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# Lack of phlorotannin induction in the brown seaweed *Ascophyllum nodosum* in response to increased copper concentrations

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ABSTRACT: Heavy metal resistance in plants and algae may involve an internal detoxification mechanism, where toxic metal ions are chelated to substances that are stored in special compartments within the cells. Phlorotannins (brown algal polyphenolics) are strong chelators to heavy metals in solution and are found in special compartments (physodes) within the cells, and have therefore been suggested to function as an internal detoxification mechanism in brown seaweeds. Phlorotannins are also believed to function as a chemical defence against herbivory and UV radiation, and the production of phlorotannins has been shown to be induced by increased intensities of these factors. In the present study, we investigated the possible role of phlorotannins as an inducible and internal detoxification mechanism in the brown seaweed *Ascophyllum nodosum* (L.) Le Jol., in response to increased copper (Cu) concentrations in the surrounding water. The phlorotannin content, tissue Cu content and growth of Cu-exposed seaweeds from areas with different levels of contamination were compared in an observational field study and 2 manipulative induction experiments. The phlorotannin content and growth of the seaweeds were not affected by high Cu concentrations, although *A. nodosum* plants exposed to high Cu levels accumulated high concentrations of Cu in their tissues. The accumulated Cu was not specifically associated with phlorotannins, since only a small part of the total tissue Cu content of the seaweeds was found in the extracted phlorotannin fractions. The results of the present study show that phlorotannin production in *A. nodosum* is not induced by high Cu concentrations and that, although plants accumulate high levels of Cu in the tissues, binding of Cu to phlorotannins is probably not an important internal detoxification mechanism in *A. nodosum*. Since plants were not negatively affected by elevated Cu levels in terms of growth, substances other than phlorotannins are probably more important in the metal resistance mechanism of *A. nodosum*.

KEY WORDS: Phlorotannins · Induction · Heavy metal · Cu · Detoxification · *Ascophyllum*

## INTRODUCTION

Brown seaweeds contain a class of polyphenolic compounds called phlorotannins (Ragan & Glombitza 1986). These are acetate-malonate derived polymers of phloroglucinol (1,3,5-trihydroxybenzene) with a wide range of molecular sizes (400 to 400 000 Da). Phlorotannins can be present in high concentrations (>10% of dry weight) in brown seaweeds, where they are located in membrane-bound vesicles called physodes. The physodes are found in high numbers in cells of the epidermal cortex of the algae (Ragan & Glombitza 1986). A number of studies have found that phlorotannins can deter feeding by different herbivore species such as fishes, urchins, gastropods and amphipods, although other experiments have yielded mixed results and the deterrent properties of phlorotannins are far from universal (see Targett & Arnold 1998 for references). From these results, phlorotannins have primarily been regarded as defence chemicals against herbivory (Targett & Arnold 1998). However, since phlorotannins have antimicrobial properties, absorb light in the UV range and chelate heavy metal ions (see Ragan & Glombitza 1986 for references), protective and defensive functions other than defence against herbivory have been suggested, e.g. defence against bacterial and fungal infections (Sieburth & Conover

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1965) and fouling organisms (Sieburth & Conover 1965, Lau & Qian 1997, but see Jennings & Steinberg 1997) and protection against harmful UV-B radiation (Pavia et al. 1997) and toxic heavy metal ions (Lignell et al. 1982, Smith et al. 1986, Karez & Pereira 1995). Induction of increased phlorotannin levels has been observed as a response to natural herbivory (Pavia & Toth in press), mechanical simulations of herbivory (Van Alstyne 1988, Yates & Peckol 1993, Hammerstrom et al. 1998) and increased intensities of UV-B radiation (Pavia et al. 1997). Other investigations have failed to detect increases in the phlorotannin content of seaweeds in response to natural herbivory (Steinberg 1995) and simulated herbivory (Pfister 1992, Steinberg 1994, Pavia et al. 1997). To our knowledge, no previous experiments have been conducted concerning the effect of increased heavy metal concentrations on phlorotannin production in brown seaweeds.

