# Impacts of Natural and Induced Abiotic Factors on Phlorotannins in Brown Algae

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# Abstract

Phlorotannins are a group of highly bioactive marine polyphenols found exclusively in brown algae. They play multiple ecological roles in algae inhabiting stressful environments and, as a result, their natural concentrations display a high degree of variability, making the determination of the specific factors driving their metabolism challenging. Their reactivity, which has hindered their elucidation to date, is largely responsible for the unprecedented commercial attention they have received over the last few decades. Owing to their structural complexity, little is known regarding their biosynthesis, as well as the chemical properties responsible for their activity, such as degree of polymerisation and isomerisation, which can influence the availability of active hydroxyl units. Moreover, the use of several nonstandardised methods has prevented cross-comparison of studies, thus the factors responsible for their natural variation are still uncertain.

This study aimed to assess the natural variability of low molecular weight phlorotannin-enriched fractions for selected species of brown algae, in an effort to clarify the primary environmental factors causing changes in their profiles. An integrated approach of both field sampling and laboratory-controlled experiments was employed to attain a more in-depth understanding of the effects abiotic factors impose on phlorotannin chemistry. This involved the comparative investigation of season, location, inter-species and intra-thallus variability within four brown algae; *Pelvetia canaliculata*, *Ascophyllum nodosum*, *Fucus vesiculosus* and *Himanthalia elongata.* Specifically, due to the potential it displays for commercial development, *F. vesiculosus* was further examined under short-term culture conditions, to assess the specific effects of irradiance, nitrogen and temperature on LMW phlorotannin profiles. The effects of experimentally-induced abiotic factors on the overall algal physiology were assessed, through measuring Chl *a* fluorescence, along with changes incurred to the LMW phlorotannin chemical composition, content and associated antioxidant activity, to better understand the mechanisms behind their variability.

The results of this study demonstrate LMW phlorotannins to be effective radical scavengers and promising candidates for commercial integration. The degree of activity varies between species, owing to their distinct chemical profiles, possibly arising through genetic evolution. However, these compounds display rapid turnover rates, with shifts in the concentrations observed in response to environmental parameters, particularly light and temperature. The impact of such factors on phlorotannin metabolism is likely to stem from their effect on key components involved in the expression and regulation of their biosynthetic pathway, as well as their subsequent degradation and oxidation. The results highlight the ecological importance of these compounds, particularly within intertidal species, functioning to provide a heightened degree of tolerance of diurnal fluctuations in irradiance and temperature levels. The results attained here provide a solid foundation of valuable scientific information on which future research can be readily established.

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**Chapter 9. Supplementary material**

# Communications

**Publications:**

The following chapters of this thesis have been published in or prepared for submission to international peer-reviewed journals:

**Chapter 7:** Kirke, D.A, Smyth, T.J, Rai, D.K, Kenny, O and Stengel, D.B. 2017. "The chemical and antioxidant stability of isolated low molecular weight phlorotannins." Food Chemistry, *221: 1104-12.*

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**Kirke, D.A**, Rai, D.K., Smyth, T.J and Stengel, D.B. Understanding natural variation in low molecular weight phlorotannins in Irish seaweeds - a fundamental step towards future exploitation. 44$^{th}$ Food Research Conference. Cork, Ireland.*

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**Kirke, D.A,** Rai, D.K, Smyth, T.J and Stengel, D.B. The single and interactive effects of PAR and UV on low molecular weight phlorotannin profiles in *Fucus vesiculosus* using mass spectrometry. 6$^{th}$ International Society of Applied Phycology (ISAP) Congress. Nantes, France.*

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**Kirke, D.A**, Rai, D.K., Smyth, T.J and Stengel, D.B. Optimisation of phlorotannin profiles for the enrichment of seaweed-based food products. IFTS 43rd Annual Food Research Conference. Dublin, Ireland.

**Kirke, D.A**, Rai, D.K., Smyth, T.J and Stengel, D.B. Environmental impact on phlorotannin profiles of commercially valuable Irish brown seaweeds. 6th European Phycological Congress (EPC6). London, England.

**Kirke, D.A**, Rai, D.K, Smyth, T.J, and Stengel, D.B. The effect of storage conditions on the stability of low molecular weight phlorotannins; potential natural food preservatives. International Union of Food Science and Technology (IuFoST). Dublin, Ireland.

# Abbreviations

α – initial slope of the rETR vs PFD curve

β –- gradient at high PFD levels which is negative if photoinhibition occurs

°C – degrees Celsius

µM – micromole

µg – microgram

µL – microliter

% – percentage

Δ $F/F_{m'}$ – effective PSII quantum efficiency,

6-4PPs – pyrimidine 6-4 pyrimidone photoproducts

ANOVA – analysis of variance

APX – ascorbate peroxidase

ATP – adenine triphosphate

BDE – bond dissociation energy

BHA – butylated hydroxyanisole

BHT – butylated hydroxytoluene

C – carbon

Ca – calcium

Chl – chlorophyll

CHS – chalcone synthase

cm – centimetres

CNBH – carbon-nitrogen balance hypothesis

C:N – carbon: nitrogen ratio

$CO_2$ – carbon dioxide

CPDs – cyclobutane pyrimidine dimers

CWB – cell-wall bound

Da – daltons

DC – direct current

DNA - deoxyribonucleic acid

DOM - dissolved organic matter

DP – degree of polymerisation

DPPH• – 2, 2-diphenyl-2-picrylhydrazyl

DW – dry weight

DWE – dry weight extract

$E_k$ – light saturation point

EPA – eicosapentaenoic acid

ER – endoplasmic reticulum

ESI – electrospray ionisation

F – steady state of fluorescence in light

FC – Folin-Ciocalteu

$F_m$ – maximal Chl *a* fluorescence after dark

$F_{m'}$ – maximal Chl *a* fluorescence after light

$F_0$ – ground fluorescence value

FRAP – ferric reducing antioxidant power

$F_v$ – variable Chl *a* fluorescence

$F_v/F_m$ – maximal efficiency of PSII

FW – fresh weight

g – gram

G3P – glyceraldehyde 3-phosphate

GR – glutathione reductase

h – hour

H – hydrogen

$H_2O_2$ – hydrogen peroxide

HAT – hydrogen atom transfer

HO· – hydroxyl radical

HP – high PAR

HPLC – high performance liquid chromatography

hsp - heat shock proteins

$IC_{50}$ – concentration required to cause 50% inhibition

IR – infrared

K – potassium

kDa – kilodaltons

kHz – kilohertz

Kv – kilovolts

L – litre

LC – liquid chromatography

LMW – low molecular weight

LP – low PAR

m – metre

MAAs – mycosporine-like amino acids

Mg – magnesium

mg – milligram

MWCO – molecular weight cut off

mM – millimole

mL – millilitre

MRM – multiple reaction monitoring

MS – mass spectrometer

MS/MS – tandem mass spectrometry

mW – milliwatts

MYA – million years ago

*m/z* – mass (*m*) to charge (*z*) ratio

N – nitrogen

$N_2$ – molecular nitrogen

$Na_2CO_3$ – sodium bicarbonate

NAD(P)H – nicotinamide adenine dinucleotide phosphate

$NH_4$ – ammonium

$NH_4NO_3$ – ammonium nitrate

nm – nanometers

NMR – nuclear magnetic resonance

NP – normal-phase

NPQ – non-photochemical quenching

$NO3^-$ – nitrate

$^1O_2$ – singlet oxygen

$O^{2\bullet-}$ – superoxide radical

OH – hydroxyl unit

P – phosphorous

*p* – *p*-value, referring to the determination of statistical significance

PAM - pulse-amplitude modulated

PAR – photosynthetically active radiation

PFD – photon flux density

PGA – phosphoglycerate

PGE – phloroglucinol equivalents

PGU – phloroglucinol units

PKS – polyketide synthase

*post-hoc – posteriori homogeneous*

PRPs – proline-rich proteins

PS I – photosystem 1

PS II – photosystem 2

PTFE – polytetrafluorethylene

QQQ-MS – triple/tandem quadrupole mass spectrometer

Q-ToF-MS – quadrupole time-of-flight mass spectrometry

$R^2$ – linear regression coefficient

rETR – relative electron transport rate

rETRmax – maximum relative electron transport rate

RF – radio frequency

ROS – reactive oxygen species

RP – reverse-phase

RSA – radical scavenging activity

RT – room temperature

RTE – room temperature & exposed to air

RuBisCO – ribulose-1, 5-bisphosphate carboxylase/oxygenase

s – second

SET – single electron transfer

SOD – superoxide dismutase

TBHQ - tert-butylhydroquinone

TE – trolox equivalents

TFA – total fatty acids

TIC – total ion chromatogram

TLC – thin-layer chromatography

TPC – total phenolic content

TPTZ – trispyridyltriazine

TQD – Tandem quadrupole detector

UPLC – ultra performance liquid chromatography

UVR – ultraviolet radiation

UVA – ultraviolet radiation A

UVB – ultraviolet radiation B

W – watts

# Chapter One

General Introduction

## 1.1. Alga - the what, when and how

### 1.1.1. Algal evolution

The term “algae” describes a vast assemblage of aquatic photosynthetic organisms, from unicellular forms only a few microns in size, to multicellular, morphologically complex species that can span over 30 m in length (Tirichine and Bowler, 2011). The origin of the photosynthetic trait stems from primary endosymbiosis in which a cyanobacterium was engulfed by a phagotrophic eukaryote leading to the integration of cellular material resulting in the formation of the plastid (Fig. 1.1; De Clerck *et al.*, 2012). This was thought to have occurred over 1,500 million years ago (MYA), giving rise to the first alga (Yoon *et al.* 2004); the Glacophytes and the green and red lineages (Fig. 1.2). Therefore, the red (Rhodophyta) and green (Chlorophyta) algae are closely related to one another belonging to the group Plantae, which is also comprised of a large assemblage of terrestrial species (Baldauf, 2003).

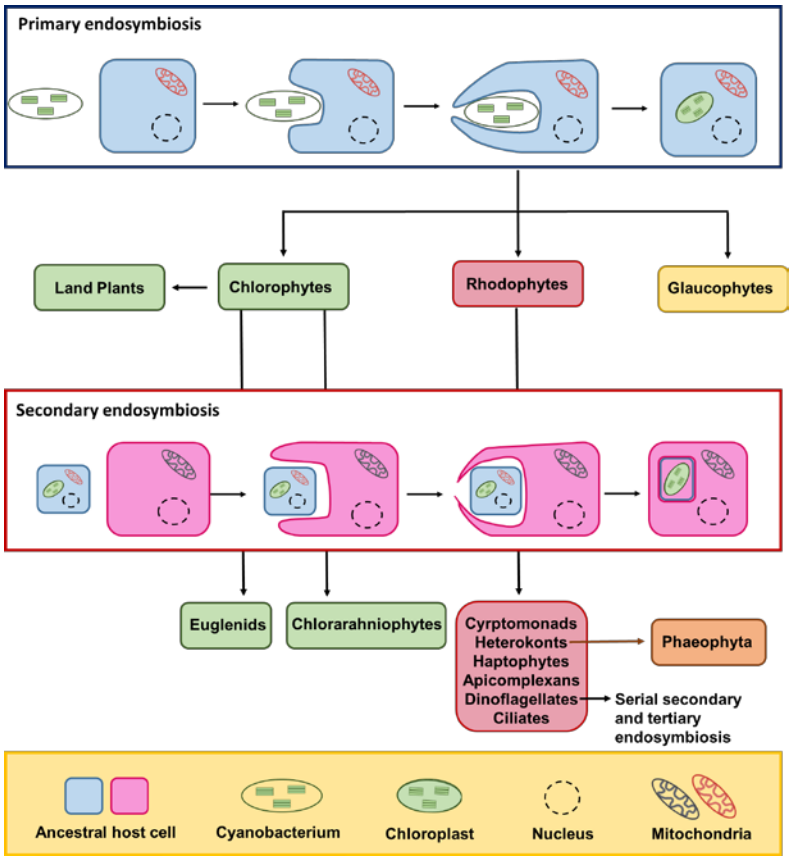



**Fig. 1.1**. The primary and secondary endosymbiotic events that resulted in the divergence of the algal groups, Chlorophyta, Rhodophyta and Phaeophyta. Adapted from Ravi *et al.* (2008).

However, there was a secondary occurrence of endosymbiosis, wherein it is thought that a red or green photosynthetic eukaryote was engulfed and incorporated into a second heteroptrophic eukaryote (Fig. 1.1; Tirichine and Bowler, 2011). It is suggested that this occurred several times giving rise to a variety of new divisions including the Cryophytes, Haptophytes and Stramenopiles (Fig. 1.2). The evolution of the brown macroalgae (Phaeophyceae) occurred from the secondary endosymbiosis and therefore, they are evolutionary different from the red (Rhodophyta) and green (Chlorophyta) algae (Fig. 1.1; Fig. 1.2). Collectively, there are approx. 10,000 species of macroalgae worldwide (Guiry, 2012).

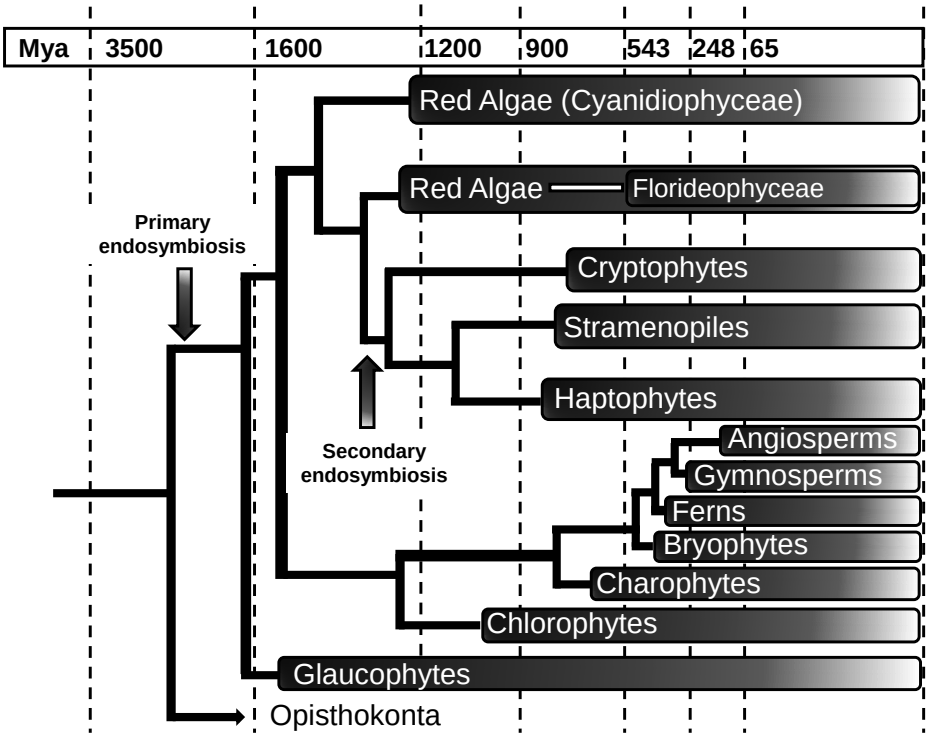



**Fig. 1.2.** Schematic representation of the evolutionary relationships and divergence times (million years ago - Mya) for the red, green, glaucophyte, and chromist algae. The branches on which the primary and secondary endosymbioses occurred are indicated by arrows. Figure redrawn from Schmid (2016), a modified adaptation of Yoon *et al.* (2004).

Traditionally, seaweed classification between red, green and brown species, was based largely on differences observed in pigmentation, but more recent research has shown that seaweeds display an extensive degree of diversity in relation to morphology, life strategy and chemical integrity, both between and within taxonomic entities (Table 1.1; Stengel *et al.*, 2011).

**Table 1.1.** The chemodiveristy between Chlorophyta, Rhodophyta and Phaeophyceae.

| | **Chlorophyta** | **Rhodophyta** | **Phaeophyceae** | **Ref.** |
|---|---|---|---|---|
| **Pigments** | Chl *a*, *b*<br>α-, β-carotenes<br>lutein<br>siphonaxanthin<br>siphonein | Chl *a*, (*d*)<br>β-carotene<br>phycoerythrins<br>phycocyanins<br>allophycocyanins | Chl *a*, $c_1$, $c_2$<br>β-carotene<br>fucoxanthin<br>violaxanthin<br>zeaxanthin | **a, b, c** |
| **Polysaccharides** | Mannan<br>Ulvan<br>Xylan | Agars<br>Carrageenans<br>Mannan<br>Xylan | Alginates<br>Fucans<br>Laminaran | **d, e, f, g** |
| **Lipids*** <br>* Fatty acids found in highest abundance | Palmitic acid (PA, C16:0)<br>Linoleic acid (LA, C18:2, n-6)<br>α-linolenic acid (ALA, C18:3 n-3) | Arachidonic acid (ARA, C20: n-3)<br>Eicosapentaenoic acid (EPA, 20:5 n-3) | α-linolenic acid (ALA, C18:3 n-3)<br>Arachidonic acid (ARA, C20: n-3)<br>Eicosapentaenoic acid (EPA, 20:5 n-3)<br>Stearidonic acid (SDA, C18:4 n-4), | **h, i, j** |
| **Other** | | Mycosporine-like amino acids (MAA's) | Phlorotannins (Polyphenolic compounds) | **k, l** |

**Reference (Ref.) Key: a –** Larkum and Barrett (1983), **b –** Prasanna *et al.* (2007), **c –** Schubert *et al.* (2006), **d –** Li *et al*. (2008), **e** – Percival (1979), **f** – Rioux *et al*. (2010), **g** – Usov (1998), **h** – Khotimchenko *et al*. (2002), **i** – Schmid *et al*. (2014), **j** – Van Ginneken *et al*. (2011), **k** – Karentz *et al*. (1991), **l** – Ragan and Glombitza (1986).

The extensive chemodiversity of macroalgae, as seen in Table 1.1, offers great physiological variability, allowing for their vast biogeographical distribution. Seaweed communities are reported from polar, temperate and tropical regions (Bartsch *et al.*, 2012). Wherever the location, seaweed populations are of great ecological importance; macroalgal communities often function as nurseries for juvenile fish and intertidal organisms, offering refuge from predators and environmental stressors, as well as being a primary source of food (Hurd *et al.*, 2014). In fact many macroalgal species have been identified as "foundation species" whereby their presence greatly affects the physical and biological aspects of the surrounding ecosystem (Bruno and Bertness, 2001). Out of all the environments they inhabit, which span from the open ocean to estuaries to tidal pools, the most interesting, from both an ecological and phycological point of view, is the coastal rocky shore intertidal

zone, the interface between terrestrial and marine world's (Hurd *et al.*, 2014; Lüning, 1990).

### 1.1.2. Rocky intertidal zone

Where the land meets the ocean makes for an intriguing ecosystem to study for ecologists. None have been more investigated than rocky intertidal shores, offering a variety of unique organisms living in a world half way between marine and terrestrial (Lobban and Harrison, 1997). One main characteristic is the pattern of zonation of species along a shores gradient and tide-marks have usually been used to describe zones with one being above the other up the slope, each of them characterized by distinctive features of its own (Fig. 1.3; Stephenson and Stephenson, 1949).

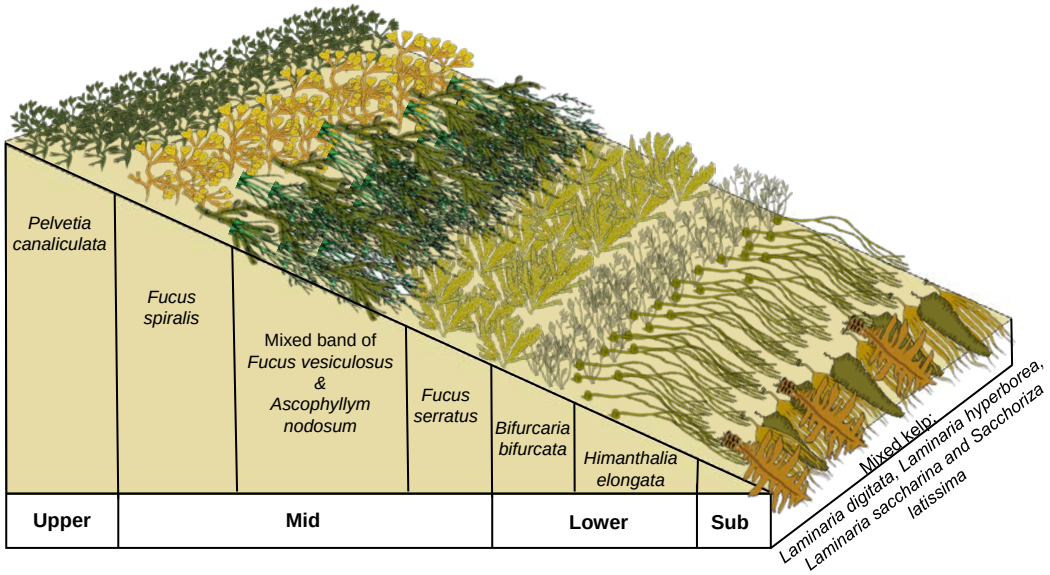



**Fig.1.3.** Illustration of typical brown algal zonation on a sheltered shore along sloping gradient comprised of the three major zones based on tidal marks (upper-, mid-, lower- and sublittoral zones).

According to Stephenson and Stephenson (1949) a "standard zonation" of a sheltered shore is comprised of the following main zones (Fig. 1.3):

**Supra-littoral**. The area of the shore which lies above tide marks yet is still subject to some marine influence, mostly spray of the waves in rough weather. Characteristic of this area, are the grey and yellow lichens such as *Xanthoria parientina* (Lüning, 1990).

**Upper littoral**. This area spreads from the upper limit of *Littorina* barnacles down to the lower limit. The lower section of this belt is invaded at high spring tides. Algae

well-adapted to prolonged periods of dessication inhabit this region such as *Pelvetia canaliculata.*

**Mid-littoral**. Also known as the intertidal zone, this area is subject the most fluctuating conditions due to tidal coverage varying progressively every day. Fucoids such as *Fucus* spp. and *Ascophyllum nodosum* dominate this area.

**Lower littoral.** Comprising the upper limit of any convenient dominant organism such as *Laminaria* spp. etc. to extreme low-water levels of spring tides. This area is only uncovered at major spring tides and in calm weather. Brown macroalgal species found here include *Bifurcaria bifurcata* and *Himanthalia elongata.*

**Sublittoral**. This area has been categorized as the area from the extreme low waters of spring tides to a depth which may be to the lower limit of seaweed vegetation. Most commonly dominated by kelp species e.g. Laminariales.

### 1.1.3. Seaweeds - Harsh habitats call for adaptive responses

Due to the extreme environments that they are found inhabiting, algae must deal with a multitude of stresses of both abiotic and biotic origin. Abiotic factors such as light intensity, temperature, nutrient supply and wave exposure (Lüning, 1984, 1990; Seymour *et al.*, 1989) coupled with biotic interactions such as grazing, fouling and competition (Horn, 1989; Schonbeck and Norton, 1980; Steinberg and De Nys, 2002) influence the limits of algal zonation along a gradient as clearly illustrated in Fig.1.3 (Lewis, 1964). However, stress must be defined as a measure of an individual rather than the value of a specific environmental parameter as optimum conditions for different species from different geographical backgrounds can vary (Davison and Pearson, 1996). Grimes (1979) characterised stress as “external constraints limiting the resource acquisition, growth or reproduction of organisms”. The overall ability of an organism to cope with adverse conditions, known as stress resistance, is a function of stress tolerance at a cellular level and/or avoidance mechanism that prevent such stress acting upon cells (Davison and Pearson, 1996). Such adaptations have evolved morphologically and/or physiologically. Morphological adaptations vary from the air bladders *Fucus vesiculosus* possess along the thallus, allowing the plants to float at the surface of the water column enabling photosynthesis to continue at a productive rate (Garbary *et al.*, 2006), to the curled fronds of *Pelvetia canaliculata*, allowing them

endure long periods of emersion avoiding osmotic damage onset by desiccation (Schonbeck and Norton, 1979). Physiological adaptations, however, in algae occur from the synthesis and regulation of biochemical components, allowing specific species to withstand certain environments (Stengel *et al.*, 2011).

### 1.1.4. Secondary metabolites - key for survival

The compounds within living organisms may be divided into two major groups; primary and secondary metabolites (Seigler, 2012). Primary metabolism encompasses all processes essential for a plants growth and development including glycolysis, the Krebs Cycle, and photosynthesis and so primary metabolites, limited to several dozen molecules, include nucleic acids, proteins, enzymes, light-harvesting antenna pigments, fatty acids and carbohydrates (Maschek and Baker, 2008; Seigler, 2012). Secondary metabolites arise from primary metabolic pathways and hence are so-called "secondary". These compounds were once considered as "errors" in primary metabolism, being generally accepted "metabolic waste products" being perceived as non-essential, inert end products (Hartmann, 2007). However, since then, several studies have come forth disproving this theory and acknowledging secondary compounds as dynamic components of plant metabolism, being essential elements in the mechanisms to cope with the adversities of a hostile environment (Fraenkel, 1959).

The biosynthesis of secondary metabolites appears highly complex with enzymes specific for each pathway. In general, it is found that tissues and organs vital for survival and reproduction, such as epidermal tissues, flowers, fruits and seeds in terrestrial plants, all possess distinctive profiles of secondary metabolites that are stored in large quantities within (Wink, 2010). All the processes and structures necessary to express these traits are considerably expensive, in terms of adenine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NAD(P)H). Therefore, it would be highly unlikely that secondary metabolites were waste products or possessed no ecological function at all, as previously suggested in older literature. Costly traits without a function do not survive in evolution, as those with them would be at a disadvantage compared to those without. Rather favoured by natural selection, the fitness of individuals that possess secondary metabolites is increased (Rosenthal and Janzen, 1979).

The fundamental characteristic of secondary metabolites is their high genetic plasticity and diversity that guarantees flexible adaptation to the demands of the environment (Hartmann, 2007; Wink, 2010). The main roles of plant and algal secondary metabolites include defence against predators and competitors (Hartmann, 2007), signal compounds (see review by Cipollini and Levey, 1997) and protection from abiotic stresses (see review in Wink, 2010). Algal secondary metabolites, in particular, account for 3,000 natural products and so they are classified according to their chemical structure or by the biosynthetic pathway from which they are derived (Maschek and Baker, 2008).

More than half of these compounds belong to the isoprenoid class (steroids, terpenes, carotenoids, etc.) and have been reported to possess several physiological roles. For example, the carotenoids, comprised of the carotenes and the xanthophylls, are important light-harvesting pigments in photosynthesis (Gantt and Cunningham, 2001) but also function in preventing the photo-oxidation of the PS II Chl reaction centres (Vílchez *et al.*, 2011). Gevaert *et al.* (2002) found a seasonal correlation between irradiance and the abundance of xanthophyll pigments in the kelp *Saccharina latissimia.* An increase in these pigments occurred in spring as a mechanism to cope with strong irradiance. The photo-protective function of these compounds stems from their ability to dissipate excess energy involving the rapid and reversible de-epoxidation of violaxanthin to antheraxanthin to zeaxanthin (Schofield *et al.*, 1998).

Mycosporine-like amino acids (MAAs) are another common sunscreen metabolised in intertidal macroalga. Present within red and a few green algal species, MAAs are a group of small (<400 Da) and highly polar intracellular compounds, characterised by either a cyclohexanone or cyclohexenamine cycle with one or two amino acids and display a absorption maxima between 310 and 360 nm, lying within the ultraviolet radiation (UVR) region, specifically UVA (Favre-Bonvin *et al.*, 1976; Bhatia *et al.*, 2011). Their role as UV-screening agents has been supported by an apparent correlation between their accumulation and increasing UV exposure. Karsten *et al.* (1998) found, upon investigating their levels in several red algae, that algae from warm-temperate regions had higher concentrations than those from Polar regions. Furthermore, their concentrations were inverse related to the shore height, decreasing with increasing depth. Species occupying higher shore positions, being consequently

exposed to a higher degree of UVR, appeared to be capable of synthesising and accumulating a higher level of MAAs in response.

Such functional groups only comprise a fraction of that discovered so far. Over 1,500 compounds have been reported within the red alga (Rhodophyta), making them the most chemically rich (Blunt *et al.*, 2007).While producing similar compounds to the red species, less than 300 known compounds are reported for the green algae (Chlorophyta). All major classes of secondary metabolites are represented within the red and green seaweed, with the exception of the phlorotannins, which are exclusive to the brown algae (Phaeophyceae) (Maschek and Baker, 2008).

Brown algae, being the predominant algal group occupying temperate rocky intertidal shores, have acquired specific physiological traits during their independent evolution from the other major eukaryotic groups. This independent evolution has given way to many novel features with regard to their chemical metabolism (Stengel *et al.*, 2011). One such being the synthesis of a particular group of polyphenols, which are exclusive to Phaeophyceae. Phlorotannins are the one of the main secondary metabolites found in brown algae, often accounting for 5-12% of dry weight (DW) biomass (Ragan and Glombitza, 1986; Targett *et al.*, 1992). This group of tannins are exclusive to brown algae where they function as chemical defence compounds against an array of external stressors.

### 1.1.5. Phlorotannins - brown algal polyphenols

The term “phenolic compounds” embraces a large collection of chemical compounds possessing an aromatic ring bearing one or more hydroxyl groups together with a number of other substituents (Levin, 1971). Phenolic compounds, or polyphenols, constitute one of the most abundant and vastly distributed groups of biomolecules within the plant kingdom, being only second to carbohydrates (Cheynier, 2012; Levin, 1971). Among them are the tannins, a group of highly hydroxylated polyphenols found in both terrestrial plants and brown algae (Ragan and Glombitza, 1986; Salminen and Karonen, 2011). In vascular plants, they are divided into two classes: condensed tannins and hydrolysable tannins (Fig. 1.4).

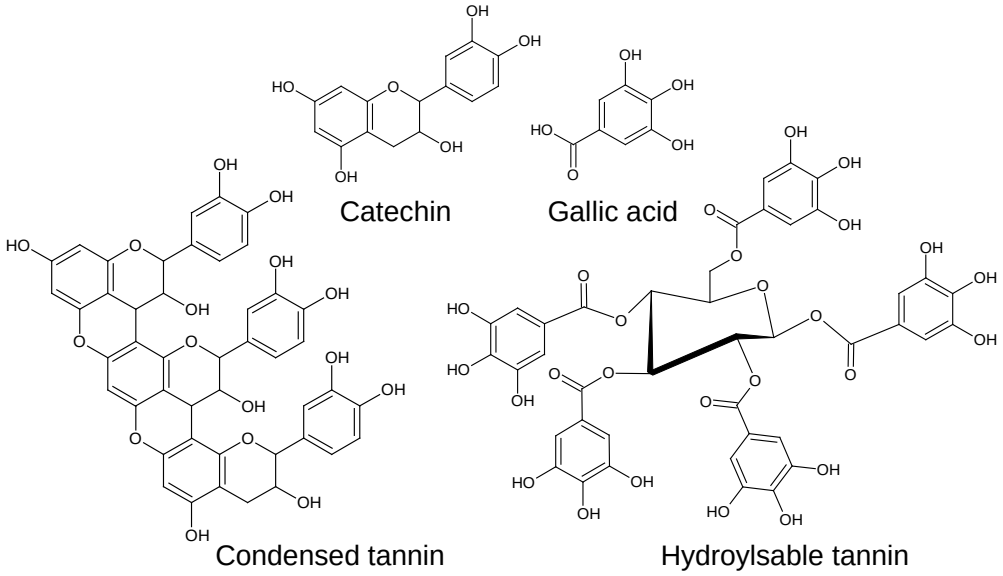



**Fig. 1.4.** Chemical structures of condensed and hydrolysable tannins (gallo- and ellagitannins) and the monomers of their composition. The hydrolysable tannins are here represented by the gallotannins and their monomeric unit gallic acid. Adapted from Salminen and Karonen (2011) and re-drawn using Chemsketch (ACD/Labs, Toronto, Canada).

However, in marine brown algae they occur as a single structural class, the phlorotannins (structural analogs of condensed tannins). Phlorotannins are derived through the oligo- and polymerization of the monomer compound, phloroglucinol (1, 3, 5 trihydroxybenzene, molecular weight 126.1 Da) and are thought to be synthesized via the acetate-malonate pathway (Herbert, 1989; Meslet-Cladière *et al.*, 2013). Despite the importance of phlorotannins in brown algae ecology, very little else is known about their biosynthesis. However, recently, Meslet-Cladière *et al.* (2013) made a major advance in this area by identifying and characterising PKS1, a type III polyketide synthase, in the brown alga *Ectocarpus siliculosus*, demonstrating that PKS1 catalyzes the synthesis of phloroglucinol monomers from malonyl-CoA.

Phlorotannins encompass an extensive range of molecular weights, from 126 Da to 650 kDa (Ahn *et al.*, 2011). Based on the type of structural linkages between monomeric units, phlorotannins can be categorised into three main structural groups: fucols, phlorethols and fucophlorethols, with fuhalols, eckols and carmalols being sub-groups of the phlorethols (Table 1.2; Fig. 1.5; Isaza Martínez and Torres Castañeda, 2013). Fucols are composed of aryl-aryl (phenyl) bonds while the hyrdoxyl units in phlorethols are linked only by diaryl ether bonds. Fuhalols, structural derivatives of phlorethols, are categorised by the presence of an additional hydroxyl group on the terminal phloroglucinol monomeric unit while the eckols and carmalols possess

characteristic 1, 4 dibenzodioixn linkages (Table 1.2; Fig. 1.5). The third phlorotannin group, the fucophlorethols possess both direct carbon to carbon and diaryl ether bonds (Table 1.2; Fig.1.5; Amsler and Fairhead, 2005; Isaza Martínez and Torres Castañeda, 2013).

**Table 1.2.** Description of the three main (and their sub-groups) structural categories of phlorotannins, as described by Isaza Martínez and Torres Castañeda (2013).

| **Category** | **Linkage type** | **Other characteristic properties** | **Example structure (as seen in Fig. 1.5)** |
|---|---|---|---|
| **i) Fucols** | Aryl-aryl (1) | Inter-phloroglucinol linkages can only be in a meta- relative position | Trifucol A |
| **ii) Phlorethols** | Diaryl ether (2) | Ortho-, meta-, or para- oriented linkages (and combinations thereof) | Diphlorethol |
| Fuhalols | | One (or more) additional hydroxyl group on the terminal monomeric unit | Trifuhalol A |
| Eckols and Carmalols | 1,4-dibenzodioxin (3) | Eckols possess phenyl-substitutions at positions 4 and 8 of the benzene ring while in carmalols it occurs at positions 3 and 7 | Dieckol and Diphlorethohydroxycarmalol |
| **iii) Fucophlorethols** | Combination of aryl and ether linkages (1, 2 and 3) | Linear and branched in structure as well as heterocyclic (e.g. dibenzodioxin or furan rings) | Diphlorethol A |

*In situ*, phlorotannins are stored in specialised membrane-bound vesicles called physodes, that are produced in the perinuclear region, in vesicles near the endoplasmic reticulum (ER) and Golgi bodies (Schoenwaelder, 2002). These subcellular bodies vary in size from 0.1-10 µm in diameter (Schoenwaelder, 2002; Schoenwaelder, 2008) and their abundance has been reported in both reproductive and vegetative tissues with studies confirming their presence in gametes, zygotes and embryos (Clayton and Schoenwaelder, 2000; Schoenwaelder and Clayton, 1998a; Schoenwaelder and Clayton, 1998b) as well as epidermal, cortical, apical and meristematic cells in various brown algal species (reviewed in Ragan and Glombitza, 1986). However,

phlorotannins can also be found within the cell wall. Soluble phlorotannins released from physodes can become deposited within the secondary cell wall where they form complexes with alginic acids (Wallace and Fry 1994; Koivikko *et al.*, 2005).

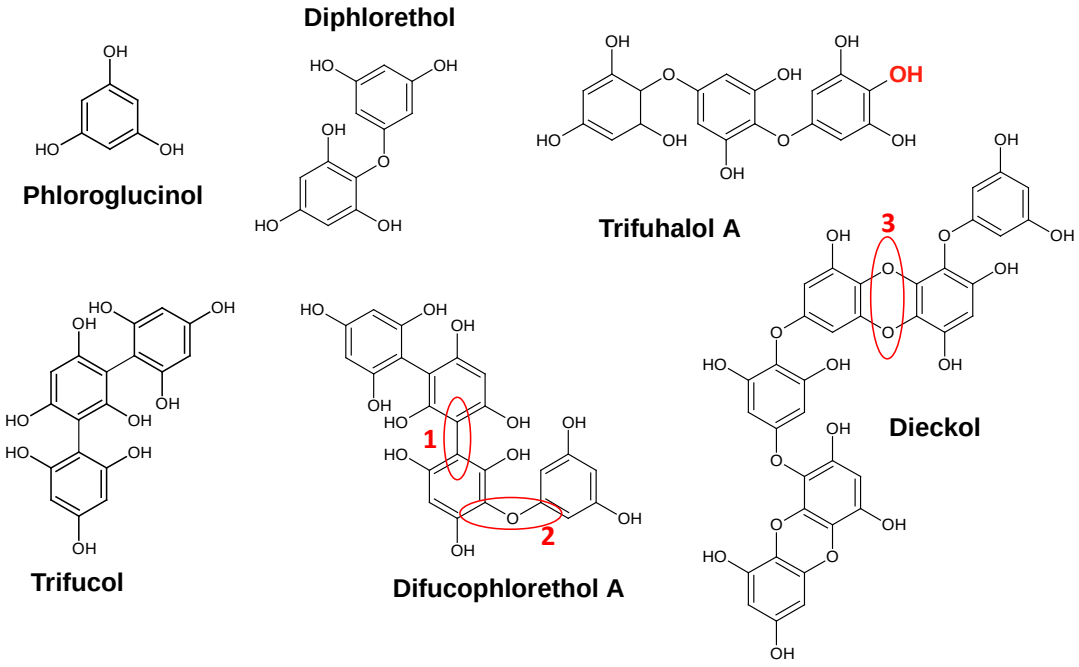



**Fig. 1.5.** Chemical structures of phlorotannins, highlighting the varying sturctural properties: additional hydroyxl group found in Fuhalols and varying linkage types (as described in Table 1.2): **1**- aryl-aryl, **2** – diaryl ether and **3** – 1, 4 -dibenzodioxin linkages. Adapted from Isaza Martínez and Torres Castañeda (2013) and re-drawn using Chemsketch (ACD/Labs, Toronto, Canada).

## 1.2. The ecological functions of brown algal phenolics

Phlorotannins are exclusively produced by brown seaweeds, where they can, in some species, account for 5-12% DW biomass (Amsler and Fairhead, 2006; Ragan and Glombitza, 1986). They have been reported from almost all brown algal orders with specificity within particular generas as seen with fucols in *Fucus* and eckols in *Ecklonia.* Once considered to be strict secondary metabolites, phlorotannins are now considered to function in both primary and secondary roles (Arnold and Targett, 2003). They have been reported to play several ecological roles including wound healing (Lüder and Clayton, 2004), herbivore and fouling deterrence (Pavia and Toth, 2000; Steinberg *et al.*, 1998) and photo-protection (Abdala-Díaz *et al.*, 2006; Gomez and Huovinen, 2010) as well as being potent radical scavengers (Ahn *et al.*, 2007; Kang *et al.*, 2004). However, they are also vital in primary functions including major components of cell wall structure and putative roles in brown algal reproduction

(Ragan and Glombitza, 1986; Schoenwaelder and Wiencke, 1999; Schoenwaelder and Clayton, 1998).

### 1.2.1. Polyspermy and zygote formation

Polyspermy results in an egg being fertilised by more than one sperm and in algae this can lead to abnormalities and even embryo death (Brawley, 1987). In *Fucus* and *Pelvetia* species, sperm entry causes a change in membrane potential which lasts for several minutes, acting as a fast block to polyspermy. However, in many species there is a short time frame during which the newly fertilised zygote has no protection against polyspermy (Brawley, 1990). Phenolics were suggested to act as an intermediate block, preventing polyspermy from occurring within this vulnerable time frame (Schoenwaelder and Clayton, 1998) and ultimately increasing survival rates. Clayton and Ashburner (1994) reported on a mass-scale secretion of phenolics from *Durvillaea potatorum* zygotes at the time of spermatozoid entry. This was also observed in *Acrocarpia paniculata* where the large scale release of phenolics caused a distortion of the cell membrane as well as decreasing the motility of the near egg spermatozoids. The accumulation of physodes at the zygote periphery is reported to occur early in development, immediately after fertilisation being secreted into the primary wall (Schoenwaelder and Clayton, 1998a; Schoenwaelder and Clayton, 1998b; Schoenwaelder, 2002). This is thought to occur in one of two ways; the entire physode undergoes exocytosis and the phenolic content penetrates the wall or small phenolic-containing vesicles break off from the physodes and then are integrated into the zygote cell wall (Arnold and Targett, 2003; Schoenwaelder, 2002).

### 1.2.2. Cell wall integrity

Phlorotannins have been observed to become deposited in the secondary cell walls, where they are implicated in thickening**,** but they are also thought to be biologically active constituents of the primary wall where, they cross-link with carbohydrates (Wallace and Fry, 1994). Generally, smaller amounts of cell wall-bound (CWB) phlorotannins are detected compared to physode-bound (PB), soluble phlorotannins (Kovikko *et al.*, 2005). This is most likely due to the complexity of the bonds phlorotannins create with other cell wall components, making them highly difficult to extract and thus quantify (discussed in Arnold and Targett, 2003). Koivikko *et al.*

(2005) found alkaline degradation was most effective in releasing cell wall-bound phlorotannins after three consecutive treatments.

Intra-specific studies have shown phlorotannin levels and composition change with thallus age. For instance, in young thallus parts, they can be found in short oligomeric forms (Koivikko *et al.*, 2005). As thallus age increases, so too does phenol content (Pedersen, 1984), with the presence of longer and more complex forms difficult to exude or degrade and so they accumulate. This thickening of the cell wall may be interpreted as a function of defence against herbivory. For intertidal species it is also suggested to be a protective mechanism against extreme wave exposure and periodical desiccation (Koivikko *et al.*, 2005; Schoenwaelder and Wiencke, 1999).

Not only do phlorotannins function as a key compound in cell wall structure but studies have shown phlorotannins to aid in wound healing (Halm *et al.*, 2011; Lüder and Clayton, 2004). Halm *et al.* (2011) observed a rapid induction of phlorotannin-rich physodes to cells near the site of injury upon mechanical wounding of *Laminaria hyperborea*, increasing over time until the wound was repaired. The functions of phlorotannins in wound healing have been suggested to be three-fold; firstly, as a "clotting" mechanism at the wound site complexing with proteins acting as a disinfectant (Fagerberg and Dawes, 1976). This was also thought to be done extracellularly with the exudation of phenolics (Fulcher and McCully, 1971). Then, as a structural function, with the deposition of phlorotannins into cell walls aiding reconstruction and finally as an anti-herbivory defence during this period of vulnerability (Van Alstyne, 1988). In fact, the preference of herbivorous snails for freshly wounded *Fucus distichus* over uninjured thalli was shifted with the accumulation of phlorotannins at the ruptured area.

### 1.2.3. Photo-protection

Owing to the diurnal tidal recession and subsequent inclination of coastlines, intertidal brown algae experience diurnal succession periods of emersion and immersion, wherein the prior they can be subject to direct solar radiation (Connan *et al.*, 2007). Both daily and seasonal differences in the intensity as well as the spectral composition (PAR, UVR) of the sunlight to which the algae can be in direct contact with can have many variable outcomes on the algae's overall health. For example, exposure to elevated irradiance levels, particularly to the shorter, more energetic wavelengths of

UVB, can be detrimental for several key biological (and vulnerable) components including (but not limited to) DNA, lipid membranes, $D_1$ and $D_2$ proteins of PS II reaction centre, ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCO) of the Calvin cycle (Holzinger and Lütz, 2006; Karsten, 2008), as well as accelerating the catalytic generation of reactive oxygen species (Das and Roychoudhury, 2014; Krumova and Cosa, 2016; Lesser, 2006).

Abdala-Díaz *et al.* (2006) observed both daily and seasonal variations in the levels of phenolic compounds in *Cystoseira tamariscifolia* to correspond with incident irradiance levels, suggesting a photo-regulatory role of these compounds. In fact, inter-specific differences in UV-tolerance has been described as a function of depth (Roleda, 2005) with species inhabiting higher shore positions being capable of doing so due to the higher abundance of phlorotannin-containing physodes (Schoenwaelder *et al.*, 2003; Schoenwaelder and Clayton, 2000). Schoenwaelder *et al.* (2003) upon examining the effects of UV radiation on the zygotes and embryos of *Fucus spiralis*, *Fucus distichus* and *Fucus serratus*, concluded that successful development is attributed to higher levels of physodes, which appear to function as a photo-protective screen. Therefore, certain species e.g. *Fucus spiralis*, displaying higher levels are capable of colonising a higher position on the shore.

However, phlorotannin levels while appearing as a constitutive defence mechanism vital for the survival of juvenile stages, are also thought to function as a means of inductive defence, increasing in response to acute periods of photo-stress (Henry and Van Alstyne, 2004; Pavia *et al.*, 1997; Swanson and Druehl, 2002). For instance, *Lessonia nigrescens*, a south Pacific kelp, was observed to accumulate higher concentrations of phlorotannins during high exposure to UV radiation during the summer months (Gomez and Huovinen, 2010). The positive correlation between the induction of phlorotannins and the reduction in the damage of photosynthetic apparatus and DNA observed was an important confirmation of the protective function of intracellular phlorotannins.

The presence of high concentrations of phlorotannins in the outer cell layers of the intertidal *Hormosira banksii* protect against strong photo-damage during the Australian summer (Schoenwaelder, 2002). After short exposure to solar radiation, parts of the thallus appeared dark brown. This was reportedly the result of soluble

phlorotannins being released from the physodes into the cystoplasma where they are easily oxidised. As a consequence, they become brownish in appearance and from a photo-protective cellular layer for the photosynthetic tissue beneath.

Yet, some reports have shown that phlorotannins UV screening role may be of greater ecological importance, extracellularly. Brown algae are known to release phlorotannins into seawater directly via exudation, indirectly via tissue erosion or through cell damage (Carlson and Carlson, 1984; Jennings and Steinberg, 1994; Ragan and Jensen, 1978). Exuded phlorotannins in the alkaline medium of seawater, rapidly react with both proteinaceous and carbohydrate substances to form UV absorbing complexes (Dujmov *et al.*, 1997). Both intense irradiance (Ragan and Jensen, 1978) and emersion (Carlson and Carlson, 1984) have been associated with increasing phlorotannin exudation rates.

### 1.2.4. Prevention of epiphytic settlement

Most, but not all, macroalgae possess some kind of host-like relationship with epiphytic, endophytic or parasitic organisms (Potin, 2012). This relationship can impact the algae either positively, such as those involved in symbiotic interactions, with both partners benefiting from it, or negatively whereby the association promotes unfavourable changes to the physical and chemical properties of the algae, such as those observed with epiphytic and parasitic colonisation (Wahl *et al.*, 2010). Often these associations can impair the hosts fitness through a number of means such as increasing drag and susceptibility to tissue breakage, reducing light penetration by shading and increasing herbivore attraction (D'Antonio, 1985; Dixon *et al.*, 1981; Wahl and Hay, 1995). However, macroalgae have evolved several mechanisms to prevent and/or reduce the settlement of epibionts. One strategy is to disrupt colonization or growth by physical means, including the production of a mucilaginous covering, outer cell layer shedding and erosion of the distal ends of blades (Moss, 1982; Nylund and Pavia, 2005). Another mechanism is the use of secondary metabolites as chemical defences (Engel *et al.*, 2006; Puglisi *et al.*, 2007; Wikström and Pavia, 2004).

It has been suggested that the occurrence of phlorotannins at high concentrations at the very outer layers of the thallus, as well as their secretion into the surrounding seawater, enables algae to deter foulers (Brock *et al.*, 2007). The settlement of

epiphytes on seaweed fronds occurs in the narrow boundary layer surrounding the algal surface, where the concentration of exuded metabolites is exponentially higher than in the adjacent water column. This is because, as water-soluble compounds, phlorotannins dilute in the water column as they travel away from the algal surface (Steinberg *et al.*, 1998). Thus, their effectiveness as deterrents depends on their *in situ* concentrations near the surface of the host. Brock *et al.* (2007) found higher concentrations of phlorotannins at the algal surface in 100 µL water samples than in 10 mL samples. Environments in which the exuded phlorotannins can accumulate such as those of rock pools, can reach levels high enough to kill settling propagules (Conover and Sieburth, 1966). Furthermore, the effectiveness of phlorotannins as anti-foulants may be more significant in intertidal species such as *F. vesiculosus*, which undergo cycles of emersion and immersion and so can exude 'spikes' of high levels of phlorotannins due to desiccation (Brock *et al.*, 2007; Jennings and Steinberg, 1997).

### 1.2.5. Herbivore deterrence

Probably the most studied biological functions of phlorotannins is their role in herbivore deterrence (Pavia and Toth, 2000; Van Alstyne, 1988; Van Alstyne and Paul, 1990; Steinberg, 1984), first suggested by Hunger (1902). The mechanism through which phlorotannins inhibit herbivore feeding is two-fold; first, by a characteristic astringent taste (a common feature possessed by all tannins), affecting the palatability of the algae, thus discouraging future feeding and secondly, through binding with proteins in the herbivores gut after ingestion, thereby decreasing their assimilation efficiency (Stern *et al.*, 1996). This has been attributed to the ability of polyphenols to form covalent, ionic and hydrogen bonds with proteins, carbohydrates and other macromolecules inhibiting their absorption and/or by binding to herbivore digestive enzymes hindering them inactive (Boettcher and Targett, 1993; Oh and Hoff, 1986; Rosenthal and Janzen, 1979).

However, the reactivity of phlorotannins is greatly dependent on the chemical environment of an herbivores gut, as well as the structural properties of the compound itself (Boettcher and Targett, 1993; Martin *et al.*, 1985; Stern *et al.*, 1996). As these bonds that polyphenols create with other macromolecules only occur at pH levels less than 8.5, organisms with alkaline digestive tracks may be able to inhibit the formation of such insoluble complexes. Also, the presence of surfactants in herbivorous digestive

fluids can also prevent polyphenolic-protein complexation (Martin and Martin, 1984; Martin *et al.*, 1985). Other evolutionary adaptations of herbivores have been proposed including saliva abundant in proline-rich proteins (PRPs). These have been reported to bind to and block the polyphenols, preventing their anti-nutritional effects (McArthur *et al.*, 1995).

From a structural aspect, size has been considered the predominant characteristic affecting phlorotannin activity in deterring herbivores. Many studies support the generalisation that high molecular weight phlorotannins (≥ 10 kDa) are more active than low molecular weight phlorotannins (Geiselman and McConnell, 1981). High molecular weight polymers reduced grazing to a greater extent than the low weighted polymers by both herbivorous marine snails and fishes (Boettcher and Targett, 1993; Targett *et al.*, 1992). For example, phlorotannins <5 kDa extracted from *F. vesiculosus* and *A. nodosum* had no effect on nutrient assimilation by the marine fish, *Xiphister mucosus.* Yet, larger phlorotannins, *i.e.* >10 kDa, proved successful in reducing nutrient assimilation efficiency by over 50% (Boettcher and Targett, 1993).

However, this protective function of phlorotannins is by no means unanimous. Several cases exist where the deterrence or negative effects of phlorotannins on performance were not been observed (Jormalainen *et al.*, 2001; Pavia and Toth, 2000; Targett *et al.*, 1995; Van Alstyne *et al.*, 2001; Van Altena and Steinberg, 1992) and sometimes even a preference for phlorotannin rich algae was found (Jormalainen *et al.*, 2001; Pavia *et al.*, 1997). This suggests that even if the original adaptive function of phlorotannins was the defence against herbivory, a number of herbivore species have evolved and are now physiologically adapted to utilize phlorotannin-rich algae (Horn, 1989; Targett *et al.*, 1995).

However, other studies believe that herbivore presence affects these carbon-based compounds through the manipulation of resources rather than through direct induction as defence chemicals. Jormalainen *et al.* (2003) argued that it was the feeding of gastropods on the epiphytes growing on the surface of the algae that caused an indirect increase in phlorotannin concentrations. The carbon:nitrogen ratio, which is presumed to affect phlorotannin production, was thought to be affected through increased light penetration upon the removal of shading organisms, as well as the decrease in nutrient uptake onset by the cutting of hyaline hairs upon feeding, thus creating conditions

under which growth is limited and resources can be allocated to carbon-based chemical defences (Bryant *et al.*, 1983).

### 1.2.6. Reactive oxygen species (ROS) scavengers

While the production of reactive oxygen species (ROS) is a normal part of algal metabolism, their accumulation can be detrimental if they reach a level that surpasses the alga's ability to quench them (Lesser, 2006). Several studies have shown that besides oxygenic photosynthesis, most environmental factors contributing to physiological stress such as excessive irradiation and UVR exposure (Dahms and Lee, 2010), freezing (Kendall and McKersie, 1989), desiccation (Burritt *et al.*, 2002), hypersalinity levels (Kumar *et al.*, 2010), heavy metals (Pereira *et al.*, 2009) and wounding (Weinberger *et al.*, 2002), all of which may accelerate the cellular formation of reactive oxygen compounds such as superoxide radical ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($HO^{\bullet}$). Under such conditions, accumulative ROS become auto-destructive, disrupting normal functioning of photosynthesis and other vital metabolic systems through the oxidation of lipids, proteins and nucleic acids (Lesser, 2006).

*In vitro* analysis has suggested that in brown algae, phlorotannins have been reported to function as potent biological antioxidants. Their strong radical scavenging ability has been reported to be associated with their unique molecular skeleton, being highly related to phenol rings which act as electron traps to scavenge radicals (Ahn *et al.*, 2007). Many studies applying *in vitro* assays to determine the antioxidant activity of both crude phlorotannin extracts and individual structures, observed a high capacity for inhibition against a range of oxidants. In fact, phlorotannins have been suggested to be even more efficient radical scavengers than some polyphenols derived from terrestrial plants due to the higher abundance of interconnecting aromatic rings (Hemat, 2007).

The antioxidant activity of phlorotannins is largely concentration-dependent with several studies finding a positive correlation between radical inhibition and phlorotannin levels (Kang *et al.*, 2004; Tierney *et al.*, 2013; Wang *et al.*, 2012). However, it is also suggested to be reliant on the chemical composition. The activity of phlorotannins has been suggested to be heavily influenced by the degree of

polymerisation, with species possessing higher molecular weight phlorotannins generally displaying a greater level of radical scavenging. For example, *A. nodosum* and *F. spiralis* were reported to have high radical scavenging activity owing to the presence of high molecular weight phlorotannin structures (Audibert *et al.*, 2010; Ferreres *et al.*, 2012). This is thought to be due to the higher abundance of phenol rings and thus more attached hydroxyl units that are available for electron donation and thus radical stabilisation. Conversely, Heffernan *et al.* (2015) found in <3.5 kDa semi-purified phlorotannin fractions that *F. vesiculosus*, a species with a high abundance of lower molecular weight structures had significantly higher activity compared to *F. serratus* and *H. elongata,* two species with much higher molecular weight phlorotannins, concluding that it may be that a lower degree of polymerisation, less hydroxyl units are involved in monomer-monomer linkages and therefore there are more free for electron donation.

However, many reports exist in which no clear relationship between activity and molecular weight was found. Wang *et al.* (2012) found no distinct association between antioxidant capacity and the degree of polymerisation of phlorotannins in *F. vesiculosus*. Similarly, Cerantola *et al.* (2006) reported two isolated phlorotannin structures from *F. spiralis*, differing in their structural complexity, to be equal in their scavenging ability. More recent studies seem to indicate that it is the number and arrangement of the hydroxyl groups in the phlorotannin frame that influences their antioxidant potential (Ferreres *et al.*, 2012; Shibata and Ishimaru, 2008). Li *et al.* (2009) found phlorotannin structures with more hydroxyl groups to be more active than those with fewer. Furthermore, they concluded the various linkages between the phloroglucinol monomeric units play an important role. For example, they found eckol classed compounds possessed a heightened activity due to the unique linkages between the monomeric units. These conflicting results suggest the relationship between their structure and radical scavenging activities still remains unclear due to the complexity of phlorotannin chemistry and so further research is required before becoming elucidated.

### 1.2.7. Metal Chelators

Several metals are essential micronutrients required for algae development. However, when their levels exceed that of which is needed, the metals can interfere with the

cellular metabolism and become toxic (Bertrand and Poirier, 2005; Clemens, 2006). Therefore, the range between metal deficiency and toxicity is rather narrow. To ensure a state of homeostasis is maintained, plants and algae have developed several regulatory mechanisms in which both physical and physiological adjustments protect against metal hyper-accumulation. Such methods include: 1) changes to root pH levels to depress metal mobility (Jackson *et al.*, 1990), 2) the alteration of the membrane potential to limit metal uptake (Kenderesová *et al.*, 2012), 3) complexation with metal-binding cell wall compounds (Bringezu *et al.*, 1999; Kupper *et al.*, 2000) and/or 4) sequestration into intracellular vacuoles for inactivation (Bertrand and Poirier, 2005). However, at high concentrations, such methods are rendered insufficient as the influx of metals is too great. The accumulative metals can become involved in chemical reactions propagating the formation of ROS (Fenton reaction), triggering oxidative stress. Therefore, alternative and/or additional mechanisms are required to deter these excess metals.

Phlorotannins have been suggested to be involved in metal chelation, both extracellularly in surrounding seawater (Connan and Stengel, 2011; Gledhill *et al.*, 1999) and intracellularly in the physodes (Lignell *et al.*, 1982). Lignell *et al.* (1982) reported the physodes of *F. vesiculosus* to contain cadium as well as the outer-cell layers using x-ray micro-analysis. Connan and Stengel (2011) observed an increase in cell wall-bound phlorotannins and exudation levels associated with elevated copper levels. It is though that exudates observed in metal contamination studies, offer protection by binding to the metal ions detoxifying them, reducing the negative impact they may incur. However, Toth and Pavia (2000) found no effect of copper on the phenolic content of *A. nodosum.* Although this may suggest that the effect is concentration-dependent as the copper concentrations used here were 10-100 times lower than in Connan's and Stengel's investigation. Wang *et al.* (2009) investigated the role of metal chelation in the antioxidant activity of several brown algae, concluding phenolic compounds as ineffective chelators, suggesting that perhaps the synergistic effects of other scavenging compounds (in crude extracts) may cause an overestimation of phlorotannins abilities to chelate metals.

### 1.2.8. Factors controlling phlorotannin abundance

One of the obstacles in phlorotannin research, to date, is largely associated with the high degree of variability observed *in situ*, making an arduous task of determining the exact parameters that drive their metabolism. Due to the vast range of ecological function they possess, as discussed above, their levels are continuously varying in response to several stimulants and are thought constantly "in a state of flux" (Koivikko *et al.*, 2005). Their variability can be seen on numerous levels. For example, they are highly differentiable between brown algal species occupying the same shore. Previous studies showing the Fucales to possess the highest concentrations, constituting up to 12% DW in some species, while the Laminariales possess the lowest with observed levels ranging between 0.1-1% DW (Connan *et al.*, 2004; Targett *et al.*, 1992). Furthermore, phlorotannin levels within a given species show a high degree of phenotypic plasticity with both seasonal and temporal variability reported. For instance, certain species from the temperate and tropical Atlantic have been reported be more abundant in phlorotannins, with concentrations of up to 5-12% DW, whereas populations from the tropical Indo-Pacific possess characteristically lower levels, rarely exceeding 3% DW (Targett *et al.*, 1992).

This is further complexed as while being integral to the constitutive defence of a species, these compounds are also considered as inducible, triggered by several environmental stimuli (as seen above). Their induction can occur in response to several parameters of both biotic and abiotic origin, all impacting on their levels on both a diurnal, seasonal and spatial scale. Some of the more prominent factors reportedly linked with their variability are: irradiance (Abdala-Díaz *et al.*, 2006; Connan *et al.*, 2007; Gomez and Huovinen, 2010), nutrient availability (Hemmi *et al.*, 2005; Koivikko *et al.*, 2008; Van Alstyne and Pelletreau, 2000), salinity (Connan and Stengel, 2011; Munda, 1964; Pedersen, 1984), herbivore intensity (Lüder *et al.*, 2004; Pavia and Toth, 2000; Van Alstyne *et al.*, 2001). Moreover, even within an individual algal specimen, the phlorotannin content can differ. The distribution within any individual seaweed is reportedly dependent on the plant size (Denton *et al.*, 1990), age (Pedersen, 1984), type (Connan *et al.*, 2006; Steinberg, 1984; Tugwell and Branch, 1989) as well as reproductive status (Plouguerné *et al.*, 2006; Ragan and Jensen, 1978). Therefore, it appears both extrinsic and intrinsic factors control their *in vivo* concentrations.

## 1.3. Methods employed for phlorotannin analysis

Aside from a few studies employing microscopic analysis (e.g. Halm *et al.*, 2011; Schoenwaelder and Clayton, 1998; Schoenwaelder, 2002), most ecological studies investigating phlorotannins, and their variability, have been reliant on the quantification of their concentrations (Pelletreau, 2008), by means of colorimetric measurement of total phenolic content (Amsler and Fairhead, 2006). The lack of more sophisticated analytical methods is largely due to high reactivity and polarity of phlorotannins coupled with their high molecular size and structural similarity to one another (Tierney *et al.*, 2014).

Additionally, variability in reported values for phlorotannins results from the inconsistencies between methods chosen for quantification, conditions in which samples are prepared, choice of solvents for extraction, cofactors used in the extraction and the desired phlorotannin fraction to extract and reference standards used. Small changes in these variables can result in large differences in recorded phlorotannin values, thus preventing intra-study comparisons (reviewed in Amsler and Fairhead, 2006; Arnold and Targett, 2003; Pelletreau and Targett, 2008). Here, we discuss both the colorimetric and mass spectrometric methods employed in this study for the quantification, molecular profiling and assessment of the antioxidant activity of phlorotannins.

### 1.3.1. The Folin-Ciocalteau assay for the quantification of total phlorotannin content

The most common method used to quantify phenolics was initially developed by Folin and colleagues to study the metabolism of proteins in humans Folin and Denis (1912) and then further improved by Folin and Ciocalteau (1927). The reaction is based on the transfer of electrons, from phenolic compounds, in an alkaline environment, to the phosphomolybdic - phosphotungstic acid, resulting in a blue-coloured complex that can be measured at 735 nm (Fig. 1.6; Prior *et al.*, 2005; Singleton and Rossi, 1965). The maximum absorption is dependent upon the concentration of phenolics within the sample (Fig. 1.6; Blainski *et al.*, 2013).

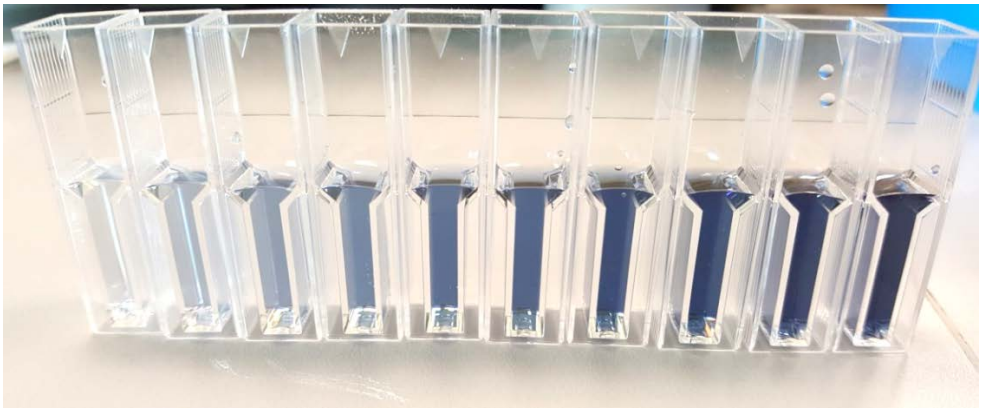
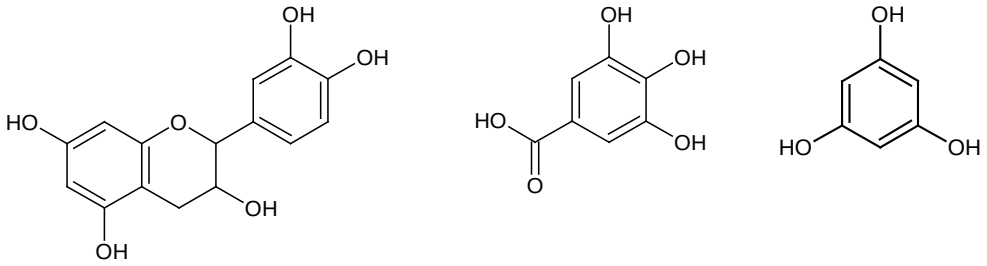


**Fig. 1.6.** Concentration-dependent colour change during a Folin- Ciocalteu (FC) assay to determine the total phenolic content (TPC) of a semi-purified phlorotannin extract. Picture taken by Dara Kirke.

Comparative quantification is then calculated through the use of a standard compound. Most commonly, gallic acid or catechin, are used as reference standard compounds when quantifying polyphenols (Fig. 1.7). However, the specific phenolics within an extract and their structural integrity should be considered when selecting a compound for standardisation. For the quantification of phlorotannins, phloroglucinol (1,3,5 – trihydroxybenzene), the monomer upon which phlorotannin polymers are synthesised, has been commonly accepted, due to its structural similarity to the target compounds (Fig.1.7; Arnold and Targett, 2003; Connan *et al.*, 2007; Connan *et al.*, 2006; Stern *et al.*, 1996).

It must be noted that this method is non-specific for phenolics and thus detects all hydroxyl containing groups in the extract, desired or otherwise, particularly sugars, aromatic amino acids and ascorbic acids (Dai and Mumper, 2010; Singleton *et al.*, 1999). Furthermore, interfere can also stem from the presence of inorganic substances within the extract, a detailed list of such compounds is described in Prior *et al.* (2005).This interference can have an additive effect with the detection of other reducing substances, contributing to the total value (Dai and Mumper, 2010) and causing an overestimation of phenolic content. Alternatively, the presence of such compounds can also act to dilute the concentration of phenolics in a crude extract and so reduce the content detected.




Catechin ($C_{15}H_{14}O_6$) | Gallic acid ($C_7H_60_5$) | Phloroglucinol ($C_6H_3O_3$)

**Fig. 1.7.** Chemical structures of varying tannin monomers used as standards in quantifying phenolic content. Adapted from Salminen and Karonen (2011) and redrawn using Chemsketch (ACD/Labs, Toronto, Canada).

This highlights the need for purification steps before quantification is carried out. If such corrective steps are taken prior to its employment, this method can be an ideal and simplistic assay for the estimations of phlorotannin levels. Overall, there are many advantages associated with this assay. Firstly, it is operationally simple and convenient, being applicable in a common laboratory environment. The results are obtained cheaply and quickly with high reproducibility (Blainski *et al.*, 2013; Huang *et al.*, 2005; Prior *et al.*, 2005; Stratil *et al.*, 2006).

### 1.3.2. Phlorotannin characterisation

Although colorimetric assays, such as the Folin-Ciocalteu (FC) assay, are capable of detecting variations in phlorotannin levels between extracts, these colorimetric assays give little to no information on the chemical composition of individual oligo-polymers. The activity of phlorotannins has been suggested to be affected by the degree of polymerisation, indicated by the molecular weight, and the structural arrangement of the phlorotannin frame, indicated by the degree of isomerisation (Ahn *et al.*, 2007; Boettcher and Targett, 1993). Thus current research is now more focused on the use of innovative techniques to extract, isolate and identify individual phlorotannins and their structures so as to better evaluate their biological and ecological value (Audibert *et al.*, 2010; Ferreres *et al.*, 2012; Heffernan *et al.*, 2015; Steevensz *et al.*, 2012; Tierney *et al.*, 2014). Such analytical techniques include both liquid chromatography and mass spectrometry.

#### 1.3.2.1. Liquid chromatography – a brief introduction

Liquid chromatography (LC), in the simplest of definitions, is “the separation of components from a mixture based on the rate at which they elute from a stationary

phase, typically over a mobile phase" (Allwood and Goodacre, 2010). The principle of liquid chromatography is based on the flow of a mobile phase *i.e*. liquid solvent, containing the sample mixture, through a stationary phase *i.e*. paper in thin-layer chromatography (TLC), or a column filled with a solid adsorbent material in column chromatography. Each component within the sample interacts differently with the stationary phase, depending on its chemistry. For example, in column chromatography, some components will be more attracted to the mobile phase and so elute through the column quicker while others may be more attracted to the stationary phase, retaining on the column for longer and thus eluting much slower *i.e.* they vary in their retention times (Allwood and Goodacre, 2010). Column chromatography e.g. solid-phase extraction, commonly employs gravity or the use of a vacuum to pull the mobile phase through columns that are unable to withstand high pressures. These columns employed large solid-phase particles (>50 μm) with low flow resistance in order to prevent the build-up of pressure.

The introduction of high performance liquid chromatography (HPLC) was classified with improvements three-fold; the use of columns packed with smaller particle sizes (<10μm) improved chromatographic separation by an overall increase in solid-phase surface area. However, this would result in a greater flow resistance and so the employment of pumps capable of generating high pressures (500 psi) was implemented to drive the mobile phase through the column (Nováková *et al.*, 2006). Finally, the new tightly packed columns were encased in metal, making them capable of enduring such high pressures (Allwood and Goodacre, 2010). These modifications have made HPLC one of the most utilised tools in analytical chemistry, to date. The mechanism behind a HPLC system is illustrated in Fig. 1.8.

HPLC has been commonly employed for the separation of phlorotannins (Koivikko *et al.* 2007; Wang *et al.*, 2012) with both reverse-phase (RP) and normal-phase (NP) - LC being employed. Both methods work on the basis of adsorption chromatography – dependant on the interaction of a metabolite with a silica packed column, composed of either polar or non-polar particles.

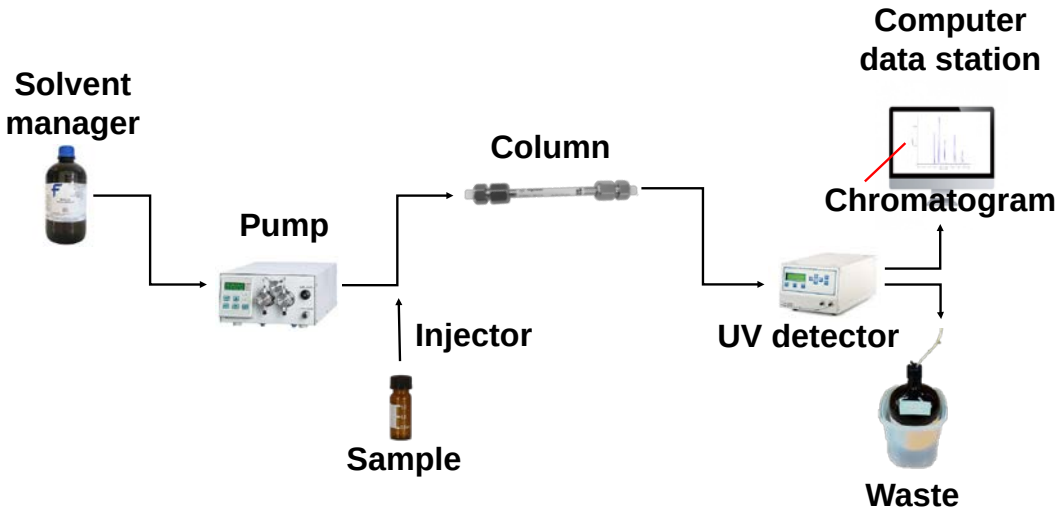



**Fig. 1.8.** Schematic illustration of the process through which a sample is analysed through a HPLC system. Adapted from Allwood and Goodacre (2010).

NP-LC involves the use of a polar stationary phase and an elution gradient beginning with a non-polar mobile phase, removing non-polar compounds first and polar compounds thereafter. RP-LC, is the absolute reverse of NP (Snyder *et al.*, 1997). Koivikko *et al.* (2007) assessed the use of both reverse-phase (RP) and normal-phase (NP) HPLC in the analysis of phlorotannins, determining that normal phase gave a more effective separation of shorter oligomers in *F. vesiculosus* purified extracts, yet a large "hump" was observed for larger phlorotannin polymers. This was also the case for Wang *et al.* (2012) who also observed a large "hump" in the *F. vesiculosus* chromatogram, indicating no separation had occurred.

Ultra-Performance Liquid Chromatography (UPLC) was developed to endure much higher system back-pressures (15,000 psi) than in conventional HPLC systems (500 - 6,000 psi), allowing the use of shorter, narrower columns with much smaller particle sizes (< 2 µm). These adjustments gave rise to increased sensitivity, better resolution and shorter, more efficient analytical times (Nováková *et al.*, 2006). Further modifications to the injection cycle and the type of detector used can increase sensitivity even further (Gumustas *et al.*, 2013).

### 1.3.2.2. Mass spectrometry (MS) - a brief introduction

Mass spectrometry is a powerful analytical technique offering both qualitative and quantitative information regarding the analyte under examination. This technique is commonly applied in order to identify unknown compounds, quantify selected compounds within a mixture and elucidate on the chemical and structural properties

of a given compound (Watson and Sparkman, 2007). A mass spectrometer is composed of three basic components; an ion source, a mass analyser and a detector, operating under high vacuum conditions (Fig. 1.9).

Briefly, a sample is introduced into the ion source where it is ionised *i.e.* converted into charged particles, ions. These ions are then passed through an electrical or magnetic field. The speed at which the ions travel and/or the degree of deflection, within the mass analyser is dependent on the ions mass (*m*) to charge (z) ratio (*m/z*), that is, the mass of the ion divided by the number of charges it possesses (Fig. 1.9). The *m/z* is the principle factor affecting the manner in which ions can be separated and subsequently detected. The detector records the current produced by an ion when it passes by or hits a surface (Fig. 1.9; Ho *et al.*, 2003).

Amplification is required for a signal to be detected as the number of ions leaving the mass analyser at one particular time is often too small. The output is a graphical representation of the ion intensity, a mass spectrum. The x-axis is the mass-to-charge ratio, *m/z* (dimensionless value) while the y-axis represents the intensity (%). The most intense peak is called the base peak and is assigned the relative abundance of 100%. The abundance of all other peaks within the spectrum are represented relative to the base peak and so are represented as percentages of the base peak (de Hoffman and Stroobant, 2007).

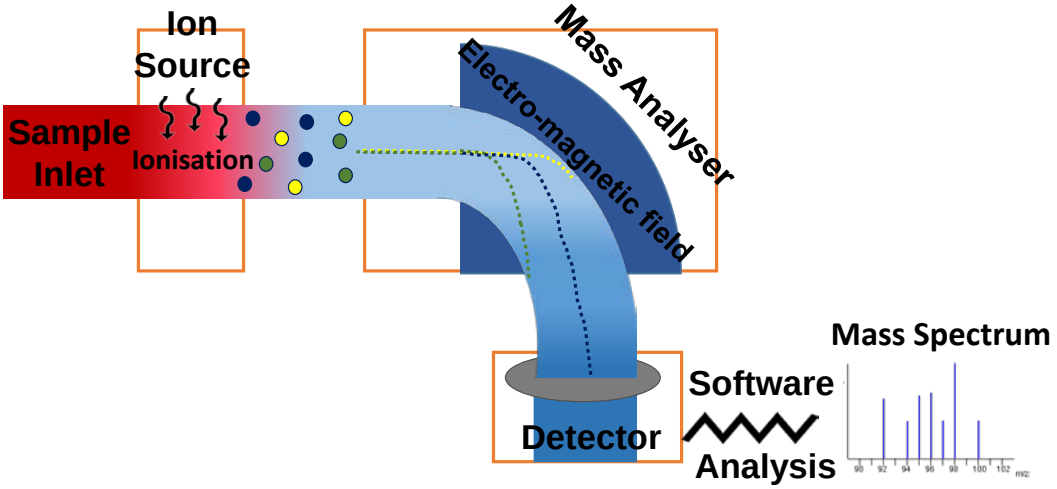



**Fig. 1.9.** Schematic Illustration of the fundamental processes that occur within the three main components of a mass spectrometer (MS). Adapted from Gross (2004).

### 1.3.2.3. Tandem mass spectrometry (MS/MS)

A tandem mass spectrometer, often denoted by MS/MS or $MS^2$, is one capable of multiple, sequential MS steps (Fig. 1.10). In MS/MS, ions are formed through the ion source and separated by their *m/z* in the first mass analyser. Thereafter, ions of a selected mass-to-charge ratio (precursor ions) undergo fragmentation into smaller (product) ions by collision with neutral gas atoms, most commonly nitrogen, argon or helium (Madeira and Florencio, 2012). When the accelerated ions collide with inert gas atoms, some of the kinetic energy is converted to internal energy leading to bond dissociation. This process is referred to as collision-induced dissociation (CID; Fig. 10). These product ions are then mass-analysed in the second MS (McLafferty, 1980).

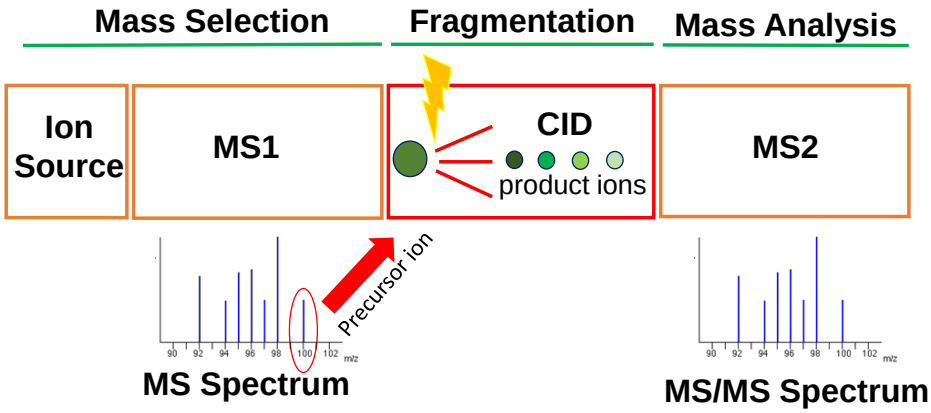



**Fig. 1.10.** Schematic Illustration of tandem mass spectrometry (MS/MS) whereby precursor ions undergo fragmentation to form product ions for mass analysis. Adapted from McLafferty (1980).

### 1.3.2.4. Selection of appropriate MS methods for phlorotannin analysis

#### 1.3.2.4.1. Electrospray ionisation

The selected method of ionisation will differ depending on the state of the sample under investigation *i.e.* solid, liquid or gas, as well as the chemistry of the target compound(s) (Gross, 2004). Previous studies on phlorotannin characterisation, have highlighted the effectiveness of electrospray ionisation (ESI) (Tierney *et al.*, 2014; Heffernan *et al.*, 2015), particularly due to the ease of transition it offers between LC and MS analysis.

The introduction of electrospray ionisation (ESI) by Yamashita and Fenn in 1984 allowed for a simple and capable interface between the liquid phase of LC systems

and the required gas phase of mass spectrometers (Pitt, 2009). The transfer of sample ions by ESI involves the dispersal of a fine spray of charge droplets, followed by solvent evaporation and finally ionic ejection from the highly charged droplets (Fig. 1.11). ESI applies a high voltage (2.5-6 kV) in a metal capillary through which the liquid sample flows, resulting in the transfer of ions from a liquid to gas phase (Ho *et al.*, 2003) which results in a mist of highly charged droplets being generated at the exit of the electrospray tip (Fig. 1.11). An elevated ESI-source temperature coupled with a stream of nitrogen drying gas leads to solvent evaporation resulting in an increase of surface charge density and a decrease of the droplet radius *i.e*. the charged droplets are continuously reducing in size. Finally, the electric field strength within the charged droplet reaches a critical point at which it becomes possible for the ions at the surface of the droplets to be ejected into the gaseous phase via electrostatic repulsion (Ho *et al.*, 2003). The emitted ions are then accelerated through the mass analyser where they are subsequently analysed for both molecular mass and measurement of ion intensity.

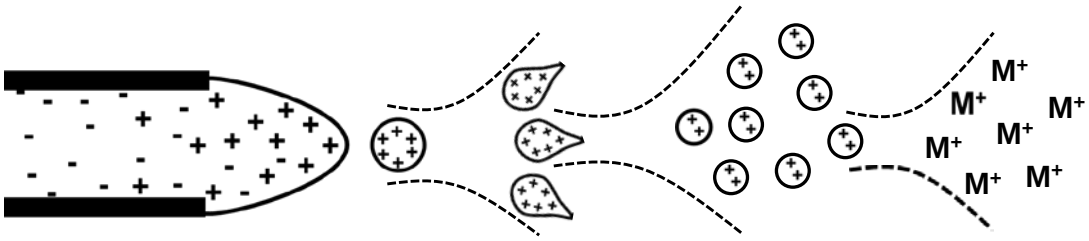



**Fig. 1.11**. Mechanism of electrospray ionisation. Adapted from Ho *et al.* (2003).

ESI is a "soft ionization" technique due to the relatively low degree of energy imparted on the sample, resulting in little fragmentation (Pitt, 2009). However, this can be counteracted with the use of tandem mass spectrometry. Another advantage of ESI, particularly for phlorotannin mass analysis, is the broad range of molecular mass that can be analysed due to the characteristic multiple charging (Gross, 2004; Smyth *et al.*, 2015).

#### 1.3.2.4.2. Triple (tandem) quadrupole (QQQ) mass analyser

There are multiple different ways through which mass spectrometers can separate and analyse ions of different masses. Relative to phlorotannin detection and characterisation, both time of flight (ToF) and triple (tandem) quadrupole (QQQ) mass analysers have been employed (Tierney *et al.*, 2013; Tierney *et al.*, 2014).

A quadrupole mass analyser consists of four conducting rods arranged parallel to one another, with opposing rod pairs being electrically connected to each other (Fig. 1.12). This type of mass analyser separates/filters ions based on the stability of their flight trajectory through an oscillating electric field, created by a radio frequency (RF) voltage applied between one rod pair and a direct current (DC) offset voltage between the other (Pitt, 2009). Only ions with a certain *m/z* will have a stable flight through the quadrupole of a given voltage combination, while all other ions will have unstable trajectories and collide with the rods, not reaching the detector (Fig.1.12). However, as only ions of a narrow *m/z* range will successfully pass through the quadrupole to the detector, single quadrupole mass analysers do not provide a large amount of structural information

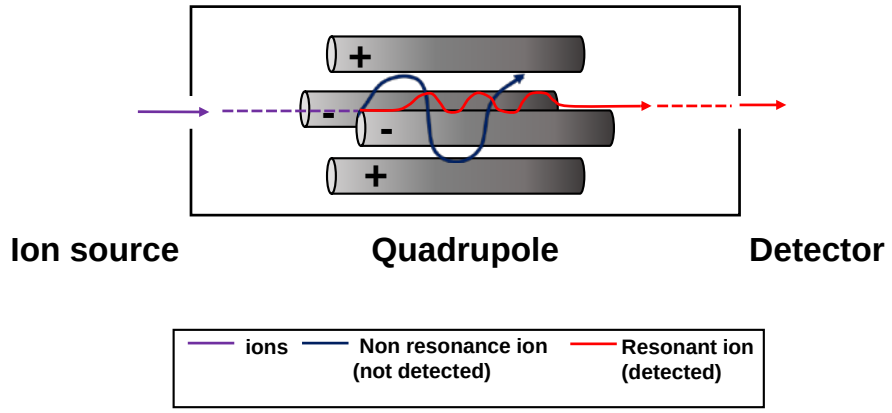



**Fig. 1.12.** Ion separation in a single quadrupole mass analyser. Adapted from Ho *et al.* (2003).

A triple (or tandem) quadrupole mass analyser employs the principle of quadrupole mass filtration but in tandem sequence for heightened ion separation, as described above in Section 1.3.2.3. Triple quadrupole mass analysers are composed of three quadrupoles in series. The first and third quadrupole act as mass filters as described above while the second (and middle) quadrupole, possessing radio frequency only, functions as a "field-free region" in which CID occurs (Fig. 1.13; Gross, 2004).

Multiple Reaction Monitoring (MRM) is the most common mode of using a triple quadrupole MS for quantitative analysis, allowing enhanced sensitivity and selectivity. Within such a process, the first and third quadrupoles can be optimised to filter for different, selected *m/z* values, with various precursor/product ion combinations being created, allowing for the specific detection of a large number of targeted analytes (Madeira and Florencio, 2012). The first quadrupole (Q1) filters a

specific precursor ion of interest. Within Q2, the collision cell, optimised parameters ensure that CID generates characteristic product ions which are then transferred into the third quadrupole (Q3). Here, only a specific *m/z* is allowed to pass with all other product ions being filtered out (Fig. 1.13; Pitt, 2009).

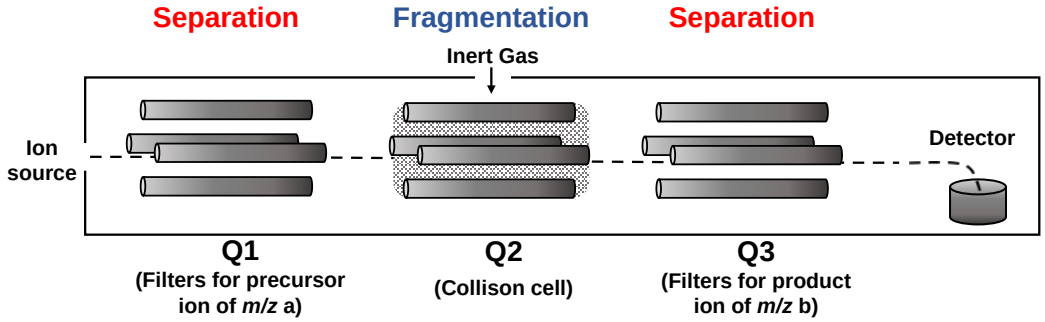



**Fig. 1.13.** Multiple monitoring mode (MRM) employed in a triple quadrupole mass analyser. Adapted from Pitt (2009).

The selective bias offered by QQQ-MS, in MRM, ensures that only desired analytes will be detected. While this can often be seen as an associated disadvantage, it is an ideal method for the characterisation of phlorotannins from semi-purified extracts. Furthermore, the high sensitivity offers more information regarding compound structure such as the degree of isomerisation for each pre-selected, targeted molecular ion transition (Tierney *et al.*, 2014; Heffernan *et al.*, 2015).

### 1.3.2.4.3. Time of flight mass (ToF) analyser

Conceptually, ToF analysers are the most simplistic mass spectrometers as unlike methods such as triple quadrupoles, ToF analysers do not employ complicated ion-focusing devices to deviate ions along a particular flight trajectory. Rather, ToF analysers separate ions by time without the use of an electrical or magnetic field (Allwood and Goodacre, 2010). Briefly, ions arrive to the mass analyser from the ion source possessing a similar initial kinetic energy. They are then accelerated through a high voltage along a field free drift path of a known length. Provided that all ions start their journey at the same time, the velocity of the ions, and hence the time taken to travel down a flight path, depends on their *m/z* values. As an ions velocity is mass-dependent, the mass of the ion can be determined by its time of flight *e.g.* lighter ions will travel at a high speed than heavier ones and so reach the detector at an earlier time (Fig. 1.14).

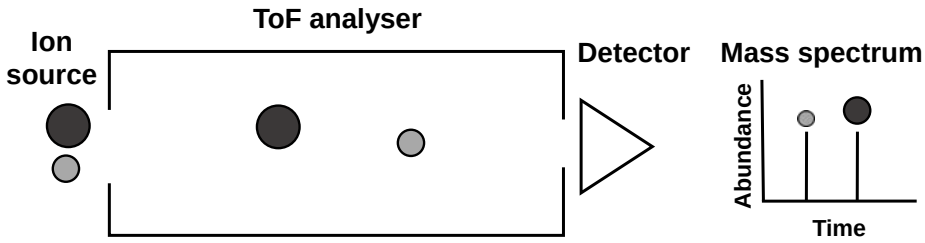



**Fig. 1.14.** The fundamental principle underlying a (linear) time of flight (ToF) mass analyser. Adapted from Allwood and Goodacre (2010).

Often a reflectron, commonly an ion mirror, is fitted to a linear ToF analyser (now orthogonal) to compensate for minor differences in the initial kinetic energy between ions of the same *m/z i.e.* ions of the same *m/z* that travel along a ToF tube at different velocities (Allwood and Goodacre, 2010). The reflectron consists of a series of ring electrodes of very high voltage placed at the end of the flight tube, where it functions as a focusing mirror (Fig. 1.15). Ions that travel into the reflectron are reflected in the opposite direction due to the high voltage. However, faster ions will travel further into the reflectron while slower ions will travel less. In this manner, ions of the same *m/z* value, regardless of kinetic energy, will reach the detector at the same time rather than at different times, increasing resolution by narrowing the bandwidth for a single *m/z* value (Fig.1.15; Gross, 2004).

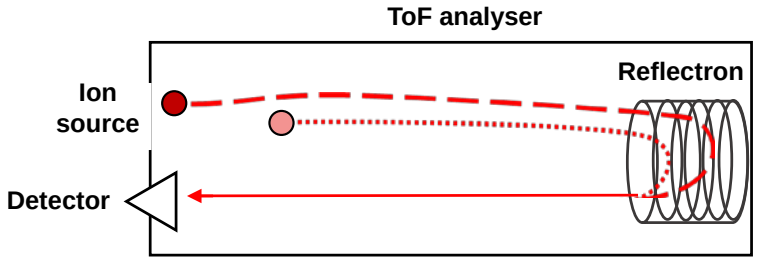



**Fig. 1.15.** The addition of a reflectron to an orthogonal ToF analyser to compensate for distribution of initial velocities between ions of the same *m/z*. Adapted from Allwood and Goodacre (2010).

The manner in which ToF-MS can rapidly acquire full mass spectral screening with such high precision and sensitivity make it an excellent choice for both qualitative and quantitative analyses across broad range of masses in complex matrices. In fact, it is often feasible to directly inject samples into the ion source without any chromatographic separation (Pitt, 2009). In recent studies, ToF-MS has been employed for the detection and identification of phlorotannin compounds with a semi-purified extract allowing for the additional assessment of sample purity (Tierney *et al.*, 2013).

### 1.3.3. Determination of *in vitro* antioxidant activity

Phlorotannin extracts are known to possess a high capacity for antioxidant activity, attracting a high level of commercial attention. A biological antioxidant can be defined as "any substance that, when present in low concentrations compared to those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate" (Halliwell and Gutteridge, 1995). At present, there are several methods practised to determine the antioxidant capacity of botanicals, all differing in their scientific approach; reagents, standards and oxidative substance (Dai and Mumper, 2010; Stratil *et al.*, 2006). The selection of a method must take into consideration, the overall purpose of the assay, the natural chemistry of the sample and the presence of interfering compounds as well as the reference standard used (Dai and Mumper, 2010). To date, the majority of studies have examined the activity of phlorotannin extracts using *in vitro* colorimetric assays.

There are two major categories of *in vitro* assays; hydrogen atom transfer (HAT) and single electron transfer (SET). HAT reaction occurs when an antioxidant component quenches a free-radical species through the donation of a hydrogen (H) atom (Craft *et al.*, 2012; Huang *et al.*, 2005).

$$X\bullet + AH \rightarrow XH + A\bullet$$

Whereby X• represents the free radical component and AH represents the anti-oxidant with a hydrogen for donation. Through this process, the free-radical becomes more stable and less likely to propagate further radical reactions (Craft *et al.*, 2012). The reactivity of the reaction is largely associated with the bond dissociation energy (BDE) of the antioxidants H-donating group. That is, the energy required to fracture a chemical bond to result in separated atoms. The greater the BDE, the less active a component will be in free-radical quenching reactions via HAT (Wright *et al.*, 2001). HAT reactions are pH and solvent independent and quite rapid, being complete in minutes (Prior *et al.*, 2005). HAT assays include: ORAC and β-Carotene bleaching.

