Journal Of Applied Phycology
April 2014, Volume 26 Issue 2 Pages 1215-1230
http://dx.doi.org/10.1007/s10811-013-0198-x
http://archimer.ifremer.fr/doc/00171/28221/

Assessment of the spatial variability of phenolic contents and associated bioactivities in the invasive alga Sargassum muticum sampled along its European range from Norway to Portugal

Tanniou A. [1, *], Vandanjon L. [2, 3], Incera M. [4], Serrano Leon Esteban [1], Husa V. [5], Le Grand Jacqueline [6], Nicolas Jean-Louis [6], Poupart N. [1], Kervarec N. [7], Engelen A. [8], Walsh R. [9], Guerard F. [1], Bourgougnon N. [10], Stiger-Pouvreau Valerie [1]

[1] UBO, IUEM, LEMAR UMR CNRS UBO IRD Ifremer 6539, F-29280 Plouzane, France.
[2] Univ Nantes, GEPEA UMR CNRS 6144, CRTT, Lab GEPEA, F-44602 St Nazaire, France.
[3] Univ Bretagne Sud, F-56321 Lorient, France.
[4] CETMAR, Bouzas Vigo 36208, Spain.
[5] Inst Marine Res, N-5817 Bergen, Norway.
[6] IFREMER, Ctr Brest, LEMAR UMR CNRS UBO Ifremer IRD 6539, F-29280 Plouzane, France.
[7] UBO, UFR Sci & Tech, Serv RMN RPE, F-29200 Brest, France.
[8] Univ Algarve, CCMAR, Faro, Portugal.
[9] Natl Univ Ireland, Ryan Inst, Irish Seaweed Res Grp, Galway, Ireland.
1 [0] UBS, IUEM, EA3884, Lab Biotechnol & Chim Marines, F-56000 Vannes, France.

* Corresponding author : Anaelle Tanniou, email address : anaelle.tanniou@univ-brest.fr

Abstract :

Sargassum muticum, an invasive brown macroalga presently distributed along European Atlantic coasts from southern Portugal to the south coast of Norway, was studied on a large geographical scale for its production of phenolic compounds with potential industrial applications and their chemical and biological activities. S. muticum can produce high biomass in Europe, which could be exploited to supply such compounds. S. muticum was collected in Portugal, Spain, France, Ireland and Norway (three sites/country) to examine the effect of the latitudinal cline and related environmental factors. Assays focused particularly on polyphenols and their activities. Crude acetone–water extracts were purified using solid phase extraction (SPE) and antioxidant and antimicrobial activities of crude extracts and semi-purified fractions measured. Total phenolic content was assessed by colorimetric Folin–Ciocalteu assay and reactive oxygen species activities by 2,2-diphenyl-1-picrylhydrazyl, reducing power, β-carotene bleaching method and xanthine oxidase assay. Antibacterial activities were tested on terrestrial and marine strains to evaluate potential use in biomedical and aquaculture fields. Purified

active phlorotannins, isolated by SPE, were identified using NMR. Phenolic contents differ clearly among countries and among sites within countries. Quality did not change between countries, however, although there were some slight differences in phlorethol type. Additionally, some fractions, especially from the extreme north and south, were very active. We discuss this in relation to environmental conditions and the interest of these compounds. S. muticum represents a potential natural source of bioactive compounds and its collection could offer an interesting opportunity for the future management of this species in Europe

Keywords : Antioxidant activities, Antibacterial activities, Latitudinal gradient, Phenolic compounds, Solid phase extraction, Sargassum muticum

1. Introduction

Seaweeds are used in many countries as a source of food and molecules, with many industrial applications, mostly involving phycocolloid extraction and, to a lesser extent, isolation of certain biomolecules with pharmaceutical, medicinal and other industrial uses (Bourgougnon and Stiger-Pouvreau *2011*). In the last few years, natural antioxidants from plant and animal sources have been actively investigated as replacements for synthetic antioxidants currently used as food additives. This recent interest in natural antioxidants as food additives has increased partly because of the restriction in synthetic antioxidant utilization in the food industry due to their long-term toxicological effects, including carcinogenicity (Ito et al. *1986*; Aruoma et al. *1997*; Bandonienė et al. *2000*). Brown algal species are known to naturally produce antioxidant compounds in great quantities: up to 20 % DW in Fucales (Ragan and Glombitza *1986*; Targett et al. *1995*). Indeed, in coastal ecosystems, biotic and abiotic factors are known to affect algae species. To defend themselves against these stresses, seaweeds are known to produce a great variety of defensive metabolites. Consequently, they can be a very interesting source of new substances for industry. Among such defensive substances, phenolic compounds (PC), also known as phlorotannins, are secondary metabolites synthesized during development as components of algal cell walls (Schoenwaelder and Clayton *1998*) or as a chemical defence in response to abiotic or biotic stress conditions, such as UV radiation, grazing, bacterial infection or epiphytism, as well as for intra- and interspecific communication (Ragan and Glombitza *1986*; Connan et al. *2004*; Stiger et al. *2004*; Koivikko et al. *2005*; Plouguerné et al. *2006*; Bourgougnon and Stiger-Pouvreau *2011*). As these compounds are produced in response to the production of reactive oxygen species (ROS), PC exhibit anti-ROS, i.e. antioxidant, properties (Nakai et al. *2006*; Kuda et al. *2007*; Kumar Chandini et al. *2008*). Structurally, phlorotannins are oligomers and polymers of 1,3,5-trihydroxybenzene (phloroglucinol) (Ragan and Glombitza *1986*; Targett and Arnold *2001*; Koivikko et al. *2007*) and can be considered as a pool of PC with different natures and/or polarity. Within a species, phenolic compounds can also vary greatly both spatially (Steinberg *1986*, *1989*, *1992*; Van Alstyne and Paul *1990*; Targett et al. *1992*, *1995*; Steinberg et al. *1995*; Pavia and Aberg *1996*; Van Alstyne et al. *1999*; Stiger et al. *2004*; Le Lann et al. *2012a*) and temporally (Stiger et al. *2004*; Connan et al. *2004*; *2007*; Plouguerné et al. *2006*). However, few studies have examined this spatial variation in connection with the activities displayed by these compounds though large-scale sampling of a single species.

In Brittany, native marine algae species are already harvested for industrial applications (agri-food, cosmetics and thalassotherapy). In some places, native species are in competition with introduced species that have

proliferated. Invasive seaweeds are often very promising for industrial applications as the chemical defences they have developed that allow them to overcome geographical barriers and colonise new environments, make them a source of interesting active molecules. In this context, *Sargassum muticum* (Yendo) Fensholt, which is an invasive species in Europe living on rocky shores, was chosen as a model organism to find applications for its phlorotannin pool. This brown macroalga, native to Japan, has spread widely along the European Atlantic coasts since its introduction on the Atlantic coast (Plouguerné et al. 2006; Kraan 2008; Engelen et al. 2008; Incera et al. 2009; Olabarria et al. 2009; Le Lann et al. 2012); it is currently one of the most readily available Sargassaceae species on European shores. Its large sustainable biomass could represent a viable biotechnological asset in European resource development programs as it is known to produce phlorotannins of interest (Tanniou et al. 2013).

The aim of this work was therefore to study the chemical plasticity, i.e. the spatial variability, of *Sargassum* phenolic compounds in Europe and to see how the quantity and quality of the compounds vary according to country and, finally, to see how *Sargassum muticum* can be usefully exploited in Europe. We assessed the antioxidant and antibacterial activities of extracts obtained from specimens established in five countries along a latitudinal gradient: Norway, Ireland, France, Spain and Portugal, from North to South of the North-East Atlantic coast. Antioxidant activities of extracts were characterized by four biochemical methods (DPPH radical-scavenging activity, reducing activity, xanthine oxidase inhibition and β-carotene–linoleic acid system), and their total phenolic contents were quantified. This led us to further select some crude extracts for fractionation by a solid phase extraction (SPE) device in order to determine the antioxidant activity and total phenolic content of each fraction. Antibacterial assays were also conducted with six bacterial strains, three marine and three terrestrial, to determine the antibacterial activities of the crude and purified extracts. Proton Nuclear Magnetic Resonance (^{1}H NMR) analysis were carried out on active fractions to determine compounds responsible for the activities detected.

2. Materials and methods

2.1. Sampling: algal material and abiotic parameters

Thalli of *Sargassum muticum* were collected between March and May 2011 in three sites in each of five countries along a latitudinal gradient in Europe. Samples were collected, from South (March) to North (May), in Portugal, Spain, France, Ireland and, finally, Norway (Figure 1). The sampling time was chosen according to the physiological state of the algae in each country. In these periods, *Sargassum muticum* was still immature in all sites. The sampled countries were also chosen according to the chronology of expansion of *Sargassum muticum* along Atlantic coasts; of the countries our sampling, France was the first colonized and Ireland was the most recently colonized.

Collection was made at low tide on semi-exposed or exposed sites in all countries except Norway, where one site was qualified as “sheltered”. The hydrodynamic conditions of the sites were determined according to their topography and the flora present during sampling. Other environmental parameters, such as

seawater temperature, photosynthetically available radiation and water salinity, were determined from measurements made by satellites as part of the AQUA Modis and Aquarius missions of NASA (National Aeronautic and Space Administration). These data are presented as data ranges for the sampling periods considered (Table 1) according to country (see Tanniou et al. 2013, in review).

During collection, only the apical and median parts of the thalli were taken and the holdfast was left in place to allow regrowth and thus minimize collection impact. Immediately after collection and epiphyte removal, the seaweeds were washed first with filtered seawater then distilled water in order to remove residual sediments and salts. The cleaned algal materials were then surface dried with blotting paper towel, chopped into fragments, pooled by site, freeze-dried, reduced to powder with a Waring Blender and, finally, sieved at 250 µm.

2.2. Solid/liquid extraction of phenolic compounds

Two gram dry weight (DW) of finely ground algal material was placed in 250 mL flasks, and extracted using 150 mL of an acetone−water mixture (50:50 v/v in distilled water). Each preparation was left under stirring at 40°C for 3 h in the dark. The mixtures were then centrifuged using an Eppendorf 5810 R centrifuge (Eppendorf A.G., Hamburg, Germany) at 4000 *g* for 10 min at 4°C. The supernatants were then filtered on cotton wool and concentrated to dryness under reduced pressure at 40°C using a rotary evaporator (R-3000, Büchi, Flawil, Switzerland). About 10 mL Milli-Q water were then added to each residue to give the crude extract. All extracts were then freeze-dried prior to further analyses (phenolic content quantification and NMR analyses), apart from 4mL that were used directly for the purification. All the extracts were prepared in triplicate: three extractions were made per site and then pooled for further analyses.

2.3. Purification of crude extracts

Crude extracts were purified by solid phase extraction (SPE) following a modified protocol published by Zubia et al. (2009). The SPE cartridges (HF Bond Elut C18, 5mg, Varian) were used in combination with an under partial vacuum system (Vac Elut SPS 24, Varian) at a pressure of 15 inHg. After conditioning, successively, with methanol (20 mL) and distilled water (20 mL), the SPE cartridge was loaded with the crude extracts (4 mL). After adsorption, fractionation was performed by stepwise elution with 40 mL of each of the following solvents: distilled water, 50% methanol (v/v in distilled water), 100% methanol, dichloromethane : methanol 50:50 (v/v) and 100% dichloromethane. Each crude extract was purified four times to accumulate fractions in sufficient weight: the same eluted fractions were pooled and evaporated under reduced pressure at 40°C using a rotary evaporator (R-3000, Büchi, Flawil, Switzerland). Each fraction was re-dissolved in the appropriate solvent and freeze-dried prior to further analyses.

