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# Bioactive compounds and antioxidant activity from *Alaria esculenta* cultivated on long-lines in Bantry Bay: effect of cultivation and processing method

**UNIVERSIDADE DO ALGARVE**

**FACULDADE DE CIÊNCIAS E TECNOLOGIA**

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# Bioactive compounds and antioxidant activity from *Alaria esculenta* cultivated on long-lines in Bantry Bay: effect of cultivation and processing method

Thesis for Master's Degree in Aquaculture and Fisheries

Specialization Aquaculture

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**2020**

“ I declare that this thesis was composed by myself, that the work contained herein is my own work, except where indicated by referencing and the work presented in it has not been submitted for any other degree or profesional qualification.”

“Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto econstam da listagem de referências incluída.”

Silvia Blanco González

# Acknowledgements

The clock is constantly ticking, and this demanding world never waits for us. However, it is through this beautiful journey of life that we experience emotions in form of happiness, sadness, and love from all the people that share our same journey, and from who eventually, we have to heard a goodbye. During the development of this thesis, I´ve been able to understand the extremely lucky that I have been for being surrounded by the most inspirational women I have ever met in my whole life, and to who I cannot be more thankful for all the provided support along these years: **Freddie**, who allowed me to cry on her shoulder (just seaweed queens do that!); **Fiona**, who picked me up as a student and guided me into the fascinating seaweed world; **Luísa**, who put her confidence and time on me (despite not knowing each other personally); **Julie**, who always trusted me with blind eyes; and **Sophie**, who has been my Irish mother in Bantry, always ready to cope with my stressing life. But overall, this would not have happened without **my parent's** hard work and perseverance in pursuing a better life for their children, one much better than the one they had, and to whom I own all I have and cannot be more proud of them. While I was on my emotional rollercoaster, my niece **Alba** and **my brother** kept looking for seaweed for me, **Max**, **Jake** and **Hugo** gave me strength in our walks around the beach and Bantry House gardens while enjoying a few ice creams and pancakes (they were just 3, 8 and 1 years old!). They all provided me with a fresh breath when I needed the most during my learning-by-doing process.

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# Abstract

The demand for seaweed-based products for food, pharmaceuticals and fish and cattle feed has been rising with a significant expansion of the global seaweed cultivation industry. The growing demand is driven for contaminant-free seaweed and by the commercial sector requiring seaweed-derived products for biotechnological and medical applications in countries with little traditional interest in seaweed aquaculture.

Brown macroalgae contain a wide variety of bioactive compounds beneficial to health with therapeutic properties such as anticancer, anti-obesity, antidiabetic, antiviral or antibacterial activity. However, these compounds can show seasonal variations and its amount can also be affected by the extraction, isolation, and biomass processing methods used. This work aims to extract, produce fractions enriched in the compounds of interest, characterize, and analyse the antioxidant activity of extracts rich in fucoxanthin and phlorotannins, determining the effect of the drying method (oven and freeze-drying), the deployment date and the sampling date on these bioactive compounds. To achieve this, *Alaria esculenta*, a winter and fast-growing species, was cultivated on long-lines in Bantry Bay, Ireland. Biomass yield was calculated to assess seaweed growth and the characterization and quantification of fucoxanthin and phlorotannins was performed using Ultra-High-Performance Liquid Chromatography, followed by DPPH scavenging capacity in order to assess the antioxidant activity of the extracts. The results demonstrate that the bioactive compounds concentration is dependent on the deployment and sampling dates and that these compounds have a strong relationship with the antioxidant activity of the extracts. However, no significant differences were found when applying the two drying methods: oven-drying and freeze-drying. We have concluded that a later deployment (December) will produce a higher biomass yield in less time, with levels of fucoxanthin and phlorotannins peaking on May 5$^{th}$, probably related with the presence of epiphytes and grazers. However, high variability was observed, suggesting that further studies need to be carried out to improve the sampling method.

**Key words:** *Alaria esculenta*; drying method; fucoxanthin; phlorotannins; antioxidant activity.

# Resumo

A procura por produtos à base de macroalgas para alimentação humana, desenvolvimento de produtos farmacêuticos e/ou rações para animais tem aumentado causando uma expansão significativa da indústria global de cultivo de algas marinhas. A crescente demanda exige algas livres de contaminantes e é impulsionada por um setor comercial que exige produtos derivados de algas marinhas para aplicações biotecnológicas e médicas em países com pouco interesse ou tradição na aquicultura das macroalgas.

As macroalgas castanhas contêm uma grande variedade de compostos bioativos benéficos para a saúde com propriedades terapêuticas, como anticancerígenos, anti-obesidade, antidiabética, antiviral ou antibacteriana. No entanto, esses compostos podem apresentar variações sazonais e sua quantidade também pode ser afetada pelos métodos de extração, isolamento e processamento de biomassa utilizados. Assim, este trabalho tem como objetivo a produção de frações enriquecidas em compostos de interesse como a fucoxantina e os florotaninos, sua caracterização, e análise da atividade antioxidante dos extratos preparados, determinando o efeito do método de secagem (estufa e liofilização), assim como a data de inicio de cultivo e de colheita. A espécie emestudo, *Alaria esculenta* é uma espécie de inverno e de crescimento rápido, e foi cultivada em cordas em Bantry Bay, na Irlanda. O rendimento de biomassa foi calculado para avaliar o crescimento das algas e a caracterização e quantificação da fucoxantina e dos florotaninos foi realizada por Cromatografia Líquida de Alta eficiência, seguida da determinação da sua atividade antioxidante (métodod do DPPH). Os resultados demonstram que a concentração dos compostos bioativos é dependente das datas de cultivo e amostragem e que esses compostos possuem forte relação com a atividade antioxidante dos extratos. Não foram encontradas diferenças significativas ao aplicar os dois métodos de secagem: estufa e liofilização. Assim, os resultados sugerem que um inicio de ciclo de cultivo em dezembro produzirá maior rendimento de biomassa em menos tempo, apresentando níveis de fucoxantina e florotaninos com valores máximos a 5 de maio, estando estes provavelmente relacionados com a presença de organismos epifíticos e *grazers*. Porém, foi observada alta variabilidade, sugerindo que mais estudos precisam ser realizados para aprimorar o método de amostragem.

**Palavras-chave:** *Alaria esculenta*; método de secagem; fucoxantina; florotaninoss; atividade antioxidante.

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# Abbreviations and acronyms

**ANOVA –** Analysis Of Variance

**BIM –** Bord Iascaigh Mhara

**BMRS –** Bantry Marine Research Station Ltd

**CAD –** Charged Aerosol Detector

**dm –** Dry Matter

**dw –** Dry Weight

**FAO –** Food and Agricultural Organization

**GRAS –** Generally Recognized As Safe

**HPLC –** High-Performance Liquid Chromatography

**$IC_{50}$ –** Half maximal inhibitory concentration

**kDa –** Kilodalton

**m –** Metres

**PVC –** Polyvinyl Chloride

**RDI** – Recommended daily intake

**rpm –** Revolutions Per Minute

**SD –** Standard Deviation

**UHPLC –** Ultra-High-Performance Liquid Chromatography

**UV-vis –** Ultraviolet-visible

**vv –** Volume to Volume

**ww –** Wet Weight

# 1. Introduction

## 1.1. Seaweed aquaculture

It is estimated that 1888 different brown macroalgae, 6200 red macroalgae and 1800 green macroalgae are found in the marine environment. Seaweeds, both from wild stocks and from aquaculture, play an important role in $CO_2$ sequestration from the atmosphere, and new evidence suggests that seaweeds are global contributors to oceanic carbon sinks (Krause-Jensen & Duarte, 2016).

To avoid the pressure on natural populations, seaweed aquaculture is undergoing global expansion raising new challenges for producers and the environment. Seaweed farming is widely perceived as one of the most environmentally benign types of aquaculture activity as it does not require additional feed or fertilisers (Cottier-Cook *et al.*, 2016), and its carbon sequestration potential, thus directly mitigating global climate change (Duarte *et al.*, 2017).

Seaweed aquaculture contributed ~49% to the global mariculture production of 27.3 million tonnes fresh weight in 2014 and is undergoing a rapid global expansion. As can be seen in **Figure 1**, world seaweed aquaculture production had increased from 13.5 million tonnes in 1995 to over 30 million tonnes in 2016 (FAO, 2018).

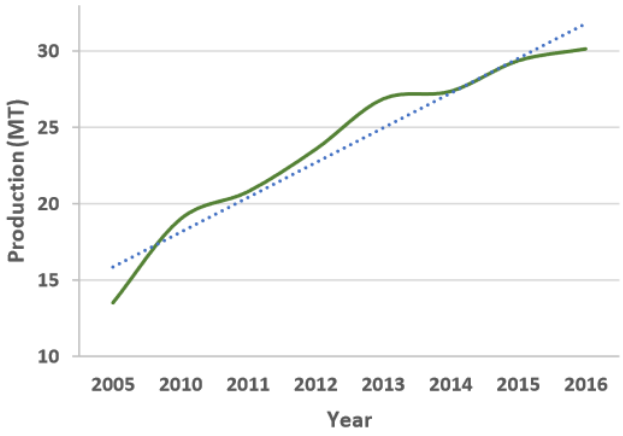


**Figure 1**: Global seaweed aquaculture production (2005-2016). Source: FAO (2018).

Furthermore, while wild harvesting remained unchanged between 1950 and 2010, aquaculture production has increased exponentially from 1970 (Cottier-Cook *et al.*, 2016) (**Fig. 2**). Also, industrial use of seaweed biomass has shifted over the years, from exploiting beach-

cast seaweeds as fertilizers and a source of potash, via iodine production, to hydrocolloid extraction (Synytsya *et al.*, 2015).

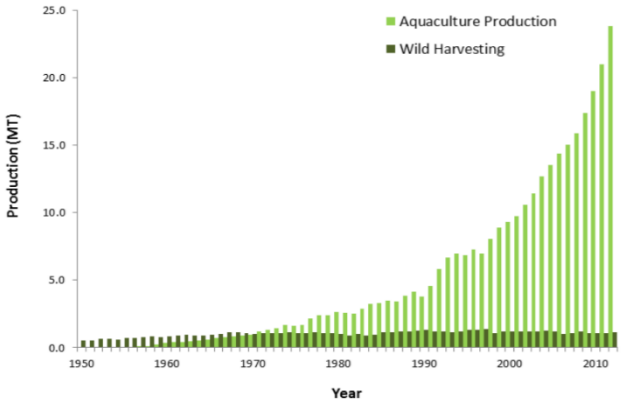


**Figure 2:** Global seaweed aquaculture and wild harvesting (1950-2014). Source: FAO (2014).

China and Indonesia are the major seaweed producers, accounting for 87% of the total global production (wet weight) (FAO, 2018) as shown in **Figure 3**. Species like *Undaria pinnatifida* or *Porphyra spp*. are the most produced species in Asia for human consumption. In 2013, it was reported that 99.9% of the utilized seaweed biomass in Asia was cultivated, whereas in Europe only 0.1% was cultivated in that same year (Skjermo *et al.*, 2014). However, different trends and uses of seaweed are increasing the number of seaweed farms in countries like Norway, England and Ireland, where the licenses applications are on the rise. Currently, cultivation and the use of biomass can be better controlled than in the past, with important factors being the harvest depth and time, and the effects of this on the amount of different compounds in specific species (Sharma *et al.*, 2018).

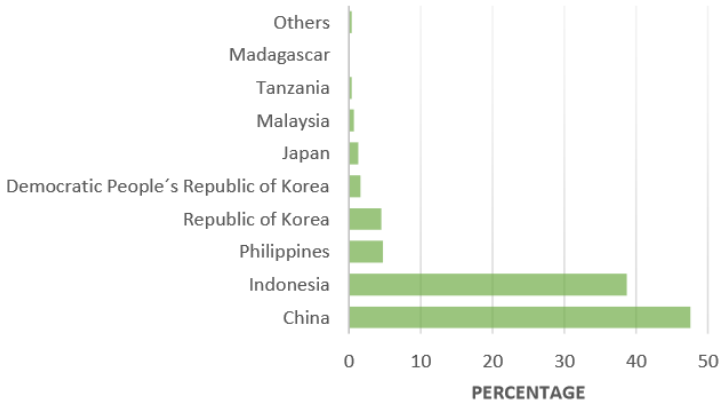


**Figure 3**: Major farmed seaweed producers in 2016. Source: FAO (2018).

Seaweed farming can contribute positively to the environment, e.g. providing oxygenation and uptake of nutrients (Vásquez *et al.*, 2014) and is cost-effective as food supply, animal feed and high-value biochemical products (bio-actives) (Buschmann *et al.*, 2017). In particular, demand for kelp (large, brown seaweeds) cultivation is expected to continue to rise (FAO, 2016), and seaweed aquaculture licenses for species such as *Alaria esculenta* and *Saccharina latissima* have recently increased significantly in Ireland (Department of Agriculture, Food and the Marine, 2020). However, some issues need to be challenged such as algal diseases, epibionts and grazers, which can considerably reduce the productivity and quality of derived products (Kuschel & Buschmann, 1991; Fletcher, 1995; Neill *et al.*, 2008; Cottier-Cook *et al.*, 2016).

## 1.2. *Alaria esculenta*

*Alaria esculenta* (**Fig. 4**), known as dabber lock, or Atlantic Wakame, is an edible common brown seaweed found in exposed shores in the Atlantic Ocean where the temperature does not exceed 16ºC. *Alaria* is found in northern Europe and North America (Irish Seaweed, 2018) in lower shores and rocky substrates. This species can grow over 2 m in length and is usually characterised by an upright stipe and a long blade along the midrib (Fredersdorf *et al.*, 2009) that can vary between individuals due to wave exposure. However, degeneration of most plants occurs progressively from spring to summer, when the sea temperature rises, giving way to epiphytes that degrade the individuals.

**Figure 4**: *Alaria esculenta*

### 1.2.1. *Alaria esculenta* epibiont fouling

A major challenge to the development and growth of the seaweed sector is the undesirable attachment of fouling organisms on kelp fronds (Walls *et al.*, 2017), which greatly increases the potential loss of biomass as a result of frond breakage. Also, a very high percentage (50-100%) of seaweed production is consumed by herbivores (Hay, 1991; Cyr & Pace, 1993), and arising tissue loss due to weakening and deterioration of the thallus (Viejo & Åberg, 2003; Toth & Pavia, 2006). Fouling organisms can affect the nutrient uptake by the seaweed (Hurd *et al.*, 2000), diminish photosynthesis by blocking the surface of the seaweed

fronds (Hepburn *et al.*, 2006), giving rise to pathogenic infections through grazing wounds (Schmitz & Lobban, 1976; Honkanen & Jormalainen, 2002).

Both abiotic and biotic factors are known to influence the occurrence and degree of fouling on the seaweed (Vairappan, 2006; Peteiro & Freire, 2013b). Sousa (2001) found that abiotic disturbances, mainly in the form of wave energy, counteract competitive exclusion, thereby enhancing species richness. Generally, kelp species are deployed in Autumn/Winter and harvested in Spring/early Summer, to match the natural wild kelp growing season, corresponding with the increase of temperature and light, which also bolster the growth of epibiotic organisms (Walls *et al.*, 2017). Market wise, heavily fouled fronds have a lower value as they are considered to be unsuitable for human consumption due to modification of taste, used instead for animal feed (Bruton *et al.*, 2009).