In general, there have been few previous studies investigating the mechanisms of heavy metal resistance in seaweeds (Lobban & Harrison 1994). However, a large number of studies have revealed such mechanisms in phytoplankton and higher plants (Shaw 1990), and these mechanisms may also apply to seaweeds. Two different ways of achieving resistance to heavy metals, avoidance and tolerance, have been recognized (Shaw 1990). The avoidance strategies include exclusion mechanisms which limit the metal uptake into the cells, e.g. alteration of membrane permeability and production of extracellular or cell wall associated metal binding compounds. The tolerance strategy involves several internal detoxification mechanisms which aim at the binding of metals within the cells. The binding of metals results in a complex that can either be stored in compartments within the cell or excluded from the cell to the surrounding environment. Other tolerance strategies involve alterations of membrane structure and cellular metabolism, although the last mechanism is not supported by any evidence (Shaw 1990) and regarded as unlikely by the fact that a mechanism of this type would involve gross changes in the metabolism (Hall 1981).

On a global scale, the main part of the input of copper (Cu), as well as other metal species, to the marine environment is anthropogenic (Nriagu & Pacyna 1988), and the sources of contamination are often concentrated in estuaries and harbours. Cu reaches the marine environment through either rivers and freshwater run-off, atmospheric deposition (Nriagu & Pacyna 1988) or directly via Cu-based antifouling products (Claisse & Alzieu 1993). Cu is regarded as 1 of the most toxic trace metals to marine algae (Gledhill et al. 1997) and there have been several reports on the negative effects of Cu on seaweeds, e.g. induced loss of potassium from the cells (Hall 1981) and reduction of growth (Strömgren 1979). The toxic effects of Cu on algae are related to $Cu^{2+}$ activity in the water rather than to the total concentration of Cu (Sunda & Guillard 1976). Therefore, heavy metal resistance mechanisms in algae are likely to involve compounds, e.g. polyphenols, that chelate the free metal ions, either in the external medium or inside the cells, resulting in Cu complexes that are less toxic to the algae. Studies concerning the metal chelation of polyphenols originating from higher plants (e.g. chestnut and pine tannins) suggest that the metal ions bind to the *o*-dihydroxyphenyl- and carboxyl-groups of the tannin molecules (McDonald et al. 1996). Brown algal phlorotannins have free *vic*-triol groups at chain termini or at branch points which may chelate metal ions in a similar fashion (Ragan et al. 1979). Ragan et al. (1979) found that brown algal phlorotannins have a high capacity to chelate divalent metal ions (especially $Cu^{2+}$ and $Pb^{2+}$), implying that they may be important chelators to heavy metals in seawater and *in vivo* in the physodes of the seaweeds. Metal-sensitive phytoplankton species displayed an increased resistance to elevated zinc (Zn) concentrations when phlorotannins were present in the medium (Ragan et al. 1980), demonstrating the ability of phlorotannins to decrease the toxicity of metal ions in seawater Skipnes et al. (1975) found that the major part of the Zn ions accumulated in *Ascophyllum nodosum* (L.) Le Jol. was transported through 1 or more membranes to compartments within the cells. The metal ions were irreversibly bound to metal binding substances; the authors suggested that these substances could be polyphenols (phlorotannins) located in physodes. Furthermore, high concentrations of cadmium (Cd) and Cu have been found in the physodes of *Fucus vesiculosus* L. (Lignell et al. 1982, Smith et al. 1986) and in extracted phlorotannin fractions from *Padina gymnospora* (Kützing) (Karez & Pereira 1995), supporting the model that phlorotannins can function as an internal detoxification mechanism in these species.

In this study, we have focused on the model that phlorotannins in the brown seaweed *Ascophyllum nodosum* function as an internal and inducible detoxification mechanism through storage of Cu-phlorotannin complexes in the physodes. From this model we derived a number of hypotheses that were tested in an observational field study and in 2 manipulative induction experiments. *A. nodosum* plants from areas with different levels of contamination were used to find out if the seaweeds' responses to high Cu levels depend on previous exposure to contaminated water. The hypotheses tested in the present study were: (1) that seaweed plants exposed to high Cu levels in the surrounding water will have higher phlorotannin and tissue Cu content compared to unexposed plants, (2) that previously Cu-exposed plants will have higher

phlorotannin and tissue Cu content compared to previously unexposed plants also after transfer to clean water, and (3) that a significant part of the total seaweed Cu content will be associated with the phlorotannins. Furthermore, to investigate if the *A. nodosum* plants were negatively affected by the increased Cu concentrations, the growth of plants subjected to different Cu concentrations was compared.