Single electron transfer (SET) based reactions measure the capacity of an antioxidant in reducing an oxidant through the transfer of an electron. This reaction results in a colour change, the intensity of which is correlated with the concentration of the

antioxidant component in the sample (Craft *et al.*, 2012; Huang *et al.*, 2005; Prior *et al.*, 2005).

$$M(III) + AH \rightarrow AH^{+} + M(II)$$

Here, the oxidant M(III), in the presence of an antioxidant compound, has been reduced to M(II). As SET reactions occur through deprotonation, the ionization potential (IP) of an antioxidant compound is a parameter in determining its capability to scavenge free radicals, *i.e.* the amount of energy required to remove an electron from a molecule (Craft *et al.*, 2012; Huang *et al.*, 2013; Prior *et al.*, 2005; Wright *et al.*, 2001). The higher the amount of energy needed, the more reluctant an antioxidant compound is to donate an electron. SET reactions are often slow in their completion and so calculations are often based on percentage decrease rather than on kinetic curves as in HAT reactions (Prior *et al.*, 2005). SET assays include: FRAP and CUPRAC. Many assays exist in which both HAT and SET reactions are measured such as DPPH.

#### 1.3.3.1. Radical scavenging activity (RSA) against the radical DPPH•

DPPH• (2, 2-diphenyl-2-picrylhydrazyl) is a stable free radical due to the delocalisation of an unpaired electron (Molyneux, 2004). In the presence of a hydrogen donor, such as an anti-oxidant compound, DPPH• is reduced to a stable non-radical form, DPPH (diphenylpicrylhydrazine). This is associated with a change in colour of the solution from a deep violet to pale yellow, and the loss of absorption at 515 nm (Fig. 1.15; Brand-Williams *et al.*, 1995). Hence, the activity of the antioxidant compound can be determined by its ability to decrease its absorbance. Steric accessibility is reported to play an important role within this assay with smaller molecules having better access to the radical site which may cause underestimation of activity in extracts with larger molecules present (Prior *et al.*, 2005). Despite these drawbacks, this method is simple, rapid, inexpensive and highly reproducible giving rise to the use of DPPH as a useful reagent for investigating the free radical scavenging activities (RSA) of plant and food based extracts (Duan *et al.*, 2006).

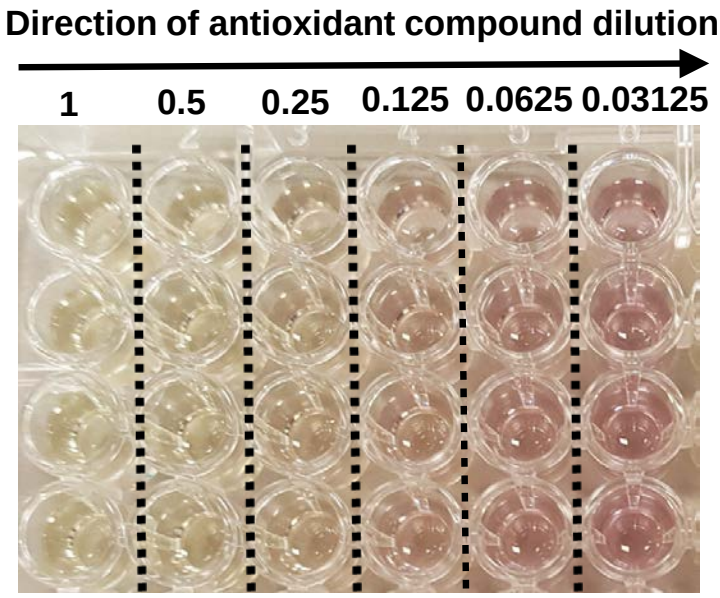



**Fig. 1.16**. The colorimetric reaction in which the radical DPPH• becomes stabilised in the presence of an antioxidant (phlorotannin extract) resulting in a colour change from purple to yellow, in a concentration-dependent manner. Picture taken Dara Kirke.

### 1.3.3.2. Ferric-reducing anti-oxidant power (FRAP).

This assay, too, is simple and inexpensive to perform and so has been used as one of the main *in vitro* determinants of antioxidant activity for a multitude of biologically-derived extracts (Benzie and Strain, 1996). In this assay trispyridyltriazine (TPTZ) forms a complex with the excess ferric ion to give rise to [Fe(III)-TPTZ] (Benzie and Strain, 1996). At low pH values (3.6), in the presence of an antioxidant, this complex is reduced to [Fe (II)]-TPTZ via electron transfer, forming an intense violet colour, which can be read at 593 nm (Fig. 1.17). The reduction that occurs is non-specific and so any compound within the extract that has a redox potential less than that of [Fe(III)-TPTZ] will cause its reduction to [Fe(II)-TPTZ]. Incubation time is an important factor for consideration when applying this assay. Some phenols have been reported to react rather quickly with the reagent within only 4 to 6 minutes needed for the reaction to be complete (Prior *et al.*, 2005). However, other polyphenols can take up to 30 minutes to be detected. These variations were concluded by Pulido *et al.* (2000) to be based on the degree of hydroxylation and their position within the compounds structure.

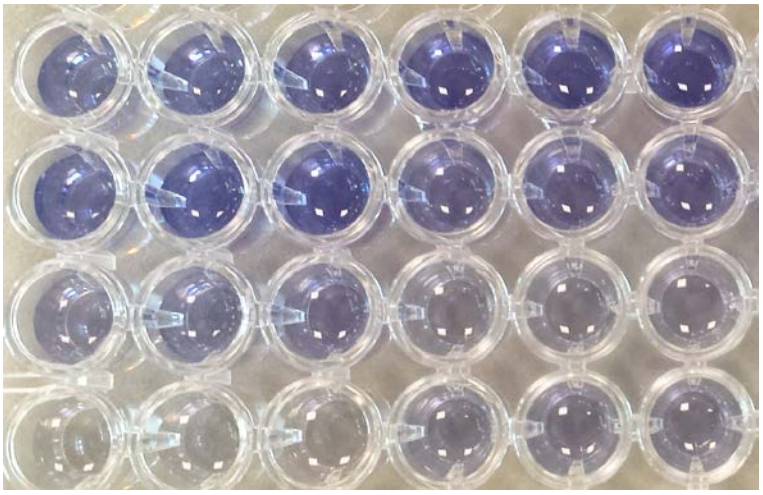


**Fig. 1.17**. The redox reaction between the FRAP reagent (Fe(III) and TPTZ) and an antioxidant compound (phlorotannin extract), resulting in a concentration-dependent formation of an intense violet colour. Picture taken by Dara Kirke.

## 1.4. The beneficial role of phlorotannins for human health

Chronic diseases, on a global level, are increasing exponentially. It is projected that by 2020, chronic diseases will account for a staggering 75% of deaths worldwide, with the three major contributors being heart disease, strokes and diabetes (WHO and FAO, 2003). Over the past fifty years, in developed countries, there has been a negative dietary shift to high calorie, high sugar, high saturated fat and low dietary fibre foods. Such poor diet choices coupled with a decline in physical activity, has led to chronic diseases such as obesity, diabetes mellitus and cardiovascular-related diseases being more prevalent than ever in our society.

Research has shown that nutrition has a strong effect, either positive or negative, on a person's level of health and that adjustments in feeding habits can reduce and/or even prevent the development of chronic diseases such as those above (Plaza *et al.*, 2008). Food has finally been recognized as a source of health improvement, so much so that an entirely new discipline of food research has been established for the development of health promoting food products, so called "functional foods". Although the concept of functional foods has many definitions and is still awaiting a universal terminology (Alzamora *et al.,* 2005), it can be perceived as a food product that demonstrates a beneficial effect on human health, aside from its basic nutritive value, to a satisfactory level. That can occur through the enhancement of a biological function to offer an improved state of well-being or in the prevention and/or treatment of a disease/ health disorder, all the while remaining within its normal dietary form, *i.e.* it cannot be a pill or capsule (Diplock *et al*., 1998; Roberfroid, 1999, 2002).

Now with a heightened awareness of the nutritional value of our food, consumer trends have started to slowly shift, with interests now leaning toward the use of more naturally-derived foods and ingredients that promote good health (Deliza *et al.*, 2003). In turn, to meet these demands, the investigation into and application of novel bioactive compounds in both food preservation and processing stages has grown dramatically. Whether it is in the search for natural alternatives to replace previously used synthetic, potentially harmful products and/or ingredients, as health-boosting additives in functional foods aimed at achieving and/or maintaining good health, or to specifically treat or prevent certain health disorders in nutraceutical products, plant-based secondary metabolites have been at the centre of the hunt (Cheynier, 2012;

Pandey and Rizvi, 2009; Scalbert *et al.*, 2005). Plant-derived secondary metabolites have received unprecedented industrial attention due to the high level of biological activity they demonstrate. Polyphenols, in particular, have received considerable attention within food research, owing to their high propensity for radical scavenging, highlighting their potential as preventive nutrients against an array of diseases including but not limited to, cancer, neurodegenerative and cardiovascular diseases (Pandey and Rizvi, 2009).

The search for beneficial bioactives has now expanded out toward the coastlines with many marine-derived compounds now recognised as valuable ingredients with potential food applications due to their potential health effects (Kim, 2012; Stengel and Connan, 2015). The role that seaweed and other marine-based foods plays in eastern diet and health, has highlighted marine-derived compounds as highly valuable ingredients due to the associated health benefits (Brown *et al.*, 2014). The consideration of phlorotannins as food ingredients is a novel concept as research on these compounds is still very much in its infancy. However, that said, research carried out to date highlights the potential of these polyphenols to promote good health and reduce or deter the occurrence of some of the most rampant health disorders in our society (Catarino *et al.*, 2017; Jung *et al.*, 2013; Lee *et al.*, 2012; Li *et al.*, 2011; Lopes *et al.*, 2012).

### 1.4.1. Health benefits and pharmaceutical applications – Diabetes Mellitus

The high bioactivity displayed by phlorotannins, *in vitro*, has led to an increase in research concerned with elucidating their potential in tackling some of the more prevalent health disorders facing modern society. For the purpose of highlighting their potential success in pharmaceutical products, we will specifically discuss their role in preventing and/or reducing the adverse health effects associated with diabetes mellitus.

Diabetes mellitus is a metabolic disease characterised by high blood glucose levels resulting from an irregularity in the production or absorption of insulin, the hormone responsible for blood glucose regulation. The disease can be classified into two forms; Type-1, which is characterised by the immunological destruction of pancreatic β cells resulting in insulin deficiency and, based on current knowledge, cannot yet be prevented (Daneman, 2006). Type-2 diabetes, is caused by the inability of target

tissues to uptake insulin efficiently leading to elevated hepatic glucose production and hyperglycaemia (DeFronzo, 1999). While the disorder is known to arise from both genetic and environmental factors (Neel, 1962), excess body weight linked with obesity has been considered the most common factor contributing to the prevalence of the disease (Goran *et al.*, 2003; Stern *et al.*, 1992).

The management of the disease involves the tight control and maintenance of normal blood glucose levels. While there are several synthetic drugs currently used in the treatment of diabetes, their continued use should be limited due to the associated adverse gastro-intestinal effects such as abdominal cramps, flatulence and diarrhoea (Kellogg *et al.*, 2014; Krentz and Wheeler, 2005). This has led to the interest in utilising naturally-derived compounds. Phlorotannin-enriched extracts have been highlighted as potential alternatives as a result of their capability to lower blood glucose levels, through a range of mechanisms involving their ability to modulate the activity of the principal enzymes involved in carbohydrate digestion, uptake and metabolism (Kang *et al.*, 2013; Kellogg *et al.*, 2014; Lee *et al.*, 2010; Lopes *et al.*, 2016; Okada *et al.*, 2004).

The observed activity of phlorotannins is thought to reside with their binding properties of the hydroxyl units, as seen with the protein-polyphenolic complexes they form (Hagerman *et al.*, 1998). Perhaps through similar hydrogen bond formations and hydrophobic interactions the hydroxyl units alter the active site dynamics, affecting the enzymes bioactivity (Heo *et al.*, 2008). The inhibitory effects are largely owed to the hydrogen bonds that form between the hydroxyl groups of the phlorotannins and the carboxylate groups of catalytic residues at the active site of the enzyme, in a similar manner to that of flavonoids (Piparo *et al.*, 2008). Okada *et al.* (2004) concluded that the activity of phlorotannins was positively correlated to molecular weight and the number of free hydroxyl units. The anti-diabetic effects of phlorotannins are numerous, displaying a heightened level of activity compared to the currently used commercial compounds, without any cytotoxic effects. This highlights them as promising natural alternatives to replace synthetic drugs in the future.

### 1.4.2. Cosmeceuticals

Cosmeceuticals are cosmetic products with biologically active ingredients claiming to have medical or drug-like benefits while still satisfying the needs of beauty and health (Gao *et al.,* 2008). Recent trends in research suggest that phlorotannins from brown algae could be ideal naturally bioactive ingredients for the integration into cosmeceutical products (Sanjeewa *et al.,* 2016; Thomas and Kim, 2013; Wijesekara *et al.,* 2010).

Human skin acts as a protective barrier shielding us from external pathogens and environmental damage. However, whilst doing it so it is left vulnerable to UV radiation and its consequential photo-oxidative stress (Heo *et al.,* 2009; Pallela *et al.,* 2010). Chronically UV-irradiated skin is often associated with abnormal cutaneous reactions, including DNA damage, carcinogenesis, epidermal hyperplasia, accelerated collagen breakdown and inflammatory responses (Armstrong and Kricker, 2001; Heo *et al.,* 2009). Melanin is essential for protecting human skin against radiation but the accumulation of abnormal melanin induces pigmentation disorders such as melasma, freckles and senile lentigines (Kim and Uyama, 2005; Wang *et al.,* 2006).

Melanogenesis is controlled by tyrosinase a key enzyme in melanin synthesis (Aroca *et al.,* 1993). Tyrosinase catalyzes two oxidative reactions leading to the production of quinones, highly reactive compounds that react with proteins and amino acids causing the formation of brown pigments (Kang *et al.,* 2004). Yoon *et al.* (2009) observed that phlorotannins derived from an ethyl acetate extract from *Ecklonia cava* had a marked inhibitory effect on on mushroom tyrosinase, with 7-phloroeckol being quite potent in its anti-tyrosinase activity with $IC_{50}$ values of 0.85 µM, more effective than the positive controls of arbutin (243.16 µM).

Cha *et al.* (2012) applied the photoprotective function of phlorotannins to zebrafish embryos, they discovered that not only did pre-treatment with phlorotannins (triphlorethol A and eckstolonol) significantly reduce UVB induced oxidative damage, cell death, melanin content and hyperpigmentation but did so while causing no adverse effects on the host themselves. Similarities to mammalian skin make the zebrafish a good alternate model as effects on human cells had not yet been carried out. However, Jang *et al.* (2012) carried out further studies on eckstolonol isolated from *E. cava* on human keratinocytes and verified its antioxidant defence against UVB induced

reactive oxygen species (ROS). A dose of 200µM of eckstonol was sufficient in increasing cell viability and decreasing ROS production by UVB irradiation. These studies show the promising potential phlorotannin extracts have for the application in the cosmeceutical field in photoprotective and anti-aging effects in skin, even in the skin-whitening in eastern Asia, which has become a fashionable trend, of late (Heo *et al.*, 2009).

### 1.4.3. Phlorotannin commercial applications: role in expanding Irish industry

The global seaweed industry is estimated to be worth over US$ 6 billion, 90% of which is owed to Asian cultivation (Bixler and Porse, 2011). Of the total biomass harvested and cultured, over 83% is for human consumption (Craigie, 2011). At present, Ireland accounts for a very small portion of the global industry (Morrissey *et al.*, 2011) and is very much underdeveloped, predominantly characterised by high volume, low value products that yield low returns such as those in agricultural applications (Walsh and Watson, 2011). Nutraceutical, pharmaceutical and cosmeceutical applications offer high value-added products for consumers with low biomass requirements yet generate high revenue.

Yet, this area remains very much underexplored in Ireland with only a small percentage of the industry involved. For example, currently in Ireland roughly 70% of the overall revenue is generated by the agri-products, requiring nearly 100% of the resources attained while the other 30% generated value is attributed to therapeutics, in which only ~ 1% of the raw material is used (Walsh and Watson, 2011). This highlights an area of missed opportunity upon which the industry should focus more of its efforts on. This underutilisation has been acknowledged and has been corroborated as a key area of research in the Marine Functional Foods Research Initiative (NutraMara); an initiative established by the Marine Institute and the Department of Agriculture, Food and the Marine (DAFM) for the development of marine-based functional food ingredients. It coincides with the incentive given to the Irish seaweed industry to increase its worth from €18 million per annum to €30 million per annum by 2020, under the Sea Change Strategy (2007-2013).

Particularly as a north Atlantic island nation, Ireland stands at an advantageous position being surrounded by coastlines dominated by brown algae, the exclusive source of phlorotannins. While research on phlorotannins is still very much in its

infancy, there is much evidence to support a successful integration of these bioactives into highly profitable sectors such as those in pharma- and nutraceuticals. The application of phlorotannins has the ability to be advantageous to both our health as well as our economy. However, to do so, more scientific information regarding their metabolism, stability and reactivity is crucial.

Although the introduction and employment of such innovative methods has accelerated the rate of progress on phlorotannin research, in a relatively short amount of time, there still remains a need for a standardised method for their extraction, isolation and evaluation, if their full exploitation is to be achieved. By doing so, cross comparison of studies will finally be possible, furthering the progression that has already been made in this area of research toward a future in which phlorotannins can be potentially used as food ingredients, benefiting human health.

## 1.5. Project Objectives

Phlorotannins show promising potential for the application in an array of therapeutic fields beneficial to human health. High levels of antioxidant, anti-inflammatory, anti-diabetic and anti-microbial activity have attracted an unprecedented level of commercial interest leading to an exponential increase in the research carried out on elucidating the parameters responsible for such high activity. Their industrial application, to date, has been hindered partially due to the difficulty their structural similarity and complexity pose, preventing adequate separation and isolation and thus subsequent characterisation. Today, such limitations can be tackled with the development of purification methods and analytical tools that offer increased sensitivity. Previous studies have demonstrated how the purification of low molecular weight (LMW; <3.5 kDa) phlorotannins can enhance the observed *in vitro* activity, highlighting such fractions as ideal for the development of commercial products. Yet there remain several factors that have not yet been elucidated upon, concerning their production, variability and stability. All of which need to be clarified on before their integration into commercial products can be realised.

These marine polyphenols are highly responsive to a wide array of both extrinsic and intrinsic factors synergistically acting on the seaweed, at any one time, in any one population. Such a strong degree of natural variability makes an arduous task of determining the degree and nature of individual parameters on their *in situ* levels. This is partially responsible for the mechanisms behind phlorotannin biosynthesis remaining unknown. The primary concern of this research was thus concerned with building a platform of scientific knowledge regarding the driving factors of LMW phlorotannin variability. This was set to be achieved through assessing the effects of natural abiotic factors through field sampling, as well as within laboratory-controlled induction of abiotic factors.

To date, the use of several protocols for the extraction, separation and analysis of phlorotannins has led to incomparable values. By employing a routine and comprehensive method, a conclusive understanding of their variability can be achieved. A previously optimised method for the extraction, purification and characterisation of low molecular weight (<3.5 kDa) semi-purified phlorotannins was employed by Tierney *et al.* (2014), throughout the entire project.

The potential outcomes were considered as three-fold; 1) a more accurate and in-depth understanding of the environmental factors that are fundamental to their metabolic rates, potentially unveiling mechanisms responsible for their biosynthesis, 2) knowledge gained as to how optimised phlorotannin levels could be achieved within commercial cultivation conditions for the future application in the food industry and 3) clarification on the *ex vivo* stability of such bioactive phlorotannins, to validate their use as potential food ingredients. This was carried out through; 1) the monitoring of their natural variability in several Irish brown seaweeds, 2) laboratory controlled experimental manipulation of specific abiotic factors considered relevant to their metabolism and 3) an investigation into the effect of storage conditions on phlorotannin polymeric fractions, post-extraction.

In **Chapter 2** the natural variability of LMW phlorotannin fractions was of primary concern. To date, phlorotannins and other phenolic compounds, have displayed substantial seasonal and spatial variability. However, full elucidation on the specific elemental factors driving such changes in their concentrations, has not yet been achieved. Furthermore, the effect of environmental factors on the phlorotannin chemical composition, in terms of molecular weight and isomeric abundance, has also not yet been assessed. By investigating the naturally-occurring variability of LMW phlorotannin profiles in four brown seaweeds, differing in their vertical distribution, from two sites along Galway Bay, over six seasons, it was hypothesised that further clarification could be attained on their variation along environmental gradients such as inter-specific, spatial and seasonal patterns.

The natural levels of phlorotannins, while being reported to change in response to environmental shifts in abiotic factors, has also been suggested to stem from ontogenetic changes such as those involving reproductive maturity and vegetative development, which also occurs along a seasonal scale, depending on the growth pattern of the species. It was hypothesised that thallus parts, varying in their physiological function, will display differing biochemical profiles. Therefore, **Chapter 3** was concerned with investigating the intra-thallus differentiation of LMW phlorotannins within a given species, specifically between the reproductive and vegetative sections. This was carried out on four brown seaweeds displaying both perennial and biennial growth patterns.

For a more in-depth understanding of the effect specific factors, particularly those of abiotic origin, impose on LMW phlorotannin profiles, laboratory-based experiments were carried out. **Chapters 4**, **5** and **6** aimed at elucidating on the specific impacts of individual environmental parameters under controlled culture conditions in short-term investigations (7 days). Abiotic factors such as irradiance, nutrients and temperature all display a high degree of seasonal and spatial variability, influencing physiological responses and, in turn, algal biochemical composition. However, while receiving considerable attention in relation to phlorotannin research, to date, their effects have not yet been examined for changes in phlorotannin chemistry.

**Chapter 4** was concerned with investigating the specific effects of irradiance, in particular light intensity and quality. Differing combinations of the two spectral components of natural sunlight, photosynthetically active radiation (PAR) and ultraviolet radiation, were examined for their effects on LMW phlorotannin profiles. **Chapter 5** focused on elucidating on the effects of seawater nitrogen concentration, examining the natural minimum and maximum nitrogen levels observed in Galway Bay in previous studies, as well treatment with nitrogen enrichment. The interaction between nitrogen and irradiance levels were also investigated on inducing variability in LMW phlorotannin profiles. The effect of seawater temperature on LMW phlorotannin profiles was examined in **Chapter 6**, again employing both the minimum and maximum levels observed in seasonal data from Chapter 2, as well as a higher level, just above the natural range. The interactive effect of temperature and irradiance was also assessed on incurring variations on LMW phlorotannin profiles.

The increase in phlorotannin research in the past few decades has been reliant on their potential for incorporation into novel commercial products, particularly within the food sector. However, before their application can be realised, several aspects of their chemistry must be thoroughly analysed. **Chapter 7** was primarily focused on deciphering the *ex vivo* chemical and antioxidant stability of LMW phlorotannin-enriched fractions to validate their future role as potential, natural alternatives to synthetic food preservatives. A broad range of storage conditions were investigated for their effect on varying the molecular distribution and/or arrangement of phlorotannins compounds post-harvest. This involved a 10-week period in which fractions of both powder and liquid-suspended phlorotannins were stored under different conditions, varying in both temperature and oxidative exposure.

The combined aspects of this research were central to revealing the factors responsible for the synthesis, induction, regulation and/or degradation of LMW phlorotannins in brown seaweed, both *in vivo* and *ex vivo*, in an effort to clarify on both their ecological importance and commercial relevance.

## 1.6. Species investigated

To attain a more in-depth knowledge regarding the factors responsible for the natural variability of low molecular weight phlorotannins, as was the primary focus of **Chapters 2** and **3**, four brown algal species were investigated, each differing in both their vertical shore position, life strategy and morphology.

### 1.6.1. *Pelvetia canaliculata* (Linneaus) Decaisne et Thuret

*P. canaliculata* (Fucaceae, Fucales, Phaeophyceae) is a brown macroalga dominating the high tide level of north Atlantic shores (Lüning, 1990). It is found as dense tufts forming a band just above *Fucus spiralis* on Irish coasts. Due to its high position on the shore, it has been referred to as maritime rather than marine, enduring extensive emersion periods (Rugg and Norton, 1987). The fronds, growing to a maximum of approx. 15 cm, are curled longitudinally, in a channel-like manner. It is irregularly and dichotomously branched, with no midrib or air vesicles. Upon reaching reproductive maturity, swollen receptacles are found on the forked tips of the thalli (Fig. 1.18).

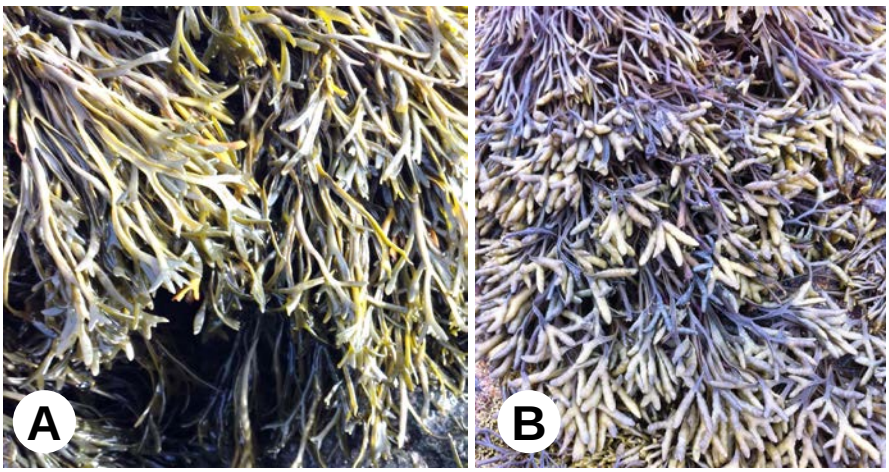



**Fig. 1.18.** The brown alga *P. canaliculata;* displaying (B) swollen receptacles during the reproductive stage**.**

### 1.6.2. *Ascophyllum nodosum* (Linneaus) Le Jolis

Currently confined to the north Atlantic basin, the fucoid species, *A. nodosum* (Fucaceae, Fucales, Phaeophyceae) is a dominant fucoid in the intertidal zone along sheltered, rocky shores (Stengel and Dring, 1998). Here, it can be found alone or in mixed bands with *F. vesiculosus*. *A. nodosum* is comprised of long strap-like fronds, along which air bladders are positioned at regular intervals (Stengel and Dring, 1997). The meristematic tissue of this species is positioned at the apical tip of the thallus,

generating dichotomous branching (Fig. 1.19). During the reproductive stage of the life cycle, yellow oval shaped receptacles can be seen sprouting from the sides of the thallus. The species occasionally bears characteristics tufts of the small reddish-brown filamentous epiphytic red macro alga *Vertebrata lanosa* (Linnaeus) T.A. Christensen.

**Fig. 1.19.** The brown macroalga *A. nodosum;* displaying oval yellow receptacles along the strap-like thallus during the reproductive stage.

### 1.6.3. *Fucus vesiculosus* (Linneaus)

*F. vesiculosus* (Fucaceae, Fucales, Phaeophyceae) is most distinguishable by the uniform presence of two oval air bladders positioned at either side of a prominent midrib along the centre of the flat thallus (Fig. 1.20). This species displays a broad geographical distribution across the north-western Atlantic (Lüning, 1990), forming distinct zones, sometimes mixed with *A. nodosum*. It is often found occupying the zone just below *Fucus spiralis* and above *Fucus serratus*. This perennial species forms swollen warty receptacles at the ends of their thalli upon reaching reproductive maturity (Knight and Parke, 1950).

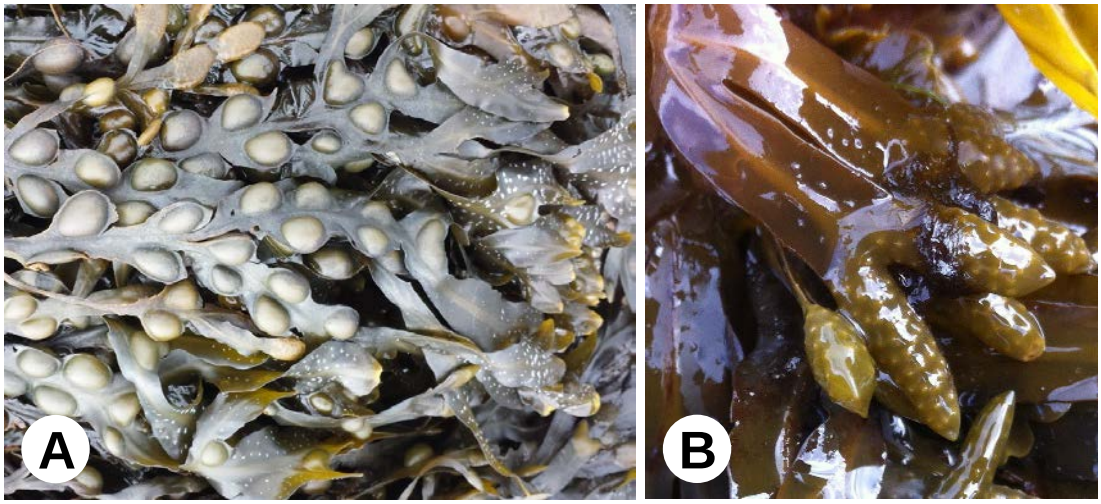



**Fig. 1.20.** The brown macroalga *F. vesiculosus;* displaying (B) swollen receptacles during the reproductive stage.

### 1.6.4. *Himanthalia elongata* (Linneaus) S.F. Gray

*Himanthalia elongata* is a brown seaweed limited to semi-exposed, gently-sloping shores on the north-west Atlantic (Moss *et al.*, 1973), forming a distinct zone below the low-intertidal *Fucus serratus* and above the subtidal *Laminaria*. It is commonly known as "sea spaghetti" due to the long, thin, strap-like receptacles produced from the small button-shaped vegetative form (Fig. 1.21). The reproductive receptacles account for up to 98% of the total biomass, growing up to 5 m length. This high investment in its reproductive tissue distinguish it from Fucales. It also differs, as it is not perennial, but rather biennial, taking two years to complete its life cycle (Stengel *et al.*, 1999).

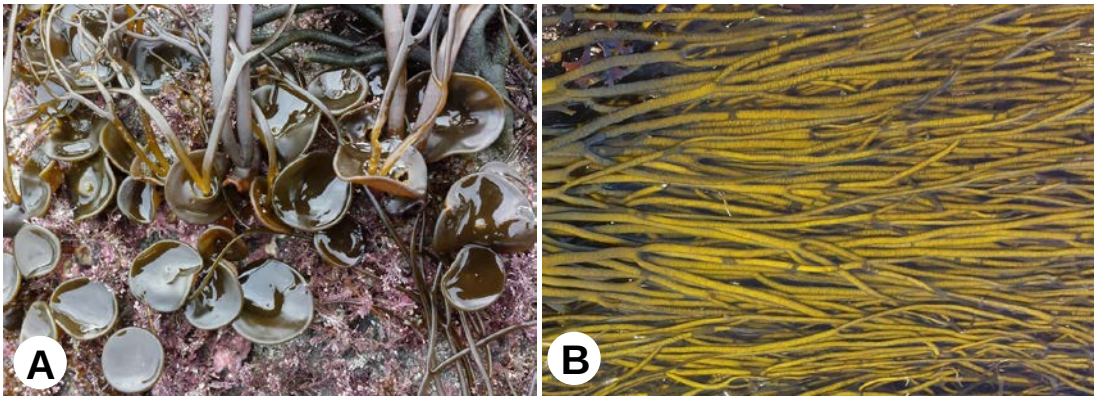



**Fig. 1.21**. Different life stages of the brown macroalga *Himathalia elongata*; (A) the strap-like receptacles and (B) the button-shaped vegetative stage.

# Chapter 2

## Interspecific, seasonal and spatial variability of LMW phlorotannin profiles in four brown seaweed

## 2.1. Introduction

Algal zonation patterns display distinct “bands” along a vertical shore slope (Lewis, 1964). For a detailed description of the different zones, see Chapter 1, Section 1.1.2, as well as Fig. 1.3. The phenomenon of zonation is complex, influenced by several factors synergistically. The distribution range displayed by a given algal species inhabiting a typical rocky shore is fundamentally determined by both biotic relationships and abiotic factors (Lüning, 1990). The lower limits are largely reliant on interspecific competition. For example, based on both culture and transplant studies, Schonbeck and Norton (1980) concluded that *Pelvetia canaliculata* is confined to the supralittoral fringe due to its inability to compete with *Fucus spiralis*, occupying the zone immediately below. However, the upper limits of such intertidal species has been linked to the overall ability tolerate and recover from abiotic stressors associated with periodic emersion, the duration of which will increase at higher shore positions (Dring and Brown, 1982; Schonbeck and Norton, 1978).

The evolution of morphological and physiological traits contribute to the ability of a given species to successfully occupy a specific shore level by heightening their ability to withstand, or even prefer, the associated environmental conditions (Schonbeck and Norton, 1978; Schonbeck and Norton, 1980; Stengel and Dring, 1997). For example, the fronds of *Pelvetia canaliculata* are stunted, thick and curled to minimise water loss during the extensive periods of emersion, to which they are constantly subjected (Schonbeck and Norton, 1979). In kelp species, increasing depth is generally correlated with increased light-harvesting pigments, such as fucoxanthin, to maximise light absorption, while the proportion of pigments, such as the carotenoids, involved in photo-protection increase with decreasing depth, to reduce photo-damage (Colombo-Pallotta *et al.*, 2006; Gévaert *et al.*, 2003).

Furthermore, algae also exhibit the ability to acclimate to temporal shifts in their surrounding environment by adjusting the content and composition of intracellular metabolic compounds, accordingly (Stengel *et al.*, 2011). For example, adjustments in biochemical properties have been associated with seasonal changes in irradiance levels,

the most notable of which, is alterations to the relative proportion and concentration of photosynthetic pigments (Esteban *et al.*, 2015). In general, maximum levels of pigments are observed in winter months to compensate for the reduced light availability. Conversely, in summer, to reduce photo-damage, the abundance of light-absorbing pigments will decline while those involved in photo-protection will generally increase (Aguilera *et al.*, 2002; Martínez and Rico, 2002; Sampath-Wiley *et al.*, 2008; Stengel and Dring, 1998). For example, a decrease in chlorophyll (Chl) *a* and accessory pigment, fucoxanthin, was observed, in *Ascophyllum nodosum* during months of high irradiance (Stengel and Dring, 1998). Gevaert *et al.* (2002) found an increase in xanthophyll pigments (involved in heat dissipation) in *Laminaria saccharina* occurred as an acclimation response to high irradiance levels in spring and summer months. Conversely, higher levels of chlorophyll *c* content were recorded during winter months, allowing for more efficient light absorption. However, the production of such pigments and other nitrogen-based compounds, is dependent on the level of nitrogen available and thus their levels are also subject to seasonal shifts in external nitrogen concentrations (Aguilera *et al.*, 2002; Korbee *et al.*, 2005; Pereira *et al.*, 2012).

In response to seasonal fluctuations in temperature, the composition of fatty acids, involved in the lipid bilayer of cellular membranes, are known to vary (Gosch *et al.*, 2015; Schmid *et al.*, 2014). The degree of saturation exhibited by the fatty acids is suggested to function in the regulation of the membrane fluidity, maintaining efficient transfer of ions. For example, under low temperatures, an increase in poly-unsaturated fatty acids (PUFA) prevents the solidification of the lipid bilayer while an increase in saturated fatty acids (SFA) in higher temperatures improves the rigidity of the membrane (Gombos *et al.*,1994; Moon *et al.*, 1995).

Differences in physio-chemical properties on a geographical scale is another source of variability impacting algal chemistry (Stengel *et al.*, 2011). Such spatial variability can occur both on a global level, as observed in biogeographical distribution patterns, as well as over a span measurable in metres and kilometres. The establishment of such microclimates can lead to different chemical compositions being observed between populations from closely situated sites. Long-term adaptation to particular niches can

result in the evolution of genetically different strains, generating ecotypes. That is, a particular form or subspecies arising through adaptation to a specific set of environmental conditions, possessing unique phenotypic characteristics (Karsten, 2012). A good, and extreme, example of such ecotypic differentiation is *Fucus radicans* in the Baltic Sea, where specialisation to extreme salinity regimes led to speciation (Bergström *et al.*, 2005; Karsten, 2012; Schagerström, 2016).

One particular location known for such strong spatial differences in algal biochemistry is the Baltic Sea, wherein many ecotypes have arisen, particularly within *Fucus* spp. (Graiff *et al.*, 2015; Kalvas and Kautsky, 1993). In fact, in certain areas with a narrow and very specific environmental regime, new species have evolved e.g. *Fucus radicans* (Bergström *et al.*, 2005).

Seasonal and spatial assessment of algal metabolic compounds, and variations therein, provide valuable information allowing for a more in-depth understanding of their ecological significance and clarity on the factors promoting their production. Such information can also be of great benefit to industry, providing scientific knowledge that will under pin optimal screening selection of species, harvest times and locations offering maximal productivity and/or activity (Connan and Stengel, 2011; Stengel and Connan, 2015).

The vast ecological functions displayed by phlorotannins in brown algae lends to the high degree of variability observed within the literature, making arduous work of elucidating on the principle factors involved in their production (Pelletreau, 2008). For example, phlorotannin concentrations are reported to vary significantly between species. Previous studies have shown the Fucales and Dictyotales to possess the highest concentrations, constituting up to 12% dry weight (DW), while the Laminariales possess the lowest levels, displaying levels between 0.1 and 1% DW (Connan *et al.*, 2004; Targett *et al.*, 1992). Even the abundance of specific phlorotannin structures is suggested to occur between species, with eckols being abundant in *Ecklonia* spp. and fucols in *Fucus* spp. (Ragan and Glombitza, 1986). Moroever, their concentrations are subject to geographical variability. For instance, species from the temperate and tropical Atlantic have been reported to exhibit phlorotannin concentrations of up to 2-15% DW, whereas populations from the

tropical Indo-Pacific possess characteristically lower levels, rarely exceeding 3% DW (Targett *et al.*, 1992).

Moreover, their levels have been reported to shift in response to several abiotic parameters including irradiance (Cruces and Huovinen, 2012; Gomez and Huovinen, 2010), nutrient availability (Hemmi *et al.*, 2005; Koivikko, *et al.*, 2008; Van Alstyne and Pelletreau, 2000), salinity (Connan and Stengel, 2011; Munda, 1964; Pedersen, 1984) and temperature (Cruces and Huovinen, 2012, 2013), as well as with biological relationships such as epiphytic settlement (Brock *et al.*, 2007; Steinberg and De Nys, 2002) and herbivore deterrence (Pavia and Toth, 2000; Van Alstyne, 1988; Van Alstyne and Paul, 1990; Steinberg, 1984), all of which are subject to seasonal and spatial differentiation. This is further complexed by their association with ontogenetic development and maturity (Plouguerné *et al.*, 2006; Ragan and Jensen, 1978).

The difficulty in clarifying on their natural variability, often prompted by contradicting and conflicting results, has resulted in a lag in progression within phlorotannin research, concerning their biosynthetic clarification. To date, the majority of our knowledge on phlorotannin derives from quantification studies on crude extracts, with chemical characterisation being limited due to their structurally complexity and reactivity (Ragan and Glombitza, 1986; Tierney *et al.*, 2014). For example, the molecular range of phlorotannins has been suggested to extend to over 100 kDa (Audibert *et al.*, 2010; Hagerman *et al.*, 1998). As the degree of polymerisation increases, so too does the level of intricacy phlorotannins display with other compounds, as well as with one another (Ferreres *et al.*, 2012; Steevensz *et al.*, 2012; Tierney *et al.*, 2014).

However, chemical characterisation is an essential part of understanding the ecological relevance of phlorotannins. For instance, their activity is reported to be dependent on the molecular weight of the compound, as a higher abundance of phenol rings will be coupled with a greater number of hydroxyl units (Audibert *et al*., 2010; Ferreres *et al.*, 2012). Moreover, the manner in which these hydroxyl units are arranged around the phlorotannin frame will affect their reactive availability (Li *et al.*, 2009). Therefore, an improved insight into their chemical characteristics would aid in elucidating on the effects the surrounding environment impose on their metabolic profiles and improve our

understanding of the mechanisms involved in their biosynthesis. This might, in turn, at least theoretically, also support the selection of optimal harvesting times for the collection of natural resources for high value applications.

The last few decades have seen an increase in research focusing on the development of methods capable of successfully characterising target bioactives such as phlorotannins (Stengel and Connan, 2015). A recent study demonstrated the ability to enhance the level of antioxidant activity in low molecular weight (<3.5 kDa) fractions through enriching the phlorotannin concentration (Tierney *et al.,* 2013). This was achieved through the employment of reverse phase (RP) flash chromatography, which successfully removed polysaccharides from the desired phlorotannin oligomer fraction. In doing so, a high degree of sample purity was achieved and characterisation of phlorotannins up to 16 phloroglucinol units, in their degree of polymerisation, was possible employing UPLC-MS/MS (Tierney *et al.*, 2014).

The research presented here sought to investigate the effects of both sampling season and site on the natural fluctuations of low molecular weight phlorotannin profiles in four Irish intertidal species, *Pelvetia canaliculata, Ascophyllum nodosum, Fucus vesiculosus* and *Himanthalia elongata*, employing the method of Tierney *et al.* (2014). These species were selected for investigation due to the higher phlorotannin abundance reported within the order of Fucales, as well as their already established use in commercial products (Catarino *et al.*, 2017). Seasonal shifts in irradiance and temperature within Galway Bay were monitored to assess the effect of changes in abiotic parameters on the LMW phlorotannin profiles and related *in vitro* activity. The results obtained from this study were expected to provide novel information regarding the impact extrinsic factors impose on the chemical variability of these bioactive compounds.

Furthermore, owing to the several purification steps involved in attaining LMW fractions of high purity, the chosen method employed here (Tierney *et al.,* 2014) is both solvent- and time-consuming. In an effort to reduce resource consumption, *i.e.* solvent, consumables and time, single-sample extraction was considered as a possible alternative to the use of biological replicates. Typically, a large amount of biomass is required for the initial extraction (30-50 g DW). Considering the fresh weight equivalent is approximately

between 150 and 250 g FW (80% water loss upon drying), samples from several individual plants could be pooled and assessed as one representative sub-sample. As a result, any bias that may arise from the use of several biological replicates, owing to intra-individual variations, could be deterred. Therefore, such an approach could, potentially, give a better over-view of the population, as a whole. However, this theory required testing and validation prior to use. In this chapter, prior to seasonal and spatial analysis on LMW phlorotannins, this hypothesis was tested for its degree of variability.

## 2.2. Materials and Methods

### 2.2.1. Reagents and materials

1, 3, 5-trihydroxybenzene (phloroglucinol), 2, 2-diphenyl-1-picryhydrazyl (DPPH), 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox®), formic acid (MS grade) and 0.22 µm polytetrafluorethylene (PTFE) filters, Iron (III) chloride hexahydrate, sodium carbonate ($Na_2CO_3$), Folin- Ciocalteu (2N) were all sourced from Sigma-Aldrich Chemical Ltd. (Co. Wicklow, Ireland). HPLC grade methanol, ethyl acetate, acetonitrile, water, and BioDesign Dialysis Tubing™ with 3.5 kDa cut-off were obtained from Fischer Scientific Ltd. (Loughborough, Leicestershire, UK).

### 2.2.2. Selected sampling sites

Two sites along Galway Bay (Fig. 2.1) differing regarding their topography and degree of exposure were selected for spatial investigation of LMW phlorotannin profiles in the four brown seaweed species; Flaggy Shore, Finavarra, Co. Clare (53°09'11.7"N 9°07'07.0"W) and Mace Head, Co. Galway (53°19'30.0"N 9°54'21.7"W).

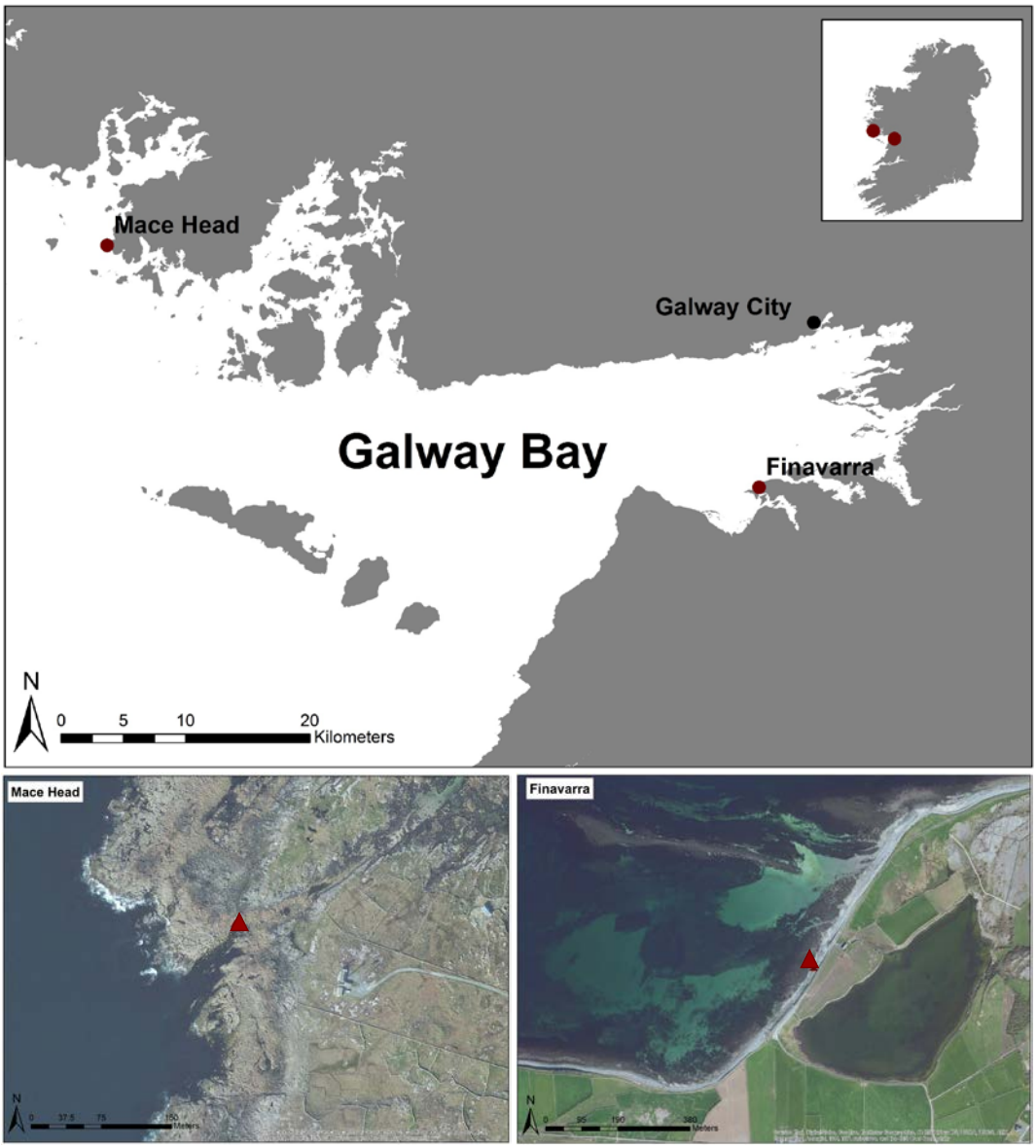



**Fig. 2.1.** Sites selected along Galway Bay for spatial investigation of phlorotannins in four brown algal species; Mace Head, Co. Galway and Finavarra, Co. Clare. Map created by Tom Rossiter, NUI Galway.

### 2.2.2.1. Finavarra, Co. Clare

Finavarra, Co. Clare (53°09'11.7"N 9°07'07.0"W), is a sheltered/semi-exposed site, located approximately 45 km southeast of Galway city centre (Fig. 2.1; 2.2). The specific location where samples were collected from, displayed a mixed substratum composed of sand and rock. A typical vertical zonation pattern was observed, with broad species-specific "bands". The site was fully marine with a salinity regime of 30-35.

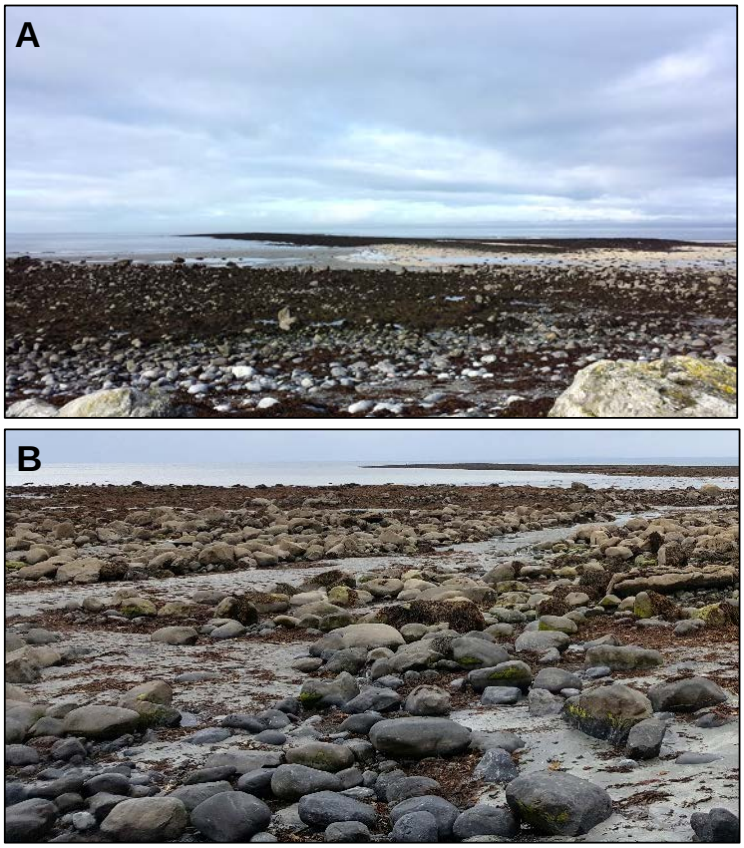



**Fig. 2.2.** An overview (A) and close-up (B) of the primary sampling site, Finavarra, Co. Clare where samples were collected, at low tide.

### 2.2.2.2. Mace Head, Co. Galway

Mace Head, Co. Galway (53°19'30.0"N 9°54'21.7"W), was a fully exposed marine site located at the most western tip of Connemara, approximately 82 km to the north-west of Galway City (Fig.2.3). The shore gradient, here, was relatively short, spanning over approximately 50 m. The substratum was composed of large boulders with small areas of sand. Alga were mixed with no clear zonation patterns, exhibiting a scattered and scarce distribution. The abundance of each species appeared to considerably lower than observed in Finavarra, Co. Clare.

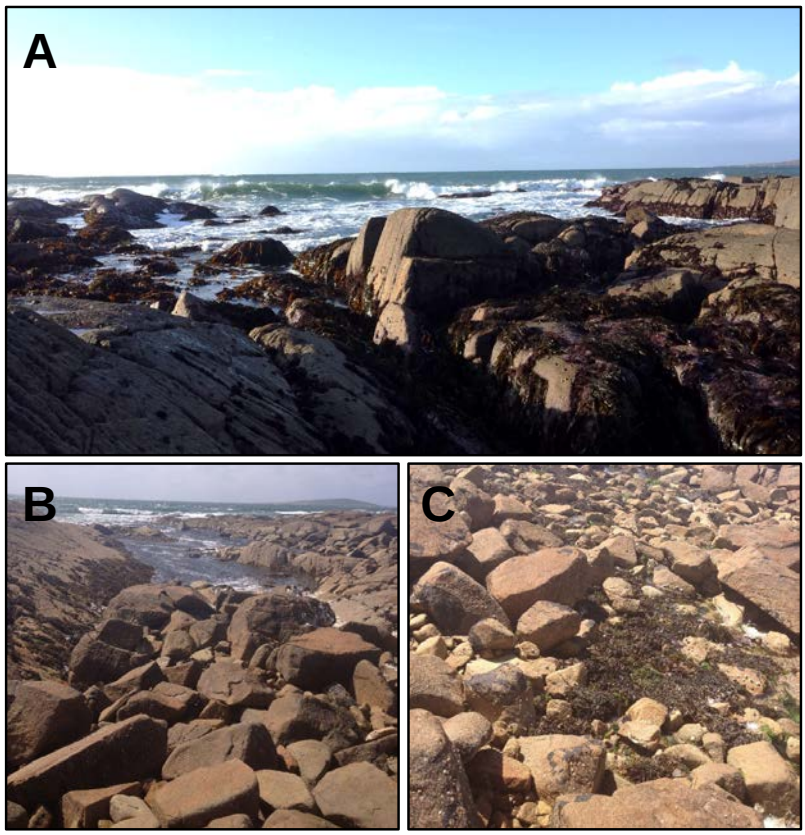



**Fig. 2.3.** Secondary sampling site, Mace Head, Co. Galway, where algae were collected. Different sections of location are shown in (A), (B) and (C).

### 2.2.3. Measurements of environmental parameters

#### 2.2.3.1. Irradiance

Annual fluctuations in irradiance levels within Galway Bay were provided by a light meter (Li-1400, Li-Cor, USA) placed on the roof of the Ryan Institute, NUI Galway (53°16'39.4"N 9°03'33.6"W). Levels of photosynthetic radiation (PAR) in µmol photons $m^{-2}$ $s^{-1}$ were recorded every hour from January 2014 – November 2015. The average monthly irradiance level was calculated by determining the mean daily irradiance received over a 24 h cycle, irrespective of photoperiod, for each day of each month. Data are represented as mean ± standard deviation.

#### 2.2.3.2. Seawater temperature

Monthly surface (1 m) seawater temperatures (SSTs) were obtained from the Marine Institutes data set for the Galway Bay wave buoy, off the coast of Spiddal, Co. Galway,

for each month from January 2014 to November 2015 and are expressed in degrees Celsius (°C). Data are presented as mean ± standard deviation.

### 2.2.4. Collection and preparation of seaweed samples

Four species differing in their vertical position along a shore gradient were selected for investigation; *Pelvetia canaliculata*, *Fucus vesiculous, Ascophyllum nodosum* and *Himanthalia elongata.* For most species, the whole frond (minus stipe) was taken for analysis, including the vegetative thallus and receptacle-containing tips. However, for *H. elongata*, only the long, strap-like receptacles were collected, as this thallus part comprise the majority of this species biomass. Samples were collected every 2-3 months between August 2014 and November 2015. A minimum of 40 individual thalli were haphazardly selected and cut at each sampling date. They were then brought back to the Algal Biosciences lab at the National University of Galway (NUI Galway), where they were cleaned (manually) of any epiphytes or other external impurities (e.g. sediment) present. Following this, (within a few hours) they were transported in cooler boxes to Teagasc Food Research Centre, Ashtown (TRFCA). Here, samples were frozen at -20°C and thereafter freeze-dried (A12/60 Freeze Dryer, Frozen in Time Ltd., York, England), before being ground into one homogenous powder for each species. Once vacuum-packed, samples were stored at -20°C until further required.

### 2.2.5. Validating the approach of a single-sample methodology

Prior to seasonal and spatial analysis, the previously discussed approach of single-sample extractions was tested, using an initial 30 g DW of homogenous algal powder. It was surmised that such a modification of the method, while removing any bias that may rise from biological replicates, could also, potentially, minimise the exhaustion of resources. Each species collected from Finavarra, Co. Clare, in May 2014. For each species, fresh weight samples were prepared and freeze-dried, as described above to attain a dry weight homogenous powder. From this powder, three 50 g DW pseudo-replicates (n=3) were taken, upon which phlorotannin extraction, purification and analysis was carried out, as described below in Sections 2.2.6 to 2.2.11. The degree of variability was statistically tested for variation between samples for the total phlorotannin content (TPC), ferric-

reducing antioxidant power (FRAP) and the radical scavenging activity (RSA) of the low molecular weight phlorotannin-enriched replicates. IBM® SPSS® statistical software (version 24) was used for all statistical tests. Normality was tested using the Shapiro-Wilk test for normal distribution and Levene's test for homogeneity of variances. In samples where homogeneity of variances was not met, the Welch ANOVA was employed (Tomarken and Serlin, 1986). Significant differences between treatments were determined at a significance level of 95% ($p<0.05$), as determined by a Tukey *post-hoc* test.

### 2.2.6. Extraction and purification of phlorotannin fractions

The method employed for the extraction, purification and characterisation of low molecular weight (<3.5 kDa) phlorotannin-enriched fractions was a slightly modified version of that described by Tierney *et al.* (2014), a schematic overview of which is illustrated in Fig. 2.4.

Finely ground algal powder was exhaustively extracted using 80% methanol at a ratio of 10:1 v/w, in an orbital shaker (MaxQ 6000 Shaker, Thermo Fisher Scientific, MA, USA) set at 160 rpm, for 24 h at room temperature. Extracts were filtered periodically (three times in total) with the solvent refreshed each time. Pooled extracts were filtered through a Büchner funnel and methanol was removed using a rotatory evaporator (Heidolph Rotatory Evaporator, Germany) at 40°C. Extracts were then frozen (-20°C) and freeze-dried to attain a dried sample. A sample (0.5 g DW) was taken at this stage to test the TPC, FRAP and RSA of the crude extract following the steps outlined in Section 2.2.10 and 2.2.11.

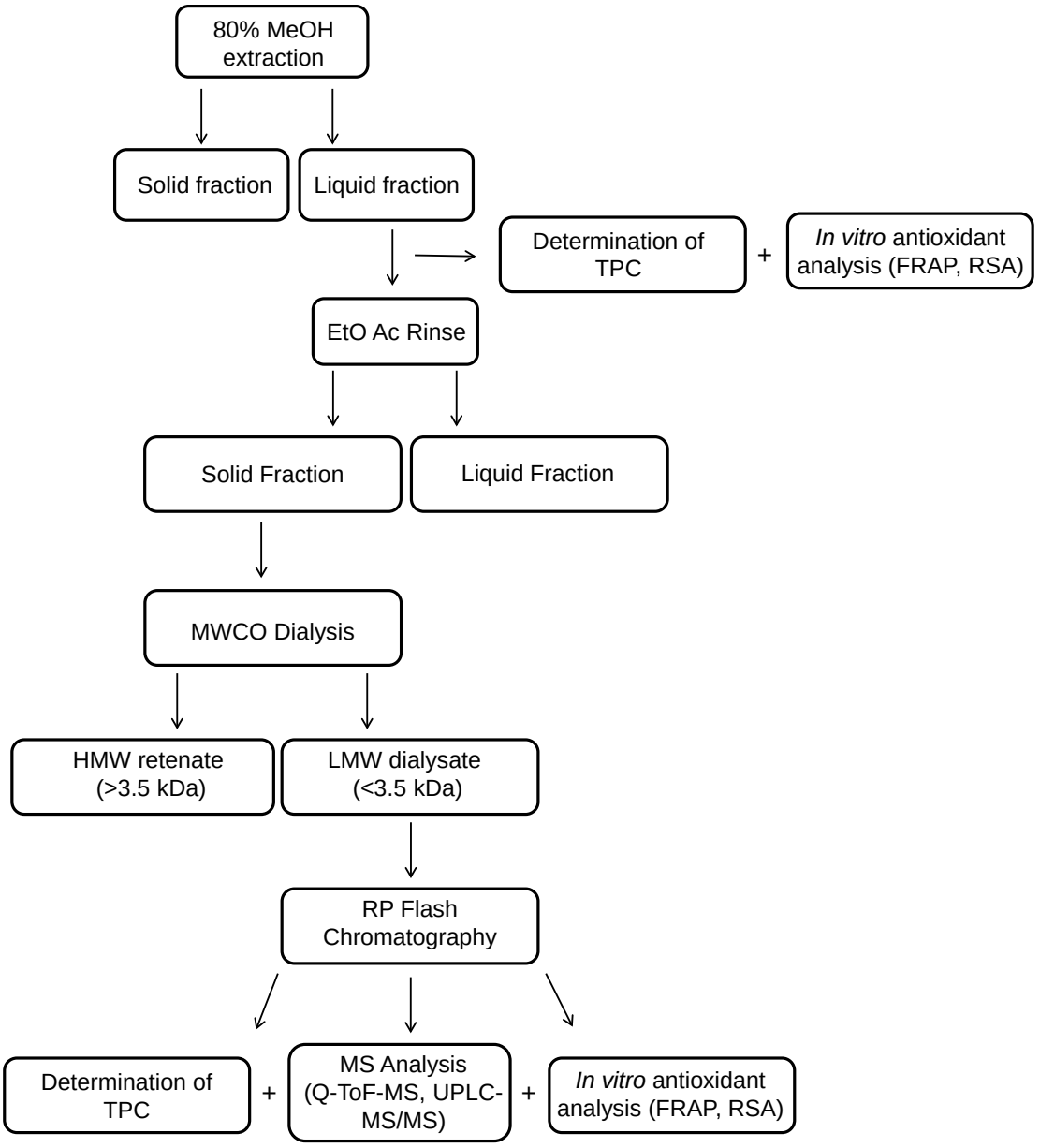



**Fig. 2.4**. Schematic representation of the steps involved in the extraction, purification and analysis of LMW phlorotannin-enriched fractions, after Tierney *et al.* (2014).

### 2.2.7. Purification and isolation of low molecular weight (LMW) phlorotannins

Dried extracts were washed with ethyl acetate to remove pigments and once colourless, they were filtered using a Buchner funnel. To achieve a low molecular weight (LMW) fraction (<3.5 kDa), and to remove any salts present, the retentate was subjected to molecular weight cut off (MWCO) dialysis. The minimal amount of deionised water

required for complete solubility was added to the samples. They were then sonicated and centrifuged before being placed in 3.5 kDa MWCO dialysis tubing, clamped at both ends, and immersed in a reservoir of deionised water (undefined volume - sufficient to completely emerse the dialysis tubing) in an orbital shaker set at 20°C for 48 h. The reservoir of deionised water was periodically refreshed until the dialysate ran clear. The pooled low molecular weight (LMW) dialysate was frozen and then freeze-dried.

To isolate phlorotannins from other polar compounds such as low molecular weight β-glucan polysaccharide and sugar alcohols, reversed-phase (RP) flash chromatography was employed using an Varian Intelliflash 310 flash system (Analogix Semiconductor Inc., California, USA.) fitted with a Reveleris® C18 RP 40 g column. 1 g DW of LMW sample was re-suspended in a minimal volume of deionised water required for complete solubility, then sonicated and centrifuged before being loaded onto the column. A two-step elution phase was employed, where the mobile phases consisted of deionised water for the primary eluent and 100% methanol for the secondary. The flow rate was set at 40 mL $min^{-1}$ and UV detection was observed at 210, 250 and 278 nm. The phlorotannin polymer fraction was collected between 10-20 min in the secondary phase (Fig 2.5). Methanol was removed from the phlorotannin fractions using a rotatory evaporator (Heidolph Rotatory Evaporator, Germany) at 45°C. The LMW phlorotannin polymer fractions were frozen at -20°C and then freeze-dried.

For MS analysis, dried (powder) samples were re-suspended in deionised water at a concentration of 20 mg $mL^{-1}$. Samples were then sonicated (42 kHz) and centrifuged at 13,000 rpm for 3 min and filtered using 0.22 µm polytetrafluorethylene (PTFE) filters.

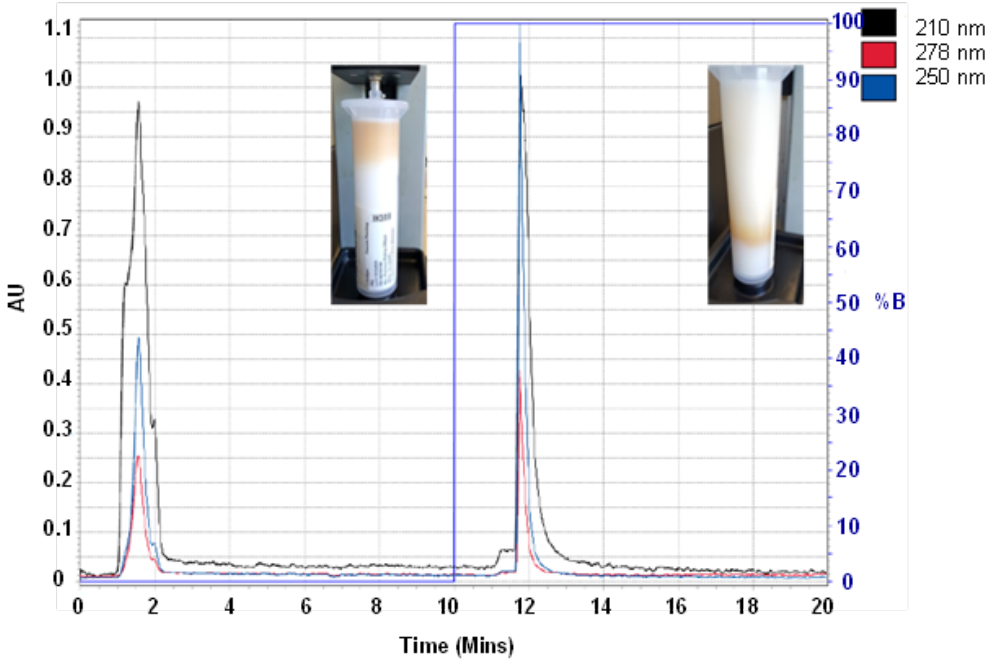



**Fig. 2.5.** Reverse phase (RP) flash chromatogram of *Fucus vesiculosus*, collected from Finavarra, Co. Clare, in August 2014, illustrating the separation of phlorotannins (second peak) from other polar compounds, such as sugar alcohol, mannitol (first peak).

### 2.2.8. Q-ToF-MS detection of LMW phlorotannins

Confirmation and detection of phlorotannin oligomer presence was achieved by direct infusion of the samples into the electrospray ionisation source of the Q-ToF-MS. This non-specific screening allowed for all compounds present to be detected and thus confirm sample purity. The method employed was that of Tierney *et al.* (2014). Mass spectral data was obtained in negative electrospray ion mode (ES-) from molecules of 300 to 3,000 *m/z*. The capillary and cone voltages were set at 2.6 and 35 kV, respectively. The desolvation gas was set at 800 L $h^{-1}$ and the cone gas at 50 L $h^{-1}$. The samples were infused at 1 µL $min^{-1}$ for a total run time of 2 min.

### 2.2.9. UPLC-MS/MS characterisation of LMW phlorotannins

Samples were analysed using the method previously described by Tierney *et al.* (2014). Ultra Performance Liquid Chromatography (UPLC) was carried out on the <3.5 kDa flash phlorotannin polymer samples using an Acquity™ UPLC System (Waters Corporation, Milford, MA, USA). The system consisted of a binary pump solvent manager coupled

with an Acquity™ TQD-MS (Waters Corporation, Milford, MA, USA). It was operated in negative electrospray ion (ES-) mode with multiple reaction monitoring (MRM) mode. The MRM method was developed and optimized with a phlorotannin-enriched sample using Intellistart™ software (Waters Corporation) according to molecular masses of phlorotanins containing 3-16 phloroglucinol units (maximum permitted by TQD). Conditions for the MRM were set following Tierney *et al.* (2014) and are outlined in Table 2.1. A Waters Acquity™ UPLC HSS PFP column was used (100 Å, 1.8 µm particle size, 2.1 mm × 100 mm) at 40°C.

Mobile phases consisted of distilled water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B). The flow rate was 0.5 mL $min^{-1}$ and the injection volume set at 1 µL. The elution gradient was set as follows: 0.5% B from 0 to 10 min, 0.5-30% from 10 to 26 min, 30-90% B from 26 to 28 min, and 0.5% B from 28 to 30 min. The capillary voltage was set at 2.8 kV. The desolvation gas and cone gas were set at 1200 L $h^{-1}$ and 50 L $h^{-1}$, respectively. The source temperature and desolvation temperature were set at 450°C and 150°C, respectively, for maximum sensitivity. Methanol blanks were injected between samples to ensure that the peaks observed where in fact those of the phlorotannin sample and not resultant from the retention of compounds on the column and subsequent carry-over from previously analysed samples.

**Table 2.1.** MRM conditions for TQD-MS analysis of phlorotannins consisting of 3-16 phloroglucinol units (PGUs) determined by a combination of Intellistart™ and manual tuning, as described in Tierney *et al.* (2014).

| PGUs | Precursor Ion (*m/z*) | Product Ion (*m/z*) | Cone Voltage (V) | Collision Voltage (V) |
|---|---|---|---|---|
| 3 | 373.1 | 139 | 40 | 34 |
| 4 | 497.2 | 139 | 40 | 40 |
| 5 | 621.2 | 139 | 42 | 42 |
| 6 | 745.3 | 139 | 40 | 40 |
| 7 | 869.3 | 139 | 52 | 52 |
| 8 | 993.4 | 229.1 | 50 | 50 |
| 9 | 1117.4 | 229.1 | 50 | 50 |
| 10 | 1241.4 | 229.1 | 52 | 52 |
| 11 | 1365.4 | 229.1 | 52 | 52 |
| 12 | 1489.5 | 229.1 | 54 | 54 |
| 13 | 1614.0 | 229.1 | 54 | 54 |
| 14 | 1738.3 | 229.1 | 56 | 56 |
| 15 | 1862.3 | 229.1 | 56 | 56 |
| 16 | 1986.4 | 229.1 | 56 | 56 |

### 2.2.10. Determination of total phlorotannin content (TPC)

The total phenolic content (TPC) of the <3.5 kDa phlorotannin-enriched fractions were determined using a slightly modified version of the Folin - Ciocalteu assay by Singleton and Rossi (1965) as described by Kenny *et al.* (2015). Dry weight phlorotannin fractions were re-suspended in methanol to a concentration of 200 µg $mL^{-1}$. 100 µL of each extract was placed in a 1.5 mL eppendorf tube along with 100 µL of methanol, 100 µL of Folin - Cioacalteu (2N) and 700 µL of 20% Sodium carbonate, to a final volume of 1 mL. Three aliquot samples were used for each analysis. Samples were vortexed and immediately after, placed in darkness to incubate for 20 min at room temperature. Samples were then centrifuged at 13,000 rpm for 3 min before the supernatant was poured off and the absorbance measured in a spectrophotometer (U-2900 spectrophotometer, Hitachi high technologies corporation, Tokyo, Japan) at 735 nm. Methanol was used as a blank. Phloroglucinol, the monomer unit of all phlorotannin compounds, was used as the external standard. A calibration curve generated by the serial diluton of a stock 2 mg $mL^{-1}$ stock sample (10, 20, 50, 80, 120, 160 µg $mL^{-1}$) was used to quantify the total phenolic content, expressed as microgram of phloroglucinol equivalents (PGE) per milligram of dry weight extract (µg PGE $mg^{-1}$ DWE).

### 2.2.11. Assessment of *in vitro* antioxidant activity

#### 2.2.11.1. Ferric Reducing Antioxidant Power (FRAP)

The ferric reducing antioxidant power (FRAP) of each extract was determined according to a slightly modified method of Stratil *et al.* (2006) as described by Kenny *et al.* (2015). The assay's oxidant consisted of a mixture of a ferric chloride aqueous solution (54 mg $mL^{-1}$), a TPTZ solution (31.2 mg $mL^{-1}$ in 40mM HCl) and an acetate buffer (pH 3.6) in the ratio of 1:1:10, respectively. Prior to its use, the reagents ingredients were mixed and heated to 37°C in the dark in an incubator (INCU-Line® IL 53, VWR, USA) until further required. 20 µL of sample (200 µg $mL^{-1}$) was pipetted into individual wells across the plate in triplicate and 180 µL of FRAP reagent added. Plates were placed in the incubator for 40 min before the absorbance of each well was measured using a plate reader (FLUOstar Omega Microplate Reader, BMG Labtech GmbH, Offenburg, Germany) at

593 nm. Methanol was used as a blank. Trolox was used as an external standard. A calibration curve, generated by the serial dilution of a 2 mg $mL^{-1}$ methanolic Trolox stock solution (150, 120, 100, 80, 60, 40, 20 µg $mL^{-1}$) was used to calculate the activity of each sample and was expressed in terms of Trolox equivalents (TE) per gram of dry weight extract (µg TE $mg^{-1}$ DWE).

### 2.2.11.2. Radical Scavenging Ability (RSA)

A modified version of the DPPH· (2, 2 Diphenyl-1-picrylhydrazyl) assay described by Kenny *et al.* (2015) was used to assess the free radical scavenging capacity of the samples. A working DPPH· solution was attained through a 1 in 5 dilution of a methanolic DPPH stock solution (23.8 µg $mL^{-1}$). 200 µl of sample (200 µg $mL^{-1}$) was pipetted onto a 96 well flat-bottom microplate (Greiner Bio-one, Germany). Each sample was serial diluted across the plate with methanol (200, 100, 50, 25, 12.5, 6.25 µg $mL^{-1}$). Three aliquot samples were used for each analysis (n=3). 100 µL of working DPPH· solution was added to each well before being incubated in the dark for 30 min at room temperature. After incubation, the absorbance of each well was measured using a plate reader (FLUOstar Omega Microplate Reader, BMG Labtech GmbH, Offenburg, Germany) at 515 nm. The radical scavenging activity (RSA) of each sample was calculated by determining the concentration of the extract needed to cause a 50% inhibition of the initial DPPH· absorbance, known as the $IC_{50}$ value. This was calculated using the below equation (Eq 1.);

$$\text{Eq 1. } IC_{50} = C_1 - [(C_1 - C_2) \times (PI_1 - 50)] / (PI_1 - PI_2)$$

Where $C_1$ and $C_2$ are the sample concentration factors at which an absorbance immediately below ($PI_1$ %) and above ($PI_2$ %) the 50% absorbance of the DPPH· control, at 515 nm, was read.

### 2.2.12. Statistical analyses

For each species, the effects of sampling site and season on the total phlorotannin content (TPC), ferric-reducing antioxidant power (FRAP) and the radical scavenging activity (RSA) of the LMW phlorotannin-enriched fractions were statistically tested using a two-

way variance of ANOVA ($p$<0.05). IBM® SPSS® statistical software (version 24) was used for all statistical tests. Normality was tested using the Shapiro-Wilk test for normal distribution and Levene's test for homogeneity of variances. Data which did not meet normality requirements were transformed using log, natural log, square root, or reciprocal transformations, prior to ANOVA analysis. In samples where homogeneity of variances was not met, the Welch ANOVA was employed (Tomarken and Serlin, 1986). Tukey-tests were performed to find *posteriori homogeneous* (*post-hoc*) sub-groups that differed significantly ($p$<0.05).

## 2.3. Results

### 2.3.1. Seasonal variations in irradiance and temperature

Both daily and monthly averages in irradiance intensity levels exhibited lowest in November through to January, in both years (2014 and 2015), coupled with the shortest photoperiods (10 h). Average monthly irradiance intensities here ranged from 37- 71 µmol photons $m^{-2}$ $s^{-1}$ while daily maximum levels were limited to 170 µmol photons $m^{-2}$ $s^{-1}$ at midday (Fig. 2.6). After Januray, the irradiance intensity levels began to increase, peaking in June and July in both 2014 and 2015 (404-477 µmol photons $m^{-2}$ $s^{-1}$; Fig. 2.6) with daily levels reaching up to 1,100 µmol photons $m^{-2}$ $s^{-1}$, peaking midday. This was coupled with a longer day length of 17 h.

The lowest temperatures, as attained through the Marine Institutes Galway Bay wave buoy data, located at Spiddal, Co. Galway, were recorded in January and February (6-7°C) in both years (2014 and 2015), increasing thereafter and displaying maximum temperatures of 14-16°C in July and August, again, in both years (2014 and 2015; Fig. 2.6)

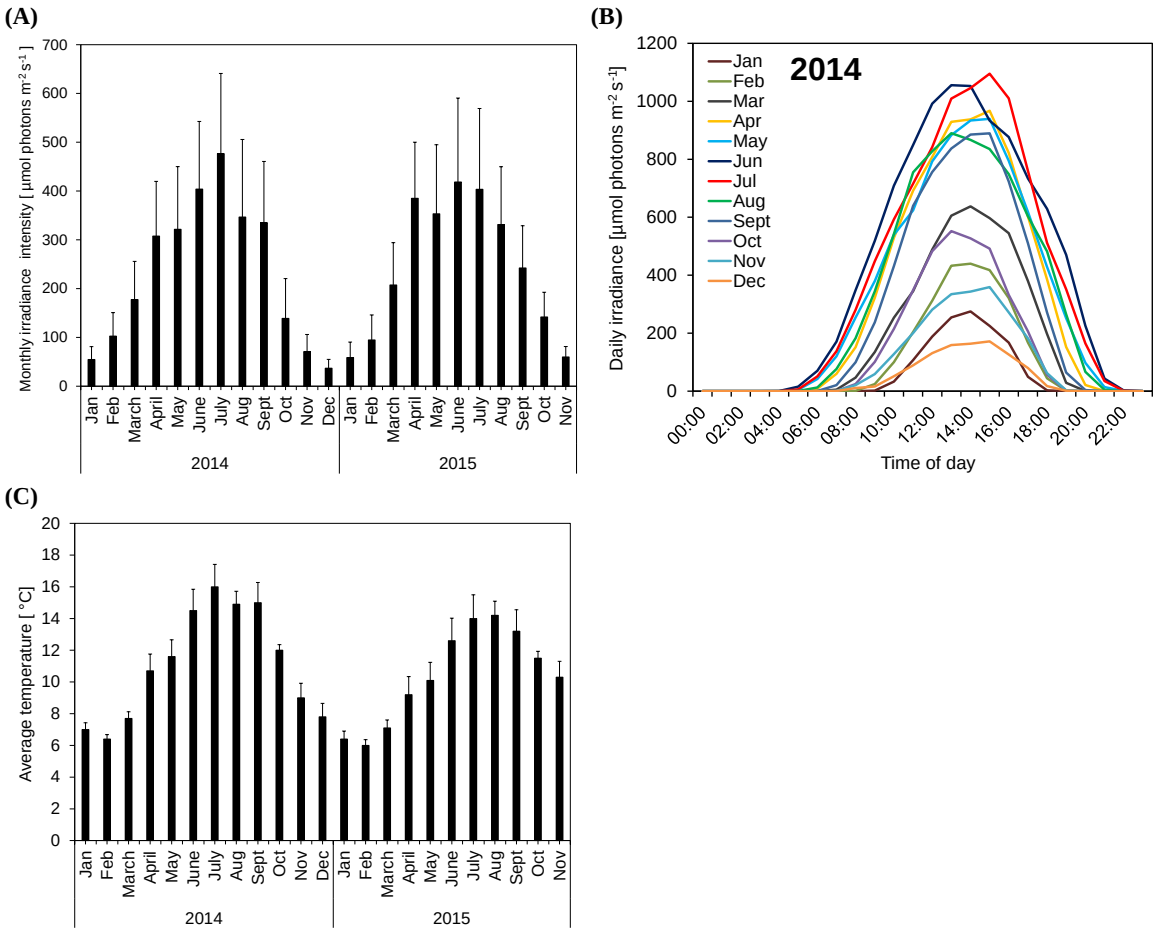



**Fig. 2.6.** Seasonal variations in abiotic factors in Galway Bay. (A) Average monthly light intensity (µmol photons $m^{-2}$ $s^{-1}$) for each month from January 2014 to November 2015. (B) Averaged daily light intensity (µmol photons $m^{-2}$ $s^{-1}$) for each month in 2014, illustrating seasonal variability in photoperiod of the irradiance, measured on the roof on the Ryan building, NUI Galway and (C) average surface seawater temperature (°C) as obtained from the Marine Institutes Galway Bay wave buoy.

### 2.3.2. Single-sample extraction validation

Within each species, little to no variability was observed between the three pseudo-replicate samples in terms of their chemical profiles, total phlorotannin content and *in vitro* antioxidant activity. Within each species, a high level of similarity was observed between the phlorotanin profiles of the pseudo-replicates, in respect to the chemical composition (see Supplementary Section 9.2, Table 9.2.1).

For the colorimetric analyses, *i.e.* TPC, FRAP and RSA, where analytical triplciates (n=3) were used for each of the three pseudo-replicates, significant differences were tested by employing a one-way variance of ANOVA ($p$<0.05), the details of which are outlines in

Supplementary Section 9.2, Table 9.2.2. The degree of reproducibility for TPC, FRAP and RSA activity was adequate, with no significant differences being observed between replicates ($p$>0.05). These results suggest that, due to the samples being comprised of a homogenous collection of several thalli, from several individual plants from the population, the extraction, purification and analysis on a single sample (30 g DW) would be sufficient for future analysis, and therefore was subsequently employed here and throughout the thesis.

### 2.3.3. Phlorotannin composition and relative abundance

LMW reverse phase (RP) flash fractions were directly injected on to the electrospray ionization source of the Q-ToF-MS and ran in negative mode (ES-). This allowed for the preliminary assessment of sample purity and/or detection of interfering impurities, thus helping to direct further separation approaches, if required. As seen in Fig. 2.7, even after several purification steps, non-phlorotannin (NP) compounds, presumably polysaccharides, were detected within the <3.5 kDa fractions of *P. canaliculata* and *A. nodosum* (Fig. 2.7, 2.8).

The degree of interference polysaccharide presence cause, which was substantially higher in *A. nodosum* than in *P. canaliculata*, also varied seasonally and spatially. Phlorotannin fractions in *A. nodosum* were continuously contaminated with the presence of such compounds (Fig. 2.7, 2.8). In an effort to further purify these fractions, prior to UPLC-MS/MS analysis, normal-phase (NP) flash separation was employed. However, this resulted in a further loss of phlorotannins and so its use was discontinued. In fractions obtained from *F. vesiculosus* and *H. elongata* such interference was not an issue, as seen in Fig. 2.7 and 2.8. The purity of the LMW fractions from these species was considerably high throughout all six seasons investigated.

The employment of UPLC-MS/MS in MRM mode allowed for the specific detection of molar masses corresponding to phlorotannin oligomers between 3-16 phloroglucinol units (PGU) in their degree of polymerization. *m/z* of 1986 (16 PGU) was the maximum permitted due to the high sensitivity of the equipment.

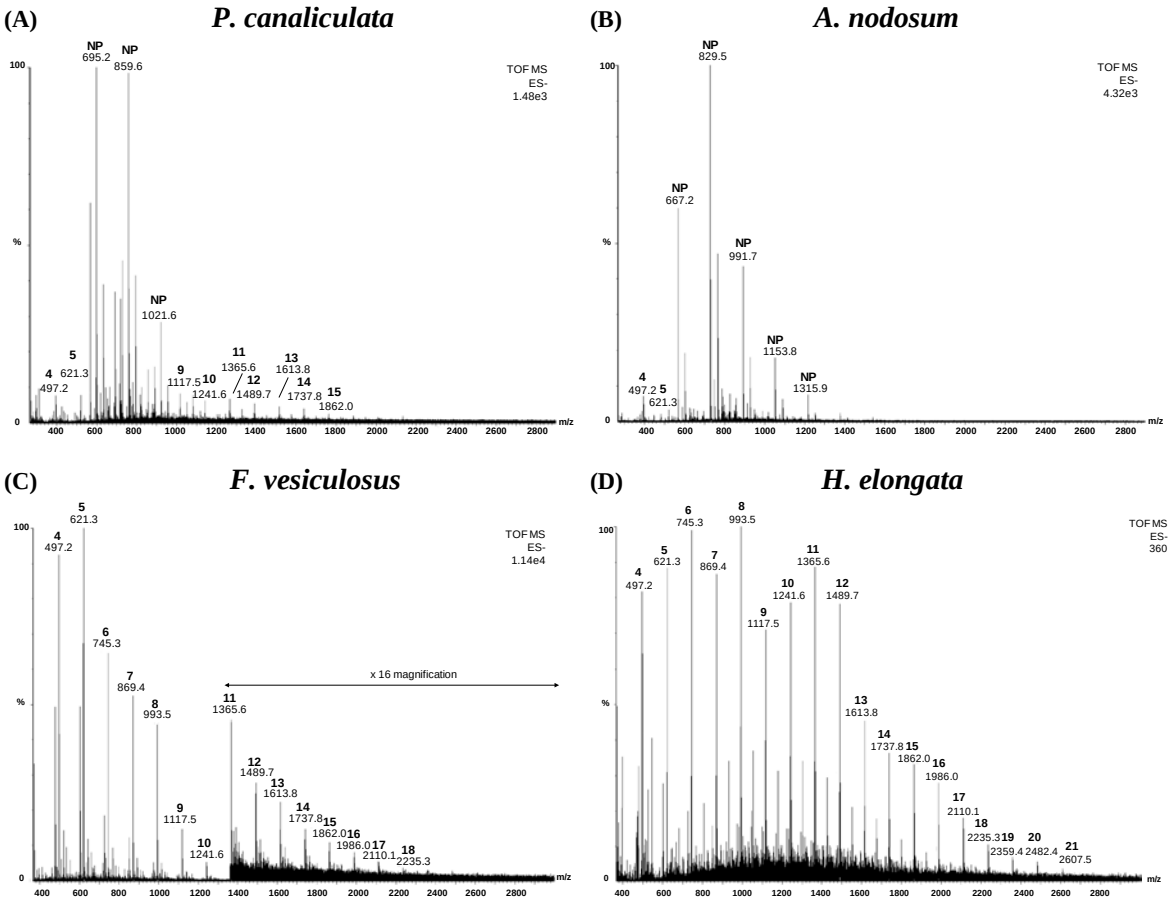



**Fig. 2.7.** Q-ToF-MS spectra, in negative mode (ES-), of LMW phlorotannin-enriched fractions from (A) *P. canaliculata*, (B) *A. nodosum*, (C) *F. vesiculosus* and (D) *H. elongata* collected from Finavarra, Co. Clare, in November 2015. Molecular mass (*m/z*) is indicated as the numbers above peaks and the degree of polymerization as the most upper numbers (bold) above peaks. Other compounds detected within the fractions not representative of phlorotannins were labelled as non-phlorotannin (NP) above the corresponding peaks.

### 2.3.3.1. *P. canaliculata*

The phlorotannin profiles obtained for this species displayed a high abundance of structures composed of 8-15 PGUs, corresponding to *m/z* 993-1865 (Fig. 2.9, 2.10). Oligomers within this range accounted for 82-97% in samples collected from Finavarra and 76-88% in samples from Mace Head. Those composed of 11 and 12 PGUs represented the most abundant compounds contributing between 15-17% to the total detected. The chemical composition of this species was relatively stable at both sites. However, in August 2014, samples collected from Mace Head exhibited a very different profile with low molecular weight structures of 4 and 5 PGUs accounting for the majority of the phlorotannins detected (31 and 27 %, respectively; Fig. 2.11).

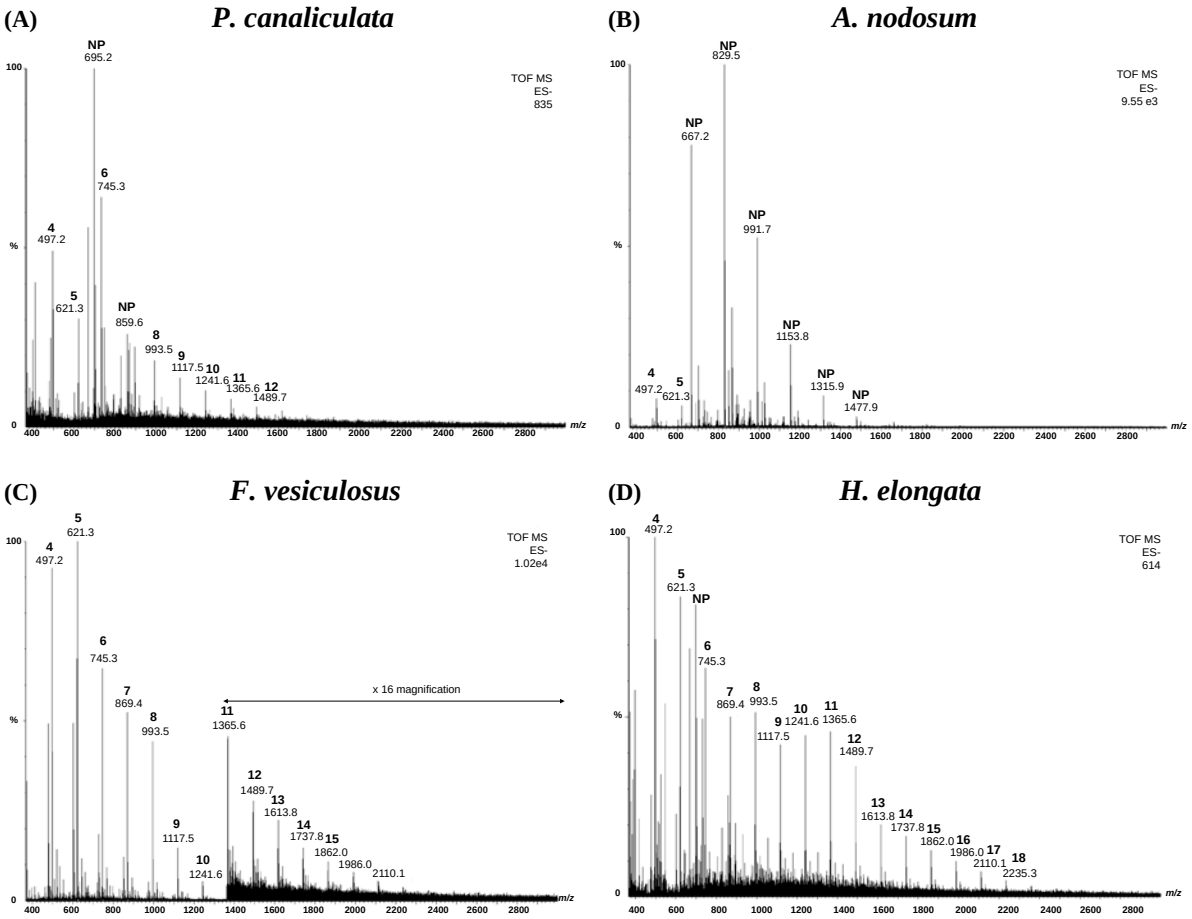



**Fig. 2.8.** Q-ToF-MS spectra, in negative mode (ES-), of LMW phlorotannin-enriched fractions from (A) *P. canaliculata*, (B) *A. nodosum*, (C) *F. vesiculosus* and (D) *H. elongata* collected from Mace Head, Co. Galway, in November 2015. Molecular mass (*m/z*) is indicated as the numbers above peaks and the degree of polymerization as the most upper numbers (bold) above peaks. Other compounds detected within the fractions not representative of phlorotannins were labelled as non-phlorotannin (NP) above the corresponding peaks.

### 2.3.3.2. *A. nodosum*

As previously stated, a high degree of interference from other compounds occurred in the LMW fractions from this species, even after RP flash separation (Fig. 2.7, 2.8). These were presumed as polysaccharides, specifically β-glucan derivatives, due to the molar distribution of the non-phlorotannin compounds detected (Fig. 2.7, 2.8). The phlorotannin profiles obtained from this species varied between the two sites with specimens collected from Mace Head exhibited a more uniform composition. Despite considerable seasonal variations, Mace Head fractions were, overall, dominated by phlorotannins composed of 4-5 PGUs as well as 8-12 PGUs (Fig. 2.10, 2.11). Structures of 5, 9 and 10 PGUs were the most dominant observed but their percentage contribution to the total varied

substantially (12-25%; 10-18%; 8-20%, respectively). Fractions derived from samples collected from Finavarra exhibited a lower degree of stability with significant seasonal shifts observed (Fig. 2.11). However, similar to Mace Head, the most abundant phlorotannins were found in the range of 8-12 PGUs (Fig. 2.11).