Epibiotism has generally been considered harmful to seaweeds due to the light and nutrients competition with the host, thereby decreasing the species growth and reproduction (D'Antonio, 1985; Cebrian *et al.*, 1999; Honkanen & Jormalainen, 2005). Furthermore, epibiota on the host algae may modify its susceptibility to herbivores, attracting grazers to the host macroalga (Bernstein & Jung 1979; Wahl & Hay, 1995; Wahl *et al.*, 1997). Lüder & Clayton (2004) found that small scale accumulation of phlorotannin vesicles occurs within a few days following wounding, indicating that phlorotannins have anti-herbivore activity. Macroalgae commonly contain metabolites with antifouling properties, but it is not known whether the compounds have an antifouling role during real ecological interactions with their natural enemies (Steinberg *et al.*, 2001).

### 1.2.2. *Alaria esculenta* chemical composition

The kelp *Alaria esculenta* is an economically valuable seaweed which is mainly cultivated for human consumption as it is rich in sugars, vitamins and proteins (Kraan *et al.*, 2000). Seaweeds are natural sources of iodine, an essential element that is primarily associated with thyroid hormone production, which is important for human health and metabolism. However, iodine ingestion at levels above the Reference Daily Intake (RDI) can also have a negative effect on human health (Leung & Braverman, 2014). Despite having less iodine content than the kelp *Saccharina latissima* , Roleda *et al.* (2018) found that 283 mg of dried *A. esculenta* meets the required levels of daily consumption for most healthy people. The composition of vitamins and minerals makes *A. esculenta* a nutritionally important food source. However, the composition of vitamins and minerals varies significantly between seasons,

influenced by both biotic and abiotic factors (Vairappan, 2006; Peteiro & Freire 2013b). **Table 1** presents the annual range of nutrients found in *A. esculenta*. Other uses of the species include biochemical extracts used in cosmetic products, as fertilizer, and the production of alginates as it contains up to 42% alginic acid (Kraan & Guiry, 2001). Seaweeds, including *A. esculenta*, can also provide the micronutrients needed for microbial production, such as those seen in fermentation (Schiener *et al.*, 2015).

In general, protein content in brown seaweeds is typically lower (9-20% of the dry weight) when compared to red and green seaweed (Fleurence, 1999), and have a higher ash content (around 30%) compared to terrestrial plants (1-10%; Grohmann & Bothast, 1994; Lynd, 1996).

**Table 1:** *Alaria esculenta's* nutritional information (Morrissey *et al.*, 2001).

| | |
|---|---|
| **Protein** | 9-20% |
| **Fat** | 1-2% |
| **Carbohydrates** | 46-51% |
| **Vitamin C** | 100-500 ppm |
| **β-Carotene** | 41 ppm |
| **Retinol** | 0.7-0.8 ppm |
| **Vitamin B1** | 5.5 ppm |
| **Vitamin B2** | 0.3-1 ppm |
| **Vitamin B3** | 5 ppm |
| **Vitamin B6** | 62 ppm |
| **Vitamin B12** | 50 ppb |
| **Calcium** | 11,670-12,900 ppm |
| **Iodine** | 165-275 ppm |
| **Iron** | 50-126 ppm |
| **Magnesium** | 8,960 ppm |
| **Manganese** | 1-14ppm |
| **Sodium** | 4-6% |
| **Copper** | 6.8% |
| **Potassium** | 7.4% |

## 1.3. Bioactive Compounds

### 1.3.1. Fucoxanthin

#### General description

Fucoxanthin is a xanthophyll and shares chemical and physical properties with carotenes, such as lipophilicity and antioxidant activity due to their ability to quench reactive oxygen and nitrogen species (Shannon & Abu-Ghannan, 2016). The presence of an unusual allenic bond and 5,6 monoepoxide in its molecule make it a unique structure (Maeda *et al.*, 2008). As determined by Holdt & Kraan (2011), fucoxanthin is one of the most abundant carotenoids in nature accounting for up to 10% of total carotenoid production. It is responsible for the brown to yellow colour of brown macroalgae, where it masks the green color of chlorophyll *a* and *c* (Hurd *et al.*, 2014; Peng *et al.*, 2011). Fucoxanthin is found in all brown macroalgae (Phaeophyceae) (Shannon & Abu-Ghannam, 2016) and also in microalgal diatoms. The pigment is primarily produced in the blade of seaweeds, where most of the photosynthesis occurs due to maximum light exposure at the ocean surface, resulting in a significantly higher level of fucoxanthin compared to the stipe and holdfast (Lobban & Wynne, 1981).

Fucoxanthin can be found in two different configurations: *cis* and *trans*, where *trans* (**Fig. 5**) comprises 90% of all isomers found in nature. The *trans* isomer is more chemically stable and a more potent antioxidant than the *cis* form (Holdt & Kraan, 2011; Nakazawa *et al.*, 2009). Unlike carotenes, fucoxanthin polarity is partly explained by the presence of oxygen in the hydroxyl and epoxide groups contained by xanthophylls (Landrum, 2009).

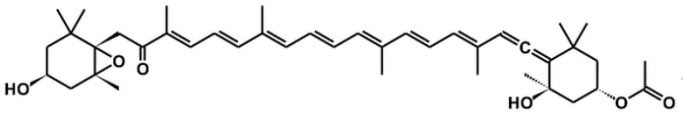


**Figure 5**: Fucoxanthin *trans* chemical structure configuration.

As a xanthophyll, fucoxanthin behaves like violaxanthin, neoxanthin and lutein found in terrestrial plants (Kotate-Nara & Nagao, 2011). The pigment is found between the thylakoids, a membrane-bound compartment present in the chloroplasts of the Chromalveolata eukaryotic algal cells (Phylum Ochrophyta) as well as in microalgal diatoms (Bacillariophyceae) (Cavalier-Smith & Chao, 2006). Fucoxanthin binds to apoproteins and chlorophylls *a* and *c* to form complexes, aiming to absorb the light in the blue-green regions to

provide energy to the algae. Moreover, fucoxanthin absorbs light from 449 to 540 nm, a broader absorption band than chlorophyll *a* and *c* alone, which makes it more effective regarding photosynthesis (Kim *et al.*, 2010; Pyszniak & Gibbs 1992). Fucoxanthin also protects algal cells from damage caused by reactive oxygen species due to excessive oxygen and light penetration in the ocean (Galasso *et al.*, 2017). Exposure to sunlight, the seasonal variation, geographic location, nutrient availability, ontogenetic effects and the extraction methods also influences the amount of the compound in the species (Fung *et al.*, 2013; Gosch *et al.*, 2015; Terasaki *et al.*, 2016).

The content of fucoxanthin among macro- and microalgae varies widely. Many diatoms and other microalgae have a higher fucoxanthin content than brown seaweeds (Kawee-ai *et al.*, 2013), but are less widely used commercially for extraction due to the need for photobioreactors and strict cultivation conditions (Xia *et al.*, 2013). Some studies have reported fucoxanthin in eight species of diatoms, with values ranging from 2.24 mg/g (dry weight; dw) in *Chaetoceros gracilis* to 18.47 mg/g (dw) in *Odontella aurita* and to 26.1 mg/g (dw) in *Phaeodactylum tricornutum* (Kim *et al.*, 2012; Derwenskus *et al.*, 2019). In macroalgae, Mori *et al.* (2004) reported 2.56 mg/g (dw) of fucoxanthin in the macroalgae *Undaria pinnatifida*, much higher than those reported in *Fucus serratus* (0.751mg/g dw) and *Alaria esculenta* (0.870 mg/g dw) (Shannon & Ghannam, 2016).

## Applications

Carotenoids are used as additives in poultry and aquaculture industries due to its modulatory effect on specific gene and protein expression in biological systems (Myashita, *et al.*, 2011). Fucoxanthin has already been incorporated as an ingredient in products such as pasta, biscuits, and dips by several food companies worldwide (Prabhasankar *et al.*, 2009; Oryza, 2015). Although its presence and isolation in seaweed was realised over a hundred years ago, this pigment has been underutilised in food and few pharmaceutical applications are found (Abu-Ghannam, 2017). Fucoxanthin is insoluble in water and the incorporation of it into water-based beverages or sauces would require emulsification or dispersion using appropriate colloids (Socaciu, 2007). Additionally, due to its unstable chemical structure, low or high pH, UV light, and long storage periods can degrade the pure form due to oxidation (Kawee-ai *et al.*, 2013; Mise *et al.*, 2011). Its organoleptic characteristics, such as texture, taste, appearance, and smell deteriorates because of chemical and/or enzymatic reactions with other ingredients (Shannon & Abu Ghannan, 2018).

Epidemiological results have suggested that the regular consumption of seaweeds can prevent pathologies linked to oxidative stress, thanks to the strong antioxidant properties of the compound (Okuzumi *et al.*, 1993, Yan *et al.*, 1999). Woo *et al.* (2010) reported improvement in plasma and liquid hepatic profiles when adding 0.05% of fucoxanthin supplement in mice diets and as stated by Takahashi *et al.* (2015), fucoxanthin and fucoxanthinol (a fucoxanthin derivative) have strong effects against colorectal cancer cell lines. A daily dosage ranging from 0.5 to 1.0 mg of pure fucoxanthin extract was found to have a significant effect on blood serum parameters related to metabolic syndrome (Oryza, 2015). Along with other carotenoids such as astaxanthin, fucoxanthin is a more powerful antioxidant than many other natural or synthetic antioxidants with stronger oxyradical scavenging activities than ascorbic acid, gallic acid, lutein or other synthetic antioxidant compounds such as BHT or BHA (Miyashita & Hosokawa, 2007). A dietary combination of fucoxanthin isolated from brown seaweeds or diatoms with edible oils could increase the absorption rate of fucoxanthin and has potential as a marine natural product. Heo & Jeon (2009) reported an 81.47% cell survival rate when cells were exposed to UV-B for cells pre-treated with fucoxanthin at a concentration of 100µM, indicating that the natural compound has protective effects against UV-B induced cell damage.

## Economic value

The potential application and commercial production of fucoxanthin is increasing due to its antioxidant properties. As mentioned before, seaweed fucoxanthin content varies seasonally; consequently, its nutritional efficacy concerning health relies on when the seaweed is harvested. Analytical grade fucoxanthin (≥ 95% pure) is produced for laboratories and retails at €681 euros per 50 mg (Sigma-Aldrich, 2020). Fucoxanthin production in 2015 reached 500 tonnes with an expected increase of at least 5.3% per annum between 2016 and 2021 (Joel, 2016). The total value of the carotenoid market rose from US$1.20 billion in 2010 to US$1.50 billion in 2014 (Ulrich, 2015). Moreover, the wholesale market price of fucoxanthin was estimated at US$ 2000 per gram and like many natural food extracts, is widely available wholesale online from many companies, primarily based in China. It is sold in the form of dried seaweed extract, generally from wakame or kombu with stated percentages of fucoxanthin purity ranging from 10% to 98%. Price ranges vary widely as do claims of purity and certification (Kyndt & D´Silva 2013).

As a supplement, fucoxanthin has a high cost due to the energy required for the extraction and seaweed freeze-drying process (Billakinti *et al.*, 2013). Despite its potential, fucoxanthin remains understudied for applications in nutraceutical, cosmetic and feed/food

industries (Shannon & Abu-Ghannam, 2016), which could be explained by the costly and inefficient extraction methods.

### 1.3.2. Phlorotannins

#### General description

Phlorotannins are a group of phenolic compounds restricted to polymers of phloroglucinol (**Fig. 6**) that have been identified in several brown seaweed families such as Alariaceae, Fucaceae, and Saargassaceae (Wang *et al.*, 2009). Concerning their chemical properties and physiological roles, algal phlorotannins are like those present in vascular plants; however, some studies have shown that phlorotannins are the only phenolic group found in brown algae (Jormalainen & Honkanen, 2004; Koivikko *et al.*, 2007). Its structure may additionally contain halogens in replacement of some hydroxyl groups.

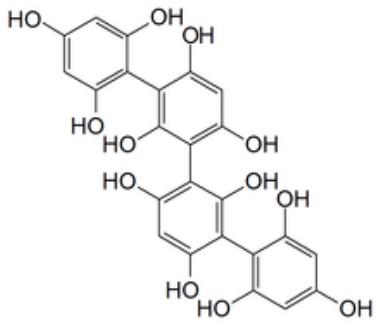


**Figure 6**: Phlorotannins chemical structure configuration.

Phlorotannins are highly hydrophilic compounds with a wide range of molecular sizes, between 126 and 650 kDa (Ragan & Glombitzka, 1986). Their unique molecular skeleton (Ahn *et al.*, 2007) with up to eight interconnected aromatic rings makes these compounds more potent free radical scavengers than polyphenols derived from terrestrial plants, including green tea catechins, which only have three to four rings (Hemat, 2007). Phenol rings act as electron traps to scavenge peroxy, superoxide anions and hydroxyl radicals (Sathya *et al.*, 2017).

Algal phlorotannins are stored in membrane-bound vesicles called physodes and when those fuse with the cell walls they form a complex with alginic acid present in the cell wall. Higher levels of physodes are found in the meristematic and cortical tissues and spores, gametes and zygotes from brown seaweed. As a defence mechanism, macroalgae produce phlorotannins that can be distasteful to potential predators. Phlorotannins are considered part

of the defence system of algae against herbivores, amphipods, gastropods and isopods (Targett & Arnold, 1998) and indeed, phlorotannin concentration varies with the presence of grazers. Moreover, geographic location, stage, size, age and reproductive status of the algal dictate their phlorotannins content. Also, environmental factors such as salinity, nutrient level, light availability, ultraviolet radiation are key factors.

This secondary metabolite also plays an important role in brown algal cell wall biosynthesis (Ragan & Glombitzka, 1986; Amsler & Fairhead, 2006). *Alaria esculenta* may produce phlorotannins and oxidized lipids as protective functions against high photosynthetically active and UV radiation. Accumulation of polyphenols starts with the onset of the growth phase and declined towards Autumn months as studied by Schiener *et al*. (2015). Inthe species *Ascophyllum nodosum*, Parys *et al.* (2009) showed higher concentrations of polyphenols in summer when compared with winter. As the day length increases, phlorotannins levels also rise in response to increased photosynthesis as reported by Fairhead *et al.* (2006).

## Applications

Brown macroalgae are rich in phenolic content, varying from 1% to 14% of dry macroalgae biomass. Phlorotannins, in term of structure and degree of polymerization, are an extremely heterogeneous group of phenolic compounds with a wide range of biological activities (Holdt & Kraan, 2011). Reported pharmacological activities of phlorotannins include antioxidant, anti-HIV, antidiabetic, anticancer, anti-inflammatory and enzyme inhibitory activities (Singh & Sidana, 2013). Some polyphenols are well established and are administered as supplements or with food, to improve health status (Scalbert *et al.*, 2005). Furthermore, polyphenols are the most abundant antioxidants in our diet, with the total dietary intake as high as 1g/d, which is much higher than of all other classes of phytochemicals and known dietary antioxidants.

The difficulty of separating and identifying the different phlorotannins species due to the low efficiency of current extraction processes makes the scaled-up production of this high-value bio-active on an industrial scale very difficult. Phenolic compounds and phlorotannins are easily soluble in solvents less polar than water (i.e. methanol, ethanol, acetone or aqueous mixtures of these) (Catarino *et al.*, 2019). While the compound is easily extracted, a variety of factors can complicate the efficiency of the extraction and potentially affect its bioactivity. Phlorotannins form complexes readily with alginates and proteins, chelate metal ions and oxidize easily. Thus, since free phlorotannins are not stable, a stabilization step is required to avoid oxidation and polymerisation (Singh & Sidana, 2013).

