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S. Andersson · L. Kautsky

Copper effects on reproductive stages of Baltic Sea *Fucus vesiculosus*

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Abstract Copper is an active ingredient in many antifouling products, and pleasure boats are estimated to be the major single source of copper pollution in Swedish coastal waters. For this reason, the effects of copper were studied on egg volume, fertilization, germination and development of apical hairs of Baltic Sea *Fucus vesiculosus* L. Germination was the most sensitive stage and was studied at different concentrations of copper, different salinities and different ages of zygotes. Low concentrations of copper, 2.5 $\mu g\, Cu\, l^{-1}$, added to natural brackish water before fertilization, adversely affected germination at the ambient, suboptimal salinity of 6 ‰, suggesting that as little as a doubling of the copper levels in the studied area will severely affect the germination frequency of *F. vesiculosus*. The addition of 20 $\mu g\, Cu\, l^{-1}$ caused about 70 to 80% decline in germination at 6 ‰ S but also at 20 ‰ S which is higher than optimum. At a salinity close to optimum (14 ‰ S) no negative effect was noticed on germination when 20 $\mu g\, Cu\, l^{-1}$ was added. The results suggest that the degree of salinity stress acting upon the zygotes is a more important factor for the response to copper than the influence of salinity on metal availability. When 2.5 to 60 $\mu g\, Cu\, l^{-1}$ was added to the medium 24 h after fertilization, the zygotes were more resistant, resembling the response of adult marine fucoid tissue.

Introduction

In the larger part of the Baltic Sea, the bladderwrack, *Fucus vesiculosus*, usually dominates the total plant biomass and is a "key-species" of great importance to the hard bottom ecosystem (Kautsky 1989; Kautsky et al. 1992). Since there is no other perennial alga that can replace this beltforming seaweed in its ecological function, the disappearance of the bladderwrack would most likely result in long lasting and severe problems to the Baltic Sea. The *F. vesiculosus* belt offers a place for foraging and shelter for many organisms, including fish, and the plants also function as substratum for epiphytes. A decline in population size, and even disappearence at some localities, has been noticed during recent years, and chemical pollution is one of the presumed possible reasons (Kautsky et al. 1992; Andersson et al. 1992).

Intertidal Fucales were observed to be as sensitive to copper as they are to mercury, and both these metals were far more toxic than zinc, lead or cadmium (Strömgren 1980a). Therefore, although copper is not among the most critical metals in the Baltic Sea as identified by the anthropogenic loading factor (Lithner et al. 1990), it nevertheless seems to be a critical one to brown algae.

Russian, eastern Baltic and Polish rivers are major sources for transport of copper into the Baltic Proper (Lithner et al. 1990). Copper is used in antifouling products, that is to control growth on underwater surfaces, and the Swedish National Chemical Inspectorate (Debourg et al. 1993) has estimated that in Swedish inland and coastal waters pleasure boats are the major single source (about 30%) of the total copper burden. Newly painted pleasure boats are launched in large numbers just before or during the time for major gamete release of Baltic Sea *Fucus vesiculosus* in May–June (Andersson et al. 1994). Prior to the present study, nothing was known about how elevated copper concentrations may affect the reproductive processes of the species. In addition, there are reasons to believe that young zygotes are more sensitive to heavy metal pollution than adult plants. In a study of the localization of cadmium in *F. vesiculosus*, Lignell et al. (1982) found the highest metal concentrations in the physodes

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S. Andersson (✉)
Department of Systems Ecology, Stockholm University, S-106 91 Stockholm, Sweden

L. Kautsky
Department of Botany, Stockholm University, S-106 91 Stockholm, Sweden

and in the cell walls. The cell walls of brown algae contain alginate and fucoidan, polysaccharides that can bind cations. Alginic acid has a strong affinity to copper (see Lobban et al. 1985), and it can be expected that the effect of copper is more detrimental to zygotes which are exposed to metals before the development of a protecting cell wall that can adsorb some of the copper and thereby reduce the amount of metal taken up.