2.4. Quantity and quality of extracted phenolics

2.4.1. Determination of the total phenolic contents and Folin-Ciocalteu assay

The total phenolic content of all extracts was determined colorimetrically (Labsystems, Multiskan MS, Finland) with a microplate-adapted Folin–Ciocalteu assay (Sanoner et al. 1999), which is known to be little affected by interfering compounds. Interfering substances, however, are thought to account for less than 5% of the Folin–Ciocalteu-reactive compounds in brown seaweeds (Toth and Pavia 2001). Phloroglucinol (1,3,5-trihydroxybenzene, Sigma, Saint Quentin Fallavier, France) was used as a standard, and concentrations were determined in each extract by freeze-drying three aliquots of 1 mL. Total phenolic contents (TPCs) were expressed as percentages of phenolic compounds in the dry weight (DW) of the aliquot or the algae.

2.4.2. NMR analysis of extracts

The overall structural composition of crude extracts was assessed by means of ^{1}H NMR analyses on a Bruker Avance 400 using the standard pulse sequences available in the Bruker software (Bruker, Wissembourg, France). All spectra were recorded in MeOD, at room temperature. Chemical shifts were expressed in ppm. NMR profiles unambiguously indicate the PC within the extracts, with peak(s) between 5.5 and 6.5 ppm. The nature of the phlorotannins present in the MeOH-water fractions was established using heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond correlation (HMBC) experiments and comparison of the chemical shifts of the ^{1}H and ^{13}C resonances with literature data (Cérantola et al. 2006). All spectra were recorded in MeOD, at 25°C.

2.5. Activity measurements on extracts

From a general point of view, the antioxidant / radical scavenging tests carried out on algal crude extract and/or semi-purified fractions are based on both the determination of products resulting from the oxidation and the measurement of the efficiency of a substance to trap radicals (Huang et al. 2005).

We used several different tests in our study, with the aim of revealing different types of mechanisms in the action of phenolic compounds: DPPH radical scavenging assay, reducing power, superoxide anion-scavenging capacity assay (XO activity assay) and β carotene bleaching method,. Our assays included those based on electron-transfer reactions (DPPH and reducing power) together with an assay involving hydrogen atom transfer reactions (β carotene bleaching method), as described by Huang et al. (2005). The first two types measure the capacity of an antioxidant to reduce an oxidant, which changes colour when reduced, while the second type uses a competitive reaction scheme in which antioxidant and substrate compete for thermally-generated peroxyl radicals through the decomposition of azo compounds (Huang et al. 2005). DPPH and reducing power tests give insight into the levels of the lipophilic and hydrophilic compounds, whereas BCBM assesses the levels of lipophilic compounds alone (Chew et al. 2008). The XO activity assay measures another ROS scavenging capacity: the superoxide anion ($O_2{\cdot}^-$) is generated by a xanthine oxidase/hypoxanthine system and reduces nitroblue tetrazolium (NBT) to blue formazon if no competitor, i.e. an antioxidant substance (the sample) is added (Huang et al. 2005). Even though $O_2{\cdot}^-$ is not the most damaging ROS, it is an initiator of highly detrimental ROS production such as singlet oxygen, hydroxyl radical (HO●) and peroxynitrate (OONO-) and could thus increase the oxidation risk indirectly (Huang et al. 2005).

2.5.1. DPPH radical scavenging assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay, modified according to Le Lann et al. (2008), was used to determine the radical scavenging activities in extracts and purified SPE fractions. The positive controls used were ascorbic acid, also known as vitamin C (Sigma, Saint Quentin Fallavier, France), α-tocopherol also known as vitamin E (Sigma, Saint Quentin Fallavier, France), butylated hydroxyl-anisole (BHA), butylated hydroxyl-toluene (BHT) (respectively 2(3)-*t*-Butyl-4-hydroxyanisole, 2,6-Di-*tert*-butyl-4-methylphenol, Sigma, Saint Quentin Fallavier, France). The protocol was microplate-adapted for faster use and to decrease sample preparation time. Briefly, five dilutions from 0.1 to 2 mg.mL^{-1} of the extracts were prepared in triplicate before addition to 12 µL aliquots of 150 µM DPPH radical (222 µL). The mixture was stored in the dark for 60 min prior to absorbance measurement at 540 nm. Distilled water was used as a negative control. All samples were assayed in triplicate. Antioxidant activity was expressed as the IC50 (the concentration of substrate that causes a 50% loss of DPPH activity): the lower the IC50 is, the stronger the antioxidant activity. Extracts with an IC50 higher than 10 mg/mL were considered as non-active extracts.

2.5.2. β-carotene bleaching method

The antioxidant activities of extracts and controls were measured by the β-carotene bleaching microplate adapted method, modified in accordance with Kaur and Kapoor (2002) and Koleva et al. (2002). Two mL of a β-carotene solution in chloroform (0.1 mg/mL) were added to round-bottom flasks containing 20 mg of linoleic acid and 200 mg of Tween 40. After evaporation with a rotavapor, oxygenated distilled water (50 mL) was added, and the mixture was shaken to form a liposome solution. This mixture was added to each of the following: 12 µL of the extracts, positive controls (α-tocopherol, BHA and BHT) and negative controls (distilled water and ethanol). The absorbance of the solution at 450 nm was measured immediately (t = 0 min) and after 2 h at 50°C (t = 120 min). All samples were assayed in triplicate. Antioxidant activity was expressed through the antioxidant activity coefficient (AAC), calculated as follows (equation 1):

$$AAC = \left[\frac{As_{(120)} - Ac_{(120)}}{Ac_{(0)} - Ac_{(120)}}\right] \times 1000 \ (eq.1)$$

where $As_{(120)}$ is the absorbance of the antioxidant mix at t = 120 min, $Ac_{(120)}$ is the absorbance of the control at t = 120 min, and $Ac_{(0)}$ is the absorbance of the control at t = 0 min. Since the positive controls, BHA, BHT and vit E, had an average AAC of 700, this value was arbitrarily chosen to express the antioxidant activity as AAC_{700}, as in Le Lann et al. (2008). So, AAC_{700} was the concentration of substrate needed to obtain an AAC value of 700 (Tanniou et al. 2013). A high AAC_{700} was therefore considered as indicative of a weak antioxidant activity.

2.5.3. Reducing power

The reducing capacity of each extract was assessed by the method adapted by Zubia et al. (2009) and Kuda et al. (2005). In a 96-well microplate, aliquots of extracts (25 µL) were mixed with phosphate buffer (25 µL, 0.2 M, pH 6.6) and potassium ferricyanide ($K_3Fe(CN)_6$) (25 µL, 1%). After incubation at 50°C for 20 min, the microplate was cooled down prior to the addition of 25µL trichloroacetic acid (10%). Then, 25 µL 0.1% $FeCl_3$ $6H_2O$ and 100 µL water were added to each well. The increase of absorbance, which indicates an increase in reducing activity, was read at 620 nm after 10 minutes at room temperature. Results were expressed as the EC50 value (mg/mL) (Oueslati et al. 2012), which is the effective concentration at which the absorbance was 0.5 for reducing power. This assay was carried out in triplicate for each sample and the positive controls (BHA, BHT, vit E and vit C).

2.5.4. Superoxide anion-scavenging activity

The superoxide anion-scavenging assay was carried out according to the method of Nagai et al. (2003). The reaction mixture consisted of 203 µL 0.05M Tris HCl buffer (pH 7.5), 57 µL 5 mM hypoxanthine, 30 µL 0.33 mM NBT and 13 µL of the sample. After incubation at 25 °C for 10 min, the reaction was started by adding 30 µL xanthine oxidase and keeping the temperature at 25°C for 21 min. The absorbance was measured every 3 min for 21 min at 560 nm. The inhibition ratio (%) was calculated from the following equation (Chua et al. 2008):

$$\%\ inhibition = \left[\frac{rate\ of\ control - rate\ of\ test\ sample}{rate\ of\ control}\right] \times 100\ (eq.2)$$

Results were then expressed as IC50 (the concentration of substrate that causes a 50% inhibition).

2.6. Antibacterial tests

Algal extracts at five different concentrations, from 50 to 150 $\mu g.mL^{-1}$, were tested for inhibitory activity against three marine bacterial strains obtained from the Ifremer-collection: *Vibrio aestuarianus* (S02-041), *Vibrio anguillarum* (S11-054) and *Vibrio parahaemolyticus* (S12-011); and three terrestrial strains: *Escherichia coli* (T05-006, ATCC8739), *Staphylococcus aureus* (T05-007, ATCC 65388) and *Pseudomonas aeruginosa* (T05-005, ATCC15442). Each treatment and control was replicated four times. Extracts were incubated for 24 h with the bacteria in exponential growth phase (at 2.10^8 UFC/mL) in 96-well plates (VWR) in LB medium (Luria Hinton Broth, Sigma, Andover, UK), supplemented with NaCl (35 $g.L^{-1}$) for the marine strains, at 21 or 37°C for marine or terrestrial strains, respectively. The antibiotic chloramphenicol was used as a control, at the same concentration as the tested extracts. Only the results for the smaller concentration (50 $\mu g.ml^{-1}$) are presented here.

2.7. Statistics

All analyses were carried out in tri- or quadruplicate, and their results are presented as mean values ± standard deviation (SD), using Statistica 8 (StatSoft ®) software for PC. Depending on the variable, means were calculated using three or four values/measurements per extract and were used for the statistical analysis. Homogeneity of variance was tested with the Brown-Forsythe test at the 0.05 error risk. Data that did not satisfy the criteria of normality and homoscedasticity for parametric tests were square root transformed before further analyses. one-way nested ANOVAs, with sites nested within countries, were performed on the data concerning the crude extract. For the semi-purified extract, simple one-way ANOVAs were performed to compare differences among sites in each country. When ANOVA demonstrated significant difference, post-hoc Tukey HSD tests were carried out to identify which means contributed to the effect.

Chemicals

All reagents used in the experiments were of analytical grade and most were obtained from Sigma. Solvents used for extraction of algae samples were purchased from Fisher Scientific. Water used was of Millipore quality.

3. Results

3.1. Distinction between countries regarding the crude extracts

Country of sampling had a significant effect on the TPCs found in *S. muticum* (one-way ANOVA, $p < 0.001$, Figure 2). *S. muticum* populations from the different sampled countries were ranked by their level of phenolic content as follows: Portugal>Norway>France=Ireland=Spain (post-hoc Tukey). The highest TPCs were observed at the extremes of the latitudinal gradient, which correspond to the edges of the distribution range of the species, i.e. Norway (1.79 ± 0.28 $\%DW_{algae}$) and Portugal (3.40 ± 0.91 $\%DW_{algae}$). Phenolic content reached 1.26 ± 0.11 for France and the minimum concentration is observed for Spain with 0.66 ± 0.15 $\%DW_{algae}$. Inter-site variations was also observed, especially in Portugal, where phenolic content varied from 2.46 ± 0.16 to 4.28 ± 0.26$\%DW_{algae}$.

In addition to the algal phenolic concentration, it is interesting to examine an extract containing the maximum PC. Based on such extracts the results are expressed in %DW where the dry weight is that of the concentrated extract. As above, great differences in maximum PC were observed among the countries (one-way nested ANOVA, $p<0.001$, Figure 3a)). Thus, the maximum is observed for Portugal where the total phenolic content reached more than 17% of the fraction dry weight (17.49 ± 0.49 $\%DW_{fraction}$ for site 2): in Portugal phenolics represent more than 15.85 % on average, whereas this content reached 13.06, 10.69, 8.25 and 7.29 % in Norway, France, Ireland and Spain, respectively (Figure 3a).