## MATERIALS AND METHODS

**Study organism and sites.** *Ascophyllum nodosum* is a common brown seaweed found in the intertidal zone on sheltered rocky shores in the Northern Atlantic. In tidal areas it can form zones >100 m wide, but in atidal areas the zone is usually <1 m (Åberg & Pavia 1997). The seaweeds used in the present study were collected from atidal areas of the Swedish west coast. *A. nodosum* is attached to the substratum with a holdfast from which 1 to several primary shoots arise. It shows apical growth with lateral and dichotomous branching and new airbladders are formed at the tip of the shoots every spring, which allows for separation of the primary shoots of *A. nodosum* into age classes (Pedersen 1984). *A. nodosum* has a slow growth rate and a long lifespan. Primary shoots >20 yr old (i.e. with >20 bladders) are not uncommon (Toth & Pavia pers. obs.). In the present study, only the outermost tips of the seaweeds, i.e. the 1 yr old shoots, were used for chemical analyses.

The seaweeds were collected from different sites along the Swedish west coast, from Göteborg (57° 42' N, 11° 58' E) to the Norwegian border. The sites were chosen according to their different levels of Cu contamination. Harbours for pleasure boats were chosen as contaminated sites and clean reference sites were chosen at least 3 km from the nearest harbour. At the Swedish west coast, pleasure boats are mainly painted with Cu-based antifouling paints, which are allowed a total maximum leakage of 350 µg Cu $cm^{-2}$ during the first month after launching. The boats are launched within a restricted period of time (a few weeks) in the spring and the Cu contamination in pleasure-boat harbours can be substantial, especially in the weeks following initial launching (S.-Å. Wängberg, S. Alexandersson & M. Hellgren unpubl.).

**Observational study.** Tissue samples were collected in April 1998 from *Ascophyllum nodosum* plants growing in 2 clean, Långholmen (58° 54' N, 11° 07' E) and Tjurpannan (58° 43' N, 11° 10' E), and 2 contaminated, Smithska Udden (57° 38' N, 11° 53' E) and Skjærhalden (59° 01' N, 11° 03' E), sites. Ten undamaged annual shoots, without visible epiphytes or particulate material, were collected from each of 3 haphazardly chosen plants in each site. The seaweed samples were freeze dried and all shoots from each plant were homogenized and mixed before phlorotannin and tissue Cu content were determined according to the methods described below in the 'Chemical analyses' section.

**Induction experiments.** Two manipulative induction experiments were carried out, the first in March 1997 at Kristineberg Marine Research Station (58° 01' N, 11° 28' E) and the second in May 1998 at Tjärnö Marine Biological Laboratory (58° 54' N, 11° 07' E). In the 1997 induction experiment, 12 *Ascophyllum nodosum* plants of similar age and size were haphazardly collected from the intertidal zone near Kristineberg Marine Research Station. In the 1998 induction experiment, 15 *A. nodosum* plants were collected from each of 2 clean sites, Råssö (58° 51' N, 11° 10' E) and Koster (58° 55' N, 11° 00' E), and 2 contaminated sites, Strömstad harbour (58° 56' N, 11° 10' E) and Koster harbour (58° 53' N, 11° 01' E) in the Tjärnö archipelago. The holdfasts of the seaweeds were carefully detached from the substratum with a knife and the plants were transported to the laboratory in a cooling bag. At the laboratory, the plants were cleaned of all visible epiphytes and particulate material, washed several times in clean seawater and placed separately in acid-washed 9 l glass aquaria. The aquaria were placed outdoors in a basin with cooling seawater and the water in the aquaria was changed twice every day in order to avoid nutrient depletion. Natural, unaerated seawater with ambient temperature and salinity was used. The different Cu treatments, with final concentrations of 10 (low) and 50 (high) µg Cu $l^{-1}$ water, were added in the form of concentrated $CuCl_2$ (aq) at the time of water exchange. In the 1997 induction experiment, only the low Cu treatment was used. The water in the aquaria was stirred directly after Cu addition in order to mix the concentrated Cu solution with the seawater. The control aquaria received seawater without the addition of Cu in order to control for changes in the background levels of Cu. The wet weights (WW) of the seaweeds were determined using a standard blotting procedure at both the start and the end of the experiment and the growth of the plants was calculated as % of initial WW. In the 1997 induction experiment, tissue samples from the *A. nodosum* plants were taken on 6 occasions (Days 0, 1, 7, 13, 19 and 30) for phlorotannin and tissue Cu analyses. The 1998 induction experiment was terminated after 21 d when tissue samples for phlorotannin and Cu analyses were collected. The plants originating from a single clean site (Råssö) in the 1998 induction experiment were transferred to new acid-washed glass aquaria supplied with running seawater but without addition of Cu. After 11 d, tissue samples were collected from the plants. All seaweed samples from the induction experiments were freeze dried, and

