Ulva*, and induction was reattempted (Niesenbaum, 1988; Hiraoka and Enomoto, 1998; Kuwano et al., 2012; Gao et al., 2017b, 2017c; He et al., 2019). This was carried out as follows:

1. *Ulva* thalli were collected from the incubator in the afternoon, consistent with the protocol by Stratmann and colleagues (1996) and the known time course for induction of differentiation. Thalli were washed thoroughly with water and blotted dry with a paper towel.
2. A small fragment of tissue (1-2 cm²) was cut from the apical region of fronds
3. Fragments were placed in individual wells of a 6 well plate (Sarstedt) with 5 mL Provasoli's enriched seawater
4. Plates were incubated at 21°C under a standard 16 L: 8 D photoperiod
5. Fragments were monitored by visual inspection and microscopy over several weeks. Based on the literature (Vesty et al., 2015; Katsaros et al., 2017), *Ulva* fragments entering a reproductive phase are identifiable by the appearance of white (sporophyte) or brown regions at the tip (gametophyte).

6. Fragments were removed from wells as seedlings appeared over the course of a few weeks. However, in a few instances swarmers adhered to the original thalli, in which case they were kept in wells
7. Media was replenished weekly once seedlings had attached and grown to a size which was not disrupted by media removal.

This protocol was initially performed with 6 thalli collected from Seaham beach. Material was washed, and fragments cut and transferred to a 21°C incubator with a 16 L: 8: D photoperiod. Material had been kept at 8°C under standard culture conditions until this point. After two weeks incubation at this elevated temperature, it was observed that thalli had differentiated and released swarmers in the wells, which had adhered to the bottom of the wells and had developed into filamentous seedlings (Figure 6.3). From this point onwards, photos of wells were taken daily to monitor growth. *Ulva* samples that were growing too large for wells were transferred to petri dishes containing 50 mL media for continued incubation (Figure 6.4). At this stage it was hypothesised that the replacement of media may induce another round of sporulation in undifferentiated thalli fragments in the original 6 well plates. However, no further evidence of sporulation was observed after this was performed. Once cultures transferred to petri dishes were large enough, they were moved to Erlenmeyer flasks or culture flasks in the standard incubator (Figure 6.4).



Figure 6.3. *Ulva* seedlings growing following swarmer release and settlement.

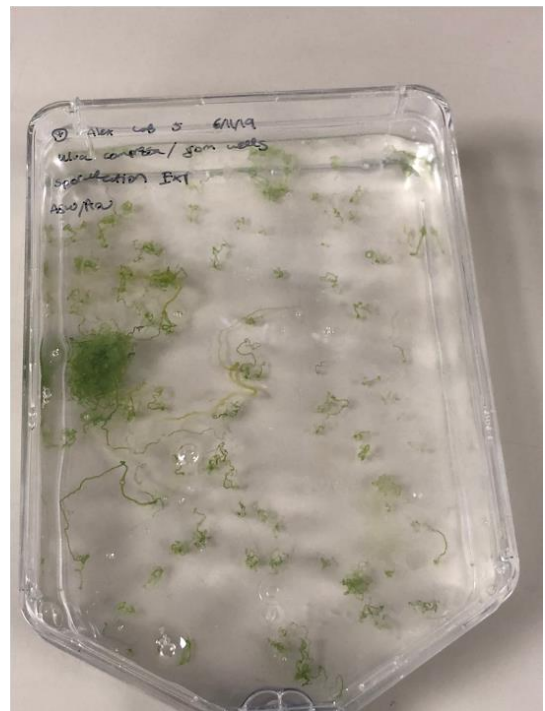
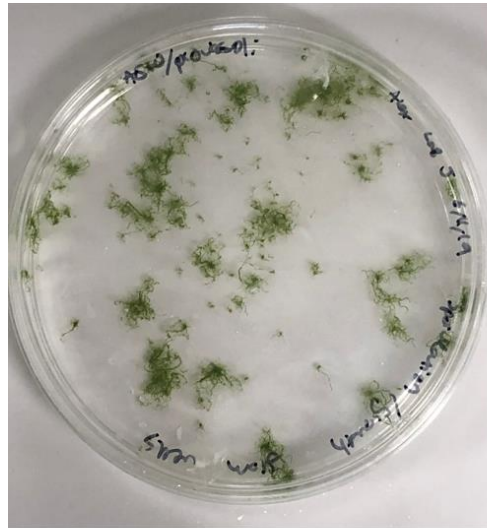


Figure 6.4. Induced *Ulva* cultures were transferred to petri dishes, Erlenmeyer flasks, and culture flasks to facilitate further growth.

This trial was then repeated with fragments from various samples of different morphologies (Figure 6.5), to better characterise the timings of spore release, as the previous experiment was monitored too infrequently to make conclusions. Here, reproduction was also successfully induced with evidence of release observed in 8 of the 12 tested samples after 24 h incubation. Sporulation was successfully induced in both morphotypes of *Ulva*. Evidence of seedling development was observed in 5 of the samples after 10 days, either attached to the bottom of the wells or to the old thallus.

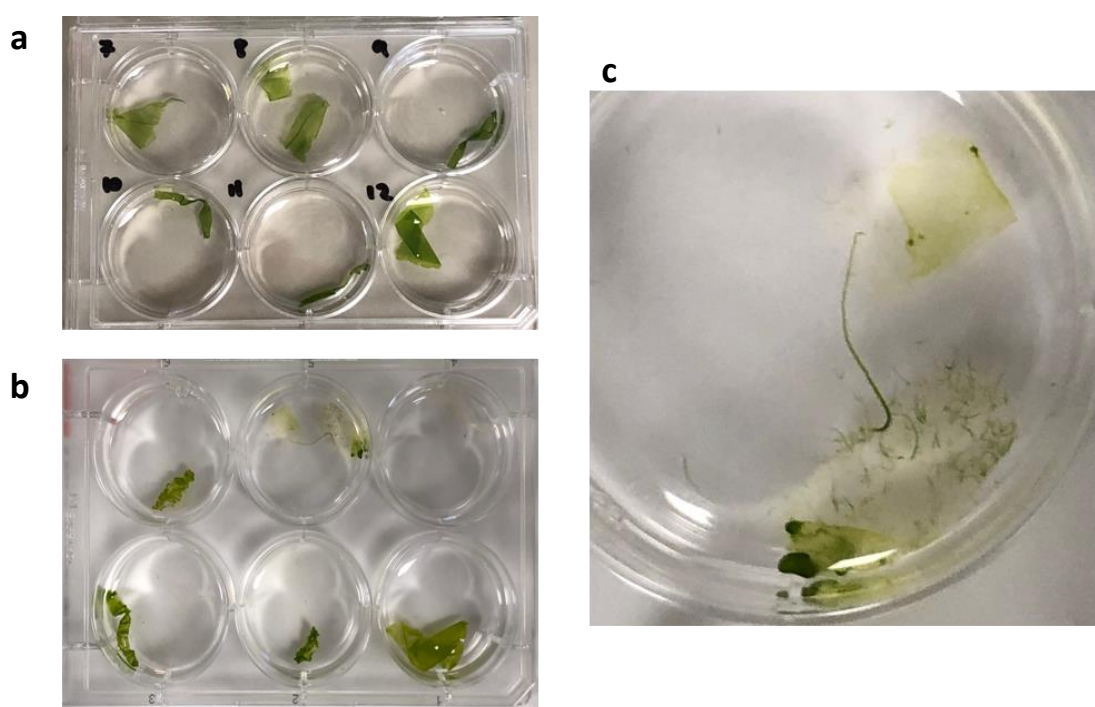


Figure 6.5. *Ulva* samples were induced in 6 well plates containing 5 mL Provasoli's Enriched Seawater (a). Successful swarmer growth and settlement growth was observed after 10 days incubation at 20°C (b, c).

This protocol was also repeated with whole thalli instead of fragments to scale up the sporulation experiment in four Petri dishes containing 50 mL media in an attempt to purify swarmers from solution before settlement (Figure 6.6). Two Petri dishes contained Provasoli's enriched seawater, and the other two were set up with standard seawater to assess if enrichment was required. No spore or gamete release was induced after two weeks for any of the samples, as a result thalli were fragmented and placed in the same Petri dishes, and media was replenished. This successfully induced release in two of the four samples, corresponding to those in enriched solution, indicating that

fragmentation was required for induction, as well as enrichment. However, swarmer release did not appear to occur to the reported time course (Stratmann, Paputsoglu and Oertel, 1996; Spoerner *et al.*, 2012; Katsaros *et al.*, 2017), and swarmers were released and settled on the expended fragments before they could be purified.

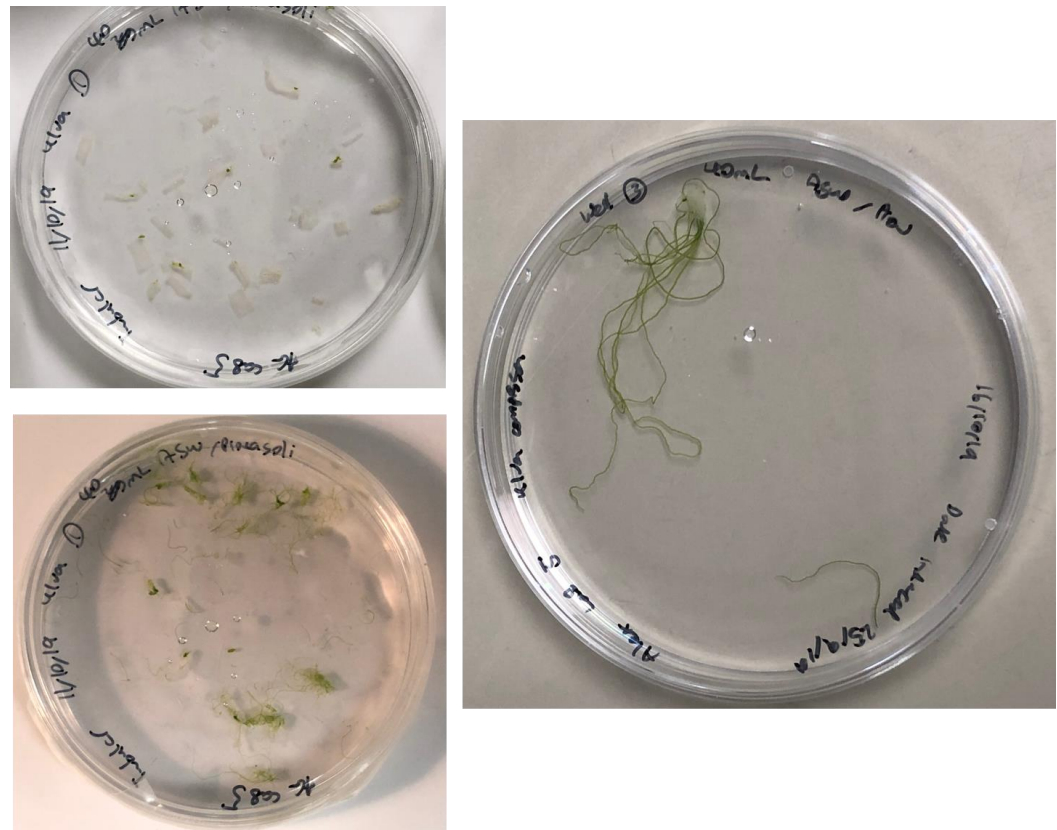


Figure 6.6. Induction was successfully performed in Petri dishes using multiple *Ulva* fragments. Empty sporophyte tissue (a). Released spores did not appear to adhere to the dish, and rather, they formed 'free floating' cultures which adhered preferentially to expended thallus.

The colouration of thalli and rapid release observed throughout all the experiments was consistent with sporophyte tissue, with gametophyte tissue typically turning 'brown' preceding gamete release. Unlike gametes, spores do not exhibit significant phototaxis, and instead they appear to move downwards from light to facilitate rapid adhesion to a substrate (Fletcher and Callow, 1992; Thompson *et al.*, 2007; Oertel, Wichard and Weissgerber, 2015). Therefore, protocols for spore purification require isolation via centrifugation of media post-release, which requires reliable prediction when spores are being released. However, this was not possible during these investigations.

The induction trial was also tested in different flasks for scale-up, including adhesive Nunc cell culture flasks (Figure 6.7) and Erlymener flasks (Figure 6.8) which were trialled to promote adhesion of swarmer. Notably, higher levels of spore adhesion were observed on the base of Nunc flasks (Figure 6.7) than on Petri dishes, where spores appeared to adhere preferentially to old thalli, most likely due to the lack of a charged surface to facilitate spore attachment (Figure 6.6). Interestingly, juvenile *Ulva* propagated from both blade and tubular thalli all assumed a tubular form as they developed (Figure 6.4). This phenomenon is also reported in the literature for generational cycling experiments performed in *U. lactuca* (Bonneau, 1977), where the morphology of the progeny was not necessarily consistent with that of the original thallus.

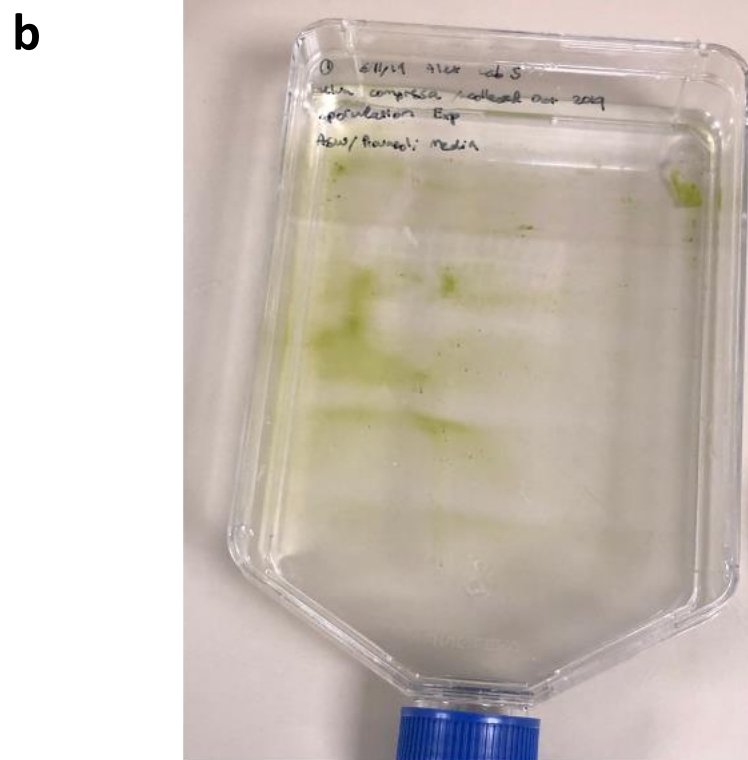


Figure 6.7. Adhesion of *U. compressa* swimmers occurs on adhesive Nunc flasks containing Provasoli's enriched seawater following induction. Fragments are placed in media (a), and successful swarmer release is observed (b).

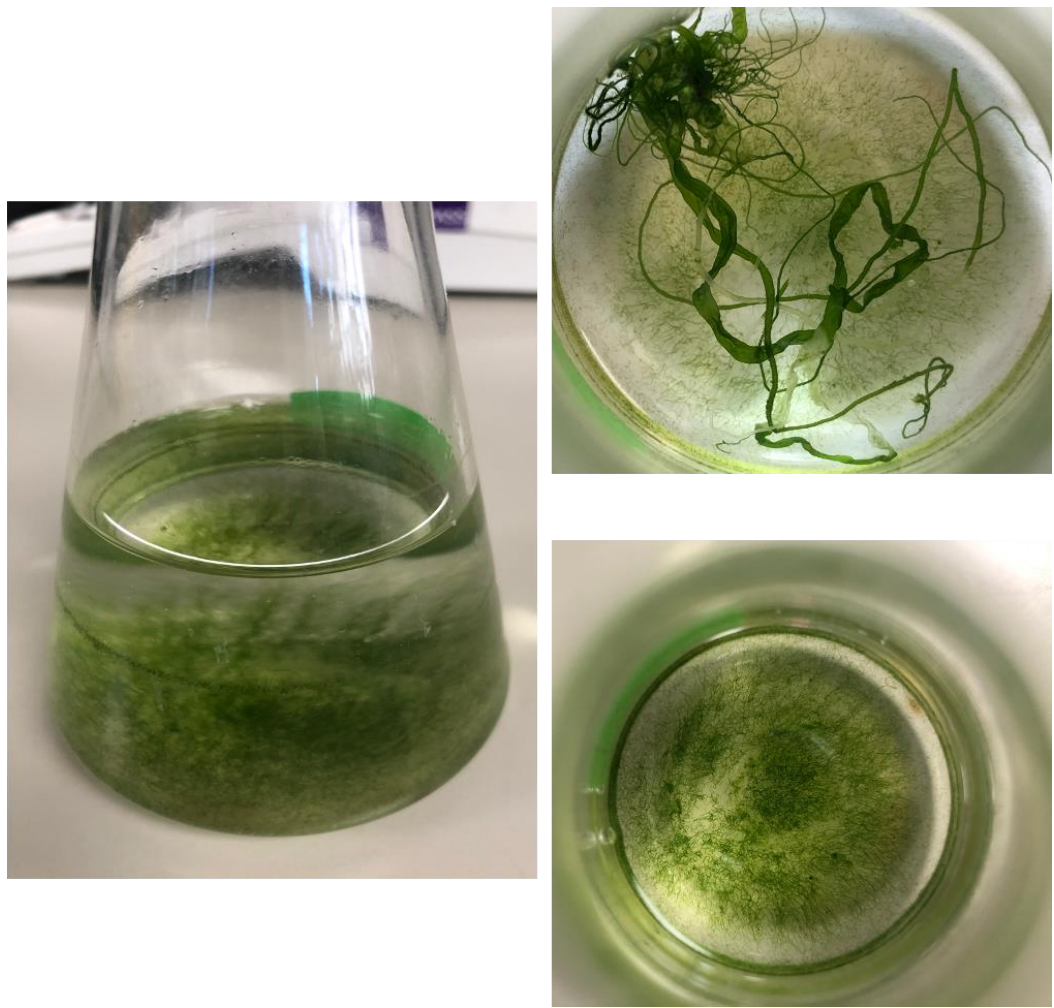


Figure 6.8. *Ulva* swarmer successfully settle and develop in Erlymener flasks containing Provasoli's enriched seawater.

As the time of spore release could not be predicted effectively during this research to enable isolation of spores before settlement, a different approach was proposed, where cover slips were placed at the bottom of dishes during induction (Figure 6.9). This would facilitate a simple assessment of the post-settlement development of *Ulva* seedlings. However, spores did not adhere to cover slips, even when swarmer release was observed. Methods to improve the adhesiveness of glassware to monitor the dynamics of macroalgal spore settlement are reported for *U. linza*, *U. rigida* and kelps, for example, by polylysine and polyethylene glycol (PEG) pre-treatment (Santelices and Aedo, 1999; Heydt et al., 2012). Modification of this protocol will likely enable experiments of the kind to be carried out.

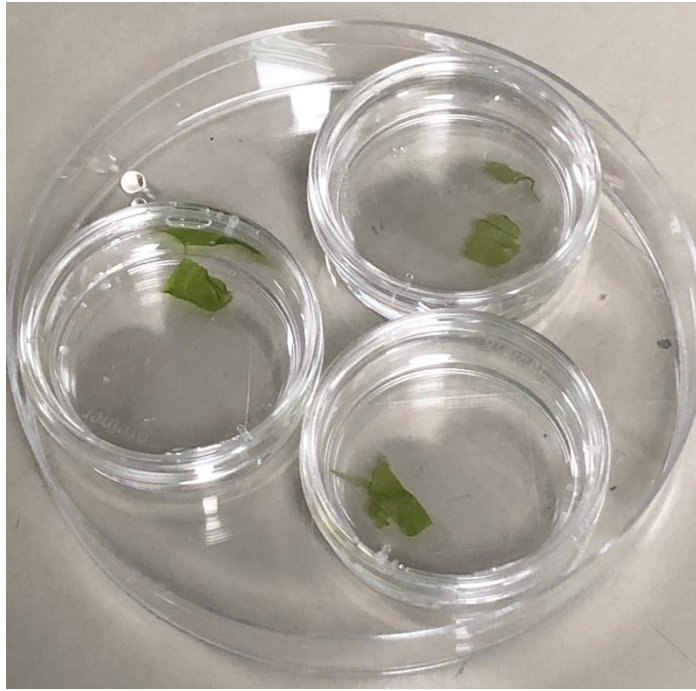


Figure 6.9. Induction experiments were set up in small flasks to facilitate swarmer attachment to glass slides for imaging.

Finally, gamete release was observed as described for a small cutting of gametophyte tissue collected during spring (Figure 6.10), with gametes demonstrating positive phototaxis and migrating to the brightest end of the dish. Further optimisation of this protocol with gametophyte tissue will enable gamete purification and transformation experiments by the PEG-mediated protocol described by Oertel and colleagues (2015).

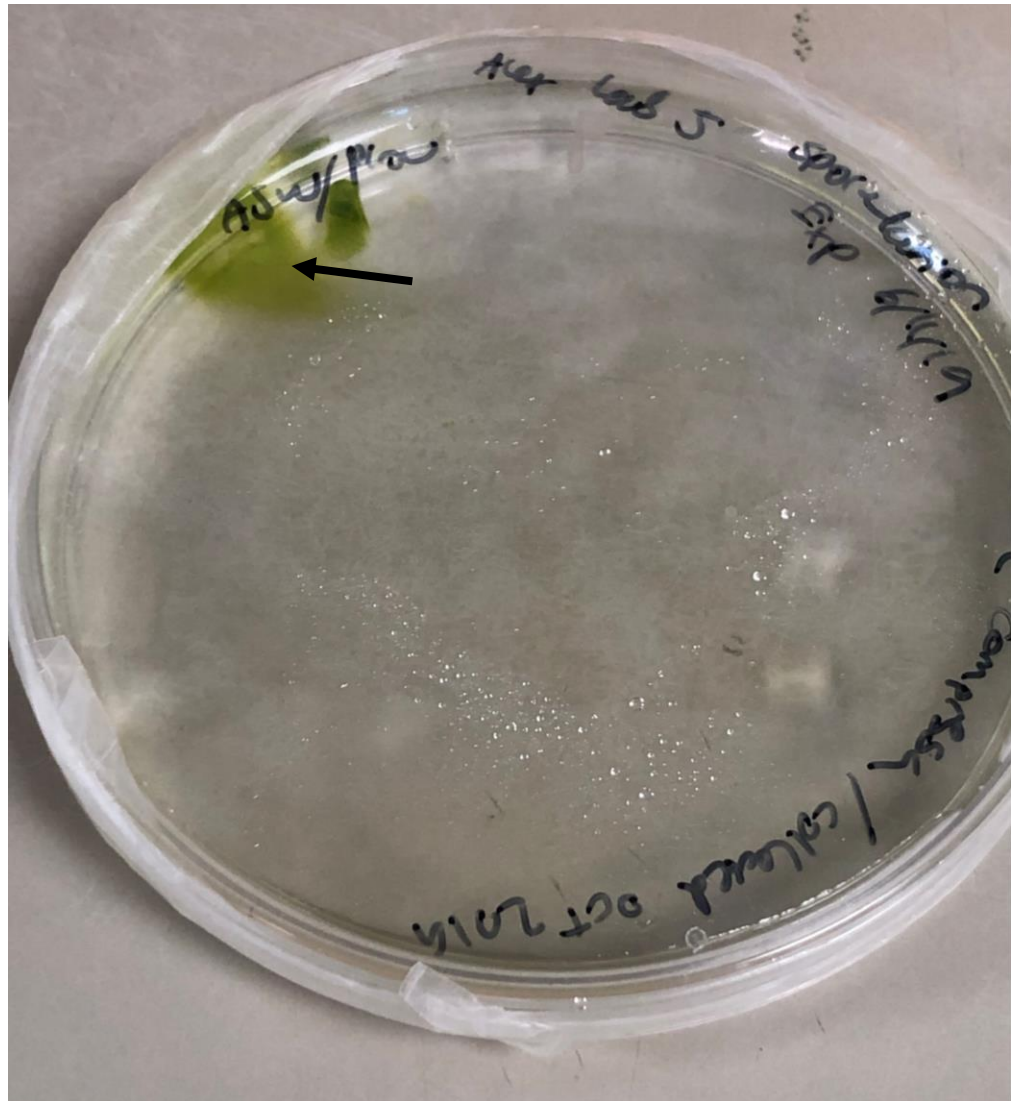


Figure 6.10. *Ulva* gametes (see arrow) display positive phototaxis and settle at the brightest spot in the Petri dish.

Further investigation into *Ulva* reproduction here was prevented by the COVID pandemic, where other research (i.e., the previously cell wall analysis) was prioritised. Nonetheless, these observations here indicate that a reproducible induction protocol is possible with this population of *Ulva*, with only a few modifications appearing necessary. Mainly, the ability to purify swimmers would enable transformation protocols to be carried out as described in the literature (Oertel, Wichard and Weissgerber, 2015). Alternatively, genetic transformation could be trialled on seedlings before the cell wall fully forms. The ability to get spores to adhere and develop on glass slides, Petri dishes and 6 well plates would also enable various imaging applications, for example, imaging the early development of *Ulva* in real-time. There are relatively few modern reports that investigate the early development of *Ulva* (Spoerner *et al.*, 2012; Vesty *et al.*, 2015;

Wichard, 2015; Ghaderiardakani, Coates and Wichard, 2017), as such this approach may facilitate the application of more modern techniques, enabling assessment of how early *Ulva* development is impacted by different environmental conditions. One option, related to the research performed in chapters 3-5 could be to apply the cell wall imaging techniques discussed in chapter 3, to assess how the cell wall develops in *Ulva* seedlings under different conditions.

