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Phlorotannins from northern Icelandic seaweeds grown in an integrated multi-trophic aquaculture system for sustainable production of constitutional food supplements

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Report Summary

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Title	Phlorotannins from northern Icelandic seaweeds grown in an integrated multi-trophic aquaculture system for sustainable production of constitutional food supplements (Einangrun á Phlorotannins úr ræktuðu þangi til framleiðslu á fæðubótarefnum)	
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Summary: Seaweeds constitute one of the commercially important renewable marine living resources in coastal areas. To cope to extreme conditions, they have evolved unique compounds not found in higher plants. These compounds are group specific, and their concentrations vary depending on seasons and grade of exposure in wild seaweeds. Phlorotannins are a group of complex polymers of phloroglucinol (1, 3, 5-trihydroxybenzene) unique to brown macroalgae. These phenolic compounds are integral structural components of the cell wall but can also play many secondary ecological roles such as protection from UV radiation. The present study focused on exuded phlorotannins during UV exposure, utilizing the Folin–Ciocalteu as well as DPPH and FRAP activity assays to quantify the yields. Testing initially seven seaweed species (*Saccharina latissima, Ascophyllum nodosum, Alaria esculenta, Laminaria digitata, Fucus evanescens*, *F. serratus* and *F. elongatus*) in 17 L tanks, a pre-selection of effective UV settings as well as suitable species was conducted. In the further, optimizations targeting sampling, harvest and extraction processes as well as the increase of the phlorotannin yield by variation of the UV exposure lengths and quality, yielded to results in which *S. latissima* and *L. digitata* showed the highest total phlorotannin contents (TPC) in the UVB150 16:08 h and UVB200 20:04 h assays, respectively, reflecting summer conditions in northern Icelandic coastal areas. In this context, the application of macroporous resins to concentrate the exuded phlorotannins in the seawater provides the opportunity to harvest phlorotannins without the time-consuming need to process high volumes. Different extraction methods were tested (particularly for the biomasses) and among them, phlorotannin extraction using ethanol (80%, v/v) solution was prioritized, as it allows separation of phenolic compounds, prior to extracting the macroalgal polysaccharides in a future biorefinery sequence. Successful identification of compounds from the purified extract was accomplished using UHPLC/MS2, which verified the presence of seven phlorotannins in the concentrated seawater samples. Finally, to simulate the co-culture of seaweeds with aquatic animals and to assess the impact of the organic fraction in aquacultural wastewater on the TPC yield in the seawater, overall, two species (*Saccharina latissima* and *Laminaria digitata*) were cultivated in three different concentrations of fish and shrimp wastewater as well as natural surface seawater (each 25, 50, 100%). The results showed a gradual decline of the yields with increase of the portion of the experimental mixtures was detected, being most distinctive in the assay using 100% fish sea-wastewater. In any case, a co-culture of the seaweeds with aquatic animals would be not desirable since also medications and other chemicals may intervene with the phlorotannin capture by adsorbents.

Keywords: *Brown Seaweeds*, *phlorotannins*, *cultivation*

Contents

1. Introduction

Seaweeds are known for their richness in polysaccharides, minerals and certain vitamins (Arasaki & Arasaki 1983), but they also contain bioactive substances like polysaccharides, proteins, lipids and polyphenols, with antibacterial, antiviral and antifungal properties, as well as many others both *in vitro* and *in vivo* (Kumar et al. 2008, Audibert et al. 2010, Li et al. 2011, Thomas & Kim 2011, Wang et al. 2012, Lopes et al. 2012). The traditional use of seaweed as food has been traced back to the fourth century in Japan and the sixth century in China. Today those two countries and the Republic of Korea are the largest consumers of seaweed as food (McHugh 2003). Whilst food has long been used to improve health, our knowledge of the relationship between food components and health is now being used to improve food. Although most foods can be considered "functional", in the context to seaweed utilization, the term is reserved for foods and food components that have been demonstrated to provide specific health benefits beyond basic nutrition. Widespread interest in select foods that might promote health has resulted in the use of the term "functional foods". Food and nutrition science has moved from identifying and correcting nutritional deficiencies to designing foods that promote optimal health and reduce the risk of disease. Today's science and technology can be used to provide many additional functional foods, and future scientific and technological advances promise an even greater range of health benefits for consumers. Functional foods can provide health benefits by reducing the risk of chronic diseases and enhancing the ability to manage chronic diseases, thus improving the quality of life (e.g., Holdt & Kraan 2011).

Various red and brown seaweeds are used to produce food additives such as agar, alginate and carrageenan (hydrocolloids). Seaweed meal, used an additive to animal feed, has been produced in Norway, where its production was pioneered in the 1960s. Other seaweed products comprise fertilizers and cosmetic products, such as creams and lotions. Over the last twenty years there have been some large projects that investigated the possible use of seaweeds as an indirect source of fuel. The idea was to grow large quantities of seaweed in the ocean and then ferment this biomass to generate methane gas for use as a fuel. The results showed the need for more research and development, that it is a long-term project and is not economic at present. In addition, there are potential uses for seaweed in wastewater treatment. Some seaweeds are able to absorb heavy metal ions such as zinc and cadmium from polluted water. The effluent water from fish farms usually contains high levels of waste that can cause problems to other aquatic life in adjacent waters. Seaweeds can often use much of this waste material as nutrient, so trials have been undertaken to farm seaweed in areas adjacent to fish farms.

In the last three decades the discovery of metabolites with biological activities from macroalgae has increased significantly. However, despite the intense research effort by academic and corporate institutions, very few products with real potential have been identified or developed. Substances that currently receive most attention from pharmaceutical companies for use in drug development, or from researchers in the field of medicine-related research include sulphated polysaccharides as antiviral substances, halogenated furanones from *Delisea pulchra* as antifouling compounds, and kahalalide F from a species of *Bryopsis* as a possible treatment of lung cancer, tumours and AIDS. Other substances such as macroalgal lectins, fucoidans, kainoids and aplysiatoxins are routinely used in biomedical research and a multitude of other substances have known biological activities (e.g., Redmond et al. 2014).

For instance, the storage polysaccharide of brown algae is laminarin, a vacuolar b-1,3-glucan with occasional b-1,6-linked branches (Percival & Ross 1951). This polysaccharide is polydisperse, consisting of a minor G-series with polymers containing only glucose residues, and a more abundant M-series with glucans terminated with a 1-linked d-mannitol residue (Read et al. 1996, Michel et al. 2010). Experiments with radioactive C demonstrated that laminarin and mannitol are interchangeable storage compounds in phaeophytes, as are sucrose and starch in higher plants (Yamaguchi et al. 1966) or saccharose in Chlorophyceae. In kelps, mannitol can be remobilized and translocated via the sieve tubes from mature tissues to supply the rapidly growing parts of the alga with C (Schmitz & Lobban 1976, Lobban & Harrison 1994). In contrast, the heteroside floridoside (a-D-galactopyranosyl-[1–2]-glycerol) is considered to be the main photosynthetic and reserve product in all orders of the Rhodophyta except the Ceramiales. In general, members of the Ceramiales synthesize and accumulate the chemically related digeneaside (a-D-mannopyranosyl-[1–2]-glycerate) (Karsten et al. 1999, Fig. 1).

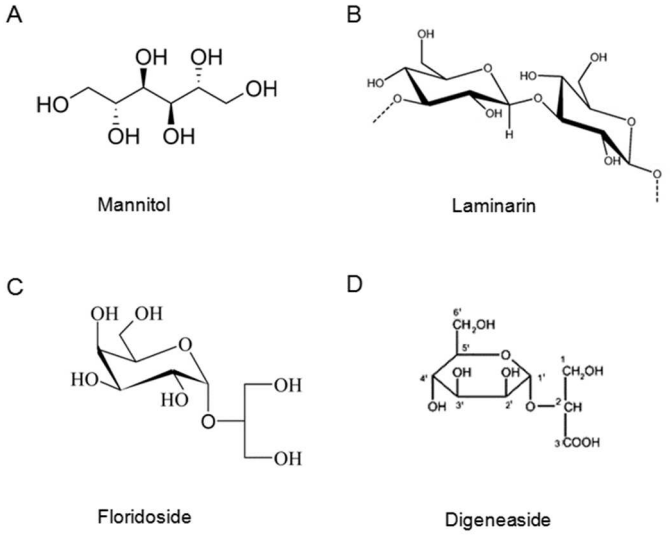


Figure 1. Chemical structures of seaweed storage compounds. A) and B) are found in brown seaweeds, whereas C) and D) are accumulated in red seaweeds.

Other bioactive compounds are originating from the need of the seaweeds to cope to environmental conditions such as high ultraviolet radiation (UVR). For algae exposed to enhanced UVR for substantial parts of their life cycles, strategies that passively screen this waveband will contribute to prevent UV-induced damage to essential bio molecules such as DNA and proteins (Bischof et al. 2006, Karsten et al. 2009). In addition, UV-screening may also save metabolic energy by reducing the need for constantly active avoidance and repair processes. The most common photo protective sunscreens in many, but not all algal taxa and cyanobacteria are the mycrosporine-like amino acids (MAAs), a suite of chemically closely related, colourless, water-soluble, polar and at cellular pH uncharged or zwitterionic amino acid derivatives. MAAs are related to fungal mycosporines which were first isolated from sporulating mycelia (Karsten et al. 2009) and consist of aminocyclohexenone or aminocyclohexenimine ring systems.

Brown algae (class Phaeophyceae), are a large group of multicellular algae, including many seaweeds located in colder waters within the Northern Hemisphere. Most brown algae live in marine

environments, where they play an important role both as food and as a potential habitat. Brown macroalgae are a well-known source of structurally unique polyphenols known as phlorotannins, derived from the oligomerization and decoupling of the monomer phloroglucinol (1,3,5-trihydroxybenzene), with molecular weights ranging from 126 Da to 100 kDa (Boettcher & Targett 1993, Sailler & Glombitza 1999, McClintock & Baker 2001; Fig. 2). Phlorotannins are synthesized via the acetate-malonate pathway and are stored in special vesicles (physodes). Research suggests that these compounds act as a defense mechanism within macroalgae against herbivores (Steinberg 1984, Schoenwaelder & Clayton 1998, Targett & Arnold 2001, Mazid et al. 2011), microbes (Waterman & Mole 1994, Pavia & Toth 2000), and the detrimental effects of ultraviolet (UV) radiation (Pavia et al. 1997). Phlorotannins also have allelopathic activity against epibionts (Davis et al. 1989) and are important for cell wall development at early phases of zygote growth in the Fucaceae family (Schoenwaelder 2002, Arnold 2003). The relative abundance of phlorotannins ranges from 5% to 30% of the dry weight of the brown algae (Heffernan et al. 2015).

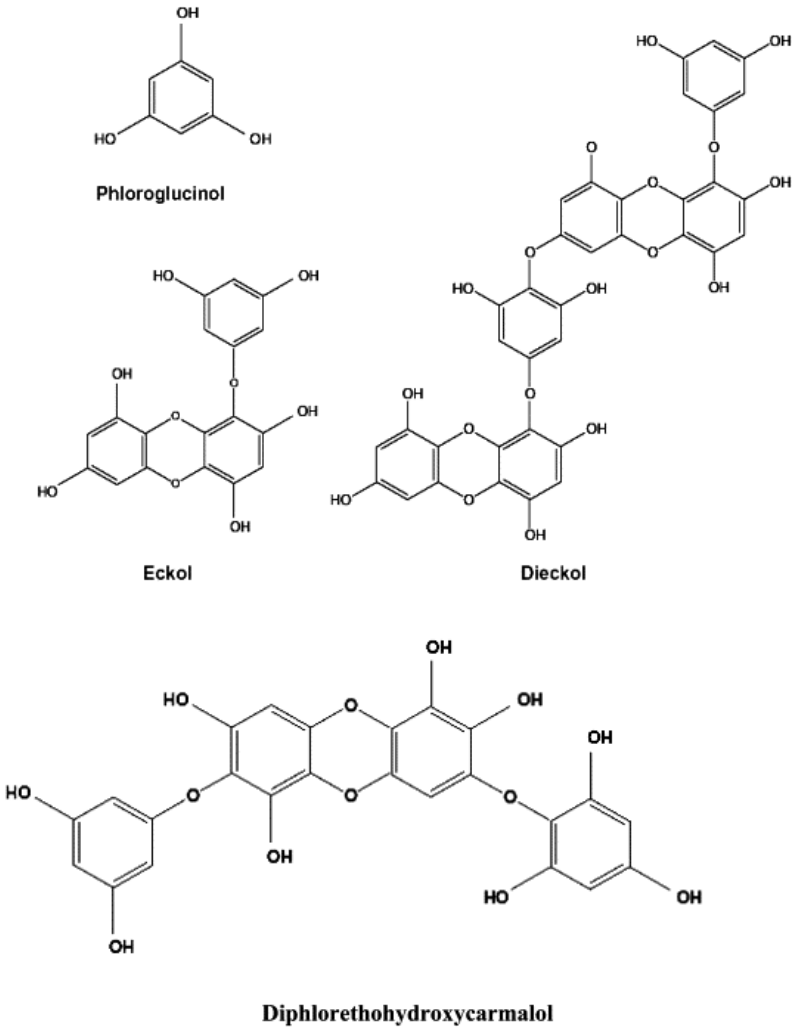


Figure 2. Examples of known and characterised phlorotannins.

It has been demonstrated that phlorotannins can have anti-diabetic (Lee et al. 2004), anti-cancer (Kong et al. 2009, Yang et al. 2010), anti-bacterial (Wang et al. 2009), anti-allergic (Le et al. 2009), anti-oxidation and anti-HIV properties (Ahn et al. 2004) (cf. Fig. 3). Phlorotannins are mostly located at the

periphery of the cells, as components of the cell wall. They also contribute to absorption of UV-B radiation (between 280 and 320 nm) and show absorbance maxima at 200 and 265 nm, corresponding to UV-C wavelengths (Pavia et al. 1997). Studies also demonstrated that sunlight intensity is related to phlorotannins production in *Ascophyllum nodosum* and *Fucus vesiculosus* natural populations (Pavia & Toth 2000). For these reasons, it has been suggested that phlorotannins act as photoprotective substances (Gómez *&* Huovinen 2010*).* Further studies with *Lessonia nigrescens* and *Macrocystis integrifolia* demonstrated that both UV-A and UV-B radiation can induce soluble phlorotannins and that there is a correlation between induction of phlorotannins and reduction in the inhibition of photosynthesis and DNA damage, two major effects of UV radiation on vegetal tissues (Swanson & Druehl 2002). The multifunctional antioxidant activity of polyphenols is highly related to phenol rings which act as electron traps to scavenge peroxy, superoxide anions and hydroxyl radicals. Phlorotannins from brown algae have up to eight interconnected rings and are therefore more potent free radical scavengers than polyphenols derived from terrestrial plants, including green tea catechins, which only have three to four rings (Hemat 2007).

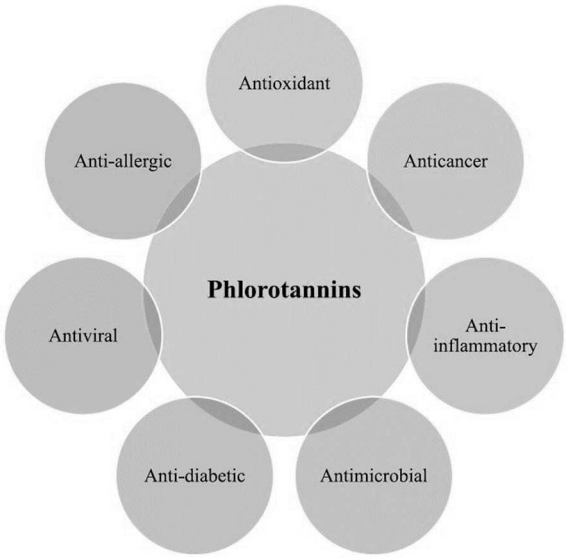


Figure 3. Examples of health beneficial properties of phlorotannins (from Cassani et al. 2020).

In addition to changes in phlorotannin content caused by UV radiation, levels of phlorotannins are also found to vary as a response to other environmental factors such as salinity, light variability and nutrients (reviewed by Targett & Arnold 1998, Amsler & Fairhead 2006, Jormalainen & Honkanen 2008). Thus, it has been reported that the concentration of phlorotannins in brown algae is reported to be highly variable among different taxa of brown seaweeds as well as among different geographical areas (Heffernan et al. 2015). Among local populations of algae, variation in phlorotannin contents may arise from plastic responses to environmental variation or from genetic divergence among populations, or both. Although the role of genetic differentiation in the among-population variation of phlorotannins has not been directly tested, phlorotannin content, for instance, in *F. vesiculosus* is found to be genetically variable (Jormalainen & Honkanen 2004).

