



## Influence of light and nitrogen on the phlorotannin content of the brown seaweeds *Ascophyllum nodosum* and *Fucus vesiculosus*

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### Abstract

Phlorotannins, C-based defence compounds in brown seaweeds, show a high degree of spatial and temporal variation within seaweed species. One important model explaining this variation is the Carbon Nutrient Balance Model (CNBM), which states that the relative supply of carbon and limiting nutrients will determine the level of defence compounds in plants. Nitrogen is often considered to be the limiting nutrient for marine macroalgal growth and the CNBM thus predicts that when the carbon:nitrogen ratio is high, photosynthetically fixed carbon will be allocated to production of phlorotannins. In the present study, we evaluated the effects of light (i.e. carbon) and nitrogen on the phlorotannin content of two intertidal brown seaweeds, *Ascophyllum nodosum* and *Fucus vesiculosus*. This was done in an observational field study, as well as in a manipulative experiment where plants from habitats with different light regimes were subjected to different nitrogen and light treatments, and their phlorotannin content was measured after 14 days. The results showed that there was a negative relationship between tissue nitrogen and phlorotannin content in natural populations of *F. vesiculosus*, but not in *A. nodosum*. In the short term, the phlorotannin content in both algal species was not affected by changes in nitrogen availability. Exposure to sunlight had a positive effect on the phlorotannin content in natural populations of both algal species but, in the manipulative experiment, only *F. vesiculosus* showed a rapid response to changes in light intensities. Plants subjected to sunlight contained higher phlorotannin content than shaded plants. In conclusion, the results imply that nitrogen availability explains some of the natural variation in the phlorotannin content of *F. vesiculosus*, but the light environment has greater importance than nitrogen availability in predicting the phlorotannin content of each species.

### Introduction

Polyphenolic compounds are ubiquitous in plants and have been a major focal point in plant chemical ecology during the last two to three decades. The single group of polyphenolics that has attracted most attention from ecologists is tannins, which are defined as polyphenolic compounds with the ability to precipitate proteins from solutions. The least complex group of tannins is the phlorotannins, which are polymers of phloroglucinol derived entirely from acetate. Phlorotannins are restricted to brown algae (Phaeophyta) where they are found in special vesicles (physodes) within the cells (Ragan & Glombitza, 1986). Phlorotannins have been shown to have an-

tifeeding activity towards several different types of herbivores, although their effectiveness as herbivore deterrents is far from universal (see reviews by Hay & Steinberg, 1992; Targett & Arnold, 1998). It has also been suggested that phlorotannins may be important in the defence against pathogenic organisms (Sieburth & Conover, 1965), as antifouling agents (Sieburth & Conover, 1965; Lau & Qian, 1997; but see Jennings & Steinberg, 1997), as chelators of toxic heavy metal ions (Pedersen, 1984; Karez & Pereira, 1995; but see Toth & Pavia, 2000), in the protection from harmful solar radiation (Berthold, 1882; Pavia et al., 1997), and in the cell-wall construction of brown seaweeds (Crato, 1896; Schoenwaelder & Clayton, 1998). Phlorotannin concentrations vary greatly among different taxa of brown seaweeds, as well as among different geographical areas. Concen-

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trations are generally higher in furoid species than in kelps, and higher in species from the Atlantic and the temperate Pacific than in species from the tropical Pacific (see Steinberg, 1992; Targett & Arnold, 1998 for reviews). Besides taxonomic and geographical variation, substantial intraspecific and intraplant variation in phlorotannin content has been documented for several brown algal species (reviewed in Targett & Arnold, 1998; see also Hammerstrom et al., 1998; Van Alstyne et al., 1999a&b).