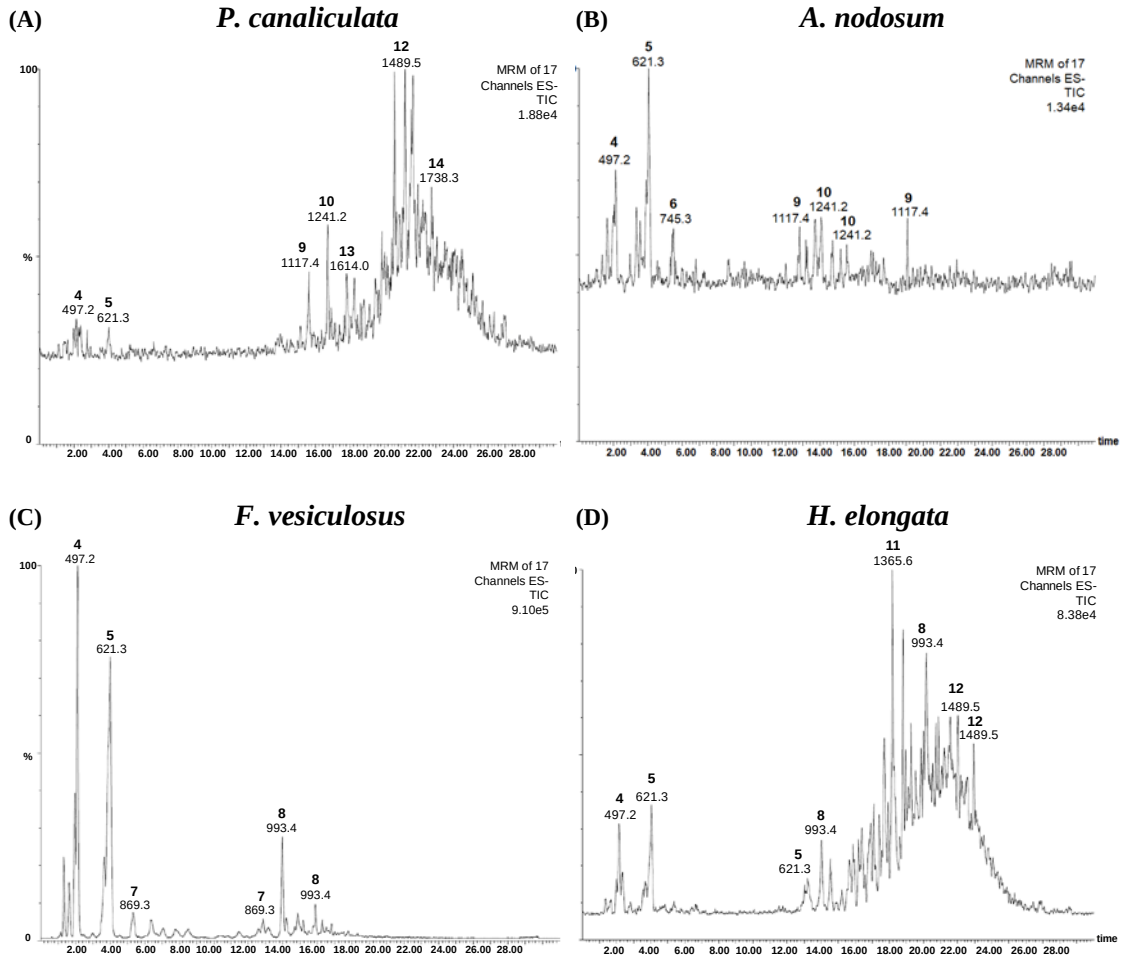



**Fig. 2.9.** UPLC-MS/MS total ion chromatograms (TIC), in negtiave mode (ES-), of LMW phlorotannin-enriched fractions from (A) *P. canaliculata*, (B) *A. nodosum*, (C) *F. vesiculosus* and (D) *H. elongata* collected from Finavarra, Co. Clare, in November 2015. Molecular mass (*m/z*) is indicated by numbers above peaks and the degree of polymerization by the most upper numbers (bold) above peaks.

### 2.3.3.3. *F. vesiculosus*

Samples collected from both sites displayed profiles highly abundant in relatively low molecular weight phlorotannins with the majority of phlorotannins within the range of

*m/z* 497-993 (4-8 PGUs), accounting for 86-89% in Finavarra and 85-95% in Mace Head (Fig. 2.11). The chemical profile within each site remained rather uniform over all six seasons, with only minor shifts detected for each individual MRM transition (Fig. 2.11). However, variations in the molecular composition were observed between the two sites (Fig. 2.9, 2.10, 2.11). For example, in samples collected in Finavarra, the most abundant phlorotannins were detected at, in order of decreasing dominance, *m/z* 621, 497 and (5, 4 and 8 PGUs), accounting for 30-38%, 15-19% and 14-17%, respectively (Fig. 2.11). Similarly, in Mace Head, phlorotannins corresponding to 5 and 4 PGUs were the two most dominant detected, but those of 5 PGUs accounting a higher % to the total than in Finavarra samples, with 35-45%. Furthermore, structures detected at *m/z* 993, corresponding to phlorotanins of 8 PGUs displayed a lower abundance than in Finavarra specimens, with 3-8% (Fig. 2.11). At this site, those composed 8 PGUs were more abundant (third most dominant detected) varying between 12 and 21%.

#### 2.3.3.4. *H. elongata*

The phlorotannin profiles obtained from this species appeared to be abundant in higher molecular weight structures (Fig. 2.9, 2.10). The most dominant phlorotanins were detected between *m/z* 993-1614, corresponding to phlorotannins composed of 8-13 PGUs. This range accounted for 76-86% in both samples collected from Finavarra and Mace Head (Fig. 2.11). Phlorotannin structures composed of 10 and 11 PGUs were the most abundant phlorotannins detected, accounting for 13-20% and 14-21%, respectively, in samples collected from Finavarra, and 14-20% and 17-21%, respectively, in those from Mace Head (Fig. 2.11). The overall molecular make-up of this species displayed a high degree of uniformity between the two sites but within each site the relative abundance was subject to small, but site-specific, seasonal shifts.

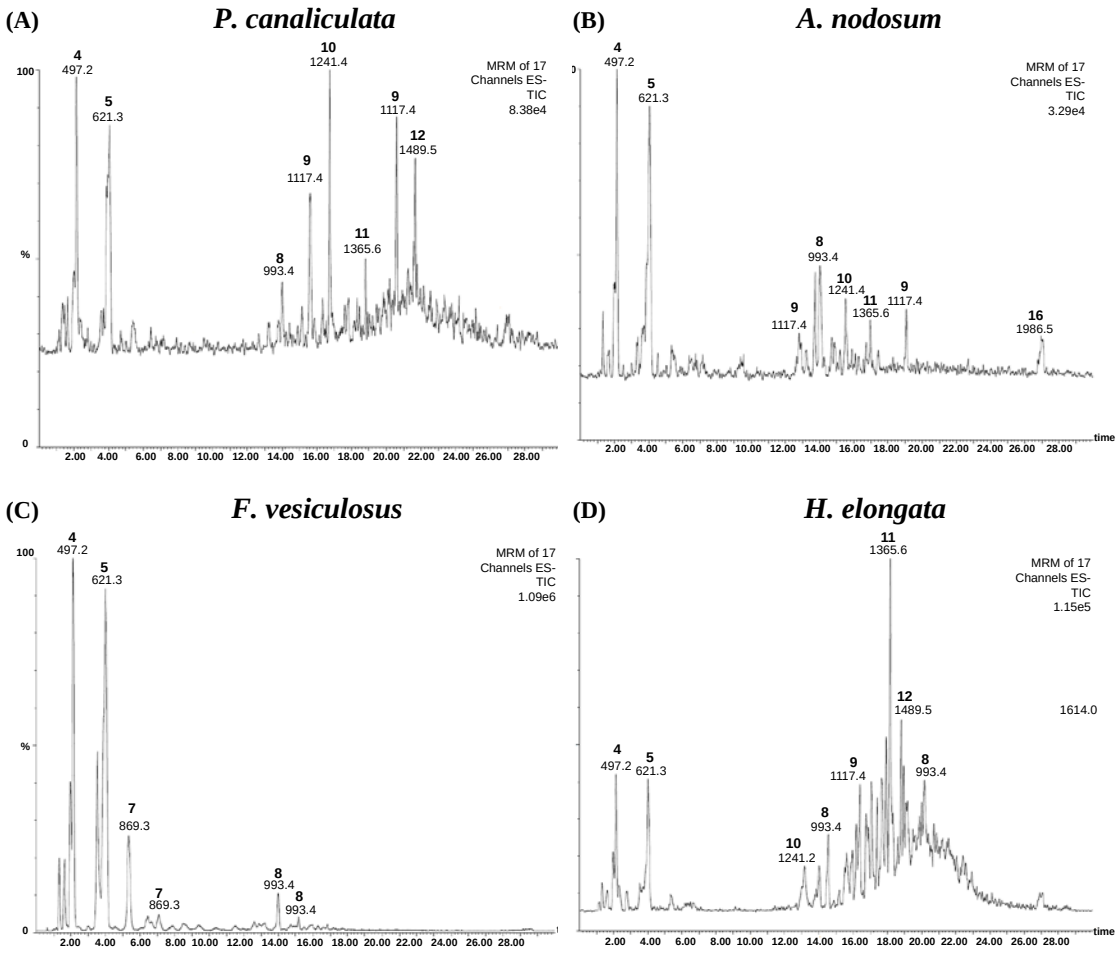



**Fig. 2.10**. UPLC-MS/MS total ion chromatograms (TIC), in negtiave mode (ES-), of LMW phlorotannin-enriched fractions from (A) *P. canaliculata*, (B) *A. nodosum*, (C) *F. vesiculosus* and (D) *H. elongata* collected from Mace Head, Co. Galway, in November 2015. Molecular mass (*m/z*) is indicated by numbers above peaks and the degree of polymerization by the most upper numbers (bold) above peaks.

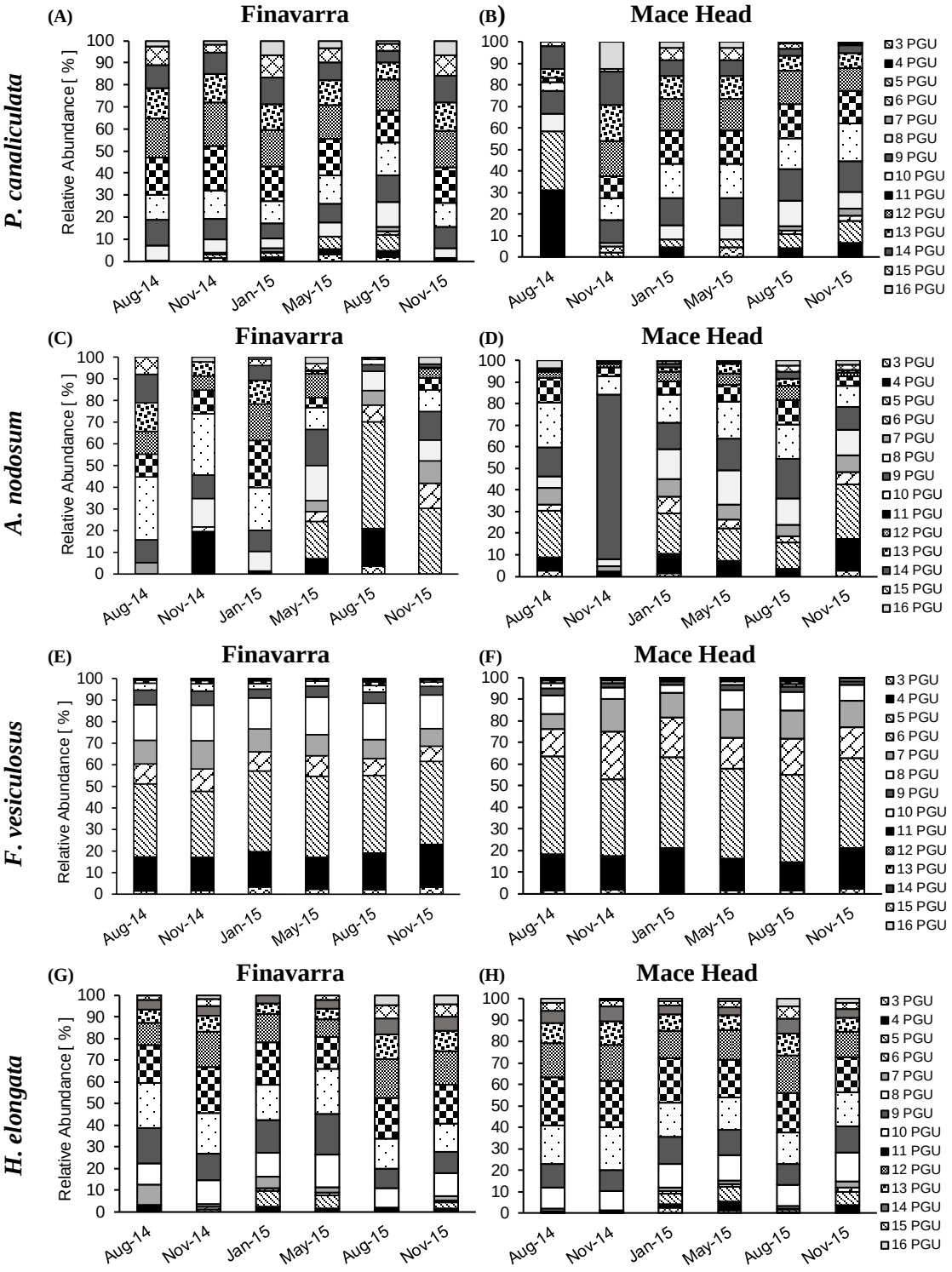



**Fig 2.11.** The relative abundance (%) of phlorotannins between 3 and 16 phloroglucinol units (PGU) as detected by the UPLC-MS/MS for (A, B) *P. canaliculata*, (C, D) *A. nodosum*, (E, F) *F. vesiculosus* and (G, H) *H. elongata* collected from Finavarra, Co. Clare and Mace Head, Co. Galway, from August 2014 to November 2015.

### 2.3.4. Degree of isomerisation

The employment of UPLC-MS/MS utilising a tandem quadrupole mass spectrometer (TQD-MS) in multiple reaction monitoring (MRM) mode allowed for increased sensitivity and resolution, thus aiding the detection and quantification of isomers for each individual molecular ion transition between 3-16 PGUs (Tierney *et al.* 2014). A signal: background noise ratio of five was set as the minimum threshold, and only peaks displaying a ratio higher were considered in the isomeric determination for each MRM transition. The total number of isomers was derived by the sum of all isomers detected from all 13 MRM transitions. Fig. 2.12 illustrates the seasonal variability detected for the total number of isomers for each species collected from both sites; the specific variability within each MRM ion transition is outlined in Tables 2.2-2.5, for each species, for the six seasons investigated.

#### 2.4.3.1. *P. canaliculata*

A high degree of isomerization was associated with this species, from both sites, as demonstrated in Fig. 2.9 and 2.10. This was depicted as a large “hump” in the total ion chromatogram (TIC). The majority of isomers were detected for phlorotannins composed of 9-14 PGUs (Table 2.2). This was observed for specimens collected from both sites. *P. canaliculata* collected from Finavarra displayed the highest isomeric abundance in November 2015, with 220 individual isomers being detected. In Mace Head samples the maximum amount was found in samples collected in May and August 2015 with 254 and 250, respectively (Fig. 2.12; Table 2.2).

#### 2.4.3.2. *A. nodosum*

This species displayed the lowest degree of structural variability out of the four species investigated. This was most likely associated with the difficulty in attaining a high level of fraction purity for UPLC-MS/MS analysis. Finavarra samples exhibited a considerably lower level of isomers (18-58) compared to those from the Mace Head (50-234). In the former samples, the number of isomers detected for an individual MRM transition did not exceed 11, while in Mace Head samples up to 38 individual isomers were observed. The majority of isomers, for the latter, were observed in phlorotannins ranging from 8-12

PGUs, while in Finavarra samples, the variability in isomeric abundance was sporadic with no one particular molecular range responsible for the isomeric total abundance (Fig. 2.12; Table 2.3).

#### 2.3.4.3. *F. vesiculosus*

The isomeric variability observed for this species was substantial, particularly considering the relative stability observed in the chemical composition. Total isomers detected in samples collected from Finavarra ranged from 138-342, with maximum levels observed in both January and August 2015 (Fig. 2.12), while in samples from Mace Head the variability observed between seasons was much greater, with total isomers ranging from 133-518 (Fig. 2.12; Table 2.4). Here, a significantly higher peak in the total isomeric abundance (total of 518) occurred in August 2015.

#### 2.3.4.4. *H. elongata*

Out of the four species investigated, the highest abundance of isomers was observed in *H. elongata* ranging between 200-400 isomers being over the six seasons. This high isomeric abundance was observed as a large “hump-like” structure in the TIC (Fig. 2.9, 2.10). At both sites, structural variability was highest in phlorotannins composed of 8-16 PGUs with as much as 50 individual isomers detected in a single MRM transition (Table 2.5). The lowest degree of isomerization was observed in November 2014 and January 2015 for both sites while maximum total isomers were detected in August 2015 and November 2015. Overall, the isomeric abundance was slightly higher in samples collected from Finavarra than in those from Mace Head and generally less variable (Fig. 2.12).

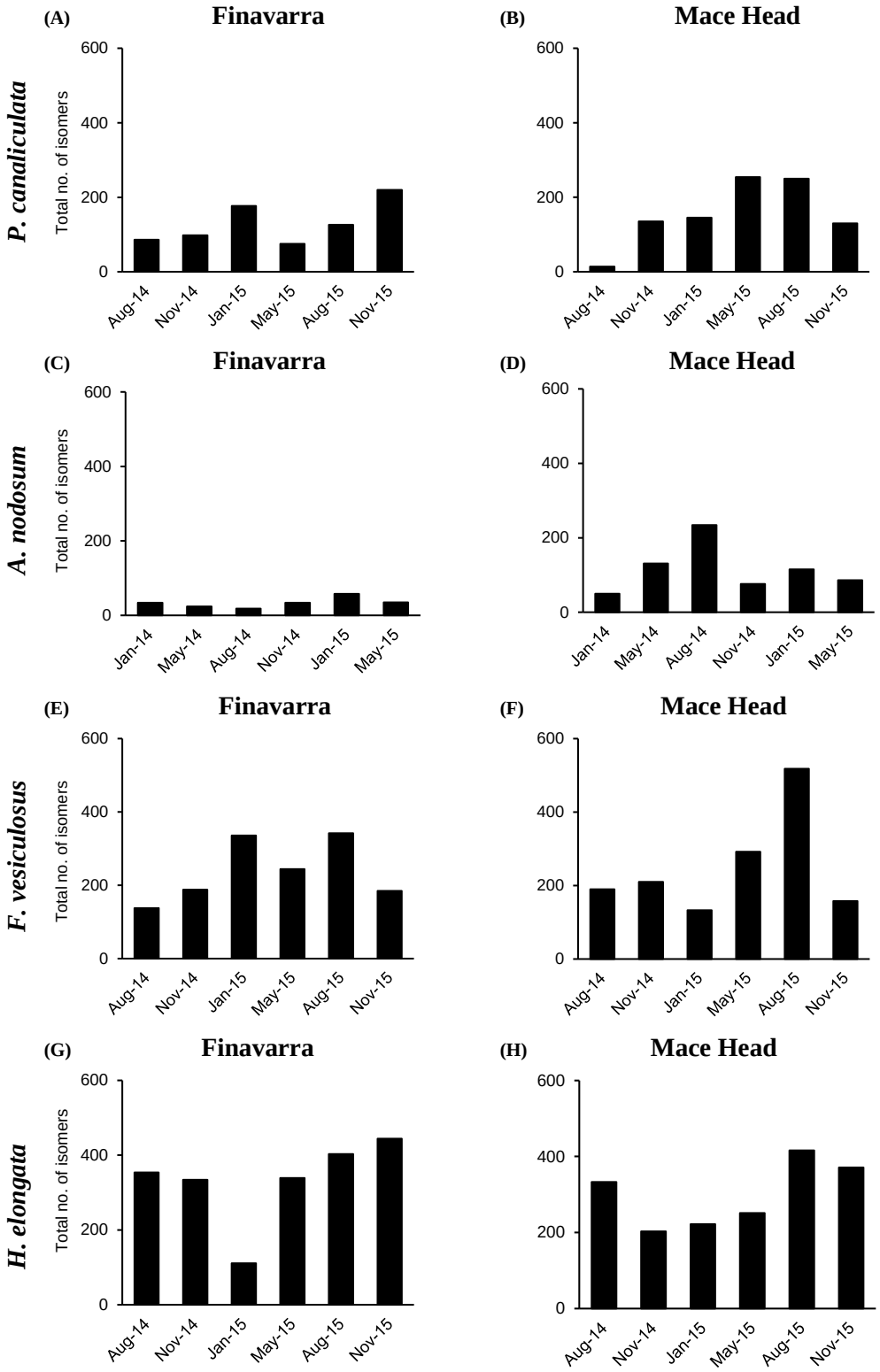



**Fig. 2.12.** Total number of isomers, as detected by UPLC-MS/MS, for phlorotannins between 3-16 phloroglucinol units (PGU) in the LMW phlorotannin-enriched fractions of (A, B) *P. canaliculata*, (C, D) *A. nodosum*, (E, F) *F. vesiculosus* and (G, H) *H. elongata* collected from Finavarra, Co. Clare and Mace Head, Co. Galway, over six seasons.

**Table. 2.2.** List of isomers detected for each MRM transition corresponding to 3-16 PGUs for ***P. canaliculata*** collected from (A) Finavarra, Co. Clare and (B) Mace Head, Co. Galway, for the six seasons investigated. Where 10 or more individual isomers were detetced for an individual molecular ion transition the number is highlighted in bold.

| | Degree of polymerization (PGU) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(A)** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **Total** |
| **Aug 2014** | 3 | 1 | 2 | 1 | 0 | 6 | 6 | **10** | **13** | **11** | **14** | **11** | 6 | 2 | 86 |
| **Nov 2014** | 0 | 1 | 2 | 0 | 1 | 5 | 7 | **12** | **17** | **21** | **16** | **13** | 2 | 1 | 98 |
| **Jan 2015** | 1 | 3 | 5 | 2 | 1 | 5 | **11** | **15** | **24** | **27** | **21** | **25** | **25** | **12** | 177 |
| **May 2015** | 4 | 4 | 8 | **10** | 9 | 9 | **10** | 6 | 7 | 3 | 2 | 2 | 0 | 1 | 75 |
| **Aug 2015** | 1 | 5 | 4 | 7 | 5 | **13** | **14** | **20** | **21** | **19** | **10** | 4 | 3 | 0 | 126 |
| **Nov 2015** | 0 | 2 | 4 | 1 | 0 | 8 | **23** | **22** | **32** | **23** | **34** | **31** | **21** | **19** | 220 |

| **(B)** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **Total** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Aug 2014** | 1 | 2 | 3 | 1 | 3 | 2 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 14 |
| **Nov 2014** | 0 | 2 | 3 | 2 | 2 | 0 | 6 | **10** | **17** | **21** | **20** | **17** | **19** | **16** | 135 |
| **Jan 2015** | 3 | 4 | 4 | 4 | 3 | 8 | **13** | **23** | **24** | **16** | **23** | 7 | 7 | 6 | 145 |
| **May 2015** | 0 | 3 | 6 | 5 | 3 | **19** | **27** | **38** | **35** | **31** | **29** | **25** | **17** | **16** | 254 |
| **Aug 2015** | 3 | 4 | 6 | 7 | 7 | **28** | **28** | **47** | **37** | **33** | **29** | **12** | 6 | 3 | 250 |
| **Nov 2015** | 2 | 3 | 7 | 7 | 6 | 9 | **14** | **21** | **25** | **21** | 8 | 3 | 2 | 2 | 130 |

**Table 2.3.** List of isomers detected for each MRM transition corresponding to 3-16 PGUs for ***A. nodosum*** collected from (A) Finavarra, Co. Clare and (B) Mace Head, Co. Galway, for the six seasons investigated. Where 10 or more individual isomers were detetced for an individual molecular ion transition the number is highlighted in bold.

| | Degree of polymerization (PGU) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(A)** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **Total** |
| **Aug 2014** | 2 | 2 | 0 | 1 | 1 | 4 | 1 | 6 | 5 | 4 | 1 | 2 | 2 | 3 | 34 |
| **Nov 2014** | 1 | 2 | 1 | 1 | 1 | 3 | 6 | 5 | 2 | 1 | 0 | 0 | 0 | 1 | 24 |
| **Jan 2015** | 1 | 1 | 0 | 1 | 1 | 1 | 4 | 6 | 1 | 2 | 0 | 0 | 0 | 0 | 18 |
| **May 2015** | 2 | 6 | 5 | 2 | 2 | 4 | 3 | 4 | 2 | 0 | 0 | 1 | 0 | 0 | 34 |
| **Aug 2015** | 2 | 6 | 6 | 10 | 9 | 11 | 8 | 3 | 1 | 1 | 0 | 0 | 1 | 0 | 58 |
| **Nov 2015** | 2 | 3 | 7 | 3 | 4 | 6 | 3 | 4 | 3 | 0 | 0 | 0 | 0 | 0 | 35 |

| **(B)** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **Total** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Aug 2014** | 1 | 3 | 4 | 6 | 5 | 5 | 6 | 5 | 5 | 2 | 3 | 1 | 2 | 2 | 50 |
| **Nov 2014** | 1 | 4 | 4 | 8 | 6 | **20** | **27** | **24** | **14** | **10** | 5 | 4 | 1 | 3 | 131 |
| **Jan 2015** | 2 | 4 | 9 | **16** | **21** | **38** | **33** | **39** | **26** | **25** | **11** | 6 | 3 | 1 | 234 |
| **May 2015** | 1 | 3 | 8 | 5 | 6 | **15** | **12** | **10** | 7 | 5 | 3 | 1 | 0 | 0 | 76 |
| **Aug 2015** | 1 | 4 | 6 | 5 | 4 | **17** | **15** | **25** | **14** | 9 | 4 | 4 | 3 | 4 | 115 |
| **Nov 2015** | 3 | 5 | 5 | 9 | 7 | **17** | **12** | **12** | 3 | 2 | 2 | 5 | 2 | 2 | 86 |

**Table 2.4.** List of isomers detected for each MRM transition corresponding to 3-16 PGUs for ***F. vesiculosus*** collected from (A) Finavarra, Co. Clare and (B) Mace Head, Co. Galway, for the five seasons investigated. Where 10 or more individual isomers were detetced for an individual molecular ion transition the number is highlighted in bold.

| **(A)** | **Degree of polymerization (PGU)** | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **Total** |
| **Aug 2014** | 2 | 2 | **10** | **17** | **26** | **27** | **16** | **14** | 8 | 7 | 2 | 3 | 3 | 1 | 138 |
| **Nov 2014** | 1 | 5 | **10** | **24** | **32** | **27** | **25** | **22** | **16** | **15** | 4 | 4 | 2 | 1 | 188 |
| **Jan 2015** | 3 | **14** | **21** | **28** | **35** | **34** | **56** | **38** | **37** | **20** | **22** | **15** | 9 | 4 | 336 |
| **May 2015** | 4 | 9 | **16** | **22** | **38** | **33** | **40** | **30** | **24** | **16** | 8 | 2 | 1 | 1 | 244 |
| **Aug 2015** | 5 | 7 | **24** | **30** | **44** | **55** | **39** | **43** | **27** | **26** | **17** | **10** | 7 | 8 | 342 |
| **Nov 2015** | 3 | 7 | **13** | **21** | **36** | **33** | **25** | **20** | **11** | 7 | 3 | 3 | 2 | 1 | 185 |

| **(B)** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **Total** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Aug 2014** | 4 | 6 | **12** | **13** | **16** | **6** | **10** | **19** | **29** | **32** | **20** | **13** | 8 | 2 | 190 |
| **Nov 2014** | 2 | 4 | 9 | **19** | **26** | **34** | **32** | **27** | **19** | **14** | **10** | 5 | 6 | 3 | 210 |
| **Jan 2015** | 1 | 6 | 8 | **19** | **31** | **25** | **12** | **15** | **10** | 3 | 2 | 1 | 0 | 0 | 133 |
| **May 2015** | 2 | **12** | **19** | **36** | **36** | **29** | **33** | **36** | **26** | **27** | **17** | **10** | 7 | 2 | 292 |
| **Aug 2015** | 4 | **17** | **38** | **33** | **50** | **73** | **52** | **58** | **45** | **51** | **38** | **27** | **15** | **17** | 518 |
| **Nov 2015** | 3 | 6 | **11** | **16** | **23** | **39** | **21** | **18** | **11** | 4 | 4 | 2 | 0 | 0 | 158 |

**Table 2.5.** List of isomers detected for each MRM transition corresponding to 3-16 PGUs for ***H. elonagata*** collected from (A) Finavarra, Co. Clare and (B) Mace Head, Co. Galway, for the six seasons investigated. Where 10 or more individual isomers were detetced for an individual molecular ion transition the number is highlighted in bold.

| | **Degree of polymerization (PGU)** | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(A)** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **Total** |
| **Aug 2014** | 2 | 5 | 5 | 5 | 7 | **30** | **35** | **47** | **49** | **49** | **38** | **38** | **26** | **18** | 354 |
| **Nov 2014** | 3 | 8 | 7 | 7 | 7 | **33** | **35** | **47** | **52** | **41** | **10** | **38** | **29** | **17** | 334 |
| **Jan 2015** | 4 | **15** | **29** | **34** | **31** | **41** | **44** | **34** | **25** | **20** | 7 | 7 | 3 | 0 | 297 |
| **May 2015** | 4 | 9 | 9 | **15** | **14** | **30** | **40** | **39** | **43** | **36** | **34** | **32** | **17** | **17** | 339 |
| **Aug 2015** | 3 | 3 | 4 | 6 | 9 | **37** | **33** | **45** | **48** | **37** | **44** | **47** | **41** | **46** | 403 |
| **Nov 2015** | 4 | 6 | 7 | **11** | **17** | **38** | **51** | **52** | **54** | **42** | **38** | **42** | **44** | **38** | 444 |

| **(B)** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **Total** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Aug 2014** | 2 | 4 | 4 | 6 | 9 | **27** | **30** | **47** | **43** | **44** | **43** | **31** | **28** | **15** | 333 |
| **Nov 2014** | 2 | 1 | 5 | 2 | 3 | **16** | **26** | **30** | **31** | **28** | **23** | **23** | **10** | 3 | 203 |
| **Jan 2015** | 4 | 6 | **10** | **10** | 8 | **26** | **28** | **25** | **33** | **24** | **21** | **16** | 6 | 5 | 222 |
| **May 2015** | 4 | 3 | 5 | 4 | **11** | **24** | **36** | **39** | **30** | **28** | **27** | **20** | **15** | 5 | 251 |
| **Aug 2015** | 3 | 4 | 4 | 4 | **15** | **40** | **40** | **45** | **47** | **39** | **45** | **51** | **40** | **39** | 416 |
| **Nov 2015** | 3 | 9 | 9 | **14** | **19** | **33** | **43** | **46** | **38** | **37** | **39** | **34** | **26** | **21** | 371 |

### 2.3.5. Total phlorotannin content (TPC) of LMW phlorotannin-enriched fractions

The total phlorotannin content (TPC) of the crude methanolic extracts was determined and are shown in the Supplementary Section 9.2, Fig. 9.2.1 – Fig. 9.2.4. The steps employed to enrich the LMW phlorotannin-enriched fractions were successful in increasing the phlorotannin content as the crude extracts displayed considerably lower phlorotannin concentrations than observed for the LMW phlorotannin-enriched fractions (results below), by 10-fold, in some species. Moreover, the two fractions (crude extract and LMW enriched fraction) displayed weak or no correlations with one another, displaying dissimilar seasonal trends, at both sites, for each of the assays employed.

The TPC of the LMW phlorotannin-enriched fractions, from each species, was tested for statistical differences occurring between thallus parts and season by employing a two-way variance of ANOVA ($p$<0.05), the details of which are described in Supplementary Section 9.2., Table 9.2.3.

#### 2.3.5.1. *P. canaliculata*

The total phlorotannin content of *P. canaliculata* collected from both sites were significantly affected by sampling season ($p$<0.001) but not the sampling site ($p$=0.95; Fig. 2.13) with both sites displaying a similar range of phlorotannin content. In samples collected from Finavarra, a relatively stable phlorotannin level was observed (294.7±10.4 to 348.1±18.4 µg PGE $mg^{-1}$ DWE; Fig. 2.13) throughout the six seasons, with the exception of May and August 2015 when they significantly decreased to 187 and 213 µg PGE $mg^{-1}$ DWE. At Mace Head, however, all six seasons differed ($p$<0.05). Maximum levels were detected in November 2015, peaking at 422.5± 23.6 µg PGE $mg^{-1}$ DWE, while lowest levels were found in May 2015, similar to those from Finavarra (211.5±12.9 µg PGE $mg^{-1}$ DWE; Fig. 2.13).

#### 2.3.5.2. *A. nodosum*

The TPC of *A. nodosum* samples displayed both significant seasonal ($p$<0.001) and spatial variations ($p$<0.001: Fig. 2.14). Algae from both sites displayed maximum levels in

August 2014 (Finavarra; 245.7±7.0 µg PGE $mg^{-1}$ DWE, Mace Head; 312.2±10.4 µg PGE $mg^{-1}$ DWE, Fig. 2.14) yet samples collected from Mace Head displayed a slightly higher level of phlorotannins detected than in those collected from Finavarra (Fig. 2.14). Seasonal minima differed between sites. In Finavarra samples minimum phlorotannin levels occurred in August in 2015, while for Mace Head samples the lowest content was observed in May 2015 (71.9±2.5, 69.5±2.1 µg PGE $mg^{-1}$ DWE, respectively; Fig 2.14).

#### 2.3.5.3. *F. vesiculosus*

Both sampling season ($p<0.001$) and site ($p<0.001$) significantly affected the TPC observed in LMW fractions of *F. vesiculosus* samples. The degree of seasonal variability, however, was more apparent in samples from Finavarra, than those from Mace Head (Fig. 2.15). Samples from Mace Head displayed a relatively stable phlorotannin level, with a narrow range of seasonal variability observed (370 to 470 µg PGE $mg^{-1}$ DWE; Fig. 2.15). Conversely, Finavarra samples demonstrated a highly variable phlorotannin content, with a minimum of 157.9±5.9 µg PGE $mg^{-1}$ DWE in August 2014, increasing to a maximum of 474.1±3.3 µg PGE $mg^{-1}$ DWE in May 2015, where after it remained stable ($p<0.05$; Fig. 2.15).

#### 2.3.5.4. *H. elongata*

The phlorotannin content of the LMW phlorotannin-enriched fractions in *H. elongata* samples displayed both seasonal ($p<0.001$) and spatial variability ($p=0.002$; Fig. 2.16). Finavarra samples contained high phlorotannin levels in both August and November 2015 (429.7±7.0 and 438.1±7.7 µg PGE $mg^{-1}$ DWE, respectively; Fig. 2.16). Similarly, Mace Head samples also displayed highest phlorotannin content in August 2015, which was similar to those in Finavarra samples (449.5±12.6 µg PGE $mg^{-1}$ DWE; Fig. 2.16). Moreover, samples from both sites contained the lowest phlorotannin content in May 2015, with, again, similar levels being observed in both sites (Finavarra; 140.9±1.2 µg PGE $mg^{-1}$ DWE, Mace Head; 197.5±2.5 µg PGE $mg^{-1}$ DWE; Fig. 2.16).

### 2.3.6. *In vitro* antioxidant activity

Similar to the phlorotannin content, the *in vitro* antioxidant activity observed was 7-10 fold higher in the LMW phlorotannin-enriched fractions, compared to the crude extracts (Section 9.2, Fig. 9.2.1 - 9.2.4). The correlation between the fractions, similar to that observed for TPC, were weak in most species. *P. canaliculata*, however, exhibited a mild correlation between the two fractions regarding the RSA ($R^2$=0.43, Supplementary Section 9.2, Fig. 9.2.1.).

The ferric reducing antioxidant power (FRAP) and the radical scavenging activity (RSA) of the LMW phlorotannin-enriched fractions, from each species, was tested for statistical differences occurring between thallus parts and season by employing a two-way variance of ANOVA ($p$<0.05), the details of which are described in Supplementary Section 9.2, Table 9.2.3. In most species, the FRAP and RSA of the LMW phlorotannin-enriched fractions investigated was significantly affected by both sampling site ($p$<0.001) and season ($p$<0.001) as well as the interaction between the two factors ($p$<0.001).

#### 2.3.6.1. *P. canaliculata*

The FRAP activity of the LMW phlorotannin-enriched fractions exhibited both seasonal ($p$<0.001) and spatial ($p$<0.001) variations (Fig. 2.17). FRAP activity observed in the LMW phlorotannin-enriched fractions in samples from Finavarra were relatively stable. Similar levels observed in all seasons, between 195.5±12.0 to 220.0±20.3 µg TE $mg^{-1}$ DWE, with the exception of August 2015, when activity decreased to 154.0±8.3 µg TE $mg^{-1}$ DWE ($p$<0.05; Fig. 2.17). In Mace Head, a slightly broader range of seasonal variability was observed (151-251 µg TE $mg^{-1}$ DWE; Fig. 2.15). The FRAP activity exhibited by samples at both sites displayed a positive correlation with the phlorotannin content ($R^2$=0.52; Fig. 2.17).

The radical scavenging activity (RSA) of the LMW fractions also displayed both seasonal ($p$=0.019) and spatial ($p$<0.001) variations (Fig. 2.13). However, it did not appear to be associated with the overall phlorotannin content ($R^2$=0.07). Minimum RSA levels were found in January 2015 at both sites although the RSA in Finavarra samples was slightly lower (Finavarra; 56.7±6.5 µg $mL^{-1}$, Mace Head; 43.2±7.3 µg $mL^{-1}$; Fig. 2.13). Highest

RSA levels were detected in August 2014 in samples from Mace Head with $IC_{50}$ values of 18.9±2.5 μg $mL^{-1}$, while in Finavarra samples, peak values were found in November 2015 (21.3±1.7 μg $mL^{-1}$; Fig. 2.13).

#### 2.3.6.2. *A. nodosum*

Both sampling season ($p$<0.001), site ($p$<0.001) and their interaction ($p$<0.001), were observed to significantly affect the FRAP activity of *A. nodosum* (Fig. 2.14). Mace Head samples displayed a higher capacity for ferric reduction, as reflected in the range of FRAP values observed (Fig. 2.14). LMW fractions derived from Finavarra samples exhibited FRAP activity ranging from 92.6±7.5 to 178.7±26.0 μg TE $mg^{-1}$ DWE, while those from Mace Head displayed a much broader range varying from 81.0±3.1 to 232.9±10.0 μg TE $mg^{-1}$ DWE across the six seasons ($p$<0.05; Fig. 2.14). The FRAP activity of *A. nodosum* was strongly correlated with the observed TPC ($R^2$=0.80).

Similar to the FRAP activity, the RSA exhibited both seasonal ($p$<0.01) and spatial ($p$<0.01) variations (Fig. 2.14). The LMW phlorotannin-enriched fractions from *A. nodosum* samples displayed the lowest activity of all species investigated, with $IC_{50}$ values of up to 158.3±10.2 μg $mL^{-1}$ in samples collected from Finavarra in May 2015. The lowest RSA for Mace Head samples was also observed in May 2015, but not to the same extent of those in Finavarra (91.4±3.9 μg $mL^{-1}$; Fig. 2.14). At both sites, the highest RSA was observed in fractions collected in August 2014, again with Mace Head fractions having an elevated activity compared to those from Finavarra (15.2±0.5, 32.7±5.7 μg $mL^{-1}$, respectively; Fig. 2.14). RSA levels displayed a positive correlation with the phlorotannin content ($R^2$=0.48), however this was not as strong as the relationship observed between FRAP and TPC (Fig. 2.14).

#### 2.3.6.3. *F. vesiculosus*

The FRAP activity exhibited by *F. vesiculosus* LMW fractions was considerably higher than all other species examined, with samples at both sites displaying highest levels of up to 717.9±12.2 and 726.01±17.8 μg TE $mg^{-1}$ DWE in November 2015 (Fig. 2.15). This was over three times the level observed for *P. canaliculata* and *A. nodosum*. Within *F. vesiculosus*, however, FRAP was significantly affected by sampling season ($p$<0.001),

site ($p$<0.001) and their interaction ($p$<0.001; Fig. 2.15). The seasonal variability of FRAP was more pronounced in samples collected from Finavarra (314.1±31.27 - 717.9±12.19 µg TE $mg^{-1}$ DWE) than those from Mace Head (508.2±18.3 - 726.1±17.8 µg TE $mg^{-1}$ DWE), in a similar manner to that observed for TPC ($R^2$= 0.71; Fig. 2.15).

In contrast to FRAP, the RSA of *F. vesiculosus* samples did not display a positive correlation with phlorotannin levels ($R^2$=0.013; Fig. 2.15), but it did appear to be significantly affected by season ($p$<0.001), site ($p$<0.001) and their interaction ($p$<0.001; Fig. 2.15). In Finavarra, a relatively high and stable level of RSA was observed over the seasons (23.0±0.7 - 29.4±2.8 µg $mL^{-1}$), with the exception of November 2014, when a significant decrease occurred ($p$<0.05; 40.7±6.0 µg $mL^{-1}$). Samples from Mace Head, while displaying seasonal differences, also displayed a narrow range in RSA levels (24.9±1.5 - 35.8±1.3 µg $mL^{-1}$; Fig. 2.15).

#### 2.3.6.4. *H. elongata*

The FRAP values of LMW phlorotannin-enriched fractions from *H. elongata* were strongly correlated with the observed phlorotannin levels, mirroring a similar degree of seasonal variability ($R^2$=0.74; Fig. 2.16). However, the observed FRAP, while displaying seasonal variation ($p$<0.001), it did not differ significantly between sites ($p$=0.964; Fig. 2.16), with samples from Finavarra and Mace Head both displaying similar levels of FRAP activity, following a similar seasonal trend (Fig.2.16).

However, the RSA varied significantly, both between seasons ($p$<0.001) and site ($p$=0.006; Fig.2.16). A significant decrease in the observed $IC_{50}$ values occurred in January 2015, with levels up to 79.2±2.4 µg $mL^{-1}$ in Finavarra samples and 71±5.7 µg $mL^{-1}$ in Mace Head samples (Fig 2.16). Such low activity was also seen in May 2015 for Mace Head (67.1±8.1 µg $mL^{-1}$; Fig. 2.16). Highest RSA was observed in fractions from Mace Head collected in August 2014 (15.3±2.3 µg $mL^{-1}$) while in Finavarra-derived fractions highest activity coincided with lowest $IC_{50}$ values observed in November 2015 (27.9±0.9 µg $mL^{-1}$). This was significantly similar to those collected in August and November 2014 (Fig. 2.16).

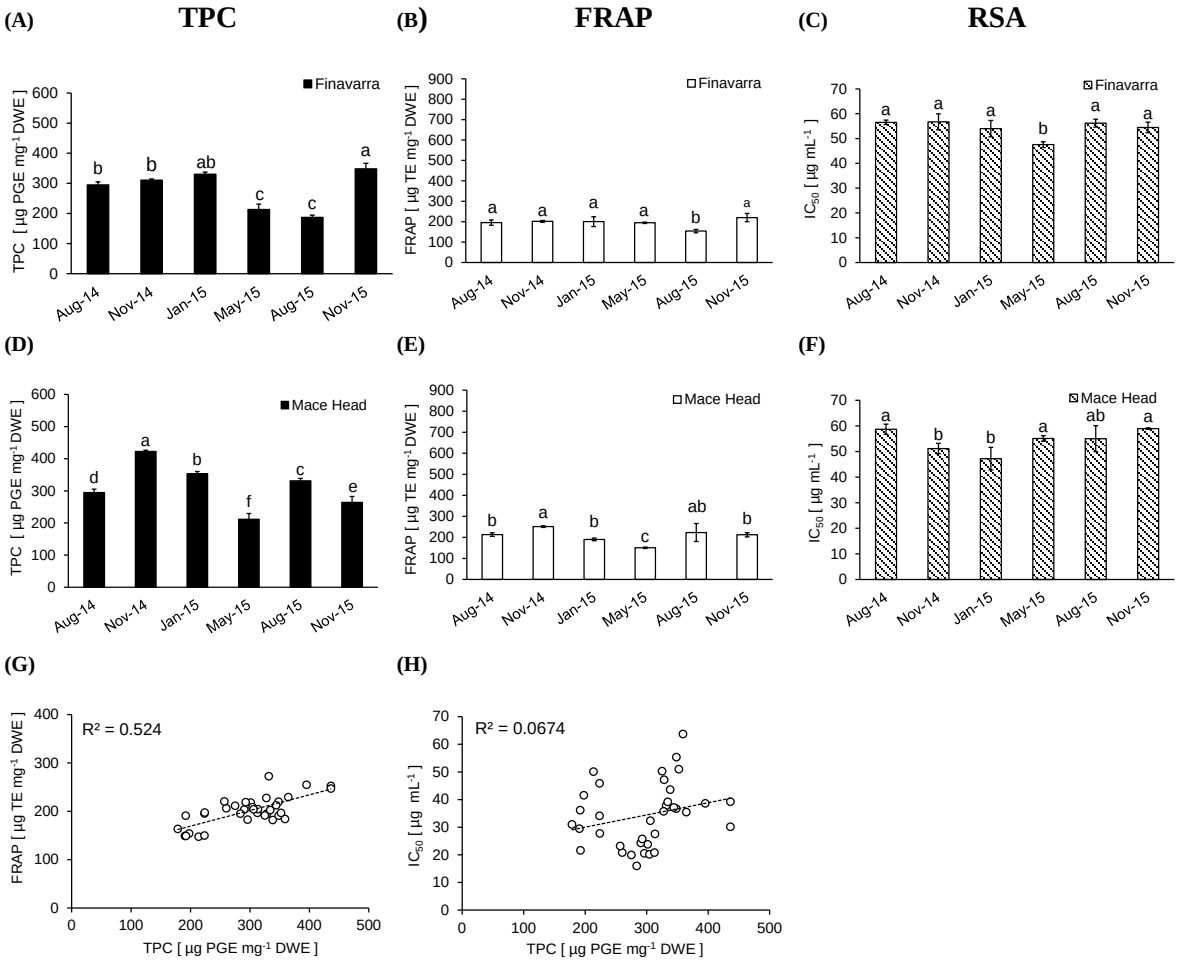



**Fig. 2.13.** Seasonal variation in the total phlorotannin content (TPC) of LMW phlorotannin-enriched fractions from ***P. canaliculata*** collected from (A) Finavarra, Co. Clare and (D) Mace Head, Co. Galway, expressed as microgram phloroglucinol equivalents per milligram of dry weight extract (µg PGE $mg^{-1}$ DWE). The seasonal variation in the ferric reducing antioxidant power (FRAP) of the LMW phlorotannins-enriched fractions from samples collected from (B) Finavarra, Co. Clare and (E) Mace Head, Co. Galway, expressed as microgram Trolox equivalents per milligram of dry weight extract (µg TE $mg^{-1}$ DWE). The seasonal variations of the radical scavenging activity (RSA) of LMW phlorotannin-enriched fractions from samples collected from (C) Finavarra, Co. Clare and (F) Mace Head, Co. Galway, expressed as $IC_{50}$ values in microgram per millilitre (µg $mL^{-1}$). Correlation coefficient ($R^2$) is given for the linear relationship between (G) FRAP and TPC and (H) $IC_{50}$ and TPC. Data are represented as mean ± standard deviation (n=3). Significant effects of the sampling season and site on the TPC, FRAP and RSA on the LMW fractions were tested using a two-way ANOVA ($p$<0.05). Superscripts identify between significant differences between samples using Tukey's *post-hoc* test.

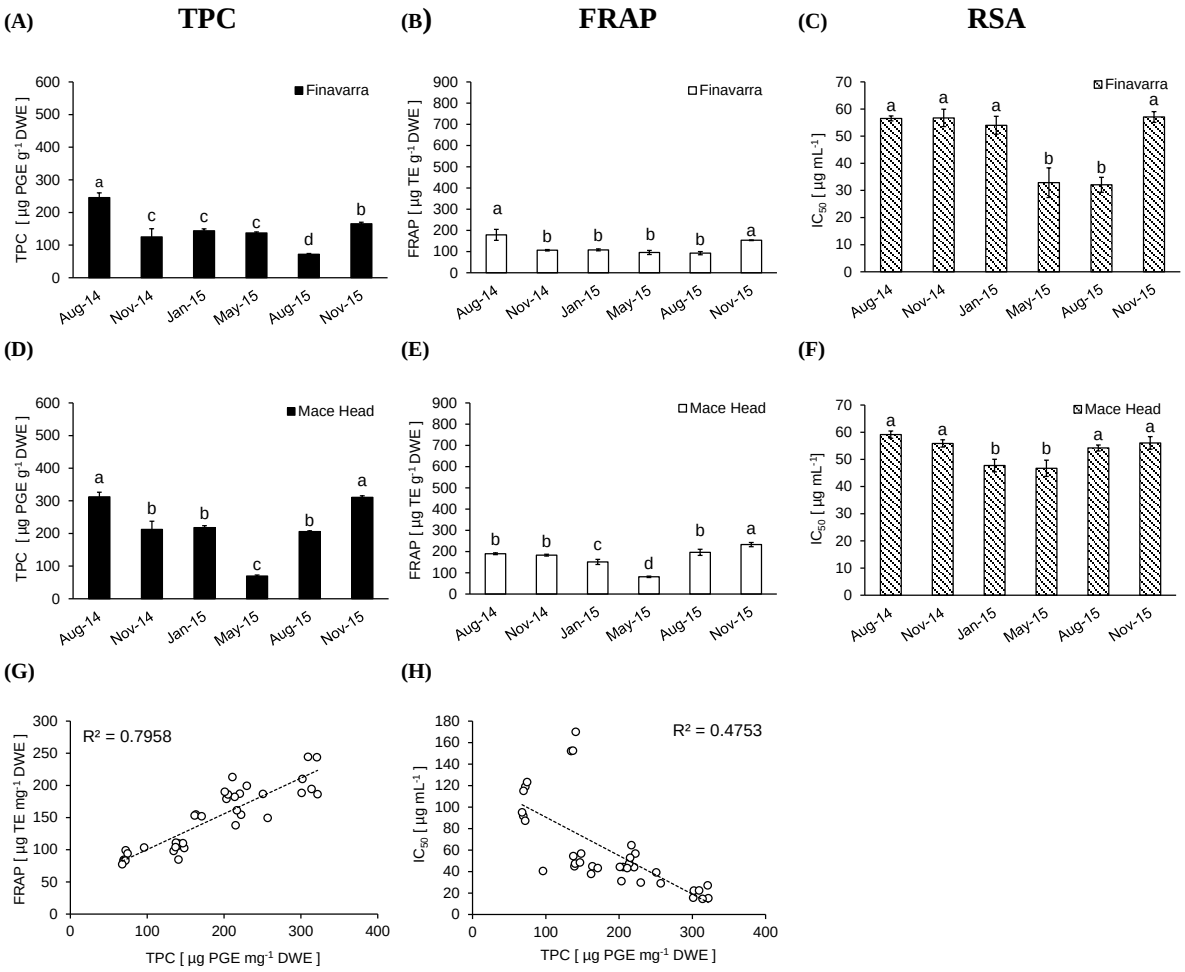



**Fig. 2.14.** Seasonal variation in the total phlorotannin content (TPC) of LMW phlorotannin-enriched fractions from ***A. nodosum*** collected from (A) Finavarra, Co. Clare and (D) Mace Head, Co. Galway, expressed as microgram phloroglucinol equivalents per milligram of dry weight extract (µg PGE $mg^{-1}$ DWE). The seasonal variation in the ferric reducing antioxidant power (FRAP) of the LMW phlorotannins-enriched fractions from samples collected from (B) Finavarra, Co. Clare and (E) Mace Head, Co. Galway, expressed as microgram Trolox equivalents per milligram of dry weight extract (µg TE $mg^{-1}$ DWE). The seasonal variations of the radical scavenging activity (RSA) of LMW phlorotannin-enriched fractions from samples collected from (C) Finavarra, Co. Clare and (F) Mace Head, Co. Galway, expressed as $IC_{50}$ values in microgram per millilitre (µg $mL^{-1}$). Correlation coefficient ($R^2$) is given for the linear relationship between (G) FRAP and TPC and (H) $IC_{50}$ and TPC. Data are represented as mean ± standard deviation (n=3). Significant effects of the sampling season and site on the TPC, FRAP and RSA on the LMW fractions were tested using a two-way ANOVA ($p$<0.05). Superscripts identify between significant differences between samples using Tukey's *post-hoc* test.

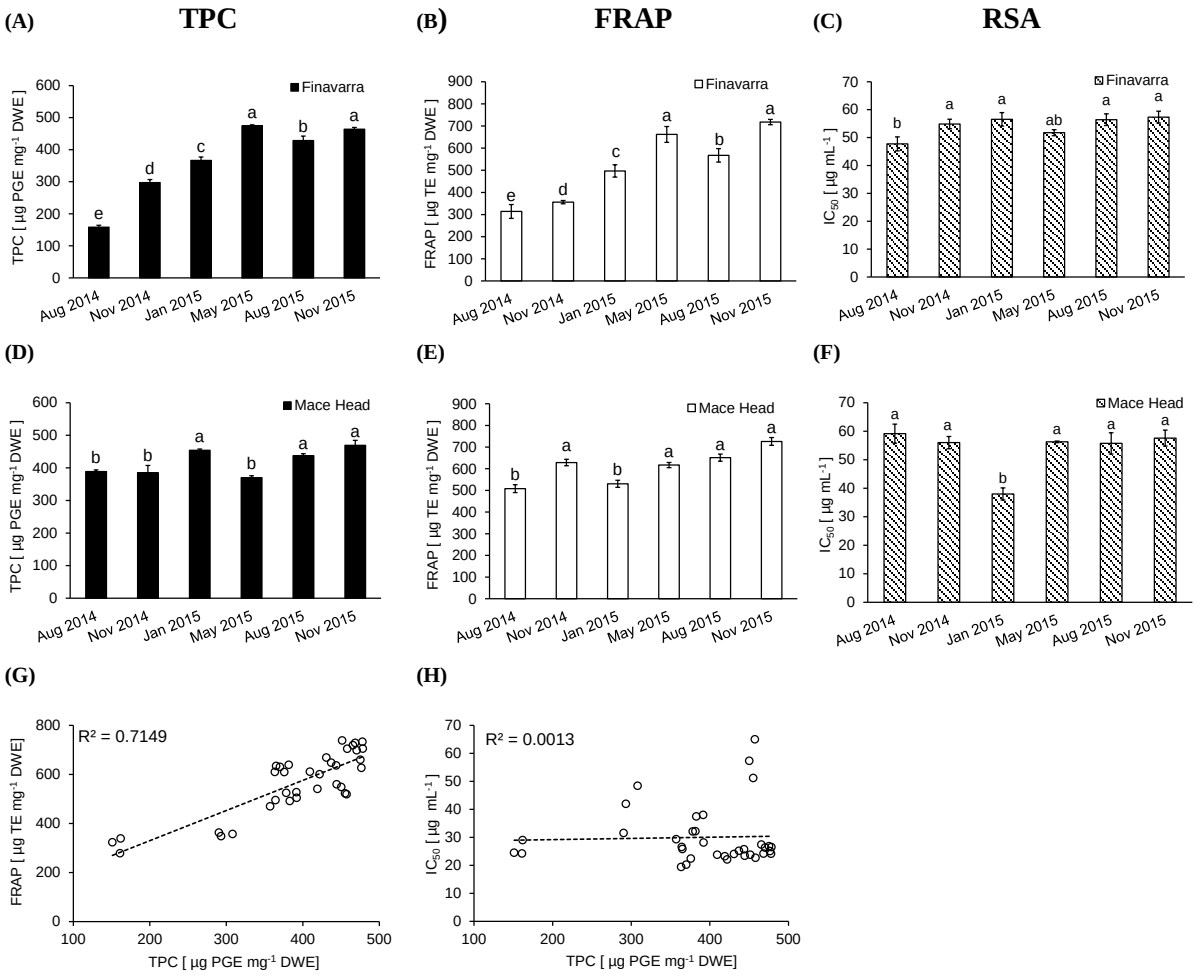



**Fig. 2.15.** Seasonal variation in the total phlorotannin content (TPC) of LMW phlorotannin-enriched fractions from ***F. vesiculosus*** collected from (A) Finavarra, Co. Clare and (D) Mace Head, Co. Galway, expressed as microgram phloroglucinol equivalents per milligram of dry weight extract (µg PGE $mg^{-1}$ DWE). The seasonal variation in the ferric reducing antioxidant power (FRAP) of the LMW phlorotannins-enriched fractions from samples collected from (B) Finavarra, Co. Clare and (E) Mace Head, Co. Galway, expressed as microgram Trolox equivalents per milligram of dry weight extract (µg TE $mg^{-1}$ DWE). The seasonal variations of the radical scavenging activity (RSA) of LMW phlorotannin-enriched fractions from samples collected from (C) Finavarra, Co. Clare and (F) Mace Head, Co. Galway, expressed as $IC_{50}$ values in microgram per millilitre (µg $mL^{-1}$). Correlation coefficient ($R^2$) is given for the linear relationship between (G) FRAP and TPC and (H) $IC_{50}$ and TPC. Data are represented as mean ± standard deviation (n=3). Significant effects of the sampling season and site on the TPC, FRAP and RSA on the LMW fractions were tested using a two-way ANOVA ($p$<0.05). Superscripts identify between significant differences between samples using Tukey's *post-hoc* test.

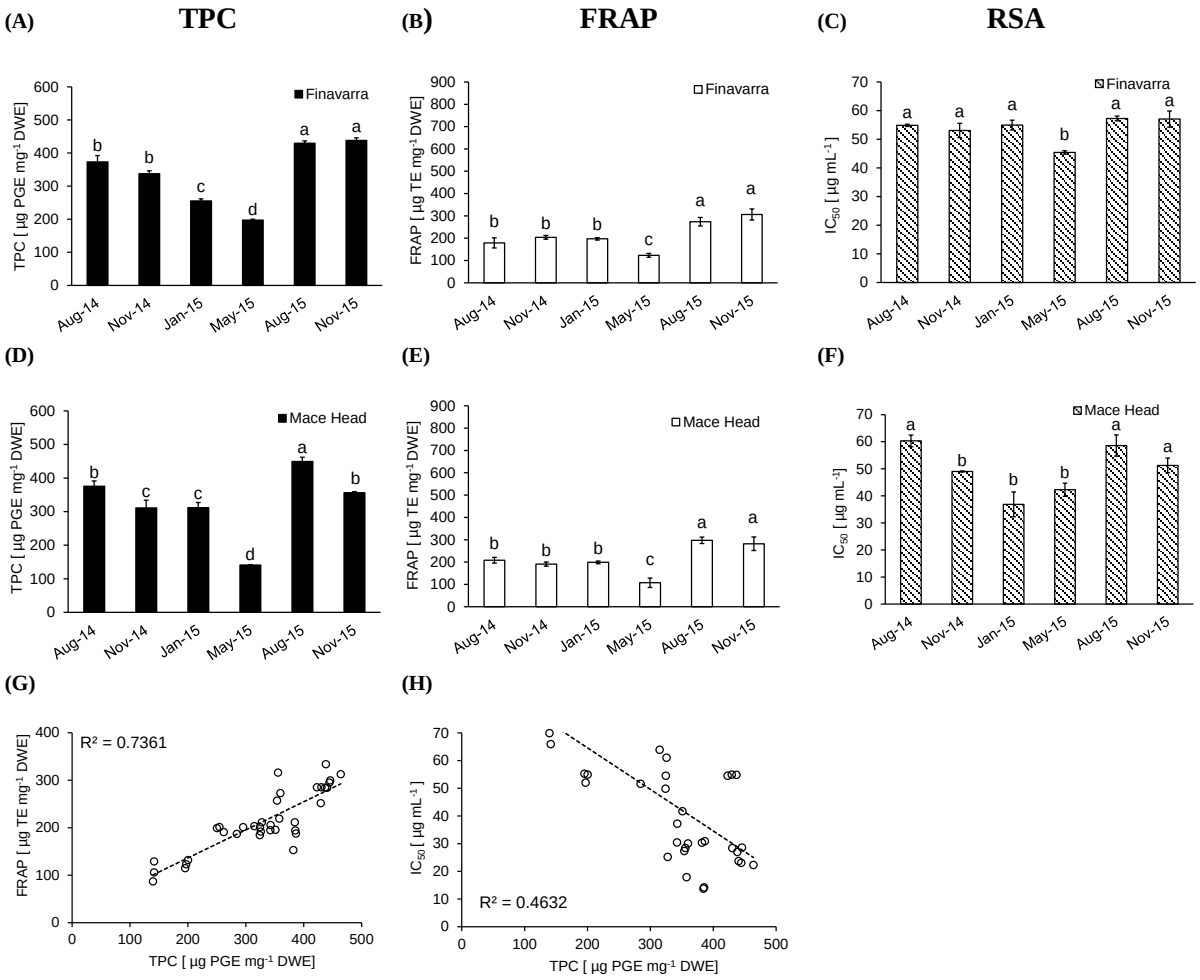



**Figure 2.16.** Seasonal variation in the total phlorotannin content (TPC) of LMW phlorotannin-enriched fractions from ***H. elongata*** collected from (A) Finavarra, Co. Clare and (D) Mace Head, Co. Galway, expressed as microgram phloroglucinol equivalents per milligram of dry weight extract (µg PGE $mg^{-1}$ DWE). The seasonal variation in the ferric reducing antioxidant power (FRAP) of the LMW phlorotannins-enriched fractions from samples collected from (B) Finavarra, Co. Clare and (E) Mace Head, Co. Galway, expressed as microgram Trolox equivalents per milligram of dry weight extract (µg TE $mg^{-1}$ DWE). The seasonal variations of the radical scavenging activity (RSA) of LMW phlorotannin-enriched fractions from samples collected from (C) Finavarra, Co. Clare and (F) Mace Head, Co. Galway, expressed as $IC_{50}$ values in microgram per millilitre (µg $mL^{-1}$). Correlation coefficient ($R^2$) is given for the linear relationship between (G) FRAP and TPC and (H) $IC_{50}$ and TPC. Data are represented as mean ± standard deviation (n=3). Significant effects of the sampling season and site on the TPC, FRAP and RSA on the LMW fractions were tested using a two-way ANOVA ($p$<0.05). Superscripts identify between significant differences between samples using Tukey's *post-hoc* test.

## 2.4. Discussion

This chapter aimed to elucidate on the natural variability in the LMW phlorotannin profiles of four intertidal brown algal species. To date, the chemical characterisation of phlorotannins has been limited due to their structurally complexity. The high activity associated with phlorotannins is reportedly owed to their structural size and arrangement (Ferreres *et al.*, 2012; Li *et al.*, 2009). Therefore, chemical elucidation is essential to understand both their ecological importance as well as commercial relevance. Through seasonal and spatial sampling, it was hypothesised that the effect of extrinsic factors, and the natural variability therein, on the content, as well as biochemical composition and associated *in vitro* antioxidant activity, of LMW phlorotannin fractions, would be revealed. Samples of *P. canaliculata*, *A. nodosum*, *F. vesiculosus* and *H. elongata* were collected over six seasons, between 2014 and 2015, from two sites along Galway Bay that differed in both their topography and degree of exposure.

Furthermore, due to the high antioxidant activity and potential commercial utilisation of low molecular weight phlorotannin fractions (Tierney *et al.*, 2013), this research was specifically interested in investigating the factors that contribute toward the variability of low molecular weight (<3.5 kDa) phlorotannin fractions. By employing the method of Tierney *et al.* (2014), LMW phlorotannin-enriched fractions were successfully attained. In comparison to crude extracts, this heightened degree of purification significantly elevated the concentration of phlorotannins and thus the level of *in vitro* antioxidant activity. While interspecific variability of LMW phlorotannin-enriched fractions has been previously carried out (Heffernan *et al.*, 2015; Tierney *et al.*, 2014), this is, to our knowledge, the first time such specific fractions have been assessed for spatial and seasonal variability.

It should be noted that as it was only phlorotannins within one fraction (<3.5 kDa) that were investigated, the natural variability observed here may not, from an ecological point of view, be representative of the entire natural phlorotannin pool within certain species. For example, phlorotannins within *A. nodosum* are thought to be of high molecular weight

compounds (over 50 kDa) (Audibert *et al.*, 2010). Therefore, shifts observed here will not reflect deviations that may arise in these larger phlorotannins.

### 2.4.1. Interspecific variability

The LMW phlorotannin profiles of the four brown algae investigated were strongly species-specific. The degree of fraction purity also varied between species owing to other aspects of their biochemistry. For example, the use of reverse phase (RP) flash chromatography was employed for adequate separation of phlorotannins from other polar compounds, such as polysaccharides, with which phlorotannins are closely associated. This was successfully achieved in fractions extracted from *F. vesiculosus* and *H. elongata.* Within these two species, LMW phlorotannin oligomers were the most dominant compounds detected in the Q-ToF-MS spectra, indicating of a high level of purity. *P. canaliculata* and *A. nodosum*. However, were associated with separation issues. Mannitol and β-glucan, in particular, often prove difficult to separate from phlorotannins (Tierney *et al.,* 2014). Such interference in the LMW fractions of *P. canaliculata* and *A. nodosum* was observed but to a greater extent in the latter. The high abundance of such compounds often masked the presence of phlorotannins in the Q-ToF-MS spectra. This "contamination" also weakened the detection of phlorotannins in UPLC-MS/MS analysis. In previous studies, further separation utilising normal phase (NP) flash chromatography, prior to UPLC-MS analysis, achieved satisfactory purity (Tierney *et al.,* 2014). Yet, here the use of NP flash separation resulted in a further loss of phlorotannins and so its use was ceased. This shows how strong the association is between these compounds and highlights the need for conditions capable of breaking their interlinking bonds before phlorotannin purity can be achieved (Salgado *et al.,* 2007). Such associations with polysaccharides has been seen in the complexation of phlorotannins with alginic acids in the cell wall, whereby they form covalent bonds, requiring strong alkaline conditions to degrade (Koivikko *et al.*, 2005). Therefore, the naturally high abundance of alginates in *A. nodosum* (Moen *et al.*, 1997) may be fundamental to the difficulty in extracting and isolating phlorotannins. The impact of these polysaccharides on phlorotannin extractability may also imply that the differences observed, both in the content and chemical composition of fractions from

*A. nodosum*, were caused by variations in polysaccharide concentrations, rather than the phlorotannins themselves.

Moreover, within the LMW fractions of *P. canaliculata* and *A. nodosum*, a high occurrence of non-phlorotannin compounds was observed, thought to be LMW β-Glucan oligomers, due to the uniform deviations in their molar distribution of *m/z* 162. The high abundance of these compounds in these two particular species is possibly linked to the presence of an endophytic fungus, *Stigmidium ascophylli* (Cotton), with which these species possess a symbiotic relationship (Garbary and Gautam, 1989; Rugg and Norton, 1987). This would explain the high abundance of β-glucan compounds, being a main component of the fungal cell wall (Fleet, 1991). Furthermore, in *P. canaliculata*, the presence of the obligate fungus has been reported to cause shifts in mannitol concentrations, with levels decreasing in the absence of the fungus (Kingham and Evans, 1986). Perhaps, through the manipulation of this fungus' abundance, adjustments to the chemical properties of these species could be achieved, aiding phlorotannin separation. For these two species to become viable sources of bioactive phlorotannins in the future, further work is needed on enhancing their extractability, potentially through the dissociation with biologically present polysaccharides.

However, out of the four species investigated the purification of *F. vesiculosus* and *H. elongata* was achieved readily. *F. vesiculosus* exhibited a high abundance of low molecular weight structures (4-8 PGUs) while *H. elongata* was predominantly composed of larger compounds (8-13 PGUs). Similar profiles for these two species have been reported on recently (Heffernan *et al.*, 2015). The LMW phlorotannin profiles derived from *H. elongata* exhibited a "hump-like" formation in the total ion chromatogram. This is due to the structural similarity within the dominant range of phlorotannins, displaying a high degree of conformational arrangements. This in agreement with Heffernan *et al.* (2015) who also observed a high degree of isomerisation for *H. elongata*. Between 200-440 isomers were detected with over 50 individual isomers within a single MRM transition. In *F. vesiculosus*, the total number of isomers detected for each season was found within a lower range of 130-330, in both sites. However, in August 2015, fractions from *F. vesiculosus* collected in Mace Head displayed over 500 isomers, highlighting that

certain environmental conditions can exacerbate the mechanisms that lead to elevated isomeric production in this species.

Despite the stark differences between their LMW profiles between *H. elongata* and *F. vesiculosus*, at certain seasons they displayed a similar level of antioxidant activity. Similarly, Cerantola *et al.* (2006) reported two isolated phlorotannin structures from *F. spiralis*, differing in their structural complexity, to be equal in their scavenging ability. This highlights how the degree of anti-radical activity observed is not just simply by way of higher phlorotannin concentrations but rather it is also the arrangement of the phlorotannin structure and the position of the active hydroxyl groups that influences their antioxidant potential (Ferreres *et al.*, 2012; Shibata and Ishimaru, 2008). This is further supported by the relatively high RSA in *F. vesiculosus*, which displayed a weak correlation with the corresponding TPC. However, this also depends on the activity under investigation (Prior *et al.*, 2005). For example, in most species, the FRAP activity displayed a stronger association with phlorotannin levels than RSA suggesting that depending on the activity in question, different parameters of the phlorotannin profile, such as the degree of polymerisation or the isomerisation, will influence the outcome. For example, phlorotannin activity has been previously suggested to increase with molecular weight (Boettcher and Targett, 1993; Hagerman *et al.*, 1998). However, Heffernan *et al.* (2015) suggested that perhaps high concentrations of low molecular weight compounds could allow for a higher number of free hydroxyl units that in larger structures could be involved in the oligomeric linkages. Furthermore, a higher degree of structural complexity, as represented in the isomeric abundance, may also lead to lower activity, again, from the use of hydroxyl units in the structural bonds between monomeric units, preventing their involvement in radical stabilisation (Kirke *et al.*, 2017).

However, the factors behind phlorotannin isomeric behaviour are still largely unknown. Previous studies comparing phlorotannin profiles of brown algae have reported on species-specific variations in isomerization, suggesting that the specific structural properties of different phlorotannin polymers present in the various species *i.e.* linkage type, maybe responsible for the differences observed (Heffernan *et al.*, 2015; Tierney *et al.*, 2014). Apart from the natural biosynthetic pathways for the production of

phlorotannin isomers, the extent of these could be increased by oxidative reactions, created by the structural recombination of molecules, most likely through intra- and/ or intermolecular bonding (Poncet-Legrand *et al.*, 2010). Therefore, variations in the observed degree of isomerization could reflect upon the effects of the surrounding environment to which the alga was exposed to at the time of collection. However, the degree of polymerization will also affect the level of isomers detected. A higher abundance of phloroglucinol monomers (i.e. increased degree of polymerization) will allow for increased opportunities for structural recombination's (Tierney *et al.*, 2014). This may also contribute to the higher isomeric abundance in *H. elongata,* compared to *F. vesiculosus.*

### 2.4.2. Spatial variability

Spatial variations in polyphenol levels of brown seaweeds have been previously investigated between hemispheres (Steinberg, 1989, 1992; Van Altena and Steinberg, 1992) as well as between temperate and tropical regions (Van Alstyne and Paul, 1990; Targett *et al.*, 1992). However, the spatial variability in phlorotannin chemical profiles has not yet been addressed. It was hypothesised that chemo-physio differences between sites may result in various profiles, dependent on the degree of defence required to cope and/or recover from the specific set of environmental parameters. The coastal serrations of the west of Ireland support a wide range of geographical niches, between which environmental parameters can vary considerably. Two sites were selected for investigation due to their differences in degree of wave exposure as well as topography, both of which can, indirectly, affect algal biochemistry. Located at the very western tip of Connemara in Co. Galway, Mace Head was a fully exposed site, characteristic of a higher degree of turbulence and mechanical stress caused by water movement. Finavarra, however, was a more sheltered site location found within the inner most part of Galway Bay. The degree of exposure exhibited by a site has been reported to affect both the morphology and physiology of the inhabiting algae (Kalvas and Kautsky, 1993; Rönnberg and Ruokolahti, 1986).

Regardless of the different environments exhibited by the two sites, the phlorotannin molecular composition of each species did not appear to vary between sites, with species-

specific metabolic profiles being observed. This suggests that differences between species, in the fundamental phlorotannin composition, arise from genetic evolution rather than physiological adaptation to local conditions. It could also suggest that while exhibiting differences in their degree of wave exposure and slope gradient, the different sites did not vary from another to the extent of generating biochemical ecotypes. However, within each site, slight shifts in the relative abundance varied. This may indicate that while imposing no major effects on the overall metabolic profile, slight differences in the environment possibly influences seasonal adjustments in the chemical proportions. However, deciphering the manner in which is difficult with these shifts occurring displaying no particular seasonal trends.

Seasonal variations between the two sites were also observed in the overall phlorotannin content, with most species collected from Mace Head, Co. Galway, displaying slightly higher levels of LMW phlorotannins, compared to those from Finavarra, Co. Clare. Van Alstyne *et al.* (1999) found polyphenolic variations within a single kelp species to vary considerably over a scale over tens of metres. Similarly, Pavia and Åberg (1996) found the spatial variability of polyphenols in *A. nodosum* to be more significant at a smaller, local scale than between distances spanning over 1,000 km. However, the manner in which these site-specific seasonal variations arose is not fully understood.

Rönnberg and Ruokolahti (1986) observed that *F. vesiculosus* occupying a more sheltered site in the Baltic Sea displayed a broader seasonal variability in the polyphenol content compared to those from an exposed site. This was suggested to relate to the differences in epiphytic settlement and seasonal variations in their abundance. The higher degree of variability observed in the phlorotannin content of *F. vesiculosus* fractions collected from Finavarra were thought to be related to such a factor. In May 2014, a significant bloom in the abundance of the filamentous epiphyte, *Pylaiella littoralis*, covered the majority of the *F. vesiculosus* population in Finavarra, making sampling impossible for that month (Fig. 2.17). Such a thick coverage would have a considerable effect on the light and resource availability for the underlying plants and thus could potentially have led to reduced metabolic rates, including the synthesis of phlorotannins. This may explain the significantly lower phlorotannin content observed in the following month (August 2014),

which was followed by a gradual increase, levelling off to a stable level similar to that of Mace Head by May 2015. This is further supported by the high phlorotannin levels observed in January 2014 (intra-thallus assessment, Chapter 3), prior to the bloom, which were similar to those observed in and after May 2015.

Such epiphytic abundance would be lowered in exposed sites due to an inability to settle in high water velocities. This highlights how biotic relationships can alter phlorotannin concentrations, potentially through manipulation of abiotic factors. Jormalainen *et al.* (2003) also shared a similar theory whereby decreases in phlorotannin levels were also thought result from epiphytic shading and only increased once again by the simultaneous removal of the epibiota and hyaline hairs by grazers, resulting in increased light availability as well as reduced nutrient uptake. They concluded phlorotannin variation was encountered through the manipulation of resources rather than as defensive induction. However, while this may explain the observed differences for *F. vesiculous,* it is does not transfer across all the species.

The effect of wave exposure on grazing pressure is another major aspect for consideration for phlorotannin variability. It is likely that, similar to its effects of epiphytic abundance, the higher degree of wave exposure associated with Mace Head would lower the grazing pressure for species here. However, past studies have suggested that phlorotannins are induced by increases in grazer presence (Pavia and Toth, 2000; Van Alstyne, 1988; Van Alstyne and Paul, 1990; Steinberg, 1984). The results here would suggest otherwise with samples from Mace Head, overall, possessing higher phlorotannin concentrations. However, the effect of such biotic factors require further extensive research before a conclusion on their effect can be made.

**Fig. 2.17.** Photo of the thick coverage of epiphyte, *Pylaiella littoralis*, on a mixed patch of *A. nodosum* and *F. vesiculosus* in Finavarra, Co. Clare, in May 2014.

Other studies investigating spatial variations in phenolic levels reported the chemistry of the site to play a significant role. For example, Ilvessalo and Tuomi (1989) found three populations of *F. vesiculosus* in the Baltic Sea to differ in their phenolic concentrations dependent on the nitrogen regime of the site. A similar effect of nitrogen has been reported on wherein phlorotannin levels decreased with increasing nitrogen concentrations (Arnold *et al.*, 1995; Koivikko *et al.*, 2008, 2005; Yates and Peckol, 1993). Salinity is another factor reported to cause polyphenolic variations (Connan and Stengel, 2011; Munda, 1964; Pedersen, 1984; van Hees *et al.*, 2017) with decreasing levels and increased exudation rates often associated with decreasing salinity. However, as full marine sites, the salinity levels within these two sites was considered as relatively stable, at a level of 30-35. These was observed in previous measurements taken in 2013 and 2014 for Finavarra, Co. Clare by (Schmid, 2016) and Gietl (2016). Similarly, Schmid (2016) observed a narrow range for seasonal nitrogen levels in Finavarra. Yet, as no measurements were taken for either factor within this study, no correlations can be made with the data obtained here.

### 2.4.3. Seasonal variability

Owing to their antioxidant and photo-protective functions, phlorotannin are subject to variability, responding to temporal environmental changes in temperature, irradiance and, in particular, UVR (Cruces and Huovinen, 2012; Gomez and Huovinen, 2010; Pavia and

Brock, 2000; Schoenwaelder, 2002). Therefore, they display a high degree of seasonality (Connan *et al.*, 2004; Kamiya *et al.*, 2010; Parys *et al.*, 2009; Plouguerné *et al.*, 2006; Ragan and Jensen, 1978). Abdala-Díaz *et al.* (2006) found a positive correlation existed between the accumulation of phenolics in *C. tamariscifolia* and seasonal increases in irradiance, with levels being two-four fold greater in summer than in autumn and winter. Short-term fluctuations in the phenolic content of *P. canaliculata* and *A. nodosum* was associated with increased irradiance and air temperatures during emersion periods (Connan *et al.*, 2007). Ragan and Glombitza (1986) observed a high correlation between the surface water temperature and the phenolic content in *F. vesiculosus.*

Seasonal variations in irradiance and temperature within Galway Bay were monitored over the two years of sampling. This was in an effort to elucidate upon any correlations between these abiotic factors and variations in the LMW phlorotannin profiles. Both parameters displayed seasonal variations with maximums observed in summer months and minimums in the winter. Phlorotannin levels, as well as the associated degree of antioxidant activity, also exhibited seasonal variations ($p<0.001$; see Section 9.2, Table 9.2.2) but no apparent correlation with the external environmental factors could be made, in any of the species investigated. In fact, both maximum and minimum phlorotannin levels were shared over several seasons.

Moreover, the seasonal differences observed in TPC, FRAP and RSA, as well as in the relative abundance of chemical profiles, appeared to be site-specific, suggesting that the response of phlorotannins to environmental changes is more strongly reliant on a local scale rather than seasonal shifts in parameters such as irradiance and/or temperature.

Previous studies have suggested that ontogenetic changes could be responsible for the observed seasonal variations rather than inducible defences (Kamiya *et al.*, 2010; Plouguerné *et al.*, 2006; Ragan and Jensen, 1978; Stiger *et al.*, 2004). Seasonal maximums were reported to coincide with the reproductive maturation of the species in question. For example, Ragan and Jensen (1978) reported the maximum phenolic levels for *F. vesiculosus* and *A. nodosum* decreased, following conceptacle development toward a summer minimum just before mature gametes were released. Phlorotannin levels in *P. canaliculata, A. nodosum* and *H. elongata* all displayed a lower phlorotannin levels in

May 2015, which could be associated with dispersal of reproductive conceptacles. This is particularly evident in *A. nodosum*, with no receptacles remaining attached to the thallus. Conversely, phenolic levels in *Sargassum* species were observed to peak in the summer months (Kamiya *et al.*, 2010; Stiger *et al.*, 2004). This was thought to coincide with the beginning of the maturity period (Plouguerné *et al.*, 2006) and was suggested to function as a protective mechanism to ensure successful dispersal of conceptacles and subsequent colonisation. However, development stages within a single species can be staggered across different locations (Åberg and Pavia, 1997), and their differences in environmental cues upon which maturation depends. Developmental maturity may have contributed to the relative changes in the abundance of different-weighted phlorotannins which were observed to vary between seasons. However, thus far, this has not been examined.

## 2.5. Conclusion

LMW Phlorotannin metabolic profiles appear to stem from genetic evolution rather than physiological local adaptation to a specific environment, particularly concerning the differences in physical properties such as the degree of wave exposure. Yet, the effect of site chemistry (e.g. salinity, nutrients) cannot be ruled out. Species-specific profiles, while displaying small seasonal shifts in the relative abundance, displayed relatively uniform chemical compositions. *F. vesiculosus*, in particular, owing to its high abundance of LMW phlorotannins and relatively high degree of stability, was revealed as a good source of highly active phlorotannins. This highlights its potential as suitable candidate for the extraction and application of phlorotannins in future commercial ultisation, as well as a model species to further investigate phlorotannin variability. However, species, such as *A. nodosum*, due to the high complexity they display with intracellular polysaccharides, are unlikely to be considered for commercial phlorotannin uses, at least not until a method can be developed that allows for a higher degree of separation. Moroever, the interspecific variations in molecular weight and isomerisation highlighted that phlorotannins are not the only factor that need to be considered when determining the factors controlling antioxidant activity, as observed between *F. vesiculosus* and *H. elongata*.

Phlorotannin levels and the associated *in vitro* antioxidant activity of the LMW fractions varied significantly over the six seasons investigated. However, the manner in which these changes were observed did not correspond to the seasonal shifts in irradiance or temperature regimes of Galway Bay, as a whole. It is likely that changes in phlorotannin profiles will be more reliant on local variations. The rapid of response of these polyphenols to an array of external stimuli may mean that seasonal sampling will not aid in elucidating the primary factors causing their variability and that perhaps they vary on a smaller-scale *i.e.* hours, or days. Such short-term shifts have been previously reported (Abdala-Díaz *et al.*, 2006; Connan *et al.*, 2007). Moreover, the possible effect of co-occuring ontogenetic development may serve to interfere with seasonal assessment of abiotic factors on phlorotannin variability and thus must be considered/investigated separately. This was addressed in the following chapter (Chapter 3), while individual and

combined effects of abiotic factors that may have contributed to the seasonal and spatial variations observed were addressed in Chapters 4-6.

# Chapter 3

Intra-thallus variability in the low molecular weight phlorotannin profiles of four brown seaweeds

## 3.1. Introduction

In response to diurnal and seasonal variations in physio-chemical regimes, most macroalgae possess the ability to acclimate to their local environment by adjusting their chemical composition (Stengel *et al.*, 2011). Such temporal tuning includes alterations in both the concentration and composition of pigments (Aguilera *et al.*, 2002; Pereira *et al.*, 2012), shifts in the proportional ratios of fatty acids (Gombos *et al.*,1994; Schmid *et al.*, 2014) and the induction of various defence metabolites (Karentz *et al.*, 1991; Pavia and Toth, 2000). However, as various thallus parts, within an individual plant, differ in their physiological function, it is also likely that different chemical compositions can also exist between thallus sections or developmental state.

The Laminariales, in particular, display a high degree of morphological distinction between thallus parts, all with very specific physiological functions *i.e.* structure, growth and photosynthesis. Such morphological differentiation is associated with varied metabolic activity (Lüning *et al.*, 1973) and thus is likely to exhibit strong diversification in the biochemical composition. One good example of this is the gradient that exists within the thalli, wherein different functions of carbon metabolism occur (Gómez *et al.*, 2005; Kremer, 1981). For instance, carbon assimilation is higher in the distal non-growing regions while the apical region is involved with photosynthesis and the meristematic region, located at the base of the lamina, shows higher rates of light-independent carbon fixation, powering biomass production. Due to these differing morpho-physio functions, intra-thallus variations would be expected in pigment concentrations, being higher in the apical areas, higher carbon stores (laminarian and mannitol) in the distal regions, and an increased abundance of carbon-fixing enzymes, in the meristem (Cabello-Pasini and Alberte, 2001; Gómez and Wiencke, 1998).

Within the Fucales, intra-thallus morphological differentiation is less extensive, and distinctions between physiological functions can be difficult to separate, particularly as most of the thallus is involved in photosynthesis. However, most species, particularly *Fucus* spp., still display distinctions between the holdfast, stipe and frond. Depending on their position, the degree of exposure differs between thallus parts and so may result in differing physio-chemical profiles. For example, Stengel and Dring (1998) found that in

*Ascophyllum nodosum*, a canopy-forming, mid-intertidal species, the tips of the plants were generally higher in pigments while the older basal parts, that are continuously shaded by the canopy, had much lower levels. This was also reflected in seasonal variation of pigment content, with the tips displaying winter maxima and subsequent losses of up to 50% in summer. In the older, more shaded parts, less seasonal variability was observed. A similar trend was also observed for both the pigment and total fatty acid (TFA) content in *A. nodosum* and *F. serratus* from Galway Bay. Schmid and Stengel (2015) found the tips and middle parts of *A. nodosum* thallus to be higher in fucoxanthin and β-carotene than the holdfast or stipe. Similarly, the chlorophyll *a* and carotenoid levels were higher in the blade of *F. serratus* than the stipe or holdfast. This was also found for the TFA content with both species displaying significantly higher TFA in the upper sections of the thallus compared to the lower holdfast and stipe (up to 2-4 times higher). As well for photosynthetic purposes, the variability of TFA's was suggested to be linked to different structural properties of the thallus.

Moreover, the accumulation of trace elements such as copper, iron, manganese and arsenic required in low amounts for metabolically important processes e.g. electron transport in photosynthesis as well as enzymatic activity, have also been observed to vary between thallus parts in the Fucales *A. nodosum* and *F. vesiculosus* and the kelp, *L. digitata* (Ronan *et al.*, 2017; Stengel *et al.*, 2005). Metal concentrations generally increased with increasing tissue age with the differences suggested to be influenced by thallus gradients in metal-binding compounds such as alginates and phenolics (Pedersen, 1984).

The intraspecific variability of phenolic compounds, however, within brown algae is complex and reportedly influenced by an array of intrinsic factors including plant size (Denton *et al.*, 1990), tissue age (Pedersen, 1984) and reproductive status (Plouguerné *et al.*, 2006; Ragan and Jensen, 1978). Due to their role in defence, the majority of studies have deemed the intra-thallus distribution of phenolics to function in deterring herbivore grazing in tissues that contribute the most to the overall fitness of the algae. Depending on the physiological function of each thallus part, some may be considered as ecologically more important and thus natural selection may favour a higher expression of defence traits

to be localised within *i.e*. constitutive rather than inducible defence (Rhoades, 1979). For example, meristematic regions and receptacles are thought to be fundamental to the fitness of an individual due to their contribution to growth and reproduction thus more vital to defend over the non-meristematic, vegetative tissues (Cronin and Hay, 1996; Pavia *et al.*, 2002; Rhoades, 1979; Toth *et al.*, 2005; Van Alstyne *et al.*, 2001).

However, defining the fitness value of different thallus parts may be difficult due to the high degree of diversity exhibited between different species of brown algae, in terms of both morphology and growth patterns. For example, in *A. nodosum* and *F. vesiculosus*, the older basal parts rather than the reproductive or meristematic tissues are considered to contribute most to the fitness (Jormalainen and Honkanen, 2004; Pavia *et al.*, 2002). These areas are thought to be of higher importance due to their role in supporting the entire plant within which there are several apices that can support new growth and receptacle production. This was validated by a higher level of phenolic content observed in these thallus parts (Hemmi *et al.*, 2005; Pavia *et al.*, 2002; Tuomi *et al.*, 1989). Conversely, Laminariales have only one basal meristematic zone thus being critical to defend (Steinberg, 1984; Tugwell and Branch, 1989; Van Alstyne *et al.*, 2001). The optimal defence theory (ODT) is further complicated due to the synergistic presence of mechanical defence traits. For example, receptacles have been thought to be protected by mechanical rather than chemical defences such as thallus toughness (Tuomi *et al*., 1989). For example, the shedding of outer epidermal layers by certain Fucales, such as *A. nodosum* and *H. elongata*, is associated with the prevention of epiphytic accumulation (Filion-Myklebust and Norton, 1981; Russell and Veltkamp, 1984).

The chemistry of phenolics is complex and is likely to be influenced by far more intricate mechanisms that the conventional assumption of fitness value (Jormalainen and Honkanen, 2008). For example, in young thallus parts, phenolic compounds are reported to occur in short oligomeric forms (Koivikko *et al.*, 2005). As tissue age increases, so too does the phenolic content (Pedersen, 1984). The presence of longer, more complex forms, thought to be difficult to exude or degrade, accumulate and contribute to thallus thickening, cross-linking with cell wall carbohydrates (Wallace and Fry, 1994). These cell wall bound (CWB) phenolics, depending on the extent of the integration, are likely

to be difficult to extract and thus quantify (Arnold and Targett, 2003), requiring alkaline degradation to be released Koivikko *et al.* (2005).

Therefore, correlating with algal development, the molecular composition of phenolic compounds, such as phlorotannins, may vary considerably. Such shifts in chemical profiles will significantly impact the observed phenolic content as variations in the degree of polymerisation and/or isomerisation will affect the amount of free hydroxyl units available for detection. In turn, such chemical divergence will also affect the level of defence a particular tissue exhibits as phlorotannin activity has been suggested to increase with molecular weight (Boettcher and Targett, 1993; Hagerman *et al.*, 1998). To complicate matters further, the reactivity and thus deterrence of the phlorotannins against grazers is also reliant by the chemistry of the grazers gut (Boettcher and Targett, 1993; Stern *et al.*, 1996).

To date, the majority of studies investigating the intra-thallus variability, most often in herbivore-plant interactions, have focused on determining the differences observed in total phlorotannins levels (Connan *et al.*, 2006; Duarte *et al.*, 2011; Fairhead *et al.*, 2005; Pavia *et al.*, 2002; Toth *et al.*, 2005). However, as discussed, the chemical composition is of great significance to both the detection and activity exhibited by specific thallus parts and has yet to be assessed.

This study aimed to elucidate on the intra-specific variability of LMW phlorotannin chemical profiles as well as in adjunction with content and *in vitro* antioxidant activity. It was anticipated that the localisation of these polyphenols and the elucidation on their distribution patterns within a given individual will allow the detection of more subtle changes in their content and/or composition in response to a given stimulus, which may remain undetected when only whole thalli are taken into consideration (Pelletreau and Targett, 2008). Ragan and Jensen (1978) observed the seasonality of phenolic content in *F. vesiculosus* and *A. nodosum* varied inverse to the reproductive maturity of the algae, with maxmiums occurring in the winter sterility period, decreasing toward a minimum just before gamete release. Therefore, the results observed for seasonal variation, described in Chapter 2, may have masked specific changes that occurred in different tissues, particularly those involved in reproductive maturity. In an effort to test this, in

this chapter, particular focus was placed on thallus parts differing regarding their physiological function in relation to the overall development *i.e.* vegetative versus reproductive thallus sections. Understanding the role of algal development, if any, on such fractions, may aid our understanding of the factors behind their natural variability. Variations that may arise in species differing in both their morphology and growth patterns *i.e.* perennial versus biennial were of key interest. For example, seasonal changes in the biochemistry of perennial species such as *A. nodosum* are largely related to both physiological changes in the vegetative thalli as well as reproductive sections (Åberg and Pavia, 1997) while in bi-annual species such as *Himanthalia elongata* seasonal shifts in chemical composition have been attributed to the progressive development of the receptacles, which comprise the majority of the alga's morphological structure (Brenchley *et al.*, 1997; Stengel *et al.*, 1999). Therefore, seasonal analysis was carried out investigating the effect of development over six seasons on LMW phlorotannin content, chemical profiles and associated *in vitro* antioxidant activity.

## 3.2. Materials and Methods

### 3.2.1. Reagents and materials

1, 3, 5-trihydroxybenzene (phloroglucinol), 2, 2-diphenyl-1-picryhydrazyl (DPPH), 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox®), formic acid (MS grade) and 0.22 µm polytetrafluorethylene (PTFE) filters, Iron (III) chloride hexahydrate, sodium carbonate ($Na_2CO_3$), Folin- Ciocalteu (2N) were all sourced from Sigma-Aldrich Chemical Ltd. (Co. Wicklow, Ireland). HPLC grade methanol, ethyl acetate, acetonitrile, water, and BioDesign Dialysis Tubing™ with 3.5 kDa cut-off were obtained from Fischer Scientific Ltd. (Loughborough, Leicestershire, UK).