2. Objectives and Aims of the Study

Utilizing the fact that phlorotannins are released by seaweeds into the surrounding medium during environmental stress (e.g., Heffernan et al. 2015), the aim of the present project was to induce the accumulation and release of the desired compounds by exposure of the plants to ultraviolet radiation (UVR, UVA and UVB) of different intensities and wavelengths. Furthermore, variations of nutrient concentrations and composition were also tested to simulate the co-culture with aquatic animals, utilizing fish and shrimp wastewater as well as natural surface seawater.

In detail, the aims of the present study were:

1. to maintain a culture collection of Icelandic macroalgae as a resource for both academic and applied users.

2. to induce the accumulation of phlorotannins, extract, screen and analyse both exuded (soluble phlorotannins in the seawater) and biomasses (cell wall bounded phlorotannins) from different brown seaweeds, including their antioxidant activities.

3. to optimize extraction and purification procedures.

4. to establish a photobioreactor (PBR)/ tank based closed culture system for the sporophytes, to obtain biomasses for further studies.

5. to investigate the impact of organic matter from aquatic animals and in natural surface seawater on the yield of exuded phlorotannins in the seawater.

6. .to identify the phlorotannins released from seaweeds.

3. Material and Methods

3.1 Seaweed Culture Collection – Species Isolation and Cultivation

After the reconstruction works at BioPol laboratory (build-up of a farmers kitchen) and reinitiating the climate chamber, the seaweed culture collection and cultivation were started again in June 2017. Although most red seaweeds were stable, all specific nutrient concentrations requiring green and brown algae were suddenly struggling. A series of optimizations of nutrient compositions and growth conditions to safe the algae were conducted under the support of international experts with no positive effects. As it turned out by accident (the PI needed to prepare the stock solutions for the standard medium Provasoli´s on short-term by herself and one salt in the receipt was missing in the BioPol laboratory) the technician K. Zech (Biochemist), who was asked to prepare the stock solutions, had decided not to use the provided media receipt, omitting one of the salts and changed thereby the required nutrient composition. This had led to nutrient depletion in the seaweeds, leaving the plants literally starving to death. Due to the incident besides other irregularities, the PI decided to prepare all stock solutions and media by herself and also conducted the maintenance of the species in biweekly intervals. No further problems regarding the media compositions occurred after that. Unfortunately, the cooling system of the climate chamber broke down and temperatures raised up to 35°C over a period of ca. 24 h, resulting in the death of all seaweeds inside the chamber from overheating in the beginning of April 2018. After several further malfunctions of the climate chamber and extraordinary high pollution levels from the farmers kitchen (smoker) as well as car painting activities in the first level of the building, it was decided by the PI in consent with Prof. U. Karsten that the remaining species of the seaweed culture collection had to be moved to an external facility. In December 2018 it was tested if a cultivation at Verið (Hólar University College) would be possible. But due to restricted space in combination with lack of free infrastructure for sterile working and proper low temperature control during the stay in addition to the negative impacts of the oxygen-free seawater used at Verið on the seaweeds, the algae had to be moved back again. Finally, in April 2019 both culture collections (microalgae and seaweeds) were moved to an external private facility where they remain until this day. The seaweed culture collection had to be rebuilt again but in order to avoid former delays, fresh collected seaweeds were used during all experiments. Since work package (WP 1) in the original proposal comprised the build-up and maintenance of the seaweed culture collection, the individual isolation and cultivation steps conducted during the project are described in the following.

3.1.1 Collection of Wild Material

Two sites, located in the north-western part of Iceland, were used for the collection of wild seaweed material (Fig. 4). The first side is a steep coast characterised by cliffs and rock pools (Figs. 5A, B), whereas the second one is a tidal flat in which stones functioning as a sand trap (Figs. 5C, D). Seaweeds were sampled during low tide. Only healthy and strong coloured plants were collected, targeting Phaeo- Rhodo- and Chlorophyceae. Whole plants from different species were collected and cultured, using artificial seawater salt (Tropic Marin© Reef Salt) enriched with Provasoli´s Enrichment Solution (PES). To obtain spores for seeding, reproductive sorus tissue from wild plants were carefully collected and processed in the laboratory.

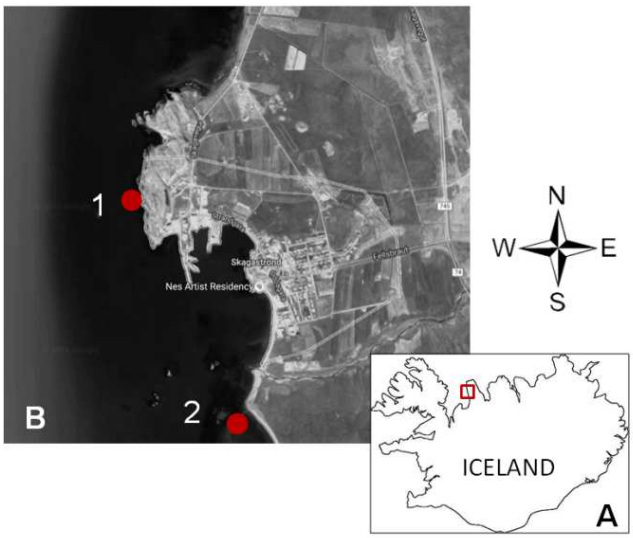


Figure 4. Sampling locations in the north-western part of Iceland neat Skagaströnd. A) Survey map; B) Sampling sites (1. rock pools, 2. tidal flat behind the stream)

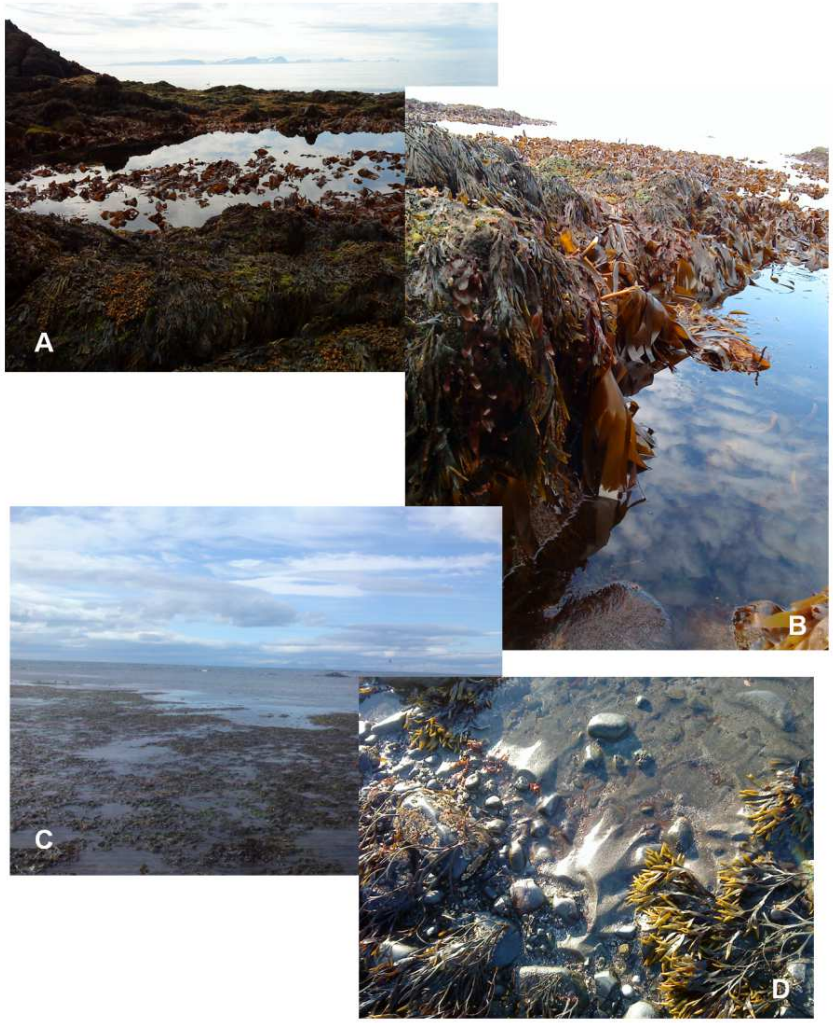


Figure 5. Seaweeds at the two different sampling locations A, B) rock pool, C, D) tidal flat.

3.1.2 Spore and Tip Isolation

Overall, three methods were used for the spore isolation of the different seaweed taxa. In the case of species of the order Laminariales (e.g., *Saccharina latissima*) the most important step in culture was the cleaning process of the collected material. Contaminated material (e.g., protozoans, fungi, other micro- and macroorganisms, or cyanobacteria) were found to overgrow the seaweeds in between weeks and finally led to the death of the plant. The cleaning process involved a series of rinses and wipes to remove microscopic contaminants on the surface of the blades. Sorus tissue was excised from all non-reproductive tissue and cut into manageable strips. Both sides were carefully and gently scraped with the edge of a sterile scalpel. This was followed by a series of rinsing and wiping down of the blades in a shallow tray and wiping with sterile paper towels, including a final rinse on both sides with a squirt bottle. After cleaning, the sorus tissue was placed between damp sterile paper towels and refrigerated overnight at 10°C in an autoclaved plastic bag. This step allowed the sorus tissue to undergo a gentle desiccation period to stimulate spore release with re-immersion in artificial seawater (Tropic Marin Reef Salt®, Aquarientechnik, Wartenberg, Germany; Fig. 6).

Figure 6. Sorus tissue preparation and spore release (Redmond et al. 2014).

The second method for spore isolation, which was used for *Dictyosiphon foeniculaceus, Vertebrata lanosa, Fucus, Cladophora, Odonthalia* and *Ulva* included the use of autoclaved Pasteur pipettes which were heated above a flame and pulled to a fine tip. This fine tip allowed individual spores to be picked up by using vacuum pressure created by placing the thumb over the end of the pipette to seal it, locate an individual spore under the dissecting microscope, carefully place the pipette adjacent to the spore and slowly release the thumb from the end of the pipette to allow the spore to be collected in the pipette. The spores were transferred into a single well of a 96 well culture plate with approximately 1 of sterile PES. The third method was used for *Palmaria*. During this procedure individual spores were isolated by the transfer of the spores into sterile shallow Petri dishes containing pieces of sterilized glass microscope slides. The spores settled onto the glass surface to grow, allowing an ease of transfer and manipulation.

3.1.3 Cultivation of Gameto- and Sporophytes

The three most important components of a culture system are seawater media (seawater and nutrients), temperature and light. In most cases the sporulation is close related to optimum temperature and light conditions. These optima are highly group specific and vary with geographical area. The optimum cultivation parameter for gameto- and sporophytes of different northern Icelandic representatives of Phaeo-, Rhodo- and Chlorophyceae are given in Table 1. While the cultivation of sporophytes was conducted in 2 L Erlenmeyer flasks, gametophytes were first grown in sterile Petri disks and later transferred into 50- and 100-mL flasks. Cultures were initially supplied in weekly intervals with fresh medium and later, after the movement to the new facility, in bi-weekly intervals. For medium preparation, the artificial seawater salt (Tropic Marin Reef Salt®) was dissolved in tap water (salinity of 30 and 32 Practical Salinity Units (=PSU), pH 8.2). For the light intensities, full spectra daylight bulbs (Phillips, Germany, Master TL-D 18W/840) were used. Conductivity, temperature, dissolved oxygen and pH were measured using handheld probes (YK-31SA, YK-200PATC, YK-200PCD and YK-2001PH, SI Model 33, Engineered Systems and Designs-Model 600, Philips W9424). Photosynthetic active radiation (PAR, 400–700 nm) was measured with an underwater spherical quantum sensor LI-193SA connected to a Licor Data Logger LI-250A.

Table 1. Optimum culture parameter for gametophyte and sporophyte culture systems adapted to northern Icelandic climate conditions.

Culture Conditions	**Gametophyte Phase**		**Sporophyte Phase**	**Additional Remarks**
	Release and settlement of Spores	Sporophyte formation	Juvenile Sporophytes	
Temperature [°C]	5-8	8-10	8-12	
Light [µmol photons m^{-1} s^{-1}]				
Phaeophyceae	200	500	500	Use of full spectra
Rhodophyceae	30	30	50	day light bulbs
Chlorophyceae	25	30	30	
Photoperiod (light:dark)				
Phaeophyceae	8:16	8:16	8:16	Use of timer
Rhodophyceae	8:16	12:12	12:12	
Chlorophyceae	8:16	12:12	12:12	
Salinity	30	30	32	
Seawater	artificial	artificial	artificial	Tropic Marin©
Nutrient Media				
Phaeophyceae	PES 0.5 strength	PES 0.75 strength	PES 0.75 strength	PES = Provasoli´s
Rhodophyceae	PES 0.25 strength	PES 0.5 strength	PES 0.75 strength	
Chlorophyceae	PES 0.5 strength	PES 0.5 strength	PES 0.75 strength	
Aeration level				
Phaeophyceae	low	medium	medium	Using a pump
Rhodophyceae	low	medium	strong	
Chlorophyceae	low	medium	medium	

3.1.4 Preservation and Identification of Seaweeds

For preservation and identification of seaweeds, specimens were washed in a tray containing fresh water (half filled) and were spread on paper sheets with the help of a brush to minimize the overlapping of the specimen. In the next step, the sheets were removed, and the seaweeds were properly arranged using forceps or needles if required. To blot dry, sheets were placed on carton sheets to remove the remaining water from the herbarium. Then a plastic cloth was placed on the top of the individual seaweed in such a way that it covered the entire specimen. After that, another sheet was placed over the herbarium sheet. Once, all the seaweeds were prepared, the herbaria were piled one above the other and then placed into a wooden press. After 24 h drying at room temperature the papers were replaced. This process was repeated until the seaweeds were free of moisture. This method still allows to extract DNA even after preservation. The following literature was used for identification of the taxa: Brodie & Irvine (2003), Dixon (2011), Fletcher (2011), Irvine (2011a, b), Burrows (2013), Christensen (2013) and Maggs (2013).

3.1.5 Determination of Growth

Growth rate was calculated for each species (replicated) by the increase in wet weight and presented as percentage growth per week using the formula of Penniman et al. (1986):

$$SGR = \% [(Gt/Go)1/t - 1] \times 100$$

SGR = specific growth rate (% in wet weight per week), Gt = weight after t weeks,
Go = initial weight, t = time in weeks

3.2 Experimental Designs

3.2.1 First Screening for Phlorotannins and Scavenging Activities

The screening experiments were conducted in five 17 L tanks in batches of one species per run in combination with four different PAR/UV sets and a PAR control, using field collected adult *Saccharina latissima, Ascophyllum nodosum, Alaria esculenta, Laminaria digitata* and *Fucus* sp (*F. evanescens*, *F. serratus* and *F. elongatus*). Field collected specimens were cleaned and acclimatized for one week to the conditions in the climate chamber prior to the experiments (cf. Figs. 7D-F). The culture parameter were adapted to the natural conditions the specimens were collected from and were the following: temperature 8-10 °C (depending on species); salinity 32 PSU using artificial seawater as described in 3.1.3; pH 8.4; 900 µmol photons m^{-1} s^{-1}; PES full strength; and 12:12 h PAR light to dark cycle with 6 hours supplementation of UVR during the light photoperiod. Nutrients were added every 7 days to prevent nutrient depletion. For the different light sets, five lamps from Exo Terra© (PT2225 Mini Compact Top Canopy, 30 cm, Figs. 6G-I) were used. The lamps were equipped with bulbs with different UV spectra (Fig. 8), each 25 W, such as Exo Terra©UVB100 PT2187 (Fig. 8A)**,** UVB150 PT2189 (Fig. 8B), UVB200 PT2341 (Fig. 8C), UVA Reptile Vision PT2346 (Fig. 8D) and natural light PT2191 (Fig. 8E) (Rolf C. Hagen Inc., Montreal, Canada).