One influential model proposed to explain intraspecific phenotypic variation in secondary metabolites of plants is the carbon–nutrient balance model (CNBM) (Bryant et al., 1983, 1989). The CNBM states that the production of secondary metabolites is governed by the relative supply of photosynthetically fixed carbon and limiting nutrients. The model assumes that plant growth has higher priority than the production of defence compounds and that excess photosynthate is allocated to carbon-based defence compounds when growth is nutrient limited (Bryant et al., 1983, Karban & Baldwin, 1997, Koricheva et al., 1998). Consequently, the CNBM predicts that environmental conditions that give rise to a high carbon/nutrient ratio (e.g. high light and low nutrient availability) will result in an increased production of carbon-based secondary metabolites. The empirical evidence for the CNBM from studies on vascular plants is mixed, with predictions being either supported or rejected in numerous studies (Herms & Mattson, 1992; Berenbaum, 1995). Most support for the CNBM seems to come from studies on the production of phenolic metabolites in early successional plants (Reichardt et al., 1991; Fajer et al., 1992). For brown algal phlorotannins, there are still only a few studies that have addressed the CNBM, showing that nutrient (nitrogen) availability may (Ilvessalo & Tuomi, 1989; Yates & Peckol, 1993; Arnold et al., 1995; Peckol et al., 1996) or may not (Cronin & Hay, 1996; Pavia et al., 1999; Pavia & Brock, 2000) be related to phlorotannin concentration. Cronin & Hay (1996) found that plants of *Sargassum filipendula* grown in experimentally shaded environments had lower concentrations of phlorotannins, in accordance with the predictions of the CNBM. The experimental data available so far are much too scarce to produce any general conclusions about taxonomic and geographical variation in the relevance of the CNBM to explain phenotypic variation in phlorotannin content of brown macroalgae.

In the present study, we tested the hypotheses, derived from the CNBM, that the availability of light and nutrients (i.e. nitrogen) can affect, separately or interactively, phlorotannin production in the brown seaweeds *Ascophyllum nodosum* and *Fucus vesiculosus*. These two species were chosen since they are dominating and often competing macroalgae in the intertidal zone of temperate rocky shores in the northern Atlantic, but still they have different life spans, with *A. nodosum* being an extremely long-lived species. Nitrogen was chosen as the experimental nutrient since it is commonly the most limiting nutrient for temperate marine macroalgae (Chapman & Craigie, 1977; Hanisak, 1983). The effects of light and N on phlorotannin levels were tested both in an observational field study and in a manipulative aquarium experiment.

## Materials and methods

### *Study organisms and sites*

*Ascophyllum nodosum* and *Fucus vesiculosus* are common perennial seaweeds in the intertidal zone on temperate rocky shores where they can form zones ranging from <1 m in atidal areas to >100 m in tidal areas. Both species have apical growth and are attached to the substratum with a holdfast from which one to several primary shoots arise. In the present study, only the outermost tips of the vegetative shoots were used for chemical analyses. The seaweeds were collected from atidal sunexposed and shaded sheltered sites in the Tjärnö (58° 54' N, 11° 07' E) and Strömstad (58° 56' N, 11° 10' E) Archipelagos of the Swedish west coast. Low, flat skerries, where the seaweeds are subjected to sunlight most of the day, were chosen as sunexposed sites. Islands with steep, vertical rocks that shade the plants from sunlight were chosen as shaded sites.

### *Observational field study*

Tissue samples were collected in May (1998) from *A. nodosum* and *F. vesiculosus* plants growing in sun-exposed and shaded habitats. Ten apical shoots were sampled from each of 16 plants of each species. The seaweed samples were freeze dried, and the shoots originating from the same plant were ground, mixed and analysed for phlorotannin and tissue N content as described below.

### Manipulative experiment

The manipulative experiment was carried out in June (1998) at Tjärnö Marine Biological Laboratory. *Ascophyllum nodosum* and *Fucus vesiculosus* plants originating from sunexposed and shaded sites were collected by carefully removing their holdfasts from the substratum with a knife. The plants were transported to the laboratory and placed immediately in Plexiglas aquaria with fresh seawater. The aquaria were placed in a basin with cooling seawater. The basin was built outdoors on a sunexposed bridge where the aquaria were subjected to natural ambient sunlight. Three plants of each species and light origin were subjected to one of the following treatments: (1) shading and additional N, (2) shading and ambient N, (3) ambient sunlight and additional N, and (4) ambient sunlight and ambient N. Shading was achieved by covering the aquaria with black plastic bags, which excluded most of the ambient sunlight. During the day, the plants in the aquaria were supplied continuously with ambient seawater. In the evening, the seawater supply was turned off and N was added in the form of aqueous ammonium nitrate ( $\text{NH}_4\text{NO}_3$ ) at a concentration of  $20 \mu\text{m}$ . The seaweeds were allowed to accumulate N during the night and in the morning the seawater supply was turned on again. The same procedure, except for the addition of N, was applied to plants in aquaria subjected to ambient N treatment. Tissue samples, collected after 14 days in the same way as described for the observational field study, were freeze dried and analysed for phlorotannin content as described below.

