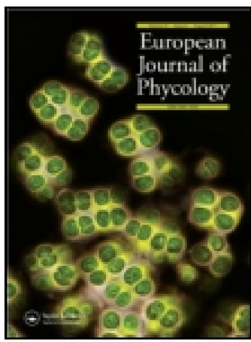

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Seasonal variation of polyphenolics in *Ascophyllum nodosum* (Phaeophyceae)

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Thalli of the brown alga *Ascophyllum nodosum* were collected on the Scottish west coast and the phlorotannin content was measured once a month between January and December 2005. Due to the potential use of *A. nodosum* extract as a nutritional supplement or for medical purposes, our aim was to determine the most favourable time for collection of the algal material. For this purpose two different methods for quantitative phenol determination were used, i.e. the well-known spectrophotometric measurements with Folin–Ciocalteu's phenol reagent (FC-method) and for the first time quantitative ^{1}H NMR spectroscopy (qHNMR). Qualitative variations regarding the composition of the polyphenolic fraction were analysed employing RP-HPLC and ^{1}H NMR spectroscopy. Both quantitative and qualitative analysis of phlorotannins revealed variations over the course of the year. The highest phenol content during the year was reached in July.

Key words: *Ascophyllum nodosum*, Folin–Ciocalteu's phenol reagent, phlorotannin content, quantitative ^{1}H NMR spectroscopy, seasonal variation, statistical evaluation

Introduction

The brown alga *Ascophyllum nodosum* (L.) Le Jol. (Fucales) grows on rocky mid-littoral/intertidal temperate coasts, especially in rather sheltered sites, where it constitutes much of the seaweed biomass. Like *Fucus vesiculosus* and *F. spiralis*, *A. nodosum* occurs on the coasts of the North Sea, the Celtic and Norwegian Sea and it is present on both sides of the North Atlantic (Lüning, 1985).

Ascophyllum and other brown algae contain polyphenols composed of phloroglucinol subunits, termed phlorotannins. The phloroglucinol subunits are connected via aryl-aryl bonds (fucols), ether bonds (phlorethols, hydroxyphlorethols, fuhalols) or both (fucophlorethols) (Ragan & Glombitza, 1986). Phlorotannins are credited with an ecological role for the producing organisms such as protection from UV radiation (Pavia *et al.*, 1997; Swanson & Druehl, 2002) and deterrence of herbivores (Boettcher & Targett, 1993; Pavia *et al.*, 1997; Schoenwaelder, 2002). The pharmacological activities observed include inhibition of a number of enzymes such as hyaluronidase, phospholipase A, lipoxygenase, cyclooxygenase-1, glycosidases, tyrosinase, HIV-1 reverse transcriptase and HIV-1 protease (e.g. Shibata *et al.*, 2002*a*; Shibata *et al.*, 2002*b*; Shibata *et al.*, 2003; Ahn *et al.*; 2004, Kang *et al.*, 2004*b*), antioxidative effects (e.g. Nakamura *et al.*, 1996; Chkhikvishvili & Ramazanov, 2000; Jimenez-Escrig *et al.*, 2001; Kang *et al.*, 2004*a*) and antibacterial activity (Nagayama *et al.*, 2002; Sandsdalen *et al.*, 2003).

The concentration of polyphenols in brown algae is usually determined using Folin-Denis reagent and it has been shown that concentrations can reach values of up to 15% calculated as phloroglucinol of the dry mass (Ragan & Glombitza, 1986). The concentration has been shown to depend on the season of harvesting (Ragan & Jensen, 1978; Rönnberg & Ruokolahti, 1986; Peckol *et al.*, 1996), the habitat (Jormalainen & Honkanen, 2004) and other extrinsic factors, e.g. light intensity and ambient nutrients (Pavia & Brock, 2000; Pavia & Toth, 2000; Toth & Pavia, 2002; Jormalainen & Honkanen, 2004; Svensson *et al.*, 2007). Seasonal variations in the brown algae *A. nodosum* and *F. vesiculosus* were investigated by Ragan & Jensen (1978) and Rönnberg & Ruokolahti (1986). Both studies found a summer and winter maximum for phlorotannin contents, which correlated to the reproductive stage of the algae.

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For *F. vesiculosus* the winter and summer maxima have been shown to depend on the degree of wave exposure of the habitat (Rönnberg & Ruokolahti, 1986). Moreover, investigations of the geographical variation in *F. vesiculosus* by Kalvas & Kautsky (1993) showed that differences between sheltered and exposed study sites did not only affect phlorotannin content, but also the morphology of the algae. The phenolic content can also vary within an individual algal plant (Tuomi *et al.*, 1989; Connan *et al.*, 2006) and between different plants of an algal population (Pavia *et al.*, 2003).

Due to the potential use of *A. nodosum* extract as nutritional supplement or for medical purposes, the most favourable time for collection of the algal material should be determined. To date, all algal species investigated for annual variation in polyphenol content originated from areas around Trondheimsfjord (Norway), Åland (Finland), Rhode Island (USA) and Australia (Steinberg, 1989), whereas our samples were obtained from the Scottish west coast near Oban. Also, in most studies, phenolic compounds were merely quantified without analysing the qualitative pattern of phenolics. In the current study the quantitative determination of phlorotannin content was done using both the well-known colorimetric method with Folin–Ciocalteu's phenol reagent (FC-method) and quantitative ^{1}H NMR spectroscopy (qHNMR). The latter method was used for the first time for this purpose. For the qualitative determination RP-HPLC and ^{1}H NMR analysis were performed to get fingerprint chromatograms and spectra, respectively. The aim of the present study was to examine quantitative and qualitative variations in polyphenol content in extracts of *A. nodosum* throughout the year 2005.