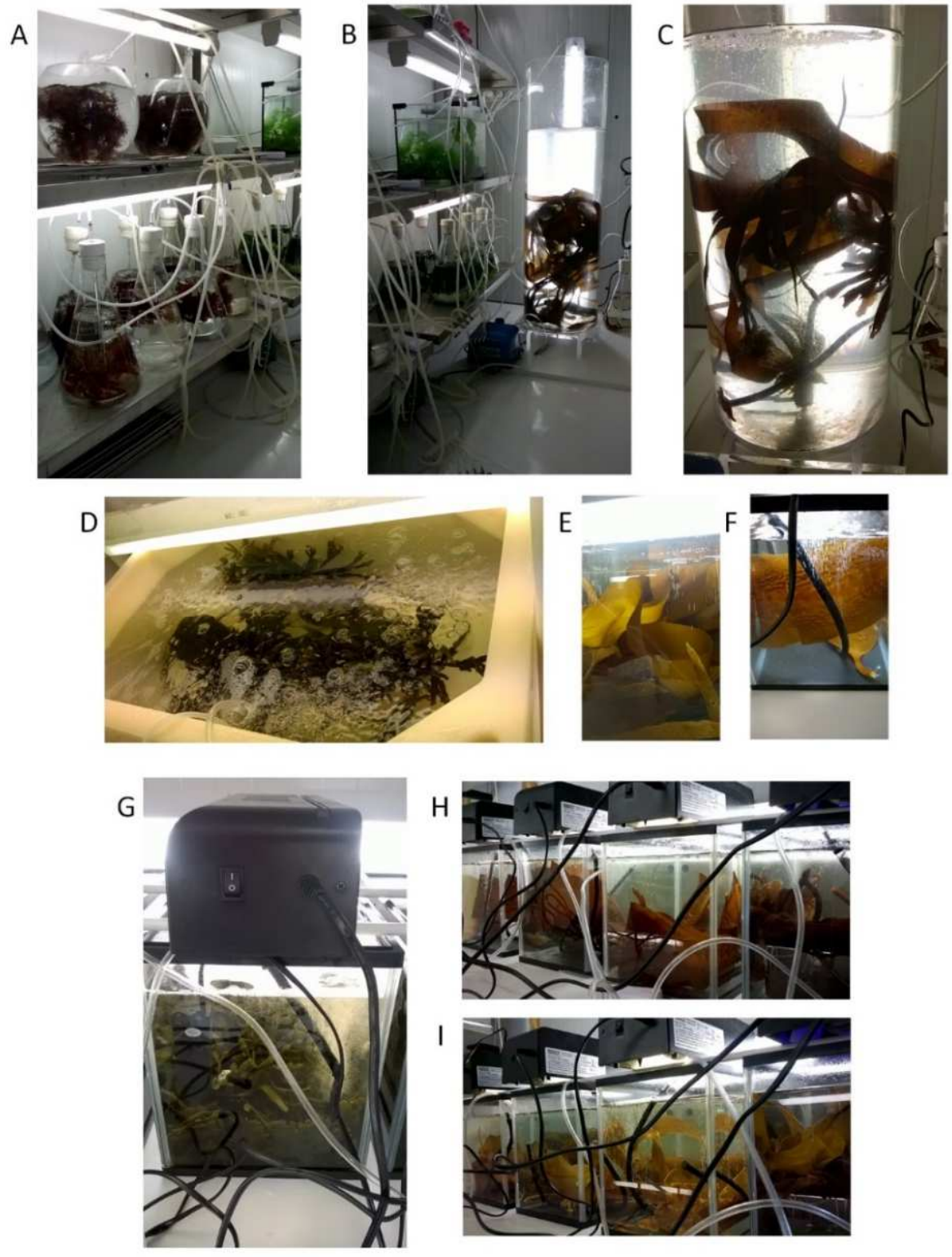


Figure 7. Pictures of the seaweed culture collection after the over-heating incident, showing new established red (A), green (B) and brown seaweeds in the climate chamber at BioPol laboratory in October 2018 (C) as well as cultures and specimens prior to the experiments using *Fucus* sp. (D), *Laminaria digitata* (E) and *Saccharina latissima* (F) collected from the field and acclimatized for one week to the conditions in the climate chamber. Overall, three examples of the experimental set up for WP 2 in 17 L tanks are depicted (G-I), showing *Fucus* sp. (G), *S. latissima* (H) and *L. digitata* (I).

UVR was measured using a handheld probe (OAI Model 308 UV Light Meter, OAI Instruments, Milpitas, U.S.A.). Specimens were weighted prior to the experiments and the trials were started by filling in 16 L fresh prepared medium and the installment of the light equipment as described above. Seawater and biomass samples were taken at t=0 h, t=168 h, t=336 h, t=504 h and t=672 h, processed as described in 3.3.1, and the total phenolic/phlorotannin content as well as antioxidant activities were analysed (cf. paragraphs 3.4.1 and 3.4.3). After 672 h incubation, all test and control assays were harvested and weighted to determine the growth (cf. 3.1.5).

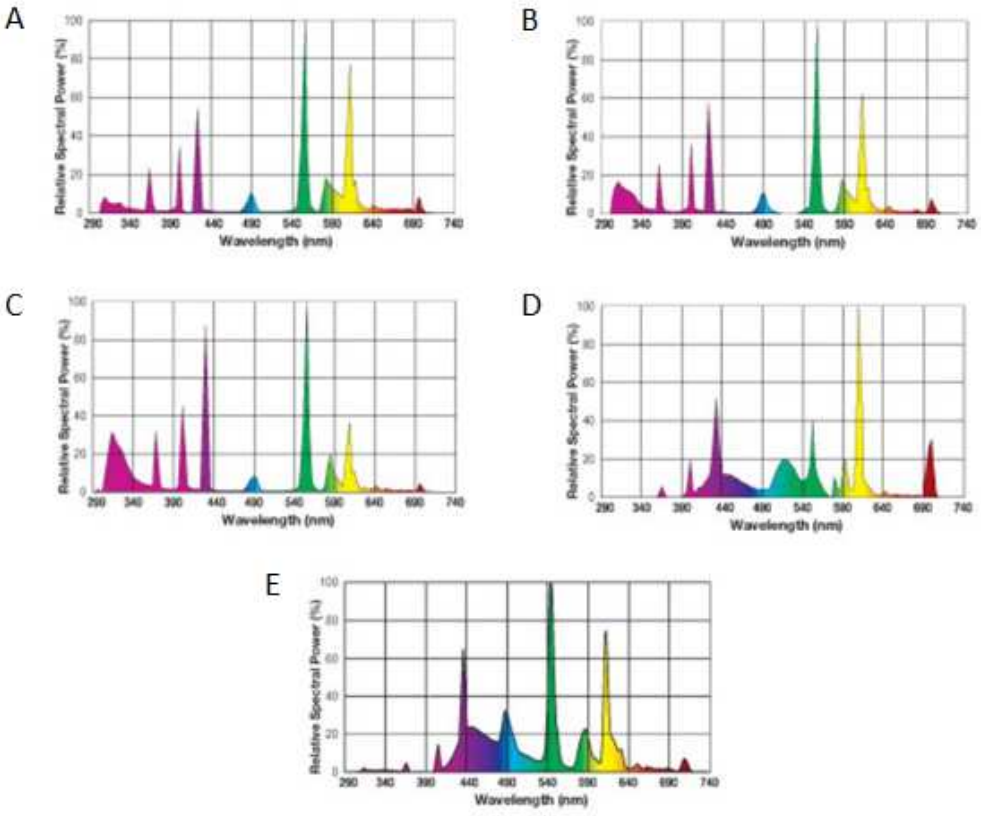


Figure 8. Spectrum of the combined UV and PAR bulbs from Exo Terra© (A, UVB100 PT2187; B, UVB150 PT2189; C, UVB200 PT2341; D, UVA Reptile Vision PT2346 and E, natural light PT2191) used during the first screening experiments. The UV-light equipment applied during the experiments is commonly used for terrarium illumination and was employed in this study due to a) safety issues, b) easy handling and purchase as well as c) already pre-assembled defined UV spectra which are similar to the natural light spectrum.

3.2.2 Increase of the Phlorotannin Yield by Variation of UV-Exposure

The optimized phlorotannin accumulation trials were conducted by variation of the duration of UV exposure times (12 h, 16 h and 24 h) and the use of different UV/PAR sources, utilizing in the first run field collected *Saccharina latissima, Ascophyllum nodosum, Laminaria digitata* and *Fucus evanescens*. Overall, three UV/PAR sets with different spectra were applied. The experiments were conducted in parallel in three 100 L bubble column reactors (for *S. latissima* and *L. digitata*) and a 200 L tank (100 L culture volume; for *A. nodosum* and *F. evanescens*) (cf. Figs. 10B, E, H). For illumination three Exo Terra© Dual Top Canopy PT2233 lamps, 90 cm (Rolf C. Hagen Inc., Montreal, Canada) were utilized, each equipped with two linear bulbs. As PAR standard an Exo Terra© NATURAL Light Linear PT2377 bulb

was used in each lamp (cf. Fig. 9A for the light spectrum), whereas the second bulb consisted of different UVR intensities which varied during each experimental run (Exo Terra© Reptile UVB100 PT2387, Fig. 9B; Reptile UVB150 PT2397, Fig. 9C and Reptile UVB200 PT2353, Fig. 9D; 25W - 75cm / 30").

Prior to the accumulation experiments, trials were conducted with the aim to optimize some of the analytical processes (e.g., drying of biomasses and concentration of exuded phlorotannins with selected adsorbents, cf. 3.3.2). The accumulation trials were started by weighting the specimens (4-8 per run), filling 100 L fresh prepared artificial seawater medium into the PBRs(cf. 3.4.1 for the culture conditions) and the installment of the light equipment described above. For the sampling of the seawater, nylon sachets were filled with 10 g of Amberlite XAD-7 (cf. 3.3.2) and used in all test assays as solid-phase adsorbents. Sachets were processed as described in 3.3.2, and the total phenolic/phlorotannin content as well as antioxidant activities were determined (cf. paragraphs 3.4.1 and 3.4.3). Biomass sub-samples were taken at t=0 h, t=168 h, t=336 h and t=504 h, processed as described in 3.3.2, and the total phenolic content (TPC) as well as antioxidant activities were analysed (cf. paragraphs 3.4.1 and 3.4.3). Samples from the t= 504 h assays were used for further optimizations of the analytical processes (cf. 3.3.3) as well as identification of the exuded phlorotannins (3.4.2). As control specimens were cultivated in 3 L flasks without UVR exposure. All specimens from the test and control assays were kept in culture and used in 3.2.3.

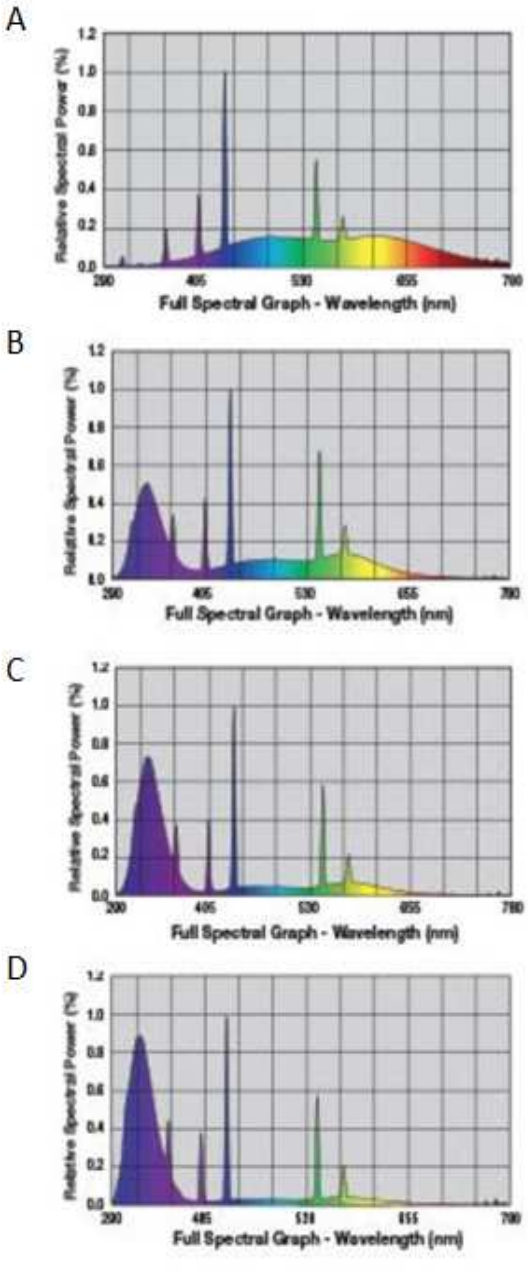


Figure 9. Spectra of the linear fluorescent bulbs from Exo Terra© used in the experiments with 100 L PBRs. NATURAL Light Linear PT2377 (A) Reptile UVB100 PT2387 (B), Reptile UVB150 PT2397 (C) and Reptile UVB200 PT2353 (D).

3.2.3 Effects of Organic Matter on the TPC yield

To simulate the co-culture of seaweeds with aquatic animals and to assess the impact of the organic fraction in aquacultural wastewater on the TPC yield in the seawater, overall, two species (*Saccharina latissima* and *Laminaria digitata*) were cultivated in three different concentrations of fish and shrimp wastewater as well as natural surface seawater (each 25, 50, 100%). The experiments were conducted in parallel in two 100 L bubble column PBRs. Ventilators were placed on one side of the experimental set up to prevent overheating of the test assays. The culture conditions were as follows: pH 8.4, salinity 32

Figure 10. Pictures showing the current culture collection of northern Icelandic seaweed species at the private external facility, with different Chloro- (A, F) and Rhodophytes (C, D, G) as well as *L*. *digitata* and *S*. *latissima* in 100 L PBRs (B, E) and *F*. *evanescens* in the 200 L tank during the experiments (B, E, H).

PSU (using Tropic Marin© Reef Salt to up salt the tap and wastewater), temperature 8-12 °C (depending on species); 900 µmol photons m^{-1} s^{-1}; PES full strength. A photoperiod of 12 h was chosen as exposure length to PAR with 6 h exposure to UVR, utilizing three Exo Terra© Dual Top Canopy PT2233 lamps, 90 cm (Rolf C. Hagen Inc., Montreal, Canada), each equipped with two linear bulbs. As PAR standard an Exo Terra© NATURAL Light Linear PT2377 bulb (spectrum Fig. 9A) was used in each lamp, whereas a Reptile UVB150 PT2397 bulb (spectrum Fig. 9C) was inserted as the second one. Nutrient concentrations as well as organic matter in the waste- and natural seawater were determined as described in paragraph 3.4.5. Turbidity was measured using a handheld probe (Trilux Fluorometer connected to a CTG HAWK, Chelsea Technologies, UK). As control, specimens were cultivated in 3 L flasks with UVR exposure. For the sampling of the liquid phase, nylon sachets were filled with 10 g of Amberlite XAD-7 (cf. 3.3.2.4.3) and used in all test assays as solid-phase phlorotannins adsorbents. After 504 h incubation time, all test and control assays were harvested and weighted to determine the growth (cf. 3.1.5). Sachets were processed as described in 3.3.2, and the total phenolic/phlorotannin content as well as antioxidant activities were determined (cf. paragraphs 3.4.1 and 3.4.3).

