

Do plant density, nutrient availability, and herbivore grazing interact to affect phlorotannin plasticity in the brown seaweed *Ascophyllum nodosum*

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Abstract Plants have different strategies to cope with herbivory, including induction of chemical defences and compensatory growth. The most favourable strategy for an individual plant may depend on the density at which the plants are growing and on the availability of nutrients, but this has not been tested previously for marine plant–herbivore interactions. We investigated the separate and interactive effects of plant density, nutrient availability, and herbivore grazing on the phlorotannin (polyphenolic) production in the brown seaweed *Ascophyllum nodosum*. Seaweed plants grown at low or high densities were exposed either to nutrient enrichment, herbivorous littorinid gastropods (*Littorina obtusata*), or a combination of nutrients and herbivores in an outdoor mesocosm experiment for 2 weeks. Seaweeds grown at a low density tended to have higher tissue nitrogen content compared to plants grown at a high density when exposed to elevated nutrient levels, indicating that there was a density dependent competition for nitrogen. Herbivore grazing induced a higher phlorotannin content in plants grown under ambient, but not enriched, nutrient levels, indicating either that phlorotannin plasticity is more costly when nutrients are abundant or that plants responded to herbivory by compensatory growth.

However, there were no significant interactive or main effects of plant density on the seaweed phlorotannin content. The results indicate that plants in both high and low densities induce chemical defence, and that eutrophication may have indirect effects on marine plant–herbivore interactions through alterations of plant chemical defence allocation.

Introduction

Several seaweed species contain secondary metabolites that can function as herbivore deterrents (Hay 1996), and the production of at least some of these compounds may be induced in response to herbivore grazing (e.g. Cronin and Hay 1996; Pavia and Toth 2000). One of the possible advantages with inducible, as opposed to constitutive, chemical defences is that the mobility of herbivores increase as they are forced to search for more palatable food (e.g. Edwards and Wratten 1987; Edwards et al. 1991). This, in turn, may lead to increased plant fitness by dispersing plant damage and/or exposing the herbivore to an increased risk of predation/parasitism (Agrawal and Karban 1999). Otherwise, it may be more advantageous for the plant to tolerate herbivory through tissue re-growth, i.e. to allocate resources to civilian rather than defensive responses (cf. Karban and Baldwin 1997). Moreover, the availability of resources will not only be important for the plant's ability to repair tissue damage, but may also affect the production of defence chemicals (e.g. Herms and Mattson 1992). The recently questioned (e.g. Nitao et al. 2002) carbon-nutrient balance hypothesis states that photosynthates will be allocated to the production of carbon-based secondary metabolites when plant growth is limited by nutrients. Nitrogen is generally considered to be the limiting nutrient in

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marine environments (e.g. Hanisak 1983), and has been shown to affect the constitutive production of polyphenolic secondary metabolites (phlorotannins) in brown seaweeds (e.g. Peckol et al. 1996).

Ascophyllum nodosum (L.) Le Jol. is a fucoid seaweed species that grows in dense stands in the rocky intertidal, but solitary plants are commonly found especially at the lower limit of the species' depth distribution (personal observation). *Ascophyllum nodosum* produces a class of carbon-based secondary metabolites called phlorotannins (polyphenolics) that may function as chemical deterrents towards herbivores (e.g. Pavia and Toth 2000; Toth et al. 2005), although the role of phlorotannins as defence compounds is disputed (e.g. Deal et al. 2003; Amsler and Fairhead 2006). Nonetheless, the phlorotannin concentration is highly variable on different spatial and temporal scales (Pavia et al. 2003), and the production of phlorotannins can be increased in response to herbivory (Pavia and Toth 2000; Toth and Pavia 2000; Toth et al. 2005). This relationship has earlier been shown independent of nitrogen (e.g. Pavia and Brock 2000) or nutrient (Toth et al. 2006) availability. Furthermore, herbivore-induced responses in *A. nodosum*, correlated to changes in phlorotannin content, have been shown to increase the mobility and feeding dispersal of the specialized herbivorous gastropod *Littorina obtusata* (Borell et al. 2004). However, it is not known whether other compounds associated with the phlorotannins could be responsible for the positive correlation between phlorotannin concentration and defence (Deal et al. 2003).

