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Influence of combined changes in salinity and copper modulation on growth and copper uptake in the tropical green macroalga *Ulva reticulata*

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ABSTRACT

The influence of salinity on growth and Cu uptake in the green macroalga *Ulva reticulata* collected from the intertidal area in the Western Indian Ocean was studied under controlled laboratory conditions. Exposure concentrations ranged from 5 to 500 μg Cu l^{-1} at five salinities (ranging 20–40). The accumulation of Cu increased with decreasing salinity, so that the uptake at 500 μg Cu l^{-1} was approximately 2.7, 2.4 and 2.0 times higher at salinities of 20, 25, and 30 respectively, than uptake at salinity of 35, and with uptake being lowest at salinity of 40. *Ulva* maintained a positive growth rate over the whole salinity range (20–40), with highest rates at salinity of 35. When exposing to Cu at low salinities (20 and 25), the growth rate of *Ulva* was strongly inhibited suggesting an increase in toxicity of Cu with decreasing salinity. EC50 and NOEC increased with increase in salinity, implying a reduced Cu toxicity at high salinities. It was concluded that salinity needs to be considered when using macroalgae, such as *U. reticulata*, as a bioindicator of heavy metals in areas with heavy rainfall, underground fresh water intrusion or in estuaries, as they might accumulate more metals and be more negatively affected.

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1. Introduction

Seawater has normally a rather constant salinity of approximately 35. However, in many areas the salinity varies considerably due to dilution, for example, in areas with a high amount of freshwater intrusion, such as estuaries, areas close to sewage outfalls, and river mouths, particularly after heavy rainfalls. In shallow areas with high evaporation, hypersalinity can occur. Many such areas of variable salinity sometimes harbor rich flora and fauna (Degobbis et al., 1986; Ravera et al., 1995; Favero et al., 1996); in addition, most such areas are known to receive significant amounts of land-based anthropogenic inputs (Eklund and Kautsky, 2003; Mremi and Machiwa, 2003).

Macroalgae are known for their ability to accumulate heavy metals in their cells, and many species, especially various brown algae, are widely used as bioindicators (Bryan, 1983; Haritonidis and Malea, 1995; Malea et al., 1995; Misheer et al., 2006). Some species of the brown algal genus *Padina* are commonly used as bioindicators in warmer waters (Burdon-Jones et al., 1982; Karez et al., 1996), and have also been used in the Western Indian Ocean region (Ferletta et al., 1996; Engdahl et al., 1998; Dulymamode et al., 2001). However, *Padina* species are restricted to a narrow range of salinity fluctuations, and do not grow well in low salinity waters or in areas with unstable substratum (Subbaraju et al., 1982; Einav et al., 1995).

Like *Padina*, many seaweeds are unable to withstand large fluctuations in salinity (Leet, 1998), but several species of the genus *Ulva* have been found to tolerate, and even flourish, in such environments (Kakinuma et al., 2006) and in the presence of high pollutant levels (Degobbis et al., 1986; Ravera et al., 1995; Ho, 1990). Furthermore, *Ulva* species have often been reported to be suitable bioindicators of heavy metal pollution in such waters (Ho, 1990; Haritonidis and Malea, 1999; Wang and Dei, 1999; Villares et al., 2001). The accumulation of metals in the algal thalli depends on the availability of dissolved free heavy metal ions in the surrounding water, which in turn depends on factors such as the amount of suspended organic matter, pH, dissolved organic carbon, and dissolved natural ligands, salinity, and water temperature (Luoma, 1983; Ansari et al., 2004). Salinity both directly and indirectly influences the heavy metal uptake of many bioindicators. Most studies of how salinity influences heavy metal uptake have been performed on marine fauna, such as crabs, fish, and bivalves (Wright, 1977; Bjerregaard and Depledge, 1994; Fischer, 1986).

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Previous studies have reported that changes in salinity directly affect metal accumulation in algae (e.g. Wang and Dei, 1999; Rijstenbil et al., 1998; Seferlis and Haritonidis, 1995; Munda, 1984). It appears that even though different macroalgal species might respond differently to salinity in terms of accumulating various heavy metals, the accumulation in *Ulva* is normally negatively affected by increased salinity (Wang and Dei, 1999). However, since both salinity and heavy metal accumulation also affect the growth rate of seaweeds (e.g. Amado Filho et al., 1997; Mamboya et al., 2007), which probably in turn affects the accumulation (Wang and Dei, 1999), it is difficult to predict how these parameters induce changes in uptake patterns were acting together. Therefore, this work aims to contribute to the understanding of the tolerance of *Ulva reticulata* at various salinity levels and at various concentrations of copper. In addition, this study contributes information on the influence of diluting seawater which causes salinity and nutrient concentration changes, on copper uptake and toxicity in *U. reticulata*.