### 3.2.2. Collection and preparation of seaweed samples

Four species of Fucales were selected for intra-thallus investigation; *Pelvetia canaliculata*, *Fucus vesiculous*, *Ascophyllum nodosum* and *Himanthalia elongata.* Samples were collected from Finavarra, Co. Clare, over six seasons between January 2014 and May 2015. To ensure a representative subsample of each population was taken and not just individual specimens, a minimum of 40 individual thalli were haphazardly selected and cut at each sampling date. They were then brought back to the Algal Biosciences lab at the National University of Galway (NUI Galway), where they were immediately manually cleaned of any epiphytes or other external impurities (e.g. sediment) present. Here, each species was sectioned into vegetative and reproductive regions, according to species;

***P. canaliculata***

1) The tips, where the receptacles develop *i.e.* the reproductive organs, were considered as the most upper longitudinal 0.5 cm section of the thallus while the 2) thallus (vegetative) was sectioned in between the tips and the basal 1 cm of the thallus.

***F. vesiculosus***

1) The tips where the receptacles develop, were sectioned from the thallus by cutting the frond and taking the upper 2 cm while the 2) thallus included the remaining frond displaying air bladders, *i.e.* without the short, thick stipe.

***A. nodosum***

1) The apical tips, representing the region of newest growth, were cut at the most upper dichotomous section occurring just after the most recent air bladder. 2) The receptacles, green when unfertile and yellow when mature, were the vesicles sprouting laterally from the 3) thallus, which was considered the remainder of the frond after these two prior sections has been cut (without the bottom 15 cm left attached to the hold fast in field).

***H. elongata***

1) The vegetative buttons, *i.e.* immature, would be considered as those not sprouting any receptacle growth while the collected 2) receptacles, comprising the majority of the total biomass, were the long strap-like fronds growing from fertile discoid buttons.

Immediately after sample were cleaned and sectioned, they were transported in cooler boxes to Teagasc Food Research Centre, Ashtown (TRFCA). Here, samples were frozen and thereafter freeze-dried before being ground into one homogenous powder upon which extraction and analysis would be carried out on. Once vacuum packed, samples were stored at -20°C until further required.

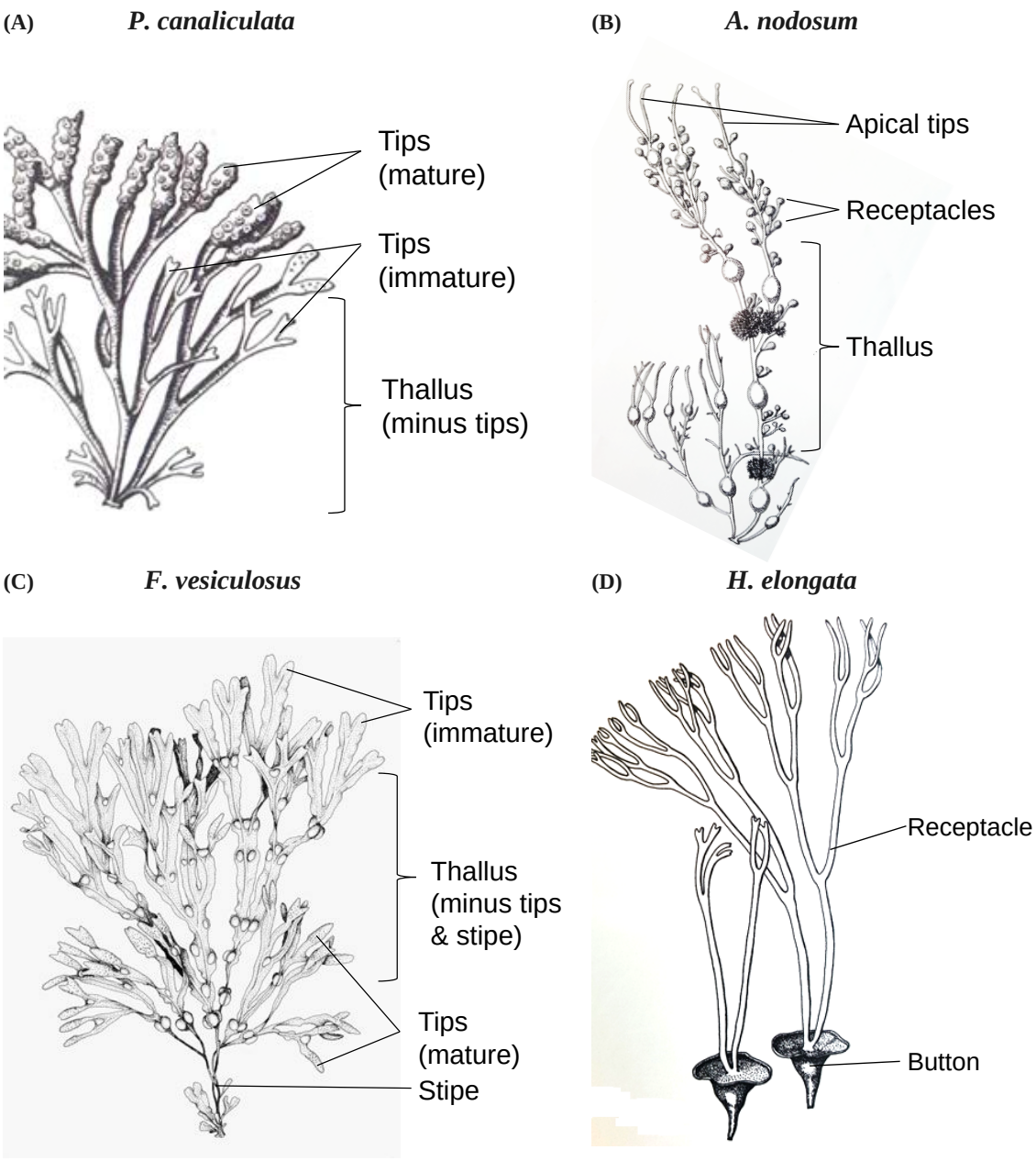



**Fig. 3.1.** Intra-thallus sections investigated for phlorotannin variation in four species of Fucales (A) *P. canaliculata*, (B) *A. nodosum*, (C) *F. vesiculosus* and (D) *H. elongata* from Finavarra, Co. Clare. Within each species, vegetative (apical tips, thallus and buttons) and reproduction sections (tips and receptacles) were examined over six seasons from January 2014 to May 2015. Diagrams have been modified from their orginal sources: Loiseaux-de Goër and Noailles (2008) and Guiry and Nic Dhonncha (2001).

**Table 3.1.** The life strategy and stage which was displayed and collected by the four species – *P. canaliculata, A. nodosum, F.vesiculosus* (all three displaying perennial growth) and *H. elongata* (biennial growth) at sampling site Finavarra, Co. Clare for each of the six sampling seasons between 2014 and 2015.

| | **Jan 2014** | **May 2014** | **Aug 2014** | **Nov 2014** | **Jan 2015** | **May 2015** |
|---|---|---|---|---|---|---|
| ***Pelvetia canaliculata*** | Receptacle formation initiated | Receptacles begin to develop & mature | Receptacles ripen & begin to release gametes | Receptacles are not present | Receptacle formation initiated | Receptacles begin to develop & mature |
| ***Ascophyllum nodosum*** | Development & mature of reproductive receptacles | Receptacles have released gametes & have been shed | No receptacles present | Formation of new receptacles | Development & maturation of reproductive receptacles | Receptacles have released gametes & have been shed |
| ***Fucus vesiculosus*** | Receptacles begin to develop & mature | Receptacles ripen & begin to release gametes | Receptacles still present in some specimen | Ripe receptacles no longer present | New receptacles form & begin to develop | Receptacles ripen & begin to release gametes |
| ***Himanthalia elongata*** | *Growth of immature buttons<br><br>*Elongation of receptacles | Development of new recruits (Feb/Mar)<br><br>*Growth of immature buttons<br><br>*Elongation of receptacles | *Growth of Immature buttons<br><br>*Fertile receptacles (established the previous year) release gametes beginning in June | Mature buttons begin to produce receptacles<br><br>*Immature button growth continues<br><br>*Elongation of receptacles<br><br>Some mature receptacles continue to release gametes | *Growth of Immature buttons<br><br>*Elongation of receptacles | Development of new recruits<br><br>*Growth of immature buttons<br><br>*Elongation of receptacles |

*life stage actually collected

### 3.2.3. Characterisation of low molecular weight (LMW) phlorotannin profiles

An initial 50 g DW of ground algal powder was used in the extraction and purification of low molecular weight (LMW) phlorotannin-enriched fractions, on which mass spectrometric characterisation (Q-ToF-MS and UPLC-MS/MS) was carried out on as well as colorimetric quantification of the total phenolic content (TPC). The method of Tierney *et al.* (2014) was employed and the details of the steps involved are outlined in Chapter 2 Section 2.2.6 to 2.2.10.

### 3.2.4. Assessment of *in vitro* antioxidant activity

To examine the degree of *in vitro* antioxidant activity displayed by the LMW fractions obtained from different sections of the four species, two colorimetric assays were employed, the ferric reducing antioxidant power (FRAP) and the radical scavenging activity (RSA) against the radical DPPH·. Each fraction was analysed in triplicate (n=3). The details of each assay are outlined in Chapter 2 Section 2.2.11.

### 3.2.5. Statistical analyses

For each species, the effects of thallus part and season on the total phlorotannin content (TPC), ferric-reducing antioxidant power (FRAP) and the radical scavenging activity (RSA) of the LMW phlorotannin-enriched fractions, from each species, were statistically tested using a two-way variance of ANOVA ($p<0.05$). IBM® SPSS® statistical software (version 24) was used for all statistical tests. Normality was tested using the Shapiro-Wilk test for normal distribution and Levene's test for homogeneity of variances. Data which did not meet normality requirements were transformed using log, natural log, square root, or reciprocal transformations, prior to ANOVA analysis. In samples where homogeneity of variances was not met, the Welch ANOVA was employed (Tomarken and Serlin, 1986). Tukey-tests were performed to find *posteriori homogeneous* (*post-hoc*) sub-groups that differed significantly ($p<0.05$).

## 3.3. Results

### 3.3.1. Phlorotannin composition and relative abundance

The degree of purity observed in the low molecular weight (LMW) reverse phase (RP) flash fractions across thallus section, from the four species investigated, was highly variable. This was particularly apparent within *P. canaliculata* and *A. nodosum*, where separation issues, previously observed in Chapter 2, make it difficult to obtain a high level of purity. Similar to Chapter 2, the degree of interference in these two species and its subsequent effect on phlorotannin purification was subject to seasonal variability for each thallus part and overall was more pronounced in *A. nodosum*.

#### 3.3.1.1. *P. canaliculata*

The observed profiles of the different thallus sections of *P. canaliculata* exhibited seasonal variability but were relatively similar. The majority of phlorotannins detected in tips and thallus were those ranging from *m/z* 1117 to 1614, corresponding to 9-14 PGUs (Fig. 3.2). In the tip sections, the contribution of this range to the total abundance varied from 50-79% while in the thallus a similar range of 53-77% was observed (Fig. 3.2). The overall dominance of a given molecular weight was variable on a seasonal scale, with no one structure being continuously observed as the most dominant. Furthermore, no apparent seasonal trend according to which the composition differed was observed as, over the two years sampling period, the same season yielded different profiles.

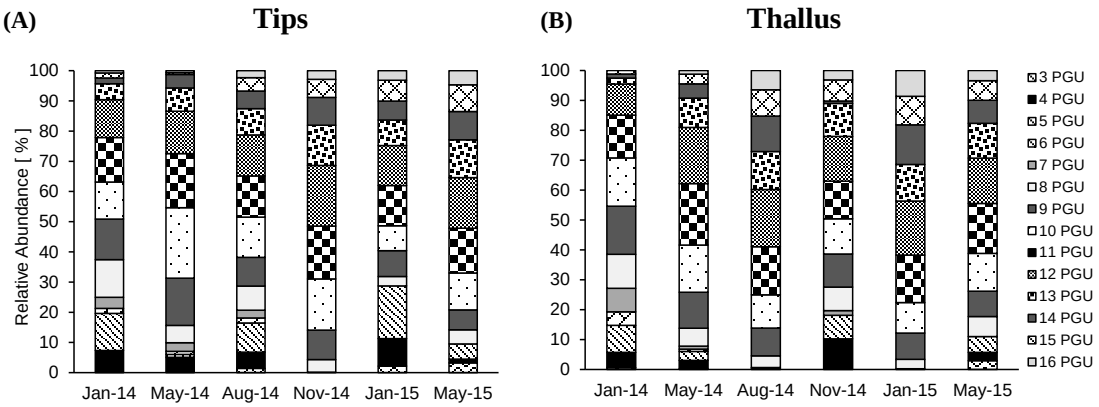



**Fig. 3.2.** The relative abundance (%) of phlorotannins between 3 and 16 phloroglucinol units (PGU) as detected by the UPLC-MS/MS for the various thallus sections; (A) tips and (B) thallus sections of ***P. canaliculata***, collected from Finavarra, Co. Clare, from January 2014 to May 2015.

#### 3.3.1.2. *A. nodosum*

For *A. nodosum*, a high degree of interference from other polar compounds was observed, similar to that seen in Chapter 2. However, the degree of interference varied between each section and fluctuated over the six seasons. Overall, this species, and the thallus sections thereof, displayed a higher abundance of phlorotannins corresponding to 870-1366 Da (7-11 PGUs). In the apical tips, the majority of detected phlorotannins were found ranging between *m/z* 993-1365 (8-11 PGUs), accounting for 51-88%, with the exception of those collected in May 2015. Here, a distinctively different profile was observed, predominantly composed of the lower molecular weighted phlorotannins of 622 Da (5 PGUs), accounting for over 37% of the total (Fig. 3.3). This particular molecular weight was also accounted for a high percentage of the total detected in other sections, at specific seasons, such as in the thallus sections in August 2014 (23%) and May 2015 (23%) and within the receptacles also in August 2014 (20%).

The receptacles, however, due to their degradation and shedding after gamete release in May of each year, could only be analysed for 4 of the 6 seasons, displaying a strong degree of seasonal variability in their chemical composition (Fig. 3.3). The abundance of phlorotannins between *m/z* 993-1365 varied considerably. In samples collected in January, in both years, this range of phlorotannins accounted for 72-80% of the total detected while in August and November 2014, this range only contributed 47% to the total. Within the thallus' sections, the contribution of phlorotannins ranging between *m/z* 869-1365 (7-12 PGUs) also varied seasonally, accounting for 55-86%. Similar to the other sections of this species, the composition observed in the thallus section displayed changes with which no specific trend could be associated with sampling season (Fig. 3.3).

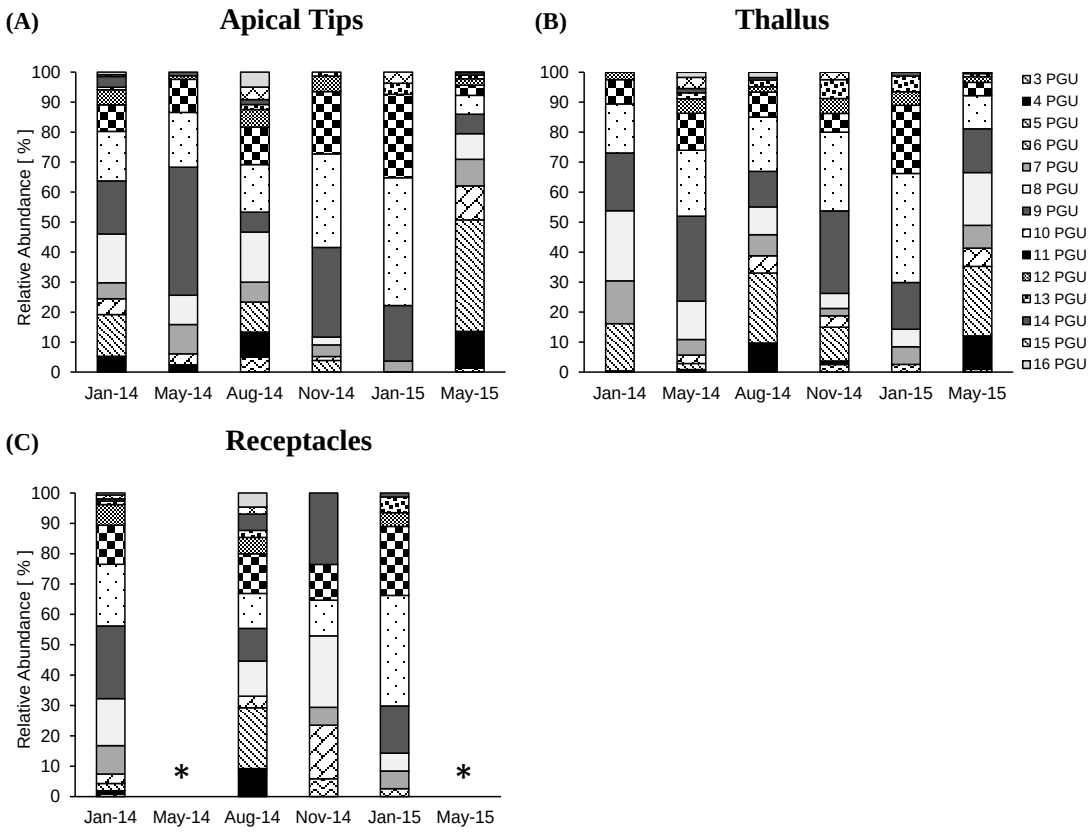



**Fig. 3.3**. The relative abundance (%) of phlorotannins between 3 and 16 phloroglucinol units (PGU) as detected by the UPLC-MS/MS for the various sections; (A) apical tips (B) thallus and (C) receptacles of ***A. nodosum***, collected from Finavarra, Co. Clare from January 2014 to May 2015. The receptacles had been shed in May of both years and so were unable to be collected for analysis, as indicated by *.

### 3.3.1.3. *F. vesiculosus*

Due to a large scale bloom of the epiphyte, *Pylaiella littoralis* (Phaeophyceae), which covered the majority of the *F. vesiculosus* population in Finavarra in May 2014, thalli could not be collected. Therefore, the seasonal variation of 5 rather than 6 seasons is described.

Both tips and thallus Sections of *F. vesiculosus* displayed a high degree of uniformity regarding their chemical composition, with little variability observed. A high abundance in relatively low molecular weight phlorotannins within the range of *m/z* 497-993 (4-8 PGUs), accounting for 86-91%, of the overall total detected, in both sections (Fig. 3.4). The chemical profile within each thallus part was exceptionally uniform over all 5 seasons, with only minor shifts detected for each individual MRM transition. The most abundant phlorotannins were detected at, in order of decreasing dominance, *m/z* 621, 497 and (5, 8 and 4 PGUs), accounting for 30-38%, 14-18 and

15-16% respectively (Fig. 3.4). Within each thallus part, at each sampling season, this distinct profile was observed.

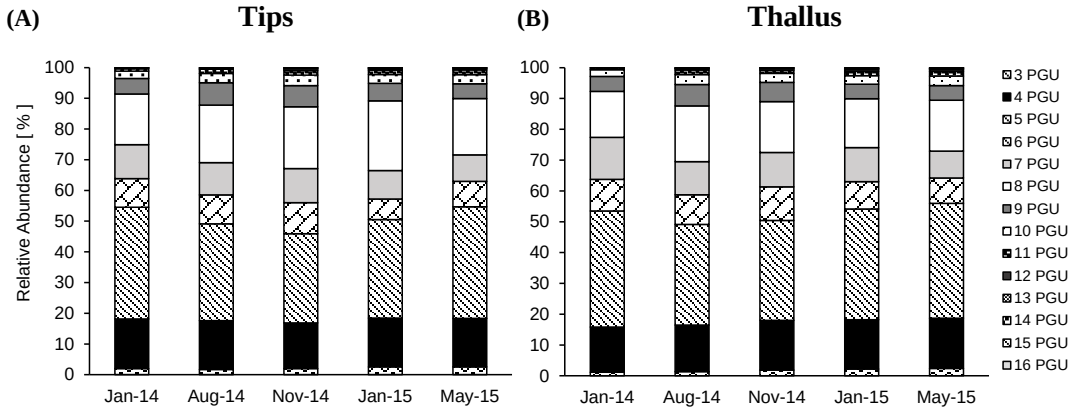



**Fig. 3.4**. The relative abundance (%) of phlorotannins between 3 and 16 phloroglucinol units (PGU) as detected by the UPLC-MS/MS for the various sections; (A) tips and (B) thallus of ***F. vesiculosus***, collected from Finavarra, Co. Clare, from January 2014 to May 2015.

### 3.3.1.4. *H. elongata*

The vegetative buttons *i.e*. buttons that had not produced receptacles were investigated and compared to the long strap-like receptacles, over six seasons. The two morphologically distinct sections demonstrated very different phlorotannin profiles. A relatively similar composition was observed in the receptacles, abundant in phlorotannin composed of 8-13 PGUS (994-11615 Da; Fig. 3.5). This range accounted for 82-93% of the total phlorotannin abundance detected. Those composed of 10, 11 and 12 PGUs were the most abundant phlorotannins detected, accounting for 16-23%, 14-23% and 10-18, respectively. The buttons, however, exhibited a more varying profile composed of lower weighted structures, particularly those composed of 5-11 PGUs (622-1366 Da), comprising between 71-88% of the total phlorotannins detected (Fig. 3.5). However, due to the high fluctuations observed between seasons no one molecular weight could be described as more dominant than another within this range.

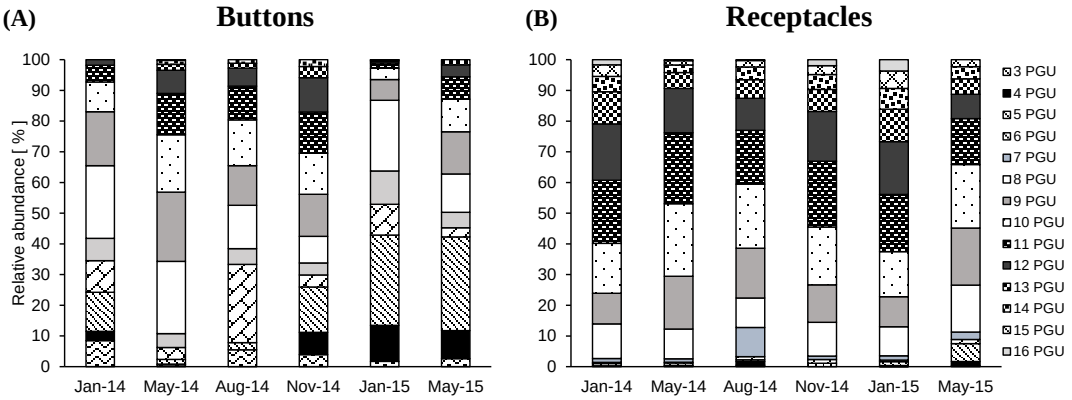



**Fig. 3.5.** The relative abundance (%) of phlorotannins between 3 and 16 phloroglucinol units (PGU) as detected by the UPLC-MS/MS for the various sections; (A) buttons and (B) receptacles of *H. elongata*, collected from Finavarra, Co. Clare, from January 2014 to May 2015.

### 3.3.2. Degree of isomerisation

#### 3.3.2.1. *P. canaliculata*

A similar level of isomeric variability was detected for both thallus sections analysed of *P. canaliculata*. In most seasons, between 100 and 200 individual isomers over the 16 MRM transitions were detected. Both the tips and thalli displayed the highest total number of isomers in August 2015, with a total of 217 in the prior and 278 in the latter (Table 3.2). The lowest levels were detected in May 2015 with the tips displaying 108 and the thalli only 33. However, this decrease in isomers is most likely to be associated with a low level of resolution in the signal for this sample due to poor separation. A similar effect was observed in November 2015. The majority of isomers were detected within the range of 9-14 PGUs, corresponding to 1118-1738 Da (Table 3.2) for both thallus parts.

#### 3.3.2.2. *A. nodosum*

This species displayed the lowest level of isomers out of the four species investigated, as previously seen from samples previously described in Chapter 2. Isomeric totals ranged from as little as 3 in the receptacles (due to poor resolution in the detection using UPLC-MS/MS) to 130, again in receptacles in January 2014 (Table 3.3). For the same season, both the apical tips and thalli displayed higher total levels with 91 and 117, respectively. However, the sections differed in respect to the time of year that the lowest isomer levels were observed. In the apical tips this occurred in May 2014, with 36 being detected in total; in the vegetative thalli both November 2014 and January

2015 displayed the lowest isomeric totals with 39 and 38, respectively. Overall, in the three thallus parts, isomers were most abundant within phlorotannins composed of 8-11 PGUs (Table 3.3).

#### 3.3.2.3. *F. vesiculosus*

Isomeric abundance varied seasonally in a similar manner between the tips and the thalli of *F. vesiculosus*, with both sections displaying a similar seasonal shift in the levels detected (Table 3.4). For example, samples of tips and thalli displayed the overall highest isomeric abundance in January 2015, with the tips exhibiting a total of 283 and the thalli displayed a substantially higher total of 410. Similarly, the lowest levels in both thallus parts was observed in August 2015, with a total of 142 detected in the tips and 163 in the thalli (Table 3.4). The majority of isomers detected were found for phlorotannins within the range of 5-12 PGUs. Again, this was observed for both sections investigated.

#### 3.3.2.4. *H. elongata*

The difference in the degree of isomerisation observed between the two thallus parts in this species was the most apparent (Table 3.5). In the receptacles, between 280 and 450 individual isomers were observed, these levels are substantially higher than those observed in the vegetative button form. Maximum levels of 447 were detected in January 2014 while the lowest levels of 289 were observed in May 2014 (Table 3.5). The maximum isomeric levels for the buttons, recorded at 176, was also found in this month, while minimum levels detected were found in January 2014 and May 2015. Similar to the other species, isomeric trends did not appear to follow a specific seasonal trend, with no particular season over the two years displaying similar levels.

**Table. 3.2.** Full list of isomers detected for each MRM transition corresponding to 3-16 PGUs for the (A) tips and (B) thalli of ***P. canaliculata*** collected from Finavarra, Co. Clare, over six seasons from January 2014 and May 2015. Where 10 or more individual isomers were detetced for an individual molecular ion transition the number is highlighted in bold.

| | **Degree of polymerization (PGU)** | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(A) Tips** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **Total** |
| **Jan 2014** | 1 | 2 | 6 | 6 | **14** | **24** | **37** | **48** | **27** | **12** | 4 | 3 | 0 | 1 | 185 |
| **May 2014** | 0 | 5 | 3 | 4 | 3 | **12** | **25** | **26** | **27** | **26** | **15** | **13** | 5 | 1 | 164 |
| **Aug 2015** | 4 | 6 | **12** | **19** | **26** | **11** | **25** | **23** | **25** | **23** | **18** | **10** | **13** | 4 | 217 |
| **Nov 2014** | 1 | 3 | 1 | 0 | 1 | 9 | **18** | **22** | **30** | **31** | **25** | **23** | **18** | 2 | 184 |
| **Jan 2015** | 1 | 4 | 2 | 5 | 14 | 7 | **21** | **18** | **22** | **23** | **11** | 9 | 5 | 1 | 143 |
| **May 2015** | 2 | 4 | 5 | 4 | 2 | 6 | 6 | **13** | **19** | **16** | **12** | **10** | 6 | 3 | 108 |
| **(B) Thalli** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **Total** |
| **Jan 2014** | 0 | 1 | 5 | 3 | 9 | **11** | **19** | **42** | **37** | **12** | 1 | 0 | 0 | 0 | 140 |
| **May 2014** | 0 | 1 | 4 | 2 | 3 | 8 | **24** | **33** | **38** | **40** | **24** | **31** | **13** | 4 | 225 |
| **Aug 2015** | 0 | 2 | 3 | 1 | 1 | **15** | **23** | **33** | **36** | **35** | **38** | **30** | **33** | **28** | 278 |
| **Nov 2014** | 0 | 2 | 4 | 1 | 5 | 7 | 8 | 8 | 6 | **11** | 3 | 2 | 3 | 3 | 63 |
| **Jan 2015** | 1 | 1 | 5 | 1 | 0 | 6 | **15** | **26** | **27** | **35** | **25** | **28** | **33** | **20** | 223 |
| **May 2015** | 1 | 3 | 3 | 0 | 1 | 3 | **10** | 4 | 5 | 0 | 1 | 2 | 0 | 0 | 33 |

**Table. 3.3.** Full list of isomers detected for each MRM transition corresponding to 3-16 PGUs for the (A) apical tips, (B) thalli and (C) receptacles of ***A. nodosum*** collected from Finavarra, Co. Clare, over six seasons from January 2014 and May 2015. Receptacles had been shed by May in 2014 and 2015 and so could not be examined. Where 10 or more individual isomers were detetced for an individual molecular ion transition the number is highlighted in bold.

| | **Degree of polymerization (PGU)** | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(A) Apical** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **Total** |
| **Jan 2014** | 2 | 6 | 6 | 9 | 3 | **12** | **11** | **11** | **16** | 4 | 0 | 6 | 2 | 3 | 91 |
| **May 2014** | 0 | 2 | 2 | 3 | 2 | 4 | **10** | 9 | 2 | 0 | 0 | 0 | 1 | 1 | 36 |
| **Aug 2015** | 3 | 3 | 3 | 3 | 5 | 9 | 7 | **10** | 3 | 3 | 3 | 1 | 1 | 2 | 56 |
| **Nov 2014** | 0 | 1 | 4 | 4 | 3 | 9 | 7 | **10** | 8 | 3 | 2 | 1 | 0 | 0 | 52 |
| **Jan 2015** | 1 | 3 | 2 | 3 | 4 | 5 | 5 | 9 | 4 | 3 | 2 | 2 | 0 | 0 | 43 |
| **May 2015** | 3 | 4 | 6 | **11** | **16** | **19** | **21** | **18** | 9 | 5 | 3 | 3 | 0 | 0 | 118 |
| **(B) Thalli** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **Total** |
| **Jan 2014** | 2 | 6 | 10 | 1 | 7 | **31** | **23** | **22** | **14** | 1 | 0 | 0 | 0 | 0 | 117 |
| **May 2014** | 1 | 3 | 1 | 4 | 3 | **11** | **16** | **28** | **14** | 3 | 5 | 3 | 2 | 3 | 97 |
| **Aug 2015** | 1 | 5 | 7 | 7 | 4 | **13** | **22** | **23** | 6 | 2 | 0 | 1 | 0 | 0 | 91 |
| **Nov 2014** | 1 | 1 | 2 | 2 | 2 | 3 | 8 | 8 | 3 | 5 | 1 | 1 | 1 | 1 | 39 |
| **Jan 2015** | 3 | 1 | 2 | 2 | 1 | 4 | 4 | 8 | 5 | 3 | 4 | 1 | 0 | 0 | 38 |
| **May 2015** | 2 | 4 | 7 | 5 | 9 | **14** | **11** | **10** | 1 | 3 | 2 | 0 | 0 | 0 | 68 |
| **(C) Recep.** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **Total** |
| **Jan 2014** | 2 | 2 | 8 | **11** | 9 | **12** | **30** | **22** | **12** | **17** | 3 | 0 | 2 | 0 | 130 |
| **May 2014** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **Aug 2015** | 2 | 2 | 4 | 5 | 4 | 9 | 8 | 1 | 3 | 3 | 2 | 2 | 2 | 5 | 52 |
| **Nov 2014** | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 |
| **Jan 2015** | 1 | 1 | 3 | 3 | 2 | 4 | 8 | **12** | 9 | 3 | 4 | 2 | 1 | 0 | 53 |
| **May 2015** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

**Table. 3.4.** Full list of isomers detected for each MRM transition corresponding to 3-16 PGUs for the (A) tips and (B) thalli of ***F. vesiculosus*** collected from Finavarra, Co. Clare, over six seasons from January 2014 and May 2015. *Samples could not be collected in May 2014. Where 10 or more individual isomers were detetced for an individual molecular ion transition the number is highlighted in bold.

| | **Degree of polymerization (PGU)** | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(A) Tips** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **Total** |
| **Jan 2014** | 3 | 5 | **10** | **13** | **31** | **38** | **33** | **16** | **26** | **23** | 0 | 0 | 1 | 0 | 199 |
| **May 2014*** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **Aug 2015** | 1 | 2 | **12** | **21** | **27** | **15** | **22** | **21** | 8 | 7 | 5 | 1 | 0 | 0 | 142 |
| **Nov 2014** | 2 | 9 | **14** | **39** | **32** | **44** | **29** | **24** | **29** | **15** | **11** | 3 | 2 | 2 | 255 |
| **Jan 2015** | 4 | **10** | **15** | **27** | **40** | **53** | **39** | **30** | **23** | **22** | **12** | 6 | 2 | 0 | 283 |
| **May 2015** | 5 | **10** | **19** | **20** | **41** | **46** | **36** | **32** | **23** | **12** | 7 | 5 | 4 | 0 | 260 |
| **(B) Thalli** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **Total** |
| **Jan 2014** | 3 | 9 | **15** | **25** | **47** | **41** | **49** | **43** | **27** | **30** | 2 | 0 | 0 | 0 | 291 |
| **May 2014*** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **Aug 2015** | 0 | 6 | **10** | **21** | **30** | **28** | **21** | **23** | 8 | 6 | 5 | 1 | 1 | 3 | 163 |
| **Nov 2014** | 1 | 5 | **20** | **22** | **33** | **38** | **43** | **27** | **21** | **15** | 8 | 3 | 2 | 2 | 240 |
| **Jan 2015** | 6 | **19** | **37** | **30** | **45** | **61** | **56** | **37** | **34** | **27** | **23** | **19** | **10** | 6 | 410 |
| **May 2015** | 3 | 8 | **23** | **20** | **39** | **45** | **42** | **39** | **24** | **16** | **14** | 8 | 4 | 1 | 286 |

**Table. 3.5.** Full list of isomers detected for each MRM transition corresponding to 3-16 PGUs for the (A) buttons and (B) receptacles of ***H. elongata*** collected from Finavarra, Co. Clare, over six seasons from January 2014 and May 2015. Where 10 or more individual isomers were detetced for an individual molecular ion transition the number is highlighted in bold.

| | **Degree of polymerization (PGU)** | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **(A) Button** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **Total** |
| **Jan 2014** | 2 | 3 | 3 | 4 | 2 | 7 | **11** | **13** | 4 | 1 | 0 | 1 | 0 | 0 | 51 |
| **May 2014** | 2 | 2 | 3 | 9 | **15** | **23** | **35** | **39** | **27** | **14** | 4 | 1 | 2 | 0 | 176 |
| **Aug 2015** | 2 | 2 | 2 | 1 | 7 | **13** | **18** | **18** | **13** | 5 | 2 | 0 | 0 | 0 | 83 |
| **Nov 2014** | 3 | 5 | 6 | 8 | **10** | **20** | 8 | 6 | **12** | 9 | 5 | 1 | 1 | 2 | 96 |
| **Jan 2015** | 5 | 7 | 7 | **12** | **10** | **15** | **16** | **13** | **10** | 8 | 6 | 2 | 0 | 0 | 111 |
| **May 2015** | 3 | 6 | 7 | 4 | 3 | 5 | 4 | 5 | 6 | 1 | 1 | 0 | 0 | 0 | 45 |
| **(B) Recep.** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** | **12** | **13** | **14** | **15** | **16** | **Total** |
| **Jan 2014** | 2 | 3 | 5 | 5 | **13** | **45** | **43** | **55** | **50** | **57** | **49** | **49** | **42** | **29** | 447 |
| **May 2014** | 2 | 2 | 2 | 3 | 5 | **37** | **37** | **46** | **46** | **48** | **29** | **19** | 9 | 4 | 289 |
| **Aug 2015** | 2 | 5 | 5 | 5 | 7 | **30** | **35** | **47** | **49** | **49** | **38** | **38** | **26** | **18** | 354 |
| **Nov 2014** | 3 | 8 | 7 | 7 | 7 | **33** | **35** | **47** | **52** | **41** | **10** | **38** | **29** | **17** | 334 |
| **Jan 2015** | 4 | **15** | **29** | **34** | **31** | **41** | **44** | **34** | **25** | **20** | 7 | 7 | 3 | 0 | 294 |
| **May 2015** | 4 | 9 | 9 | **15** | **14** | **30** | **40** | **39** | **43** | **36** | **34** | **32** | **17** | **17** | 339 |

### 3.3.3. Total phlorotannin content (TPC) of LMW phlorotannin-enriched fractions

The TPC of the LMW phlorotannin-enriched fractions, from each species, was tested for statistical differences occurring between thallus parts and season by employing a two-way variance of ANOVA ($p$<0.05), the details of which are described in the Supplementary Section 9.3.1.

#### 3.3.3.1. *P. canaliculata*

The total phlorotannin content (TPC) detected within in the LMW phlorotannin-enriched fractions did not differ significantly between the two thallus sections ($p$=0.068) but it did display variability in relation to the sampling season ($p$<0.001) and the interaction between the two parameters ($p$<0.001; Fig. 3.6). Both the tips and thalli exhibited maximum, and similar, phlorotannin levels in January 2014 (326.7±7.6 and 347.4±4.8 µg PGE $mg^{-1}$ DWE, respectively). In both thallus parts the phlorotannin content decreased in August 2014, after which the tips displayed a significant increase in November 2014, with which the thalli did not follow, remaining low ($p$<0.05; Fig. 3.6). However, in January 2015, the phlorotannin content in the thalli increased to levels to that of the previous January while the tips levels had decreased once more. Both sections displayed their overall lowest phlorotannin levels in May 2015, with the tips exhibiting a higher abundance (198.7±4.2 and 136.7±3.8 µg PGE $mg^{-1}$ DWE, respectively; $p$<0.05; Fig. 3.6).

#### 3.3.3.2. *A. nodosum*

A high degree of intra-thallus ($p$<0.001) and seasonal ($p$<0.001) variation was observed for the phlorotannin content between the three thallus parts investigated for this species (Fig. 3.7). Overall, the LMW fractions derived from the thalli exhibited the highest phlorotannin content (63.4±4.7 to 281.8±1.7 µg PGE $mg^{-1}$ DWE) which differed significantly between sampling seasons ($p$<0.001; Fig. 3.7). The maximum levels were observed in May 2014, while the minima were found in the following May, with a four-fold decrease in phlorotannin content (Fig. 3.7). The phlorotannin content detected in the apical tips also varied seasonally, displaying the highest level in Jan 2014, with 216.3±5.6 µg PGE $mg^{-1}$ DWE, decreasing toward a minimum in August 2014 (41.1±4.9 µg PGE $mg^{-1}$ DWE) and increasing thereafter. The receptacles, during the four seasons they could be collected, contained the overall lowest levels,

with the maximum reaching 157.9±15.7 µg PGE $mg^{-1}$ DWE in August 2014, and the overall minimum level detected in November 2014, with 32.4±1.5 µg PGE $mg^{-1}$ DWE (Fig. 3.7).

#### 3.3.3.3. *F. vesiculosus*

The phlorotannin levels of *F. vesiculosus* did not differ significantly between thallus parts, with both sections displaying similar to one another ($p$=0.188). However, they were affected by both season ($p$<0.001) and the interaction between the two variables ($p$<0.001; Fig. 3.8). Both thallus parts followed a similar seasonal trend but within each sampling season, the tips continuously displayed a slightly, yet not significant (except for January 2014), higher TPC. In the LMW fractions extracted from the tips, the overall highest phlorotannin content was observed in January 2014 (490.7±28.5 µg PGE $mg^{-1}$ DWE; Fig. 3.8). This, along with the content of the thalli-derived fractions, had decreased significantly in August 2014 when the lowest levels for both sections was seen (233.3±34.4 and 205.4±10.6 µg PGE $mg^{-1}$ DWE, respectively; $p$<0.001; Fig. 3.8). A steady increase in the TPC in both thallus parts was observed towards January 2015, following this, with higher and stable levels reinstated in both the tips and thalli fractions (426.8±24.2 and 400.3±20.1 µg PGE $mg^{-1}$ DWE; Fig. 3.8).

#### 3.3.3.4. *H. elongata*

The phlorotannin content of the LMW phlorotannin-enriched fractions extracted from the buttons and receptacles of *H. elongata* displayed significant variation in respect to thallus part ($p$<0.001), season ($p$<0.001) and their interaction ($p$<0.001; Fig. 3.9). Both buttons and receptacle fractions had the highest phlorotannin content in May 2014 (461.5±4.9 and 481.6±4.9 µg PGE $mg^{-1}$ DWE, respectively; $p$<0.05; Fig. 3.9), displaying similar levels to one another. From here onwards, TPC decreased linearly in the receptacles, toward the lowest level which occurred in the following May, in 2015 (197.5±2.5 µg PGE $mg^{-1}$ DWE; Fig. 3.9). TPC in the buttons, however, displayed a high level of fluctuation, peaking in May 2014 (as mentioned) and then again, but not to the same extent, in January 2015 (353.5±23.1 µg PGE $mg^{-1}$ DWE; $p$<0.05). The lowest levels of TPC in buttons was detected in samples collected in November 2014 (156.1±2.4 µg PGE $mg^{-1}$ DWE; $p$<0.001; Fig. 3.9).

### 3.3.4. *In vitro* antioxidant activity

The FRAP and RSA of the LMW phlorotannin-enriched fractions, from each species, was tested for statistical differences occurring between thallus parts and season by employing a two-way variance of ANOVA ($p$<0.05), the details of which are described in the Supplementary Section 9.3.1.

#### 3.3.4.1. *P. canaliculata*

The ferric reducing antioxidant power (FRAP) of the two sections from *P. canaliculata* was positively correlated with the phlorotannin content ($R^2$=0.56; Fig. 3.6), displaying a similar trend in most seasons investigated. Yet, the FRAP activity of the LMW fractions was, unlike the TPC, significantly affected by thallus part ($p$<0.001), as well as season ($p$<0.001) and their interaction ($p$<0.001; Fig. 3.6). Fractions derived from the thalli displayed the overall highest levels, with a maximum of 340.1±9.0 and 322.3±4.8 µg TE $mg^{-1}$ DWE being detected in January and May 2014, respectively ($p$<0.05; Fig. 3.6). Similarly, the tips also displayed maximum levels within the same sampling seasons, with 255.9±18.2 and 235.5±9.2 µg TE $mg^{-1}$ DWE, respectively. A significant decrease ($p$<0.001) in both thallus sections followed in August 2014 after which levels remained at their lowest. Absolute minimum levels were detected in May 2015 for both tip- and thalli-derived fractions, with the two parts exhibiting a similar level of FRAP activity (158.7±5.1 and 166.9±2.9 µg TE $mg^{-1}$ DWE).

Similar to FRAP, the radical scavenging activity (RSA) of the LMW fractions of the two thallus sections was also positively correlated to the TPC ($R^2$=0.59; Fig. 3.6). However, unlike FRAP, the RSA of *P. canaliculata* did not differ in relation to thallus part ($p$=0.519; Fig. 3.6), although it was significantly affected by season ($p$<0.001) and the interaction of season and thallus part ($p$<0.001; Fig. 3.6). Maximum activity was observed in January and May in 2014 for both tips (24.7±1.6 and 27.3±1.7 µg $mL^{-1}$, respectively) and thallus fractions (21.1±1.9 and 23.3±1.4 µg $mL^{-1}$, respectively) as indicated by the low $IC_{50}$ values (Fig. 3.6). Thereafter, activity levels continuously decreased to a minimum RSA for both tips and thalli in May 2015 (50.7±3.9 and 64.1± 4.5 µg $mL^{-1}$), with the thalli displaying a significantly lower RSA (Fig. 3.6).

### 3.3.4.2. *A. nodosum*

The FRAP activity of the three different thallus parts of *A. nodosum* displayed a positive correlation with TPC ($R^2$=0.49; Fig 3.7) and was significantly affected by thallus part ($p$<0.001), season ($p$<0.001) and their interactive effect ($p$<0.001). Overall, the LMW fractions derived from thalli displayed the highest FRAP activity, following the same seasonal trend as that observed for TPC; highest levels were detected in samples collected in May 2014, while the lowest were seen in the following May, in 2015 (231.7±34.4 and 88.5±3.8 µg TE $mg^{-1}$ DWE, respectively; $p$<0.05; Fig. 3.7). Apical tips were second after the thalli, in terms of activity, again following the trend displayed by TPC, with highest levels observed in January 2014 (201.9±26.2 µg TE $mg^{-1}$ DWE), yet the lowest activity was detected in the following January, in 2015 (96.7±7.7 µg TE $mg^{-1}$ DWE; $p$<0.05; Fig. 3.7), unlike the TPC. The FRAP activity displayed by the LMW fractions of the receptacles was highest in January 2014 (the overall highest observed of all samples), with 254.5±21.5 µg TE $mg^{-1}$ DWE while the lowest levels were detected in November 2014 (35.7±5.5 µg TE $mg^{-1}$ DWE; $p$<0.05; Fig. 3.7).

Similar to that of FRAP, the RSA values also displayed a positive correlation with the phlorotannin content ($R^2$=0.41; Fig. 3.7). Again, LMW fractions derived from the thalli had the highest activity overall, with maximum activity detected in January 2014 (24.17±0.8 µg $mL^{-1}$; $p$<0.05; Fig. 3.7). Apical tips and receptacles also showed maximal activity in this month, with similar values to that of the thalli (22.6±1.7 and 36.4±5.9 µg $mL^{-1}$, respectively; $p$>0.05; Fig. 3.7). Similar to TPC and FRAP, the RSA of both the thalli and apical tips decreased in May 2015, with both sections exhibiting lowest levels at this season, but this decrease was more pronounced for the thalli (176.5±6.2 and 103.0±0.6 µg $mL^{-1}$, respectively; $p$<0.05; Fig. 3.7). The receptacles exhibited the overall lowest level of RSA in November 2014, following that of TPC and FRAP, with an $IC_{50}$ value of 183.9±11.1 µg $mL^{-1}$, which was similar to the lowest level found for thalli ($p$>0.05; Fig. 3.7).

### 3.3.4.3. *F. vesiculosus*

The FRAP activity of LMW fractions for each thallus part from *F. vesiculosus* was significantly affected by thallus part ($p<0.001$), season ($p<0.001$) and their interaction ($p<0.001$; Fig. 3.8). It was also exhibited a strongly positive correlation to TPC ($R^2=0.73$; Fig. 3.8), displaying similar seasonal trends. FRAP activity significantly decreased in August 2014, with the overall lowest levels of activity being observed, for both the tips and thalli fractions (371.0±24.3 and 380.6±12.3 µg TE $mg^{-1}$ DWE, respectively; $p<0.05$: Fig. 3.8). After this, a steady increase toward May 2015 in which the highest FRAP in thalli-derived fractions was observed (604.5±20.98 µg TE $mg^{-1}$ DWE). However, a higher level for that of the tips (and the overall highest) was observed initially in January 2014 with 751.0±17.1 µg TE $mg^{-1}$ DWE; $p<0.001$; Fig. 3.8).

Unlike most other species, and unlike the FRAP activity for this species, the RSA for *F. vesiculosus* thallus sections, did not display any kind of correlation with the phlorotannin content ($R^2=0.005$; Fig. 3.8), but it should be noted that seasonal differences were observed within a narrow range (20.57±0.37 to 35.7±3.1 µg $mL^{-1}$; Fig. 3.8). Overall the highest RSA was observed for both sections in January and August 2014 after which a slight, but significant, decrease was between November 2014 and May 2015 ($p<0.001$; Fig. 3.8).

### 3.3.4.4. *H. elongata*

Similar to all other species, the FRAP activity of the LMW fractions derived fom the different thallus sections of *H. elongata*, followed a similar seasonal trend to that of TPC ($R^2=0.42$; Fig. 3.9). The observed FRAP levels of the vegetative buttons and receptacles differed significantly from another ($p<0.001$) as well as between seasons ($p<0.001$). The overall highest FRAP activity was observed in fractions extracted from the vegetative buttons, with maximum levels observed in January 2015 (338.3±16.4 µg TE $mg^{-1}$ DWE; $p<0.05$; Fig. 3.9). A similar, yet slightly lower level, was also observed in May 2014 (302.2±7.7 µg TE $mg^{-1}$ DWE). The receptacle-derived fractions, however, displayed a similar pattern to that of the TPC where a maximum level of FRAP activity was detected in January and May 2014 (258.2±26.6 and 251.4±7.1 µg TE $mg^{-1}$ DWE, respectively);

thereafter a steady decrease was observed, to the lowest level occurring in May 2015 (123.2±8.6 µg TE $mg^{-1}$ DWE; $p<0.05$: Fig. 3.9).

Similar to the FRAP, the RSA of the LMW fractions displayed a positive correlation with TPC ($R^2=0.69$; Fig 3.9). LMW fractions, from both the vegetative buttons and reproductive receptacles, exhibited maximum ($p<0.05$) RSA in May 2014, displaying similar levels to one another (20.4±0.6 and 18.6±3.0 µg $mL^{-1}$, respectively; Fig. 3.9). However, the lowest ($p<0.05$) RSA values for the buttons occurred over 3 sampling seasons; January 2014, November 2014 and January 2015 (56.7±3.5, 49.5±5.3 and 46.4±1.5 µg $mL^{-1}$; respectively) while the lowest RSA for receptacles, and lowest overall, was observed in samples collected in January 2015, with 79.2±2.4 µg $mL^{-1}$ ($p<0.05$; Fig. 3.9).

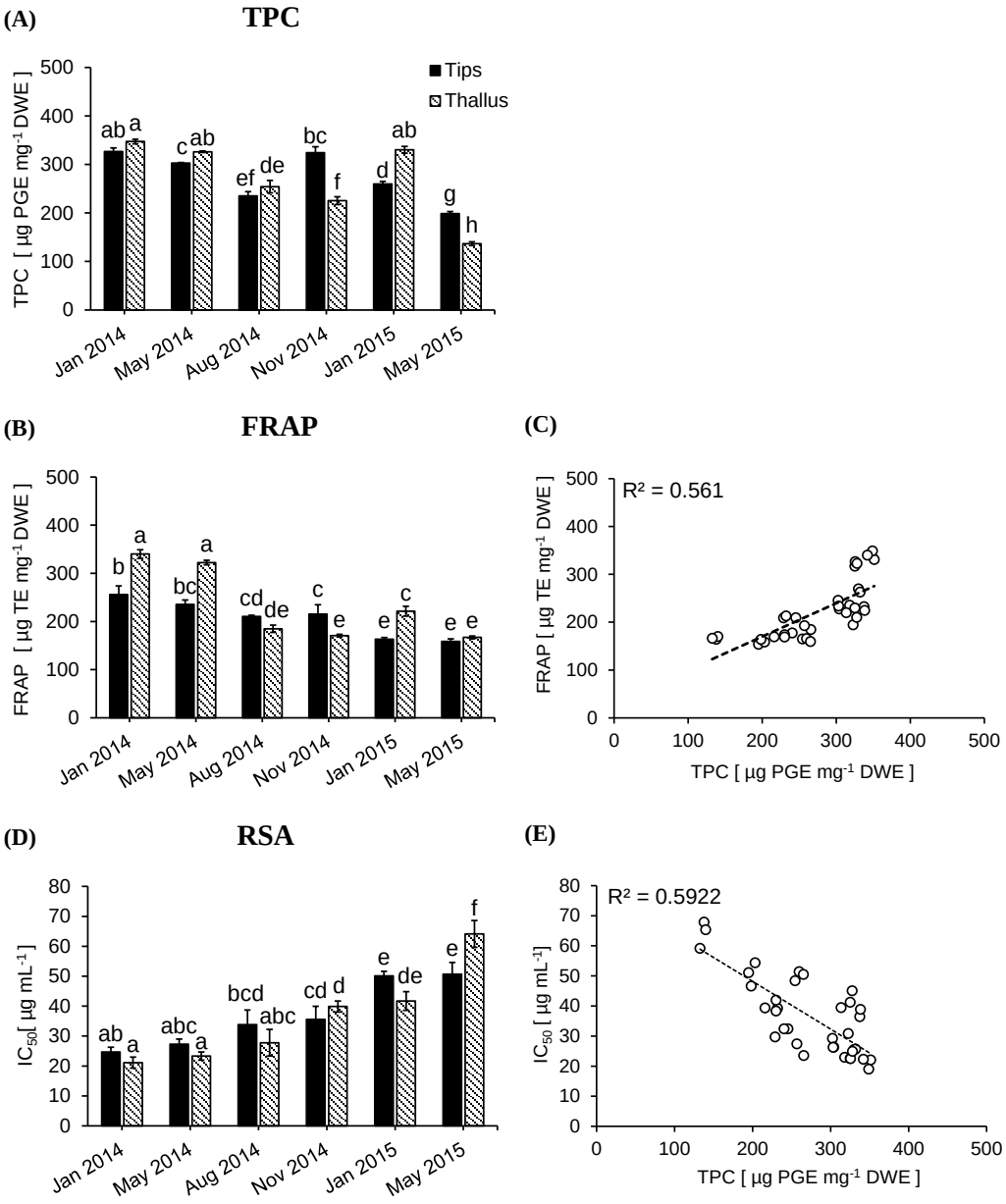



**Fig. 3.6.** Seasonal variation in the (A) total phlorotannin content (TPC) of LMW phlorotannin-enriched fractions from the tips and thallus of ***P. canaliculata***, expressed as microgram phloroglucinol equivalents per milligram of dry weight extract (µg PGE $mg^{-1}$ DWE). (B) The seasonal variation in the ferric reducing antioxidant power (FRAP), expressed as microgram Trolox equivalents per milligram of dry weight extract (µg TE $mg^{-1}$ DWE). (D) The seasonal variations of the radical scavenging activity (RSA) expressed as $IC_{50}$ values in microgram per millilitre (µg $mL^{-1}$). Correlation coefficient ($R^2$) is given for the linear relationship between (C) FRAP and TPC and (E) $IC_{50}$ and TPC. Data are represented as mean ± standard deviation (n=3). Significant effects of thallus part and sampling season were tested using a two-way ANOVA ($p$<0.05). Superscripts identify between significant differences between samples using Tukey's *post-hoc* test.

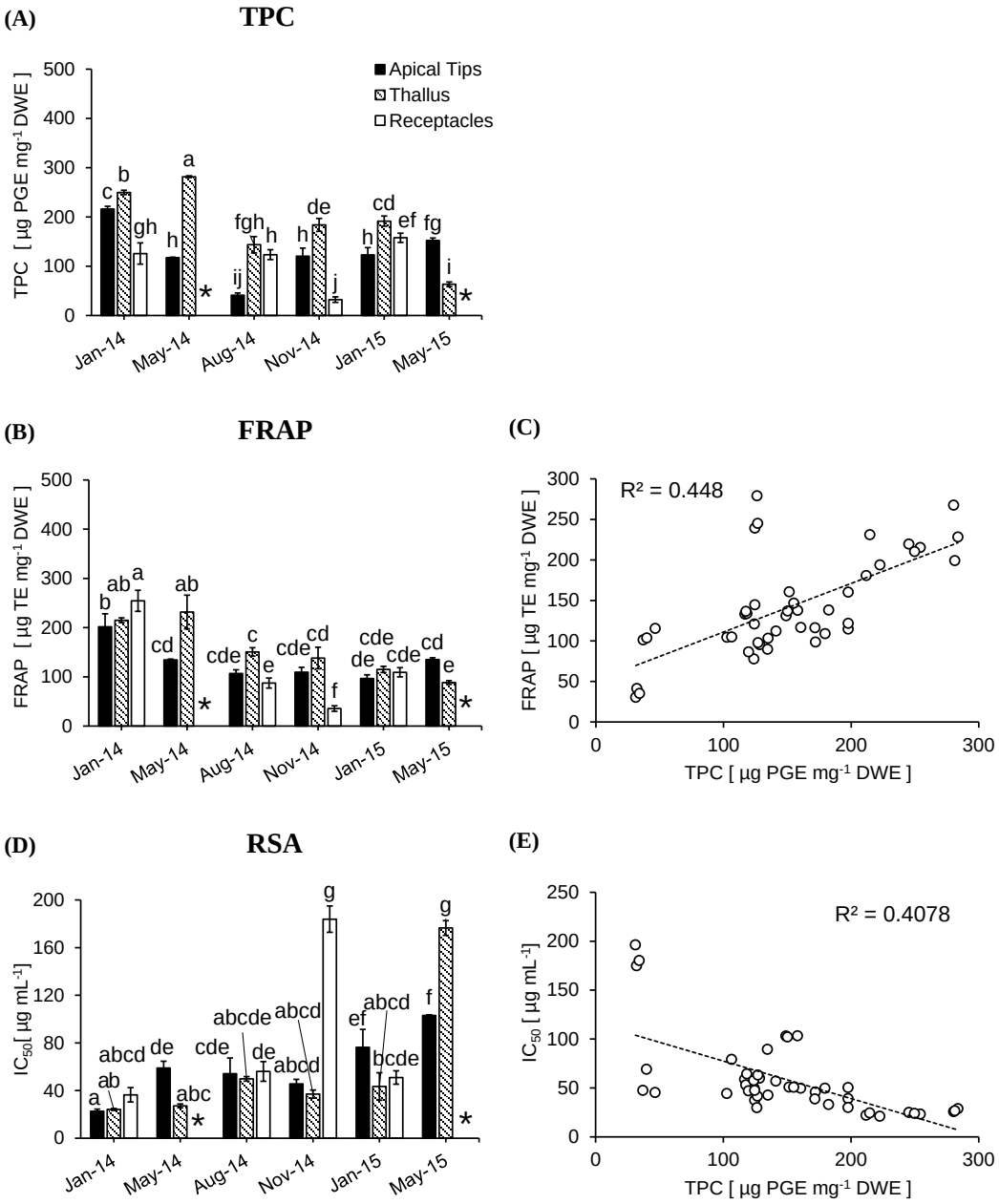



**Fig. 3.7.** Seasonal variation in the (A) total phlorotannin content (TPC) of LMW phlorotannin-enriched fractions from the apical tips, thallus and receptacles of ***A. nodosum***, expressed as microgram phloroglucinol equivalents per milligram of dry weight extract (µg PGE $mg^{-1}$ DWE). (B) The seasonal variation in the ferric reducing antioxidant power (FRAP), expressed as microgram Trolox equivalents per milligram of dry weight extract (µg TE $mg^{-1}$ DWE). (D) The seasonal variations of the radical scavenging activity (RSA) expressed as $IC_{50}$ values in microgram per millilitre (µg $mL^{-1}$). Correlation coefficient ($R^2$) is given for the linear relationship between (C) FRAP and TPC and (E) $IC_{50}$ and TPC. In May 2014 and 2015 receptacles had been shed and so were not able to be collected, as indicated by *. Data are represented as mean ± standard deviation (n=3). Significant effects of thallus part and sampling season were tested using a two-way ANOVA ($p$<0.05). Superscripts identify between significant differences between samples using Tukey's *post-hoc* test.

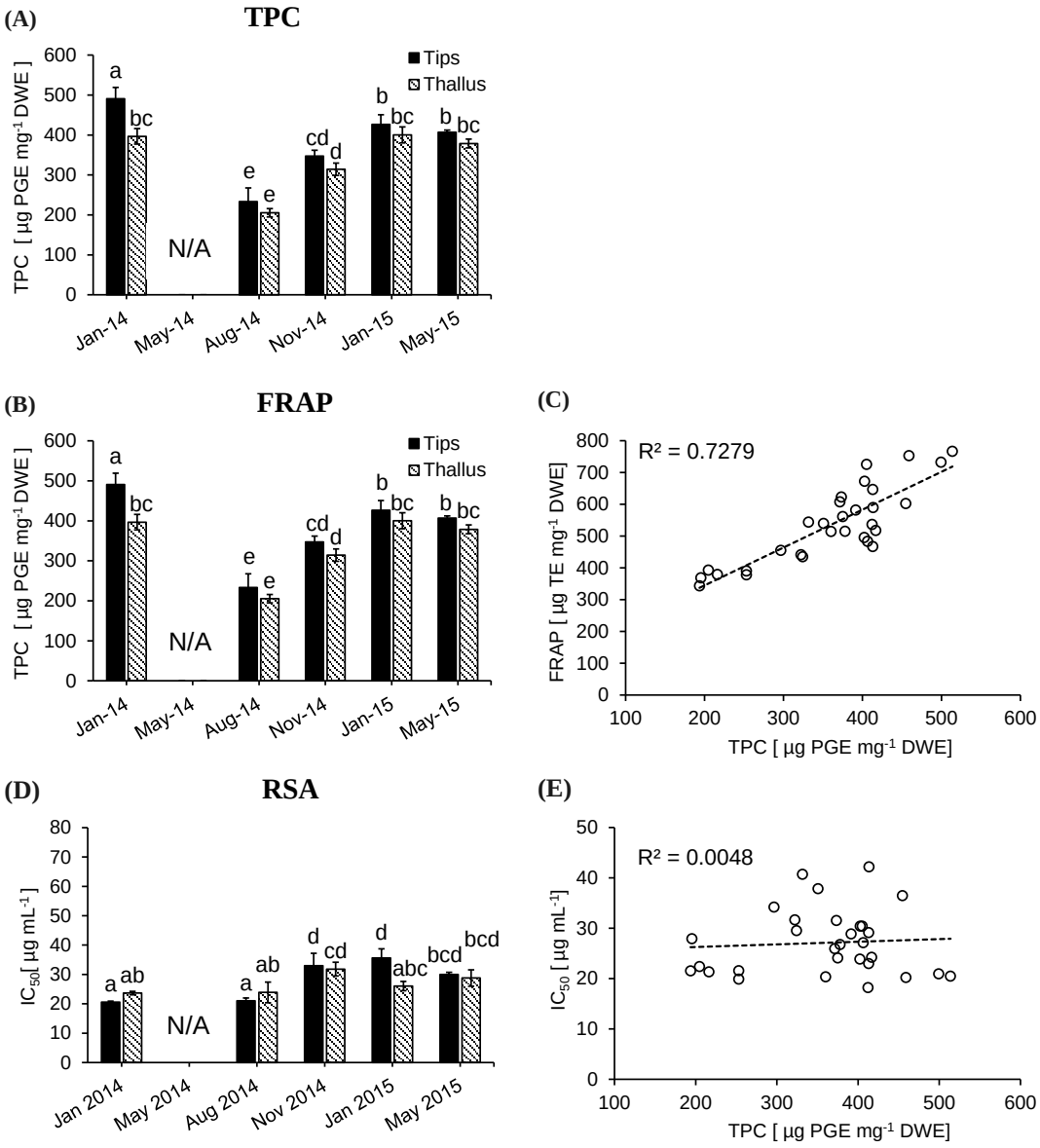



**Figure 3.8.** Seasonal variation in the (A) total phlorotannin content (TPC) of LMW phlorotannin-enriched fractions from the tips and thallus sections of ***F. vesiculosus***, expressed as microgram phloroglucinol equivalents per milligram of dry weight extract (µg PGE $mg^{-1}$ DWE). (B) The seasonal variation in the ferric reducing antioxidant power (FRAP), expressed as microgram Trolox equivalents per milligram of dry weight extract (µg TE $mg^{-1}$ DWE). (C) The seasonal variations of the radical scavenging activity (RSA) expressed as $IC_{50}$ values in microgram per millilitre (µg $ml^{-1}$). Correlation coefficient ($R^2$) is given for the linear relationship between (G) FRAP and TPC and (H) $IC_{50}$ and TPC. Data are represented as mean ± standard deviation (n=3). Significant effects of thallus part and sampling season were tested using a two-way ANOVA ($p$<0.05). Superscripts identify between significant differences between samples using Tukey's *post-hoc* test.

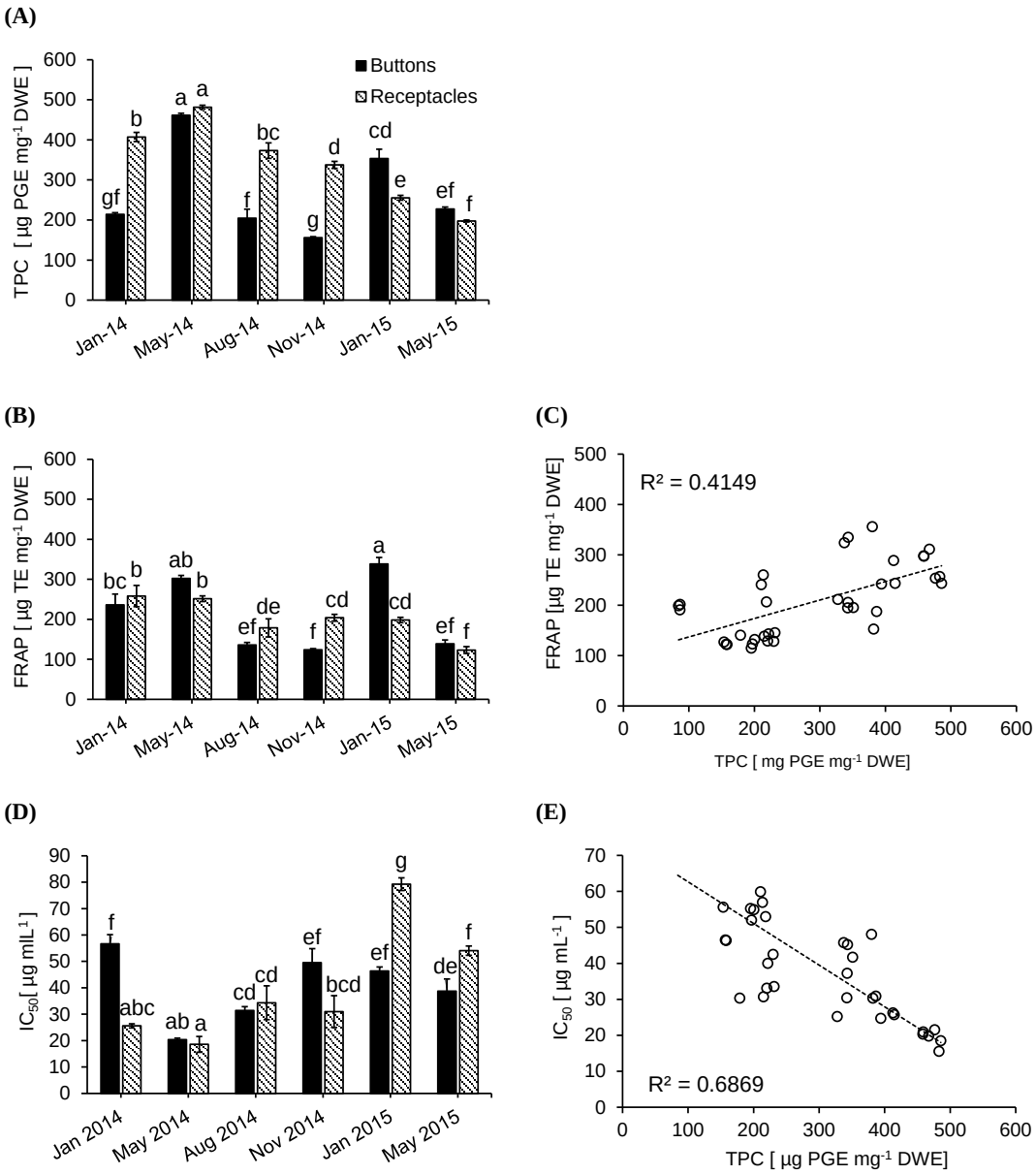



**Fig. 3.9.** Seasonal variation in the (A) total phlorotannin content (TPC) of LMW phlorotannin-enriched fractions from the buttons and receptacles of *H. elongata*, expressed as microgram phloroglucinol equivalents per milligram of dry weight extract (µg PGE $mg^{-1}$ DWE). (B) The seasonal variation in the ferric reducing antioxidant power (FRAP), expressed as microgram Trolox equivalents per milligram of dry weight extract (µg TE $mg^{-1}$ DWE). (D) The seasonal variations of the radical scavenging activity (RSA) expressed as $IC_{50}$ values in microgram per millilitre (µg $mL^{-1}$). Correlation coefficient ($R^2$) is given for the linear relationship between (C) FRAP and TPC and (E) $IC_{50}$ and TPC. Data are represented as mean ± standard deviation (n=3). Significant effects of thallus part and sampling season were tested using a two-way ANOVA ($p$<0.05). Superscripts identify between significant differences between samples using Tukey's *post-hoc* test.

## 3.4. Discussion

Intra-thallus variability was examined within four species, belonging to the Fucales, which differ in their vertical shore position, growth pattern and morphology. By examining individual thallus sections involved in vegetative growth and reproduction, as well as different ages, this study aimed at elucidating any potential effects of algal development on LMW phlorotannins. Both the vegetative thallus (or tips or buttons) and the reproductive receptacles were analysed over six seasons to attain a better understanding of the effect of development may have over their content, composition and associated in vitro antioxidant activity, if any.

As discussed in Chapter 2, the specificity involved in the analysis of phlorotannins from a single sized fraction (<3.5 kDa) may interfere in revealing the overall natural variability occurring in some species, particularly those known to have a high abundance of higher-weighted phlorotannin compounds such as those found in *A. nodosum* (Audibert *et al.*, 2010). However, the purpose of this work was not to decipher the ecological role phlorotannins in development but rather the opposite, to investigate whether or not LMW phlorotannin fractions vary, intra-specifically, as a result of developmental changes occurring throughout algae lifecycle. However, the novelty associated with these analyses make the comparison of results with past studies difficult, as previously crude extracts, containing all phenolic compounds, were assessed. Here, intra-thallus sections were collected over six seasons and their LMW phlorotannin-enriched fractions were assessed for variations in their metabolic profiles, content and antioxidant activity.

### 3.4.1. Intra-thallus variability in the total phlorotannin content of the LMW phlorotannin-enriched fractions

For all species investigated, considerable intra-thallus differences were observed in the total phlorotannin content (TPC). In most species, seasonal fluctuations were also seen, the manner of which differed between thallus parts. However, seasonal differences were not uniformly detected, with levels displaying inter-annual variations rather than specific seasonal trends. For example, in *P. canaliculata*, the tips displayed maximum phlorotannin levels in January 2014, but significantly lower levels were observed in the

following January, in 2015. Similarly, in *A. nodosum*, thallus maxima in TPC were seen in May 2014 but displayed the lowest levels the following May, in 2015. The TPC of *H. elongata* also displayed significant seasonal fluctuations in both thallus parts. Surprisingly, both the vegetative buttons and reproductive receptacles displayed maximal phlorotannin levels in May 2014 but otherwise differed during most other seasons. However, due to the high degree of morphological differentiation within this species, such intra-thallus variability was anticipated.

In general, regardless of seasonal fluctuations, the thallus parts that contributed most to the total biomass displayed the highest levels of phlorotannins, in most species. This was the vegetative thallus in *P. canaliculata* and *A. nodosum* and the reproductive receptacles in *H. elongata.* Similar findings have been reported suggesting that the higher abundance of phlorotannins in particular section stems from a defensive trait, protecting the region in which the highest energy investment has been placed (Hemmi *et al*., 2005; Pavia *et al*., 2002; Tuomi *et al*., 1989) and thus validating the theory of optimal defence and contribution to fitness (Cronin and Hay, 1996; Rhoades, 1979). On the other it is conceivable that the higher amount of biomass may serve to increase the yield of extracted soluble phlorotannins, thus misinterpreting localised concentrations.

While the thallus parts of most species investigated showed varying seasonal trends in phlorotannin content to one another, the thallus sections of *F. vesiculosus* appeared to correlate with one another. While the tips displayed a slightly higher level of phlorotannins compared with the thalli, *F. vesiculosus* was the only species in which both thallus parts shared a similar seasonal trend. This may be either due to the lack of morphological differentiation observed by this species or potentially, due to its dominant position along the intertidal, suggest that both thallus parts require similar and high constitutive levels of defence.

The dramatic decline in phlorotannin content observed in both thallus sections of *F. vesiculosus* in August 2014 was unlikely to be associated with developmental variations, but rather the potential cause of a previous epiphytic bloom that engulfed the entire *F. vesiculosus* population of Finavarra in May 2014 (hence why no sampling was conducted for this period). It was surmised that the presence of *Pylaiella littoralis,* a filamentous

epiphyte, could have affected the light and nutrient availability of *F. vesiculosus*. This, in turn, could have reduced several aspects of its metabolic activity, ultimately lowering the production of numerous metabolites including phlorotannins (Jormalainen *et al.*, 2003). Therefore, the gradual re-establishment of natural high and relatively stable phlorotannin concentrations in *F. vesiculosus*, thereafter, may be responsible for the observed "seasonal" changes, as discussed in Chapter 2.

The large variability observed in the phlorotannin content within the different thallus parts of all species implies that intrinsic factors strongly impact phlorotannin levels. However, due to the complex nature of these compounds, great difficulty is associated with deciphering which specific factors are responsible *i.e.* tissue age, reproductive status, plant size, fitness value, if any. Phlorotannin levels have been suggested to vary on a seasonal scale inversely correlated to the reproductive development of the individual (Ragan and Jensen, 1978). They reported that the phenolic content of *A. nodosum* and *F. vesiculosus* was inverse to the species reproductive maturity, with winter maxima and summer minima being observed. The synergistic effect of both intrinsic and extrinsic factors, concurrently, on algae biochemistry, is most likely responsible for the varied, and poorly understood, fluctuations observed in phlorotannin concentrations. Moreover, these two sets of parameters are mechanistically joined. For example, it is through environmental cues that certain aspects of algal development are triggered. For example, the photoperiod has been largely associated with receptacle initiation in some Fucales (Terry and Moss, 1980). Such an interaction will only further complex matters.

Furthermore, the rapid turnover rates exhibited by phlorotannins previously reported, will make it difficult for seasonal data to provide clarity on a given metabolic process in which they may be involved in. For example, Connan *et al.* (2007) observed the phlorotannin levels of *P. canaliculata* and *A. nodosum* to vary on a diurnal scale, in response to emersion/ immersion periods. This was also observed by Abdala-Díaz *et al.* (2006) with phlorotannin levels following daily fluctuations in irradiance. Cruces and Huovinen (2012) found short-term shifts in phlorotannins in response to increased UVR exposure, with induction detected after just 3 h.

### 3.4.2. Intra-thallus variability in the *in vitro* antioxidant activity of the LMW phlorotannin-enriched fractions

Despite the difficulty in elucidating the factors behind the intra-thallus variability of the phlorotannin content, a strong association between the phlorotannin levels and the observed degree of activity existed for each species. In general, the FRAP activity displayed a positive correlation ($R^2$=0.41-0.73) with the TPC detected, suggesting that the overall phlorotannin abundance was significant in ferric reducing activity. Furthermore, the RSA results were also positively correlated with TPC (inverse correlation with $IC_{50}$ values; $R^2$=0.41-0.69). The antioxidant activity of phlorotannins has been considered as largely concentration-dependent, with several previous studies finding a positive linear relationship between the biological activity and the overall phlorotannin content (Kang *et al*., 2004; Tierney *et al*., 2013; Wang *et al*., 2012). However, no such correlation was observed between the TPC and RSA of *F. vesiculosus* thallus parts ($R^2$=0.005). This may have stemmed from the considerably narrow range of RSA variation observed for this species for each thallus part. A similar correlation was observed for this species previously in samples collected from different locations and sites (Chapter 2). This lack of variability in RSA may also stem from the relative stability in the chemical make-up of the LMW phlorotannin profiles observed between, and within, the different thallus parts of F. *vesiculosus*.

### 3.4.3. Intra-thallus variability in the chemical composition of the LMW phlorotannin-enriched fractions

As well as concentrations, the chemical properties of phlorotannins has also previously been reported to affect the degree of bactivity observed (Ahn *et al*., 2007; Ferreres *et al*. 2012; Li *et al*., 2009). For example, the differences in the overall level of activity observed between species was thought to be related to the differences in the phlorotannin metabolic profiles (Heffernan *et al.*, 2015) as the degree of polymerisation and isomerisation are likely to, synergistically, effect the amount of free hydroxyl units available for reaction (Shibata and Ishimaru, 2008). However, as far as we are aware, intra-specific elucidation of phlorotannin metabolic profiles has not yet been carried out.

Overall the different thallus part of *P. canaliculata* exhibited a similar chemical composition. Each section was predominantly comprised of phlorotannins with molecular weights between 1118 and 1615 Da (8-13 PGUs). While overall the relative abundance of individually-sized structures varied between the sections, this particular range displayed a similar seasonal trend between the sections. With regard to structural variability, the two thallus parts demonstrated a similar degree of isomerisation. This was observed throughout most seasons, except for two (November 2014 and May 2015), during which the thallus sections displayed considerably lower isomeric levels. The lack of isomers observed was associated with a reduction in the resolution of the total ion chromatogram onset by a high polysaccharide presence, highlighting how seasonality in phlorotannin characterisation is also subject to the seasonal variability in carbohydrate chemistry.

As outlined in Chapter 2, the LMW phlorotannin-enriched fractions of *P. canaliculata*, as well as *A. nodosum*, were associated with a high degree of interference from co-extracted polar polysaccharides, such as β-glucan, from which they were difficult to separate (Tierney *et al.*, 2014). The high abundance of such polar polysaccharides is possibly derived from the presence of an obligate endophyte, *Stigmidium ascophylli* (Cotton), with which these species form a symbiotic relationship (Garbary and Gautam, 1989; Rugg and Norton, 1987).

The abundance of these "contaminant" compounds differed between thallus parts, particularly so within *A. nodosum*. The level of purity permitted by reverse phase (RP) flash separation varied considerably for both thallus part and season. For example, the receptacles in January of each year allowed for an adequate separation while in November 2014, the total ion chromatogram (TIC) for this thallus section resembled that of a methanol blank, with very little phlorotannins detected. It is difficult to determine whether or not this decline occurred simply due to a decrease in phlorotannin abundance at this particular season, owing to the maturation of the receptacles, or if an increase in the polysaccharide content at this time of year made the detection of LMW phlorotannins problematic. As a result, it is likely that *A. nodosum* will become utilised for commercial extraction and application of these bioactives only after the development of a method capable of disintegrating the adjoining bonds between the two compounds.

It is not surprising that the most distinct intra-thallus variability observed within the four species investigated was found for *H. elongata*. This species displays the most distinct intra-thallus morphological differentiation between thallus parts; thus, the chemical composition between thallus parts is also likely to vary considerably, as indeed observed in their phlorotannin profiles. The receptacles, accounting for the majority of this species biomass, displayed a relatively uniform composition throughout the six seasons investigated with small seasonal changes observed within the relative abundance of the dominant phlorotannin range (8-13 PGUs). However, the vegetative buttons appeared to vary considerably, with no specific range of phlorotannins being abundant consecutively across all seasons. This is likely to occur in conjunction with the development of vegetative buttons into a fertile form from which the receptacles are produced. The changes in the overall chemistry of the alga with maturation will undoubtedly affect the phlorotannin metabolic profiles. The ever-changing composition and content exhibited by the buttons could also imply that as they develop, taking up to two years (Stengel *et al.*, 1999), they may acquire varying phlorotannin compositions as a defensive trait due to their particular vulnerability to grazing pressure, thus enabling them to reach maturation.

A high level of uniformity was observed within the chemical composition of the two thallus parts investigated in *F. vesiculosus*, with an almost identical profile observed for the two sections. This species displays a molecular distribution highly abundant in lower molecular weight phlorotannin structures (5, 8 and 4 PGUs, in order of abundance). Such a profile has been previously observed (Heffernan *et al.*, 2015). Even over the six seasons, apart for minute variations, the chemical composition of this species remained relatively stable. This highlights the attraction of such a species for future work regarding the commercial application of LMW phlorotannins.

In summary, while intra-thallus variability was observed for most of the species examined, the synergistic effect of both intrinsic and extrinsic factors in the field acting upon the algae at any one time, makes it challenging to decipher the specific parameters driving the observed differences. Clearly, the physiological plasticity these compounds offer algae may contribute to their ability to inhabit the differing environmental gradients to which they are exposed to, enabling them to dominant the shore zones they occupy.

The results from this study also highlight how an assessment of phlorotannin variability in field-collected samples alone will not suffice in elucidating on the natural variability of these bioactives. To become capable of fully exploiting their multi-commercial benefits, a better insight into the specific factors contributing to their synthesis, as well as degradation, is required. Only then will commercial cultivation even be considerable. In order to achieve a better knowledge on the influence of such parameters, investigation into individual environmental factors is key. Therefore, the work described in the subsequent chapters concentrated on the manipulation of abiotic factors under laboratory-controlled conditions with the aim to elucidate on the mechanisms involved in phlorotannin metabolism. The data from this chapter, as well as Chapter 2 suggest that the use of *F. vesiculosus* as model species may be beneficial. The high degree of purity within the LMW phlorotannin-enriched fractions not only permits a high level of antioxidant activity, highlighting their potential for commercialisation but also allows for detailed assessment of incurred variations imposed by cultivation conditions.

# Chapter 4

The single and interactive effects of PAR and UVR on low molecular weight phlorotannin profiles in *F. vesiculosus* (L.)

## 4.1. Introduction

In the process of photosynthesis, algae utilise light energy from the sun to chemically fix inorganic carbon into energy-rich organic compounds (Karsten, 2008). In short, located in the chloroplast, specifically the thylakoids, the light-harvesting complex (LHC) of photosystem II (PSII) chlorophyll (Chl) *a* and accessory pigments (Chl *b*, *c*, carotenes and xanthophylls; see Chapter 1, Section 1.1, Table 1.1) absorb photons (Gantt and Cunningham, 2001). This energy, now in an excited state, is transferred through a series of redox reactions between electron acceptors and donors until it reaches the PSII reaction centre, where it is used to oxidise water molecules (photolysis) from the lumen (Gomez and Chitnis, 2000). The water-derived electrons are then carried toward photosystem I (PSI) via a chain of electron transporters, where they ultimately result in the formation of energy storage and transporter molecules, adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADP(H); Fig 4.1).

ATP and NADP(H) are energy storage and transporter molecules which drive the dark reactions in the Calvin-Benson cycle, in the thylakoid stroma of the chloroplast. Here, the enzyme ribulose bisphosphate carboxylase (RuBisCo) catalyses the carboxylation of the $CO_2$ acceptor, ribulose 1, 5 bisphosphate (RuBP), producing 3-phosphoglycerate (PGA). Each PGA is then converted into glyceraldehyde 3-phosphate (G3P) promoted by the release of energy through the reduction of ATP back to ADP and NADPH to $NADP^+$. The majority of G3P remains in the cycle to regenerate RuBP for the cycle to continue and a small amount exits from the cycle where they are used to synthesis sugar and starch compounds (Raines, 2003; Fig 4.1).

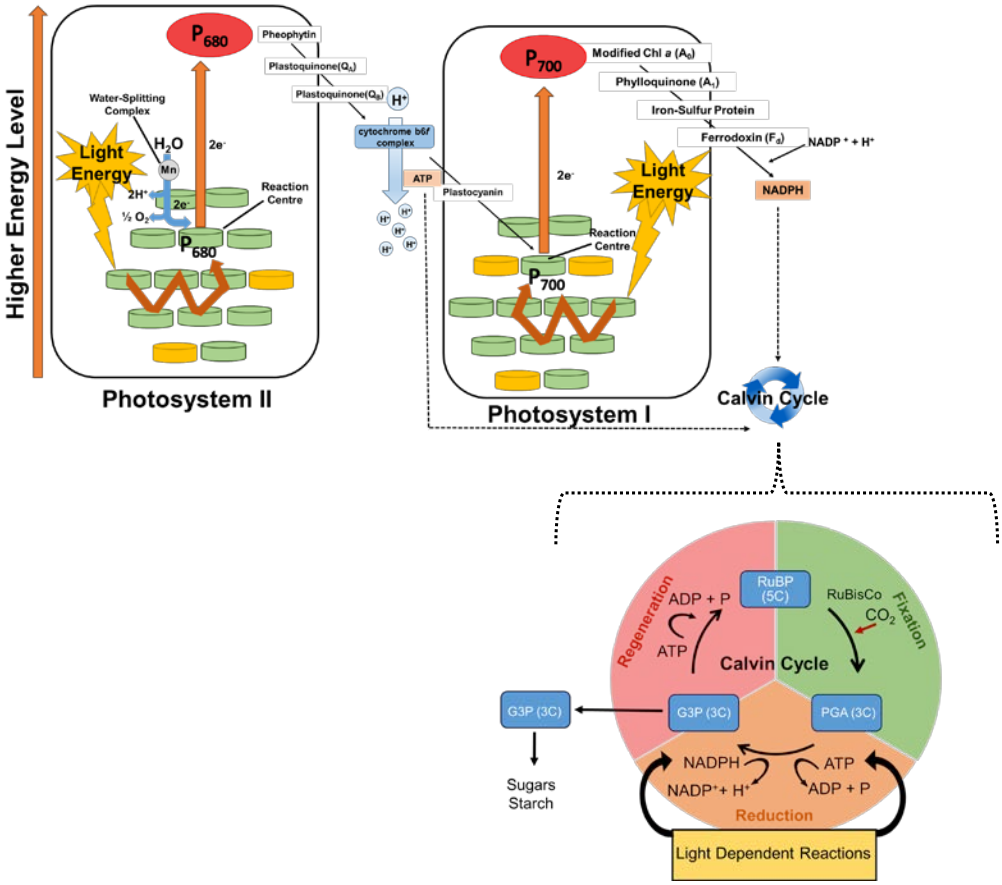



**Fig. 4.1.** Overview of the light and dark reactions involved in photosynthesis. Adapted and modified from http://www.majordifferences.com/2013/12/difference-between-photosystem-i-and.htmL#.WbQAgrKGPcs.

Chlorophyll and the other light-harvesting pigments display a high absorption across a broad spectral range of 400 to 700 nm, maximising photosynthetic efficiency. Thus, this band of light is defined as photosynthetically active radiation (PAR; Fig. 4.2). However, solar radiation is also composed of both infrared (IR; 700 nm-1,000 nm) and ultraviolet (UVR; UVC; 190-280 nm, UVB; 280-315 nm, UVA; 315-400 nm) radiation. Generally, the total solar energy reaching the Earth is composed of 8 % UVR, 46.5 % PAR and 45.5 % IR (Iqbal, 2012; Sen, 2008), with longer IR wavelengths being perceived as heat rather than light. While UVR is not considered as photosynthetically active, except for a small portion near the UVA band, it is regarded as very biologically active (Bhatia *et al.*, 2011). This is in respect to the higher energy associated with the shorter wavelengths of UVR and the adverse implications they can impose on biological organisms (Holzinger and

Lütz, 2006). Despite the fact that UVB only accounts for ~ 1 % of the total solar energy (Rastogi *et al.*, 2010), it has been extensively reported as the most detrimental (Sinha and Häder, 2002).

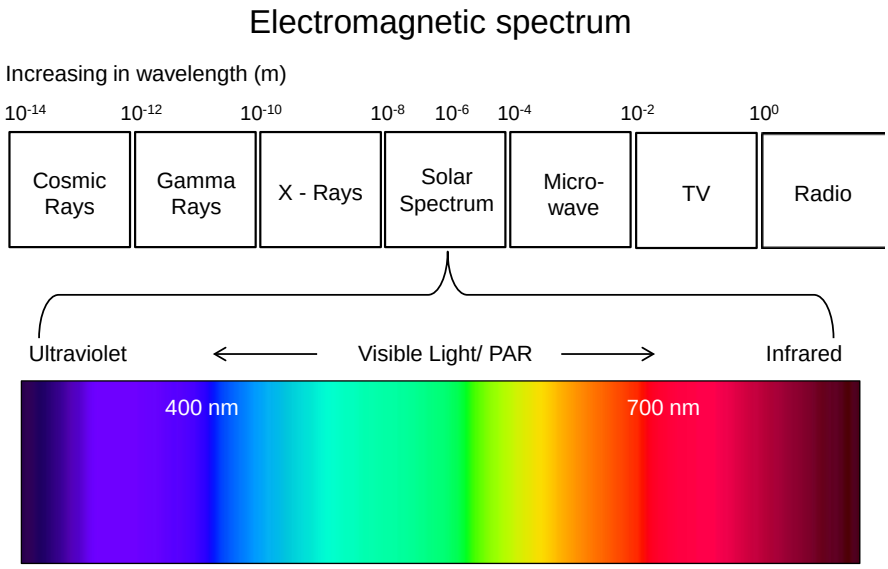



**Fig. 4.2.** Illustration of the various components within the electromagnetic spectrum including the solar spectrum composed of ultraviolet radiation (190-400nm; UVR), photosynthetically active radiation (400-700 nm; PAR) and infrared radiation (700-1000 nm; IR).

Specifically, the direct absorption of UVB by cellular DNA can cause conformational alterations including single and/or double strand breaks as well as the formation of lesions such as cyclobutane pyrimidine dimers (CPDs) and pyrimidine 6-4 pyrimidone photoproducts (6-4PPs; Gill *et al.*, 2015; Rastogi *et al.*, 2010). Such structural distortions can severely impede accurate DNA transcription and replication, leading to an increased rate of mutagenesis, reduced gene expression and elevated cell mortality (Holzinger and Lütz, 2006; Karsten, 2008; Sinha and Häder, 2002).

Although PAR and UVA are not directly absorbed by DNA components, pronounced and/or prolonged exposure to these wavelengths (as well as UVB) can incur cellular damage via other means. The majority of them is initiated by their involvement in accelerating the generation of reactive oxygen species (ROS). The evolution of oxygen ($O_2$) within the process of photosynthesis means that ROS formation, at basal levels, is an unavoidable part of plant and algal metabolism (Tripathy and Oelmüller, 2012).

However, under environmental conditions that sanction their overproduction, ROS can become auto-destructive, targeting a range of vulnerable biomolecules such as unsaturated fatty acids, proteins and DNA and thus impair normal physiological functions (Das and Roychoudhury, 2014; Krumova and Cosa, 2016; Lesser, 2006).

The extent of the biological damage inflicted by PAR and UVR, however, will vary depending on the degree of exposure, a factor that is greatly influenced by both atmospheric and geographical parameters (Godar, 2005). For example, UVC never reaches the Earth's surface, being completely absorbed by $O_2$ as it travels through the atmosphere and therefore, is considered biologically irrelevant (Madronich *et al.*, 1998). UVA is less attenuated by ozone particulate than UVB and can penetrate greater depths of the water column (e.g. 40-60 m; 20-30 m, respectively; Ban *et al.*, 2007). Yet this depth will also vary, depending on the optical properties of the water column with waves, currents, dissolved organic matter (DOM) and salinity, all affecting the UV penetration (Pessoa, 2012). However, even along the intertidal zone, the degree of exposure algae will endure will vary depending on a number of factors from the topography of the shore, their vertical position, the extent of the diurnal tidal recession as well as canopy shading.

In general, turbid coastal waters display a higher degree of UV attenuation than the clear polar waters. However, the fluctuating tidal cycles of coastal shores can place algal species inhabiting the upper sublittoral and intertidal zones in direct contact with high PAR and UVR levels for extended periods of time (Holzinger and Lütz, 2006; Hurd *et al.*, 2014), the extent of which will depend on shore level. Therefore, for a species to successfully occupy such an environment, physiological adaptations, enabling them to withstand such strong light exposure, are required.

Several physiological mechanisms have evolved to mitigate the absorption, transfer and quenching of excessive excitation energy under periods, or in locations, of intense solar irradiance. Many of these adaptations are associated with protecting photosynthetic apparatus from long-term (chronic) damage such as those involved in dynamic photo-inhibition, the short-term, reversible decrease in photosynthetic capacity (Hanelt, 1998; Powles, 1984). Such mechanisms can include the dissipation of excessive excitation energy as heat (Krause and Weis, 1991) as well as shifts in the abundance and composition

of photosynthetic pigments (Bischof *et al.*, 2002). Others photo-protective responses involve the increased production of both enzymatic (e.g. superoxide dismutase; Bischof *et al.*, 2003) and non-enzymatic radical scavengers such as mycosporine-like amino acids (MAAs) in red algae (Karsten *et al.*, 1998) and phlorotannins in brown algal species (Pavia *et al.*, 1997) to reduce cellular oxidative stress onset by elevated irradiance exposure.