Material and methods

Algal material

Algal material for the analysis was collected at approximately monthly intervals (Table 1) from an intertidal, sheltered site at Dunstaffnage Marine Laboratory (56°28.9N; 05°30.1W), Oban, Scotland from January to December 2005. For every sampling date (January 23, February 19, March 31, April 29, May 26, June 27, July 25, August 30, September 21, October 20, November 16 and December 21), a total of 5–10 thalli were collected around midday, with thalli usually cut approx. 10 cm above the holdfast, and all thalli with their different tissues pooled together. The fresh material was immediately frozen and stored at −20°C. Hydrographic parameters (conductivity, temperature and depth) were measured with a CTD probe (Seabird 19, Sea-Bird Electronics, Inc, USA) for the period of the study. Chlorophyll *a* (Chl *a*) data were obtained from the same location and was measured with a Turner TD-700 fluorometer (Turner Design, Sunnyvale, CA, USA) after extraction in 90% acetone overnight. The Chl *a* concentrations were determined according to Jeffery & Humphrey (1975).

Table 1. Used material of *A. nodosum* and obtained extracts.

Month	Algal material [g fresh weight]	Date of collection	Extract I [g]	Extract II [g]
January 2005	336	23.i.2005	9.57	4.45
February 2005	318	19.ii.2005	6.11	3.11
March 2005	442	31.iii.2005	12.53	7.12
April 2005	501	29.iv.2005	12.42	8.60
May 2005	510	26.v.2005	18.73	11.57
June 2005	663	27.vi.2005	21.59	12.29
July 2005	425	25.vii.2005	17.83	15.53
August 2005	928	30.viii.2005	28.44	14.00
September 2005	695	21.ix.2005	31.10	13.87
October 2005	805	20.x.2005	28.78	12.40
November 2005	780	16.xi.2005	21.15	9.86
December 2005	950	21.xii.2005	34.37	6.12

Extraction

Extracts obtained from *A. nodosum* (Asco I/II) were prepared according to the extraction procedure shown in Fig. 1. For this purpose deep frozen algal fragments (318–950 g fresh weight, for individual quantities see Table 1) were pulverized and extracted on ice and, in order to prevent autoxidation, under N_2-gassing with ethanol 96% (700 ml–1500 ml, extract I = Asco I) employing an Ultra Turrax (Ika T 25) for 2 h. The solid residue was removed by centrifugation and extracted a second time (extract II = Asco 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) and the residual aqueous phase till the organic layer was slightly yellow. Subsequently, the aqueous phases were freeze-dried to yield the extracts.

Quantitative and qualitative determination of polyphenols

The quantitative measurements were done using the FC-method and qHNMR, as described below. All measurements were done in triplicates with a standard deviation $< 4.0\%$. Qualitative analyses were done by ^{1}H NMR spectroscopy and HPLC.

FC-method. Freeze-dried algal extract (50.0 mg) was dissolved in 25.0 ml of demineralised (dem.) water and 10.0 ml of this solution was diluted with water (dem.) up to 25.0 ml. From this diluted solution 2.0 ml were mixed with 10.0 ml of water (dem.), 1.0 ml of Folin–Ciocalteu's phenol reagent (Merck, Germany), and 12.0 ml of a $Na_2CO_3 \times 10\,H_2O$ (29% m/V) to a final volume of 25.0 ml (= reaction volume). The mixture was incubated for 30 min in the dark at room temperature. Thereafter the absorbance was measured at 760 nm against the blank (measurement 1). The blank was prepared in the