In the present study we investigated the individual and interactive effects of plant density (high or low), nutrients (ambient or enriched) and herbivore pressure (present or absent) on the production of phlorotannins in *A. nodosum*. We chose to focus on the production of phlorotannins, because estimates of compensatory growth in herbivore-exposed seaweeds were confounded by tissue loss due to grazing. We specifically tested the hypotheses (1) that *A. nodosum* would induce higher tissue content of phlorotannins in response to grazing by the littorinid snail *L. obtusata*, (2) that the induction of phlorotannins would be lower when plants were grown at a low density and/or under elevated nutrient conditions, and (3) that the constitutive phlorotannin production (i.e. when herbivores are not present) would be lower in plants grown at high densities and/or under elevated nutrient levels.

Materials and methods

Induction experiment

This study was conducted during summer 2005 at Tjärnö Marine Biological Laboratory (TMBL) on the west coast of

Sweden. The experiment was conducted under laboratory conditions in outdoor mesocosms consisting of transparent plastic aquariums (20 l) with a constant flow (100 l min^{-1}) of unfiltered sea surface water. Approximately 100 *A. nodosum* individuals were randomly collected from five locations in the Tjärnö archipelago. The holdfasts of the seaweeds were carefully detached from the substratum and the plants were transported to the laboratory where they were cleaned from grazers and epiphytes. Genetically independent shoots ($\approx 20 \text{ g}$) were placed individually in aquariums by attaching the algal stipes to plastic nets in the bottom of the aquariums with plastic cable ties. High seaweed densities were prepared by attaching genetically independent *A. nodosum* plants closely around the experimental plant to a total weight of about 120 g wet weight in each high-density aquarium. High nutrient load was achieved by attaching a fly-mesh bag (mesh size 1 mm^2) containing approximately 150 g of a commercial slow-release NPK-fertilizer (Plantacote™ Depot 6M, Urania Agrochem, Hamburg) consisting of pellets with a semi-permeable polyurethane layer (for the kinetics of nutrient release in situ from this material, see Worm et al. 2000) inside each aquarium. Adult ($>1 \text{ cm}$) *L. obtusata* gastropods were added at a density of 1 grazer per 10 g of algae to the treatments with herbivores. The density of *L. obtusata* in the study area is very variable both among different *A. nodosum* populations and among different individuals within populations, with maximum observed mean densities of 8 snails per 100 g algal wet weight (Pavia and Toth 2000). Each of the 8 treatment combinations (i.e. a high or a low density of plants with either ambient or added nutrient levels, with or without grazing) was replicated 6 times (i.e. a total of 48 aquariums were prepared). The experiment was terminated after 14 days, by collecting one apical shoot from each experimental *A. nodosum* individual.

Chemical and statistical analyses

The *A. nodosum* tissue samples were freeze-dried, homogenised, and extracted in aqueous acetone (60%) in room temperature on a shaking table for 1 h. After the extraction, samples were centrifuged, and the acetone was evaporated in vacuo at less than 40°C . The samples were filtered to remove precipitated lipophilic material and diluted to a known volume using distilled water. The concentration of phlorotannins in the samples was determined using the Folin-Ciocalteu analysis of phenolic compounds (Van Alstyne 1995). The nitrogen content in the seaweed tissues was measured rather than the concentration in the surrounding water, because it is the adsorbed rather than the dissolved nutrients that may affect the seaweed phlorotannin production/induction. Samples for tissue nitrogen content were freeze-dried, homogenized and analysed in an elemental

analyser (Model: EA 1108 CHNS-O, Fison, Italy) using 2,5-Bis-[5-tert.-butyl-benzoazol-2-yl]-thiophen (BBOT) as a standard. Data on the tissue phlorotannin and nitrogen content were statistically analysed using a 3-way analysis of variance (ANOVA) with density, nutrients and grazing as fixed orthogonal two-level factors. Prior to all statistical analyses, data were tested for homogeneity of variances with Cochran's test (Underwood 1997). All variances were homogeneous and data were not transformed. Significant differences between mean-values were detected using the Student–Newman–Keul's (SNK) multiple-comparisons test (Underwood 1997).