### Chemical analyses

Phlorotannins were extracted from freeze dried, homogenised seaweed tissue in aqueous acetone (60-vol%) in the dark at  $4^\circ\text{C}$  for 24 h. The acetone was evaporated in vacuo and the remaining water fraction was filtered to remove precipitated lipophilic material. The concentration of phlorotannins was determined using the Folin-Ciocalteus method for quantification of total phenolic compounds (Van Alstyne, 1995). Phloroglucinol (Merck art. 7069) was used as a standard. Samples for tissue N content were ground and analysed in an elemental analyser (FISONS Instruments NA 1500 NC).

### Statistical analyses

Analysis of covariance (ANCOVA) was used to test for interactions between the fixed factor Light origin

(2 levels) and the covariate N content in the observational field study. No significant interaction was detected for either *A. nodosum* (ANCOVA,  $F_{1,12} = 0.67$ ,  $p = 0.43$ ) or *F. vesiculosus* (ANCOVA,  $F_{1,12} = 1.81$ ,  $p = 0.20$ ), implying that the relationship between tissue N and phlorotannin content was not significantly different amongst sites. Consequently, data of plants from sites with different light conditions were pooled and regression analyses, separate for each species, were used to analyse the relationship between phlorotannin and tissue N content. Data on the phlorotannin content of *A. nodosum* and *F. vesiculosus* plants were also analysed in a one-way analysis of variance (ANOVA) with Light origin (2 levels) as a fixed factor. Data on the phlorotannin content of *A. nodosum* and *F. vesiculosus* plants from the manipulative experiment were analysed in a three-way ANOVA with Light origin (2 levels), Light treatment (2 levels) and N treatment (2 levels) as fixed orthogonal factors. Prior to all statistical analyses, the homogeneity of variances was tested with Cochran's test (Underwood, 1997).

### Results

The regression analyses showed that there was a significant negative relationship between phlorotannin and tissue N content in natural populations of *F. vesiculosus* (Fig. 1b), but not in *A. nodosum* (Fig. 1a). The ANOVA revealed that there was no statistically significant difference in the mean phlorotannin content of *A. nodosum* from sunexposed (4.32% dw) and shaded (3.82% dw) sites (ANOVA,  $F_{1,14} = 1.35$ ,  $p = 0.26$ ), but a significant difference was found for *F. vesiculosus* (ANOVA,  $F_{1,14} = 7.36$ ,  $p = 0.02$ ). *Fucus vesiculosus* plants exposed to sunlight in the field had a mean phlorotannin content of 4.88% dw, while shaded plants contained only 3.70% dw.

There were no significant interactions between the factors Light origin, Light treatment and N treatment for any of the species in the manipulative experiment (Table 1, Fig. 2). There was a significant difference in phlorotannin content of *A. nodosum* plants originating from sites with different light conditions (Table 1), with plants from sunexposed and shaded sites having a phlorotannin content of 4.26 and 3.49% dw, respectively (Fig. 2a). The light and N treatments had no apparent effect on the phlorotannin content of *A. nodosum* (Table 1) but, for *F. vesiculosus*, the light treatment had a significant effect (Table 1); plants exposed to ambient sunlight had a higher phlorotan-

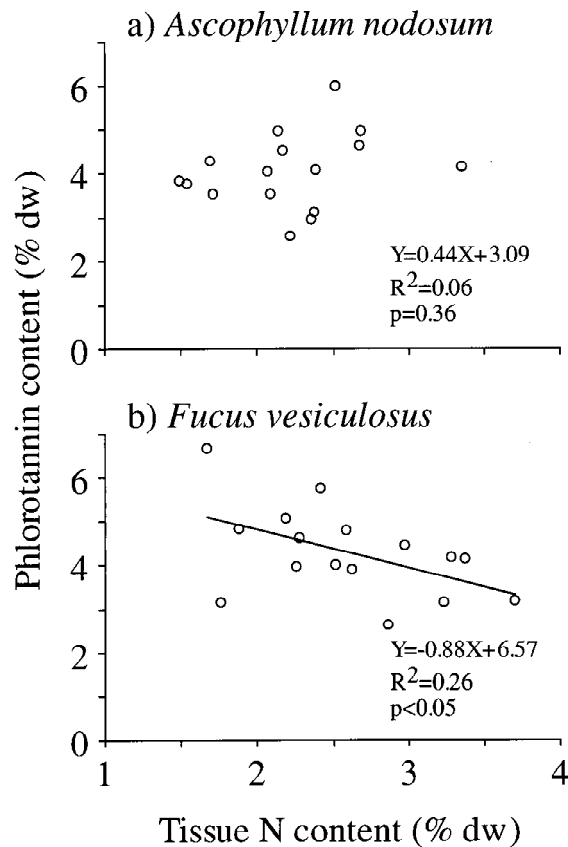


Figure 1. The relationship between phlorotannin and tissue N content (% dw) in natural populations of (a) *Ascophyllum nodosum* and (b) *Fucus vesiculosus* collected from sunexposed and shaded sites in the Tjörn and Strömstad Archipelagos.

