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Phlorotannins in intertidal brown algae: inducing factors and ecological roles

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2006

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Ph D Thesis

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Akademisk avhandling för filosofie doktorsexamen i Marin Botanik vid Göteborgs universitet (examinator: Prof. Inger Wallentinus), som enligt beslut av Naturvetenskapliga fakulteten kommer att försvaras offentligt fredagen den 13 oktober 2006 kl. 10.00 i föreläsningssalen på Tjärnö Marinbiologiska Laboratorium, 452 96 Strömstad.
Fakultetsopponent: Prof. Peter Steinberg, School of Biological, Earth & Environmental Sciences, The University of New South Wales, Sydney, Australia

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To My Father

and

Gunnar, Kristoffer, Katarina, Pontus

Abstract: Phlorotannins are polyphenolic compounds found exclusively in brown algae. Fucoids (Fucales) generally contain high levels (up to 20% of algal wet weight) of these polyphenolic metabolites. The concentrations, however, can vary considerably.

One objective of this thesis was to examine how environmental factors will affect phlorotannin concentrations in fucoids. The results from an experiment with the gastropod grazer *Littorina obtusata* and the fucoid *Ascophyllum nodosum* showed, for the first time, that natural grazing can induce increased phlorotannin production in brown algae. *Fucus vesiculosus* and *Ascophyllum nodosum* grow intertidally on the Swedish west coast, and these algae are subjected to highly variable environmental conditions. At low sea water levels, they can naturally become emerged for several days, with increased exposure to elevated irradiance and harmful UV radiation as a consequence. The results of studies in this thesis showed that UV-B radiation increased the amount of phlorotannins within the algal tissue. Furthermore, algae that had been exposed to emersion had lower concentrations of tissue phlorotannins, than those that were constantly submerged. However, phlorotannins are also known to exude into the seawater, and the results from studies of the effects of emersion on exudation of phlorotannins showed that previously desiccated algae exuded significantly more phlorotannins when re-immersed, compared to non-desiccated algae. Overall, the results suggest that there is a re-location of metabolites to the surface of the algal thallus when the plants are emerged at low water levels. Since phlorotannins have UV-absorbing properties, this re-distribution of phlorotannins could possibly generate an added shield against harmful radiation.

A second objective of this thesis was to examine the role of phlorotannin as an antifouling agent, by testing their settlement deterrence on larvae of the barnacle *Balanus improvisus*. Phlorotannins were shown to inhibit larval settlement, both as algal tissue extracts and as exudates in seawater. The deterrent effect was demonstrated both quantitatively, for different concentrations of phlorotannins, and qualitatively, for two different molecular size-fractions of tissue phlorotannins. Furthermore, natural field sampling of seawater close to recently emerged algae, showed that phlorotannin levels were close to settlement inhibiting concentrations. Altogether, the results imply that phlorotannins may function as inhibiting compounds against fouling on intertidal fucoids. This could possibly explain the observed settlement preference of barnacle larvae for rock, rather than algae, and also the greater natural abundance of adult *B. improvisus* on rock, compared to algae.

Through a combination of manipulative experiments and field observations, the studies of this thesis have assessed natural patterns of variation in phlorotannin levels, both as algal tissue content and as exudates. The thesis has furthermore demonstrated that phlorotannins in fucoids can vary in response to both abiotic and biotic factors, and that the compounds can affect the settlement of fouling organism. Overall, the combined results from the experiments of this thesis strengthen the notion that phlorotannins are ecologically important metabolites in the intertidal rocky shore community.

Keywords: Phlorotannins, *Fucus vesiculosus*, *Ascophyllum nodosum*, inducing factors, herbivory, *Littorina obtusata*, emersion, desiccation, UV-radiation, algal tissue, exudation, molecular size-fraction, fouling, *Balanus improvisus*, settlement.

List of papers:

This thesis is based on the following papers, referred to by their Roman numbers:

I Pavia H, Brock E (2000) Extrinsic factors influencing phlorotannin production in the brown alga *Ascophyllum nodosum*. Marine Ecology Progress Series 193: 285-294

II Brock E, Pavia H (manuscript) Effects of light and desiccation on phlorotannin tissue concentration and exudation in the brown alga *Fucus vesiculosus*.

III Pavia H, Brock E, Jonsson PR (submitted manuscript) Effects of quantitative and qualitative variation in brown algal phlorotannins on settlement of barnacle larvae.

IV Brock E, Nylund GM, Pavia H (conditionally accepted) Chemical inhibition of barnacle larval settlement by the brown alga *Fucus vesiculosus*. Marine Ecology Progress Series

A doctoral thesis at a university in Sweden is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarises the accompanying papers. These have already been published or are manuscripts at various stages (in press, submitted or in manuscript).

1. INTRODUCTION

1.1. Chemical response to environmental factors in brown algae

1.1.1. Background

Intertidal canopy forming brown algae have an important function as primary producers in coastal marine areas, constituting food for a variety of herbivores. They are furthermore vital as habitat for many fouling invertebrates and epiphytic micro- and macroalgae. Grazing and fouling organisms can have a negative impact and potentially interfer with growth and survival of these brown algae. Furthermore, seaweeds living in the intertidal zone are subjected to a fluctuating physical environment, e.g. changes in water levels, irradiance and temperature. As for terrestrial plants, benthic macroalgae cannot escape unfavourable conditions and potential danger. They have to either adjust and tolerate the unfavourable conditions or to make use of defence mechanisms that can be morphological (structural), mechanical through shedding of epithallial layers, physical through changes in surface wettability and/or via chemical substances (see Nylund 2005 for references). There is growing evidence that strategies used by algae can be complex, but also highly specialized, in order to target specific biotic or abiotic threats (see McClintock & Baker 2001, Amsler & Fairhead 2006, Paul & Puglisi 2004, Hay 1996, Arnold & Targett 2002, for references).

During the last decades, extensive research in marine chemical ecology has focused on secondary metabolites (natural products), and their defence functions (see Hay 1996 and McClintock & Baker 2001 for references). Secondary metabolites in plants were in contrast to primary metabolites, long considered to be of little importance, since they did not have any apparent role in the maintenance and growth of living cells. It was only in 1959, when Fraenkel put forward their defence function, as a justification of their existence (Fraenkel 1959), that the secondary metabolites began to be of interest, and discussed as having important functions, through their mediation of information within and between individuals and species. Secondary metabolites are also produced in marine algae, and during the last decade, research in marine chemical ecology suggests, that algae can actively respond and regulate their secondary metabolism in response to external threats and conditions (see Paul & Puglisi 2004 and Amsler & Fairhead 2006 for references). The distinction between primary and secondary functions is sometimes difficult to make, and many metabolites can have both primary and secondary functions. Thus, the dynamics of these chemical metabolites is often the result of complex trade-offs between factors affecting fitness and the benefit of primary or secondary functions of the metabolites.

1.1.2. Phlorotannins

Polyphenolic compounds, such as tannins, are ubiquitous in terrestrial plants. Tannins are also found in marine algae and have been the focus of many studies in marine chemical ecology (see Paul & Puglisi 2004 and Amsler & Fairhead 2006 for references).