Phlorotannins play multiple roles in the photo-protection of brown algae; firstly, their position along the peripheral of the epidermal cells enable these compounds to directly intercept harmful solar rays, acting as a physical barrier and thereby protecting intracellular components (Schoenwaelder, 2002). Phlorotannins are also regarded as highly potent antioxidant compounds, capable of significantly reducing the degree of oxidative stress via ROS scavenging (Ahn *et al.*, 2007; Heo *et al.*, 2009; Shibata and Ishimaru, 2008). Moreover, their abundance has been suggested to be central to the successful development in early life stages (Clayton and Ashburner, 1994; Schoenwaelder and Clayton, 1998; Schoenwaelder and Wiencke, 1999), with species possessing higher phlorotannin levels capable of inhabiting elevated shore positions (Schoenwaelder *et al.*, 2003). While integral to the successful development of juvenile stages of several species, they are also inducible in response to photo-stress in adult stages. Shifts in natural phlorotannin levels have been correlated to both daily and seasonal increases in solar intensity (Abdala-Díaz *et al.*, 2006; Connan *et al.*, 2004, 2007; Gomez and Huovinen, 2010; Kamiya *et al.*, 2010; Pavia and Brock, 2000; Plouguerné *et al.*, 2006), further suggesting that these compounds are involved in photo-protective mechanisms. Yet, conclusions from laboratory-controlled studies are not consistent, with some supporting UVR induction (Henry and Van Alstyne, 2004; Pavia *et al.*, 1997; Swanson and Druehl, 2002), while others found no effect (Cronin and Hay, 1996; Fairhead *et al.*, 2006). On the other hand, some studies have suggested that ontogenetic changes, such as reproductive maturity, are responsible for the observed seasonal variations rather than inducible defences (Creis *et al.*, 2015; Parys *et al.*, 2009; Ragan and Jensen, 1978; Stiger, *et al.*, 2004).

Therefore, one of the main aims of this work was to to determine whether or not phlorotannin induction functions of phlorotannins as a protective response to photo-stress. In this case, photosynthetic measurements can provide valuable information regarding the physiological effect specific environmental factors can impose on the "health" of algae (Tait *et al.*, 2017). This may allow for correlations to be made between the "resilience" of the algae displayed towards different light regimes and the level of phlorotannins detected, therefore, elucidating on their potential physiological role.

During the light-dependent reactions of photosynthesis, light energy is absorbed by antenna pigments in PSII and is transferred to the reaction centre, comprised of a photoactive Chl *a* molecule which, through receiving the transported energy, becomes excited. To quench this excitation three processes can occur; 1) photochemical redox reactions, through which electrons are transferred through a series of electron acceptors and donors (electron transport chain) until they are eventually utilized in ATP and NADPH formation. 2) Energy dissipation through non-photochemical quenching processes *i.e.* heat dissipation (NPQ) and 3) re-emitted as light – Chl *a* fluorescence. These three processes are directly linked to one another in a competitive manner, such that an increase in the efficiency in one will be relative to the decrease in the other two. Therefore, Chl *a* fluorescence measurements can indirectly yield information on both the photochemical and non-photochemical reactions occurring and thus the overall functioning of photosynthesis (Maxwell and Johnson, 2000; Schreiber *et al.*, 1986).

Regardless of how they are induced *in vivo*, the ability of phlorotannins to inhibit photo-damage has highlighted their potential application within the field of cosmeceuticals. Human skin, functioning as a physical barrier to external stressors, is extremely vulnerable to UVR and consequential photo-oxidative stress (Heo *et al.*, 2009; Pallela *et al.*, 2010). Cha *et al.* (2012), upon applying the photo-protective function of phlorotannins to zebrafish embryos, discovered that not only did pre-treatment with isolated phlorotannin compounds (triphlorethol A and eckstolonol) significantly reduce UVB induced oxidative damage, cell death and hyperpigmentation but did so while imposing no toxic effects on the host. Jang *et al.* (2012) investigated the effect of eckstolonol, isolated from *Ecklonia cava,* on human keratinocytes, verifying its potent antioxidant defence against UVB induced ROS. These results highlight the potential of phlorotannin

extracts within cosmeceutical products, as photo-protective, anti-aging and even skin-whitening compounds (Heo *et al.*, 2009).

Before their inclusion in such commercial products can be realised, clarification is needed on the specific effects of irradiance on their variability, particularly information regarding their structural properties, an aspect that has not yet been investigated. Such information would function as a source of novel knowledge furthering our understanding on their natural variability that could be exploited to develop uniform biologically active ingredients for commercial products. This chapter focused on elucidating the effects of irradiance, in terms of intensity and spectral diversity, on inducing variations in the molecular weight and degree of isomerisation, as well as the subsequent *in vitro* antioxidant activity, in the <3.5 kDa phlorotannin-enriched fractions of *F. vesiculosus*. This species was selected due to its potential as natural resource for commercial extraction and application of phlorotannins, as observed in Chapters 2 and 3. *F. vesiculosus* samples were exposed to nine light regimes for 7 days. The physiological role of phlorotannins in response to irradiance was assessed by correlating the photosynthetic activity, as determined by Chl *a* fluorescence measurements, with the variations observed in the LMW phlorotannin profiles at different time points, specifically, 3 and 7 days, under laboratory controlled conditions.

## 4.2. Materials and Methods

In order to meet the aims of this study, as outlined above, *F. vesiculosus* was subjected to nine various irradiance conditions, differing in both their both their light intensity and spectral quality, for a total of 7 days. Both the photosynthetic performance, as determined by Chl *a* fluorescence measurements, and corresponding LMW phlorotannin profiles were assessed for any variations that may have been induced by the surrounding light regime.

### 4.2.1. Reagents and chemicals

1, 3, 5-trihydroxybenzene (phloroglucinol), 2, 2-diphenyl-1-picryhydrazyl (DPPH), 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox®), formic acid (MS grade) and 0.22 µm polytetrafluorethylene (PTFE) filters, Iron (III) chloride hexahydrate, sodium carbonate ($Na_2CO_3$), Folin- Ciocalteu (2N), were all sourced from Sigma-Aldrich Chemical Ltd. (Co. Wicklow, Ireland). HPLC grade methanol, ethyl acetate, acetonitrile, water, and BioDesign Dialysis Tubing™ with 3.5 kDa cut-off were obtained from Fischer Scientific Ltd. (Loughborough, Leicestershire, UK).

### 4.2.2. Collection and preparation of algal material

Approximately 15-20 individual plants (1 plant = all fronds growing from the one holdfast) of *Fucus vesiculosus* (L.) were collected from Flaggy Shore, Finavarra, Co. Clare (53°09'11.7"N 9°07'07.0"W) on the 6$^{th}$ of November 2016 and were brought back to the Algal Bioscience lab in NUI Galway where they were cleaned (manually) of any epiphytes and external impurities (e.g. sediment) present. They were stored at 12°C (mimicking natural temperature of site at sampling date), under 20 µmol photons $m^{-2}$ $s^{-1}$ (cool white fluorescent tubes, General Electric Company, Fairfield, USA) in filtered natural seawater (salinity of 35), obtained from the Ryan Institute Research Centre at Carna, Co. Galway, fitted with aeration apparatus. Samples were kept under these specific conditions for 24 h prior to the experiment to acclimate to pre-experimental laboratory conditions.

### 4.2.3. Culture conditions

After 24 h lab acclimation, 100 g fresh weight (FW) of seaweed, consisting of between 15-20 randomLy selected thalli (belonging to different individuals) were frozen. This sample would serve as a control sample with which all lab-controlled treatments would be compared to. Thereafter, 50 g FW, comprised of 8-10 individual thalli, was placed in an open-top 2.5 L plastic bowl filled with filtered natural seawater and placed under one of nine various light conditions; complete darkness, UVA, UVAB, low-PAR (LP), low-PAR+UVA, low-PAR+UVAB, high-PAR (HP), high-PAR+UVA and high-PAR+UVAB, at a constant temperature of 12°C (natural temperature in sampling site). Under high-PAR conditions, due to the high number of light bulbs required to achieve this light intensity, fans were fitted to blow in cool air and remove the build-up of heat. For each treatment, a total of 8 replicates (bowls) were used (n=8).

UVA and UVB were provided using UVA-340 lamps (Q-Lab Corporation, Cleveland, USA). To specifically exclude UVB, Folanform (Folex GmbH, Dreich, Germany) cut-off filters were placed between the UV lamps and the algae, with only PAR and UVA being transmitted. The spectral distribution of the UVR and PAR treatments are outlined in Fig. 4.3. The total energy emitted by each light environment as well as the contribution of each spectral band to that total energy, in terms of $Wm^{-2}$, as recorded by a TriOS hyper-spectral irradiance sensor (RAMSES-ACC-2 UV/VIS), are shown in Table 4.1 and Fig. 4.4. Samples were exposed to the different light regimes continuously for 7 days under a 12 h: 12 h light: dark cycle. The media was refreshed every second day.

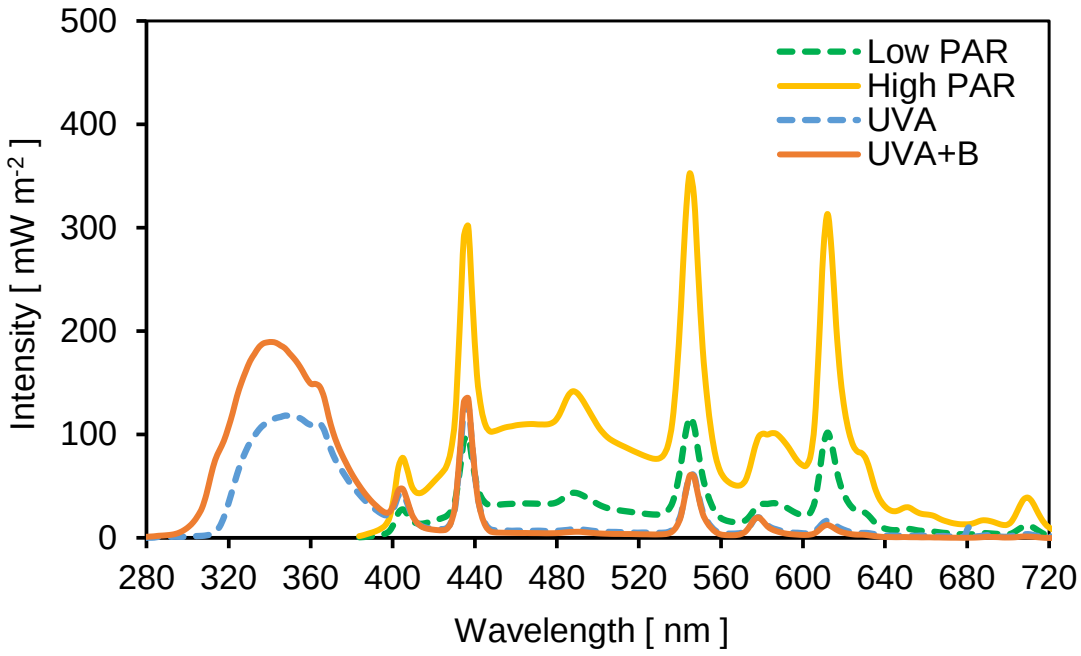



**Fig. 4.3**. The various light spectra, represented as milliWatt per metre squared ($mWm^{-2}$) to which the algae were exposed to, either singly or in combination. PAR levels were achieved using different amounts of cool white fluorescent tubes (General Electric Company, Fairfield, USA) while UVA+B were provided by UVA-340 lamps (Q-Lab Corporation, Cleveland, USA). To exclude UVB, Folanform (Folex GmbH, Dreich, Germany) cut-off filters were used.

The UVA: UVB ratio was similar, at ~ 2.5: 1, between treatments (Table 4.1; Fig. 4.4). However, the PAR: UVR ratio varied as did the contribution of each spectral band to the total energy output (Fig. 4.4). Although these intensity levels and spectral ratios may not have been representative of those found in the field, most likely being much lower than those experienced in the field, they allowed for the physiological effects of each spectral bands to be assessed.

**Table. 4.1.** Energy levels ($mWm^{-2}$) and spectral composition (UVR and PAR) of each light treatment applied to *F. vesiculosus* samples for a total for 7 days.

| | UVA | UVAB | LP | LP+UVA | LP+UVAB | HP | HP+UVA | HP+UVAB |
|---|---|---|---|---|---|---|---|---|
| **UVR** | 5.3 | 7.1 | - | 5.3 | 7.1 | - | 5.2 | 7.1 |
| **PAR** | - | - | 7.2 | 7.2 | 7.2 | 27.9 | 27.9 | 27.9 |
| **Total** | 5.3 | 7.1 | 7.2 | 12.4 | 14.2 | 27.9 | 33.2 | 35.0 |

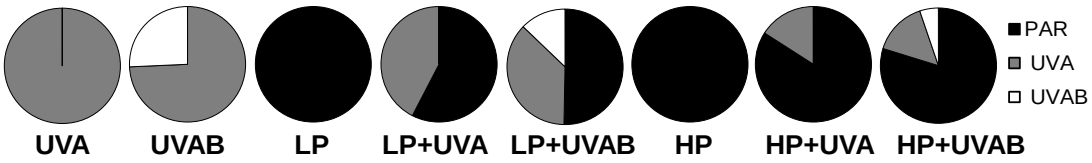



**Fig. 4.4.** The percentage contribution of each spectral component to the total energy of each light treatment applied to *F. vesiculosus* samples were maintained for a total of 7 days.

### 4.2.4. Chlorophyll *a* fluorescence measurements

*In vivo* chlorophyll *a* fluorescence measurements were carried out on days 3 and 7 of the experiment using a diving pulse-amplitude modulated fluorometer (Diving-PAM, Heinz Walz GmbH, Effeltrich, Germany). The method of Nitschke *et al.* (2012) was followed, utilizing the measuring principle of Schreiber *et al.* (1986). Prior to measurements, all samples were dark acclimated for 20 min in a glass beaker containing the seawater in which they were kept, at 12°C (experimental temperature) to allow PSII reaction centres to open. Here, the ground fluorescence state ($F_0$) was determined using a 1.6 kHz pulsed far red light (~ 0.2 µmol photons $m^{-2}$ $s^{-1}$; 650 nm). The maximal Chl *a* after dark acclimation ($F_m$) and the maximal Chl *a* fluorescence after light acclimation ($F_{m'}$) were determined with a saturating white light pulse (20 kHz; 9200 µmol photons $m^{-2}$ $s^{-1}$). The algal samples were exposed to 11 sequentially increasing photon flux densities (PFDs) of actinic light within the range of 0-1150 µmol photons $m^{-2}$ $s^{-1}$, provided by the internal halogen lamp of the PAM-2000, for 3 min each. The PFD levels received by the algae were determined using a calibrated Li-Cor LI-1400 (Li-Cor Inc., Lincoln, USA) data logger equipped with a planar quantum sensor prior to measurements. Measurements were taken triplicates for each treatment (n=3).

After each light exposure, a saturating pulse was given to determine $F_{m'}$ and $\Delta F/F_{m'}$, the effective PSII quantum efficiency. Thereafter, a 5s far-red pulse (~ 1 µmol photons $m^{-2}$ $s^{-1}$; 735 nm) was given to re-oxidise the electron transport chain. The relative electron transport rate of PSII (rETR) was calculated according to Genty *et al.* (1989). Photosynthesis-irradiance (*P/E*) curves were plotted as a function of rETR vs PFD (Fig. 4.5) and were first computed and then plotted according to the mathematical model of Walsby (1997). The various parameters involved in the determining photosynthetic performance are described in Fig. 4.5 and Table 4.2.

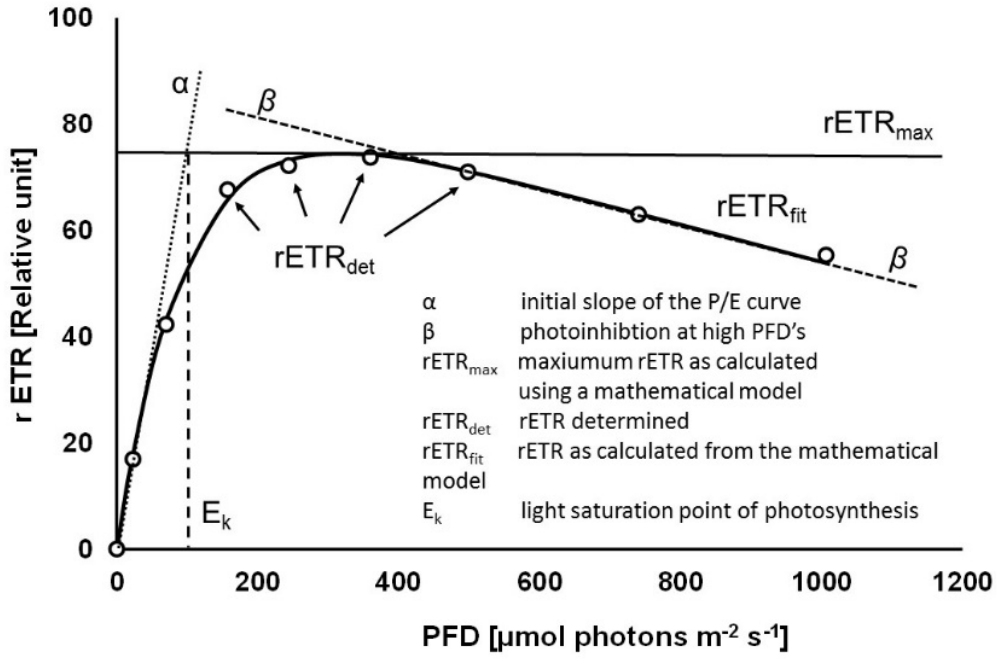



**Fig. 4.5**. Example of *P/E* curve of *F. vesiculosus* after 7 days of exposure to 200 µmol photons $m^{-2}$ $s^{-1}$, illustrating the various photosynthetic parameters determined, each of which is described further in Table 4.2.

**Table 4.2**. Overview of the parameters involved in the determination of photosynthetic performance of *F. vesiculosus* under each light treatment; the maximal efficiency of PSII ($F_v/F_m$), the maximum relative electron transport rate ($rETR_{max}$) and the light saturation point ($E_k$).

| ***Photosynthetic parameter*** | ***Equation for parameter calculation*** | ***Explanations of abbreviations*** |
|---|---|---|
| *Maximal PSII efficiency, $F_v/F_m$* **(Genty *et al., 1989)*** | $F_v/F_m = \frac{F_m - F_0}{Fm}$ | *$F_0$ - ground fluorescence value* |
| *Effective PSII quantum efficiency, $\Delta F/F_{m'}$* **(Genty *et al., 1989)*** | $\Delta F/F_{m\prime} = \frac{F_{m\prime} - F}{F_{m\prime}}$ | *$F_m$ - maximal Chl a fluorescence after dark* |
| *Relative electron transport rate of PSII, rETR* **(Genty *et al., 1989)*** | $rETR = \Delta F/F_{m'} \times PFD$ | *$F_v$ - variable Chl a fluorescence* |
| *rETR, as a function of P/E curves, computed and fitted according to the mathematical model of Walsby (1997), $rETR_{walsby}$* **(Walsby, 1997)** | $rETR_{walsby} = rETR_{max} \times \left(1 - exp^{\frac{-\alpha \times PAR}{rETR_{max}}}\right) + \beta \times PFD$ | *F - steady state of fluorescence in light* |
| *$rETR_{max}$ using the analytical maximum of the model (Walsby, 1997) when overestimated as a result of high β, according to* **(Nitschke *et al.*, 2012)** | $rETR_{max} = rETR_{max\,Walsby} + \left(rETR_{max\,Walsby} \times \left(\frac{\beta_{walsby}}{\alpha_{Walsby}}\right) \times \left(1 - Ln\left(\frac{-\beta_{Walsby}}{\alpha_{Walsby}}\right)\right)\right)$ | *$F_{m'}$ - maximal Chl a fluorescence after light* |
| *Light saturation point of P/E curves, $E_k$* **(Henley, 1993)** | $E_k = \frac{rETR_{max}}{\alpha}$ | *PFD - photon flux density of each PAR level applied to the algae* |
| | | *α - initial slope of the rETR vs PFD curve* |
| | | *β - gradient at high PFD levels which is negative if photoinhibition occurs* |
| | | *$rETR_{max}$ - maximum relative electron transport rate of PS II* |

### 4.2.5. Characterisation of low molecular weight (LMW) phlorotannin profiles

After 3 days of exposure, half of the replicate samples (n=4) under each treatment were collected and immediately frozen while the remaining samples were collected and frozen after 7 days of exposure. Thereafter, the samples were freeze-dried using a Labconco Freezone® (Kansas City, MO, USA) freeze-dryer system and ground into a fine algal powder using a coffee bean grinder (DeLonghi, America Inc., New Jersey, USA). The samples collected at each time point were pooled into one homogenous powder sample (100 g DW), for each treatment and subsequently vacuum-packed. They were then transported to Teagasc Food Research Centre, Ashtown, Dublin where they were stored in the dark at -20°C until further required.

The extraction and purification was carried out on 50 g DW algal powder to obtain LMW phlorotannin-enriched fractions, on which mass spectrometric (Q-ToF-MS and UPLC-MS/MS) analysis was performed, following the method of Tierney *et al.* (2014). Determination of the total phlorotannin content (TPC) was also carried out on the LMW phlorotannin-enirched fractions. The details of the steps involved are outlined in Chapter 2, Sections 2.2.6 to 2.2.10.

### 4.2.6. Assessment of *in vitro* antioxidant activity

To examine the effects of the different irradiance regimes on the LMW phlorotannin-enriched fractions *in vitro* antioxidant activity, two colorimetric assays were employed; the ferric reducing antioxidant power (FRAP) and the radical scavenging activity (RSA) against the radical DPPH·. The details of each assay are outlined in Chapter 2, Section 2.2.11.

### 4.2.7. Statistical analyses

The potential effect of UVR and PAR on the photosynthetic parameters obtained from Chl *a* fluorescence measurements on *F. vesiculosus* thalli at each sampling point, *i.e.* day 3 and day 7, was tested using a two-way ANOVA at a significane level of 95% ($p<0.05$). IBM® SPSS® statistical software (version 24) was used for all statistical tests. Normality was tested using the Shapiro-Wilk test for normal distribution and Levene's test for homogeneity of variances. Significant differences between treatments were determined

by a Tukey *posteriori homogeneous* (*post-hoc)* test ($p<0.05$). To determine if any significant differences arose within each treatment as a result of time, a paired *t*-test was employed ($p<0.05$) to compare day 3 and day 7.

A three-way mixed ANOVA ($p<0.05$) was conducted to analyse the effects of UVR and PAR (between-subject factors) over time (within-subject factor) on the total phlorotannin content (TPC), the ferric reducing antioxidant power (FRAP) and the radical scavenging activity (RSA) of the LMW phlorotannin-enriched fractions of *F. vesiculosus*. Normality was tested using the Shapiro-Wilk test for normal distribution and Levene's test for homogeneity of variances while sphericity was tested for using Maulchy's test of sphericity. Individual one-way ANOVA's ($p<0.05$) were conducted for each sampling point, *i.e.* after 3 and 7 days, to identify any significant diffeences between the experimental treatments, compared to the control at day 0. Tukey-tests were performed to find *posteriori homogeneous* (*post-hoc*) sub-groups that differed significantly ($p<0.05$).

## 4.3. Results

Samples of *F. vesiculosus* were subjected to nine varying light conditions, differing in both light intensity and spectral composition, for a total of 7 days. The effects of irradiance on the overall health of the algae as well as on LMW phlorotannin profiles were determined after 3 and 7 days of exposure to each treatment. Details of the statistical tests performed on the photosynthetic data are given in Supplementary Section 9.4 in Table's 9.4.1 and 9.4.2 while the statistical tests performed on TPC, FRAP and RSA are outlined in Table 9.4.3.

### 4.3.1. Chl *a* fluorescence measurements

The photosynthetic performance of *F. vesiculosus* exposed to the different UVR and PAR combinations were measured through Chl *a* measurements. Effects on photosynthetic parameters ($F_v/F_m$, $rETR_{max}$, $E_k$) were determined through photosynthesis-irradiance (*P/E*) curves, plotting rETR as a function of PFD (photon flux density; see Supplementary Section 9.4; Fig. 9.4.1).

After 3 and 7 days of exposure, the maximal efficiency of PSII ($F_v/F_m$) was significantly affected by both the UVR ($p<0.001$; $p<0.001$, respectively) and PAR ($p=0.003$; $p<0.001$, respectively) regime to which the algae were exposed, as well as their interaction ($p=0.006$; $p<0.001$, respectively; Table 4.3). At both time points, the $F_v/F_m$ of the samples appeared to decrease with increasing PAR exposure as all samples under high-PAR conditions displayed the lowest $F_v/F_m$ values ($p<0.05$; Table 4.3).

After 3 days of exposure, the maximum relative electron transport rate, $rETR_{max}$, of the samples was only affected by the PAR level ($p<0.001$) of the treatment to which the algae were subjected, with both UVR ($p=0.117$) and its interaction with PAR ($p=0.804$) imposing no significant effects. Samples placed in environments without PAR *i.e.* dark, UVA and UVAB, yielded the highest values for $rETR_{max}$ (236.16±66.5, 254.09±42.7 and 204.05±6.5 rel. units, respectively; Table 4.3) while samples placed under high PAR levels displayed the lowest values (124.73±43.11, 135.93±31.3 and 101.0±11.9 rel. units, respectively). A similar PAR-related trend was observed again after 7 days of exposure

($p$<0.05; Table. 4.3). However, now, UVR ($p$<0.001) and the interaction between UVR and PAR ($p$=0.008) also imposed significant effects on the observed $rETR_{max}$ values (Table 4.3).

The light saturation point, $E_k$, was also unaffected by UVR ($p$=0.107) and its interaction with PAR ($p$=0.543) after 3 days of exposure (Table 4.3). In a similar manner to $rETR_{max}$, the $E_k$ of the samples after 3 days of exposure, were only affected by the PAR level ($p$<0.001) to which the algae were subjected to, with $E_k$ values decreasing with increasing PAR. For example, samples under conditions without PAR displayed the highest values (Dark; 456.31±124.7, UVA; 497.83±70.5 and UVB; 362.03±44.6 μmol photons $m^{-2}$ $s^{-1}$) while those exposed to high PAR conditions displayed the lowest (HP; 235.87±68.3, HP+UVA; 264.45±61.9 and HP=UVAB; 212.06±22.1 μmol photons $m^{-2}$ $s^{-1}$, respectively). Again, this trend was also observed after 7 days of exposure to the different treatments ($p$<0.05; Table 4.3). Yet, now, UVR ($p$=0.001) and the interaction between UVR and PAR ($p$=0.013) also imposed significant effects on the observed $rETR_{max}$ values (Table 4.3).

Aside from the influence of PAR intensity, the overall spectral composition of the light conditions to which the algae were subjected also appeared to affect the photosynthetic activity. For example, the addition of UVA (without UVB), under both low- and high-PAR levels increased both $rETR_{max}$ (31, 8%, respectively) and $E_k$ levels (35, 12%, respectively) after 3 days of exposure, compared to PAR alone (Table 4.3). This enhancing effect of UVA was also seen after 7 days of exposure and was particularly pronounced under high-PAR with an increase of 29% in $rETR_{max}$ and 47% increase in $E_k$, compared to PAR alone (Table. 4.3). However, these differences were not deemed significant ($p$>0.05; Table 4.3). Despite its positive effects, by day 7, the addition of UVA under low- and high-PAR simultaneously yielded the lowest $F_v/F_m$ levels at 0.664 relative units in both treatments ($p$<0.05; Table 4.3).

After 3 and 7 days of exposure, the further addition of UVB (*i.e*. +UVAB), either without any PAR or coupled with high-PAR, led to an observed reduction in most of the parameters measured. However, low-PAR appeared to mitigate such negative effects at both time points, with low-PAR and low-PAR+UVAB exhibiting similar values for

$rETR_{max}$ after 3 days (176.18±41.8, 192.74±47.6 rel. units, respectively) and 7 days (208.78±34.0, 207.42±72.4 rel. units, respectively) of exposure ($p$=0.74; Table 4.3). This was also observed for $E_k$, with again similar values observed between the two light treatments after 3 days (312.64±83.8, 328.21±61.9 μmol photons $m^{-2}$ $s^{-1}$, respectively) and 7 days (364.0±56.9, 369.58±133.3 μmol photons $m^{-2}$ $s^{-1}$, respectively) of exposure ($p$=0.64; Table 4.3).

Even when compared to samples kept in complete darkness, exposure to UVAB alone resulted in a decrease in both $rETR_{max}$ and $E_k$ after 3 (14, 21% respectively) and 7 (47, 51%, respectively) days of exposure. Under high-PAR, the addition of UVAB led to a decrease of 19% in $rETR_{max}$ and 10% decrease in $E_k$, after 3 days, compared to PAR alone (Table 4.3). However, after 7 days of exposure, this decrease became lessened with only a 3% decrease observed for $rETR_{max}$ and 6 % decrease in $E_k$ (Table 4.3).

**Table 4.3.** Photosynthetic parameters – $F_v/F_m$ (Relative units), $rETR_{max}$ (Relative units) and $E_k$ (µmol photons $m^{-2}$ $s^{-1}$) of *F. vesiculosus* grown under nine different light treatments for 3 or 7 days. Data are represented as mean ± standard deviation (n=3). Significant effects of the UVR and PAR on the samples were tested using a two-way mixed ANOVA for each day investigated. Data are represented as mean ± standard deviation (n=3). Details of the statistical tests are outlined in Supplementary Section 9.4, Table 9.4.1 and Table 9.4.2. Superscripts distinguish significant differences between treatments at each sampling point *i.e.* after either 3 (lowercase) or 7 (uppercase) days of exposure, for each parameter measured, as given by Tukey's *post-hoc* test.

| | **Day 3** | | | **Day 7** | | |
|---|---|---|---|---|---|---|
| | **$F_v/F_m$** | **$rETR_{max}$** | **$E_k$** | **$F_v/F_m$** | **$rETR_{max}$** | **$E_k$** |
| **Dark** | $0.752 \pm 0.01^{a}$ | $236.16 \pm 66.5^{ab}$ | $456.31 \pm 124.7^{a}$ | $0.751 \pm 0.01^{A}$ | $348.22 \pm 62.2^{A}$ | $663.97 \pm 160.0^{A}$ |
| **UVA** | $0.731 \pm 0.01^{abc}$ | $254.09 \pm 42.7^{a}$ | $437.83 \pm 70.5^{a}$ | $0.744 \pm 0.01^{AB}$ | $373.36 \pm 10.3^{A}$ | $640.23 \pm 14.8^{A}$ |
| **UVAB** | $0.743 \pm 0.01^{ab}$ | $204.05 \pm 6.5^{abc}$ | $362.03 \pm 44.6^{abc}$ | $0.711 \pm 0.01^{D}$ | $187.70 \pm 13.9^{BC}$ | $327.49 \pm 24.8^{BC}$ |
| **LP** | $0.75 \pm 0.01^{ab}$ | $176.18 \pm 41.8^{abc}$ | $312.64 \pm 83.8^{abc}$ | $0.718 \pm 0.00^{CD}$ | $208.78 \pm 34.0^{BC}$ | $364.00 \pm 56.9^{BC}$ |
| **LP+UVA** | $0.731 \pm 0.01^{abc}$ | $232.39 \pm 32.2^{ab}$ | $424.4 \pm 49.5^{ab}$ | $0.664 \pm 0.01^{E}$ | $265.97 \pm 30.0^{AB}$ | $476.99 \pm 37.5^{AB}$ |
| **LP+UVAB** | $0.732 \pm 0.01^{abc}$ | $192.74 \pm 47.6^{abc}$ | $328.21 \pm 61.9^{abc}$ | $0.741 \pm 0.01^{ABC}$ | $207.42 \pm 72.4^{BC}$ | $369.58 \pm 133.3^{BC}$ |
| **HP** | $0.719 \pm 0.01^{cd}$ | $124.73 \pm 43.11^{bc}$ | $234.87 \pm 68.3^{bc}$ | $0.722 \pm 0.00^{BCD}$ | $111.22 \pm 40.4^{C}$ | $214.8 \pm 79.0^{C}$ |
| **HP+UVA** | $0.725 \pm 0.01^{bc}$ | $135.393 \pm 31.3^{bc}$ | $264.45 \pm 61.9^{abc}$ | $0.664 \pm 0.01^{E}$ | $144.00 \pm 5.0^{C}$ | $315.27 \pm 72.7^{BC}$ |
| **HP+UVAB** | $0.697 \pm 0.01^{d}$ | $101.00 \pm 11.9^{c}$ | $212.06 \pm 22.1^{c}$ | $0.705 \pm 0.01^{D}$ | $108.85 \pm 35.0^{C}$ | $227.42 \pm 48.5^{C}$ |

### 4.3.2. Total phlorotannin content (TPC) of LMW phlorotannin-enriched fractions

The control sample, representing the natural phlorotannin profile after 24 h lab acclimation prior to experimental manipulation *i.e* at day 0, displayed a high level of phlorotannins with 551.1±4.0 µg PGE $mg^{-1}$ DWE (Fig. 4.6). Relative to this value, TPC decreased in all experimental treatments after both 3 and 7 days of exposure ($p$<0.001; Fig. 4.6). Rather than being influenced by the total energy level emitted, the phlorotannin content was observed to vary depending on the spectral composition of the light environment under which the algae were grown (Fig. 4.7).

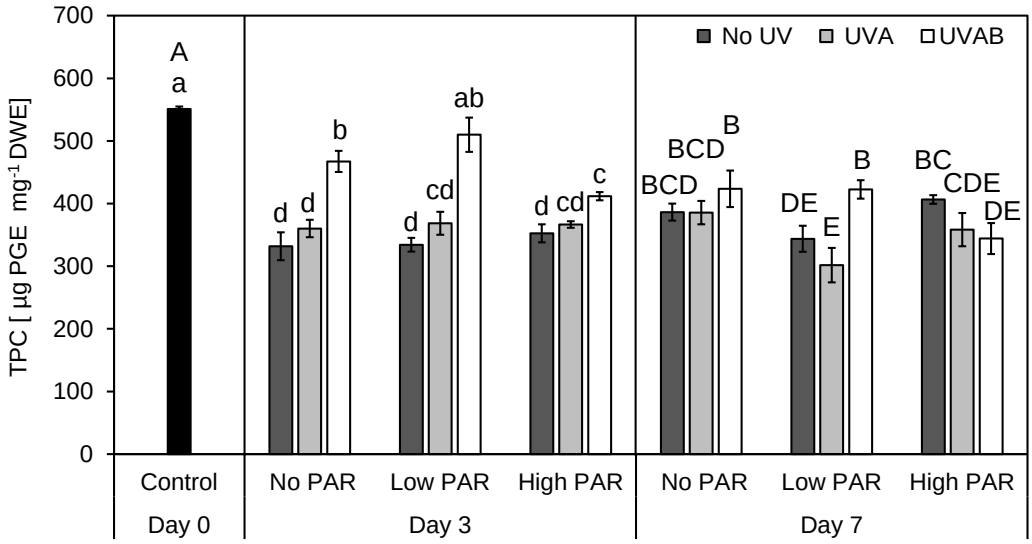



**Fig. 4.6.** The total phlorotannin content of the LMW phlorotannin fractions extracted from *F. vesiculosus* exposed to different PAR (No PAR, low PAR and high PAR) and UVR (No UV, UVA and UVAB) combinations after 3 and 7 days, compared to the control. Samples denoted by "No PAR" in combination with "No UV" correspond to those kept in complete darkness. The control is representative of a collection of samples that were frozen after 24 h lab acclimation prior to experimental manipulation. Data are represented as mean ± standard deviation (n=3) expressed as microgram phloroglucinol equivalents per milligram dry weight extract (µg PGE mg $DWE^{-1}$). Significant effects of the UVR and PAR levels to which the algae were exposed were tested using a three-way mixed ANOVA ($p$<0.05), details of which are outlined in Supplementary Section 9.4 in Table 9.4.3. Superscripts distinguish significant differences between treatments after either 3 (lowercase) or 7 (uppercase) days of exposure, compared to the control sample at day 0, using Tukey's *post-hoc* test ($p$<0.05).

Specifically, UVR ($p$<0.001) was observed to impose a stronger effect on the observed TPC levels than PAR ($p$=0.046) with increasing UVR exposure associated with increasing phlorotannins levels ($R^2$=0.17; Fig. 4.6; Fig. 4.7). UVB, in particular, was associated with the highest phlorotannin levels detected across all PAR levels ($p$<0.05;

Fig. 4.6) displaying the strongest correlation with phlorotannin content, compared to all other spectral components ($R^2$=0.46; Fig. 4.7).

After 3 days, the presence of UVB under low-PAR (LP+UVAB) yielded the highest phlorotannin content out of all the experimental treatments (510±27.4 μg PGE $mg^{-1}$ DWE), displaying a level similar to that of the control at day 0 ($p$>0.05; Fig. 4.6). This was followed by samples exposed to no PAR *i.e.* UVAB alone (467.3±16.8 μg PGE $mg^{-1}$ DWE; 84.8% of the control), and then high-PAR (HP+UVAB) with a level of 411.8±6.4 μg PGE $mg^{-1}$ DWE (74.6% of the control; Fig. 4.6). However, while these levels decreased with time ($p$<0.001; Fig. 4.6), samples grown under LP+UVAB maintained the highest levels observed after 7 days along with those grown under UVAB (422.5±14.7, 423.6±29.1 μg PGE $mg^{-1}$ DWE, respectively; Fig. 4.6).

The same effect was not recorded for samples exposed to UVA (without UVB), where a relatively stable level of phlorotannins was observed, irrespective of PAR intensity and time (301.7 -385.6 μg PGE $mg^{-1}$ DWE; $p$>0.05; Fig. 4.6). It was only after 7 days of exposure that samples under UVA, coupled with low PAR, displayed a significant decrease ($p$<0.05; Fig. 4.6).

Conditions void of UVR, *i.e.* PAR alone, displayed the lowest phlorotannin levels after 3 days of exposure ($p$<0.05; 331.9-352.6 μg PGE $mg^{-1}$ DWE; Fig. 4.6), with no significant differences observed between the different PAR intensity levels. However, it was only in samples exposed to PAR alone that an increase in phlorotannin content over time was observed ($p$<0.05; Fig. 4.6). This trend was detected even in samples left in complete darkness. Furthermore, PAR intensity appeared to contribute to the effects of UVAB, particularly in high-PAR exposure, which resulted in a lowered phlorotannin content compared to low- and no-PAR exposure after a 3-day exposure. High-PAR conditions also resulted in the largest decrease seen under UVAB exposure with time ($p$<0.05; Fig. 4.6).

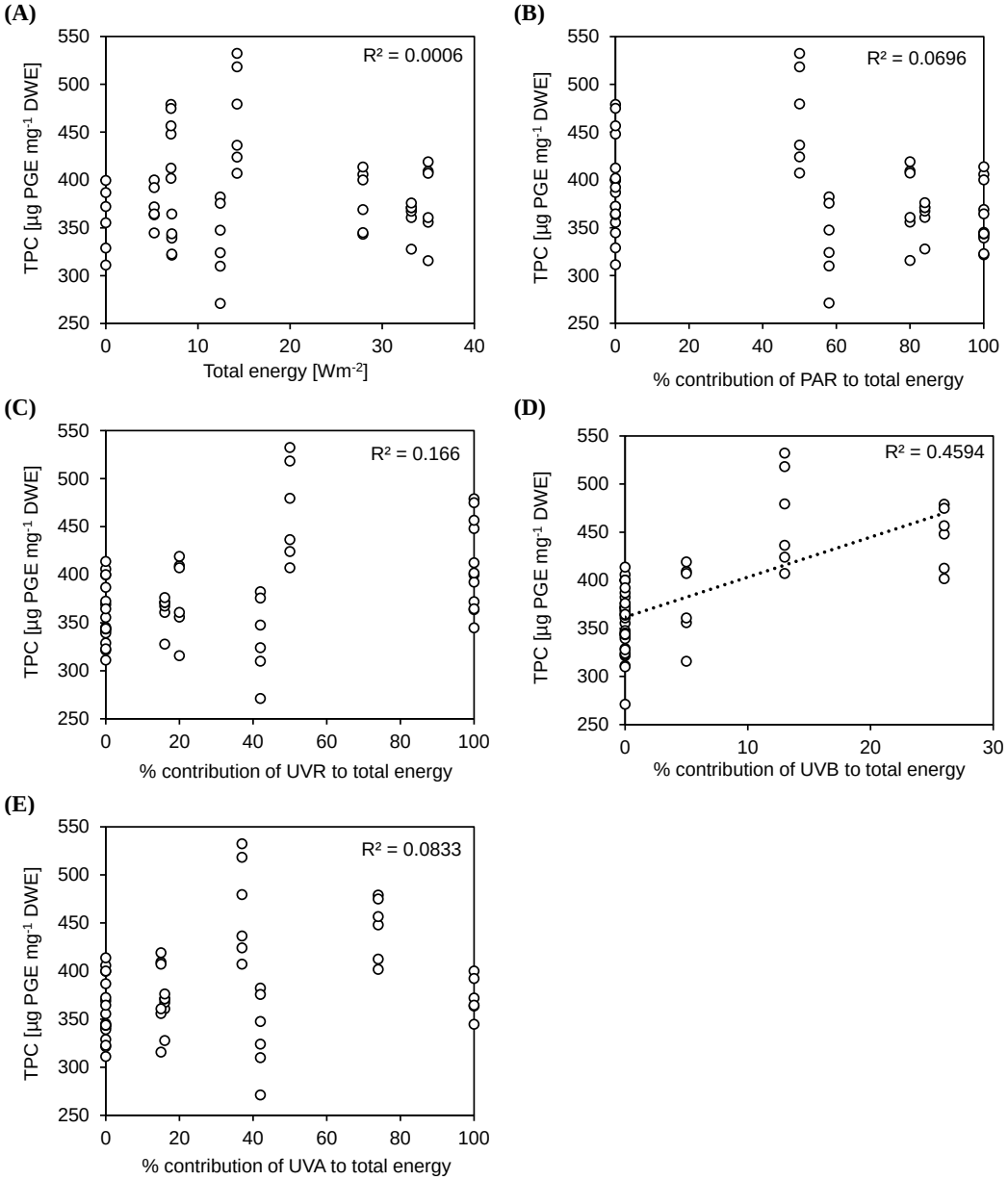



**Fig. 4.7.** Linear regression between the total phlorotannin content observed in the LMW phlorotannin-enriched fractions of *F. vesiculosus* under each light treatment and (A) the total energy ($Wm^{-2}$), (B) the percentage contributed by PAR to the total energy, (C) the percentage contributed by UVR, (D) the percentage contributed by UVA and (E) the percentage contributed by UVB. Linear regression coefficient, $R^2$, is presented.

### 4.3.3. LMW phlorotannin profile characterisation

Within the control sample, the predominant phlorotannins observed were those between 4 and 8 PGUs, corresponding to 498 and 994 Da in molecular weight (Fig. 4.8; Fig. 4.9). While the presence of larger structures (up to 2238 Da, 18 PGUs) was observed, they were only present in small amounts and magnification (x16) was required to allow their identification (Fig. 4.8). Phlorotannins of *m/z* 1241 and 1986,

corresponding to 10-16 PGUs, had a collective contribution of ~2.5% to the total abundance. Phlorotannins within the range of 4-8 PGUs accounted for ~91%, with the most dominant structures being those of 5, 8 and 4 PGUs (622, 994 and 498 Da) in order of descending abundance (37, 20 and 16%, respectively; Fig.4.9).

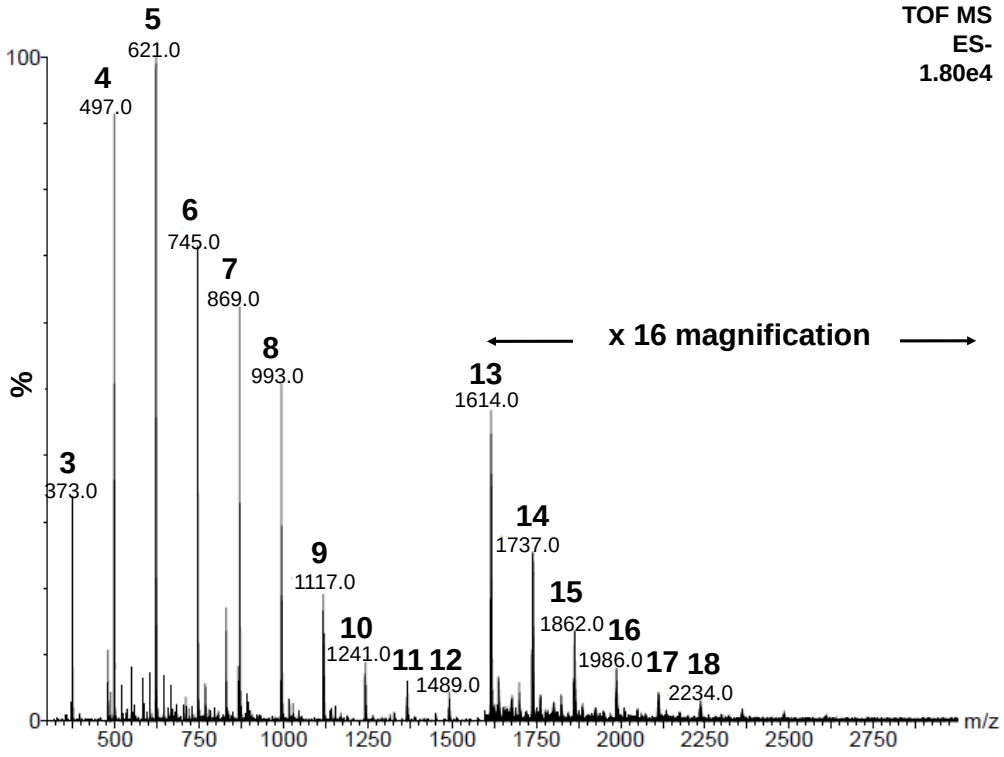



**Fig. 4.8**. Q-ToF-MS spectrum of the control sample of *F. vesiculosus*, after 24 h lab acclimation. $[M-H]^-$ ions are indicated as the numbers above peaks and the degree of polymerisation, expressed in terms of phloroglucinol units (PGUs), as the most upper bold numbers above peaks.

While variations in the relative abundance of all MWs were observed, respective to both the light condition and exposure time (Fig. 4.9), this composition remained relatively uniform with structures corresponding to 5 PGUs (*m/z* 621) being the most abundant phlorotannins detected throughout (34-41%). This was followed by compounds composed of either 4 (15-21%) or 8 (15-21%) PGUs. The contribution of polymers within the range of 4-8 PGUs also varied, ranging from 87 to 95% of the total phlorotannin abundance. The only distinguishable variation that could be seen between the treatments was the higher abundance of phlorotannin oligomers composed of 5 PGUs (622 Da) associated with exposure to UVAB, compared to PAR alone and/or with UVA. This was observed regardless of PAR intensity and exposure time, accounting for up to 41% of the total phlorotannins detected in some treatments

(Fig. 4.9). However, this was only observed as a 1-3% increase in most fractions. Under high-PAR conditions, however, after 3 days, the addition of UVAB led to an increased abundance of phlorotannins with a molecular weight of 622 Da (5 PGUs) of up to 7%, compared to high-PAR alone, and 5% compared to PAR+UVA (Fig. 4.9).

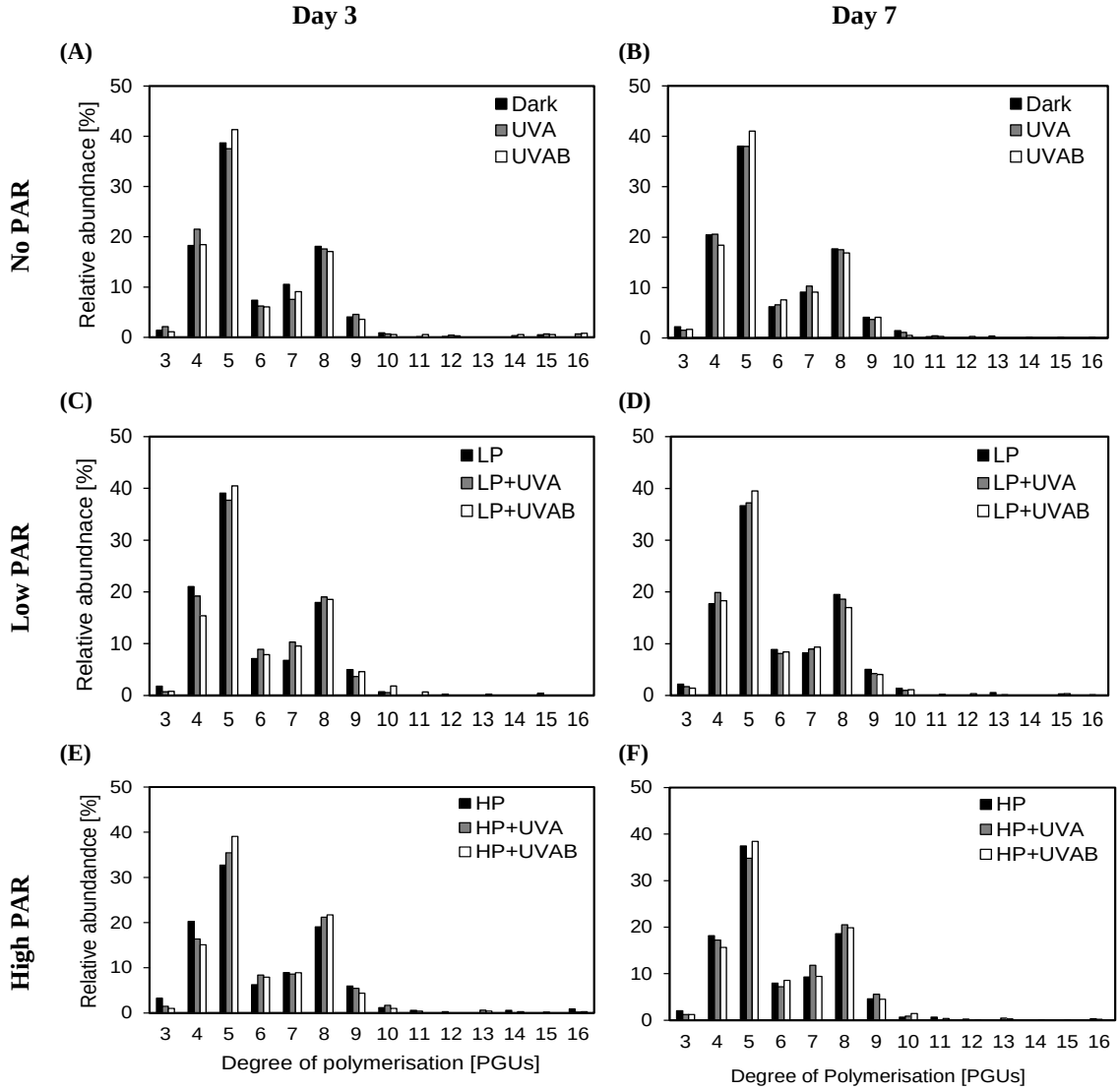



**Fig. 4.9**. The relative abundance (%) of phlorotannins between 3-16 phloroglucinol units (PGUs, corresponding to 374-1897 Da, as detected by UPLC-MS/MS, in the LMW phlorotannin-enriched fractions of *F. vesiculosus* exposed to irradiance conditions with no PAR for (A) 3 or (B) 7 days, irradiance conditions of low-PAR for (C) 3 or (D) 7 days and irradiance conditions of high-PAR for (E) 3 or (F) 7 days of exposure.

### 4.3.4. Degree of isomerisation

The control sample displayed a total isomeric count of 179 (Table 4.4). This was the highest count observed, with all experimental conditions resulting in lower/reduced levels. The majority of isomers were observed in structures corresponding to 5-9 PGUs (622-1118 Da) which accounted for 74% of the total detected. This was characteristic under all experimental light conditions (Table 4.4). Between the various experimental light environments, some short-term trends could be seen relating to the spectral composition. For example, after 3 days of exposure, the degree of isomerisation was observed to decrease with the increased UV intensity and samples exposed to UVAB, under all PAR levels, possessed the lowest isomeric levels (Table 4.4). Moreover, samples grown under PAR alone exhibited the highest isomeric levels. However, after 7 days a higher degree of variability was observed with no one particular trend evident relating to spectral composition or intensity. A decrease occurred under both PAR levels while samples kept in complete darkness exhibited an increase from day 3 (Table 4.4). Samples exposed to LP+UVAB, which after 3 days displayed the lowest number of isomers (total of 55; Table 4.4), now displayed a ~50% increase with a total of 106 (Table 4.4).

**Table 4.4.** List of phlorotannin isomers as detected by UPLC-MS/MS in MRM mode for each molecular ion transition between *m/z* 373 and 1986, in negative ion mode, corresponding to 3-6 phloroglucinol units (PGUs), in the LMW phlorotannin-enriched fractions of *F. vesiculosus* exposed to nine different light regimes for 3 or 7 days of exposure, compared to the control at day 0. The control are representative of a collection of samples that were frozen after 24 h lab acclimation prior to experimental manipulation. Where 10 or more individual isomers were detetced for an individual molecular ion transition the number is highlighted in bold.

**Day 3**

| | | No PAR | | | Low PAR | | | High PAR | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **PGU** | **Control** | **Dark** | **UVA** | **UVAB** | **PAR** | **UVA** | **UVAB** | **PAR** | **UVA** | **UVAB** |
| 3 | 3 | 1 | 1 | 1 | 2 | 3 | 1 | 1 | 2 | 1 |
| 4 | 5 | 3 | 8 | 4 | 8 | 5 | 3 | 5 | 5 | 3 |
| 5 | **20** | 6 | **13** | 8 | **24** | **15** | 5 | **17** | **12** | **11** |
| 6 | **32** | **17** | **25** | **12** | **32** | **25** | **13** | **28** | **19** | **18** |
| 7 | **32** | **18** | **15** | **18** | **28** | **27** | **11** | **21** | **21** | **19** |
| 8 | **30** | **17** | **23** | **15** | **24** | **14** | 9 | **26** | **20** | **18** |
| 9 | **26** | **12** | **13** | **10** | **14** | **17** | 8 | **23** | **16** | **14** |
| 10 | **23** | 8 | 9 | 7 | **12** | 9 | 4 | **16** | **12** | 8 |
| 11 | 6 | 4 | 5 | 2 | **11** | 5 | 1 | 5 | 7 | 6 |
| 12 | 2 | 1 | 2 | 2 | 1 | 1 | 0 | 4 | 1 | 1 |
| 13 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 2 | 0 | 1 |
| 14 | 0 | 3 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 |
| 15 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 1 | 0 |
| 16 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 1 |
| **Total** | **179** | **90** | **116** | **79** | **160** | **122** | **55** | **148** | **117** | **101** |

**Day 7**

| | | No PAR | | | Low PAR | | | High PAR | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **PGU** | **Control** | **Dark** | **UVA** | **UVAB** | **PAR** | **UVA** | **UVAB** | **PAR** | **UVA** | **UVAB** |
| 3 | 3 | 4 | 3 | 1 | 1 | 1 | 2 | 2 | 2 | 1 |
| 4 | 5 | 4 | 7 | 4 | 5 | 3 | 6 | 5 | 3 | 5 |
| 5 | **20** | **10** | **12** | **12** | **11** | **13** | **12** | **14** | **16** | **12** |
| 6 | **32** | **20** | **26** | **19** | **19** | **21** | **19** | **14** | **24** | **21** |
| 7 | **32** | **23** | **20** | **14** | **17** | **15** | **22** | **16** | **19** | **16** |
| 8 | **30** | **22** | **17** | **12** | **21** | **15** | **19** | **16** | **24** | **15** |
| 9 | **26** | **17** | **12** | 9 | **12** | **11** | **12** | **14** | **13** | **15** |
| 10 | **23** | **12** | 8 | 6 | 7 | 6 | 8 | 7 | **11** | 9 |
| 11 | 6 | 7 | 4 | 2 | 4 | 3 | 4 | 2 | 5 | 5 |
| 12 | 2 | 2 | 3 | 3 | 0 | 1 | 0 | 2 | 1 | 1 |
| 13 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 0 |
| 14 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| 15 | 0 | 1 | 1 | 0 | 1 | 2 | 0 | 0 | 0 | 0 |
| 16 | 0 | 0 | 0 | 1 | 2 | 0 | 1 | 0 | 0 | 0 |
| **Total** | **179** | **122** | **114** | **84** | **100** | **91** | **106** | **93** | **120** | **100** |

### 4.3.5. *In vitro* antioxidant activity

#### 4.3.5.1. Ferric reducing antioxidant power (FRAP)

The control sample, representing the natural phlorotannin profile after 24 h lab acclimation prior to experimental manipulation *i.e.* day 0, displayed a high level of activity in regard to ferric reduction with an observed FRAP level of 654.9±7.2 μg TE $mg^{-1}$ DWE (Fig. 4.10). Relative to this value, exposure to all light treatments resulted in decreased activity ($p<0.05$; Fig. 4.10) after both 3 and 7 days of exposure. Both the UVR ($p<0.001$) and PAR ($p=0.006$) regime of the light treatments as well as the duration of exposure ($p<0.001$) and the interaction of the three factors ($p<0.001$), all significantly affected the FRAP observed between treatments.

After 3 days of exposure, samples grown under high-PAR conditions displayed the lowest activity of which those grown under (high-) PAR alone had the overall lowest (307.1±3.5 μg TE $mg^{-1}$ DWE: $p<0.05$; Fig. 4.10). By contrast, after 7 days of exposure to the various light environments, the activity of most samples grown under high-PAR had increased, and now samples exposed to all three PAR levels displayed a similar level of activity, with little variation observed between treatments, regardless of UVR exposure. However, samples exposed to HP+UVA had significantly lower activity than any other treatments, decreasing (by 11%) from that observed after 3 days of exposure ($p<0.05$; Fig. 4.10).

#### 4.3.5.2. Radical scavenging activity (RSA)

The radical scavenging activity (RSA) of the LMW phlorotannin-enriched fractions of *F. vesiculosus*, unlike TPC and FRAP, was not significantly affected by the UVR (p=0.989), PAR regime ($p=0.065$) or their interaction ($p=0.212$; Fig. 4.10). Rather the only impacting factor on the observed $IC_{50}$ values was time ($p<0.001$) and its interaction with UVR alone ($p<0.001$) and in combination with PAR ($p<0.001$). The $IC_{50}$ value of the control sample, at day 0, was detected as 33.6±3.7 μg $mL^{-1}$ (Fig.4.10). After 3 days of exposure, similar values were observed between samples exposed to UVR (both UVA and UVAB), regardless of the associated PAR level of the treatment. However, under PAR-only environments, a significant decrease in activity was observed, with samples exposed to high-PAR displaying the lowest activity detected overall (53.7±6.7 μg $mL^{-1}$; $p<0.05$; Fig. 4.10).

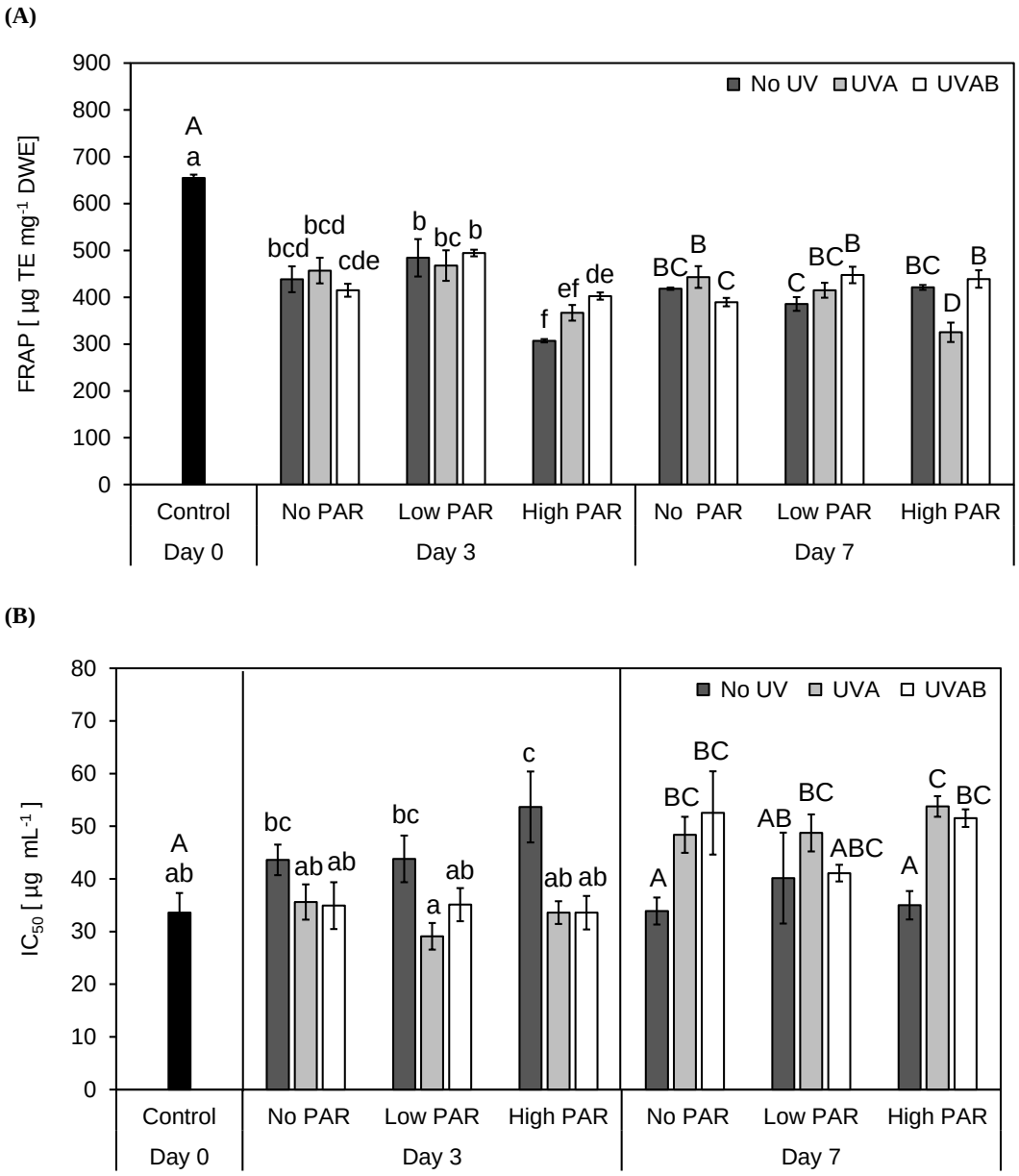



**Fig. 4.10.** (A) FRAP and (B) RSA ($IC_{50}$) levels detected for LMW phlorotannin fractions from *F. vesiculosus* exposed to different PAR (No PAR, low PAR and high PAR) and UVR (No UV, UVA and UVAB) combinations after 3 and 7 days, compared to the control. Samples denoted by "No PAR" in combination with "No UV" correspond to those kept in complete darkness. The control is representative of a collection of samples that were frozen after 24 h lab acclimation prior to experimental manipulation. Data are represented as mean ± standard deviation (n=3). FRAP values are expressed as µg TE $mg^{-1}$ DWE while $IC_{50}$ value are expressed as microgram per millilitre (µg $mL^{-1}$). Significant effects of the UVR and PAR levels to which the algae were exposed were tested using a three-way mixed ANOVA ($p$<0.05), details of which are outlined in Supplementary Section 9.4, Table 9.4.3. Superscripts distinguish significant differences between treatments after either 3 (lowercase) or 7 (uppercase) days of exposure, compared to the control sample at day 0, using Tukey's *post-hoc* test ($p$<0.05).

After 7 days, however, this trend was reversed ($p$<0.05; Fig. 4.10), as now all samples grown without UVR displayed an increase in RSA ($p$<0.05; Fig. 4.10) from day 3,

with all samples subjected to PAR only now exhibiting $IC_{50}$ values similar to one another, regardless of PAR intensity, as well as the control ($p<0.05$; Fig 4.10). Furthermore, the majority of samples grown under UVR, both UVA and UVAB, displayed significant reductions in RSA levels ($p<0.05$; Fig. 4.11). This was not observed, however, under LP+UVAB wherein the RSA remained similar to that detected after 3 days of exposure (Fig. 4.10).

## 4.4. Discussion

Due to their shore position, intertidal seaweeds, such *as F. vesiculosus*, require and possess several physiological mechanisms that enable them to tolerate extended periods of emersion and intense solar exposure (Helmuth *et al.,* 2006). The ability of higher-positioned species to withstand such harsh conditions has previously been partially accredited to a higher abundance of intracellular phlorotannins (Schoenwaelder *et al.,* 2003). Phlorotannins, suggested to be photo-protective compounds, are reportedly inducible in environments promoting photo-stress, particularly under elevated and/or prolonged exposure to UVR (Cruces and Huovinen, 2012; Pavia *et al.*, 1997; Schoenwaelder, 2002; Schoenwaelder *et al.,* 2003). Yet, to date, despite its relevance, no information exists regarding the associated effects of irradiance on the chemical properties of these polyphenols, such as structural composition and isomerisation. Therefore, this research focused on elucidating upon the impact of both PAR and UVR, both alone and in combination with one another, on LMW phlorotannin metabolic profiles. Additionally, Chl *a* fluorescence measurements and antioxidant activity assessment were carried out to determine the effects of different light regimes on the *in vivo* physiological response of the algae as well as the effects on the fractions *in vitro* activity.

The photosynthetic performance, as measured via Chl *a* fluorescence, of *F. vesiculosus* was, overall, more affected by the PAR level to which the algae were subjected to, rather than the presence of UVR. High-PAR conditions imposed the highest degree of photo-stress, with decreases observed in $F_v/F_m$, $rETR_{max}$ and $E_k$ after both 3 and 7 days of exposure. While the adverse effects of high PAR over UVR have been reported previously this has been mainly in relation to the more light sensitive polar macroalgae (Bischof *et al.*, 1999; Dring *et al.*, 2001) with fewer studies focusing on its effect on intertidal species. In fact, UVR exposure has been known to impose positive effects on photosynthesis.

UVA exposure has been reported to be beneficial, providing additional energy for photosynthesis and increasing rates of $CO_2$ fixation (Gao *et al.* 2007; Xu and Gao, 2010a). A similar effect was observed here, with all samples exposed to UVA displaying enhanced $rETR_{max}$ and $E_k$, after both 3 and 7 days of exposure. Even UVB has also been suggested to possess advantageous effects, aiding in the recovery of

dynamic photo-inhibition by functioning as a signal in photo-repair processes, potentially via stimulating the synthesis of photo-protective compounds (Bischof *et al.*, 1999; Cruces and Huovinen, 2013; Flores-Moya *et al.*, 1999; Xu and Gao, 2010b). The elevated degree of photo-stress imposed by high PAR may have been caused by damage to key components involved in their metabolic pathway, the specific mechanisms of which are still poorly understood. This reduced production, coupled with an increase in ROS formation, is likely to have caused the reduced levels of phlorotannins observed, stemming from an imbalance between the rate of their synthesis and subsequent oxidation. The enhanced carbon fixation observed under UVA may have counteracted this, promoting a state of equilibrium between pro- and anti-oxidants, yielding the relatively stable phlorotannin levels recorded.

The results obtained here agree with previous studies regarding an increase in phlorotannin levels appearing to be inducible by exposure to UVR, particularly UVB. After 3 days of exposure, regardless of PAR intensity, the presence of UVB resulted in the highest (experimentally-induced) level of phlorotannins. However, this induction may have been more rapid than observed in the present experiment. For example, increased phenolic accumulation has been reported after just 2-3 h of exposure UVR in kelp species such as *Lessonia nigrescens*, *Macrocystis pyrifera* and *Durvillaea antarctica* (Cruces and Huovinen, 2012; 2013). Abdala-Díaz *et al.* (2006) observed rapid fluctuations in phenolic levels in *Cystoseira tamariscifolia* following diurnal variations in solar irradiance. A similar trend was observed by Connan *et al.* (2007) when measuring the effect of tidal cycles on the phenol content of the intertidal species, *A. nodosum* from Brittany.

Exposure to low doses of UVB has been associated with the accumulation of several secondary metabolites within terrestrial plants. Suggested to function as an informational signal, UVB reportedly stimulates the gene expression of compounds involved in UV protection and repair (Jenkins and Brown, 2001). For example, low levels of UV have been seen to stimulate the phenylpropanoid pathway, resulting in the accumulation of flavonoids, a group of phenolic compounds ubiquitous in higher plants (Winkel-Shirley, 2001). These compounds, located in upper epidermal cell layer, protect intracellular components by specifically absorbing at peaks of 290-320 nm (UV region). The regulation of flavonoids in plants involves the UVB-induced (as well as other environmental factors) expression and activation of chalcone synthase

(CHS), a type III polyketide synthase (PKS) which is involved in catalysing the early reactions of their synthesis (Jansen *et al.*, 1998). Recently, Meslet-Cladière *et al.* (2013) revealed the significance of a type III PKS in the synthesis of phloroglucinol, the monomeric unit of all phlorotannins, from malonyl co-A within the acetate malonate pathway in *Ectocarpus siliculosus*. Due to the similarity in both the location, function and absorption range (phloroglucinol; 274 nm) of these two phenolic groups, it seems plausible that UVB may trigger the production of phlorotannins, in a similar manner to terrestrial phenolics.

The stimulation of phenolic compound production is likely to be responsible for the observed maintenance in photosynthetic activity under UVB over time here. *F. vesiculous* samples exposed to UVB displayed a constant and unchanged rate of photosynthetic activity after both 3 and 7 days. An increase in phlorotannin levels would have aided in a heightened absorption of UVB, preventing any UV-induced damage acting upon important (and vulnerable) photosynthetic machinery. In doing so, they would have become oxidised resulting in a decrease in their detected levels, as seen after 7 days of exposure. These results therefore support the theory that phlorotannins function as UV defence compounds, but not as general photoprotective compounds, as high PAR levels resulted in their decline most likely through, or in combination with, reduced photosynthesis.

With respect to the effects of phlorotannin structures, exposure to UVB was associated with an increase in the abundance in phlorotannin oligomers composed of 5 PGUs (622 Da), compared to treatment of PAR alone, or PAR+UVA. Creis *et al.* (2015) also observed an increase in the abundance of this particular MW in semi-purified phlorotannin fractions extracted from *F. vesiculosus* after two weeks exposure to UVB, compared to the control. This increase may be a reflection on the elevated phlorotannin synthesis stimulated by UVB, as phlorotannins of this particular MW have been universally detected as the most abundant structures in this particular seaweed species, regardless of environment (as seen in Chapters 2 and 3). The naturally high occurrence of structures with such a low degree of polymerisation would allow a higher number of free hydroxyl units (OH) that in larger polymers, would be involved in monomer-monomer linkages. Therefore, a further increase in the abundance of such structures, however slight, would only serve to increase the

availability of OH constituents for redox reactions aiding radical scavenging (Dai and Mumper, 2010; Singleton *et al.*, 1999).

The structural arrangement of the compounds and variations therein were also seen to be affected by the different spectral bands of irradiance, however the manner in which this occurred over time is not clear. After 3 days, increased exposure to UVR was correlated with lowered levels of isomers while PAR alone was associated with the highest degree of isomerisation. This is considered unusual as a higher level of phlorotannins would be assumed to provide an increased opportunity for structural rearrangements to occur (Tierney *et al.*, 2014). However, after 7 days of exposure, the isomeric abundance in all light treatments were not associated with any particular trend, but, the mechanisms behind phlorotannin isomeric behaviour are still largely unknown.

UVB exposure was hypothesised to cause structural variability over time due to the high energy of this spectral band. UVB wavelengths are capable of breaking strong hydrogen bonds interconnecting the molecules of compounds that absorb strongly in this spectral range. This has been reported for the effects of UV on nucleic acid conformation during DNA damage (Rastogi *et al.*, 2010a; Wilson *et al.*, 1995; Xue *et al.*, 2005). These conformational arrangements could then lead to an increase in free hydroxyl units that could have been involved in the bridging link and make them available for electron donation against radical species. However, complete oligomeric breaks would most likely result in degradation products or an increased abundance of lower MW compounds, both of which were not observed by MS analysis here. It could also lead to the formation of new and potentially more intricate linkages through intra- and intermolecular bonding. However, an increase in isomeric abundance was only observed in samples exposed to LP+UVAB after 7 days, and no increase in the abundance of higher molecular weight phlorotannin was observed, as might be expected if inter-molecular bonds can be formed. The behaviour of isomeric generation under different irradiance conditions appears complex and suggests that the effect of both UV absorption as well as radical scavenging do not affect phlorotannin structures in a uniform and organised manner; this in turn may be due to their destructive role in the oxidation of these compounds. Therefore, further work is required to fully understand the means through which irradiance exposure manipulates

their structural diversity. The use of analytical tools, such as nuclear magnetic resonance (NMR) spectroscopy would be beneficial on elucidating upon the matter.

The degree of antioxidant activity exhibited by the different fractions was also influenced by the light treatment to which the algae were exposed, corresponding to the respective phlorotannin concentrations. However, the specific effects are likely to vary depending on the assay employed as the specific parameters affecting the redox reaction will also differ (Dai and Mumper, 2010; Singleton *et al.*, 1999). For example, the RSA of the radical DPPH· exhibited a positive correlation to the phlorotannin levels while FRAP appeared to be less variable, the latter perhaps being associated with the chemical composition of the fraction, the uniformity of which remained relatively stable throughout regardless of light regime. This is an important aspect to consider, particularly where the role of phlorotannins in commercial applications is concerned. Ideally culture conditions offering maximal phlorotannin concentrations of high activity are most desirable. In this case, a light treatment composed of LP+UVAB would be preferential.

Overall, the effect of UVB on stimulating the synthesis of highly active LMW phlorotannin oligomers may explain the dominance of *F. vesiculosus* in the intertidal zone. The high plasticity allowed by these polyphenols offers a higher degree of tolerance of this species to fluctuating UV exposure through their role in protecting photosynthetic apparatus from UV-related damage. However, their biosynthesis appears vulnerable to high PAR levels, the specific mechanisms of which require further investigation. Moreover, the rapid plasticity observed in their concentrations may offer an explanation as to why seasonal sampling revealed little information regarding their natural variability. Phlorotannins display a high turnover rate, with both synthesis and oxidation occurring over a matter of days, possibly even hours. This is further complicated as different spectral components, which in the field are often experienced at a higher magnitude than in this experiment, act upon the phlorotannin profiles in a different manner to one another. For example, at any particular time, both PAR and UVR can act upon algae. The opposing effects of these factors may then lead to a neutral effect being observed in seasonal sampling. This suggests that once-off sampling may be obsolete in deciphering the factors affecting their profiles. Short-term time-series could potentially offer a solution to this. Furthermore, the decline in phlorotannin concentrations observed from the control

(samples without light treatment) may also suggest that other factors, which are not captured within a laboratory setting, may be involved in determining phlorotannin concentrations. This idea is even further supported by the fact that samples kept in complete darkness displayed an increase in phlorotannin concentrations, isomerisation and radical scavenging activity over time.

In summary, this study highlights the ability of UVB to enhance phlorotannin production, potentially at a transcription level activating the gene expression of catalysing enzymes involved in their metabolic pathway. While, here, sampling was carried out after 3 days, potentially higher phlorotannin levels may have been observed at an earlier time, perhaps even after a few hours. However, irradiance does not appear to be the sole cause of phlorotannin variability and the results here highlight the need to investigate additional abiotic factors that have also been reported on drive their metabolism such as nutrients and temperature (Cruces and Huovinen, 2012; Koivikko *et al.*, 2008). The effects of these factors are further addressed in further chapters.

# Chapter 5

The effect of nitrogen on low molecular weight phlorotannin profiles in *F. vesiculosu*s (L.)

## 5.1. Introduction

Light availability governs rates of photosynthesis, influencing rates of ATP production and carbon fixation, two main factors affecting growth. Light can also alter the abundance of nitrogen-containing components involved in the photosynthetic process such as light-harvesting pigments and key enzymes including ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCO; Lapointe and Duke, 1984). Therefore, via both direct and indirect mechanisms, light ultimately determines the required N supply for growth (Hurd *et al.*, 2014).

Nitrogen (N), along with phosphorus (P), is deemed as the most fundamental element required for algal and plant growth. Some minerals and vitamins are also essential but are required in smaller quantities (Goldman *et al.*, 1971). Therefore, the availability and uptake of such nutrients are vital for the normal functioning of metabolic processes involved in growth and development (Hurd *et al.*, 2014).

Liebig's 'law of the minimum' states that when all other factors are optimal, the nutrient that is available in the lowest concentrations, in respect to the requirements of the plant (or alga), will limit the growth rate (Harrison and Hurd, 2001). As the required concentrations of N and P often exceed those present in the water column, they are generally regarded as the two most limiting factors in algal productivity (DeBoer and Ryther, 1977; Ryther and Dunstan, 1971; Lynby, 1990). Particularly in marine waters, N is generally, but not universally, reported to be more limiting than P, compared to freshwater systems. This is reportedly due to a lower rate of planktonic N-fixation, resulting from a reduced availability of iron and molybdenum in seawater (Howarth *et al.*, 1988; 2000).

As well as being essential for the production of key fundamental compounds such as protein and DNA, N is also involved in the formation of light-harvesting pigments (Martínez and Rico, 2002; Pereira *et al.*, 2012). Therefore, both seasonal and spatial variations in the N concentration to which seaweed are exposed, can result in alterations in the chemical composition and physiological responses (Martínez and Rico, 2002; Sampath-Wiley *et al*., 2008; Stengel and Dring, 1998). This has also been reported for phycobiliproteins and mycosporine-like amino acids (MAAs) in red algae (Aguilera *et al.*, 2002; Figueroa *et al.*, 2010; Korbee *et al.*, 2005).

The main marine forms of bioavailable N include nitrate ($NO_3^-$) and ammonium ($NH_4^+$) with the latter, for most algal species, being the preferred ion for assimilation due to a more efficient rate of uptake and lower energy requirements for transport (Phillips and Hurd, 2003; Zehr and Ward, 2002). However, in macroalgae, this preference varies between species, with some showing no preference (Hanisak, 1983). The availability of different N sources can vary both seasonally and spatially (Hurd *et al.*, 2014; Phillips and Hurd, 2003), influenced by several processes, as illustrated in Fig. 5.1; (i) physical supply, mainly of nitrate, by vertical mixing, advection and horizontal transport (Mahaffey, *et al.*, 2004; Oschlies, 2002), (ii) fixation of $N_2$ by diazotrophs (Capone *et al.*, 2005; Karl *et al.*, 2002) and (iii) the de-nitrification of organic matter (Deutsch *et al.*, 2001; Gruber and Sarimento, 1997; Gruber, 2005). In coastal areas, anthropogenic influence through land drainage, sewage and/or agricultural run-off can also provide another source of N to the water column (Howarth *et al.*, 2012; Howarth, 2008).

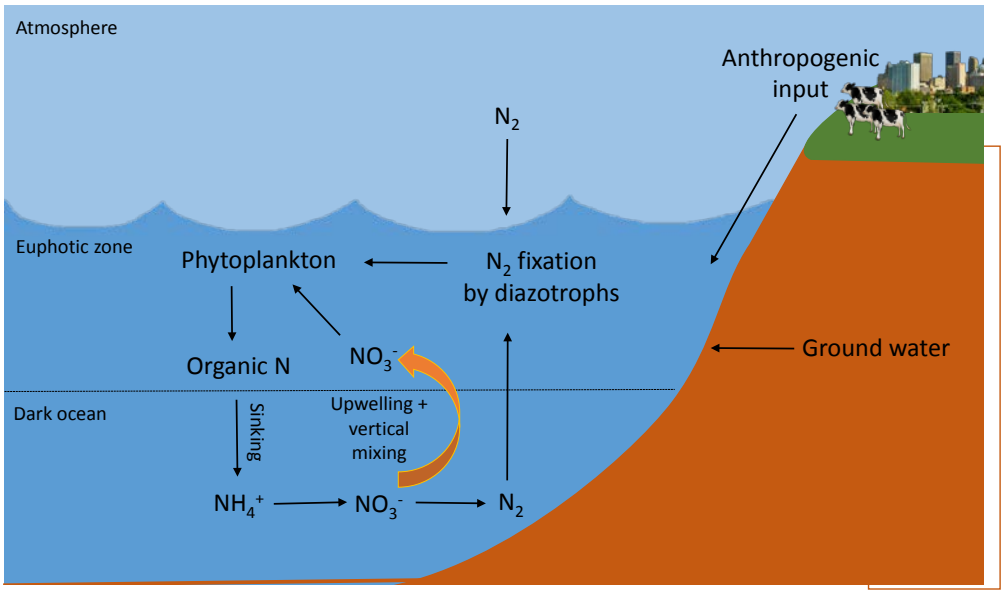



**Fig. 5.1.** A simplified illustration of the main processes within the marine nitrogen cycle, adapted from Arrigo (2005).

The uptake of N by algae, however, is not only affected by external concentrations, it can also vary depending on the both the morphology of the species (Hein *et al.*, 1995; Rosenberg, 1984) as well as its position along a vertical shore gradient (Phillips and Hurd 2004). Interaction with other abiotic factors can also mediate the rate of N uptake including water velocity, salinity, temperature and irradiance (Choi *et al.*, 2010;

Gordillo *et al.*, 2002; Harlin and Craigie, 1978; Hurd, 2000; Kaine and Norton, 1990; Kregting *et al.*, 2015). However, overall light availability has been suggested to have the most significant interaction with the uptake, storage and assimilation of N (Hurd *et al.*, 2014).

On a diurnal scale, fluctuations can occur in the uptake and assimilation of N by intertidal macroalgae, mitigated by shifts in irradiance levels. For example, Gordillo *et al.* (2002) reported that for *Fucus serratus*, grown in culture, nitrate uptake peaked during the light period and decreased toward the later part of the dark phase. Gevaert *et al.* (2007) found, for the green alga *Ulva pertusa*, maximum rates in ammonium uptake and assimilation occurred in the late morning and declined toward sunset. This was followed by a peak in cellular sucrose, suggesting a shift in energy contribution between the mechanisms involved in N assimilation and C fixation. Competition between N and C metabolism has been previously reported (e.g. Gao *et al.* 1995). Shifts between C- and N-based compounds (commonly referred to as the C: N ratio) can be observed on a seasonal scale. In general, during winter months algae exhibit low C:N ratios, due to the light-limited photosynthetic fixation of inorganic C coupled with elevated external N concentrations (Hurd *et al.*, 2014; Lapointe and Duke, 1984). This leads to increased levels of internal N and N-based pigments, as previously discussed (Martínez and Rico, 2002; Stack *et al.*, 2017; Stengel and Dring, 1998). In summer, when N resources decline, and irradiance levels elevate photosynthetic rates, C: N ratios are then increased.

Shifts in C: N ratios have been investigated in an effort to elucidate on naturally occurring variations observed in C-based defence compounds such as phlorotannins as, to date, little progress has been made in uncovering the mechanisms involved in their synthesis. Temporal variations in external N concentrations have been inversely correlated with the phlorotannin content of several brown algal species, suggesting that the effect of N availability on C: N ratios could impact phlorotannin biosynthesis. Temporal variations in the phlorotannin content in different populations of *Fucus vesiculosus* displayed inverse correlations with the external N availability and tissue N concentrations (Ilvessalo and Tuomi, 1989; Koivikko *et al.*, 2008; Yates and Peckol, 1993). A trade–off between primary (growth) and secondary (defence) metabolism was suggested to explain the variations observed, similar to those assumed by the carbon-nitrogen balance hypothesis (CNBH; Bryant *et al.*, 1983). This was further

supported by laboratory controlled studies whereby N enrichment resulted in a decreased phenolic content (Arnold *et al.*, 1995; Koivikko, *et al.*, 2005).

However, several studies exist where the authors propose alternative explanations to resource allocation for the decreased phlorotannin content observed (Arnold and Targett, 2003; Jormalainen *et al.*, 2003). For example, no effect of N on phlorotannin levels was found by Hemmi *et al.* (2005) while others have reported on the greater influence of light on inducing phlorotannin compounds than N deficiency (Pavia and Toth, 2000; Pavia *et al.*, 1997). These contradictory results, as well as a growing lack of confidence in the CNBH (Hamilton *et al.*, 2001), has led to uncertainty regarding the significance of N on phlorotannin metabolism.

Seasonal and spatial variations in seawater N concentrations have been previously reported for sites along Galway Bay (Gietl, 2016; Schmid *et al.*, 2017). Such fluctuations may, potentially, have contributed to the observed differences in phlorotannin levels observed within previous seasonal and spatial sampling (Chapter 2). However, the effects of a single environmental factor are difficult to interpret based on seasonal field studies alone. While irradiance has been considered as the primary factor influencing *in situ* phlorotannin concentrations, also exhibiting seasonal fluctuations, it appears that it may not be the only factor to impact LMW phlorotannins, as seen in Chapter 4. In an effort to clarify the underlying causes of phlorotannin variability, as well as the contribution of N toward such differences, the primary concerns of this chapter were two-fold; 1) to investigate the effect of external N concentrations on phlorotannin metabolism, keeping irradiance, along with all other factors fixed as well as 2) the interaction of N and irradiance *i.e*. the effect of C: N ratios on phlorotannins. Experiments were carried out on the intertidal fucoid, *F. vesiculosus*, due to the potential this species exhibits as a source of the commercial integration of highly bioactive phlorotannins. This species has displayed potential as an excellent model on which to study variations incurred by external parameters due to its ease of extraction and characteristic profile, which if any variations occurred would be easily noticed.

The effect of N concentrations was investigated by placing *F. vesiculosus* under a controlled and consistent irradiance and temperature regimes while the media differed in its N%. Within the seasonal minimum (0 µM) and maximum (2 µM) of seawater N concentrations previously observed for Finavarra. Co. Clare, were investigated (Gietl

2016), as well as enhanced N levels (100 μM and 1 mM). The interaction of N and irradiance was investigated through subjecting the algae to both low and high levels of photosynthetically active radiation (PAR) as well as low and high concentrations of N, simultaneously. In an effort to achieve a better understanding of how N and irradiance might affect phlorotannin profiles, Chl *a* fluorescence measurements were taken to examine the combined effect of the two factors on the algal physiology. The effects on their chemical composition, levels and *in vitro* antioxidant activity were all assessed subsequently.

## 5. 2. Materials and Methods

### 5.2.1. Reagents and chemicals

1, 3, 5-trihydroxybenzene (phloroglucinol), 2, 2-diphenyl-1-picryhydrazyl (DPPH), 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox®), formic acid (MS grade) and 0.22 µm polytetrafluorethylene (PTFE) filters, ammonium nitrate ($NH_4NO_3$), Iron (III) chloride hexahydrate, sodium carbonate ($Na_2CO_3$), Folin-Ciocalteu (2N), were all sourced from Sigma-Aldrich Chemical Ltd. (Co. Wicklow, Ireland). HPLC grade methanol, ethyl acetate, acetonitrile, water, and BioDesign Dialysis Tubing™ with 3.5 kDa cut-off were obtained from Fischer Scientific Ltd. (Loughborough, Leicestershire, UK). Coral Pro Salt (Red Sea) was obtained from Seahorse Aquariums (Galway, Ireland).

### 5.2.2. Preliminary assessment of temporal N variations on phlorotannin content

Two sites along Galway Bay, thought to differ in the nitrogen levels (Gietl, 2016) were investigated for natural variations in carbon, nitrogen and the subsequent effect of C:N ratios on the natural phlorotannin content of *Fucus vesiculosus* (L.) - Oranmore, Co. Galway (53°16'15.8"N 8°59'35.0"W; Fig. 5.2) and Finavarra, Co. Clare (53°09'11.7"N 9°07'07.0"W; Fig. 5.2). Approximately 10-20 individual thalli were collected on 3rd October 2016 from each site. All thalli were cut at the stipe, leaving approx. 15 cm. attached. Specimens were brought back to the Algal Biosciences lab at NUI Galway where they were cleaned manually of any epiphytes and other external impurities (e.g. sediment) and immediately frozen. Thereafter, the samples were freeze-dried using a Labconco Freezone® (Kansas City, MO, USA) freeze-dryer system and ground to a fine powder using a coffee bean grinder (De'Longhi, Italy) and subsequently vacuum-packed. They were then transported to Teagasc Food Research Centre, Ashtown, Dublin, where they were stored in the dark at -20°C until further required. An initial 50 g DW algal powder sample was used for the extraction and purification of LMW phlorotannin enriched fractions, the phlorotannin content of which was quantified. The method of Tierney *et al.* (2014) was employed. The details of the steps involved are outlined in Chapter 2 Section 2.2.6-2.2.10.

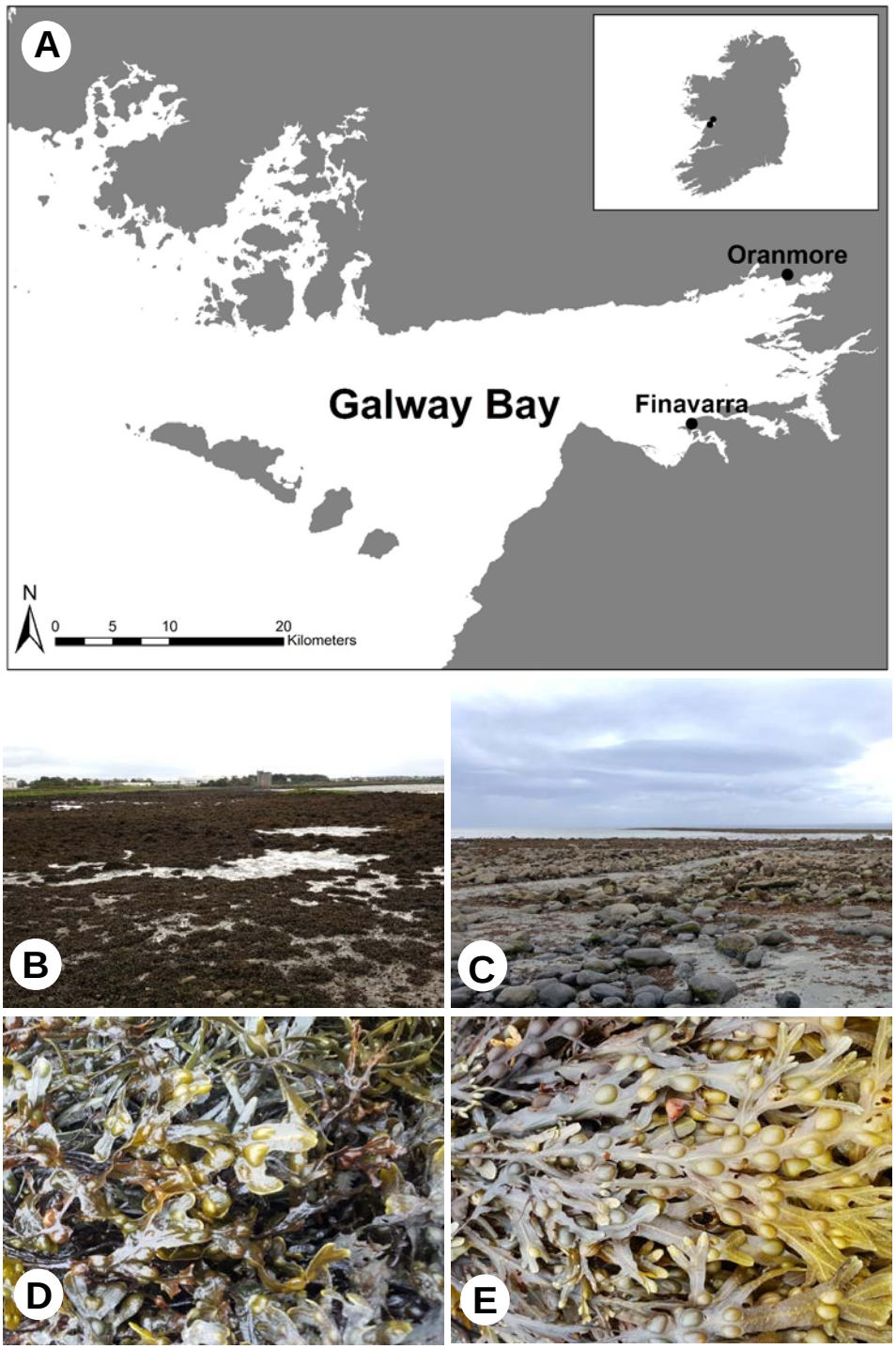



**Fig. 5.2.** (A) Map of Galway Bay depicting the two sites investigated for natural differences in C, N and phlorotannin content; (B) Oranmore, Co. Galway, and (C) Finavarra, Co. Clare, that were investigated for natural differences in the internal nitrogen levels of *F. vesiculosus* and the effect on LMW phlorotannin levels. The morphological differences observed in *F. vesiculosus* between sites is illustrated; *F. vesiculosus* collected from (D) Oranmore, Co. Galway and (E) Finavarra, Co. Clare. Map created by Tom Rossiter.