6.3 Nucleic Acid Isolation

Obtaining pure 'PCR quality' nucleic acid from seaweeds is often challenging due to the abundance of sulphated polysaccharides, proteins, and secondary metabolites present in macroalgal biomass, which are inhibitory to most enzymatic assays (Jin *et al.*, 1997; Wattier, Prodöhl and Maggs, 2000; Joubert and Fleurence, 2005; Varela-Alvarez *et al.*, 2006; Fort, Guiry and Sulpice, 2018). For example, in two-step qPCR, co-precipitation of contaminants during RNA isolation may inhibit the reverse transcription reaction (required to generate cDNA), leading to inaccurate or failed quantification when samples are assayed. Therefore, the protocol used for RNA isolation must have effective purification steps for removing cellular debris and inhibitory components. Proper sample handling is also necessary, as, during RNA isolation RNase can be unintentionally introduced into the sample – causing degradation (Bustin *et al.*, 2009; Nolan, Huggett and Sanchez, 2013; Mommaerts *et al.*, 2015). Therefore, the quality and integrity of *U. compressa* RNA isolates were investigated before progressing with cDNA synthesis and qPCR.

A series of trial RNA isolations on untreated *U. compressa* thalli were carried out using two commercial RNA isolation kits: the RNeasy PowerPlant Kit (Qiagen) and the FavorPrep Plant Total RNA Kit (Favorgen), to determine an appropriate kit for use with *U. compressa*, to facilitate gene expression analysis via qPCR. Extractions were optimised for *U. compressa* samples specifically rather than the predicted *U. linza* or *U. intestinalis* identified at the sampling site due to the availability of the *U. compressa* (syn. *U. mutabilis*) genome, which greatly assisted experiment design (De Clerck *et al.*, 2018). However, a comparison between yields for *U. linza* and *U. compressa* was performed (Figure 6.12, b), with *U. linza* being used as a model for various transcriptomic studies (Dong *et al.*, 2012; X. Zhang *et al.*, 2012; Guan *et al.*, 2016; Y. Wang *et al.*, 2019).

Both the Qiagen kit and the Favorgen kit use a silica-gel membrane spin column-based isolation protocol, with this technology used almost universally in commercial nucleic acid isolation kits (Karp, Isaac and Ingram, 1998). The Qiagen kit is marketed as being suitable for plant material containing high levels of phenolics and polysaccharides, whereas the Favorgen kit contains two lysis buffers – one for regular plant material and the other for plants containing high levels of secondary metabolites (for this work, the latter was used). Both kits employ an on-column DNase digestion step to remove genomic DNA (gDNA) from isolates, with co-precipitated gDNA being a significant interferent in qPCR assays (Taylor *et al.*, 2019). As no bespoke kits for nucleic acid isolation in macroalgae exist, these kits appeared appropriate for performing initial isolation trials in *Ulva*.

Due to the difficulties in homogenising macroalgal tissue, extractions were performed manually using seaweed tissue ground to a fine powder under liquid nitrogen with a mortar and pestle. As an initial test of RNA quality, RNA yield and purity were estimated using Nanodrop absorbance readings at 230, 260, and 280 nm. Here, nucleic acid concentration is estimated by absorbance at 260 nm, whereas A260/A230 nm and A260/A280 nm ratios are estimates of RNA purity. Expected ratios for RNA are ~ 2.0 (for the A260/A280 ratio) and > 2.0 (for A260/A230), with lower values indicating possible contamination by co-precipitated proteins, polyphenols, polysaccharides, or kit reagents (Desjardins and Conklin, 2010; Page, 2010).

Absorbance ratios were comparable between the two kits (Figure 6.11, b, c), with both kits appearing to produce sufficiently pure RNA from *U. compressa*. For the Favorgen kit, ratios were 2.23 (A260/A280) and 2.75 (A260/A230). With the Qiagen kit, mean ratios of 2.22 (A260/A280) and 2.23 (A260/A230) were obtained. However, yields were consistently higher using the Qiagen kit (82.2 µg RNA per g biomass) than the Favorgen kit (25.7 µg RNA per g biomass, Figure 6.11, a). As a result, the Qiagen kit was selected for further optimisation.

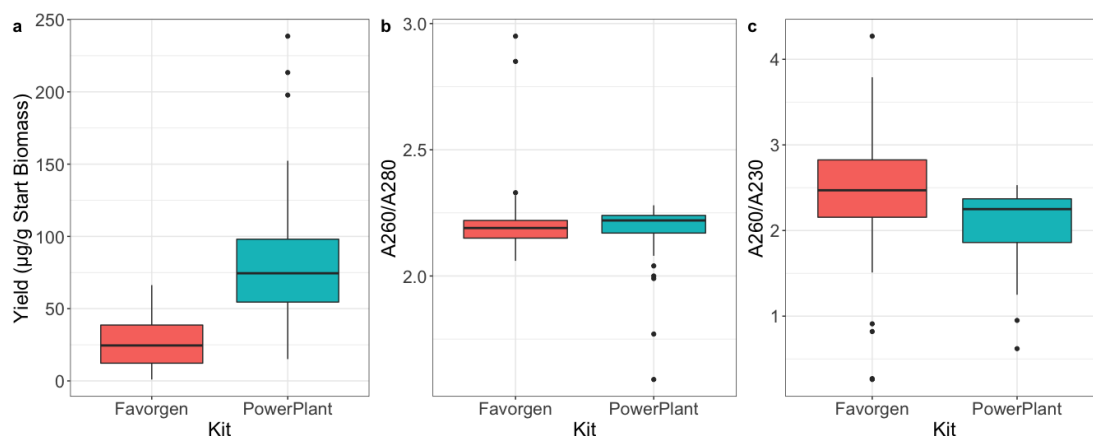


Figure 6.11. Comparison of RNA yields from the two commercial RNA isolation kits as determined by Nanodrop absorbance measurements. Yields (a), A260/A280 ratio (b), and the A260/A230 ratio are presented. Boxplots represent minimum and maximum values (whiskers), interquartile range (box, from 25th to 75th percentile), median value (line), and outliers (dots).

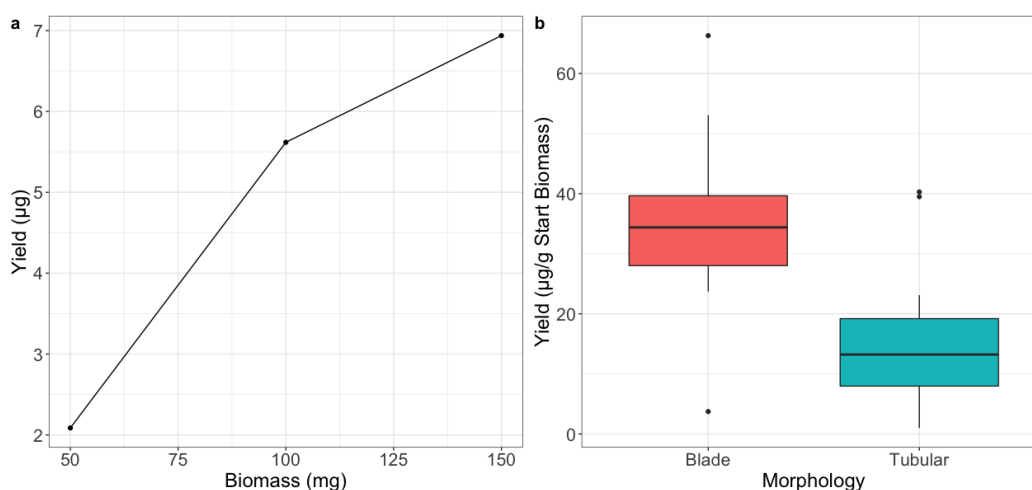


Figure 6.12. RNA yields increase using the Qiagen RNeasy Power Plant Kit using increasing amounts of *U. compressa* biomass (a), and higher RNA yields are obtained from 'blade' type *Ulva* corresponding to *U. linza* than *U. compressa* (tubular). Boxplots represent minimum and maximum values (whiskers), interquartile range (box, from 25th to 75th percentile), median value (line), and outliers (dots).

As shown in Figure 6.12 (a), increasing the amount of biomass for RNA isolation increased yield. The Qiagen protocol recommends using 50 mg plant biomass, whereas Favorgen recommends 100 mg. However, seaweed tissue is tougher to homogenise than most plant species due to a more robust cell wall and the presence of extensive extracellular mucosa (Boney, 1981; Lahaye and Robic, 2007; Baweja *et al.*, 2016). The use of 50 mg biomass here resulted in a total RNA yield of 2.1 µg, which was deemed too low for the reverse transcription protocol required to generate cDNA samples for qPCR. For the reverse transcription kit used in this research (ReadyScript cDNA Synthesis Mix, Sigma), 1 µg RNA was required in a total reaction volume of 20 µL. Based upon this, I set a minimally acceptable RNA yield from *U. compressa* samples as 5 µg, which corresponded to an RNA concentration of ~100 ng/µL in the 50 µL sample eluted at the final stage of the RNA isolation process. In this scenario, a maximum volume of 10 µL RNA sample would be used in the reverse transcription reaction, with the rest of the volume made up by the other reaction components. Increasing starting biomass to 100 mg and 150 mg successfully increased yield to exceed this limit from 2.1 µg to 7.95 µg and 9.81 µg respectively (Figure 6.12, a).

Increasing biomass in the Favorgen kit also increased yield, but not to the same extent as the Qiagen kit, with an average total yield of 3.12 µg obtained using 100 mg biomass (not plotted) and with a total yield of 2.1 µg obtained using 50 mg biomass. Further increases in biomass visibly clogged the Favorgen spin column (Figure 6.13). Purity ratios for the Qiagen kit did not appear to be affected by the increase in biomass to 150 mg, with ratios of 2.22 (A260/A280) and 2.44 (A260/A230) obtained. However, increases in biomass using the Favorgen kit to 100 mg and 200 mg reduced the A260/A230 ratio to 1.64 and 1.76 respectively, corresponding with the increased cellular debris observed on the spin column during the isolation procedure. A low A260/A230 ratio is often indicative of polysaccharide contamination (Fort, Guiry and Sulpice, 2018), providing further support that the Qiagen protocol was more suited for mucilaginous seaweed biomass than the Favorgen kit.



Figure 6.13. Visible clogging of the Favorgen spin column occurs with increased amounts of starting biomass.

RNA integrity from extraction trials appeared to be acceptable using the Qiagen kit, with no significant RNA degradation apparent, nor residual genomic DNA, with intact ribosomal RNA bands visible on a denaturing MOPS-agarose gel (Figure 6.14, a). Here, eukaryotic 28S and 18S rRNA subunits are visible, in addition to chloroplast 23S and 16S subunits (Claros and Cánovas, 1999). Isolations were also trialled with and without the toxic β -mercaptoethanol (β -ME), which is added to lysis buffer during the Qiagen protocol as a reducing agent to inactivate RNase, preventing premature RNA degradation (Mommaerts *et al.*, 2015). Removal of β -ME from the protocol did not appear to impact RNA yield, purity, or integrity (with the samples shown in Figure 6.14 generated without β -ME), with proper sample handling ensured. Therefore, β -ME was omitted from the protocol to improve throughput - as the addition of β -ME to lysis buffer prevented me from extracting RNA on the lab bench (necessitating a fume hood), increasing the duration of the protocol as samples had to be homogenised manually. Therefore, RNA extractions for the stress experiments carried out in this chapter (6.4.2) were performed using this slightly modified Qiagen protocol with 150 mg *U. compressa* biomass.

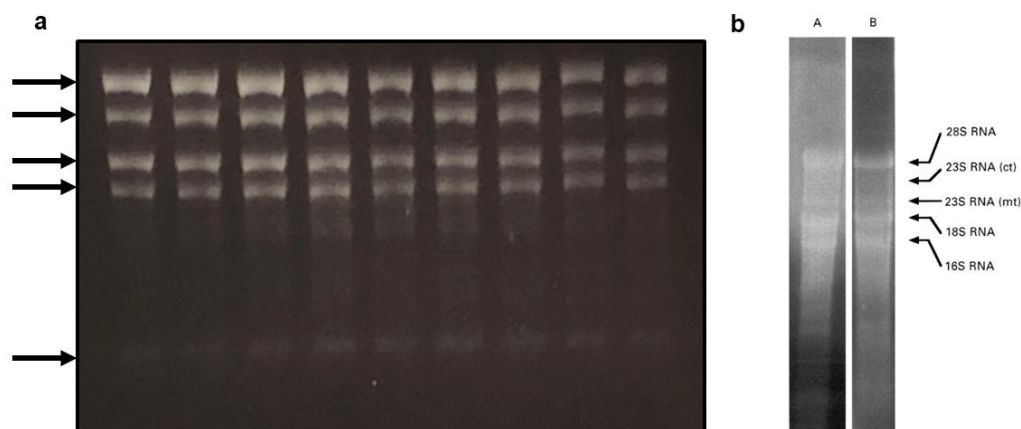


Figure 6.14. Denaturing gel of RNA isolates. (a) 300 ng RNA was loaded with 5 μ L RNA loading buffer (see Materials and Methods, Table 2.16). Samples were incubated at 65°C for 10 minutes, then were loaded onto a 1.2% (w/v) agarose-MOPS (1X) gel. Gels were run in 1X MOPS running buffer for 30 minutes at 75 V before imaging. Gels were inspected for evidence of RNA degradation under UV illumination, indicated by a low molecular weight smear on the gel and the absence of visible ribosomal RNA (rRNA) bands (28S and 18S), as indicated by (b). Image from (b) reprinted from Figure 1, Claros, M. G., & Cánovas, F. M. (1999). RNA isolation from plant tissues: A practical experience for biological undergraduates. *Biochemical Education*, 27(2), 110–113. [https://doi.org/10.1016/S0307-4412\(98\)00289-1](https://doi.org/10.1016/S0307-4412(98)00289-1)

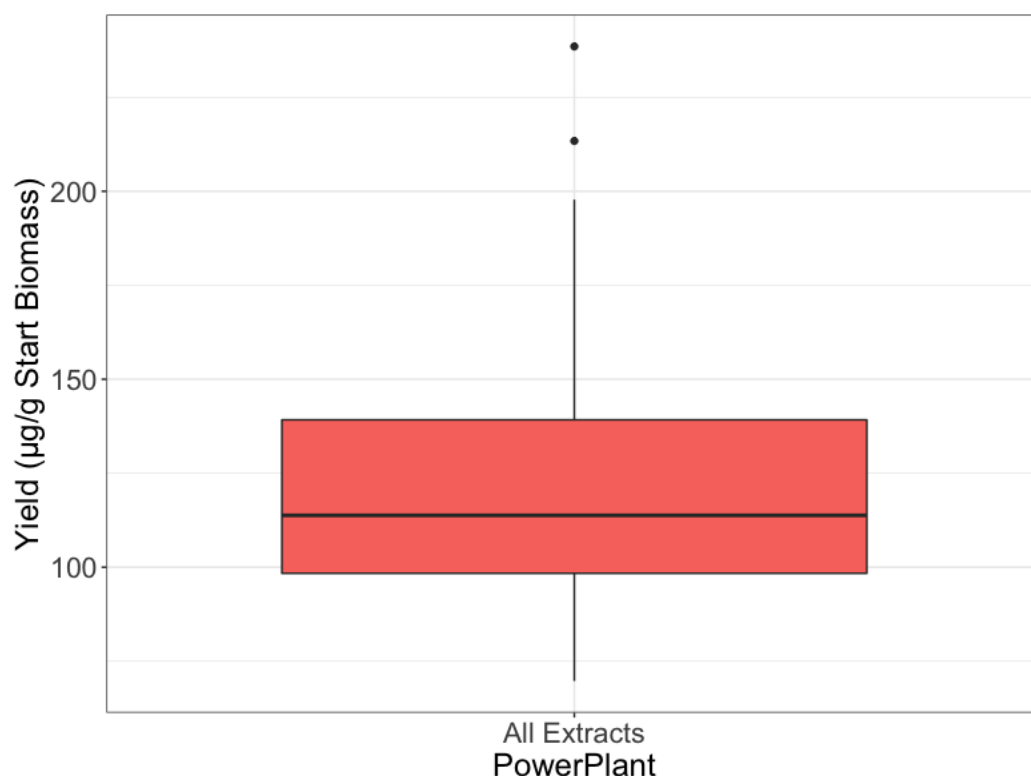


Figure 6.15. Boxplot of the yield of RNA isolations performed in this thesis. Boxplot represents the minimum and maximum values (whiskers), interquartile range (box, from 25th to 75th percentile), median value (line), and outliers (dots).

For these subsequent stress studies, RNA appeared again to be satisfactorily pure, with average absorbance ratios of 2.19 (A260/A280) and 2.23 (A260/A230) recorded. The yields here were within the expected range for RNA yield from plant material as quoted by Qiagen who estimate yields of 60-1000 µg RNA per gram of plant biomass (QIAGEN, 2017), whereas here, 69.73-238.53 µg RNA per gram of biomass was obtained (Figure 6.15), corresponding to total yields of 10.46-35.78 µg RNA. The suitability of these extracts for molecular biology applications was verified by the successful reverse transcription of RNA into cDNA using a commercial cDNA synthesis kit (ReadyScript cDNA Synthesis Mix, Sigma-Aldrich) and analysis of corresponding 'no reverse transcriptase' controls prepared for each cDNA sample by qPCR (Figure 6.16).

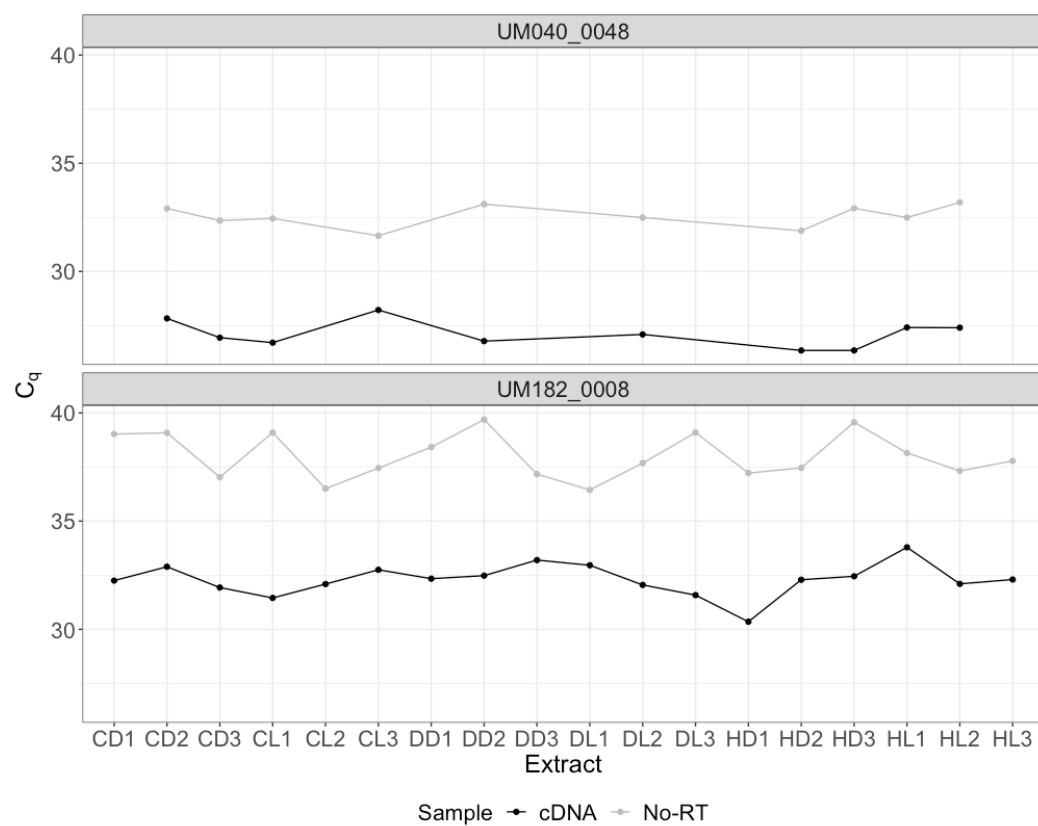


Figure 6.16. C_q of cDNA samples compared to the C_q of the corresponding no-reverse transcriptase control. Extract identifiers are summarised in Table 6.2.

6.3.1 QPCR Method Development

The isolation of molecular biology quality RNA from *U. compressa* now facilitated gene expression analysis. However, before the intended gene expression study could be performed, an appropriate method for data analysis (curve fitting for C_q determination) and data normalisation (establishing appropriate reference genes) was required. Both are discussed and verified within this section.

6.3.2 Introduction to Quantitative PCR

Quantitative PCR (qPCR) is an effective tool for monitoring differential gene expression in developmental, biotic, and abiotic stress studies. In qPCR, amplification of a target gene during a PCR reaction is monitored in real-time using a DNA binding fluorophore such as SYBR Green (Mao, Leung and Xin, 2007). Fluorescence intensity is recorded after each qPCR cycle, with fluorescence corresponding to the amount of template in a reaction, as a result the kinetics of the PCR reaction can be monitored. The most common utility for qPCR is the comparative gene expression study (or relative gene expression), which is used to determine whether a gene is upregulated or downregulated under certain biological conditions by comparing the reaction profile of an experimental sample to that of a biological control (Livak and Schmittgen, 2001; Pfaffl, 2001; Vandesompele *et al.*, 2002; Derveaux, Vandesompele and Hellemans, 2010). qPCR, therefore, provides a simple and versatile tool for assessing cellular stress responses. However, proper assessment of assay design is crucial to ensure that qPCR data is accurate. The MIQE guidelines (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) outline recommended assessments of qPCR assay performance to ensure data is of a suitable standard for publication (Bustin *et al.*, 2009). Considerations include assessment of RNA isolation, reverse transcription, primer design, reference gene selection and validation, and data analysis procedures (Rutledge, 2004; Spiess, Feig and Ritz, 2008; Bustin *et al.*, 2009; Untergasser *et al.*, 2012; Bustin and Huggett, 2017). This is particularly important when designing a qPCR assay in a new biological model – e.g., wild-type *U. compressa*.

Various qPCR studies have been carried out in green seaweed to date, including studies in *U. linza*, *U. limnetica*, *U. profilera*, *U. pertusa*, *U. fasciata*, and also *U. compressa* (Contreras-Porcia, Dennett, *et al.*, 2011; Fu *et al.*, 2011; Ichihara, Mineur and Shimada, 2011; Dong *et al.*, 2012; H. Zhang *et al.*, 2012; Tominaga *et al.*, 2012; He *et al.*, 2018; Fan *et*

al., 2020). However, due to the absence of genomic information, the scope of these studies has until recently been restricted compared to studies carried out on other model organisms for plant and algal research, such as *Arabidopsis* and *Chlamydomonas*. In these studies, typically, the expression of only a handful of genes that are well characterised in other organisms has been assessed, most likely due to the difficulties of identifying organism-specific genes of interest without significant genomic information being available (Tominaga *et al.*, 2010, 2012; Dong *et al.*, 2012; H. Zhang *et al.*, 2012; Guan *et al.*, 2016). Due to the publication of various Ulvophyte genomes (De Clerck *et al.*, 2018; Arimoto *et al.*, 2019; He *et al.*, 2021), there is now an opportunity to identify more interesting targets for green seaweed gene expression studies for seaweed-specific analyses (Zúñiga *et al.*, 2020; González *et al.*, 2021; Yu Wang *et al.*, 2021), and also the opportunity for phycologists to take a closer look at qPCR assay design enabling higher quality data and publications to be generated. However, as molecular genetics has only been recently applied to seaweed genetics, there are still a series of fundamental experiments that need to be carried out before more comprehensive transcriptomic studies can be planned as described in microalgal and land plant models. Steps to optimise a protocol to carry out the gene expression study described in 6.4 are now discussed.

6.3.3 Curve Fit Modelling

6.3.3.1 Curve Fit Theory

Inaccurate estimation of the efficiency of a qPCR reaction may result in under or overestimation of fold differences during relative gene expression calculation, where the amplification efficiency (E) is defined as “the increase in amplicon per cycle” (Bar, Kubista and Tichopad, 2012). In the commonly used delta-delta C_t method for relative gene expression calculation, it is assumed that the E of different amplicons is the same (Livak and Schmittgen, 2001). However, the E of a PCR reaction will differ from primer to primer – the different amplicons and primer properties will affect primer specificity, affinity and the degree of mispriming that occurs, consequently affecting reaction kinetics (Svec *et al.*, 2015). This is demonstrated for this data set in Figure 6.17. Differences in E between a gene of interest and a reference gene will introduce a bias in the calculated fold change, leading to incorrect interpretation of expression data. Some relative quantification methods, such as the ones described by Pfaffl and Vandesompele,

correct for differences in E , where the efficiency for an amplicon must be determined experimentally before analysis (Pfaffl, 2001; Vandesompele *et al.*, 2002).