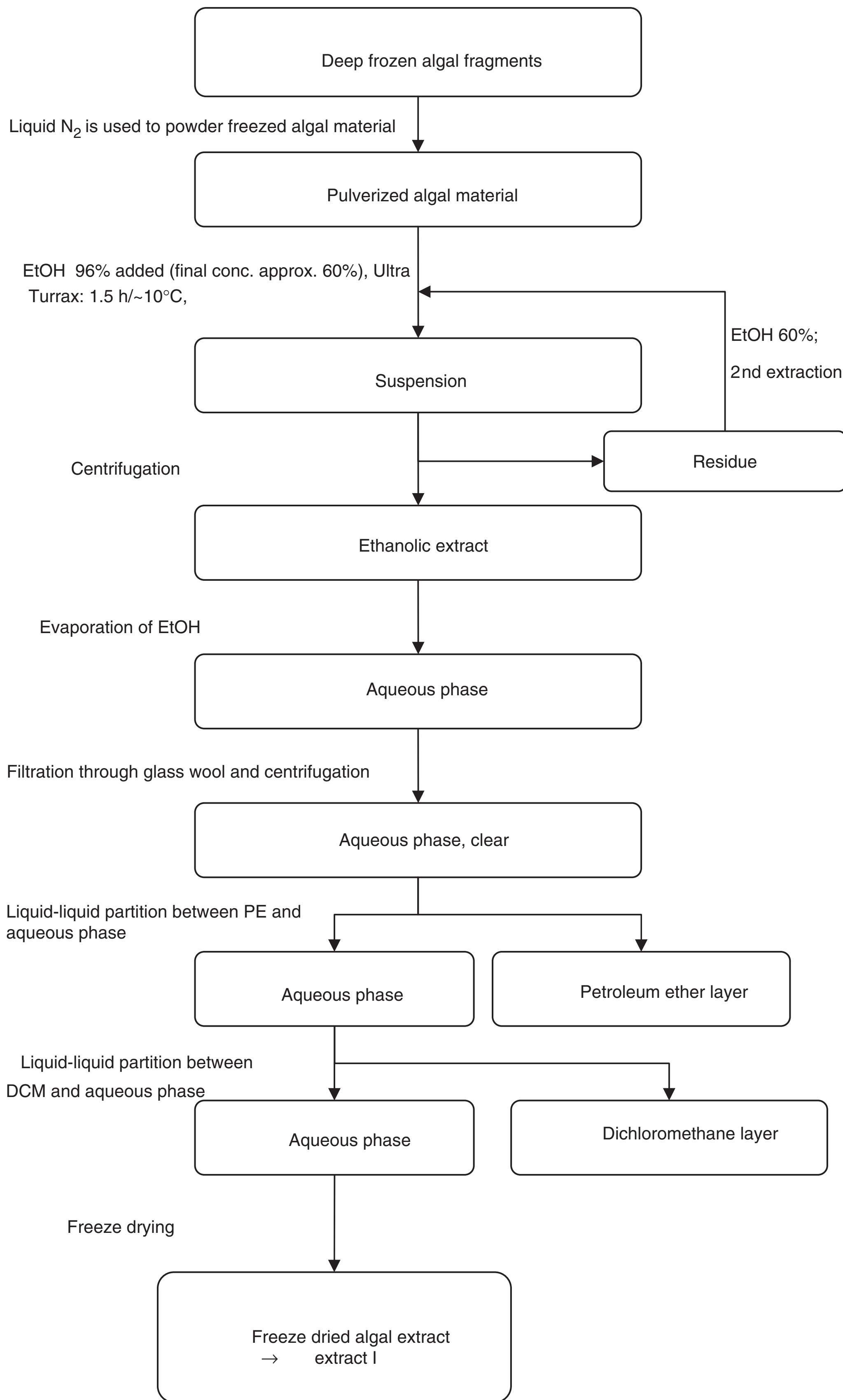

Fig. 1. Scheme illustrating the procedure for obtaining extracts of *Ascophyllum nodosum*.

same way as described above and contains water (dem.), Folin–Ciocalteu's phenol reagent, and sodium carbonate solution. After 30 min in the dark, the blank remained colourless.

In order to prepare a calibration curve, 200.0 mg of phloroglucinol (Merck, Germany) was dissolved in 100.0 ml of water (dem.). The following concentrations of phloroglucinol were used: 0.0 ml, 3.0 ml, 4.0 ml, 5.0 ml

and 6.0 ml of solution of phloroglucinol were each diluted with water (dem.) up to 100.0 ml and 2.0 ml of this solution was treated in the same way as described above.

In a second experiment 10 mg/ml insoluble polyvinylpolypyrrolidone (PVPP; Sigma-Aldrich, Germany) was added to the sample or the phloroglucinol solutions, in order to remove all polyphenolics able to adsorb on PVPP. Sample or phloroglucinol solutions were stirred for 10 min with a magnetic stirrer. Afterwards PVPP was removed by centrifugation and the supernatant carefully pipetted into another reaction vessel. The pH of this solution was in the range between 6 and 7. The sample was treated once again with PVPP in the same manner. After this the remaining supernatant was treated as described for the freeze-dried algal extract (measurement 2). The difference in absorbance between measurement 1 and 2 is equivalent to the content of phenolic compounds able to interact with PVPP.

qHNMR. Extract (20 mg) was dissolved in 0.5 ml standard solution. Standard solution was prepared by dissolving 2.0 mg trimesic acid (Merck, Germany) in a mixture of 0.8 ml MeOH-d_4 (99.8%; Deutero GmbH, Germany) and 0.2 ml deuteriumoxide (99.9%; Deutero GmbH). Trimesic acid was recrystallised twice from ≥97% before use and had a purity of 99.2%.

For validation 20.0 mg phloroglucinol was dissolved in 2.0 ml D_2O or 1.0 ml MeOH-d_4. Afterwards 0.5 ml of standard solution was added to phloroglucinol solution obtaining concentrations of 0.5 mg/ml, 1 mg/ml, 2 mg/ml, 3 mg/ml and 4 mg/ml of phloroglucinol. Each sample solution was transferred to a 5-mm NMR tube. The measurements were done at room temperature. The following acquisition parameters were used: number of scans 256, no-spinning, acquisition time 9.1 sec, relaxation delay (d1) 30 sec, pulse angle 30°, pulse width 7.8 µsec, spectrum data point and time domain 64 k. All ^{1}H NMR spectra were referenced to the resonances of the residual solvent signals (HDO in D_2O at δ 4.68 ppm, CH_3OD in CD_3OD at δ 3.35 ppm).

The data were processed with Bruker XWin-NMR software 3.5, using a line broading parameter of 0.2 Hz, manual phasing and Bruker 'basl' command for baseline correction.

