**MADALENA CARIA MENDES**

# Antioxidant Contents of *Fucus vesiculosus* L., in Response to Environmental Parameters

**UNIVERSIDADE DO ALGARVE**
Faculdade de Ciências e Tecnologia

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# Antioxidant Contents of *Fucus vesiculosus* L., in Response to Environmental Parameters

**Mestrado em Aquacultura e Pescas**

**Trabalho efetuado sob a orientação de:**

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**UNIVERSIDADE DO ALGARVE**
Faculdade de Ciências e Tecnologia

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Madalena Caria Mendes

# Abbreviations

| | |
|---|---|
| BHA | Butylated hydroxyl anisole |
| BHT | Butylated hydroxytoluene |
| DAD | Diode array detector |
| DPPH | α,α-diphenyl-β-picrylhydrazyl |
| EtOH | Ethanol |
| *FC* | Folin-Ciocalteu's phenol reagent |
| GAE | Gallic acid equivalent |
| HCl | Hydrochloric acid |
| HPLC | High performance liquid chromatography |
| MCX | Mixed-mode cation exchange |
| MeOH | Methanol |
| MS | Mass spectrometry |
| nm | Nanometers |
| NMR | Nuclear magnetic resonance |
| PAR | Photosynthetically active radiation |
| PG | N-propyl gallate |
| PSU | Practical Salinity Unit |
| qHNMR | Quantitative H nuclear magnetic resonance spectroscopy |
| ROS | Reactive oxygen species |
| RP-HPLC | Reverse phase high performance liquid chromatography |
| SLE | Solid liquid extraction |
| TBHQ | Tertiary butyl hydroquinone |
| TPC | Total phenolic contents |
| UV | Ultraviolet |

# Nomenclature

| | |
|---|---|
| $A_0$ | Absorbance of sample control |
| $A_b$ | Absorbance of sample blank |
| $A_s$ | Absorbance of sample |
| $A^c$ | Peak area of the internal standard in the extraction solvent |
| $A^{Pi}$ | Peak area |
| $A^s$ | Peak area of the internal standard in the sample |
| *ae* | Acetone extracts |
| $C_{phenolic}$ | Concentration of each individual peak of phenolic compound |
| *cpi* | Individual peak of pigment |
| *dw* | Dry weight |
| *dm* | Dry matter |
| ECxx | Effective concentration to inhibit xx% |
| *eaf* | Ethyl acetate fractions of liquid-liquid partitioning |
| *ee* | Ethanol extracts |
| *me* | Methanol extracts |
| Mf | Weight of the sample |
| Pi | Pigment |
| RPi | Response factor of pigment Pi |
| T | Time in days |
| Vc | Amount of sample injected onto the column |
| Vx | Extraction volume |
| *we* | Water extracts |
| *ww* | Wet weight |
| $ww_i$ | Final wet weight |
| $ww_i$ | Initial wet weight |

# Abstract

Seaweed are a diverse group of algae with great value and vast application possibilities. The seaweed *Fucus vesiculosus*, stands out for having elevated concentrations of polyphenols and antioxidant activities. Polyphenols possess many biological properties including antioxidant, antimicrobial, anti-inflammatory, and free radical scavenging activities. Therefore, this species is a possible candidate as natural source of bioactive compounds for the development of novel products to be applied in the nutraceutical, pharmaceutical, and cosmetic industries, in the future. This project aimed to investigate the effects of seasonality, and culture conditions such as salinity and ultraviolet radiation (UV) on the antioxidant contents and *in vitro* properties of *F. vesiculosus*. This was carried out through spectrophotometric analysis, namely total phenolic contents (TPC), high performance liquid chromatography (HPLC), α,α-diphenyl-β-picrylhydrazyl (DPPH) radical scavenging activity and iron chelating capacity. Furthermore, the epiphytic algae coverage was determined and physodes (structures containing phenolics) were investigated microscopically through vanillin-HCL staining.

This information was used to identify the best harvest time targeting the highest concentration of antioxidants, which was **hypothesized** to be in late summer-early autumn, due to the protection against UV-irradiation over summer by phenolic accumulation. Significant differences supporting seasonality in antioxidants were found. Late Spring to early Fall had the highest extraction yields ($P \approx 0.0045$) and concentrations ($P < 0.0010$) in contrast to the lowest winter concentrations. A total of 13 monophenolic acids were detected through RP-HPLC, with September samples being the most diverse in compounds and March samples the least. Gallic acid was identified in all analyzed months. Furthermore, antioxidant contents and activity was linked to environmental parameters, namely positive correlations between temperature, TPC ($P= 0.0391$) and DPPH ($P= 0.0016$); likewise, between sunlight hours per day, TPC ($P= 0.0458$) and DPPH ($P=0.0060$). During experimental growth trials, three salinity concentrations and presence of UV light were tested, to verify or reject the **second hypothesis:** increasing salinity and exposure to UV light would yield higher polyphenolic concentrations. No significant differences were observed between the TPC and salinity ($P=0.0525$), and light ($P=0.2443$), under the experimental conditions tested. Regarding growth rate there were significant differences between different salinities ($P=0.0216$). Lastly, physodes were observed to test a **third hypothesis** inquiring on their location within tissues. Microscopic images revealed that physodes were distributed mainly in the outer layers of the blades, epidermis and outer cortical layers, serving as a shield effect.

*Keywords*: *Seaweed, phenolic compounds, HPLC, seasonal variation, antioxidant assays*

# Resumo

As algas são um grupo diversificado de organismos muito valorizado com várias aplicações, que atualmente constituem um recurso marinho sub-explorado. O grupo das algas castanhas é conhecido pelo seu elevado valor nutricional incluindo vários compostos bioativos, na sua composição. Dentro deste grupo, a espécie *Fucus vesiculosus,* ou Bodelha*,* destaca-se por ter uma atividade antioxidante forte, associada a elevados níveis de polifenóis. Os polifenóis pertencem a uma classe de moléculas que possuem muitas propriedades biológicas, incluindo atividade antioxidante, antimicrobiana, anti-inflamatória e de eliminação de radicais livres. Assim, esta espécie representa uma escolha plausível para o desenvolvimento de novos produtos a serem aplicados na indústria nutracêutica, farmacêutica e cosmética, futuramente. Esta tese de mestrado teve como objetivo analisar a variação de conteúdos antioxidantes, através da identificação, quantificação e caracterização das propriedades *in vitro* de polifenóis, na macroalga *F. vesiculosus.* Nomeadamente através de uma análise espectrofotométrica, HPLC e TPC, do método do radical DPPH e da medição da atividade quelante de ferro. O projeto dividiu-se em duas etapas principais: sendo o primeiro o mapeamento das variações sazonais no conteúdo de polifenóis e atividade antioxidante em amostras de populações naturais. E a segunda, a identificação das condições de cultura ótimas, nomeadamente, salinidade e exposição a radiação UV, numa experiência à escala de laboratório, de forma a otimizar a concentração de polifenóis. Adicionalmente, as algas epífitas e os físodos foram observados ao microscópio ótico. Os físodos são vesículas que contém os polifenóis na célula vegetal e a técnica de coloração com vanilina-HCL permite a sua observação.

A alga *F. vesiculosus* foi amostrada mensalmente da praia Bellevue, a norte de Copenhaga, na Dinamarca, contemplando as diferentes estações de um ano completo. Pigmentos e polifenóis foram extraídos de algas previamente liofilizadas. Estes compostos foram identificados (sempre que possível), quantificados e caracterizados através de ensaios antioxidantes. Esta informação foi utilizada para identificar o melhor período de colheita desta espécie de alga, visando uma maior concentração em compostos antioxidantes. Este período foi considerado como hipótese sendo no final de verão - início de outono, devido à acumulação destes compostos durante o verão, que servem como proteção contra as radiações ultravioletas. Foram obtidas diferenças significativas que evidenciam a sazonalidade em antioxidantes nesta espécie. Os produtos de extração ($P \approx 0.0045$) e concentração de polifenóis ($P < 0.0010$) mais elevados foram obtidos desde o final da primavera até ao início de outono,

em contraste com os meses de inverno, com os valores mais baixos. O número e tipo de pigmento manteve-se constante ao longo de todas as estações, estando sempre oito picos em cada cromatograma. Os pigmentos foram identificados como duas clorófilas, (clorofila $C_2$ e clorofila-a) e seis carótenoides (fucoxantina, Prasinaxantina, dinoxantina, Diatoxanthin, Zeaxantina a carotenos a+b). Os pigmentos demonstraram sazonalidade quantitativamente, sendo que a sua concentração aumenta do verão para o inverno e primavera, de forma a compensar a diminuição de luz disponível. Foram detectados 13 ácidos monofenólicos, através da análise de UV RP-HPLC, setembro foi o mês com maior diversidade de compostos e março o de menor diversidade. Ácido gálico foi identificado e estava presente em todos os meses analisados. Os conteúdos antioxidantes e a atividade antioxidante demonstraram correlação positiva com parâmetros ambientais, nomeadamente correlações positivas entre a temperatura, TPC (P= 0,0391) e DPPH (P= 0,0016); bem como entre as horas de luz solar, TPC (P= 0,0458) e DPPH (P= 0,0060).

Durante o ensaio experimental, foram testadas três concentrações de salinidade (≈13, 28 e 40 PSU) e ainda a exposição a radiação UV, para verificar ou rejeitar a segunda hipótese deste projeto, de que com o aumento das variáveis em questão, seria possível otimizar a concentrações de polifenóis. Não foram observadas diferenças significativas entre os tratamentos para a TPC relativamente à salinidade (P=0.0525) e à luz (P=0.2443), nas condições experimentais testadas. Houve diferenças significativas entre a taxa de crescimento dos tratamentos para diferentes salinidades (P=0.0216). No decorrer da experiência, problemas em manter os níveis de salinidade constante poderão ter influenciado os resultados obtidos. Por último, físodos foram observados microscopicamente para testar a terceira hipótese, que inquiria sobre a sua localização nos tecidos. Imagens microscópicas revelaram um efeito de escudo, uma vez que os físodos se encontram distribuídos principalmente nas camadas externas das algas: na epiderme e camadas corticais externas. Os meses de verão coincidiram com a altura de maior incidência de algas epífitas, das quais três espécies foram encontradas a crescer sobre *F. vesiculosus* (verde, castanha e vermelha). A herbivoría e o parasitismo de organismos epífitos sobre esta alga é também um dos motivos descritos noutros estudos para justificar o aumento das concentrações de polifenóis durante os meses de verão.

*Palavras chave*: *Algas, compostos fenólicos, HPLC, variação sazonal, ensaios antioxidantes*

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# Introduction

## 1.1 Seaweed biology and distribution

Seaweed are a diverse group of marine macroalgae, divided into three major groups: Chlorophyta (Green), Rhodophyta (Red) and Phaeophyceae (Brown), depending on their dominant pigments and preferred wavelength absorption (Makkar et al., 2016, Baweja et al., 2016). They are key elements in aquatic ecosystems given their role as primary producers, utilizing carbon dioxide, water and light energy for photosynthesis, yielding organic carbohydrates, and making biomass building blocks by assimilating nutrients such as nitrogen and phosphorous. Not only are they responsible for producing a large percentage of atmospheric oxygen, but also sustaining complex food webs (Roff et al., 2011).

Seaweed typically inhabit the rocky shores of coastal ecosystems, including the intertidal and the shallow subtidal, within the temperate and polar regions (Guiry, 2015). In these zones, they are faced with extreme conditions as a result of the tidal cycle, which they endure by anchoring themselves to the substrate by means of holdfasts (Guiry, 2015, Baweja et al., 2016). Their vertical distribution is closely related to photosynthesis light requirements; thus, most are constricted from 8 to 40 m in depth. Still, in very clear waters they can go up to 250 m deep, this is the case in some regions of the Mediterranean, Caribbean and Brazil (Guiry, 2015). The photosynthetic pigments of each algal group, spectral distribution and light intensity are limiting factors in growth and decisive for zonation (Kanaizuka et al., 2002). Characteristically, green seaweed inhabit the upper shallow zones, because of their greater need for light availability; brown seaweed tolerate less light than green, so they are placed in the middle, although many possess gas filled pouches to float at the surface; red seaweed can withstand the greatest depths, since they have adapted pigments to make use of blue light, which penetrates deeper in the water column (Baweja et al., 2016, Guiry, 2015, Kanaizuka et al., 2002).

## 1.2 Seaweed aquaculture

Seaweed have been used worldwide for thousands of years, predominantly in Asian countries, and are currently estimated in a total annual value of US$ 5.5-6 billion corresponding to 7.5-8 million tonnes of wet seaweed (McHugh, 2003). Seaweed farming has been growing rapidly, and is expected to continue, to meet the increasing demand for food, fuels, bioactive compounds and enhanced health functional food products (Tiwari and Troy,

2015, FAO, 2016). In China, Japan, and the Republic of Korea, seaweed have been a traditional food source for over 2000 years, contrarily to western countries, where their utilization is mainly for non-food applications (Tiwari and Troy, 2015). Asian countries dominate the worldwide production of seaweed (99%) being China the main producer, followed by Korea and Japan. In European countries seaweed are largely underexploited resources: wild harvesting and aquaculture is only 0.009% of the total worldwide production, and accounts to 0.08% of the annual value, the main producers are from France and Norway (Farvin and Jacobsen, 2013, Hoefnagel, 1991, FAO, 2017).

### 1.3 The search for novel bioactive compounds

Antioxidants are widely used by the food, cosmetic and nutraceutical industries to enhance the oxidative stability of lipid rich products. The use of synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), has raised some concerns due to their health risks and toxicity. This has led to the search of novel plant-based natural sources of antioxidants (Hermund et al., 2016). Seaweed are sources of polysaccharides, minerals, vitamins and bioactive compounds, such as sulphated polysaccharides, peptides, amino acids, lipids and polyphenols (Holdt and Kraan, 2011). Therefore, they hold a great potential for the extraction of high-value products with biological activities, that can be applied in the nutraceutical, pharmaceutical, and cosmetic industries (Fernando et al., 2016). Research on the factors contributing to bioactive compounds variation, in natural and cultivated algae, is important since bioactivity is many times extract-specific and varies with location, growth conditions, seasonality and species (Stengel et al., 2011) .

Brown algae are particularly interesting for having a wide range of bioactive compounds and high nutritional value, and are recognized as rich sources of biologically active phenolic compounds (Hermund et al., 2016, Fernando et al., 2016). Currently, they are most known for their alginates (polysaccharides), used for commodities such as stabilising agents, but also in the pharmaceutical and health industries (McHugh, 2003). Worldwide biomass collected from the wild or cultivated is coming from a few species of the orders Laminariales and Fucales, and are valued in about US$ 300 million per year (Guiry, 2015). *Fucus vesiculosus* is one of the most common species in the North Sea and Danish inner waters and is a promising resource due to elevated levels of total phenolic compounds, and higher antioxidant activities, compared to both red or green algae (Hermund et al., 2016).

## 1.4 Study species

The study species, *F. vesiculosus*, also known as Bladderwrack (this species) or Rockweed (*Fucus* species in general), belongs to the brown algal class, a group with high nutritional value and content of a wide range of bioactive compounds (Holdt and Kraan, 2011). It is especially rich in polyphenolic compounds and excels in scavenging activities (Wang et al., 2009). The basic form of the brown alga is the thallus, a simple, relatively undifferentiated vegetative body, divided into three basic structural units, holdfast, stipe and blades (Figure 1). The disc shaped holdfast is firmly attached to a substrate; the stipe is prominent, flexible, and connects the holdfast to the blades and fronds, which are dichotomously branched and have a tough leather-like surface to endure wave action. In the blades, there are characteristic gas vesicles or air bladders (usually paired) that may be absent in smaller individuals and in exposed shores. They help maintain the blades at the surface for better light exposure and improve photosynthesis. Fertile fronds have fruiting bodies, the receptacles, with several conceptacles inside (Evert and Eichhorn, 2013, Guiry and Guiry, 2016, White, 2008).

The presence of this species is predominantly along rocky and stony coasts, usually appearing highly concentrated and widely distributed. It is one of the most important phytobenthic species of the Baltic, providing habitat for species-rich epiphytic and epibenthic communities (Torn et al., 2006). It prefers salty, brackish waters and sheltered rocky shores, and is also associated to cold water patches from spring–summer upwelling (Guiry, 2015, Viana et al., 2015, Plass, 2013).

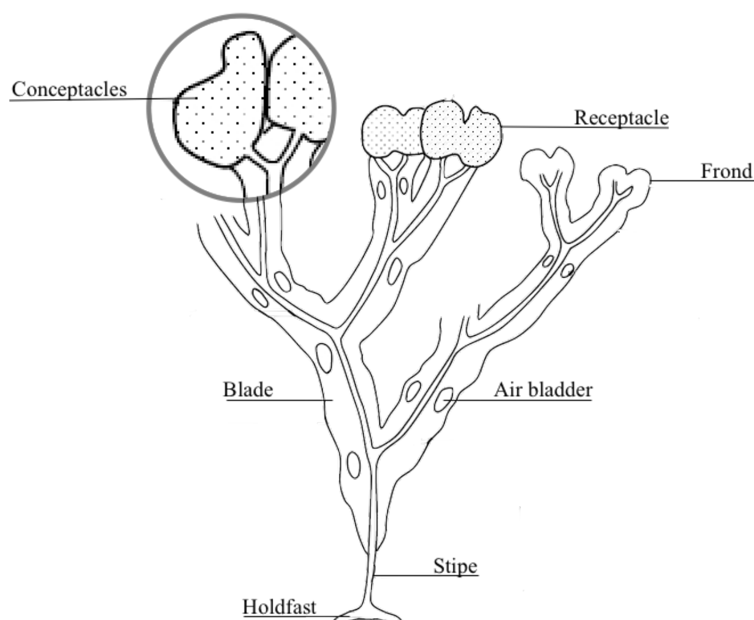



*Figure 1 Structure of a mature F. vesiculosus thallus. Adapted from (Evert and Eichhorn, 2013, Hermund et al., 2016).*

### 1.4.1 Life cycle

In the Baltic Sea, *F. vesiculosus* exhibits two periods of reproduction, the most pronounced is during early summer (May to June) but can also occur in late autumn (September to November) (Berger et al., 2001). The life cycle of *F. vesiculosus* is portrayed in Figure 2, they are dioecious (male and female individuals) and have a gametic life cycle, meiosis occurs before the gamete formation and the zygote and adult are diploid (2n) (Evert and Eichhorn, 2013). Gametes are produced in specialized chambers, the conceptacles, located in the receptacles at the tips of fertile fronds (Pearson and Serrão, 2006). During reproductive periods, eggs and sperm are released only in calm waters (Serrao et al., 1996a, Serrão et al., 1999), resulting in up to a million fertilized eggs, per individual (Pearson and Serrão, 2006, Serrao et al., 1996a), except when salinity is limiting to gamete viability (Serrão et al., 1996b, Serrão et al., 1999). The zygote attaches to the substrate within a few hours after release and then grows directly into a young diploid seaweed. Seaweed enter a period of dormancy in winter and new biomass grows slowly, until spring arrives again, and receptacles mature (Serrão et al., 1999). After the reproductive period, the receptacles and parts of the fronds that support it are usually abscised (Berger et al., 2001). The lifespan of this species is around 2 to 3 years in Spain (Viana et al., 2015). Some individuals can have clonal propagation especially in the sheltered conditions of the Baltic, where vegetative propagules can develop rhizoids and attach to substrate given enough time (Tatarenkov et al., 2005).