### Economic value

As reported by Grand View Research Incorporation (2020) the global market size of polyphenols is projected to hit USD 2.08 billion by 2025 with Asia leading the global market, followed by North America. BioPure Ltd. supplies capsules of pure polyphenols and phlorotannins (98.8% pure extracts) from brown seaweed under the name "PC-Ecklonia cava" at a price of US$51 per 300mg (BioPure, 2020). Supersmart also sells dietary supplements made with extracts of the same species, *Ecklonia cava*, under the patent SEANOL-F® containing at least 15% total polyphenolic compounds, including phlorotannins, at €71.29 for 400mg (Supersmart, 2020).

# 2. Objectives of the work

The main objective of this work was to characterise, compare and test the antioxidant activity of two highly bioactive compounds/group of compounds, fucoxanthin and phlorotannins, from the species *Alaria esculenta* cultivated on long-lines. The effect of the cultivation methods implemented on the yields of the bioactives as well as their antioxidant activity was used to identify best dates for the initial deployment of the long-lines as well as harvesting of the cultivated seaweed. It also determines the effect of initial pre-processing methods by comparing freeze-drying and oven drying on the yields of the bioactives and their antioxidant activity.

# 3. Material and methods

## 3.2. *Alaria esculenta* seed

*Alaria esculenta* mass cultivation was based on the manual "Cultivation of the brown seaweed *Alaria esculenta*" written by Arbona & Molla, (2006) and carried out by Freddie O´Mahony from Carton Point Shellfish Ltd. The "free-living technique" used involves fertilisation inhibited strains and is an adaptation of the Korean method used for other species, such as *Laminaria japonica* and *Undaria pinnatifida.* With this method, fertilisation can be induced at any moment just by changing temperature and light regimes.

***Preparation of the mature sporophylls:***

*Alaria esculenta* mature sporophylls were collected in spring 2017 from a natural bed in Gearhies Bantry (Ireland; 51°38'49.8"N 9°34'49.5"W). A total of 40 sporophylls were collected from 20 mature individuals, cleaned and placed in a 2L beaker. The temperature was kept at 10ºC and after 18-20 hours, 1.5 litres of distilled water was added into the beaker and shaken. Sporulation started just a few minutes after the water was added and the obtained solution was filtered through different filter sizes (250µm and 60µm). 350 mL of the obtained solution was inoculated in a Pyrex flask containing filtered seawater and growth media. A 1ºC increment per day was performed until the temperature reached 14ºC. Light intensity was set between 27 and 34 µmol/$m^2$ s in a 24-hour period with air passed through a membrane air filter (0.2µm) to aerate the culture. The culture was kept at 14ºC until the fertilisation induction took place.

## 3.3. *Alaria esculenta* cultivation

Temperature manipulation, irradiance and photoperiod are the factors responsible for fertilisation induction. Parameters used are shown in **Table 2**.

**Table 2:** *Alaria esculenta* fertilisation induction parameters

| Temperature | Irradiance | Photoperiod | Duration |
|---|---|---|---|
| 10ºC [a] | 34 µmol/$m^2$ s | 12:12 | 8 days |

[a] Temperature and photoperiod were gradually decreased from the initial 14ºC and 24-hour period.

***Nursery culture of plantets indoors:***

Collectors were made of a string used as a substrate for zygote settlement. The collector consisted of PVC bars 50 cm long. The string was 1mm in diameter made of polyamide and coiled around the collector. The solution was blended by stirring for 25 seconds to allow for a

better settlement on the collector and sprayed on the string. A total of 35 metres of settlement string allows for around 30 linear metres on the culture rope at sea. The collectors were then placed in 500 litres white fibre glass tanks for approximately 24 days with artificial fluorescent daylight with a light intensity of 40 µmol/$m^2$ s before being transferred to the sea.

***The culture at sea:***

The culture rope was prepared, and a collector was inserted. The gentle motion of the boat backwards and forward allowed for the automatic coiling of the collector around the culture rope, uncoiling the seeded string around th culture ropes at sea. Buoys were placed at several points along the rope to keep the rope at 1 to 1.5 metres below the surface.

## 3.4. *Alaria esculenta* sampling

Seaweed lines were seeded in Bantry Bay, West Cork, Ireland between October and December 2017. A total of 11 lines were deployed, three of which were used for repeated sampling. The deployed lines were 110 meters long and hung to a maximum depth of 3 meters (ideally between 1 to 1.5 meters). These lines ran from WSW to ESE as shown in **Figure 7**. The seaweed seed used on the long lines was from Carton Point Shellfish funded by BIM operating from the BMRS hatchery in Gearhies. The sampled long lines were No. 2 (deployed on the 25th of October), No. 4 (deployed on the 1st of November) and No. 8 (deployed on the 9th of December). **Figure 8** shows a scheme of the seaweed farm with the lines of interest and the sampling points along the lines. Sampling took place at five separate times: March 29th, April 19th, April 24th, May 5th and May 28th, 2018, and were collected from three different points across the rope, and a total of 10 cm sampled per point.

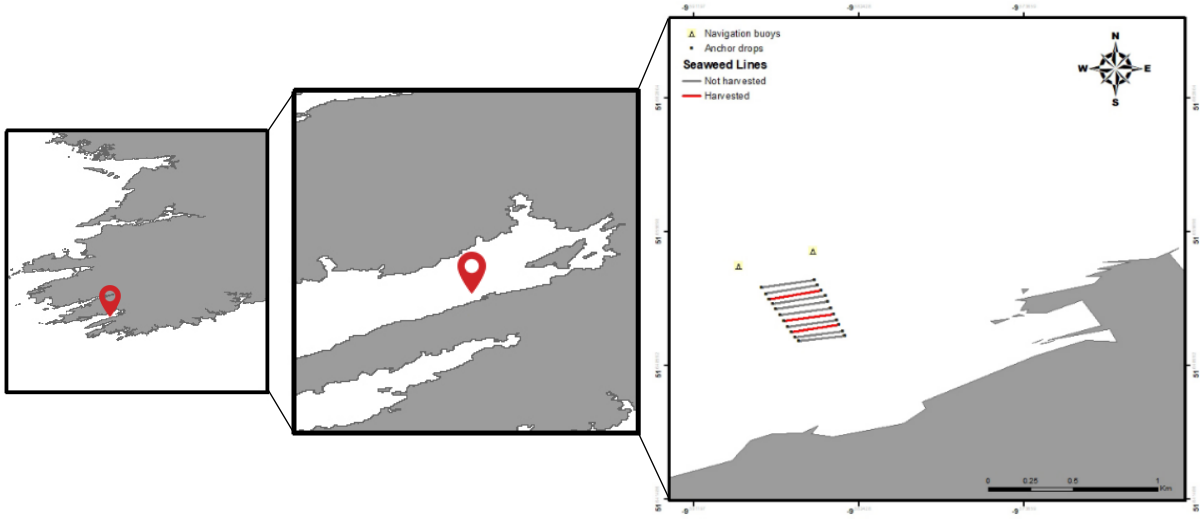


**Figure 7**: Map of *Alaria esculenta* seaweed farm located in Bantry bay.

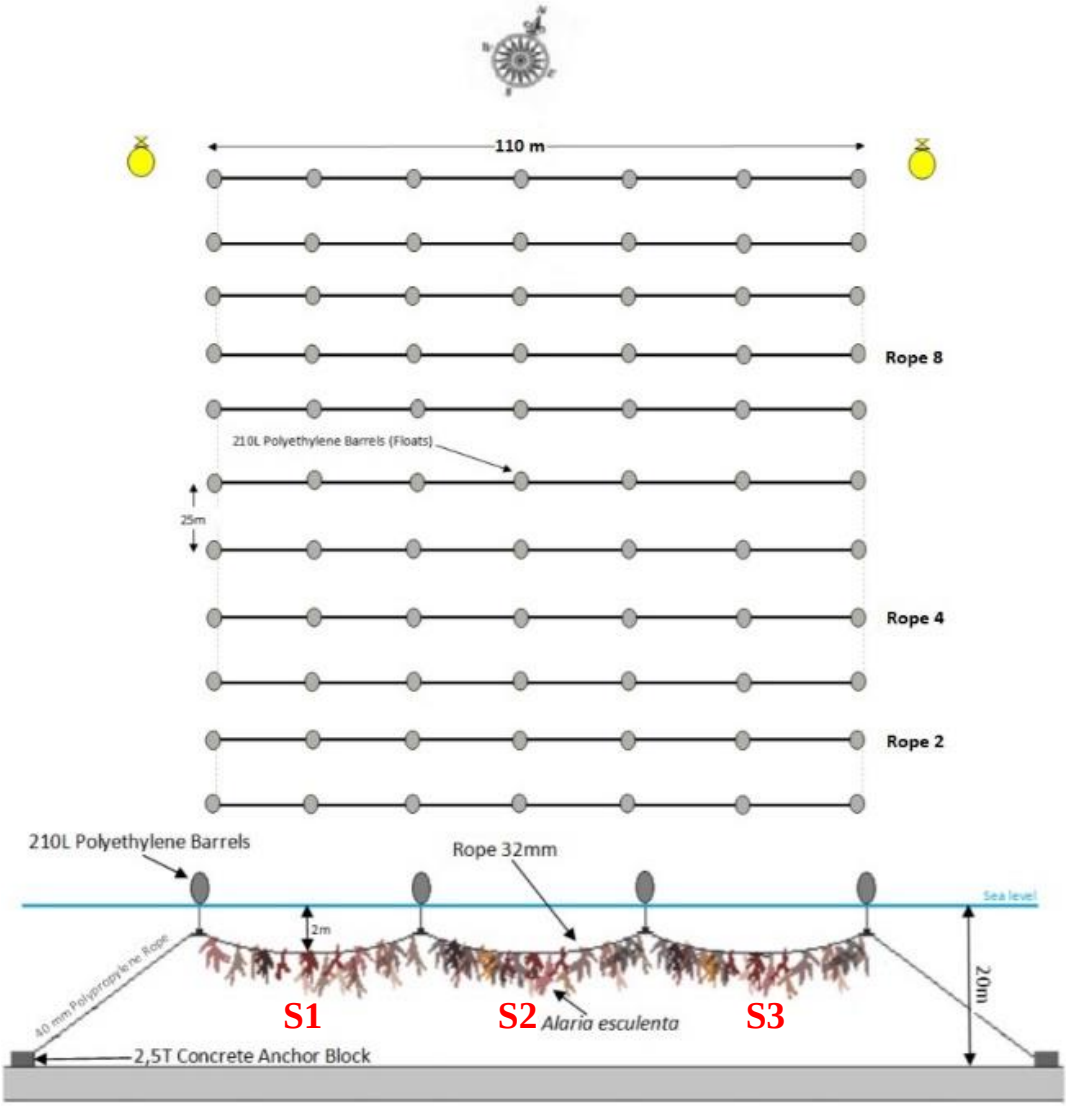


**Figure 8**: *Alaria esculenta* ropes scheme.

Samples (n=45) were hand-cut from the long lines, placed in labelled plastic bags and brought to the lab at ambient temperature. In the laboratory, samples were shaken 20 times with the aid of a sieve to get rid of all surface water. The samples were weighed, and 7 individuals were randomly chosen for length measurement with a measuring tape and placed back with the rest of the seaweed. The samples were then stored at -80ºC until the drying procedure. Exposure to light was avoided and all processing took place in a low light environment. For drying, the samples were divided in half, one for freeze-drying and one for oven drying.

## 3.5. Drying procedures

Kelp species are characterized by high moisture content and rapid microbial decomposition, once harvested (Enríquez *et al.*, 1993). Dried biomass of *A. esculenta* is reported to range from 8-20% weight with a maximum observed at wintertime and minimum towards summer (Adams *et al.*, 2011b). The effect of preservation treatments (including drying) on the quality parameters of seaweed biomass is a major issue which has been studied only partially.Previous studies have shown that many of the nutritionally important molecules in seaweed have therapeutic benefits (Ganesan *et al.*, 2018; Holdt & Kraan, 2011; Mohamed *et al.*, 2012). However, some of the compounds are heat-labile and may be lost or depleted when the seaweed is processed, meaning that some important chemical components might be lost (Sappati & Nayak, 2018; Chan *et al.*, 1997).

Drying can have severe consequences to the chemical profile of the seaweeds. The disintegration of the vegetal matrix because of the drying method used results in greater or lesser exposure of antioxidant compounds to oxidation reactions decreasing the commercial value of the seaweed (Nguyen *et al.*, 2016). Hence, two drying methods were tested: freeze-drying and oven drying.

### 3.5.1. Freeze-drying

Seaweed was weighed before freeze-drying it. A 2-minute wash with sterilised water was carried out to get rid of the salts that could affect subsequent UHPLC analysis. Seaweed was chopped and freeze-dried (LABCONCO FreeZone 7754030) for 36 hours. These samples were washed with sterilised water to get rid of the salts and placed for further 36 hours in the freeze dryer. The resulting dry weight of the material was taken. To avoid degradation of the bioactive compounds by light exposure, the samples were wrapped in aluminium foil, vacuum-packed, labelled and stored at -20ºC.

### 3.5.2. Oven-drying

Seaweed was weighed before placing it inside the oven ( Memmert IN 55). A 2-minute wash with sterilised water was carried out to get rid of the salts that could affect subsequent UHPLC analysis. The samples were chopped and placed in trays. A three-day oven drying method was carried out; the temperature was first set at 30ºC for 24 hours and then these samples were washed with sterilised water again and placed in the oven for further 42 hours at

40ºC. The oven fan was set at 30%. The dried material was weighed, wrapped in aluminium foil, vacuum-packed, labelled and placed inside the freezer at -20ºC until extraction.

## 3.6. High-value bioactive compounds extraction

### 3.6.1. Fucoxanthin extraction

The protocol used was first described by Wright *et al.* 1991, modified by Bidigare *et al.* (2005), and improved by Schmid & Stengel (2015) and Shannon & Abu-Ghannam (2016). The fucoxanthin extraction protocol for this work is based on the Shannon & Abu-Ghannan method as described in their 2016 paper.

2 g of dried seaweed was weighed on an analytical scale (OHAUS Pioneer). The seaweed was then ground into a powder for more efficient solvent extraction. The prepared seaweed was placed for 1 hour at 30ºC in an orbital shaker at 100 rpm (Mini orbital SO5) with acetone (20mL, >99.5%) in the dark. The flasks were covered by aluminium foil to avoid any light degradation of the pigment.

The content of the flask was transferred to falcon tubes and centrifuged (Eppendorf 5702R) at 12000 rpm for 10 minutes at 4ºC. The supernatant was collected, and the remaining seaweed biomass was washed another 3 times with 20 mL of acetone. The supernatants were pooled, and a total of 80 mL was concentrated to 5mL in a rotary evaporator (Stuart RE300DB) at 30ºC under vacuum with the help of a mini vacuum pump (KNF Labopor). Extracts were stored at -80ºC freezer for subsequent characterisation.

### 3.6.2. Phlorotannins extraction

Around 15 g of dried seaweed was placed in 250 mL flasks covered by aluminium foil, and 200 mL of solvent methanol/water (v/v) was added. The flasks were placed in a magnetic stirrer (Bibby HC502) for 90 minutes at 160 rpm at 35ºC, followed by another 90 minutes in a rotary shaker. Samples were kept in the dark during the whole process. Flasks were carefully stoppered to avoid evaporation. The samples were filtered through a Buchner ceramic funnel with filter paper Grade 1 (Whatman Dia. 70 mm). The residue was discarded, and the solvent was concentrated to 40 mL in a rotary evaporator at 40ºC under vacuum.

The extract was purified in a separating funnel by two consecutive additions of 40 mL of hexane and by two consecutive additions of 40 mL of dichloromethane. Each time the organic phase was discarded, and the aqueous phase maintained at -20ºC for phlorotannins content analysis.