Calculation of polyphenol content (Malz, 2003):

$$K_{Anl} = \frac{m_{Anl}}{m_{Matrix}} = \frac{I_{Anl} \times N_{Std} \times M_{Anl} \times m_{Std}}{I_{Std} \times N_{Anl} \times M_{Std} \times m_{Matrix}} \times f_{Std}$$

m = weighed sample [mg]
I = intensity
N = number of ^{1}H/ring – $N_{Std} = 3$; $N_{Anl} = 1.7$
M = molar mass – $M_{Std} = 210.14$; $M_{Anl} = 124$
f = grade of purity
Anl = analyte
Std = standard

Spectroscopic measurements

Spectrophotometric measurements (FC-method) were done with a Perkin Elmer Lambda 40 UV-VIS spectrometer with UV WinLab software Version 2.80.03. The ^{1}H NMR spectra were recorded on a Bruker Avance 300-DPX spectrometer operating at 300.13 MHz.

HPLC-analysis

HPLC was undertaken using a Waters system controlled by Waters Millennium™ software consisting of a Waters controller 600 with inline degasser, an autosampler 717 plus, a 996 photodiode array detector (DAD detection) and a fraction collector III. As stationary phase an Aqua reversed-phase C_{18} column (Phenomenex™, 200 Å, 5 µm, 250 × 10 mm) was used. The column was eluted using (A) 1% acetic acid in water (dem.) and (B) 1% acetic acid in acetonitrile as linear gradient (1% (B) up to 100% (B) in 30 min). UV detection was at 254 nm, 270 nm and 280 nm.

Extract (0.250 g) was dissolved in 2.0 ml water (dem.) and filtrated through a syringe filter (CME, 0.22 µm, Ø 25 mm, Carl Roth GmbH & Co, Germany). The filters were washed with 0.5 ml dem. water and 100 µl of the affiliated filtrates were injected. HPLC analysis was done twice for each extract. The composition of the algal extracts was analysed by interpretation of the resulting chromatograms (retention time intervals: 7–9 min and 16–20 min; UV detection at 270 nm).

Statistical evaluation

The statistical evaluation was done using (non-paired) two-sided t-test with a probability value (p-value) of $\alpha = 0.05$.

Results

The study area was characterized in terms of its hydrography. Temperature followed a seasonal pattern ranging from 7.9°C to 13.9°C. The coldest water was found in February and March, whereas the highest temperature occurred in August and September. Salinity varied from 32.9 to 33.9 psu and was typical of a well-mixed water column. At the end of January 2005, the water column was marked by a noticeable low salinity event that was related to intense rainfall. Chl *a* ranged from 1.3 to 38 mg Chl *a* equivalent m^{-2} with the highest value found in April (19 mg Chl *a* equivalent m^{-2}), June (38 mg Chl *a* equivalent m^{-2}) and July (17 mg Chl *a* equivalent m^{-2}).

The quantitative measurements were performed using FC-method and qHNMR, which has not been used for the determination of algal phenols before. Fig. 2 shows the variation in polyphenol content calculated for the fresh weight of *A. nodosum* from January until December 2005. Values determined by the FC-method and those from qHNMR comply with each other; however,

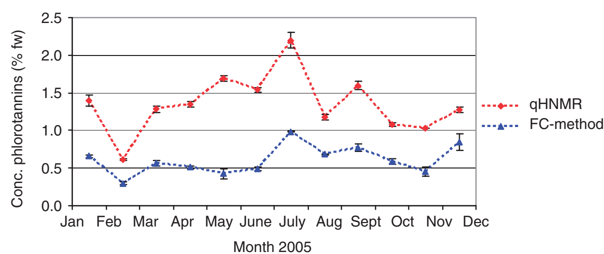

Fig. 2. Seasonal variation in polyphenol content calculated as phloroglucinol with trimesic acid as internal standard for qHNMR and phloroglucinol for FC-method based on the fresh weight of *A. nodosum.*

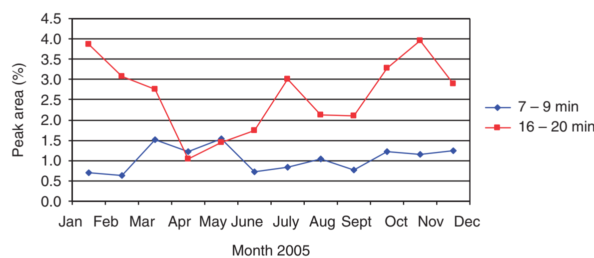

Fig. 4. Seasonal variation of UV active compounds eluting around 8 and between 16 and 20 min in extracts of *A. nodosum* (peak area [%]).

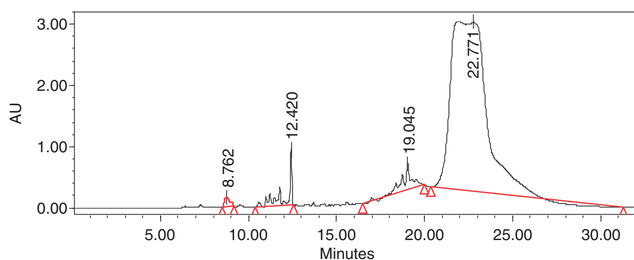

Fig. 3. Typical HPLC chromatogram of extract Asco I for January 2005 (UV detection at 270 nm).