The antioxidant activity of *S. muticum* extracts differed among countries (one-way ANOVA, $p<0.001$). According to the ANOVA post-hoc Tukey test ($p < 0.05$), the AAC700 following the β-carotene bleaching

method (AAC_{700}) in the extracts was superior to those of all the positive controls, showing that those extracts have very low antioxidant activity ($p < 0.001$, Figure 3b). In the same way, radical scavenging activity differed among countries (one-way ANOVA, $p < 0.001$). Activity of crude extracts differed from the positive controls (post-hoc Tukey test, $p < 0.001$). However, some extracts showed activities close to those of the positive controls: the radical scavenging activity determined by DPPH method (IC50) was the highest in extracts from Norway and Portugal, with 0.44 ± 0.03, 0.41 ± 0.03 and 0.46 ± 0.01 $mg.mL^{-1}$ for the sites 2 and 3 of Norway and the site 1 of Portugal, respectively (Figure 3c). As previously seen for the phenolic content, the most active extracts are those of the extreme countries, followed by France, Spain and finally the less active extracts, those from algae collected in Ireland (0.75 ± 0.01, 0.94 ± 0.02 and 0.84 ± 0.02 $mg.mL^{-1}$ for sites 1, 2 and 3, respectively). This tendency is confirmed by the results of the reducing activity assay (Figure 3d) showing that the highest reducing activity was displayed by the extracts from Norway and Portugal (0.079 ± 0.01, 0.074 ± 0.01, 0.082 ± 0.01 and 0.086 ± 0.01, 0.082 ± 0.01, 0.079 ± 0.01 $mg.mL^{-1}$ for the sites 1, 2 and 3 of Norway and Portugal, respectively). Moreover, according to the statistical analysis, this reducing activity is equivalent to those displayed by the positive controls (0.10± 0.01; 0.086 ±0.01; 0.092± 0.01; 0.13± 0.01 $mg.mL^{-1}$ for BHA, BHT, vit E and vit C, respectively). Finally, xanthine oxidase (XO) inhibition also depends on the country of origin of the samples (Figure 3e). Only extracts from Norway and Portugal displayed activities that can be compared to the positive controls (0.20 ± 0.01, 0.19 ± 0.01, 0.17 ± 0.01 and 0.29 ± 0.01, 0.21 ± 0.02, 0.19 ± 0.01 $mg.mL^{-1}$ for sites 1, 2 and 3 of Norway and Portugal, respectively).

3.2. Inter-country differences in the purified extracts

Figure 4 shows the phenolic polarity repartition in fractions obtained after an SPE procedure. Depending on the country of origin, the majority of the phenolics were found in the water, Methanol-water or Methanol fraction. Thus, for Norway, France and Portugal, compounds were present in the water fraction in small quantity (means: 0.53 ± 0.11, 0.095 ± 0.03 and 0.56 ± 0.25 $\%DW_{algae}$ for Norway, France and Portugal, respectively) but with the majority in the MeOH-water fraction (means: 0.82 ± 0.19, 0.44 ± 0.06 and 1.45 ± 0.37 $\%DW_{algae}$ for Norway, France and Portugal, respectively). For Ireland, apart from the site 1, the great majority of the phenolic compounds were found in the MeOH fraction. For Spanish extracts, the same quantities were present in the water and MeOH-water fractions, representing approximately 0.15 $\%DW_{algae}$.

Only the results of antioxidant assays and radical scavenging activities will be presented for the purified fractions, other anti-ROS activities were not measured on purified extracts. Only *S. muticum* from Norway and Portugal contained high amounts of active phenolics, so only the results for these two countries are presented in Table 2. Radical scavenging activities (determined by the DPPH method and using IC50 to calculate the activity), were always better in purified fractions than in the crude extracts and were close to those of the positive controls. In addition, the MeOH-water fraction was more active than the MeOH one in almost all cases. Thus, the best radical scavenging activities were displayed by MeOH-water fraction from sites 2 and 3 in Norway (0.28 ± 0.02; 0.27 ± 0.02 $mg.mL^{-1}$ respectively), by the MeOH fraction from site 2 in Portugal (0.25 ± 0.03 $mg.mL^{-1}$) and by the MeOH-water fraction from site 3 in Portugal (0.25 ± 0.01 $mg.mL^{-1}$). As for the crude extracts, for both these countries, antioxidant activities determined by the β-carotene bleaching method

(ACC_{700}) were very low and dissimilar to the positive controls. From here on we will focus on the most active fractions (MeOH-water and MeOH), which contain sufficient PC.

Antibacterial activities were dependent on country and varied strongly among sites within country (Table 3). Thus, if we focus on marine strains, almost all extracts, i.e. both crude and purified, possessed activities against *Vibrio aestuarianus* and *V. anguillarum*. Conversely, few extracts were active against *V. parahaemolyticus*: only crude extracts from Ireland (all sites) and from sites 2 and 3 in Portugal displayed good activities (>50% bacterial growth inhibition) against this strain. In many cases, the purified extracts were less active than the crude extracts, depending both on the country and on the studied strain. When purified extracts showed higher activity, it was always from the MeOH fractions. For the terrestrial strains, in the case of *Staphylococcus aureus* strains, the purified extracts were more active than the crude ones for all the countries tested, except Ireland. Norwegian and Irish extracts showed very high activities against *Escherichia coli*. Interestingly, all the crude extracts were active against *Pseudomonas aeruginosa*.

3.3. Identification of active phlorotannins from Norway, France and Portugal

Figure 5 shows the spectrum obtained using ^{1}H NMR to analyze active fractions from Norway, France and Portugal. In crude and purified extracts, PCs are visible between 5.5 and 6.5 ppm. In both purified extracts the two phlorotannin profiles seem to be very similar (Figure 5b). In the chosen active purified fractions, i.e. for the MeOH-water ones, phenolics were always present and the two profiles were always quite similar. In addition, the separation by polarity allows the removal of a great amount of mannitol, which is prevalent in crude extracts.

Results from the two dimensional NMR analysis (HMBC) showed that the purified extracts from *Sargassum muticum* collected in Norway, France and Portugal were all rich in phlorethol(s) (Figure 6) and that these compounds seem not to be linear (Cerantola et al. 2006).

For these two types of analyses (^{1}H NMR and 2D NMR), the small quantity of compound present in extracts of samples from Ireland and Spain did not allow clear signals to be obtained in the aromatic area. These minor signals are masked by other more abundant molecules. Despite the low antiROS activities of the extracts from France, these results are presented here for information purposes, to see whether phenolic compounds produced by French populations are of the same type as from Portuguese and Norwegian populations. Some small differences were visible between spectra obtained from French, Portuguese and Norwegian populations (Figure 6). Using HMBC, Norwegian and French populations can be seen to be similar and separated from the Portuguese population; using HMQC, the three populations appear quite similar, but with differences remaining in the general phlorotannin profiles.

4. Discussion

4.1. Activities of the crude extracts

This study examined the effect of the country and site of sampling on the TPC, antioxidant and antibacterial activities of *Sargassum muticum* extracts. The screening procedure also provided us with a way to identify the best extracts for later application, and also to know whether exploitation of this species would be viable all the countries in the study.

The antiROS tests showed that at the selected sampling period, the extracts were quite active. Crude extracts had very good reducing activities and antiXO comparable to activities measured for positive controls used in industry (BHA, BHT, vit E and vit C for the reducing power and Trolox for the XO test). The best activities were shown by the extracts from the extremities of the gradient, i.e., Norway and Portugal; this was also where the phenolic compound concentrations were highest, showing the important role of these compounds in the detected activities. Moreover, extracts from samples taken in France also showed good reducing activities, in accordance with the phenolic concentrations in the extracts. Even though the PC concentration was lower in France than in these two other countries, it was still more than 1 % of the dry weight of the seaweed. Here, only a small quantity of phenolics was extracted from seaweeds compared with what has been shown to be possible in other studies on the same species (Connan et al. 2004; Plouguerné et al. 2006; Parys et al. 2009), it can be understandable by the chosen sampling period. Indeed it has been known for a long time that the quantity of phenolics contained in seaweeds is largely dependent on the season and environmental parameters in general (Jormalainen and Honkanen 2001; Hemmi and Jormalainen 2004; Fairhead et al. 2005; Connan et al. 2007). Here, the collection period was chosen to sample individuals of *Sargassum muticum* in a same physiological state along the European gradient studied. This approach was taken to enable the comparison of results and to avoid bias caused by reproduction; indeed, only immature individuals were collected. However, this period is not the most convenient for the production of a large quantity of PC by seaweeds, as shown by numerous authors (Connan et al. 2004; Plouguerné et al. 2006; Le Lann et al. 2012). Nevertheless, crude extracts showed interesting antioxidant and antibacterial activities. Some extracts inhibited the growth of five bacterial strains by more than 50% and some have activities equivalent to the one detected by the antibiotic, the chloramphenicol at the same concentration (50 $\mu g.mL^{-1}$).

4.2. Activities of the purified extracts

The compound distribution by polarity seems rather similar from one country to another. The PCs were found for the greater part in the polar or quite apolar fractions, such as aqueous, methanol-water and methanolic fractions. This distribution was not identical among samples, which seems to indicate that the pool of extracted compounds does not consist of a single phenolic type. Indeed, as we have already shown for *S. muticum*, the pool of compounds can vary according to different parameters (Tanniou et al. 2013). The SPE is used as an assay here to separate molecules contained in the extract by polarity but also, therefore, to obtain one or several "cleansed" fractions concentrating the compounds of interests (here the PCs). One can note that there is an increase of the radical scavenging activities after purification, showing that phenolic compounds are responsible for the

measured activities. These activities are comparable to those obtained with the positive controls, which are molecules used in industry. For the antioxidant activity results measured by the β-carotene bleaching method, no activity was measured after purification. As this test measures the activity of lipophilic molecules (Koleva et al. 2002; Le Lann et al. 2008) then the active compounds here would thus tend to be polar, or slightly apolar.

Extracts from all five countries showed evidence of bacterial inhibition. Extracts were active against three marine bacterial strains (*Vibrio aestuarianus*, *V. anguillarum* and *V. parahaemolyticus*) and three terrestrial strains (*Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*). In this test, the most active extracts were no longer those from the gradient extremities. Indeed, if we look at the crude extracts, it was especially in those from Ireland and Portugal that we found the highest activities. For the purified extracts, only those with high antiROS activities and a large phenolic content were tested here. Yet, in the course of our experiments we found that many extracts were less active after purification. This would suggest that the compound(s) active against bacteria is/are not necessarily phenolic. Preliminary studies have described antibacterial activities for *S. muticum* extracts (Hellio et al. 2002, 2004a,b). For the same genus, some authors also demonstrated antibacterial activity of chloroform extracts (Sastry and Rao 1994). Plougerné et al. (2008, 2010) also showed that non-polar extracts of *Sargassum muticum* were the most active against bacterial strains. In our study, apolar extracts were not tested. The goal was to determine wherher fractions containing PCs had antibacterial activities. In the literature, we find that phlorotannins isolated from *Sargassum vestitum* and *S. natans* also have antifouling activity (Sieburth and Conover 1965; Jennings and Steinberg 1997). Our results showed that only a few extracts keep their antibacterial activities after purification. This is the case of the site 1 Portuguese extract tested against *V. aestuarianus* and *E. coli*, for example.