Results and discussion

Grazing damage was found on all *A. nodosum* plants exposed to herbivores, showing that the *L. obtusata* snails were actively feeding during the experiment. The ANOVA showed that there was a statistically significant relationship between shoot nitrogen content and treatments with NPK-fertilizer pellets (Table 1A, Fig. 1). The plants grown under ambient and enriched nutrient concentrations had average tissue nitrogen contents of 1.53 ± 0.05 and 0.87 ± 0.03 (mean \pm SEM)% dry weight, respectively. Furthermore, there was a trend towards a significant interaction effect of the main factors density and nutrients on tissue nitrogen content ($P = 0.06$, Table 1A). The seaweeds grown at a low density tended to contain slightly more nitrogen than the plants grown in a high density in the enriched, but not in the ambient, nutrient treatments (Fig. 1). These results indicate that there may be a density dependent effect on the nitrogen uptake of the experimental plants, i.e. that the seaweeds in the high-density treatment were competing for nitrogen. However, there were no statistically significant main or

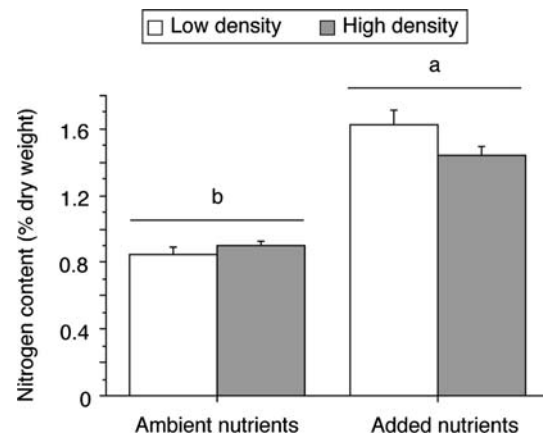


Fig. 1 Tissue nitrogen content (% dry weight) in *A. nodosum* plants grown at low and high densities, and exposed to ambient (Ambient nutrients) or elevated (Added nutrients) nutrient concentrations in the surrounding water. Letters above bars indicate significant differences between mean values based on the Student–Newman–Keul's multiple comparisons test (SNK, $P < 0.05$). Error bars show SEM ($n = 12$)

interactive effects of the factor herbivore on the tissue nitrogen content of the plants (Table 1A), indicating that the grazing snails did not have a fertilizing effect on the plants.

Grazing by *L. obtusata* induced an increased production of phlorotannin in *A. nodosum* as shown by the statistically significant effect of the factor herbivore on the phlorotannin content of the seaweeds (Table 1B, Fig. 2). However, there were no statistically significant main or interactive effects of the factor density on the phlorotannin content of the plants (Table 1B). These results show that the presumed increase in resource competition at higher densities does not affect the constitutive phlorotannin production. Furthermore, they show that *A. nodosum* induce phlorotannin production in response to grazing by *L. obtusata* independent

Table 1 Analysis of variance of the (A) tissue nitrogen content (% dry weight), and (B) tissue phlorotannin content (% dry weight) in *A. nodosum* plants grown either in high and low densities (density, D), and

exposed either to ambient or elevated nutrient concentrations in the surrounding water (nutrients, N) with or without the presence of *L. obtusata* (grazing, G)

Source of variation	df	(A) Nitrogen content			(B) Phlorotannin content		
		MS	F	P	MS	F	P
Density (D)	1	0.05	1.18	0.28	0.04	0.21	0.65
Nutrients (N)	1	5.27	119.54	<0.01	2.38	11.61	<0.01
Grazing (G)	1	<0.01	0.02	0.89	4.28	20.93	<0.01
D \times N	1	0.17	3.81	0.06	<0.01	0.01	0.92
D \times G	1	0.01	0.14	0.71	0.17	0.82	0.37
N \times G	1	<0.01	0.04	0.84	2.10	10.26	<0.01
D \times N \times G	1	0.04	1.27	0.27	0.13	0.66	0.42
Residual	40	1.76	0.04		8.19	0.21	

Data are mean values and SEM for the interaction between density and nutrients for the tissue nitrogen content and between grazing and nutrients for the tissue phlorotannin content are presented in Figs. 1 and 2, respectively