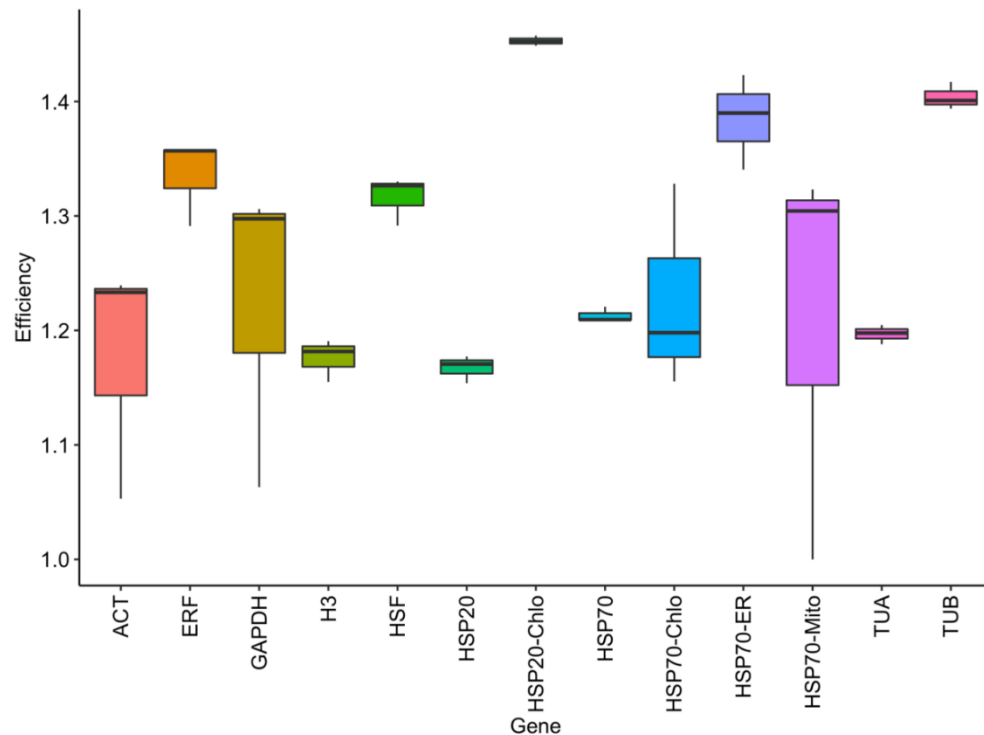


Figure 6.17. Amplification efficiency of all primers tested. Amplification efficiency calculated from efficiency curve derived by fitted model (see Figure 6.18, b), from cDNA diluted 1 in100. Efficiency value is taken from the cycle corresponding to C_q as determined by the C_{y0} method reported by Guescini *et al.* (2008).

Researchers have typically used linear quantification methods to interpret qPCR data, for example, the threshold method (C_t). Here a fluorescence threshold value is set which corresponds to where the reaction reaches an ‘inflection’ point (as demonstrated by the explanatory plot in Figure 6.18, a). To calculate E for this method, researchers typically use the ‘standard curve method’ where efficiency for each primer tested is calculated based on a linear regression of a dilution series (Swillens, Dessars and Housni, 2008). This facilitates primer-to-primer efficiency corrections. However, numerous assumptions are still made when using the standard curve method. Firstly, this method assumes that E at C_t (the threshold) remains constant for every reaction using a set of primers, and that amplification efficiency is constant at each step of the dilution series (corresponding to different amounts of starting template). Standard curves will typically be manufactured from an independent sample to the test samples, as such biological variability is not considered (Rutledge and Stewart, 2008; Bar, Kubista and Tichopad, 2012; Tellinghuisen and Spiess, 2014; Svec *et al.*, 2015; Tellinghuisen,

2020). It reported that PCR kinetics are affected by many factors – including biological variability observed from sample to sample, inhibitors, and also variability in reagent and sample loading (Schrader *et al.*, 2012; Svec *et al.*, 2015; Taylor *et al.*, 2019). As discussed previously (6.3), inhibition of PCR and other molecular biology assays is a common issue when working with recalcitrant biological material such as seaweed tissue due to the high risk of polysaccharide or phenolic contamination in nucleic acids (Jin *et al.*, 1997; Greco *et al.*, 2014). E also changes throughout the PCR reaction (Figure 6.18, b), with maximum efficiency observed during the exponential phase (Bar, Kubista and Tichopad, 2012). Due to the dynamic change in E , the C_t method may be too inflexible for highly accurate estimations of efficiency and quantifying gene expression.

A more accurate quantification and estimation method is facilitated by the non-linear fitting (curve fitting) of qPCR data (Liu and Saint, 2002; Rutledge, 2004; Spiess, Feig and Ritz, 2008b). Here, the returned fluorescence data for a qPCR reaction is modelled – typically using a logistic (sigmoidal) fit, using multiple parameters to describe the data. For example, a four-parameter logistic for qPCR data includes the minimum asymptote (baseline fluorescence of reaction), slope, the inflection point, and the maximum asymptote (maximum fluorescence of reaction) – as such, the model considers the whole reaction profile (as shown in Figure 6.18, a). This enables more accurate quantification than the C_t method, which considers a user defined ‘inflection point’ of the qPCR reaction only. A more appropriate C_q to be used in relative gene expression calculation can therefore be determined.

Most importantly, curve fitting enables calculation of how E changes throughout the reaction (Rutledge and Stewart, 2008; Bar, Kubista and Tichopad, 2012; Tellinghuisen and Spiess, 2014; Tellinghuisen, 2020). As such, reaction efficiency can be determined based on the kinetics of individual reactions rather than from the universal estimation of efficiency for an amplicon by the standard curve method. As curve-fitting enables more accurate quantification due to better interpretation of the qPCR amplification data, it was decided that a curve-fitting method would be used to analyse qPCR data in this thesis.

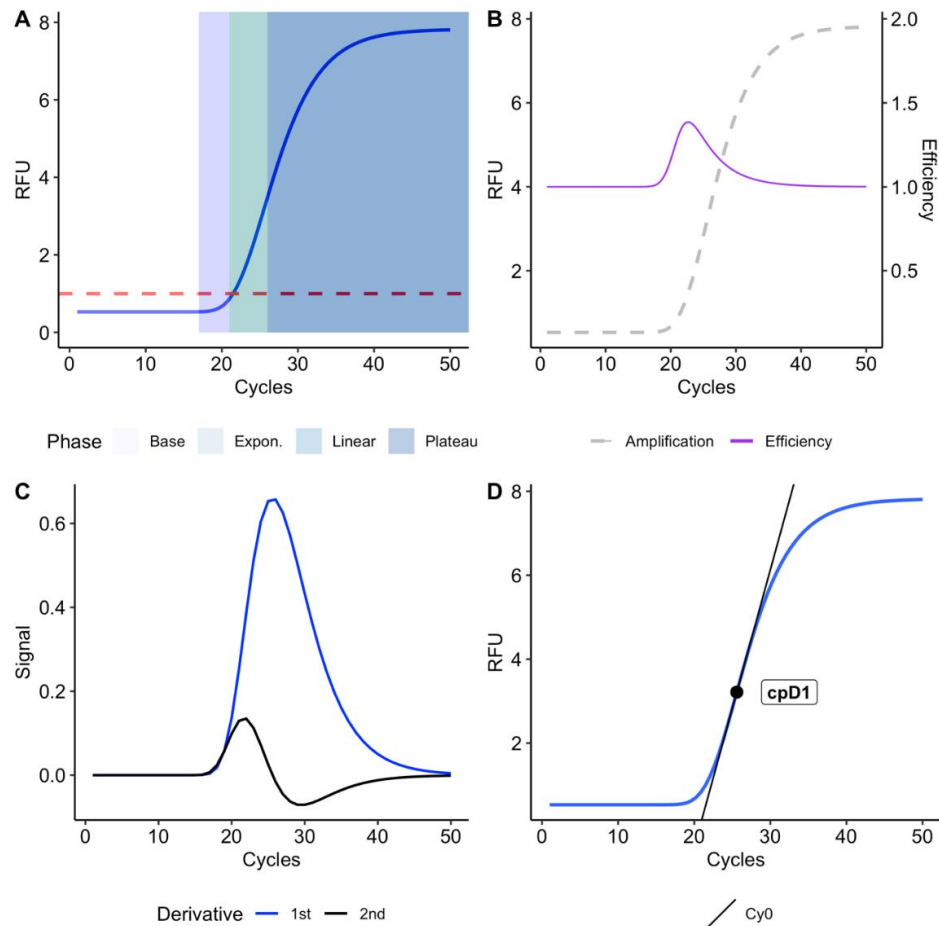


Figure 6.18. qPCR kinetics – summary of key concepts.

a) Different phases of the qPCR reaction are highlighted on an example amplification curve, including 1) baseline, 2) exponential, 3) linear, and 4) plateau phases. The red dashed line is a visual representation of the threshold (or C_t) quantification method, where C_t is taken as the assay cycle where the threshold fluorescence is reached (indicated by the red line). The set threshold value should correspond with the exponential phase of the qPCR reaction.

(b) Cycle-dependent efficiency. Amplification efficiency changes throughout the reaction. qPCR efficiency here is calculated by the equation $E_n = \frac{F_n}{F_{n-1}}$, with F representing fluorescence.

(c) Use of first and second derivative of fitted curves is a commonly used method of C_q determination. The 'cpD1' method takes the cycle corresponding to the maximum of the first derivative as C_q , whereas 'cpD2' takes the second derivative maximum. The Corbett 'CQ' method takes C_q at the cycle where fluorescence is at 20% of the second derivative maximum

(d) The Cy_0 method, as described by Guescini *et al.* (2008). This is performed by the function $Cy_0 = cpD1 - \frac{F_{cpD2}}{slope}$, which fits a tangent to the 'infection' point of the fitted curve, and C_q is taken as the cross point on the x -axis.

6.3.3.2 Data Generation

A test data set was generated to compare the performance of different curve fitting models (6.3.3.3) to establish a method to analyse data from the planned gene expression study (6.4). Here, a five-point linearity series was manufactured from a pool of cDNA samples reverse transcribed from multiple *U. compressa* RNA isolates across various abiotic stress conditions (Table 6.2). Dilutions were performed in nuclease-free water, with the dilution series ranging from neat cDNA to cDNA diluted 1 in 10,000. Samples were pooled for this analysis to ‘average’ out the effect of biological variation, with some of the tested amplicons (i.e., the heat-shock proteins) likely to have low or no expression in ‘unstressed’ tissue (Gallup and Ackermann, 2008). The pooled samples were then assayed in triplicate using a selection of qPCR primers (listed in Methods and Materials, Table 2.9) designed for the subsequent reference gene selection experiments and ‘dual stress’ experiments (6.3.4 and 6.4.2).

In addition to providing a test data set for model selection, this experiment was also performed to determine the linear range and sensitivity of this assay for each amplicon, with non-linearity indicating that the selected primers may require rework, or that low levels of the PCR target were present in samples (Liu and Saint, 2002; Vandesompele *et al.*, 2002; Svec *et al.*, 2015). Determination of assay sensitivity was required to identify an appropriate pre-dilution factor for assaying cDNA samples, with it being impractical to assay cDNA neat as only 20 µL of sample was generated per reverse transcriptase reaction, which would limit the number of tests that could be performed per sample, with a minimum of 3 technical replicates required per test for qPCR (Bustin *et al.*, 2009). This analysis was also performed to assess whether inhibitory components persisted in samples post reverse transcription, and if this was the case, an appropriate dilution factor at which the effect of inhibition was diluted out could be determined (Svec *et al.*, 2015).

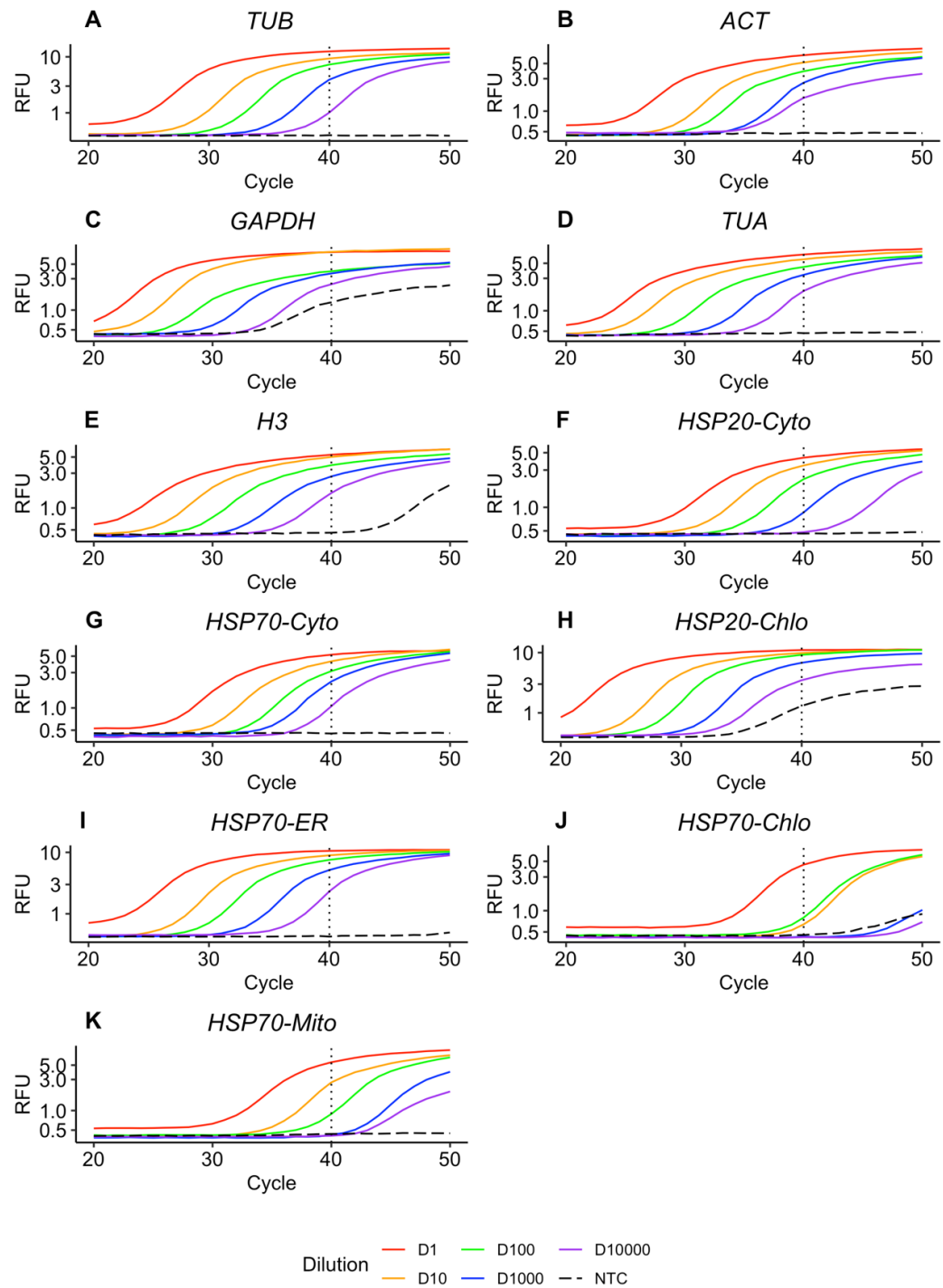


Figure 6.19. qPCR amplification curves from linearity series for all primers tested. A pooled cDNA sample was assayed neat and at serial dilutions from 1/10-1/1000 in nuclease-free water. Amplification curves from this dilution series and a no template control (black dashed line) are shown for (a) β -tubulin, (b) actin, (c), GAPDH, (d) α -tubulin, (e) histone 3, (f) cytoplasm localised HSP20, (g) cytoplasm localised HSP70, (h) chloroplast localised HSP20, (i) chloroplast localised HSP70, (j) endoplasmic reticulum localised HSP70, and (k) mitochondria localised HSP70 amplicons.

Amplification curves with a sigmoidal curve shape and strong fluorescent signal consistent with the qPCR reaction profile were observed in neat cDNA, and cDNA diluted with nuclease-free water from 1 in 10 to 1 in 10,000 for all primers except the *HSP70-Chlo* set (Figure 6.19). Here (Figure 6.19, j), the 'shift' in the cycle of the amplification curves at each serial dilution as observed for all other primers was not observed, instead the 1 in 10 and 1 in 100 samples had a similar threshold cycle (C_t), and further sample dilutions were indistinguishable from the no template control. This likely indicated that the expression of the *HSP70-Chlo* target was low in the pooled sample, and fell below the linear range of the assay as the sample was diluted further. As this was not observed in any other primer set, a cDNA pre-dilution factor of 1 in 100 was decided for the subsequent experiments (§6.3.4 and §6.4.2).

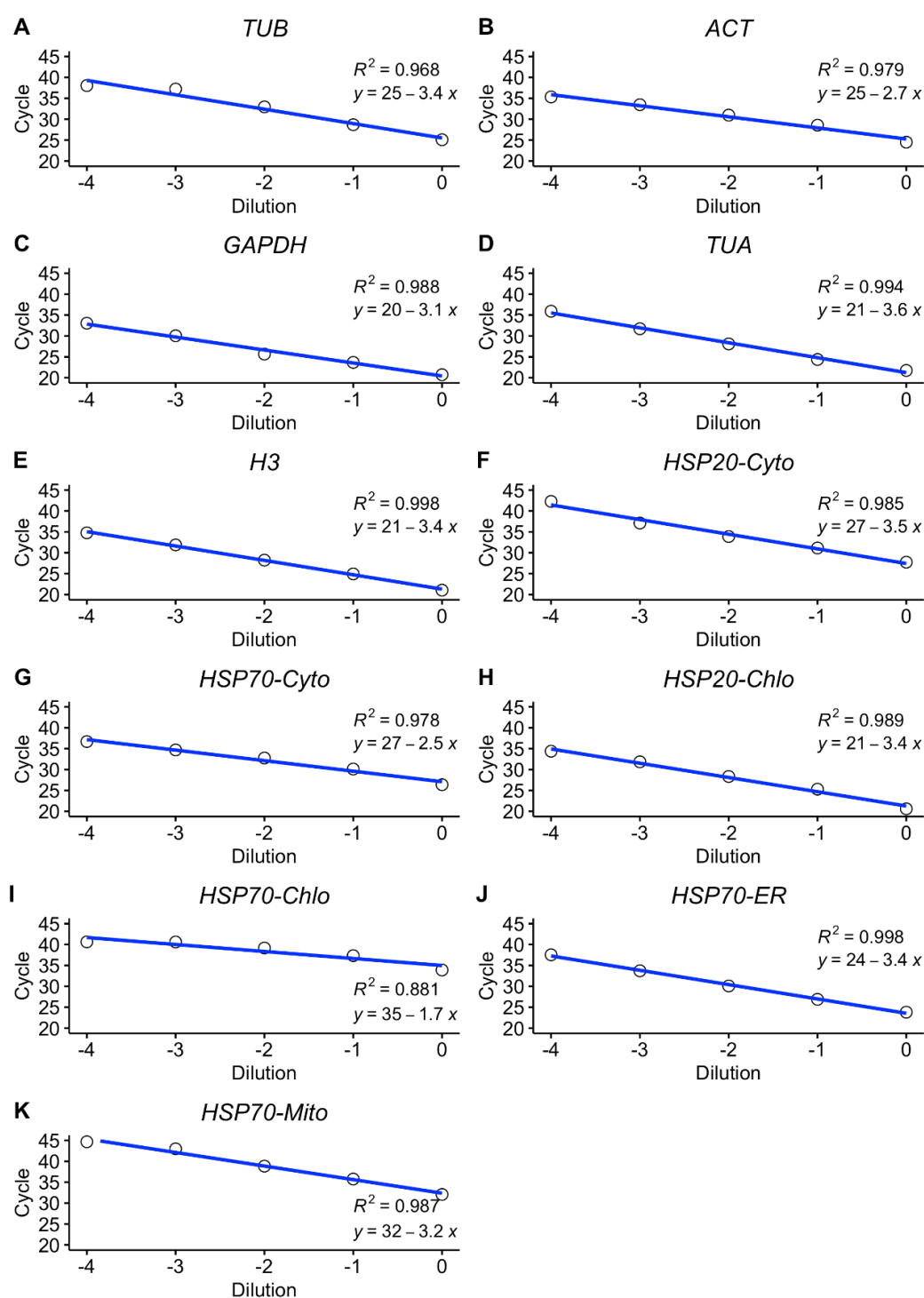


Figure 6.20. Standard curves from linearity series for all primers tested. C_q for the amplification curves shown in Figure 6.19 determined by the C_{y0} method reported by Guescini *et al.* (2008). R-squared, intercept, and the gradient is reported for each primer set.

A linear regression analysis (Figure 6.20) was performed on the determined C_q values from each linearity series, calculated by the C_{y0} method (Guescini *et al.*, 2008, 2013), where correlation coefficients (R^2) were determined as 0.9788 (*ACT*), 0.9878 (*GAPDH*), 0.9978 (*H3*), 0.9853 (*HSP20*), 0.9784 (*HSP70*), 0.9942 (*TUA*), 0.9675 (*TUB*), 0.9890 (*HSP20-Chlo*), 0.8812 (*HSP70-Chlo*), 0.9978 (*HSP70-ER*), and 0.9873 (*HSP70-Mito*). The assay therefore appeared linear using these primer sets, however, the *HSP70-Chlo* data set (Figure 6.20, i) had notably higher variance than the others with an $R^2 = 0.8812$ (all others had an $R^2 > 0.9675$), consistent with the observations from the amplification curves.

6.3.3.3 Model Selection

Using the 'qpcR' package on R (Ritz and Spiess, 2008), a four-parameter, five-parameter, and 6-parameter logistic model was fitted to the preliminary qPCR data set (Figure 6.19). Here, the data generated was fitted to a model by the non-linear least squares method that identifies parameters that describe the data best, i.e., by finding parameters values that give the lowest possible residual sum of squares (RSS, Table 6.1), which is a measure of the amount of variance in a data set that cannot be fitted by the model (Cobbs, 2012; Hsu, Sherina and McCall, 2020). The 'mselect' function of the package was then used to calculate and compare various 'goodness-of-fit' measures from the different models tested, to determine the 'best-suited' model to use for the analysis of qPCR data in this chapter. Three 'goodness-of-fit' measures were used to compare the different curve fit models, bias-corrected Akaike information criterion (AICc), residual variance, and log-likelihood (Table 6.1).

Akaike's information criterion (AIC) is a model selection method used to estimate the goodness of fit in multiple models (Burnham and Anderson, 2004; Wagenmakers and Farrell, 2004; Spiess, Feig and Ritz, 2008; Lauritano *et al.*, 2019; Wahl *et al.*, 2020). When fitting data, whilst using an increased number of parameters will improve how a model describes a data set, this increases the risk of 'overfitting' the data set. 'Overfitting' a data set requires more parameters to be estimated from the same amount of data, which makes the model more subject to random error, as whilst the model increases in complexity (i.e., more variables to predict), the data set stays the same (Hurvich and Tsai, 1989; Boggy and Woolf, 2010). The AIC considers the number of the parameters of a model (k), meaning the likelihood of overfitting is accounted for during model selection, with more complex models being 'penalised'. The AICc is a modified

version of AIC, which always corrects for sample size (n), with the AIC more likely to select overfitting models when n is small (Burnham and Anderson, 2004; Wagenmakers and Farrell, 2004). With smaller data sets, overfitting is more likely, as with limited data, a model is more likely to attempt to fit the variance observed in the data set, resulting in an overcomplicated model (Hurvich and Tsai, 1989). With a higher n a more accurate model can be determined, as the effect of sample-to-sample variation is filtered out, with more general trends in the dataset being fitted. By accounting for sample size, the AICc facilitates a more appropriate comparison of models when less data is available.

According to Burnham and Anderson (2004), the corrected method (AICc) should always be employed preferentially over the original method (AIC), as when n increases (i.e., $\frac{n}{k} < 40$) the differences between the outcomes of the two methods are negligible, meaning that the original AIC offers no advantage in large data sets. Based upon this, the AICc method was selected as the primary goodness-of-fit measure.

For comparison, residual variance provides a similar but simpler measure of 'goodness of fit' by summarising the differences observed between fitted data and actual data (i.e., the residual value), summarised in Table 6.1. Log-likelihood (required for calculation of AIC) represents the natural logarithm of the likelihood function (Bates and Watts, 1988; Boos and Stefanski, 2013).

Method	Equation	Reference
Residual Variance	$\sigma = \frac{rss}{n-k}$ where: k = the number of parameters in the model n = sample size rss = residual sum of squares calculated by: $rss = \sum_{i=1}^n (y_i - \hat{y}_i)^2$ where: y = actual fluorescence at i $\hat{y}$ = fitted fluorescence at i	(Tellinghuisen and Spiess, 2014)
AICc	$AICc = AIC + \frac{2k^2 + 2k}{n - k - 1}$ where: $AIC = -2(\log\text{-likelihood}) + 2k$	(Burnham and Anderson, 2004)

Table 6.1. Methods used to determine ‘goodness-of-fit’ in curve fit models

Here, the outcomes of all goodness-of-fit analyses were identical, and outcomes were not influenced by the amplicon assessed (Figure 6.21). The addition of the parameter f improved curve fit (see Materials and Methods, §2.1.5.5 for equation), with improved goodness-of-fit in the five-parameter logistic observed relative to the four-parameter logistic model. This is demonstrated visually (Figure 6.22, a), with the five-parameter logistic more accurately fitting *GAPDH* amplification data than the four-parameter model around the ‘inflection point’ of the qPCR reaction, and the respective root mean square error (i.e., the standard deviation of the residuals, Figure 6.22, b) from each of the three models at each cycle. The addition of a further parameter (i.e., the 6-parameter logistic) did not improve the fit further. As a result the five-parameter logistic model was selected for data analysis.