2. Materials and methods

2.1. Macroalgal collection

Healthy submerged thalli of *Ulva reticulata* were collected from the intertidal area during spring low tide in Oyster Bay, Dar es Salaam, Tanzania. Oyster Bay is located away from the major pollution sources of Dar es Salaam and consequently the background levels of copper in *Ulva* thalli was below 10 $\mu g\, g^{-1}$ dwt. The salinity of the area is 35. The algae were washed thoroughly with seawater and packed in plastic containers for transport to Stockholm University; the samples were left to acclimate in a temperature-controlled culture room (25 °C, 90 μmol photons $m^{-2}\, s^{-1}$) for at least 2 weeks before starting the experiments under natural seawater collected from the West coast of Sweden, with salinity of 33.

2.2. Experimental set-up

Natural seawater was diluted with distilled water, to mimic rainwater dilution, so that the desired salinity levels were achieved. To prepare a salinity of 40, NaCl (Merck, Darmstadt, Germany) was added. Before starting the experiments, the algal thalli were left to acclimate to the different salinities for 24 h prior to exposure to different concentrations of Cu, added as $CuCl_2$ (Sigma Aldrich, Steinhelm, Germany). The experimental design employed was simple factorial design also termed as 'two-factor-factorial design' where salinity and copper concentrations were the two variables involved. *Ulva* thalli were cultured at different concentrations of Cu with a control (5, 50, and 500 plus a control of 0 $\mu g\, l^{-1}$) and at different salinities (20, 25, 30, 35 and 40). Six replicates were made of controls and treatments for each experiment, and exposure duration was 7 days.

All experimental containers were covered with transparent plastic film to avoid evaporation, which might change the salinity and also to avoid foreign particles. Cultures were aerated and temperature maintained at 25 °C with 12:12 h of light and dark. Light intensity was approximately 90 μmol photons $m^{-2}\, s^{-1}$, while algal stocking density was 5 g l^{-1} fresh weight. After 7 days of experimental treatment, algal wet weights were measured; then the algae were washed with distilled water and left to dry at 60 °C in an oven for 3 days in preparation for Cu analysis.

2.3. Determination of growth rate

The daily growth rate (DGR) of *Ulva reticulata* was estimated according to Lignell et al. (1987) and Haglund et al. (1996) as per cent growth per day using the following formula:

$$\mathrm{DGR} = \left[(Wt/Wo)^{1/t} - 1\right] \times 100,$$

where DGR represents daily growth rate in per cent, *Wt* the fresh weight at time *t*, *Wo* the initial fresh weight, and *t* the time in days.

2.4. Determination of EC50 and NOEC values

In this study, EC50 was defined as the concentration of Cu that caused a decrease in growth to 50% of that of the control, while NOEC was the maximum exposure concentration of Cu at which no effect was observed on the growth. A dose-response diagram was plotted using growth rates as per cent of the controls' growth rates vs. logarithm to base ten of the Cu exposure concentrations; a regression line was fitted.

2.5. Analysis of Cu concentration in the algal thalli

Each dried macroalgae sample was ground in a porcelain mortar with a pestle to a fine homogenous powder. Approximately 0.1 g of the powder was put into a digesting glass test tube and 15 ml of 7:3 (v/v) hydrochloric acid (HCl): perchloric acid ($HClO_4$) were added; the mixture was subjected to wet digestion using a hot plate at different ramping temperatures were maximum temperature used was 225 °C for 21 h. After digestion, each extract was brought to 10 ml volume using deionized water. Copper analysis was then performed using a Spectra AA-100 atomic absorption Spectrophotometer (Varian, Springvalve, Australia) using a standard addition method (Harris, 2003). Analytical AAS grade of Cu standard solution was used as an internal standard by using 'the standard addition method', the r^2 value was greater than 0.99 but less than 1. For each sample analyzed, Cu standard was used as internal standard to check the recovery and to avoid the matrix effect.

3. Results

Both salinity and Cu exposure concentration were found to have significant influence on the Cu uptake pattern (two-way ANOVA, $p < 0.05$). The highest accumulation of Cu occurred at the lowest salinity, and as salinity increased the uptake decreased (Fig. 1). The