nin content (4.98% dw) than shaded plants (4.23% dw) (Fig. 2b). In the manipulative experiment, the phlorotannin content in *F. vesiculosus* plants was not affected by N availability (Table 1).

## Discussion

The results of the present study indicate that, in general, light has greater influence than N availability on the phlorotannin content of both *A. nodosum* and *F. vesiculosus*. Effects of sunlight intensity on phlorotannin production were found in natural populations of both species. *Ascophyllum nodosum* plants collected from sunexposed sites in the manipulative experiment had higher phlorotannin content than plants from shaded sites, as had *F. vesiculosus* plants collected from sunexposed sites in the observational field study. No statistically significant difference in phlorotannin

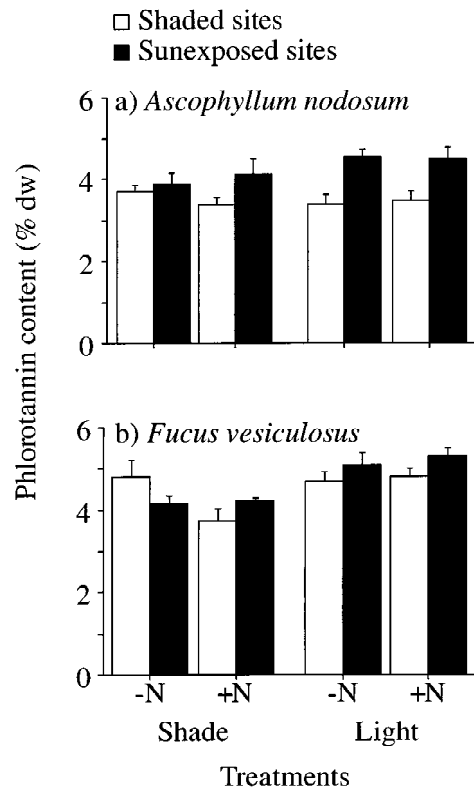


Figure 2. The phlorotannin content (% dw) in (a) *Ascophyllum nodosum* and (b) *Fucus vesiculosus* plants collected from sunexposed and shaded sites in the Tjörn Archipelago and subjected to light (shade and ambient sunlight) and N treatments (additional and ambient N) in a manipulative aquarium experiment. ANOVA results are presented in Table 1. Error bars show  $\pm 1$  SE ( $n = 3$ ).

content was found between *A. nodosum* plants collected from sites with different light conditions in the observational field study. This may be due to the fact that fewer replicates were used in the observational survey than in the manipulative experiment and, thus, the statistical analysis was less powerful. Light intensity had no significant short-term effects on the phlorotannin content of *A. nodosum*, but a significant effect was found for *F. vesiculosus*. In a previous study on *A. nodosum*, increased concentrations of phlorotannins were found in response to additional UV-B radiation after 2 weeks (Pavia et al., 1997). In the present study, however, the light exposure of plants in aquaria covered with black plastic bags was decreased compared to natural levels. Since it is regarded as unlikely that phlorotannins can be re-metabolised (Targett & Arnold, 1998), a decrease in phlorotannin content would implicate that phlorotannins are released to the surrounding water, either by

Table 1. Three-way analysis of variance (ANOVA) of the phlorotannin content (% dw) of *Ascophyllum nodosum* and *Fucus vesiculosus* plants collected from sunexposed and shaded sites, and subjected to light (shade and ambient sunlight) and N treatments (additional and ambient N) in a manipulative aquarium experiment