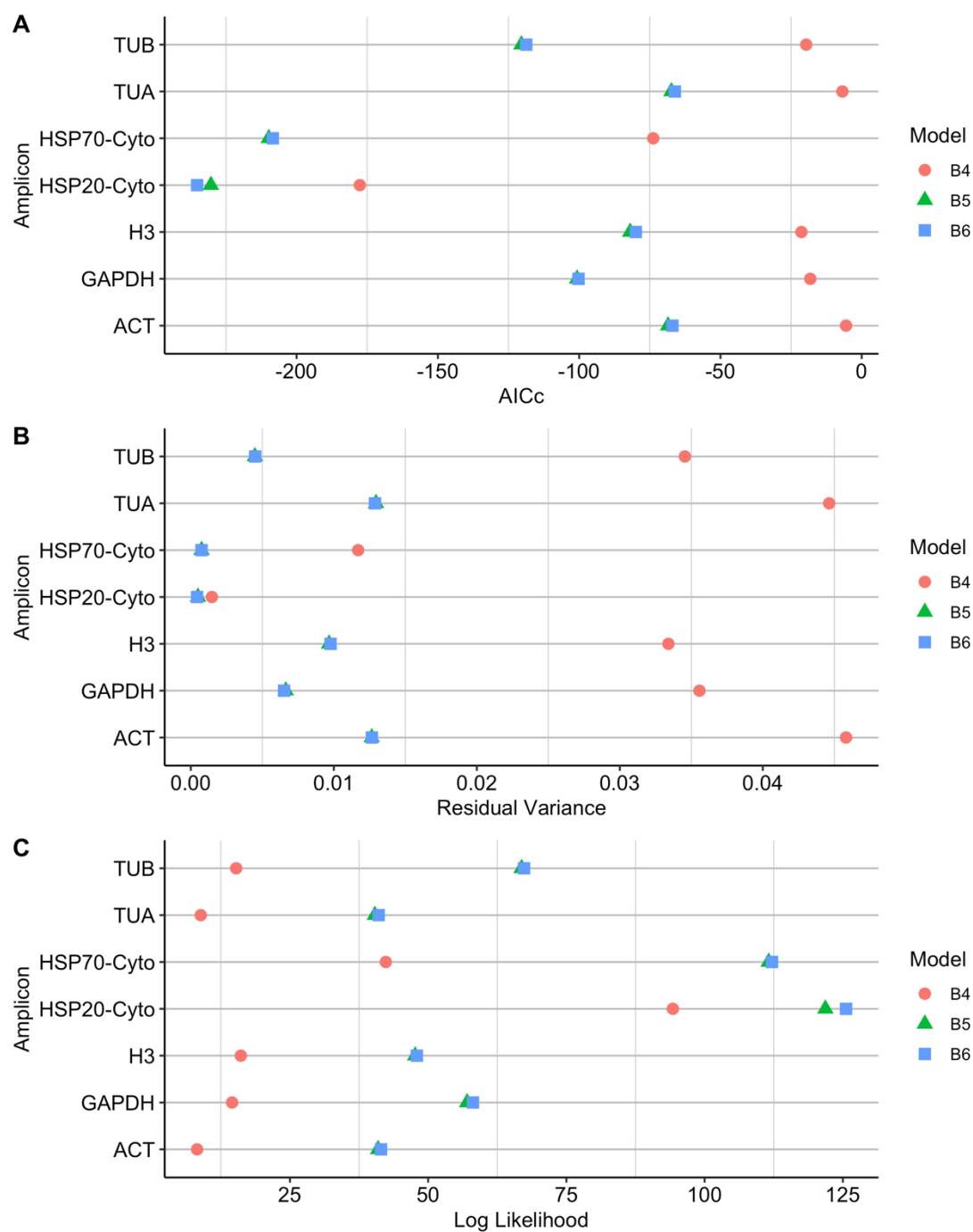


Figure 6.21. The goodness of fit measures for curve fit model selection. (a) AICc, (b) Residual variance, and (c) maximum log-likelihood. The goodness of fit was assessed per amplicon, with 15 replicate curves assessed per amplicon. For AICc and residual variance, a model with a lower value than another is indicative of a better fit. With log-likelihood, a higher value suggests the model is more accurate than another. Analysis was performed using the 'mselect' function in the qpcR package by Ritz and Spiess (2008).

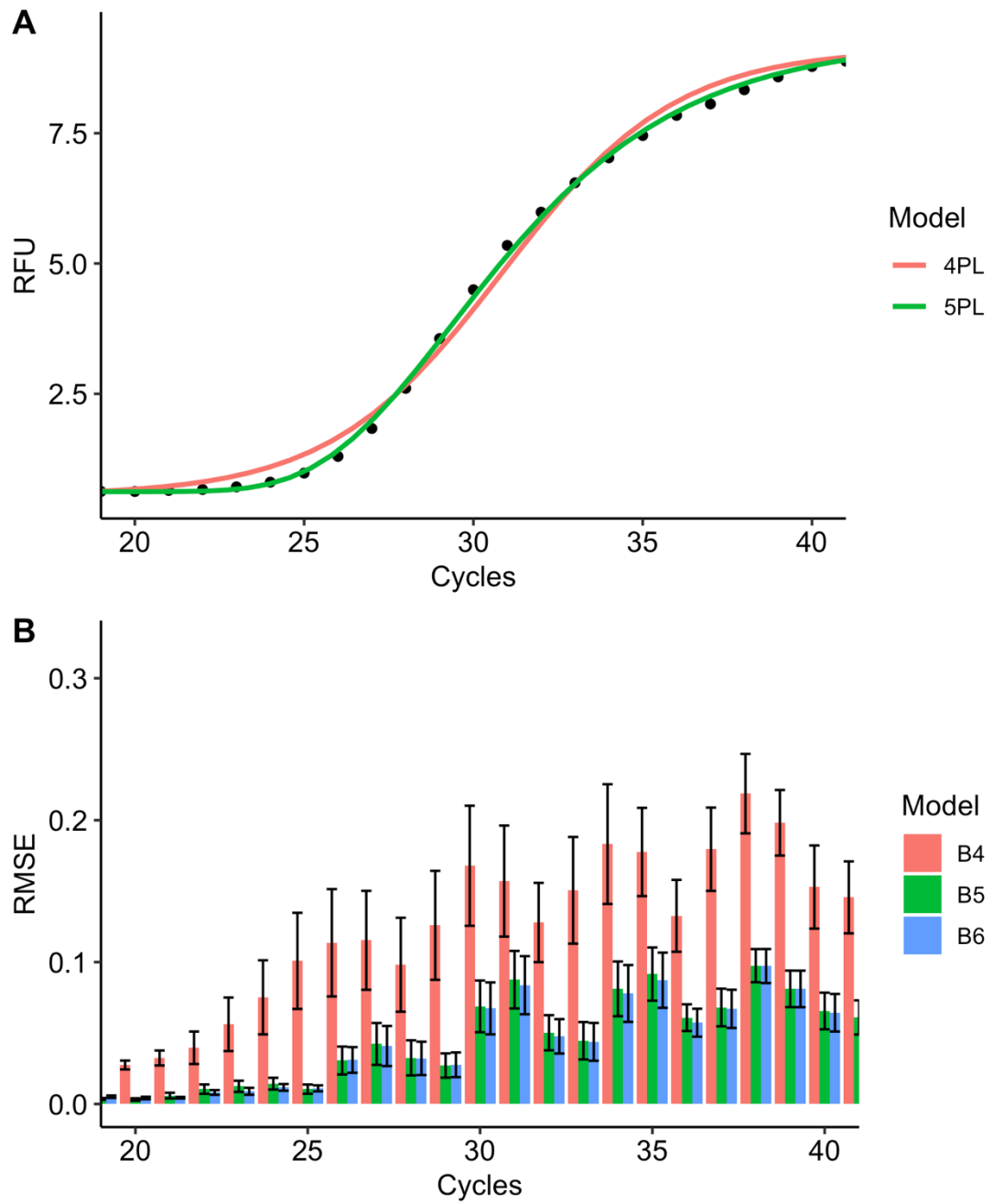


Figure 6.22. Comparison of the goodness of fit of different curve fit models at the exponential and linear phase of qPCR. (a) Plotted comparison of a four- and five-parameter logistic from a representative amplification curve using the *GAPDH* primers (b) root mean square error across *GAPDH* amplification curves by cycle (bars are mean results from $n = 15$ curves, with SE bars)

6.3.4 Reference Gene Selection

Reference genes are essential internal controls for quantitative PCR (Kozera and Rapacz, 2013; Kong et al., 2015; Andrade et al., 2017; D. Gao et al., 2018; Joseph, Poolakkalody and Shah, 2018; J. Li et al., 2019). Appropriate reference genes represent those with expression profiles that do not vary significantly across the experimental conditions tested in a study, historically referred to as ‘housekeeping’ genes (McCurdy, McGrath and Mackay-Sim, 2008; Jain, Vergish and Khurana, 2018). These controls correct for reaction-to-reaction variation introduced in the sampling (RNA isolation and cDNA synthesis) and sample loading process that will impact qPCR reaction kinetics. Reference genes are required to normalise relative gene expression data. However, the method by which this is performed (and how many genes are required) depends on the protocol (Livak and Schmittgen, 2001; Vandesompele *et al.*, 2002; Pfaffl, 2004). A staple component of the qPCR literature is the reference gene validation study, where authors test and recommend reference genes for a model organism subject to different experimental conditions (Bustin *et al.*, 2009; Taylor *et al.*, 2019). Here, a reference gene validation study was performed for *U. compressa*, to identify stable reference genes for when *U. compressa* samples are exposed to various combinations of light, heat, and desiccation stress, as described in Table 6.2. For this purpose, the stability of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), histone 3 (*H3*), α -tubulin (*TUA*), β -tubulin (*TUB*), and actin (*ACT*) gene expression was assessed, with all these genes frequently cited as internal controls for qPCR experiments in various model systems (McCurdy, McGrath and Mackay-Sim, 2008; Tichopad *et al.*, 2009; Dong *et al.*, 2012; Liu *et al.*, 2012; Ji *et al.*, 2015; Andrade *et al.*, 2017; Chen *et al.*, 2017; Joseph, Poolakkalody and Shah, 2018).

Two algorithms were used to identify the most appropriate reference genes for *U. compressa* across various combinations of abiotic stress, representing conditions that seaweeds would be exposed to in tide pools, including heat-shock, dehydration, and light variability (described in Table 6.2). These algorithms were geNorm and Normfinder (Vandesompele *et al.*, 2002; Andersen, Jensen and Ørntoft, 2004), which are both regularly cited in the literature for analyses of reference gene stability (Dong et al., 2012; Liu et al., 2012; Kianianmomeni and Hallmann, 2013; Kowalczyk et al., 2014; Ji et al., 2015; Alves-Lima et al., 2017; Chen et al., 2017; D. Gao et al., 2018; Panina et al., 2018; J. Li et al., 2019; Lauritano et al., 2019; Linardić and Braybrook, 2021).

In the case of geNorm (Vandesompele *et al.*, 2002), reference gene stability is evaluated by a stability measure denoted M . To calculate M , the pairwise variation (i.e., the standard deviation of expression data generated across genes) from all combinations of genes tested in the reference gene study is calculated for each individual gene, with M calculated as the mean of all pairwise variations calculated for a gene. The M scores of the candidate reference genes are then compared, and the least stable gene is eliminated. This process is repeated until the most stable combination of genes is identified.

NormFinder is an ANOVA-based algorithm that calculates a stability score for gene expression data by taking into consideration different biological variables in the data set (e.g., sample type, experimental treatment), determining intra- and inter- group variability (Andersen, Jensen and Ørntoft, 2004). This provides an advantage over the geNorm method which does not facilitate analysis of reference gene stability by group. With grouping, NormFinder facilitates the determination of whether some of the reference genes tested are suitable for normalising some of the experimental conditions tested in a study but not others, or whether certain reference genes are more appropriate for certain sample types in the data set. NormFinder determines gene stability for individual genes, and as performed by the geNorm method also calculates the stability of multiple combinations of gene pairs. In both methods, a lower score indicates a more stable gene. As required by both protocols, C_q data was converted to relative quantity values before analysis by the equation $E^{-\Delta C_q}$, with E taken as the mean amplification efficiency for each gene calculated in Figure 6.17 for efficiency correction (Rao *et al.*, 2013). Here, C_q was determined from qPCR data fitted with the five-parameter logistic model determined previously (6.3.3.3), using the C_{y0} method (Figure 6.18, d) as the measure of qPCR reaction kinetics (Guescini *et al.*, 2013).

Experiment ID	Condition 1	Condition 2	Condition 3
Control-Darkness (CD)	10°C		
Control-Light (CL)	10°C	Light	
Desiccation-Darkness (DD)	10°C		Desiccation
Desiccation-Light (DL)	10°C	Light	Desiccation
Heat-Desiccation (HD)	37°C		
Heat-Light (HL)	37°C	Light	

Table 6.2. Experimental conditions for reference gene selection study. *U. compressa* was cultured for 1 day in artificial seawater at 10°C in constitutive darkness prior to the stress experiments, where samples were exposed to the above combinations of stress for 3 hours. 3 biological replicates used per sample.

Using Normfinder, the most stable reference genes across all stress conditions were determined as *GAPDH* and *ACT*, both with stability scores of 0.06, followed by *H3*, *TUA*, and *TUB*, all with stability scores of 0.07 (Figure 6.23, a). The most stable gene combination (for when multiple reference genes were required in analysis) was calculated to be *GAPDH* and *H3* (0.03), as shown by Figure 6.25. This was followed by *TUB* and *TUA* (0.04), then by various equally ranked combinations of genes – including *GAPDH* and *TUA* (0.05), then *TUA* and *H3* (0.06).

The geNorm rankings were generally comparable to Normfinder. From analysis of genes individually (not plotted), the most stable gene was *ACT* with an *M* score of 0.156, followed by *GAPDH* (0.160), *TUA* (0.166), *H3* (0.169), and *TUB* (0.190). As stated, geNorm also performs a pairwise variation analysis, which determines how many reference genes are needed to analyse the data set. Here, a pairwise variation score (*V*) is assessed by comparing the effect of increasing the number of reference genes on the stability score by V_n/V_{n+1} , with *n* representing the number of reference genes. A cut-off value of 0.15 was established by the authors, i.e., if V_n/V_{n+1} is lower than 0.15, there is no benefit to using another reference gene (*n*+1) rather than *n* (Vandesompele *et al.*, 2002). Here, V_2/V_3 had a value well below the cut-off (0.0463), indicating that there was no statistical benefit to using three reference genes rather than two (Figure 6.24). As such, a gene pair was determined as sufficient to analyse the data set. The most stable gene pair by geNorm was determined to be *GAPDH* and *TUA*, with an *M* score of 0.137 (Figure 6.23, b).

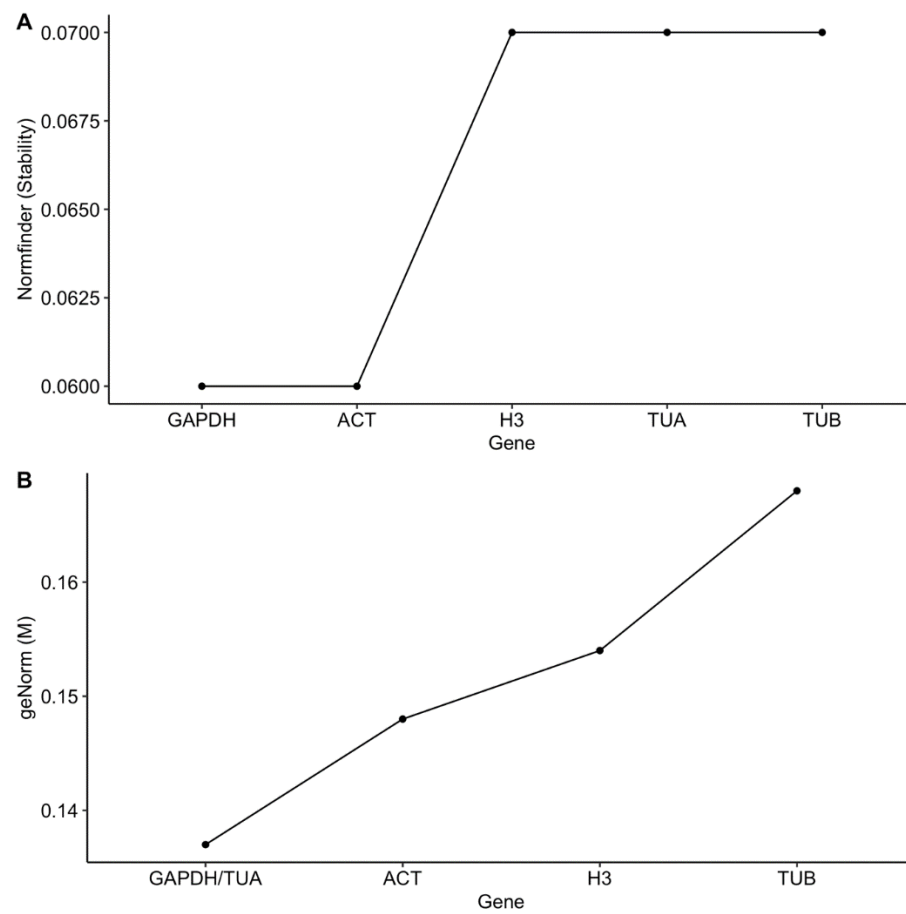


Figure 6.23. Ranked reference gene stability. The (a) Normfinder and (b) geNorm algorithms were used to identify the most stable reference genes across all stress condition.

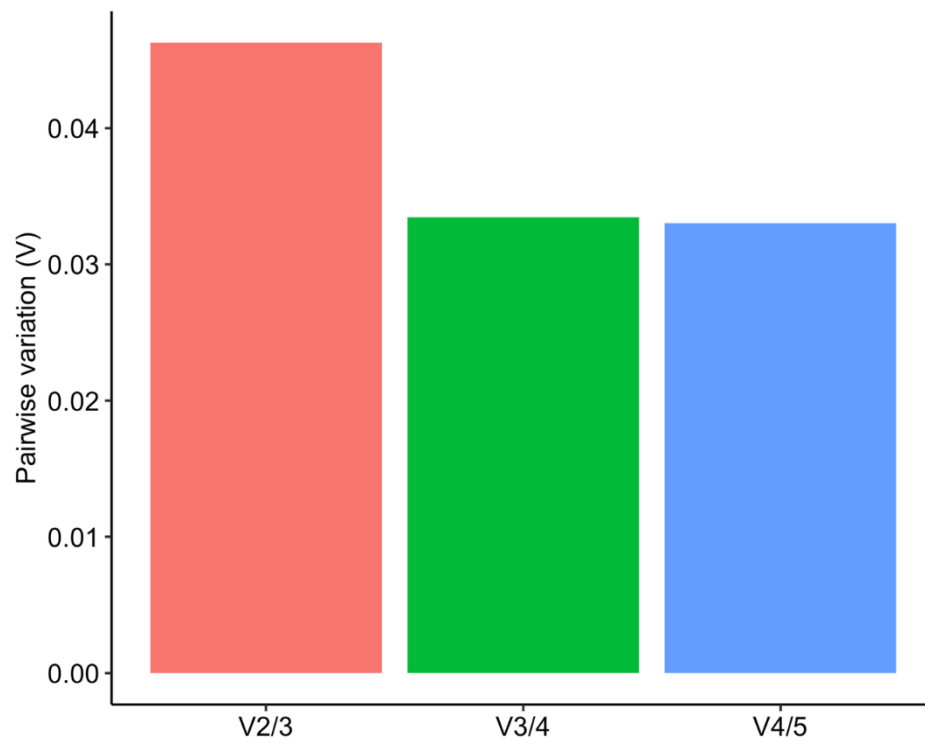


Figure 6.24. Calculated pairwise variance (V) by geNorm. The appropriate number of reference genes to use in a study is established by comparing the use of 2 vs 3, 3 vs 4, and 4 vs 5 reference genes for analysis. If $V < 0.15$, there is no benefit to using $n+1$ genes for analysis rather than n .

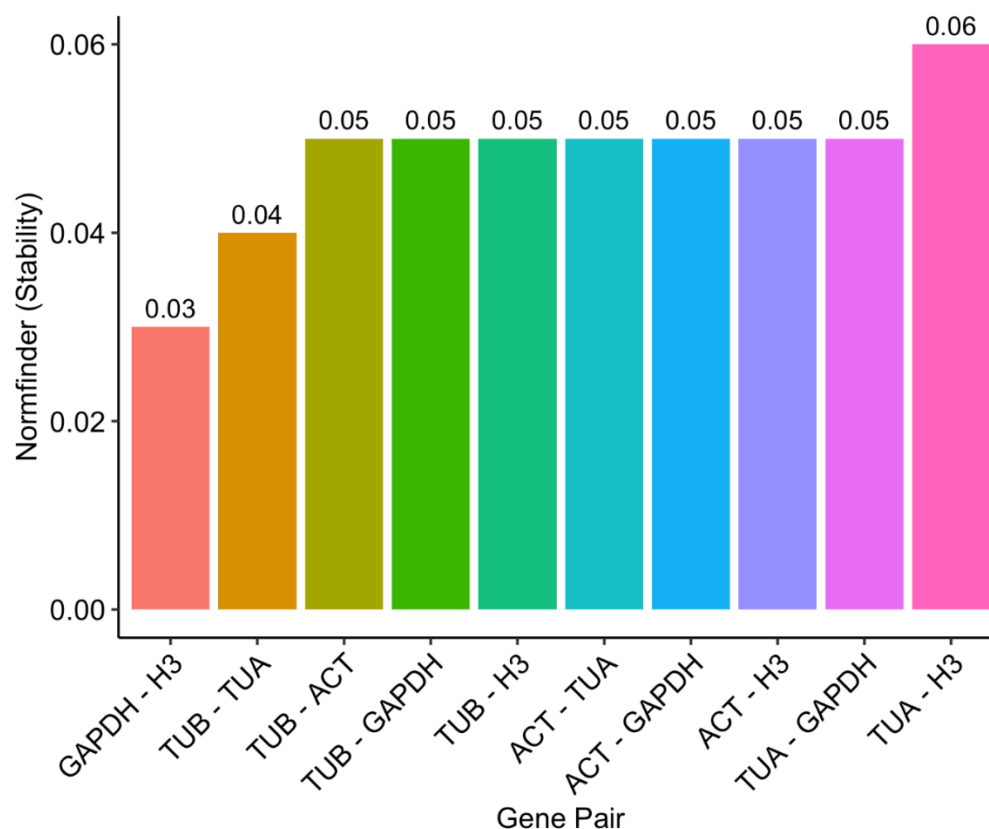


Figure 6.25. Most stable combinations of reference genes as determined by Normfinder.

6.4 Response of Molecular Chaperones to Dual Stress

In the previous sections (6.3.1), I established a protocol for performing quantitative PCR (qPCR) in *U. compressa*, and appropriate reference genes for intertidal stress studies. This now facilitated the differential gene expression studies described in this section, where the expression of components of the *U. compressa* stress response were monitored in response to various tidal stress conditions. This work also functions as proof-of-principle for the qPCR method development described previously.

The design of this experiment was based upon the observation that exposing organisms to stress *in vitro* does not accurately reflect how they respond to stress in the field. In environmental stress studies carried out under laboratory conditions, the effect of a single stress at a time on the organism of interest is often assessed, which has been the case for *Ulva* (Wu *et al.*, 2009; Tominaga *et al.*, 2010, 2012; Fu *et al.*, 2011; Gómez *et al.*, 2015; Fan *et al.*, 2017; Zúñiga *et al.*, 2020). However, this is not a reflection of conditions in the field, where organisms are exposed to multiple environmental factors that act concurrently (Morris and Taylor, 1983; Davison and Pearson, 1996; Smit and Glassom,

2017). Intertidal seaweeds such as *Ulva* must withstand constant changes in environmental conditions, and are exposed to simultaneous environmental stressors for which stress intensity varies with tide cycle, time of day, and season (Morris and Taylor, 1983; Davison and Pearson, 1996; Gracey *et al.*, 2008; van Hees and van Alstyne, 2013; Smit and Glassom, 2017). At low tide, seaweeds are exposed to high irradiance, desiccation stress, and extremes of temperature which they are sheltered from when immersed. In previous studies of stress responses in *Ulva* (*U. pertusa*, *U. prolifera*), researchers have assessed how chaperone expression changes in seaweed exposed to a single stress for increasing amounts of time (Tominaga *et al.*, 2010, 2012; Fu *et al.*, 2011; H. Zhang *et al.*, 2012). Whilst such experiments are a good measure of the dynamics and energetic requirements of the stress response, they do not necessarily reflect true environmental conditions – where the intensity and types of stress exposed to changes frequently. Additionally, as different stress factors interact (e.g., heat exasperates dehydration and hypersaline stress through evaporation), stress responses in seaweed to a single controlled stress are not an accurate representation of stress tolerance. As a result, testing the effect of different combinations of stress on an organism likely has more ecological relevance than studying stresses in isolation.