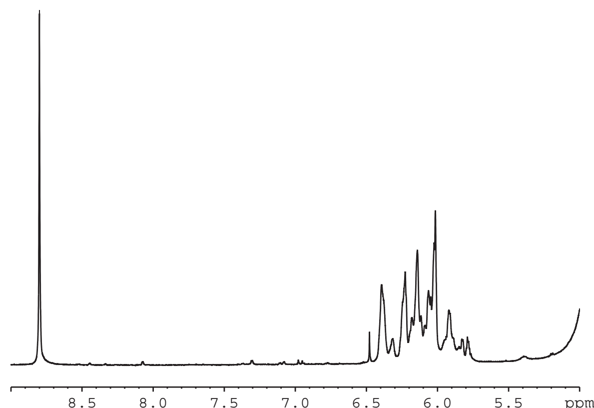

Fig. 5. Typical ^{1}H NMR spectrum of extract Asco I for January 2005 measured in $CDCl_3$.

values determined with qHNMR are approx. 1.5- to 4-fold higher than those measured using the FC-method. The highest phenol content during the year is reached in July (summer maximum). Further maxima, but not as prominent as the one in July, were observed in December/ January (winter maximum) and in September. The lowest concentration of polyphenols was found in February, followed by minima in August and November. Between the lowest value in February and the maximum in July there is an increase from 0.3% to 1.0% or 0.6% to 2.2% of polyphenols determined with the FC-method and qHNMR, respectively.

All values obtained using the FC- as well as qHNMR-method were treated using the (non-paired) two-sided *t*-test (*p*-value $\alpha = 0.05$). The test of significance showed that values for the maxima (January, July and December) and the minimum (February) were significantly different to data found for other months.

The composition of the algal extracts was analysed by HPLC separation, which resulted in characteristic fingerprint chromatograms (Fig. 3). Phenols were characterized by their UV absorption maximum at approx. 270 nm with a shoulder at 285 nm, deduced from DAD detection (Pavia *et al.*, 1997). From 5.5 to 19 min retention time resolved peaks were discernable, whereas from 19 to 29 min an unresolved broad peak eluted from the column. In previous work with extracts of *A. nodosum* from the French coast (unpublished data) MALDI-TOF-MS and LC-ESI-MS analyses (data not shown), demonstrated that the molecular weight of compounds increased with elution time. According to those analyses, compounds with retention time between 6 and 12 min were shown to have a lower molecular weight ($\leq$498; four units of phloroglucinol) than compounds with retention time between 15 and 25 min (molecular weights of up to 2110; corresponds to 17 units of phloroglucinol). The fingerprint chromatograms vary considerably over the course of the year depending on the month of collection. Changes are mainly noticeable for the compounds eluting around 8 and between 16 and 20 min retention time (Fig. 3). As each HPLC-analysis was done using identical injection volumes and concentrations, chromatograms are directly comparable. Peak areas (detected at 270 nm) for compounds eluting between 7 and 9 min and between 16 and 20 min were thus determined (Fig. 4). According to the observed peak areas phenols with a retention time between 7 and 9 min are hardly present at the beginning of the year (January–February) followed by an abrupt rise in March which lasts until May. After a sharp decline in June a minimum is reached, and then the values were more or less constant until the end of the year (November–December). For compounds eluting between

16 and 20 min, a maximum appears in January, July and November, whereas between February and June and from August until October lower contents were seen.

Similar to HPLC chromatograms, ^{1}H NMR spectra of crude extracts were suitable to obtain fingerprints. ^{1}H NMR signals for phlorotannins appear between 6.0 and 6.3 ppm (Fig. 5). Previous ^{1}H NMR analysis with acetylated fractions of *A. nodosum* of the French coast evidenced the predominant structural type as phlorotannins, and due to characteristic ^{1}H NMR shifts ($CDCl_3$: δ 6.2-7.7 ppm, acetone-d_6: δ 6.3-7.3 ppm) structural elements with diaryl-ether linkages were proposed indicating the presence of fucophlorethols. Spectra of extracts from January to December clearly revealed changes in the composition of the monthly extracts of *A. nodosum* due to differences in the pattern of the resonance signals in the spectra. Since the resonance signals of the aromatic protons were broad and overlapping, an examination of individual signals was not possible. Nevertheless, changes were visible when integrating all resonance signals of aromatic protons and in the multiplicity of these signals. Thereby, variability of the composition of phenolic compounds during the course of the year was obvious.

Discussion

In this study we investigated the seasonal variation in phlorotannin content in *A. nodosum* thalli from the Scottish west coast from January to December 2005. Earlier, similar studies on *A. nodosum* and *F. vesiculosus* have concentrated on the coastal waters around Scandinavia and the east coast of USA (Steinberg, 1989). Quantitative as well as qualitative analysis was carried out using the FC-method, qHNMR spectroscopy, HPLC-DAD and ^{1}H NMR spectroscopy, respectively. The latter techniques have never been used for this purpose – this study provides a pioneering test of their effectiveness as an alternative method.

Phlorotannin contents determined by qHNMR are higher than those determined with the FC-method (Fig. 2). qHNMR is a direct determination method of phenols, in which resonance signals of all phenolic compounds contained in the algal extracts are integrated and calculated as phloroglucinol with trimesic acid as internal standard. In contrast to that, the FC-method only measures phenolic compounds able to bind to polyvinylpolypyrrolidone, and which react in a redox type reaction with Folin–Ciocalteu's phenol reagent. This reaction is an indirect measurement, unspecific and based on the reductive potential of the phenolic compounds (Stern *et al.*, 1996). Due to these principle differences the results of the two methods are not strictly comparable.