Taking into account all results, Norway and Portugal are the two countries where active phenolic compounds could be obtained in great quantity after separation. One could hypothesize that "extreme" environmental conditions along the Atlantic coasts could stress *Sargassum muticum*, thus forcing it to produce more phenolic compounds to act as a defence against UV radiation and seawater temperature changes, for example. Indeed, if we take into account the tendencies observed during the study of the environmental parameters, we can propose some hypotheses regarding the factors that could influence phenolic content. In the chosen sampling periods, we noticed that the quantity of available radiation for photosynthesis was higher where we found the largest quantities of active CP, i.e. in the extremities of the gradient and in France. Other authors observed an effect of season, and thus probably of light levels (Connan et al. 2004; Plouguerné et al. 2006), on the production of CP as photoprotective molecules (de la Coba et al. 2009). Other parameters seem less correlated with the observed variations, although salinity has been cited by other authors as a factor that can influence and reduce phenolic content (Ragan and Glombitza 1986; Connan and Stengel 2011). Here, salinity was not correlated with lowered PC concentration, although the smallest content was for Spain, where salinity is the highest (up to 37 psu). The dates of colonization of *Sargassum muticum* could also be taken in consideration. Indeed, this species spread northward and southward from the south of England starting in 1973. It has been post recently found on the north Portuguese coast (by 2002-2010) and in Norway around 2000. It is thus possible that at first, early in their arrival in new area, these seaweeds produce more defensive compounds to colonize their novel environment. In any case, it seems a little dangerous to draw general conclusions on the effect of geographical position and thus environmental parameters on the production of PC by *S. muticum*; indeed,

numerous authors having demonstrated the existence of very small scale sources of variation, such as day/night or seasonal variation (Connan et al. 2004; 2007). Also, the expression of genes involved in PC biosynthesis occurs only a few hours after a light stimulation (E. Creis, personal communication). It would thus be necessary to repeat this study at another period of the year and/or make several samplings per day, to be able to identify the sources of variation in total phenolic content.

4.3. Identification of active phlorotannins from Norway, France and Portugal

The ^{1}H-NMR profiles show a great similarity between the spectra of Norway, France and Portugal. The 2D spectra allowed us to identify the phlorotannins produced by *Sargassum muticum* as being of the phlorethol type, and HMBC revealed that they are rather not linear (Cerantola et al. 2006). It seems that *S. muticum* does not produce different compounds according to geographical zones studied. According to quantitative analyses, only the amount varies. It is possible, considering phenolic content as a pool, that the relative proportions of the different compounds vary. This is easiest to see on the ^{1}H NMR spectra: the French PC profile (with visible peaks) is different from that of Portuguese and Norvegian populations in the aromatic area. Phenolic compounds have already been isolated and identified in certain brown seaweeds (Kang et al. 2005; Cerantola et al. 2006; Singh and Bharate 2006). Here, even if we know the overall structure of the isolated compounds, we still need to find out the number of repeated units within the molecules to really discern the structure of the present compounds. Mass spectrometry analyses are therefore currently in progress at the laboratory, which should provide us with answers regarding the various sizes of phlorethols present in extracts and also give an idea of the category of compounds varying according to the environmental conditions and thus the geographical position of *S. muticum*.

Spatial variation in levels of brown algal phenolic compounds has been examined at very different scales (Table 4). Van Alstyne et al. (1999) highlighted this by presenting number of various studies made in the field. Among these studies on the brown algae, *Sargassum* is a model classically studied for the variability of its phenolic content (Plouguerné *et al.* 2006; Le Lann *et al.* 2011; 2012), but, to our knowledge no previous study has focused on the spatial variability of both the quantity and the quality of phlorotannins on a large scale (Atlantic coasts). Therefore, the present study allowed us to gain a first idea of the variability of this type of compound produced by a species on a large geographical scale. Studies on the variability of macromolecules (carbohydrates, lipids and proteins) of *S. muticum* along to the same gradient are now in progress. The results should provide an overview of compartments subject to variation for the same species according to its geographical position, especially in a context of global change.

4.4. Future research

Phenolics or phlorotannins can have many applications in industry, correlated with their numerous properties: anti-diabetic, anti-cancer, anti-oxidation, antibacterial, radioprotective and anti-HIV (Kohen and Nyska 2002; Naikai et al. 2006; Kuda et al. 2007; Kumar et al. 2008; Gupta and Abu-Ghannam 2011; Yong-Xin

et al. 2011). Phloroglucinol can be used as a coupling agent in printing, in explosives and for the industrial synthesis of pharmaceuticals (Flopropione). The interest of this molecule makes it important to try to find ways of exploiting natural marine resources like seaweeds. However, numerous studies show that these compounds can vary a great deal in terms of polarity and structure, according to species of seaweed and even within the same species according to the environmental conditions (Jormalainen and Honkanen 2001; Hemmi and Jormalainen 2004; Fairhead *et al.* 2005; Connan et al. 2007). It is thus rather difficult to find a standard extraction protocol for all these compounds. Also, if we wanted to use them in the food or pharmaceutical industries, extraction and purification processes would need to comply with European requirements. In a previous study, Tanniou et al. (2013) demonstrated for the first time the unquestionable usefulness of using modern, solvent-free and environmentally-friendly methods for the non-denaturing extraction of brown algal polyphenols, using *S. muticum* as a model. In particular, CPE and PLE seemed to be the most promising methods for the extraction of polyphenols with useful antioxidant potential. It will thus be necessary to replace the acetone–water extraction by one of these processes, for example. Finally, large biomasses of *S. muticum* are present at the northern and southern extremities of Europe. This available biomass contains a large amount of phenolics, which can be purified to a point where phenolic structure can be determined. The purified extracts have good antiROS and antibacterial activities and, consequently, could be very useful for future industrial applications. As we find *S. muticum* in great quantities on the Atlantic coasts, algal harvesting could be possible in Europe. However, the particular weather conditions of each country could represent an additional barrier. For example, it is difficult to have access to seaweeds all year long in Norway. The exploitation of this species would thus be easier in southern Europe, e.g. in Portugal. Besides being more easily accessible, our study showed seaweeds in this country are richer in phenolics. However, in countries such as Ireland, France and Spain, where the algae did not produce the highest amount of phenolics in our results, such exploitation would be a compromise. A solution could be the cultivation of *S. muticum* under controlled conditions which mimic natural stresses conditions (light intensity, temperature,..) to force *S. muticum* to produce phenolics in large quantities (metabolic forcing), thus making the exploitation of Irish, French and Spanish biomasses possible.

Conclusion

Our paper presents the first study taking into account the spatial variability of the phenolic compounds for one species along a large spatial scale and the potential of this species for a future use in industry as antiROS or antibacterial molecules. The most interesting extracts were those from Portuguese populations, which display the best antiROS activities after purification and contain a very large amount of PCs. *S. muticum* biomass is available in considerable quantities on Portuguese coasts where the environmental conditions (especially weather) allow it to be harvested easily for a large part of the year. This brown invasive macroalga, which caused environmental disturbance when it arrived, could finally become a valuable marine resource for European countries.

Acknowledgments

This study is part of the PhD. thesis work carried out by the first author at the Laboratoire des Sciences de l'Environnement Marin (LEMAR UMR 6539), which is part of the IUEM (UBO-UEB). It was supported by the Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche (UBO grant to the first author). The authors would like to thank M.A. Poullaouec, I. Bihannic, students and staff of the Research Group for their help. The authors sincerely thank H. McCombie for fruitful assistance in writing this manuscript in English. This study is related to two research projects: BIOTECMAR (Interreg IVB, 2009-2011) and INVASIVES (Era-net, 2012-2016).

References

Aruoma OI, Spencer JPE, Warren D, Jenner P, Butler J, Halliwell B (1997) Characterization of food antioxidants, illustrated using commercial garlic and ginger preparations. Food Chemistry 60: 149-156.

Bandoniené D, Pulaskas A, Veskutonis PR, Gruzdiené D (2000) Preliminary screening of antioxidant activity of some plant extracts in rapeseed oil. Food Research International 33: 785-791.

Bourgougnon N, Stiger-Pouvreau, V (2011). Chapter 4: Chemodiversity and bioactivity within red and brown macroalgae along the french coasts, metropole and overseas departements and territories. In: Handbook of Marine Macroalgae: Biotechnology and Applied Phycology (ed S.-K. Kim), pp. 58-105

Cérantola S, Breton F, Ar Gall E, Deslandes E (2006) Co-occurrence and antioxidant activity of fucol and fucophlorethol classes of polymeric phenols in *Fucus spiralis*. Bot. Mar. 49: 347-351.

Chua MT, Tung YT, Chang ST (2008) Antioxidant activities of ethanolic extracts from the twigs of *Cinnamomum osmophloeum*, Bioresource Technology 99: 1918-1925.

Connan S, Deslandes E, Ar Gall E (2007) Influence of day–night and tidal cycles on phenol content and antioxidant capacity in three temperate intertidal brown seaweeds. J Exp Mar Biol Ecol 349: 359-369.

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

Engelen A, Espirito-Santo C, Simoes T, Monteiro C, Serrao EA, Pearson GA, Santos ROP (2008) Periodicity of propagule expulsion and settlement in the competing native and invasive brown seaweeds, *Cystoseira humilis* and *Sargassum muticum* (Phaeophyta). European Journal of Phycology 43: 275-282.

Fairhead VA, Amsler CD, McClintock JB, Baker BJ (2005) Variation in phlorotannin content within two species of brown macroalgae (*Desmarestia anceps* and *D. menziesii*) from the Western Antarctic Peninsula. Polar Biol 28: 680-686.

Gupta S, Abu-Ghannam N (2011) Bioactive potential and possible health effects of edible brown seaweeds. Trends in Food Science & Technology 22 (6): 315-326.

Hellio C, Marechal JP, Véron B, Bremer G, Clare A, Le Gal Y (2004)a Seasonal variation of antifouling activities of marine algae from the Brittany coast (France). Mar. Biotechnol. 6: 67-82.

Hellio C, Simon Colin C, Clare AS, Deslandes E (2004)b Anti-settlement activities of *Grateloupia turuturu* hydroalcoholic extracts on cyprids of Balanus amphitrite. Biofouling 20: 139-145.

Hellio C, Berge JP, Beaupoil C, Le Gal Y, Bourgougnon N (2002) Screening of marine algal extracts for antisettlement activities against microalgae and macroalgae. Biofouling 18: 205-215.

Hemmi A, Jormalainen V (2004) Geographic covariation of chemical quality of the host alga *Fucus vesiculosus* with fitness of the herbivorous isopod *Idotea baltica*. Mar Biol 145:759–768.

Incera M, Olabarria C, Troncoso JS, Lopez J (2009) Response of the invader Sargassum *muticum* to variability in nutrient supply. Marine Ecological Progress Series 377: 91-101.

560 Ito N, Hirose M, Fukushima H, Tsuda T, Shirai T, Tatenatsu M (1986) Studies on antioxidants: Their
561 carcinogenic and modifying effects on chemical carcinogens. Food and Chemical Toxycology 24: 1071-1092.

562 Jennings JK, Steinberg PD (1997) Phlorotannins versus other factors affecting epiphyte abundance on the kelp
563 *Ecklonia radiata*. Oecologia 109: 461-473.

564 Jormalainen V, Honkanen T (2001) Multiple cues for phenotypic plasticity in phlorotannin production of the
565 bladder wrack *Fucus vesiculosus*. Phycologia 40: 59-60.

566 Kang KA, Lee KH, Chae S, Zhang R, Jung MS, Lee Y, Kim SY, Kim HS, Joo HG, Park JW, Ham YM, Lee NH,
567 Hyun JW (2005) Eckol isolated from *Ecklonia cava* attenuates oxidative stress induced cell damage in lung
568 fibroblast cells. FEBS Letters 579: 6295-6304.