In land plants, the effect of different combinations of stress on the stress response are not easily predicted – as different stressors may have different effects on the cell (e.g., inducing heat shock, osmotic, and oxidative stress), therefore inducing stress responses by different stress signals. More simply put, the nature and intensity of the stress response is not necessarily correlated to the total amount of stress an organism is under, but instead corresponds to the effects of the different types of stress the cell withstands (Rasmussen *et al.*, 2013; Pandey *et al.*, 2017; Berens *et al.*, 2019). Studying the effect of different stress factors on gene expression will help researchers understand how the cell coordinates the stress response with input from different stress signals. Land plants are known to have highly complex signalling networks that regulate stress responses (Sanchez, Shin and Davis, 2011; ben Rejeb, Pastor and Mauch-Mani, 2014; Nouri, Moumeni and Komatsu, 2015; Haak *et al.*, 2017; M. Li *et al.*, 2019). By comparison, how intertidal *Ulva* regulates the stress response has not been looked at in much detail, with a few reports monitoring stress signals (e.g., MAPK and Ca²⁺) in *U. compressa* following exposure to copper (Laporte *et al.*, 2016; Celis-Plá *et al.*, 2019). In the present work, testing the expression of genes associated with the cellular stress response following exposure to simultaneous stress will provide early evidence for mapping out

how *Ulva* perceives, signals for, and responds to changing environmental conditions in the intertidal zone.

To better assess these dynamics in *Ulva*, I selected the molecular chaperone family (heat-shock proteins) as a biomarker, with these proteins representing universal and highly conserved components of the cellular stress response, characterised not only by their response to 'heat shock', but to their responses to general disruption of protein homeostasis (Kültz, 2003, 2005; Wang *et al.*, 2004; Schroda and Vallon, 2009; Chen, Feder and Kang, 2018). These proteins have general chaperone activity, being responsible for refolding misfolded proteins, protecting proteins from aggregation, and regulating protein synthesis, import, and degradation (Wang *et al.*, 2004; Haslbeck *et al.*, 2005; Swindell, Huebner and Weber, 2007; Hilton *et al.*, 2013; Balchin, Hayer-Hartl and Hartl, 2016; Jacob, Hirt and Bendahmane, 2017; Chen, Feder and Kang, 2018; Rigg, Mccarty and Aslan, 2018). Both selected families, the small heat shock proteins (HSP20s) and HSP70s, are cited frequently in the literature as biomarkers for environmental stress for many model organisms (Ireland *et al.*, 2004; Cruces, Huovinen and Gómez, 2012; Smolina *et al.*, 2016; Tang *et al.*, 2016; Sathasivam and Ki, 2019). Furthermore, the subcellular localisation profile of HSPs enables a comparison of how different combinations of stress impact different compartments of the cell (e.g., chloroplast), providing evidence for how different cellular stress responses are integrated (Renner and Waters, 2007; Waters and Rioflorido, 2007; Siddique *et al.*, 2008; Waters, 2013).

6.4.1 Identification of Molecular Chaperones

The publication of the *U. mutabilis* genome will facilitate more comprehensive phylogenetic studies to be performed, for evolutionary studies comparing genomic information with better-studied microalgal chlorophytes (e.g., *Chlamydomonas reinhardtii*) and land plants. This will provide us with a better understanding of the genetic basis of multicellularity in diverse habitats. Whilst such investigations are beyond the scope of this thesis, here I outline the steps I performed to identify the molecular chaperones present in the *U. mutabilis* genome.

The major heat-shock protein (HSP) families (HSP20, HSP33, CPN60, HSP70, HSP90, and HSP100) were identified in *U. mutabilis* by the methods described in the Materials and Methods (§2.1.5.5, 'Primer Design') using a combination of reciprocal BLAST searches using the corresponding HSP families from *A. thaliana* and *C. reinhardtii*, and protein domain searches based upon the conserved structures of HSPs

against the *U. mutabilis* proteome using HMMER and PROSITE (Eddy, 1998; de Castro et al., 2006; ‘Database resources of the National Center for Biotechnology Information’, 2016). The full list of the *U. mutabilis* HSPs identified is attached in the Appendices (Appendix I and II). As shown in Figure 6.26 (a), a total of 47 HSPs were identified in *U. mutabilis*, with the small HSP (HSP20) and HSP70 families having the most representatives (n = 13 and n = 15 respectively). Interestingly, *U. mutabilis* appeared to retain a gene for HSP33, which is present in various unicellular chlorophytes but not in the macroalga *Caulerpa* (Arimoto et al., 2019). Previous phylogenetic analysis in the literature demonstrates that this gene was lost in higher plants (Segal and Shapira, 2015).

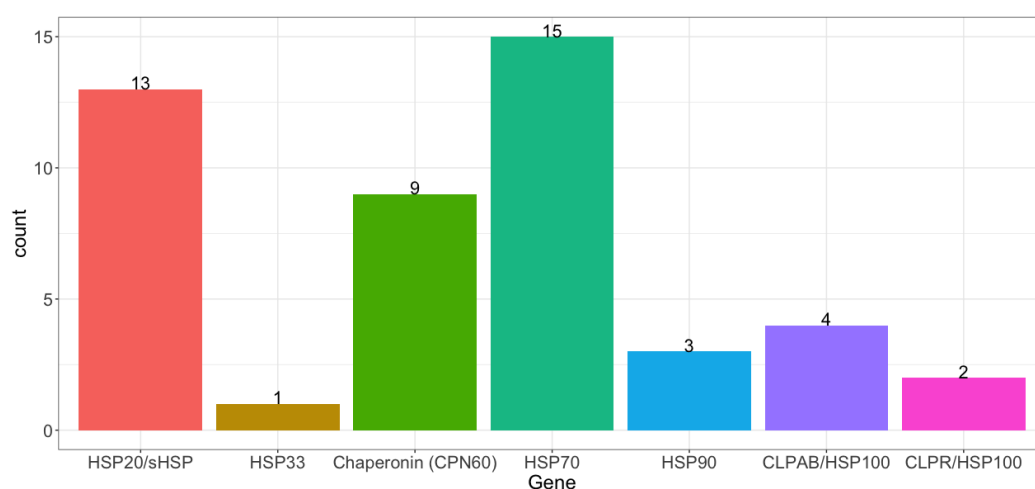


Figure 6.26. Number of heat shock proteins in the *U. mutabilis* genome

Following identification, the subcellular localisation of HSPs was predicted using three different prediction servers, DeepLoc, BUSCA, and PredAlgo (Tardif et al., 2012; Almagro Armenteros et al., 2017; Savojardo et al., 2018). A comparison of these methods was performed with my data set (Figure 6.27), with the PredAlgo neural-network trained using *C. reinhardtii* proteins in an attempt to develop a more accurate tool for predictions in algae, with contemporary localisation prediction tools reported to mispredict algal protein localisation (Waters and Rioflorida, 2007; Tardif et al., 2012). The BUSCA was selected as this method also predicts membrane proteins (both plasma and organelle membrane), with some of the chloroplast localised HSPs in plants and algae known to be associated with the thylakoid membrane (Rütgers et al., 2017; Theis et al., 2020).

The expected difficulty in predicting the localisation of *U. mutabilis* HSPs using this approach is demonstrated by the different outcomes observed across the different

methods (Figure 6.27). Notably, whilst the DeepLoc algorithm predicted that 5 of the HSP sequences were plastid localised, the BUSCA predictions identified 10 chloroplast localised proteins, and PredAlgo identified 16. For mitochondrial HSPs, the corresponding predictions were 8, 4, and 2 for each method respectively. Additionally, the BUSCA tool appeared to identify ER-targeted proteins being extracellular instead of just being 'endomembrane system' proteins, with the DeepLoc method annotating the same proteins as endoplasmic reticulum-localised (two HSP70s with the ORCAE identifiers: UM012_0036.1 and UM001_0412.1, and one HSP90: UM012_0034.1). These three proteins are also positively identified as ER proteins by PredAlgo from the N-terminal signal sequence, however, this method also attributed three other proteins as having this sequence, one SHSP which was identified as nuclear HSP by both the other methods (UM005_0210.1), and a HSP70 (UM012_0035.1) and a HSP100 (UM001_0583.1) protein where no consensus was obtained. Extracellular HSPs have been reported in mammalian models but not plants, as a result it appears these predictions by the BUSCA method appears to be a misattribution of ER proteins (Calderwood, Gong and Murshid, 2016). Nonetheless, more experimental evidence is needed to assess which of these tools is most accurate, and whether the PredAlgo tool is in fact more suitable for predictions of proteins from the Ulvophyceae, rather than just microalgal chlorophytes. At the outset of this research there were no other reliable methods to verify protein localisation in *Ulva*, however, more recently, Blomme and colleagues (2021) have recently demonstrated a robust workflow for introducing fluorescently tagged transgenes into *U. mutabilis* cultures, which has been used to image various *U. mutabilis* proteins *in vivo*, confirming localisation. The use of this protocol in the future will help verify the predictions made in this work.

However, from the reported predictions for the HSP families selected for qPCR (HSP20 and HSP70), there were a selection of organelle-localised proteins where a consensus prediction was achieved across the three methods (Table 6.3). These corresponded to one mitochondrial-localised (UM039_0067.1), one ER-localised (UM012_0036.1), and two chloroplast-localised proteins (UM003_0083.1 and UM002_0182.1). In the case of the ER-localised protein (UM012_0036.1), this assignment was supported by the positive identification of a C-terminal tetrapeptide sequence (HDEL), with a HDEL sequence also identified in the sequence for the previously discussed ER-localised HSP90 (UM012_0034.1), but not the other HSP70 where a consensus prediction on ER localisation was determined (UM001_0412.1).

Primers for qPCR were designed for these genes, as well as two cytosolic chaperones, including a HSP70 protein (UM041_0091.1) homologous to HSP70E in *C. reinhardtii* and HSP70-14 in *A. thaliana*, which represents a higher molecular weight representative of the HSP70 family homologous to the HSP110 proteins in mammals (Easton, Kaneko and Subjeck, 2000; Lin *et al.*, 2001; Dragovic *et al.*, 2006; Schroda and Vallon, 2009), and the p23 co-chaperone for the HSP90 family which was initially misidentified as a HSP20 protein (UM038_0098.1), and was labelled with the primer identifier of *HSP20-Chlo* during the previous method development work (6.3.1). p23 forms a complex with HSP90 and other HSP90-associated co-chaperones (e.g., Hop) during the stress response, which subsequently interact with HSP70 complexes. The p23 protein has also been associated with various developmental processes in plants, including root development (Felts and Toft, 2003; D'Alessandro *et al.*, 2015, 2019; Biebl *et al.*, 2021). As a result, it was hypothesised that the expression of this gene would be correlated with general HSP activity during the described experiments.

For these selected genes, the subcellular localisation prediction was also supported by reciprocal BLAST searches against the *A. thaliana* (TAIR) and *C. reinhardtii* genomes in the instance that subcellular localisation of the homologous proteins was known. This was the case for the predicted mitochondrial HSP70 (*HSP70-Mito*), chloroplast HSP70 (*HSP70-Chlo*) and ER-localised HSP70 (*HSP70-ER*), which corresponded with proteins in *A. thaliana* and *C. reinhardtii* with matching subcellular localisation. For the chloroplast localised HSP70, the *C. reinhardtii* homolog was identified as the HSP70B, which has been positively identified as a chloroplast protein in the literature (Surpin, Larkin and Chory, 2002; Willmund and Schroda, 2005), as was the identified HSP70 protein in *A. thaliana*. The mitochondrial HSP70 was identified as a homolog of mitochondrial heat shock cognate in *A. thaliana*, with heat shock cognates representing a group of HSP70 proteins that are expressed constitutively in cells rather than in the cellular stress response exclusively (Jacob, Hirt and Bendahmane, 2017; Rigg, Mccarty and Aslan, 2018). The ER-localised HSP70 was identified as a homolog of binding protein 1 from both searches, with BIP proteins representing a highly conserved component of the unfolded protein response (Cho and Kanehara, 2017).

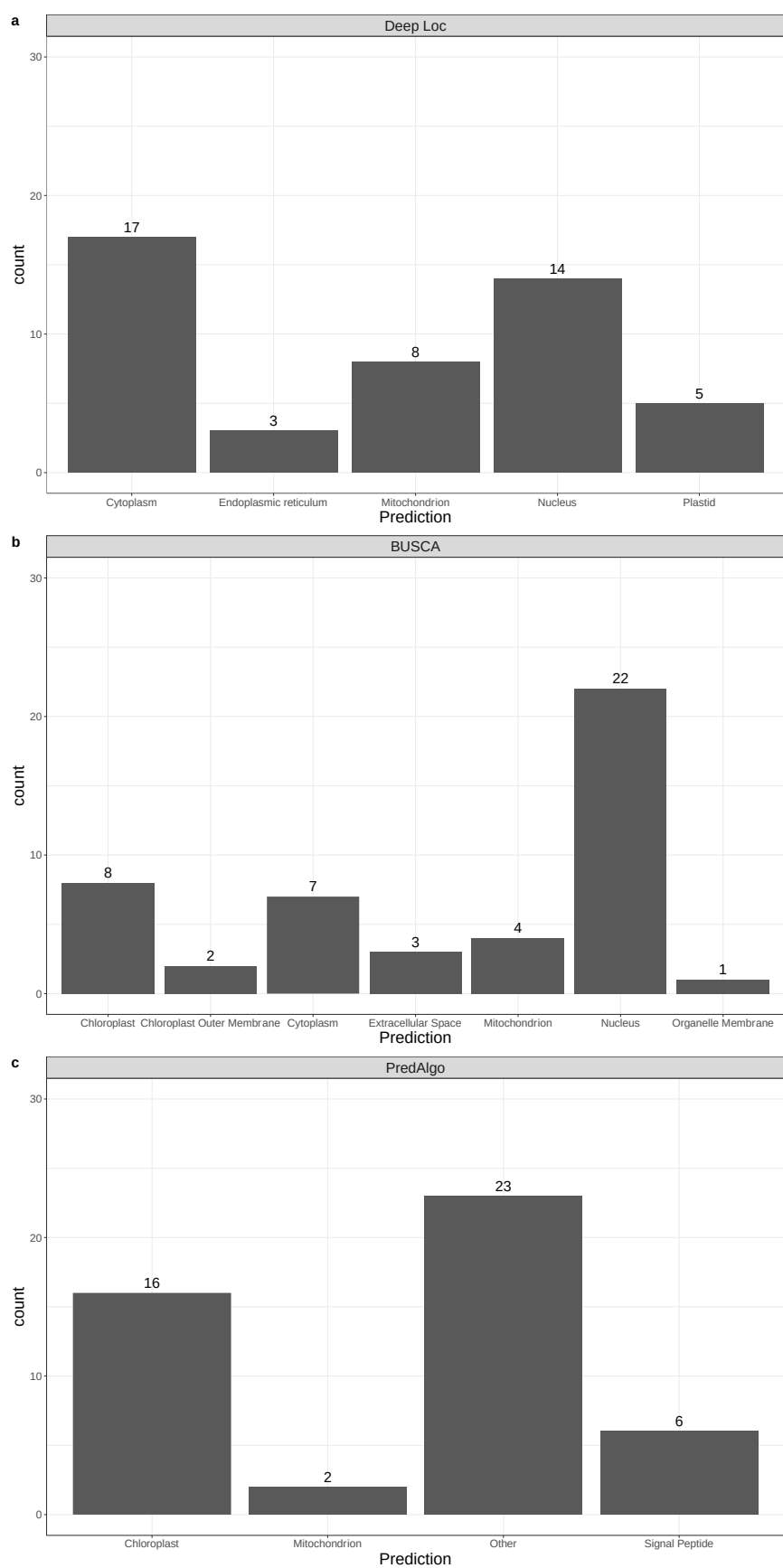


Figure 6.27. Subcellular localisation of all heat-shock proteins identified in the *U. mutabilis* genome using DeepLoc, BUSCA, and PredAlgo

Localisation Prediction				<i>Chlamydomonas reinhardtii</i> Genome			<i>Arabidopsis thaliana</i> Genome		
ID	Deep Loc	BUSCA	PredAlgo	Best Hit	Accession	E	Best Hit	Accession	E
UM038_0098.1: p23	Cytoplasm (0.76)	Nucleus (1)	NA	p23 co-chaperone of HSP90 system	XP_001692527.1	7E-26	P23-1	AT4G02450	6E-14
UM003_0083.1: HSP20- Chloroplast	Plastid (0.89)	Chloroplast Outer Membrane (0.82)	Chloroplast (1.08)	hypothetical protein CHLRE_14g617400v5	PNW73064.1	1E-7	HSP17.6A HSP20-like chaperones superfamily protein	AT1G59860	6E-19
UM041_0091.1: HSP70 (HSP110)	Cytoplasm (0.91)	Cytoplasm (0.7)	Chloroplast (0.69)	heat shock protein 70E	XP_001695858.1	0	ATHSP70-14, HEAT SHOCK PROTEIN 91, HSP91	AT1G79930	0
UM002_0182.1: HSP70- Chloroplast	Plastid (0.72)	Chloroplast Outer Membrane (0.67)	Chloroplast (1.65)	heat shock protein 70B	XP_001696432.1	6E-60	HSC70-7, cpHsc70-2 chloroplast heat shock protein 70-2, HEAT SHOCK PROTEIN 70-7	AT5G49910	2E-57
UM012_0036.1: HSP70-ER	Endoplasmic reticulum (0.73)	Extracellular Space (0.86)	Signal Peptide (1.11)	binding protein 1	XP_001701685.1	0	BIP1	AT5G28540	0
UM039_0067.1: HSP70- Mitochondria	Mitochondrion (0.93)	Mitochondri on (0.6)	Mitochondri on (1.98)	hypothetical protein CHLRE_09g393200v5	PNW78536.1	0	HEAT SHOCK COGNATE, HSC70-5, MITOCHONDRIAL HSO70 2, MTHSC70-2	AT5G09590	0

Table 6.3. Predictions for the HSP genes selected for qPCR and corresponding homologs from the *C. reinhardtii* and *A. thaliana* genomes.
BLAST searches performed on Phytozome (*C. reinhardtii*) and TAIR (*A. thaliana*) databases (Merchant *et al.*, 2007; Goodstein *et al.*, 2012; Berardini *et al.*, 2015)

6.4.2 Responses of Molecular Chaperones to Dual Stress

Relative gene expression in *U. compressa* was assessed for the selected HSPs and the co-chaperone p23 across the described combinations of stress (Table 6.2), using *GAPDH* and *H3* as reference genes, both of which were verified experimentally by the previously described reference gene study (§6.3.4), and the five-parameter logistic model as determined by the model selection study combined with the Cy_0 quantification method to fit and quantify raw data (§6.3.3.3).

As shown in Table 6.4, the outcomes of two-way ANOVA analysis indicated that the main effect of condition 1 (i.e., *U. compressa* exposed to control conditions, dehydration, or heat shock) had a significant effect on p23 ($F = 39.69$, $p < 0.001$), chloroplast HSP20 ($F = 22.1$, $p < 0.001$), HSP70 ($F = 41.59$, $p < 0.001$), and ER HSP70 expression ($F = 10.69$, $p < 0.01$), but not chloroplast HSP70 ($F = 1.42$, $p = 0.280$) or mitochondrial HSP70 ($F = 0.12$, $p = 0.886$). The main effect of condition 2 (i.e., stress performed in the light or dark) appeared to have a significant effect on the expression of all genes (p23: $F = 8.82$, $p < 0.05$, HSP70: $F = 17.75$, $p < 0.01$, chloroplast HSP70: $F = 7.22$, $p < 0.05$, mitochondrial HSP70: $F = 7.59$, $p < 0.05$, and ER HSP70: $F = 10.09$, $p < 0.01$) except the chloroplast localised HSP20 ($F = 3.97$, $p = 0.070$). ANOVA also indicated that there was a significant interaction between condition 1 (stress) and condition 2 (light) that influenced p23 ($F = 0.92$, $p < 0.01$) and chloroplast HSP20 expression ($F = 7.71$, $p < 0.05$). This interaction had a non-significant effect on all other genes.

Gene	Condition 1 (Control or Dehydration or Heat)	Condition 2 (Light or Dark)	Interaction of Condition 1 and Condition 2
p23	$F = 39.69$, $p < 0.001$	$F = 8.82$, $p < 0.05$	$F = 9.52$, $p < 0.01$
HSP20-Chlo	$F = 22.1$, $p < 0.001$	$F = 3.97$, ns ($p = 0.070$)	$F = 7.71$, $p < 0.05$
HSP70 (HSP110-Like)	$F = 41.59$, $p < 0.001$	$F = 17.75$, $p < 0.01$	$F = 2.33$, ns ($p = 0.139$)
HSP70-Chlo	$F = 1.42$, ns ($p = 0.280$)	$F = 7.22$, $p < 0.05$	$F = 2.91$, ns ($p = 0.093$)
HSP70-Mito	$F = 0.12$, ns ($p = 0.886$)	$F = 7.59$, $p < 0.05$	$F = 3.34$, ns ($p = 0.070$)
HSP70-ER	$F = 10.69$, $p < 0.01$	$F = 10.09$, $p < 0.01$	$F = 3.61$, ns ($p = 0.280$)

Table 6.4. Two-way ANOVA analysis of HSP expression following abiotic stress in light or dark conditions. DF for condition 1 = 2, condition 2 = 1, interaction of condition 1 and condition 2 = 2, total = 12.

Post hoc pairwise analysis (Tukey HSD) confirmed the outcomes of ANOVA and is presented in Figure 6.28 with corresponding log₂ fold changes summarised in Table 6.5. Figure 6.28 (a) and demonstrates that whilst p23 expression appeared to be upregulated compared to the control (dark) sample following heat stress, this upregulation was only significant with additional light stress. As indicated by the compact lettering in Figure 6.28 (a), summarising the outcomes of Tukey HSD for the various stress interactions (where shared letters between groups indicate there is no significant difference between them), the upregulation observed following combined heat-light stress (group a) was significantly greater than sole heat-induction (Tukey HSD: group b). The expression profile and statistical outcomes were near identical for the cytosolic HSP70 (Figure 6.28, c), with a non-significant increase in expression observed relative to the control (group bc) observed following sole heat stress (group b) and a significant increase observed following combined heat and light stress (group a). Interestingly, this gene appeared to be slightly downregulation following dehydration in the dark (log₂ fold change = -0.33). However, this change in expression was deemed non-significant compared to the control samples.

For the chloroplast localised HSP20 (Figure 6.28, b) and ER HSP70 (Figure 6.28, d), only combined heat and light stress appeared to have a significant effect on gene expression. In the case of the ER HSP70, sole heat shock did not appear to induce a major change in gene expression relative to the control (log₂ fold change = 0.14). For the chloroplast and mitochondrial HSP70 genes (Figure 6.28, e, f), no stress condition appeared to induce a significant change in gene expression relative to the control. However, for both, the relative gene expression of combined heat-light stress was significantly higher than heat shock performed in the dark, which appeared to downregulate expression slightly (log₂ fold change = -0.09 and -0.23 for *HSP70-Chlo* and *HSP70-Mito* respectively).

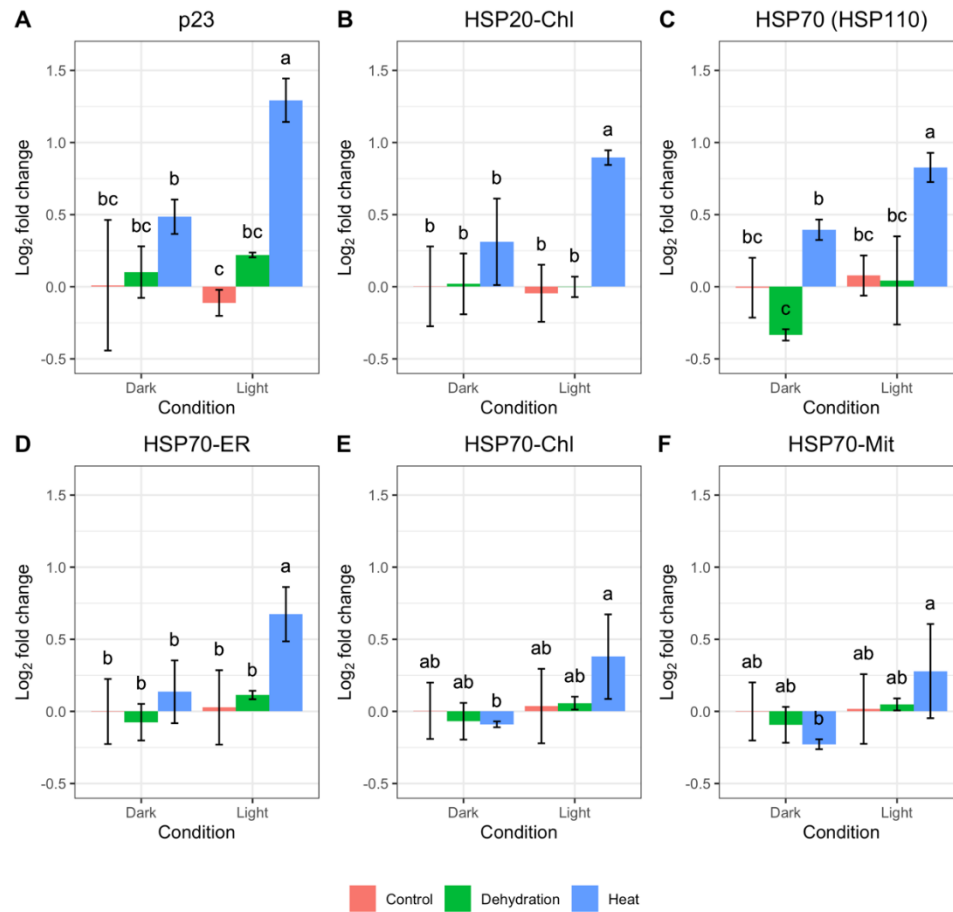


Figure 6.28. Barplot of HSP expression following abiotic stress in light or dark conditions. HSP expression in *U. compressa* following cultivation for 3 h under control conditions (10°C, within 35 ppt seawater), dehydration stress, or heat stress in the presence and absence of light. Relative gene expression, expressed as log₂(fold change), was calculated by the Vandesompele method (Vandesompele *et al.*, 2002) using H3 and GAPDH reference genes. Expression is calculated relative to the mean result of the control-dark sample. Error bars represent 95 CI. Data was analysed by two-way ANOVA, followed by Tukey's HSD post hoc test. Letters represent outcomes of post hoc analysis, here treatments that share a common letter are not statistically different ($p > 0.05$).