## 3.7. High-value bioactive compounds characterization

The solvents used were all HPLC grade and supplied by Honeywell. The fucoxanthin analytical standard (Sigma-Aldrich), chemicals and material for the analysis were purchased from Reagecon Diagnostics Ltd. All samples were analysed in triplicate.

A method for fucoxanthin was established in a Thermo Fisher Ultimate 3000 UHPLC equipped with a Dionex UltiMate 3000 Rs Pump, an UltiMate 3000 Rs Auto-sampler, an UltiMate 3000 Rs Column Compartment and a charged aerosol detector (CoronaVeo Rs). The UHPLC is controlled through Chromaleon 7 software.

### 3.7.1. Fucoxanthin

The analytical standard (>99% purity established by HPLC) was prepared in MilliQ® water at 1mg/mL. The UHPLC parameters for the analysis of fucoxanthin content from *Alaria esculenta* were optimized and are shown in **Table 3**. The gradient program was as follows: 0-5 min, 10% B; 5-10 min, change to 95% B; 10-15 min, 95% B.

**Table 3:** UHPLC Parameters for the Analysis of Fucoxanthin Content

| Parameter | Conditions |
|---|---|
| **Mobile Phase A** | 95% MilliQ® water, 4.6% acetonitrile, 0.4% formic acid |
| **Mobile Phase B** | 95% acetonitrile, 4.6% MilliQ® water, 0.4% formic acid |
| **Flow rate** | 0.800 ml / min |
| **Injection volume** | 10 µl |
| **Column** | BDS Hyper Sil C18 |
| **Column temperature** | 35ºC |
| **Detector** | CAD (CoronaVeo Rs) |
| **Run time** | 15 min |

### 3.7.2. Phlorotannins

The phlorotannins analysis protocol by UHPLC is shown in **Table 4**. The gradient program was as follows: 0-5 min, 1% B; 5-15 min, change to 95% B; 15-25 min, 95% B.

**Table 4:** UHPLC Parameters for the Analysis of Phlorotannins Content

| Parameter | Conditions |
|---|---|
| **Mobile Phase A** | 95% MilliQ®water, 4.6% acetonitrile, 0.4% formic acid |
| **Mobile Phase B** | 95% acetonitrile, 4.6% MilliQ®water, 0.4% formic acid |
| **Flow rate** | 0.300 ml / min |
| **Injection volume** | 10 µl |
| **Column** | Acclaim ™ 120 C18 |
| **Column temperature** | 40ºC |
| **Detector** | CAD (CoronaVeo Rs) |
| **Run time** | 25 min |

## 3.8. Antioxidant activity

DPPH assay is one of the most extensively used methods to evaluate the antioxidant activity of plant extracts, foods and single compounds, thanks to its stability and ease of use (Krishnaiah *et al.*, 2011). The DPPH radical (1,1 – diphenyl-2-picrylhydrazyl) is a stable and commercially available organic nitrogen radical, which reacts with hydrogen/electron donor compounds and has a maximum UV-Vis absorption within the range of 515-520 nm (Chen *et al.*, 2012).

The ability of the extracts prepared for the fucoxanthin and phlorotannins analyses to scavenge the DPPH radical was analysed. A total of 22µl of the extract was used in 96-well plates with butylated hydroxytoluene (BHT) as the positive control with four replicates per sample. The negative control was prepared with methanol instead of the extract. A colour control was made containing only the extract and methanol. The antioxidant activity was calculated with the following equation:

$$Scavenging\ capacity\ (\%) = \left[100 - \left(\frac{100 \times A_{corrected}}{A_{Negative\ control}}\right)\right]$$

where $A_{corrected}$ is the absorbance of the sample corrected for the colour control.

DPPH , MeOH, and BHT HPLC grade were purchased from Reagecon. BHT was diluted in MeOH. Fresh stock solutions were prepared before the analyses and the DPPH was prepared in MeOH to a concentration of 120µM.

# 4. Statistical analysis

The statistical analysis was performed with R software, and the results expressed as mean±standard deviation (SD). Data were tested for normal distribution and variance homogeneity (e.g. parametricity of the data). Non-parametric Kruskal-Wallis and Wilcoxon paired tests were performed to assess significant differences between obtained values in those cases where parametricity of the data did not prevail.

# 5. Results & discussion

## 5.1. *Alaria esculenta* morphometrical analysis

To evaluate the biomass yield of each long-line and the impact of the deployment and harvesting dates, the morphometric parameters of *Alaria esculenta* were assessed.

### 5.1.1. Biomass and length

By 2014, kelp farming produced approximately 54,000 tons of seaweed (FAO, 2016), mainly in raceways and long-lines. Kraan & Guiry (2001) reported a production of the kelp species *Alaria esculenta* from 5-14 kg up to 45 kg wet weight per m rope, depending on the temperature and daylength, as these factors play an important role in the metabolism, reproduction and spatial dispersion of the species Fredersdorf *et al.* (2009).

By the end of March 2018, ropes No. 1, 9, and 10 were lost due to heavy storms, and rope No. 11 was displaced to another location on the seaweed farm. Consequently, lines No. 2 and No. 8 became the ends of the seaweed farm, making them more vulnerable to early spring currents. As observed during the samplings, the individuals on line No. 4 were the first to be degraded at the end of April, something that could be explained by the increase in temperature and the weakness of individuals due to tangling.

**Figure 9** shows that a clear positive linear relationship exists between the individual's length and algal biomass, revealing that the biomass increase can be explained by the individual's length increasing. In the same graph, specific values can be identified (surrounded by a red circle) showing high biomass with a relatively small length. This can be explained due to the fact that some seaweed individuals showed a growth in width, as wave exposure can affect blade growth (McLeod *et al.*, 2014). This was followed by significant degradation, inducing these individuals to lose a significant part of biomass at their edges (oldest parts of the macroalga), increasing their width while reducing their length.

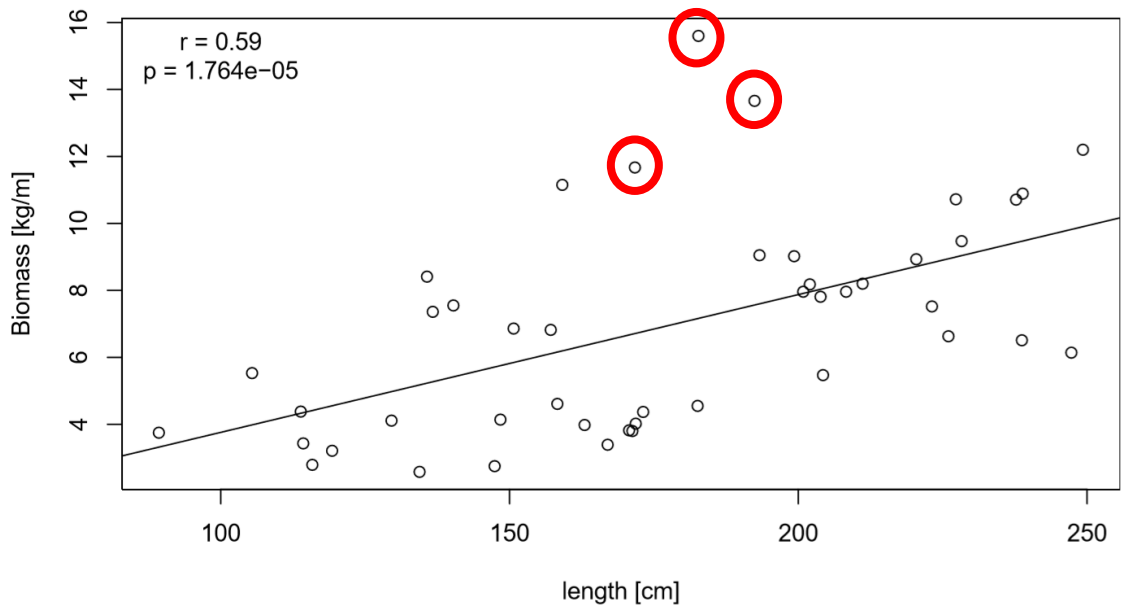


**Figure 9**: Graph representing the correlation between *Alaria esculenta* individual length and the biomass. The red circles show high biomass and short length individuals.

Biomass of *Alaria esculenta* from line No. 2 (deployed on the 25th of October 2017) remained unchanged at around 4 kg/m during early Spring (between March 29 and April 19 2017) but significantly increased in late Spring to values close to 9 kg/m in May. However, the amount of biomass along the rope showed high variability (6.82 – 15.6 kg/m), which accounts for the high standard deviation observed for this rope at the May 28 sampling date (**Fig. 10**).

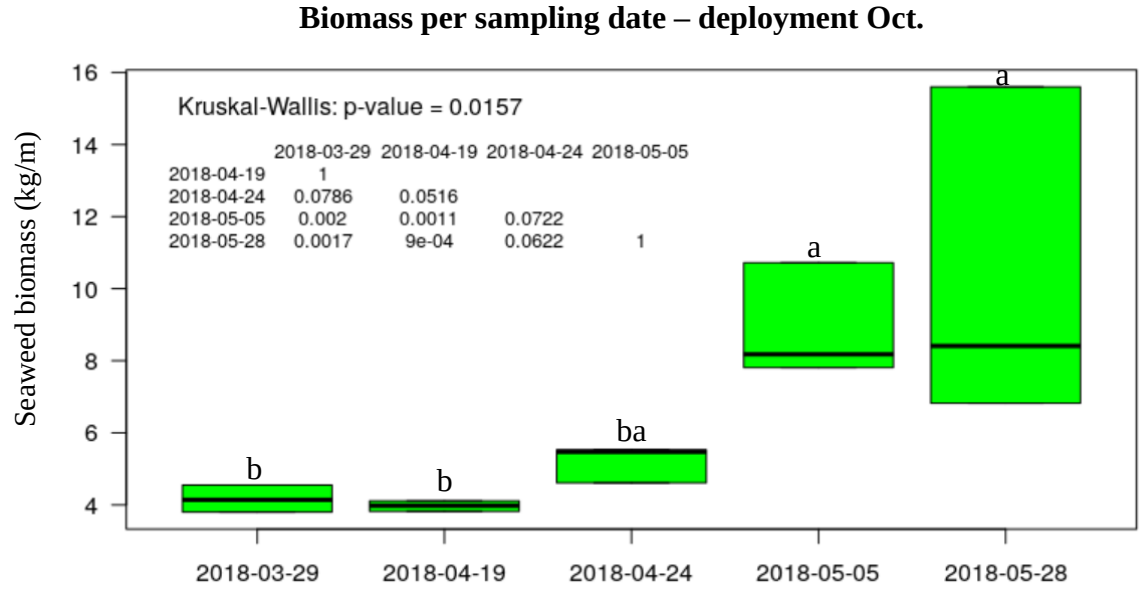


**Figure 10**: *Alaria esculenta* biomass (kg/m) along with the sampling dates on rope No. 2 (Deployed on the 25th of October). Different letters indicate significant differences between the samples taken on different dates (Kruskal-Wallis test, $p<0.05$).

**Figure 11** reflects seaweed biomass on rope No. 4 (deployed 1st November 2017). The seaweed biomass showed values of around 4 kg/m during early Spring – between March 29 and April 19. However, the results showed variability among the samples on April 19, ranging from 2.79 to 7.55 kg/m. Within the sampling dates, no significant differences were noted. There is nonetheless, a trend towards growing biomass from April 19 to April 24 (average values of 4.90 – 9.50 kg/m). The amount of biomass attached to the rope appears to fall on May 5, followed by an increase on May 28, with high sample variability (3.75-13.6 kg/m). This might be explained by the fact that, from March 24, rope No. 4 was found notably tangled with rope No. 5 due to adverse weather conditions, limitating therefore light penetration and absorbance of nutrients. Thus, this may have had a significant effect on the seaweed growth on this rope. *Alaria esculenta* is a winter species found frequently on exposed rocky shores so its growth is directly influenced by exposure to currents and waves.

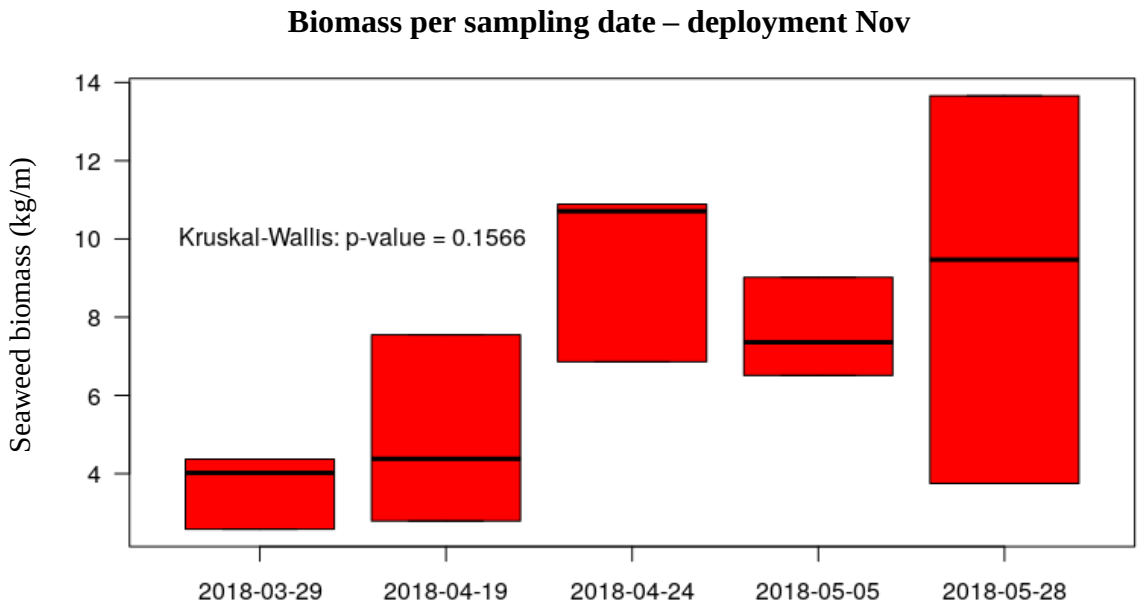


**Figure 11:** *Alaria esculenta* biomass along the sampling dates on rope No. 4 (Deployed on the 1st of November). Statistical analysis performed using the Kruskal-Wallis test.

Rope No. 8 (deployed on 9th December 2017) (**Fig. 12**) was similar to rope No. 4, and no significant differences were found in seaweed biomass from different sampling dates ($p >$

0.05). From March to May, however, an increasing trend in growth can be observed. May 28 had the lowest variability among samples, ranging from 9 to 11.8 kg/m.

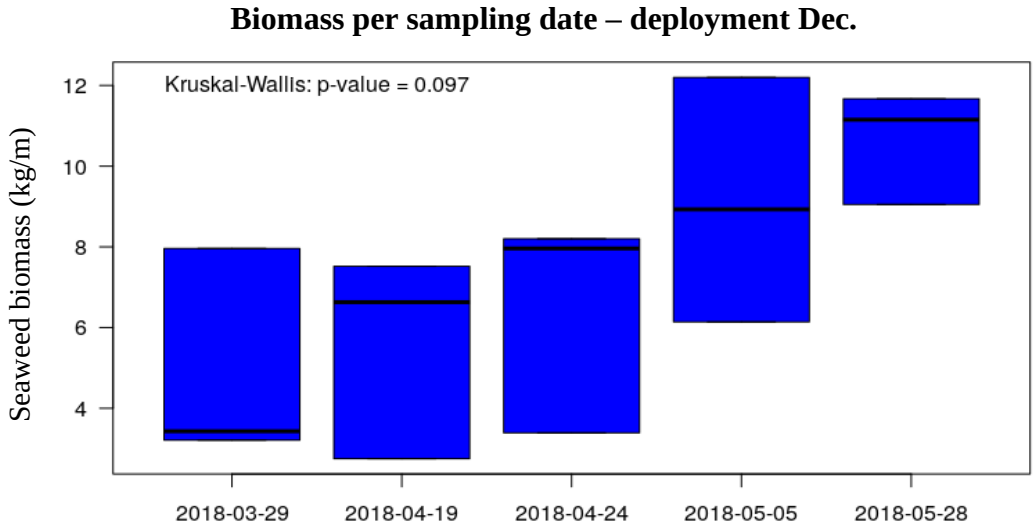


**Figure 12**: *Alaria esculenta* biomass along the sampling dates on rope No. 8 (Deployed on the 9th of December). Statistical analysis performed using the Kruskal-Wallis test.