569 Kaur C, Kapoor HC (2002) Anti-oxidant activity and total phenolic content of some Asian vegetables. Int.
570 J.Food Sci. Tech. 37: 153-61.

571 Kohen R, Nyska A (2002) Oxidation of biological systems: Oxidative stress phenomena, antioxidants, redox
572 reactions and methods for their quantification. Toxicol. Pathol., 30: 620-650.

573 Koivikko R, Loponen J, Pihlaja K, Jormalainen V (2007) Highperformance liquid chromatographic analysis of
574 phlorotannins from the brown alga *Fucus vesiculosus*. Phytochem Anal 18: 326-332.

575 Koivikko R, Loponen J, Honkanen T, Jormalainen V (2005) Contents of soluble, cell-wall-bound and exuded
576 phlorotannins in the brown alga *Fucus vesiculosus*, with implications on their ecological functions. J. Chem.
577 Ecol. 31: 195-212.

578 Koleva II, Van Beek TA, Linssen JPH (2002) Screening of plant extracts for antioxidant activity: a comparative
579 study on three testing methods. Phytochem. Anal.13: 8-17.

580 Kraan S (2008) *Sargassum muticum* (Yendo) Fensholt in Ireland: an invasive species on the move. Journal of
581 Applied Phycology 20: 825-832.

582 Kuda T, Kunii T, Goto H (2007) Varieties of antioxidant and antibacterial properties of *Ecklonia stolonifera* and
583 *Ecklonia kurome* products harvested and processed in the Noto peninsula, Japan. Food Chem. 103: 900-5.

584 Kuda T, Tsunekawa M, Hishi T, Araki Y (2005) Antioxidant properties of dried 'kayamo-nori', a brown alga
585 *Scytosiphon lomentaria* (Scytosiphonales, Phaeophyceae). Food Chem. 89: 617-622.

586 Kumar Chandini S, Ganesan P, Bhaskar N (2008) *In vitro* antioxidant activities of three selected brown
587 seaweeds of India. Food Chem. 107: 707-13.

588 Le Lann K, Ferret C, VanMee E, Spagnol C, Lhuillery M, Stiger-Pouvreau V (2012) Total phenolic, size-
589 fractionated phenolics and fucoxanthin content of tropical Sargassaceae (Fucales, Phaeophyceae) from the South
590 Pacific Ocean: Spatial and specific variability. Phycol. Res. 60(1): 37-50.

591 Le Lann K, Connan S, Stiger-Pouvreau V (2012) Phenology, TPC and size-fractioning phenolics variability in
592 temperate Sargassaceae (Phaeophyceae, Fucales) from Western Brittany: Native versus introduced species.
593 MERE 80: 1-11.

594 Le Lann K, Jégou C, Stiger-Pouvreau V (2008) Effect of different conditioning treatments on total phenolic
595 content and antioxidant activities in two Sargassacean species: Comparison of the frondose *Sargassum muticum*
596 (Yendo) Fensholt and the cylindrical *Bifurcaria bifurcata* R. Ross. Phycol. Res. 56: 238-245.

597 Nagai T, Inoue R, Inoue H, Suzuki N (2003) Preparation and antioxidant properties of water extracts of propolis.
598 Food Chemistry 80: 29-33.

599 Nakai M, Kagayama N, Nakahara K, Miki W (2006) Phlorotannins as radical scavengers from the extract of
600 *Sargassum ringgoldianum*. Mar. Biotechnol. 8: 409-14.

601 Olabarria C, Rodil IF, Incera M, Troncoso JS (2009) Limited impact of *Sargassum muticum* on native algal
602 assemblages from rocky intertidal shores. Marine Environmental Research 67: 153-158.

603 Oueslati S, Trabelsi N, Boulaaba M, Legault J, Abdelly C, Ksouri R (2012) Evaluation of antioxidant activities
604 of the edible and medicinal *Suaeda* species and related phenolic compounds. Industrial Crops and Products 36:
605 513-518.

606 Parys S, Kehraus S, Pete R, Kupper FC, Glombitza KW, Konig GM (2009) Seasonal variation of polyphenolics
607 in *Ascophyllum nodosum* (Phaeophyceae). Eur J Phycol 44:331-338.

608 Pavia H, Aberg P (1996) Spatial variation in polyphenolic content of *Ascophyllum nodosum* (Fucales,
609 Phaeophyta). Hydrobiologia 326/327: 199-203.

610 Plouguerné E, Georgantea P, Ioannou E, Vagias C, Roussis V, Hellio C, Kraffe E, V Stiger-Pouvreau (2010)
611 Anti-microfouling activity of lipidic metabolites from the invasive brown alga *Sargassum muticum* (Yendo)
612 Fensholt. Mar Biotechnol 12(1): 52-61.

613 Plouguerné E, Hellio C, Deslandes E, Véron B, Stiger-Pouvreau V (2008) Anti-microfouling activities in
614 extracts of two invasive algae: *Grateloupia turuturu* and *Sargassum muticum*. Botanica Marina 51: 202-208.

615 Plouguerné E, Le Lann K, Connan S, Jechoux G, Deslandes E, Stiger-Pouvreau V (2006) Spatial and seasonal
616 variation in density, reproductive status, length and phenolic content of the invasive brown macroalga
617 *Sargassum muticum* (Tendo) Fensholt along the coast of Western Brittany (France). Aquat. Bot. 85: 337-4.

618 Ragan MA, Glombitza KW (1986) Phlorotannins, brown algal polyphenols. Prog. Phycol. Res. 4: 129-241.

619 Sanoner P, Guyot S, Marnet N (1999) Polyphenol profiles of French cider apple varieties (*Malus domestica* sp.).
620 J. Agric. Food Chem. 47: 4847-53.

621 Sastry VMVS, Rao GRK (1994) Antibacterial substances from marine algae: successive extraction using
622 benzene, chloroform and methanol. Bot. Mar. 37: 357-360.

623 Schoenwaelder MEA, Clayton MN (1998) Secretion of phenolic substances into the zygote wall and cell plate in
624 embryos of *Hormosira* and *Acrocarpia* (Fucales, Phaeophyceae). J. Phycol. 34: 969-80.

625 Sieburth JM, Conover JT (1965) *Sargassum* tannin, an anti-biotic which retards fouling. Nature 208: 52-53.

626 Singh IP, Bharate SB (2006) Phloroglucinol compounds of natural origin. Natural Product Reports, 23, 558-591.

627 Steinberg PD, Estes JA, Winter FC (1995) Evolutionary consequences of food chain length in kelp forest
628 communities. Proc natn Acad Sci USA 92: 8145-8148.

629 Steinberg PD (1992) Geographical variation in the interaction between marine herbivores and brown algal
630 secondary metabolites. In: Paul VJ (ed) Ecological roles of marine natural products. Comstock Publishing
631 Associates, Ithaca, New York, pp 51-92.

632 Steinberg PD (1989) Biogeographical variation in brown algal polyphenolics and other secondary metabolites:
633 comparison between temperate Australasia and North America. Oecologia 78: 374-383.

634 Steinberg PD (1986) Chemical defenses and the susceptibility of tropical brown algae to herbivores. Oecologia
635 69: 628-630.

636 Stiger V, Deslandes E, CE Payri (2004) Phenolic contents of two brown algae, *Turbinaria ornata* and *Sargassum*
637 *mangarevense* on Tahiti (French Polynesia): interspecific, ontogenic and spatio-temporal variations. Bot. Mar.
638 47(5): 402-409.

639 Tanniou A, SerranoLeon E, Vandanjon L, Ibanez E, Mendiola JA, Cerantola S, Kervarec N, LaBarre S, Marchal
640 L, Stiger-Pouvreau V (2013) Green improved processes to extract bioactive phenolic compounds from brown
641 macroalgae using *Sargassum muticum* as model. Talanta 104: 44-52.

642 Targett NM, Arnold TM (2001) Effects of secondary metabolites on digestion in marine herbivores. In:
643 McClintock JB, Baker BJ (eds) Marine chemical ecology. CRC, Boca Raton, pp 391-411.

644 Targett NM, Boettcher AA, Targett TE, Vrolijk NH (1995) Tropical marine herbivore assimilation of phenolic-
645 rich plants. Oecologia 103: 170-179.

646 Targett NM, Coen LD, Boettcher AA, Tanner CE (1992) Biogeographic comparisons of marine algal phenolics:
647 evidence against a latitudinal trend. Oecologia 89: 464-470.

648 Toth GB, Pavia H (2001) Removal of dissolved brown algal phlorotannins using insoluble
649 polyvinylpolypyrrolidone (PVPP). J. Chem. Ecol. 27: 1899-910.

650 Van Alstyne KL, McCarthy JJ, Hustead CL, Duggins DO (1999) Geographic variation in polyphenolic levels of
651 Northeastern Pacific kelps and rockweeds. Marine Biology 133: 371-379.

652 Van Alstyne KL, Paul VJ (1990) The biogeography of polyphenolic compounds in marine macroalgae:
653 temperate brown algal defenses deter feeding by tropical herbivorous fishes. Oecologia 84: 158-163.

654 Yong-Xin L, Isuru W, Yong L, Se-Kwon K (2011) Phlorotannins as bioactive agents from brown algae. Process
655 Biochemistry 46 (12): 2219-2224.

656 Zubia M, Fabre MS, Kerjean V, Le Lann K, Stiger-Pouvreau V, Fauchon M, Deslandes E (2009) Antioxidant
657 and antitumoural activities of some Phaeophyta from Brittany coasts, Food Chemistry 116: 693-701.