Gene	Stress					
	Control		Dehydration		Heat	
	Dark	Light	Dark	Light	Dark	Light
p23	0.01 (-0.44,0.46) (bc)	-0.11 (-0.20, -0.02) (c)	0.10 (-0.08, 0.28) (bc)	0.22 (0.20, 0.24) (bc)	↑ 0.48** (0.37, 0.60) (b)	↑ 1.29*** (1.14, 1.44) (a)
HSP20-Chlo	0.00 (-0.27,0.28) (b)	-0.05 (-0.24, 0.15) (b)	0.02 (-0.19, 0.23) (b)	0.00 (-0.07, 0.07) (b)	0.31 (0.01, 0.61) (b)	↑ 0.90*** (0.84,0.95) (a)
HSP70 (HSP110- Like)	-0.01 (-0.21, 0.20) (bc)	0.08 (-0.06, 0.22) (bc)	-0.33 (-0.37, -0.29) (c)	0.04 (-0.25, 0.35) (bc)	0.39 (0.32, 0.47) (b)	↑ 0.83*** (0.73, 0.93) (a)
HSP70-Chlo	0.00 (-0.19, 0.20) (ab)	0.04 (-0.22, 0.30) (ab)	-0.07 (-0.20, 0.06) (ab)	0.06 (0.01, 0.10) (ab)	-0.09 (-0.11, -0.07) (b)	0.38 (0.09, 0.67) (a)
HSP70-Mito	0.00 (-0.20, 0.20) (ab)	0.02 (-0.22, 0.26) (ab)	-0.09 (-0.22, 0.03) (ab)	0.05 (0.01, 0.09) (ab)	-0.23 (-0.26, -0.19) (b)	0.28 (-0.05, 0.61) (a)
HSP70-ER	0.00 (-0.23,0.22) (b)	0.03 (-0.23, 0.29) (b)	-0.08 (-0.20, 0.05) (b)	0.11 (0.08, 0.14) (b)	0.14 (-0.08, 0.35) (b)	↑ 0.67** (0.49, 0.86) (a)

Table 6.5. Summary of HSP expression following abiotic stress in light or dark conditions. HSP expression in *U. compressa* following cultivation for 3 h under control conditions (10°C, within 35 ppt seawater), dehydration stress, or heat stress in the presence and absence of light. Relative gene expression expressed as log₂(fold change), was calculated by the Vandesompele method (Vandesompele *et al.*, 2002) using H3 and GAPDH reference genes. Expression is calculated relative to the mean result of the control-dark sample. Mean log₂(fold change) values are reported with 95 CI reported in brackets. Data was analysed by two-way ANOVA, followed by Tukey's HSD post hoc test. Letters represent outcomes of post hoc analysis, here treatments that share a common letter are not statistically different ($p > 0.05$). Significance values are indicated by asterisks, with $p < 0.001 = ***$, $p < 0.01 = **$, and $p < 0.05 = *$.

A repeat of the heat and combined heat-light stress experiment was performed with an increased number of biological replicates (five rather than three) to improve statistical power due to the relatively low fold changes in gene expression observed, particularly for the sole heat stress condition, and the high variance observed (as indicated by the error bars denoting 95 confidence intervals in Figure 6.28). Additionally, as combined heat-desiccation stress was not assessed in the initial study, this was also tested, with this representing a challenge that seaweed would withstand at low tide during summer months. As there was no evidence of a significant HSP response to sole light and desiccation stress in the first experiment, they were not re-tested.

Interestingly, no expression was observed for the mitochondrial HSP70 and chloroplast HSP70 genes in the second experiment, either in control tissue or following combined heat stress (Table 6.6). For the other genes, except the chloroplast HSP20 ($F = 1.81$, $p = 0.409$), one way ANOVA analysis indicated that there were significant differences between the relative gene expression under the tested stress conditions (heat, heat-light, and heat-dehydration), corresponding to p23 ($F = 8.7$, $p < 0.01$), HSP70 ($F = 29.06$, $p < 0.001$), and the ER HSP70 ($F = 4.95$, $p < 0.05$).

Gene	Condition (Control, Heat, Heat-Light, or Heat-Dehydration)
<i>p23</i>	$F = 8.7$, $p < 0.01$
<i>HSP20-Chlo</i>	$F = 1.81$, ns ($p = 0.409$)
<i>HSP70 (HSP110-Like)</i>	$F = 29.06$, $p < 0.001$
<i>HSP70-Chlo</i>	ns (no expression observed)
<i>HSP70-Mito</i>	ns (no expression observed)
<i>HSP70-ER</i>	$F = 4.95$, $p < 0.05$

Table 6.6. One way ANOVA analysis of HSP expression following heat shock, combined light and heat shock stress, and combined dehydration and heat shock stress.

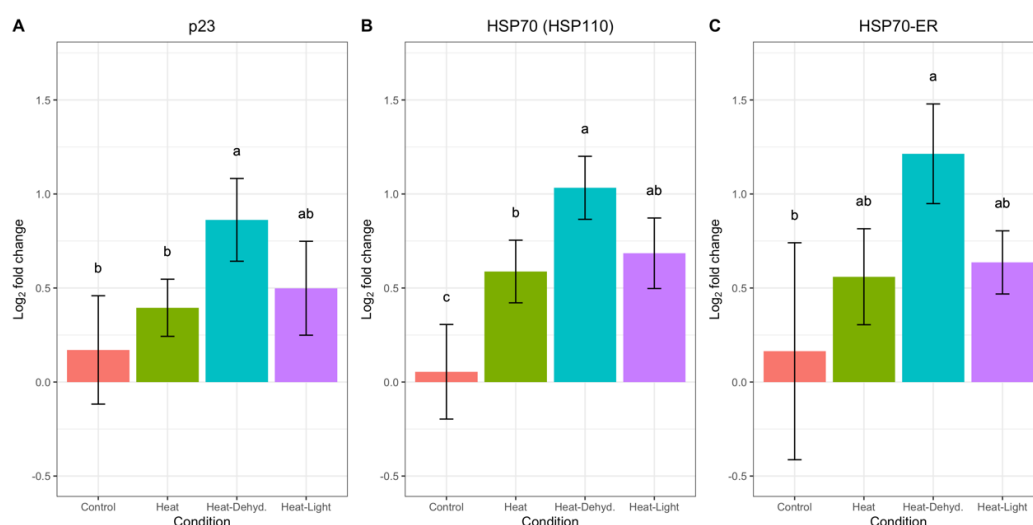


Figure 6.29. Summary of HSP expression following abiotic stress. HSP expression in *U. compressa* following cultivation for 3 h under control conditions (10°C, within 35 ppt seawater), heat stress in the presence and absence of light, and combined heat and dehydration stress. Relative gene expression, expressed as $\log_2(\text{fold change})$, was calculated by the Vandesompele method (Vandesompele et al., 2002) using *H3* and *GAPDH* reference genes. Expression is calculated relative to the mean result of the control-dark sample. Mean $\log_2(\text{fold change})$ values are reported with 95 CI reported for error bars. Data was analysed by one-way ANOVA, followed by Tukey's HSD post hoc test. Letters represent outcomes of post hoc analysis, here treatments that share a common letter are not statistically different ($p > 0.05$). Significance values are indicated by asterisks, with $p < 0.001 = ***$, $p < 0.01 = **$, and $p < 0.05 = *$.

As demonstrated in Figure 6.29, in this repeat experiment, the light-induced increase in gene expression when combined with heat shock was no longer observed for the assessed genes (p23, HSP70, and ER localised HSP70). For p23 (Figure 6.29, a), neither sole heat shock (Tukey group b) nor combined light-heat shock (group ab) induced a significant increase in gene expression relative to the control (group b), but combined heat-dehydration stress did (group a). The same outcomes were observed for the ER localised HSP70 (Figure 6.29, c), with Tukey HSD groupings of b, ab, ab, and a reported for control, heat shock, combined light-heat shock, and combined dehydration-heat shock stress respectively. For HSP70, all heat-shock-induced increases were significant relative to the control (group c), with combined heat-dehydration stress (group a) having a significantly greater effect on gene expression than sole heat stress (group b). High variance was observed under control conditions for all genes (Table 6.6), particularly in the ER localised HSP70, possibly reflective of the low expression of HSPs expected when stress responses had not been induced.

Gene	Stress			
	Control	Heat	Heat-Light	Heat-Dehydration
<i>p23</i>	0.17 (-0.12,0.46) (b)	0.39 (0.24,0.55) (b)	0.50 (0.25, 0.75) (ab)	↑ 0.86** (0.64,1.08) (a)
<i>SHSP-Chlo</i>	0.05 (-0.76, 0.86) (a)	0.54 (0.15, 0.94) (a)	0.69 (0.17, 1.21) (a)	0.54 (0.28, 0.80) (a)
<i>HSP70 (HSP110)</i>	0.05 (-0.20, 0.31) (c)	↑ 0.59* (0.42, 0.75) (b)	↑ 0.68** (0.50, 0.87) (ab)	↑ 1.09*** (0.87, 1.20) (a)
<i>HSP70-ER</i>	0.16 (-0.41, 0.74) (b)	0.56 (0.30, 0.82) (ab)	0.64 (0.47, 0.80) (ab)	↑ 1.21** (0.95, 1.48) (a)

Table 6.7. Summary of HSP expression following abiotic stress. HSP expression in *U. compressa* following cultivation for 3 h under control conditions (10°C, within 35 ppt seawater), heat stress in the presence and absence of light, and combined heat and dehydration stress. Relative gene expression, expressed as $\log_2(\text{fold change})$, was calculated by the Vandesompele method (Vandesompele et al., 2002) using *H3* and *GAPDH* reference genes. Expression is calculated relative to the mean result of the control-dark sample. Mean $\log_2(\text{fold change})$ values are reported with 95 CI reported in brackets. Data was analysed by one-way ANOVA, followed by Tukey's HSD post hoc test. Letters represent outcomes of post hoc analysis, here treatments that share a common letter are not statistically different ($p > 0.05$). Significance values are indicated by asterisks, with $p < 0.001 = ***$, $p < 0.01 = **$, and $p < 0.05 = *$.

6.5 Discussion

6.5.1 Sigmoidal Curve Fitting Enables Accurate Quantification of qPCR Data

Commercial curve fitting software that automatically fit qPCR data once generated are available and are integrated with thermal cycler hardware. For example, the comparative quantification method can be used to analyse fitted data generated with Rotorgene (QIAGEN) thermal cyclers (McCurdy, McGrath and Mackay-Sim, 2008). However, within this thesis, I decided not to use commercial software, and instead I critically evaluated different methods for fitting qPCR data to ensure that the data I generated here was quantified accurately.

There were several reasons for this decision. Firstly, different methods for fitting qPCR data exist (Pfaffl, 2001, 2004; Spiess, Feig and Ritz, 2008; Rutledge, 2011; Tellinghuisen and Spiess, 2014; Svec *et al.*, 2015; Hsu, Sherina and McCall, 2020; Tellinghuisen, 2020), and the model used to fit will impact the outcomes of analysis, that is the calculation of amplification efficiency and the cycle of quantification (C_q). Furthermore, whilst researchers working with well-characterised model organisms have a wealth of prior literature and data to benchmark their qPCR assay performance against, and as such can be more confident in selecting an analysis method without the need for extensive validation, this is not the case for field collected *Ulva*. Due to this, and the known difficulties of isolating nucleic acid from seaweed, the risk of suboptimal assay performance using *U. compressa* samples was anticipated, as despite the promising nucleic acid isolations described (6.3), assay inhibition can only be determined definitively when samples are tested on the assay in question. Furthermore, compared to model plants and there are also fewer tools available to ensure high-quality primer design. For example, there are no databases documenting previously validated primers for target genes as reported for *Arabidopsis thaliana* (Han and Kim, 2006), *Chlamydomonas* and various other plants and algae (Lu *et al.*, 2018). Fewer well-characterised gene models are reported for *Ulva*, making designing highly specific and strongly performing primers slightly more challenging (for example, identifying appropriate exon-exon junctions when selecting an amplicon). However, primer design in this thesis was for the most part successful, as discussed previously, with acceptable primers designed for most of the intended targets.

Additionally, accurate quantification of genes with low levels of expression is challenging, as it depends on the sensitivity of the assay. For qPCR, it is important that the quantification method successfully distinguishes background noise (identified in no template controls) and actual gene expression. This presents a challenge for quantifying

some of the 'stress responsive' genes assessed in this thesis (e.g., heat shock proteins), some of which exhibit 'all or nothing' expression – i.e., are not expressed (or are expressed at a very low level) unless the cellular stress response is activated, whereas other may have relatively high basal expression profiles (Kruse, Liu and Kloppstech, 1993; Wunderlich, Werr and Schoffl, 2003; Rampino et al., 2009; Halter et al., 2017; Chen, Feder and Kang, 2018; K. Li et al., 2021). No obvious low signal was seen in the *U. compressa* amplification curves for the linearity experiment, with all primers demonstrating good linearity except the chloroplast HSP20, indicating that there were no major issues with sample preparation. However, as expected, all heat shock proteins appeared to have low expression under standard conditions – evidenced by the relatively high C_q values and variance observed in control samples. In the case of the mitochondrial HSP70 and the chloroplast HSP70 tested, no expression was observed in the second experiment. Here, the two experiments were performed on field-collected *Ulva* collected in different months, as a result this discrepancy was attributed to possibly biological variability within the *Ulva* population at the sampling site. This inconsistency could be limited by establishing defined cultures of *U. compressa* in the lab as discussed in §6.2 rather than assessing field-collected material; however, the establishment of clonal cultures may limit the ecological relevance of investigations, with biological variability an essential component of experiment design. However, regardless of signal strength, by comparing the performance of different curve fit methods, it could be ensured that the qPCR data generated during this thesis was quantified accurately in lieu of these mentioned challenges.

Spiess and colleagues (2008) demonstrate that a five-parameter logistic (and log-logistic) more accurately describes qPCR data than the respective four-parameter model due to the inclusion of an extra parameter f to account for asymmetry in the qPCR amplification curve, which is not accounted for in a four-parameter model (that assumes qPCR data is symmetrical both sides of the 'take-off' point). The superiority of a five-parameter fit over the four-parameter model for fitting biological assays which produce asymmetric data sets is also illustrated by Gottschalk and Dunn (2005). Due to slightly reaction-to-reaction differences in qPCR kinetics, amplification curves can be non-symmetrical about the take-off point (i.e., when the reaction shifts from the lag phase to the exponential phase) (see Figure 6.18). In the publication by Spiess and colleagues (2008b), the authors identify little evidence of amplification curve symmetry in the extensive qPCR data sets they use for model comparison. This supports the observations from my data set where a five-parameter model describes data around the exponential phase accurately, whilst the four-parameter model

describes it relatively poorly (Figure 6.22). This was confirmed quantitatively by various goodness-of-fit analyses (Figure 6.21). As no obvious advantages to using a 6-parameter logistic were observed (and I could find no references justifying using a more complex model than this in the literature), the 5-parameter logistic was selected for analysis based upon the outcomes of model comparison.

Following curve fitting, different methods are available to quantify data (Guescini et al., 2008, 2013; Sisti et al., 2010; Svec et al., 2015; L. Zhang et al., 2019; Tellinghuisen, 2020). Quantification cycle (C_q) estimation can be performed using various outputs from a fitted model, typically concerning the exponential phase of the qPCR reaction, which corresponds to the reaction cycle with the highest amplification efficiency (Figure 6.18, b, c). Following a literature review, I selected the Cy_0 method (as described in Figure 6.18, d) reported by Guescini and colleagues (2008, 2013) to estimate C_q in this thesis. Guescini and colleagues (2008) compared the performance of this quantification method to other commonly used quantification methods (including the well-characterised threshold and second-derivative maximum methods of C_q determination). The authors demonstrated that whilst the accuracy and precision of data analysed by the other commonly cited methods were compromised when PCR was inhibited artificially, the Cy_0 method remained robust. The ability to quantify inhibited qPCR data accurately may help researchers working with tough samples such as seaweed, where suboptimal assay conditions are likely due to difficulties in sample preparation. The Cy_0 method is considered to provide greater accuracy as it accounts for reaction-to-reaction differences in amplification efficiency – as C_q is calculated from the slope at the exponential phase (Figure 6.18, d), which is influenced by reaction-specific qPCR kinetics (Sisti *et al.*, 2010; Bar, Kubista and Tichopad, 2012). The Cy_0 was also shown to be more precise than other C_q determination methods from standard curves generated in more recent method comparison studies (Tellinghuisen and Spiess, 2014; Tellinghuisen, 2020). As such, it appeared to be a suitable choice for analysing the qPCR data in this chapter.

6.5.2 Identification of Stable Reference Genes Facilitates Relative Gene Expression Studies in *U. compressa*

In addition to the reference gene study described in this chapter (6.3.4), to date, there is only one other documented report of a reference gene validation study for an *Ulva* species (Dong *et al.*, 2012). By contrast, there are multiple reports advising on reference gene selection for plants (Lilly *et al.*, 2011; Kudo *et al.*, 2016; Andrade *et al.*, 2017; Chen *et al.*, 2017; Joseph, Poolakkalody and Shah, 2018; Jin *et al.*, 2019), chlorophyte microalgae including *Volvox carteri* (Kianianmomeni and Hallmann, 2013) and *Chlamydomonas* ICE-L (Liu *et al.*, 2012), and various brown and red seaweeds, including representatives from the *Chondrus*, *Porphyra*, *Pyropia*, *Gracilaria*, *Undaria* and *Fucus* genera (Wu *et al.*, 2012; Kowalczyk *et al.*, 2014; Kong *et al.*, 2015; Alves-Lima *et al.*, 2017; D. Gao *et al.*, 2018; J. Li *et al.*, 2019; Linardić and Braybrook, 2021). In the paper by Dong and colleagues (2012), the stability of candidate reference genes in *U. linza* was assessed in response to various abiotic stressors, including heat, light, salt, and drought. From the literature, however, it appears researchers working with other *Ulva* species often report the use of the 18S rRNA gene as an internal control, with primers (Tominaga *et al.*, 2010; Ichihara, Mineur and Shimada, 2011; X. Zhang *et al.*, 2012; Yang *et al.*, 2019). This is most likely due to the lack of genomic information available for green seaweeds until recently, which precluded more rigorous selection of reference genes. In fact, Dong and colleagues identified that within the experimental design tested for *U. linza* the 18S rRNA gene displayed relatively low stability compared to the other genes tested. Improper reference gene selection can change the biological interpretation of data, particularly when looking at a system where small fold changes in gene expression are expected. For example, in *Fucus distichus*, the statistical significance of salinity induced HSP90 upregulation was demonstrated to be lost when using ‘unstable’ reference genes to normalise data instead of experimentally validated genes (Linardić and Braybrook, 2021). For more accurate gene expression analysis, the stability of the intended reference genes must first be determined experimentally, as performed here.

The ability to perform more in-depth reference gene selection studies is now facilitated by the publication of the *U. mutabilis* genome. As such, this work provides a contribution to the literature by identifying candidate reference genes for researchers working with *U. compressa* (syn. *U. mutabilis*) that might inform reference gene selection in their own ‘in-house’ reference gene validation studies. The genes selected here represented typical ‘housekeeping genes’ (e.g., structural- and metabolism-associated genes) that are expected to be expressed constitutively in the cell and are not expected to be influenced by

stress. However, the ‘housekeeping gene’ nomenclature is now considered outdated, as tissue-specific variability in the expression of these genes is likely, as is disruption of normal cell physiology by stress (Kozera and Rapacz, 2013), hence the need to validate reference genes before qPCR analysis. The use of my selected candidates for normalising qPCR data, GAPDH, histones, tubulin (α and β), and actin proteins are widely reported in animal, plant, and algal models (Barber et al., 2005; Murthi et al., 2008; Li et al., 2011; Dong et al., 2012; Liu et al., 2012; D. Gao et al., 2018; Kang et al., 2018; Jin et al., 2019; Linardić and Braybrook, 2021). From my selection of candidate genes, all genes except α -tubulin were tested in the published *U. linza* reference study (Dong et al., 2012). This offers an (albeit limited) opportunity for comparison between *U. linza* and *U. compressa*, however, the nature of the stress experiments between the two studies was different.

Disagreement in ranking between reference gene selection algorithms are frequently reported in reference gene validation studies (Kianianmomeni and Hallmann, 2013; Ji et al., 2015; D. Gao et al., 2018; Jin et al., 2019). However, here, outcomes of the analysis for individual genes were generally comparable between the two methods. From the assessment of individual genes, GAPDH and actin were ranked as the top two most stable reference genes respectively by both methods. Both are classical ‘housekeeping genes’, with GAPDH functioning as a key glycolysis enzyme and actin being a key cytoskeleton protein. In some systems however (for example, mammals), the use of these genes is often not recommended – due to observed tissue-to-tissue variability, and the impact of certain stress conditions on gene expression (McCurdy, McGrath and Mackay-Sim, 2008; Brattelid et al., 2010). In the various brown and red seaweed species, actin and GAPDH expression is shown to be highly variable across multiple stress conditions, including metal exposure, hypersaline conditions, drought, and irradiance stress (le Bail et al., 2008; Kong et al., 2015; Alves-Lima et al., 2017; J. Li et al., 2019). Actin-associated genes were also demonstrated as unstable in virus-infected *Arabidopsis thaliana* (Lilly et al., 2011). Furthermore, these genes ranked poorly in the *U. linza* study across all stress conditions tested (Dong et al., 2012).

However, in other organisms and experimental designs these genes appeared suitably stable. For example, in *Fucus distichus*, GAPDH and actin expression appeared highly stable across multiple environmental stress conditions (Linardić and Braybrook, 2021). GAPDH and/or actin stability was also highly ranked across multiple experimental conditions in *Arabidopsis pumila* (Jin et al., 2019), the chlorophyte *Volvox carteri* (Kianianmomeni and Hallmann, 2013), and the red seaweed *Chondrus crispus* (Kowalczyk et al., 2014). In different model organisms, and different experimental designs, different genes may be appropriate,

hence the need for pre-validation. For example, differences in gene stability were observed in wild-collected and lab-cultivated *Chondrus crispus* (Kowalczyk *et al.*, 2014), and also in various algal species at different life phases (Wu *et al.*, 2012; Kianianmomeni and Hallmann, 2013; Kong *et al.*, 2015), and plants of different genotype (Andrade *et al.*, 2017). As such, comparisons between systems regarding gene stability are often inappropriate – and it is often recommended in the literature that the suitability of reference genes are assessed before new experimental conditions (or model organisms) are tested (Bustin *et al.*, 2009; Taylor *et al.*, 2019). For the purposes of the stress conditions tested in the present work, these two genes seem suitably stable.

Due to likely variability in gene expression observed across an experiment, even from conventional ‘housekeeping’ genes it is often recommended multiple reference genes are used to normalise data. As a result, GAPDH and actin were not used in isolation to normalise data. This is often a requirement for calculating relative gene expression, for example, the method employed in this chapter normalises gene expression data based upon the geometric mean of multiple reference genes (Vandesompele *et al.*, 2002). The use of multiple reference genes, and experimental determination of the correct number of reference genes needed to accurately analyse a dataset is also often a requirement for submitting qPCR data in high-impact journals (Bustin *et al.*, 2009). To date, most qPCR studies reported for *Ulva* tend to report the use of a sole reference gene to normalise data. With more genomic information now available, phycologists will find it easier to screen appropriate reference genes for a qPCR study and determine empirically how many are required for accurate data analysis. This is demonstrated in more recent qPCR analyses in *Ulva*, where the use of multiple reference genes is now cited (Blomme *et al.*, 2021).