analyzed for phlorotannin and tissue Cu content as described below in the 'Chemical analyses' section.

**Chemical analyses.** Phlorotannins were extracted from freeze-dried, homogenized seaweed material in aqueous acetone (60 vol%) in the dark at 4°C for 24 h. The acetone was evaporated *in vacuo* and the remaining water fraction was filtered in order to remove precipitated lipophilic material. The concentration of phlorotannins was determined using the Folin-Ciocalteus (F-C) method for quantification of total phenolic compounds (Van Alstyne 1995). Phloroglucinol (Merck art. 7069) was used as a standard. For the Cu analyses, freeze-dried tissue samples were digested in concentrated nitric acid (100 mg sample $ml^{-1}$ acid) under high temperature and pressure in a Microwave Solvent Extraction System (CEM MES 1000). The Cu concentrations in the digested samples were measured in a GBC GF 932/933 Atomic Absorption Spectrophotometer. Samples with concentrations <300 µg $l^{-1}$ were measured using a System 3000 Graphite Furnace and samples with concentrations >300 µg $l^{-1}$ with a 932/933 Flame AAS. In order to investigate if the Cu present in the tissues of *Ascophyllum nodosum* was associated with phlorotannins, the extracts were divided into 2 parts, and 1 part was washed 3 times with insoluble polyvinylpolypyrrolidon (PVPP, Sigma P-6755), which selectively removes phenolic compounds (Van Alstyne 1995). The crude, untreated extract (with phlorotannins) and the PVPP-treated extract (without phlorotannins) were freeze dried and the dry weight (DW) was determined. The dry extracts were dissolved in concentrated nitric acid (20 mg sample $ml^{-1}$ acid), digested and analysed for Cu content in the same way as the seaweed tissue samples described above. The Cu content of the extracts was calculated as µg $g^{-1}$ DW, both on the basis of the extract DW and of the phlorotannin DW, estimated from the F-C analysis. The removal of phlorotannins with PVPP and the following analyses and calculations were performed on extracts from *A. nodosum* plants in the observational study and on plants from a single clean site (Råssö) in the 1998 induction experiment.

**Statistical analyses.** Data on the phlorotannin and tissue Cu content in *Ascophyllum nodosum* plants from the observational study were analysed using nested analysis of variance (ANOVA) with Area (2 levels) as a fixed factor and Site (2 levels) as a random factor nested within Area. The phlorotannin and tissue Cu content in the plants from the 1997 induction experiment were analyzed using univariate analysis of variance with repeated measures (ANOVAR; Potvin et al. 1990) with Time (6 levels) as within subject factor and Treatment (2 levels) as between subject factor, since samples from different times were not independent. The growth of the seaweeds in the 1997 induction experiment was tested using a 1-way ANOVA with Treatment (2 levels) as a fixed factor. Data on phlorotannin and tissue Cu content and growth of *A. nodosum* plants from the 1998 induction experiment were analysed using mixed model ANOVA with Treatment (3 levels) and Area (2 levels) as fixed orthogonal factors and Site as a random factor nested within Area. Data on the phlorotannin and tissue Cu content of the seaweeds transferred to clean water were analysed using 1-way ANOVA with Treatment (3 levels) as a fixed factor. In order to establish if data on Cu content in extracts with and without phlorotannins from different areas and sites in the observational study and the different treatments in the induction experiment could be combined, the ratios between the Cu content in the extracts originating from the same seaweed sample were analysed using mixed model ANOVA with Area (2 levels) as a fixed factor and Site (2 levels) as a random factor nested within Area for the observational study, and using 1-way ANOVA with Treatment (3 levels) as a fixed factor for the induction experiment. When it had been established that the data could be combined, i.e. when no significant difference in Cu content ratios was found between areas or sites in the observational study or among treatments in the induction experiment ($p > 0.30$, see 'Results'), data on the Cu content for each extract (crude phlorotannin and PVPP treated) originating from the same seaweed sample, were paired and analysed with a paired-samples *t*-test.