3.3 Sampling and Processing

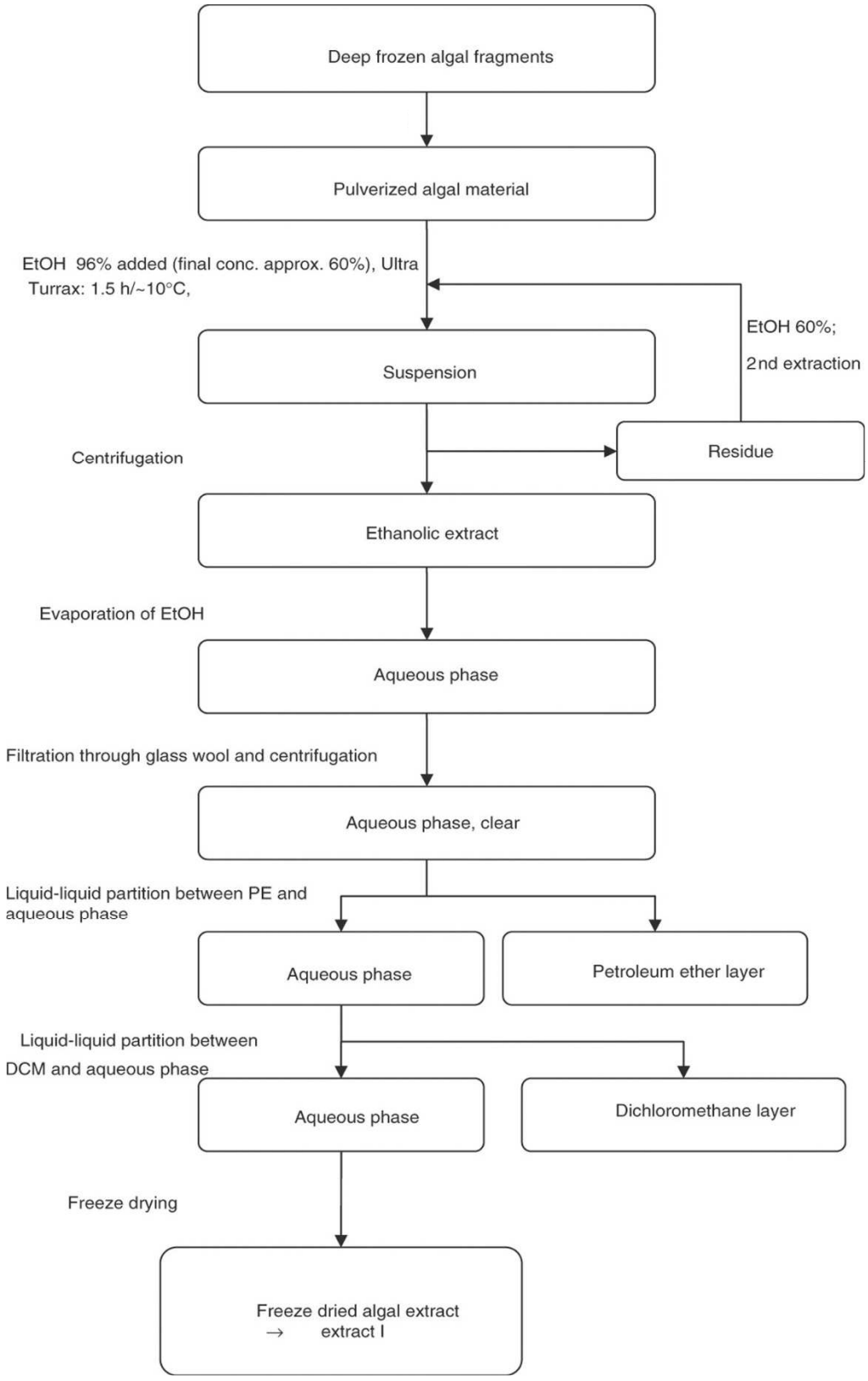


Figure 11. Scheme illustrating the initial procedure for obtaining extracts of the tested seaweed species in the first year.

3.3.1 Initial Procedure

Sub-samples of 1-2 g were cut from the tested specimens, whereas liquid samples (seawater) from the experiments were sampled with 50 mL centrifuge tubes (25 mL sample volume). Samples were lyophilized (Modulyo desk top lyophilisator 3981, Edwards High Vacuum Int, Sussex, England) and stored at -20°C until analysis. The phlorotannin extraction followed the method presented by Parys et al. (2009) in which *Ascophyllum nodosum* was studied.

Samples obtained from the first screening experiments were prepared according to the extraction procedure shown in Fig. 11. For this purpose, deep frozen lyophilized algal fragments from *Saccharina latissima, A. nodosum, Alaria esculenta, Laminaria digitata, Fucus evanescens*, *F. serratus* and *F. elongatus* were pulverized and extracted on ice with ethanol (96%) employing a dispersing machine (Ultra Turrax Ika T 25) for max. 1.5 h (extract I). The solid residue was removed by centrifugation and extracted a second time (extract II). After evaporation of ethanol under reduced pressure chlorophyll and lipophilic substances were removed by liquid-liquid partitioning three to nine times between petroleum ether or dichloromethane (each 300 ml). Subsequently, the aqueous phases were freeze-dried and stored at -20 °C until further analysis. In contrast to the process of the seaweed biomass, the lyophilized supernatants (seawater samples) from the experiments were only extracted twice on ice with ethanol (60%), centrifuged, evaporated and the aqueous phases were freeze-dried.

3.3.2 First Optimizations of the Initial Procedure

During pre-experiments to the effects of variations of UV exposure on the phlorotannin yield (3.2.2), different drying temperatures (30, 40, 50, 60 and 70°C) were tested regarding their effects on the total polyphenol/phlorotannin yield (TPC, 3.4.1) as well as total antioxidant activity (TAA, 3.4.3.1) from the biomass samples, utilizing a horizontal dryer with hot air flow (Tray dryer, model no. FDTHQQZ). In addition, different macroporous resins were tested for their ability to adsorb phlorotannins from the seawater and thereby concentrate the samples. Overall, six macroporous resins (Diaion HP-20, Sepabeads SP-850, Amberlite XAD-7, XAD-16N, XAD-4 and XAD-2) were tested to choose the one with the best TPC adsorption/desorption behaviour in seawater samples according to the methods presented by Kim et al. (2014).

Table 2. Physicochemical properties and kinetic parameters of phlorotannin adsorption on XAD-7, XAD-16N, HP-20, XAD-4, XAD-2 and SP-850 at 25 °C (from Leyton et al. 2017).

Physiochemical properties	**HP-20**	**SP-850**	**XAD-7**	**XAD-16N**	**XAD-4**	**XAD-2**
Structure	SDVB	SDVB	Acrylic ester	SDVB	SDVB	SDVB
Porosity [mL/g]	1.3	1.2	1.14	0.55	1.0	0.65
Surface area [m^2/g]	600	930	450	800	725	330
Pore radius [A]	260	38	90	200	20	90
Particle size [mm]	0.25-0.60	0.30-0.80	0.25-0.84	0.56-0.71	0.4-0.6	0.25-0.84

Abbreviations: SDVB, styrene divinyl-benzene (information obtained from provider)

The physicochemical characteristics of the resins are comprised in Table 2. All resins were washed with ethanol 70% at 25 °C for 12 h prior to use. Overall, 10 g of each pre-weighed resin was put into a nylon

sachet (mesh size 100 µm, 5 x 5 cm) and hang into 2 L Erlenmeyer flasks during the UV pre-experiments (12:12 h, spectrum Fig. 9D), utilizing field collected specimens of *Saccharina latissima, Ascophyllum nodosum, Laminaria digitata* and *Fucus evanescens.* After 168 h, the sachets were removed, and the resins filled into 500 mL beakers. Then 200 mL of an ethanol solution (80%) were added to the beakers containing the resins. The beakers were shaken (180 rpm) at 25 °C for 12 h to reach desorption equilibrium. After desorption, the solutions were filtered and the TPC was analysed. The recovery rates were calculated and presented as % phloroglucinol equivalents (PGEs) against the control (3.1.1).

During the subsequent experiments (cf. 3.2.2), 2.5 g of the chosen resin (XAD-7) were harvested at t=0 h, t=168 h, t=336 h and t=504 h, placed into 250 mL beakers and 40 mL of an ethanol solution (80%) were added. As before, the beakers were shaken (180 rpm) at 25 °C for 12 h and after desorption, the solutions were filtered and the total phenolic/phlorotannin content (TPC) as well as antioxidant activities were determined (cf. 3.4.1 and 3.4.3). In addition, the t=504 h samples were used after dialysis of the solutions (3.3.3.5) for qualitative analysis of the phlorotannins (3.4.2).

3.3.3 Further Optimizations of the Initial Procedure

The most commonly used methods for the purification of crude extracts of phlorotannins are liquid-liquid or solid-liquid separation based on the polarity of the molecules (Kubanek et al. 2004, Cérantola et al. 2006, Zubia et al. 2009), and discrimination of molecular size through dialysis (Arnold & Targett 1998, Breton et al. 2011, Le Lann et al. 2012, Tierney et al. 2013). The use of macroporous resins for the purification of phlorotannins from biomasses is proposed as an environmentally friendly alternative due to the use of food-grade organic solvent, low cost due to the reuse of the resins, and safety due to their compatibility with food products (Leyton et al. 2017). Thus, subsequently to the phlorotannin accumulation trials (3.2.2), a number of optimization trials considering different extraction solvents (3.3.2.1, 3.3.2.2), supportive extraction techniques (3.3.2.3) as well as purification and fractionation of the phlorotannins by solid-phase methods (3.3.2.4) were conducted utilizing the collected biomass samples of *Saccharina latissima, Ascophyllum nodosum, Laminaria digitata* and *Fucus evanescens*.

3.3.3.1 Lipid Removal prior to Extractions

To maximize the efficiency of the phlorotannin extraction from the biomass samples, lipids were removed from aliquots of 0.5 g of powdered material with n-hexane prior to extraction according to the method given by Koivikko et al. (2005). The n-hexane treatment was repeated three times with 1 mL of solvent followed by centrifugation (5 minutes at 4000 rpm, 300 × g). After evaporation, the extracts were subjected to different solvent extractions.

3.3.3.2 Solvent Extractions

3.3.3.2.1 Water

Extraction of phlorotannins by water was performed using different solid (sample powder):liquid ratios and temperatures according to the methods given by Machu et al. (2015). In the first procedure a solid:liquid ratio of (1:100) was used. Briefly, 10 mL of distilled water were added to 0.1 g of either seaweed or supernatant sample powder from the first experiment in a 20-mL glass bottle with screw cap. The bottle was covered with foil and put in a heating block at 70 °C for one hour with constant shaking at 700 rpm. A second assay was carried out at a solid:liquid ratio of (1:10). 5 mL of distilled

water were added to 0.5 g sample in a 10-mL glass bottle with screw cap. Then the bottle was covered with foil and put in a shaker incubator at 65 °C for one hour with constant shaking at 150 rpm. After that the samples from both experiments were centrifuged (3890 x g, 10 min, 4 °C) to separate the supernatants from the residues. The supernatants were freeze-dried and kept in a freezer at -20 °C until further analysis.

3.3.3.2.2 Ethanol

Extraction of phlorotannins from the dried biomass samples by ethanol was performed using three different aqueous concentrations (30%, 50% and 80% v/v).

The procedure with 30% ethanol (v/v) was conducted according to the method given by Fu et al. (2017) and carried out with a solid:liquid ratio of 1:5 in separate glass bottles with screw cap. After introducing the samples to the ethanol, the bottles were covered with foil and placed in a shaker incubator at 25 °C for 30 min with constant shaking at 150 rpm. Subsequently, the samples were centrifuged (3890 x g, 10 min, 4 °C) and the supernatant was separated from the residues. Then, the ethanol was removed from supernatants by evaporation and the supernatants were freeze-dried and kept frozen at -20 °C until further analysis.

Phenolic compounds from the seawater were extracted with an ethanol/water (50/50, v/v) mixture in the dark at 4°C for 6 h under rotary agitation (60 rpm). Samples were centrifuged at 6500 × g and evaporated at 30 °C under vacuum.

The procedure with 80% ethanol (v/v) was carried out at a solid:liquid ratio of (1:10). Five millilitres of ethanol (80%, v/v) was added to 0.5 g sample in a 100-mL glass bottle with screw cap. Then the bottle was covered with foil and put in the shaker incubator at 25 °C for 20 h and then at 65 °C for 5 h with constant shaking at 150 rpm. Then the sample was centrifuged (3890 x g, 10 min, 25 °C) and the supernatant was separated from the solid part and collected. Another 50 mL of ethanol (80%, v/v) was added to the solid part and the bottle was covered with foil and put in the shaker incubator at 65 °C for extra 5 h with constant shaking at 150 rpm. After that the sample was centrifuged at (3890 x g, 10 min, 4 °C), the supernatants from both steps were mixed and, after removal of ethanol by evaporation, the supernatants were freeze-dried and kept in a freezer (-20 °C) until further analysis.

3.3.3.2.3 Acetone

Defatted material was extracted four times with 10 mL of acetone:water (7:3), for 1 hour at 400 rpm, followed by centrifugation for 5 minutes at 4000 rpm (Lopes et al. 2012). The organic fractions were combined and evaporated to dryness under reduced pressure, at 30°C.

3.3.3.3 Supportive Extraction Techniques

Ethanol/water (80%) mixtures were subjected to novel extraction techniques including

- ultrasound-assisted extraction (UAE),
- microwave-assisted extraction (MAE),
- ultrasound–microwave-assisted extraction (UMAE),
- hydrothermal-assisted extraction (HAE) and
- high-pressure-assisted extraction (HPAE).

All extraction procedures were performed in duplicate and repeated twice (n = 4). UAE treatments were performed using a semi-industrial UIP500hdT 26 kHz (Hielscher Ultrasound Technology, Teltow, Germany) at 100% ultrasonic amplitude. MAE was performed in a microwave oven (Panasonic NN-

CF778S0, Bracknell, UK; 2450 MHz) at 250 W. UMAE was performed by coupling the devices, UIP500hdT 26 kHz and Panasonic NN-CF778S0 2450 MHz, keeping the ultrasonic power (100%) and microwave power (250 W) constant through the extraction process. HAE was performed using an autoclave (Panasonic, MLS-3020U, Japan) at 100 °C and 15 psi. HPAE was performed in the HPP Tolling facility (Dublin, Ireland) using a 200 L Hiperbaric HPP (Hiperbaric, Burgos, Spain) at 600 MPa.

3.3.3.4 Solid-Phase Purifications

All extracted liquid samples from solvent extractions were filtered through 0.2 µm polypropylene syringe filters before purification.

3.3.3.4.1 Cellulose

The method described by Fairhead et al. (2005) modified by Lopes et al. (2012) was applied. Briefly, the dried acetone:water extract was resuspended in 30 mL methanol, adsorbed into cellulose (approximately 2× the residue weight) and then dried under reduced pressure, at 30°C. This mixture was washed with toluene to remove pigments, until the filtrate run clear. Cellulose was rinsed with acetone:water (7:3) to release the phlorotannins. After centrifugation (5 minutes at 4000 rpm), the purified extract was evaporated to dryness under reduced pressure, at 30°C. The purified samples were stored at -20 °C until further analysis.

3.3.3.4.2 SPE Cartridges

In addition, a two-step purification was tested using SPE cartridges according to the method given by Gall et al. (2015). The first purification step was done using the Sep-pak-C18 SPE end capped cartridge (10 g sorbent mass, 55–105 µm, Waters), which was initially conditioned with a full bed volume of 100% acetonitrile and 0.25% v/v aqueous acetic acid. The filtered aqueous sample was loaded onto the column and allowed to flow through under gravity. After that the co-extracted compounds such as mannitol and other polar compounds were eluted by 100 mL of 0.25% v/v aqueous acetic acid. Then the phenolic compounds were eluted using approximately 200 mL of acetonitrile:water (80:20%, v/v) containing 0.25% v/v acetic acid. After that, the acetonitrile in the collected fractions was removed by evaporation and the remaining liquid fraction was filtered through 0.2 µm polypropylene syringe filters before the second purification step. The second purification step was carried out using DSC-18 SPE cartridge (Supelco) with the same protocol as above. After removing acetonitrile from the collected fraction of second purification step, the fraction was freeze-dried and kept at -20 °C until further analysis.

3.3.3.4.3 Adsorbents

Based on the results presented by Leyton & Lienqueo (2017) Amberlite XAD-4 and XAD-7 were used for TPC purification from the defatted biomass samples. The resins were washed with ethanol 70% at 25 °C for 24 h prior to use. For the phlorotannin purification 2 g of the resins were put into tubes with 30 mL of the ethanolic phlorotannin biomass extracts. Phloroglucinol (PGE) was used as standard control. The tubes were shaken at 300 rpm, at 25 °C to reach adsorption equilibrium. According to Leyton et al. (2017) the best desorption of phlorotannins was achieved with ethanol at 90% v/v for both resins; hence this concentration was employed for the desorption of the phlorotannins. Subsequently, 30 mL of solvent were added to the resins, and shaken at 300 rpm and 25 °C to reach desorption equilibrium. After desorption, the supernatants were filtered and the TPC in the extracts were measured (3.4.1).

3.3.3.5 Dialysis

The phenolic pool of the aqueous ethanolic extracts from each species at the end of the optimization trials (cf. 3.2.2) were fractionated in function of their sizes as described in Le Lann et al. (2012), utilizing three successive dialysis steps (corresponding to three size classes of cuts) on molecular weight cut off (MWCO) 2000 Da, 5000 Da and 12–14,000 Da cellulose membranes (Spectra/Por dialysis tubing, Spectrum Europe, Breda, The Netherlands). The steps were carried out in the dark at 4°C. Between each dialysis, an aliquot of the fraction was taken to determine the phenolic content using the Folin-Ciocalteu procedure (cf. 3.4.1). For each fraction, results were expressed in % of the total phenolic content of the extract.

3.4 Analysis

If not otherwise mentioned all chemicals used in this project were of highest purity and purchased from Sigma-Aldrich©.