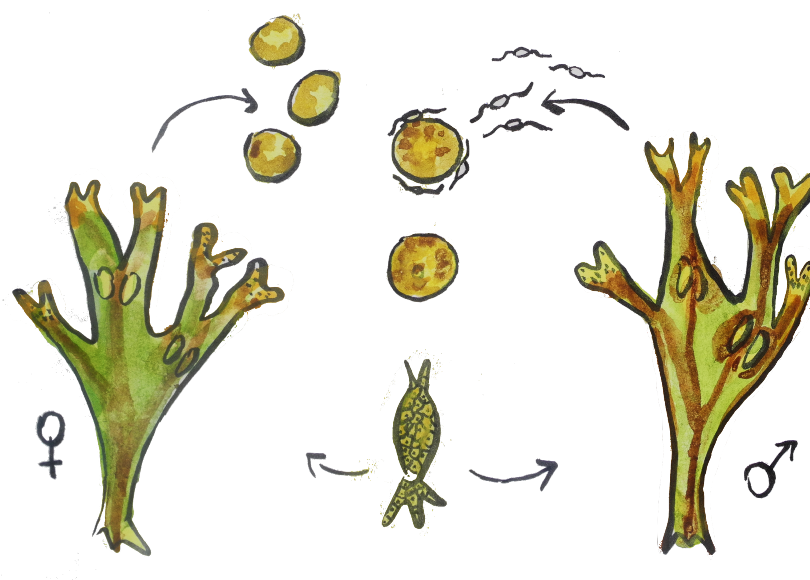



*Figure 2 The life cycle of Bladderwrack (F. vesiculosus). Sperm from the male thallus fertilizes the egg that is released from the female thallus and forms a zygote that will attach to a substrate and grow into a new individual. ©Madalena Mendes*

### 1.4.2 Geographic distribution

This species occurs in the North Atlantic in coastal regions: in the eastern coast of Canada and North America; around the coastlines of the British Isles, Faroe Islands, Iceland, Morocco, Iberian Peninsula, France, Belgium, the Netherlands and Germany; in Scandinavia and the Baltic sea, as well as some regions of the Arctic including Greenland (Guiry and Guiry, 2016, White, 2008, *OBIS*, 2017).

### 1.4.3 Cultivation

There are few experimental data and studies on the cultivation of *F. vesiculosus,* it has been possible to produce sporophytes from spores in lab cultures, but they die once deployed in the field making it a challenging species to work with. The majority of *Fucus* species are manually harvested from shores and are currently not farmed at an industrial scale (NetAlgae, 2012). At this stage, offshore cultivation is described by Hermund et al., (2016), as a plausible option for this species. Nevertheless, onshore tank cultivation is also a possibility; it allows a higher control over culture conditions and access to seaweed is easier, which may be very useful if the biomass is meant for high-end applications. Hermund et al., (2016) findings reveal that apical fronds of *F. vesiculosus* generally have higher polyphenolic contents, which suits a more sustainable cultivation because only young parts need to be harvested, instead of a full removal of the seaweed, and then there is continued vegetative growth of the remaining thalli.

In reference to culture conditions, optimal growth consist of cold water (<18°C), a minimum 8-hour photoperiod, salinities vary from 18-40 PSU, accessible nitrogen, and a suitable substrate for attachment in a sheltered or semi exposed areas, (Hermund et al., 2016, White, 2008). There seem to be no specific nutrient requirements, a temperature of 15 ºC promotes rapid growth; and increased biomass densities in a culture tank significantly limit growth rate (Fulcher and McCully, 1969, McLachlan et al., 1971). The highest relative growth rate of vegetative branches of *F. vesiculosus* has been reported in the summer months (up to 0.7% per day) compared to winter growth (less than 0.3% per day) in the Northern Baltic (White, 2008). Fulcher and McCully (1969) documented small and slow growth in isolated thalli apices of mature individuals, as well as small portions of thalli lacking apices regenerating with shoots. Individuals are known to easily regenerate new fronds from the holdfast after destructive events (Viana et al., 2015). Fulcher and McCully (1969) further reported improvements in growth by designing an apparatus that enabled alternating periods

of immersion and exposure, just like a tidal cycle in natural conditions. They also obtained low levels of contamination in the system, which they attributed to the antibiotic properties of produced polyphenols. On the other hand, McLachlan et al., (1971) obtained algae comparable in size in less time, when using a submerged culture, finding that the absence of a tidal cycle did not restrain from a successful cultivation. It is relevant to note that within the Baltic Sea there is little or no influence of tidal cycles (Pearson and Serrao, 2006).

## 1.5 Antioxidant compounds

Bioactive compounds from seaweed that display antioxidant properties are valuable in the sense that they can replace synthetic antioxidants, such as n-propyl gallate, monoglyceride citrate, butylated hydroxyanisole, butylated hydroxytoluene (BHT) and butylated hydroxyquinone, many of which are restricted due to health concerns and toxicity (Cérantola et al., 2006). Moreover, they may be used to replace available natural mono-compound antioxidants, such as tocopherol and ascorbic acid, which are insufficient in most fish-oil-enriched foods (Farvin and Jacobsen, 2013, Hermund et al., 2016). Listed below are some of the commercially relevant applications of seaweed antioxidant extracts. They can be applied in food to avoid lipid oxidation and prolong shelf life, already shown in muesli bars (Hermund et al., 2016). They are fit to be used as supplements in functional foods, pharmaceutical drugs, in cosmetics and skin care industries, for example, as radiation protection agents (Holdt and Kraan, 2011, Parys et al., 2010). The seaweed antioxidants can be beneficial to human health by regulating the balance between reactive oxygen species (ROS) production and scavenging. ROS are highly reactive towards essential biomolecules, hence damaging the integrity of cells (Belda et al., 2016). Lastly, antioxidants serve as defense mechanisms against many diseases, such as cardiovascular diseases, diabetes, cancer, atherosclerosis, aging, and other degenerative diseases (Fernando et al., 2016).

### 1.5.1 Pigments

Pigments selectively absorb light giving colour to the algae. They also possess antioxidant properties (Raposo et al., 2015). Brown seaweed major accessory pigments are chlorophyll c1 + c2 and xanthophylls, such as fucoxanthin. The abundance of xanthophylls is responsible for the brownish colour and masks the other pigments, chlorophyll-a and –b; β-carotene and other carotenoids (Holdt, 2011). Chlorophyll-a and –b, are responsible for the green colour in plants, and are essential to photosynthesis, since they absorb light and are primary electron donors. Additionally, in certain marine algae, including brown algae, exist

chlorophyll-c, accessory pigments with a blue-greenish colour. Carotenoids are a family of red yellow and orange lipophilic tetraterpenoids that protect plants against photo-oxidative processes. They are effective free radical scavengers and deactivators, and promote antioxidant interactions with other compounds, which enhances this effect. They are divided into xanthophylls, with oxygen, and carotenes, hydrocarbons without oxygen (Hermund et al., 2016, Safafar et al., 2015).

### 1.5.2 Polyphenols

Phenolic compounds are a diverse class of biological molecules, produced as secondary metabolites and present in most algal groups (Stengel et al., 2011, Schoenwaelder, 2008, Tsao, 2010). They comprise around 8000 natural occurring compounds, with a hydroxyl group (-OH) bonded directly to an aromatic hydrocarbon group (Fernando et al., 2016, Holdt and Kraan, 2011). They are classified into different groups as a function of the number of phenol rings they contain and on the structural elements that bind these rings to each other (Pandem et al., 2009). The main classes include phenolic acids, flavonoids, stilbenes and lignans (Figure 3).

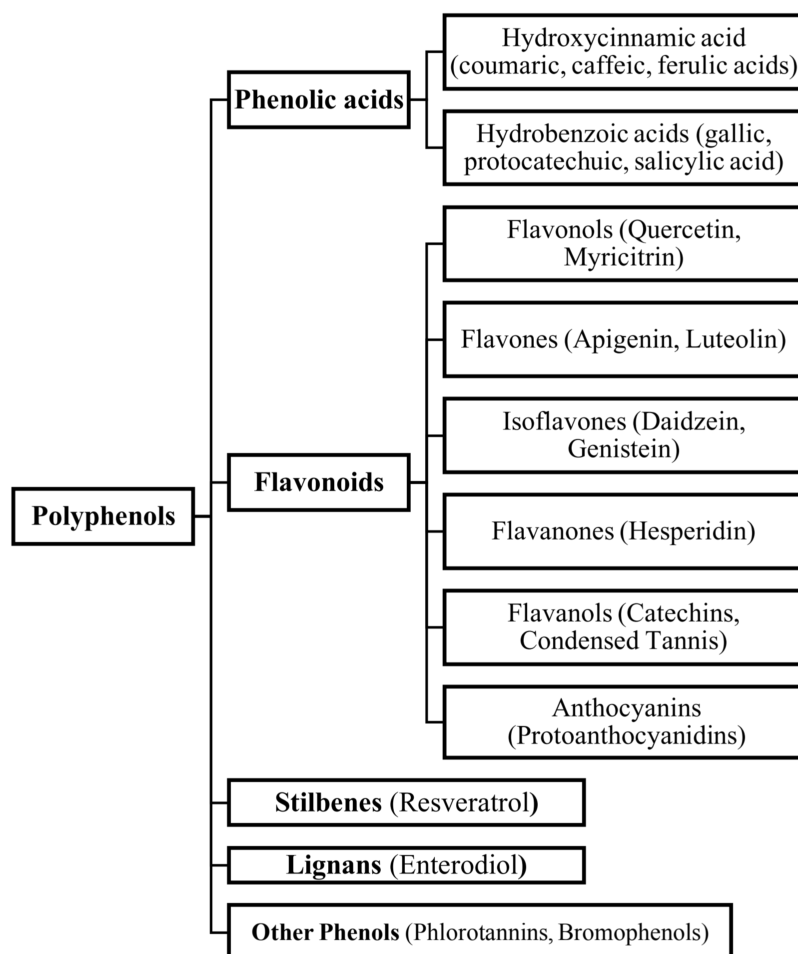



*Figure 3 Polyphenols classification based on Pandem et al. (2009), Tsao (2010) and IUPAC (2014).*

Often acting as stress compounds and involved in chemical protective mechanisms, they display antioxidant, antimicrobial, anti-inflammatory, and free radical scavenging activities, through single electron transfer and through hydrogen atom transfer (Belda et al., 2016, Fernando et al., 2016, Safafar et al., 2015, Schoenwaelder, 2008). They play an important role in diverse biological processes, including UV photo-protection, polyspermy blocking, trace metal bounding, protection against herbivores, oxidative stress, and injuries (Belda et al., 2016, Pandem et al., 2009, Salgado et al., 2007). Hence, their concentration can be increased by various parameters, both biotic, grazing, settlement of bacteria and other fouling organisms; and abiotic, excessive irradiance from UVA and UVB, metal contamination, as well as osmoregulatory stress from increasing rain or salinity (Ragan and Jensen, 1978, Schoenwaelder, 2008, Stengel et al., 2011). Lower salinity has been associated to a higher concentration of secondary metabolites, and consequently greater antioxidant potency; while higher salinities promote higher concentration of polysaccharides, and less antioxidant power in the species *Fucus ceranoides*, Linnaeus (Cotas, 2015).

Brown seaweed are rich in different phenolic compounds, some examples are described in Table 1. Bromophenols are common to most seaweed, though it is phlorotannins that make up most of the phenolic compounds of brown algae, going up from 5-30% of the dry weight (*dw*) of the seaweed (Hefernan et al., 2015, Stengel et al., 2011). Phlorotannins are exclusive to brown seaweed and are divided into numerous classes, depending on the type of linkage between phloroglucinol units (Cérantola et al., 2006, Hefernan et al., 2015). They are characterized by having a molecular mass from 126 Da to 100 kDa; a polymeric structure formed by phenolic oxidative coupling; 14–24 phenolic hydroxyl groups and 5–8 aromatic rings per 1,000 units of relative molecular mass (Hefernan et al., 2015, Martinez and Castaneda, 2013). Exceptionally, they also display primary functions within growth and cell wall development in Fucales, where they are very abundant (Cérantola et al., 2006). Martínez & Castañeda, (2013) divide three main groups of phlorotannins: fucols, phloroethols and fucophloroethols. The first two are constituted of only aryl-aryl and aryl-ether bonds respectively and the latter contains both (Cérantola et al., 2006).

*Table 1. Distribution of phenolic compounds in Phaeophyceae. Adapted from (Stengel et al., 2011, Parys et al., 2010, Fernando et al., 2016, Wang et al., 2009).*

| **Phenolic compounds** | Examples |
|---|---|
| **Bromophenols** | 2-bromophenol, 4-bromophenol, 2,4-dibromophenol, 2,6-dibromophenol, 2,4,6-tribromophenol |
| **$C_6$-$C_4$-$C_6$ metabolite** | Colpol, 8,9-dihydrocolpol |
| **Meroditerpenoids** | Plastoquinones, sargaquinoic acid, sargachromanols, chromene derivatives. |
| **Phenolic acids** | Gallic, protocatechuic, gentisic, chlorogenic, vanillic, caffeic |
| **Phlorotannins** | Phloroglucinol, phloroglucinol with a $C_{20}$ acyl side chain, fucols, fucophlorethols, fuhalols, phlorethols, eckols, eckstolonol, phloroeckol, phlorofucofuroeckol-A, triphlorethol-A, trifucodiphloroethol-A, dioxinodehydroeckol, carmalol, diphlorethohydroxycarmalol |

#### *1.5.2.1 Physodes*

The distribution of phenolics in plants is not uniform at the tissue, cellular and sub cellular levels. Insoluble phenolics are found in cell walls, while soluble phenolics are present within the plant cell vacuoles (Pandey et al., 2009). Polyphenolic compounds are stored in membrane bound vesicles called physodes (Ragan, 1976). Looking into the specific location of phenolic compounds, in the cell tissue, gives us some insight into understanding the ecological and physiological functions of these compounds (Ragan and Jensen, 1978). Physodes are present in many stages of the algal life cycle, from the early development (spores, gametes, zygotes) up to the adult plant, in cell wall formation, in vegetative tissue, for adhesion, polyspermy prevention, defence and UV protection (Ragan, 1976, Schoenwaelder, 2008). They are found in the periphery of cells and perinuclear regions, where they are most likely produced, their size varies between 0.1-10 μm in diameter (Schoenwaelder, 2008). In the newest shoots, where there is more active growth, they seem to be richer in total phenols (Ragan, 1976).

Physodes can be stained and observed in fresh or fixed tissue, under light or electron microscopy (Schoenwaelder, 2008). Vanillin-HCl colours the physodes red, which is described as an efficient method by Ragan (1976) and can specifically detect phlorotannins (Shibata et al., 2004). Nevertheless, there are many histochemical staining methods specific

for  available including osmium tetroxide, neutral red, toluidine blue O, fast red GG, and cresyl blue (Schoenwaelder, 2008). By light microscopy with vanillin-HCl staining, it has become clear that phlorotannins accumulate primarily within the vegetative cells of the outer cortical layer of brown algae, regardless of the variety of tissue, stage of growth or organ (Shibata et al., 2004). A few authors have contributed to the study of the polyphenols and physodes in *F. vesiculosus* (Baardseth, 1958, Ragan, 1976, Ragan and Jensen, 1977, 1978). However, considering that many used indirect methodologies of detection dependent on colorimetric reagents, for example, the Folin-Denis; a fresh overtake of this topic would be useful, to determine distribution and secretion of phlorotannis and phenolic substances in brown algae (Shibata et al., 2004). The morphology of the cell tissue in *Fucus* fronds is displayed in Figure 4. There are three main sections: the inner most layer is called the medulla, its filamentous cells are fully covered in mucilage; the middle layer is the cortex, comprised of cortical cells loosely arranged in some mucilage, lastly the epidermis, with photosynthetic cells closely packed together. The central area in the medulla midrib contains longitudinal filaments tightly packed together.

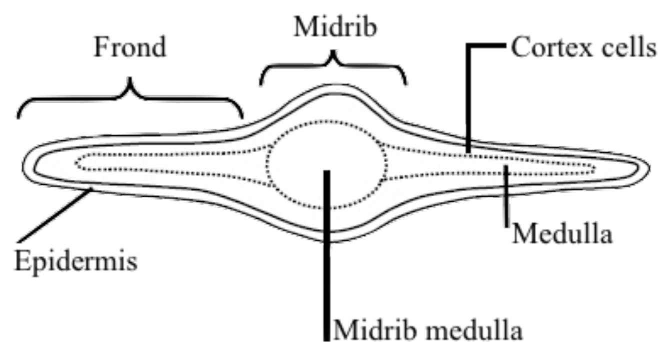



*Figure 4 Arrangement of tissues in the F. vesiculosus frond, seen in a transversal section. Adapted from* (Evert & Eichhorn 2013; Hermund et al. 2016).

### 1.5.3 Extraction methods

Polyphenols are polar molecules that must be extracted from inside the cell wall, with minimum risk of damage to the compounds, by different means (Martínez and Castañeda, 2013, Safafar et al., 2015). Solvent polarity is very defining for the extraction yield and antioxidant activity. It interferes with the qualitative and quantitative analysis of extracted compounds and must have the ability to penetrate the cell wall and dissolve compounds of interest (Sineiro et al., 2008). In plant studies, highest yields are obtained with ethanol, methanol, and their mixtures with water, other utilized solvents are ethyl acetate or acetone (Sineiro et al., 2008).

Water (*we*), acetone (ae) ethanolic (*ee*), and methanolic (*me*) extracts have presented different results, on both TPC and antioxidant activities in the extraction of polyphenols from Icelandic seaweed (Wang et al., 2009). Water has been described as being efficient in extracting iron chelating compounds, in spite of being a poor solvent for phlorotannis (Farvin and Jacobsen, 2013). Meanwhile, acetone, ethanol and methanol are highly effective and yield large polyphenolic contents, in addition to having good radical scavenging capacity and reducing power results (Farvin and Jacobsen, 2013, Hermund et al., 2016, Wang et al., 2009). However, environmental concerns urge industries to switch from conventional solvents to more sustainable methods, for instance, the pressurized liquid extraction, when producing natural novel antioxidants (Hermund et al., 2016). There is a need for the improvement of fractionation and purification of bioactive components, which is certain to increase the activity and other potential health benefits, launching polyphenols as natural sources of antioxidants for commercial use (Wang et al., 2009).

### 1.5.4 Identification and quantification

Polyphenols are quantified through TPC usually determined by the FC assay (Singleton and Rossi, 1965). This method consists in the spectrophotometric detection between 725 and 765 nm, of a blue complex and molybdenum oxide, from the oxidation of phenolic rings, phosphotungstic and phosphomolybdic acids (Hermund et al., 2016). TPC units are expressed as phloroglucinol or Gallic Acid Equivalents (GAE) (Martínez and Castañeda, 2013, Farvin and Jacobsen, 2013). Phlorotannins tend to dominate within polyphenols, and consequently, the total phenolic content is often related to them (Wang et al., 2009, Farvin and Jacobsen, 2013). In Hermund et al., (2016) TPC also varied within the tissue location of the algae, younger tips had higher values than the older ones. Sometimes this method causes

uncertainty because non-phenolic substances can interfere and fall into the spectrum of phenolic compounds, causing an overestimation of TPC (Hermund et al., 2016).

HPLC is ideal to separate polyphenols, due to their polar nature and high solubility, and gives a rapid structural identification (Hermund et al., 2016). In order to fully identify and characterize polyphenols, advanced analytical methods must be applied, for example, tandem mass spectrometry (MS) and nuclear magnetic resonance (NMR) and could even be combined with HPLC (Hermund et al., 2016). In previous studies with *F. vesiculosus*, 13 phlorotannins isomers with molecular weights between 374 and 870 Da were identified by mass spectrometry, and the antioxidant activity decreased with increasing molecular weight (Hermund et al., 2016).

#### 1.5.5 Antioxidant activity assays

Antioxidant assays have been used to characterize the antioxidant properties of polyphenols in *F. vesiculosus* (Arnold et al., 1995, Farvin and Jacobsen, 2013, Hermund et al., 2016, Wang et al., 2009). They can be determined by *in vitro* studies, which are simple, cheap and fast methods to characterize antioxidants (Hermund et al., 2016). This study focuses on two antioxidant activity mechanisms which will hereby briefly be discussed.