Tannins are substances with hydroxyl substituents bonded to aromatic (carbon) rings with the ability to bind to proteins and other macromolecules (Stern et al. 1996a). Phlorotannins, the least complex group of the tannins, are exclusively found in brown algae (Ragan & Glombitza 1986). Phlorotannins are mainly considered to be synthesised via the acetate-malonate (polyketide) pathway, and are polymers of phloroglucinol (1,3,5-trihydroxybenzene) (Ragan & Glombitza 1986, Waterman & Mole 1994, Arnold & Targett 1998). These water-soluble polar metabolites are for the most part stored in specialized, highly mobile vesicle-like bodies in the cytoplasm, called physodes (Ragan 1976, Schoenwaelder 2002a). The amount of physodes is denser in the outer layers of the algal fronds (Ragan & Glombitza 1986, Tugwell & Branch 1989). Phlorotannins are also known to exude into the seawater (Conover & Sieburth 1966, Sieburth & Jensen 1969, Langlois 1975, Ragan & Glombitza 1986, Jennings & Steinberg 1994 and see **Paper II** and **IV**).

Phlorotannins have been demonstrated to have primary functions (Vreeland & Laetsch 1990, Schoenwaelder & Clayton 1998). As primary metabolites they can be cell-bound, being involved in cell wall constructions (Schoenwaelder & Clayton 1998) and also in embryo development (Schoenwaelder & Wiencke 2000). Arnold and Targett (2003) argued that phlorotannins are primary metabolites with multiple transitional secondary roles. However, a recent study on the fucoid *Fucus vesiculosus* suggests that most of the phlorotannins present in the algal tissues have secondary functions (Koivikko et al. 2005). Soluble phlorotannin can constitute up to 20 % of the algal dry weight (DW), but levels are highly variable between and within brown algal species, populations, individuals and even parts of algal thalli (Steinberg 1989, Pavia & Åberg 1996, Van Alstyne et al. 1999, 2001, Pavia et al. 2003).

Current research suggests that phlorotannins serve multiple functions (Schmitt et al. 1995 and see Amsler & Fairhead 2006 for references). Most ecological studies have been examining their role as herbivore deterrents, although they are also discussed as having antifouling properties (see **Paper III** and **IV**). Furthermore, phlorotannins have been shown to offset bacterial infections (Sieburth & Conover 1965, Lau & Qian 1997), which might also be a reason for their participation in wound healing (Fagerberg & Dawes 1976, Lüder & Clayton 2004). Phlorotannins absorb UV radiation, with the absorption maxima 260-280 nm (Ragan & Glombitza 1986, Swanson & Druehl 2002), and more recently a role of phlorotannins as a protection against harmful radiation has been suggested (Pavia et al. 1997, Schoenwaelder 2002b and see **Paper I** and **II**).

The great majority of ecological studies on phlorotannins, concerning extrinsic factors affecting production as well as the effects of phlorotannins on other organisms, have analyzed the quantity of total phenolics and few have examined qualitative differences in terms of molecular size-fractions of phlorotannins (but see van Altena & Steinberg 1992, Boettcher & Targett 1993 and **Paper III**).

1.1.3. Extrinsic factors influencing tissue content and exudation of phlorotannins

Brown algae growing on rocky shores near the seawater level are subjected to highly variable environmental conditions, which in their turn can affect the production of secondary metabolites. Accordingly, the amount of brown algal phlorotannins, both in algal tissue and as exudates, has been demonstrated to be exceedingly plastic in response to several factors, both abiotic and biotic.

Nutrients

Variation in nutrient availability has been suggested to influence phlorotannin concentrations, and the most commonly addressed theory to explain this influence, is the carbon-nutrient balance model CNBM (Bryant et al. 1983). The CNBM was proposed by Bryant and co-workers (1983), and it states that the relative resource availability of carbon and nutrients determines the production of secondary metabolites. When nutrients are more limiting than ambient light for algal growth, excess carbon from photosynthesis can be allocated to the production of secondary metabolites. The reasoning behind the CNBM often implies that carbon based secondary compounds mainly are waste products (Haslam 1985, Waterman &Mole 1989). However, the majority of recent studies on marine algae propose that secondary compounds serve important ecological functions, and are not primarily driven by a source-based model like the CNBM. Generally, studies have demonstrated a lack of change in phlorotannin concentrations in response to nutrient enrichment (see Amsler & Fairhead 2006 for references, and see Paper I), although it has been observed, in some studies, that natural phlorotannin levels in algal tissue correlate negatively with nutrient supply (Ilvessalo & Tuomi 1989, Yates & Peckol 1993).

Desiccation

Algae living in the intertidal zone are subjected to the risk of becoming severely desiccated at low water levels, with added effects of elevated temperatures or frost and high intensities of irradiance, including harmful UV radiation. Some physical conditions, such as tidal changes of water levels, and exposure to waves, are for the most part predictable, but others can be more uncertain and unpredictable e.g. changes in water levels due to atmospheric pressure.

Densities of phenolic vesicles in *Fucus serratus* have been observed to be higher in plants emerged at low tide, which suggests an effect of desiccation on the phenolic content (Pontillon 1926). Emersion has further been suggested to increase the exudation of brown algal polyphenolic metabolites in seawater (Carlson & Carlson 1984 and see **Paper II** and **IV**). Craigie and McLachlan (1964) proposed, that exudation of phenolic compounds from *Fucus vesiculosus* was due to an increase in temperature. However, an effect of temperature might be related to emersion, especially at intense sunlight. Research on the effects of emersion and desiccation on phlorotannin variation is so far limited, both concerning the effects of emersion per se, as well as the potential interactive effects of emersion and light (but see **Paper I, II** and **IV**).

Light

Peckol and co-workers (1996) found a positive seasonal correlation between phlorotannins and light in *Fucus vesiculosus* and correspondingly, Steinberg (1995) reported higher levels of phlorotannins in several Australian brown algae in spring compared to autumn. With a combination of observational field studies and mesocosm experiments, Pavia and Toth (2000b) showed that *Fucus vesiculosus* exposed to direct sunlight contained higher concentrations of phlorotannins than shaded algae. Furthermore, Cronin and Hay (1996) observed that spores from the fucoid *Sargassum filipendula* that settled and grew naturally in a shaded environment contained lower levels of phlorotannins compared to less-shaded plants. In all studies described above, variation in phlorotannins has been examined solely within the algal tissue. However, Ragan and Jensen (1979) suggested that light equally can affect exudation of phenolic metabolites.

Phlorotannins, being able to absorb UV radiation (UVR), have also been suggested to vary in response to UVR. In the first direct test of this hypothesis, Pavia and co-workers (1997) demonstrated that added UVBR increased concentrations of phlorotannins in the algal thallus of the fucoid *Ascophyllum nodosum*. Enlargement of phenolic vesicles, physodes, in kelp spores was found as a response to UVR, and this was specifically pronounced in the most shallow growing species (Wiencke et al. 2004), Furthermore, in a study from the Baltic sea, Jormalainen and Honkanen (2004) found higher concentrations of phlorotannins in *F. vesiculosus* grown under added UVR.

UV absorbing phlorotannins might additionally be of importance as a protection against harmful radiation, when they are exuded in seawater. Exuded phenolic compounds have been shown to act as a shield against UVR. Embryos of *Fucus spiralis* grew better under a filter with phloroglucinol, when exposed to elevated levels of UVR (Schoenwaelder et al. 2003), and Swanson and Druehl (2002) observed that exuded kelp phlorotannins reduced transmission of UVBR, and that kelp exudates in seawater significantly increased the survivorship of germinating spores of *Laminaria groenlandica*. To my knowledge Swanson and Druehl is the only study examining the dynamics between algal tissue content of phlorotannins and exudation of the compounds in relation to UVR (but see **Paper II**).