Both the FC-method and qHNMR clearly showed that the polyphenolic content in *A. nodosum* fluctuated throughout the year. During the year maxima occurred in summer (July) and to a lesser extent in late summer (September) and winter (December/January). The lowest concentration of phenols was observed in February. Since the content of polyphenols in extracts of *A. nodosum* correlates with their potential use as a nutritional supplement or for medical purposes the most favourable time for collection of the algal material is in summer (July).

Similar to former studies of brown algae from other locations it could be shown that the concentration of phenols in *A. nodosum* from the Scottish west coast is dependent on the season. In our and all other studies (Ragan & Jensen, 1978; Rönnberg & Ruokolahti, 1986; Peckol *et al.*, 1996) maxima occurred in January, September and December. The summer maximum was found in June in the study of Ragan & Jensen (1978) and in the present study in July. Furthermore, the data of Ragan & Jensen (1978) showed only one strong minimum, that was between April to May (1977), about 2 months later than in our study.

Various factors have to be taken into account for a possible explanation of the observed patterns. Herbivore grazing was found to lead to increased phlorotannin levels in *Ascophyllum* (Svensson *et al.*, 2007): grazing pressure tends to be highest in the summer (especially in northern latitude locations such as Scandinavia or Scotland) which coincides with one of the observed phlorotannin maxima and reduced growth with more carbon being made available for defence chemicals. In contrast, herbivore mortality in winter is high and consequently, grazers are less abundant in spring. Also, it should be highlighted that *A. nodosum* releases relatively high, constitutive levels of hydrogen peroxide (which serves as a co-substrate for the cross-linking of phenolic compounds) compared to other brown algae (Küpper *et al.*, 2002), which is likely increased in the summer due to photo-oxidative and heat stress. Furthermore, increased irradiance in summer will increase the need for UV-screening and antioxidant chemicals in intertidal seaweeds (Häder *et al.*, 1998; Henry *et al.*, 2004), especially as other antioxidant systems such as iodide may become depleted during this season, which has recently been shown in *Laminaria* (Ar Gall *et al.*, 2004; Küpper *et al.*, 2008). Indeed, Pavia *et al.* (1997) and Pavia & Brock (2000) found a significant increase in phlorotannin content of *A. nodosum* after exposure to ambient or increased UV-B radiation. It was thus discussed that phenols through their ability to

absorb UV light protect spermatozoids, eggs and zygotes against photo destruction. This was supported by Clayton & Ashburner (1994) who observed that soon after fertilisation phenolic bodies were detectable over the entire surface of the zygote. Other environmental conditions such as wave exposure may influence a combination of multiple factors including desiccation, irradiance exposure, salinity and grazing. Data of Rönnberg & Ruokolahti (1986), which were obtained for the period between May 1983 and May 1984, showed that the degree of wave exposure of a site (i.e. sheltered versus exposed locations) resulted in different curves concerning the polyphenol content. In this respect, they were able to show that the fluctuation of phenolic content at an exposed site was smaller and that summer and winter maxima occurred about two months later than at a sheltered site. Light conditions and wave action were discussed as a reason for these differences. Similar conclusions were drawn in the study of Pavia *et al.* (1999), which was done with *A. nodosum* collected in October 1992 and October 1993, in each case at two different study sites. Further investigations of polyphenol contents also demonstrated that year-to-year variations (Van Alstyne *et al.*, 1999; Van Alstyne *et al.*, 2001) occurred, and that differences in the habitat (Jormalainen & Honkanen, 2004), such as salinity, temperature, light intensity and ambient nutrients (Lüning, 1985; Pavia & Brock, 2000; Pavia & Toth, 2000; Toth & Pavia, 2002; Jormalainen & Honkanen, 2004) play a role. The differences in the polyphenol content between our study and that of Ragan & Jensen (1978) may have been caused by different conditions at collection sites.

Throughout the year, *A. nodosum* goes through different phases of growth and development. It is fertile from April to June, and at the end of June fruit bodies are shed. In September, development of conceptacles at the end of the short shoots starts (Kornmann & Sahling, 1993; Kraberg & Norton, 2007). Ragan & Jensen (1978) and Rönnberg & Ruokolahti (1986) discussed a correlation between variations in polyphenol content and the reproductive stage of the alga. From Fig. 2 it can be seen that there are lower polyphenol concentrations throughout the phase of fertility (April to June), whereas the maximum is reached during the period of shedding fruit bodies. Thus, variation of polyphenol content throughout the year may correlate with the reproductive stage of *A. nodosum*.

The composition of *A. nodosum* extracts was determined by RP-HPLC analysis and resulted in characteristic fingerprint chromatograms, which differed throughout the year. Differences were particularly prominent for compounds eluting between 7 and 9 min and between 16 and 20 min. The intensity of absorption of UV-active compounds at these eluting times was compared throughout the year. For lower molecular weight phenols (around 8 min), the maximum concentration was found from March to May reaching approximately twice to threefold higher values than during the months before and after this period. The amount of low molecular weight phenols (< 500) thus increased during the period of fertility. ^{1}H NMR experiments which were used to indicate changes in polyphenol content resulted also in the conclusion that phenol structures vary throughout the year.

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References

Ahn, M.J., Yoon, K.D., Min, S.Y., Lee, J.S., Kim, J.H., Kim, T.G., Kim, S.H., Kim, N.G., Huh, H. & Kim, J. (2004). Inhibition of HIV-1 reverse transcriptase and protease by phlorotannins from the brown alga *Ecklonia cava*. *Biol. Pharm. Bull.*, **27**: 544–547.