Pairwise analysis using geNorm confirmed that two reference genes were sufficient to analyse the data generated in this chapter and provided GAPDH and α -tubulin as the most stable pair of genes to use in combination. For pairwise analysis, there was some disagreement between the Normfinder and geNorm methods– with the most stable gene pair determined by Normfinder to be GAPDH and histone 3, followed by β -tubulin and α -tubulin. Interestingly, two independent β -tubulin genes were deemed to be the most stable combination across multiple stress conditions in *Ulva linza* (Dong *et al.*, 2012), however, as previously stated, direct comparisons between different experimental setups are often flawed. As stated previously (see section 6.3.4), both the geNorm and Normfinder algorithms are widely cited in the literature for model organisms, as such despite their slight discrepancies the reference genes selected by either method appear suitable for qPCR data

analysis. Additionally, compared to other cited reference gene studies in the literature, the range in stability scores observed between the most and least stable genes was relatively low (0.034 for geNorm and 0.01 for Normfinder). This most likely indicates that all the genes tested have similar stability under the experimental conditions tested – and outcomes of data analysis are unlikely to be affected to a great extent if different combinations were selected. In this work the Normfinder outcomes were chosen (GAPDH and histone 3).

However, there are limitations to the study which share a commonality with other reference gene selection studies. Due to reagent and sampling limitations, there were other candidate genes that could not be tested, which may or may not have had better performance than the genes selected. For example, genes such as elongation factor 1- α , elongation factor 1- β , and polyubiquitin were highly stable in other intertidal seaweeds but were not tested here (Dong *et al.*, 2012; Kowalczyk *et al.*, 2014; Alves-Lima *et al.*, 2017; Linardić and Braybrook, 2021). Researchers may want to consider these genes as well when performing reference gene selection for other *Ulva* species and experimental conditions. The use of elongation factor 1- α and ubiquitin was recently reported for *U. mutabilis* mutants, but the purpose of the qPCR experiment was to verify the expression of a transgene, rather than to normalise data collected across multiple stress conditions (Blomme *et al.*, 2021). My study also did not consider the life cycle phase, as referenced occasionally in seaweed gene expression studies in the literature (Wu *et al.*, 2012; Kong *et al.*, 2015), as during my research, I worked with wild-collected tissue rather than tissue cultivated and propagated in the lab – as such distinguishing between sporophyte and gametophyte tissue was not possible. Additionally, attempts to induce sporulation and gametogenesis in *Ulva* did not yield enough material to facilitate molecular work (6.2), meaning that microscopic phases of the *Ulva* life cycle could not be assessed. Environmental stress may impact seaweed gametes and spores differently to mature tissue, as they occupy a distinct microhabitat, and the ratio of energy dedicated to stress tolerance as opposed to developmental processes will change with life cycle phase (Lichtenthaler, 1998; Kranner *et al.*, 2010). Furthermore, as previously stated, the reference genes here are only relevant for the stress conditions tested. More intense stress may induce more variability in the genes tested.

Finally, I did not consider time-dependent expression as a variable. In land plants it is reported that approximately 90% of the transcriptome is subject to diurnal oscillations (Michael *et al.*, 2008). For chronobiology (i.e., circadian) studies, where gene expression is compared at different points of the day a new set of reference genes may be required that only demonstrate minor diurnal variation (Jain, Vergish and Khurana, 2018). In the case of

seaweed, we hypothesise that a 'circatidal' influence has a major influence on physiology and development (Titlyanov, Titlyanova and Lüning, 1996; Lüning, Kadel and Pang, 2008; Wilcockson and Zhang, 2008; Tal *et al.*, 2011; Goto and Takekata, 2015). Regardless, the reference genes selected in this chapter were deemed appropriate for the intended 'dual stress' study.

6.5.3 Characterisation of Dual Stress Response Dynamics

In the final set of experiments in this thesis, I opted to assess the effects of different combinations of stress on *U. compressa* HSP expression for a fixed period of time rather than perform a conventional time-course study, to better resolve how different stress factors interact. Despite their universal role in the stress response, few reports document the role of molecular chaperones in *Ulva*. In *Ulva*, increased transcription, or abundance of cytoplasmic HSP70, HSP90, and HSP100 proteins occurred following various environmental stress conditions (Tominaga *et al.*, 2010; Fu *et al.*, 2011; Sung *et al.*, 2011; H. Zhang *et al.*, 2012; X. Zhang *et al.*, 2012; Fan *et al.*, 2017). The same was observed for a mitochondrial HSP60 and chloroplast localised HSP90C in *U. pertusa* and *U. profilera* (Tominaga *et al.*, 2012; Huan *et al.*, 2016). To date, no reports document qPCR of *Ulva* sHSP (HSP20) specifically apart from general transcriptomic studies (Contreras-Porcia, Dennett, et al., 2011), but these proteins have been shown to be stress-responsive in other algae, including the chlorophytes *Chlamydomonas reinhardtii* and *Tetraselmis suecica* (Lauritano et al., 2019; Sathasivam and Ki, 2019), and in the red seaweed *Pyropia yezoensis* (Uji et al., 2019), and have been identified as differentially expressed genes for various RNA sequencing studies in performed in *Ulva* (X. Zhang *et al.*, 2012; Fan *et al.*, 2017). Additionally, to my knowledge, no reports documenting the response of any *Ulva* molecular chaperone (or stress response) to dual abiotic stress, nor have expression profiles from different subfamilies and subcellular compartments been directly compared.

The most notable observations from this work were the synergistic effect of combined light and heat stress on gene expression for all the tested HSPs and p23, and the lack of response observed following sole dehydration stress. In the case of the HSPs predicted to have subcellular localisation, no significant increase in expression was observed under sole heat stress, either combined heat-light or heat-dehydration stress was required to induce a significant upregulation.

Light-induced induction of HSP expression is reported in the literature in algae, seaweeds, and plants, indicating that this response is likely conserved in the green lineage

(Kropat *et al.*, 1995, 1997; Fu *et al.*, 2011; Dickinson *et al.*, 2018; Theis *et al.*, 2020). In the case of intertidal seaweeds, light represents an environmental variable that is influenced by both photoperiod and tide cycle, with the intensity of light that seaweeds are exposed to varying with water depth (Leukart and Luning, 1994)

Light-induced induction of HSP expression is thought to occur via retrograde signalling from the chloroplast (e.g., ROS), which activates heat-shock factor (HSF), enabling it to bind to promoter elements and induce the appropriate stress responses (Miller and Mittler, 2006; Dickinson *et al.*, 2018). HSP70 is known to have both light-responsive, dehydration-responsive, and heat-responsive promoter elements, with the heat-responsive element (HSE) being successfully cloned in *U. prolifera* (Wu *et al.*, 2019). Interestingly, the authors note that dehydration-responsive elements were quite rare in algal HSP promoters, perhaps indicating that HSP expression does not comprise part of the cellular stress response to emersion. Notably, the reported responses of *Ulva* HSPs to emersion are variable in the literature, with HSP expression not always observed after emersion, or only being observable after extended periods of dehydration (Fu *et al.*, 2011; Sung *et al.*, 2011; H. Zhang *et al.*, 2012).

Due to the frequency of emersion, and the energetic cost of the stress response, it is likely that macroalgae use less energetically demanding ways of protecting the cells against desiccation stress, with the rehydration-dehydration cycle the major environmental factor in the *Ulva* life cycle. Cited examples for how this may occur include the flexibility of the cell wall under dehydration stress as discussed by Holzinger (2015), and the biosynthesis of osmoprotectants that maintain intracellular homeostasis for extended periods of time when seaweeds are emersed, both of which may negate the requirement for a comprehensive stress response at low tide (Gupta and Kushwaha, 2017). Water retention is also determined by morphology, with filamentous species having a lower surface area compared to 'blade type' *Ulva*, meaning rates of water loss will be lower. Filamentous *Ulva* are reported as having remarkable levels of stress tolerance, including to heat shock and dehydration (Choo, Snoeijs and Pedersén, 2004; Luo and Liu, 2011; Carl, de Nys and Paul, 2014; Holzinger *et al.*, 2015), being identified more frequently in the upper intertidal zone and in more diverse habitats than *Ulva* with 'sea lettuce' morphology (Einav and Israel, 2007; Ichihara, Mineur and Shimada, 2011; Carl *et al.*, 2014; Flores-Molina *et al.*, 2014b).

Whilst HSPs are characterised for their role in the 'heat shock' response, the mechanism behind their activation is based upon a general response to the disruption of protein homeostasis (i.e., aggregation and denaturation). Whilst severe desiccation will

induce protein aggregation (Chakrabortee *et al.*, 2007; Holzinger and Karsten, 2013; López-Cristoffanini *et al.*, 2015), as demonstrated by significant upregulation in HSP expression observed by the dual heat-dehydration experiment, it does not appear that the short-term dehydration performed here does, consistent with the ability to maintain osmotic homeostasis for extended periods of time until high tide returns. Interestingly, a 'circatidal clock' is hypothesised to synchronise the biological activity of marine invertebrates to the tide cycle (Takekata *et al.*, 2012, 2020; Zhang *et al.*, 2013; Cheeseman, Fewster and Walker, 2017; Satoh and Terai, 2019). With the stress responses of land plants known to be under circadian control, including HSP expression, one unexplored area of research is whether the endogenous clock of seaweeds synchronises mechanisms of stress tolerance to the tide cycle rather than the photoperiod (as in plants), with the tide cycle the predominant environmental factor. To date, the endogenous clock has not been reported for any intertidal alga. However, the reproductive cycle of seaweeds is known to be synchronised with tidal and lunar events, for example the release of swarmer in intertidal *Ulva* populations predominantly occurs at spring tide, when the tidal range is greatest (Lüning, Kadel and Pang, 2008). Additionally, semi-lunar rhythms of the content of individual metabolites in *Ulva* have been characterised (Tal *et al.*, 2011). Therefore, it is likely that there is some molecular mechanism in *Ulva* that synchronises physiology to environmental conditions.

The observed heat-light interaction was not replicated to statistical significance in the repeat study. However, it must be noted that collections were performed in different months. As a result, seasonal and biological variation is likely a factor in the discrepancy. Seasonal variation in the observed stress responses are reported in various organisms (Manitašević *et al.*, 2007; Singh *et al.*, 2021). Here, differences in light exposure between the different months may explain the observed differences. However, this will need to be verified experimentally to be conclusive.

Overall, the profile of what stresses were responded to did not differ too much between the different genes, with the localised responses appearing more subtle, most notably the lack of a response to sole heat stress. This may indicate that the genes predicted to be localised have a higher threshold for induction, or have low expression under the tested conditions. More intense or bespoke types of stress may induce stronger responses in the mitochondria and chloroplast. For example, the mitochondria can be inhibited specifically by various compounds in cellular research (Sako *et al.*, 2020). Light should induce chloroplast stress (Kropat *et al.*, 1995; Mullineaux *et al.*, 2018), although as *Ulva* has notable tolerance to variable light conditions (Murthy *et al.*, 1987; Guan *et al.*, 2016; Y. Wang

et al., 2016), higher light intensities may be required to induce a more robust response. Finally, it was somewhat surprising that a stronger response wasn't observed for sole heat shock for the endoplasmic reticulum localised HSP70, due to the role of BiP in the unfolded protein response (Mittler, Finka and Goloubinoff, 2012; Cho and Kanehara, 2017) .

For this reason, it appears adding a time course variable to such dual stress experiments will be important for understanding the dynamics of the *Ulva* stress response further. This will help us understand whether *Ulva* responds intensely in the short term to stress and stress responses 'tail off' under extended stress, or whether stress responses are maintained under prolonged stress (Lichtenthaler, 1998). This will also provide ecological context for determining whether *Ulva* responds differently to predictable stress events in the tide zone (i.e., immersion) to unpredictable stress events such as heat shock. Combining gene expression analysis with a proteomic approach will help us map out how *Ulva* maintains protein homeostasis with tide (Fan *et al.*, 2018).

Despite this, the localisation of these proteins needs to be confirmed by the fluorescence-tagging methods described by Blomme and colleagues (2021). This work nonetheless provides a good platform for future investigations. As gene expression studies have only recently been applied to seaweed research, experiments such as the ones described in this thesis provide important information about the fundamental biological processes of seaweeds. Establishing 'seaweed specific' knowledge of stress tolerance and physiology, and how it differs from land plants and microalgae will help us identify 'seaweed specific' processes that will enable more comprehensive transcriptomic and proteomic studies to be carried out in these organisms in the future.

6.5.4 Conclusions

Here, a protocol for performing gene expression analysis in *U. compressa* was established successfully, with the objectives outlined at the beginning of the chapter all successfully achieved (6.1.2), except for the successful isolation of gametes from *Ulva* to facilitate transformation experiments.

- An effective sampling protocol has been established for gene expression studies
- The outcomes of model selection ensure accurate quantification of the qPCR data generated in this chapter
- The outcomes of the reference gene selection study are a contribution to the current literature; researchers can use gene stability data from this study to help them inform decisions about the best-suited genes to screen in their own qPCR studies.
- The dynamics of molecular chaperone gene expression in *U. compressa* have been characterised in response to various abiotic stressors, creating hypotheses for follow-up research.

The development of new tools for molecular phycology now enables more comprehensive investigations into *Ulva* physiology, development, and stress tolerance, all of which will facilitate the improvement of *Ulva* as a bioenergy crop and help identify mechanisms of stress tolerance in seaweeds that could possibly be engineered into land plants to generate more 'hardy' land crops.

Here, the demonstration of a concise gene expression study in *Ulva* in response to various abiotic stressors offers a novel insight into how *Ulva* regulates stress responses to simultaneous environmental stressors in the intertidal zone, using the highly conserved HSP family. With further genomic investigations, seaweed-specific processes may be identifiable to be tested for future gene expression and transcriptomic studies. This, combined with the ability to propagate *Ulva* cultures in the lab will also enable comparison of individuals at different stages of the life cycle, to better understand the genetic basis of morphogenesis and early seaweed development.

7. Discussion

7.1 Thesis Outcomes

7.1.1 An Improved Model of the *Ulva* Cell Wall

No 'Phycolignin' in the *Ulva* Cell Wall

At the outset of this thesis, I defined my primary aim as identifying and characterising the structure of the 'lignin-like' fraction of the *Ulva* cell wall (Chapter 1, §1.5). The presence of a 'phycolignin' structure in *Ulva* had been proposed in the literature but not definitively characterised (Yaich *et al.*, 2011, 2015; Gupta *et al.*, 2013; Fernandes *et al.*, 2019; Moral *et al.*, 2019), as performed for the coralline red seaweed *Calliarthron* and charophytes (Martone *et al.*, 2009; Sørensen *et al.*, 2011).

To do so, I used a 'non-invasive' biophysical analysis rather than other frequently cited 'destructive' methods such as the gravimetric Klason lignin approach and mass-spectrometry via DFRC, which I suspected would generate too many artefacts to provide conclusive evidence as observed in other biomass sources with 'low lignin' content (Bunzel, Seiler and Steinhart, 2005; Schäfer *et al.*, 2015). In particular, a lot of the evidence for 'phycolignin' in *Ulva* has been provided by the Klason lignin and acid-detergent lignin methods (Yaich *et al.*, 2011; Yoza and Masutani, 2013; Li *et al.*, 2016; Moral *et al.*, 2019), which is known to overestimate lignin content with certain types of biomass. For example, an overestimation of lignin content is observed by the Klason method in leaf tissue relative to wood tissue due to the presence of 'non-cell wall' associated components in biomass (Toda *et al.*, 2015). Furthermore, the presence of protein in cell wall extract has been demonstrated experimentally to cause Klason lignin overestimations (Kim *et al.*, 2017), consistent with the identification of proteinaceous cell wall in this thesis for *Ulva*. However, no steps to limit interference during this approach are reported with *Ulva*; hence I conclude that these previous observations are inaccurate.

As expected, attempts to use derivatisation-based methods in this thesis (i.e., acetyl bromide soluble lignin assay) had extensive interference and, as such, were uninterpretable. Other methods of analysis that were considered but not trialled in the search for 'phycolignin' in this thesis included immunolabelling techniques that could have provided supporting *in vivo* evidence as performed for *Calliarthron* and *Coleochaeteles* (Martone *et al.*, 2009; Sørensen *et al.*, 2011), as would fluorescence lifetime-imaging (FLIM) of the *Ulva* cell wall autofluorescence in Chapter 3 to better characterise

the dynamics of the observed stress-induced changes (Donaldson and Radotic, 2013; Chacko and Eliceiri, 2019). However, neither of these approaches provides high-resolution structural information, as observed in combined FTIR and NMR analyses.

Nonetheless, the outcomes of the investigations outlined in this thesis were not consistent with a 'lignin-like' fraction being associated with the *Ulva* cell wall. The biochemical verification of a 'lignin-free' cell wall in *Ulva*, therefore, is a new contribution to the literature, supplementing the latest genomic information we have for various macroalgae (De Clerck et al., 2018; Nishiyama et al., 2018; Arimoto et al., 2019; S. Wang et al., 2020; He et al., 2021), that indicates that the biosynthetic pathways required to synthesise phenylpropanoids are absent outside the streptophyte lineage (De Vries et al., 2017, 2021). This may have implications for understanding how multicellularity evolved independently multiple times in diverse algae, and in land plants, with phenylpropanoid biosynthesis and lignification considered key adaptations that contributed to the success and increased complexity of plants on land (Umen, 2014; Coates, Umm-E-Aiman and Charrier, 2015; Fürst-Jansen, De Vries and De Vries, 2020).

In terrestrial habitats, lignin provides structural support to an organism, and the development of vascular tissue enables water supply in a water-depleted environment (Bhardwaj et al., 2014). The requirement for vascular tissue is unnecessary for the primarily marine intertidal seaweeds, which absorb water throughout the whole structure when submerged. However, it is likely that some degree of structural support is required to withstand the dynamic variations in the environmental conditions they are exposed to, particularly the hydrodynamic forces experienced when submerged (Boller and Carrington, 2006). Interestingly, seagrasses, a group of monocots that reverted to the marine ecosystem after plants had colonised land, have a notably lower lignin content than their terrestrial counterparts, consistent with a reduced need for this polymer in an aquatic environment (Pfeifer and Classen, 2020).

Concerning the literature, the presence of 'phycolignin' in the streptophyte algae (i.e., the charophytes) is explainable due to the identification of a proto-phenylpropanoid pathway in several representatives, with these algae being the most closely related algal lineage to land plants, diverging before the terrestrialisation of plants (Domozych, Popper and Sørensen, 2016; De Vries and Archibald, 2018; Fürst-Jansen, De Vries and De Vries, 2020). Furthermore, lower plants (i.e., the bryophytes) have lignin-like material in their cell walls (Renault et al., 2017; Davies et al., 2020). Here,

a clear path defining the evolution of phenylpropanoid biosynthesis and lignification is evident from the aquatic ancestors of land plants to vascular plants.

However, the presence of lignin in the divergent red seaweed *Calliarthron* is more challenging to explain, as, during my research, no evidence could be provided of an equivalent structure in another red seaweed *Mastocarpus stellatus*. The lack of a 'lignin-like' fraction being present in *M. stellatus* is also supported by prior evidence in the literature, using various biochemical tests such as those described in chapter 3 (Gómez-Ordóñez, Jiménez-Escrig and Rupérez, 2010; Espiñeira *et al.*, 2011). As with the chlorophyte seaweeds, the red rhodophyte seaweeds are also highly diverse, with the majority of red seaweeds being classified as part of the diverse Florideophyceae group, which includes the calcified coralline algae (including *Calliarthron*) as well as carrageenan producing algae such as *Chondrus*, *Kappaphycus*, and *Mastocarpus* (Yoon, Zuccarello and Bhattacharya, 2010; Rosas-Alquicira *et al.*, 2011).

From a molecular perspective, recent transcriptomic and genomic analysis of *Calliarthron tuberculatum* has confirmed that the mechanism of lignin biosynthesis in coralline algae is ammonia-lyase independent, with no phenylalanine-ammonia lyase (PAL), tyrosine-ammonia lyase (TAL), or phenylalanine/tyrosine-ammonia lyase (PTAL) being identified (Xue *et al.*, 2022). Whilst a selection of other phenylpropanoid-associated genes were identified in this publication, no alternative pathway for the fundamental step of phenylpropanoid biosynthesis (i.e., the deamination of an aromatic amino acid to coumaric acid) in this alga was determined in the absence of PAL.

Furthermore, a proportion of these genes are also identified in chlorophyte genomes (Labeeuw *et al.*, 2015; De Vries *et al.*, 2017, 2021), indicating that lignin in *Calliarthron* is synthesised by an independent mechanism to that characterised in plants, as despite the presence of these genes, lignin is apparently not synthesised by these species. Unfortunately, no *Mastocarpus* genome is currently available for cross-reference, but in the closely related *Chondrus crispus* genome, a subset of traditional phenylpropanoid-associated genes are also present, whilst genes that encode ammonia-lyases are not (Collén *et al.*, 2013; Labeeuw *et al.*, 2015). However, whilst the observation that phenylpropanoids and lignin can still be synthesised in PAL mutants could be an artefact of 'leaky expression', it could possibly indicate that there are aspects to this process in plants that are still not fully understood, which may have implications for the presence of this structure in seaweed (Rohde *et al.*, 2004; Huang *et al.*, 2010; Cass *et al.*, 2015).

As a result, more recent evolutionary events such as horizontal gene transfer into different lineages may explain the differences observed in the apparent ability to synthesise lignin and phenylpropanoids between other algae, rather than lignin biosynthesis being a conserved mechanism from an ancestral alga, which has since been lost in the various algal groups (Labeeuw *et al.*, 2015). This is consistent with how the phenylpropanoid pathway was hypothesised to originate in plants, with PAL thought to have been transferred to the ancestor of modern plants by a horizontal gene transfer event, but it is unclear whether this was pre- or post-transition to land via an aquatic or terrestrial soil bacterium (Emiliani *et al.*, 2009). The observation that PAL and other essential components of the phenylpropanoid pathway are absent from some charophyte genomes such as *Penium margaritaceum* but present in others such as *Klebsormidium flaccidum* and *Chara braunii* is perhaps indicative of an aquatic origin (Hori *et al.*, 2014; Nishiyama *et al.*, 2018; Jiao *et al.*, 2020). A similar hypothesis is proposed for the presence of flavonoid biosynthesis in algae, which is also PAL-dependent (Goiris *et al.*, 2014; Wen, Alseekh and Fernie, 2020).

Based upon the available genomic information, we cannot yet establish why *Calliarthron* can synthesise lignin, but other seaweed species apparently cannot, as there appear to be no major differences in biosynthetic capabilities between the different genomes. However, we can perhaps hypothesise why *Calliarthron* needs to synthesise lignin, but other seaweeds do not from an ecological perspective. Therefore, whether coralline algae were subjected to different evolutionary pressures to the other characterised seaweed lineages that would require a 'phycolignin' in an aquatic environment must be considered.

Here, the observation of lignin in coralline algae specifically may be a function of different organism complexity rather than significant differences in metabolic machinery between algae. The geniculate coralline algae such as *Calliarthron* have a relatively complex morphology relative to *Ulva* and *Mastocarpus*, consisting of an inflexible, rigid, calcified structure with a 'stem-like' stipe formed of numerous segments, in addition to a series of branched appendages resembling leaves. Within a cross-section of this stipe, we observe clear differentiation of layers, consisting of distinct primary and secondary cell walls, analogous to vascular tissue in plants (Martone and Denny, 2008; Martone *et al.*, 2019).

By contrast, 'blade-like' seaweeds such as *Ulva* and *Mastocarpus* have remarkably low differentiation of tissue, which for *Ulva* comprises blade tissue constructed as a

bilayer of cells and a small holdfast, whilst the blade morphology *Mastocarpus* is slightly more complex, consisting of numerous flattened fronds protruding from a stipe. These species also lack a distinction between primary and secondary cell walls. The more flexible structure of these seaweeds is hypothesised to enable them to withstand mechanical stress from the variable water motion experienced whilst emersed, which is impacted by wave-action and local environmental conditions (Carrington, 1990; Boller and Carrington, 2006). This flexibility allows macroalgae to move freely with the flow of water, preventing physical damage to the alga from hydrodynamic forces (Vogel, 1984; Denny and Gaylord, 2002).

The rigidity of coralline algae appears as an alternative yet equally successful approach to tolerating intertidal stress, for which flexibility is conferred by 'joints' between the different segments of the stipe, which allow it to 'bend' with flowing water (Martone and Denny, 2008). In general, intertidal organisms either exhibit high rigidity or extreme levels of flexibility, which both appear as alternative strategies to the same environmental problem (Koehl, 1984).

These differences in morphology and lignin content between coralline and flexible 'blade-type' seaweeds might parallel the differences in lignin content observed in different components of a vascular plant, where a lower lignin content is observed in leaf tissue than vascular tissue, with a higher lignin content required in the vasculature tissue for structural rigidity and water conductance, whereas the same rigidity is not needed for the functions performed in the blade of a leaf (Brinkmann, Blaschke and Polle, 2002; Jin *et al.*, 2003). As a result, the strategy of 'rigidity' for stress tolerance in *Calliarthron* likely required the convergent biosynthesis of lignin, whereas, in the flexible *Ulva*, it did not. Furthermore, I speculate that a more 'malleable' cell wall may be preferable within intertidal habitats due to the extreme osmotic fluctuations they experience through the tide cycle. Whilst lignin deposition would protect the cell during rehydration (or hypo-osmotic conditions), the 'rigidity' of cell walls would need to be reversible on a semi-diurnal basis as seaweeds are dehydrated at low tide. Notably, seaweeds such as *Ulva* and *Mastocarpus* are typically located in the mid to upper intertidal zone, whereas coralline algae are found lower down (Gómez and Huovinen, 2011; Kokabi *et al.*, 2016). As a result, coralline algae likely populate a slightly more stable environment which does not require this flexibility.