When the factors or interaction between factors in the ANOVAs had p-values >0.30, a pooling procedure was applied where the mean-square for the factor was pooled with the residual mean-square (Underwood 1997). This was done with the nested factor Site for data on phlorotannin and tissue Cu content in the observational study and for data on the Cu content ratios between the extracts prepared from seaweeds in the observational study, with the interaction between the factor Treatment and nested factor Site for data on phlorotannin content and seaweed growth in the 1998 induction experiment, and with the interaction between Treatment and Area for data on phlorotannin content in the 1998 induction experiment. The pooled factors are not shown in the tables. Prior to all statistical analyses, data were tested for homogeneity of variances with Cochran's test and transformed when required (Underwood 1997). Multiple mean comparisons were made with the Student Newman Keuls (SNK) test (Underwood 1997).

## RESULTS

There was no significant difference in the phlorotannin content between *Ascophyllum nodosum* plants

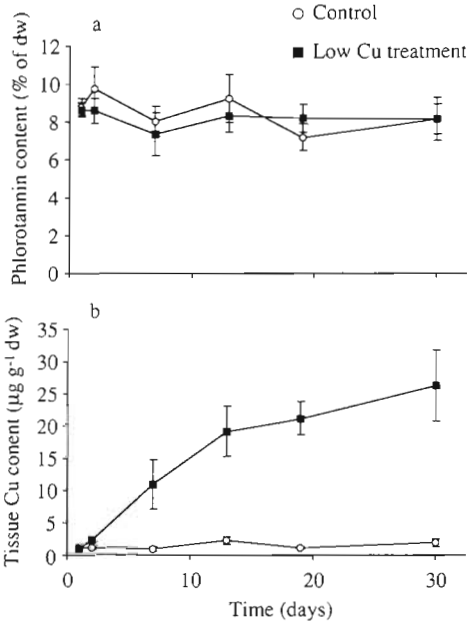


Fig. 1. 1997 induction experiment. (a) Phlorotannin content (% DW) and (b) tissue Cu content (µg $g^{-1}$ DW) of *Ascophyllum nodosum* plants exposed to ambient seawater without added Cu (Control) and with 10 µg Cu $l^{-1}$ added (Low Cu treatment). Samples were collected on 6 occasions: Days 1, 2, 7, 13, 19 and 30. Error bars show +95% confidence limits (n = 6)

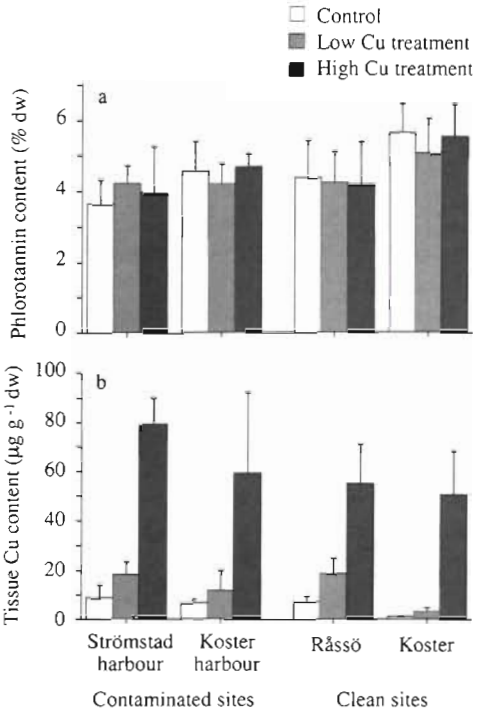


Fig. 2. 1998 induction experiment. (a) Phlorotannin content (% DW) and (b) tissue Cu content (µg $g^{-1}$ DW) of *Ascophyllum nodosum* plants from 2 contaminated sites (Strömstad harbour and Koster harbour) and 2 clean sites (Råssö and Koster) exposed to ambient seawater without added Cu (Control) and with 10 µg Cu $l^{-1}$ (Low Cu treatment) and 50 µg Cu $l^{-1}$ added (High Cu treatment). Samples were collected after 21 d. Error bars show +95% confidence limits (n = 5)