### 5.2.3. Culture conditions

For experimental treatment, only *Fucus vesiculosus* (L.) collected from Finavarra, Co. Clare were investigated (based on preliminary results shown below).

#### 5.2.3.1. Effect of media N concentration only

Approximately 10-15 individual plants (1 plant = several fronds growing from the one holdfast) were collected from Finavarra, Co. Clare, on 26$^{th}$ of October 2016. Specimens were brought back to the Algal Bioscience lab at NUI Galway where they were cleaned manually of any epiphytes and other external impurities and placed in UV-treated, filtered seawater (salinity of 35) with aeration fitted, under low light at 20 µmol photons $m^{-2}$ $s^{-1}$ (cool white fluorescent tubes, General Electric Company, Fairfield, USA), at 13-14°C (the natural seawater temperature at time of collection). Algae were kept under the above conditions for 24 h to acclimate to laboratory conditions. After 24 h, prior to any experimental manipulation, 100 g fresh weight (FW) of seaweed, consisting of between 15-20 randomly selected thalli (belonging to different individuals) were frozen with which all lab-controlled treatments would be compared to after analysis *i.e.* the control.

**Table 5.1.** Partial elemental make-up of Red Sea Coral Pro Salt (as given by manufacturer) at a desired salinity level of 35 and the recommended dosage to achieve this composition. *Does not contain nitrates or phosphates according to the manufacturer.

| Salinity | pH | Ca (mg $L^{-1}$) | Mg (mg $L^{-1}$) | K (mg $L^{-1}$) | Dosage (g $L^{-1}$) |
|---|---|---|---|---|---|
| 35.0 | 8.2-8.4 | 455-475 | 1360-1420 | 390-410 | 38.2 |

Thereafter, 50 g FW, comprised of 8-10 individual thalli, was placed in 2.5 L open-top plastic bowls filled with artificial medium composed of distilled water with 38.2 g $L^{-1}$ of Red Sea Coral Pro Salt (Table 5.1), containing no N or P sources, made up to a salinity level of 35 (the natural salinity level recorded at the time collection). Four N concentrations were investigated; 0 µM, 2 µM, 100 µM and 1 mM. 0 µM N was simply the artificial seawater medium without any nitrogen enrichment, representing the summer minimum observed from previous seasonal field measurements (Gietl, 2016). 2 µM N, representing the natural winter maximum for this site, was achieved by the addition of 10 mL of a 1 mM $NH_4NO_3$ stock solution while 100 µM concentrations

were reached by the addition of 5 mL 100 mM stock and 1 mM with 5mL of 1 M stock. The media for each treatment were refreshed every 2 days. The algae which were placed under a constant PAR intensity of 50-60 μmol photons $m^{-2}$ $s^{-1}$ with a light: dark cycle of 12 h: 12 h, with aeration fitted (Fig. 5.3). The temperature was kept constant at 13-14°C (natural temperature at sampling date). For each treatment, a total of 8 replicates (bowls) were used (n=8).

#### 5.2.3.2. Interactive effect of media N concentrations and irradiance

Approximately 10-15 individual plants were collected from Finavarra, Co. Clare, on 29th of November 2016. Collected material was cleaned and prepared in a similar manner as above, with a control samples also being frozen after 24 h lab acclimation prior to experimental manipulation. Here, 50 g FW of seaweed (comprised of 8-10 individual thalli) was placed a 2.5 L open-top plastic bowl filled with medium composed of either a lower (ambient) concentration of 2 μM N or a higher (enrichment) concentration of 100 μM and placed under two light levels; low-PAR (15-20 μmol photons $m^{-2}$ $s^{-1}$) and high-PAR (110-115 μmol photons $m^{-2}$ $s^{-1}$), making a total of four experimental conditions (Fig. 5.3); high light + high N (HL+HN), high light + low N (HL+LN), low light + high N (LL+HN) and low light + low N (LL+LN). For each treatment, a total of 8 replicates (bowls) were used (n=8).

##### 5.2.3.2.1. Chl *a* fluorescence measurements

The interactive effect of N and light, if any, on photosynthetic performance was assessed after 3 and 7 days of exposure. *In vivo* chlorophyll *a* fluorescence measurements were taken using a Diving Pulse-Amplitude Modulated fluorometer (Diving-PAM, Heinz Walz GmbH, Effeltrich, Germany) on days 3 and 7 in the middle of the light period, for each interactive treatment. The method of Nitschke *et al.* (2012) was followed, utilizing the measuring principle of Schreiber *et al.* (1986), the details of which are outlined previously in Chapter 4 Section 4.2.4.

### 5.2.4. Characterisation of low molecular weight (LMW) phlorotannin profiles

After 3 days of exposure, half of the replicate samples (n=4) under each treatment were collected and immediately frozen while the remaining samples were collected and frozen after 7 days of exposure. Thereafter, the samples were freeze-dried using a Labconco Freezone® (Kansas City, MO, USA) freeze-dryer system and ground into a fine algal powder using a coffee bean grinder (DeLonghi, America Inc., New Jersey,

USA). The samples collected at each time point were pooled into one homogenous powder sample (100 g DW), for each treatment, and subsequently vacuum-packed. They were then transported to Teagasc Food Research Centre, Ashtown, Dublin where they were stored in the dark at -20°C until further required.

The extraction and purification was carried out on 50 g DW algal powder to obtain LMW phlorotannin-enriched fractions, on which mass spectrometric (Q-ToF-MS and UPLC-MS/MS) analysis was performed, following the method of Tierney *et al.* (2014). Determination of the total phlorotannin content (TPC) was also carried out on the LMW phlorotannin-enirched fractions. The details of the steps involved are outlined in Chapter 2, Sections 2.2.6 to 2.2.10.

### 5.2.5. Assessment of *in vitro* antioxidant activity

To examine the effects of N concentration, or N concentration in combination with different irradiance levels, on the LMW phlorotannin-enriched fractions *in vitro* antioxidant activity, two colorimetric assays were employed; the ferric reducing antioxidant power (FRAP) and the radical scavenging activity (RSA) against the radical DPPH·. Each fraction was analysed in triplicate (n=3). The details of each assay are outlined in Chapter 2, Section 2.2.11.

### 5.2.6. Determination of total carbon (C) and nitrogen (N) content

The determination of total C and N was carried out on the pooled algal powder samples using an Exeter Analytical CE 440 Elemental Analyser, a Varian 55B Spectra AA Atomic Absorption Spectrometer and various Metrohm titrators. This was conducted at the microanalytical laboratory within the School of Chemistry and Chemical Biology, University College Dublin (UCD). Analysis was carried out in triplicate for each sample. Results are expressed as C or N percentage of dry weight (% DW) and represented as mean ± standard deviation (n=3).

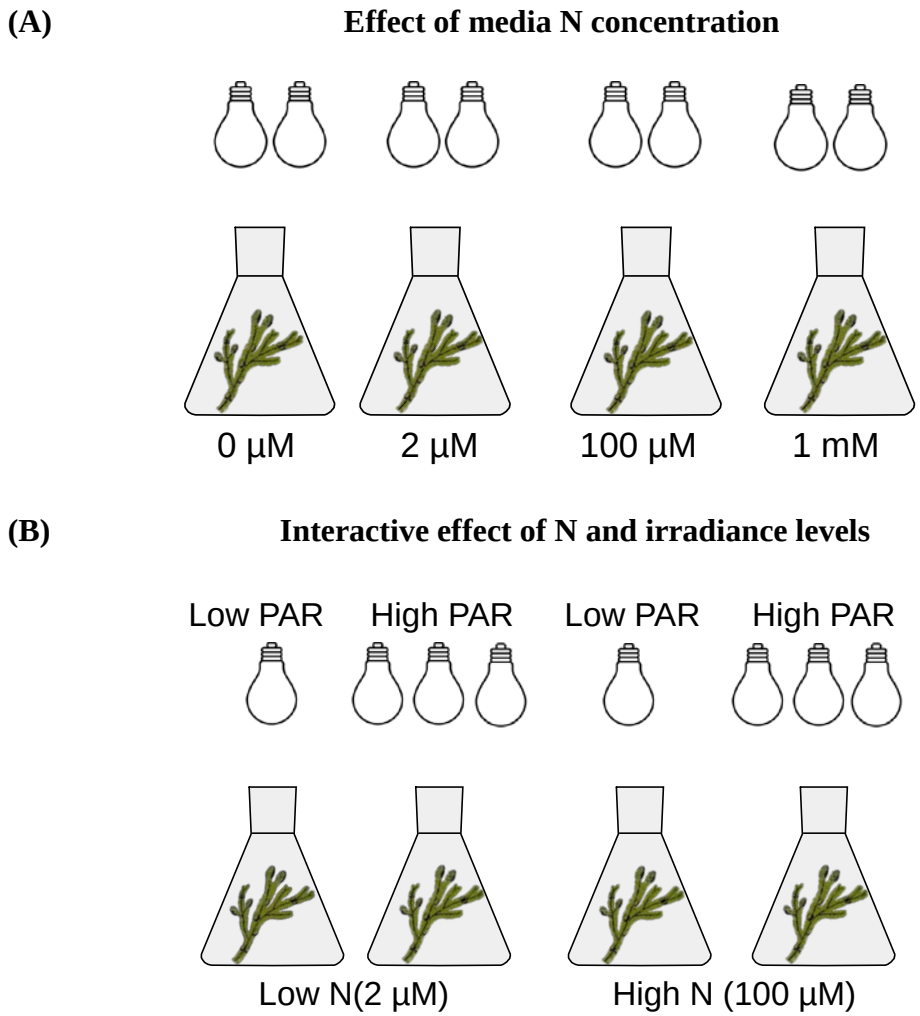



**Fig 5.3.** The culture conditions employed to investigate (A) the effect of media N concentrations under one uniform PAR level (50-60 µmol photons $m^{-2}$ $s^{-1}$) or (B) the interactive effect of both PAR - low (15-20 µmol photons $m^{-2}$ $s^{-1}$) and high (110-115 µmol photons $m^{-2}$ $s^{-1}$), and N levels - low (2 µM) and high (100 µM) on the C and N content and Chl *a* fluorescence of *F. vesiculosus* thalli, as well as the phlorotannin composition, content, FRAP and RSA of the LMW phlorotannin-enriched fractions extracted from the treated individuals, compared to the control.

### 5.2.7. Statistical analyses

Significant differences between the total C, N levels as wellas the C:N ratios of the *F.vesiculosus* thalli collected from the two sites – Finavarra, co. Clare and Oranmore, co. Galway were tested using a one-way ANOVA at a significance level of 95% ($p$<0.05). IBM® SPSS® statistical software (version 24) was used for all statistical tests. Normality was tested using the Shapiro-Wilk test for normal distribution and Levene's test for homogeneity of variances. A Tukey-test was performed to find *posteriori homogeneous* (*post-hoc*) sub-groups that differed significantly ($p$<0.05).

A two-way mixed ANOVA ($p$<0.05) was conducted to analyse the effects of different media N concentrations (between-subject factors) over time (within-subject factor) on the total phlorotannin content (TPC), the ferric reducing antioxidant power (FRAP) and the radical scavenging activity (RSA) of the LMW phlorotannin-enriched fractions of *F. vesiculosus*. Normality was tested using the Shapiro-Wilk test for normal distribution and Levene's test for homogeneity of variances while sphericity was tested for using Maulchy's test of sphericity. Individual one-way ANOVA's ($p$<0.05) were conducted for each sampling point, *i.e.* after 3 and 7 days, to identify any significant diffeences between the experimental treatments, compared to the control at day 0. Tukey-tests were performed to find *posteriori homogeneous* (*post-hoc*) sub-groups that differed significantly ($p$<0.05).

The potential effect of different media N concentrations and irradiance levels on the photosynthetic parameters obtained from Chl *a* fluorescence measurements on *F. vesiculosus* thalli at each sampling point *i.e.* day 3 and day 7 was tested using a two-way ANOVA ($p$<0.05). Normality was tested using the Shapiro-Wilk test for normal distribution and Levene's test for homogeneity of variances. Significant differences between treatments were determined by a Tukey *posteriori homogeneous* (*post-hoc)* test ($p$<0.05). To determine if any significant differences arose within each treatment as a result of time, a paired *t*-test was employed ($p$<0.05) to compare day 3 and day 7.

A three-way mixed ANOVA ($p$<0.05) was conducted to analyse the effects of different media N concentrations and irradiance levels (between-subject factors) over time (within-subject factor) on the total phlorotannin content (TPC), the ferric reducing antioxidant power (FRAP) and the radical scavenging activity (RSA) of the LMW phlorotannin-enriched fractions of *F. vesiculosus*. Normality was tested using the Shapiro-Wilk test for normal distribution and Levene's test for homogeneity of variances while sphericity was tested for using Maulchy's test of sphericity. Individual one-way ANOVA's ($p$<0.05) were conducted for each sampling point, *i.e.* after 3 and 7 days, to identify any significant diffeences between the experimental treatments, compared to the control at day 0. Tukey-tests were performed to find *posteriori homogeneous* (*post-hoc*) sub-groups that differed significantly ($p$<0.05).

## 5.3. Results

### 5.3.1. Preliminary assessment of temporal N variations

The total C and N content of *F. vesiculosus* collected from both sites differed significantly ($p<0.001$). Finavarra displayed a slightly higher total C than Oranmore. However, in relation to total N content, a stark difference was observed between the two sites (Fig. 5.4; $p<0.001$). Samples collected from Oranmore displayed an almost three-fold increase in the N content compared to those collected from Finavarra. This spatial variation in N resulted in very different C: N ratios for samples from the sites, with Finavarra displaying a ratio of 70 while in samples collected from Oranmore, it was 26 ($p<0.001$; Fig. 5.4).

The phlorotannin content of the LMW phlorotannin-enriched fractions also differed between sites ($p<0.001$). *F. vesiculosus* collected from Finavarra displayed significantly higher levels with a total content of 372.5±10.3 µg PGE mg $DWE^{-1}$, compared to Oranmore (258.4±6.3 µg PGE mg $DWE^{-1}$; Fig. 5.4; $p<0.001$). Due to the higher levels detected in Finavarra, as well as its use as a reference site in other chapters, only *F. vesiculosus* from this site were selected for further investigation.

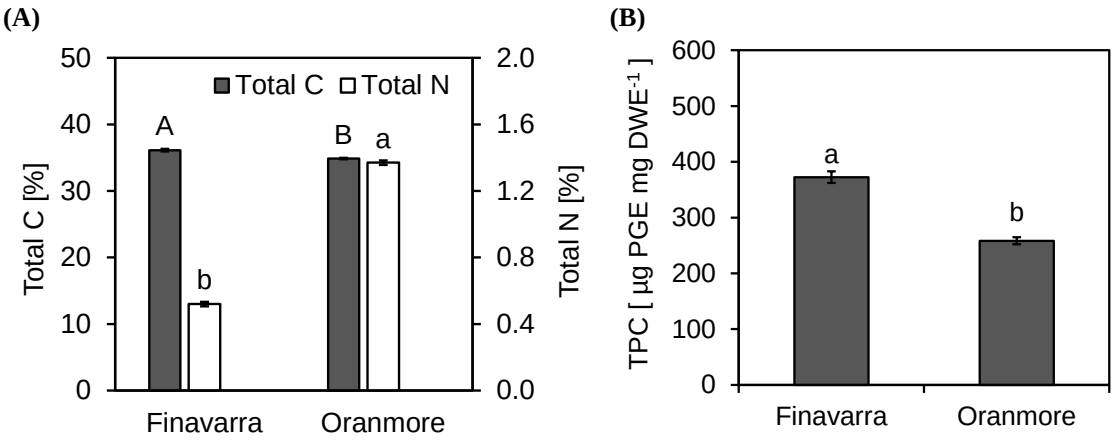



**Fig. 5.4.** The total C and N content (%) of *F. vesiculosus* collected from Finavarra, Co. Clare, and Oranmore, Co. Galway. Data are represented as mean ± standard deviation (n=3). Significant differences between the two sampling sites were tested using a one-way ANOVA ($p<0.05$). Details of the statistical test are outlined in Supplementary Section 9.5, Table 9.5.1.Superscripts identify significant differences between samples using Tukeys *post-hoc* test.

### 5.3.2. Effect of N concentration in media

#### 5.3.2.1. Carbon: nitrogen ratios

The total C and N content observed in *F. vesiculosus* samples frozen prior to experimental manipulation (control) were 33.5±0.3% and 0.64±0.01%, respectively (Fig. 5.5). While a narrow range (33.3-34.5%) for the total C % was observed between the different N treatments, these values differed significantly from one another as a result of both the media N concentration ($p$=0.014), exposure time ($p$<0.001) as well as the interaction of the two factors ($p$<0.001).

The internal total N content of the algae was strongly affected by the N concentration of the media ($p$<0.001) in which they were grown, with a positive correlation observed between the two parameters ($R^2$=0.91; Fig. 5.5). Exposure time ($p$<0.001) and the interaction between N concentration and time ($p$<0.001) also had significant effects on the total N content. Increases over time were observed in samples grown in 2 µM and 1 mM while those in 100 µM displayed no change. Those grown in media with N enrichment (0 µM) exhibited a slight decrease overtime (Fig. 5.5).

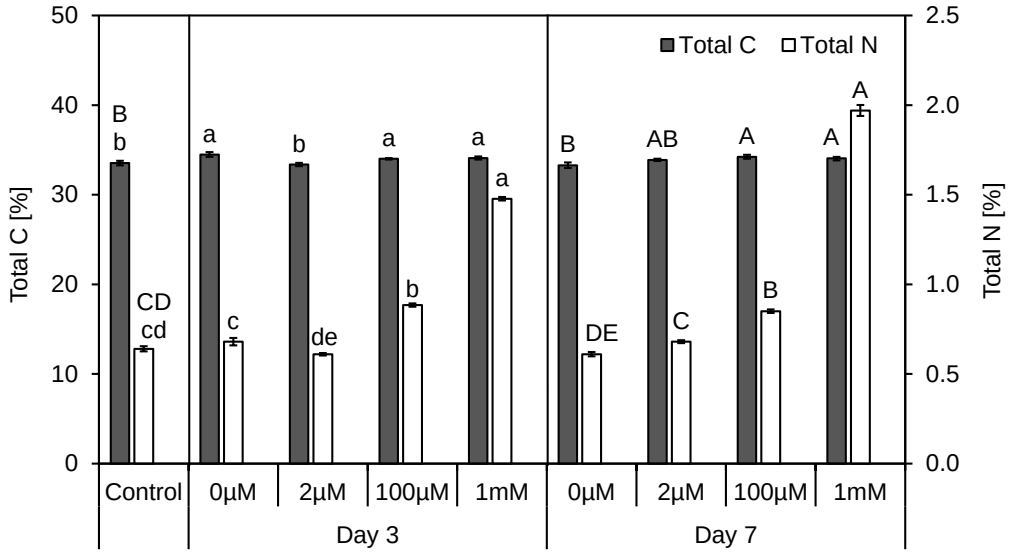



**Fig. 5.5.** The total C and N content (%) of *F. vesiculosus* grown under 50-60 µmol photons $m^{-2}$ $s^{-1}$ PAR in different N concentrations, after 3 and 7 days of exposure, compared to the control. The control sample was representative of a collection of individual thalli that were frozen after 24 h lab acclimation, prior to experimental manipulation. Data are represented as mean ± standard deviation (n=3). The effect of media N concentration over time was tested using a two-way mixed ANOVA ($p$<0.05), details of which are outlined in Supplementary Section 9.5 in Table 9.5.2. Superscripts distinguish significant differences between treatments after either 3 (lowercase) or 7 (uppercase) days of exposure, compared to the control sample at day 0, using Tukey's *post-hoc* test ($p$<0.05).

The media N concentration ($p$<0.001) significantly affected the C:N ratios in the algal thalli, in an inverse manner ($R^2$=0.88: $p$<0.001; Table 5.2) with samples grown in the higher N concentrations displaying the lowest C:N ratios. For example, samples grown in the highest N concentration of 1 mM possessed the lowest C: N ratio at both time points, decreasing over time ($p$<0.05; Table 5.2). This was then followed by samples grown in 100 µM, displaying a rather stable C: N ratio after both 3 and 7 days (Table 5.2). Media N concentrations of 0 and 2 µM resulted in C: N ratios similar to that of the control, with slight shifts observed over time ($p$<0.001; Table 5.2).

**Table 5.2.** The carbon: nitrogen (C: N) ratio of *F. vesiculosus* samples grown under 50-60 µmol photons $m^{-2}$ $s^{-1}$ PAR in different N concentrations after 3 and 7 days of exposure, compared to the control. The control sample was representative of a collection of individual thalli that were frozen after 24 h lab acclimation, prior to experimental manipulation. Data are represented as mean ± standard deviation (n=3). The effect of media N concentration over time was tested using a two-way mixed ANOVA ($p$<0.05), details of which are outlined in Supplementary Section 9.5 in Table 9.5.2. Superscripts distinguish significant differences between treatments after either 3 (lowercase) or 7 (uppercase) days of exposure, compared to the control sample at day 0, using Tukey's *post-hoc* test ($p$<0.05).

| | **Control** | **0µM** | **2µM** | **100µM** | **1mM** |
|---|---|---|---|---|---|
| **Day 0** | $51.9\pm0.9^{b,B}$ | - | - | - | - |
| **Day 3** | - | $51.1\pm1.6^{b}$ | $55.03\pm0.9^{a}$ | $38.0\pm0.5^{c}$ | $23.1\pm0.2^{d}$ |
| **Day 7** | - | $54.9\pm1.5^{A}$ | $49.8\pm0.7^{B}$ | $40.3\pm0.7^{C}$ | $17.3\pm0.2^{D}$ |

### 5.3.2.2. Total phlorotannin content (TPC) of LMW phlorotannin-enriched fractions

The phlorotannin content of the control sample of *F. vesiculosus* from Finavarra, Co. Clare, prior to experimental exposure to different media N concentrations was measured as 442.7±14.2 µg PGE mg $DWE^{-1}$ (Fig. 5.6). The TPC levels between the four treatments were observed to differ significantly as a result of the media N concentration ($p$<0.001) in which the algae were grown. Thalli exposed to the higher concentrations of 100 µM and 1 mM displayed higher phlorotannin levels after both 3 and 7 days compared to those grown in the lower concentrations of 0 and 2 µM. Yet, overall, a universal decrease in phlorotannin content was observed across all treatments after both 3 and 7 days, compared to the control at day 0 ($p$=0.003; Fig. 5.6). However, the interaction between media N concentration and exposure time

appeared not to impose any significant effects as little variation was observed within each treatment between day 3 and day 7 ($p$=0.050; Fig. 5.6).

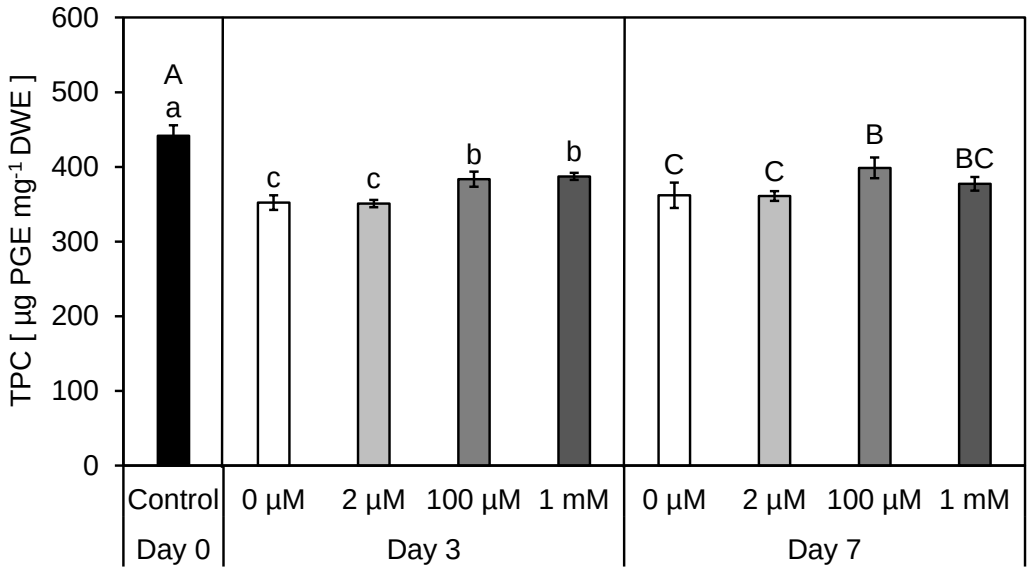



**Fig. 5.6.** The phlorotannin content of the LMW phlorotannin fractions extracted from *F. vesiculosus* grown in different media N concentrations; 0 µM, 2 µM, 100 µM and 1mM, for 3 or 7 days of exposure, compared to the control. The control sample was representative of a collection of individual thalli that were frozen after 24 h lab acclimation, prior to experimental manipulation. Data are represented as mean ± standard deviation (n=3) and are expressed as microgram phloroglucinol equivalents per milligram dry weight extract (µg PGE mg $DWE^{-1}$). Significant effects of the experimental conditions were tested using a two-way mixed ANOVA ($p$<0.05). Details of the statistical test are outlined in Section 9.5, Table 9.5.3. Superscripts distinguish significant differences between treatments after either 3 (lowercase) or 7 (uppercase) days of exposure, compared to the control sample at day 0, using Tukey's *post-hoc* test ($p$<0.05).

### 5.3.2.3. LMW phlortannin profile characterisation

All phlorotannin-enriched fractions of samples exposed to the different N concentrations for 3 and 7 days were characterised using Q-ToF-MS followed by UPLC-MS/MS. Direct infusion using Q-ToF-MS allowed for the detection of all compounds present within the sample. All samples displayed a high degree of purity with LMW phlorotannins being the predominant compounds present (Fig. 5.7), thus no further separation steps were required. The use of multiple reaction monitoring (MRM) mode in the UPLC-MS/MS allowed for the detection of selected molecular weights corresponding to phloroglucinol polymers, specifically those comprised of 3-16 phloroglucinol units (PGUs).

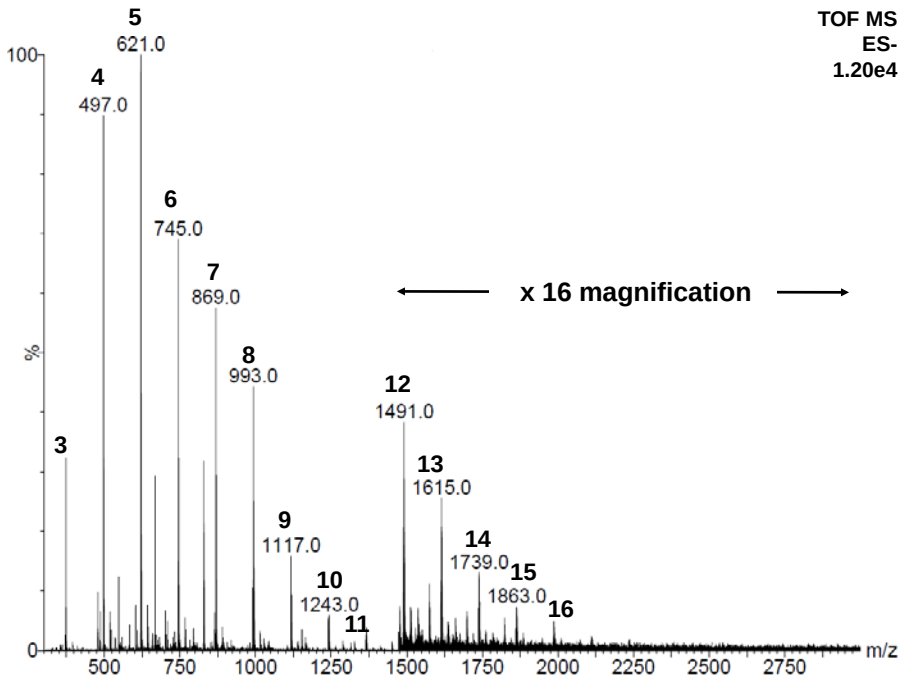



**Fig. 5.7.** Q-ToF-MS spectrum, acquired in negative ion mode, of *F. vesiculosus*, after 24 h lab acclimation prior to experimental manipulation, displaying a high abundance of compounds with molecular weights; (number immediately above each *m/z* peak) that correspond to a degree of polymerisation of between 3-16 phloroglucinol units (PGUs; most upper bold numbers above peaks). The presence of larger structures of between 12-16 PGUs, are shown through magnification (x 16).

The LMW phlorotannin fractions obtained from samples grown in different N concentrations exhibited relatively similar profiles, displaying a high abundance of structures between *m/z* 497 and 993, corresponding to 4-8 PGUs (Fig. 5.8). This range of phlorotannins accounted for over 88% of the total abundance. Structures composed of 4, 5 and 8 PGUs, corresponding to 498, 622, and 994 Da, were the most dominant, collectively accounting for 71-75% of the total. Structures of 5 PGUs (622 Da) were the most abundant in all samples, accounting for 36-38% followed by either those corresponding to 498 Da (4 PGUs) or 994 Da (8 PGUs) with each comprising between 16-20%. Larger phlorotannins detected (10-16 PGUs) accounted for a smaller percentage, accumulatively representing 2-3%. Q-ToF-MS analysis also confirmed the presence of polymers up to 20 PGUs on thorough scrutiny of the mass spectra data (Fig. 5.7).

The exposure of *F. vesiculosus* to different media N concentrations over a total of 7 days, however, appeared to induce little to no distinguishable variations in the chemical composition (Fig. 5.8). While slight shifts did occur, no particular trend was observed in response to an increased internal N content.

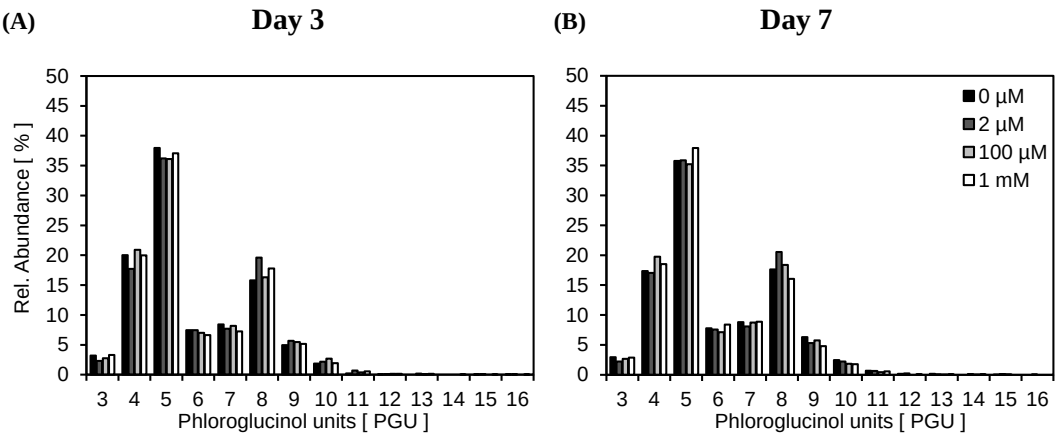



**Fig. 5.8.** The relative abundance (%) of phlorotannins structures between 3-16 phloroglucinol units (PGUs), as detected by UPLC-MS/MS, in the LMW phlorotannin fractions derived from *F. vesiculosus* grown in different N concentrations (0 µM, 2 µM, 100 µM and 1 mM) after (A) 3 or (B) 7 days.

### 5.3.2.4. Isomeric variation

The total number of isomers for the LMW phlorotannins was determined by the sum of all isomers detected for the 13 MRM transitions (3-16 PGUs; Table. 5.3). The total isomeric count prior to experimental manipulation with N was observed as 156. In all fractions, the highest abundance of isomers was found in structures composed of 5-10 PGUs with the majority of fractions displaying the highest number of isomers for phlorotannins of 6 PGUs, the most dominant structures. Regardless of the N concentration of the media, isomeric levels displayed an overall 15-22% decrease compared to the controls after 3 days. This further declined after 7 days (23-37%), yet now an N-dependent decrease was observed, with samples exposed to 1 mM N displaying the lowest level of isomers (Table 5.3).

**Table 5.3.** List of isomers for each individual MRM transition between 3-16 phloroglucinol units (PGUs), as detected by UPLC-MS/MS, in the LMW phlorotannin fractions derived *F. vesiculosus* grown in different N concentrations (0 µM, 2 µM, 100 µM and 1 mM) for 3 or 7 days, compared to the control at day 0. The control sample was representative of a collection of individual thalli that were frozen after 24 h lab acclimation, prior to experimental manipulation. Where 10 or more individual isomers are present for an individual molecular ion transition the number is highlighted in bold.

| | **Day 0** | **Day 3** | | | | **Day 7** | | | |
|---|---|---|---|---|---|---|---|---|---|
| **PGUs** | **Control** | **0 µM** | **2 µM** | **100 µM** | **1 mM** | **0 µM** | **2 µM** | **100 µM** | **1 mM** |
| **3** | 2 | 3 | 2 | 3 | 2 | 1 | 2 | 1 | 2 |
| **4** | 8 | 5 | 6 | 4 | 6 | 5 | 4 | 6 | 4 |
| **5** | **18** | **12** | **12** | **16** | **13** | **12** | **10** | **10** | 9 |
| **6** | **32** | **24** | **21** | **25** | **20** | **28** | **22** | **26** | **20** |
| **7** | **30** | **19** | **18** | **20** | **18** | **23** | **21** | **17** | **18** |
| **8** | **24** | **20** | **20** | **24** | **19** | **19** | **21** | **17** | **18** |
| **9** | **22** | **15** | **15** | **22** | **17** | **15** | **12** | **15** | **14** |
| **10** | **11** | **11** | **11** | **11** | 9 | **10** | **11** | 6 | 6 |
| **11** | 5 | 5 | 6 | 4 | 6 | 4 | 7 | 3 | 3 |
| **12** | 3 | 2 | 1 | 0 | 1 | 1 | 3 | 2 | 0 |
| **13** | 1 | 1 | 3 | 2 | 1 | 0 | 0 | 1 | 2 |
| **14** | 0 | 1 | 1 | 1 | 0 | 0 | 2 | 0 | 1 |
| **15** | 0 | 1 | 3 | 0 | 1 | 1 | 0 | 1 | 1 |
| **16** | 0 | 2 | 1 | 0 | 1 | 1 | 0 | 0 | 0 |
| **Total** | **156** | **121** | **120** | **132** | **114** | **120** | **115** | **105** | **98** |

### 5.3.2.5. *In vitro* antioxidant activity

#### 5.3.2.5.1. Ferric reducing antioxidant power (FRAP)

The FRAP activity of the LMW phlorotannin-enriched fractions appeared to be unaffected by the media N concentration in which the algae were grown ($p$=0.146; Fig 5.9). The only significant difference observed was the universal decline displayed by all experimental samples, compared to the control at day 0 (735.3±21.3 µg TE $mg^{-1}$ DWE; $p$<0.001; Fig. 5.9). Furthermore, there was no significant affect of the interaction of media N concentration and time ($p$=0.529) as within each treatment no differences were observed in the FRAP values between day 3 and day 7 (p>0.05; Fig. 5.9). Overall, the FRAP levels displayed a positive correlation with the observed TPC levels ($R^2$=0.53).

#### 5.3.2.5.2. Radical scavenging activity (RSA)

The RSA, expressed as $IC_{50}$ values, of the control sample corresponded to 22.4±1.9 µg $mL^{-1}$ (Fig. 5.9). Exposure to different N concentrations appeared to have no effect on the RSA of the LMW phlorotannin fractions ($p$=0.148; Fig. 5.9). However, the duration of exposure to different N concentrations appeared to induce differences ($p$<0.001; Fig. 5.9). This was particular apparent in samples exposed to the higher concentrations of 100µM and 1mM, with both tretaments displaying a significant decrease in activity after 7 days of exposure ($p$<0.05; Fig. 5.9). Such a decline in RSA, however, was not seen in samples grown in 0 and 2 µM N, with relatively similar values being observed after 3 and 7 days of exposure ($p$>0.05; Fig. 5.9). Moreover, the interaction between the media N concentration of the treatment and exposure time was not significant ($p$=0.232), as all treatments, after either 3 or 7 days, displayed similar $IC_{50}$ values to one another (Fig. 5.9). Overall, the RSA of the LMW fractions displayed a weak correlation with the phlorotannin levels ($R^2$=0.16).

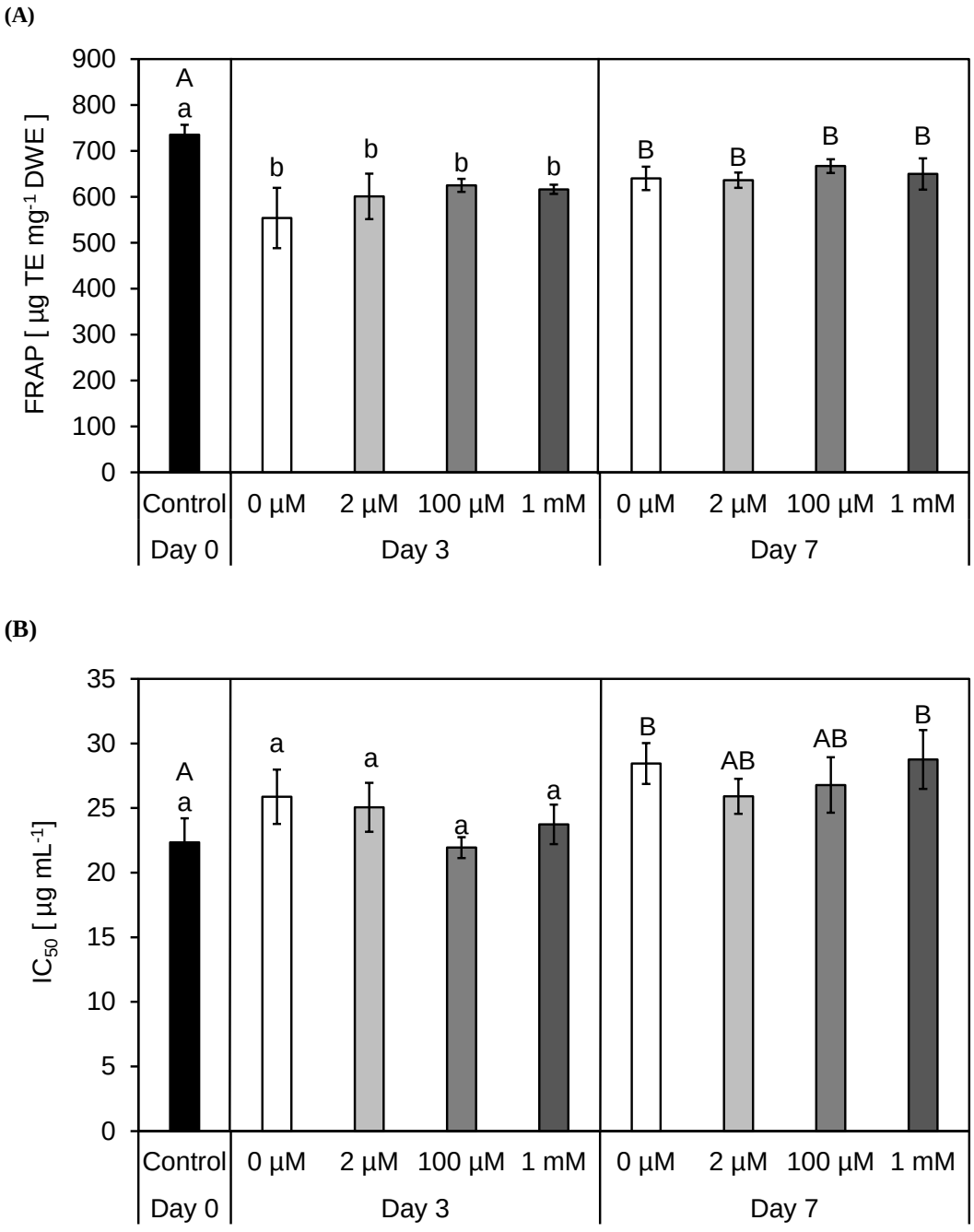



**Fig 5.9.** (A) The FRAP and (B) RSA ($IC_{50}$) levels obtained for LMW phlorotannin fractions derived from *F. vesiculosus* grown in different N concentrations (0 µM, 2 µM, 100 µM and 1 mM) for 3 or 7 days, compared to the control. The control sample was representative of a collection of individual thalli that were frozen after 24 h lab acclimation, prior to experimental manipulation. Data are represented as mean ± standard deviation (n=3). FRAP values are expressed as microgram Trolox equivalents per milligram dry weight extract (µg TE $mg^{-1}$ DWE) while $IC_{50}$ values are expressed as milligram per millilitre (µg $mL^{-1}$). The effect of media N concentration over time was tested using a two-way mixed ANOVA ($p$<0.05), details of which are outlined in Supplementary Section 9.5 in Table 9.5.3. Superscripts distinguish significant differences between treatments after either 3 (lowercase) or 7 (uppercase) days of exposure, compared to the control sample at day 0, using Tukey's *post-hoc* test ($p$<0.05).

### 5.3.3. Interaction of N with light

#### 5.3.3.1. Carbon: nitrogen ratios

The total internal C and N content of the control sample *i.e*. thalli frozen after lab acclimation prior to experimental manipulation, were observed as 36.1 ± 0.1% and 0.52 ± 0.01%, respectively (Fig. 5.10). The total C content of *F. vesiculosus* appeared to be affected by all three impacting factors, the N concentration of the media ($p$<0.001), the irradiance level of the culture condition ($p$<0.001) and the duration of exposure ($p$<0.001; Fig. 5.10).

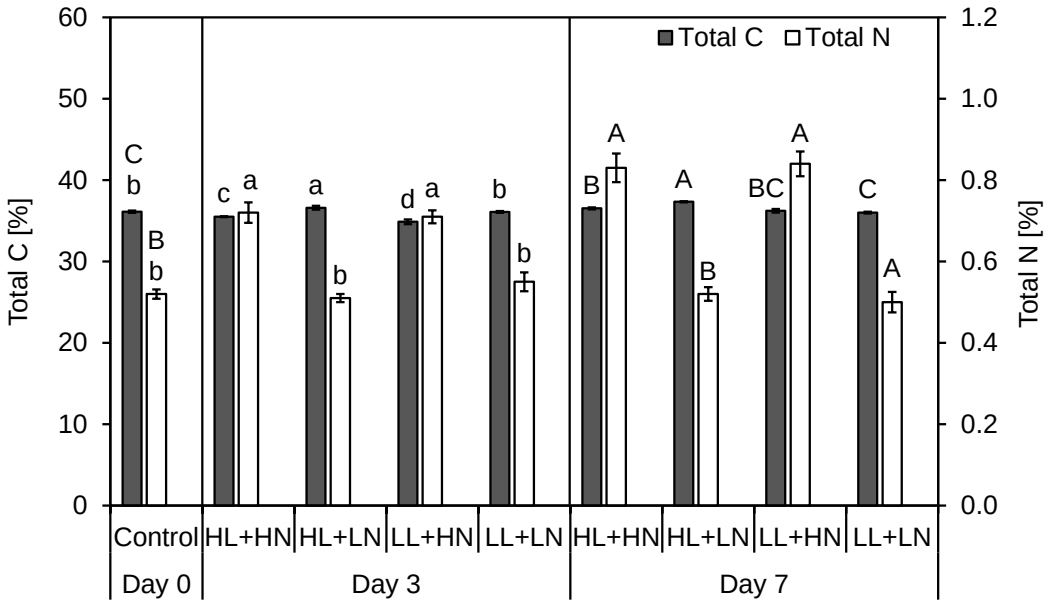



**Fig. 5.10.** The total carbon and nitrogen content (%) of *F. vesiculosus* samples grown under different combinations of N and PAR levels (HL+HN, HL+LN, LL+HN and LL+LN) after 3 and 7 days, compared to the control. The control sample was representative of a collection of individual thalli that were frozen after 24 h lab acclimation, prior to experimental manipulation. Data are represented as mean ± standard deviation (n=3). Significant effects of the experimental conditions were tested using a three-way ANOVA ($p$<0.05). Details of the statistical test are outlined in Section 9.5, Table 9.5.4. Superscripts distinguish significant differences between treatments after either 3 (lowercase) or 7 (uppercase) days of exposure, compared to the control sample at day 0, using Tukey's *post-hoc* test ($p$<0.05).

The total N content of the algae, however, while being affected by the media N concentration ($p$<0.001) as well as the duration time of exposure ($p$<0.001), it was not significantly affected by the PAR level ($p$=0.691; Fig. 5.10). The highest N % was displayed by samples exposed to the high-N conditions of 100 µM (0.7%), under both PAR levels. After 7 days, this had significantly increased ($p$<0.05; 0.83-0.85%), again, irrespective of irradiance (Fig. 5.10). Those grown in low-N (2 µM) displayed a lower

internal N content, similar to that of the control (0.51-0.55%; Fig 5.10). The N % within these samples did not vary over time, displaying similar values after both 3 and 7 days ($p$>0.05; Fig. 5.10).

The C: N ratios of *F. vesiculosus* were significantly influenced by the N concentration of the media in which the algae were grown ($p$<0.001; Table 5.4). An inverse correlation was observed between the media N concentration and the observed C: N ratios of the algae ($R^2$=0.86). Samples grown under low-N concentrations, which possessed similar C: N ratios to the control (69.9±1.8), displayed significantly higher C: N ratios compared to those grown in high-N concentrations ($p$<0.001; Table 5.4). Similarily to the C% and N%, C: N ratios were not significantly affected by the irradiance of the treatment ($p$=0.262) with samples exposed to the same N concentration displaying similar values to one another, regardless of PAR level after either 3 or 7 days ($p$>0.05; Table 5.4). Exposure time also imparted a significant affect on the C: N ratios ($p$<0.001). This was particularly noticeable in samples grown in high-N conditions, with a decrease observed after 7 days of treatment (Table 5.4).

**Table 5.4.** The carbon: nitrogen (C: N) ratios of *F. vesiculosus* grown under different combinations of N and PAR levels (HL+HN, HL+LN, LL+HN and LL+LN) for 3 or 7 days, compared to the control. The control sample was representative of a collection of individual thalli that were frozen after 24 h lab acclimation, prior to experimental manipulation. Data are represented as mean ± standard deviation (n=3). Significant effects of the experimental conditions were tested using a three-way mixed ANOVA ($p$<0.05). Details of the statistical test are outlined in Supplementary Section 9.5, Table 9.5.4. Superscripts distinguish significant differences between treatments after either 3 (lowercase) or 7 (uppercase) days of exposure, compared to the control sample at day 0, using Tukey's *post-hoc* test ($p$<0.05).

| | **Control** | **HL+HN** | **HL+LN** | **LL+HN** | **LL+LN** |
|---|---|---|---|---|---|
| **Day 0** | $69.9 \pm 1.8^{ab, A}$ | - | - | - | - |
| **Day 3** | - | $49.6 \pm 1.8^{c}$ | $71.9 \pm 1.7^{a}$ | $49.3 \pm 0.7^{c}$ | $65.9 \pm 2.6^{b}$ |
| **Day 7** | - | $44.2 \pm 1.8^{B}$ | $72.4 \pm 2.5^{A}$ | $43.4 \pm 1.8^{B}$ | $72.6 \pm 3.6^{A}$ |

### 5.3.3.2. Chl *a* fluorescence measurements

The photosynthetic performance of *F. vesiculosus* exposed to different light and nitrogen combination treatments were measured through Chl *a* measurements. Effects on photosynthetic parameters ($F_v/F_m$, $rETR_{max}$, $E_k$) were determined through

photosynthesis-irradiance (*P/E*) curves, plotting rETR as a function of PFD (photon flux density; see Supplementary Section 9.5, Fig. 9.5.1).

After 3 days of exposure, the maximal efficiency of PS II ($F_v/F_m$) was observed to be affected by both the N concentration of the media ($p$=0.029) and the irradiance level ($p$=0.048) of the treatment in which samples were grown as well as by their interaction ($p$=0.039; Table 5.5). Almost all treatments displayed a similar value, except those under LL+HN, in which a marked decrease was observed ($p$<0.05; Table 5.5). However, after 7 days, it was only the interactive effect of the media N concentration and irradiance level of the treatment had imparted any significant differences on the $F_v/F_m$ values between the treatments ($p$<0.001; Table 5.5). In fact, after 7 days, the $F_v/F_m$ of those under LL+HN and HL+LN had significantly increased ($p$=0.022, 0.002, respectively) and now both treatments yielded the highest $F_v/F_m$ values overall ($p$<0.05; Table 5.5).

**Table 5.5.** Photosynthetic parameters – $F_v/F_m$ (Relative units), $rETR_{max}$ (Relative units) and $E_k$ (µmol photons $m^{-2}$ $s^{-1}$) of *F. vesiculosus* grown under different combinations of N and PAR levels (HL+HN, HL+LN, LL+HN and LL+LN) for 3 or 7 days. Data are represented as mean ± standard deviation (n=3). Significant effects of the experimental treatments on the samples were tested using a two-way ANOVA at each time point *i.e.* after either 3 or 7 days of exposure. Data are represented as mean ± standard deviation (n=3). A paired *t*-test was carried out to identify significant differences arising over time within each treatment. Details of the statistical test are outlined in Supplementray Section 9.5, Table 9.5.5 and Table 9.5.6. Superscripts distinguish significant differences between treatments after either 3 (lowercase) or 7 (uppercase) days of exposure using Tukey's *post-hoc* test ($p$<0.05).

| | **Day 3** | | | **Day 7** | | |
|---|---|---|---|---|---|---|
| | $F_v/F_m$ | $rETR_{max}$ | $E_k$ | $F_v/F_m$ | $rETR_{max}$ | $E_k$ |
| **HL+HN** | 0.62 ± 0.03$^{a}$ | 127.4 ± 7.6 | 262.0 ± 26.1 | 0.62 ± 0.01$^{B}$ | 103.8 ± 22.8 | 214.5 ± 40.5 |
| **HL+LN** | 0.63 ± 0.02$^{a}$ | 94.3 ± 28.7 | 241.58± 17.7 | 0.66 ± 0.02$^{A}$ | 108.2 ± 6.2 | 231.2 ± 4.2 |
| **LL+HN** | 0.55 ± 0.02$^{b}$ | 144.3 ± 24.5 | 306.5 ± 17.6 | 0.68 ± 0.01$^{A}$ | 154.3 ± 26.1 | 286.7 ± 35.2 |
| **LL+LN** | 0.63 ± 0.04$^{a}$ | 165.3 ± 44.5 | 324.9 ± 39.4 | 0.62 ± 0.01$^{B}$ | 81.5 ± 13.2 | 203.3 ± 24.0 |

After 3 days of exposure to the different treatments, the maximum relative electron transport rate, $rETR_{max}$, appeared to be affected by the irradiance level of the treatment ($p$=0.032). However, neither the N concentration of the media or the interaction between nitrogen concentration and irradiance level had no effect ($p$=0.73, 0.15, respectively) and so no significant differences in $rETR_{max}$ were observed between treatments or over time ($p$>0.05; Table 5.5).

Similarly, the light saturation point, $E_k$, was only affected by the irradiance level of the treatment ($p$=0.034) after 3 days of exposure, with samples exposed to low-PAR generally displaying higher $E_k$ values than those under high PAR (Table 5.5). However, as no significant effects were incurred by either the media N concentration ($p$=0.554) or the interaction between the two factors ($p$=0.947; Table 5.5), overall there was no significant differences observed for the $E_k$ values between the different treatments (Table 5.5).

After 7 days of exposure the difference treatments, neither of the experimental factors or their interaction imposed any significant effects on the $rETR_{max}$ or $E_k$ values observed between the different treatments ($p$>0.05; Table 5.5). Furthermore, no effect of time (p>0.05) was observed with rETRmax and Ek values remaining unchanged after both 3 and 7 days of exposure (Table. 5.5).

### 5.3.3.3. Total phlorotannin content (TPC) of LMW phlorotannin-enriched fractions

The phlorotannin content in the control, *i.e.* thalli frozen after 24 h lab acclimation, prior to treatment, was determined as 424.5±24.9 µg PGE $mg^{-1}$ DWE (Fig. 5.11). The observed phlorotannin levels were not significantly affected by the media N concentration ($p$=0.873) in which the algae were grown but rather the irradiance level of the treatment ($p$<0.001), the interaction between N concentration and irradiance ($p$<0.001; Fig. 5.11). This was particularly apparent for samples grown in low-N concentrations. For example, under high-N conditions, differences in the PAR level appeared to impose no effect, with samples under both HL+HN and LL+HN displaying similar phlorotannin levels to one another after 3 (384.62± 18.40, 380.57± 6.48 µg PGE $mg^{-1}$ DWE, respectively) and 7 (387.76± 24.11, 373.49±12.10 µg PGE $mg^{-1}$ DWE, repstively; Fig. 5.11) days of exposure.

However, in samples grown in low-N medium, exposure to high PAR resulted in a significant decrease after 3 days compared to those under low PAR ($p$<0.05; 369.67±23.63, 429.18±15.69 µg PGE $mg^{-1}$ DWE, respectively; Fig. 5.11). A similar effect was observed after 7 days ($p$<0.05) but now a further decrease in TPC had occurred in samples exposed to HL+LN (Fig. 5.11) while those under LL+LN had maintained a level similar to that of day 3 ($p$>0.05; Fig. 5.11).

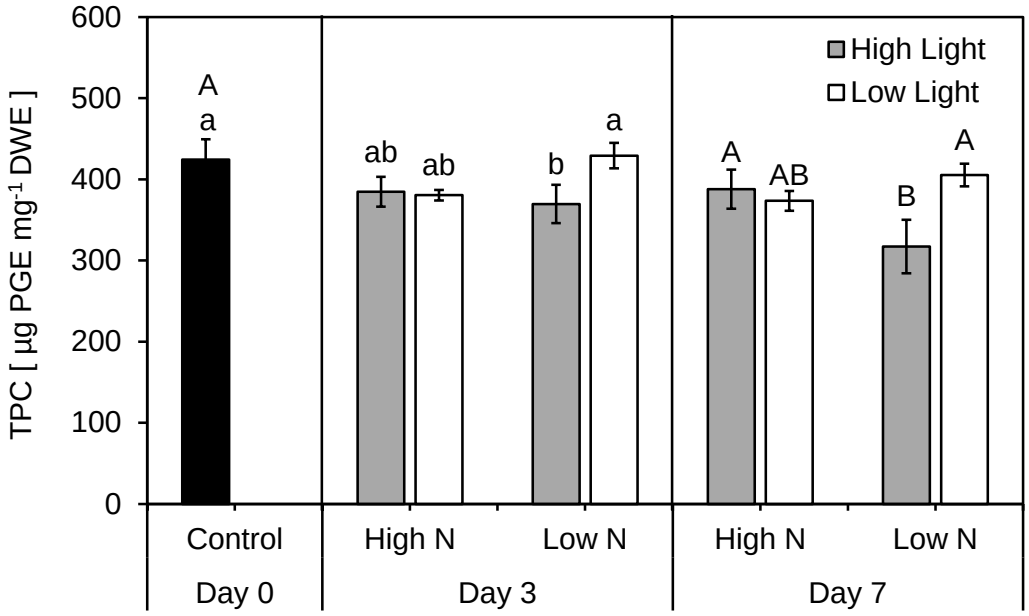



**Fig. 5.11.** The total phlorotannin content of the LMW phlorotannin fractions extracted from *F. vesiculosus* grown under different combinations of N and PAR levels (HL+HN, HL+LN, LL+HN and LL+LN) for 3 or 7 days, compared to the control. The control sample was representative of a collection of individual thalli that were frozen after 24 h lab acclimation, prior to experimental manipulation. Data are represented as mean ± standard deviation (n=3) expressed as microgram phloroglucinol equivalents per milligram dry weight extract (µg PGE mg $DWE^{-1}$). Significant effects of the experimental conditions were tested using a three-way ANOVA ($p$<0.05). Details of the statistical test are outlined in Supplementary Section 9.5, Table 9.5.7. Superscripts distinguish significant differences between treatments after either 3 (lowercase) or 7 (uppercase) days of exposure, compared to the control sample at day 0, using Tukey's *post-hoc* test ($p$<0.05).

### 5.3.3.4. LMW phlorotannin profile characterisation

The relative abundance of certain phlorotannin structures appeared to vary according to both external N concentration as well as the PAR level, with a time-dependent differentiation in the effect of each of these factors. For example, the abundance of phlorotannins composed of 5 PGUs (622 Da) appeared to increase with decreasing PAR after 3 days. Yet after 7 days, this trend was more associated with the N concentration than PAR (Fig. 5.12). Conversely, a higher abundance of phlorotannin structures composed of 8 PGUs (994 Da) was associated with higher N levels after 3 days. However, after 7 days, the presence of 8 PGUs appeared to correlate with PAR level, as samples grown under high PAR displayed a higher abundance, regardless of N concentration (Fig. 5.12).

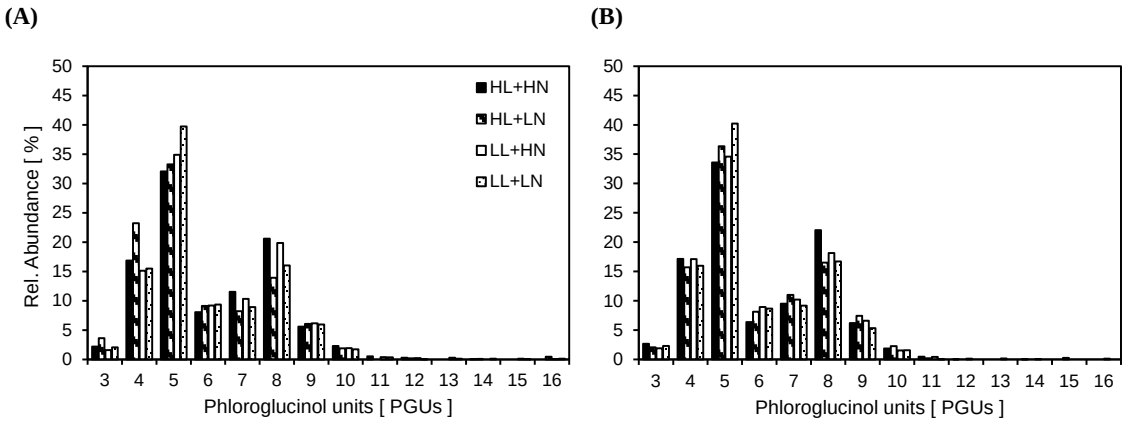



**Fig. 5.12**. The relative abundance (%) of phlorotannins structures between 3-16 phloroglucinol units (PGUs), as detected by UPLC-MS/MS, in LMW phlorotannin fractions derived from *F. vesiculosus* grown under different combinations of N and PAR levels (HL+HN, HL+LN, LL+HN and LL+LN) for (A) 3 or (B) 7 days, compared to the control.

### 5.3.3.5. Isomeric variation

The degree of isomerisation after 3 days appeared to relate to PAR level, with samples grown under low-PAR, regardless of external N concentration, displaying an increased number of isomers. Under high-PAR, however, a decrease in the degree of isomerisation was seen (Table 5.6). After 7 days of exposure, while all samples displayed a decrease total compared to the control, isomeric abundance appeared to have a greater dependency on N concentration than PAR level, with samples grown in high N possessing higher isomeric counts than those in low N (Table 5.6).

**Table 5.6.** List of isomers for each individual MRM transition between 3 – 16 phloroglucinol units (PGUs), as detected by UPLC-MS/MS, in LMW phlorotannin fractions derived from *F. vesiculosus* grown under different combinations of N and PAR levels (HL+HN, HL+LN, LL+HN and LL+LN) for 3 or 7 days, compared to the control. The control sample was representative of a collection of individual thalli that were frozen after 24 h lab acclimation, prior to experimental manipulation. Where 10 or more individual isomers are present for an individual molecular ion transition the number is highlighted in bold.

| | | Control | Day 3 | | | | Day 7 | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **[M-H]-** | **PGUs** | | **HL+HN** | **HL+LN** | **LL+HN** | **LL+LN** | **HL+HN** | **HL+LN** | **LL+HN** | **LL+LN** |
| **373** | **3** | 1 | 2 | 1 | 2 | 1 | 1 | 1 | 1 | 3 |
| **497** | **4** | 2 | 3 | 3 | 3 | 5 | 3 | 2 | 2 | 2 |
| **621** | **5** | 8 | 9 | 8 | 9 | **14** | 7 | 7 | 9 | 7 |
| **745** | **6** | **27** | **27** | **20** | **19** | **27** | **22** | **18** | **21** | **18** |
| **869** | **7** | **20** | **11** | **9** | **21** | **24** | **14** | **12** | **16** | **16** |
| **993** | **8** | **20** | **16** | **14** | **24** | **25** | **22** | **15** | **16** | **14** |
| **1117** | **9** | **18** | **11** | **12** | **16** | **16** | **14** | **11** | **14** | **11** |
| **1241** | **10** | 8 | 9 | 6 | **17** | 9 | 5 | 5 | 7 | 6 |
| **1365** | **11** | 5 | 6 | 1 | 6 | 3 | 5 | 2 | 4 | 2 |
| **1489** | **12** | 1 | 3 | 0 | 2 | 1 | 2 | 1 | 1 | 0 |
| **1614** | **13** | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 1 | 0 |
| **1738** | **14** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **1869** | **15** | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 |
| **1986** | **16** | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 |
| **Total** | | **110** | **94** | **79** | **127** | **123** | **95** | **72** | **92** | **79** |

### 5.3.3.6. *In vitro* antioxidant activity

#### 5.3.3.6.1. Ferric reducing antioxidant power (FRAP)

The control sample, at day 0, exhibited a high degree of FRAP activity with 703±25.3 µg TE $mg^{-1}$ DWE (Fig. 5.12). Simialr to the TPC, the FRAP activity was significantly affected by the irradiance level of the treatment ($p$<0.001) and its interaction with media N concentration ($p$=0.002) rather than the media N concentration alone ($p$=0.538; Fig. 5.13). Time was also an impacting factor ($p$<0.001; Fig. 5.12). After 3 days, all treatments except for LL+LN (701.50± 9.72 µg TE $mg^{-1}$ DWE) resulted in a decline in activity (by 4-18%) from the control ($p$<0.05; Fig. 5.11). However, by day 7, all treatments displayed a reduced activity compared to the control, with samples grown under HL+LN representing the lowest overall FRAP activity ($p$<0.05; 505.87±17.90 µg TE $mg^{-1}$ DWE; Fig. 5.12). The observed FRAP levels were positively correlated with the phlorotannin levels detected ($R^2$=0.62).

#### 5.3.3.6.2. Radical scavenging activity (RSA)

The RSA of the control sample corresponded to 33.2±2.1 µg $mL^{-1}$ (Fig. 5.12). However, after either 3 or 7 days exposure to either of the four experimental treatments failed to result in any significant differences ($p$=0.271, 0.071, respectively; Fig. 5.12). Neither the media N concentration ($p$=0.071), the irradiance level ($p$=0.873) of the treatment, not their interaction ($p$=0.684) imposed any significant effect (Fig. 5.12).

The only factor that imparted any significant effect on the RSA levels was exposure time ($p$=0.002; Fig. 5.12). However, as the interaction between all three factors (N concentration, irradiance and time) was insignificant ($p$=0.546), overall there were no significant differences were observed in the RSA of LMW phlorotannin fractions between treatments (Fig. 5.12). Furthermore, no correlation was observed between the RSA exhibited by the LMW phlorotannin fractions and the detected phlorotannin content ($R^2$=0.022).

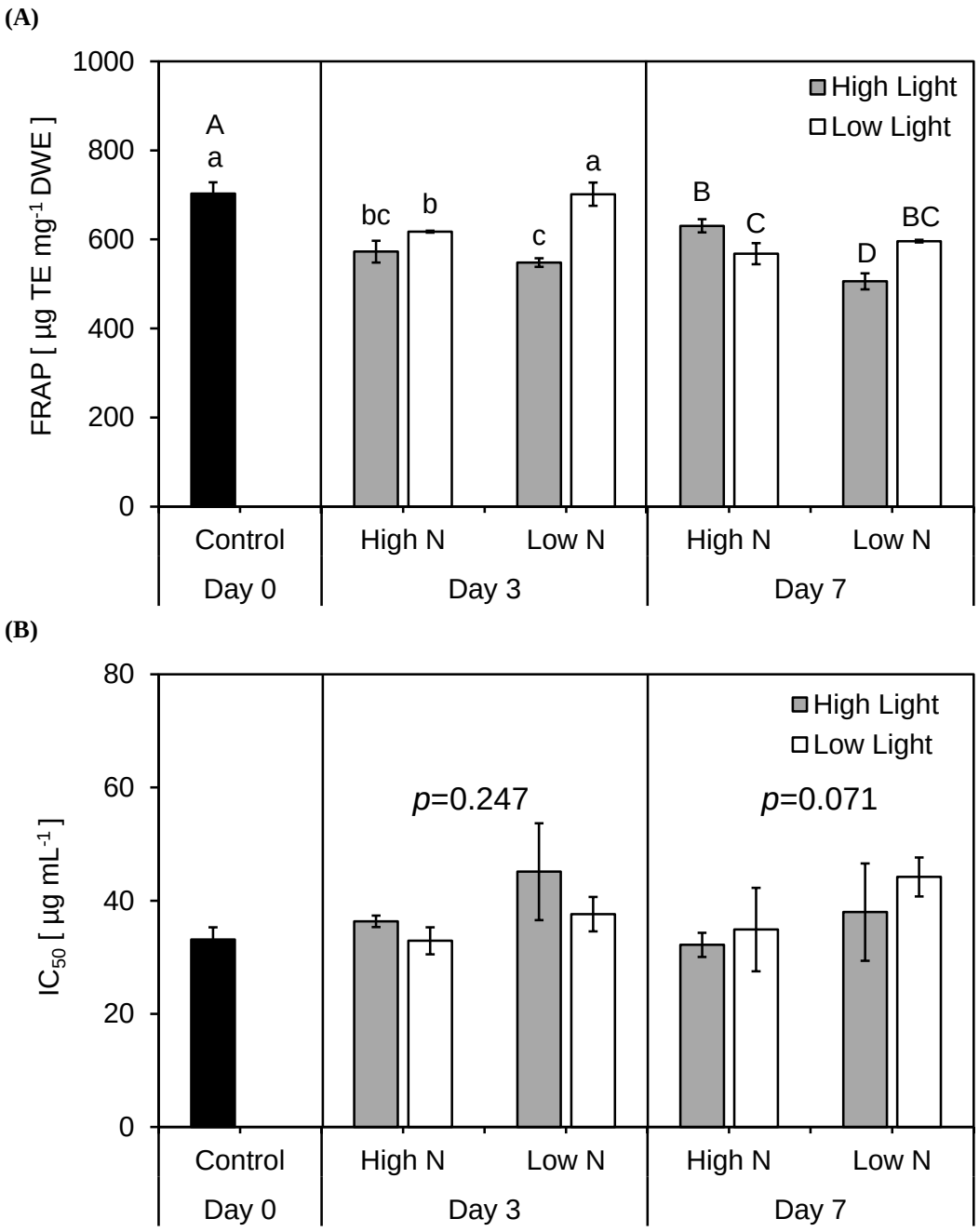



**Fig 5.13.** The FRAP levels obtained for LMW phlorotannin fractions derived from *F. vesiculosus* grown under different combinations of N and PAR levels (HL+HN, HL+LN, LL+HN and LL+LN) for 3 or 7 days, compared to the control. The control sample was representative of a collection of individual thalli that were frozen after 24 h lab acclimation, prior to experimental manipulation. Data are represented as mean ± standard deviation (n=3). FRAP values are expressed as µg TE $mg^{-1}$ DWE while $IC_{50}$ value are expressed as microgram per millilitre (µg $mL^{-1}$). Significant effects of the experimental conditions to which the algae were exposed were tested using a three-way mixed ANOVA ($p$<0.05). Details of the statistical test are outlined in Supplementary Section 9.5, Table 9.5.7. Superscripts distinguish significant differences between treatments after either 3 (lowercase) or 7 (uppercase) days of exposure, compared to the control sample at day 0, using Tukey's *post-hoc* test ($p$<0.05).

## 5.4. Discussion

To date, contradictions exist within the literature regarding the degree of influence nitrogen may have over *in vivo* phlorotannin concentrations. Many studies have suggested an inverse relationship exists between the nitrogen concentration of the surrounding environment and intracellular phlorotannin levels. This has been assumed to be related to the effect of N on the relationship between C: N ratios and phlorotannin synthesis. Natural variations within the two parameters between two sites in Galway Bay were investigated and an inverse correlation was observed whereby spatial differences observed in the phlorotannin content of *F. vesiculosus* were negatively correlated to the C: N ratio.

It was thought that seasonal differences in seawater N levels, as observed for Finavarra previously by Gietl (2016) and Schmid *et al.* (2017), may be responsible for the seasonal variability observed within Fucale species in Chapter 2. However, it is thought that due to the rapid turnover these compounds display, elucidation on specific factors is made difficult by seasonal sampling. Therefore, it was hoped that by monitoring the effects of N, if any, over a scale of days and not months, clarity on its effects on phlorotannin variability would be achieved. Therefore, the aim of this work was to investigate the effect of external N concentrations on C: N ratios and the subsequent effect this may have on both the overall algal physiology (as determined by Chl $\alpha$ fluorescence measurements), LMW phlorotannin profiles and associated *in vit*ro antioxidant activity. The effect on N alone was examined by placing *F. vesiculosus* thalli in media of different N concentrations under a controlled and uniform irradiance level. Thereafter, the synergistic interaction of N and irradiance on phlorotannins was also investigated over a 7 day period.

While manipulation of external N concentrations did incur shifts in the observed C: N ratios, no major effect was observed on the phlorotannin content after either 3 or 7 days. Even when N concentrations of 1 mM resulted in a threefold increase in total N content and a significant decline in the C: N ratio to below 20 *i.e*. no longer considered N limited (Hurd *et al.*, 2014), no further decrease in phlorotannin content was observed, in comparison to all other treatments. However, removal of nutrients from media is not always associated with their direct incorporation in the production of organic compounds (Gordillo *et al*., 2002). The kinetics involved in N uptake and

assimilation differ, with rates of the latter often becoming saturated regardless of a linear uptake. This excess N was most likely taken up and stored in vacuoles, for when N becomes limiting, rather than being utilised in the immediate synthesis of N-based compounds. This seems particularly plausible considering that *F. vesiculosus* is a slow-growing perennial species and thus possesses a low N demand for growth (Pedersen and Borum, 1996).

Therefore, if N concentrations were rendered ineffective in varying phlorotannin content perhaps it is shifts in the internal carbon levels and their subsequent effect on C: N ratios that impacts phlorotannin production. The effect of light intensity on C fixation rates was investigated as it was hypothesised that environments that yield higher rate of electron transport could potentially increase the internal carbon levels. The rate of electron transfer in PS II has been reported to be linearly related to $CO_2$ fixation rates (Baker and Oxborough, 2004; Genty, Briantais and Baker, 1989). However, while the highest rates of $rETR_{max}$ were observed under low PAR environments, particularly when coupled with high nitrogen, there was no observed correlation between $rETR_{max}$ and C %, with total C displaying a narrow range between treatments (34.8-37.3%). However, this may imply that the experimental irradiance levels employed here, being considerably lower than experienced in the field, were not high enough to incur such an increase in $CO_2$ fixation rates.

Total C % also appeared to have no effect on the phlorotannin content. Phlorotannin abundance under various PAR and N levels displayed a low degree of variability. It was only under conditions of high PAR coupled with low N that rendered a decrease in phlorotannins. However, this may be more of a reflection on the adverse effects that high PAR levels impose on these compounds rather than N, as seen in other chapters (4 and 6).Yet, when coupled with a high N level no such decrease occurred. This may suggest that through the effect of N on some other aspect of algal physiology the effect of high PAR exposure on phlorotannins was mitigated. Perhaps, allowed by a higher N availability, the concentrations of enzymes involved in phlorotannin synthesis will be increased, therefore maintaining a balanced metabolic rate. Enzymatic activity is thought to be a related to its concentration (nitrogen dependent) and turnover rate (temperature-dependent)(Hurd *et al.*, 2014). Wheeler and Weidner (1983) found that the activity of several enzymes within the brown algal kelp, *Laminaria saacharina* (ribulose-1, 5 bisphosphate carboxylase, phosphoenolpyruvate carboxykinase,

mannitol-1-phosphate dehydrogenase, nitrate reductase and glutamine synthetase) all increased with an increase in nitrate availability. Meslet-Cladière *et al.* (2013) recently revealed the contribution of a type III polyketide synthase in the conversion of malonyl co-A to the phlorotannin monomeric unit, phloroglucinol. However, very little else is known regarding the mechanism involved in the biosynthesis, therefore, this theory would require further research to be clarified.

The carbon-nitrogen balance hypothesis (CNBH), first put forward by Bryant, Chapin III, and Klein (1983), attempts to explain the natural variation of defence metabolites, in respect to growth demands. This theory has also been applied to phlorotannins, in the hope of explaining their natural variability (Arnold *et al.*, 1995; Ilvessalo and Tuomi, 1989; Jormalainen *et al.*, 2003; Pavia *et al.*, 1999; Steinberg, 1995; Van Alstyne and Pelletreau, 2000; Yates and Peckol, 1993). The hypothesis suggests that the resource requirements for primary metabolism *i.e.* growth, will have a higher priority over those for defence and therefore surmises that only when growth is limited by N availability (as mirrored in high C: N ratios) that excess resources can be allocated to production of carbon-based secondary metabolites, which otherwise would be too costly to produce.

Here, treatment with HL+LN yielded a high C: N ratio yet also resulted in the lowest phlorotannin levels, compared to the other experimental treatments. The C: N ratio exhibited in thalli under this treatment was similar to the control which displayed significantly higher level of phlorotannins and so these results contradict the assumption that phlorotannin levels will increase under such conditions. In fact, the results obtained in both experiments render the use of C: N ratios as a predictive tool inadequate, with no correlation between the two parameters being observed. In fact, the integrity of the CNBH has already been heavily criticised (Hamilton *et al.*, 2001; Koricheva, 2002; Nitao *et al.*, 2002) and largely discredited. The biosynthesis of these polyphenols and the metabolic pathways therein are undoubtedly complex and are unlikely to be elucidated upon through such simple correlations with C: N ratios, as seen here.

However, exposure to different treatments did result in variations being observed within the chemical composition of the phlorotannin profiles. Yet, there were more pronounced changes seen in samples exposed to combination treatments of both PAR

and N. The most striking was the increased abundance of phlorotannin structures with a molecular weight of 622 Da, corresponding to 5 PGUs in samples exposed to LL+LN. This may explain the high phlorotannin content and activity observed as such LMW compounds may contribute to a higher number of free hydroxyl units that could aid in stabilizing radical species. Such an increase in phlorotannins of this size was also seen in Chapter 4. Exposure to UVB led to a similar increase in the abundance of phlorotannins, primarily those comprised of 5 PGUs. This shift in the molecular distribution was also associated with an increased activity. Yet, exactly how or why this shift occurs is still unknown.

The inverse effect of N on phlorotannins observed in past studies (Arnold *et al.*, 1995; Ilvessalo and Tuomi, 1989; Koivikko *et al.*, 2008; Svensson *et al.*, 2007; Van Alstyne and Pelletreau, 2000; Yates and Peckol, 1993) may stem from the use of crude extracts. Here, LMW phlorotannin-enriched fractions were assessed displaying with a high degree of purity, as detected by the Q-ToF-MS. The potential of other compounds, aside from phlorotannins, to interfere with phenolic quantification has been reported (Prior *et al.*, 2005). Therefore, through the possible effect of N on these compounds, the estimated impact on phlorotannins may have been skewed.

In this study, however, such interference cannot explain the inverse relationship between C: N ratios and phlorotannin content observed between sites. The high N % of *F. vesiculosus* collected in Oranmore was associated with a low phlorotannin content while the low N % of Finavarra samples was coupled with a higher phlorotannin abundance, which would appear to support the CNBH. This spatial variation in phlorotannin concentrations may be derived on a genetic level with levels reflecting evolution responses to the local environment. This can be seen even within the differences in morphology. Therefore, such physiological adaptation cannot be mimicked in just 7 days and experiment run times of several months would be needed. Furthermore, these two sites are also known to differ in another abiotic factor, salinity. Oranmore, due to the input of freshwater, represents a low salinity environment (8-14) while Finavarra is a fully marine site (30-35). Salinity is reported to affect phlorotannin concentrations considerably (Pedersen, 1984). Munda (1964) reported the phenolic content of *A. nodosum* and *F. vesiculosus* of low salinity environments to increase when transplanted into locations displaying near oceanic salinity levels. Meslet-Cladière *et al.* (2013) found phloroglucinol synthesis to increase with

increasing salinity level, correlating to PKS1 gene expression. They suggested that through acclimation and adaptation to increased salinity levels, the pathway of phlorotannin biosynthesis is activated. Such an impact of salinity may explain the spatial differences observed in this work, over variations in nitrogen regime.

In preliminary work carried out in this research, salinity levels were examined on phlorotannin levels in *F. vesiculosus*. The results (not shown) did not show any effect on phlorotannin oligomers after 7 days of exposure to salinity levels of 15, 35 and 55. Therefore, the theory of genetic variability is further supported to explain the spatial variability with C: N ratios and phlorotannin levels being separate to one another. Regardless, in respect to environmental factors driving the natural variability of phlorotannins, nitrogen is not considered to be of primary importance, particularly in comparison to other abiotic factors such as light and salinity. Furthermore, if phlorotannin metabolism is affected by the effects of abiotic factors on enzymatic activity as suggested here, one environmental factor that should be investigated is temperature, due to the fundamental role it plays on enzymatic and thus metabolic rates.

# Chapter 6

The effect of seawater temperature on low molecular weight phlorotannin profiles in *Fucus vesiculosus* (L.)

## 6.1. Introduction

Within the intertidal zone, due to daily fluctuations in tidal coverage, seaweed populations regularly experience rapid changes in both the degree of irradiance and temperature. As the tide recedes, the environment in which the algae are surrounded shifts from marine to terrestrial. This can be associated with temperature changes of up to 10-15°C (Pearson and Davison, 1993). Prolonged exposure to temperatures above or below optimal levels can have significant implications on the rate of algal metabolism, including the biochemical processes involved in the uptake and conversion of nutrients into compounds required for growth, reproduction and defence (Beardall and Raven, 2001). Dependent on both temporal and seasonal variations, algae can be exposed to extreme temperatures at both ends of the spectrum and additionally potential exposure to either rapid cooling and freezing (Dudgeon *et al.*, 1990; Pearson and Davison, 1993), or exposure to elevated heat and dehydration (Dring and Brown, 1982; Karsten, 2012). The frequency and severity of such thermally-induced stress, typically increasing with shore height, is considered as a prime determinant of algal vertical distribution (Helmuth and Hofmann, 2001; Pearson and Davison, 1993), along with photo-tolerance and the ability to withstand dehydration (Dring and Brown, 1982; Schonbeck and Norton, 1978). Therefore, zonation patterns are largely based on the degree of temperature tolerance exhibited by a given species, of which is thought to be directly relative to thermal sensitivity at a cellular level (Davison and Pearson, 1996; Lüning, 1984).

In particular, adaptive variations in both the structural and functional properties of proteins have been observed in species adapted to different environmental temperatures (Somero 2010). Increased thermal stability and functioning of proteins has been observed with minor changes in amino acid sequences, potentially contributing to interspecific differences in biogeographic distribution (Somero 1995, 2010). Such responses are typically reflective of evolutionary adaptation with genetic information no longer required for certain environments or temperatures being “lost” through DNA decay (Hoffmann and Willi, 2008).

The acclimation of enzymes to different temperatures is considered as the most important adjustment in thermal adaptation (Eggert, 2012). Due to their fundamental role in regulating/catalysing metabolic reactions, the degree of enzymatic adaptation will determine the overall sensitivity of algal physiological processes to changes in temperature, ultimately defining the thermal range of the environment in which a species can occupy (Hall 1985). Often the range of temperatures between which sufficient photosynthesis can occur, follows that of the local environment, reflecting such enzymatic adaptation (Eggert *et al.*, 2003). For example, in temperate regions where seawater temperatures display broad seasonal fluctuations, eurythermal species are likely to be dominant, exhibiting a higher degree of temperature tolerance. Conversely, regions where a constant or narrow temperature range exists, will mostly be inhabited by stenothermal species (Bischoff *et al.*, 1993; Eggert, 2012; Eggert and Wiencke, 2000; Pakker *et al.*, 1995; Wiencke *et al.*, 2000).

Similarly, following longitudinal shifts in temperature, species inhabiting cold-water environments are likely to display optimum metabolic rates at much lower temperatures than those native to warm water regions. Antarctic species display optimum rates of photosynthesis between temperatures of 10-20°C while the optimum range for tropical species lies between 25-35°C (Bischoff *et al.*, 1993; Eggert and Wiencke, 2000; Pakker *et al.*, 1995; Terrados and Ros, 1992). For example, the activity of ribulose-l, 5-bisphosphate carboxylase, (RuBisCO, catalysing enzyme in the Calvin cycle) in Antarctic diatoms species was observed to function efficiently at much lower temperatures compared to temperate species (Descolas-Gros and de Billy, 1987). However, similar acclimation responses can also be induced on a daily or seasonal scale to compensate for temperature changes, being mirrored in the metabolic processes of which they regulate. For example, seasonal variability in the activity of glyceraldehyde-3-phosphate dehydrogenase (GADPH) and RuBisCO, involved in carbon metabolism in the brown algal kelp, *Laminaria saccharina*, displayed seasonal peaks in their activity with which the photosynthetic capacity was correlated (Davison and Davison, 1987).

In addition to proteins, the physical properties of several biological membranes are also temperature sensitive (Schmid *et al.*, 2014). Alterations in the composition of fatty acids

comprising the lipid bilayer are reported to occur in response to temperature changes. This is considered as an adaptive mechanism to maintain an optimum phase state for membrane associated activities such as ion permeability and electron transfer, among others (Becker *et al.*, 2010; Schmid, 2016). At a given temperature, the degree of unsaturation of fatty acids will determine the membrane fluidity. For example, at low temperatures, an increase in the proportion of unsaturated fatty acids supports membrane fluidity, preventing solidification. Conversely, high temperatures are known to cause fluidization of membranes which can lead to the disintegration of the lipid bilayer (Los and Murata 2004). To counteract this, an increase in the proportion of saturated fatty acids is observed in an effort to the adjust the membrane's rigidity (Becker *et al.*, 2010; Gombos *et al.*, 1994; Moon *et al.*, 1995; Schmid *et al.*, 2014).

However, pronounced, and/or prolonged, exposure to extreme temperatures *i.e*. those significantly lower or higher than the natural range experienced by the algae, can impose adverse effects on key physiological processes. For example, the integrity and functionality of the photosynthetic machinery can be damaged by both low and high temperatures (Berry and Bjorkman 1980). In the literature three major physiological temperature sensitive sites are reported; the oxygen evolving complex, ATP generation and the Calvin cycle (Allakhverdiev *et al.*, 2008; Eggert, 2012). Adverse effects on membrane fluidity, protein stability, water-splitting complexes, electron transport chain, phosphorylation and enzymatic inactivation are all incurred by exposure to both low and high temperatures. The exact mechanisms of which have been outlined in detail in Berry and Bjorkman, (1980), Allakhverdiev *et al.* (2008) and Yadav (2010). Such adverse alterations of photosynthetic machinery, onset by either low or high temperatures, can have significant knock-on effects on the cellular oxidative state, particularly when considering the interaction with irradiance and their synergistic effects on the photosensitivity of a species.

For example, low temperatures typically result in a reduced rate of enzymatic activity of the Calvin Cycle (Becker *et al.*, 2010; Davison, 1991). As a result, under high irradiance levels, the light absorbed will exceed that which can be utilized, resulting in photo-inhibition (Berry and Bjorkman 1980). Moreover, high temperatures may cause heat labile

enzymes involved in $CO_2$ fixation to become inactivated and/or denatured leading to a similar fate (Feller *et al.*, 1998; Law and Crafts-Brandner, 1999). This limitation in electron flow will increase the pressure of the electron transport chain, supplying more electrons for the Mehler reaction, yielding an increased reduction of oxygen ($O_2$,) to the superoxide anion ($O_2^{\bullet-}$) (Das and Roychoudhury, 2014). However, owing to its relatively short half-life, $O_2^{\bullet-}$ does not induce extensive damage by itself. Rather through its sequential transformation into more reactive forms, $^1O_2$ (singlet oxygen) and $H_2O_2$ (hydrogen peroxide) can the elevated production of $O_2^{\bullet-}$ incur oxidative damage. The subsequent reactivity of these molecules with other radicals, as well as metal ions (e.g. Fenton reaction), can trigger a further cascade in radical formation such as hydroxyl radicals (OH•). the This highly aggressive species disturb normal biochemical functions due to its role in denaturing nucleic acids and proteins and oxidising lipids (Bischof and Rautenberger, 2012; Das and Roychoudhury, 2014; Halliwell, 2006).

However, most intertidal seaweeds, particularly in temperate regions, have evolved a heightened tolerance to rapid fluctuations in temperature, enabling them to cope with acute periods of thermal stress (Eggert, 2012). Such thermal acclimation involves several processes which function to mitigate the negative effects rapid or extreme changes in temperature can impose on vital metabolic processes. For example, the rapid induction of heat shock proteins (hsp) function to protect against the folding and degradation of cellular proteins during short-term periods of cold or heat stress (Ireland *et al.*, 2004). An increase in the production of low molecular weight compatible solutes such as mannitol has been seen to protect against the osmotic stress during freezing (Pearson and Davison, 1994). The increased activity of antioxidant enzymes, superoxide dismutase (SOD), glutathione reductase (GR) and ascorbate peroxidase (APX) are reportedly positioned at “the front line of defence” against ROS damage in some polar species (Aguilera *et al.*, 2002).

Phlorotannins also function as potent antioxidants in brown algae, reducing the degree of oxidative stress imposed by external stressors and have previously been reported to correspond to the vertical distribution of brown species along a shore gradient (Schoenwaelder *et al.*, 2003). However most related stuides, to date, have associated their abundance with exposure to elevated irradiance, particularly ultraviolet radiation (UVR)

(Abdala-Díaz *et al.*, 2006; Connan *et al.*, 2007; Connan *et al.*, 2004; Gomez and Huovinen, 2010; Kamiya *et al.*, 2010; Pavia and Brock, 2000; Plouguerné *et al.*, 2006), with the effect of temperature alone receiving minimal attention. Particularly considering how little information is available regarding the biosynthesis of phlorotannins, it is perceived to be of benefit, both from an ecological and commercial aspect, to understand the potential effects temperature and its role in regulating phlorotannin metabolism. However, the naturally occurring correlation between irradiance and temperature currently make it difficult to separate their synergistic effects on algal physiology.

The specific effects of irradiance were investigated and discusses in Chapter 4. Here, in this work, the focus was placed on the effect of temperature on LMW phlorotannin profiles, including molecular composition, content and *in vitro* antioxidant activity. Based on the results obtained in the Chapter 4, it was hypothesised that temperatures capable of inducing cellular stress, such as those above the natural range experienced by this population, may act in a similar manner to UVB, stimulating an enhanced synthesis of phlorotannins to aid in the quenching of radical species, the generation of which may also be triggered under elevated temperatures. However, their production was also observed to be sensitive to variations in light conditions. Furthermore, in Chapter 5, the addition of high N concentrations (100 μM) enabled phlorotannin levels to remain stable after 7 days of exposure to high-PAR. This was thought to be linked to the increasing effect of N on enzyme concentrations involved in their production. Due to the effect of temperatures on enzymatic regulation, thermal variations may function to either reduce or increase the rate of phlorotannin synthesis. This abiotic factor is assumed to play a significant role in their natural variability, however, the exact mechanisms through which phlorotannins may be affected, is lacking as a consequence of other factors, such as irradiance and nutrients, being considered more influential. Here, we aimed to address this gap by elucidating on the effect of temperature on algal physiological responses, such as photosynthesis, through Chl *a* fluorescence measurements, as well as the implications, if any, on LMW phlorotannins in the intertidal species *Fucus vesiculosus*. This species makes for an ideal model to investigate the short-term effects of abiotic factors on phlorotannin metabolism due to the ease of extraction and purification as well as the high degree of stability observed in its chemical profile, making variations within highly noticeable. For the same

reasons, it is also an ideal candidate for utilisation in the future commercial application of these compounds.

## 6.2. Materials and methods

### 6.2.1. Reagents and chemicals

1, 3, 5-trihydroxybenzene (phloroglucinol), 2, 2- diphenyl-1-picryhydrazyl (DPPH), 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox®), formic acid (MS grade) and 0.22 µm polytetrafluoroethylene (PTFE) filters, iron (III) chloride hexahydrate, sodium carbonate ($Na_2CO_3$), Folin- Ciocalteu (2N), were all sourced from Sigma-Aldrich Chemical Ltd. (Co. Wicklow, Ireland). HPLC grade methanol, ethyl acetate, acetonitrile, water, and BioDesign Dialysis Tubing™ with 3.5 kDa cut-off were obtained from Fischer Scientific Ltd. (Loughborough, Leicestershire, UK).

### 6.2.2. Collection and preparation of seaweed samples

Approximately 15-20 individual plants (1 plant = all fronds growing from the one holdfast) of *Fucus vesiculosus* (L.) were collected from Flaggy Shore, Finavarra, Co. Clare (53°09'11.7"N 9°07'07.0"W) on the 28$^{th}$ of May 2017 and were brought back to the Algal Bioscience lab in NUI Galway where they were manually cleaned of any epiphytes and external impurities (e.g. sediment) present. They were stored at 11°C (mimicking natural temperature of site at sampling date), under 20 µmol photons $m^{-2}$ $s^{-1}$ (cool white fluorescent tubes, General Electric Company, Fairfield, USA) in filtered natural seawater (salinity of 35), obtained from the obtained from the Ryan Institute Research Centre at Carna, Co. Galway, fitted with aeration apparatus. Samples were kept under these specific conditions for 24 h prior to the experiment to acclimate to pre-experimental laboratory conditions.

### 6.2.3. Culture conditions

After lab acclimation, 100 g fresh weight (FW) of seaweed, consisting of between 15-20 randomLy selected thalli (belonging to different individuals) were frozen. This sample would serve as a control sample with which all lab-controlled treatments would be compared to. Thereafter, 50 g FW of seaweed, comprised of 8-10 individual thalli, was placed in a 2.5 L open-top plastic bowl filled with filtered natural seawater and placed in one of the following three temperatures (7, 17 or 24°C) representing the minimum (7°C) and maximum (17°C) monthly averages in seawater temperature observed for Galway

Bay, as seen in Chapter 2, as well as an elevated temperature (24°C), which during periods of emersion were measured in shallow pools in the intertidal zone at this collection site. Additionally, at each temperature, algae were also placed under 3 different PAR intensities – low (15-20 µmol photons $m^{-2}$ $s^{-1}$), medium (50-60 µmol photons $m^{-2}$ $s^{-1}$) and high (110-115 µmol photons $m^{-2}$ $s^{-1}$) for 7 days. Therefore, in total, 9 experimental conditions were investigated (Fig. 6.1). The media in which they were maintained (natural filtered seawater) were refreshed every second day. For each treatment, a total of 8 bowls was used.