Polyphenols contain many hydroxyl groups bound to an aromatic ring making them very good candidates for donating protons to a radical, acting as chain breaking molecules or antioxidant upon secondary oxidation (Sineiro et al., 2008). Screening of substances with potential antioxidant activity and scavenging of free radicals is widely performed by using DPPH, a stable radical (Yang et al., 2008). Studies have shown that algal polyphenols are mainly responsible for the free radical scavenging activities of extracts, since high TPC correlates with high DPPH (Wang et al., 2009, Hermund et al., 2016). Though sometimes co-extracted active compounds also contribute to the overall scavenging effect, as is the case with fucoxanthin and sterols from 70% *ae*, and sulphated polysaccharides, proteins or peptides in *we* (Wang et al., 2009). Ferrous iron chelating indicates how antioxidants reduce oxidized intermediates into a more stable form, two neighboring OH groups (o-diphenol) are required in the structure, an essential characteristic for food and skin care products (Hermund et al., 2016).

# Objectives

The aim of this project was to analyze the antioxidant variation in the seaweed F. vesiculosus, this was done through physical location of polyphenols, quantification of polyphenols and pigments, and characterization of antioxidant activities, by DPPH and iron chelating method. Fucus vesiculosus was furthermore evaluated as a potential source in the future development of natural antioxidants which is of interest to industries. This study was divided in three stages (summarized in a Fluxogram illustrated in Figure 6), the first being the mapping of the seasonal polyphenolic and pigment variations, by monthly samples throughout a year of natural populations, which was used to determine the optimal season for biomass harvesting and utilization. The second stage was the identification of optimized culture conditions in lab scale experiments, regarding salinity concentration and UVA light, to yield higher polyphenolic concentrations to be potentially used industrially. The third stage was imaging of the polyphenol content in F. vesiculosus by vanillin HCl staining and microscopy. The physical location of polyphenols gives information on the extraction methods and possibilities. As a complement to this work, the epiphyte coverage was evaluated qualitatively to gather knowledge on what species occur in the sampling region and at what time of the year they are most present.

## 1.1 Hypotheses

This study tested the following hypotheses:

**H1** - There are seasonal differences in the polyphenolic content and activity of wild *F. vesiculosus,* more specifically, late summer and early autumn months present the highest polyphenol values.

**H2** – Increased salinity and UVA light increases the antioxidant yield, and therefore it is possible to optimize *F. vesiculosus* antioxidant contents, in respect to these parameters.

**H3**- The physodes can be stained by vanilin HCl and are located in the outer cells.

The hypotheses led to the more specific tasks that were carried out:

- Extraction and profiling of polyphenols and pigments from *F. vesiculosus.*
- Characterization of antioxidant activities from *F. vesiculosus.*
- Evaluation of seasonal changes in the polyphenolic content of wild *F. vesiculosus.*
- Testing the optimization of antioxidant contents in *F. vesiculosus* cultured under different environmental parameters, such as salinity concentration and UVA light exposure.
- Microscopic evaluation of epiphytes and physodes in *F. vesiculosus*.

## 1.2 Learning objectives

- General skills on sampling and identification of seaweed.
- Conduct a lab-scale seaweed culture trial, including management and manipulation of seaweed.
- Identification of optimized conditions, regarding salinity concentrations and UVA light presence.
- Quantification of antioxidants: polyphenols and pigments
- Biochemical analysis of seaweed biomass: antioxidant extraction, profiling and antioxidant capacity essays.
- Determine the optimal season for biomass utilization in *F. vesiculosus.*
- Microscopic observation techniques, including staining of algal physodes.
- General skills on data analysis (including statistics), interpretation, and discussion of scientific results.
- Presentation for and discussion of data with the Bioactive Research Group

*Figure 5. Collection of biomass at Bellevue beach, Denmark. © Madalena Mendes*

## 1.3 Fluxogram

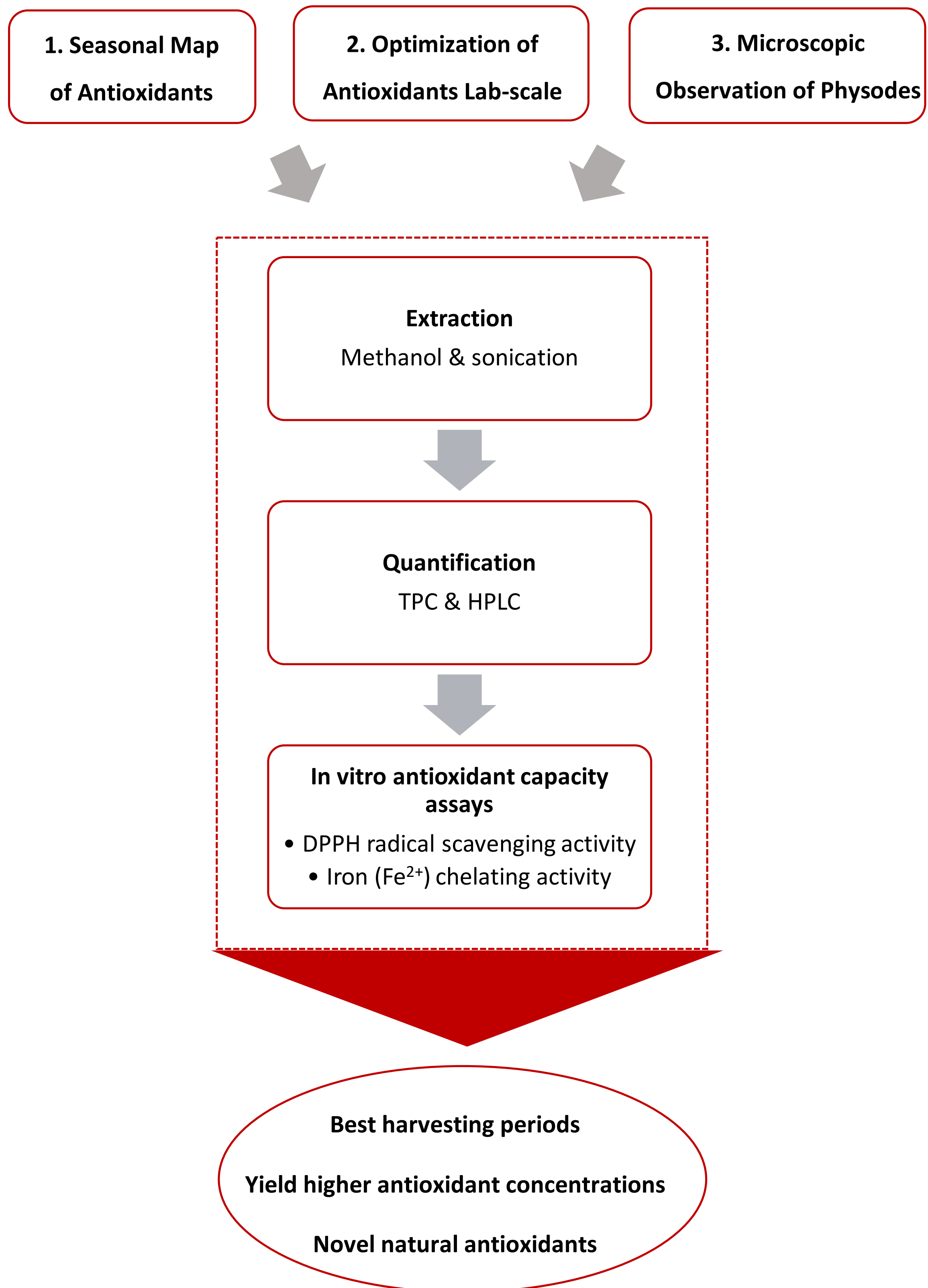



*Figure 6 Overview of the main stages of this thesis.*

# Methodology

## 1.1 Sampling

Wild *F. vesiculosus* (Figure 7*a*) was collected by hand, monthly, from June 2016 to May 2017. Additionally, fresh samples of *F. vesiculosus* were collected in March, to be used in polyphenol optimization experiments. They were conditioned at 10°C, with a 12h light photoperiod, 73,8 ± 14,4 $\mu mol.s^{-1}.W^{-1}$ photosynthetically active radiation (PAR) and ≈15 PSU salinity (Hermund et al., 2016, McLachlan et al., 1971). The collection site was along a 25 meter transect at the intertidal zone of Bellevue beach (55°46'17.4"N 12°35'48.4"E), North of Copenhagen, Denmark (Figure 7*b*). Samples were stored in a freezer room at -40°C, until further processing and analysis.

*Figure 7 a) Wild F. vesiculosus frond © Madalena Mendes. b) Sampling location, Bellevue beach, Denmark © Google.*

## 1.2 Environmental data

Environmental data was obtained from weather archives of the Danish Meteorological Institute (Cappelen, 2017). Monthly averages of mean temperature (ºC), precipitation (mm) and sunlight (h) were taken from June to August 2016 (Denmark) and from September 2016 to June 2017 (Lyngby-Tårbæk, the sampling location). Additionally, UV-dose 2017 measurements from Copenhagen and yearly projections were used in this study (Cappelen, 2017).

## 1.3 Dry matter and ash contents

Samples were thawed overnight in a cool room and cleaned to remove holdfasts, epiphytes and bryozoans. Wet weight (*ww*) was determined gravimetrically after biomass was dapped with a paper towel, followed by freeze drying for 48 hours, using a Heto Drywinner (DW8, ThermoFisher Scientific), and *dw* measured gravimetrically. Then, each sample (representative of a month) was divided into triplicates of pooled individuals (Figure 8) and reduced to a fine powder using a sample mill (FOSS Tecator), for 30 to 40 seconds. Dry biomass was calculated as the dry to wet weight ratio multiplied by 100 as following:

$$Dry\ biomass\ (\%) = \frac{dw}{ww} \times 100 \qquad (1)$$

*Figure 8 Freeze dried F. vesiculosus collected at Bellevue beach in June 2016.*

Dry matter (*dm*) was determined after drying the samples in an oven at 105 °C for 24 hours, until constant weight and ash content was determined by incineration in a muffle furnace at 550 °C for 6 h (Figure 9). Dry matter and ash content were calculated as following:

$$Dry\ matter\ (g\ dm.100g\ dw^{-1}) = \frac{dm}{dw} \times 100 \qquad (2)$$

$$Ash\ (g\ ash.100g\ dw^{-1}) = \frac{ash}{dw} \times 100 \qquad (3)$$

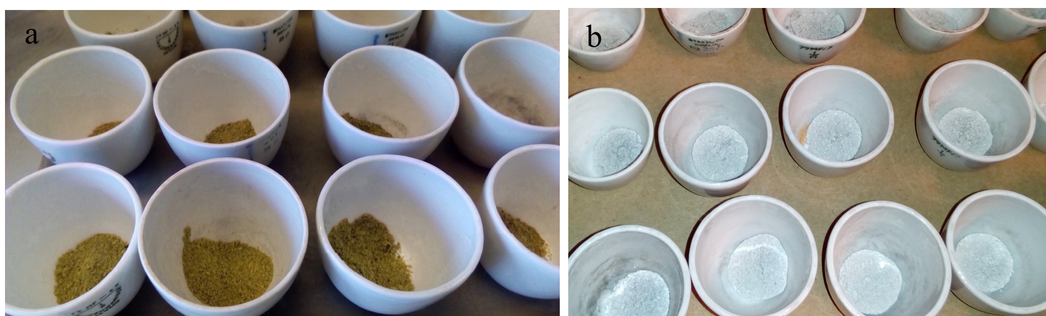



*Figure 9 Crucibles with dried seaweed powder biomass: a) dry matter and b) ash*

## 1.4 Antioxidant profiling

### 1.4.1 Extraction

Polyphenols were extracted with a solid liquid extraction (SLE) using methanol and sonication, according to (Farvin and Jacobsen, 2013). A total of 0.600 g of powdered seaweed were added to previously weighed centrifuge tubes. Then, 5 mL of methanol were added and mixed at room temperature. The mixture was placed in the sonicator, for 30 min (Branson Ultrasonics, CA, USA) and centrifuged at 3500 rpm for 10 min (Sigma 4-16ks, Germany). The supernatant content was collected to a separate tube, filtered (0.45 μm), and evaporated under nitrogen flow (Figure 10a). Remaining residue was re-extracted around 9 times, under the same conditions. After evaporation, *me* were weighed and the absolute yield was calculated as following:

$$Absolute\ yield\ \left(g\ me.100g\ dm^{-1}\right) = \frac{me}{dm} \times 100 \quad (4)$$

Furthermore, the *me* of four months (2016: September; November; 2017: January and March) were analyzed by RP-HPLC, to identify simple phenolic compounds such as phenolic acids and flavonoids. After the previously described extraction process, the extracts were re-diluted in MeOH and loaded to a sulfonic acid 6cc mixed-mode cation exchange (MCX) cartridge (OASIS Waters, USA), to remove pigments. The cartridge was conditioned and equilibrated with 3 mL of MeOH, 5 mL of sample were loaded, and finally the cartridge was washed with 4 mL of MeOH. The volume was collected and evaporated under the nitrogen flow (Figure 10b). The new extracts were weighed and the absolute yield (g *me*. 100g $dm^{-1}$ of seaweed) was calculated.

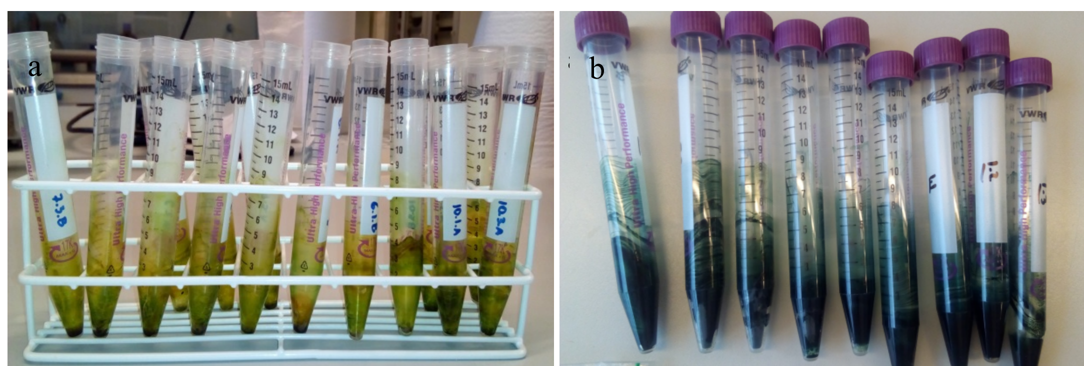



*Figure 10 Polyphenol extraction process after nitrogen flow: a) test tubes with me b) Test tubes with me after removal of pigments with the OASIS MCX cartridge.*

### 1.4.2 Identification and quantification of Pigments

Pigments were analyzed bimonthly in triplicate samples, from July 2016 to May 2017, according to the method described in Safafar et al., (2015) with some modification. Pigment extraction was performed with methanol and sonication; and their quantification and identification by RP-HPLC. A total of 0,100 g of dried seaweed were weighed, and mixed with 3 mL of methanol (with 0.025 $\mu g.ml^{-1}$ BHT). The test tubes were kept in a beaker with ice, covered with aluminum foil, and placed in a sonication bath, for 15 min (Branson Ultrasonics, CA, USA). The samples were then centrifuged at 5000 rpm for 10 minutes (Sigma 4-16ks, Germany), and the supernatant filtered (0.45µm) and collected to a separate tube. The remaining residue was re-extracted three times, under the same conditions, concluding a final volume of 10mL.

A total of 1 mL of each sample was placed in HPLC vials and analyzed immediately or kept in the freezer at -18° without light, and analyzed in the following morning. RP-HPLC analysis was performed with an Agilent 1100 series HPLC (Agilent Technologies, CA, USA) equipped with Diode Array Detector (DAD) (Agilent G13158). Moreover, a Zorbax Eclipse C8 column 150 mm×46 mm×3.5 µm from Phenomenex was used for the separation. Elution was performed with a mixture of solvent A (70% MeOH + 30% of 0.028 M tertiary butyl ammonium acetate in water) and solvent B (MeOH) at a flow rate of 1.1 $mL.min^{-1}$, chosen as the mobile phase. Retention times and peak were monitored and computed automatically by Chem32 integrator (Agilent, USA). DHI pigment standard mix (DHI LAB Products) was used as a pigment standard for the identification of peaks, and the pigments were detected at 440 nm.

Calculation of concentration of each individual peak of pigment ($cpi$):

$$Cpi = Response\ Factor \times Dilution\ Factor \times Recovery\ Factor \times Calibration\ Factor$$

The peak areas and pigment identities were transferred to an excel file, and based on the response factors, the pigment concentrations were calculated:

$$Cpi = \left(\frac{Vx}{Mf}\right) \times \left(\frac{A^c}{A^s}\right) \times \left(\frac{A^{Pi} \times RPi}{Vc}\right) \tag{5}$$

Where $A^{Pi}$ and RPi are the peak area and the response factor of pigment Pi, respectively; Vx is the extraction volume; Mf is the weight of the sample; Vc is the amount

of sample injected onto the column; $A^c$ is the peak area of the internal standard in the extraction solvent; and $A^s$ is the peak area of the internal standard in the sample.

### 1.4.3 Identification and quantification of Phenolic compounds

Phenolic acids and flavonoids were identified through a chromatographic qualitative analysis, HPLC, by a modified method of Safafar et al., (2015). RP-HPLC was performed with an Agilent 1100 series HPLC (Agilent Technologies, CA, USA), equipped with a DAD (Agilent G13158). The separation was carried out on a Prodigy ODS-3 column 250 mm x 46 mm with 5 µm particle size from Phenomenex (Torrance, CA, USA).

Elution was performed with a mixture of solvent A (Phosphoric acid in deionized water, pH=3) and solvent B (MeOH + Acetonitrile, 50:50 v/v) at a flow rate of 0.9 $mL.min^{-1}$, chosen as the mobile phase, and the injection volume was 20µl. Detection was done using a DAD with reference wavelength of 255 nm. Retention times and peak were monitored and computed automatically by Chem32 integrator (Agilent, USA) and manually integrated when necessary. Identification of individual phenolic acids was done by the retention time of sample chromatographic peaks, being compared with the authentic standards under the equivalent HPLC operating conditions (Figure 11). Individual phenolic acids were identified by the retention time of sample chromatographic peaks being compared with those of authentic standards using the same HPLC operating conditions. Standards were Caffeic acid; Catechol; Cathechin; Chlorogenic; Coumaric; Ferulic; Gallic acid; Gentisic; Hesperidin; Hydroxybenzoic; Morin; Myricetrin; Protocatechuic; Quercitrin; Rutin; Salicylic; Syringic; and Vanilic.