Herbivores

Biotic factors, such as grazing, have also been shown to affect variation in the production of phlorotannins, and many studies on phlorotannins have in the past focused on their role as herbivore deterrents (see Amsler & Fairhead 2006 for references). Phlorotannins have been shown to decrease herbivore grazing from fishes, urchins and mesoherbivores (i.e. grazers <2.5 cm), although the effects are suggested to be dependent of both algal and herbivore species (see Hay & Steinberg 1992, Steinberg 1992, Targett & Arnold 1998, 2001 and Amsler & Fairhead 2006 for references).

Herms and Mattson (1992) suggested that secondary compounds function as defence metabolites, but that their production is costly, since they utilize resources otherwise allocated to growth and survival. The Induced Defence Model (IDM) predicts an optimal use of these costly metabolites (Karban & Meyers 1989, Harvell 1990). According to the IDM, production of defence metabolites will only be induced by direct damage or when there are evident and distinct cues of attacks, that plants can respond to (Karban & Baldwin 1997). To function as a defence, a substance needs to be produced in time to act as a deterrent, and present in significant concentrations to have an impact on the attacking or damaging organism. Hay (1996) argued that inducible defences in seaweeds against grazing are likely to be more efficient as response to mesoherbivores, than to larger grazers such as fish and urchins. These smaller herbivores stay and feed on the algal fronds long enough for an induced defence to be effective.

Grazing on fucoids in the temperate waters of the Swedish west coast is mainly performed by mesoherbivores such as gastropods, amphipods and isopods. Although many of these herbivores mostly graze by scraping the algal surface, some, like the littorinid *Littorina obtusata* equipped with radula teeth, will penetrate tough fucoid fronds (Viejo & Arrontes 1992), and can incur severe damage to algal thallus (Pavia & Toth 2000a). Some studies have demonstrated artificial wounding (clipping) of algal thallus to induce phlorotannin production (Van Alstyne 1988, Yates & Peckol 1993, Hammerstrom 1998) whereas others failed to find increase in phlorotannins of simulating herbivory (Steinberg 1994, Pavia et al. 1997). Pavia and Toth (2000a) observed elevated phlorotannin levels in natural populations of *Ascophyllum nodosum* plants with a high number of grazing marks. However, at the time of the induction experiment in **Paper I**, no other study had experimentally demonstrated an induction of phlorotannins to directly be caused by natural grazing.

Studies of grazing effects on the variation of phlorotannins have mainly focused on changes of the compounds in algal tissue however, the isopod *Idotea baltica* has been reported to induce exudation of phlorotannins in *Fucus vesiculosus* (Koivikko et al. 2005).

1.1.4. Antifouling activity of phlorotannins

Many brown algae have large leafy thalli that can represent suitable habitats for epiphytic organisms, and excess overgrowth might ultimately restrain photosynthesis and nutrient uptake in the algal plant. Many species, including hydroids and bryozoans as well as filamentous macroalgae, can cover large parts of the surface of seaweeds. Sessile crustaceans in the tidal zone, such as barnacles are likewise known to settle, attach and live as adults on the algal surface (Barnes & Barnes 1962). Foliose brown algae also represent an important substrate for microorganisms such as bacteria, microalgae and algal propagules (Wahl 1989).

Several studies have demonstrated antifouling qualities of phlorotannins (Sieburth & Conover 1965, Lau & Qian 1997, 2000, and see Steinberg et al. 1998 and Amsler & Fairhead 2006 for further references) although Jennings and Steinberg (1997) pointed out that many antifouling studies, have not measured natural concentrations of these polyphenolics, that are likely to deter foulers in the field.

Jennings and Steinberg (1997) suggested that, for algal chemical compounds to inhibit fouling they need to be present at or near the surface of algae, in sufficient concentrations to deter fouling organisms. They found no effect of exudates from the subtidal kelp *Ecklonia radiata,* but they suggested that exudation of phlorotannins might be ecologically more important on intertidal shores. Conover and Sieburth (1966) observed that phenolic substances in tide pools can accumulate and become toxic, and Langlois (1975) showed that seawater from rock pools with fucoids, inhibited population growth of ciliates. However, there is still limited data on effects of naturally exuded of phlorotannins as antifouling compounds (but see **Paper II** and **IV**).

1.2. Study area

Field studies and laboratory experiments were all performed at Tjärnö Marine Biological Laboratory (TMBL), and in the archipelago around Tjärnö. The laboratory is located on an island on the Swedish west coast (58°52´ N, 11°09´ E). The archipelago is situated in the North-Eastern Skagerrak, part of the North Sea. Much of the shoreline consists of rocky shores, with many small islands, bays and straits, which for living organisms create large differences in exposure to wave action and currents. Furthermore, the environment is characterized by a high degree of stochasticity for intertidal organisms (Åberg 1992). The tidal range is only between 20-40 cm, but climatic factors, such as changes in wind conditions and fluctuations in atmospheric pressure, can cause a difference in water levels of up to 2 m (Johannesson 1989, Fonselius 1995). These climatic changes in seawater level are unpredictable, and can exert extra stress on sessile organisms living in the upper tidal zone. Winter conditions often include ice and very low water levels, leaving sessile organisms in the upper tidal zone dried and frozen, for up to more than a week at a time, and in late winter also with exposure to ice abrasion. During the summer-season, changes in water level are usually less dramatic, but differences in wind and air-pressure will nevertheless commonly leave near-shore organisms emerged for days. All field studies, and all but one manipulative experiment were carried out during the summer months (May – September). One settlement experiment (**Paper IV**) was performed in March.

1.3. Study organisms

Fucus vesiculosus *Ascophyllum nodosum* *Halidrys siliquosa*

Fig. 1 (illustrations Helena Samuelsson)

1.3.1. Algae

For this thesis three different species of brown algae (order Fucales, class Phaeophyceae) have been studied: *Fucus vesiculosus* L., *Ascophyllum nodosum* (L.) Le Jol. and *Halidrys siliquosa* (L.) Lyngbye (Fig. 1). All plants were collected on rocky shores in the Tjärnö archipelago. *A. nodosum* and *F. vesiculosus* grow intertidally, while *H. siliquosa* mainly grows subtidally. Most work has been performed using *F. vesiculosus* (**Papers II-IV**). However, in **Paper I** the induction experiments were carried out on *A. nodosum* and in **Paper III** repellent effects of phlorotannins from *H. siliquosa*, as well as from *F. vesiculosus* and *A. nodosum*, were tested. The focus of the studies has been to investigate factors causing variation in concentrations of phlorotannins in algal fronds, the exudation of these polyphenolic metabolites in seawater, and the potential role of phlorotannins in the interactions between brown algae and fouling organisms.

Fucus vesiculosus, bladder wrack, is a widespread fucoid on rocky shores of the North Atlantic (Lüning 1990), and is commonly found on the west coast of Sweden. The yellow-green to dark brown plants are perennial and can reach 1 m in length. *F. vesiculosus* can adjust to environmental conditions such as variances in salinities (Russell 1988, Kalvas & Kautsky 1998, Bergström 2005), depths (Waern 1952, Wallentinus 1979, Kautsky et al. 1986, Pedersen & Snoeijs 2001) and wave exposure (Kalvas &

Kautsky 1993, Eriksson & Bergström 2005). On the Swedish west coast *F. vesiculosus* grows in the intertidal zone down to a depth of about 1.5 m, for the most part in sheltered areas, but is in addition found on wave exposed shores, where the plants generally are smaller in size and lack air bladders.