3.4.1 Total Phenolic/Phlorotannin Contents (TPC)

Total phenolic content of the seaweed extracts was determined by the Folin–Ciocalteu (F-C) (Fig. 12) assay described by Wang et al. (2012) with the modifications given by Li et al. (2015). The TPC analysis was conducted from extracted samples. An aqueous solution with a concentration of 1 g/L of the lyophilized extract was prepared and filtered through 0.2 µm syringe filters. Then 0.2 mL of the prepared solution from the lyophilized extract or the liquid supernatant was added to 1.3 mL of distilled water and 0.5 mL of Folin–Ciocalteu's phenol reagent. After mixing, 1 mL of 7.5% (w/v) Na_2CO_3 was added to the solution. The solution was mixed on a vortex mixer and incubated for 1 h at 18 °C in darkness. Then, the absorbance of the sample was measured at 770 nm using a UV/Vis spectrophotometer (ATI Unicam 5625 UV/VIS Spectrometer, Richmond Scientific Ltd., Chorley, UK). The compound phloroglucinol was used as standard and a standard curve was made with serial phloroglucinol dilutions (5–100 µg/mL). The result was obtained as milligrams of phloroglucinol equivalents (PGEs) per gram extract.

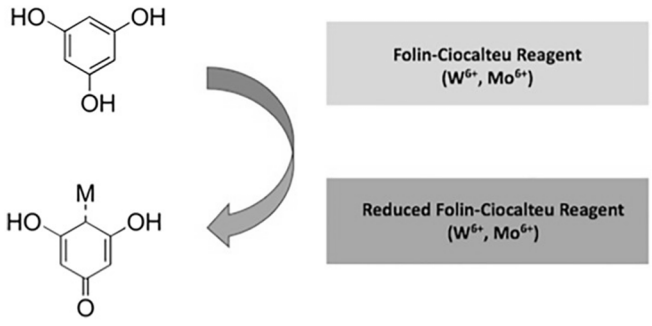


Figure 12. Diagram showing the reduction of the Folin–Ciocalteu reagent caused by the oxidation of the phenolics in a sample. This assay relies on the transfer of electrons from phenolic compounds to phosphomolybdic/ phosphortungstic acid complexes in alkaline conditions (from Ford et al. 2019).

3.4.2 Qualitative Phlorotannin Analysis

The analysis of phlorotannins was conducted according to the method described by Sardari et al. (2021). In brief, all samples were injected into a Thermo Scientific Accela UHPLC system equipped with an Accela Autosampler, an Accela 600 pump, and an Accela PDA detector (Thermo Scientific, Waltham, MA, USA). The UHPLC system was connected to an LTQ Orbitrap Velos Pro mass spectrometer equipped with a heated electrospray ionisation source (Thermo Scientific). An ACQUITY CSH Phenyl-hexyl UPLC column (2.1 mm x 100 mm, 1.7 µm, 130 Å) protected with an ACQUITY UPLC VanGuard pre-column (CSH Phenyl-hexyl (2.1 mm x 5 mm, 1.7 µm, 130 Å) were purchased from Waters (Milford, MA, USA). For centrifugation of the samples a 5424R Eppendorf centrifuge (Eppendorf, Hamburg, Germany) was used.
A Xcalibur 2.2 (Thermo Fisher Scientific) was used to operate the UHPLC/MS system and for data acquisition. The open-source software MZmine 2 and Xcalibur 2.2 were used for MS data evaluation. The lyophilized samples were dissolved in water/acetonitrile (95/5, v/v) with a final concentration of 10 mg/mL. Then the samples were centrifuged for 10 min at 14.000 rpm and 20 °C and then, five microliters of each sample were injected. After each injection the syringe, injection needle and the injection transfer tubes were flushed with 1 mL with an acetonitrile/water (50/50, v/v) flush solution. As mobile phase solvent (A) water and (B) acetonitrile/water (95/5, v/v), both containing 10 mM ammonium formate were used. A gradient elution was performed starting with 5% B at 0 min, and was then hold up to 15 min, then ramped up to 30% B until 30 min, then ramped up to 100% B until 35 min, then hold up 47 min. Then the mobile phase composition was ramped down to 5% B until 48 min and hold until 58 min to equilibrate the column for the next injection. The column temperature was 40 °C and a flow rate of 0.3 mL/min was used. UV/Vis spectra were collected at 210 nm, 254 nm and 600 nm, each with a filter band width of 9 nm and a sample rate of 20 Hz. Spectral data were collected with a photo diode array (PDA) detector from 200 nm to 600 nm using a wavelength step of 1 nm, a sample rate of 20 Hz and a filter band width of 9 nm. For the ionisation a heated electrospray ionisation source was used in negative mode, using a spray voltage of 3 kV, a heater temperature of 275 °C, a sheath gas flow of 70 (arbitrary units), an auxiliary gas flow of 10, a sweep gas flow rate of 0, a capillary temperature of 275 °C, and a source fragmentation voltage of 0 V. The mass spectrometer was used in data dependent MS2 mode, performing a MS2 for the three most abundant ions in every scan event. Each MS1 scan event was performed in the Orbitrap mass analyser with a MS resolution of 100.000 and every MS2 scan event was performed using the linear quadrupole ion trap. The m/z scan range was set to m/z 120 to 1500. Collision induced dissociation was performed using a default charge state of 2, activation time of 10 ms, an isolation with of m/z 2.0, an activation q of 0.250 and a normalized collision energy of 35%. Before every sample batch the mass spectrometer was calibrated using an external calibration standard. To create sample peak lists and to determine chemical formulas, including C, H and O, and the ring double bond (RDB) equivalents MZmine 2 was used.

The identification confidence level system introduced by Schymanski et al. (2014) was used to categorize the identification confidence of identified phlorotannins. The system by Schymanski et al. consists in brief of five identification confidence levels, where level 1 represents the highest identification confidence and level 5 the lowest. Level 1 is given if an identified compound can be compared with a reference standard with respect to retention time, m/z and MSn fragmentation (Sardari et al. 2021).

3.4.3 Scavenging Activity

3.4.3.1 DPPH Radical Scavenging Activity

The DPPH was determined according to Tierney et al. (2013) with slight modifications presented by Li et al. 2017. Prior to analysis, a working DPPH solution (0.10 $mg \cdot mL^{-1}$) and appropriate serial of the sample solutions were prepared using ethanol. In all experiments, Trolox, a synthetic antioxidant analogous to vitamin E, was used as the standard. The standard or sample (500 µL) and DPPH working solution (500 µL) were added to a quartz cuvette and then left in the dark for 30 min at room temperature (18 °C). The absorbance was measured at 515 nm. The decrease in absorbance of the sample extract was calculated by comparison to a control. where sample A is the absorbance of the test sample (500 µL DPPH and 500 µL sample), sample A blank is the absorbance of the sample only (500 µL sample and 500 µL ethanol), A control is the absorbance of the control (500 µL DPPH and 500 µL ethanol), and A control blank is the absorbance of the ethanol only (1000 µL ethanol). The IC50 value was calculated as the concentration of sample or standard antioxidant ($\mu g \cdot mL^{-1}$) requiring scavenging 50% of the DPPH in the reaction mixture.

3.4.3.2 Ferric-reducing Antioxidant Power (FRAP)

FRAP assay was modified from that reported by Tierney et al. (2013). The oxidant in the FRAP assay consisted of a reagent mixture that was prepared by mixing acetate buffer (pH 3.6), ferric chloride solution (20 mM), and 2,4,6-tripyridyl triazine (TPTZ) solution (10 mM TPTZ in 40 mM HCl) in the ratio of 10:1:1. A Trolox stock solution (2 mM) was prepared and diluted with ethanol to give concentrations ranging from 0.1 to 0.5 mM. Prior to analysis, the FRAP reagent was heated and protected from light, until it had reached a temperature of 37 °C. 180 µL of freshly prepared FRAP reagent at 37 °C was pipetted into quartz cuvettes with either a 20 µL test sample or standard (or ethanol for the blank). The absorbance was measured at 593 nm after 50 min incubation at 18°C. The Trolox standard curve was used to calculate the antioxidant activity of the samples which was expressed as milligram Trolox equivalents (TE) per milligram of sample ($mg\ TE \cdot mg^{-1}$).

3.4.4 Nutrient and Organic Matter Analysis

Samples for inorganic nutrient analyses were collected unfiltered in duplicate polyethylene 12 ml tubes and stored at −20°C until analysis. Concentrations of phosphate (PO_4^{3-}), ammonium (NH_4^+), nitrate (NO^{3-}), and nitrite (NO^{2-}) were determined using colorimetric methods according to the procedures given by Grasshoff et al. (1983)

Samples (3 L) for particulate organic carbon (POC), particulate organic nitrogen (PON) and particulate organic phosphorus (POP) were filtered onto pre-combusted (450°C for 4 h) Whatman GF/F filters and stored at −20°C until analysis. POP filters were rinsed with 5 ml of 0.17 M Na_2SO_4 to remove traces of dissolved phosphorus from the filter. All filters were stored in pre-combusted aluminium packets. Particulate Organic Carbon/Nitrogen: Prior to analysis, the filters for POC and PON were dried according to the JGOFS protocol (Knap et al. 1996). The protocol has a detection range of 0.43-43.13 µM for POC and 0.037-7.39 µM for PON in sea water (Knap et al. 1996). First, the filters were dried in an incubator at 55°C for 24-48 h and then stored in a desiccator with concentrated HCl fumes for 24 h to remove inorganic carbonates. Secondly, the filters were dried again at 55°C for 48 h before being folded and packed into pre-combusted tin capsules. The packaged filters are analysed on a CN FlashEA 1112 Elemental Analyzer (Thermo Scientific) against an atropine standard curve (chemical formula $C_{17}H_{23}NO_3$). Particulate Organic Phosphorus: Particulate organic phosphorus (POP) were analysed according to a modified ash-hydrolysis protocol (Lomas et al. 2010). Thawed filters were placed in along with a corresponding standard curve of KH_2PO_4. 2 mL of 0.017M $MgSO_4$ was added to the acid-washed glass

vials containing filters and covered with pre-combusted aluminium foil. The vials were placed in an incubator at 90°C for 24 h and then combusted (500°C, 2 h). Once cooled, 5 mL 0.2 M HCl was added and incubated at 90°C for at least 30 min. Next, the supernatant plus 5 mL milli-Q water was mixed with 2:5:1:2 parts ammonium molybdate tetrahydrate, 5N sulfuric acid, potassium antimonyl tartrate, and ascorbic acid for 30 min. Finally, the standards and samples were analysed on a spectrophotometer at a wavelength of 885 nm to determine POP concentration with an assay detection limit ~0.1 nmol l^{-1}.

3.4.5 Statistical Analysis

Measures were carried out in triplicate (n = 3), and the results are given as mean values and standard deviations. Data were analysed by using SPSS software (Statistical Package for the Social Sciences) (version 18 for Windows). One-way analysis of variance (one-way ANOVA), using the Dunnett Multiple Comparison test, was carried out on data obtained from four assays performed in duplicate for each sample. A level of statistical significance at $p<0.05$, $p<0.01$ and $p<0.001$ was used.

4. Results

4.1 First Screening for TPC and Scavenging Activities

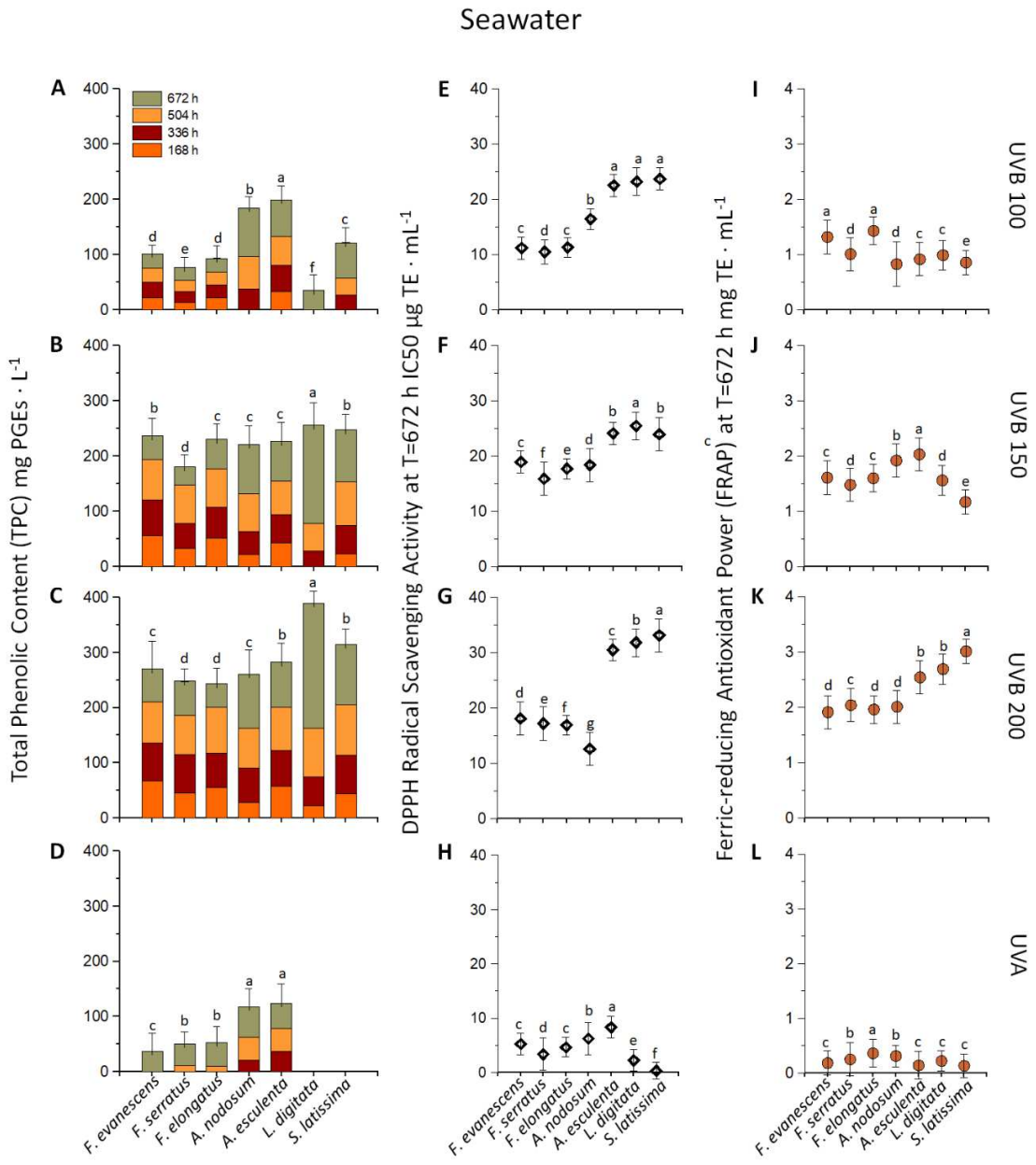


Figure 13. First part of the results of the first screening experiments, utilizing seven field collected seaweed species (*Saccharina latissima, Ascophyllum nodosum, Alaria esculenta, Laminaria digitata, Fucus evanescens*, *F. serratus* and *F. elongatus*) and four PAR/UV sets. Depicted are the results for the TPC (A-D), DPPH radical scavenging activity (EC_{50}, E-H) and the ferric reducing antioxidant power (FRAP) assays (I-L) recorded for the seawater extracts prepared as described in 3.3.1. Data are means ± SD (n=3). TPC was measured for t=168 h, 336 h, 504 h and 672 h samples, whereas DPPH and FRAP were assessed from t= 672 h samples only. Different letters indicate significant difference among treatments.