Calculation of concentration of each individual peak of phenolics ($C_{phenolic}$):

(6)

$$Cphenolic = Area \times Response\ Factor \times Dilution\ Factor \times Recovery\ Factor \times Calibration\ Factor$$

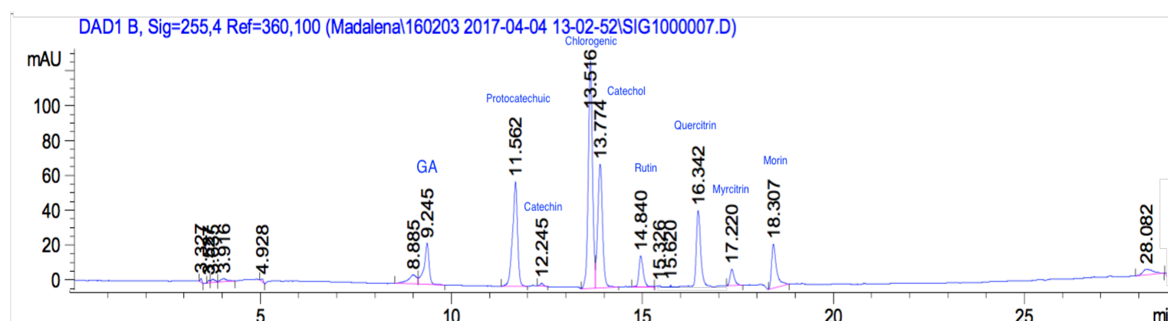



*Figure 11 HPLC-DAD analysis (255 nm) of standard mix. UV chromatogram with labeled peaks according to the compound they represent.*

### 1.4.4 Determination of TPC

Polyphenols were quantified spectrophotometrically by the determination of TPC, with the Folin-Ciocalteu (FC) assay method Singleton and Rossi (1965) according to Farvin and Jacobsen (2013) with some modifications. TPC were expressed as GAE mg.100 $mg^{-1}$ of dried seaweed, and samples were run in analytical duplicates. An aliquot (100 µL) of extract (dissolved in methanol, concentration range from 0.6 to 3.8 mg. $mL^{-1}$) was mixed with 0.75 mL of FC reagent (10 % in distilled water) and incubated, at room temperature, during 5 minutes. Then, 0.75 mL of sodium carbonate (7.5% in distilled water) was added to the mixture and incubated in darkness during 90 minutes. Total phenols were measured by the absorbance in a spectrophotometer at a wavelength of 725 nm (Shimadzu UV mini 1240, Duisburg, Germany). A standard curve plot with serial gallic acid solutions (0-100 µg. $mL^{-1}$) was used for calibration.

### 1.4.5 In vitro antioxidant capacity assays

To determine the antioxidant properties of the *me*, two antioxidant activity assays were used, the DPPH scavenging activity and the iron chelating activity.

#### *1.4.5.1 DPPH radical scavenging activity*

DPPH was used to evaluate the efficiency of the antioxidant scavenging effect of the extracts on free radicals and H-atoms donation, measured by the method described in Yang et al., (2008) and Farvin and Jacobsen (2013) with some modification. The activity of each extract was measured in serial dilutions, with analytical triplicates, at a concentration range of 0.0003-0.97 mg. $mL^{-1}$. An aliquot (100 µl) of DPPH solution (0.1mM in 96% ethanol) was added to extract solution (100 µl). The mixture was agitated (600 rpm), and incubated in the dark, for 30 min at room temperature. The absorbance of the resulting solution ($A_s$) was measured at 517 nm using a microtiter plate reader spectrophotometer (Biotek, Shimadzu, Holm & Halby, Denmark). The following controls were used: sample control ($A_0$), 100 µl extract + 100 µl EtOH; a sample blank ($A_b$), 100 µl DPPH + 100 µl MeOH; and finally, a positive control (100 µl DPPH + 100 µl BHT).

Radical scavenging activity was calculated as follows and given as inhibition percentage (Farvin and Jacobsen, 2013). $EC_{50}$ and $1/EC_{50}$ values were determined by plotting dose-response curves (GraphPad Prism 6), present in Annex C. Antioxidant Profiling.

$$Radical\ scavenging\ activity\ (\%) = \left[1 - \frac{(A_s - A_0)}{A_b}\right] \times 100$$

(7)

*1.4.5.2 Iron chelating activity*

The iron ($Fe^{2+}$) chelating activity of the extracts was estimated by the method described in Farvin and Jacobsen (2013), with some modification. The activity of each extract was measured in serial dilutions, with analytical triplicates, at a concentration range of 0.084-10.95 $mg.ml^{-1}$. An aliquot of extract solution (100 µl) and distilled water (110 µl) were transferred into the microtiter plate. To start the reaction, ferrous chloride (20 µl; 0.5mM) was added and mixed (600 rpm), for 3 minutes. Then, ferrozine (20 µl; 2.5mM) was added, mixed again, and left at room temperature, during 10 minutes. The absorbance of the resulting solution ($A_s$) was read at 562 nm using a using a spectrophotometer (Biotek, Shimadzu, Holm & Halby, Denmark). The following controls were used: sample control ($A_0$), 100 µl extract + 150 µl $H_2O$; a sample blank ($A_b$), reagents + 210 µl $H_2O$; and finally, a positive control with 100 µ of the metal chelator ethylenediaminetetraacetic acid (50 mM; EDTA) + 150 µl $H_2O$.

The iron chelating capacity was calculated as follows and given as inhibition percentage (Farvin and Jacobsen, 2013). $EC_{50}$, $1/EC_{50}$ $EC_{30}$ and $1/EC_{30}$ values were determined by plotting dose-response curves (GraphPad Prism 6), present in Annex C. Antioxidant Profiling.

$$Iron\ chelating\ activity\ (\%) = \left[1 - \frac{(A_s - A_0)}{A_b}\right] \times 100 \quad (8)$$

## 1.5 Optimization of Polyphenols

During a four-week trial, pooled individuals of *F. vesiculosus* were grown under two different lighting conditions (**L1**: PAR; **L2**: PAR+UVA); and three salinity concentrations (**S20:** 13.4±2.6; **S30:** 28.5±6.2; **S40:** 41.0±3.6 PSU). L1 and S20 represent control groups; L2 measures the influence of UVA light and S2 and S3 measure the influence of an increasing gradient of salinity. The combination of different light and salinities resulted in six treatments, each with triplicates (n=3). The experimental setup is displayed in Figure 12.

Seawater was collected from Bellevue beach and contamination was minimized by vacuum filtration (90 mm filter paper), salinity was adjusted with synthetic sea salt (Blue Treasure, Qingdao). The experiment was setup in a cold room, with fixed temperature of 10 ºC and the photoperiod was increased throughout the trial, from 12h to 16h light, to simulate the transition of seasons into summer. PAR light was provided by Grolux F36W lamps (Sylvania, Australia), UVA light by a 40 W UVA sunlamp (Philips, Holland), and foil paper enclosed the experimental area, to help distribute light evenly. Fresh thali of *F. vesiculosus* (10.38 ± 0.57 g) were sectioned, weighed, and equally distributed in 500 mL Erlenmeyer flasks (DURAN, Germany), sealed with parafilm and connected by 3 mm diameter tubing to an air flow supply system (Eheim 400, Germany). The flasks were randomly distributed and changed positions throughout the experimental trial (Figure 12).

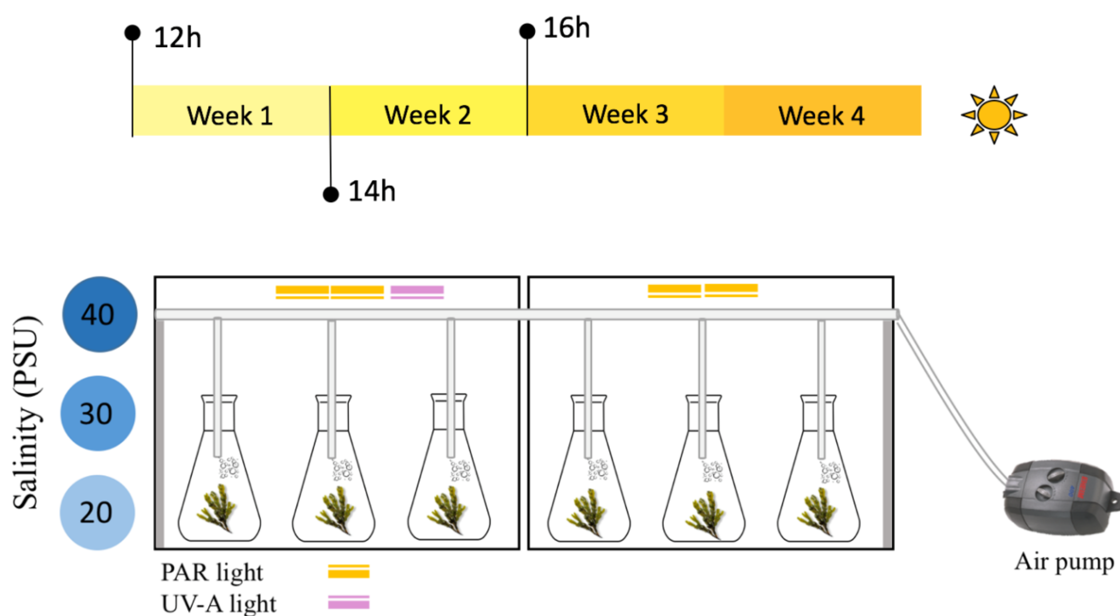



*Figure 12 Experimental setup for the optimization of polyphenols in F. vesiculosus, regarding photoperiod, type of lighting (**L1**: PAR and **L2**: UV-A) and salinity gradient (**S20**; **S30**; **S40** PSU).*

Cultures were maintained on a weekly basis, with monitoring of salinity, light, changes of water and addition of Cell-hi F2 (Varicon aqua) medium based on the Guillard F/2 medium. At the end of the trial, biomass was weighed, before and after being freeze-dried, to determine the growth rate, which was calculated as follows:

$$Growth\ rate\ (\%, day^{-1}) = \frac{\ln(ww_{f)} - \ln(ww_i)}{T} \times 100 \quad (9)$$

Where $ww_f$ is the final wet weight, $ww_i$ is the initial wet weight and T is time in days.

Samples were milled and phenolic compounds were extracted, as described previously. Given the small quantity of biomass per sample, only one method of phenolic quantification was performed, the FC assay and results were expressed as GAE.

## 1.6 Microscopic observation

### 1.6.1 Physodes

Fresh thalli of *F. vesiculosus* were hand-sectioned in the apical regions, using a sharp razor blade, and immersed in vanillin-HCL reagent for a few minutes. The vanillin-HCl reagent was prepared as follows: 10% vanillin dissolved in a freshly mixed 2:1 solution of 95% EtOH plus concentrated hydrochloric acid (Shibata et al., 2004). Sections were observed under light microscopy and photographed (Eclipse 80i, Nikon) as can be seen in Figure 13. The location and distribution of physodes within the cell tissue was assessed.

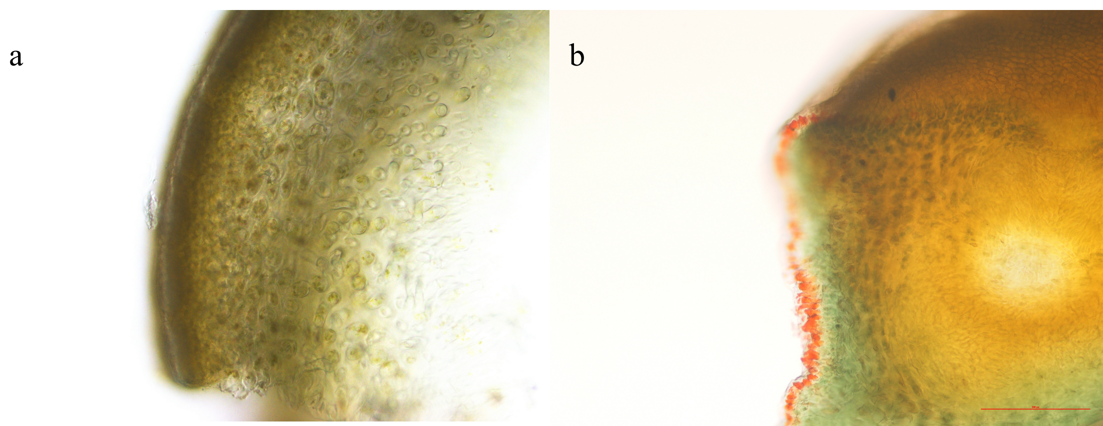



*Figure 13 Transversal section of F. vesiculosus apical region: a) before vanillin-HCL staining and b) after vanillin-HCL staining observed under light microscopy. Scale in pixels.© Madalena Mendes*

### 1.6.1 Epiphytes

Epiphyte coverage was registered qualitatively throughout the sampling period. Three species of epiphytes were collected from May samples and observed under light microscopy and photographed (Eclipse 80i, Nikon) for identification purposes.

## 1.7 Data treatment

Results were expressed as mean ± standard deviation (SD) and experiments were conducted in triplicates. Data was analyzed statistically through Excel and Prism 6 (GraphPad Software). Normality was checked by the D'Agostino & Pearson omnibus test followed by the appropriate parametric or non-parametric test. Comparison between groups was done via post hoc tests and statistical significance was taken as $p < 0.05$. Kruskal-Wallis tests were performed to investigate the effect of seasonality on ash contents, absolute yield of *me* and UV HPLC phenolic compounds. A one-way ANOVA and Tukey's multiple comparisons test were performed to determine the effect of seasonality, in the TPC, radical scavenging activity; and iron chelating capacity. Furthermore, correlation between TPC, radical scavenging activity, iron chelating capacity and environmental parameters was investigated by a Pearson r test. The effects of light and salinity on the TPC and growth rate, of seaweed from the experimental trial, were tested by a two-way ANOVA. Significant difference was determined with >95% confidence level.

# Results and discussion

1.1 Dry matter and ash contents

Dry biomass was approximately a quarter of initial fresh seaweed (26.53%), after freeze drying. The *dm* ranged from values of 87.4±2.9 to 92.9±0.3% in the freeze-dried samples. The standards for kelp production in *Laminaria* are of maximum water content for fresh-dried plants 22% and maximum water content for salt-dried plants 32% (FAO, 1989).

The *dm* and ash contents are represented in Figure 14. Ash content ranged between 15.4±0.3 to 19.9±0.3% and was not found to vary with any seasonal pattern like other studies. Ash content has been reported to range from 4.2 to 21.4% in *F. vesiculosus* (Truus et al., 2001, Balina et al., 2016). There were significant differences (P≈0,0008) in the ash content in May samples compared to June and October, which could be related to environmental causes. An increase in salinity and in mineral levels in seawater would increase ash content, for example (Balina et al., 2016).

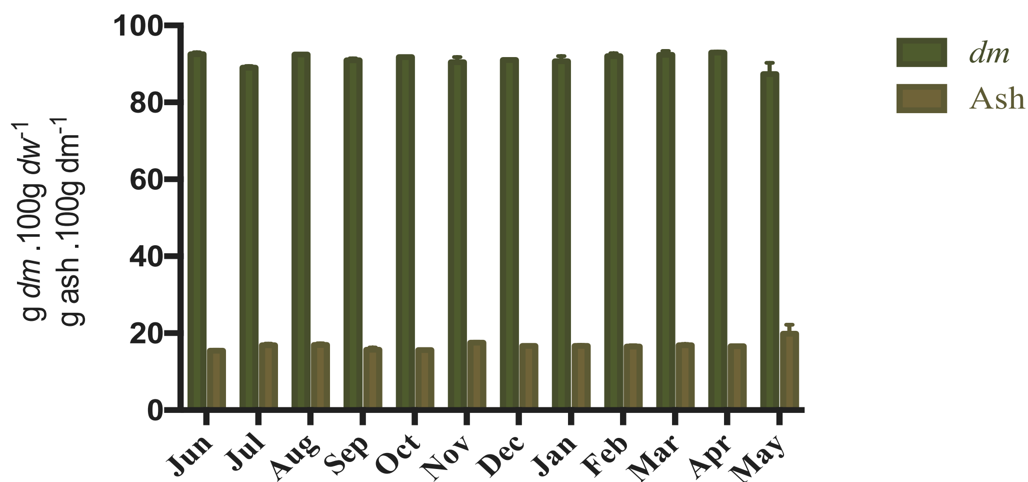



*Figure 14 Biomass characterization of F. vesiculosus seasonal samples, regarding dry matter and ash contents.*

## 1.2 Antioxidant profiling

### 1.2.1 Extraction

The absolute yield of *me* per *dm* of seaweed was in average 10.22%±0.03. A Kruskal-Wallis test was performed and results showed significant differences among means throughout the year (P≈0.0045) specifically in March, compared to June and May. The highest yields of *me* were found in the late spring and summer months, June and May (15.57%±0.00 *dm*; 14.08%±0.01 *dm*), also February (11.72%±0.01 *dm*). March had the lowest yield (6.58%±0.00 *dm*; Figure 15). Yields obtained in Farvin and Jacobsen (2013) were within the same range, *we* 15.7%±2.9 *dm* and *ea* 10.4%±0.5 *dm*, regarding April to September extracts.

The chemical composition of seaweed is affected by many factors, geographic location, season, wave exposure and sea temperature, mineral levels in seawater, pH level and salinity (Balina et al., 2016). *Fucus vesiculosus*, has a high capacity to absorb environmental pollution, such as heavy metals, which are much higher in the Baltic Sea than in the North Atlantic. Therefore, it is preferred to extract algal compounds (proteins, minerals, fatty acids and antioxidant compounds) instead of direct consumption as a food (Balina et al., 2016).

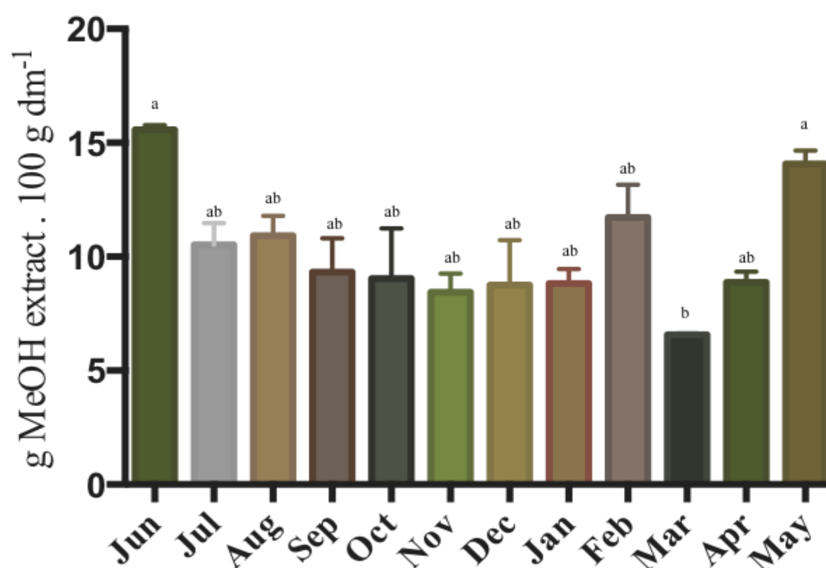



*Figure 15 Fucus vesiculosus me absolute yield from initial dm of seaweed over the season June 2016 to May 2017.*

### 1.2.2 Identification and quantification of pigments

Pigments showed consistency seasonally, 8 peaks were present in all months analyzed, two chlorophylls and six carotenoids (Figure 16). In general, they increased concentration from July to March, and then decreased to May. July had the lowest pigment concentrations (0.37 ±0.05 µg.mg dm$^{-1}$), and March the highest (1.71 ±0.25 µg.mg dm$^{-1}$). The most abundant pigments were chlorophyll-a, fucoxanthin, and prasinaxanthin, detailed data concerning pigment concentration is displayed in Table 2. Previous studies have reported pigments within the brown algal class. They are chlorophylls (a, $c_1$, $c_2$); main accessory pigments (β-carotene, fucoxanthin, zeaxanthin, violaxanthin); and also minor pigments (antheraxanthin-like, cryptoxanthin-like, cryptoxanthin-5,6-epoxide-like, latoxanthin-like, and mactraxanthin-like carotenoids, and neoxanthin) (Stengel et al., 2011).