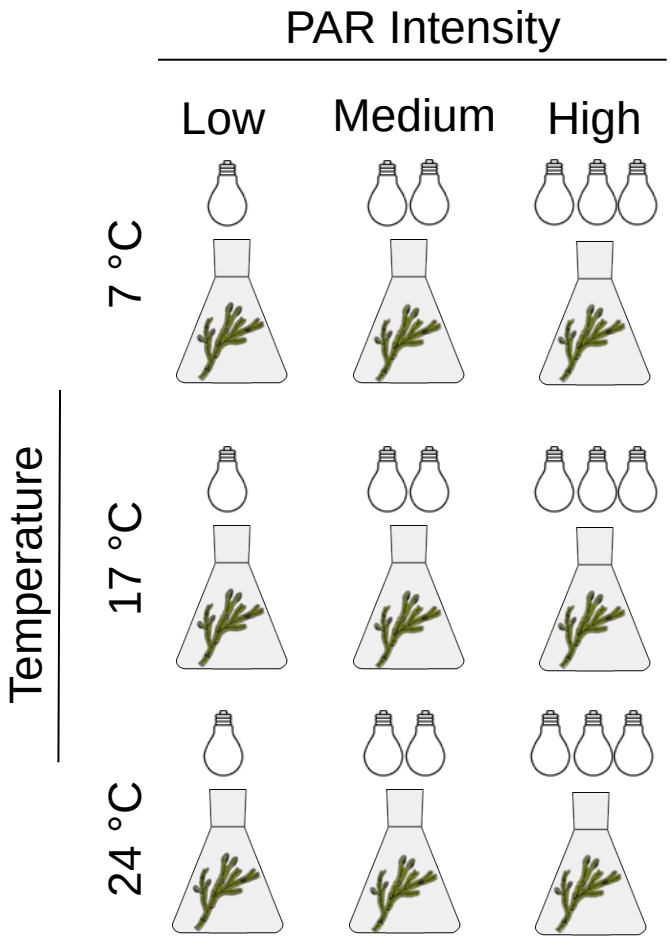



**Fig. 6.1.** The culture conditions employed to investigate the interactive effect of temperature and light on Chl a fluorescence measurements and phlorotannin profiles in *F. vesiculosus*. Samples were exposed to different PAR intensities - low (15-20 µmol photons $m^{-2}$ $s^{-1}$), medium (50-60 µmol photons $m^{-2}$ $s^{-1}$) or high (110-115 µmol photons $m^{-2}$ $s^{-1}$) under three temperature regimes (7, 17 and 24°C) for a total of 7 days.

### 6.2.4. Chl *a* fluorescence measurements

The physiological status of the algae under each treatment was assessed by monitoring the photosynthetic activity after 3 and 7 days of exposure. This was carried out through *in vivo* chlorophyll *a* fluorescence measurements using a Diving Pulse Amplitude Modulated fluorometer (Diving-PAM, Heinz Walz GmbH, Effeltrich, Germany). The method of Nitschke *et al.* (2012) was followed utilizing the measuring principle of Schreiber *et al.* (1986), the details of which are outlined previously in Chapter 4 Section 4.2.4.

### 6.2.5 Analysis of low molecular weight (LMW) phlorotannin profiles

After 3 of exposure, samples from half of the bowls (n=4) in each treatmnent were collected and immediately frozen, while the remaining samples were collected after 7 days of exposure. Thereafter, the samples were freeze-dried using a Labconco Freezone® (Kansas City, MO, USA) freeze-dryer system and ground into a fine algal powder using a coffee bean grinder (DeLonghi, America Inc., New Jersey, USA). The four biological replicates were pooled into one homogenous powder sample (100 g DW) and subsequently vacuum-packed. They were then transported to Teagasc Food Research Centre, Ashtown, Dublin where they were stored in the dark at -20°C until further analysed.

The extraction and purification was carried out on 50 g DW algal powder to obtain LMW phlorotannin-enriched fractions, on which mass spectrometric (Q-ToF-MS and UPLC-MS/MS) analysis was performed, following the method of Tierney *et al.* (2014). The details of the steps involved are outlined in Chapter 2, Section 2.2.6 to 2.2.10.

### 6.2.6 Assessment of *in vitro* antioxidant activity

To examine the effects of the different temperature and irradiance levels on the LMW phlorotannin-enriched fractions *in vitro* antioxidant activity, two colorimetric assays were employed; the ferric reducing antioxidant power (FRAP) and the radical scavenging activity against the radical DPPH·. Each fraction was analysed in triplicate (n=3). The details of each assay are outlined in Chapter 2, Section 2.2.11.

### 6.2.7 Statistical analyses

The potential effect of different temperature and irradiance levels on the photosynthetic parameters obtained from Chl *a* fluorescence measurements on *F. vesiculosus* thalli at each sampling point, *i.e.* day 3 and day 7, was tested using a two-way ANOVA at a significance level of 95% ($p<0.05$). IBM® SPSS® statistical software (version 24) was used for all statistical tests. Normality was tested using the Shapiro-Wilk test for normal distribution and Levene's test for homogeneity of variances. Significant differences between treatments were determined by a Tukey *posteriori homogeneous* (*post-hoc)* test ($p<0.05$). To determine if any significant differences arose within each treatment as a result of time, a paired *t*-test was employed ($p<0.05$) to compare day 3 and day 7.

A three-way mixed ANOVA ($p<0.05$) was conducted to analyse the effects of different temperature and irradiance levels (between-subject factors) over time (within-subject factor) on the total phlorotannin content (TPC), the ferric reducing antioxidant power (FRAP) and the radical scavenging activity (RSA) of the LMW phlorotannin-enriched fractions of *F. vesiculosus*. Normality was tested using the Shapiro-Wilk test for normal distribution and Levene's test for homogeneity of variances while sphericity was tested for using Maulchy's test of sphericity. Individual one-way ANOVA's were conducted for each sampling point, *i.e.* after 3 and 7 days, to identify any significant diffeences between the experimental treatments, compared to the control at day 0. Tukey-tests were performed to find *posteriori homogeneous* (*post-hoc*) sub-groups that differed significantly ($p<0.05$).

## 6.3. Results

### 6.3.1. Chl *a* fluorescence measurements

The photosynthetic performance of *F. vesiculosus* exposed to different temperature and light combination treatments were measured through Chl *a* measurements. Effects on photosynthetic parameters ($F_v/F_m$, $rETR_{max}$, $E_k$) were determined through photosynthesis-irradiance (*P/E*) curves, plotting rETR as a function of PFD (photon flux density; see Supplementary Section 9.6, Fig. 9.6.1).

The maximal efficiency of PS II, $F_v/F_m$, of *F. vesiculosus* thalli grown under the different treatments appeared to be unaffected by either temperature ($p$=0.208), irradiance ($p$=0.22) or their interaction ($p$=0.897; Table 6.1) after 3 days of exposure. However, the light level of the treatment was observed to cause significant differences after 7 days ($p$=0.034) with samples grown under high-PAR at either 7 or 24°C, displaying a lowered $F_v/F_m$ value compared to all other treatments (0.698, 0.708 rel. units; respectively; Table 6.1). Such a decline was not observed in the samples grown at 17°C exposure. However, as no significant effects were not incurred by the temperature level of the treatment ($p$=0.272) or the interaction between temperature and irradiance ($p$=0.217), overall no significant differences were observed between treatments after 7 days of exposure (Table 6.1).

The maximum relative electron transport rate, $rETR_{max}$, displayed by the algae was significantly affected by both the temperature ($p$=0.016), irradiance ($p$<0.001) as well as the interaction between the two factors ($p$=0.038; Table 6.1) after 3 days of exposure. The $rETR_{max}$ levels appeared to increase with increasing temperature ($p$<0.001; Table 6.1). For example, individuals grown at 7°C displayed the lowest $rETR_{max}$, ranging between 129.5±20.9 to 158.0±14.2 rel. units, while those grown at 24°C displayed the highest $rETR_{max}$ values (294.5±9.0 to 336.7±60.8 rel. units; $p$<0.001; Table 6.1). Within most temperature treatments $rETR_{max}$ appeared inverse to the associated PAR level of the treatment ($p$<0.001; Table 6.1) with the $rETR_{max}$ displayed by the algae decreasing with increasing PAR exposure. A similar significant effect of temperature ($p$<0.001) and irradiance ($p$<0.001) was also observed after 7 days of exposure. After 7 days, the majority of treatments displayed a similar $rETR_{max}$ value to that of day 3, with time imposing no significant effect ($p$>0.05; Table 6.1). However, in one treatment, when the lowest

temperature (7°C) was coupled with the highest irradiance level (110-115 µmol photons $m^{-2}$ $s^{-1}$) a significant decrease of 46 % was observed after 7 days ($p$=0.007; Table 6.1).

**Table 6.1.** Photosynthetic parameters – $F_v/F_m$ (relative units), $rETR_{max}$ (relative units) and $E_k$ (µmol photons $m^{-2}$ $s^{-1}$) for *F. vesiculosus* grown in 3 different temperatures (7, 17 and 24°C) under 3 different PAR levels – low (15-20 µmol photons $m^{-2}$ $s^{-1}$), medium (50-60 µmol photons $m^{-2}$ $s^{-1}$) and high (110-115 µmol photons $m^{-2}$ $s^{-1}$) for 3 or 7 days. Data are represented as mean ± standard deviation (n=3). The effect of temperature, irradiance and exposure time on each parameter was tested using a three-way ANOVA ($p$<0.05). Superscripts are used to identify significant differences observed between samples for each parameter measured, as given by Tukey's *post-hoc* test. The details of the statistical tests are outlined in Supplementary Section 9.6, Table 9.6.1.

**$F_v/F_m$ [relative units]**

| | Day 3 | | | Day 7 | | |
|---|---|---|---|---|---|---|
| **PAR** | **7 °C** | **17 °C** | **24 °C** | **7 °C** | **17 °C** | **24 °C** |
| **Low** | 0.731 ± 0.02 | 0.746 ± 0.01 | 0.719 ± 0.02 | 0.740 ± 0.01$^{a}$ | 0.745 ± 0.02 | 0.744 ± 0.00 |
| **Med** | 0.713 ± 0.03 | 0.724 ± 0.02 | 0.715 ± 0.02 | 0.731 ± 0.01$^{a}$ | 0.727 ± 0.04 | 0.736 ± 0.01 |
| **High** | 0.722 ± 0.02 | 0.725 ± 0.01 | 0.709 ± 0.02 | 0.698 ± 0.03 | 0.743 ± 0.02 | 0.708 ± 0.01 |

**$rETR_{max}$ [relative units]**

| | Day 3 | | | Day 7 | | |
|---|---|---|---|---|---|---|
| **PAR** | **7 °C** | **17 °C** | **24 °C** | **7 °C** | **17 °C** | **24 °C** |
| **Low** | 129.5 ± 20.9$^{e}$ | 263 ± 38.0$^{bcde}$ | 336.7 ± 60.8$^{ab}$ | 121.8 ± 27.9$^{d}$ | 264.4 ± 53.6$^{ab}$ | 317.7 ± 39.1$^{a}$ |
| **Med** | 130 ± 39.0$^{e}$ | 241.5 ± 5.5$^{bcd}$ | 355.5 ± 37.2 $^{a}$ | 171.4 ± 44.1$^{bcd}$ | 237.5 ± 42.4$^{abc}$ | 316.3 ± 31.7$^{a}$ |
| **High** | 158 ± 14.2$^{cde}$ | 144.5 ± 49.3$^{de}$ | 294.5 ± 9.0$^{abc}$ | 85.5 ± 21.1$^{d}$ | 151.3 ± 1.6$^{cd}$ | 238.0 ± 21.7$^{abc}$ |

**$E_k$ [µmol photons $m^{-2}$ $s^{-1}$]**

| | Day 3 | | | Day 7 | | |
|---|---|---|---|---|---|---|
| **PAR** | **7 °C** | **17 °C** | **24 °C** | **7 °C** | **17 °C** | **24 °C** |
| **Low** | 346.3 ± 86.3$^{c}$ | 477.9 ± 68.7$^{bc}$ | 688.4 ± 57.4$^{a}$ | 312.8 ± 66.5$^{bcd}$ | 464.6 ± 19.1$^{ab}$ | 389.3 ± 69.7$^{abc}$ |
| **Med** | 298.4 ± 88.5$^{c}$ | 456.7 ± 27.3$^{bc}$ | 581.6 ± 67.6$^{ab}$ | 333.5 ± 18.3$^{bcd}$ | 436.7 ± 95.2$^{abc}$ | 539.9 ± 65.0$^{a}$ |
| **High** | 318.4 ± 11.6$^{c}$ | 282.0 ± 93.0$^{c}$ | 521.2 ± 9.4$^{abc}$ | 199.1 ± 20.2$^{d}$ | 280.8 ± 14.6$^{cd}$ | 405.5 ± 29.3$^{abc}$ |

After 3 days of exposure, the light saturation point, $E_k$, appeared to be affected by both the temperature ($p$=0.007) and irradiance level ($p$<0.001) to which the algae were exposed

to but not the interaction between the two factors ($p$=0.15; Table 6.1). Similarly to $rETR_{max}$, $E_k$ values were observed to increase with increasing temperatures ($p$<0.05; Table 6.1). After 3 days, samples grown at 7°C displayed the lowest $E_k$ values (298.4±88.5 to 346.3±86.3 µmol photons $m^{-2}$ $s^{-1}$) while those at 24°C displayed the highest (521.2±9.4 to 688.4±57.4 µmol photons $m^{-2}$ $s^{-1}$). Furthermore, again similar to $rETR_{max}$, the $E_k$ values displayed by the algae under different treatments also appeared to decrease with increasing PAR exposure ($p$<0.05; Table 6.1). However, this was more pronounced in samples grown in the higher temperatures of 17 and 24°C as those grown in 7°C displayed a similar $E_k$ level in all PAR levels. After 7 days, temperature ($p$<0.001) and irradiance ($p$<0.001) maintained their significant effects on the $E_k$ values in a similar manner to that observed at day 3. In fact, within most treatments, no significant differences had arisen over time ($p$>0.05). However, now the effect of increasing PAR on decreasing the $E_k$ values of the algae under the lower temperature of 7°C had become more pronounced, now displaying a significant decrease of 37% from day 3 ($p$=0.004; Table 6.1).

### 6.3.2. Total phlorotannin content (TPC) of LMW phlorotannin-enriched fractions

The control sample, representing the natural phlorotannin profile after 24 h lab acclimation prior to experimental manipulation, displayed a high level of phlorotannins (568.8±13.7 µg PGE mg $DWE^{-1}$; Fig. 6.2). The TPC levels appeared to be affected by the temperature ($p$=0.009) of the treatment in which the algae were grown. This was particularly evident after 3 days of exposure as while all treatments had resulted in a decline from the control at this time point, the samples grown at the higher temperature of 24°C, regardless of the associated PAR level, possessed the lowest phlorotannin levels (424.0±23.3 - 430.6±18.9 µg PGE mg $DWE^{-1}$; Fig. 6.2).

Time also appeared to affect the observed phlorotannin levels ($p$<0.001) as by day 7, the phlorotannin levels in samples grown at 24°C had increased, displaying levels similar to the two lower temperatures under all PAR level (Fig. 6.2). The increase observed in samples grown in 24°C, over time, however, appeared to relate to the PAR level. For example, samples under low-PAR displayed a 21% increase, under intermediate levels a 18% increase was seen while those under high-PAR displayed the smallest increase over time (11%; Fig 6.2). Overall, after 7 days exposure, phlorotannin levels were more

affected by the degree of PAR exposure ($p$=0.040) than the temperature, with all temperatures treatments resulting in uniform phlorotannin levels at each PAR level, the highest of which was seen under low-PAR (499.5±18.3 to 541.5±12.01 µg PGE mg $DWE^{-1}$ and the lowest levels was observed under high-PAR treatment (479.6±12.6 to 490.7±13.7 µg PGE mg $DWE^{-1}$; Fig. 6.2).

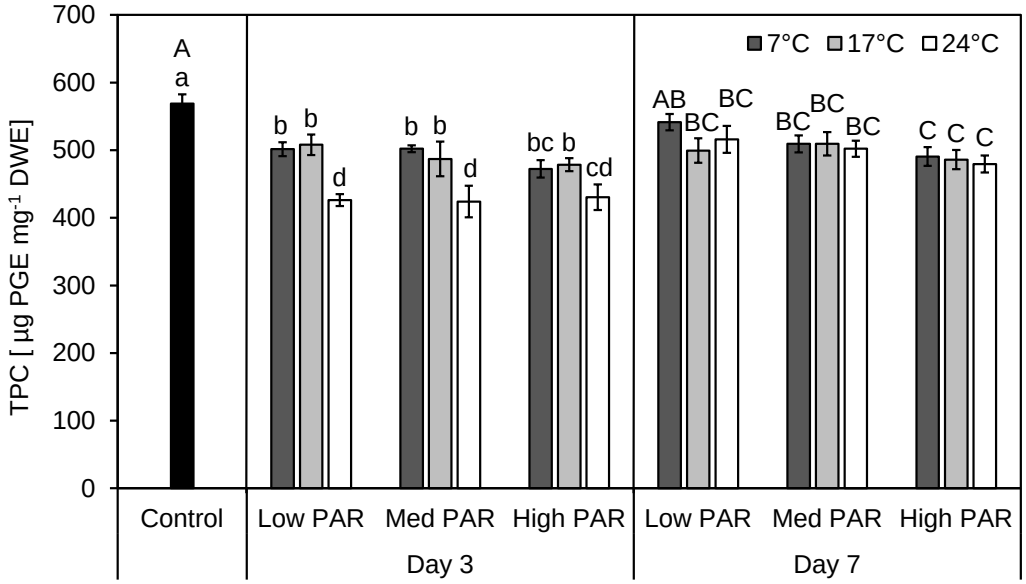



**Fig. 6.2.** The total phlorotannin content (TPC) of the LMW phlorotannin fractions derived from *F. vesiculosus* grown in 3 different temperatures (7, 17 and 24°C) under 3 different PAR levels – low (15-20 µmol photons $m^{-2}$ $s^{-1}$), medium (50-60 µmol photons $m^{-2}$ $s^{-1}$) and high (110-115 µmol photons $m^{-2}$ $s^{-1}$) for 3 or 7 days, compared to the control. The control sample was representative of a collection of individual thalli that were frozen after 24 h lab acclimation, prior to experimental manipulation. Data are represented as mean ± standard deviation (n=3) expressed as microgram phloroglucinol equivalents per milligram dry weight extract (µg PGE mg $DWE^{-1}$). The effects of temperature, irradiance and exposure time were tested using a three-way mixed ANOVA ($p$<0.05). Details of the statistical test are outlined in Supplementary Section 9.6, Table 9.6.3. Superscripts distinguish significant differences between treatments after either 3 (lowercase) or 7 (uppercase) days of exposure, compared to the control sample at day 0, using Tukey's *post-hoc* test ($p$<0.05).

### 6.3.3. Characterisation of LMW phlorotannin profiles

The control, *i.e.* a collection of individual thalli that were frozen after 24 h lab acclimation, prior to experimental manipulation, displayed a high abundance of LMW phlorotannins, particularly those between *m/z* 497-993 corresponding to 4-8 PGUs. Phlorotannins within this molecular mass range accounted for 89% of the total abundance. Polymers of 5, 4 and 8 PGUs were the three most dominant detected, in order of descending abundance (Fig.

6.3; Fig. 6.4). Phlorotannins composed of 5 PGUs (*m/z* 621) were most abundant, comprising 34% of total phlorotannins detected. This was followed by structures composed of 4 PGUs (*m/z* 497; 22%) and 8 PGUs (*m/z* 993; 17%). Larger structures ranging between 10-16 PGUs only accounted for 1.5%, collectively. The presence of even larger polymers, up to 20 PGUs, were detected in the Q-ToF-MS spectrum, however, magnification (x16) was required to observe these compounds due to their low abundance (Fig. 6.3).

Variations in chemical composition from the control occurred in relation to both the temperature at which algae were grown, as well as PAR level. Samples grown in the lower temperature of 7°C displayed a high abundance of phlorotannins structures composed of 5 PGUs in most treatments (37-43%; Fig. 6.4). The lowest % were seen under high-PAR exposure. This was a considerable increase on the control whereby the abundance for the same structures accounted for 34% of the total. Samples grown at 17°C displayed an overall reduced contribution of 4-8 PGUs to the total phlorotannin abundance (89-92%) and thus had a composition similar to that of the control sample. Over time and under the different PAR levels, profiles observed for samples grown at 17°C appeared relatively stable (Fig. 6.4).

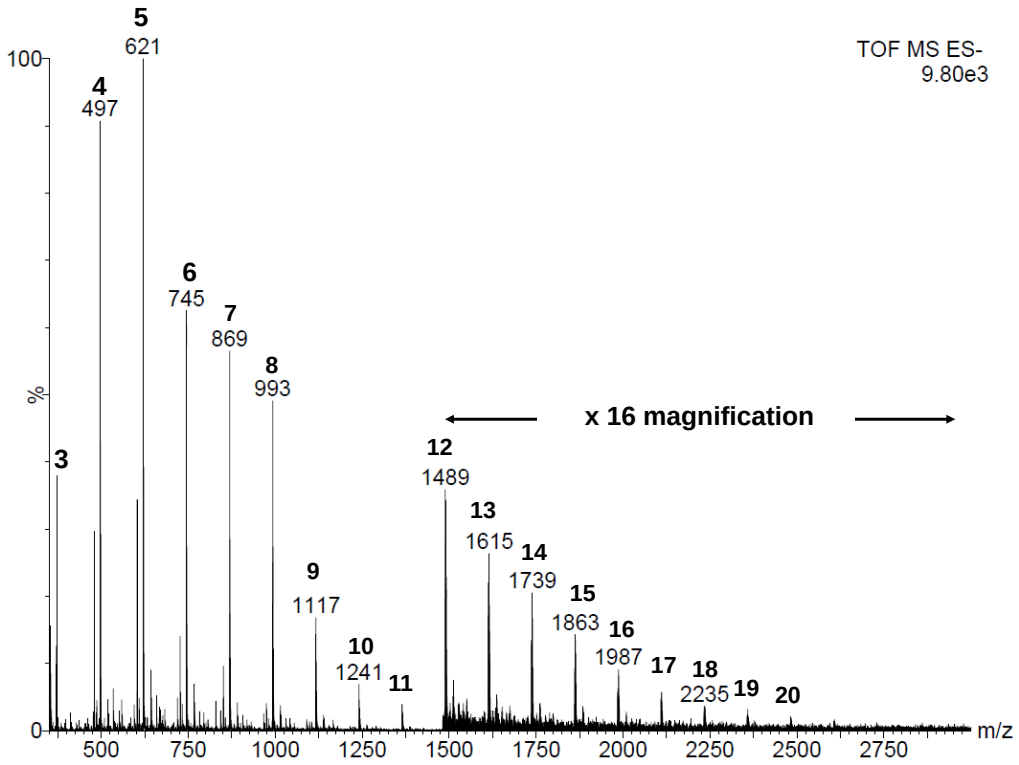



**Fig. 6.3.** Q-ToF-MS spectrum, in negative ion mode, of *F. vesiculosus*, after 24 h lab acclimation prior to experimental manipulation *i.e*. the control sample, displaying a high abundance of LMW phlorotannin compounds. $[M-H]^-$ ions (m/z) is indicated by the number immediately above each peak and the corresponding degree of polymerisation (in phloroglucinol units; PGUs) as the most upper bold numbers above. The presence of larger phlorotannin oligomers is shown with magnification (x16).

In samples grown at 24°C temperatures, phlorotannins composed of 4-8 PGUs accounted for 91-94 % of the total abundance. Short-term changes were observed relating to the PAR level under which the algae were maintained. For example, after 3 days the % contributed to the total phlorotannin abundance by structures with a MW of 498 Da (4 PGUs) decreased as PAR intensity increased (25, 22 and 19%, respectively). This was coupled with an increase in the % of structures with a MW of 622 Da (5 PGUs) with increasing PAR levels (35, 40, and 43%, respectively). However, after 7 days' exposure, the abundance of structures with both these MW levelled off and no effect of different PAR levels was detected (Fig. 6.4). A stark increase in the abundance of the larger polymers composed of 8 PGUs (994 Da) was observed under high PAR after 7 days, now accounting for 21% of the total phlorotannins detected, compared to the 15-17% observed in other light treatments at the same temperature.

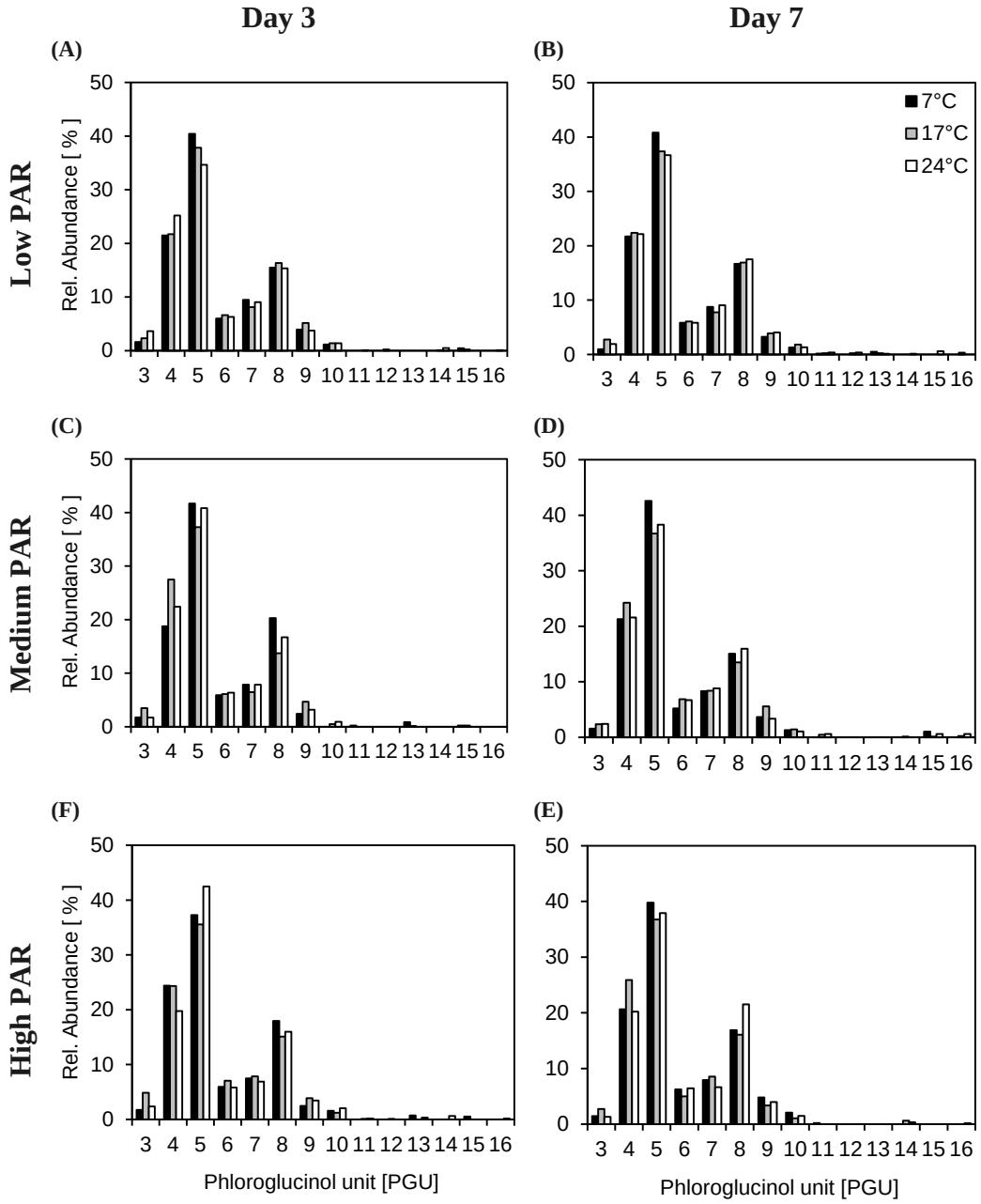



**Fig. 6.4.** The relative abundance (%) of phlorotannin structures between 3-16 phloroglucinol units (PGUs), as detected by UPLC-MS/MS, in the LMW phlorotannin fractions derived from *F. vesiculosus* grown in 3 different temperatures (7, 17 and 24°C) under 3 different PAR levels – low (15-20 µmol photons $m^{-2}$ $s^{-1}$), medium (50-60 µmol photons $m^{-2}$ $s^{-1}$) and high (110-115 µmol photons $m^{-2}$ $s^{-1}$) for 3 or 7 days.

### 6.3.4. Degree of isomerisation

The total number of isomers for all LMW phlorotanin fractions was derived through the sum of all isomers detected for the 13 MRM transitions (3-16 PGUs), as detected by the UPLC-MS/MS. The control sample displayed a total isomeric count of 136 (Table 6.2). The highest abundance of isomers was observed in structures ranging between 5-9 PGUs and polymers composed of 7 PGUs (*m/z* 869), in particular, possessed the overall highest isomer levels (Table 6.2).

After 3 days of exposure to 7°C, the total number of isomers appeared to relate to the PAR level of the treatment. For example, samples exposed to low-PAR displayed a similar total number to the control while high-PAR exposure led to a decrease compared to the control (Table 6.2). A similar pattern was also seen for samples grown at 24°C with, again, increasing PAR exposure resulting in a lower total number of isomers. However, the overall count was higher at this higher temperature (Table 6.2). Treatment with 17°C, however, yielded an opposing effect, with exposure to medium and high-PAR levels, resulting in elevated isomeric abundance after 3 days.

By day 7, samples grown at the lower temperature of 7°C displayed a decrease in the number of isomers detected under all PAR levels. This decrease was more pronounced under medium and high-PAR levels. Conversely, in samples grown at 17°C exposure, to medium and high-PAR levels led to a reduced isomeric abundance (Table 6.2). Samples grown at 24°C displayed a lower, but stable, isomeric count under all PAR levels (Table 6.2). However, this was again further reduced under low-PAR.

**Table 6.2.** List of isomers for each individual MRM transition between 3-16 phloroglucinol units (PGUs), as detected by UPLC-MS/MS, in samples of *F. vesiculosus* grown in 3 different temperatures (7, 17 and 24°C) under 3 different PAR levels – low (15-20 µmol photons $m^{-2}$ $s^{-1}$), medium (50-60 µmol photons $m^{-2}$ $s^{-1}$) and high (110-115 µmol photons $m^{-2}$ $s^{-1}$) for 3 or 7 days, compared to the control. The control sample was representative of a collection of individual thalli that were frozen after 24 h lab acclimation, prior to experimental manipulation. Where 10 or more individual isomers were detected for an individual molecular ion transition the number is highlighted in bold.

**Day 3**

| | | 7°C | | | 17°C | | | 24°C | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **PGU** | **Control** | **LP** | **MP** | **HP** | **LP** | **MP** | **HP** | **LP** | **MP** | **HP** |
| **3** | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| **4** | 4 | 3 | 3 | 3 | 3 | 2 | 3 | 3 | 2 | 2 |
| **5** | **18** | **25** | **22** | **18** | **25** | **14** | **29** | **22** | **36** | **14** |
| **6** | **24** | **21** | **16** | **11** | **22** | **16** | **24** | **19** | **22** | **14** |
| **7** | **32** | **41** | **41** | **37** | **32** | **41** | **41** | **50** | **37** | **37** |
| **8** | **28** | **28** | **25** | **22** | **34** | **22** | **28** | **25** | **19** | **19** |
| **9** | **19** | **10** | **15** | **10** | **17** | **13** | **17** | **17** | **13** | **13** |
| **10** | 6 | 3 | 5 | 5 | 6 | 3 | 6 | 6 | 3 | 5 |
| **11** | 2 | 0 | 0 | 0 | 0 | 2 | 2 | 0 | 4 | 2 |
| **12** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **13** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **14** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **15** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **16** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Total** | **136** | **134** | **129** | **108** | **117** | **153** | **145** | **145** | **139** | **109** |

**Day 7**

| | | 7°C | | | 17°C | | | 24°C | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **PGU** | **Control** | **LP** | **MP** | **HP** | **LP** | **MP** | **HP** | **LP** | **MP** | **HP** |
| **3** | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 |
| **4** | 4 | 2 | 2 | 2 | 3 | 3 | 3 | 2 | 2 | 1 |
| **5** | **18** | **18** | **18** | **18** | **22** | **14** | **14** | **14** | **18** | 7 |
| **6** | **24** | **16** | **16** | **16** | **19** | **19** | **16** | **13** | **16** | **14** |
| **7** | **32** | **37** | **23** | **41** | **46** | **27** | **27** | **27** | **41** | **50** |
| **8** | **28** | **22** | **19** | **12** | **34** | **28** | **19** | **25** | **25** | **22** |
| **9** | **19** | **19** | **8** | **11** | **13** | **15** | **10** | **15** | **13** | **15** |
| **10** | 6 | 6 | 3 | 0 | 3 | 6 | 2 | 8 | 2 | **12** |
| **11** | 2 | 0 | 0 | 0 | 2 | 4 | 0 | 2 | 2 | 4 |
| **12** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **13** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **14** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **15** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **16** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Total** | **136** | **122** | **91** | **104** | **145** | **120** | **93** | **109** | **122** | **129** |

### 6.3.5. *In vitro* antioxidant activity

#### 6.3.5.1. Ferric reducing antioxidant power (FRAP)

A high level of ferric reducing antioxidant power (FRAP) activity was observed for the control (703.6±8.7 μg TE $mg^{-1}$ DWE). Within the experimental treatments, the observed FRAP levels were significantly affected by both the temperature ($p$<0.001) and irradiance level of the treatment ($p$<0.001) as well as the interaction of the two factors ($p$<0.001: Fig. 6.5). For example, after 3 days of treatment, the activity of samples grown at 7 and 24°C appeared to be slightly reduced with increasing PAR levels while, conversely, in samples grown at 17°C, activity increased with increasing PAR exposure ($p$<0.001; Fig. 6.5). A similar trend was also evident after 7 days of exposure, with samples grown at 7 and 24°C displaying a decrease in activity with increasing PAR exposure ($p$<0.001; Fig. 6.5). However, now, the activity of samples grown at 17°C also displaying a decrease in FRAP with increasing PAR exposure ($p$<0.001; Fig. 6.5). Despite the temperature of the treatment, all samples displayed the lowest FRAP activity under high-PAR levels after 7 days of exposure ($p$<0.001; Fig 6.5).

#### 6.3.5.2. Radical scavenging activity (RSA)

The radical scavenging activity (RSA) of the LMW phlorotannin-enriched fractions from both the control and experimentally-treated samples is illustrated in Fig. 6.5, expressed in terms of $IC_{50}$ values. The control sample displayed an $IC_{50}$ value of 21.6 ± 4.3 μg $mL^{-1}$. The temperature of the treatment in which the algae were grown imposed a significant effect on the RSA levels ($p$=0.009) while the irradiance level had no effect ($p$=0.222; Fig. 6.5). However, after 3 days samples grown at the lower temperature of 7°C displayed a slightly lower activity, across all PAR levels, compared to the other two higher temperature treatments (Fig. 6.5). Yet, it was only when samples grown at 7°C were exposed to high PAR that a significant decrease from the control was seen ($p$<0.05; Fig. 6.5).

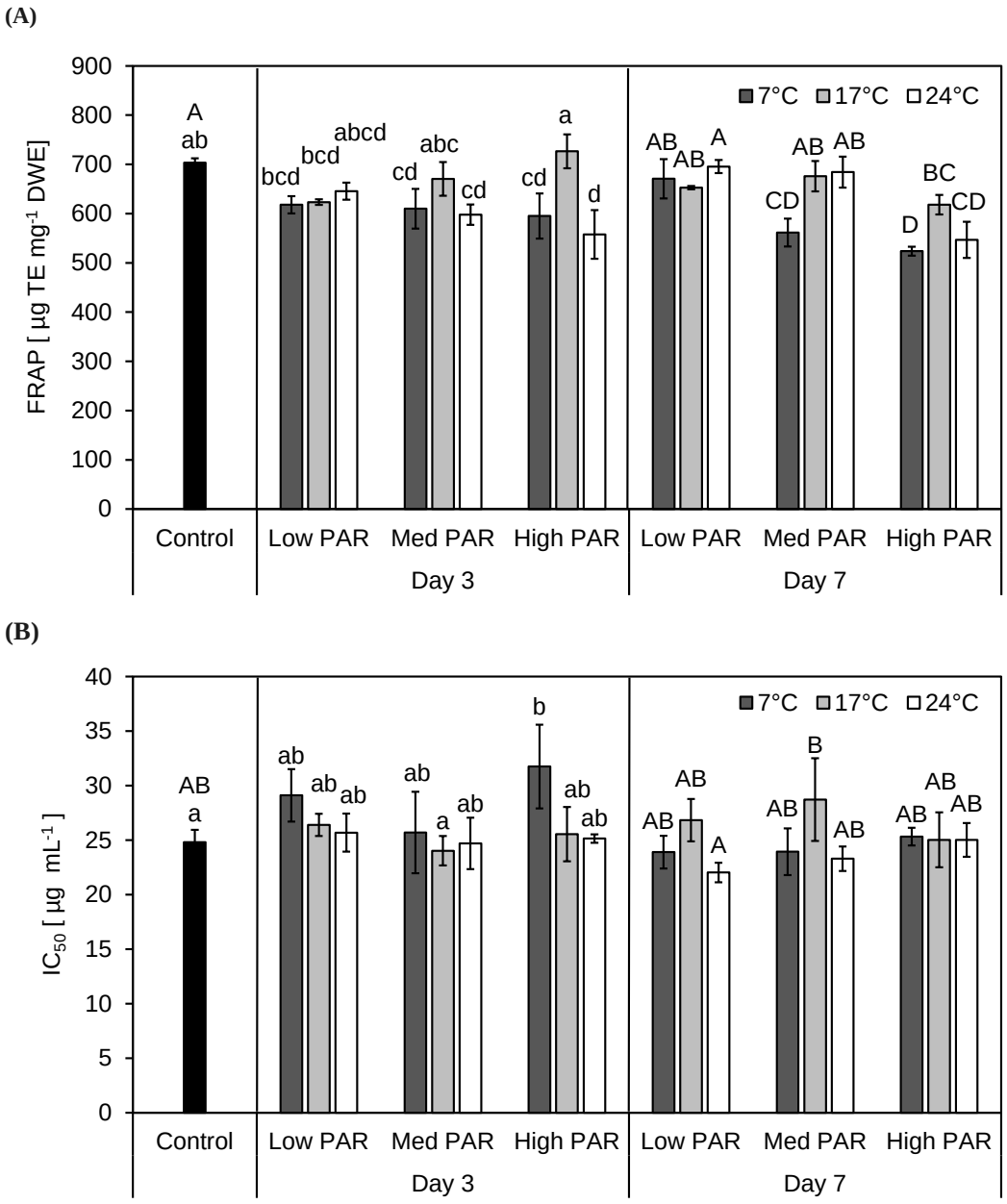



**Fig. 6.5.** (A) The FRAP and (B) RSA ($IC_{50}$) levels obtained for LMW phlorotannin fractions derived from *F. vesiculosus* grown in 3 different temperatures (7, 17 and 24°C) under 3 different PAR levels – low (15-20 µmol photons $m^{-2}$ $s^{-1}$), medium (50-60 µmol photons $m^{-2}$ $s^{-1}$) and high (110-115 µmol photons $m^{-2}$ $s^{-1}$) for 3 or 7 days, compared to the control. The control sample was representative of a collection of individual thalli that were frozen after 24 h lab acclimation, prior to experimental manipulation. Data are represented as mean ± standard deviation (n=3). FRAP values are expressed as µg TE $mg^{-1}$ DWE while $IC_{50}$ value are expressed as microgram per millilitre (µg $mL^{-1}$). Significant effects of the experimental conditions to which the algae were exposed were tested using a three-way mixed ANOVA ($p$<0.05). Details of the statistical test are outlined in Supplementary Section 9.6, Table 9.5.2. Superscripts distinguish significant differences between treatments after either 3 (lowercase) or 7 (uppercase) days of exposure, compared to the control sample at day 0, using Tukey's *post-hoc* test ($p$<0.05).

Exposure time also affected the RSA displayed ($p$=0.008), as after 7 days, samples grown at 7°C displayed an increase in RSA (from day 3) under all PAR environments with all samples, regardless of the temperature or irradiance level of the treatment, now displayed similar activity ($p$>0.05; Fig. 6.5). The only significant difference observed was between those grown at 24°C under low PAR and those at 17°C under medium PAR ($p$<0.05; Fig. 6.5).

## 6.4. Discussion

Natural seawater temperatures, as well as irradiance and nutrient concentrations, display strong seasonal variability in temperate regions. Collectively, these abiotic factors cause variations in the natural rates of algal metabolism and thereby alter the concentration and composition of several secondary metabolites. However, temperature, despite its importance in regulating the enzymatic reactions and thus controlling metabolic rates, is the least studied factor potentially impacting *in situ* phlorotannin concentrations. So far, whenever temperature effects on algal physiology were investigated, they were complexed with the simultaneous effect of both irradiance and dehydration. Therefore, the sole effect of temperature on phlorotannin metabolism requires further attention. In this study the effect of temperatures, within and just above the natural range of the sampling site were investigated on the phlorotannin chemical profiles of *F. vesiculosus*. Due to the natural co-occurrence of light and temperature, three different PAR levels were also examined. Here, both the temperature, as well as the PAR intensity to which samples of *F. vesiculosus* was subjected to, were observed to significantly affect the LMW phlorotannin profiles after both 3 and 7 days of exposure.

Previously, Nygård and Dring (2008) reported that the temperature range for optimal photosynthetic efficiency in *F. vesiculosus* inhabiting the Irish Sea lies between 15-20°C, decreasing when grown at a higher temperature of 25°C. Yet here, Chl *a* fluorescence measurements showed that temperature changes between 7-24°C did not impose any apparent physiological stress on *F. vesiculosus* populations inhabiting the west coast of Ireland. Tolerance to such a broad range of temperature mirrors the local adaptation of this species to the natural changes in seawater temperature occurring in Galway Bay. Such a high degree of thermal acclimation may also contribute to, among other factors, the dominance exhibited by *F. vesiculosus* within the intertidal zone.

However, temperature-dependent changes in photosynthetic rates were observed with both $rETR_{max}$ and $E_k$, displaying a positive correlation with temperature. This is possibly a reflection of the temperature-induced increase in the rate of photosynthesis. The $Q_{10}$, temperature coefficient of photosynthesis (among other metabolic processes) is modelled at 2 (Hurd *et al.*, 2014). That is, an increase in temperature by 10°C will result in a two-

fold increase in the rate of the chemical reactions involved. Above this, thermal inactivation of various components therein will cause a decrease in the rate of activity (Davison, 1991). Out of the three temperatures investigated, photosynthetic activity was highest in samples grown at the higher temperature of 24°C. A similar effect was also observed by Graiff *et al.* (2015) examining the temperature tolerance of *F. vesiculosus* populations of the Baltic Sea. Conversely, significantly lower $rETR_{max}$ and $E_k$ values were observed in samples exposed to low temperatures of 7°C. This reduced photosynthetic capacity was most likely attributed to a decreased rate of activity in key enzymes involved in major photosynthetic reactions such as ATP generation, phosphorylation and $CO_2$ fixation (Allakhverdiev *et al.*, 2008; Eggert *et al.*, 2003) as well as the impaired synthesis of light-harvesting pigments that can occur at low temperatures (Machalek *et al.*, 1996).

Along with temperature, the degree of PAR exposure was also a prominent factor affecting the photosynthetic state of the algae. At all temperatures, high-PAR levels decreased the photosynthetic performance. Such an adverse effect was also observed in Chapter 4, as well as in past studies carried out on both Laminariales (Dring *et al.*, 2001) and other Fucales (Abdala-Díaz *et al.* 2006), where elevated PAR levels were associated with photo-inhibition to a greater extent than UVR. In the present study, high-PAR exposure imposed the most severe effects when coupled with the lower temperature of 7°C. Typically, when high irradiance levels are coupled with low temperatures, the absorption of energy will exceed that which can be utilized (Berry and Bjorkman 1980). This is owed to the reducing effect of low temperatures on the activity of enzymes involved the catalysis of light-independent (Calvin) reactions. Therefore, amount of light required to saturate these reactions will also be lowered and the threshold over which photo-inhibition occurs, will decline. However, in relation to the LMW fractions, this reduction in photosynthetic rates did not appear to affect the corresponding phlorotannin content of samples grown at 7°C under high-PAR, compared to the two other higher temperatures. In general, phlorotannin content was reduced (by approx. 4-6%) by exposure to high-PAR, regardless of temperature, the degree of which became more pronounced over time *i.e.* after 7 days. However, high growth temperatures incurred the most significant effect.

After 3 days of exposure to the different combination treatments, the phlorotannin content of the LMW fractions displayed a significant decrease under elevated temperatures, regardless of PAR intensity. However, over time these levels increased and by day 7, the phlorotannin content of samples grown at the three temperatures were similar to one another. The fast rate at which their concentrations can be re-established may explain the lack of effect observed in previous temperature investigations (Endo *et al.*, 2013, 2015).

The few studies that have investigated the effect of temperature on phlorotannins all reported that artificial increases in temperature failed to induce any effect on their levels (Endo *et al.*, 2013, 2015; Hay *et al.*, 2010; Poore *et al.*, 2013). For example, Endo *et al.* (2013, 2015), upon finding no significant effect of temperature on phlorotannin content in *Sargassum* species, after 8 and 9 days, concluded that the effect of temperature, if any, may be too small to be observed in short-term culture experiments. However, shifts in phlorotannin concentrations have been observed elsewhere after as little as 1-3 days, in other (kelp) species (Cruces and Huovinen, 2012, 2013; Hammerstrom *et al.*, 1998) in response to other environmental factors. While differences between studies can arise due to the specific species under investigation and as well as the extraction method employed, this emphasizes the need for multiple time points for analysis in order to prevent such misinterpretation.

The decrease in phlorotannin levels after 3 days' exposure to 24°C could suggest that high temperatures, *i.e.* those outside the range of their natural environment, may incur thermal damage to structures involved in their biosynthesis, particularly if these components are heat labile. The Golgi-endoplasmic reticulum (ER) complex has been deemed as the most probable location of phlorotannin biosynthesis (Sanders *et al.*, 2005; Schoenwaelder and Clayton, 2000). Such structural damage could result in an imbalance between the rate of phlorotannin production and their subsequent oxidation, as such high temperatures may enhance the generation of intracellular ROS (Suzuki and Mittler 2006). A similar theoretical conclusion was reached by Cruces and Huovinen (2013). They suggested that temperatures of 20°C, under which lipid peroxidation was increased and photosynthetic activity was reduced in three south Pacific kelps after 3 days, could also inhibit the synthesis of phlorotannins.

Figueroa *et al.* (2014) also found a decrease in the phenolic content of *Cystoseira tamariscifolia* when exposed to a 4°C increase in the ambient seawater temperature. This was also associated with a decrease in total fatty acids (TFA). Similarly, Schmid (2016) found the TFA of *F. serratus* to be highest under the lower temperature of 10°C, compared to 15 or 20°C after 12 d of exposure. Figueroa *et al.* (2014) suggested that perhaps a link existed between these primary and secondary compounds which may explain their similar fate under elevated temperatures. Such a connection could arise from their shared metabolic derivatisation via the condensation of acetate and malonate units (Amsler and Fairhead, 2006; Maschek and Baker, 2008; Meslet-Cladière *et al.*, 2013; Pelletreau, 2008). In fact, eicosapentaenoic acid (EPA), a long chained poly unsaturated fatty acid (C20:5 n-3; PUFA) has been proposed as a precursor for phloroglucinol formation (El Hattab *et al.* 2009).

If the responses of both metabolites (within a given species) to external parameters are similar, it may be indicative of a specific effect acting on a shared section of their biosynthetic pathway. Therefore, if exposure to elevated temperatures results in a similar fate for both fatty acids and phlorotannins, it may suggest that elevated heat negatively effects an earlier section of the acetate-malonate pathway, prior to their deviation. The initial "heat shock", associated with rapid increases in temperature, may have caused a reduced/halted activity of enzymes involved in the catalysis of the acetate-malonate reactions, such as acetyl-coenzyme A and/or a specific (or multiple) polyketide synthases (Maschek and Baker 2008). However, this is merely a hypothesis and further research would be required for its confirmation.

By day 7 samples grown in all temperatures displayed uniform phlorotannin levels. This may suggest that the rate of their production was resumed and a time-dependent restoration in their metabolic balance occurred. Again, this could potentially arise from thermal acclimation of key regulatory enzymes involved in their biosynthesis (Somero 1978). Temperature effects on their synthesis is assumed over degradation as described and discussed in Chapter 7, in which isolated phlorotannin fractions from this species, *ex vivo*, displayed a high degree of stability in response to temperatures of 50°C (Kirke *et al.*, 2017). Therefore, it is likely that the effects observed here were consequential of elevated

temperatures on biological factors affecting in their *in vivo* synthesis rather than thermally-induced degradation. This theory is further supported as no degradative by-products were observed in mass spectrometry analysis.

The associated antioxidant activity of the phlorotannin-enriched fractions did not appear to correlate with the level of phlorotannins as both assays displayed either no or weak correlations with TPC (RSA; $R^2$=0.01, FRAP; $R^2$=0.12). Moreover, the observed variations between conditions, while significant, displayed a narrow range with a 25% difference between the maximum and minimum values observed, applying either assay. This may be reflective of the relatively uniform composition observed within fractions, in terms of molecular distribution as well as the degree of isomerisation, in response to temperature, both of which interactively influence the degree of antioxidant activity. While some structural variability was detected, it did not appear to onset a strong impact on either quantification or activity.

These results show, for the first time, the sole effect of seawater temperatures on extractable phlorotannin levels. Only under elevated temperatures, outside that of their natural environment, did phlorotannin levels decrease, most likely resulting from a reduced rate of production. This reduced synthesis, would result in a lowered rate of turnover, due to their continued fucntion in radical scavenging, and/or translocation *i.e.* integration into cell wall and/or exudation (Arnold and Targett, 2003; Koivikko *et al.*, 2005). However, this effect was short-lived, highlighting the rapid plasticity of these compounds, or rather the mechanisms involved in their production, in adapting to the external environment. Furthermore, the ability to recover within a matter of days (possibly fewer than here) would also explain why the effect seasonal changes in temperature would not have been detected in seasonal sampling. Pelletreau and Targett (2008) also argued the need for more short-term measurements of secondary metabolites due to the rapid response of such compounds to external stimuli. They highlighted how long-term investigations may over look certain responses. However, due to methodical constraints such short-term monitoring can be challenging.

The results presented here also re-confirm the significance of irradiance as a determinant factor of their *in vivo* concentrations, as described and discussed in Chapter 4. As well as temperature, phlorotannin biosynthesis also appears highly vulnerable to the PAR intensity to which algae are exposed. Regardless of the temperature treatment, exposure to high PAR resulted in a reduced phlorotannin content. However, both factors appear to affect phlorotannin levels in a similar manner, by causing a disproportionate rate of turnover, onset by lowered productivity and continued and/or elevated assimilation. Yet, the mechanisms through which temperature affect phlorotannin concentrations appear more plastic than those incurred by PAR.

This study, along with the previous experiments, investigating the sole effect of irradiance (Chapters 4 and 5), highlight the influence of abiotic factors on the *in vivo* synthesis of phlorotannins, causing rapid shifts in their concentrations and level of antioxidant activity. To fully exploit the potential of phlorotannins in commercial applications, a focal area for future research should be the continued elucidation of their metabolic pathway, an area already receiving much recent attention. The similarities observed between their biosynthesis and other polyketide derivatives such as fatty acids and flavonoids may offer an opportunity to utilise such compounds for comparative studies, in an effort to understand the degree of impact of specific environmental factors on their metabolism

# Chapter 7

## The chemical and antioxidant stability of isolated low molecular weight phlorotannins from *F. vesiculosus* (L.)*

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## 7.1. Introduction

Since the 1940's, synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ) have been widely used in the commercial processing of food products to reduce lipid oxidation and subsequent food spoilage (Shahidi, 2000). However, reports of carcinogenic and mutagenic effects associated with these compounds (Ahn *et al.*, 2007; Chen *et al.*, 1992) have greatly affected consumer confidence regarding their continued use in foods, leading to the preference for naturally-occurring antioxidants as novel alternatives.

The reportedly high biological activity of phlorotannins has stimulated considerable commercial interest into their potential application in range of therapeutics, from pharmaceuticals to cosmeceuticals (Lopes *et al.*, 2012; Thomas and Kim, 2013). These marine-derived polyphenols are exclusively found in brown algae (Phaeophyceae, Orchrophyta), where they can comprise up to 5-12% dry weight of the total biomass, in certain species (Ragan and Glombitza, 1986; Targett *et al.*, 1992). A higher abundance, however, is associated with species occupying higher shore positions as seen in the inter-specific differences between intertidal Fucales and sublittoral Laminairales (Connan *et al.*, 2004). This is largely related to the protective role of phlorotannins in reducing the degree of oxidative stress incurred by pronounced and/or prolonged exposure to both high temperatures and UV radiation (Cruces and Huovinen 2012; Swanson and Druehl 2002), extremes of which are frequently experienced within the intertidal zone.

As the food industry looks toward naturally-resourced ingredients in an effort to minimise the use of synthetic food additives, recent research has focused on these algal polyphenols. Numerous reports demonstrating a high capacity for scavenging free radicals have highlighted the potential use of phlorotannins in food preservation as natural antioxidants (e.g. Ahn *et al.*, 2007; Audibert *et al.*,2010; Heffernan *et al.*, 2014).

Lipid oxidation within a food system is largely responsible for the qualitative and nutritional deterioration of the food (Frankel, 2014). Through a series of catalytic reactions, the volatile compounds (alcohols, hydrocarbons and aldehydes) derived

from the breakdown of lipid hydroperoxides lead to lipid rancidity, negatively impacting on the overall quality of the product (Frankel, 2014). Novel processing techniques involving the use of lower temperatures, reducing oxygen exposure and catalytic enzyme inactivation can be implemented in order to retard the rate of oxidation within food products (Gordon, 2007) However, another method commonly used involves the use of antioxidant compounds. That is, the addition of compounds capable of, at low dosages, preventing or significantly slowing the oxidation of other, more easily oxidised materials (Chipault, 1962).

The scavenging of reactive oxygen species (ROS), as well as their interaction with proteins, is a common characteristic shared between phlorotannins and other polyphenol compounds. Similarly to other polyphenols, the radical scavenging capacity of phlorotannins has largely been attributed to their unique structural arrangements, which aid in the scavenging of peroxyl, superoxide anions and hydroxyl radicals (Shibata *et al.*, 2008). Although these properties suggest that polyphenols, including phlorotannins, are an excellent source of potential antioxidants, their use in food products is often limited by their susceptibility to degradation during postharvest storage and various processing techniques (Cheynier, 2005; Dai and Mumper, 2010).

To date, studies on the impact of processing and storage on polyphenol compounds have been mainly focussed on plant-based polyphenols (Ioannou and Ghoul, 2012; Patras *et al.*, 2010), with very few reporting on the marine-derived phlorotannins specifically (Cuong *et al.*, 2016; Kang *et al.*, 2012). Moreover, the majority of these studies have been carried out on crude extracts using non-specific spectrophotometric methods for quantitative analysis, which can lead to an over-estimation of these compounds due to the interference of other compounds present (Singleton *et al.*, 1999). Despite the recent employment of advanced LC-MS methods, only the partial characterisation of phlorotannins has currently been achieved due to the difficulty involved in separating these highly polar compounds during the extraction process (Koivikko *et al.*, 2007). However, this issue has been significantly reduced with the introduction of flash chromatography as a post-extraction clean-up procedure for the removal of simple sugars and β-glucans (Heffernan *et al.*, 2015; Tierney *et al.*, 2014). Additionally, the isolation and subsequent characterisation of individual low molecular weight phlorotannins has been further complicated by the occurrence of both structural and conformational isomers, as well as a number of pre-derivatisation,

usually preacetylation, stabilisation steps required prior to purification due to their inherent instability. As a result, only a limited number of successful characterisation studies have been carried out to date (Ahn *et al.*, 2007; Kim *et al.* 2013; Shibata and Ishimaru, 2008).

Phlorotannin polymeric fractions derived from *F. vesiculosus* have displayed a high degree of anti-radical activity under an array of environmental variables, as seen in previous chapters. Moreover, where variations were observed, it was thought to be related to environmental effects on their synthesis rather than due to their degradation. The high RSA associated with this species is linked to the high abundance of LMW phlorotannin oligomers, specifically those comprised of 4, 5 and 8 PGUs. This characteristic profile remained relatively unchanged under the most extreme of induced variations in irradiance, seawater nitrogen concentration and temperature. Such stability *in vivo*, only served to highlight this species as an ideal candidate for future use in phlorotannin extraction and application in commercial products, particularly the food sector. However, the degree of stability these compounds may display *ex vivo* is a completely different aspect, one of which that is requires extensive investigation before they can be considered as potential food ingredients. To date, no such study has been carried out.

Therefore, the purpose of this research was to investigate the stability of low molecular weight (<3.5 kDa) phlorotannin fractions, from *F. vesiculosus*, under storage conditions varying in both temperature and oxygen exposure – the two most influential parameters in terms of shelf life. Both UPLC-MS/MS and Q-ToF-MS were employed to analyse the effects of these storage conditions on phlorotannin profiles including; composition, abundance and isomerisation, while the DPPH assay was carried out to monitor their radical scavenging efficacy over time. Only through further understanding the effect of storage parameters have on the integrity of phlortannins and their subsequent radical scavenging efficacy, can these compounds be realistically considered potential alternatives to synthetic antioxidants within food products.

## 7.2. Materials and methods

### 7.2.1. Reagents and materials

2, 2- diphenyl-1-picryhydrazyl (DPPH), 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox®), formic acid (MS grade) and 0.22 µm polytetrafluorethylene (PTFE) filters, were all sourced from Sigma-Aldrich Chemical Ltd. (Co. Wicklow, Ireland). HPLC grade methanol, ethyl acetate, acetonitrile, water, and BioDesign Dialysis Tubing™ with 3.5 kDa cut-off were obtained from Fischer Scientific Ltd. (Loughborough, Leicestershire, UK).

### 7.2.2 Collection and preparation of seaweed samples

Between 10-15 individual plants of *Fucus vesiculosus* (L.) were collected from Finavarra, Co. Clare (53°09'11.7"N 9°07'07.0"W), in November 2015. Each plant was cut at the stipe, leaving approx. 15 cm for regeneration while the rest of the thallus was taken. Specimens were brought back to the Botany and Plant Science lab at the National University of Galway (NUI Galway), where the thalli were immediately cleaned of epiphytes and other impurities, before being transported, cooled and in the dark, to Teagasc Food Research Centre, Ashtown (TRFCA). There, samples were frozen (-20°C) and thereafter freeze-dried before being ground to a fine powder. Once vacuum packed, samples were stored at -20°C until further processing.

### 7.2.3 Characterisation of low molecular weight (LMW) phlorotannin profiles

The extraction, purification and analysis of the LMW phlorotannin-enriched fractions followed a slightly modified version of that described by Tierney *et al.* (2014) , the details of which have been described previously in Chapter 2 Section 2.2.6 – 2.2.9.

### 7.2.4. Assessment of *in vitro* antioxidant activity

To examine the effects of the different storage conditions on the *in vitro* antioxidant activity of the LMW phlorotannin-enriched fractions, the radical scavenging activity (RSA) against the radical DPPH· was examined, the details of which are outlined in Chapter 2, Section 2.2.11.

### 7.2.5. Experimental set-up

Aliquots of the <3.5 kDa RP flash phlorotannin polymer fraction (200 mg) were exposed to differing storage conditions for a total period of 10 weeks. The conditions were as follows: -20°C, 4°C, Room temperature and 50°C. Conditions were selected to test the stability of phlorotannins over a broad range of storage environments. Samples were frozen at -20°C and refrigerated at 4°C, mimicking commonly used methods in food processing to prevent or slow the rate of biochemical changes. Similarly, conditions expected to onset oxidation and other reactions provoking food spoilage, were also tested by storing samples at room temperature and 50°C.

Samples were placed in 10 mL glass vials with fitted cap and sealed with parafilm®. As water plays such a significant role in food production, both a powder and an aqueous fraction (200 mg in 10 mL of distilled water) were investigated for each condition. Samples stored at room temperature were further divided into two subsamples; one in which samples were sealed with a fitted cap (RT), and one where the lids were removed and samples were directly exposed to the surrounding atmosphere for the duration of the experiment. These samples will hence forth be referred to room temperature and exposed (RTE). At all temperatures samples were kept in the dark at all times; at room temperature vials were wrapped in tinfoil. Chemical profiles and associated anti-oxidant activity were analysed at week 0, 2, 4, 8 and 10. Potential changes were monitored using both mass spectrometric (UPLC-MS/MS, Q-ToF-MS) and colorimetric methods (DPPH assay) and determined by comparison to a control, *i.e*. the initial profile and radical scavenging activity recorded at week 0 before the experiment began.

### 7.2.6. Statistical analyses

A three-way mixed ANOVA ($p<0.05$) was conducted to analyse the effects of storage condition and the physical state of sample (between-subject factors) over time (within-subject factor) on the radical scavenging activity (RSA) of the LMW phlorotannin-enriched fractions of *F. vesiculosus*. IBM® SPSS® statistical software (version 24) was used for all statistical tests. Normality was tested using the Shapiro-Wilk test for normal distribution and Levene's test for homogeneity of variances while sphericity was tested for using Maulchy's test of sphericity. Individual one-way repeated measures ANOVA's were conducted for each storage condition to identify any

significant changes in the RSA over the 10 weeks of the experiment, where in Tukey-tests were performed to find *posteriori homogeneous* (*post-hoc*) sub-groups that differed significantly ($p<0.05$).

## 7.3. Results

### 7.3.1. Phlorotannin composition and relative abundance

Direct infusion into the electrospray ionization source of the Q-ToF-MS allowed for full mass spectral screening to occur, resulting in preliminary assessment of sample purity and/ or detection of interfering impurities, thus helping to direct further separation approaches, if required. Furthermore, Q-ToF-MS analysis provided preliminary information of the molecular mass distribution of the phlorotannin polymers. A high level of purity was observed in phlorotannin polymer fractions isolated from *F. vesiculosus*, adding to the potential of this particular species, due to the relative ease of the purification, for commercial consideration (Fig. 7.1). The molecular mass distribution of phlorotannins were highly abundant at relatively low degrees of polymerization (4-8 PGUs), although structures composed of up to 18 phloroglucinol units (18 PGUs) were detected in trace amounts (Fig. 7.1).

UPLC-MS/MS analysis, performed in negative mode (ES-) is restricted to the characterization of phlorotannins upto *m/z* 1986 (16 PGUs) in size. However, as seen in Fig. 7.2, *F. vesiculosus* polymer profiles were predominantly composed of low molecular weight phlorotannins, and thus such limitations posed no impact on their profiling. Structures ranging between *m/z* 497 and 993, corresponding to 4-8 PGUs, accounted for up to 90% of the overall composition (Fig. 7.2), as detected at week 0 in the control sample. Polymers of *m/z* 497 and 621 (4 and 5 PGUs) were the most dominant phlorotannins present, accounting for 20 and 42%, respectively, of the total phlorotannin abundance (Fig. 7.2), while larger polymers (9-14 PGUs) were detected, they only accounted for a small percentage (4-0.2%).

Over the 10 week storage period, the majority of samples in both powder and liquid state demonstrated a uniform composition similar to that of the control (Fig. 7.2). However, metabolic profiles of liquid samples kept at 50°C showed a decrease in phlorotannins of 6-16 PGUs after 8 weeks of storage. By week 10, 96% of the overall composition was attributed to phlorotannins comprised of 3, 4 and 5 PGU, and the complete disappearance of larger molecules (Fig. 7.2 and 7.3).

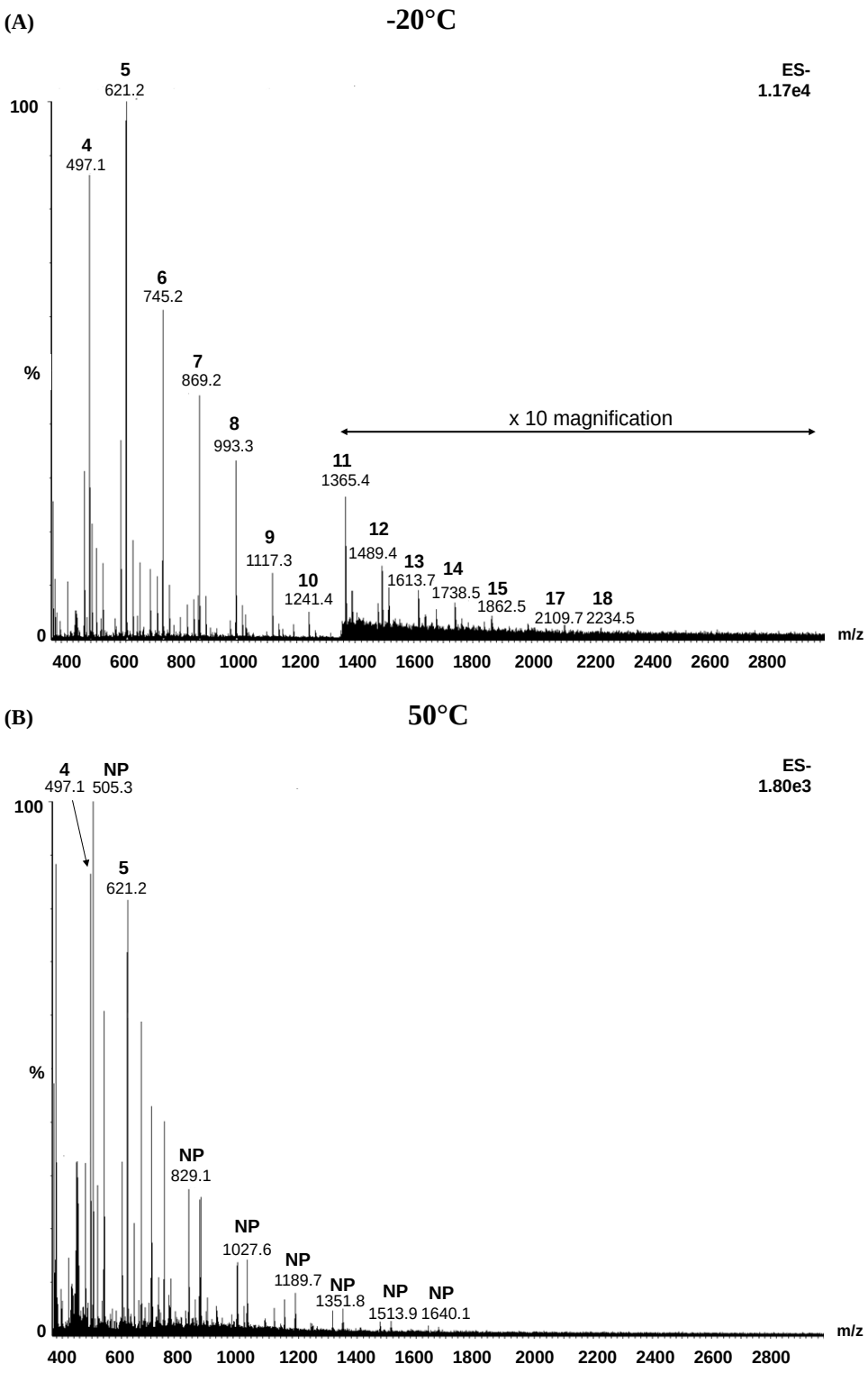


**Fig 7.1**. Q-ToF-MS spectra of *F. vesiculosus* LMW phlorotannin fractions, after 10 weeks in liquid form stored at (A) -20°C or (B) 50°C. Deprotonated molecular ions (*m/z*) is indicated as the numbers above peaks and the degree of polymerization as the most upper numbers above peaks. Compounds detected not representative of original phlorotannns, *i.e*. phlorotannin degradation products or β-glucan compounds, were labelled as non-phlorotannin (NP) above the corresponding peaks.

Q-ToF-MS spectra comparing liquid samples after 10 weeks of storage at -20°C and 50°C displayed two very different profiles (Fig. 7.3). Samples in a frozen state demonstrated a similar profile to that of the control while phlorotannin abundance at 50°C was extremely low (Fig. 7.2, 7.3). Ions related to β-glucan molecules and modified (degraded) phlorotannins, the combination of which are denoted as 'non-phlorotannin compounds' in Fig. 7.1, were the dominant compounds detected with only phlorotannins of 497 and 621 Da (4 and 5 PGUs) being observed. These non-phlorotannin compounds did not increase over time but rather appeared more abundant, without the high phlorotannin presence that once masked them. However, such a shift in composition was not observed for powder samples stored at the same temperature (Fig. 7.2, 7.3).

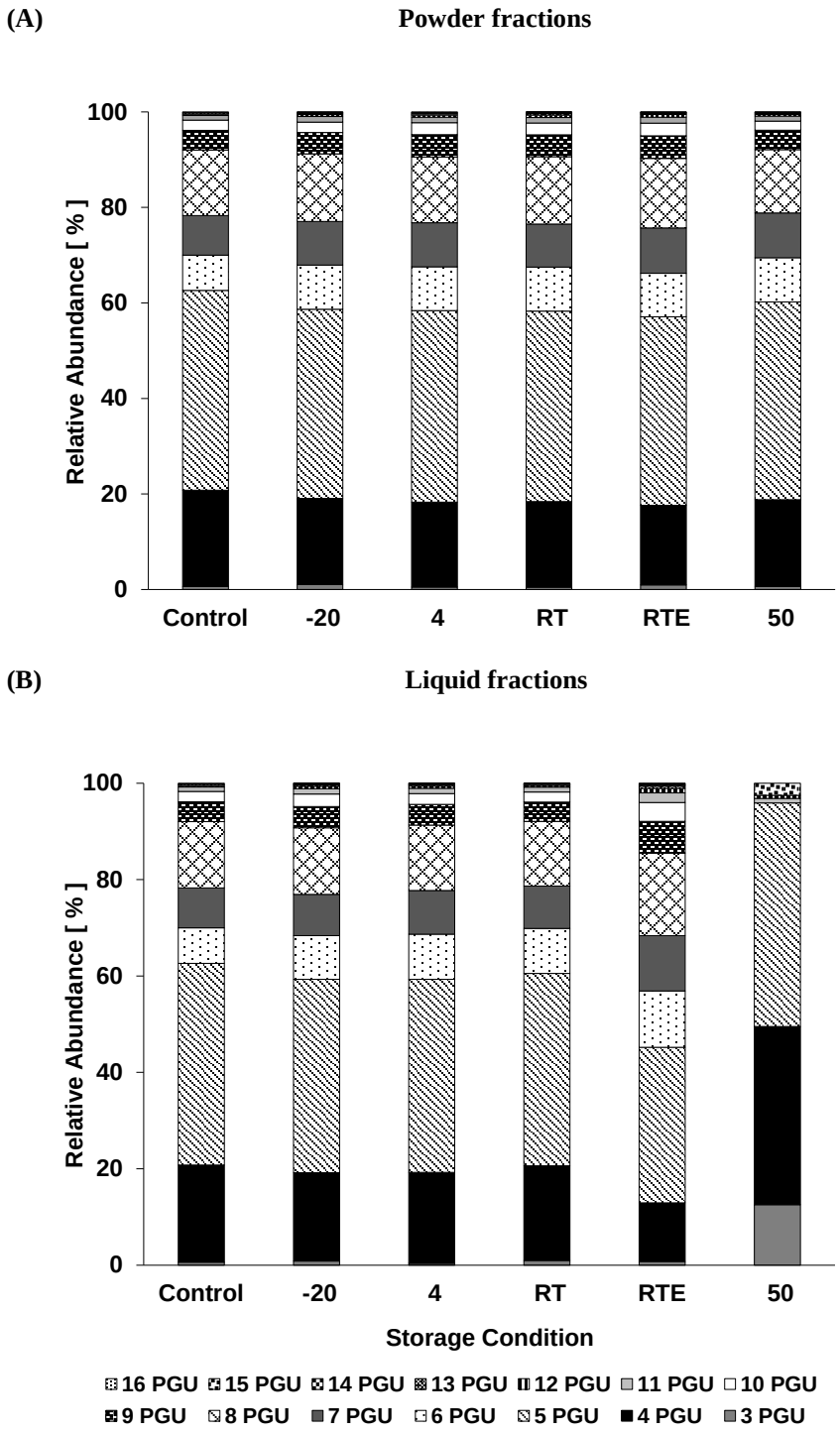


**Fig 7.2.** The relative abundance (%) of phlorotannins, in *F. vesiculosus* LMW phlorotannin fractions, between 3 and 16 phloroglucinol units (PGUs) corresponding to 374-1987 Da, as detected by UPLC-MS/MS. Samples stored in (A) powder or (B) liquid form, after 10 weeks of storage.

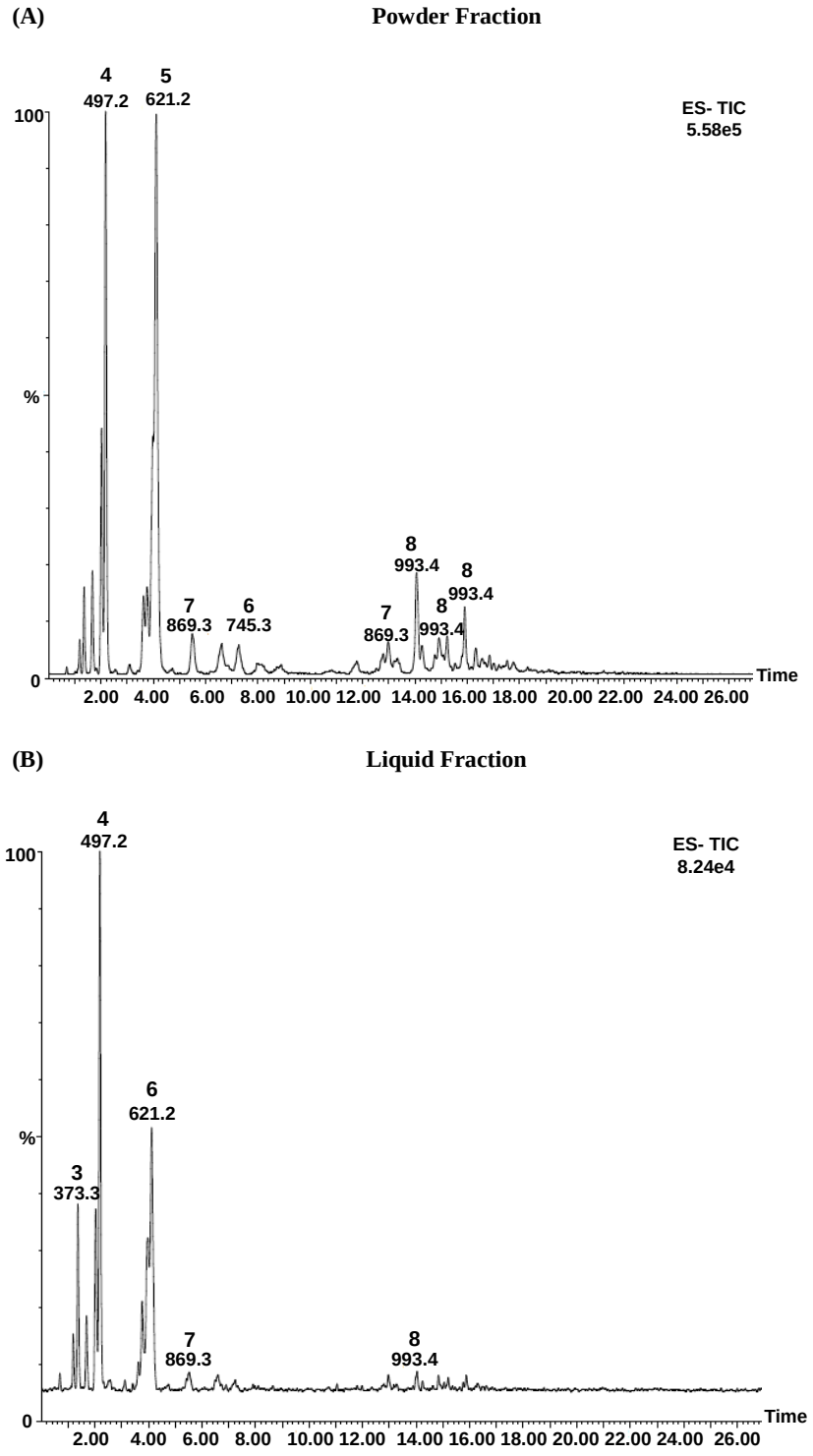


**Fig 7.3.** UPLC-MS/MS total ion chromatograms (TIC) of *F. vesiculosus* LMW phlorotannin fractions subjected to 50°C for 10 weeks in (A) powder form and (B) liquid form. Deprotonated molecular ions (*m/z*) is indicated by numbers above peaks and the degree of polymerization by the most upper numbers above peaks.

### 7.3.2. Degree of Isomerisation

The employment of UPLC-MS utilisng a tandem quadrupole mass spectrometer (TQD-MS) in multiple reaction monitoring (MRM) mode allowed for heightened sensitivity and resolution aiding the detection and quantification of isomers for each individual molecular ion transition. The method used for optimising isomeric followed that of Tierney *et al.* (2014) and is described in chapter 2. The control sample at week 0 possessed a total count of 162 isomers. Table 7.1 illustrates the variability observed in isomeric levels in all samples under different storage conditions after 10 weeks compared to the control, prior to experimental manipulation while Fig. 7.5 demonstrates the total isomer levels observed for all samples throughout the 10 weeks of storage; the majority of samples in both powder and aqueous form demonstrated a similar level of phlorotannin isomers over the 10 weeks.

**Table 7.1.** List of isomers for each individual MRM transition between 3-16 phloroglucinol units (PGUs), as detected by UPLC-MS/MS for *F. vesiculosus* LMW phlorotannin fractions. The control sample *i.e.* prior to experiment is compared to fractions after 10 weeks of continuous storage at -20°C, 4°C, room temperature (RT), room temperature exposed to air (RTE) and 50°C in both powder (P) and liquid (L) form. Where 10 or more individual isomers were detected for an individual molecular ion transition the number is highlighted in bold.

| | **Control** | **-20°C** | | **4°C** | | **RT** | | **RTE** | | **50°C** | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **PGU** | | P | L | P | L | P | L | P | L | P | L |
| 3 | 2 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 6 | 3 | 2 |
| 4 | 3 | 5 | 7 | 7 | 6 | 6 | 5 | 5 | **19** | 5 | 3 |
| 5 | 7 | 9 | **14** | **15** | **11** | **13** | **11** | **10** | **32** | **12** | 6 |
| 6 | **10** | **20** | **15** | **20** | **20** | **19** | **19** | **16** | **41** | **18** | **10** |
| 7 | **14** | **28** | **34** | **34** | **32** | **34** | **32** | **29** | **50** | **31** | 6 |
| 8 | **9** | **22** | **31** | **26** | **25** | **33** | **28** | **25** | **40** | **25** | 4 |
| 9 | **14** | **18** | **23** | **24** | **21** | **21** | **19** | **22** | **55** | **17** | 3 |
| 10 | **9** | **11** | **26** | **17** | **19** | **19** | **15** | **13** | **46** | **14** | 0 |
| 11 | **10** | 8 | **13** | **12** | **11** | **10** | **8** | **11** | **46** | 7 | 0 |
| 12 | **26** | 5 | **12** | 8 | 8 | 6 | **13** | 5 | **39** | 3 | 0 |
| 13 | **24** | 1 | 4 | 1 | 2 | 4 | 3 | 3 | **32** | 2 | 0 |
| 14 | **15** | 0 | 3 | 1 | 3 | 0 | 2 | 2 | **23** | 1 | 0 |
| 15 | **13** | 1 | 1 | 1 | 1 | 0 | 0 | 0 | **13** | 0 | 0 |
| 16 | **11** | 1 | 0 | 1 | 1 | 0 | 0 | 0 | **11** | 0 | 0 |
| **Total** | **167** | **132** | **186** | **170** | **163** | **168** | **158** | **144** | **453** | **138** | **34** |

The only significant variability observed was in samples in an aqueous solution stored at both 50°C and room temperature exposed to air (RTE). However, opposing effects were exhibited between these conditions. Aqueous samples exposed to elevated temperatures displayed a decline in the total isomer count over time, and by week 10 a total of only 34 isomers were detected (Table 7.1; Fig. 7.4). This number was

considerably low as on average 20 to 30 isomers could be observed for one individual molecular mass. Conversely, an increase in isomers over time was observed in aqueous samples exposed to air at room temperature (RTE). By the end of the experiment (week 10), the total number of isomers detected had reached 453 (Table 7.1; Fig. 7.4). The highest abundance was within the range of 6-11 PGUs, comprising 61% of the total isomers observed for this sample. The highest recorded was 55 isomers detected for the individual molecular transition corresponding to 9 PGU.

### 7.3.3 Radical scavenging activity (RSA)

Phlorotannin polymer fractions from *F. vesiculosus* had a high capacity for scavenging DPPH· radicals, with the initial control sample displaying $IC_{50}$ values of 18.2 ± 2.6 µg mL-1 (Fig. 7.5). For up to 8 weeks, regardless of the storage condition or the physical state of the sample, all the phlorotannin fractions displayed a consistent level of DPPH· inhibition, similar to that of the control, *i.e.* at day 0, prior to the experiment. However, over time, both storage condition ($p<0.001$) and the physical state of the sample ($p<0.001$) and their interaction ($p<0.001$) imparted significant effects on the observed $IC_{50}$ values. After 8 weeks, aqueous samples stored at 50°C began to decrease in activity ($p<0.001$; Fig. 7.5). By week 10, these samples had significantly decreased compared to all other samples, with $IC_{50}$ values of 75.7 ± 4.2 µg mL-1 (Fig. 7.5, $p<0.05$). Furthermore, $IC_{50}$ values for aqueous samples kept at room temperature and exposed to air (RTE), after 10 weeks, also showed a significant decrease (37.0 ± 3.4 µg mL-1, $p<0.05$), but not to the same extent as those at 50°C.

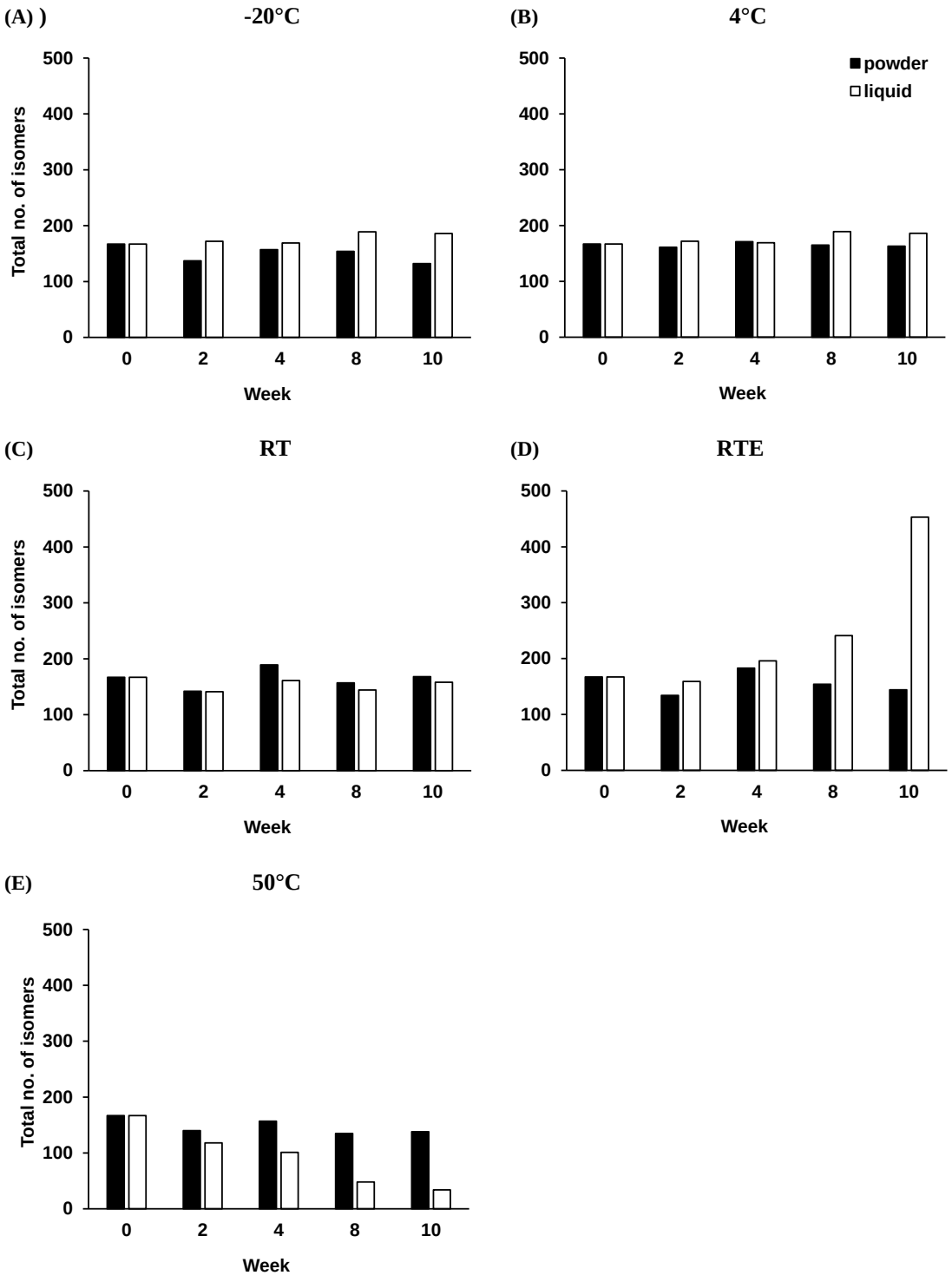


**Fig. 7.4**. Total number of phlorotannin isomers, as detected by UPLC-MS/MS, for *F. vesiculosus* LMW phlorotannin fractions kept in powder (black) and liquid (white) form stored either at (A) -20°C, (B) 4°C, (C) room temperature (RT), (D) room temperature exposed to air (RTE) or (E) 50°C, over 10 weeks of continuous storage.

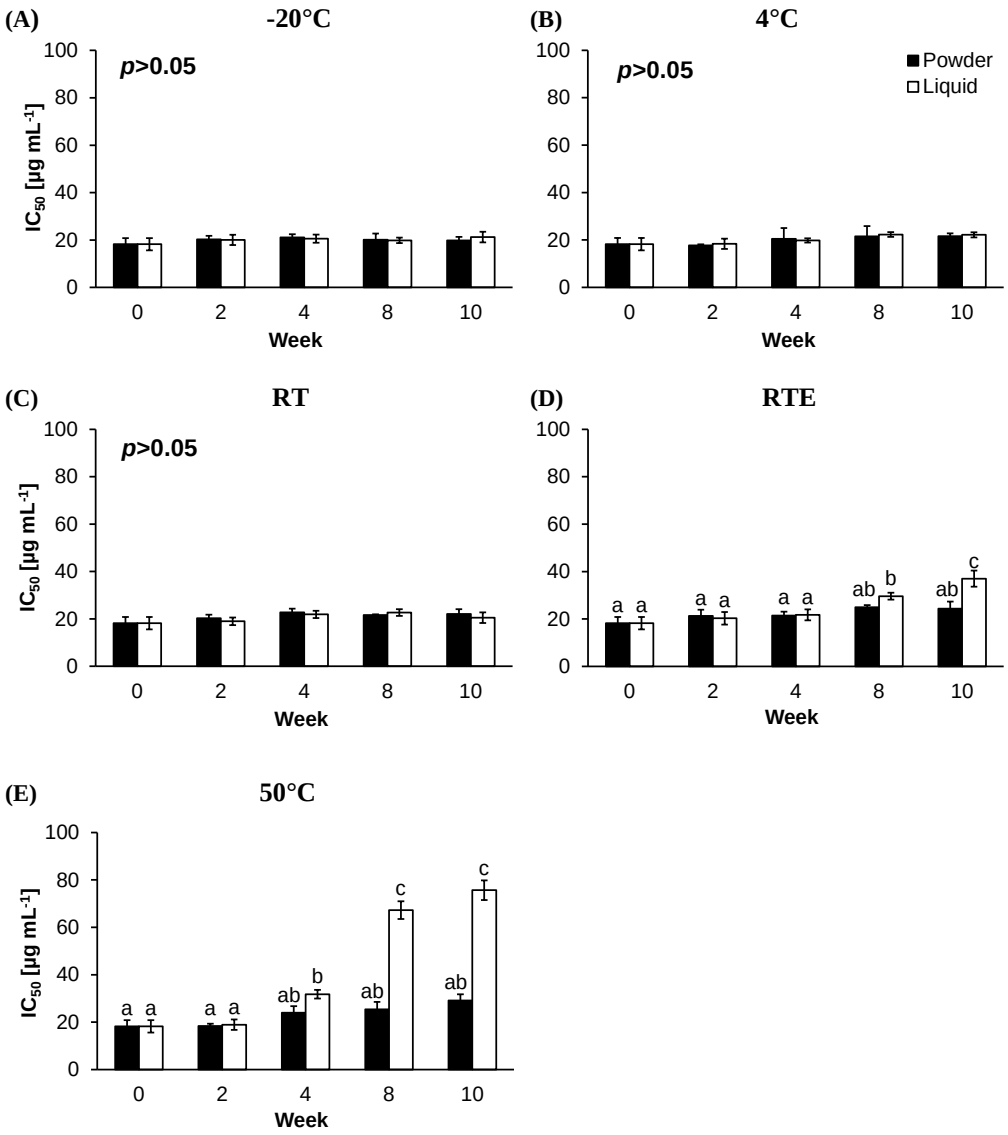


**Fig 7.5**. Radical scavenging activity (RSA) of DPPH·, presented as $IC_{50}$ values ($\mu gmL^{-1}$) of *F. vesiculosus* LMW phlorotannin fractions kept in powder (black) and liquid (white) samples powder (black) and liquid (white) form stored either at (A) -20°C, (B) 4°C, (C) room temperature (RT), (D) room temperature exposed to air (RTE) or (E) 50°C, over 10 weeks of continuous storage. Data represented as mean±standard deviation (n=3). The effects of the storage condition and the physical state of sample over time on the radical scavenging activity (RSA) of the LMW phlorotannin-enriched fractions of *F. vesiculosus* were statistically tested using a two-way mixed ANOVA ($p<0.05$). Details of the statistical test are outlined in Section 9.7, Table 9.7.1. Superscripts distinguish between significant differences arising between the physical state of the samples over time, as determined by Tukey's *post-hoc* test ($p<0.05$).

## 7.4. Discussion

The integration of phlorotannins into food products has long been considered as a future prospective due to the uncertainty associated with the biochemistry of these compounds. The primary focus of the research carried out within this thesis was placed on elucidating upon the natural variability of LMW phlorotannins within brown algae. The LMW phlorotannins derived from *F. vesiculosus*, in particular, repeatedly displayed, a high degree of purity and ease of extraction, as well as an unmatched capability in radical scavenging. The next step toward their commercial application is thorough investigation into their *ex vivo* reactivity and stability, particularly if they are to be considered as reliable ingredients for food preservation.

To date, many studies exist on the stability of other polyphenol compounds, particularly on those of terrestrial origin. However, relatively few have been carried out on phlorotannins, possibly as a result of the difficulty associated with their isolation and characterization, as has been previously seen in Chapter 2 and 3 with *P. canaliculata* and *A. nodosum*. Therefore, the aim of this work was to investigate the stability of both the chemical integrity as well as the antioxidant capacity of LMW phlorotannin-enriched fractions from *F. vesiculosus*, in an effort to assess their prospective application to food products in the future, particularly as natural replacements for synthetic chemical preservatives.

The phlorotannin fractions derived from *F. vesiculosus* demonstrated a high level of activity against DPPH radicals. However, the initial $IC_{50}$ values were not as low as those obtained by Heffernan *et al.* (2015) for the <3.5 kDa fractions of the same species. This may due to differences in the season and/or location in which sampling was carried out as shifts in environmental factors on both temporal and geographical scales are known to impact phlorotannin content and composition, and thus activity (Connan *et al.*, 2004; Parys *et al.*, 2009). Such effects have been seen time and time again within the previous chapters here, with abiotic factors, predominantly light (Chapter 4, 5 and 6) as well as developmental changes (Chapter 2, 3), playing a significant role in determining their *in vivo* concentrations.

The high activity observed for this particular species is considered to be associated with the high abundance of low molecular weight (LMW) phlorotannin oligomers, which are known to be powerful antioxidants. The low degree of polymerisation could allow for a higher number of free hydroxyl units that are not involved in monomer-monomer linkages (Heffernan *et al.* 2015). This would enable for a higher level of hydrogen donation and consequent stabilisation of radicals.