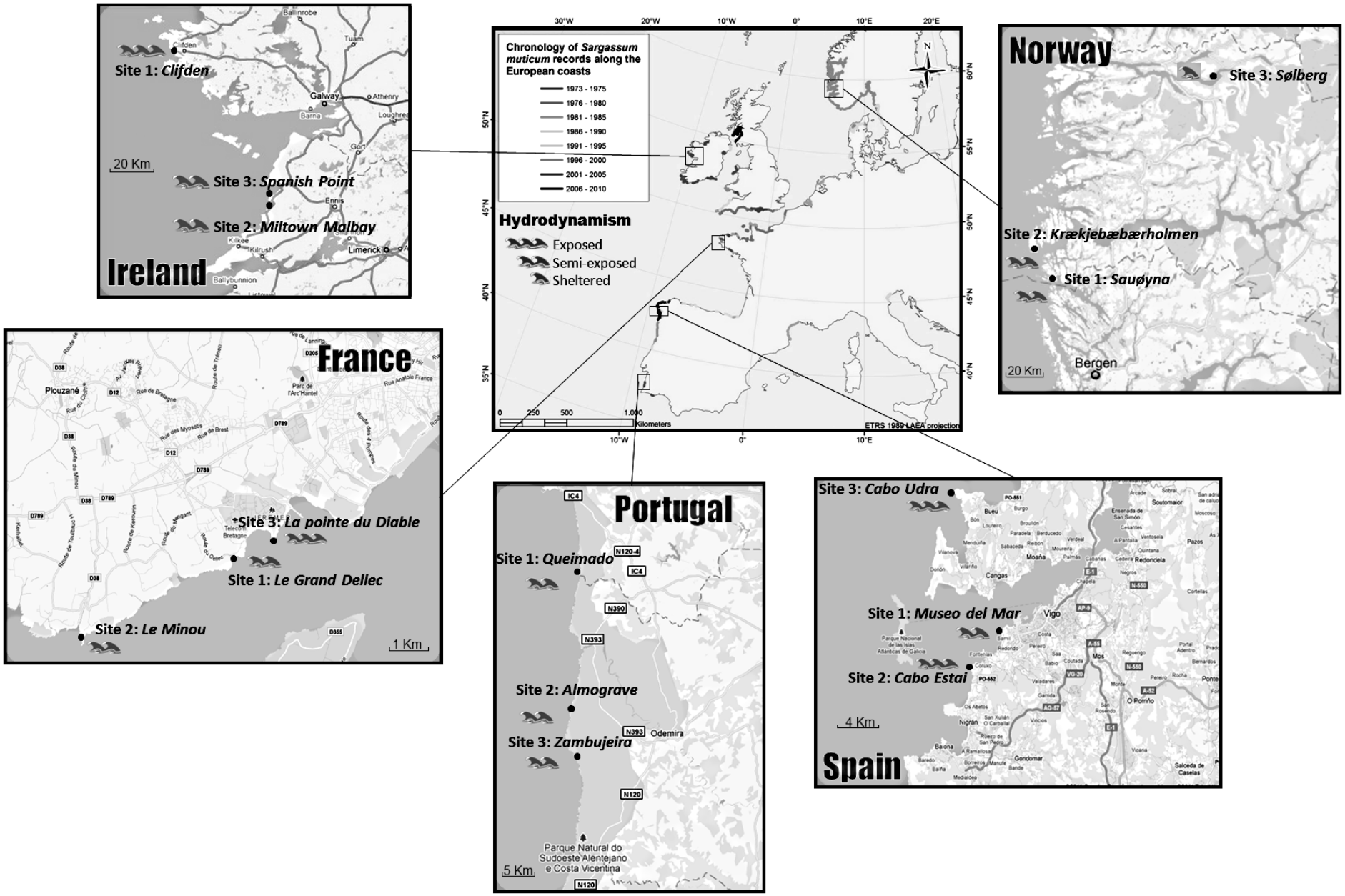


Figure 1. Sampling sites along the Atlantic coasts of Europe where *Sargassum muticum* were collected to examine variations in phenolic concentrations and activities over a large scale (3 sites per country: Norway, Ireland, France, Spain and Portugal).

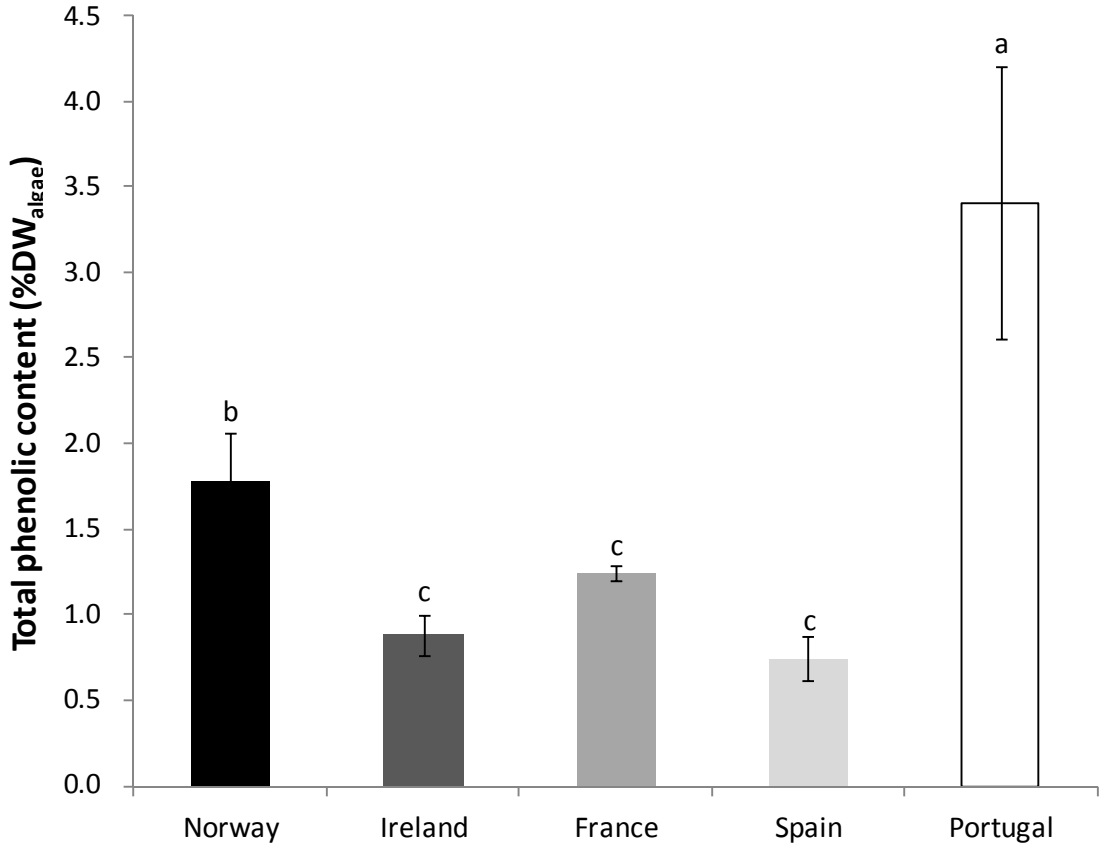
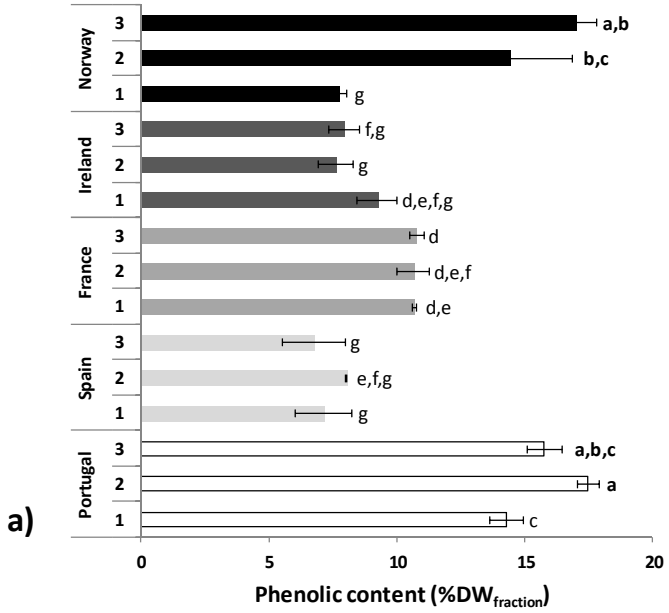
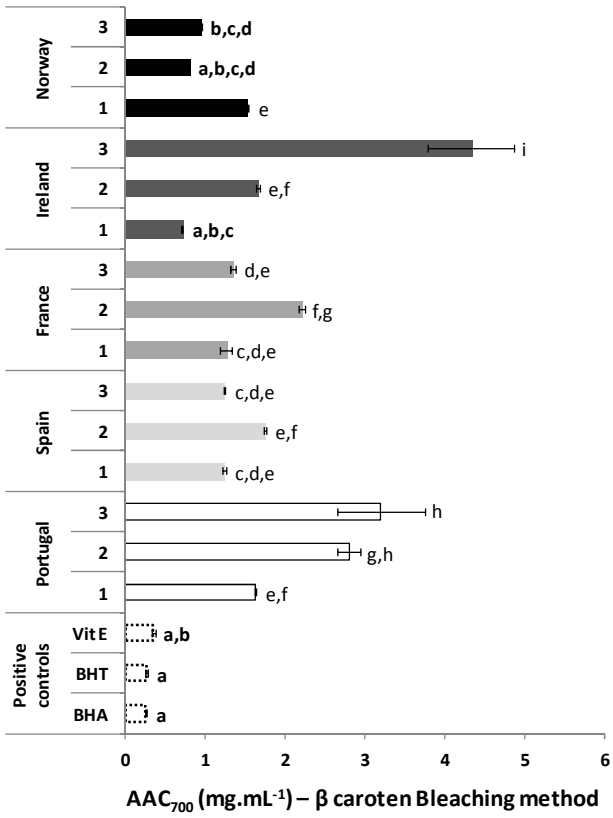


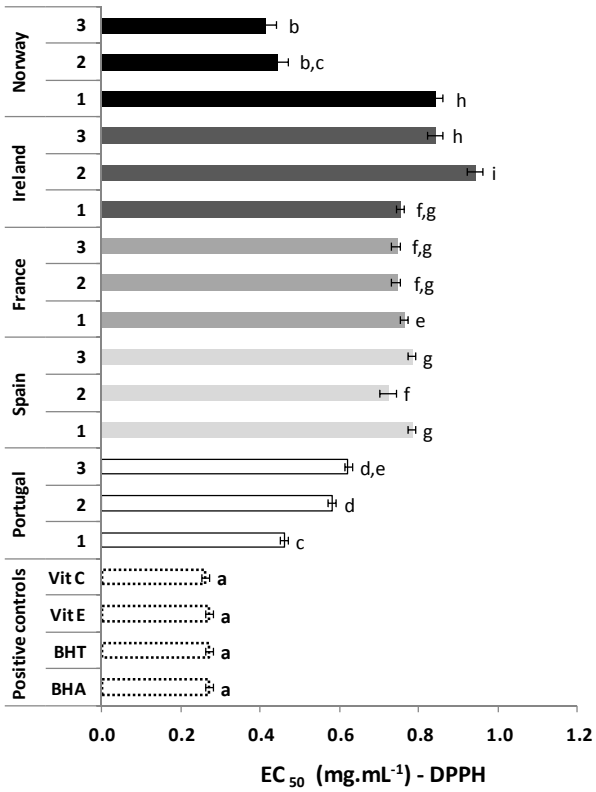
Figure 2. Spatial variability of phenolic content of crude extracts from *Sargassum muticum* collected along the Atlantic coasts of Europe from Portugal to Norway. Data (mean values per country ± standard deviation) were analyzed using one-way ANOVA followed by post-hoc Tukey tests (a-c). Different letters indicate significant differences.


Norway
Ireland
France
Spain
Portugal
3
2
1
a,b
b,c
g
f,g
g
d,e,f,g
d
d,e,f
d,e
g
e,f,g
g
a,b,c
a
c
0
5
10
15
20
Phenolic content (%DW fraction)

a)


Norway
Ireland
France
Spain
Portugal
Positive controls
3
2
1
Vit E
BHT
BHA
b,c,d
a,b,c,d
e
i
e,f
a,b,c
d,e
f,g
c,d,e
c,d,e
e,f
c,d,e
h
g,h
e,f
a,b
a
a
0
1
2
3
4
5
6
AAC700 (mg.mL-1) – β caroten Bleaching method

b)


Norway
Ireland
France
Spain
Portugal
Positive controls
3
2
1
Vit C
Vit E
BHT
BHA
b
b,c
h
h
i
f,g
f,g
f,g
e
g
f
g
d,e
d
c
a
a
a
a
0.0
0.2
0.4
0.6
0.8
1.0
1.2
EC 50 (mg.mL-1) - DPPH

c)