Ascophyllum nodosum, knotted wrack, commonly grows together with *Fucus vesiculosus* in the intertidal zone on the Swedish west coast (Lüning 1990). Knotted wrack, however, does not tolerate exposure to wave action, nor low salinities (<15 psu), and is consequently found only on sheltered shores, with good water exchange and in salinities over 15 psu. It does not grow inside the northern entrance to the Öresund. *A. nodosum* varies in colour, from yellow to olive-green and grows to a length of about 1 m, although under certain circumstances it can become over 2 m long. The individual plants can live long and sometimes reach >20 years of age (H Pavia, E Brock personal observation, through counting airbladders). Knotted wrack is found from mean sea level to a depth of 2m.

Halidrys siliquosa, sea oak, grows subtidally on the Swedish west coast on cliffs and rocks, in both wave exposed and more protected habitats (Lüning 1990). It grows from the depth of 1 m, often down to around 10 m, although it has been found down to 17m (Lunnerud & Åberg 1983). *H. siliquosa* is found in the Skagerrak and Kattegat, with a southern distribution limit at the northern part of the Öresund (Nielsen et al. 1995). The light brown thallus can grow to a maximum length of 2m.

1.3.2. Invertebrates

The intertidal algal belt where *Fucus vesiculosus* and *Ascophyllum nodosum* are two dominating fucoids, is a community with a large diversity. Many invertebrates live in, on or of these seaweeds, and both the flat periwinkle, *Littorina obtusata*, and the bay barnacle, *Balanus improvisus*, (see **Paper I** and **Papers III** and **IV**, respectively) are common in this community (Pavia et al. 1999, Wikström & Pavia 2004, 2006). The gastropod *Littorina obtusata* (L.) was employed for the herbivore treatment in the first induction experiment, in **Paper I,** and the crustacean *Balanus improvisus* Darwin for the experiments testing antifouling qualities of phlorotannins, in **Papers III** and **IV** (Fig. 2). Both invertebrates are common organisms in the intertidal algal belt on the Swedish west coast. The herbivore *L. obtusata* was used as a potential biotic factor affecting the production of phlorotannins within algal thalli, while the purpose of utilizing *B. improvisus* was to investigate a deterrent effect of exuded phlorotannins on the larvae of this fouling barnacle.

photo B. Johannesson
Littorina obtusata
(on *Ascophyllum nodosum*)

illustration H Samuelsson
Balanus improvisus, adult

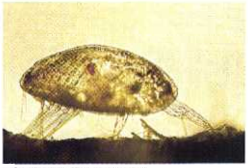

photo K Berntsson
Balanus improvisus, cyprid

Fig. 2

The flat periwinkle *Littorina obtusata* is a mesoherbivore common on *Ascophyllum nodosum* and *Fucus vesiculosus*. The littorinid is specialized to live on a few fucoids (Williams 1990, Vermeij 1992, McQuaid 1996). The radula teeth of *L. obtusata* have, unlike other littorinids, shovel-like cups well adapted for penetrating and biting through tough algal fronds (Steneck & Watling 1982, Watson & Norton 1987, Veijo & Arrontes 1992), a specialization that could support a host-specific interaction by the snail, (Watson & Norton 1987). *L. obtusata* can cause extended damage to individual plants as it feeds, by biting through the thallus of the algae (Viejo & Åberg 2003), and a recent study in natural populations, showed that grazing by *L. obtusata* can reduce net growth of *A. nodosum* plants by 50 % (GB. Toth, M. Karlson and H. Pavia unpublished). Specialized herbivores are uncommon in marine habitats where the majority of grazers are generalists (Hay & Steinberg 1992). One explanation for the dominance of generalist herbivores in the sea is the existence of planktonic larval stages in marine invertebrates, which restricts the possibility for larvae to find specific species to settle upon and feed on (Hay & Fenical 1988, Hay & Steinberg 1992). *L. obtusata,* however, has no planktonic stage and the fully developed juvenile snails are hatched from eggs, laid in jelly-like coverings directly on the algal thalli (Goodwin 1979, Williams 1990). This form of life cycle could have favoured a development towards a higher degree of host specialization.

The life cycle of the cirripede crustacean, *Balanus improvisus* includes several pelagic larval stages. When the larvae have metamorphosed into their last larval stage (cyprids), they commence to search, identify and attach to a substratum appropriate to adult life (Andersson 1994). This search has been observed to be an active process (see references in Young 1990), where the cyprids investigate surface properties and attach temporarily, while they seem to evaluate substrate qualities. They can subsequently detach for further attempts to find a preferred surface to settle upon. *B. improvisus* is one of the major fouling organisms in Swedish waters (Berntsson & Jonsson 2003), and attaches to many natural substrates, such as stone, shell and algae, but is in addition a common fouler on boat hulls and other man-made substrates (Berntsson & Jonsson 2003). *B. improvisus* is the only barnacle found in the Baltic Sea, and is then often an epibiont on *Fucus vesiculosus*. For the experiments in this thesis, cyprids were used in order to investigate a potential deterrence function of phlorotannins on the settlement of the larvae. (**Papers III** and **IV**). The larvae were reared at TMBL, where a permanent barnacle culture can supply cyprids all year round.

1.4. Objectives of the thesis

Many fucoids contain considerable amounts of phlorotannins, and one aim for the research in this thesis has been to find environmental factors that affect the production of phlorotannins (**Papers I** and **II**). Furthermore, previous research on phlorotannins has rarely tested the relationship between concentrations of phlorotannins in the thallus of algae versus the exudation of these metabolites (but see Swanson & Druehl 2002). The dynamics of this relationship was addressed in **Papers II** and **IV**. The other objective of this thesis was to focus on inhibiting qualities of phlorotannins on a fouling organism (**Papers III** and **IV**).

The experiments of the thesis are based on the hypotheses that:

1) Extrinsic factors, such as herbivory (i.e. a biotic factor), and light, nutrients, desiccation (i.e. abiotic factors) can induce changes in algal tissue concentrations of phlorotannins in *Fucus vesiculosus* and *Ascophyllum nodosum* (**Papers I** and **II**).

2) Light and desiccation can induce exudation of phlorotannins in the fucoid *Fucus vesiculosus* (**Papers II** and **IV**).

3) There are quantitative and qualitative differences in deterrent effects of phlorotannins in *Fucus vesiculosus, Ascophyllum nodosum* and *Halidrys siliquosa* on settlement by the larvae of the barnacle *Balanus improvisus* (**Papers III** and **IV**).

4) The natural settlement process by *B. improvisus* can be deterred by phlorotannins exuded by *F. vesiculosus* (**Paper IV**).

2. METHODS

In this chapter a general description of the methods for collection, experimental design and analyses is outlined. Detailed information about each experiment is given under the heading "Materials and Methods" for each paper (I-IV).

2.1. Collection of algae

The algae studied in this thesis, *Ascophyllum nodosum, Fucus vesiculosus* and *Halidrys siliquosa,* were haphazardly collected and handled only in direct conjunction with experimental set up. Whole plants were cut at their holdfast and directly transported in seawater to TMBL. The algae were kept in running seawater for no more than 24 h, before they were relocated to the aquaria used for the experiments. When fractions of algal thalli were used, fronds were cut several branches away from the parts to be studied. This was done in order to try to avoid any additional stress, which could influence the experimental results, in those parts of the plants used for examination. When identification of individual plants was of importance, fronds were placed in open plastic bags in the water. For experiments using desiccated algae, plants were emerged and dried at their growing location, and subsequently transported to the laboratory. During collection, consideration was taken to make as little damage to the natural environment as possible.