LMW (<3.5 kDa) phlorotannins were successfully fractionated for MS analysis using the method previously described by Tierney *et al.* (2014). The employment of reversed phase (RP) flash chromatography allowed for adequate separation from other polar compounds, such as polysaccharides, with which phlorotannins are largely associated. Mannitol and β-glucans, in particular, often prove difficult to separate from phlorotannins as seen in other brown macroalgal species, particularly *Ascophyllum nodosum* and *Pelvetia canaliculata* (Tierney *et al.*, 2014; Chapter 2 and 3). In some cases, further separation utilising normal phase flash chromatography is required to achieve satisfactory purity for UPLC-MS analysis.

The phlorotannin polymer fractions were highly abundant in low molecular weight structures, particularly those displaying a degree of polymerization of 4-8 PGUs. This is in accordance with previous studies on this species from Ireland and Nova Scotia (Heffernan *et al.*, 2015; Steevensz *et al.*, 2012; Tierney *et al.*, 2014). Under the majority of storage conditions, particularly when maintained in a dry powder state, the chemical composition of the phlorotannin-enriched fractions displayed a high degree of stability over time. Even when re-suspended in an aqueous matrix, most conditions yielded little to no variability over a period of 10 weeks. This was mirrored in the radical scavenging activity. These results highlight LMW phlorotannins as prospective ingredients in food preservation.

However, a significant decrease in DPPH inhibition was observed in samples maintained in an aqueous solution under excessive temperatures of 50°C, but only beginning after 8 weeks of storage. This loss of activity was associated with the thermal degradation of phlorotannin polymers. However, no such decrease was observed in powder samples under the same temperature. This is most likely due to the fact that during the heating of

aqueous solutions, due to the presence of dissolved oxygen, a range of free radicals can be generated and/or a combination of these, coupled with the presence of trace amounts of mineral ions, is known to promote radical propagation (Matsumoto *et al.* 2012). Phlorotannins within this sample, due to their free radical scavenging properties, would have likely reacted with the generated radicals resulting in their termination and the subsequent degradation of the phlorotannin structures observed.

As well as the compositional make-up, the degree of isomerisation was also an impacting factor affecting the *in vitro* radical scavenging activity. The control sample displayed a total isomer count of 162. This is, again, similar to those reported by Heffernan *et al.* (2015) for the same species, and by Tierney *et al.* (2014) for *F. spiralis*, belonging to the same algal genus. The mechanisms behind phlorotannin isomeric behaviour are still largely unknown. Previous studies comparing phlorotannin profiles of brown algae have documented species-specific variations in isomerization, suggesting that the specific structural properties of different phlorotannin polymers present in the various species *i.e.* linkage type, maybe responsible for the differences observed (Heffernan *et al.*, 2015; Tierney *et al.*, 2014). Apart from the natural biosynthetic pathways (currently unknown) for productions of phlorotannin isomer, the extent of these could be increased by oxidative reactions, created by the structural recombination of molecules, most likely through intra- and/ or intermolecular bonding (Poncet-Legrand *et al.* 2010).

It appears that when exposed to air, the aqueous fractions, possibly due to the diffusion of atmospheric oxygen into the water (Chaix *et al.*, 2014), underwent extensive oxidative reactions that led to an increase in isomer abundance over time. A slight shift in the compositional makeup of the phlorotannin profile for aqueous RTE samples was observed with an increased abundance of phlorotannin structures composed of 6-11 PGUs, which may be indicative of intermolecular bonding between phlorotannin structures, causing an increase in the degree of polymerization (Poncet-Legrand *et al.* 2010). In aqueous solutions phlorotannins would most likely hydrogen bond with each other, thus helping to stabilize them from environmental factors, explaining the increase in isomerization observed only for this sample. However, through such structural modifications of

phlorotannin molecules, free hydroxyl units, once available for radical scavenging would be significantly reduced.

This is, to our knowledge, the first study to chemically investigate LMW phlorotannin polymers for stability and antioxidant activity under varying storage conditions. The results here support the theory of isomers being products of oxidation from external constraints with an increase in isomer abundance observed only in samples stored in conditions that promote an oxidative environment. The results also highlight the significant potential of phlorotannins as preservative ingredients due to their capability to self-stabilise under such adverse conditions. Conversely, in the presence of free radicals, as would be generated in aqueous samples under elevated temperatures or exposed to atmospheric conditions, they are quickly utilised as powerful antioxidants.

This work, in adjunction the previous chapters was carried out in an effort to achieve a better understanding of the factors that affect phlorotannin variability, working toward realising a future application of these natural antioxidants into food products. The effects of both natural and induced abiotic factors were investigated and the results concluded that *Fucus vesiculosus* represents an excellent source of highly active, low molecular weight phlorotannins, requiring minimal processing. The high *in vitro* stability of the phlorotannin profile of this species is mirrored within the long shelf life exhibited by the extracts, *ex vivo*, further highlighting their potential use as natural bioactive ingredients in several therapeutics, particularly the food industry.

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# Chapter 8

General Discussion

## 8.1. Potential of macroalgal biochemistry – a recap

The global seaweed industry is estimated to be worth over US$ 6 billion, 90% of which is owed to Asian cultivation (Bixler and Porse, 2011). 83% of the total biomass, either being of wild harvest or cultivated, is for human consumption (Craigie, 2011). In eastern Asia, there is a long tradition of seaweed consumption with which a high quality of life has been associated (Brown *et al.*, 2014). Macroalgae offer high nutritional value, consisting of dietary fibres, protein, polyunsaturated fatty acids, antioxidant compounds and many vitamins and minerals (Cornish and Garbary, 2010; Osman and El-manawy, 2011; Patarra *et al.*, 2011; Schmid *et al.*, 2017; Stack *et al.*, 2017). The connection between eastern diet and health, has highlighted the potential health benefits associated with seaweed, sparking commercial attraction. Now, as a result, algal chemical research has become heavily involved in identifying, manipulating, separating and characterising specific macroagal-derived bioactives for uses in an array of therapeutic fields for the benefit of human health and well-being (Kim, 2012; Stengel and Connan, 2015; Thomas and Kim, 2013).

Now, as consumers become more health-aware, interests lean toward the use of more naturally-derived ingredients. Under pressure to meet these demands, industry have now focused their efforts toward the use of novel bioactive compounds as natural alternatives to the previously used synthetic, potentially harmful products and/or ingredients (Deliza *et al.*, 2003; Katz, 2000; Plaza *et al.*, 2008; Thompson, 1998). Among such targeted natural products, are the marine polyphenols, phlorotannins, exclusive to brown algae. Particularly owing to their potent antioxidant capacity, phlorotannins exhibit great potential for the development of novel health-promoting products with high commercial value within pharmaceutical, cosmeceutical, nutraceutical and food industries (Catarino *et al.*, 2017; Kim and Himaya, 2011; Kirke *et al.*, 2017; Thomas *et al.*, 2011; Sullivan *et al.*, 2014).

However, the very cause of their appeal is what has hindered the progression towards their commercialisation. The high affinity of phlorotannins, like all polyphenols, for reactivity and oxidation is what makes them ideal ingredients for food preservation or anti-aging products, among others (Kirke *et al.*, 2017; Sanjeewa *et al.*, 2016). However, it also central to the difficulty associated with their structural elucidation, arising from their chemical intricacy among one another or other compounds, such as

polysaccharides (Audibert *et al.*, 2010; Steevensz *et al.*, 2012). As a result, little information is known regarding their biosynthesis or the factors involved therein. Furthermore the high reactivity of these compounds has greatly impeded the assessment of their natural variability, which to date, has been fundamentally reliant on their quantification via colorimetric reactions, providing no insight into their chemical properties, such as molecular weight or conformational arrangements, which interactively influence the degree of inhibitory activity exhibited of a compound (Ferreres *et al.*, 2012; Shibata and Ishimaru, 2008). Furthermore, a lack of standardisation within the literature has led to inconsistencies and often contradictory results between studies, preventing cross-comparisons being made.

This thesis was concerned with elucidating on the variability incurred by abiotic factors on semi-purified low molecular weight (LMW) phlorotannin fractions by employing a standardised method comprised of colorimetric quantification, mass spectral characterisation and bioactive assessment. Four species belonging to the Fucales, *P. canaliculata, A. nodosum, F. vesiculosus* and *H. elongata*, differing in their shore position and life strategy, were investigated. The assessment of the natural variability in LMW phlorotannin profiles included the comparison of species collected from different sites along Galway Bay (**Chapter 2**) as well intra-thallus variability over several seasons (**Chapter 3**). Three key abiotic factors–irradiance (**Chapter 4**), nutrients (**Chapter 5**) and temperature (**Chapter 6**) were investigated for their effect on algal physiology and corresponding LMW phlorotannins. Finally, the degree of stability displayed *ex vivo* by LMW phlorotannin polymeric fractions was assessed in an attempt to validate their use within commercial applications (**Chapter 7**).

### 8.2. LMW phlorotannins exhibited a high degree of natural variability

The strong degree of variability observed in phlorotannin levels has made unearthing the factors responsible for their production and/or degradation difficult. The naturally-occurring differences in LMW phlorotannin profiles, both between and within different brown algal species may identify and characterise the impact of different environmental factors and ultimately provide relative screening for the selection of species, sites and seasons for optimal commercial harvesting and extraction.

### 8.2.1. Inter-species variations

The interspecific variability of phenolic content between brown algal species has been previously demonstrated, with the Fucales (up to 12 % DW) being deemed as the most abundant, while the Laminariales (0.1-1% DW) exhibit the lowest levels (Connan *et al.*, 2004; Targett *et al.*, 1992). Such differences have been suggested to be related to shore depth and degree of exposure to abiotic stressors (Schoenwaelder and Wiencke, 1999). In **Chapter 1**, four species belonging to the Fucales from the Irish west coast, differing in their vertical position were examined for variability in LMW phlorotannin profiles.

The results showed the content and composition of LMW phlorotannins to differ between species, ultimately affecting the observed inter-specific antioxidant activity. *F. vesiculosus* (4-8 PGUs) was highly abundant is lower molecular weights while *H. elongata* (8-13 PGUs) and *P. canaliculata* (8-15 PGUs) displayed profiles more prominent in larger structures. The latter two species also possessed a high degree of structurally similarity within its composition as seen the high degree of isomerisation. *A.nodosum* varied considerably, owing the substantial interference of polysaccharides. Despite slight changes observed in the relative abundance in the phlorotannin profile, most species displayed a rather characteristic profile, which was repeatedly observed over the six seasons as well between the two sites. This was thought to derive from differences in their genetic evolution rather than being based on physiological adaptation to local conditions.

However, it would appear that other aspects of algae chemistry play an influential role in the degree of phlorotannin separation that can be achieved. The separation issues associated with *P. canaliculata* and *A. nod*osum, more so with the latter, as mentioned was likely to be related the higher abundance of polar polysaccharides, from which the LMW phlorotannins proved difficult to isolate, even with the employment of reverse phase flash separation. Such issues have been previously reported by Tierney *et al.*, (2014), who achieved adequate separation for UPLC-MS/MS characterisation through the additional use of normal phase flash separation. However, here the application of NP flash resulted in a further loss of phlorotannins, highlighting how strong the bonds between the two molecular groups can be. Such interference was reflected in both the resolution allowed for characterisation as well quantification and

was more pronounced in *A. nodosum*. This high abundnace of LMW polysaccharides has previously been thought to be linked to the presence of an endophytic fungi (Deckert and Garbary, 2005; Rugg and Norton, 1987). Therefore, the potential use of either of this species for the commercial extraction and application of phlorotannins, is unlikely to occur until a method can be developed providing conditions capable of degrading the adjoining bonds of such complexes.

Although occupying the same shore level to *A. nodosum*, *F. vesiculosus* displayed a very different profile, exhibiting a considerably higher degree of purity and *in vitro* activity. These results were similar to those reported by Heffernan *et al.* (2015) and Tierney *et al.* (2014). Such differences between species occupying the same shore height, further suggests that phlorotannin variability is not fully determined by vertical distribution and that differences at a genetic level contribute to inter-species differentiation. The differences may result from the specificity of the fraction examined, as *A. nodosum* is reported to abundant in much larger phlorotannin polymers, up to 50 kDa in size (Audibert *et al.*, 2010). Tierney *et al.* (2013) while investigating various fraction sizes for activity, observed ethanolic fractions of over 100 kDa to be the most active in several assays. The differences these two observe in their phlorotannin structural size may indicate at differing defence mechanisms. For example, the high abundance of LMW phlorotannin oligomers allows for rapid turnover rates and potent radical scavenging, while a higher presence of phlorotannin polymers, accumulating with thallus age (Pedersen, 1984) and becoming complexed with the cell wall (Koivikko *et al.*, 2005), could suggest that it is through the mechanical rather than chemical traits of phlorotannins in thickening the thallus, that they aid in defence.

*H. elongata* is a lower-littoral species, inhabiting the zone just above the Laminariales, *L. digitata* and *L. hyperborea*. This species demonstrated a profile highly abundant in higher molecular weighted as well as a high (the highest) degree of isomerisation. A similar profile for this species from Galway Bay was found by Heffernan *et al.* (2015). However, the reasons as to why this species display such structural variability is not yet known and further studies are required to examine the ecological significance. To date, only a few compounds have been successfully isolated and characterised (Glombitza and Grobe-Damhues, 1985; Grosse-Damhues *et al.*, 1983). Here over 400

individual isomers were found suggesting full structural elcudation is unlikely to occur in this species in the near future.

*H. elongata* and *F. vesiculosus* at certain seasons displayed similar levels of antioxidant activity, despite the stark differences in their chemical composition. Similarly, Cerantola *et al.* (2006) reported two isolated phlorotannin structures from *F. spiralis,* differing in their structural complexity, to be equal in their scavenging ability. This highlights how it is not just simply by way of higher concentrations that phlorotannins can display high activity but rather the arrangement of phlorotannin structure and position of the active hydroxyl groups that influences their antioxidant potential (Ferreres *et al.*, 2012; Shibata and Ishimaru, 2008). This suggests by way of chemical rearrangements, *H. elongata* has the capacity to increase its defence levels, coping with external stressors, potentially grazing pressure.

### 8.2.2. Seasonal and spatial variability

Seasonal and spatial fluctuations in abiotic parameters can impose significant effects on algal chemical composition, stimulating acclimation responses to changes in local abiotic factors (Stengel *et al.*, 2011). In **Chapter 2** both seasonal and spatial variability was observed in the phlorotannin content, composition and relevant antioxidant activity for each species. However, the manner in which these differences occurred varied both between and within species. Overall, samples collected from Mace Head, Co. Galway, appeared to possess a higher and more stable phlorotannin content and composition. This may be due to the reduced biotic pressure that is associated with a higher degree of wave exposure rather than differences in abiotic exposure between sites (Ronnberg and Ruokolahti 1986), as was particularly evident in *F. vesiculosus*. However, spatial variation in phlorotannins is also likely to stem from genetic adaptation, the degree of which will differ in response to the extremity of the environment, as seen in **Chapter 5**, between *F. vesiculosus* samples collected from Finavarra, Co. Clare and Oranmore, Co. Galway. Here, possibly through specialised adaptation, the *F. vesiculosus* from Oranmore displayed both morphological and chemical diversity from those at Finavarra. The two sites differed in the salinity regime, the nitrogen concentration and the natural content of LMW phlorotannins. However, due to the synergistic effects of multiple factors acting upon an algal

population, at one time, at any one location, make deciphering the exact factors responsible for spatial differences difficult.

### 8.2.3. Intra-thallus variability

Morphological differentiation within an individual algae is often associated with different algal physiological function, such as growth and reproduction, and so it would seem likely that different chemical compositions will exist between thallus sections (Stengel *et al.*, 2011). In **Chapter 3**, differences in phlorotannin profiles were investigated between parts contributing to vegetative development and reproductive maturity. Moreover, species varying in their growth patterns were investigated *i.e.* those displaying perennial vs biennial.

Depending on the species under investigation, intra-thallus phlorotannin variability was either was highly differentiated or mildly apparent. For example, *F. vesiculosus* displayed a uniform composition throughout all seasons between the reproductive tips and the vegetative thallus, with a slightly higher content observed in the tips. Previous reports suggested that defence levels were thought to vary betwwen thallus parts depending on physiological function with some being considered as ecologically more important and so more vital to defend (Rhoades, 1979). In *A. nodosum* and *F. vesiculosus*, the older basal parts rather than the reproductive or meristematic tissues are considered to contribute most to the fitness (Jormalainen and Honkanen, 2004; Pavia *et al.*, 2002) due to their importance to the overall plant. Tuomi *et al.* (1989) reported higher phlorotannin levels in the vegetative tissue of *F. vesiculosus*, attributing the difference to the mechanical defence displayed by the receptacles. However, thes result here suggest that the lack of morphological distinction along the frond of *F. vesiculosus*, and other fucoids, is likely to result in a lack of specialisation in chemical defensive requirements.

*H. elongata*, however, representing the most morphologically compartmentalised of the Fucales investigated, displayed the highest degree of intra-thallus differentiation. The developmental progress of the buttons, from vegetative to fertile form, taking up to two years, may imply a differentiated phlorotannin composition at different stages offers an optimised defence system to ensure maturity is reached (Stengel *et al.*, 1999).

### 8.3. Short-term investigations are critical for elucidation of abiotic influence

Field sampling has often proven adequate in establishing correlations between algal chemical compounds and differences in environmental factors (Aguilera *et al.,* 2002; Gevaert *et al.,* 2002; Gosch *et al.,* 2015). For example, alterations to the relative proportion and concentration of photosynthetic pigments have been found to correspond to the lower irradiance levels in winter months, corresponding with higher N availability, subsequently decreasing toward the high light in summer (and depleted N) (Aguilera *et al.*, 2002; Martínez and Rico, 2002; Sampath-Wiley *et al.,* 2008; Stengel and Dring, 1998). The ratio of total fatty acids is often observed to shift with a higher proportion of PUFA during colder temperatures and SFA increases with increasing temperature (Gombos *et al.,* 1994; Moon *et al.*, 1995; Schmid *et al.*, 2017). However, this is often due to the specialised physiological functions displayed by the target compounds. For example, certain pigments function dissipate excess energy from thermal- or light-induced stress and therefore will exhibit a proportional shift in periods of such stress (Gevaert *et al.*, 2002; Stengel and Dring, 1998). Nitrogen-based photo-protective compounds have been found to vary with higher accumulation in species exposed to elevated levels of irradiance (Karsten *et al.* 1998).

Phlorotannins, however, display a high degree of plasticity, owing to their ubiquitous defensive response to an array of ecological factors. Therefore, while the results attained from monitoring the natural variability of phlorotannins within field samples function as a valuable source of novel knowledge, they also make assessing the exact factors driving phlorotannin variations difficult to determine. Pelletreau and Targett (2008) argued the need for more short-term measurements of secondary metabolites due to the rapid response of such compounds to external stimuli. They highlighted how long-term investigations may over look certain responses. Therefore, seasonal field studies alone will not suffice in providing clarity on the matter; the employment of *in vitro,* controlled experiments, spanning for a timescale of days, is necessary to investigate the impact and nature of individual environmental parameters

#### 8.3.1. Abiotic influence on phlorotannins arises from shifts in their metabolic balance

Concerning abiotic factors, the three most influential parameters affecting algal chemistry are considered as irradiance, nutrients and temperature (Hurd *et al.*, 2014).

Here, the effects of *in vitro* manipulation of each of these factors on LMW phlorotannin profiles were investigated in *F. vesiclosus*. The results from **Chapter 2** and **3** highlight this species as the most ideal candidate for future commercial extraction and application and so further elucidation on its variability is essential. The results here highlighted how such factors impart short-term effects on the phlorotannin variability, thus re-emphasising the need for more in-depth and small-scale investigation for such plastic compounds.

Variations in such environmental factors have been previously linked to both seasonal and spatial variations in the phlorotannin content of brown algae (Abdala-Díaz *et al.*, 2006; Connan *et al.*, 2007; Koivikko *et al.*, 2008) and so were hypothesised to incur variations to their biochemical properties, the effects of which had not been examined previously. The results of the culture experiments revealed that the degree of influence exhibited by various abiotic factors on phlorotannin concentrations is largely related to alterations to their metabolic balance between production and assimilated, either through oxidation, exudation and/or integration into the cell wall (Koivikko *et al.*, 2005). It was perceived that through their potential effect on the enzymes largely responsible for phlorotannin synthesis that different abiotic factors should incur a differing effect. This theory was only strengthened by the exceptional stability these compounds display *ex vivo* against an array of external stressors, as seen in **Chapter 7**. In **Chapter 4** and **5**, the different effects of irradiance and temperature were thought to stem from such different effects on the phlorotannin metabolic pathway (discussed below).

#### 8.3.1.1. Abiotic influence on concentrations via effects on metabolic pathways

Irradiance has been considered as the most influential factor on brown algal phlorotannins, as suggested by reports of a photo-protective role, and thus far has received the most attention, in terms of research. The spectral quality, in particular, is of utmost importance, with ultraviolet radiation (UVR) displaying enhancing effects on their concentrations, as reported previously (Cruces and Huovinen, 2013; Gomez and Huovinen, 2010; Pavia *et al.*, 1997). In **Chapter 4**, short-term exposure to low doses of UVB was thought to function as an informational signal, stimulating of the expression and activation of enzymes involved in phlorotannin synthesis, such as polyketide synthases. Such induced levels could then be use in deterring UV-induced

cellular damage, in a similar manner to other condensed aromatic ring systems, such as in plant flavonoids (Jenkins and Brown, 2001;Winkel-Shirley, 2001). Conversely, high-PAR exposure had deleterious effects, again thought to arise from its effects upstream on photosynthetic activity and enzymatic deactivation and/or denaturation. Similar adverse effects of high-PAR exposure were also in **Chapter 5** and **6**, also leading to lowered phlorotannin levels. As in the field, both spectral components are found (in much higher levels than here) concurrently acting on algae, their opposing effects may act to render a neutral effect on a larger time-frame. However, diurnal variations are likely to occur, particularly with tidal cycles. Such daily shifts have been previously found for intertidal brown algae (Abdala-Díaz *et al.* 2006; Connan *et al*., 2007).

When coupled with higher nitrogen concentrations (100 µM) the effects of high-PAR were mitigated, as seen in **Chapter 5**. Again, the influence of N on phlorotannin levels was suggested arise from a higher levels of enzymes (involved in phlorotannin synthesis), permitted by higher N availability. This, in turn, may have enabled the maintenance of a stable metabolic balance between their synthesis and oxidation under these harsh irradiance levels. This idea was further supported by the significant decrease observed when algae were exposed to high-PAR in low N concentrations.

Moreover, when investigating the effects of temperature, high-PAR exposure also incurred a reduction in phlorotannin levels under three different temperatures. The effect of high temperatures imparted a short-term decrease in the observed concentrations, of which, in past studies, had been overlooked due to time-related analysis (Endo *et al.*, 2013, 2015). Furthermore, these results displayed the varying kinetics of abiotic effects, as the response of phlortannins appeared more plastic with full recovery of phlorotannin concentrations was observed after 7 days in response to temperature shifts but not to high-PAR.

### 8.4. LMW Phlorotannin fractions exhibit a high degree of *ex vivo* stability – promising future in food preservation

The reportedly high biological activity of phlorotannins has stimulated considerable recent commercial interest into their potential application in range of therapeutics (Lopes *et al.*, 2012; Thomas and Kim, 2013), particularly within food products (Cheynier, 2012; O'Sullivan *et al.*, 2014). As the food industry looks toward naturally-

resourced ingredients in an effort to minimise the use of synthetic food additives, recent research has focused on these algal polyphenols. However, before they can be integrated into such products the impact of processing and storage on their chemical and antioxidant stability requires assessment. The results obtained in **Chapter 7** highlighted the potential of such fractions as potent food preservatives due to their ability to self-stabilise under an array of conditions. Only when exposed to prolonged and pronounced environments of thermal and oxidative stress, did they become degraded, being quickly utilised as powerful antioxidants.

### 8.5. Optimisation of future industrial-scale cultivation conditions

Cultuivation of seaweed in large-scale aquaculture systems can potentially provide the opportunity to manipulate and specifically tune the environmental conditions to control the biochemical quality of the treated biomass enabling maximal yields of a desired compound (Mata *et al.*, 2015). While it seems unlikely the brown algal species investigated here will be considered for land-based aquaculture cultivation in the near future, they serve as valuable model organisms, particularly *F. vesiculsosus* as mentioned previously.

The results obtained here demonstrate that by monitoring shifts in phlorotannin profiles at regular and short intervals, optimum culture conditions can be elucidated on and potentially applied on a large-scale platform for commercial prospects. Such a novel approach, in the future, could be successful in establishing maximal yields of phlorotannin extracts and/or the biochemical optimisation for maximum activity, the two of which are not always necessarily linked, as seen in throughout this thesis (**Chapters 2-6**). However, the resultant revenue of such cultivation would need to be considerable to match the high costs associated with aquaculture such as infrastructure, maintenance and energy investment (Mata *et al.*, 2015). However, the high degree of stability displayed by LMW phlorotannins in *F. vesiculosus* in the field suggests that harvesting wild populations of this species will be sufficient in attaining a high-value end-product.

### 8.6. Conclusion and future research prospectives

In summary, this research provided, for the first time, an in-depth analysis of the natural variability of phlorotannins, specifically those of low molecular weight, in four

species of Fucales. Phlorotannin diversity appears to be influenced on a number of levels, from genetic inheritance (inter-specific and spatial), to developmental maturity (intra-specific and spatial) to environmental stimulation (seasonal and experimental). Such novel information serves as a platform of scientific knowledge on which future research on phlorotannins can be built, progressing toward their commercial integration. However, further research is needed including; additional screening for selection of other species, other, more diverse sites using shorter, more frequent time-frames for assessment of phlorotannin variability.

*In vivo* phlorotannins display a high degree of plasticity offering brown algae a heightened degree of physiological flexibility, contributing to the ability of brown algae to inhabit and endure extreme environments such as the intertidal zone. However, the complexity of these polyphenols chemistry, combined with their rapid turnover rates, makes elucidating the principle factors involved in their metabolism difficult to assess, highlighting the need for small-scale, *in vitr*o investigations. Moreover, the results of this research suggest that the effect of abiotic factors on phlorotannins fundamentally derives from their influence on the mechanisms regulating their synthesis such as enzymatic activity, potentially in a similar manner to terrestrial polyphenols. Therefore, it may be possible that by cross-comparative studies with other terrestrial originating polyphenols, that future research may be capable of achieving full, if not partial, elucidation on the specific components that drive the intercellular synthesis of these highly bioactive compounds.

# Chapter 9

Supplementary material

## 9.1. Overview

Due to the volume of data obtained within this research, additional figures and tables for each chapter have been designated to a supplementary section. Data are separated into separate sections according to the chapter from which it derives and is referred to within the figure and table captions of each chapter, accordingly.

## 9.2. Supplementary data for Chapter 2

**Table 9.2.1.** The relative abundance (%) of phlorotannins between 3 and 16 phloroglucinol units (PGU) as detected by the UPLC-MS/MS for each of the three pseudo-replicate samples of *P. canaliculata* (PC), *A. nodosum* (AN), *F. vesiculosus* (FV) and *H. elongata* (HE) collected from Finavarra, Co. Clare, in May 2015.

| | PC | | | AN | | | FV | | | HE | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **PGU** | **#1** | **#2** | **#3** | **#1** | **#2** | **#3** | **#1** | **#2** | **#3** | **#1** | **#2** | **#3** |
| **3** | 0.00 | 0.00 | 0.00 | 0.20 | 0.46 | 0.45 | 1.98 | 2.64 | 2.37 | 0.00 | 0.00 | 0.00 |
| **4** | 1.88 | 1.69 | 2.00 | 0.98 | 1.10 | 1.25 | 14.35 | 15.91 | 14.28 | 0.00 | 0.63 | 0.52 |
| **5** | 1.23 | 1.36 | 1.45 | 12.87 | 12.04 | 10.88 | 37.47 | 37.89 | 37.34 | 5.15 | 5.09 | 4.81 |
| **6** | 0.31 | 0.27 | 0.18 | 0.85 | 0.69 | 1.36 | 9.50 | 9.54 | 9.40 | 3.04 | 3.05 | 2.72 |
| **7** | 1.22 | 1.32 | 1.45 | 8.40 | 9.63 | 7.27 | 9.92 | 9.57 | 9.88 | 5.61 | 6.33 | 5.54 |
| **8** | 6.78 | 5.43 | 7.37 | 18.80 | 15.60 | 15.45 | 17.92 | 16.65 | 17.66 | 23.04 | 21.59 | 23.51 |
| **9** | 9.38 | 10.47 | 8.96 | 27.06 | 27.06 | 25.45 | 5.24 | 4.65 | 5.44 | 18.83 | 19.59 | 18.50 |
| **10** | 15.88 | 16.03 | 17.39 | 11.75 | 10.55 | 12.66 | 2.38 | 2.08 | 2.29 | 16.84 | 16.54 | 17.97 |
| **11** | 24.00 | 23.88 | 21.74 | 9.86 | 10.55 | 11.02 | 0.89 | 0.80 | 1.00 | 11.93 | 12.00 | 10.76 |
| **12** | 17.00 | 17.48 | 18.99 | 2.80 | 2.90 | 4.09 | 0.27 | 0.17 | 0.21 | 6.67 | 7.38 | 6.17 |
| **13** | 7.00 | 8.20 | 7.25 | 2.20 | 2.36 | 3.47 | 0.09 | 0.04 | 0.08 | 5.26 | 4.61 | 5.22 |
| **14** | 7.20 | 6.79 | 6.00 | 0.68 | 0.92 | 0.72 | 0.00 | 0.04 | 0.02 | 2.22 | 1.64 | 2.72 |
| **15** | 4.38 | 4.08 | 5.22 | 2.04 | 4.13 | 3.64 | 0.00 | 0.00 | 0.00 | 0.70 | 0.53 | 0.84 |
| **16** | 3.75 | 2.99 | 2.00 | 1.50 | 2.01 | 2.27 | 0.00 | 0.02 | 0.02 | 0.70 | 1.02 | 0.73 |

**Table. 9.2.2.** Output of one-way ANOVA testing the reproducibility of the three pseudo-replicate extractions on the total phlorotannin content (TPC; µg PGE $mg^{-1}$ DWE), ferric-reducing antioxidant power (FRAP, µg TE $mg^{-1}$ DWE) and radical scavenging activity (RSA; $IC_{50}$, µg $mL^{-1}$) of the LMW phlorotannin-enriched fractions from *P. canaliculata, A. nodosum, F. vesiculosus* and *H. elongata* collected from Finavarra, Co. Clare, in May 2015. The F-statistic (F) and the *p*-value are reported.

| | ***P. canaliculata*** | | ***A. nodosum*** | | ***F. vesiculosus*** | | ***H. elongata*** | |
|---|---|---|---|---|---|---|---|---|
| Variable | F | *p* | F | *p* | F | *p* | F | *p* |
| TPC | (2, 6) = 1.94 | 0.244 | (2, 6) = 2.99 | 0.181 | (2, 6) = 0.109 | 0.160 | (2, 6) = 1.80 | 0.244 |
| FRAP | (2, 6) = 0.81 | 0.488 | (2, 6) = 0.52 | 0.62 | (2, 6) = 0.80 | 0.494 | (2, 6) = 0.71 | 0.957 |
| RSA | (2, 6) = 0.36 | 0.721 | (2, 6) = 2.32 | 0.179 | (2, 6) = 2.73 | 0.143 | (2, 6) = 0.41 | 0.683 |

**Table. 9.2.3**. Output of two-way ANOVA testing the effects of sampling location (L) and season (S) on the on the total phlorotannin content (µg PGE $mg^{-1}$ DWE), ferric-reducing antioxidant power (FRAP, µg TE $mg^{-1}$ DWE) and radical scavenging activity (RSA: $IC_{50}$:µg $mL^{-1}$) of the LMW phlorotannin-enriched fractions from the four brown algal species investigated (*P. canaliculata, A. nodosum, F. vesiculosus* and *H. elongata*) collected from Finavarra, Co. Clare, and Mace Head, Co. Galway, over six seasons from August 2014 to November 2015. The degrees of freedom (DF), the F-statistic (F) and the *p*-value are reported. Tested parameters for which the raw data required transformation prior to ANOVA analysis are indicated by *.

***P. canaliculata***

| | **TPC** | | | **FRAP** | | | **RSA** | | |
|---|---|---|---|---|---|---|---|---|---|
| Variable | DF | F | *p* | DF | F | *p* | DF | F | *p* |
| L | 1 | 0.004 | 0.95 | 1 | 60.88 | <0.001 | 1 | 26.4 | <0.001 |
| S | 5 | 200.92 | <0.001 | 5 | 15.2 | <0.001 | 5 | 19.29 | <0.001 |
| L x S | 5 | 221.18 | <0.001 | 5 | 57.05 | <0.001 | 5 | 8.87 | <0.001 |

***A. nodosum***

| | **TPC*** | | | **FRAP*** | | | **RSA*** | | |
|---|---|---|---|---|---|---|---|---|---|
| | DF | F | *p* | DF | F | *p* | DF | F | *p* |
| L | 1 | 472.26 | <0.001 | 1 | 63.32 | <0.001 | 1 | 303.76 | <0.001 |
| S | 5 | 243.28 | <0.001 | 5 | 162.79 | <0.001 | 5 | 279.44 | <0.001 |
| L x S | 5 | 85.31 | <0.001 | 5 | 22.23 | <0.001 | 5 | 60.83 | <0.001 |

***F. vesiculosus***

| | **TPC** | | | **FRAP** | | | **RSA** | | |
|---|---|---|---|---|---|---|---|---|---|
| | DF | F | *p* | DF | F | *p* | DF | F | *p* |
| L | 1 | 226.15 | <0.001 | 1 | 160.55 | <0.001 | 1 | 22.41 | <0.001 |
| S | 5 | 272.46 | <0.001 | 5 | 157.05 | <0.001 | 5 | 6.36 | 0.019 |
| L x S | 5 | 171.04 | <0.001 | 5 | 45.45 | <0.001 | 5 | 18.75 | <0.001 |

***H. elongata***

| | **TPC** | | | **FRAP** | | | **RSA** | | |
|---|---|---|---|---|---|---|---|---|---|
| | DF | F | *p* | DF | F | *p* | DF | F | *p* |
| L | 1 | 12.35 | 0.002 | 1 | 90.28 | <0.001 | 1 | 133.17 | <0.001 |
| S | 5 | 375.24 | <0.001 | 5 | 0.002 | 0.964 | 5 | 8.9 | 0.006 |
| L x S | 5 | 26.06 | <0.001 | 5 | 2.48 | 0.06 | 5 | 38.94 | <0.001 |

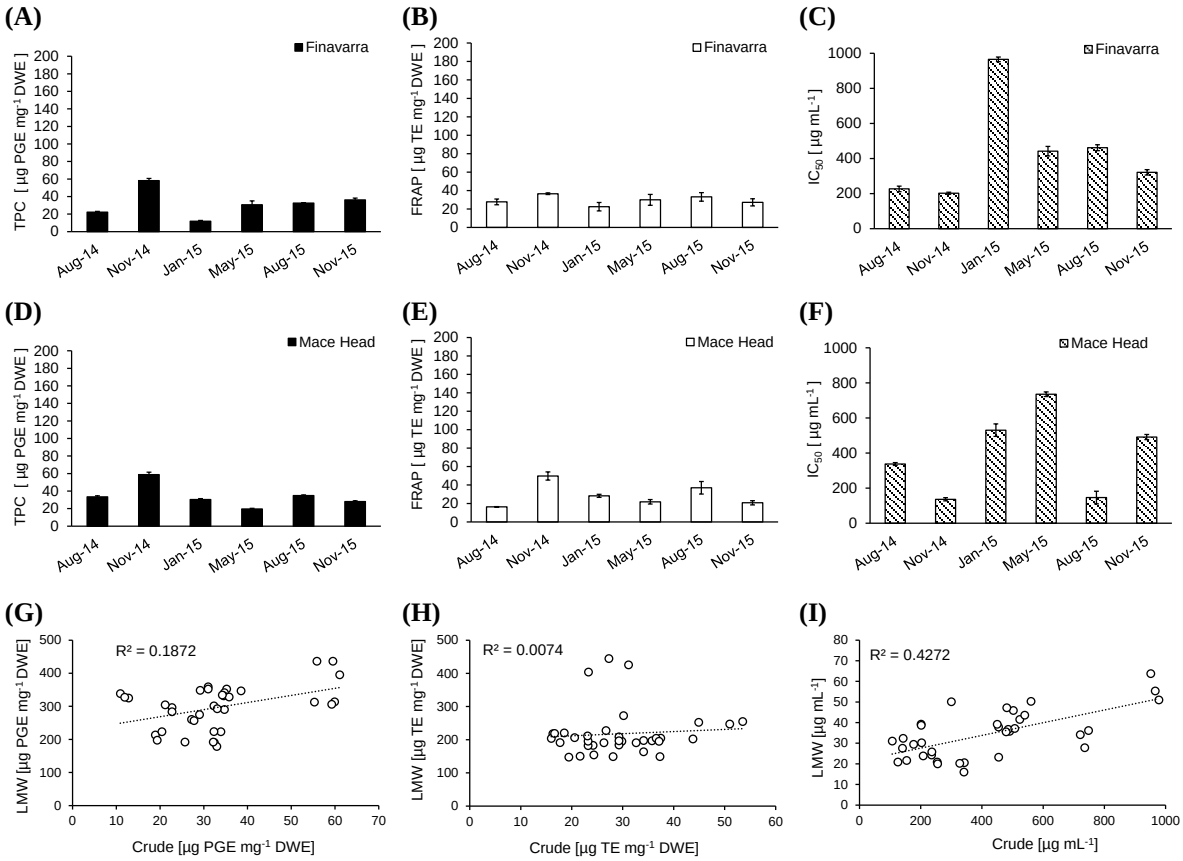


**Fig. 9.2.1.** Seasonal variation in the total phenolic content (TPC) of crude fractions from ***P. canaliculata*** collected from (A) Finavarra, Co. Clare and (B) Mace Head, Co. Galway, expressed as microgram phloroglucinol equivalents per milligram of dry weight extract (µg PGE $mg^{-1}$ DWE). The seasonal variation in the ferric reducing antioxidant power (FRAP) of the crude fractions from *P. canaliculata* collected from (B) Finavarra, Co. Clare and (E) Mace Head, Co. Galway, expressed as microgram Trolox equivalents per milligram of dry weight extract (µg TE $mg^{-1}$ DWE). The seasonal variations of the radical scavenging activity (RSA) of LMW crude fractions from *P. canaliculata* samples collected from (C) Finavarra, Co. Clare and (F) Mace Head, Co. Galway, expressed as $IC_{50}$ values in microgram per millilitre (µg $mL^{-1}$). Correlation coefficient ($R^2$) is given for the linear relationship between crude extracts and LMW fractions in (G) TPC, (H) FRAP and (I) RSA observed at both sites.

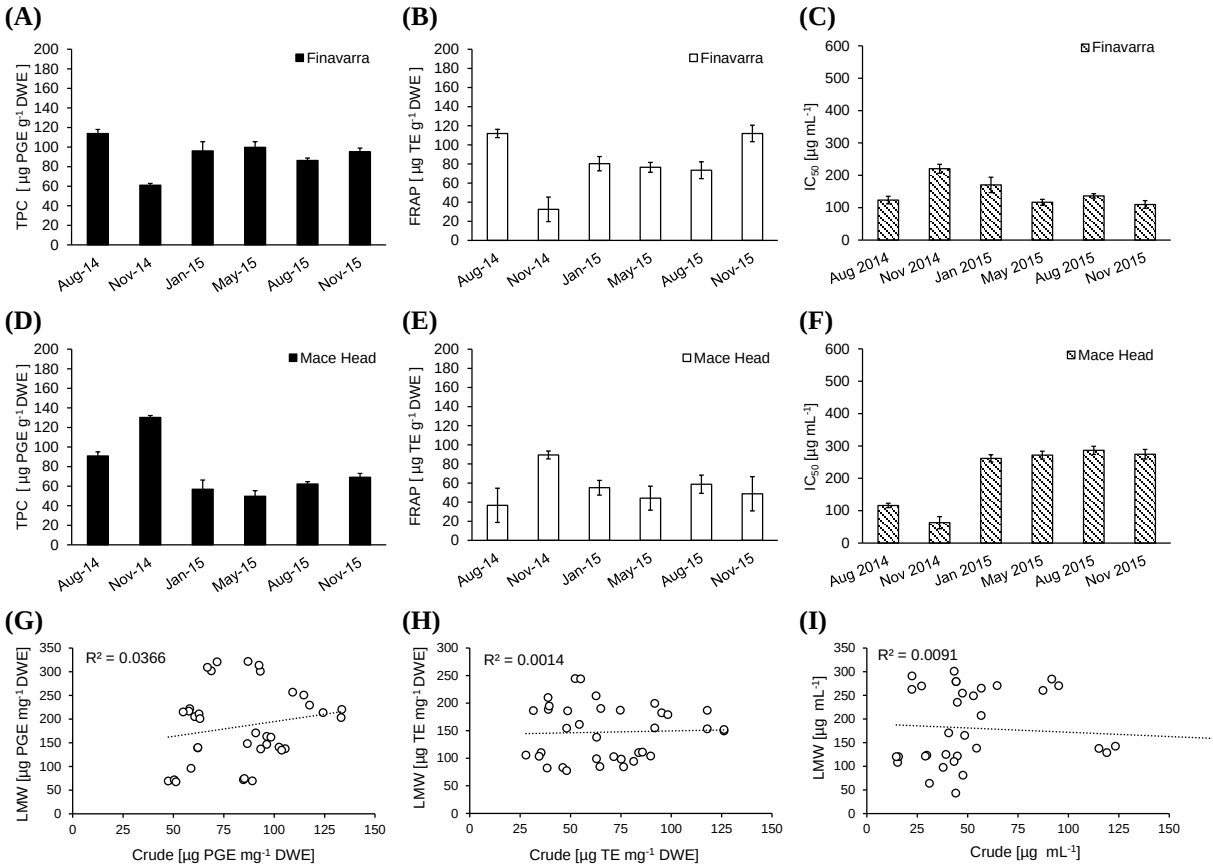


**Fig. 9.2.2.** Seasonal variation in the total phenolic content (TPC) of crude fractions from ***A. nodosum*** collected from (A) Finavarra, Co. Clare and (B) Mace Head, Co. Galway, expressed as microgram phloroglucinol equivalents per milligram of dry weight extract (µg PGE $mg^{-1}$ DWE). The seasonal variation in the ferric reducing antioxidant power (FRAP) of the crude fractions from *A. nodosum* collected from (B) Finavarra, Co. Clare and (E) Mace Head, Co. Galway, expressed as microgram Trolox equivalents per milligram of dry weight extract (µg TE $mg^{-1}$ DWE). The seasonal variations of the radical scavenging activity (RSA) of LMW crude fractions from *A. nodosum* samples collected from (C) Finavarra, Co. Clare and (F) Mace Head, Co. Galway, expressed as $IC_{50}$ values in microgram per millilitre (µg $mL^{-1}$). Correlation coefficient ($R^2$) is given for the linear relationship between crude extracts and LMW fractions in (G) TPC, (H) FRAP and (I) RSA observed at both sites.

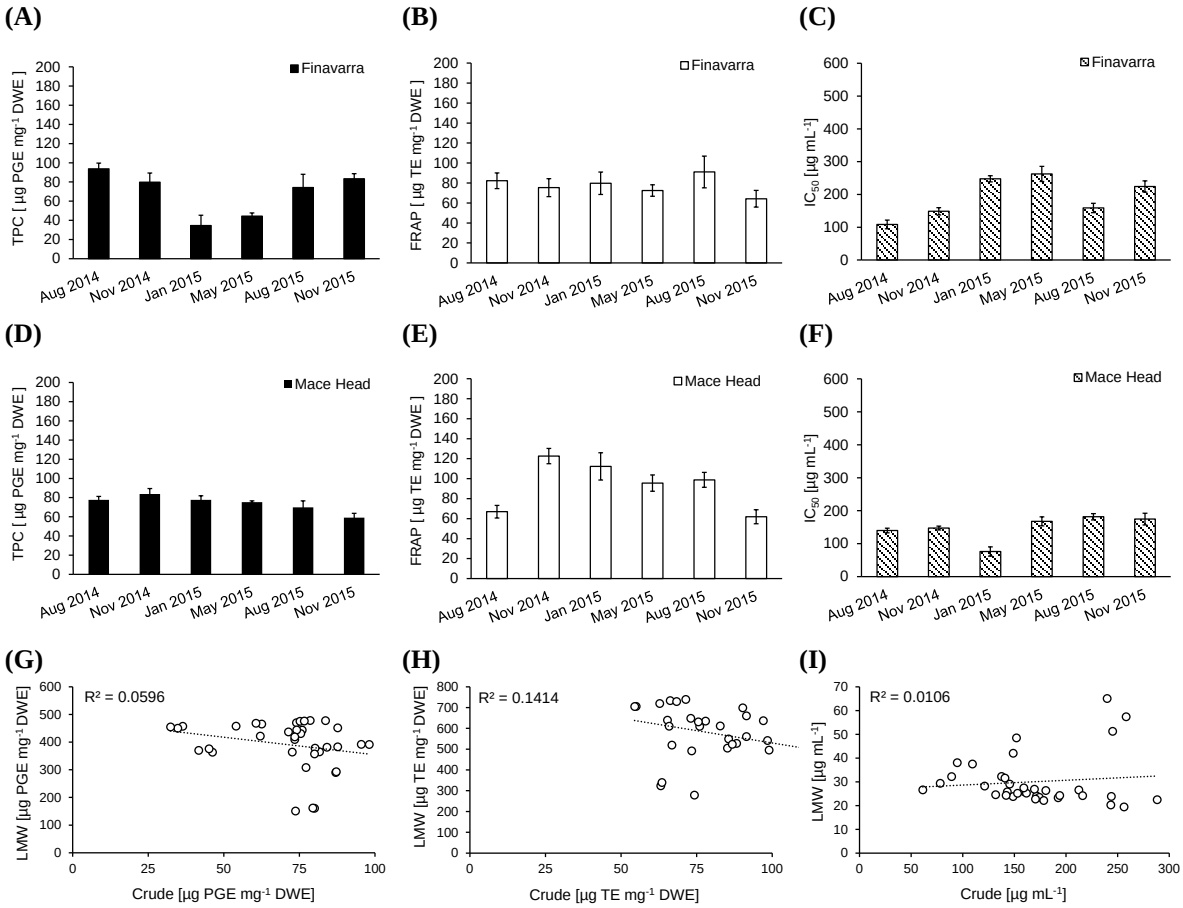


**Fig. 9.2.3.** Seasonal variation in the total phenolic content (TPC) of crude fractions from ***F. vesiculosus*** collected from (A) Finavarra, Co. Clare and (B) Mace Head, Co. Galway, expressed as microgram phloroglucinol equivalents per milligram of dry weight extract (µg PGE $mg^{-1}$ DWE). The seasonal variation in the ferric reducing antioxidant power (FRAP) of the crude fractions from *F. vesiculosus* collected from (B) Finavarra, Co. Clare and (E) Mace Head, Co. Galway, expressed as microgram Trolox equivalents per milligram of dry weight extract (µg TE $mg^{-1}$ DWE). The seasonal variations of the radical scavenging activity (RSA) of LMW crude fractions from *F. vesiculosus* samples collected from (C) Finavarra, Co. Clare and (F) Mace Head, Co. Galway, expressed as $IC_{50}$ values in microgram per millilitre (µg $mL^{-1}$). Correlation coefficient ($R^2$) is given for the linear relationship between crude extracts and LMW fractions in (G) TPC, (H) FRAP and (I) RSA observed at both sites.

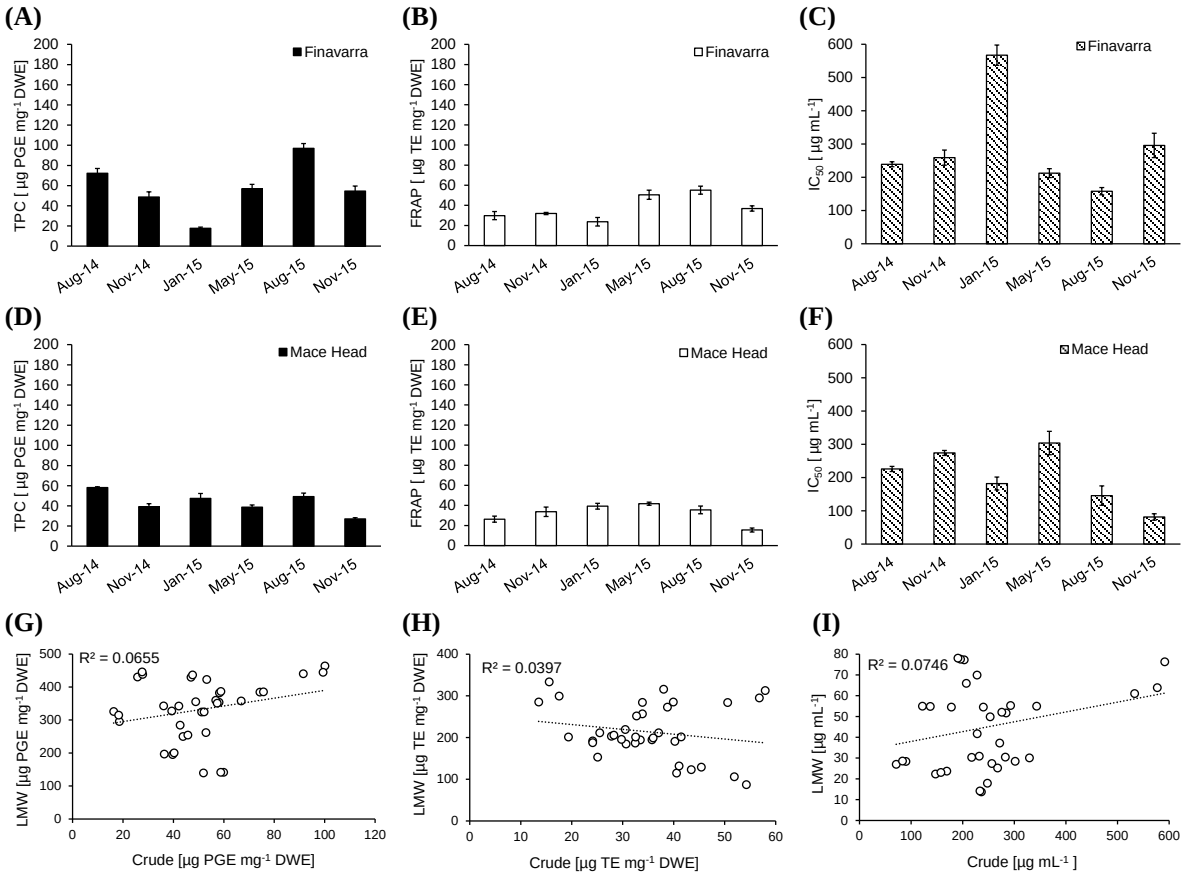


**Figure 9.2.4.** Seasonal variation in the total phenolic content (TPC) of crude fractions from ***H. elongata*** collected from (A) Finavarra, Co. Clare and (B) Mace Head, Co. Galway, expressed as microgram phloroglucinol equivalents per milligram of dry weight extract (µg PGE $mg^{-1}$ DWE). The seasonal variation in the ferric reducing antioxidant power (FRAP) of the crude fractions from *H. elongata* collected from (B) Finavarra, Co. Clare and (E) Mace Head, Co. Galway, expressed as microgram Trolox equivalents per milligram of dry weight extract (µg TE $mg^{-1}$ DWE). The seasonal variations of the radical scavenging activity (RSA) of LMW crude fractions from *H. elongata* samples collected from (C) Finavarra, Co. Clare and (F) Mace Head, Co. Galway, expressed as $IC_{50}$ values in microgram per millilitre (µg $mL^{-1}$). Correlation coefficient ($R^2$) is given for the linear relationship between crude extracts and LMW fractions in (G) TPC, (H) FRAP and (I) RSA observed at both sites.

## 9.3. Supplementary data for Chapter 3

**Table. 9.3.1**. Output of two-way ANOVA testing the effects of thallus part (T) and sampling season (S) on the on the total phlorotannin content (TPC; µg PGE $mg^{-1}$ DWE), ferric-reducing antioxidant power (FRAP; µg TE $mg^{-1}$ DWE) and radical scavenging activity (RSA; $IC_{50}$:µg $mL^{-1}$) of the LMW phlorotannin-enriched fractions from the four brown algal species investigated (*P. canaliculata, A. nodosum, F. vesiculosus* and *H. elongata*) collected from Finavarra, Co. Clare, and Mace Head, Co. Galway, over six seasons from August 2014 to November 2015. The degrees of freedom (DF), the F-statistic (F) and the *p*-value are reported. Tested parameters for which the raw data required transformation prior to analysis are indicated by *.

| ***P. canaliculata*** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **TPC** | | | **FRAP** | | | **RSA** | | |
| Variable | DF | F | *p* | DF | F | *p* | DF | F | *p* |
| T | 1 | 3.66 | 0.068 | 1 | 74.76 | <0.001 | 1 | 0.429 | 0.519 |
| S | 5 | 405.41 | <0.001 | 5 | 191.16 | <0.001 | 5 | 100.43 | <0.001 |
| T x S | 5 | 110.47 | <0.001 | 5 | 51.93 | <0.001 | 5 | 9.55 | <0.001 |
| ***A. nodosum*** | | | | | | | | | |
| | **TPC*** | | | **FRAP*** | | | **RSA*** | | |
| | DF | F | *p* | DF | F | *p* | DF | F | *p* |
| T | 1 | 270.89 | <0.001 | 1 | 21.57 | <0.001 | 1 | 60.85 | <0.001 |
| S | 5 | 171.64 | <0.001 | 5 | 105.07 | <0.001 | 5 | 149.96 | <0.001 |
| T x S | 5 | 122.7 | <0.001 | 5 | 21.34 | <0.001 | 5 | 57.05 | <0.001 |
| ***F. vesiculosus*** | | | | | | | | | |
| | **TPC** | | | **FRAP** | | | **RSA** | | |
| | DF | F | *p* | DF | F | *p* | DF | F | *p* |
| T | 1 | 1.82 | 0.188 | 1 | 5.24 | 0.030 | 1 | 24.29 | <0.001 |
| S | 5 | 32.31 | <0.001 | 5 | 89.89 | <0.001 | 5 | 1.87 | 0.186 |
| T x S | 5 | 3.13 | <0.001 | 5 | 17.15 | <0.001 | 5 | 6.88 | <0.001 |
| ***H. elongata*** | | | | | | | | | |
| | **TPC*** | | | **FRAP*** | | | **RSA*** | | |
| | DF | F | *p* | DF | F | *p* | DF | F | *p* |
| T | 1 | 327.39 | <0.001 | 1 | 109.94 | <0.001 | 1 | 91.67 | <0.001 |
| S | 5 | 322.91 | <0.001 | 5 | 4.46 | 0.045 | 5 | 0.001 | 0.979 |
| T x S | 5 | 159.61 | <0.001 | 5 | 41.87 | <0.001 | 5 | 58.51 | <0.001 |

## 9.4. Supplementary data for Chapter 4

**Table. 9.4.1.** Output of an two-way ANOVA testing the effect of UVR (U; none, UVA and UVA+UVAB) and PAR (P; none, low or high) on the photosynthetic parameters, $F_v/F_m$ (rel. units), $rETR_{max}$ (rel. units) and $E_k$ (µmol photons $m^{-2}$ $s^{-1}$) displayed by *F. vesiculosus* samples after either 3 or 7 days of exposure. The degrees of freedom (DF), F-statistic (F) and the *p*-value are reported.

| **Day 3** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Variable | **$F_v/F_m$** | | | **$rETR_{max}$** | | | **$E_k$** | | |
| | DF | F | *p* | DF | F | *p* | DF | F | *p* |
| U | 2 | 7.974 | <0.001 | 2 | 2.418 | 0.117 | 2 | 2.538 | 0.107 |
| P | 2 | 31.629 | 0.003 | 2 | 17.772 | <0.001 | 2 | 15.352 | <0.001 |
| U x P | 4 | 5.241 | 0.006 | 4 | 0.403 | 0.804 | 4 | 0.797 | 0.543 |
| **Day 7** | | | | | | | | | |
| | **$F_v/F_m$** | | | **$rETR_{max}$** | | | **$E_k$** | | |
| | DF | F | *p* | DF | F | *p* | DF | F | *p* |
| U | 2 | 51.800 | <0.001 | 2 | 12.362 | <0.001 | 2 | 9.574 | 0.001 |
| P | 2 | 47.110 | <0.001 | 2 | 47.040 | <0.001 | 2 | 27.605 | <0.001 |
| U x P | 4 | 33.755 | <0.001 | 4 | 4.776 | 0.008 | 4 | 4.334 | 0.013 |

**Table. 9.4.2.** Output of a paired *t*-test investigating the effect of sampling time, *i.e.* differences between day 3 and 7, on the photosynthetic parameters, $F_v/F_m$ (rel. units), $rETR_{max}$ (rel. units) and $E_k$ (µmol photons $m^{-2}$ $s^{-1}$) displayed by *F. vesiculosus* samples under each of the different irradiance treatments after both 3 and 7 days of exposure. The t-value, degrees of freedom (DF) and the p-value are reported.

| | **Fv/Fm** | | | **$rETR_{max}$** | | | $E_k$ | | |
|---|---|---|---|---|---|---|---|---|---|
| Treatment | t | DF | *p* | t | DF | *p* | t | DF | *p* |
| Dark | 0.38 | 2 | 0.742 | -1.51 | 2 | 0.269 | -1.26 | 2 | 0.334 |
| UA | -1.29 | 2 | 0.323 | -3.90 | 2 | 0.006 | -5.18 | 2 | 0.035 |
| UAB | 11.61 | 2 | 0.007 | 0.88 | 2 | 0.472 | 1.02 | 2 | 0.416 |
| LP | 10.73 | 2 | 0.009 | -3.33 | 2 | 0.080 | -1.86 | 2 | 0.204 |
| LPA | 8.12 | 2 | 0.015 | -3.01 | 2 | 0.095 | -5.45 | 2 | 0.032 |
| LPAB | -1.04 | 2 | 0.406 | -0.38 | 2 | 0.740 | -0.55 | 2 | 0.638 |
| HP | -0.89 | 2 | 0.469 | 0.32 | 2 | 0.780 | 0.27 | 2 | 0.813 |
| HPA | 5.64 | 2 | 0.003 | -0.48 | 2 | 0.680 | -1.43 | 2 | 0.290 |
| HPAB | -1.69 | 2 | 0.233 | -0.29 | 2 | 0.797 | -0.33 | 2 | 0.774 |

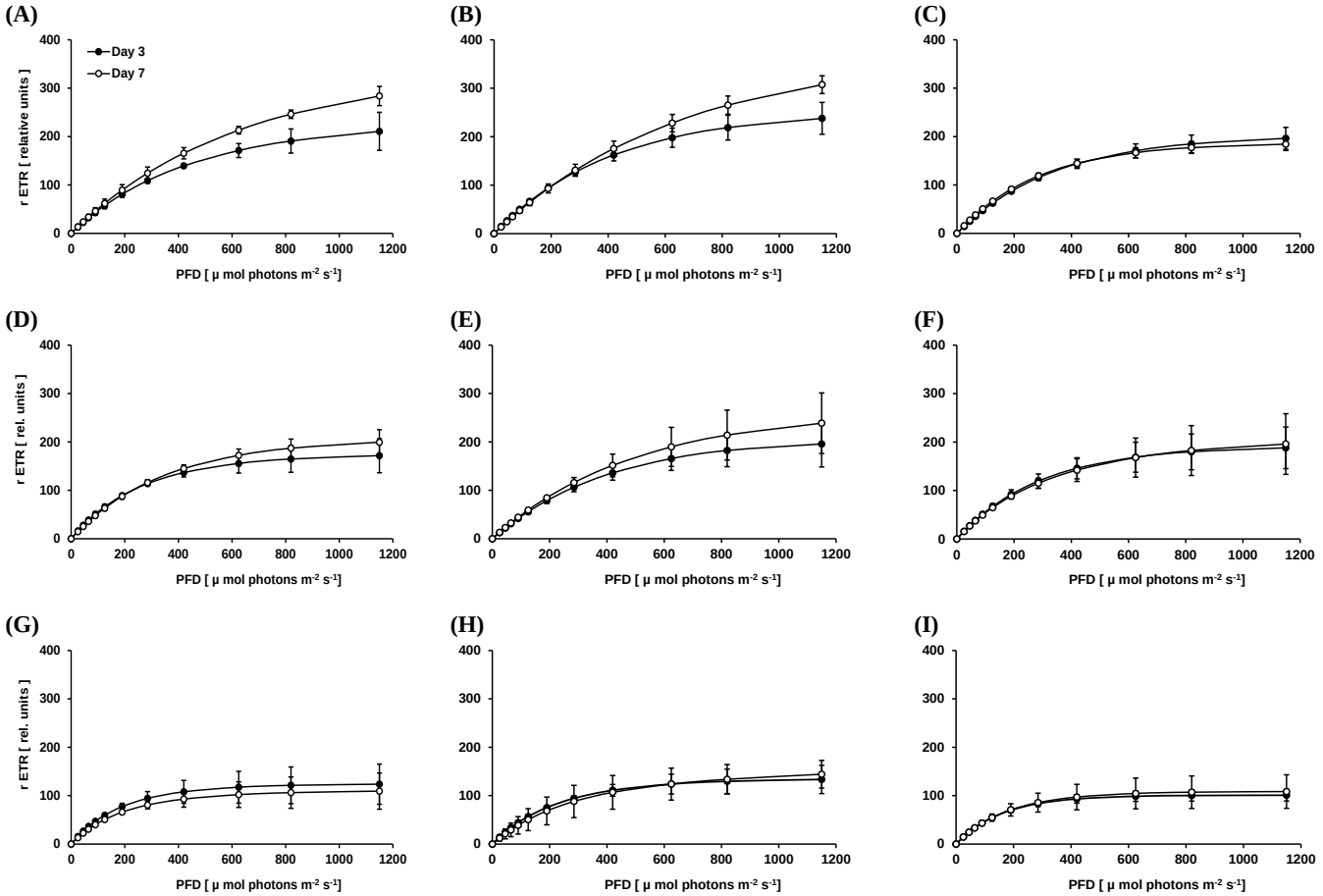


**Fig. 9.4.1**. Photosynthesis-irradiance (*P/E*) curves of *F. vesiculosus* thalli after 3 and 7 days under (A) complete darkness (no PAR, no UVR), (B) UVA, (C) UVA+B, (D) low PAR, (E) low PAR+UVA, (F) low PAR+UVAB, (G) high PAR, (H) high PAR+UVA and (I) high PAR+UVAB. Data is represented as mean ± standard deviation (n=3) of $rETR_{fit}$ calculated using the mathematical model of Walsby (1997).

**Table. 9.4.3.** Output of a three-way mixed ANOVA testing the effects of UVR (U; none, UVA, UVA+B) and PAR (P; none, low and high) on the total phlorotannin content (TPC; μg PGE $mg^{-1}$ DWE), the ferric reducing antioxidant power (FRAP; μg TE $mg^{-1}$ DWE) and the radical scavenging activity (RSA; $IC_{50}$ μg $mL^{-1}$) of LMW pholorotannin-enriched fractions of *F. vesiculosus* samples over time (T). The effect of the various interactions between the three factors was also tested. Degrees of freedom (DF), sum of squares (SS), mean of squares (MS), F-statistic (F) and the *p*-value are reported for each variable tested.

| **TPC** | | | | | |
|---|---|---|---|---|---|
| Variable | DF | SS | MS | F | *p* |
| U | 2 | 41379.089 | 20689.544 | 67.021 | <0.001 |
| P | 2 | 2265.855 | 1132.928 | 3.67 | 0.046 |
| T | 2 | 517352.374 | 258676.187 | 1170.516 | <0.001 |
| Ux P | 4 | 21106.426 | 5276.607 | 17.093 | <0.001 |
| U x T | 4 | 45786.912 | 11446.728 | 51.797 | <0.001 |
| P x T | 4 | 9674.464 | 2418.616 | 10.944 | <0.001 |
| U x P x T | 8 | 11997.425 | 1499.678 | 6.786 | <0.001 |
| **FRAP** | | | | | |
| U | 2 | 3439.575 | 1719.787 | 6.843 | <0.001 |
| P | 2 | 32792.074 | 16396.037 | 65.243 | 0.006 |
| T | 2 | 1016653.018 | 508326.509 | 1768.181 | <0.001 |
| U x P | 4 | 16246.516 | 4061.629 | 16.162 | <0.001 |
| U x T | 4 | 4534.374 | 1133.594 | 3.943 | 0.009 |
| P x T | 4 | 39983.228 | 9995.807 | 34.770 | <0.001 |
| U x P x T | 8 | 26001.846 | 3250.231 | 11.306 | <0.001 |
| **RSA** | | | | | |
| U | 2 | 0.311 | 0.156 | 0.011 | 0.989 |
| P | 2 | 89.603 | 44.802 | 3.197 | 0.065 |
| T | 2 | 1776.426 | 888.213 | 50.452 | <0.001 |
| Ux P | 4 | 90.822 | 22.706 | 1.62 | 0.212 |
| U x T | 4 | 2114.688 | 528.672 | 30.029 | <0.001 |
| P x T | 4 | 46.405 | 11.601 | 0.659 | 0.625 |
| U x P x T | 8 | 405.668 | 50.709 | 2.880 | 0.014 |

## 9.5. Supplementary data for Chapter 5

**Table. 9.5.1.** Output of one-way ANOVA testing the effect of sampling site (Finavarra, Co. Clare and Oranmore, Co. Galway) on the carbon (C) and nitrogen (N) content (% DW) and carbon: nitrogen (C: N) ratios within *F. vesiculosus* thalli as well as the total phlorotannin content (TPC) of the LMW phlorotannin-enriched fractions. The F-statistic (F) and the *p*-value are reported.

| Variable | F | *p* |
|---|---|---|
| **C** | (1, 4) = 66.315 | <0.001 |
| **N** | (1, 4) = 1246.21 | <0.001 |
| **C: N** | (1, 4) = 1644.73 | <0.001 |
| **TPC** | (1, 4) = 154.11 | <0.001 |

**Table. 9.5.2.** Output of a two-way mixed ANOVA testing the effect of media nitrogen concentrations (M; 0, 2, 100 µM and 1 mM) and time on the carbon (C) and nitrogen (N) content (% DW) and carbon: nitrogen (C: N) ratios displayed by *F. vesiculosus* samples over time (T). The interactive effect of the two factors was also tested. Degrees of freedom (DF), sum of squares (SS), mean of squares (MS), F-statistic (F) and the *p*-value are reported for each variable tested.

| **C** | | | | | |
|---|---|---|---|---|---|
| Variable | DF | SS | MS | F | *p* |
| M | 3 | 0.682 | 0.227 | 6.799 | 0.014 |
| T | 2 | 1.919 | 0.959 | 17.093 | <0.001 |
| M x T | 6 | 2.797 | 0.466 | 8.306 | <0.001 |
| **N** | | | | | |
| M | 3 | 3.166 | 1.055 | 2558.757 | <0.001 |
| T | 2 | 0.919 | 0.459 | 3339.226 | <0.001 |
| M x T | 6 | 1.876 | 0.313 | 2272.694 | <0.001 |
| **C: N** | | | | | |
| M | 3 | 2829.045 | 943.015 | 87236.162 | <0.001 |
| T | 2 | 922.788 | 461.394 | 496.769 | <0.001 |
| M x T | 6 | 1517.146 | 252.858 | 272.244 | <0.001 |

**Table. 9.5.3.** Output of a two-way mixed ANOVA testing the effects of of media nitrogen concentrations (M; 0, 2, 100 µM and 1 mM) on the total phlorotannin content (TPC; µg PGE $mg^{-1}$ DWE), the ferric reducing antioxidant power (FRAP; µg TE $mg^{-1}$ DWE) and the radical scavenging activity (RSA; $IC_{50}$ µg $mL^{-1}$) of LMW pholorotannin-enriched fractions of *F. vesiculosus* samples over time (T). The interactive effect of the two factors was also tested. Degrees of freedom (DF), sum of squares (SS), mean of squares (MS), F-statistic (F) and the *p*-value are reported for each variable tested.

| **TPC** | | | | | |
|---|---|---|---|---|---|
| Variable | DF | SS | MS | F | *p* |
| M | 3 | 3810.162 | 1270.054 | 10.958 | <0.001 |
| T | 2 | 39504.406 | 19752.203 | 132.081 | 0.003 |
| M x T | 6 | 2455.116 | 409.186 | 2.736 | 0.050 |
| **FRAP** | | | | | |
| M | 3 | 5286.756 | 1762.252 | 2.372 | 0.146 |
| T | 2 | 114347.817 | 57173.909 | 55.899 | <0.001 |
| M x T | 6 | 5419.064 | 903.177 | 0.883 | 0.529 |
| **RSA** | | | | | |
| M | 3 | 16.911 | 5.637 | 2.351 | 0.148 |
| T | 2 | 161.999 | 81.0 | 28.215 | <0.001 |
| M x T | 6 | 26.291 | 4.382 | 1.526 | 0.232 |

**Table. 9.5.4.** Output of a three-way mixed ANOVA testing the effect of media nitrogen concentration (M; low: 2 and high: 100 µM), irradiance (L; low: 15-20 µmol photons $m^{-2}$ $s^{-1}$ and high: 110-115 µmol photons $m^{-2}$ $s^{-1}$) on the carbon (C) and nitrogen (N) content (% DW) and carbon: nitrogen (C: N) ratios displayed by *F. vesiculosus* samples over time (T). The effect of the various interactions between the three factors was also tested. Degrees of freedom (DF), sum of squares (SS), mean of squares (MS), F-statistic (F) and the *p*-value are reported for each variable tested.

| **C** | | | | | |
|---|---|---|---|---|---|
| Variable | DF | SS | MS | F | *p* |
| M | 1 | 2.107 | 2.107 | 114.963 | <0.001 |
| L | 1 | 2.021 | 2.021 | 110.260 | <0.001 |
| T | 2 | 3.205 | 1.603 | 53.847 | <0.001 |
| M x L | 1 | 0.216 | 0.216 | 11.796 | 0.009 |
| M x T | 2 | 2.193 | 1.097 | 36.846 | <0.001 |
| L x T | 2 | 1.074 | 0.537 | 18.035 | <0.001 |
| M x L x T | 2 | 0.581 | 0.291 | 9.765 | 0.002 |
| **N** | | | | | |
| M | 1 | 0.258 | 0.258 | 424.998 | <0.001 |
| L | 1 | 0.000 | 0.000 | 0.170 | 0.691 |
| T | 2 | 0.146 | 0.073 | 214.894 | <0.001 |
| M x L | 1 | $7.803e^{-5}$ | $7.803e^{-5}$ | 0.128 | 0.729 |
| M x T | 2 | 0.159 | 0.80 | 235.249 | <0.001 |
| L x T | 2 | 0.001 | 0.000 | 0.947 | 0.409 |
| M x L x T | 2 | 0.002 | 0.001 | 3.338 | 0.061 |
| **C: N** | | | | | |
| M | 1 | 2315.053 | 2315.053 | 273.464 | <0.001 |
| L | 1 | 12.308 | 12.308 | 1.454 | 0.262 |
| T | 2 | 1023.586 | 511.793 | 236.155 | <0.001 |
| M x L | 1 | 5.145 | 5.145 | 0.608 | 0.458 |
| M x T | 2 | 1286.565 | 643.283 | 296.828 | <0.001 |
| L x T | 2 | 18.581 | 9.291 | 4.287 | 0.032 |
| M x L x T | 2 | 20.100 | 10.050 | 4.637 | 0.026 |

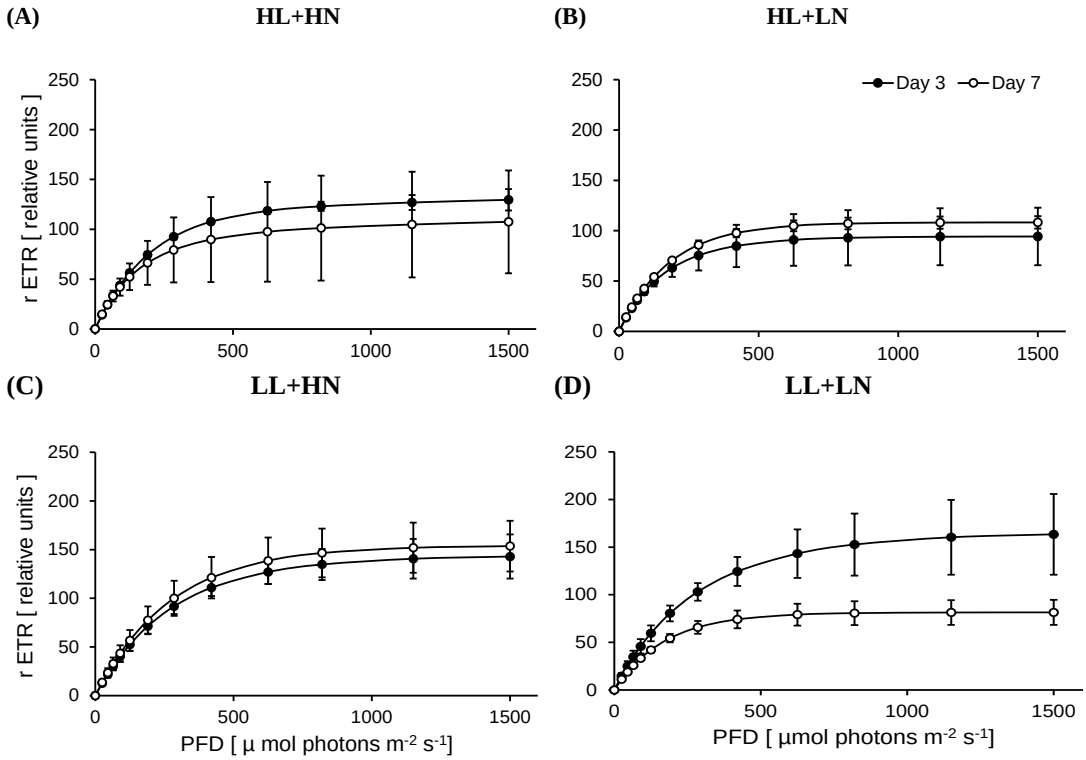


**Fig. 9.5.1.** Photosynthesis-irradiance (*P/E*) curves of *F. vesiculosus* exposed of to (A) HL+HN, (B) HL+LN, (C) LL+HN and (D) LL+LN after 3 (black) and 7 (white) days of exposure. Data are represented as mean ± standard deviation (n=3). *P/E* curves were fitted to the mathematical model of Walsby (1997).

**Table. 9.5.5.** Output of two-way ANOVA testing the effects of the nitrogen concentration of the media (M; low: 2 µM and high: 100 µM), irradiance (L; low: 15-20 µmol photons $m^{-2}$ $s^{-1}$ and high: 110-115 µmol photons $m^{-2}$ $s^{-1}$) after 3 or 7 days and the interaction of the two parameters on the carbon (C) and nitrogen (N) content (% DW) and carbon: nitrogen (C: N) ratios displayed by *F. vesiculosus* after 3 and 7 days of exposure. The degrees of freedom (DF), F-statistic (F) and the p-value are reported.

| **Day 3** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Variables | **$F_v/F_m$** | | | **$rETR_{max}$** | | | **$E_k$** | | |
| | DF | F | *p* | DF | F | *p* | DF | F | *p* |
| M | 1 | 7.040 | 0.029 | 1 | 0.128 | 0.730 | 1 | 0.381 | 0.554 |
| L | 1 | 5.415 | 0.048 | 1 | 6.713 | 0.032 | 1 | 6.476 | 0.034 |
| M x L | 1 | 6.039 | 0.039 | 1 | 2.542 | 0.150 | 1 | 0.005 | 0.947 |
| **Day 7** | | | | | | | | | |
| | **$F_v/F_m$** | | | **$rETR_{max}$** | | | **$E_k$** | | |
| | DF | F | *p* | DF | F | *p* | DF | F | *p* |
| M | 1 | 2.250 | 0.172 | 1 | 3.563 | 0.096 | 1 | 1.658 | 0.234 |
| L | 1 | 3.087 | 0.117 | 1 | 0.427 | 0.5312 | 1 | 0.731 | 0.417 |
| M x L | 1 | 40.228 | <0.001 | 1 | 4.549 | 0.066 | 1 | 3.731 | 0.090 |

**Table. 9.5.6.** Output of a paired *t*-test investigating the effect of sampling time, *i.e.* differences between day 3 and 7, on the photosynthetic parameters, $F_v/F_m$ (rel. units), $rETR_{max}$ (rel. units) and $E_k$ (µmol photons $m^{-2}$ $s^{-1}$) displayed by *F. vesiculosus* samples under each of the different combination treatments of both different nitrogen (N; low – L, high – H) concentrations and irradiance (L: low – L, high – H) levels. The t-value, degrees of freedom (DF) and the *p*-value are reported.

| | **$F_v/F_m$** | | | **$rETR_{max}$** | | | **$E_k$** | | |
|---|---|---|---|---|---|---|---|---|---|
| Treatment | t | DF | *p* | t | DF | *p* | t | DF | *p* |
| HL+HN | 0.327 | 2 | 0.775 | 0.693 | 2 | 0.560 | 0.072 | 2 | 0.949 |
| HL+LN | -6.615 | 2 | 0.022 | -0.782 | 2 | 0.516 | 1.592 | 2 | 0.252 |
| LL+HN | -24.296 | 2 | 0.002 | -0.342 | 2 | 0.765 | 0.340 | 2 | 0.767 |
| LL+LN | 0.486 | 2 | 0.675 | 4.267 | 2 | 0.051 | 2.137 | 2 | 0.166 |

**Table. 9.5.7.** Output of a three-way mixed ANOVA testing the effect of media nitrogen concentration (M; low: 2 and high: 100 µM), irradiance (L; low: 15-20 µmol photons $m^{-2}$ $s^{-1}$ and high: 110-115 µmol photons $m^{-2}$ $s^{-1}$) on the total phlorotannin content (TPC; µg PGE $mg^{-1}$ DWE), the ferric reducing antioxidant power (FRAP; µg TE $mg^{-1}$ DWE) and the radical scavenging activity (RSA; $IC_{50}$ µg $mL^{-1}$) of LMW pholorotannin-enriched fractions of *F. vesiculosus* samples over time (T). The effect of the various interactions between the three factors was also tested. Degrees of freedom (DF), sum of squares (SS), mean of squares (MS), F-statistic (F) and the *p*-value are reported for each variable tested.

| **TPC** | | | | | |
|---|---|---|---|---|---|
| Variable | DF | SS | MS | F | *p* |
| M | 1 | 6.292 | 6.292 | 0.027 | 0.873 |
| L | 1 | 4179.825 | 4179.825 | 17.970 | 0.003 |
| T | 2 | 17532.782 | 8766.391 | 14.815 | <0.001 |
| M x L | 1 | 6883.175 | 6883.175 | 29.592 | <0.001 |
| M x T | 2 | 1965.379 | 982.690 | 1.661 | 0.221 |
| L x T | 2 | 2216.640 | 1108.320 | 1.873 | 0.186 |
| M x L x T | 2 | 4006.413 | 2003.206 | 3.385 | 0.059 |
| **FRAP** | | | | | |
| M | 1 | 337.457 | 337.457 | 0.415 | 0.538 |
| L | 1 | 12747.915 | 12747.915 | 15.659 | 0.004 |
| T | 2 | 104833.049 | 52416.524 | 233.153 | <0.001 |
| M x L | 1 | 17074.649 | 17074.649 | 20.974 | 0.002 |
| M x T | 2 | 9376.029 | 4688.014 | 20.853 | <0.001 |
| L x T | 2 | 17222.511 | 8611.255 | 38.304 | <0.001 |
| M x L x T | 2 | 9267.290 | 4633.645 | 20.611 | <0.001 |
| **RSA** | | | | | |
| M | 1 | 151.413 | 151.413 | 4.339 | 0.071 |
| L | 1 | 0.951 | 0.951 | 0.027 | 0.873 |
| T | 2 | 250.314 | 125.157 | 8.925 | 0.002 |
| M x L | 1 | 6.208 | 6.208 | 0.178 | 0.684 |
| M x T | 2 | 94.064 | 47.032 | 3.354 | 0.061 |
| L x T | 2 | 78.993 | 39.497 | 2.816 | 0.090 |
| M x L x T | 2 | 17.628 | 8.814 | 0.628 | 0.546 |

## 9.6. Supplementary data for Chapter 6

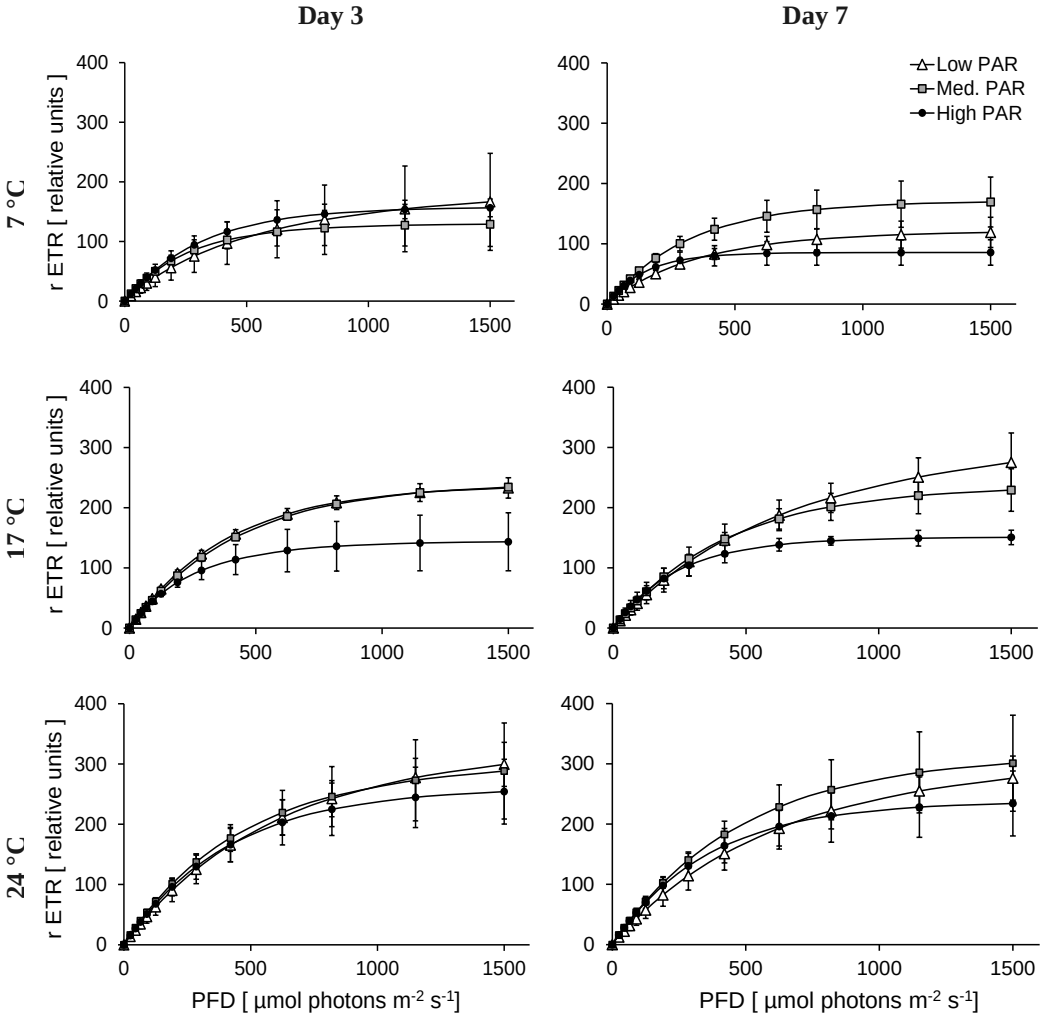


**Fig. 9.6.1** Photosynthesis-irradiance (*P/E*) curves in samples of *F. vesiculosus* exposed of to three temperatures (7, 17 and 24°C) under three different PAR intensities-low (15-20 µmol photons $m^{-2}$ $s^{-1}$; white triangle), medium (50-60 µmol photons $m^{-2}$ $s^{-1}$; grey square) and high (110-115 µmol photons $m^{-2}$ $s^{-1}$; black circle) after 3 and 7 days of exposure. Data is represented as mean ± standard deviation (n=3). *P/E* curves were fitted to the mathematical model of Walsby (1997).

**Table. 9.6.1.** Output of two-way ANOVA testing the effects of temperature (TE; low -7, med - 17 and high - 24°C) and irradiance (L; low- 15-20, med - 50-60, high - 110-115 µmol photons $m^{-2}$ $s^{-1}$) and the interaction of the two parameters on the photosynthetic parameters, $F_v/F_m$ (rel. units), $rETR_{max}$ (rel. units) and $E_k$ (µmol photons $m^{-2}$ $s^{-1}$) displayed by *F. vesiculosus* after 3 and 7 days of exposure. The degrees of freedom (DF), F-statistic (F) and the *p*-value are reported.

| **Day 3** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Variables | **$F_v/F_m$** | | | **$rETR_{max}$** | | | **$E_k$** | | |
| | DF | F | *p* | DF | F | *p* | DF | F | *p* |
| TE | 2 | 1.715 | 0.208 | 2 | 5.257 | 0.016 | 2 | 6.567 | 0.007 |
| L | 2 | 1.640 | 0.222 | 2 | 49.216 | <0.001 | 2 | 25.194 | <0.001 |
| TE x L | 2 | 0.265 | 0.897 | 2 | 3.205 | 0.038 | 2 | 1.926 | 0.150 |
| **Day 7** | | | | | | | | | |
| | **$F_v/F_m$** | | | **$rETR_{max}$** | | | **$E_k$** | | |
| | DF | F | *p* | DF | F | *p* | DF | F | *p* |
| TE | 2 | 1.402 | 0.272 | 2 | 18.354 | <0.001 | 2 | 13.186 | <0.001 |
| L | 2 | 4.106 | 0.034 | 2 | 58.392 | <0.001 | 2 | 17.671 | <0.001 |
| TE x L | 2 | 1.602 | 0.217 | 2 | 1.402 | 0.273 | 2 | 2.964 | 0.048 |

**Table. 9.6.2.** Output of a paired *t*-test investigating the effect of sampling time, *i.e.* differences between day 3 and 7, on the photosynthetic parameters, $F_v/F_m$ (rel. units), $rETR_{max}$ (rel. units) and $E_k$ (µmol photons $m^{-2}$ $s^{-1}$) displayed by *F. vesiculosus* samples under each of the different combination treatments of both different temperature (T; low–L, med–M and high–H) irradiance (L; low–L, med–M and high–H) levels. The t-value, degrees of freedom (DF) and the *p*-value are reported.

| | **$F_v/F_m$** | | | **$rETR_{max}$** | | | **$E_k$** | | |
|---|---|---|---|---|---|---|---|---|---|
| Treatment | t | DF | *p* | t | DF | *p* | t | DF | *p* |
| **LL+LT** | -1.964 | 2 | 0.188 | 0911 | 2 | 0.458 | 0.651 | 2 | 0.582 |
| **LL+MT** | -1.512 | 2 | 0.270 | -1.007 | 2 | 0.420 | -0.480 | 2 | 0.679 |
| **LL+HT** | 1.084 | 2 | 0.392 | 12.308 | 2 | 0.007 | 15.779 | 2 | 0.004 |
| **ML+LT** | 0.319 | 2 | 0.780 | -0.771 | 2 | 0.521 | -0.975 | 2 | 0.432 |
| **ML+MT** | -0.353 | 2 | 0.758 | 0.146 | 2 | 0.897 | 0.343 | 2 | 0.765 |
| **ML+HT** | -3.600 | 2 | 0.069 | -0.243 | 2 | 0.831 | 0.022 | 2 | 0.984 |
| **HL+LT** | -1.521 | 2 | 0.268 | 0.335 | 2 | 0.770 | 4.146 | 2 | 0.054 |
| **HL+MT** | -1.090 | 2 | 0.390 | 1.371 | 2 | 0.304 | 0.711 | 2 | 0.551 |
| **HL+HT** | 0.069 | 2 | 0.952 | 0.988 | 2 | 0.427 | 1.160 | 2 | 0.366 |

**Table. 9.6.3.** Output of a three-way mixed ANOVA testing the effects of temperature (TE; low -7, Med -17 and high - 24°C) and irradiance (L; low- 15-20, med - 50-60, high - 110-115 µmol photons $m^{-2}$ $s^{-1}$) on the total phlorotannin content (TPC; µg PGE $mg^{-1}$ DWE), the ferric reducing antioxidant power (FRAP; µg TE $mg^{-1}$ DWE) and the radical scavenging activity (RSA; $IC_{50}$ µg $mL^{-1}$) of the LMW pholorotannin-enriched fractions of *F. vesiculosus* samples over time (T). The effect of the various interactions between the three factors was also tested. Degrees of freedom (DF), sum of squares (SS), mean of squares (MS), F-statistic (F) and the *p*-value are reported for each variable tested.

| **TPC** | | | | | |
|---|---|---|---|---|---|
| Variable | DF | SS | MS | F | *p* |
| TE | 2 | 10623.715 | 5311.858 | 10.100 | <0.001 |
| L | 2 | 4077.569 | 2038.784 | 3.876 | 0.040 |
| T | 2 | 135978.583 | 67989.291 | 970.033 | <0.001 |
| TE x L | 4 | 669.163 | 167.291 | 0.318 | 0.862 |
| TE x T | 4 | 15817.041 | 3954.260 | 56.417 | <0.001 |
| L x T | 4 | 2608.126 | 652.031 | 9.303 | <0.001 |
| TE x L x T | 8 | 2636.011 | 329.501 | 4.701 | <0.001 |
| **FRAP** | | | | | |
| TE | 2 | 25586.062 | 12793.031 | 19.583 | <0.001 |
| L | 2 | 19909.034 | 9954.517 | 15.238 | <0.001 |
| T | 2 | 107266.452 | 53613.226 | 89.814 | <0.001 |
| TE x L | 4 | 28768.700 | 7192.175 | 11.009 | <0.001 |
| TE x T | 4 | 25541.226 | 6385.311 | 10.697 | <0.001 |
| L x T | 4 | 37726.394 | 9431.599 | 15.800 | <0.001 |
| TE x L x T | 8 | 23163.145 | 2895.393 | 4.850 | <0.001 |
| **RSA** | | | | | |
| TE | 2 | 35.049 | 17.524 | 6.221 | 0.009 |
| L | 2 | 9.218 | 4.609 | 1.636 | 0.222 |
| T | 2 | 45.610 | 22.805 | 5.482 | 0.008 |
| TE x L | 4 | 26.115 | 6.529 | 2.317 | 0.097 |
| TE x T | 4 | 98.831 | 24.708 | 5.939 | 0.001 |
| L x T | 4 | 33.795 | 8.449 | 2.031 | 0.111 |
| TE x L x T | 8 | 34.148 | 4.269 | 1.026 | 0.435 |

## 9.7. Supplementary data for Chapter 7

**Table. 9.7.1.** Output of three-way mixed ANOVA testing the effects of different storage conditions (STO; -20°C, 4°C, at room temperature in closed vials (RT), at room temperature in open vials exposed to atmospheric air (RTE) and at 50°C) and the physical state (STA: powder or liquid) on the radical scavenging activity of LMW phlorotannin-enriched fractions from *F. vesiculosus* over time (T: week 0, 2, 4, 8 and 10). The degrees of freedom (DF), F-statistic (F) and the p-value are reported.

| **RSA** | | | | | |
|---|---|---|---|---|---|
| Variable | DF | SS | MS | F | *p* |
| **STO** | 4 | 3760.133 | 940.003 | 115.939 | <0.001 |
| **STA** | 1 | 108.603 | 108.603 | 13.395 | <0.001 |
| **T** | 4 | 3641.586 | 910.396 | 92.368 | <0.001 |
| **STO x STA** | 4 | 2365.822 | 591.455 | 72.947 | <0.001 |
| **STO x T** | 16 | 4368.024 | 273.001 | 27.699 | <0.001 |
| **STA x T** | 4 | 1307.461 | 326.865 | 33.163 | <0.001 |
| **STO x STA x T** | 16 | 3138.874 | 196.180 | 19.904 | <0.001 |

# Chapter 10

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