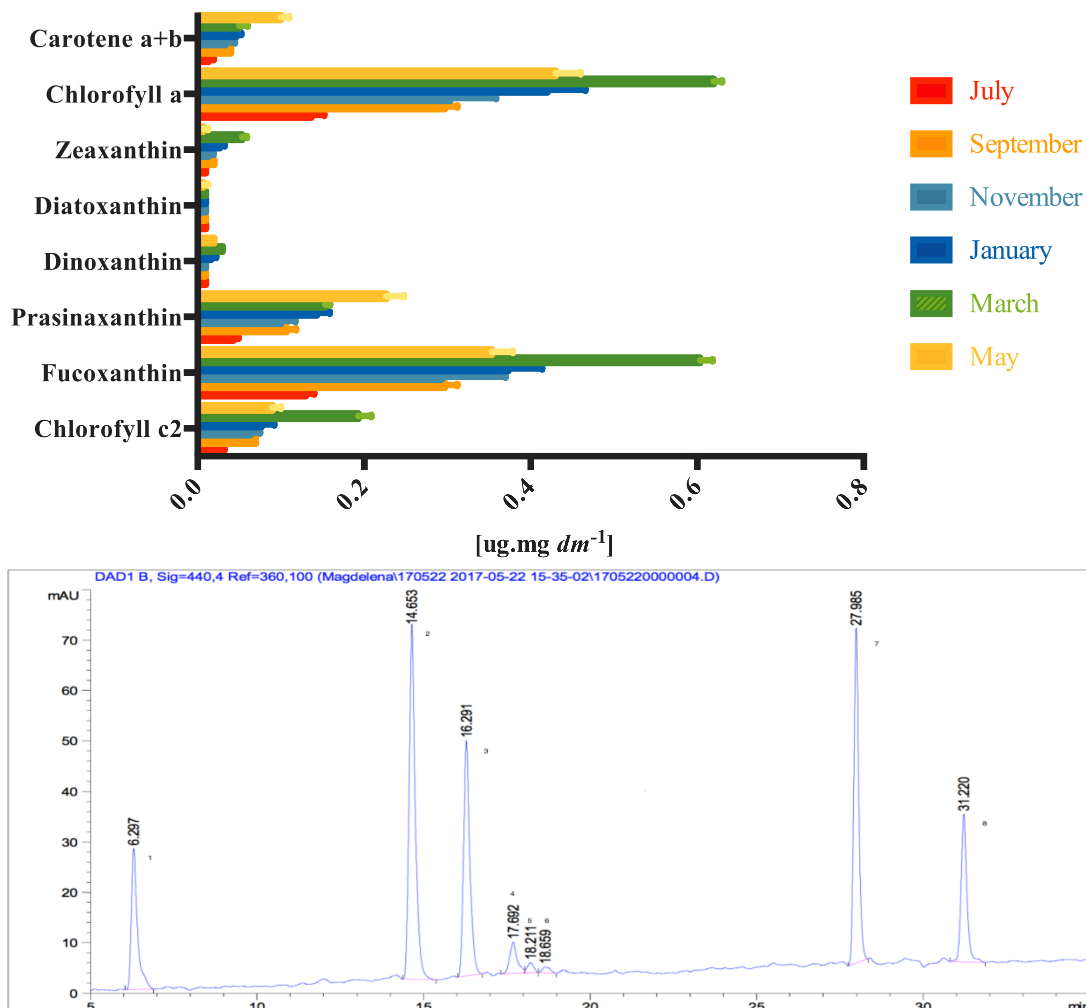



*Figure 16 Pigment contents. Top: Seasonal variation in pigments. Bottom: HPLC-DAD analysis (440 nm) of pigments in F. vesiculosus extracts, from May 2017. Peak numbers refer to the compounds in Table 2.*

*Table 2 Pigment contents expressed in µg.mg $dm^{-1}$from July 2016 to May 2017. Values are mean ± SD (n=3).*

| Peak nº | Pigment [µg.mg $dm^{-1}$] | 2016 | | | 2017 | | |
|---|---|---|---|---|---|---|---|
| | | July | September | November | January | March | May |
| 1 | ***Chlorofyll $C_2$*** | 0.03 ±0.00 | 0.07 ±0.00 | 0.06 ±0.00 | 0.08 ±0.00 | 0.19 ±0.00 | 0.09 ±0.00 |
| 2 | ***Chlorofyll-a*** | 0.14 ±0.01 | 0.29 ±0.01 | 0.30 ±0.05 | 0.42 ±0.05 | 0.62 ±0.01 | 0.43 ±0.03 |
| | ***Total** chlorophylls* | 0.16 ±0.07 | 0.36 ±0.16 | 0.36 ±0.16 | 0.45 ±0.21 | 0.79 ±0.29 | 0.44 ±0.19 |
| 3 | ***Fucoxanthin*** | 0.05 ±0.01 | 0.29 ±0.00 | 0.10 ±0.00 | 0.37 ±0.00 | 0.60 ±0.00 | 0.35 ±0.00 |
| 4 | ***Prasinaxanthin*** | 0.05 ±0.01 | 0.10 ±0.00 | 0.10 ±0.00 | 0.14 ±0.00 | 0.15 ±0.00 | 0.22 ±0.00 |
| 5 | ***Dinoxanthin*** | 0.01 ±0.00 | 0.01 ±0.00 | 0.01 ±0.00 | 0.01 ±0.00 | 0.03 ±0.00 | 0.02 ±0.00 |
| 6 | ***Diatoxanthin*** | 0.01 ±0.00 | 0.01 ±0.00 | 0.01 ±0.00 | 0.01 ±0.00 | 0.01 ±0.00 | 0.01 ±0.00 |
| 7 | ***Zeaxanthin*** | 0.01 ±0.00 | 0.02 ±0.00 | 0.30 ±0.10 | 0.03 ±0.00 | 0.05 ±0.00 | nd |
| 8 | ***Carotene* a+b** | 0.14 ±0.01 | 0.29 ±0.00 | 0.03 ±0.00 | 0.42 ±0.00 | 0.62 ±0.00 | 0.43 ±0.00 |
| | ***Total** carotenoids* | 0.22 ±0.05 | 0.48 ±0.11 | 0.47 ±0.11 | 0.66 ±0.16 | 0.92 ±0.23 | 0.79 ±0.17 |
| | ***Total** pigments* | 0.37 ±0.05 | 0.84 ±0.12 | 0.82 ±0.12 | 1.11 ±0.16 | 1.71 ±0.25 | 1.23 ±0.16 |

*nd = not detected.*

The specific pigment concentrations in *F. vesiculosus* has been previously reported for *ae* (Nygård and Ekelund, 2007, Bianchi et al., 1997); and in ethyl acetate fractions of liquid-liquid partitioning (*eaf*), *we*, *ae* and *ee*, in samples collected in May (Hermund et al., 2016). Chlorophyll-a was found in concentrations of 5 µg.mg $dw^{-1}$ (Nygård and Ekelund, 2007); 0.16 µg.mg $dw^{-1}$ (Bianchi et al., 1997); and 0, 0.1, 0.2 and 0 µg.mg $dw^{-1}$, respectively (Hermund et al., 2016). Fucoxanthin was found in the concentration of 1 (Nygård & Ekelund 2007); 0.12 (Bianchi et al., 1997); and 9.4, 0.6, 0 and 0.9 µg.mg $dw^{-1}$ (Hermund et al., 2016), regarding each extraction type. The concentration of chlorophyll-a obtained was not as high as Nygård and Ekelund, (2007) but is in accordance with Bianchi et al., (1997); and Hermund et al., (2016) for *we* and *ae*. The concentration of fucoxanthin obtained was lower than reported in other studies. It should be considered that the unit of concentration, method and solvent of extraction are distinct in the different studies.

Pigment concentration shows seasonality, with the increasing concentration from summer to winter and early spring suggesting a compensation towards decreasing light availability, in the same period. Given its role in photo-oxidation for photosynthesis, chlorophyll-a has pro-oxidative effects leading to the formation of singlet oxygen species (Hermund et al., 2016). On the other hand, fucoxanthins are recognized as antioxidants in

autoxidation because of their high radical scavenging activity, and therefore often act synergistically with other compounds (Hermund et al., 2016).

### 1.2.3 Identification and quantification of Phenolic compounds

RP-HPLC UV chromatograms are represented in Figure 17, and detailed data on peaks and their concentration expressed in GAE is detailed in Table 3. A total of 14 phenolic compounds were detected in *F. vesiculosus* extracts at a 255nm wavelength. Most were unable to be correctly identified, except for Peak 2 (9.616±0.0 min) identified as Gallic acid. A Kruskal-Wallis test was performed and significant differences were detected in the means of phenolics compounds (P≈0.0115), more specifically between September and March.

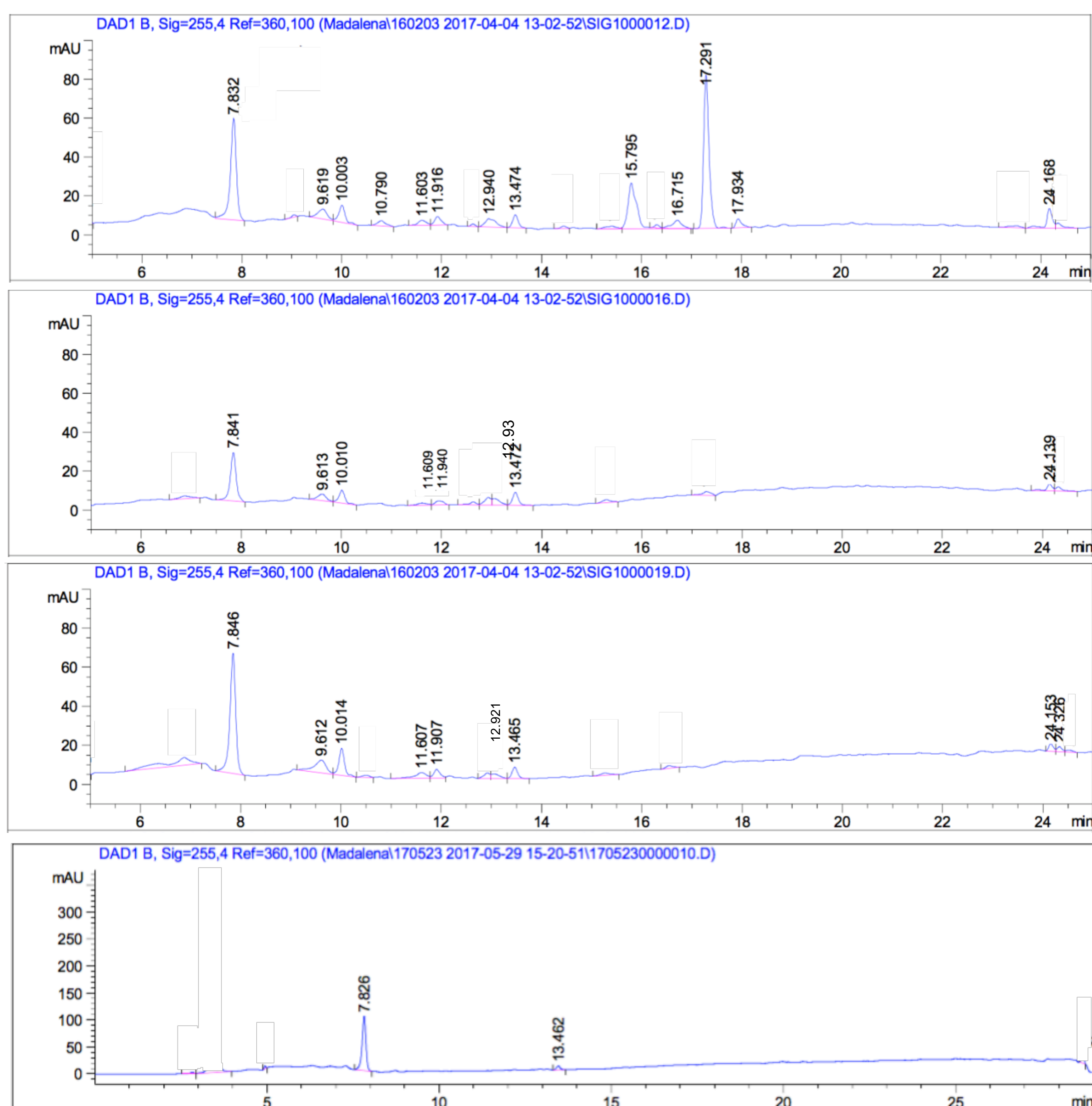



*Figure 17 HPLC-DAD analysis (255 nm) of phenolic compounds in F. vesiculosus extracts, from May 2017. Peak numbers refer to the compounds in Table 2. UV chromatograms for polyphenols. Top to bottom: September, November; January and March.*

September was the most diverse and rich month in phenolic compounds, with 13 peaks present and total sum 36.9±6.5 mg GAE.100 mg *dm*$^{-1}$. March was the least diverse, with only 3 peaks detected, although the second highest in total 28.8±3.8 mg GAE.100mg *dm*$^{-1}$. This concentration is mostly given by a singular compound, Peak 1 (7.837±0.0 min), which increased markedly its concentration from November (6.4±1.1 mg GAE.100mg *dm*$^{-1}$) to March (26.7±3.4 mg GAE.100 mg *dm*$^{-1}$). Some of the detected peaks suggest possible matches with standards, considering retention times and literature, however without certainty, and therefore were calculated as GAE. Moreover, some of the unidentified peaks might correspond to different compounds, even having removed pigments, the lack of standards and literature about seaweed phenolic acids make it a challenging task.

*Table 3 Polyphenol contents analyzed by RP-HPLC, from September 2016 to March 2017. Unidentified polyphenol peaks and respective retention time (min) phenolic contents expressed in mg GAE. 100 mg dm*$^{-1}$.

| **Peak nº** | **Phenolic Compound** | **Retention time** (min) | **TPC** [mg GAE.100 mg *dm*$^{-1}$] | | | |
|---|---|---|---|---|---|---|
| | | | September | November | January | March |
| 1 | | 7.837±0.0 | 8.5±2.3 | 6.4±1.1 | 11.4±4.7 | 26.7±3.4 |
| 2 | **Gallic Acid** | 9.616±0.0 | 2.4±1.0 | 2.9±2.9 | 1.9±0.4 | nd |
| 3 | | 10.010±0.0 | 2.2±0.9 | 2.3±0.7 | 3.1±1.9 | nd |
| 4 | | 10.661±0.2 | 0.4±0.3 | nd | nd | nd |
| 5 | Protocatechuic[1] | 11.606±0.0 | 0.9±0.7 | 1.0±1.3 | 1.4±0.7 | nd |
| 6 | Catechin[1] | 11.917±0.0 | 0.9±0.4 | 0.9±0.4 | 1.0±0.5 | nd |
| 7 | | 12.947±0.0 | 1.5±0.3 | 0.2±0.4 | 0.3±0.4 | nd |
| 8 | Chlorogenic[1] | 13.477±0.0 | 1.2±0.4 | 1.3±0.1 | 1.1±0.5 | 1.8±0.2 |
| 9 | Ferulic[1] | 15.498±0.3 | 4.6±1.0 | 0.1±0.2 | nd | nd |
| 10 | | 16.710±0.0 | 1.8±0.7 | nd | nd | nd |
| 11 | Myrcitrin[1] | 17.339±0.1 | 10±2.0 | 0.4±0.4 | nd | 0.4±0.3 |
| 12 | Morin[1] | 17.929±0.0 | 1.1±0.4 | nd | nd | nd |
| 13 | | 24.148±0.0 | 1.5±0.2 | 0.6±0.3 | 0.7±0.4 | nd |
| 14 | | 24.320±0.0 | nd | 0.3±0.3 | 0.1±0.1 | nd |
| Total | | | 36.9±6.5 | 16.5±5.2 | 21±9.2 | 28.8±3.8 |

*Values are mean ± SD (n=3).* [1]*Uncertain about identification; nd = not detected.*

A series of polyphenolic compounds have been previously documented in *F. vesiculosus* (Hermund et al., 2016, Parys et al., 2010, Singleton and Rossi, 1965, Wang et al., 2009, Farvin and Jacobsen, 2013). In Farvin and Jacobsen, (2013) six phenolic acids were identified by RP-HPLC in *we* and *ee,* at a 280nm wavelength. They obtained Gallic (*ee*: 2.9 ± 0.1; *we*: 13.5 ± 1.0 mg.g$^{-1}$ extract), Protocatechuic (*ee*: 14.0 ± 0.0; *we*: 1.7 ± 0.0 mg.g$^{-1}$

extract), Gentisic (*ee*: 29.0 ± 0.8; *we*: 19.4 ± 1.2 $mg.g^{-1}$ extract), Chlorogenic (*ee*: 0.1 ± 0.0; *we*: 0.9 ± 0.0 $mg.g^{-1}$ extract), Vannilic (*we*: 1.2 ± 0.4 $mg.g^{-1}$ extract), Caffeic (*we*: 0.9 ± 0.3 $mg.g^{-1}$ extract). Generally, values in Farvin and Jacobsen, (2013) are lower when compared to the present study in a similar unit. However, the difference in extraction methods, units used and sampling time, make a direct comparison of these results on phenolic acid constituents not feasible. HPLC is used to estimate the total simple phenolic compounds such as phenolic acids and flavonoids, whereas complex phenolics, such as phlorotannins, do not appear in the chromatogram, and require more in depth investigation with MS and NMR (Hefernan et al., 2015, Hermund et al., 2016).

### 1.2.4 TPC and *in vitro* antioxidant capacity assays

TPC, expressed as GAE, and antioxidant activity assays are shown in Table 4. A one-way ANOVA and Tukey's multiple comparisons test were performed on TPC. Results show significant differences between the means of each month ($P < 0.0010$) for $\alpha=0.05$. TPC ranged from 11±1.7 (November) to 18.4 ±1.6 mg GAE.100 mg $dm^{-1}$ (May). Overall, the lowest values of TPC were obtained during winter months (November to January) and the highest during spring, summer and fall (March to October; Figure 18).

*Table 4 Characterization of F. vesiculosus extracts by SLE from June 2016 to May 2017. TPC and in vitro antioxidant properties determined by 1/ECxx values (DPPH radical scavenging activity and iron chelating ability).*

| Month | **TPC** [mg GAE.100mg $dm^{-1}$] | **DPPH radical scavenging** [mg *dm*. $mL^{-1}$] | **Iron chelating ability** [mg *dm*. $mL^{-1}$] | |
|---|---|---|---|---|
| | | $1/EC_{50}$ | $1/EC_{50}$ | $1/EC_{30}$ |
| June | 14.89±1.91$^{abc}$ | 87.1±19.7$^{ab}$ | 0.15±0.1 | 0.14±0.1 |
| July | 16.19±1.72$^{abc}$ | 75.3±14.6$^{ab}$ | 0.11±0.1 | 0.07±0.1 |
| August | 14.98±1.07$^{abc}$ | 60.2±34.9$^{ab}$ | 0.05±0.0 | 0.69±0.9 |
| September | 15.06±1.07$^{abc}$ | 103.4±28.5$^{b}$ | 0.12±0.0 | 0.31±0.4 |
| October | 16.85±1.99$^{bc}$ | 30.9±43.6$^{ab}$ | 0.20±0.0 | 0.32±0.4 |
| November | 11.65±1.34$^{a}$ | 44.0±5.2$^{ab}$ | 0.15±0.1 | 0.15±0.1 |
| December | 12.61±2.74$^{ab}$ | 35.2±26.1$^{ab}$ | 0.07±0.1 | 0.30±0.3 |
| January | 12.55±0.48$^{ab}$ | 19.9±26.5$^{a}$ | 0.06±0.0 | 0.31±0.1 |
| February | 14.33±2.19$^{abc}$ | 47.8±19.8$^{ab}$ | 0.12±0.1 | 0.20±0.1 |
| March | 14.73±0.66$^{abc}$ | nd* | 0.17±0.0 | 0.14±0.2 |
| April | 13.49±0.38$^{ab}$ | 35.8±1.9$^{ab}$ | 0.12±0.1 | 0.12±0.1 |
| May | 18.38±1.25$^{c}$ | 62.0±23.4$^{ab}$ | 0.55±0.2 | 0.67±0.3 |

*Values are mean ± SD (n=3). *no data. Letters a–c indicate if there are significant differences between months, for each analysis (same letters not significant).*

Spectrophotometric methods to estimate TPC have been widely used to assess phenolic compounds (Hermund et al., 2016, Farvin and Jacobsen, 2013, Ragan and Jensen, 1978, Cotas, 2015, Hermund et al., 2015). In Farvin and Jacobsen (2013), TPC values were measured for April to September *F. vesiculosus* extracts. They obtained TPC for *we* 607.7±15.1, and for *ee* 1045±45.8 mg GAE. 100 $g^{-1}$ of *dw*, which are inferior to the values obtained in this study. Whereas in Hermund et al., (2015, 2016) September *F. vesiculosus* TPC values for *we* were 18.4±0.1; for *ae* 23.2±1.1; for *ee* 20.4±2.4; and for *eaf* 26.5±1.2 g GAE.100 $g^{-1}$ of dw, which are slightly superior to the ones obtained in this study for the same method. Once again, the difference in extraction methods, units used and sampling location, make a direct comparison of these results on TPC unreliable.