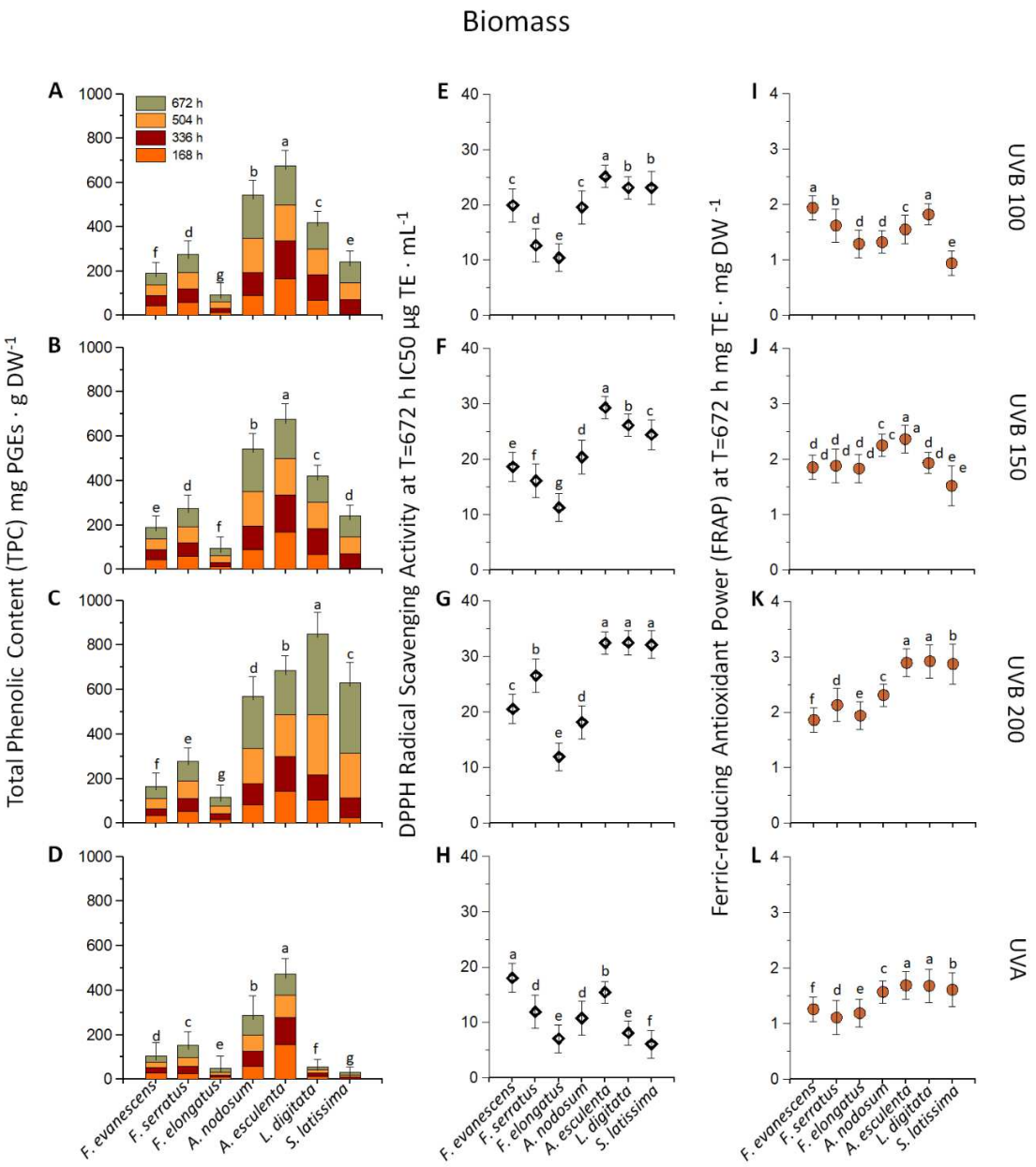


Figure 14. Second part of the results of the first screening experiments, utilizing seven field collected seaweed species (*Saccharina latissima, Ascophyllum nodosum, Alaria esculenta, Laminaria digitata, Fucus evanescens*, *F. serratus* and *F. elongatus*) and four PAR/UV sets. Depicted are the results for the TPC (A-D), DPPH radical scavenging activity (EC_{50}, E-H) and the ferric reducing antioxidant power assays (I-L) recorded for the biomass extracts prepared as described in 3.3.1. Data are means ± SD (n=3). TPC was measured for t=168 h, 336 h, 504 h and 672 h samples, whereas DPPH and FRAP were assessed from t= 672 h samples only. Different letters indicate significant difference among treatments.

Overall, seven field collected brown seaweed species (*Saccharina latissima, Ascophyllum nodosum, Alaria esculenta, Laminaria digitata, Fucus evanescens*, *F. serratus* and *F. elongatus*) were sub-cultured in 17 L tanks equipped with lamps employing different UV radiations (UVB100, 150, 200 and UVA) to test the best UV/PAR combination to facilitate the accumulation and release of phlorotannins by the test specimens. As indicator for the success total phenol/phlorotannin contents (TPC), DPPH radical scavenging activities and the ferric reducing antioxidant power (FRAP) measured in ethanolic extracts

(96%) of seawater and biomass samples after exposure at t=168 h, 336 h, 504 h and 672 h. The results presented in Figures 13 and 14, show high variability between species and applied UVR. The highest TPC was recorded for *L. digitata* in the seawater samples after 672 h exposure to UVB200 with 226.3 ± 8.6 mg PEGs · L^{-1}, whereas the highest FRAP and DPPH activities were detected in *S. latissima* with 3.01 ± 0.22 mg TE · mL^{-1} and 33.11 ± 3.1 µg TE · mL^{-1}, respectively (Figs. 13C, G, K). Specimens were not able to regenerate after this long exposure time regardless the species. Therefore, further experiments were conducted with a shorter experimental length (504 h, cf. 4.2).

4.2 Increase of the Phlorotannin Yield by Variation of the UV-Exposure

Prior to the experiments the effects of different drying temperatures on the phlorotannin yield in the biomasses after harvest as well as the use of different macroporous resins for adsorbing phlorotannins from the surrounding seawater were tested, utilizing initially filed collected specimens of *S. latissima, A. nodosum, L. digitata,* and *F. evanescens*.

Table 3. Effect of different drying temperatures on the phlorotannin yield [% from the control] obtained from the biomasses of the four tested seaweed species.

	Unit	*F. evanescens*	*A. nodosum*	*S. latissima*	*L. digitata*
30°C	[%]	48.3 ± 1.5^{d}	49.7 ± 1.0^{d}	51.3 ± 1.6^{e}	50.5 ± 0.3^{d}
40°C	[%]	54.4 ± 0.8^{c}	66.4 ± 1.4^{c}	72.4 ± 1.7^{c}	70.9 ± 2.4^{c}
50°C	[%]	83.9 ± 1.1^{a}	85.3 ± 0.9^{a}	82.9 ± 1.9^{b}	76.1 ± 1.1^{b}
60°C	[%]	79.5 ± 2.5^{b}	80.3 ± 1.0^{b}	86.5 ± 2.3^{a}	88.4 ± 1.5^{a}
70°C	[%]	46.2 ± 1.2^{d}	66.1 ± 1.9^{c}	58.2 ± 2.2^{d}	53.7 ± 1.7^{d}
TPC Control*	[mg · L^{-1}]	58.3 ± 1.0	77.9 ± 2.2	126.3 ± 0.9	153.3 ± 1.8

* Lyophilized samples.

Abbreviations: TPC = Total Phenolic/Phlorotannin Content. Different letters indicate significant difference among treatments.

Table 4. Recovery rate [%] of adsorbed phlorotannins from the artificial seawater samples after 168 h exposure to UVR, utilizing different macroporous resins.

	Control*[1] [mg · L^{-1}]	**HP-20** [%]	**SP-850** [%]	**XAD-7** [%]	**XAD-16N** [%]	**XAD-4** [%]	**XAD-2** [%]
F. evanescens	283.6 ± 2.3^{a}	76.4 ± 0.8^{e}	76.9 ± 1.1^{d}	96.3 ± 1.5^{a}	82.2 ± 2.1^{d}	89.2 ± 0.9^{d}	88.5 ± 1.5^{c}
A. nodosum	279.3 ± 1.9^{b}	80.1 ± 0.6^{b}	79.4 ± 1.7^{b}	95.8 ± 1.3^{b}	80.5 ± 0.7^{e}	90.1 ± 1.1^{b}	85.7 ± 2.9^{e}
S. latissima	199.3 ± 2.0^{d}	79.3 ± 1.3^{c}	79.0 ± 0.2^{b}	92.2 ± 1.0^{d}	85.3 ± 1.6^{b}	91.3 ± 0.7^{a}	89.4 ± 1.2^{b}
L. digitata	225.7 ± 2.1^{c}	78.5 ± 1.4^{d}	77.5 ± 0.8^{c}	94.1 ± 0.3^{c}	83.0 ± 2.0^{c}	89.9 ± 2.3^{c}	87.3 ± 0.5^{d}
Control*[2]	199.3 ± 0.1^{e}	82.7 ± 0.5^{a}	80.1 ± 1.3^{a}	98.7 ± 0.5^{c}	89.5 ± 1.5^{a}	90.7 ± 1.0^{b}	90.6 ± 0.8^{a}

*[1] TPC of known (PEG) and unknown phlorotannins (3.4.1) utilizing the standard method described in 3.3.1.

*[2] Known amount of PEG in artificial seawater (200 mg · L^{-1}].

Abbreviations: TPC = Total Phenolic/Phlorotannin Content. Different letters indicate significant difference among treatments.

The pre-experiments revealed that a drying temperature of 60°C was only favourable for biomass samples of *S. latissima* and *L. digitata*, whereas for samples from *A. nodosum* and *F. evanescens* a drying temperature of 50°C showed optimal results (Table 3). The use of adsorbents as tool to concentrate the phlorotannins in the seawater showed the best results for Amberlite XAD-7 (Table 4).

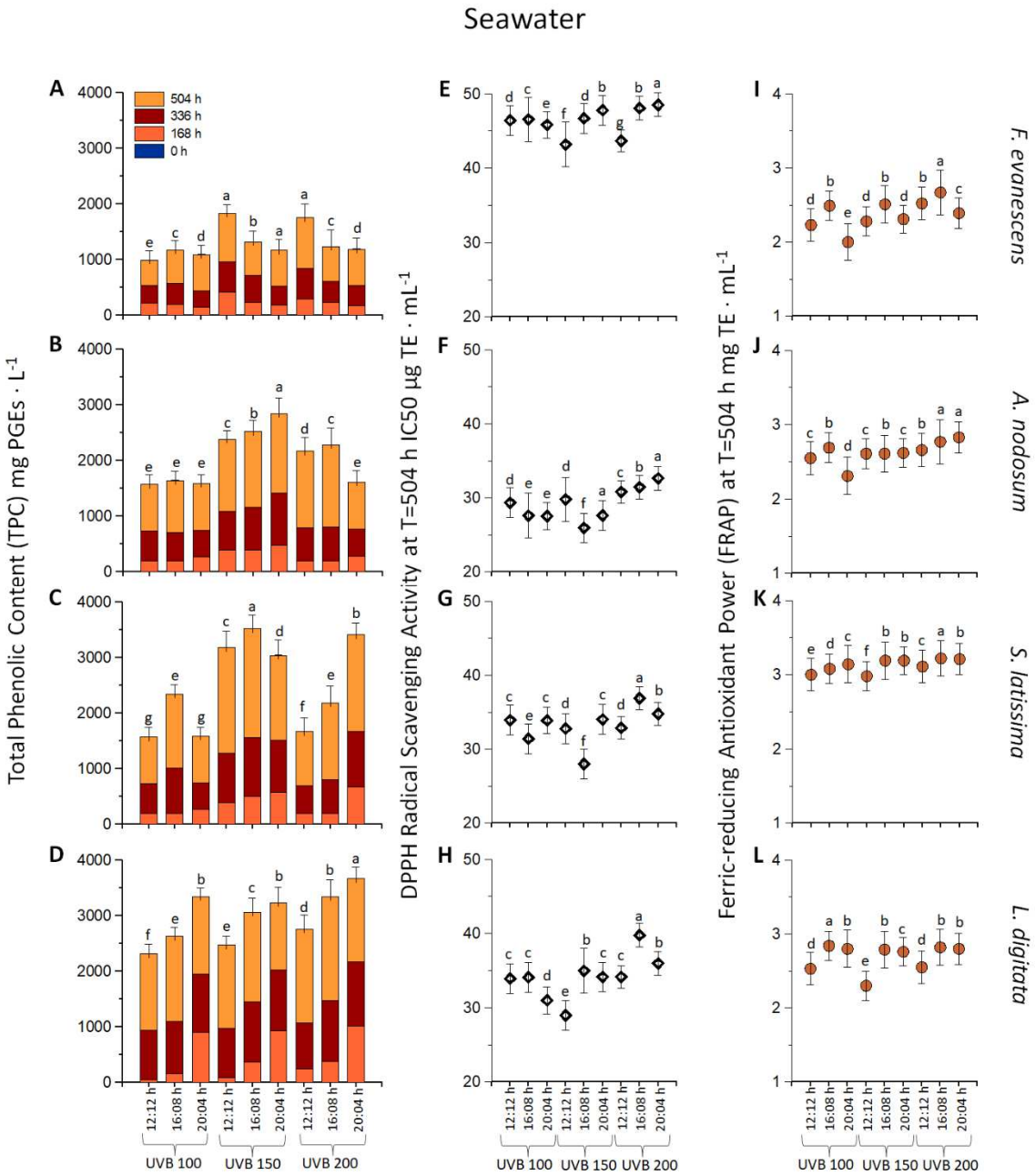


Figure 15. First part of the results of the up-scale experiments in conventional bubble column PBRs (*S. latissima*, *L. digitata*) and a 200 L tank with 100 L culture volume (*F. evanescens, A. nodosum*), utilizing different UVR intensities and exposure lengths to induce the phlorotannin accumulation and release into the seawater. Depicted are the results for the TPC (A-D), DPPH radical scavenging activity (EC_{50}, E-H) and the ferric reducing antioxidant power assays (I-L) recorded for the XAD-7 extracts from the seawater, prepared as described in 3.3.2. Data are means ± SD (n=3). TPC was measured for t=0 h, 168 h, 336 h and 504 h samples, whereas DPPH and FRAP were assessed from t= 504 h samples only. Different letters indicate significant difference among treatments.

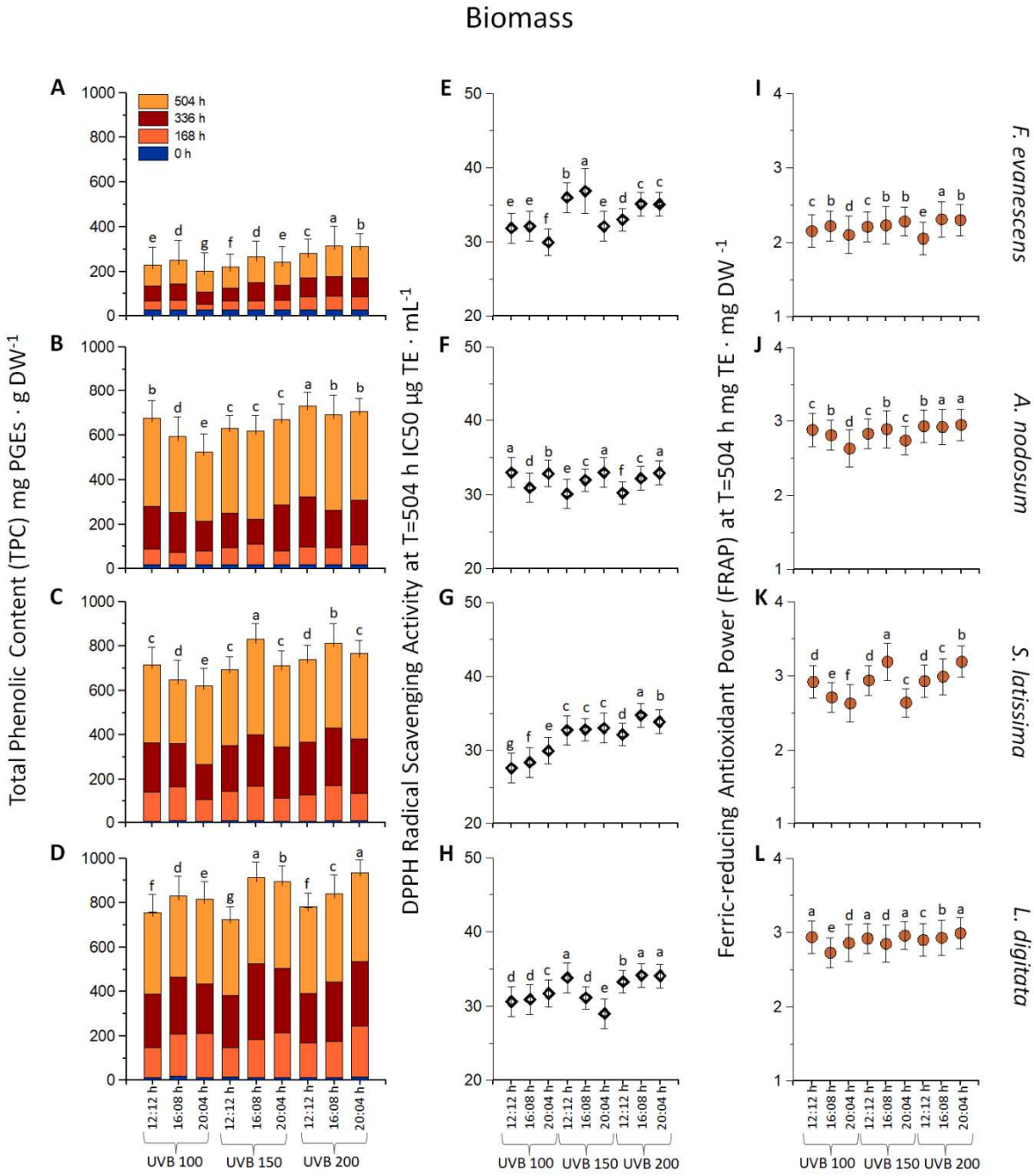
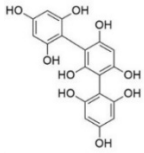
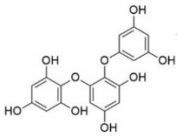
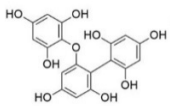
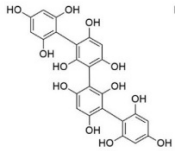
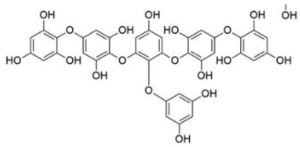
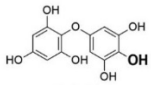
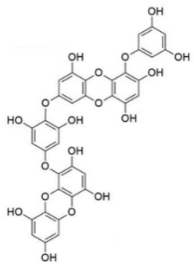


Figure 16. Second part of the results of the up-scale experiments in conventional bubble column PBRs (*S. latissima*, *L. digitata*) and a 200 L tank with 100 L culture volume (*F. evanescens, A. nodosum*), utilizing different UVR intensities and exposure lengths to induce the phlorotannin accumulation and release into the seawater. Depicted are the results for the TPC (A-D), DPPH radical scavenging activity (EC_{50}, E-H) and the ferric reducing antioxidant power assays (I-L) recorded for the biomass extracts prepared as described in 3.3.1 and 3.3.2. Data are means ± SD (n=3). TPC was measured for t=0 h, 168 h, 336 h and 504 h samples, whereas DPPH and FRAP were assessed from t= 504 h samples only. Different letters indicate significant difference among treatments.