2.2. Experimental set-up

Experiments in **Papers I** and **II** were all conducted in aquaria placed outdoors on a jetty adjacent to TMBL. For the experiments, a flow-through system of filtered seawater was used, providing continuously fresh seawater. However, for the first induction experiment in **Paper I** aquaria were closed and water changed daily. Experiments were of mainly two major categories: 1) manipulative experiments where algae were exposed to different treatments (**Papers I** and **II**) and where barnacle larvae were presented with a choice of natural settlement surface (**Paper IV**), and 2) bioassays where effects of algal metabolites were tested on *Balanus improvisus* larvae (**Papers III** and **IV**). Experimental design was mainly factorial with statistical analyses of data based on analysis of variance

(ANOVA) (Underwood 1997), although paired t-tests were used for the analysis of results from the settlement preference experiment in **Paper IV**.

2.3. Chemical analyses

The greater part of the chemical analyses consisted of the quantification of phlorotannins, although tissue nitrogen concentrations in algal thalli were analyzed, as well as extractions of surface-bound metabolites (see **Papers I** and **IV** for details). Here a general description of the method used to analyze phlorotannin levels, both in algal tissue and as exudates in seawater, is outlined.

For tissue concentrations of phlorotannins, fronds, directly after clipping, were frozen (-70° C), freeze-dried and ground to a fine powder with pestle and mortar. Aqueous acetone (60%), a solvent that would extract polar compounds, such as phlorotannins, was used and the metabolites were extracted on a shaking rack in dark and cold (0-8°C) conditions for 24 h. Crude extracts were subsequently centrifuged, the supernatant was collected, the acetone evaporated in vacuum, and the remaining extract in water solution was filtered to remove any lipophilic material. Concentrations of phlorotannins were quantified using the Folin-Ciocalteu (F-C) method (Folin & Ciocalteu 1927). The most commonly used chemical assay on phlorotannins and other polyphenolic substances is the Folin-Denis colorimetric procedure, first presented in 1912 (Folin & Denis 1912). This method has since been developed and improved in several ways. The method is based on an oxidation/reduction reaction. The polyphenolic extract is mixed with a reagent of sodium tungstate, phosphomolybdic acid and phosphoric acid in solution. The reduced reagents produce a blue coloured solution, which can be measured spectrophotometrically (λ_{max} = 725 nm). To calculate concentrations in the extracts, a standard curve for phloroglucinol (CAS no. 6099-90-7) was used. An occurring problem, when applying the Folin-Denis method, can be the formation of a white precipitate, which renders spectrophotometric readings difficult. This problem can be overcome by using the F-C method, which improves the Folin-Denis procedure through the addition to the reagent of lithium sulphate (Folin & Ciocalteu 1927, Waterman & Mole 1994, Van Alstyne 1995). The only disadvantage of using the F-C assay is the 2 h, compared to 30 min for Folin-Denis, it takes for the colour to develop in the solution.

For quantification of exuded phlorotannins, seawater samples and controls with filtered seawater, were directly mixed with F-C reagent and quantified as above, although before spectrophotometric readings the mixture had to be centrifuged in order to separate a formation of salt from the solution.

Extracts of phlorotannins can sometimes contain disturbing compounds e.g. tyrosine and ascorbic acid, which, besides phlorotannins, can react with the reagents (Stern et al. 1996b). This disturbance, however, is considered small (Steinberg 1988, Van Alstyne 1995).

In **Paper III**, a qualitative difference of phlorotannins was examined, and two molecular fractions (<30 kDa and >30 kDa) of phlorotannins were tested for effects on settlement by larvae of the barnacle *Balanus improvisus* (for detailed explanation of method see **Paper III**).

3. RESULTS AND DISCUSSION

In this chapter the main results of the different experiments in the attached papers are summarized and discussed.

3.1. Factors affecting phlorotannin concentrations

In **Papers I** and **II** separate and interactive effects of several potential factors affecting phlorotannin production were examined. In **Paper I** phlorotannin concentrations in algal tissues were studied, whereas in **Paper II** and **IV** both tissue content and exuded metabolites were discussed. Overall, there was no effect of nutrient availability on phlorotannin production. However, grazing, light and emersion were shown to separately influence the levels of phlorotannins. Furthermore, in **Paper I** there were interactive effects on phlorotannin production in *Ascophyllum nodosum* of the factors UVBR and time. Plants exposed to ambient UVBR contained higher concentrations of phlorotannins after 7 weeks, but not after 2 or 4 weeks. There were also interactive effects of UVBR and water levels, and concentrations of phlorotannins in algal tissues were found to be lower, when the algae were subjected to periodical emersion and when UVBR was screened out. In addition, there were interactive effects on growth, measured as wet weight (WW) change, caused by nutrients and grazing. A positive effect of nitrogen enrichment was found on algal growth, but only in the absence of grazers.

3.1.1. Herbivores

Herbivores have been demonstrated to have a large impact on many seaweed populations and communities (Lubchenko & Gaines 1981, Hay 1985, John et al. 1992), and the role of grazers in the regulation of brown algal phlorotannin concentrations has extensively been described in the literature, (see Amsler & Fairhead 2006 for references). According to Hay (1996), mesoherbivores, such as gastropods, are more likely to induce a chemical defence response in algae, than larger consumers. Grazers like fish or urchins are capable of damaging plants severely, before an induced response would be effective. Mesograzers, on the other hand, generally feed more slowly, but over long enough periods, allowing for an induced response to be beneficial to the plant (Cronin & Hay 1996). As the prevalent herbivores in the waters of the Swedish west coast are mesograzers, mainly gastropods and small crustaceans, the levels of phlorotannins induced in the preferred fucoids might serve as an effective defence. However, this would be true only if the grazers are deterred from feeding on the individuals with high levels of phlorotannins. In the first experiment in **Paper I**, were there was an induction of phlorotannins in response to grazing by *Littorina obtusata*. There was, however, no test for effects of increasing levels of phlorotannins on the feeding behaviour of the snails. There are, however, results from other studies suggesting that higher levels of phlorotannins can affect feeding behaviour of littorinids. Geiselman and McConnell (1981) observed a deterrent effect of phlorotannins on the littorinid *Littorina littorea* and Van Alstyne (1988) demonstrated that a ≈20% increase in phlorotannins deterred *Littorina sitkana* from feeding on *Fucus distichus*. Pavia and Toth (2000a) found that *L. obtusata* preferred ungrazed plants and artificial food of A. *nodosum*, containing lower levels of phlorotannins, and they also noticed that grazed A. *nodosum* plants were less susceptible to further grazing by the snails than ungrazed plants. Furthermore, Pavia and co-workers (2002) linked a feeding deterrence by *L. obtusata,* to elevated phlorotannin concentrations in A. *nodosum*. In a recent study, Toth and co-workers (2005) demonstrated that a decrease in reproduction of *L. obtusata* was correlated to elevated concentrations of phlorotannins.

Prior to experiment 1 in **Paper I,** most studies on the effects of herbivores on phlorotannin production in brown algae, had examined effects of artificial grazing, such as clipping and hole punching (Van Alstyne 1988, Yates & Peckol 1993, Peckol et al. 1996, Hammerstrom et al. 1998). Results have however, been inconsistent, and while artificial grazing in fucoids (Van Alstyne 1988, Yates & Peckol 1993, Peckol et al. 1996) and in kelp (Hammerstrom 1998) has been observed to induce phlorotannin production, other studies have reported no effect of simulated grazing on phlorotannin production (Pavia et al. 1997, Pavia & Toth 2000a).