**Figure 13** presents the amount of biomass collected from each rope at each of the sampling dates (averaged regardless of deployment date). Significant differences were observed, suggesting that the best harvesting date is the end of April rather than earlier (end of March/beginning of April) in order to get a higher biomass. Overall, the biomass increased from March 29 until the last sampling date in May (2.6 to 15.8 kg/m).

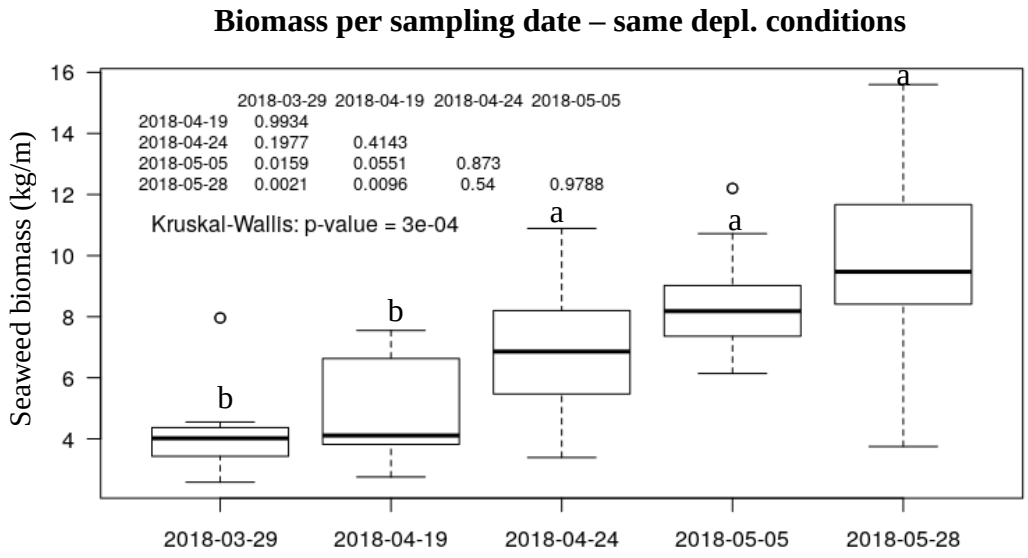


**Figure 13**: Average of *Alaria esculenta* biomass collected from each of the three ropes at the different sampling dates regardless of deployment date. Different letters indicate significant differences between the samples taken in different dates (Kruskal-Wallis test, $p<0.05$).

The strong variability among the triplicates indicates that the sampling method needs to be revised and adapted; collection of samples should probably be done in triplicate at each of the sample locations for the farmed species. When *Alaria esculenta* starts to degrade, the specimen begins to lose the end parts of the blades, and this may be the explanation for the reduction of biomass on rope No.4 from March 24 to May 5 (**Fig. 10**). This species, at that point, invests most of its energy resources increasing in width rather than length. From the first week of May, degradation in ropes No. 2 and No. 8 was observed, although not as much as was spotted on rope No. 4. The growth of seaweed along the long-line is likely to follow a different pattern, affected by the currents in the bay. On the other hand, the date of deployment does not appear to affect statiscally the production of biomass on the rope, but more research is needed.

The highest accumulation of biomass was observed from April 24 onwards, resulting in higher yields between late April and early May. Kelp species have high water content, which triggers in a fast degradation once harvested (Perez, 1968). Additionally, seaweed blades begin to be highly loaded with epiphytes as the sea temperature rises and daylength increases, which could also trigger a faster degradation. Moreover, if the seaweed is being farmed for food consumption, the presence of epiphytes may not look attractive, suggesting harvesting the seaweed for high quality products between April 24 and May 5.

**Table 5** summarizes the biomass and individual length for each of the three ropes (No. 2, No. 4 & No. 8), averaged from all the sampling dates (March 29, April 19, April 24, May 5 and May 28). The ropes followed a different pattern between each other and within line sampling points based on the results obtained in this study (**Table 5**). Results suggest that deployment date does not affect the overall biomass obtained on the lines, since no significant differences were observed between the biomass collected from each rope. However, on the line deployed in December (youngest seaweed), the individuals reached an average of 1.91±0.34 m in length, the longest of the study. This is probably related to the fact that they spent less time exposed to the storms occurring in Bantry Bay during Autumn months. Since a similar amount of biomass was obtained on all the ropes and that they rested at sea for different periods, we can say that the productivity of *Alaria esculenta* is higher when the ropes are deployed in December – same biomass in less time (rope deployed in December - 170 days; November - 208 days; and October - 215 days). An estimation of the total dry material obtained from each rope is also presented in **Table 5** (based on 90% moisture content), with values ranging from 71.7 to 80.3 kg per rope; higher in the rope deployed in December.

**Table 5:** *Alaria esculenta´s* biomass, individual´s length and productivity average by rope. Based on 90% MC.

| | **Rope 2 (depl. Oct.)** | **Rope 4 (depl. Nov.)** | **Rope 8 (depl. Dec.)** |
|---|---|---|---|
| Biomass ww (kg/m) (mean±sd) | 6.52 ± 2.89 | 6.94 ± 2.54 | 7.36 ± 2.45 |
| Biomass dw (kg/m) (mean±sd) | 0.65 ± 0.15 | 0.69 ± 0.13 | 0.73 ± 0.05 |
| Individuals length (m) (mean±sd) | 1.69 ± 0.36 | 1.71 ± 0.33 | 1.91 ± 0.34 |
| Productivity ww (kg/m d) | 0.030 | 0.033 | 0.043 |
| Productivity dw (g/m d) | 3 | 3.3 | 4.3 |
| ***Total estimated dw of Alaria esculenta per rope (Kg)*** | **71.7** | **75.9** | **80.3** |

**ww:** wet weight**; dw:** dry weight; **MC:** moisture content.

### 5.1.2. Effect of fouling species, epiphytes and grazers

The abundance of fouling species on *A. esculenta* fronds probably reflects seasonal factors influencing reproduction, dispersal and settlement (Hayward & Ryland, 2002; Park *et al.*, 2008; Park & Hwang, 2012; Førde *et al.*, 2016). The bryozoan *Membranipora membranacea* was the first observed epiphyte, significantly increasing its presence from 19$^{th}$ April (**Fig. 14a**) until the last sampling date, May 28 (**Fig. 14b**). Individuals of *Spirobranchus triqueter* and species from the Order *Amphipoda* were also detected. In mid-April, several individuals of lumpfish *Cyclopterus lumpus* (**Fig. 14c**) were attached to the macroalgae blades together with species from the Syngnathidae family (**Fig. 14d**). Blue rayed limpets or peacock's feathers (*Patella pellucida*) (Linnaeus, 1758), a grazer, was observed on the lines sampled at the end of April, matching the rise of the temperatures in the bay. McGrath (1992) found that the peacock's feathers attach to the seaweed blades to begin its life cycle when the seaweed accumulates high sugar levels and ends as the sugar content decreases, which may aid in future studies of seaweed sugars. Seaweed with high sugar content could also be attracting another species which could feed on the macroalga, however, further studies need to be carried out. As a result, the presence of epiphytes and grazers may affect the length of the individuals, and indeed the biomass. As observed on rope No. 4, from April 24 onwards, thepresence of epiphytes could trigger the weakening of the individuals and hence, the loss of the blade ends.


**Figure 14**: Fouling species observed in *Alaria esculenta*. **a:** *Membranipora membranacea* (observed on April 19); **b**: *Alaria esculenta's* blades fully covered by a bryozoan; **c:** Two individual's of *Cyclopterus lumpus* spotted on the seaweed blades; **d:** Individual of *Syngnathus spp*. (observed when sampling on April 19)

## 5.2. Moisture content using two different drying methods

The study showed that moisture analysis did not show significant differences ($p$ <0.05) when comparing the two methods of drying, freeze-drying and oven drying (**Fig. 15**). As a result, any of the drying methods can be used.

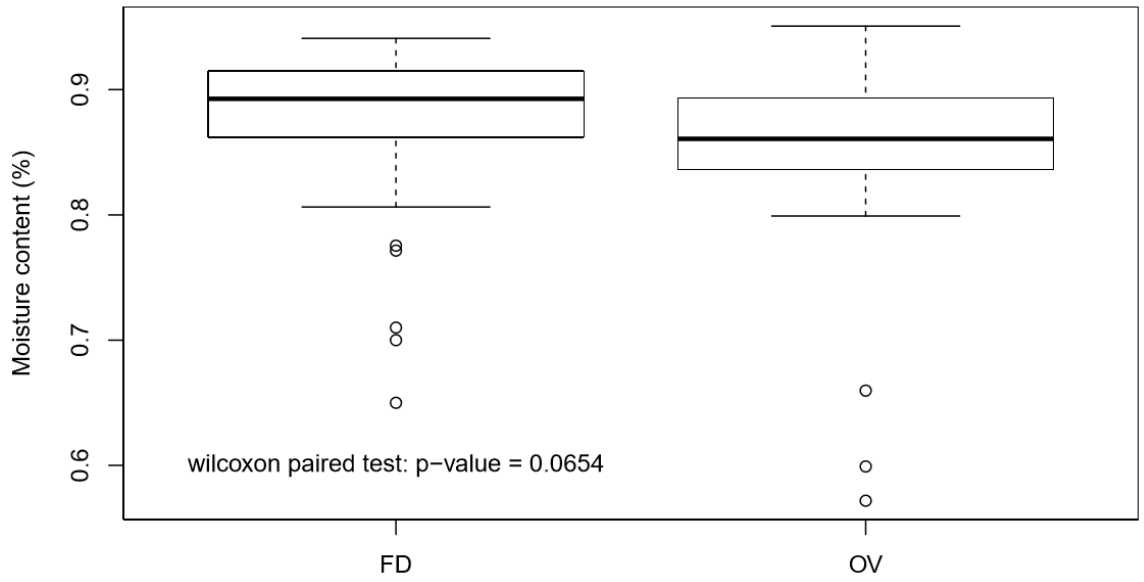


**Figure 15**: Box-plot representing the effect in moisture content of two drying methods, freeze drying (**FD**) and oven drying (**OV**) in *Alaria esculenta* samples. Statistical analysis were performed using Wilcoxon paired test.

## 5.3. Bio-active compounds quantification and the effect of the drying method in *Alaria esculenta*

### 5.3.1. Fucoxanthin content

Comparing the content of fucoxanthin on freeze-dried and oven-dried samples, significant differences were observed, with higher amounts of the compound being detected in the freeze-dried samples. The paired Wilcoxon test was conducted using the results from the three lines regardless of samplings dates (**Fig. 16**).

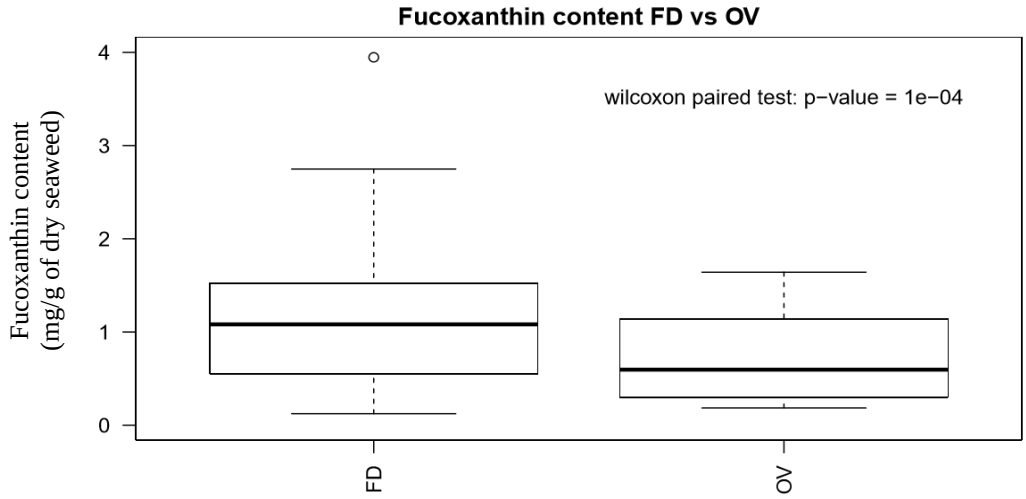


**Figure 16**: Box-plot representing fucoxanthin content in freeze-dried (FD) and oven dried (OV) samples. Statistical analysis performed using Wilcoxon paired test ($p<0.05$).

Shannon & Abu-Ghanam (2016) reported maximum levels of fucoxanthin, 0.870±0.030 mg/g (dm) in wild-harvested *Alaria esculenta*, with higher pigment concentrations during the mature sporophyte phase (from September to March) (Henley & Dunton, 1995; Fung *et al.*, 2013; Gosch *et al.*, 2015; Terasaki *et al.*, 2016). This study of farmed *A. esculenta* followed different trends as observed in **Figure 17** with fucoxanthin concentrations peaking at the end of April on rope No. 4, and reaching high values on May 5 on ropes No. 2 and 8. Although there were no significant differences when applying freeze drying, ropes No. 2 and No. 8 showed increasing fucoxanthin concentrations from March 29 to May 5 (1.76 mg/g and 2.02 mg/g respectively) followed by a decrease on May 28 (0.72 mg/g and 0.64 mg/g) **(Fig. 17 a** & **c**). Rope No. 4 shows a different trend when compared with the two other ropes in the experiment, reaching the highest value on April 24 (1.81 mg/g) (**Fig. 17b**).