Ragan and Jensen (1978) previously found seasonal fluctuations in *F. vesiculosus* at the Trondheim fjord in Norway, with the Folin-Denis, Brentamine, and vanillin-$H_2SO_4$ colometric methods. TPC was highest during the sterility phase (≈11-13% of *dw*), August through March, and minimum values (≈8-10% *dw*) were attained in the end of spring, just before the period of maximum fertility, in May to June. These results are not in accordance to the present study, it is also important to take notice that the TPC method was different.

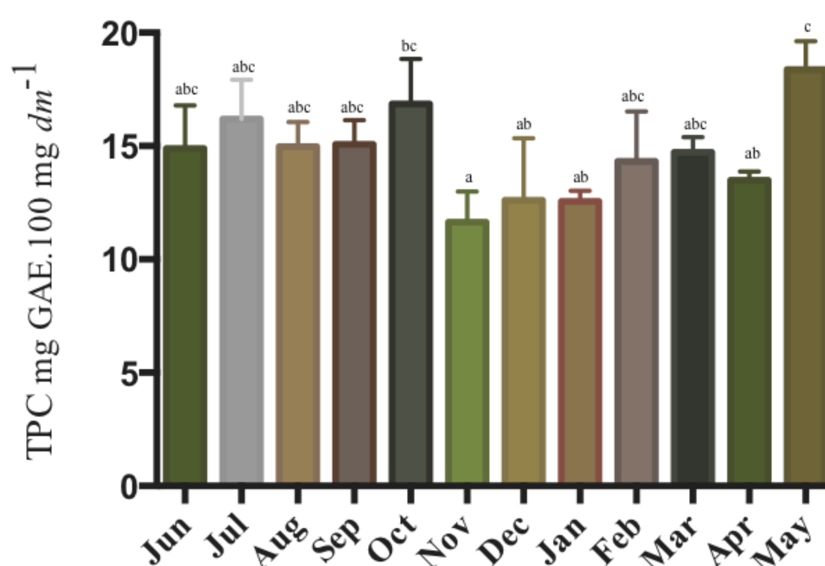



*Figure 18 TPC from F. vesiculosus extracts, expressed as GAE, from June 2016 to May 2017.*

The radical scavenging capacity 1/$EC_{50}$ displayed in Figure 19, was highest during summer (May to September). It ranged from 19.9±26.5 (January) to 103.4±28.5 (September) mg *dm*.$mL^{-1}$ and presented much higher values, when compared to the Iron chelating activity, 0.05±0 (August) to 0.55±0.2 (May). The coefficient of variation from both *in vitro* antioxidant assays 1/$EC_{50}$ and 1/$EC_{30}$ values was quite high, sample variation in iron chelation had previously been documented (Hermund et al., 2016). A one-way ANOVA test was performed, and significant differences were detected for radical scavenging (P≈0.0116) between September (highest activity) and January (lowest activity); but not for iron chelating (P≈0.4670), for α=0.05.

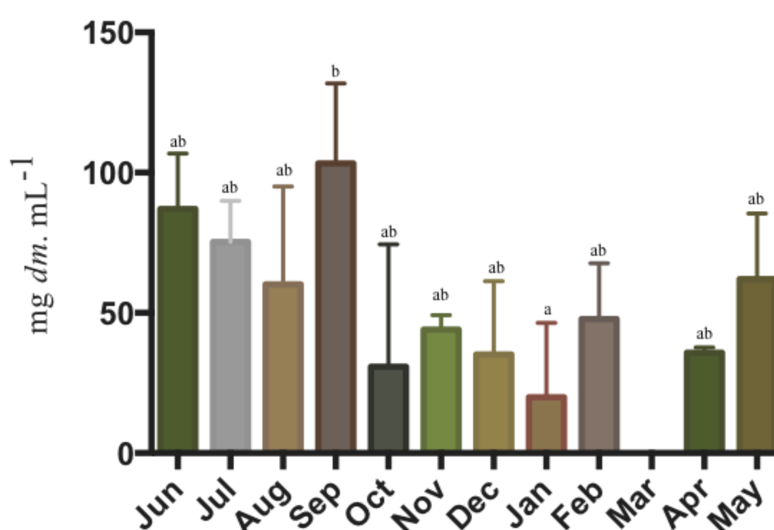



*Figure 19 DPPH radical scavenging activity expressed as 1/$EC_{50}$, from June 2017 to May 2017. There is no data for March.*

In Farvin and Jacobsen (2013), the extracts of April to September obtained DPPH $EC_{50}$ in *ee* of 9.9±1.6, and *we* 8.3±0.8 μg.$mL^{-1}$. In the same study, iron chelating $EC_{50}$ values in *ee* were 1000±75.0 and in *we* 128.6±23.0 μg.$mL^{-1}$. When converted into similar units, these results are inferior to the present study in the same range of months. In Hermund et al. (2016) September *F. vesiculosus* extracts obtained a DPPH 1/$EC_{50}$ in *ae* was 68.2±0.3; *ee* was 75.3±15.4; and *we* 53.0±3.6 mg *dw*.$mL^{-1}$. Furthermore, iron chelating 1/$EC_{30}$ in *ae* was 1.5±0.1; *ee* was 2.1±0.3; and *we* 2.5±0.2 mg *dw*.$mL^{-1}$. The DPPH radical scavenging activity was lower in Hermund et al. (2016) in comparison to the present study, suggesting that *me* are better scavengers than ae, ee and we; whereas the iron chelating capacity was higher in Hermund et al. (2016). Studies have shown that iron chelating capacity is better in *we,* since other highly polar compounds, such as dietary fibers, are co-extracted and present good metal chelating abilities (Farvin and Jacobsen, 2013, Wang et al., 2009, Hermund et al., 2016).

To investigate correlation between TPC, radical scavenging activity ($1/EC_{50}$) iron chelating activity ($1/EC_{30}$) and environmental parameters (mean temperature, precipitation and sunlight) a Pearson r test was performed. Results showed that there were significant positive correlations between mean temperature, TPC (P=0.0391) and DPPH (P=0.0016); likewise, between sunlight hours, TPC (P=0.0458) and DPPH (P=0,0060). There was no correlation between iron chelating and these factors, or for any of the antioxidant measures and precipitation. A projection of the TPC, radical scavenging activity, mean temperature sunlight and UV-dose is represented in Figure 20. Environmental data enphasise a bell shaped peak graph, with increasing values from late winter to the summer maximum and back down again. TPC and DPPH scavenging activity also have their highest values in spring, summer and fall, and lowest during the winter months.

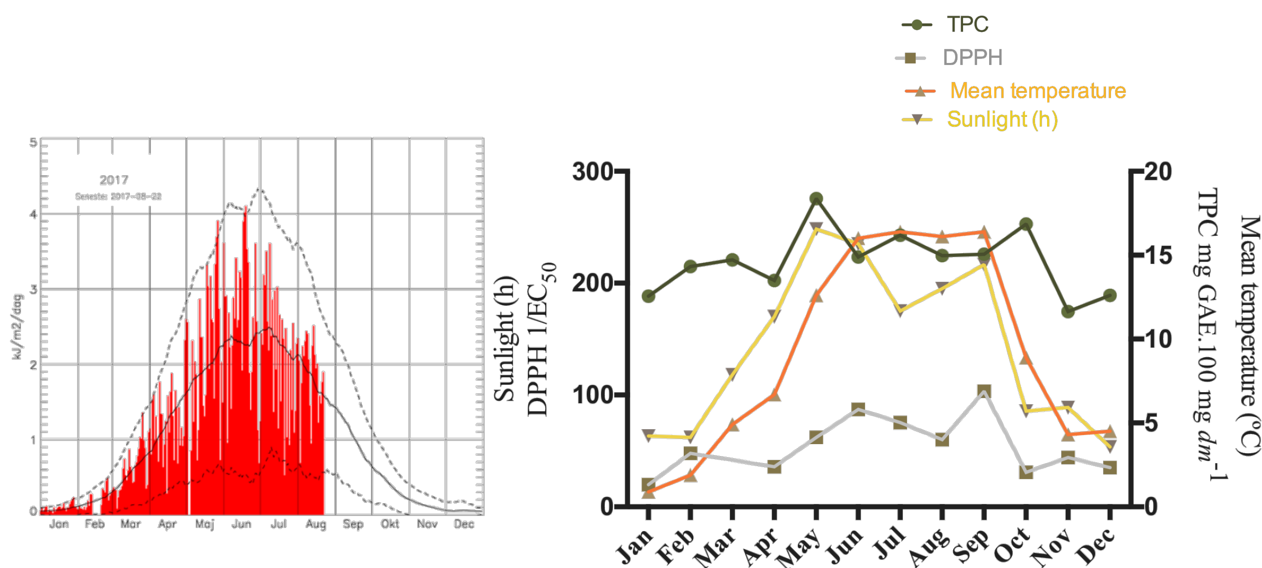



*Figure 20 Left) UV-dose index for the year 2017 retrieved from DMI* (Cappelen 2017). *Right) Representation of antioxidant assays, (TPC and DPPH radical scavenging activity) and environmental parameters (mean temperature and sunlight), corresponding to data from June 2016 to May 2017* (Cappelen 2017)

Parys et al. (2009) documented fluctuations in phenolic compounds in *Ascophyllum nodosum* L. (fucoid species) throughout the year, in Scotland. The study used both FC and quantitative H nuclear magnetic resonance spectroscopy (qHNMR) to analyse TPC. The highest TPC occurred in summer, with a maximum in July (≈1.0% FC; ≈2.2% qHNMR) while the lowest were recorded in winter, with a minimum in February (≈0.3% FC; ≈0.6% qHNMR). This seasonal pattern agrees with the present study, despite concerning a different species.

Although being species specific,  the concentration of phenolic compounds is generally highest during the summer and lowest during fall and winter (Connan et al., 2004). The increase of phenolic compounds in summer has been linked to a photoprotective mechanism with dynamic photoinhibition of photosynthesis, to tolerate light stress in response to the intensified UV radiation (Hefernan et al., 2015, Hermund et al., 2016, Abdala-Díaz et al., 2006). *Fucus vesisculosus* is an intertidal species and is often exposed to high solar radiation dosages over summer, therefore it developed an efficient physiological adaptation to tolerate the deleterious irradiances. Abdala-Díaz et al. (2006) suggested a link between the concentrations of polyphenols in brown algae to irradiance levels in the field, which indicates that the synthesis of these compounds has a very rapid turnover time. Furthermore, daily variation in phenolic compounds has been positively correlated with air temperature (low tide exposure), in *Pelvetia canaliculata, Acscophyllum nodosum,* and *Bifurcaria bifurcate* (Connan et al., 2004).

The high incidence of grazing in summer and early autumn is also considered as one of the factors contributing to increase in polyphenols, when there is less growth and more carbon available for defence chemicals (Hefernan et al., 2015, Parys et al., 2009). Interestingly enough, the seasonal pattern of polyphenols also matches the reproductive cycle of *F. vesiculosus,* with fertile periods during summer (May to June) but also late autumn (September to November) and dormancy phases in winter (Berger et al., 2001). Low salinities have a negative effect on the reproduction of marine organisms, because they decrease the motility and longevity of gametes and polyspermy is more likely to occur. *Fucus vesiculosus* is present throughout most of the Baltic, and as such had to develop unique features to successfully reproduce despite the low salinities (Serrão et al., 1999). Hence the increase in polyphenol concentration during the reproductive season, since they are known to block polyspermy (Serrão et al., 1999).

## 1.3 Optimization of Polyphenols

Phenolic compounds and growth rates were analyzed from an experimental trial on *F. vesiculosus*, to understand the effects of salinity concentration and light conditions on these parameters. TPC was similar between treatments, except in salinity 40, with a higher value for the UV treatment. The TPC concentration ranged from 2.5 to 8.4 mg GAE.100 mg *dm*$^{-1}$. Detailed results are summarized in Table 5 and displayed in Figure 21. No significant differences were found between the TPC and salinity (P=0.0525) or light (P=2443) conditions, in the experimental setting, for α=0.05. Growth rates decreased as salinity increased and overall were higher in the seaweed exposed to UV light, there were significant differences between salinities (P=0.0216), specifically between 20 and 40 for the PAR light treatment.

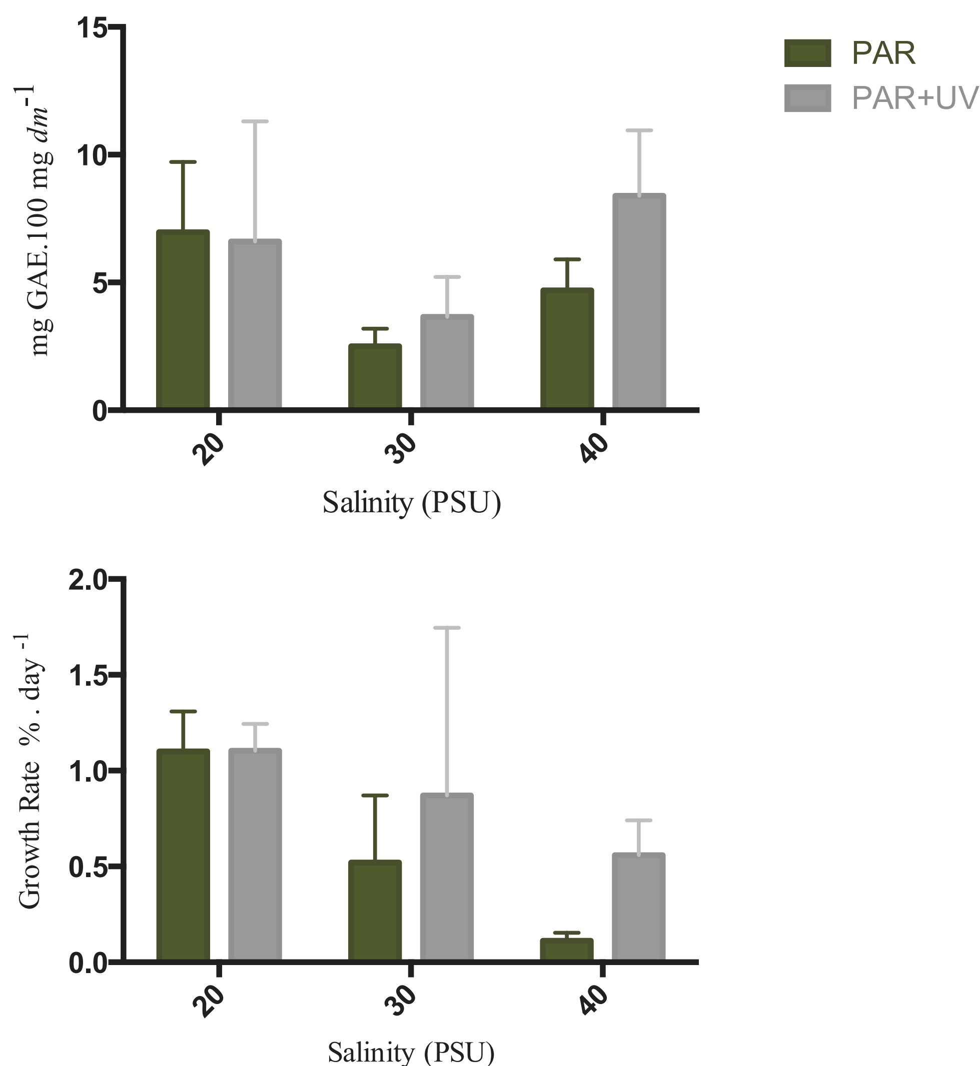



*Figure 21 TPC expressed in GAE of F. vesiculosus extracts and growth rate expressed in percentage per day during the experimental trial, where three salinity concentrations and two light conditions were tested.*

*Table 5 Experimental trial regarding light and salinity concentrations. Salinity values registered during the trial are given in PSU, growth rate in percentage and TPC in GAE. Values are mean ± SD (n=3).*

| Light | Salinity code | Salinity (PSU) | TPC [ mg GAE.100 mg $dm^{-1}$] | Growth rate (%. $day^{-1}$) |
|---|---|---|---|---|
| UV+PAR | 20 | 12.7±2.9 | 6.6±4.2 | 1.1±0.1 |
| | 30 | 28.1±3.9 | 3.7±1.5 | 0.9±0.9 |
| | 40 | 40.3±3.5 | 8.4±2.3 | 0.6±0.2 |
| PAR | 20 | 14.1±2.1 | 7.0±2.5 | 1.1±0.2 |
| | 30 | 28.8±8.0 | 2.5±0.8 | 0.5±0.4 |
| | 40 | 41.7±3.7 | 4.7±1.2 | 0.1±0.0 |

The concentrations of TPC obtained in the experiment, ranging from 2.5±0.8 to 8.4±2.3 mg GAE.100 mg $dm^{-1}$, were much lower than from natural populations. This might be explained by the milling of biomass and extraction process. For the seasonal analysis, a large quantity of biomass was milled into fine powder, contrarily to the experiment, in which each sample had around 10 g of ww. This resulted in much larger particles for the experiment biomass, and less surface for the methanol to act on during extraction. It might also be that polyphenols were released into the water as a response to the stress of the harvest or trial itself since they act as defense mechanisms (Fernando et al. 2016; Pandey et al. 2009). In the future, this could be verified by a biochemical analysis of the water.

These results were taken with a grain of salt given the errors that occurred during the experimental trial, for instance, maintaining the right salinity for each treatment during the trial. Even though flasks were closed in parafilm, every other day water would evaporate and increase salinities, when detected, fresh water was immediately added up to the 500mL mark and salinity measured (Figure 22). The instability within each salinity group could have affected the outcome of the experiment leading to the very high coefficient of variation rates for both TPC and growth rates.

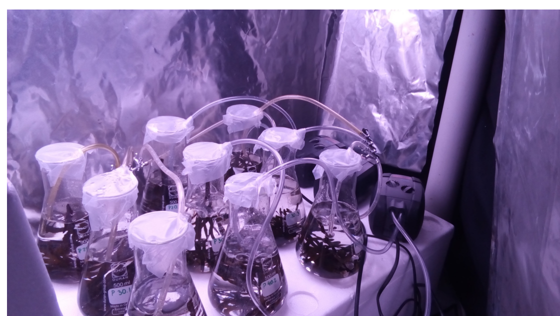


*Figure 22 Experimental setup. Erlenmeyer flasks under PAR and UVA light.© Madalena Mendes*

## 1.4 Microscopic observation

### 1.4.1 Physodes

Transversal sections of the tips of *F. vesiculosus* seaweed, from the lab-culture experiment, were observed under light microscopy. HCl-vanillin staining revealed the epidermis and outer cortical layer covered in physodes, and a few scattered in some regions of the cortex and medulla, evidenced by the red coloration, seen in Figure 23. These observations are in accordance to previous research (Ragan, 1976, Schoenwaelder, 2008) and therefore the third hypothesis was accepted. Bladderwrack seaweed inhabit the upper most regions of the littoral and possesses gas vesicles to float, therefore they are more exposed to UV radiation, tidal cycles of desiccation (even if little in the Baltic) and salinity changes (White, 2008). They are also subject of intense fouling during summer, which covers a large part of the fronds (Evert and Eichhorn, 2013). The location of the physodes in the outer layers of the tissue, suggest a barrier or shield effect against both biotic and abiotic parameters.