There are few studies on the effect of copper on brown seaweed reproduction. Chung and Brinkhuis (1986) report that 10 to 50 $\mu g\,Cu\,l^{-1}$, added to natural seawater, inhibits development of *Laminaria saccharina* (L.) Lamour gametophytes and young sporophytes. Settlement and germination of meiospores were not affected up to 500 $\mu g\,Cu\,l^{-1}$. As for adult seaweeds there are some reports concerning heavy metal effects on growth, but the use of different experimental conditions, exposure time and parameters in which the effects are measured must be taken into account, and it is therefore often difficult to compare results from different studies. However, Strömgren (1979, 1980a, b, c) studied the effect of various metals on the growth of intertidal Fucales. The metals were added to natural seawater during a period of 10 d. *Fucus vesiculosus* was found to show a significant reduction in growth rate when 50 $\mu g\,Cu\,l^{-1}$ was added to the background of 1 to 5 $\mu g\,Cu\,l^{-1}$. *Pelvetia canaliculata* (L.) Dec. et Thur., *F. spiralis* L., and *F. serratus* L. were more sensitive while *Ascophyllum nodosum* (L.) Le Jolis. showed a higher resistance capacity. When different metals are compared after 10 d exposure, a 50% reduction of growth rate occurs at 60 to 80 $\mu g\,Cu\,l^{-1}$, 100 to 200 $\mu g\,Hg\,l^{-1}$ and 5000 to 10 000 $\mu g\,Zn\,l^{-1}$, respectively. Cadmium and lead showed marked differences in toxicity between species. For example, after 10 d exposure to 810 $\mu g\,Pb\,l^{-1}$, the growth of *P. canaliculata* was significantly above the control. For *F. spiralis*, *F. serratus* and *A. nodosum*, a near 50% reduction was found at 2600 $\mu g\,Pb\,l^{-1}$. Cadmium showed a similar pattern with relatively low toxicity or, in some species, even enhanced growth.

Salinity in the archipelago of Stockholm is ≈6 ‰ during the reproduction season of *Fucus vesiculosus*. In earlier experiments (Andersson et al. 1992), a studied population of Baltic Sea *F. vesiculosus* was found to suffer from salinity stress at 6 ‰, resulting in reduced germination and a lower growth rate of young embryos. This salinity stress is likely to further increase the negative effects of additional stress factors acting upon reproductive processes. Copper release from pleasure boats coincides to a large extent with the release period of gametes, and thus the low salinity in the Baltic Sea is hypothesized to increase the effect of antifouling paints on fertilization and germination in *F. vesiculosus*. The aim of our study was to investigate the response to copper on different reproductive stages of Baltic Sea *F. vesiculosus* and also to study how different salinities affect this response.

Materials and methods

Ripe receptacles of *Fucus vesiculosus* L. were collected in the archipelago of Stockholm during the reproduction seasons of 1993 (18°23.5′; 59°8.4′) and 1994 (18°6.5′; 59°3.9′). This change of collection area was necessary since a period of extremely low water destroyed most of the plants at the locality used in 1993. The influence of year (and locality) on the results were analyzed using analysis of variance (ANOVA). As no significant influence was found, the results refer to both localities. Gamete release in Baltic Sea *F. vesiculosus* is controlled by a lunar related rhythm (Andersson et al. 1994). Therefore receptacles were collected before the expected gamete shedding, stored in moist condition at 4 °C and used within 4 d. The two gamete suspensions were filtered through 100 and 20 µm plankton nets, respectively, to separate oogonia and antheridia from free gametes. The diameters of 60 eggs were measured after 2 h exposure to different copper concentrations. The egg volumes were calculated according to the formula for a sphere and the result was analyzed using ANOVA and the non-parametric Kruskal–Wallis rank-sum test. In fertilization and germination experiments, spermatozoids were added to the eggs in excess with about 1 egg:700 spermatozoids. Germination of marine *F. vesiculosus* is reported to be "saturated" at ≈1:500 (Bolwell et al. 1977). Fertilization was determined by the development of a cell wall (see Andersson et al. 1992) and studied at different copper concentrations with and without exposure of the eggs before addition of spermatozoids. Zygotes derived from the fertile tissue were cultured at 14 °C under long-day conditions (17 light:7 dark cycle, 120 $\mu E\,m^{-2}\,s^{-1}$) in 5 cm glass petri dishes containing 10 ml of medium. The medium used was natural brackish water (6 ‰ S), and samples from 7 and 27 June 1994 were analyzed by atomic absorption spectrophotometry for background levels of copper by MILAB (Järfälla, Sweden). Increased salinities were obtained by addition of NaCl to natural brackish water, and copper was added as $CuSO_4$. To avoid density dependent differences in germination frequency and development rate, the egg density was held at about 3000 eggs per petri dish in each experiment. Except for the fertilization study, the experiments in the present study were performed in triplicate with a total number of 600 zygotes counted for each treatment. The results were analyzed using one- and two-factor ANOVA. Equal variances were tested using Cochran's test. In the fertilization study without pre-exposure of the eggs to copper, triplicates were used and the results were analyzed as above. The influence of copper on fertilization of pre-exposed eggs was studied in two different, not replicated, experiments. 400 eggs were counted for each treatment and experiment. The results were analyzed using Chi-square test. 95% confidence limit was calculated according to $t \times \sqrt{pq/n}$, t = 1.96. On the basis of the low germination frequency of eggs fertilized in the presence of copper, the effect of pre-exposure on fertilization frequency was not further investigated.