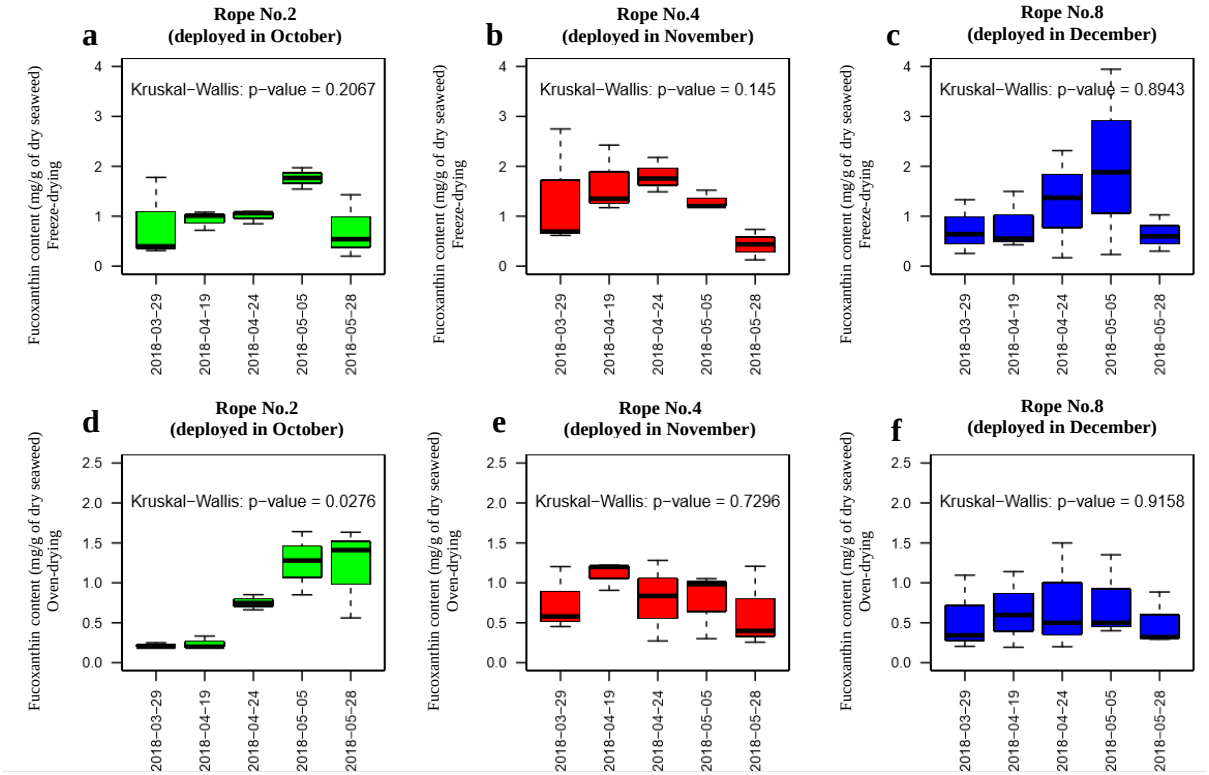


**Figure 17**: Box-plots representing the content of fucoxanthin (mg/g) in the different ropes analysed (Lines No. 2, 4 & 8) depending on the deployment month (October, November & December); **a, b & c**: variation of fucoxanthin content content using freeze-drying; **d, e & f**: variation of fucoxanthin content when using oven-drying. Statistical analysis performed using the Kruskal-Wallis test ($p$ <0.05).

Oven-dried samples from rope No. 2 were the only ones showing significant differences among the different sampling dates (**Fig. 18**). Fucoxanthin content on rope No. 2 was significantly higher in the May sampling dates with values of 1.26 mg/g and 1.20 mg/g respectively, when compared with the samples from March and April (ranging from 0.22mg/g to 0.76 mg/g). No significant differences were found between the March 29 and April 19, nor between May 5 and May 28 samples.

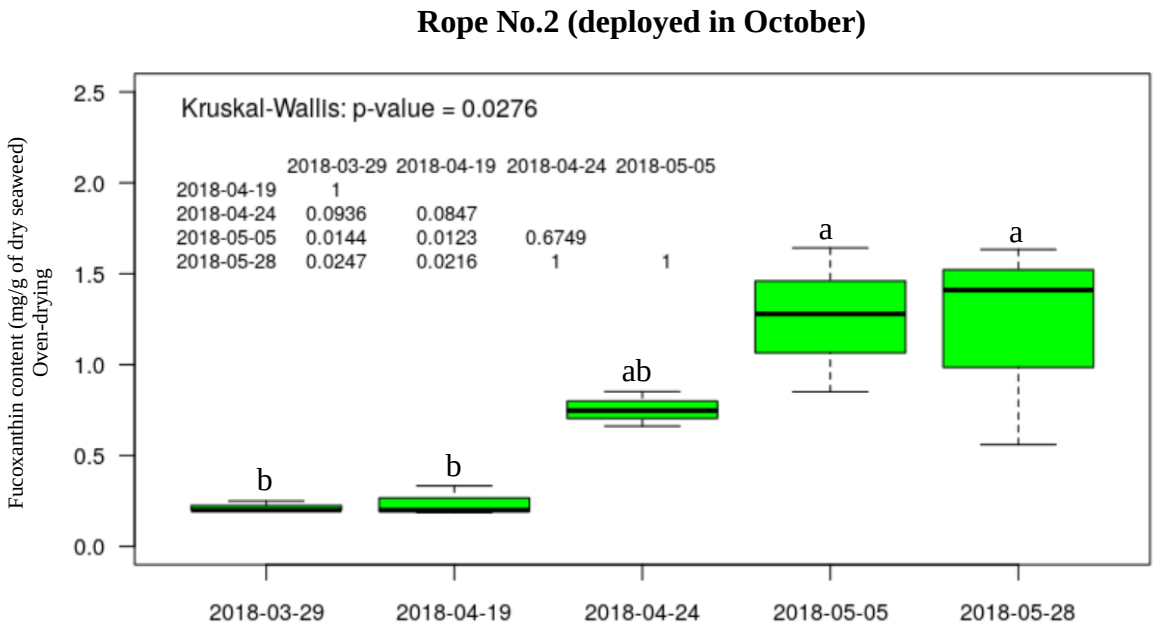


**Figure 18**: Box-plot representing fucoxanthin variation along the different sampling dates on rope No.2 when applying oven-drying. Statistical analysis performed using Kruskal-Wallis test ($p$ <0.05).

### 5.3.2. Phlorotannins

This study showed that no significant differences were observed when comparing the effect of the drying method on phlorotannins content, when analysing the ropes together (**Fig. 19**). Hence, in what concerns phlorotannin content, either method could be applied to dry the biomass.

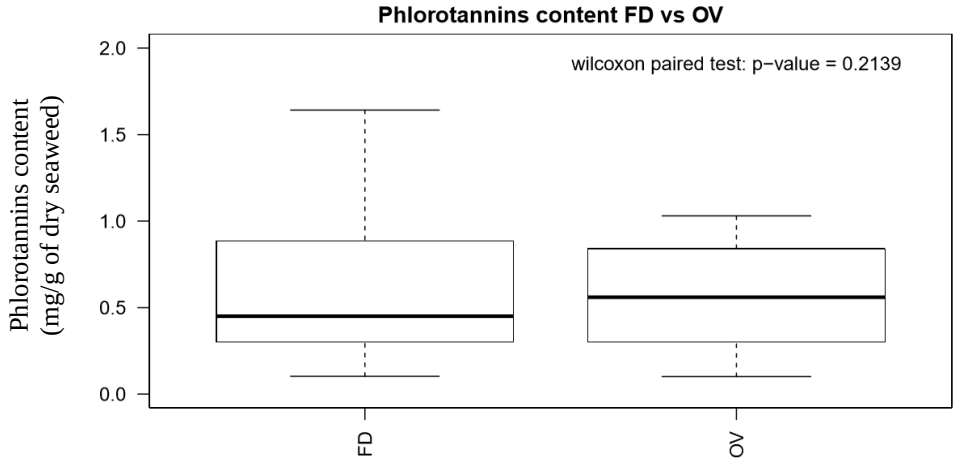


**Figure 19**: Box-plot representing phlorotannins content when using freeze-drying and oven drying. statistical analysis performed using Wilcoxon paired test ($p$<0.05).

The results from the seasonal variation of the compounds suggests that the production of phlorotannins increased as the seaweed matured on the long-lines. Ropes No. 2 and No. 8, located in the exposed areas of the farm, showed the highest quantity of the compound of interest in the May sampling dates (**Fig. 20 & 21**). Both ropes also showed a considerable rise of phlorotannins content from April 24 to May 5, probably due to the increase of the daylength and presence of epiphytes. On the other hand, there were no significant differences on rope No. 4 as observed in **Figure 20**. Overall, the results suggest that low phlorotannins content is found in early Spring samplings, with values below 0.5 mg/g of dry seaweed.

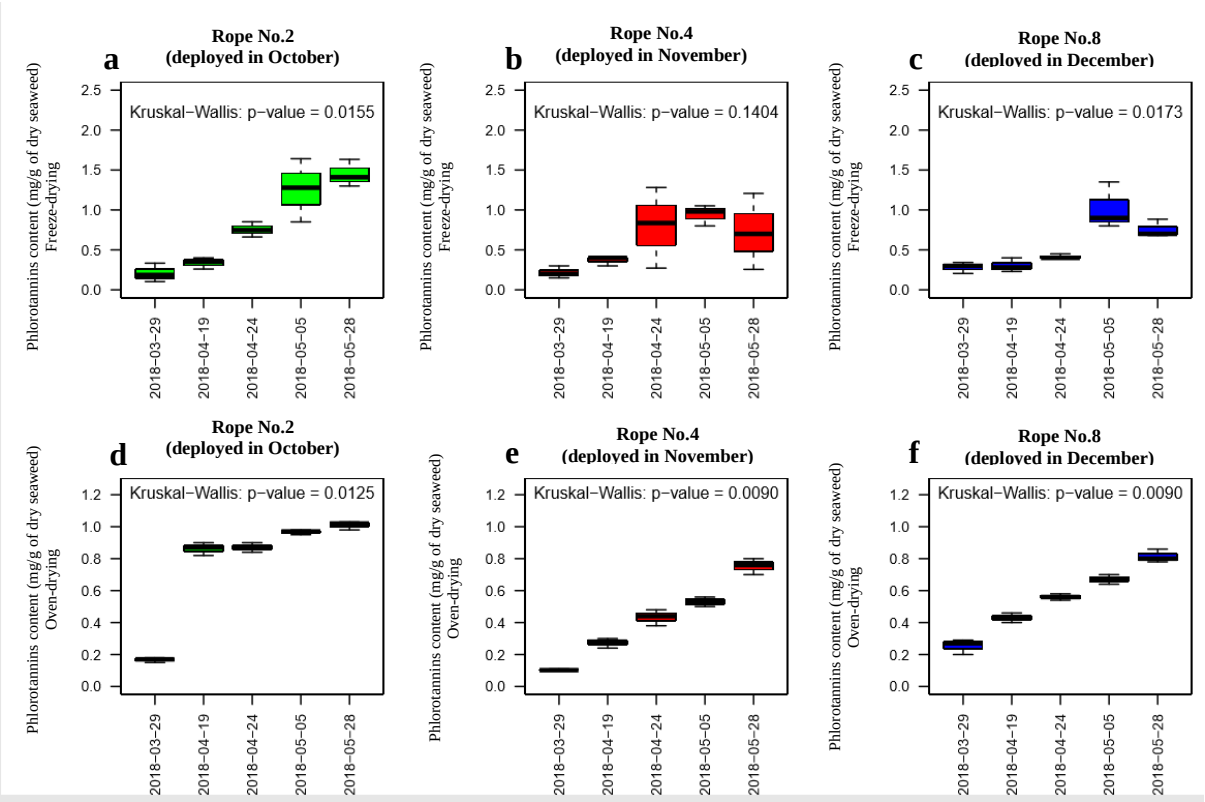


**Figure 20**: Box-plots representing phlorotannins content (mg/g) when using freeze-drying and oven drying and the content variation from March 19 to May 28. Statistical analysis performed using the Kruskal-Wallis test ($p$<0.05).

Significant differences were observed in ropes No. 2 and 8 in freeze-dried samples (**Fig. 21a** & **b**) when comparing the different sampling dates. Freeze dried samples from rope No. 2 reached its maximum amount of phlorotannins on May 28 (1.45 mg/g). **Figure 21a** shows, in more detail, the variation of phlorotannins contained in *Alaria esculenta* on the rope deployed on October 25 when looking at sampling dates. The concentration of phlorotannins increased from the first sampling date (March 29), remaining at values of <34 mg/g during early Spring (between March 29 and April 19) followed by a significant increase on April 24, reaching values of 0.75 mg/g. According to the results, the samplings carried out in May displayed the

highest quantity of the compound (1.26 mg/g and 1.45 mg/g respectively), coinciding with an increase in the amount of epiphytes and grazers spotted on the seaweed blades. This suggests that, as found by Toth & Pavia (2000), in the species *Ascophyllum nodosum*, phlorotannin production may be induced by species like the blue-rayed limpet, resulting in a 15% higher phlorotannin concentration when the seaweed is exposed to gastropod grazing, indicating induced resistance.

**Figure 21c** shows variation in phlorotannin from March 29 to May 28 on freeze-dried samples from rope No. 8 (deployed in December). Early Spring samples resulted in at least half of the concentration of phlorotannins found on May sampling dates, with values not exceeding 0.5 mg/g. Statistical differences were observed in oven-dried samples (**Fig. 21c**) on the rope deployed first (October deployment). The highest concentration of the bio-active was observed in May samplings, 0.98 mg/g and 1.05 mg/g (May 5 and May 28). The next highest concentrations were found on April, with an average of 0.85 mg/g compared to March 29 (barely exceeding 0.20 mg/g).

Significant variations were observed from the end of March until the end of May on the phlorotannins content on rope No. 4 (deployed in November) and on rope No. 8 (deployed in December) when using oven-drying, as shown in **Figure 21 d** & **e.** Both ropes followed a similar pattern, with slightly lower quantities of the bio-active compound in rope No. 4. Both ropes peaked at the last sampling date but the concentration of phlorotannins on May 28 resulted in values of 0.81 mg/g, lower than those observed at the highest peaks on lines 4 and 8 using the same drying method and thus, below the freeze-dred sample values.

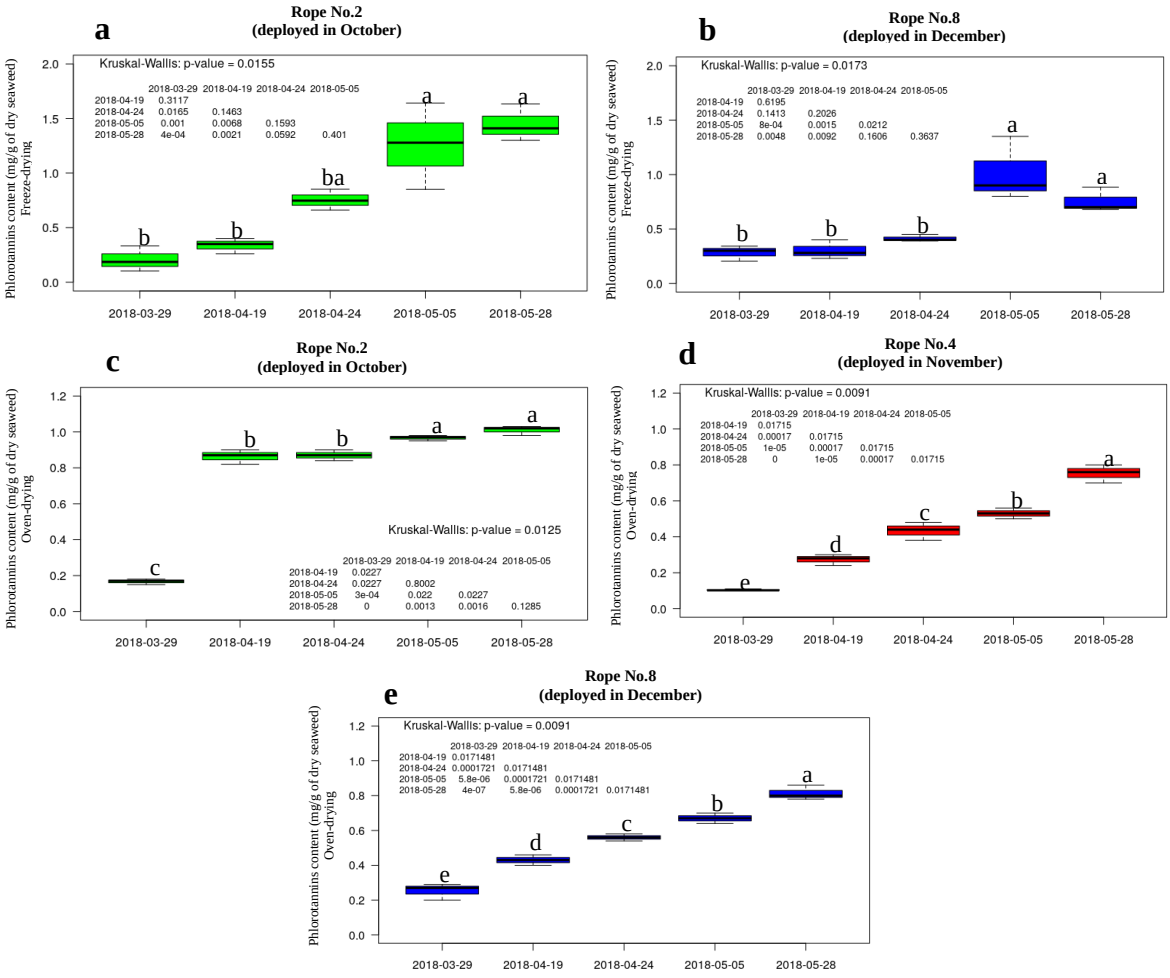


**Figure 21**: Box-plot representing phlorotannins variation on the ropes where there are statiscally differences; **a** & **b**: freeze-drying processed method -lines No. 2 & 8-; **c**, **d** & **e**: oven-drying processing method -lines No.2, 4 & 8 -. Statistical analysis performed using the Kruskal-Wallis test ($p$ <0.05).