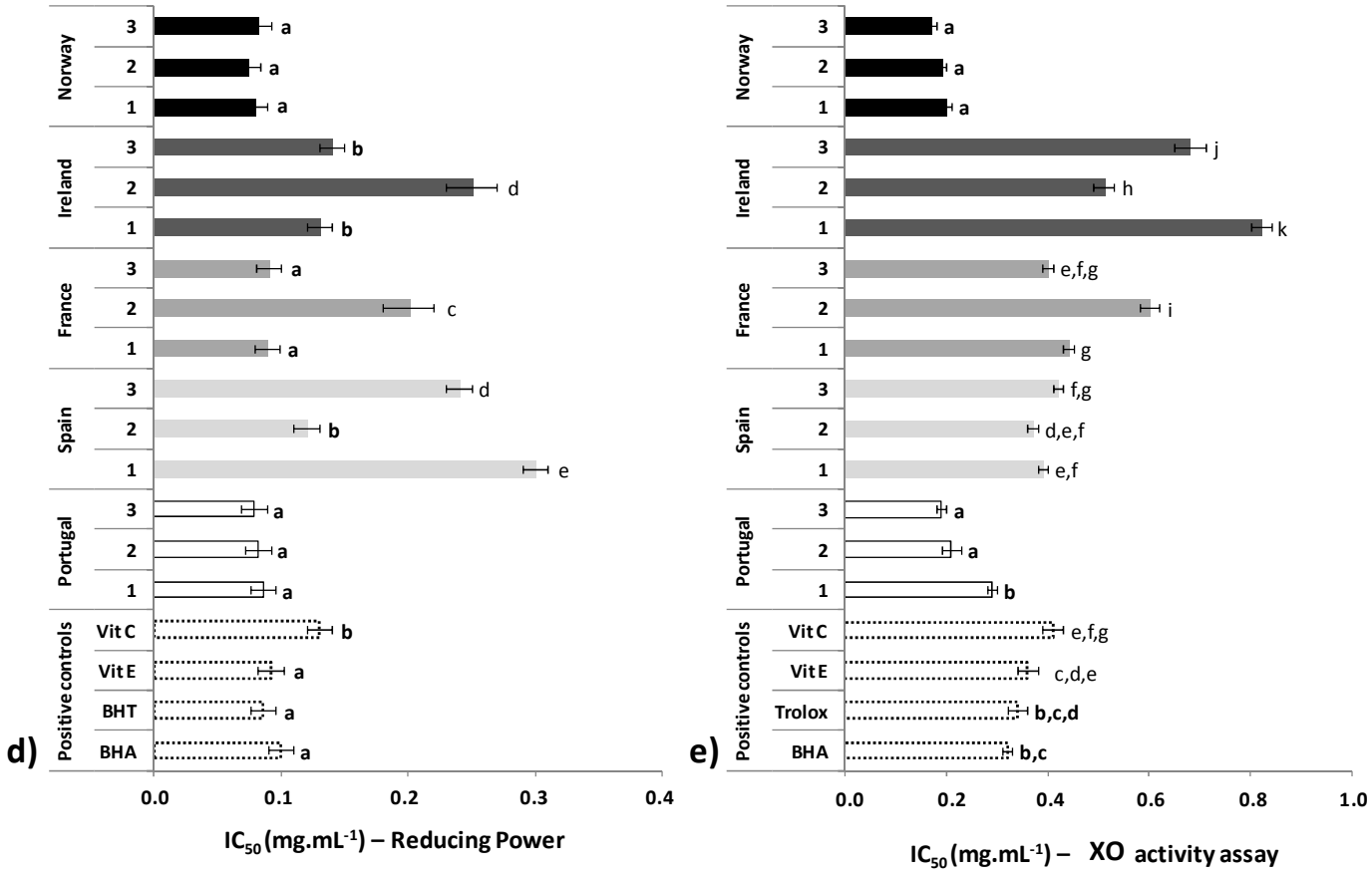


Figure 3. Phenolic content (a) and screening of several antioxidant activities measured on phenolic extracts from *Sargassum muticum* collected along the Atlantic coasts of Europe from Norway to Portugal, using the b-caroten bleaching test (b), radical scavenging activity (c), reducing power (d) and XO activity (e). Data were analyzed using one-way nested ANOVA followed by post-hoc Tukey tests (a-k). Different letters indicate significant differences.

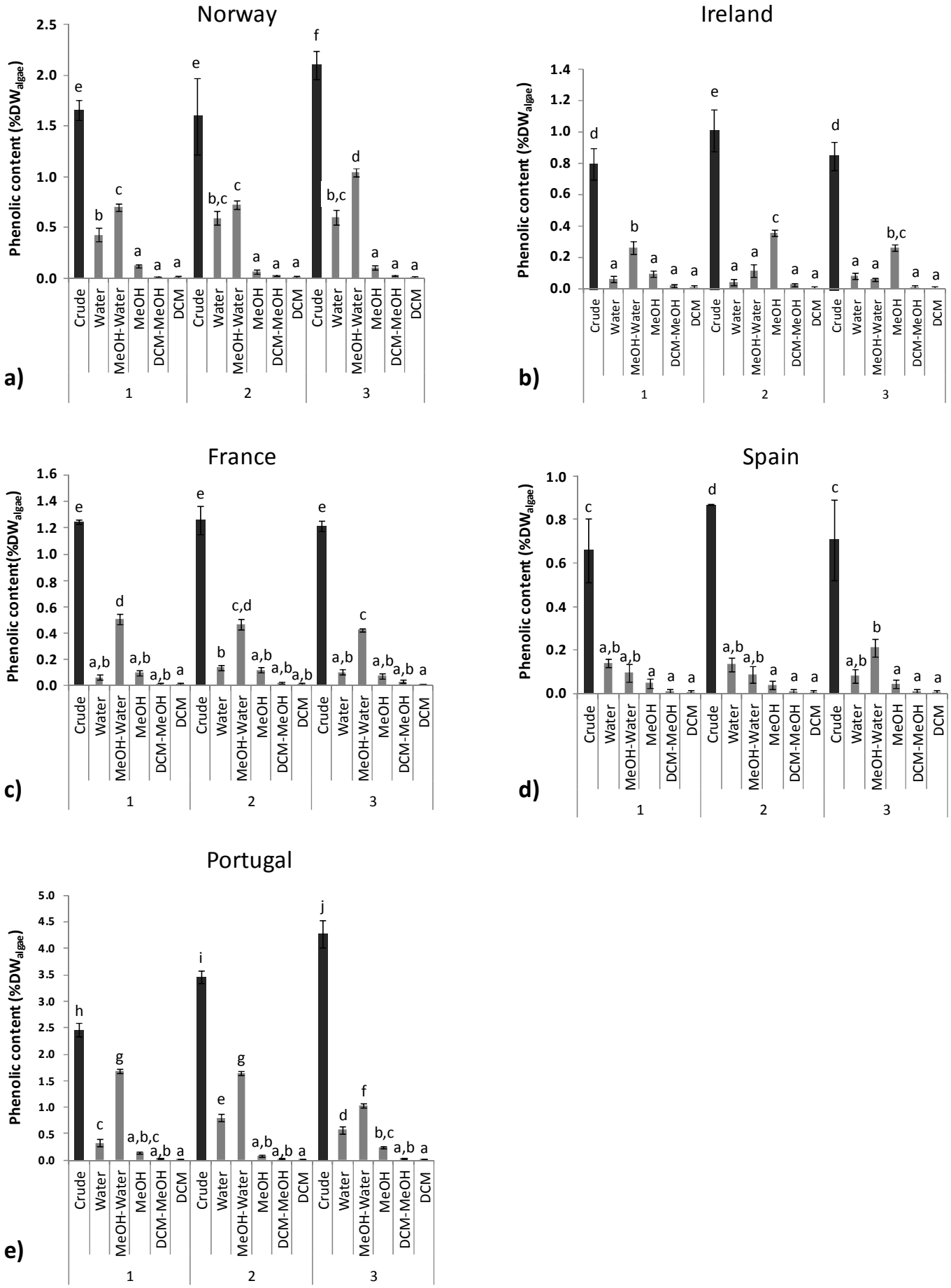


Figure 4. Spatial variability of phenolic content determined on crude and SPE-purified extracts from *Sargassum muticum* collected along the Atlantic coasts of Norway (a), Ireland (b), France (c), Spain (d) and Portugal (e). For each country, data were analyzed using one-way ANOVA followed by post-hoc Tukey tests (a-j). Different letters indicate significant differences.

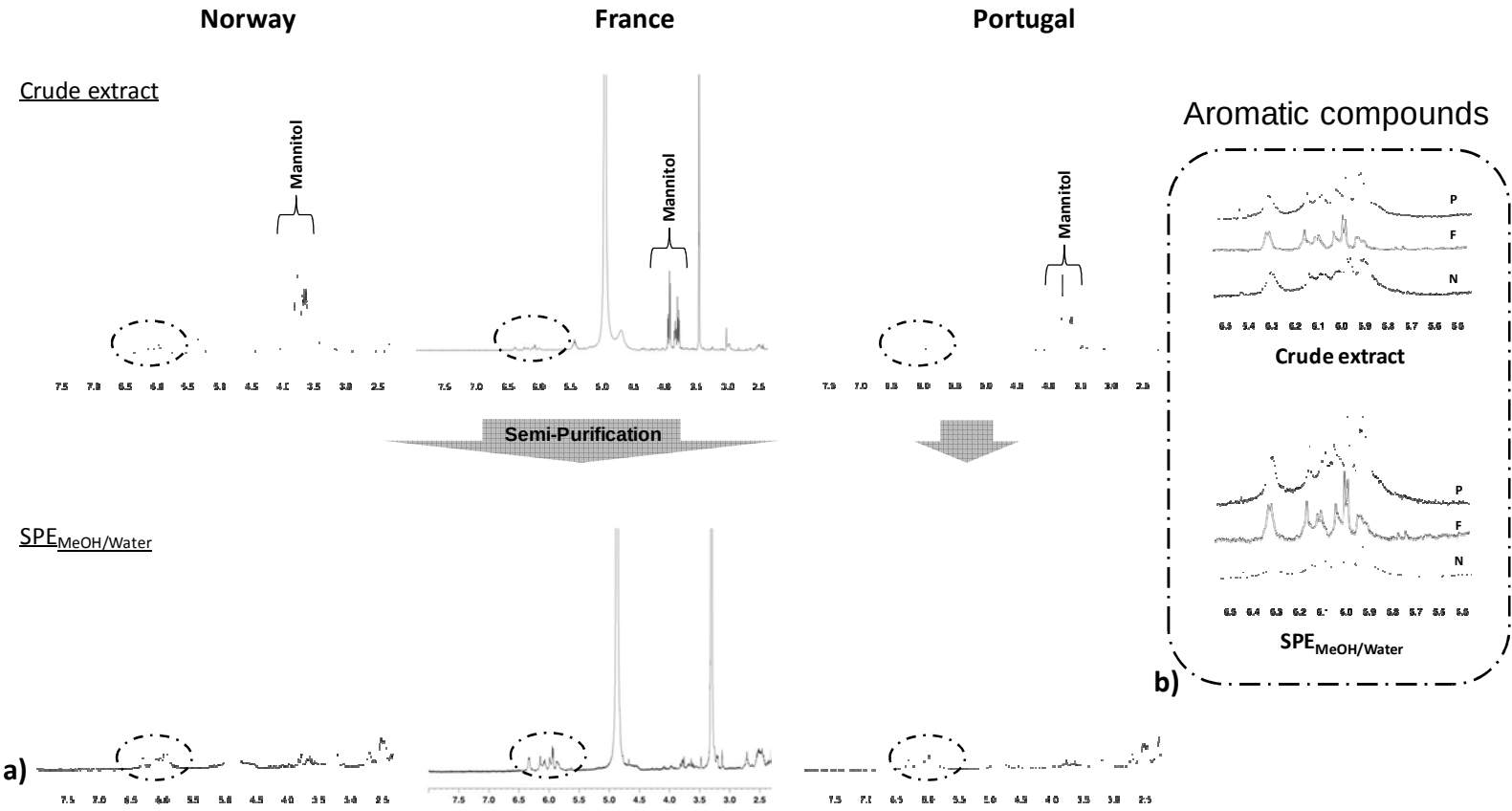


Figure 5. ^{1}H NMR analysis of active crude and SPE purified extracts from *Sargassum muticum* collected in Norway, France and Portugal (a) and enlargement of the aromatic area between 5.5 and 6.5 ppm for the crude and purified extracts (b). N: Norway; F: France and P: Portugal.