The effect of copper on germination was studied at different copper concentrations, different salinities and different ages of zygotes as follows: the effect on germination was studied at 6 ‰ S on zygotes derived from eggs fertilized in the presence of different copper concentrations and also on zygotes which were exposed to copper 24 h after fertilization. At 14 °C, 14 ‰ salinity was close to the germination optimum and 6 and 20 ‰ salinity reduced germination to about the same extent, to between 20 and 30%. Therefore, 6, 14 and 20 ‰ were used to study the effect of 20 $\mu g\,Cu\,l^{-1}$ added to these media, representing two different kinds of salinity stress and about optimum salinity conditions, respectively. Fertilization occurred in the respective media. Since the male receptacles were kept in ambient salinity (6 ‰), the addition of spermatozoids slightly lowered the final salinity to 13–14 and 19–20 ‰. Germination frequency was determined from the development of primary rhizoids, and the experiments reflect the influence on germination separately as only actually fertilized eggs were counted.

The development of apical hairs was studied at 6 ‰ S. These hairs are suggested to be involved in nutrient uptake (Hurd et al. 1993) and develop after about 14 d.

Results

The background concentration of total copper in the medium was found to be about 2 to 3 µg Cu l^{-1} (i.e. 1.7 ± 0.1, 7 June, and 3.2 ± 0.6, 27 June 1994).

No significant effect ($p = 0.7221$ Kruskal–Wallis rank-sum test, $p = 0.3137$ ANOVA) of the tested copper concentrations on egg volume ($n = 60$) was noticed after 2 h exposure. Mean egg volume ranged from 3.11×10^5 to 3.39×10^5 µm^3.

The influence of copper pollution on fertilization, measured as cell wall formation, seems to be dependent on the exposure time of the unfertilized eggs. When spermatozoids and copper were added at the same time to the egg suspension, fertilization ranged from 91.8 to 94.8% and no significant copper effect was observed. However, a 30 min copper exposure of the eggs before the spermatozoids were added, resulted in a significantly lowered fertilization frequency in both experiments with the addition of 10 µg Cu l^{-1} medium. A near 50% reduction was noticed when 10 and 20 µg Cu l^{-1} were added, respectively (Fig. 1).

Although fertilization seems to be unaffected when the unfertilized eggs are not previously exposed to the tested copper concentrations, most of the resulting zygotes will never germinate when cultured in these media. At 6 ‰ S, the addition of as little as 2.5 µg Cu l^{-1} medium, which was the lowest concentration tested, raising the total copper level by about a factor of 2, caused about 40% decline in germination frequency and when 20 µg Cu l^{-1} was added about 70 to 80% of the zygotes died (Fig. 2). Thus, among the tested young developmental stages, germination was the most copper sensitive. Eggs exposed to copper during fertilization showed a higher sensitivity than eggs which had developed a cell wall before copper was added to the medium. When zygotes were exposed after 24 h, addition of 20 µg Cu l^{-1} did not affect germination significantly. A near 50% decline in germination was noticed when 60 µg Cu l^{-1} was added (Fig. 3) which was the highest concentration tested. This is in agreement with results for adult tissue of marine fucoid plants (Strömgren 1980a). Earlier experiments at 11 °C suggested that 1-d-old zygotes are slightly more sensitive to copper pollution at lower temperatures, with a 50% decline in germination at 20 to 40 µg l^{-1} medium (Andersson unpublished data).