Overall, phlorotannin concentrations changed from the end of March to the end of May, with higher quantities being reached at the end of the study. Ropes No. 2 and No. 8 resulted in higher quantities when applying any of the drying methods, when compared with rope No. 4. Significance differences were not found among drying methods, suggesting that any of those can be used when harvesting in May. It is know that concentration of secondary metabolites such as phlorotannins are produced by the seaweeds to deter harmful UV radiation and epiphytes or grazers, but it can also be affected by temperature and nutrient availability (Puglisi & Paul, 1997; Pavia *et al.*, 1997; Sudatti *et al.*, 2011 & Kamiya *et al.*, 2010). The sea surface temperature outside Bantry Bay rose from 9.15°C in March, to 10.36°C in April and 12.56°C in May based on the Marine Institute database (2018). Daylength also rose from 12.46 light hours on March 29, to 16.14 hours on May 28, with an average of 5 to 6.5 hours of sunshine

(Met Éireann, 2018). However, very little of the external conditions were taken into account for this study, suggesting that further research may be needed to better understand the production of phlorotannins in farmed *Alaria esculenta* in Bantry Bay.

## 5.4. Antioxidant activity

### 5.4.1. Fucoxanthin-rich extracts antioxidant activity

Statistically significant differences were found both on oven-dried and freeze-dried samples when sampling at different dates (**Fig. 22**). Rope No. 4 followed a trend decreasing from April 19 to May 28. The highest antioxidant activity was observed in the samples taken May 5 from rope No. 8 (deployed in December), peaking up to levels above 50% of antioxidant activity. This can be explained by the higher amount of fucoxanthin on those dates as mentioned in the previous section. However, a substantial decrease is observed on May 28 on all lines under the different drying treatments, which can be explained by the degradation of other compounds with strong antioxidant activity in the rich-fucoxanthin extracts like vitamin E (Angerhofer *et al.*, 2009).

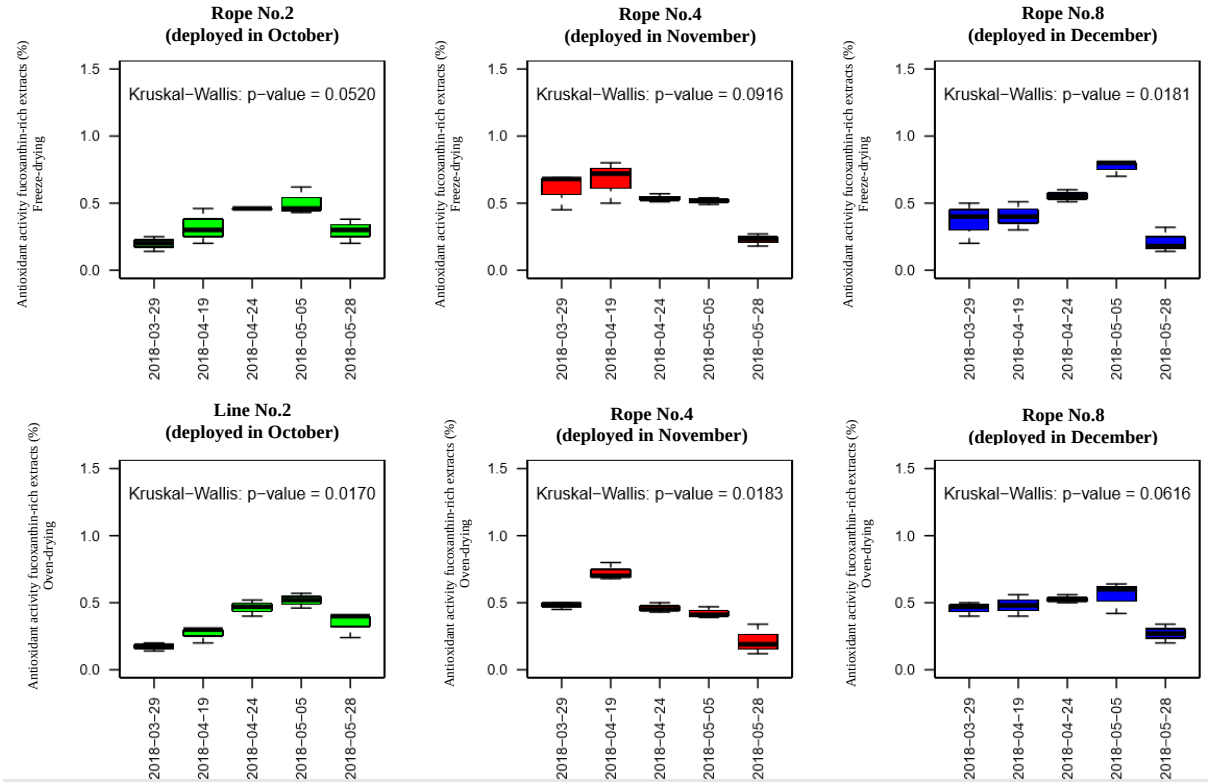


**Figure 22**: Box-plots representing fucoxanthin-rich extracts antioxidant activity (%) when using freeze-drying and oven drying and the content variation from March 19 to May 28. Statistical analysis performed using the Kruskal-Wallis test ($p$ <0.05).

More detailed box-plots from the above results are shown in **Figure 24**, just for those ropes in which significant differences were observed between samples. Freeze-dried samples on the rope that was deployed in December (Rope No. 8) resulted in the highest antioxidant activity (>75% scanvenging activity) on May 5 (**Fig. 23a**), followed by a sudden decrease on May 28. On rope No. 2 (**Fig. 23b**), fucoxanthin-rich extracts of fucoxanthin peaked on May 5, same date as on rope No. 8, corresponding with high levels of the compound. High antioxidant activity was found rope No. 4 (**Fig. 23c**) at the end of March/beginning of April, coinciding with the highest amount of the compound for that rope.

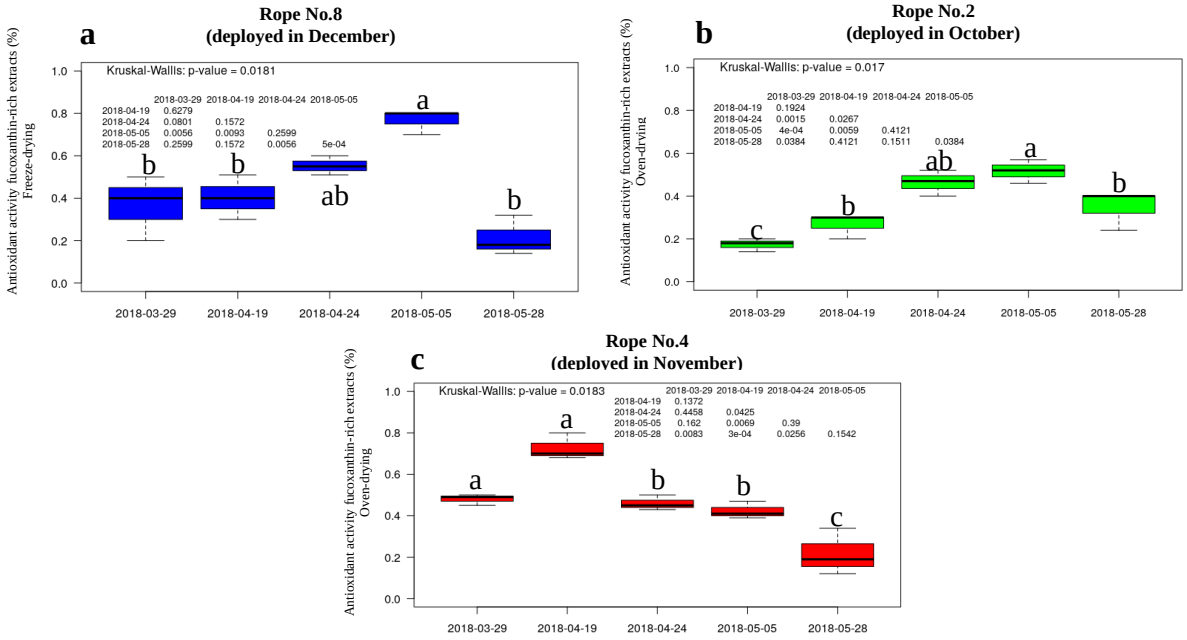


**Figure 23**: Box-plot representing fucoxanthin-rich extracts antioxidant activity (%): **a**: rope No. 8 (deployed in December) using freeze drying; **b**: rope No. 2 (deployed in October) using oven-drying; **c**: rope No. 4 (deployed in November) when using oven-drying. Statistical analysis performed using the Kruskal-Wallis test ($p$ <0.05).

Aman *et al.* (2005) found that fucoxanthin is easily degraded when heated, affecting its antioxidant activity. However, in this study no significance differences were found when using freeze-drying and oven-drying, suggesting that the processing method does not affect the overall antioxidant compounds in these extracts, even though fucoxanthin may be affected (**Fig. 24).** Thus, it is possible that some other compounds such as vitamin E and vitamin C (Le Tutour, 1990) and polyphenolic compounds (Generalic *et al.*, 2019) may also contribute to the antioxidant activity.

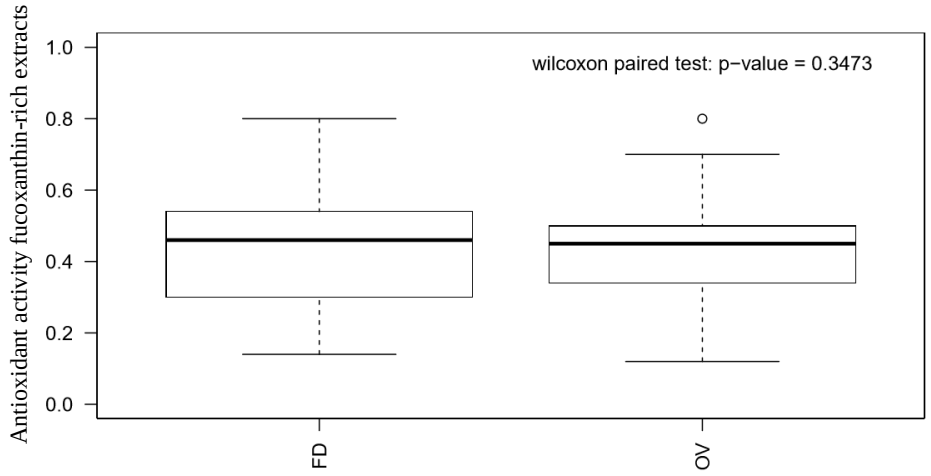


**Figure 24:** box-plot representing the effect in moisture content using two drying methods. **FD**: freeze-drying; **OV**: oven-drying in *Alaria esculenta* samples. Statistical analysis performed using Wilcoxon paired test ($p$<0.05).

The correlation of antioxidant activity and the amount of the compound was assessed, in both freeze and oven drying samples. A strong correlation (43%) suggests that the antioxidant activity is related, at least partially, to the presence of fucoxanthin in the extract, despite the possible presence of other antioxidant compounds (**Fig. 25**).

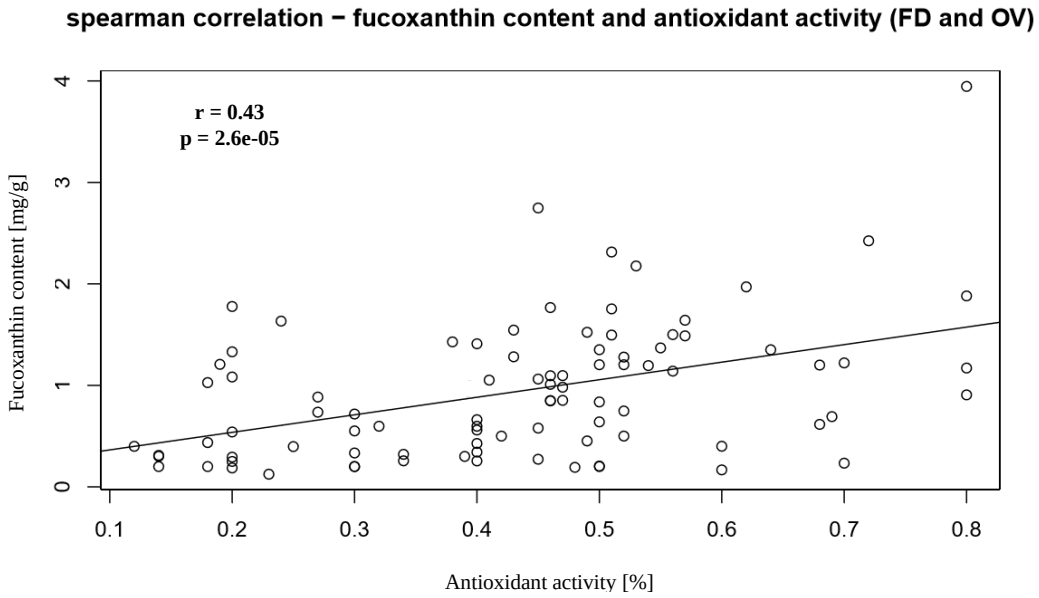


**Figure 25**: Graph representing fucoxanthin-rich extracts antioxidant activity using freeze-drying (**FD**) and oven-drying (**OV**).

### 5.4.2. Phlorotannins-rich extracts antioxidant activity

The antioxidant activity of the phlorotannins-rich extracts prepared from freeze- or oven dried biomass did not differ significantly. An increasing trend is observed from March 23 to May 5 in the ropes studied. However, the antioxidant activity on freeze-dried samples from rope No. 8 showed slightly higher levels of activity from April 19 to May 28 (40-70%), peaking on May 5. On theother hand, oven-dried samples showed high antioxidant activity (70%) on May 28 in all the ropes (**Fig. 26**). The increasing trend of the antioxidant activity in late Spring agrees with the results from the species *Fucus vesiculosus* studied by Heavisides *et al.* (2018), with higher DPPH free radical scavenging activity in April and May when compared with February and March. Several metabolites, including phlorotannis are produced by the seaweeds as a defence mechanism against biotic factors such grazing, fouling and epiphytism (Svensson *et al.*, 2007; Jennings & Steinberg, 1997), observed in this study in later Spring, but also, against abiotic factors like temperature and light (Abdala-Díaz *et al.*, 2006; Tanniou *et al.*, 2014; Le Lann *et al.*, 2012a; Yates & Peckol, 1993; Swanson & Druehl, 2002), increasing progresively from March until the end of May. Moreover, as found by Sanderson *et al.* (2008), the nutrients variability can affect the biomass biochemical content, resulting in seasonal variations of the compound.