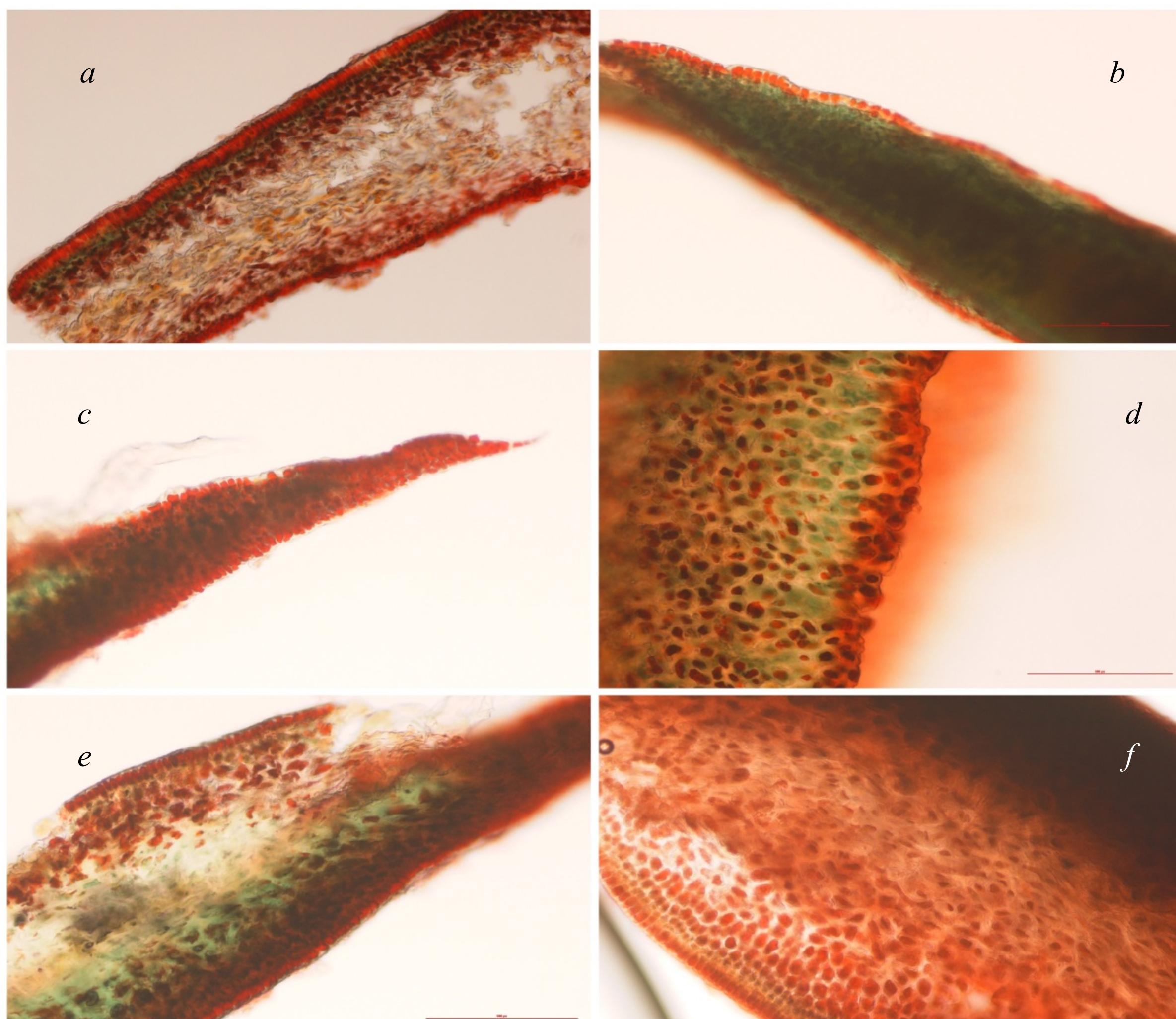



*Figure 23 Transverse sections of F. vesiculosus taken from the light and salinity experiment: a) PAR.20; b) PAR+UV.20; c-e) UV.30; f) PAR+UV.40. Scales are in pixels. © Madalena Mendes*

### 1.4.2 Epiphytes

Epiphytes were present during the warmer summer months, May to September, with a significant amount of fouling. Three species of filamentous algae, *Rhizoclonium* sp; *Ectocarpus siliculosus* (Dillwyn) Lyngbye; and *Polysiphonia* sp. (Guiry and Guiry, 2016), were found growing on *F. vesiculosus* (Figure 24). However, further investigation such as gene sequencing should be used to fully identify these species. The increased phenolic compounds, during summer, are also produced to discourage herbivores (Evert and Eichhorn, 2013, Hefernan et al., 2015). *Fucus vesiculosus* harbours many visitors, surface grazing snails, herbivorous isopods and fish, and tube worms looking for substrate and shelter (White, 2008). During winter months, the increased wave exposure and whiplash effect help control epiphytes (Torn et al., 2006).

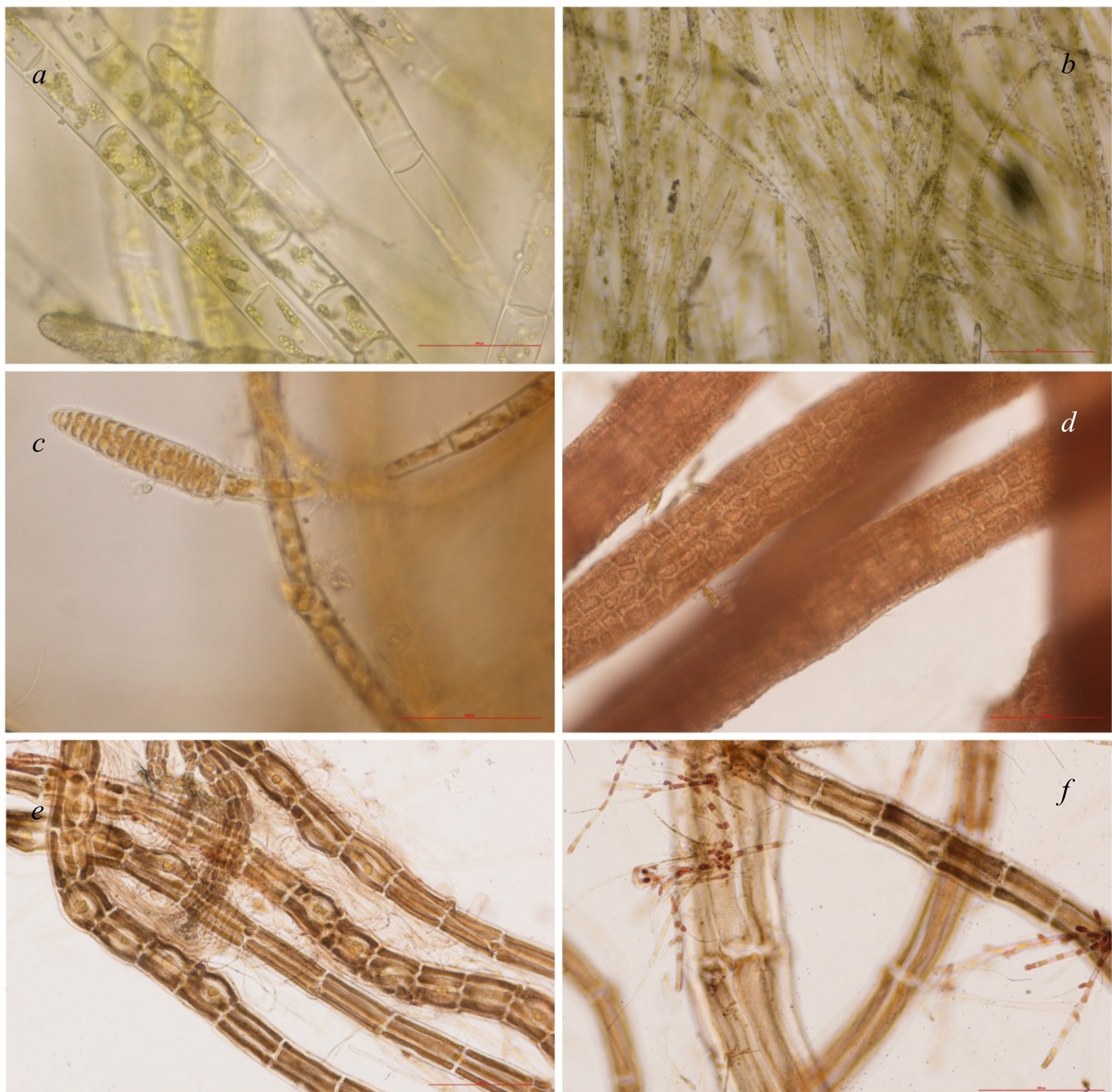



*Figure 24 F. vesiculosus epiphytes May 2017 a-b) Chlorophyta, Rhizoclonium sp; c-d) Phaeophyceae, Ectocarpus siliculosus; e-f) Rhodophyta, Polysiphonia sp. Scales are in pixels. © Madalena Mendes*

## Conclusions and Future Perspectives

The study of the polyphenolic variations within *F. vesiculosus* is necessary for the future development of natural antioxidants. Antioxidants from this species have shown to be active in food models, such as muesli bars, and in lotions making it very appealing to the food and cosmetic industries (Hermund 2016). Knowledge on seasonal patterns, and optimized culture conditions, regarding salinity and UV light, are useful to understand these variations and yield higher concentrations of polyphenolic compounds. Given the seasonal variation in polyphenol concentration, the time of the year is crucial and to be taken in consideration, if *F. vesiculosus* is to be cultivated and harvested at an industrial scale.

This study analyzed the variation in the antioxidant content and activity of the seaweed *F. vesiculosus*, which shows high potential in terms of bioactive compounds. The collective results on RP-HPLC, TPC and radical scavenging activity assays confirmed the first hypothesis, that there are seasonal differences in the polyphenolic content and activity of wild *F. vesiculosus*. More specifically, late spring, summer and early autumn months (April to October) presented the highest polyphenol content and activity, whereas winter the lowest. Seasonality was linked to environmental parameters, mean temperatures and sunlight hours, which correlated positively to the quantity (TPC) and quality (DPPH radical scavenging activity) of antioxidants. Unfortunately, UV-index data from 2016 is archived and was not available from DMI. For this reason, it could not be included in this study, apart from a graphical representation of daily UV-doses for 2017, it would however be very interesting to collaborate with DMI and compare this data to these results. Pigment contents revealed a reverse pattern of seasonality in comparison to polyphenols, due to photosynthetic requirements during lower light availability periods. HPLC alone only permits the identification of monophenolic compounds and the FC assay is a spectrophotometric analysis. In a future perspective, the use of advanced methods, such as MS and NMR, to identify and quantify complex polyphenols, mainly phlorotannis, would complement this study, since they are the most abundant polyphenols in brown seaweed. In light of this research, I would suggest that the best harvesting periods for *F. vesiculosus,* in Denmark, are from late spring, right before the reproduction peak (April-May), to early Fall (September-October), because they demonstrated high antioxidant contents and activity and therefore ideal for specific extraction of these compounds. If the biomass is intended to be used for direct consumption as food I would suggest harvesting before the summer months to avoid the intense fouling regarded negatively in a commercial perspective.

There were no significant differences between the TPC, regarding salinity and light obtained during the optimization of polyphenols experiment, therefore, the second hypothesis was rejected. The increased salinity and UVA light did not have effects on the antioxidant yield, and therefore it was not possible to optimize *F. vesiculosus* antioxidant contents, under the experimental conditions of this study. However, it would be interesting to repeat this study, but ensuring the stability of salinity conditions since this is of major importance to correctly assess its effect. It would also be beneficial to upscale the experiment from flasks to small tanks using more individuals, thus having more seaweed biomass to work with and avoid problems in the extraction process. During winter months, polyphenol levels are very low, and so it would be useful to cultivate *F. vesiculosus* in land aquaculture, under optimal conditions, to increase those levels.

Physodes were observed under the microscope and were distributed mainly in the outer layers of the blades, epidermis and outer cortical layers, confirming the third hypothesis. This distribution is likely to be a defense mechanism, against UV irradiation and herbivory, in such that it provides a shield effect.

*Figure 25 Sampling at Bellevue beach in January 2017.© Madalena Mendes*

# References


Abdala-Díaz, R. T., Cabello-Pasini, A., Pérez-Rodríguez, E., Álvarez, R. M. C., Figueroa, F. L. and Conde Álvarez, R. M. (2006) 'Daily and seasonal variations of optimum quantum yield and phenolic compounds in *Cystoseira tamariscifolia* (Phaeophyta)', *Marine Biology,* 148(3), pp. 459-465.

Arnold, T. M., Tanner, C. E. and Hatch, W. I. (1995) 'Phenotypic variation in polyphenolic content of the tropical brown alga *Lobophora variegata* as a function of nitrogen availability', *Marine Ecology Progress Series,* 123(1-3), pp. 177-183.

Baardseth, E. (1958) '*A Method of Estimating the Physode Content in Brown Algae'*, Trondheim.

Balina, K., Romagnoli, F. and Blumberga, D. (2016) 'Chemical Composition and Potential Use of *Fucus vesiculosus* from Gulf of Riga', *Energy Procedia,* 95 (Supplement C), pp. 43-49.

Baweja, P., Kumar, S., Sahoo, D. and Levine, I. (2016) 'Biology of Seaweeds': Elsevier Inc., pp. 41-106.

Belda, M., Sanchez, D., Bover, E., Prieto, B., Padrón, C., Cejalvo, D., Lloris, J. M. and Miguel, J. (2016) 'Extraction of polyphenols in Himanthalia elongata and determination by high performance liquid chromatography with diode array detector prior to its potential use against oxidative stress', *Journal of Chromatography B,* 1033 (Supplement C), pp. 334-341.

Berger, R., Malm, T. and Kautsky, L. (2001) 'Two reproductive strategies in baltic *Fucus vesiculosus* (phaeophyceae)', *European Journal of Phycology,* 36(3), pp. 265-273.

Bianchi, T. S., Kautsky, L. and Argyrou, M. (1997) 'Dominant chlorophylls and carotenoids in macroalgae of the Baltic Sea (Baltic proper): Their use as potential biomarkers', *Sarsia,* 82 (2012), pp. 55-62.

Cappelen, J. (2017) '*Guide to Climate Data and Information from the Danish Meteorological Institute'*. Retrieved from: http://www.dmi.dk/laer-om/generelt/dmi-publikationer/.

Connan, S., Goulard, F., Stiger, V., Deslandes, E., Ar Gall, E. and Gall, E. A. (2004) 'Interspecific and temporal variation in phlorotannin levels in an assemblage of brown algae', *Botanica Marina,* 47(5), pp. 410-416.

Cotas, J. F. P. (2015) '*Fucus ceranoides (Ochrophyta, Phaeophyceae): Bioatividades dependentes do gradiente salino'.*

Cérantola, S., Breton, F., Gall, E. A. and Deslandes, E. (2006) 'Co-occurrence and antioxidant activities of fucol and fucophlorethol classes of polymeric phenols in *Fucus spiralis*', *Botanica Marina,* 49(4), pp. 347-351.

Evert, R. F. and Eichhorn, S. E. (2013) '*Raven Biology of plants'.* 8 ed edn. New York: W. H. Freeman and Company.

FAO (1989) '*Culture of Kelp (Laminaria japonica) in China'.* Qingdao.

FAO (2016) '*The State of World Fisheries and Aquaculture 2016. Contributing to food security and nutrition for all'.* Rome.

FAO (2017) *'Global Aquaculture Production' 1950-2015 dataset.*

Farvin, S. K. H. and Jacobsen, C. (2013) 'Phenolic compounds and antioxidant activities of selected species of seaweeds from Danish coast', *Food Chemistry,* 138(2-3), pp. 1670-1681.

Fernando, I. P. S., Kim, M., Son, K.-t., Jeong, Y. and Jeon, Y.-J. (2016) 'Antioxidant Activity of Marine Algal Polyphenolic Compounds: A Mechanistic Approach', *Journal of Medicinal Food,* 19(7), pp. jmf.2016.3706-jmf.2016.3706.

Fulcher, R. G. and McCully, M. E. (1969) 'Laboratory culture of the intertidal brown alga *Fucus vesiculosus*', *Canadian Journal of Botany,* 47(11), pp. 219-223.

Guiry, M.D. & Guiry, G.M. 2016. *AlgaeBase*. World-wide electronic publication, National University of Ireland, Galway. Available at http://www.algaebase.org; searched on 07 October 2016.

Guiry, M. D. R. 2015. *'The Seaweed Site: information on marine algae'.* Available at http://www.seaweed.ie/; searched on 07 October 2016.

Heffernan, N., Brunton, N.P., FitzGerald, R.J., Smyth, T.J. (2015) 'Profiling of the Molecular Weight and Structural Isomer Abundance of Macroalgae-Derived Phlorotannins', *Mar. Drugs*, *13*, pp. 509-528.

Hermund, D. B., Jacobsen, C. and Nielsen, K. F. (2016) *Extraction, characterization and application of antioxidants from the Nordic brown alga Fucus vesiculosus.* National Food Institute, Technical University of Denmark.

Hermund, D. B., Yeşiltaş, B., Honold, P., Jónsdóttir, R., Kristinsson, H. G. and Jacobsen, C. (2015) 'Characterisation and antioxidant evaluation of Icelandic *F. vesiculosus* extracts in vitro and in fish-oil-enriched milk and mayonnaise', *Journal of Functional Foods,* 19, pp. 828-841.

Hoefnagel, E. (1991) 'Seaweed, status and the symbolic domain: seaweed harvesters in Portuguese coastal communities', *Maritime Anthropological Studies,* 4(2), pp. 40-61.

Holdt, S. (2011) '*Bioactive compounds in seaweed; review, with emphasis on available species in Danish waters',* Lyngby.

Holdt, S. L. and Kraan, S. (2011) 'Bioactive compounds in seaweed: Functional food applications and legislation', *Journal of Applied Phycology,* 23(3), pp. 543-597.

IUPAC. 'Compendium of Chemical Terminology', 2nd ed. (the "Gold Book"). Compiled by A. D. McNaught and A. Wilkinson. Blackwell Scientific Publications, Oxford (1997). XML on-line corrected version: http://goldbook.iupac.org (2006-) created by M. Nic, J. Jirat, B. Kosata; updates compiled by A. Jenkins. ISBN 0-9678550-9-8. https://doi.org/10.1351/goldbook.

Kanaizuka, Y., Fujioka, K. and Katayama, N. (2002) 'The vertical distribution of seaweeds and their photosynthetic characteristics', *Journal of Biological Education,* 36(2), pp. 89-93.

Makkar, H. P. S. S., Tran, G., Heuzé, V., Giger-Reverdin, S., Lessire, M., Lebas, F. and Ankers, P. (2016) 'Seaweeds for livestock diets: A review', *Animal Feed Science and Technology,* 212(Supplement C), pp. 1-17.

Martínez, J. H. I. and Castañeda, H. G. T. (2013) 'Preparation and Chromatographic Analysis of Phlorotannins', *Journal of Chromatographic Science,* 51(8), pp. 825-838.

McHugh, D. J. (2003) '*A Guide to the Seaweed Industry'.*

McLachlan, J., Chen, L. C. M. and Edelstein, T. (1971) 'The culture of four species of *Fucus* under laboratory conditions', *Canadian Journal of Botany,* 49(8), pp. 1463-1469.

NetAlgae (2012) '*Seaweed Industry in Europe'.* Available at: www.netalgae.eu; searched on 07 October 2016.

Nygård, C. A. and Ekelund, N. G. A. (2007) 'Photosynthesis and UV-B tolerance of the marine alga *Fucus vesiculosus* at different sea water salinities', in Anderson, R., Brodie, J., Onsøyen, E. & Critchley, A.T. (eds.). Dordrecht: Springer Netherlands, pp. 235-241.