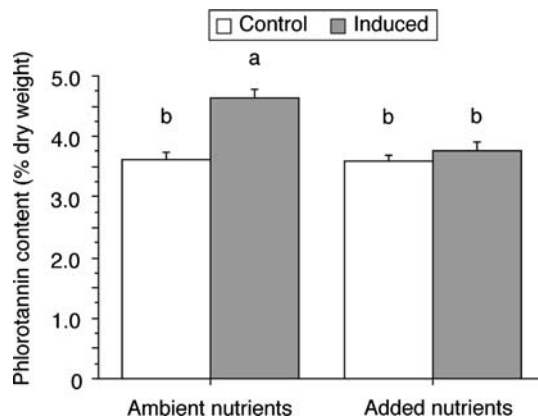


Fig. 2 Tissue phlorotannin content (% dry weight) in *A. nodosum* plants exposed to ambient (Ambient nutrients) or elevated (Added nutrients) nutrient concentrations in the surrounding water without (Control) or with (Induced) the presence of *L. obtusata*. Letters above bars indicate significant differences between mean values based on the Student–Newman–Keul’s multiple comparisons test (SNK, $P < 0.05$). Error bars show SEM ($n = 12$)

of the presence or absence of neighbouring plants. *Littorina obtusata* is specialized on *A. nodosum* and a few other fucoids, and is also sensitive to high phlorotannin levels in their diets (Pavia and Toth 2000; Toth et al. 2005). If a solitary *A. nodosum* plant induces higher phlorotannin content in the tissues in response to *L. obtusata* grazing, the snail will not be able to easily move to an alternative food source. However, *A. nodosum* plants can be large and even if the snail cannot move to another plant individual, dispersing the grazing damage to neighbouring parts of the plant will still be an advantage to the inducing *A. nodosum* plant, as this will reduce the risk of frond breakage (i.e. Borell et al. 2004). Furthermore, herbivore induced changes correlated to the phlorotannin content have a negative effect on *L. obtusata* reproduction (Toth et al. 2005), indicating that herbivore induced responses in the *A. nodosum* plants may affect the *L. obtusata* population density, thereby decreasing the herbivore pressure for these long-lived plants.

The herbivore induced phlorotannin production in *A. nodosum* was not independent of nutrient availability as shown by the statistically significant interaction effect between the main factors herbivory and nutrients (Table 1B). When means were compared in the SNK-test it was found that grazing by *L. obtusata* increased the phlorotannin content of *A. nodosum* plants by a statistically significant ($P < 0.05$) 28% when plants were grown under ambient nutrient levels (Fig. 2). However, the phlorotannin content of grazed and control plants grown in seawater enriched with nutrients was not significantly different (SNK, $P > 0.05$, Fig. 2). This is, as far as we know, the second example showing interactive effects of herbivory

and nutrient availability on the phlorotannin induction in seaweeds (see also Peckol et al. 1996). These results indicate that differences in nutrient availability may explain some of the previously observed variation in herbivore-induced phlorotannin production in this species (Pavia et al. 2003), although phlorotannin plasticity in response to grazing is also genetically determined in *A. nodosum* (H. Pavia and G.B. Toth, unpublished results). Our results contradict earlier observations (Pavia and Brock 2000; Toth et al. 2006) that did not find any effects of nitrogen addition on herbivore-induced phlorotannin induction in *A. nodosum*. However, the tissue nitrogen content of the enriched *A. nodosum* plants in the experiment by Pavia and Brock (2000) was slightly lower ($\approx 1.2\%$ dry weight) than the nitrogen content in the nutrient enriched plants in our experiment, which may explain the discrepancy between the results. Furthermore, Pavia and Brock (2000) used dissolved ammoniumnitrate rather than NPK-fertilizer to increase nutrient levels, indicating that nutrients other than nitrogen may be responsible for the significant interaction between herbivory and nutrient enrichment on the phlorotannin content of *A. nodosum*.

In conclusion, we did not find support for the hypothesis that plant density affects the production of phlorotannins in *A. nodosum*. Seaweed plants induced the production of phlorotannins when grown at both high and low densities and the constitutive phlorotannin production (i.e. when herbivores are not present) did not change with density. However, the induction of phlorotannins did depend on the nutrient availability as shown by the lack of induction when seaweeds were grown at a high nutrient concentration. These results imply that eutrophication may affect plant–herbivore interactions in marine ecosystems through alteration of chemical defence induction.

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