from clean and contaminated areas in the observational study (ANOVA, $F_{1,10} = 0.50$, p = 0.50). The mean phlorotannin contents were 3.5 and 3.2% DW for seaweeds from the clean and contaminated areas respectively. However, there was a significant and substantial difference in the tissue Cu content of the plants (ANOVA, $F_{1,10} = 12.7$, $p < 0.01$), with seaweeds from clean and contaminated areas having average tissue Cu contents of 8.0 and 18.4 µg $g^{-1}$ DW respectively.

Data on the phlorotannin content of *Ascophyllum nodosum* plants in the 1997 induction experiment were log-transformed prior to statistical analysis in order to obtain homogeneity of variances. There was no significant difference in the phlorotannin content between plants subjected to different Cu levels (ANOVAR, $F_{1,50} = 1.19$, p = 0.30, Fig. 1a). In the 1998 induction experiment, there was a significant difference in phlorotannin content between *A. nodosum* plants from different sites, but not between areas or Cu treatments (Table 1, Fig. 2a). Data on the tissue Cu content in seaweeds from both induction experiments were log-transformed prior to statistical analysis in order to obtain homogeneity of variances. In 1997, there was a significant interaction between the factors Time and Treatment (ANOVAR, $F_{5,50} = 64.9$, $p < 0.01$). The mean tissue Cu content in the control plants did not change, but the tissue Cu content in plants exposed to elevated Cu levels increased during the experiment (Fig. 1b). In 1998, seaweeds exposed to low and high Cu treatments had significantly higher tissue Cu content at the end of the experiment compared to control seaweeds from the same site. However, the relative difference in tissue Cu content among treatments varied between seaweeds from different sites, as indicated by the significant interaction between the factors Treatment and Site (Table 2, Fig. 2b). No significant differences in the growth of the seaweeds due to the Cu treatments were observed at the end of either the 1997 (ANOVA, $F_{1,10} =$

Table 1. *Ascophyllum nodosum*. ANOVA of phlorotannin content (% DW) in plants from clean and contaminated sites exposed to different Cu treatments in the 1998 induction experiment

| Source | df | MS | *F* | p | *F* vs |
|---|---|---|---|---|---|
| Treatment | 2 | 0.13 | 0.26 | 0.77 | Residual |
| Area | 1 | 6.08 | 1.00 | 0.42 | Site (Area) |
| Site (Area) | 2 | 6.10 | 12.01 | <0.01 | Residual |
| Residual | 54 | 0.51 | | | |

Table 2. *Ascophyllum nodosum*. ANOVA of tissue Cu content (µg $g^{-1}$ DW) in plants from clean and contaminated sites exposed to different Cu treatments in the 1998 induction experiment. Data were log-transformed prior to statistical analysis

| Source | df | MS | *F* | p | *F* vs |
|---|---|---|---|---|---|
| Treatment | 2 | 6.59 | 27.12 | <0.01 | Treatment × Site (Area) |
| Area | 1 | 0.88 | 0.74 | 0.48 | Site (Area) |
| Site (Area) | 2 | 1.20 | 47.24 | <0.01 | Residual |
| Treatment × Area | 2 | 0.10 | 0.42 | 0.68 | Treatment × Site (Area) |
| Treatment × Site (Area) | 4 | 0.24 | 9.58 | <0.01 | Residual |
| Residual | 48 | 0.03 | | | |

1.48, p = 0.25) or the 1998 (ANOVA, $F_{2,52}$ = 0.16, p = 0.86) induction experiments. The WW of the seaweeds increased by an average of 11% in 1997 and 12% in 1998.