To increase the phlorotannin yield in the four selected seaweed species, the duration of UV exposure times were varied (12 h, 16 h and 24 h) and three UV/PAR sets with different spectra were applied, utilizing in the first run field collected *S. latissima, A. nodosum, L. digitata* and *F. evanescens*. Between

each run the specimens were cultivated under standard conditions as described in 3.1.3 to regenerate for 4-6 weeks. Therefore, no new specimens had to be collected and the seaweeds were integrated into the culture collection. The results of these trials are depicted in Figures 15 and 16. In comparison, the TPC was significantly higher in the seawater than in the biomass samples in all tested species (up to 48%). The highest TPC was observed for *S. latissima* in the UVB150 16:08 h assay with 1961.4 ± 31.4 mg PEGs · g^{-1} at t=504 h (Fig. 15C), whereas the highest FRAP activity was found in the biomass samples from the same species in the UVB200 20:04 h assay (3.193 ± 1.13 mg TE · mg DW^{-1}; Fig. 16K). In contrast, the highest DPPH activity was detected in the concentrated seawater samples of *F. evanescens* in the UVB200 20:04 h assay (47.6 ± 1.0 µg TE · mL^{-1}; Fig. 15E).

Table 5. Identified phlorotannins in seawater samples extracted with aqueous ethanol (80%, v/v) according to the method given in 3.4.2.

Species	Name	Chemical Formula	M_R	Structure
F. evanescens	Trifucol	$C_{18}H_{14}O_9$	374	
	Triploethol	$C_{18}H_{12}O_9$	374	
	Fucoplorethol	$C_{18}H_{14}O_9$	374	
A. nodosum	Tetrafucol	$C_{18}H_{12}O_9$	498	
S. latissima	Hexaphlorethol	$C_{36}H_{26}O_{18}$	746	
	Bifuhalol	$C_{12}H_{10}O_7$	266	
L. digitata	Dieckol	$C_{36}H_{20}O_{18}$	742	

Biomass samples from the experiments were used for further refinement of the analytical procedures applying different solvents and supportive techniques as well as purification and fractionation procedures. The results are presented in the appendix (Tables A1-A4). In contrast, samples from the phlorotannin-enrichments employing Amberlite XAD-7 were used after purification via dialysis for identification of the compounds. Overall, seven phlorotannins were identified in the seawater samples from the experiments (Trifucol, Triploethol, Fucoplorethol, Tetrafucol, Hexaphlorethol, Bifuhalol and Dieckol; Table 5). In addition, two phlorotannins were only tentatively identified (Table 6, Fig. 17).

Table 6. Tentatively identified phlorotannins in seawater samples extracted with aqueous ethanol (80%, v/v) with detected m/z, theoretical m/z, difference in m/z, retention time (RT), ring double bond (RDB) equivalent for neutral compounds, detected MS^2 fragments and identification level according to Schymanski et al. (2014).

Species	Detected $[M - H]^-$	Mass Difference (mDa)	RT (min)	RDB	Detected MS^2 Fragments
A. nodosum	389.0509	−0.8	21.03	12.0	343.04 (−CO, $-H_2O$); 315.02 (−2 × CO, $-H_2O$); 264.02 ($-C_6H_5O_3$); 249.02 ($-C_6H_4O_4$)
	497.0719	0.1	9.72	16.0	479.20 ($-H_2O$); 413.02 (−3 × CO); 373.07 ($-C_6H_4O_3$); 371.03 ($-C_6H_6O_3$); 353.01 ($-C_6H_6O_3$, $-H_2O$); 265.01 ($-C_{12}H_8O_5$); 229.13 ($-C_{12}H_8O_5$, −2 × H_2O)

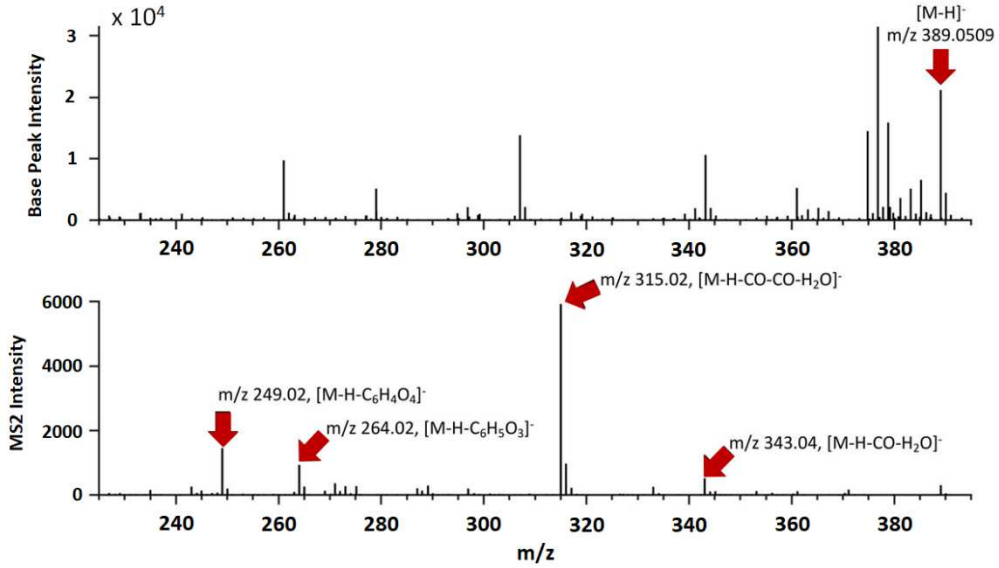


Figure 17. MS^1 and MS^2 spectra of m/z 389.0509 found in *A. nodosum*.

In the MS^2 spectrum, three fragments were identified. At m/z 315.02 a neutral loss of $C_2H_2O_3$ was observed, which most likely corresponds to a loss of two carbon monoxide and one water molecule. A neutral loss of $C_6H_5O_3$ corresponding to a loss of a phloroglucinol unit (PGU) was observed at m/z 264.02. The third fragment observed at m/z 249.02 showed a neutral loss of $C_6H_4O_4$, which corresponds to a PGU with one additional oxygen atom (Fig. 17).

4.5 Effects of Organic Matter on the TPC yield

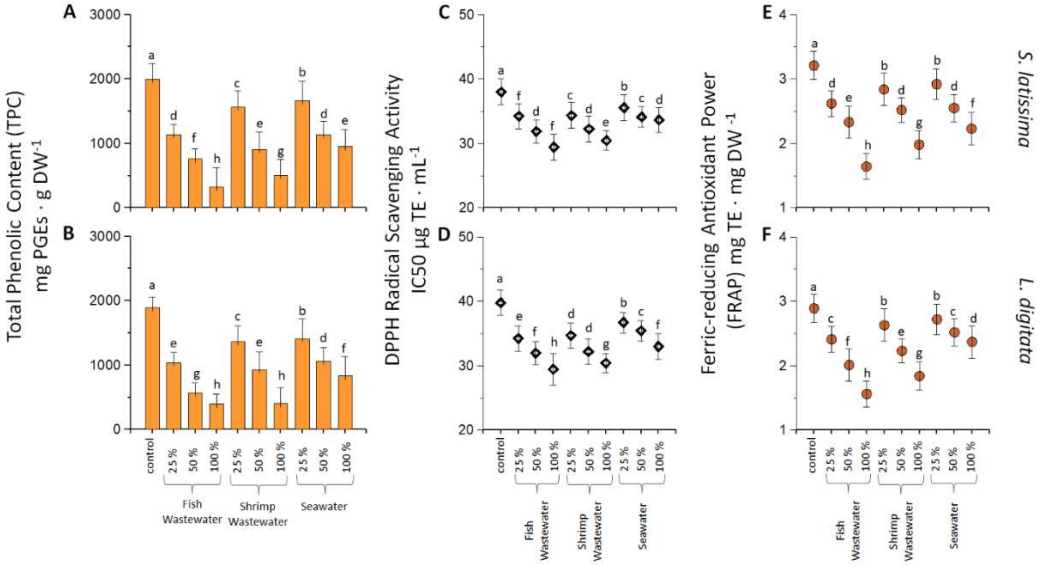


Figure 18. Results of the experiments in conventional bubble column PBRs, focussing on the cultivation of *S. latissima* and *L. digitata* in three different concentrations of fish and shrimp wastewater as well as natural surface seawater (each 25, 50, 100%) to simulate the co-culture of seaweeds with aquatic animals and to assess the impact of the organic fraction in aquacultural wastewater on the TPC yield in the seawater. Depicted are the results for the TPC (A-D), DPPH radical scavenging activity (EC_{50}, E-H) and the ferric reducing antioxidant power assays (I-L) recorded for the XAD-7 extracts from the liquid phase at t=504 h, prepared as described in 3.3.2. Data are means ± SD (n=3). Different letters indicate significant difference among treatments.

Table 9. Mean ± SD for concentrations of dissolved nutrients (µM) as well as of PON, POC, POP (µmol L^{-1}) and turbidity (ppm).

	NH_4^+	NO^{3-}	NO^{2-}	PO_4^{3-}	PON	POP	POC	Turbidity
Fish Wastewater								
25%	1.3 ± 0.3	0.22 ± 0.1	0.08 ± 0.03	0.55 ± 0.3	14.1 ± 0.9	0.26 ± 0.05	26.7 ± 2.7	409 ± 10.4
50%	2.1 ± 0.8	0.56 ± 0.1	0.17 ± 0.06	1.08 ± 0.4	29.3 ± 1.1	0.48 ± 0.09	51.3 ± 1.2	936 ± 22.7
100 %	4.4 ± 0.5	1.13 ± 0.7	0.38 ± 0.08	2.15 ± 0.3	54.2 ± 3.6	0.82 ± 0.11	109 ± 5.7	1098 ± 36.1
Shrimp Wastewater								
25%	8.2 ± 0.4	0.15 ± 0.1	0.02 ± 0.01	0.23 ± 0.3	10.2 ± 0.7	0.82 ± 0.01	20.8 ± 2.5	209 ± 6.6
50%	1.6 ± 0.2	0.41 ± 0.1	0.07 ± 0.03	0.53 ± 0.3	18.7 ± 1.5	0.16 ± 0.10	42.9 ± 3.0	421 ± 13.9
100 %	3.1 ± 0.6	0.83 ± 0.2	0.13 ± 0.09	1.05 ± 0.2	33.8 ± 2.3	0.29 ± 0.15	89.3 ± 3.8	835 ± 26.7
Coastal Seawater								
25%	0.4 ± 0.3	0.10 ± 0.1	n.d.	0.13 ± 0.1	4.8 ± 0.2	0.05 ± 0.01	93.1 ± 1.1	186 ± 8.5
50%	0.8 ± 0.3	0.23 ± 0.2	0.02 ± 0.01	0.33 ± 0.2	9.5 ± 0.2	0.08 ± 0.03	186.4 ± 2.2	303 ± 9.2
100 %	1.5 ± 0.5	0.47 ± 0.3	0.04 ± 0.01	0.72 ± 0.2	18.4 ± 0.3	0.16 ± 0.05	373.3 ± 9.9	588 ± 11.3

Abbreviations: PO_4^{3-}, phosphate; NH_4^+, ammonium; NO^{3-}, nitrate; NO^{2-}, nitrite; PON, particulate organic nitrogen; POP, particulate organic phosphorus; POC, particulate organic carbon.

To simulate the co-culture of seaweeds with aquatic animals and to assess the impact of the organic fraction in aquacultural wastewater on the TPC yield in the seawater samples, overall, two species (*S. latissima* and *L. digitata*) were cultivated in three different concentrations of fish and shrimp wastewater as well as natural surface seawater (each 25, 50, 100%). The results are presented in Figure 18 and Table 9. Beginning with the features of the employed mixtures, fish wastewater showed the highest dissolved nutrient as well as particulate organic nitrogen, phosphorous and carbon concentrations in comparison to shrimp wastewater and coastal surface seawater (Table 9). In addition, the high turbidity of the mixtures is noteworthy.

Regarding the TPC as well as DPPH and FRAP activities of the two species in relation to the applied mixtures, the best results in recovery of the phlorotannins were obtained for natural surface seawater in comparison with the control using artificial one (Fig. 18). In each case a gradual decline of the yields with increase of the portion of the experimental mixtures was detected, being most distinctive in the assay using 100% fish sea-wastewater.

5. Discussion

Polyphenols are one of the essential micronutrients in the human diet due to their health effects, antioxidant properties, and prevention of diseases associated with oxidative stress such as cancer, cardiovascular, and neurodegenerative diseases. Hence, there is increasing interest in these compounds and their exploitation as bioactive molecules from natural resources. Marine brown macroalgae (seaweeds) have been recognized as one of the main natural sources for biologically active polyphenols. In brown seaweeds, the major types of polyphenols are phlorotannins. Several studies have highlighted the influence of UV light intensity on the phenol content of seaweeds. Solar radiation is essential for photosynthesis, however, high energy UV light cause damage to DNA and other organelles (Bischof et al. 2006). Seaweeds have developed a protective mechanism against UV damage which uses cellular antioxidant systems and antioxidant compounds such as polyphenols and carotenoids. In a study by Flodin et al. (1999) levels of bromophenols and bromoperoxidases were observed to be high in the summer months in Australia. In contrast, Chung et al. (2003) observed that the levels of bromophenol in the species *Padina arborescens*, *Sargassum siliquastrum* and *Lobophora variegata* were significantly higher in the winter months in the northern hemisphere. Other studies observed a similar trend with levels of phlorotannins and polyphenols increasing in response to UV exposure during the summer months in a range of investigated species (Pavia & Brock 2000, Abdala-Díaz et al. 2006, Kamiya et al. 2010). Therefore, many studies concluded that the polyphenol levels were at a maximum during the summer and autumn months when UV radiation was at its highest. Similar observations were made in the present study, in which the highest phlorotannin values were detected in samples exposed to high UVR and a 16:08 h and 20:04 h light:dark photoperiod. Specific comparisons of the obtained data to values given in the literature showed only in some cases relatively good accordance (e.g., for TPC, DPPH and FRAP activities). In contrast, for a few of the tested species either no equivalent data were available at all (e.g., *Alaria esculenta*) or the data showed huge differences to the obtained values in the present study. In particular, the methods used for phlorotannin analysis in the literature differ substantially. A systematic search in several databases (Scopus, ScienceDirect and PubMed) and free-access repositories (Google Scholar) was carried out based on the keyword ‘phlorotannins’, revealing that 673 articles with the keyword ‘phlorotannins’ have been published. Among these documents, research articles have been mainly published (77.9%), followed by reviews (10.4%), book chapters (8.7%), conference papers (2.1%) and short surveys in minor proportion. Once harvested, fresh seaweed must overcome a sequence of crucial steps to confer their biological activity and a number of potential pre-processing operations, extraction processes, as well as qualitative analysis steps for seaweed biomasses described in the literature are manifold. Conversely, studies focusing on exudated phlorotannins, their capture, analysis and biotechnological potential are seemingly not present.