In the combined copper salinity experiment, a two-factor ANOVA showed a significant effect of both copper (***) and salinity (***), and also significant interaction between these factors (***). When fertilization occurred in media of different salinities, germination declined relative to 14 ‰ salinity in both the ambient salinity of 6 ‰ and in 20 ‰ salinity, that is, when zygotes were exposed to sub- and supraoptimal salinity conditions, respectively. Also in 6 and 20 ‰ salinity, addition of 20 µg Cu l^{-1} caused about 70 to 80% decline in germination. In 14 ‰ S, there was no effect on germination when 20 µg Cu l^{-1} was added (Fig. 4).

Apical hair frequency after 16 d was not significantly affected by copper pollution up to 20 µg l^{-1} medium under the used conditions. Mean percentage germlings with apical hairs ranged from 62.2 to 69.2.

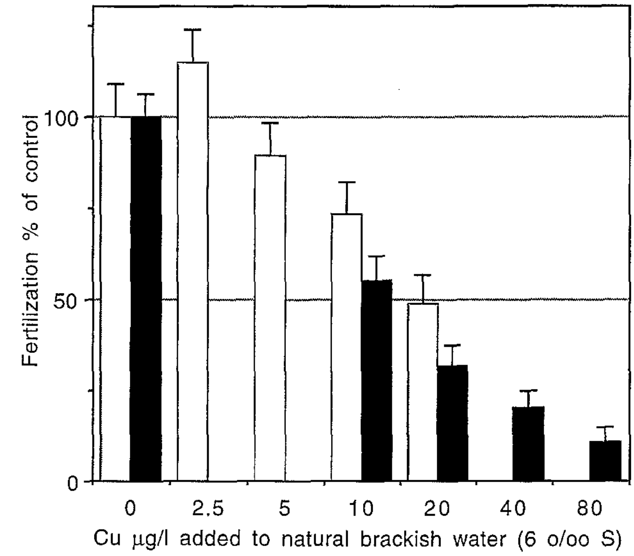

Fig. 1 *Fucus vesiculosus.* Fertilization (% of control) of eggs at different copper concentrations, 6 ‰ S. Eggs were pre-exposed to the different copper concentrations for 30 min in two different experiments. ■ Experiment 1: 0, 10, 20, 40 and 80 µg Cu l^{-1} added to natural brackish water. □ Experiment 2: 0, 2.5, 5, 10 and 20 µg Cu l^{-1} added to natural brackish water. 400 eggs were counted for each treatment and experiment. *Error bars* show 95% confidence limit

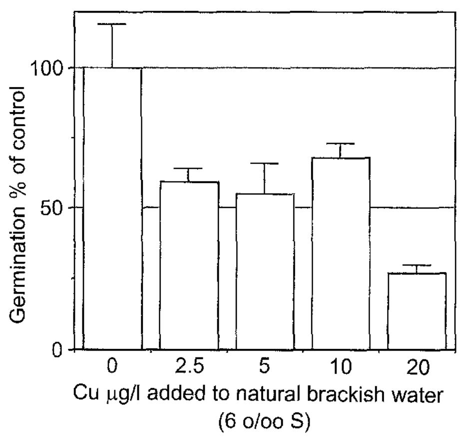

Fig. 2 *Fucus vesiculosus.* Germination of eggs (% of control), fertilized and cultured at five different copper concentrations in natural brackish water, 6 ‰ S. 600 eggs (200 for each replicate) were counted for each treatment. *Error bars* show standard error