There was no difference in phlorotannin content of the *Ascophyllum nodosum* plants previously exposed to different Cu treatments 11 d after transfer to aquaria with clean seawater (ANOVA, $F_{2,12}$ = 0.003, p = 0.99). The average phlorotannin content of the seaweeds was 3.8% DW for all treatments. There was still, however, a significant difference in the tissue Cu content of the seaweeds (ANOVA, $F_{2,12}$ = 56.5, p < 0.01). When mean values of the tissue Cu content of seaweeds from different Cu treatments were compared by SNK test, it was found that they were all significantly different. Seaweeds previously exposed to the high Cu treatment had the highest tissue Cu content (38.9 µg $g^{-1}$ DW), followed by seaweeds exposed to the low Cu treatment (17.2 µg $g^{-1}$ DW) and the control seaweeds (10.0 µg $g^{-1}$ DW).

For statistical analyses, data on the Cu content in the extracts prepared from *Ascophyllum nodosum* plants in the observational study and the 1998 induction experiment were calculated on basis of the extract DW and not on the phlorotannin DW. When the ratios between the Cu content in the crude phlorotannin and PVPP treated extracts were analysed, no significant difference was found between the areas or sites in the observational study (ANOVA, $F_{1,10}$ = 0.003, p = 0.95), or among the different Cu treatments in the 1998 induction experiment (ANOVA, $F_{2,12}$ = 0.47, p = 0.64). Therefore, samples from the observational study were combined before data on the Cu content in the extracts were analysed with a paired-samples *t*-test. The same procedure was applied to samples from the 1998 induction experiment. There was no significant difference in the Cu content between extracts prepared from seaweeds in the observational study (paired-samples *t*-test, *t* = 1.59, p = 0.14). The crude phlorotannin and PVPP treated extracts had average Cu contents of 22.5 and 16.1 µg $g^{-1}$ DW respectively. However, there was a significant difference between extracts prepared from seaweeds in the 1998 induction experiment (paired-samples *t*-test, *t* = 2.27, p = 0.04), with crude phlorotannin and PVPP treated extracts having average Cu contents of 18.6 and 9.7 µg $g^{-1}$ DW respectively. The Cu content in the crude phlorotannin extracts corresponds to only 5.8 and 3.2% of the total tissue Cu content of the seaweeds from the observational study and the induction experiment respectively. On the other hand, when the Cu content in the crude extracts was calculated on basis of the phlorotannin DW (cf. Karez & Pereira 1995), estimated from the F-C analysis, the Cu contents were on average 518 and 164 µg $g^{-1}$ DW respectively, which corresponds to 132 and 28% of the total tissue Cu content of the seaweeds.

## DISCUSSION

In the present study we have evaluated, for the first time, the possible role of phlorotannins as an inducible and internal detoxification mechanism against Cu in the brown seaweed *Ascophyllum nodosum*, using both observations and experimental manipulations. The results of the observational study and the induction experiments show that the phlorotannin production of *A. nodosum* is not affected by high Cu concentrations in the surrounding water. There were no differences in the phlorotannin content of seaweeds collected from clean and contaminated areas in the field, nor were there differences in the phlorotannin content of seaweeds subjected to exposure to high Cu concentrations in the aquaria. Furthermore, the results from the observational study and the induction experiments show that *A. nodosum* can accumulate high concentrations of Cu in the tissues. Cu seems to be irreversibly bound in the tissues, since the difference in tissue Cu content between seaweeds previously exposed to different Cu concentrations in the 1998 induction experiment persisted for 11 d after transfer to clean water. However, we found no support for the hypothesis that Cu was bound to phlorotannins in the physodes of *A. nodosum* to a large extent, although previous studies have found high concentrations of Cd and Cu in the physodes of *Fucus vesiculosus* (Lignell et al. 1982, Smith et al. 1986). To our knowledge, the only study attempting to estimate the metal content in extracted phlorotannin fractions of a brown seaweed was performed by Karez & Pereira (1995). They compared the Cu content of extracted phlorotannin fractions from the