The flexibility of the *Ulva* cell wall in response to dehydration-rehydration cycles has been demonstrated to be in part attributed to the remodelling of the amorphous

ulvan matrix, which moves with the retracting plasma membrane during desiccation to prevent plasmolysis, and retracts whilst cells are hydrated (Holzinger *et al.*, 2015). Lignin deposition will likely hinder this without an *in vivo* mechanism to depolymerise. Both of these would be energetically costly in a dynamic environment such as the intertidal zone, and lignification is largely considered an irreversible process that can only be degraded 'naturally' by fungal and microbial enzymes (Del Valle and Stanley, 1995; Zhao and Dixon, 2011; Janusz *et al.*, 2017), which contradicts the reversible changes in *Ulva* autofluorescence observed *in vivo*.

Nonetheless, assessment of a more diverse range of macroalgae may help resolve some of these questions, with genomic information now available for a broader range of species, including the freshwater charophytes and intertidal chlorophytes such as *Caulerpa*. The diverse cell walls of chlorophytes make them particularly interesting models for this purpose. For example, the absence of cellulose in the siphonous Bryopsidales representatives (e.g., *Caulerpa* and *Codium*) and the calcification of some green seaweeds such as *Halimeda* in an analogous manner to coralline species may impact whether phycolignin is present or absent (Domozych *et al.*, 2012; Vogel *et al.*, 2015). 'Lignin-like' structures are also hypothesised to be present in the furoid algae such as *Sargassum*. However, as with the chlorophytes, there are no conclusive reports to date to confirm this (Alzate-Gaviria *et al.*, 2021).

NMR of Cell Wall Proteins

As an alternative, this thesis proposes that cell wall proteins are a major structural component of the *Ulva* cell wall; however, further analysis is needed to identify the nature of these proteins and how they are associated with the *Ulva* cell wall. This has been determined by a combination of elemental analysis, FTIR, and NMR analyses which are all consistent with amine and amide-associated structures being associated in some manner with the extracted cell wall fractions.

A wealth of NMR data by multidimensional techniques has been generated during this thesis. Whilst an initial analysis of this data has been performed, in practice, these experiments are highly complicated, especially in complex biopolymers. Continued investigation of this data set may yield further insights into the structure of extracted cell wall polymers. It will require collaboration with chemists with extensive expertise in high-resolution NMR analysis. This will likely be performed in subsequent research projects based on this thesis's outcomes.

Despite the complexity of the applied technique, I still propose that NMR is an attractive 'non-invasive' approach for characterising cell wall structure in *Ulva* as opposed to conventional protein analysis techniques. The ability to study complex biopolymer mixes relatively undisturbed compared to methods such as mass spectrometry remains an advantage despite the compromise of sensitivity, as we can assess how different structures are associated with each other by characterisation of the corresponding linkages between polymers (Yuan *et al.*, 2011; Frueh, 2014; Ma, Dang and Xu, 2016; W. Wang *et al.*, 2020). Furthermore, it allows analysis of 'unextractable' proteins that are tightly associated with the cell wall, with the observations in this thesis consistent with two populations of cell wall proteins, one comprising ionically bound proteins associated with the amorphous component of the cell wall (i.e., ulvans), and another consistent with structural proteins that are strongly associated, most likely by covalent linkages. This is consistent with what is observed in the plant cell wall, with cell wall glycoproteins known to interact with pectin and hemicellulose either by adsorption or through the formation of covalent linkages (Tan *et al.*, 2013). Interestingly, the fraction that appeared most strongly associated with protein in *Ulva* here was the fibrillar component α -cellulose. It is unclear whether this is due to co-extraction of 'hemicelluloses' or whether this represents a unique interaction, warranting further investigation. The sensitivity of NMR for these investigations will likely be enhanced by the isotopic labelling methodologies proposed in chapter 5, particularly ^{15}N labelling to facilitate protein NMR experiments, with bespoke ^{15}N labelling protocols recently reported for seaweed (Legrand, Martin, et al., 2018; Bailes and Gröcke, 2020).

Overall, the cell wall proteins of *Ulva* are understudied, and their role uncharacterised, however arabinogalactan-like (AGP) proteins have recently been characterised in *Ulva lactuca*, as have corresponding genes in the *U. mutabilis* genome (De Clerck et al., 2018; Přerovská et al., 2021). Additionally, AGP-like and other hydroxyproline-rich glycoproteins (HRGPs) are reported in lower chlorophytes cell walls, particularly in the volvocine algae such as *Chlamydomonas* and *Volvox* for which the cell wall is composed almost entirely out of protein (Voigt and Frank, 2003; Hallmann, 2006; Cronmiller *et al.*, 2019). As such, it appears that the observed cell wall protein observed here may reflect the same component, especially as these proteins are characterised as generally insoluble once they cross-link cell walls (Cooper and Varner, 1984; Fritz, Hood and Hood, 1991; Basavaraju *et al.*, 2009; Chen *et al.*, 2015), consistent with the segregation of protein into the insoluble fraction as observed in this thesis

(Chapter 4). However, confirmation of amino acid analysis will be necessary for confirmation.

As stated, whilst seagrasses have a low lignin content, a series of uronic acid-rich HRGPs have recently been characterised in the cell wall of *Zostera marina*, which may indicate that these proteins have a role in the adaptation to the marine ecosystem (Pfeifer *et al.*, 2020). In various terrestrial plant models, HRGPs have been demonstrated to be important components of tolerance to both osmotic and saline stress, with the cross-linking activity exhibited by various HRGP representatives thought to modulate cell wall porosity in response to stress (Lamport, Kieliszewski and Showalter, 2006; Yang *et al.*, 2010, 2011; Zagorchev and Odjakova, 2011; Yang, Rao and Horst, 2013; Chen *et al.*, 2014). HRGPs may also stabilise the plasma membrane during these stresses at the 'anchor side' (Lamport, Kieliszewski and Showalter, 2006). A similar mechanism for cell wall remodelling is likely to be important in *Ulva*, as described previously by the observed ulvan remodelling during desiccation (Holzinger *et al.*, 2015), due to the dynamic conditions of the intertidal zone. One worthwhile follow-up to this project would be to assess whether the observed autofluorescence changes observed in the *Ulva* cell wall during this thesis (Chapter 2, §3.3.2) can be correlated with cell wall proteins, with the dynamic changes in cell wall fluorescence observed under hypersaline and hyposaline conditions possibly reflective of cell wall remodelling.

7.1.2 A General Model for Intertidal Stress Tolerance

As with investigations into cell wall proteins, a proteomic approach may also help resolve our understanding of the cellular stress response in the intertidal zone, as assessed by the gene expression study in my final research chapter. Here, the profile of heat shock protein expression provided insight into how protein homeostasis is maintained in *Ulva* during various combinations of environmental stress.

One understudied aspect of seaweed physiology is understanding how seaweeds regulate the proteome throughout the tide cycle. In addition to chaperone activity via heat shock proteins, ubiquitination, SUMOylation, and proteasome activity comprise key components of the machinery required to maintain protein homeostasis and are important areas of research in modern plant biology (Vallentine *et al.*, 2014; Morrell and Sadanandom, 2019; Xie *et al.*, 2019). However, none of these processes have been studied significantly in seaweeds. The unique environmental conditions of the intertidal zone relative to terrestrial habitats may mean seaweeds have unique regulatory networks for

stress tolerance, which may be of interest to researchers looking to engineer more stress-tolerant land crops.

In plants, the cellular stress response and protein homeostasis machinery (i.e., HSP expression) is known to be under significant circadian control (Cha *et al.*, 2017; Gil *et al.*, 2017; Blair *et al.*, 2019; Mody, Bonnot and Nagel, 2020). As stress responses are energetically costly, synchronisation of stress responses to the day-night cycle via the circadian clock enables plants to anticipate when environmental stress is likely, improving their ability to withstand environmental fluctuations. The circadian oscillator from microalgal chlorophytes such as *Ostreococcus* and *Chlamydomonas* has been studied extensively (Djouani-Tahri *et al.*, 2011; Thommen *et al.*, 2012; McClung, 2013; Matsuo *et al.*, 2020), as has the equivalent mechanism in model plants such as *Arabidopsis thaliana* (Hsu and Harmer, 2014; Shalit-Kaneh *et al.*, 2018). By comparison, our current understanding of seaweed circadian biology is limited.

One research gap for intertidal seaweeds, with the tide being the primary environmental pressure, is whether intertidal seaweeds exhibit rhythmicity to 'predict' the semi-diurnal emersion-immersion cycle or whether the endogenous clock synchronises physiology to photoperiod as observed in plants. In various intertidal invertebrates, the presence of a 'circatidal' clock is hypothesised (Wilcockson and Zhang, 2008; Takekata *et al.*, 2012, 2014, 2020; Zhang *et al.*, 2013; Cheeseman, Fewster and Walker, 2017; Satoh and Terai, 2019; Tran *et al.*, 2020). However, our understanding of the molecular mechanism behind this is limited. Additionally, there are no studies that report an extensive investigation into the molecular basis of chronobiology of seaweeds, despite there being several biological processes in seaweeds are known to be synchronised to tidal and semilunar events, indicating that an endogenous clock may have an important role in seaweed survival (Titlyanov, Titlyanova and Lüning, 1996; Lüning, Kadel and Pang, 2008; Tal *et al.*, 2011). However, to facilitate this approach, some method development would be required, with only a few proteomic and transcriptomic studies documented for green seaweeds in the literature (Fan *et al.*, 2017, 2018; Y. Wang *et al.*, 2019).

7.2 New Genetic Tools for 'Molecular Phycology'

One barrier to performing more comprehensive investigations in this thesis was the inability to perform the genetic transformation in *Ulva*. One desired approach during this research was to verify the identified cell wall modifications with a combined biochemical and molecular approach. For example, homologs to genes associated with O-acetylation in land plants have been identified in the *U. mutabilis* genome (De Clerck *et al.*, 2018) by our research group, including the reduced wall acetylation gene (*RWA*) and altered xyloglucan 9 (*AXY9*) (Pauly and Ramírez, 2018). By modulating the expression of this gene in *Ulva* and assessing the corresponding FTIR spectra of the extracted ulvans, it could be identified whether the peak attributed to 'esters' corresponds to this modification. This approach has been applied to plants to characterise cell wall structure in various genotypes and mutants (Mazurek *et al.*, 2013; Dinant *et al.*, 2019).

Compared to microalgal chlorophytes such as *Chlamydomonas reinhardtii*, for which a comprehensive molecular toolkit has been established, no equivalent amount of method development has been performed to develop genetic tools in macroalgae (Zhang, Wang and Wang, 2021). A series of genetic tools have been developed for microalgae specifically, as due to their low complexity, ease of manipulation, and fast growth rates, they function as effective proxies for studies of fundamental biological processes in plants (Couso *et al.*, 2012; Erickson, Wakao and Niyogi, 2015; Schroda, Hemme and Mühlhaus, 2015; Müller *et al.*, 2017). By comparison, macroalgae are challenging organisms to work with due to their diverse morphologies and robust extracellular matrix, which forms a direct barrier to the introduction of exogenous material.

However, since the outset of my project, various research groups have been working to establish reproducible genetic manipulation techniques for *Ulva* to facilitate a range of development studies. So far, successful 'gain of function' transgene expression has been reported for *U. mutabilis* gametes using the frequently cited GoldenGate cloning system by Blomme and colleagues (2021), with this work being a direct continuation of the previously developed protocols for purifying and transforming macroalgal gametes (Spoerner *et al.*, 2012; Oertel, Wichard and Weissgerber, 2015). A direct application for this protocol for my thesis would be confirmation of the localisation of the *U. mutabilis* heat-shock proteins in chapter 6, with authors reporting

successful introduction and imaging of transgenes tagged with yellow-fluorescent protein (YFP).

However, this method requires the ability to reliably induce differentiation in *Ulva*, which, as described in this thesis, is sometimes challenging for 'wild-collected' algae. Furthermore, large amounts of biomass may be required for biochemical analyses, which may not be obtainable from small mutant lines prepared *in vitro*. This was the case for the cell wall fractionation protocol described in this thesis. Therefore, the ability to induce transient gene expression in mature tissue may be more appropriate for certain studies where a combined molecular and biochemical approach is desired.

Agrobacterium-mediated transformation is reported for the red seaweed *Chondrus crispus* (Ramessur *et al.*, 2018), as is the gene gun approach in *Ulva prolifera* (Wu *et al.*, 2018, 2019). During my research projects, *Agrobacterium*-mediated transformation and a novel nanoparticle-mediated transformation technique which has been used to successfully introduce plasmids into mature plant tissue (Doyle *et al.*, 2019). However, due to time constraints, these avenues were not pursued further. Nevertheless, due to the success of these methods for complex algal and plant tissue, they will likely work for *Ulva* with further optimisation.

By contrast, reports of loss-of-function experiments are scarce for macroalgae; however, mutations were successful induced in *U. prolifera* via a (CRISPR)-Cas9 experiment (Ichihara, Yamazaki and Kawano, 2022). As a result, 'molecular phycology' appears as an exciting emerging field, with these recent developments likely to enable more comprehensive investigations into diverse algae over the next few years.

7.3 Concluding Remarks

The outcomes for this thesis provide a platform for future biophysical and proteomic investigations in green seaweeds, where I have proposed an updated model of the *Ulva* cell wall and assessed the dynamics of the cellular stress response to various combinations of environmental stress. Further investigation will improve our understanding of how these species tolerate highly variable environmental conditions in the intertidal zone and help decipher the evolutionary basis for multicellularity in diverse habitats. This improved understanding of seaweed biology will also enhance our ability to exploit it economically as a bioenergy crop.

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Appendices

Appendix I

<i>Family</i>	<i>ID</i>	<i>Isoelectric Point</i>	<i>Size (KDa)</i>	<i>Protein (AA)</i>	<i>CDS</i>	<i>Introns</i>	<i>Exons</i>
SHSP	UM001_0090	6.92	19.16	181	541	1	2
SHSP	UM013_0157	6.12	18.76	180	538	1	2
SHSP	UM028_0009	8.05	22.23	202	604	1	2
SHSP	UM058_0031	5.63	19.44	181	541	1	2
SHSP	UM105_0001	8.02	51.44	475	1417	7	8
SHSP	UM060_0017	7.69	57.91	536	1601	6	7
SHSP	UM001_0093	5.65	21.19	198	592	1	2
SHSP	UM001_0095	6.51	27.14	255	763	1	2
SHSP	UM001_0098	6.76	17.24	165	494	0	1
SHSP	UM001_0099	6.01	18.78	172	514	1	2
SHSP	UM001_0104	8.52	21.12	198	592	1	2
SHSP	UM001_0127	8.92	15.46	146	436	1	2
SHSP	UM003_0083	7.63	23.37	216	646	1	2
SHSP	UM005_0210	6.12	15.25	138	411	2	3
HSP33	UM030_0028	4.81	34.67	331	988	4	5
CPN60	UM001_0140	5.11	57.12	541	1615	7	8
CPN60	UM015_0132	6.37	60.66	558	1666	7	8
CPN60	UM026_0043	6.57	57.86	536	1602	5	6
CPN60	UM038_0110	4.97	59.07	537	1605	5	6
CPN60	UM042_0109	5.21	59.28	552	1653	2	3
CPN60	UM116_0035	8.46	58.87	541	1616	6	7
CPN60	UM002_0158	6.70	60.42	557	1665	5	6
CPN60	UM002_0236	4.86	62.82	597	1785	5	6
CPN60	UM003_0101	5.86	57.42	535	1598	6	7
CPN60	UM004_0333	4.77	62.11	587	1754	6	7
CPN60	UM010_0035	4.75	61.79	585	1748	6	7
CPN60	UM011_0180	4.76	61.05	576	1723	4	5
HSP70	UM001_0412	5.15	97.19	886	2649	8	9
HSP70	UM039_0067	5.14	70.32	655	1955	9	10
HSP70	UM127_0015	4.68	54.72	502	1503	2	3
HSP70	UM004_0183	9.24	62.12	561	1677	5	6
HSP70	UM004_0334	4.67	72.71	681	2038	4	5
HSP70	UM012_0035	4.72	75.46	683	2041	7	8
HSP70	UM012_0036	4.72	72.35	667	1993	7	8
HSP70	UM013_0190	4.77	70.88	655	1959	5	6
HSP70	UM023_0131	4.37	57.59	536	1603	4	5
HSP70	UM028_0130	6.11	58.75	541	1620	2	3
HSP90	UM012_0034	4.53	89.96	801	2392	10	11

HSP90	UM019_0113	4.74	85.71	764	2283	8	9
HSP90	UM060_0018	4.53	80.85	704	2106	5	6
HSP100	UM001_0159	6.06	155.84	1388	4146	17	18
HSP100	UM001_0583	8.18	85.03	820	2452	7	8
HSP100	UM009_0119	6.46	72.85	711	2123	9	10
HSP100	UM060_0114	9.10	99.21	922	2753	12	13
HSP100	UM065_0021	6.53	88.08	799	2394	2	3
HSP100	UM035_0089	6.12	99.78	906	2711	6	7
HSP100	UM065_0020	10.41	16.48	150	448	1	2

Appendix I. Heat shock proteins identified in the *U. mutabilis* (syn. *U. compressa*) genome accessed via the ORCAE database (Sterck *et al.* , 2012; De Clerck *et al.* , 2018).

Appendix II

<i>Family</i>	<i>ID</i>	<i>DeepLoc</i>		<i>BUSCA</i>		<i>PREDALGO</i>	
<i>SHSP</i>	UM001_0090	Cytoplasm	0.54	Nucleus	1.00	Chloroplast	0.82
<i>SHSP</i>	UM013_0157	Nucleus	0.53	Nucleus	1.00	Chloroplast	1.01
<i>SHSP</i>	UM028_0009	Nucleus	0.34	Chloroplast	1.00	Chloroplast	2.76
<i>SHSP</i>	UM058_0031	Nucleus	0.49	Nucleus	1.00	Other	NA
<i>SHSP</i>	UM105_0001	Nucleus	0.58	Nucleus	1.00	Other	NA
<i>SHSP</i>	UM060_0017	Cell membrane	0.63	Nucleus	1.00	Chloroplast	0.97
<i>SHSP</i>	UM001_0093	Nucleus	0.60	Nucleus	1.00	Other	NA
<i>SHSP</i>	UM001_0095	Nucleus	0.68	Nucleus	1.00	Other	NA
<i>SHSP</i>	UM001_0098	Nucleus	0.81	Nucleus	1.00	Other	NA
<i>SHSP</i>	UM001_0099	Cytoplasm	0.44	Nucleus	1.00	Other	NA
<i>SHSP</i>	UM001_0104	Nucleus	0.55	Nucleus	1.00	Other	NA
<i>SHSP</i>	UM001_0127	Cytoplasm	0.51	Nucleus	1.00	Other	NA
<i>SHSP</i>	UM003_0083	Plastid	0.89	Chloroplast Outer Membrane	0.82	Chloroplast	1.08
<i>SHSP</i>	UM005_0210	Nucleus	0.46	Nucleus	1.00	Signal Peptide	0.25
<i>HSP33</i>	UM030_0028	Mitochondrion	1.00	Chloroplast	0.79	Chloroplast	2.63
<i>CPN60</i>	UM001_0140	Cytoplasm	0.78	Cytoplasm	0.70	Other	NA
<i>CPN60</i>	UM015_0132	Cytoplasm	0.83	Cytoplasm	0.70	Other	NA
<i>CPN60</i>	UM026_0043	Peroxisome	0.49	Cytoplasm	0.63	Mitochondrion	0.82
<i>CPN60</i>	UM038_0110	Cytoplasm	0.85	Cytoplasm	0.70	Other	NA
<i>CPN60</i>	UM042_0109	Cytoplasm	0.85	Cytoplasm	0.70	Other	NA
<i>CPN60</i>	UM116_0035	Mitochondrion	0.73	Chloroplast	0.70	Other	NA
<i>CPN60</i>	UM002_0158	Cytoplasm	0.76	Cytoplasm	0.70	Other	NA
<i>CPN60</i>	UM002_0236	Cytoplasm	0.43	Cytoplasm	0.70	Mitochondrion	0.43
<i>CPN60</i>	UM003_0101	Cytoplasm	0.91	Cytoplasm	0.70	Other	NA
<i>CPN60</i>	UM004_0333	Plastid	0.38	Chloroplast	0.70	Chloroplast	2.51
<i>CPN60</i>	UM010_0035	Plastid	0.49	Mitochondrion	0.50	Chloroplast	2.77
<i>CPN60</i>	UM011_0180	Cytoplasm	0.51	Cytoplasm	0.70	Chloroplast	2.48
<i>HSP70</i>	UM001_0412	Endoplasmic reticulum	0.69	Extracellular Space	0.97	Signal Peptide	2.90
<i>HSP70</i>	UM039_0067	Mitochondrion	0.93	Mitochondrion	0.60	Mitochondrion	1.98
<i>HSP70</i>	UM127_0015	Cytoplasm	0.66	Nucleus	1.00	Other	NA
<i>HSP70</i>	UM004_0183	Cytoplasm	0.69	Nucleus	1.00	Other	NA
<i>HSP70</i>	UM004_0334	Mitochondrion	0.33	Chloroplast	0.70	Chloroplast	2.55
<i>HSP70</i>	UM012_0035	Plastid	0.38	Organelle Membrane	0.90	Signal Peptide	0.60
<i>HSP70</i>	UM012_0036	Endoplasmic reticulum	0.73	Extracellular Space	0.86	Signal Peptide	1.11
<i>HSP70</i>	UM013_0190	Cytoplasm	0.93	Cytoplasm	0.51	Other	NA
<i>HSP70</i>	UM023_0131	Mitochondrion	0.48	Chloroplast	0.54	Chloroplast	1.38
<i>HSP70</i>	UM028_0130	Cytoplasm	0.63	Cytoplasm	0.70	Other	NA
<i>HSP90</i>	UM012_0034	Endoplasmic reticulum	0.92	Extracellular Space	1.00	Signal Peptide	1.55

<i>HSP90</i>	UM019_0113	Mitochondrion	0.87	Mitochondrion	0.84	Chloroplast	2.93
<i>HSP90</i>	UM060_0018	Cytoplasm	0.64	Nucleus	1.00	Other	NA
<i>HSP100</i>	UM001_0159	Cytoplasm	0.76	Nucleus	1.00	Other	NA
<i>HSP100</i>	UM001_0583	Mitochondrion	0.62	Chloroplast	1.00	Signal Peptide	0.87
<i>HSP100</i>	UM009_0119	Plastid	0.46	Chloroplast Outer Membrane	0.69	Mitochondrion	0.69
<i>HSP100</i>	UM060_0114	Mitochondrion	0.93	Chloroplast	0.77	Chloroplast	1.31
<i>HSP100</i>	UM065_0021	Cytoplasm	0.44	Nucleus	1.00	Other	NA
<i>HSP100</i>	UM035_0089	Mitochondrion	0.69	Mitochondrion	0.84	Chloroplast	3.03
<i>HSP100</i>	UM065_0020	Cytoplasm	0.58	Nucleus	1.00	Other	NA

Appendix II. Predicted localisation of *U. mutabilis* heat shock proteins performed using the DeepLoc, BUSCA, and PredAlgo localisation prediction servers. Predicted subcellular localisation for each identified protein is presented with the corresponding prediction score from each method (Tardif *et al.* , 2012; Almagro Armenteros *et al.* , 2017; Savojardo *et al.* , 2018).

Appendix III

Appendix III. Solid-state NMR analysis of the barley straw α -cellulose fraction performed using phase-inversion CP-MAS, using the Bruker pulse program 'cpqi' (on the next page).

Current Data Parameters
NAME drh_20220307
EXPNO 4
PROCNO 1

2511/9952 Barley cell @ 10 kHz

F2 - Acquisition Parameters
Date_ 20220308
PROBHD 4 mm MAS BB/1H
PULPROG cpri
NS 900
SWH 100000.000 Hz
AQ 0.0204800 sec
TE 298.0 K
D1 4.00000000 sec

===== CHANNEL f1 =====
SFO1 100.6265980 MHz
NUC1 13C
P15 1000.00 usec
P16 40.00 usec
PLW1 81.00000000 W

===== CHANNEL f2 =====
SFO2 400.1708003 MHz
NUC2 1H
CPDPRG[2] spinal64_13
P3 3.00 usec
PCPD2 5.80 usec
PLW2 0 W
PLW12 170.00000000 W
PLW13 170.00000000 W
SPNAM[0] ramp90100.100
SPOAL0 0.500
SPOFFS0 0 Hz
SPW0 119.94999695 W
SPNAM[1] square.100
SPOAL1 0.500
SPOFFS1 0 Hz
SPW1 119.94999695 W

F2 - Processing parameters
SI 65536
SF 100.6227248 MHz
WDW EM
SSB 0
LB 25.00 Hz
GB 0
PC 1.00