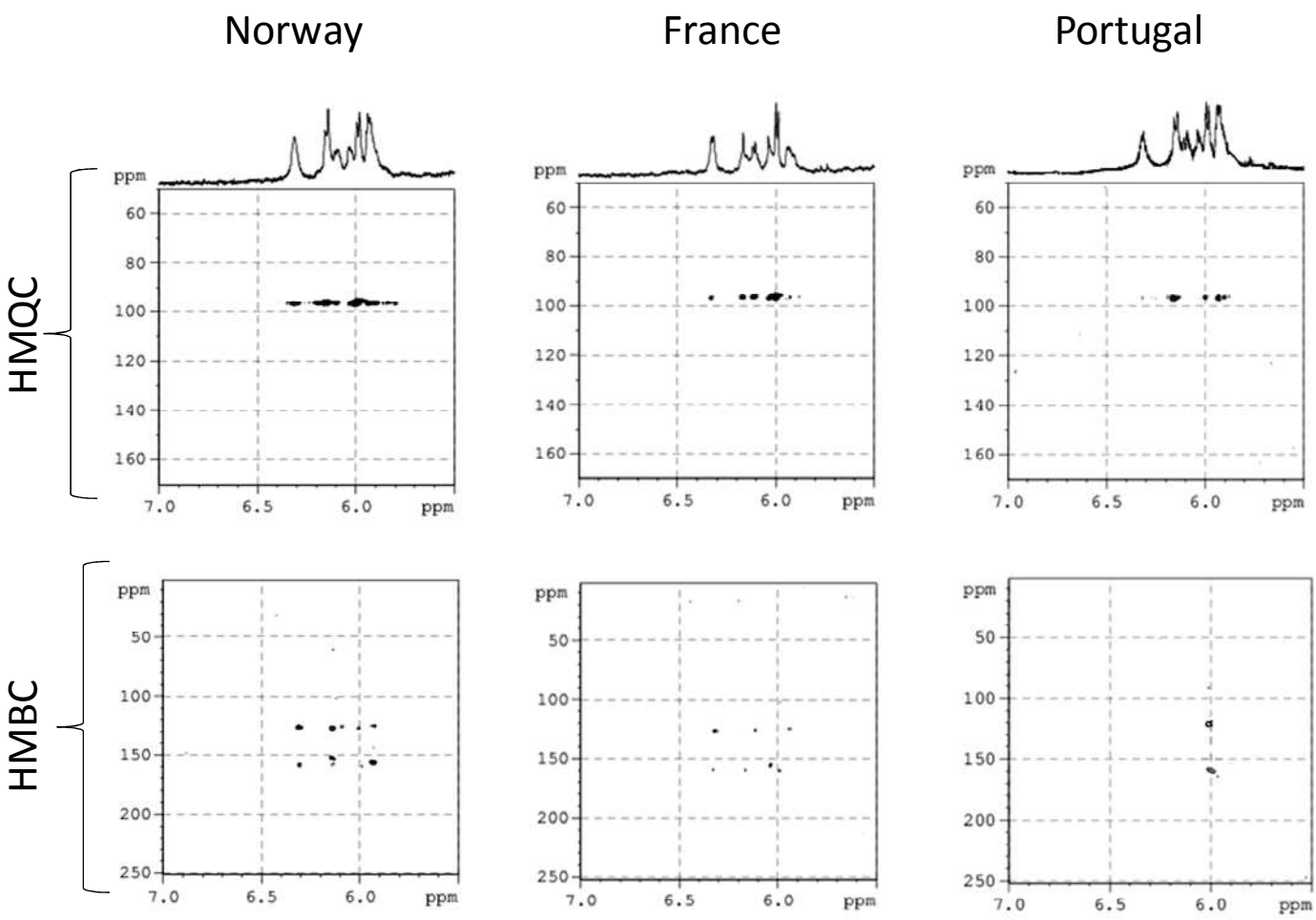


Figure 6. The nature of the phlorotannins present in the MeOH-water fractions was established using heteronuclear multiple bond correlation (HMBC) and heteronuclear multiple quantum coherence (HMQC) experiments.

Table 1. Environmental parameters according to country during the sampling period (March to May 2011; see Tanniou et al. 2013 in review): sea surface temperature, photosynthetically available radiation and sea surface salinity. Data obtained from NASA satellites during the two missions Aqua MODIS and Aquarius. The different shades correspond to low (white), average (light grey) and high (grey) values.

Abiotic Parameters	Norway	Ireland	France	Spain	Portugal
Sea Surface Temperature in °C	8-10	12-14	12-14	12-14	18-20
Photosynthetically Available Radiation in Einstein.m^{-2}.day^{-1}	35-40	30-35	35-40	20-25	40-45
Sea Surface Salinity PSU	34	35	35	36	37

Table 2. Antioxidant activities measured using solid phase extraction (SPE) purified polar extracts from *Sargassum muticum* collected along the European Atlantic coasts, from Portugal to Norway. Data were analyzed using one-way nested ANOVA followed by post-hoc Tukey tests. The most active extracts/fractions compared with the positive controls are presented in bold. Different letters indicate significant differences among extracts for each variable (i.e. activity test).

			Means values ± SD Tukey HSD (p<0.05)		
Variable	Countries	Fractions	Site 1	Site 2	Site 3
DPPH		Crude	0.84 ± 0.02 (h)	0.44 ± 0.03 (d,e)	0.41 ± 0.03 (d,e)
IC 50 (mg/mL)	Norway	MeOH-Water	0.39 ± 0.02 (c,d)	**0.28 ± 0.02 (a,b)**	**0.27 ± 0.02 (a,b)**
		MeOH	0.51 ± 0.03 (f)	0.54 ± 0.03 (f)	0.41 ± 0.03 (d,e)
		Crude	0.46 ± 0.01 (e,f)	0.58 ± 0.01 (g)	0.62 ± 0.01 (g)
	Portugal	MeOH-Water	**0.33 ± 0.01 (b,c)**	**0.33 ± 0.01 (b,c)**	**0.25 ± 0.01 (a)**
		MeOH	0.43 ± 0.03 (d,e)	**0.25 ± 0.03 (a)**	0.46 ± 0.03 (e,f)
	positive controls	BHA	0.27 ± 0.01 (a,b)		
		BHT	0.27 ± 0.01 (a,b)		
		Vit C	0.26 ± 0.01 (a,b)		
		Vit E	0.27 ± 0.01 (a,b)		
β-caroten		Crude	1.53 ± 0.01 (e)	0.79 ± 0.01 (b,c)	0.94 ± 0.02 (b,c)
AAC_{700} (mg/mL)	Norway	MeOH-Water	1.65 ± 0.01 (e,f)	1.33 ± 0.01 (e)	1.74 ± 0.01 (e,f,g)
		MeOH	1.10 ± 0.01 (c,d)	0.63 ± 0.01 (b)	1.07 ± 0.01 (c,d)
		Crude	1.63 ± 0.01 (e,f)	2.80 ± 0.14 (h)	3.20 ± 0.55 (i)
	Portugal	MeOH-Water	2.06 ± 0.01 (g)	1.63 ± 0.01 (e,f)	1.41 ± 0.01 (d,e)
		MeOH	1.93 ± 0.01 (f,g)	0.93 ± 0.01 (b,c)	1.48 ± 0.01 (e)
	positive controls	BHA	0.25 ± 0.01 (a)		
		BHT	0.26 ± 0.01 (a)		
		Vit C	0.26 ± 0.01 (a)		
		Vit E	0.27 ± 0.02 (a)		

Table 3. Bioassays for the antibacterial activities of crude or SPE-purified extracts (fractions MeOH-water and methanol) for the three sites in each of the five studied countries along the Atlantic coasts. SPE: solid phase extraction. ns: no activity.

	Norway-Site 1			Norway- Site 2			Norway-Site 3			Ireland-Site 1			Ireland-Site 2			Ireland- Site 3		
	Crude	M-W	M	Crude	M-W	M	Crude	M-W	M	Crude	M-W	M	Crude	M-W	M	Crude	M-W	M
02-041	+++	+	++	+++	+	++	+++	+	++	+++	+	+	++	+	+	+++	+	+
11-054	++	++	+	+	++	++	++	-	++	++	++	+	++	++	++	++	++	-
12-011	+	-	-	+	-	-	+	-	-	+++	-	-	++	-	-	++	-	-
T007	-	++	+	-	+	+	+	++	++	+++	+	+	+++	+	+	+++	+	+
T006	++	+++	+++	+	+	+++	++	+++	+++	-	+++	+++	-	++	+++	-	+++	+++
T005	++	ns	ns	+++	ns	ns	++	ns	ns	++	ns	ns	+++	ns	ns	+++	ns	ns
	France-Site 1			France-Site 2			France-Site 3			Spain-Site 1			Spain-Site 2			Spain-Site 3		
	Crude	M-W	M	Crude	M-W	M	Crude	M-W	M	Crude	M-W	M	Crude	M-W	M	Crude	M-W	M
02-041	-	+	++	+	++	+++	+	++	+++	+	+	+++	+	+	+	-	+	+
11-054	+	+	+	++	+	+	+	+	+	++	++	+	-	+	++	++	+	+
12-011	-	-	+	-	-	+	-	-	-	-	-	+	-	-	+	-	-	-
T007	-	++	++	-	++	+++	-	++	+	-	+++	++	-	++	+++	+	++	++
T006	-	++	-	-	+	-	-	-	-	+	+++	-	+++	++	-	+++	+	-
T005	++	ns	ns	+++	ns	ns	+	ns	ns	+++	ns	ns	++	ns	ns	++	ns	ns
	Portugal-Site 1			Portugal-Site 2			Portugal-Site 3											
	Crude	M-W	M	Crude	M-W	M	Crude	M-W	M									
02-041	+++	+++	+++	+++	++	++	++	++	++									
11-054	++	+	+	++	+	+	++	-	+									
12-011	+	-	-	++	-	-	++	+	-									
T007	+	+	+	+	+	++	-	+	+									
T006	++	++	++	+++	+	+	+++	+	-									
T005	++	ns	ns	+++	ns	ns	++	ns	ns									

Marine bacterial strains 02-041: *Vibrio aestuarianus*; 11-054: *Vibrio anguillarum*; 12-011: *Vibrio parahaemolyticus*;

Terrestrial bacterial strains T007: *Staphylococcus aureus*; T006: *Escherichia coli*; T005: *Pseudomonas aeruginosa*;

M-W and M: methanol-water and methanol fractions, respectively, after SPE purification

+++: >75%; ++: 50-75%; +: 25-50 %; -: <25% bacterial growth

Antibiotic chloramphenicol at 50 µg.mL^{-1} inhibited growth by >75% for all strains (marine and terrestrial)

Table 4. Different scales of spatial variation in levels of brown algal phenolic compounds according to published reports

Scale	Examples	Authors
Large	Interspecific variations between hemispheres	Steinberg 1989, 1992; Steinberg et al. 1995
	Interspecific variations between temperate and tropical habitats	Steinberg 1986; Van Alstyne and Paul 1990; Targett et al. 1992
Average	Inter- and intraspecific variations within a large geographical area (more than hundred km)	Pavia and Aberg 1996; Van Alstyne et al. 1999; Le Lann et al. 2011
Small	Inter- and intraspecific variations between sites in the same country or small geographical area (ten to hundred km)	Steinberg 1989; Targett et al. 1992, 1995; Plouguerné et al. 2006; Connan et al. 2007; Le Lann et al. 2012