Ar Gall, E., Küpper, F.C. & Kloareg, B. (2004). A survey of iodine contents in *Laminaria digitata*. *Bot. Mar.*, **47**: 30–37.

Boettcher, A.A. & Targett, N.M. (1993). Role of polyphenolic molecular-size in reduction of assimilation efficiency in *Xiphister mucosus*. *Ecology*, **74**: 891–903.

Chkhikvishvili, I.D. & Ramazanov, Z.M. (2000). Phenolic substances of brown algae and their antioxidant activity. *Appl. Biochem. Microbiol.*, **36**: 289–291.

Clayton, M.N. & Ashburner, C.M. (1994). Secretion of phenolic bodies following fertilization in *Durvillaea potatorum* (Durvillaeales, Phaeophyta). *Eur. J. Phycol.*, **29**: 1–9.

Connan, S., Delisle, F., Deslandes, E. & Gall, E.A. (2006). Intra-thallus phlorotannin content and antioxidant activity in Phaeophyceae of temperate waters. *Bot. Mar.*, **49**: 39–46.

Häder, D.P., Kumar, H.D., Smith, R.C. & Worrest, R.C. (1998). Effects on aquatic ecosystems. *J. Photoch. Photobio. B*, **46**: 53–68.

Henry, B.E. & Van Alstyne, K.L. (2004). Effects of UV-radiation on growth and phlorotannins in *Fucus gardneri* (Phaeophyceae) juveniles and embryos. *J. Phycol.*, **40**: 527–533.

Jeffrey, S.W. & Humphrey, G.F. (1975). New spectrophotometric equations for determining chlorophylls A, B, C1 and C2 in higher plants, algae and natural phytoplankton. *Biochem. Physiol. Pflanz.*, **167**: 191–194.

Jimenez-Escrig, A., Jimenez-Jimenez, I., Pulido, R. & Saura-Calixto, F. (2001). Antioxidant activity of fresh and processed edible seaweeds. *J. Sci. Food Agric.*, **81**: 530–534.

Jormalainen, V. & Honkanen, T. (2004). Variation in natural selection for growth and phlorotannins in the brown alga *Fucus vesiculosus*. *J. Evol. Biol.*, **17**: 807–820.

Kalvas, A. & Kautsky, L. (1993). Geographical variation in *Fucus vesiculosus* morphology in the Baltic and North Seas. *Eur. J. Phycol.*, **28**: 85–91.

Kang, H.S., Chung, H.Y., Kim, J.Y., Son, B.W., Jung, H.A. & Choi, J.S. (2004*a*). Inhibitory phlorotannins from the edible brown alga *Ecklonia stolonifera* on total reactive oxygen species (ROS) generation. *Arch. Pharmacol. Res.*, **27**: 194–198.

Kang, H.S., Kim, H.R., Byun, D.S., Son, B.W., Nam, T.J. & Choi, J.S. (2004*b*). Tyrosinase inhibitors isolated from the edible brown alga *Ecklonia stolonifera*. *Arch. Pharmacal Res.*, **27**: 1226–1232.

Kornmann, P. & Sahling, P.-H. (1993). *Meeresalgen von Helgoland – Benthische Grün-, Braun- und Rotalgen*. unveränderter Nachdruck. Biologische Anstalt Helgoland. pp 162–169.

Kraberg, A.C. & Norton, T.A. (2007). Effect of epiphytism on reproductive and vegetative lateral formation in the brown, intertidal seaweed *Ascophyllum nodosum* (Phaeophyceae). *Phycol. Res.*, **55**: 17–24.

Küpper, F.C., Carpenter, L.J., Mcfiggans, G.B., Palmer, C.J., Waite, T.J., Boneberg, E.-M., Woitsch, S., Weiller, M., Abela, R., Grolimund, D., Potin, P., Butler, A., Luther III, G.W., Kroneck, P. M.H., Meyer-Klaucke, W. & Feiters, M.C. (2008). Iodide accumulation provides kelp with an inorganic antioxidant impacting atmospheric chemistry. *Proc. Natl. Acad. Sci. USA*, **105**: 6954–6958.

Küpper, F.C., Müller, D.G., Peters, A.F., Kloareg, B. & Potin, P. (2002). Oligoalginate recognition and oxidative burst play a key role in natural and induced resistance of the sporophytes of Laminariales. *J. Chem. Ecol.*, **28**: 2057–2081.

Lüning, K. (1985). *Meeresbotanik – Verbreitung, Ökophysiologie und Nutzung der marinen Makroalgen*. Georg Thieme Verlag, Stuttgart, Germany.

Malz, F. (2003). Quantitative NMR-Spektroskopie als Referenzverfahren in der analytischen Chemie. PhD thesis. Humboldt-Universität Berlin, Berlin, Germany.

Nagayama, K., Iwamura, Y., Shibata, T., Hirayama, I. & Nakamura, T. (2002). Bactericidal activity of phlorotannins from the brown alga *Ecklonia kurome*. *J. Antimicrob. Chemother.*, **50**: 889–893.

Nakamura, T., Nagayama, K., Uchida, K. & Tanaka, R. (1996). Antioxidant activity of phlorotannins isolated from the brown alga *Eisenia bicyclis*. *Fish. Sci.*, **62**: 923–926.