Once algae are harvested from the sea, they are exposed to some pre-processing operations before being used in any analytical assay, industrial process, or storage. Among these operations, drying is the widely applied technique for extending the algae shelf-life by reducing the moisture content and thus, minimizing the algae spoilage before extraction. Besides, the seaweed volume is reduced thereby packaging, storage and transportation cost are significantly minimized (Leyton et al. 2016). Air-drying is an economical and rapid technique, chosen by industrial producers because of the high production rates are yielded. However, the air-drying process leads to a loss of phlorotannins content as a result of

exposure to high temperatures and oxidative processes. On the other hand, freeze-drying maintains the phlorotannins content at stable levels but in an expensive and time-consuming way (Chowdhury et al. 2011, Cassani et al. 2020). Leyton et al. (2016) evaluated the application of hot air-drying at different temperatures (30, 40, 50 and 60 °C) on the phlorotannins' content of *Macrocystis pyrifera*. These authors found that at the optimal drying temperature (40 °C), the content of phlorotannins was lower than that of fresh algae, possibly due to the degradation of the compounds caused by heat treatments. Chowdhury et al. (2011) compared different drying pre-treatments (sun-drying, shadow-drying, oven-drying, and freeze-drying) on the phlorotannins extraction from *Ecklonia cava* and found that drying process significantly changed the phlorotannins content in the crude extract as the freeze-dried tissue yielded the highest amount of compounds whereas sun-dried tissues showed the lowest ones. Shadow- and oven-dried tissues were extracted at almost 80% of the freeze-dried sample. Besides, these authors reported that algae washed with tap water before drying showed lower crude phlorotannins than non-washed tissue. According to these findings, the lesser pre-treatments the algae receive, the better the phlorotannins content maintenance. In the present study, TPC recovery rates of up to 85.3% were found in the 50°C drying process (Table 3).

There are a number of different parameters which need to be considered when analysing the phenolic content in the seaweeds, from collection through to measurement. Pre-treatment of seaweed prior to extraction can also make a large difference on the results, and hence standardisation of the pre-treatments when analysing phenolic content is also needed (e.g., some studies use hexane as a pre-treatment to remove lipophilic substances prior to F-C analysis). Furthermore, some studies fail to note the collection date, processing time, method and conditions of storage of seaweeds prior to analysis, all of which may influence the phenolic content. A study by Kirke et al*.,* reported that low molecular weight phlorotannins isolated from *Fucus vesiculosus* were stable up to 8 weeks in aqueous solutions at temperatures below 50°C (Kirke et al. 2017). Prior to analysis, these samples were cleaned of epiphytes and other impurities, transported in cool conditions, frozen to −20°C, freeze-dried, ground and stored in vacuum-packed packages at −20°C. It is unclear from the literature, if phlorotannins are stable under other preparatory methodologies, as few studies report sample preparation prior to storage for analysis. In terms of the extraction, there are several parameters that can influence the extraction efficiency, which include, polarity of solvent, ratio of solvents, temperature of extraction, particle size of seaweed powders used in extraction and condition of samples (i.e., wet or dry samples). Another major difference in the extraction conditions are the temperature and time of extraction. The majority of extraction conditions involve room temperature conditions and overnight extractions (e.g., Koivikko et al. 2005, 2008, Zenthoefer et al. 2017). However, these conditions could vary from laboratory to laboratory, based on the room temperature in that part of the world. It is also known that the phlorotannins in brown seaweeds readily undergo oxidation and hence overnight extraction could lead to depleted results (Ragan & Glombitzka 1986, Glombitza & Schmidt 1999a, b, Parys et al. 2010). Leyton *et al*. compared several experimental extraction conditions and found acetone/water (70:30) to be the best extraction method for highest total phenolic content (TPC) (Leyton et al. 2016). This paper also assessed the optimum temperature for drying, extraction and pre-treatment. They suggest the storage of dried seaweed at 4°C prior to use, a pre-treatment with hexane (solid/liquid ratio of 5:1, *w*/*v*) and an extraction temperature of 55°C. Leyton *et al*. compared many parameters in their study however only looked at one species; *Macrocystis pyrifera*, and hence these conditions are optimised for this species. This paper highlights how different solvent systems, extraction temperatures and particle size affect the phenolic content, thus unless exactly the same extraction protocol is used, results from different publications are not comparable. Another consideration is the seaweed species, as different

species produce phlorotannins with different linkages, functional groups, reactivity and sizes as previously mentioned. This may mean that the wide variability in structures may have different solubilities based on the structures of the polyphenols, and different polyphenols may be present in different species. Therefore, some species of seaweed may contain higher contents of smaller, less polar polyphenolic phlorotannins which would be more soluble in less polar solvents, whereas other species may contain larger, polar structures which would require more polar extraction solvents. Reactivity of the phlorotannins could also be very different from species to species and hence the temperature of extraction for one species may be optimum in terms of phenolic extraction and antioxidant activity but could cause degradation in another species leading to lower levels detected (Ford et al. 2019). Phlorotannin concentrations vary from 0.5% to 20% of their dry weight, which can fluctuate with respect to season (e.g., changes in light exposure), environment (e.g., nutrient availability in the surrounding water) and also between species (Caro et al. 2012). It is noteworthy that there are some significant discrepancies between results in the literature and their methods of calculation. This is of particular concern from an analytical chemistry perspective, as yields quoted can mislead industry who are interested in commercialising phytochemicals (Ford et al. 2019).

Numerous extraction methods have been applied with the aim to study phlorotannins from field collected brown algae biomasses, in particular solvent extractions using a hydrophilic solvents such as water, ethanol, and methanol (Generalic´ Mekinic et al. 2019). These are usually combined with analysis of the total phenolic content (TPC) by the Folin–Ciocalteu (F-C) assay, which is a simple and fast colorimetric method (Folin & Ciocalteu 1927). However, this method provides no information regarding the chemical composition of the phlorotannins and is known to oxidize several non-phenolic compounds. For instance, phenols, proteins, and thiols were all found to cause a reaction with the F-C reagent in a study testing 80 different compounds (Everette et al. 2010). In addition, many vitamins were also found to cause a response along with the inorganic ions Fe^{2+}, Mn^{2+}, I^{-}, and SO^{3+} (Everette et al. 2010). Therefore, the F-C reaction is not sensitive to phenols but a measurement of anything in the matrix that is able to reduce, and therefore be oxidised itself. Based on the assumption that the majority of reducing power in a plant matrix is due to phenolics, the F-C assay is widely used to give an approximation of the phenolic content. In this context, the main problem is the lack of fast specific test assays. The use of liquid chromatography (LC) to quantify the phlorotannin compositions in macroalgal extracts is limited by the lack of commercially available standards. The only standard for calibration that is commercially available is the monomer phloroglucinol. Therefore, further research needs to be performed with characterised standards to fully develop qualitative methods of high-performance liquid chromatography (HPLC) analysis. Nuclear magnetic resonance (NMR) spectroscopic methods have sometimes been used to obtain structural information on phlorotannin structures (Li et al. 2008, Eom et al. 2012, which is challenging since relatively large amounts of pure compound fractions need to be isolated. In this work we used instead ultra-high-performance liquid chromatography/high resolution tandem mass spectrometry (UHPLC/HRMS2), being a method for quick profiling of phlorotannins from brown seaweeds based on their degree of polymerization. This method has thus far been used for profiling of phlorotannin-enriched extracts from five brown algae species (*Ascophyllum nodosum*, *Pelvetia canaliculata*, *Fucus spiralis*, *Fucus vesiculosus*, *Saccharina longicruris* and *Saccharina latissmia*) (Tierney et al. 2014, Sardari et al. 2021). Moreover, 42 phlorotannins from *Sargassum fusiforme* have been detected and identified, based on their degree of polymerisation (DP), by Li and co-workers, including fuhalol-type phlorotannins, phlorethols, fucophlorethols, and eckol-type phlorotannins (Li et al. 2017). In the present investigation, trifucol, triploethol, fucoplorethol, tetrafucol, hexaphlorethol, bifuhalol and dieckol were identified in the exudates of the tested seaweeds. All these phlorotannins are

already described from biomass samples but not from seawater ones. For instance, fucols in which the inter-phloroglucinol links can only be in meta relative position, as found in tetrafucol-A (lineal) and B (branched) from *Fucus vesiculosus* (Glombitza et al. 1975, Truus et al. 2004). The linear phlorethols may have ortho-, meta- or para- oriented biphenyl ether bridges or combinations thereof, as observed in triphlorethol C and tetraphlorethols A and B, isolated from *Laminaria ochroleuca* (Koch et al. 1980). Other structural motifs in the phorethols arise for an additional hydroxyl group on the terminal monomer unit, as in the case of fuhalols, bifuhalol (Glombitza & Rösener 1974, Glombitza et al. 1975), trifuhalol A with para-arranged ether bridges or ortho-arranged trifuhalol B (Glombitza et al. 1978). Another subgroup of phlorethols, the eckols, includes a 1,4-dibenzodioxin system, as exemplified by the trimers eckol and dioxinodehydroeckol from *Ecklonia maxima* (Glombitza & Vogels 1985) and *Eisenia arborea* (Glombitza & Gerstberger 1985, Martınez & Castañeda 2013). In the case of bifuhalol detected in *S. latissima* in the present study only two references were found reporting structures built from two PGU (with m/z 265.0352) and three PGU (m/z 389.0509 as in the A. nodosum extract) from the brown algae *S. fusiforme* (Li et al. 2017) and *S. latissima* (Sardari et al. 2021). This shows that the different types of phlorotannin structures are conserved in the different types of brown macroalgae, although at different concentrations and with different profiles in the different species.

6. Conclusions and Perspectives

The algae industry is highly committed to find natural sources of functional rich ingredients (*e.g*., phlorotannin, fucoidans, laminarin, fucoxanthin, carrageenan, alginate, etc.) from sustainable and cost-effective raw materials useful for innovation in the food and cosmetic industries. Although algae constitute a highly widespread renewable resource in fact, most brown seaweeds are underexploited and processed primarily into fertilizers and animal feeds, more studies are mandatory to better understand the effects of seaweed cultivation and exploitation on safety, toxicity and environmental issues. Phlorotannins are structurally diverse and heterogeneous, which makes studies of their structures challenging. The production of a standardized commercial product based on algal constituents will be a challenge since their structural and pharmacological features vary depending on species, location and time of harvest. The main differences between most phlorotannin studies available to date and the present one is that they focused on phlorotannins from i) biomasses and ii) used in each case fresh collected materials from the field. Particularly in the second part of the present study, only long-term cultivated species were utilized (*F. evanescence*, *A. nodosum*, *S. latissima*, *L. digitata*), providing the option of controlled environmental conditions and reliable repeatability of the produced phlorotannin quality and quantity, including the avoidance of ecological harmful effects of wild harvests. Furthermore, the application of macroporous resins to concentrate the exuded phlorotannins in the seawater provides the opportunity to harvest phlorotannins without the time-consuming process of high volumes. Although the cause of the high decrease in total phlorotannin contents observed during the exposure of the seaweeds to fish wastewater is unclear and may be more related to the high turbidity than to the amount of dissolved and particulate nutrients in the seawater, a co-culture of the seaweeds with aquatic animals would be in any case not desirable since also medications and other chemicals may intervene with the phlorotannin capture by adsorbents. Further investigations are necessary to develop standardised protocols, assays and testing of interferences when using absorbents for the phlorotannin enrichment from seawater.

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9. Appendix

Table A1. Effect of different solvents on the phlorotannin yield (% from the control I) obtained from the biomasses of the four tested seaweed species.

Species	Control I*[1] [mg · L^{-1}]	Water*[2]	Ethanol*[2]			Acetone*[2]
			30%	50%	80%	
F. evanescens	139.1 ± 1.0	96.9 ± 0.9	86.1 ± 0.7	80.0 ± 1.1	73.8 ± 1.0	77.5 ± 1.5
A. esculenta	404.2 ± 0.2	90.3 ± 0.3	85.4 ± 0.4	78.3 ± 0.7	72.9 ± 0.9	94.8 ± 0.2
S. latissima	382.0 ± 0.9	89.5 ± 0.5	80.3 ± 0.5	95.0 ± 0.6	98.4 ± 0.3	79.4 ± 0.6
L. digitata	399.8 ± 0.5	89.7 ± 0.5	83.1 ± 0.9	99.2 ± 0.7	74.5 ± 1.5	78.1 ± 0.7
Control*[3]	195.8 ± 0.6	94.1 ± 0.2	95.3 ± 0.2	94.2 ± 0.3	99.2 ± 0.6	90.1 ± 0.6

*[1] Utilizing the initial extraction protocol with ethanol (96%) (3.3.1).

*[2] % TPC calculated from the control I value for each individual species.

*[3] Known amount of PEG in artificial seawater (200 mg · L^{-1}].

Table A2. Comparison of supportive techniques during the extraction process, using 80% ethanol biomass extracts. Presented are the phlorotannin yields (TPC) as % from control I.

Species	Control I*[1] [mg · L^{-1}]	UAE*[2]	MAE*[2]	UMAE*[2]	HAE*[2]	HPAE*[2]
F. evanescens	136.9 ± 1.0	80.2 ± 0.9	79.4 ± 0.8	83.6 ± 0.6	89.4 ± 0.5	92.8 ± 0.3
A. esculenta	403.6 ± 0.5	81.0 ± 0.5	79.2 ± 0.4	84.2 ± 0.2	90.1 ± 0.1	95.0 ± 1.2
S. latissima	381.8 ± 1.8	80.7 ± 0.7	80.1 ± 0.2	83.7 ± 0.5	90.3 ± 0.1	94.9 ± 0.6
L. digitata	389.2 ± 2.1	80.9 ± 0.3	79.9 ± 0.5	84.0 ± 0.9	89.7 ± 1.7	94.3 ± 0.5
Control*[3]	196.3 ± 0.5	83.1 ± 0.5	80.8 ± 0.3	89.7 ± 0.6	90.4 ± 0.5	96.3 ± 0.3

Abbreviations: UAE, ultrasound-assisted extraction; MAE, microwave-assisted extraction; UMAE, ultrasound–microwave-assisted extraction; HAE, hydrothermal-assisted extraction; HPAE, high-pressure-assisted extraction.

*[1] Utilizing the initial extraction protocol (3.3.1).

*[2] % TPC

*[3] Known amount of PEG in artificial seawater (200 mg · L^{-1}].

Table A3. Purifications of extracted biomass samples utilizing different solid-phase procedures. Procedures are described in 3.3.3. Presented are the phlorotannin yields (TPC) as % from control I.

Species	Control I*[1] [mg · L^{-1}]	Cellulose*[2]	Cartridges*[2,3]	Adsorbents*[2]	
				XAD-4	XAD-7
F. evanescens	139.5 ± 1.3	83.5 ± 1.1	71.4 ± 1.0	73.4 ± 0.7	74.8 ± 0.3
A. esculenta	405.8 ± 0.6	84.3 ± 0.8	75.2 ± 0.5	74.2 ± 0.5	75.7 ± 0.5
S. latissima	383.7 ± 1.0	85.1 ± 0.9	73.7 ± 1.1	72.9 ± 0.5	74.9 ± 0.2
L. digitata	392.3 ± 1.2	83.9 ± 0.7	72.5 ± 0.9	76.1 ± 0.9	72.1 ± 0.7
Control II*[4]	193.9 ± 0.8	98.3 ± 0.5	96.5 ± 0.1	99.4 ± 0.1	99.3 ± 0.1

*[1] Utilizing the initial extraction protocol (3.3.1) without purification.

*[2] % TPC

*[3] Step one: Sep-pak-C18 SPE end capped cartridge; Step two: DSC-18 SPE cartridge.

*[4] Known amount of PEG in artificial seawater (200 mg · L^{-1}].