*OBIS* (2017). '*Fucus vesiculosus* Linnaeus, 1753 dataset (444637)'. Retrieved from http://www.iobis.org/explore/#/taxon/444637.

Pandey, K. B., & Rizvi, S. I. (2009). 'Plant polyphenols as dietary antioxidants in human health and disease', *Oxidative Medicine and Cellular Longevity*, *2*(5), pp. 270–278.

Parys, S., Kehraus, S., Krick, A., Glombitza, K.-W., Carmeli, S., Klimo, K., Gerhäuser, C. and König, G. M. (2010) 'In vitro chemopreventive potential of fucophlorethols from the brown alga

*Fucus vesiculosus* L. by anti-oxidant activity and inhibition of selected cytochrome P450 enzymes', *Phytochemistry,* 71(2-3), pp. 221-229.

Parys, S., Kehraus, S., Pete, R., Küpper, F. C., Glombitza, K.-W. and König, G. M. (2009) 'Seasonal variation of polyphenolics in *Ascophyllum nodosum* (Phaeophyceae)', *European Journal of Phycology,* 44(3), pp. 331-338.

Pearson, G. A. and Serrão, E. A. (2006) 'Revisiting synchronous gamete release by fucoid algae in the intertidal zone: Fertilization success and beyond?', *Integrative and Comparative Biology,* 46(5), pp. 587-597.

Plass, M. (2013) *RSPB 'Handbook of the Seashore'.* 1st edn.: Bloomsbury Natural History.

Ragan, M. A. 1976. Physodes and the Phenolic Compounds of Brown Algae. Composition and Significance of Physodes in vivo.

Ragan, M. A. and Jensen, A. (1977) 'Quantitative studies on brown algal phenols. I. Estimation of absolute polyphenol content of Ascophyllum nodosum (L.) Le Jol. and *Fucus vesiculosus* (L.)', *Journal of Experimental Marine Biology and Ecology,* 30(2), pp. 209-221.

Ragan, M. A. and Jensen, A. (1978) 'Quantitative studies on brown algal phenols. II. Seasonal variation in polyphenol content of *Ascophyllum nodosum (*L.) Le Jol. and *Fucus vesiculosus* (L.)', *Journal of Experimental Marine Biology and Ecology,* 34(3), pp. 245-258.

Raposo, M., de Morais, A. and de Morais, R. (2015) 'Carotenoids from Marine Microalgae: A Valuable Natural Source for the Prevention of Chronic Diseases', *Marine Drugs,* 13(8), pp. 5128-5155.

Roff, J., Zacharias, M. and Day, J. (2011) '*Marine Conservation Ecology'.* London Washington: DC Earthscan.

Safafar, H., van Wagenen, J., Moller, P. and Jacobsen, C. (2015) 'Carotenoids, Phenolic Compounds and Tocopherols Contribute to the Antioxidative Properties of Some Microalgae Species Grown on Industrial Wastewater', *Mar Drugs,* 13(12), pp. 7339-56.

Salgado, L. T., Tomazetto, R., Cinelli, L. P., Farina, M. and Amado Filho, G. M. (2007) 'The influence of brown algae alginates on phenolic compounds capability of ultraviolet radiation absorption in vitro', *Brazilian Journal of Oceanography,* 55(2), pp. 145-154.

Schoenwaelder, M. E. A. (2008) 'The Biology of Phenolic Containing Vesicles', *ALGAE,* 23(3), pp. 163-175.

Serrao, E. A., Pearson, G., Kautsky, L. and Brawley, S. H. (1996a) 'Successful external fertilization in turbulent environments', *Proceedings of the National Academy of Sciences of the United States of America,* 93(11), pp. 5286-5290.

Serrão, E. A., Brawley, S. H., Hedman, J., Kautsky, L. and Samuelsson, G. (1999) 'Reproductive Success of *Fucus vesiculosus* (Phaeophyceae) in the Baltic Sea', *J. Phycol,* 35, pp. 254-269.

Serrão, E. A., Kautsky, L. and Brawley, S. H. (1996b) 'Distributional success of the marine seaweed *Fucus vesiculosu*s L. in the brackish Baltic Sea correlates with osmotic capabilities of Baltic gametes', *Oecologia,* 107(1), pp. 1-12.

Shibata, T., Kawaguchi, S., Hama, Y., Inagaki, M., Yamaguchi, K. and Nakamura, T. (2004) 'Local and chemical distribution of phlorotannins in brown algae', *Journal of Applied Phycology,* 16(4), pp. 291-296.

Sineiro, J., Franco, D., Rubilar, M., Sánchez, M., Jerez, M., Pinelo, M., Costoya, N. and Núñez, M. J. (2008) 'Polyphenols from plant materials: extraction and antioxidant power', *Electronic Journal of Environmental, Agricultural and Food Chemistry,* 7(8), pp. 3210-3216.

Singleton, V. L. and Rossi, J. A. (1965) 'Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents', *American Journal of Enology and Viticulture,* 16(3), pp. 144-158.

Stengel, D. B., Connan, S. and Popper, Z. A. (2011) 'Algal chemodiversity and bioactivity: Sources of natural variability and implications for commercial application', *Biotechnology Advances,* 29(5), pp. 483-501.

Tatarenkov, A., Bergstrom, L., Jonsson, R. B. and Serrao, E. A. (2005) 'Intriguing asexual life in marginal populations of the brown seaweed *Fucus vesiculosus'*, *Molecular Ecology,* 14(2), pp. 647-651.

Tiwari, B. K. and Troy, D. J. (2015) 'Seaweed sustainability - food and nonfood applications': Elsevier Inc., pp. 1-6.

Torn, K., Krause-Jensen, D. and Martin, G. (2006) 'Present and past depth distribution of bladderwrack (*Fucus vesiculosus*) in the Baltic Sea', *Aquatic Botany,* 84(1), pp. 53-62.

Truus, K., Vaher, M. and Taure, I. (2001) 'Algal biomass from *Fucus vesiculosus* (Phaeophyta): Investigation of the mineral and alginate components', *Proc. Estonian Acad. Sci. Chem.,* 50(2), pp. 95-103.

Tsao, R. (2010) 'Chemistry and Biochemistry of Dietary Polyphenols', *Nutrients*. 2, pp. 1231-1246.

Viana, I. G., Bode, A. and Fernández, C. (2015) 'Ecology of *Fucus vesiculosus* (Phaeophyceae) at its southern distributional limit: growth and production of early developmental stages', *European Journal of Phycology,* 50(3), pp. 247-259.

Wang, T., Jónsdóttir, R. and Ólafsdóttir, G. (2009) 'Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic seaweeds', *Food Chemistry,* 116(1), pp. 240-248.

White, N. (2008) '*Fucus vesiculosus* Bladder wrack', in Tyler-Walters H. and Hiscock K. (eds) *Marine Life Information Network: Biology and Sensitivity Key Information Reviews*, [on-line]. Plymouth: Marine Biological Association of the United Kingdom. [cited 07-10-2016]. Available at: http://www.marlin.ac.uk/species/detail/1330

Yang, J., Guo, J. and Yuan, J. (2008) 'In vitro antioxidant properties of rutin', *LWT - Food Science and Technology,* 41(6), pp. 1060-1066.

# Annex

A. Environmental data

*Table A. 1 Data from the Danish Meteorological Institute (Cappelen 2017). Monthly averages of mean temperature (ºC), precipitation (mm) and sunlight (h) were taken from June to August 2016 (*; Denmark) and from September 2016 to May 2017 (Lyngby-Tårbæk, the sampling location).*

| Month | **Mean Temperature** (°C) | **Precipitation** (mm) | **Sunlight** (h) |
|---|---|---|---|
| June* | 16 | 79 | 235 |
| July* | 16.4 | 85 | 175 |
| August* | 16.1 | 60 | 195 |
| September | 16.4 | 33.4 | 216.6 |
| October | 8.9 | 76.1 | 85.5 |
| November | 4.3 | 54.2 | 88.9 |
| December | 4.5 | 34.8 | 53.6 |
| January | 0.9 | 19.1 | 63.3 |
| February | 1.9 | 51.1 | 62.1 |
| March | 4.9 | 46.4 | 118.1 |
| April | 6.7 | 65.8 | 170.1 |
| May | 12.6 | 24 | 248.4 |

## B. Dry matter, ash contents and extraction

*Table A. 2 Biomass characterization of F. vesiculosus seasonal samples, regarding dry weight, dry matter and ash contents, expressed as percentages. Mean ±SD.*

| Year | Month | $\frac{dw}{ww}$(%) | ***dm* (%)** | **Ash (%)** |
|---|---|---|---|---|
| 2016 | June | 25.30 | 92.5±0.5 | 15.4±0.3 |
| | July | 24.85 | 89±0.5 | 16.9±0.4 |
| | August | 27.57 | 92.4±0.1 | 17±0.4 |
| | September | 28.00 | 91±0.5 | 15.7±0.5 |
| | October | 26.15 | 91.8±0.1 | 15.6±0.2 |
| | November | 31.80 | 90.5±1.3 | 17.5±0.3 |
| | December | 32.07 | 91±0.1 | 16.7±0.2 |
| 2017 | January | 36.42 | 90.7±1.4 | 16.7±0.3 |
| | February | 25.68 | 92±0.8 | 16.5±0.3 |
| | March | 24.02 | 92.4±1 | 16.9±0.3 |
| | April | 21.44 | 92.9±0.3 | 16.6±0.1 |
| | May | 15.11 | 87.4±2.9 | 19.9±2.3 |
| | M | 26.53 | 91.1±1.6 | 16.8±1.2 |

*Table A. 3 Absolute extraction yield of me (%) before and after OASIS column.*

| Month | Before OASIS MCX | After OASIS MCX |
|---|---|---|
| September | 11.9±3.9 | 6.3±0.7 |
| November | 8.4±1.9 | 5.3±0.7 |
| January | 8.1±1 | 6.5±2.6 |
| March | 8.6±0.6 | 4.1±0.5 |
| M | 9.2±2.5 | 5.6±1.6 |

## C. Antioxidant Profiling

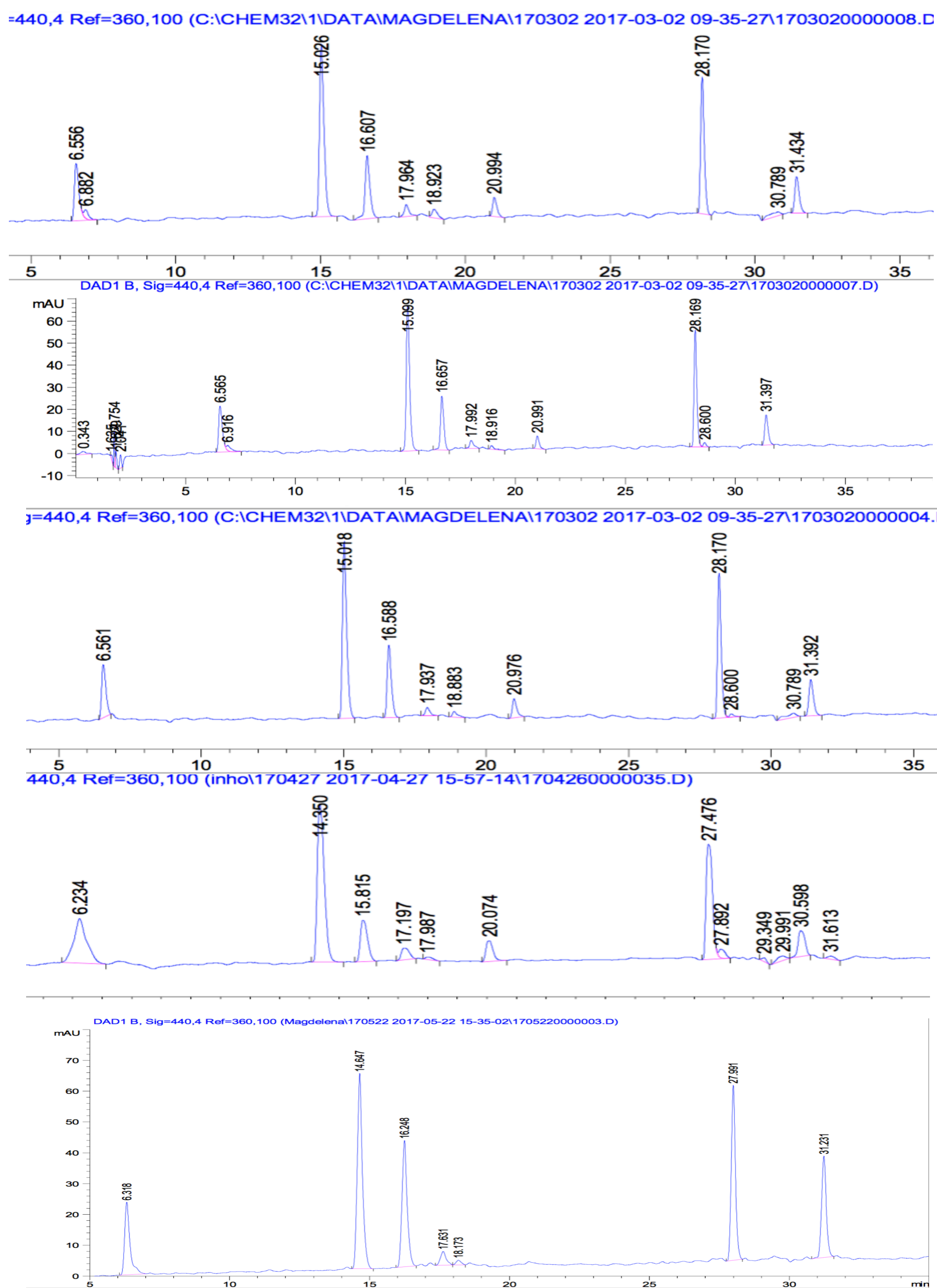



*Figure A. 1 HPLC-DAD analysis (440 nm) of pigments in F. vesiculosus extracts. Top to bottom: September, November (2016), January, March and May (2017).*

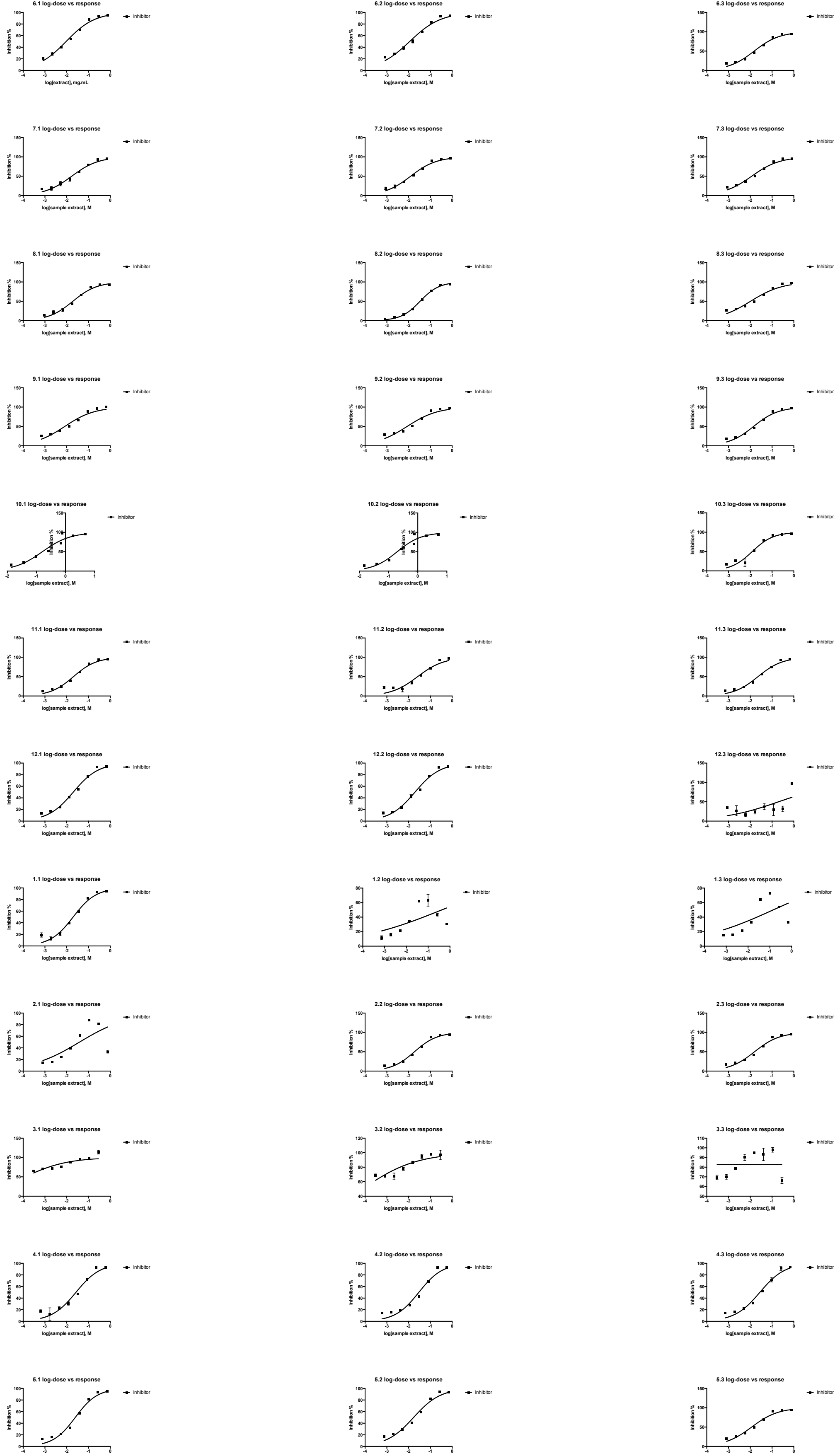
*Table A. 4 TPC, antioxidant activity assays and environmental data Pearson correlation matrix.*

| | TPC | DPPH 1/EC50 | IRON 1/EC30 | Mean temperature (°C) | Precipitation (mm) | Sunlight (h) |
|---|---|---|---|---|---|---|
| TPC | | 0.3969027 | 0.3822672 | **0.6001376** | 0.1795217 | **0.584765** |
| DPPH 1/EC50 | 0.3969027 | | -0.0657744 | **0.8300945** | 0.1914146 | **0.7655177** |
| IRON 1/EC30 | 0.3822672 | -0.0657744 | | 0.2525267 | -0.3907158 | 0.3661713 |
| Mean temperature (°C) | 0.6001376 | 0.8300945 | 0.2525267 | | 0.4009816 | **0.8542211** |
| Precipitation (mm) | 0.1795217 | 0.1914146 | -0.3907158 | 0.4009816 | | 0.1669941 |
| Sunlight (h) | 0.584765 | 0.7655177 | 0.3661713 | 0.8542211 | 0.1669941 | |

*Table A. 5 TPC, antioxidant activity assays and environmental data Pearson correlation matrix P-values. *Significant differences for α=0.05*

| | TPC | DPPH 1/EC50 | IRON 1/EC30 | Mean temperature (°C) | Precipitation (mm) | Sunlight (h) |
|---|---|---|---|---|---|---|
| TPC | | 0.2268058 | 0.2200792 | 0.03910611* | 0.5766503 | 0.04581959* |
| DPPH 1/EC50 | 0.2268058 | | 0.8476338 | 0.001563902* | 0.5728801 | 0.006030666* |
| IRON 1/EC30 | 0.2200792 | 0.8476338 | | 0.4284458 | 0.209194 | 0.2417426 |
| Mean temperature (°C) | 0.03910611 | 0.001563902 | 0.4284458 | | 0.1964169 | 4.038126e-04* |
| Precipitation (mm) | 0.5766503 | 0.5728801 | 0.209194 | 0.1964169 | | 0.6039402 |
| Sunlight (h) | 0.04581959 | 0.006030666 | 0.2417426 | 4.038126e-04 | 0.6039402 | |

# EC50 Radical scavenging activity



## EC50 Iron chelating activity