Pavia, H. & Brock, E. (2000). Extrinsic factors influencing phlorotannin production in the brown alga *Ascophyllum nodosum*. *Mar. Ecol.: Prog. Ser.*, **193**: 285–294.

Pavia, H., Cervin, G., Lindgren, A. & Aberg, P. (1997). Effects of UV-B radiation and simulated herbivory on phlorotannins in the brown alga *Ascophyllum nodosum*. *Mar. Ecol.: Prog. Ser.*, **157**: 139–146.

Pavia, H. & Toth, G.B. (2000). Influence of light and nitrogen on the phlorotannin content of the brown seaweeds *Ascophyllum nodosum* and *Fucus vesiculosus*. *Hydrobiologia*, **440**: 299–305.

Pavia, H., Toth, G. & Aberg, P. (1999). Trade-offs between phlorotannin production and annual growth in natural populations of the brown seaweed *Ascophyllum nodosum*. *J. Ecol.*, **87**: 761–771.

Pavia, H., Toth, G.B., Lindgren, A. & Aberg, P. (2003). Intraspecific variation in the phlorotannin content of the brown alga *Ascophyllum nodosum*. *Phycologia*, **42**: 378–383.

Peckol, P., Krane, J.M. & Yates, J.L. (1996). Interactive effects of inducible defence and resource availability on phlorotannins in the North Atlantic brown alga *Fucus vesiculosus*. *Mar. Ecol.: Prog. Ser.*, **138**: 209–217.

Ragan, M.A. & Glombitza, K.W. (1986). Phlorotannins, brown algal Polyphenols. *Prog. Phycol. Res.*, **4**: 129–241.

Ragan, M.A. & Jensen, A. (1978). Quantitative studies on brown algal phenols. II. Seasonal variation in polyphenol content of *Ascophyllum nodosum* (L.) Le Jol. and *Fucus vesiculosus*. *J. Exp. Mar. Biol. Ecol.*, **34**: 245–258.

Rönnberg, O. & Ruokolahti, C. (1986). Seasonal variations of algal epiphytes and phenolic content of *Fucus vesiculosus* in a northern Baltic archipelago. *Ann. Bot. Fennici.*, **23**: 317–323.

Sandsdalen, E., Haug, T., Stensvag, K. & Styrvold, O.B. (2003). The antibacterial effect of a polyhydroxylated fucophlorethol from the marine brown alga, *Fucus vesiculosus*. *World J. Microbiol. Biotechnol.*, **19**: 777–782.

Schoenwaelder, M.E.A. (2002). Physode distribution and the effect of 'Thallus sunburn' in *Hormosira banksii* (Fucales, Phaeophyceae). *Bot. Mar.*, **45**: 262–266.

Shibata, T., Fujimoto, K., Nagayama, K., Yamaguchi, K. & Nakamura, T. (2002*a*). Inhibitory activity of brown algal phlorotannins against hyaluronidase. *Int. J. Food Sci. Technol.*, **37**: 703–709.

Shibata, T., Nagayama, K., Tanaka, R., Yamaguchi, K. & Nakamura, T. (2003). Inhibitory effects of brown algal phlorotannins on secretory phospholipase A(2)s, lipoxygenases and cyclooxygenases. *J. Appl. Phycol.*, **15**: 61–66.

Shibata, T., Yamaguchi, K., Nagayama, K., Kawaguchi, S. & Nakamura, T. (2002*b*). Inhibitory activity of brown algal phlorotannins against glycosidases from the viscera of the turban shell *Turbo cornutus*. *Eur. J. Phycol.*, **37**: 493–500.

Steinberg, P.D. (1989). Biogeographical variation in brown algal polyphenolics and other secondary metabolites: comparison between temperate Australasia and North America. *Oecologia*, **138**: 373–382.

Stern, J.L., Hagerman, A.E., Steinberg, P.D., Winter, F.C. & Estes, J.A. (1996). A new assay for quantifying brown algal phlorotannins and comparisons to previous methods. *J. Chem. Ecol.*, **22**: 1273–1293.

Svensson, C.J., Pavia, H. & Toth, G.B. (2007). Do plant density, nutrient availability, and herbivore grazing interact to affect phlorotannin plasticity in the brown seaweed *Ascophyllum nodosum*. *Mar. Biol.*, **151**: 2177–2188.

Swanson, A.K. & Druehl, L.D. (2002). Induction, exudation and the UV protective role of kelp phlorotannins. *Aquat. Bot.*, **73**: 241–253.

Toth, G.B. & Pavia, H. (2002). Lack of phlorotannin induction in the kelp *Laminaria hyperborea* in response to grazing by two gastropod herbivores. *Mar. Biol.*, **140**: 403–409.

Tuomi, J., Ilvessalo, H., Niemela, P., Siren, S. & Jormalainen, V. (1989). Within-plant variation in phenolic content and toughness of the brown alga *Fucus vesiculosus* L. *Bot. Mar.*, **32**: 505–509.

Van Alstyne, K. L., Mccarthy, J. J., Hustead, C. L. & Kearns, L. J. (1999). Phlorotannin allocation among tissues of northeastern pacific kelps and rockweeds. *J. Phycol.*, **35**: 483–492.

Van Alstyne, K.L., Whitman, S.L. & Ehlig, J.M. (2001). Differences in herbivore preferences, phlorotannin production, and nutritional quality between juvenile and adult tissues from marine brown algae. *Mar. Biol.*, **139**: 201–210.