Source of variation	df	<i>Ascophyllum nodosum</i>			<i>Fucus vesiculosus</i>		
		MS	F	p	MS	F	p
Light origin (LO)	1	3.61	18.57	<0.01	0.20	1.04	0.32
Light treatment (LT)	1	0.23	1.20	0.29	3.40	17.75	<0.01
N treatment (NT)	1	<0.01	0.01	0.91	0.16	0.82	0.38
LO × LT	1	0.67	3.44	0.08	0.44	2.32	0.15
LO × NT	1	0.06	0.28	0.60	0.55	2.87	0.11
LT × NT	1	0.01	0.04	0.84	0.67	3.49	0.08
LO × LT × NT	1	0.91	0.98	0.34	0.43	2.56	0.15
Residual	16	0.19		0.19			

exudation or passive leaking, or transported to other parts of the seaweed. Seaweeds are nonvascular plants and, therefore, the possibility for efficient transport is limited, while several previous studies have found that brown seaweeds can release significant amounts of phlorotannins into the surrounding water (Sieburth, 1969; Carlson & Carlson, 1984; Jennings & Steinberg, 1994). If exudation is the primary way for metabolite turnover of phlorotannins, and if the release rates from healthy seaweed plants are low (see Jennings & Steinberg, 1994), any environmentally induced decreases in phlorotannin content would be relatively slow (cf. Pavia & Brock, 2000). This could explain the lack of a rapid response in *A. nodosum* plants subjected to changing light levels in the manipulative experiment of the present study. In contrast, there was a significant short-term effect of the light treatment on the phlorotannin content of *F. vesiculosus*, where plants subjected to sunlight in the aquaria had higher phlorotannin content than shaded plants. Lack of difference in phlorotannin content of *F. vesiculosus* plants with different light origin in the aquarium experiment could be explained possibly by the fact that the effect of the light treatment was strong enough to mask the initial difference in phlorotannin content of the *F. vesiculosus* plants.

The difference in short-term response in phlorotannin content to changes in light exposure between *A. nodosum* and *F. vesiculosus* may be due to differences in morphology and/or growth rate between the two species. The CNBM predicts that rapidly growing species should show the most pronounced changes in secondary metabolite content in response to changes in nutrient availability and light intensity (Reichardt et al., 1991). However, even if *A. nodosum* is ex-

tremely slow growing as a juvenile (Lazo et al., 1994), there does not seem to be any obvious differences in growth rate between adult plants of *F. vesiculosus* and *A. nodosum* during the season (May–June) when the present study was conducted (Carlson, 1991; Stengel & Dring, 1997; H. Pavia & G. Toth, unpublished data). We suggest that the difference in the short-term response of phlorotannin content to changes in light intensity between the two species is more likely to be due to differences in morphology. The apical shoots of *F. vesiculosus* are flat and have a higher surface:volume ratio compared to the apical shoots of *A. nodosum*. Since phlorotannins are stored primarily in the cortical layer of the seaweed thallus (Ragan & Glombitza, 1986; Tugwell & Branch, 1989; Lowell et al., 1991), a light-induced decrease in phlorotannin content, where phlorotannins are lost primarily through exudation, may appear faster in shoots with a high surface:volume ratio. Nitrogen availability had no effects on the phlorotannin content of *A. nodosum* in the present study. There was, however, a significant, negative relationship between tissue N and phlorotannin content of *F. vesiculosus* plants in the field. These results are in accordance with previously reported results on the two species. Nitrogen availability was not correlated to the phlorotannin content of *A. nodosum* in field observations performed in two different geographical areas during two consecutive years (Pavia et al., 1999). Neither did increased N concentrations result in a decreased phlorotannin production in a 4-week manipulative aquarium experiment (Pavia & Brock, 2000). In contrast, significant negative relationships between tissue N and phlorotannin content of *F. vesiculosus* were found both by Yates & Peckol (1993)

and by Peckol et al. (1996). The difference in the response to N availability between the two seaweed species is in accordance with results from terrestrial plants with different life histories, where responses in the production of C-based secondary metabolites seem to be less pronounced in long-lived species (Koricheva et al., 1998). *Ascophyllum nodosum* is an extremely long-lived seaweed species (Åberg, 1992), while *F. vesiculosus* individuals usually live for <5 years (Williams, 1990). In conclusion, the results of this study give support to the relevance of the CNBM in explaining long-term effects of N on phlorotannin levels in *F. vesiculosus*, but not in *A. nodosum*, and of light on phlorotannin levels in both species. Furthermore, the manipulative experiment showed that *F. vesiculosus*, but not *A. nodosum*, can respond by a significant short-term decrease in phlorotannin content when light intensity is reduced, in accordance with the predictions of the CNBM. Finally, neither of the two species showed any significant short-term changes in phlorotannin content as a response to increased N availability.

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