Cronin and Hay (1996) observed an increase in diterpenoid dictyols in the brown alga *Dictyota menstrualis* of natural grazing by the amphipod *Ampithoe longimana*. No previous study, however, had experimentally demonstrated effects on phlorotannin content of natural grazing prior to experiment 1 in **Paper I.** Recent studies have confirmed that *L. obtusata* can induce phlorotannin production in *Ascophyllum nodosum* (Pavia & Toth 2000a, Borell et al. 2004, Toth et al. 2005).

The aim of the first experiment in **Paper I** was to test effects of natural grazing on phlorotannin production, but also to investigate potential effects of other factors, with a possible impact of separate and/or interactive effects. Yates and Peckol (1993) and Peckol and co-workers (1996) tested the two factors; simulated grazing and nutrient availability, on phlorotannin production in *Fucus vesiculosus,* and argued that phlorotannin production is likely to be controlled by a complex interaction of environmental, developmental and defence-related factors. No study though, had examined a possible combined effect of three factors on phlorotannin content in fucoids, and experiment 1 in **Paper I** was the first to test effects of herbivores, nutrients and UVR on phlorotannin production. However, the only significant effect found on phlorotannin levels in *Ascophyllum nodosum* was of grazing by the herbivore, *Littorina obtusata*.

3.1.2. Light

Effects of light were examined at several levels. In the first experiment (**Paper I**), the light treatment consisted of both added and filtered UV-B radiation (UVBR), with a control of ambient irradiance. For the second experiment (**Paper I**) the treatment was to shield algae from UVBR and to compare with replicates receiving ambient UVBR. For all experiments in **Paper I,** phlorotannins in *Ascophyllum nodosum* were analyzed for algal tissue only. In **Paper II,** effects of ambient and filtered UVBR were tested on phlorotannin levels in the fucoid *Fucus vesiculosus*. These analyses were made for both tissue phlorotannins and, for all but experiment 1, also for exuded metabolites. For all experiments, the hypothesis was that increased levels of UVBR would induce phlorotannin production in algal thalli, and in addition that UVBR would induce exudation into the seawater.

The added UVBR treatment did not affect phlorotannin production in *A. nodosum*. Several factors could possibly explain the lack of response. The addition of UVBR might have been too insignificant, especially in relation to the relatively high natural irradiance prevailing at the time for the experiment (**Paper I**). In a study, where Pavia and co-workers (1997) observed an increase in phlorotannins after added exposure to UVBR, they had added more than twice the amount of UVBR and three times the length of exposure to UVBR, than the addition applied in experiment 1, in **Paper I**. Furthermore, Pavia and co-workers (1997) conducted their experiment in the spring, when the general levels of phlorotannins are low. The experiment in **Paper I** was set up after several weeks of summer sunlight, hence, the initial concentrations of phlorotannins were found to be higher for this study (7.0 % DW) than those measured by Pavia and co-workers (4.8 % DW). The initial levels of phlorotannins in **Paper I** might have been sufficient to build up an adequate protection against harmful radiation, with a lack of induction as a result. This is further supported by observations from the second experiment (**Paper I**), where the initial concentration of phlorotannins was lower (≈4.5% DW), and where an increase in phlorotannins was found as a response to ambient UVBR levels. However, the increase was only observed after 7 weeks of exposure to UVBR. The effect of time in this experiment, might in addition explain the lack of response in experiment 1, which was run for only 3 weeks (**Paper I**).

In **Paper II,** the effects of UVBR on phlorotannin levels in *Fucus vesiculosus* supported parts of the results from the experiment with *Ascophyllum nodosum*. In the first experiment (**Paper II**), ambient UVBR increased the amount of phlorotannins in the algal fronds. There was no effect of time, and the peak levels were here observed already after 2 weeks. The initial concentrations of phlorotannins for *F. vesiculosus* were higher than for *A. nodosum*. Earlier reasoning, where lower levels of phlorotannins more readily could have induced a response, is not fully in consistency with the results for *F. vesiculosus*. However, the response to UVBR might vary for the two species, depending on time and/or physiology (Pavia & Toth 2000b). Nevertheless, the combined results of the studies in **Papers I** and **II,** testing effects of UVBR, point towards UVBR being able to induce phlorotannin production.

There was an interaction effect of ambient UVBR and periodical submersion on phlorotannins in *Ascophyllum nodosum* (experiment 2, **Paper I**). The expected results were that effects of UVBR would be more pronounced at variable water levels, and that phlorotannin concentrations would increase at ambient UVBR and emersion. The results however, were the opposite. When UVBR was filtered out, phlorotannin levels in algal fronds were higher in the replicates with variable water levels. These results suggest that emersion and desiccation can have effects in combination with UVR, in affecting production of phlorotannins. Effects of emersion and desiccation are further discussed under paragraph 3.1.3.

3.1.3. Emersion and Desiccation

Variable water levels, with algae being emerged out of the seawater, had an effect on the algal tissue content of phlorotannins in *Fucus vesiculosus* (experiment 1, **Paper II**). Plants that had been exposed to emersion had significantly lower concentrations of phlorotannins, than those that were constantly submerged. This was not in accordance with the original hypothesis, where the predictions were an induction of phlorotannins, considering the extra stress on algal thallus from desiccation and elevated irradiance, at low water. Already in 1926, Pontillon observed a higher density of phenolic vesicles in *F. serratus*, emerged at low tide, suggesting an effect of desiccation, but he also found that the density was greater on the upper exposed side of the algal fronds (Pontillon 1926). However, he did not examine a potential relation between tissue concentrations and exudation of metabolites onto the surface of algal fronds during emersion. At present there are still limited data on the dynamics between exuded or surface located verses membrane-bound phlorotannins. Recently, Schoenwaelder (2002b) observed that a thin layer of increased amount of oxidized phenolic compounds, released from physodes, was formed in the fucoid *Hormosira banksii* after sun exposure.

If emersion can add to the stress of UVBR on algae, leading to a pronounced induction of phlorotannins, the metabolites might be translocated onto the surface of the thallus, where they would exert a protective screen. At re-immersion of the seaweeds, the compounds on the surface would subsequently be released into the seawater. In a previous study, Carlson and Carlson (1984) showed that natural levels of phenolic substances from *A. nodosum* would increase following immersion.

To study a possible relation between tissue concentrations and exuded phlorotannins, as response to emersion, further experiments in **Paper II,** in addition to analyses of tissue concentrations, also examined exuded metabolites. The results from experiment 2 and 3 (**Paper II**), testing effects of desiccation on exudation of phlorotannins, showed that previously desiccated algae exuded significantly more phlorotannins, when re-immersed, than non-desiccated algae. This observation was further confirmed in the settlement experiment with conditioned water (**Paper IV**), where desiccated plants of *F. vesiculosus* were found to exude significantly more phlorotannins than submerged algae. Furthermore, field samplings of seawater in the intertidal rockweed zone (**Paper IV**), showed higher levels of phlorotannins close to previously emerged algae, than for samples taken close to immersed algae. These results support the suggestion, that there is a re-location of metabolites to the surface of the algal thallus, possibly generating an added shield from the UV absorbing metabolites, against harmful radiation.

In order to evaluate an additional effect of UVBR on phlorotannin concentrations in desiccated algae, an experiment was conducted to test the effects of ambient UVBR on both phlorotannin tissue concentration and exudation in desiccated algae (experiment 4, **Paper II**). There was no significant effect of UVBR, although there was a tendency for lower levels of tissue concentrations, and an increase of exuded phlorotannins, for plants that had been exposed to ambient UVR. The results from the experiment with rinsing of algal fronds (**Paper II**), where very high levels of phlorotannins could be washed off the algal thallus directly after re-immersion, with a sharp decline in phlorotannin yield after only a few minutes, indicate that a large amount of phlorotannins were present at the surface of the emerged algae. If the bulk of the exudation occurs onto the algal thallus during emersion rather than after re-immersion, the exuded phlorotannins could give the alga an effective UV protective layer during periods of low water (cf Schoenwaelder 2002b).