brown seaweed *Padina gymnospora* growing in sites with different levels of metal contamination in Brazil. 100% of the Cu found in the tissues of seaweeds growing in the clean site were associated with the phlorotannin fractions. The results for seaweeds growing in the contaminated sites were more variable and ranged between 10 to 100% In the present study, we calculated the Cu content of the extracts on the basis of both the extract DW and the phlorotannin DW. When the extract DW was used, only 5.8 and 3.2% of the total tissue Cu content was associated with the extracted phlorotannin fractions in the observational study and the induction experiment. The corresponding figures when the phlorotannin DW was used were 132 and 28%. The calculations on the metal content of the phlorotannin fractions performed by Karez & Pereira (1995) were based on the total Cu content of the extracted fractions and the DW of the total polyphenols in the extracts, as estimated in the F-C analysis. The results of the present study show that such calculations can be misleading, since aqueous acetone may extract metal chelating compounds other than phlorotannins from the seaweeds. This may explain the high percentages of metals found in phlorotannin fractions of *P. gymnospora* noted by Karez & Pereira (1995), compared to the results in the present study.

There was no significant difference in Cu content between extracts with and without phlorotannins prepared from seaweeds in the observational study, but a significant difference was found between extracts prepared from seaweeds in the 1998 induction experiment. The results imply that phlorotannins chelated Cu ions, but whether the chelation of Cu to phlorotannins took place within the living cells of the seaweeds or in the solution of the extracted phlorotannins remains open for speculation. The plants exposed to the high Cu treatment in the induction experiment had high tissue Cu contents and it is possible that part of the accumulated Cu was reversibly bound in the intercellular space of the seaweeds. Skipnes et al. (1975) suggested that a small part of the Zn uptake in *Ascophyllum nodosum* was reversibly bound in the intercellular space of the seaweeds, and if this also applies to Cu, the reversibly-bound Cu may have chelated with the phlorotannins in solution during the process of phlorotannin extraction. Moreover, there is a possibility that the seaweeds exude phlorotannins with bound Cu to the surrounding water as a way of detoxifying the tissues (Ragan & Glombitza 1986). Exudation of polyphenolic substances has been thought in earlier studies to play an important ecological role (see Ragan & Glombitza 1986 for references, but see also Jennings & Steinberg 1994). To our knowledge, no studies have been carried out concerning the effect of increased heavy metal stress on the exudation rate of phlorotannins and its importance to the detoxifying ability of seaweeds. Our results imply that phlorotannin exudation, if it exists, is an insufficient mechanism for Cu detoxification in *A. nodosum*, since the Cu concentration in algal tissue increased when the seaweeds were exposed to elevated Cu levels in the surrounding water. On the other hand, the seaweed thalli appeared healthy through both experiments and the growth was not affected, suggesting that the elevated Cu concentration in the water (10 and 50 $\mu g\ l^{-1}$) was not directly harmful to the plants. This, and the fact that the plants accumulated high levels of Cu without producing higher levels of phlorotannins, indicates that there are other substances that are more important for the metal-resistance mechanisms in *A. nodosum*. Brown seaweeds contain cell wall associated polysaccharides like alginates (Haug 1961) and fucoidan (Paskins-Hurlburt et al. 1976), which have high affinities to different heavy metals, especially Cu and lead (Pb). Algae also contain phytochelatins, which are small peptides analogous to the metallothioneins found in animals (Gekeler et al. 1988). The structure of phytochelatins, $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ with $n = 2–11$, is different compared to the structure of metallothioneins, but they may have the same function of binding metal ions by mercaptide complexes (Grill et al. 1985). Gekeler et al. (1988) found the production of phytochelatins to be induced by high metal levels in all studied algal taxa, including the brown seaweed *Sargassum muticum* (Yendo) Fensholt, implying that they are important metal-sequestering compounds in algae. An interesting topic for future studies would be to find out if these substances are also present and inducible in other brown seaweeds, e.g. *A. nodosum* and other fucoids.

To our knowledge, this is the first study evaluating the possible induction of phlorotannin production in response to high Cu concentrations in a brown seaweed. The results show that, although the *Ascophyllum nodosum* plants accumulate high levels of Cu in the tissues, no induction of phlorotannin production in plants exposed to high Cu concentrations can be detected. Furthermore, binding of accumulated Cu to phlorotannins within the cells is probably not an important resistance mechanism in *A. nodosum*, since only a small part of the total tissue Cu content in the plants is associated with phlorotannins. The results suggest that other substances that bind metal ions, e.g. polysaccharides and/or phytochelatins, are more important for the Cu resistance in *A. nodosum*.

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