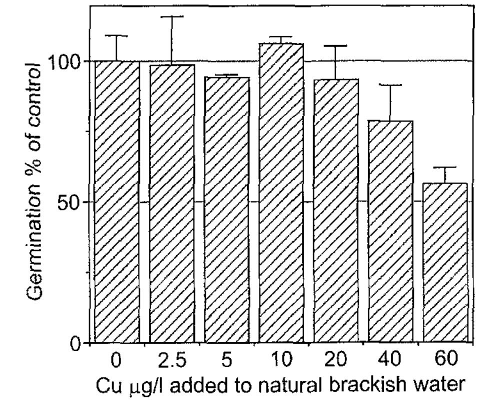

Fig. 3 *Fucus vesiculosus.* Germination of eggs (% of control), fertilized in natural brackish water (6 ‰ S) without copper addition, and exposed to seven different copper concentrations 24 h after fertilization. Only actually fertilized eggs were observed for determination of germination frequency. 600 eggs (200 for each replicate) were counted for each treatment. *Error bars* show standard error

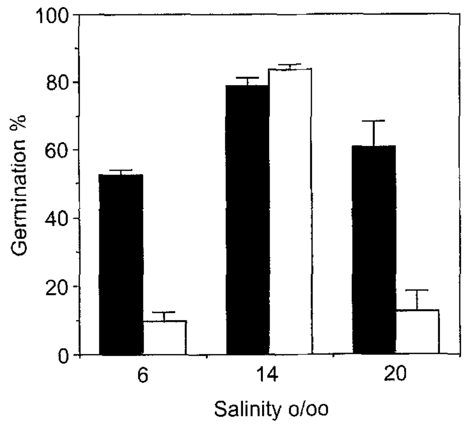

Fig. 4 *Fucus vesiculosus.* Germination of eggs (%), fertilized and cultured in media of different salinities (6, 14 and 20 ‰), with □ and without ■ addition of 20 µg Cu l^{-1}, respectively. Only actually fertilized eggs were observed for determination of germination frequency. 600 eggs (200 for each replicate) were counted for each treatment. *Error bars* show standard error

Discussion

In this work the most copper sensitive of the tested reproductive stages of *Fucus vesiculosus* was germination. The results indicate that as little as a doubling of the copper levels in the studied area (6 ‰ S) will severely affect the germination frequency of *F. vesiculosus.* Since the anthropogenic loading of heavy metals is high in the archipelago of Stockholm, this could have resulted in a change in genotypes in favour of more resistant plants, and it is possible that plants from more unpolluted areas would have shown an even higher sensitivity to copper than exhibited by the plants in this study. Also, the control plants were incubated in natural brackish water with somewhat elevated copper levels, and the effect of additional copper is compared to the effect in this control medium. Already the lowest concentration tested, i.e. 2.5 µg Cu l^{-1} added before fertilization, resulted in about 40% decrease of germination as compared to the control, and addition of 20 µg Cu l^{-1} caused 70 to 80% decline. From this result we cannot exclude the possibility that copper concentrations, in the archipelago of Stockholm, have already reached levels that affect the reproduction of *F. vesiculosus* negatively. It should also be kept in mind that, in addition to the early lethal copper effects on zygotes, there is likely to be sublethal or even lethal long term responses that might affect the plants and the population significantly. Among the possible ecological effects of copper pollution are not only a change in the genetic diversity but also a decrease in *F. vesiculosus* primary production as a result of lower recruitment and growth.

To our knowledge, there are no reports on the physiological effects of copper on seaweeds while the effects on phytoplankton have been reviewed by Sorentino (1979). Copper(II) ions cause a loss of K^+, and changes in cell volume, a response that was not detected in our study on *Fucus vesiculosus* eggs. When transported to the chloroplasts Cu^{2+} inhibits electron transport to $NADP^+$. As reviewed by Chung and Brinkhuis (1986) copper is believed to affect the iron transportation into plastids or the incorporation of iron into iron-binding enzymes and may also be responsible for peroxidative degradation of chloroplast membrane lipids. Shioi et al. (1978) suggest that copper acts by direct inactivation of ferredoxin and also by blocking a site between the reaction center of Photosystem II (PS II) and the water splitting system. Pandey et al. (1992) report a copper induced inhibition of $^{14}CO_2$-fixation and PS II activity in the cyanobacterium *Nostoc calcicola* Bréb., and a decline in the activity of PS I and ATP content was also observed.