A strategy to exude phlorotannins onto the algal surface at desiccation might, however, have additional consequences. Renaud and co-workers (1990) found that the normally unpalatable brown alga, *Padina* *gymnospora*, became susceptible to grazing, following desiccation, and that this was due to loss of chemical defence in the desiccated algae.

It is, however, difficult to distinguish effects of exposure to UVR from effects of desiccation. When algae are submerged, they are shielded from UVR in relation to the depth of the water covering them. Results from earlier studies on photosynthetic responses in macroalgae, have suggested that deeper growing algae are more sensitive to UV radiation (Karsten et al. 2001, Johansson & Snoeijs 2002). Nygård and Ekelund (2006), examining photosynthesis, observed that *Fucus vesiculosus,* growing in the northern Baltic Sea, where it only occurs in the sublittoral zone, had a lower tolerance to UVBR, than Atlantic populations. This was mainly attributed to a higher exposure to sunlight in the Atlantic and only partly due to differences in salinities.

The results from **Paper II** indicated that UVBR could induce higher concentrations of phlorotannins in algal tissues when plants are submerged. The results also demonstrated a tendency towards UVBR further increasing the exudation levels of phlorotannins observed during periods of emersion. Together with the observation of a tendency towards a decrease in tissue concentrations of phlorotannins of desiccation, enhanced by UVBR, the overall results suggest that the phlorotannin production could be influenced by increased exposure to UVBR, and that a UV-protective function of phlorotannins could be effective also when algae are emerged at low water levels (see also Ragan & Jensen 1979, Swanson & Druehl 2002).

3.2. Phlorotannins and fouling organisms

A dual role of phlorotannins related to both herbivores and fouling organisms, has been discussed in previous studies. Schmitt and co-workers (1995) raised the issue that secondary metabolites, by serving multiple functions, can deter both herbivores and fouling organisms. Marine tannins are, in addition to being inhibitors of grazing, also known to have antifouling properties, both for micro- and macroorganisms (Conover & Sieburth 1964,1966, Sieburth & Conover 1965, Lau & Qian 1997, 2000, Nagayama et al. 2003, Wikström & Pavia 2004).

The possible importance of phlorotannins reducing fouling has been investigated through correlative studies of algal tissue concentrations and the cover of fouling organisms. However, as pointed out by Jennings and Steinberg (1997), measurements of tissue concentrations of phlorotannins are probably inappropriate when evaluating antifouling activity, since fouling organisms will most likely react to chemical deterrents in the water near, or on the surface of algal fronds. An early observational field study performed at Tjärnö showed that there were no clear relationship between filamentous macroalgal epiphytes and phlorotannin concentrations in algal tissues of *Ascophyllum nodosum*.

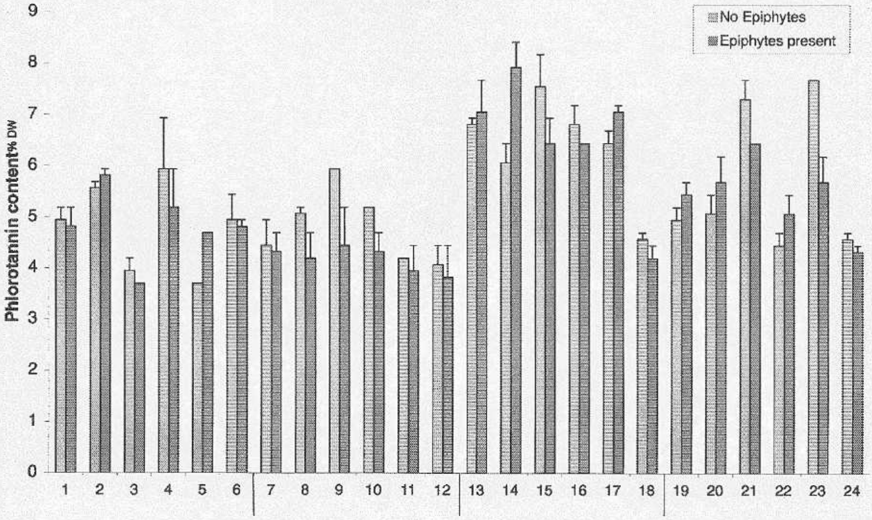

Fig. 3 Phlorotannin content in *Ascophyllum nodosum* individuals with and without macroalgal epiphytes. Data are means +SE (n=2)

Significant effects of epiphytes on phlorotannin concentrations were found for sites and individual algal plant, but there was no general effect of epiphytes on phlorotannin levels. Apical parts with epiphytes present had either higher or lower levels of phlorotannin than samples without epiphytes (Fig. 3). Lower levels of phlorotannins for plants with epiphytes could support a theory that epiphytes would prefer a habitat with less amounts of chemical deterrents. Higher levels, on the other hand, suggest that the foulers induce phlorotannin production in the host plants. However, the interpretation of the results is difficult, and epiphytic cover can in addition, have significant effects on phlorotannins through their participation in changes of light and nutrient resources. Jennings and Steinberg (1997) and de Nys and Steinberg (1999) also underlined the importance of testing appropriate concentrations of phlorotannins when searching for inhibitory effects. Hence, subsequent experiments in this thesis studying antifouling effects of phlorotannins have put greater emphasis on testing effects of exuded phlorotannins, and also strived to examine ecologically relevant concentrations of phlorotannins, when testing extracts from algae.

A field study of the natural abundance of adult *Balanus improvisus*, suggested that rock is preferred to algal thalli, as a habitat substrate

(Paper IV). The experiment testing settlement preference of the barnacle larvae, cyprids, on natural surfaces, proposed that barnacle abundance could, at least partly, be explained by a settlement process of the larvae and that this process was chemically mediated (experiment 1, **Paper IV**). Subsequent studies in this thesis examining effects of phlorotannins from fucoids have focused on testing these metabolites on larvae of *B. improvisus*.

In **Paper III** extracts from three species of fucoids: *Ascophyllum nodosum, Fucus vesiculosus and Halidrys siliquosa*, were tested for inhibitory effects on settlement by cyprids. Extracts from *F. vesiculosus* and *A. nodosum* were observed to inhibit settlement at a concentration of 0,1-1 μg ml^{-1}, whereas extracts from *Halidrys siliquosa* were less repellent, by 1-2 orders of magnitude. The results could be attributed, directly for *A. nodosum* and mostly for *F. vesiculosus,* to the activity of phlorotannins, since control tests were performed where phlorotannins had been removed from the algal extracts with pvpp.

The deterrent effect of phlorotannins tested in these experiments was exclusively quantitative and evaluated for total phenolics only. However, Geiselman and McConnell (1981) suggested that, differences in molecular structure and fractions of phlorotannins could differ in their deterrent effect, on *Littorina littorea*. Steinberg (1988) tested several fractions from different phenolic compounds for effects on two gastropods and one echinoid, and he proposed that qualitative differences among polyphenolics can have an impact on deterrence, but that quantitative variances seem to have higher importance. Boettcher and Targett (1993) observed that, the deterrent effect on the herbivore fish *Xiphister mucosus* of different molecular-sized phlorotannins from several brown algal species corresponded to the natural concentrations in the algae. Furthermore, van Altena and Steinberg (1992) tested a deterrent effect on grazers, using different molecular fractions of phlorotannins from fucoids and kelp. They concluded that the chemical differences could not adequately explain the differences in deterrence. To my knowledge, all previous research on qualitative differences in effects of phlorotannins have focused on their interaction with grazers, and the experiment in **Paper III**, is the first study testing a repellent effect on fouling of molecular size-fractions of phlorotannins. In agreement with Steinberg (1988), the main deterrent effect on cyprid settlement in the experiment in **Paper III** was quantitative, although some qualitative differences were also observed, especially for phlorotannins from *F. vesiculosus*.