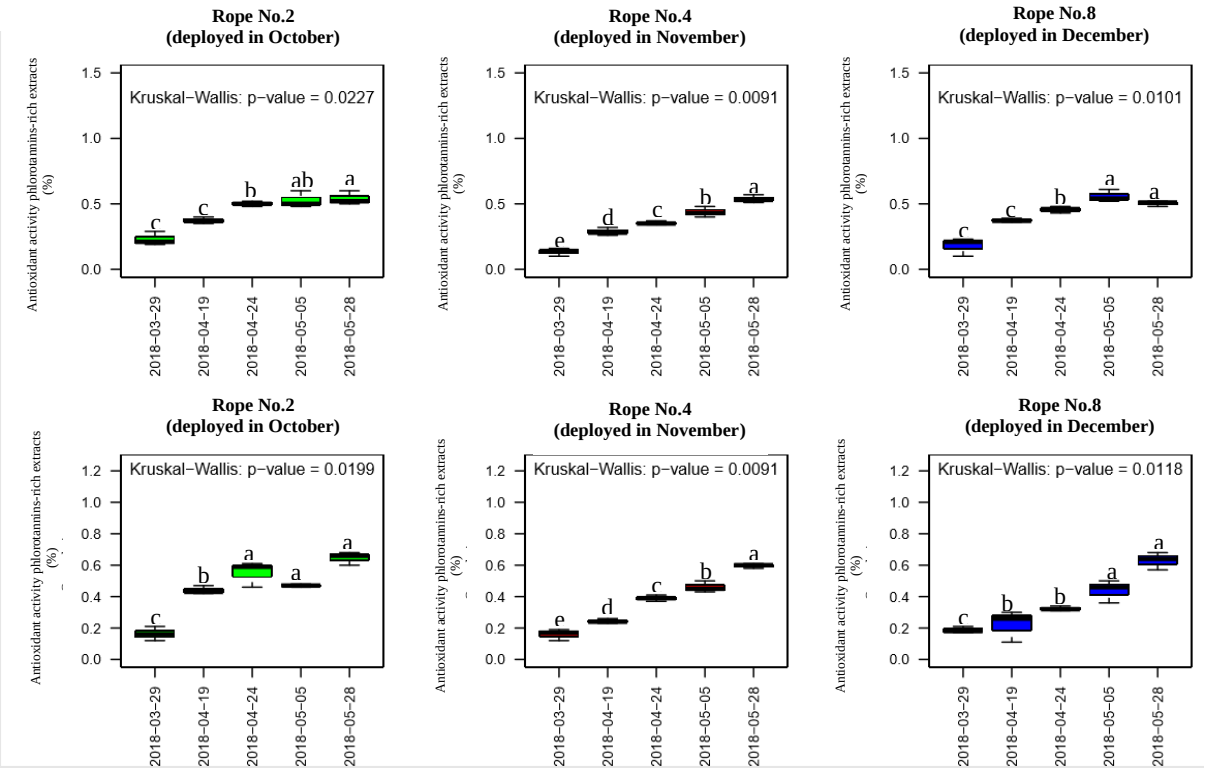


**Figure 26**: Box-plots representing phlorotannins antioxidant activity (%)when using freeze-drying and oven drying from March 19 to May 28. Statistical analysis performed using the Kruskal-Wallis test ($p$ <0.05).

No significant differences were observed when comparing antioxidant activity of the extracts prepared from biomass dried by the two different methods applied. The paired Wilcoxon test was conducted using the results from the three lines and included the different sampling dates (**Fig. 27**). As already observed for the antioxidant activity of the fucoxanthin-rich extracts, the drying method applied does not affect the antioxidant compounds of the phlorotannins-rich extract.

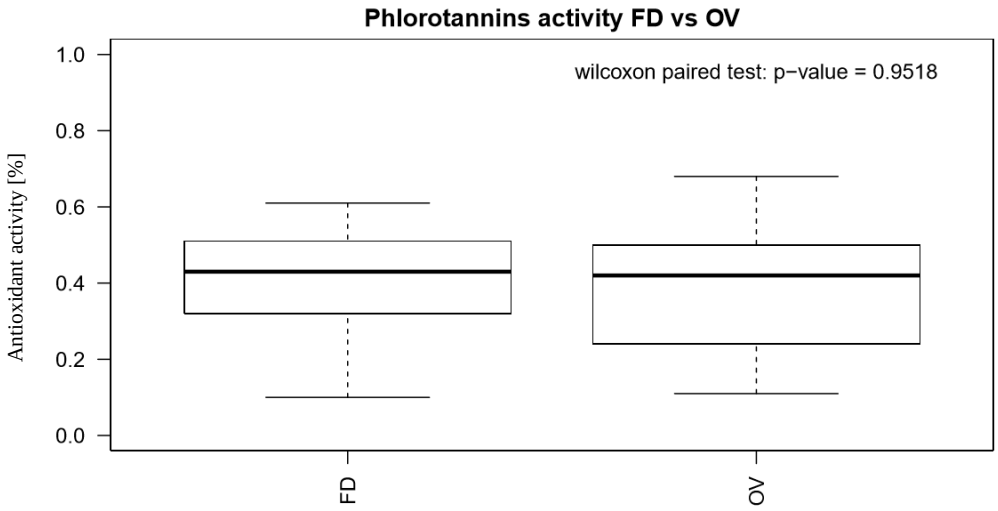


**Figure 27**: Box-plot representing phlorotannins antioxidant activity when applying freeze drying and oven drying. Statistical analysis performed using the Wilcoxon paired test ($p$<0.05).

A strong correlation of antioxidant activity and the amount of phlorotannins in the extract is represented in **Figure 28**. A strong correlation (77%) means that an increase of antioxidant activity occurs as the concentration of phlorotannins increases, suggesting that the compound of interest is one of the major compounds responsible for the antioxidant activity.

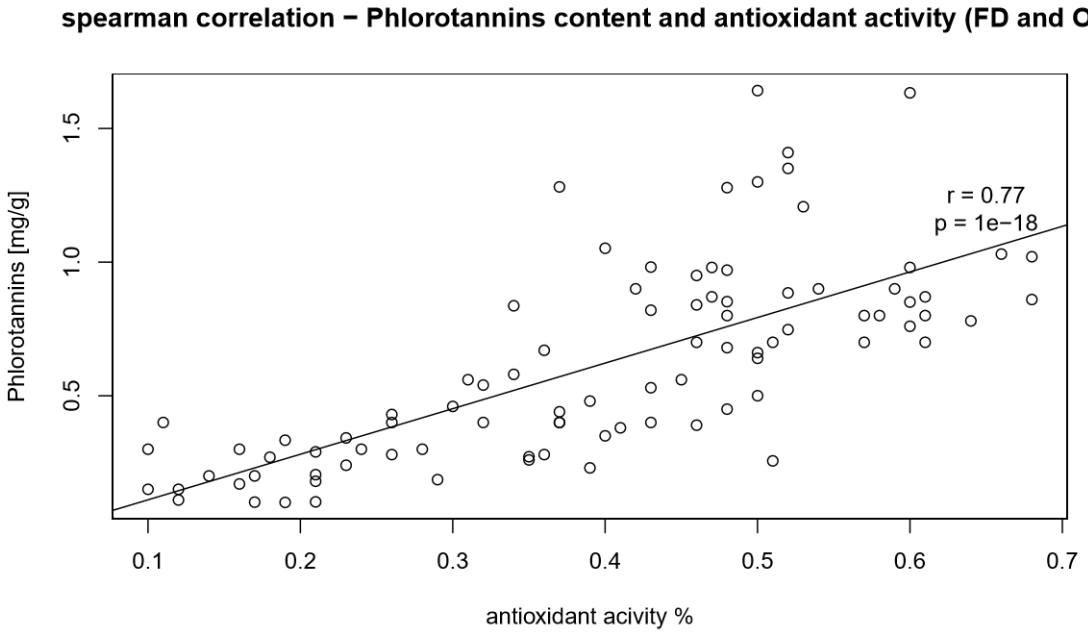


**Figure 28**: Graph representing the correlation of phlorotannins content and its antioxidant activity when applied freeze-drying (**FD**) and oven-drying(**OV**) on the samples from the study.

Ireland has one of the most established seaweed industries in Europe. In 2018, 77,000 tonnes of fresh seaweed, worth €37 million, were exported (BIM, 2020). Two of the ropes in this study reached the highest biomass on May 28 (with the exception of rope No. 4). The ropes used in this study were 110m long, resulting in an estimated production between 700-800 kgs ww of *Alaria esculenta* per rope. However, the species has high levels of water content (80-90%) as shown in this study, meaning that each rope will produce between 97.9 to 99 kg dw (**Table 5**) (90% MC).

**Table 6** gathers the overall results of the study for May 5, when maximum levels of biomass as well as the two compounds were found. Rope No. 8 (deployed in December) resulted in the highest biomass and productivity, with an estimated production of 99 kg of dry *Alaria esculenta* from the deployment date (December 9) to May 5 (147 days), followed by rope No. 4 (deployed in November) with 83.6 kg dw (in 185 days), and rope No. 2 (deployed in October) with 97.9 kg of dw (in 192 days). A late deployment has also resulted in higher contents of fucoxanthin and phlorotannins with strong antioxidant activity, both peaking on May 5. The price of fucoxanthin extracts varies widely as do claims of purity and certification (Kyndt & D´Silva 2013). Fucoxanthin-rich extract price varies depending on their purity. Based on this study, each of the ropes can produce rich-fucoxanthin extracts with strong antioxidant activity but its purity needs to be assessed in further studies to calculate its turnover. So far, based on Shandong Jiejing Group Corporation (2020), a company based in China, fucoxanthin-rich extracts range from €337.62/kg per 1% purity, €4 200/kg per 10% purity, €26 100/kg per 50% purity and €48 000/kg for 90% purity. This would correspond to a maximum value of €67.52 (1% purity), €844 (10% purity), €5 200 (50% purity) and €9 600 (90% purity) per rope (2.02 mg/g on rope No. 8 on May 5 using freeze-drying).

Overall, phlorotannin productivity does not differ much among treatment methods, with similar results when the rope was deployed on October and December. SEANOL-F® containing at least 15% of total polyphenolic compounds, including phlorotannins, is sold at €71.29 for 400mg (Supersmart, 2020) which traduces, using the results from this study, in a turnover ranging from €11 800 to €18 000,based on the maximum phlorotannin quantity found in rope No. 8, per rope. Rope No. 8 was deployed 1.5 months after rope No. 2. Consequently, as the seaweed lines need to be checked regularly, a later deployment is more efficient and

reduces costs. A later deployment date may also reduce the risk of ropes tangling to each other due to heavy Autumn storms, as was the case for rope No. 4.

**Table 6:** Results summary including seaweed biomass as ww and dw, fucoxanthin and phlorotannins rich extracts production and antioxidant activity, and overall productivity by rope in May 5th

| | **Rope 2 (depl. Oct.)** | **Rope 4 (depl. Nov.)** | **Rope 8 (depl. Dec.)** |
|---|---|---|---|
| Biomass ww (kg/m) (mean±sd) | 8.90 ± 1.29 | 7.63 ± 1.04 | 9.09 ± 2.48 |
| Biomass dw (90% MC)(kg/m) | 0.89 | 0.76 | 0.90 |
| ***Total estimated dw of Alaria esculenta per rope (kg)*** | ***97.9*** | ***83.6*** | ***99*** |
| ***Biomas productivy (kg/d from deployment to May 5th )*** | ***0.51*** | ***0.45*** | ***0.67*** |
| **Fucoxanthin** (mg/g of dry seaweed) (FD - OV) | 1.76 – 1.26 | 1.31 – 0.78 | 2.02 – 0.75 |
| Antioxidant activity fucoxanthin-rich extracts (%) (FD – OV) | 71 – 52 | 52 – 42 | 77 – 55 |
| ***Total estimated rich-fucoxanthin extract per rope (g) (FD-OV)*** | ***172 – 123*** | ***109 – 65*** | ***200 – 74.25*** |
| ***Rich-fucoxanthin extract productivity (g/d from deployment to May 5th)*** | ***0.90 – 0.64*** | ***0.59 – 0.35*** | ***1.36 – 0.50*** |
| **Phlorotannins** (mg/g of dry seaweed) (FD - OV) | 1.26 - 0.97 | 0.94 - 0.53 | 1.02 - 0.67 |
| Antioxidant activity phlorotannins-rich extracts (%) (FD – OV) | 53 - 47 | 44 - 46 | 56 - 44 |
| ***Total estimated rich-phlorotannins extract per rope (g) (FD-OV)*** | ***123 – 95*** | ***78.60 – 44.30*** | ***101 – 66.33*** |
| ***Rich-phlorotannins extract productivity (g/d from deployment to May 5th)*** | ***0.64 – 0.49*** | ***0.42 – 0.24*** | ***0.69 – 0.45*** |

**sd:** standard deviation**; ww**: wet weight; **dw**: dry weight; **MC**: moisture content; **FD**: freeze-dried; **OV**: oven-dried.

Any of the processing methods used in this study are suitable to dry *Alaria esculenta* without affecting the antioxidant activity of the bioactive compounds of interest or others also present in the extracts. However, freeze-drying is an expensive technique to use at large-scale, and hence, it is not widely used in the commercial processing of seaweed (Badmus *et al.*, 2019), suggesting that oven-drying may be easier to be scaled-up when compared to freeze-drying. Further research is needed concerning more energy efficient processing methods, such as using directly fresh seaweed in the extraction process, or using ensiling to preserve it, as oven-drying may not be sustainable in the long-term. The extractions used in this study will use large amounts of solvents which are usually hazardous for human health and for the environment (Ibañez *et al.*, 2012) suggesting that new extractions methods must be applied, such as enzyme-assisted extraction, supercritical fluid extraction, accelerated solvent extraction or microwave-assisted extraction (Zhang *et al.*, 2018).

Therefore, further research is needed for improved and increased large-scale production of bio-actives from *Alaria esculenta*, and to ensure that the compounds of interest remain available and undegraded in the final product.

# 6. Conclusion

The results suggest that *Alaria esculenta* biomass quantity and composition changes with deployment date, probably affected by abiotic factors such as currents, light penetration or nutrients availability. This suggests that the cultivation method is location-specific and would require studies to be carried out at a proposed seaweed aquaculture site prior to beginning cultivation. The study also demonstrates that *Alaria esculenta* is a fast growing kelp species, rich in high-value bio active compounds such as fucoxanthin and phlorotannins with high antioxidant activity when farmed in Bantry Bay, Ireland, with higher biomass being recovered when the ropes are deployed later in the year.

This study suggests that freeze-drying and oven-drying are both suitable processing methods to dry *A. esculenta* as the bioactive compound contents did not statistically differ between samples dried by the two methods, whilst maintaining strong antioxidant activities. The bioactive compounds, fucoxanthin and phlorotannin, showed a seasonal variation from March 29 (Spring in Ireland) to May 28 (Summer in Ireland), with higher levels of the compounds in late Spring, which can be related to the presence of epiphytes and grazers that induce the production of these compounds as protection, as well as an increase in the seawater temperature and daylength. However, more studies need to be carried out to prove this.

Overall, this study proposes to deploy the ropes in December and harvested at the beginning of May, followed by either freeze-drying or oven-drying to get high levels of fucoxanthin-rich extracts and phlorotannin-rich extracts with high antioxidant activity in less time, and reduce the risk of losing the ropes to storms.

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