When 1-d-old zygotes were exposed to copper, no significant effect was noticed on germination frequency at 20 µg Cu l^{-1}. Although much of the protein synthesis in *Fucus* spp. during the first hours after fertilization seems to rely on maternal mRNA stored in the eggs, zygotic transcription as well as translation are necessary for germination and division (Kropf et al. 1989). Inactivation of ferredoxin is not only likely to affect photosynthesis but also the process of nitrate reduction since it provides electrons used to reduce NO_2^- to NH_4^+ in the chloroplasts (Salisbury and Ross 1985). Using 5 to 50 µg Cu l^{-1}, Harrison et al. (1977) found that copper inhibits nitrate uptake and synthesis of nitrate reductase in phytoplankton. However, the degree of copper tolerance was related to ambient copper concentrations. Assuming a similar action in seaweeds, copper may affect one or several steps necessary for protein synthesis, thereby inhibiting germination. In marine *Fucus* spp. proteins required for germination are synthesized during the first 11 to 13 h after fertilization (see Kropf et al. 1989). Baltic Sea *Fucus vesiculosus* have a slow pronuclear migration rate (Serrão et al. 1994) and germinate more slowly than marine *Fucus vesiculosus* (Andersson personal observation) which could mean that a longer time is also required for protein synthesis. However, the necessary proteins are likely to be present after 24 h. This could, in addition to the presence of cell walls, be a reason for the lower sensitivity of the older zygotes.

When 20 $\mu g\,Cu\,l^{-1}$ was added to media of different salinities, again, at 6 ‰ S, germination decreased by about 70 to 80%. This was the result at 20 ‰ S as well, while at 14 ‰ S no negative effect of copper was noticed. In contrast to what was found at 11 °C (Andersson et al. 1992), 14 ‰ S was close to optimum for germination under the used conditions (14 °C), and the results suggest that the most important factor for the response to copper stress is the pre-existing stress level of the zygotes rather than the influence of salinity on metal availability. This is supported by results concerning the influence of temperature on copper toxicity on the brown alga *Undaria pinnatifida* (Harv.) Suringar (Laminariales), where Lee et al. (1989) found maximum germination of *U. pinnatifida* meiospores at 10 °C. When the effect of copper was studied under a temperature–irradiance gradient, meiospore germination was affected at all tested temperatures except for 10 °C.

The physiological reason for the higher copper tolerance of Baltic Sea *Fucus vesiculosus* at 14 ‰ S is not known. In marine *F. vesiculosus* eggs, Brawley and Bell (1987) noticed initiation of cell wall formation about 10 min after addition of sperms. This has been observed to take longer in *F. vesiculosus* from the Baltic Sea (Serrão unpublished). It is likely that the lower developmental rate of the Baltic Sea *F. vesiculosus*, including the delay in the secretion of metal binding polysaccharides during cell wall formation, is caused by unfavourable salinity conditions. If so, a relatively lower amount of copper can be expected to be taken up at optimum salinity, leading to a less detrimental effect on enzymatic activity.

Although the loading factor and sediment content of several metals is higher in the Baltic Proper, measurements of, for example, cadmium levels in biota showed higher values in Bothnian Sea organisms (see Lithner et al. 1990). The lower metal content in the Baltic Proper biota might to some extent be caused by the higher salinity but also by a "dilution" effect due to the higher productivity in the Baltic Sea. Eutrophication is one of the most significant large scale problems in the Baltic Sea today and is thought to be the reason for the declining vertical distribution of the Baltic *Fucus vesiculosus* population that has been noticed since the 1940s (Kautsky et al. 1986; Kautsky et al. 1992). Thus, an improvement of the eutrophication situation in the Baltic Sea should be combined with a decreased anthropogenic metal loading. In our study, the copper concentration in the water was elevated by about 1 $\mu g\,Cu\,l^{-1}$ from 7 to 27 June, that is, within the critical time for *F. vesiculosus* reproduction. The elevated copper concentration is most likely due to leakage from pleasure boats treated with antifouling products and might influence the reproduction of *F. vesiculosus* negatively.

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