The effects of desiccation on phlorotannins presented in this thesis, showing a significant increase of released metabolites in the seawater at the time of re-immersion, suggest that concentrations near the plants might naturally reach levels that could deter settlement by epiphytic organisms. In

Paper IV, a deterring impact of seawater with naturally exuded compounds from *Fucus vesiculosus* on settlement by larvae of *Balanus improvisus*, was examined. Water conditioned with desiccated algae contained 30 times the amount of phlorotannins compared to non-desiccated plants, and also 30 times the concentration previously shown to inhibit settlement by cyprids (0.1-1 µg ml^{-1}) (Lau & Qian 2000, Wikström & Pavia 2004). Mean concentrations in water with submerged plants was 0.85 µg ml^{-1} and although close to inhibiting levels, only seawater conditioned for 3 h exhibited a tendency towards a settlement inhibition (**Paper IV**). Furthermore, phlorotannin levels in most of the field samples were close to (for submerged algae), or just above (for recently immersed algae), the levels inhibiting settlement. Altogether, these results imply that phlorotannins, exuded and dissolved in seawater, may function as inhibiting compounds against fouling on intertidal fucoids.

4. CONCLUSION

Through a combination of manipulative experiments and field observations, the studies of this thesis have assessed natural patterns of variation in phlorotannin levels, both as algal tissue content and as exudates. The thesis has furthermore demonstrated that phlorotannins in fucoids can vary in response to both abiotic and biotic factors, and that the compounds also can affect organisms living on fucoids. The combined results from the four papers strengthen the notion that phlorotannins are ecologically important metabolites in the intertidal rocky shore community.

Biotic factors

Herbivores might exert a threatening force on the growth and survival of brown algae, such as fucoids. Phlorotannins have been reported to have an inhibitory effect on grazers such as the littorinids, and the results from this thesis, where the grazer *Littorina obtusata* was found to induce phlorotannin production, further support the hypothesis that phlorotannins have a possible role in herbivore deterrence. Fouling organisms may also constitute a significant threat to algal fitness, and the results of this thesis indicate that phlorotannins may serve a function also as inhibitors of fouling on intertidal fucoids.

Jennings and Steinberg (1994) stated that studies on effects of exuded phlorotannins lack in information on ecological relevant levels of phlorotannins. However, the results of studies in this thesis suggest that "spikes" of exuded phlorotannins, enough to deter foulers, can occur in intertidal algae, when they are re-immersed in seawater after periods of low water. The spikes could be correlated with incoming internal tidal bores, bringing larvae in shore (Shanks 1995, Pineda & Lopez 2002, Ladah et al. 2005). Although partly in contrast to the suggestions of Jennings & Steinberg (1997), that polar metabolites, such as phlorotannins, are unlikely to have defence functions against epiphytes, it is in accordance with their proposal that the deterrence factor of phlorotannins could be more important in intertidal communities.

Abiotic factors

The results from the studies in this thesis, further demonstrate that abiotic factors can have pronounced effects in determining levels of phlorotannins.

Protection against harmful radiation is important for organisms living in the intertidal zone, where there is risk of increased UVR, and studies suggest that UV absorbing polyphenolic metabolites are more abundant in high intertidal algae (Peckol et al. 1996, Van Alstyne et al. 1999, Connan et al. 2004). The fucoid species used for the studies in this thesis, are shallow living species on rocky shores, and they are often exposed to high irradiances. The increase in phlorotannin tissue concentrations in response to UVB radiation found in experiments in this thesis, further support the theory of an UV-protecting role of phlorotannins.

Furthermore, the algae used in this thesis are naturally subjected to periods of desiccation at low water levels, and the results of effects of emersion showed that algal tissue concentrations of phlorotannins decreased, and levels of exuded metabolites increased, after re-immersion in seawater. The indication of a translocation of metabolites onto the surface of algal fronds could be a response to added UV-stress from desiccation on emerged plants, already exposed to elevated UVR, and might function as an additional "sun-screen".

The suggestion of an interrelation between tissue content and exudation of phlorotannins in response to UVBR, has been considered in one study by Swanson and Druehl (2002). However, to my knowledge, there is no previous report of effects of desiccation and UVBR correlated to both tissue and exudate levels of phlorotannins in fucoids.

Furthermore, phlorotannins exuded in seawater could, irrespectively of its adaptive implication in terms of UV protection for the source alga, have important ecological effects in near shore waters, and be beneficial as well as detrimental to many organisms living in the algal belt.

In this thesis, many results indicate that phlorotannins have multiple functions. High levels of phlorotannins in algal tissues, might deter grazers, and also protect against harmful radiation. Desiccated algae might get additional protection from harmful radiation of phlorotannins on the algal surface, and exudation of metabolites into the seawater could deter foulers. One important aspect for further research is to try and determine the relative importance of phlorotannins as deterrent and/or protective agents.

5. FUTURE DIRECTIONS

There are two lines of direction I would like to follow in my prospective investigations. Both will have the aspect that exudation of phlorotannins is an ecologically important process for algae but also for other organisms in the intertidal community. I want to pursue the research on exudation from two perspectives.

Do exuded phlorotannins in intertidal fucoids have a protective function as a "sun-screen" for the producing algae?

Fucoids growing in the changing environment of intertidal shores on the Swedish west coast are adapted to withstand changes of physical conditions that sometimes are extreme. My studies indicate that phlorotannins in intertidal fucoids have a role in the ability of algae to sustain these conditions. I would like to continue to investigate the potential protection of phlorotannins in brown algae against harmful radiation. This will comprise research whereby I will study the ability of algal plants to transfer phlorotannins to areas of the thallus needing protection. It will also include further investigation of the relative roles of desiccation and UVR, and how these conditions affect, not just phlorotannin protection, but also the growth and fitness of the algae.

The brown alga, *Fucus spiralis, grows in the upper intertidal* zone, even more exposed to emersion than the species examined in this thesis. To investigate potential differences in phlorotannin production of these different species, as response to emersion and UVR, could possibly further broaden the knowledge of phlorotannins having a protective function.

As emphasised by Davison and Pearson (1996), it is important to employ both well-designed field studies and manipulative experiments, in order to relate experimental results to an ecological context. A next step forward would be to more rigorously monitor the natural variances in phlorotannin response to differences in UVR in both submerged and desiccated algae.

What ecological implications do exuded phlorotannins have in the intertidal zone?

Results from studies in this thesis indicate that levels of exuded phlorotannins can reach concentrations in seawater that may deter fouling organisms. This could be advantageous for the producing algae. However, exuded phlorotannins in seawater, absorbing UVR, could in addition protect many organisms living in close vicinity of intertidal brown algae. This process could potentially become more important in the intertidal community with increasing climatic changes. Ducrotoy (1999) pointed out that, because of their sensitivity to environmental changes, algal species ought to be considered as potential bio indicators. Monitoring of phlorotannins as protection against elevated UVR, might potentially be one parameter to include when trying to understand the impact of man-inflicted changes of the marine environment.

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