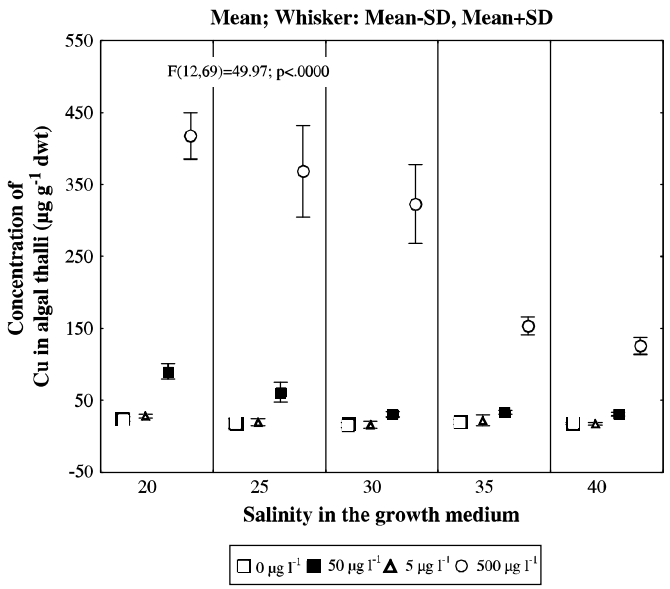


Fig. 1. Accumulation of Cu by *Ulva reticulata* at different salinities and exposed to different concentrations of Cu ($n = 6$, mean ± standard deviation).

results of multiple comparison tests (Tukey's post hoc HSD test) of the effect of Cu exposure concentrations on Cu uptake indicated that at each salinity level tested, Cu uptake was significantly changed at each exposure concentration (Tukey's post hoc HSD test, $p < 0.05$). No significant differences in Cu uptake were observed between controls and 5 μg Cu l^{-1} exposure at any tested salinity (Tukey's post hoc HSD test, $p > 0.05$). *T*-testing was used to compare levels of Cu accumulation at different salinities, to detect significant differences ($p < 0.05$); however, there were no significant differences ($p > 0.05$) in Cu accumulation patterns between salinities of 30 and 35 or between 30 and 40.

3.1. Effect of salinity on DGR without Cu addition

The growth rate was highest at a salinity of 35 and lowest at 20 and 40 (Fig. 2). The results of multiple comparison testing (Tukey's post hoc HSD test) of the effects of different salinities on the DGR of *Ulva reticulata* showed that significant differences in DGR were only observed between salinities of 20 and 35, 25 and 35, 40 and 35, and 40 and 30.

3.2. Effect of Cu on DGR at different salinities

Generally, exposure of *Ulva reticulata* to Cu caused a significant inhibition of DGR (ANOVA, $p < 0.05$, Fig. 3) at different salinities (0, 25, 30, 35 and 40). The inhibition was highest when salinity was low and in high Cu concentration. The high DGR inhibition occurred at salinities of 20 and 25, while less inhibition was observed at salinities 30 and 35. At all salinities tested, an exposure to 5 μg l^{-1} of Cu had no significant effect on the DGR (Tukey's post hoc HSD test; $p > 0.05$).

Furthermore, multiple comparison tests revealed no significant differences (Tukey's post hoc HSD test; $p > 0.05$) in DGR between exposure concentrations of 50 and 500 μg Cu l^{-1} at 25, 5 and 50 μg Cu l^{-1} at 30 salinity level, or 5 and 50 μg Cu l^{-1} at 35 salinity. Exposure of *Ulva reticulata* to Cu at a salinity of 40 caused a significant inhibition of growth only at a concentration of 500 μg l^{-1}.

The DGR of *Ulva* exposed to different concentrations of Cu at different salinities did differ significantly (*T*-test, $p < 0.05$), except between salinities of 20 and 25, 20 and 40, and 25 and 40. DGR was significantly negatively correlated with concentration of Cu

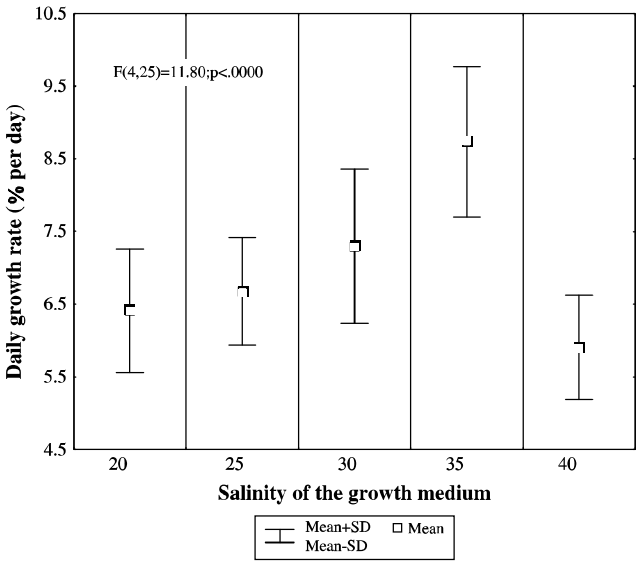


Fig. 2. Daily growth rate of *Ulva reticulata* at different salinities without Cu addition for 7 days ($n = 6$, mean ± standard deviation).

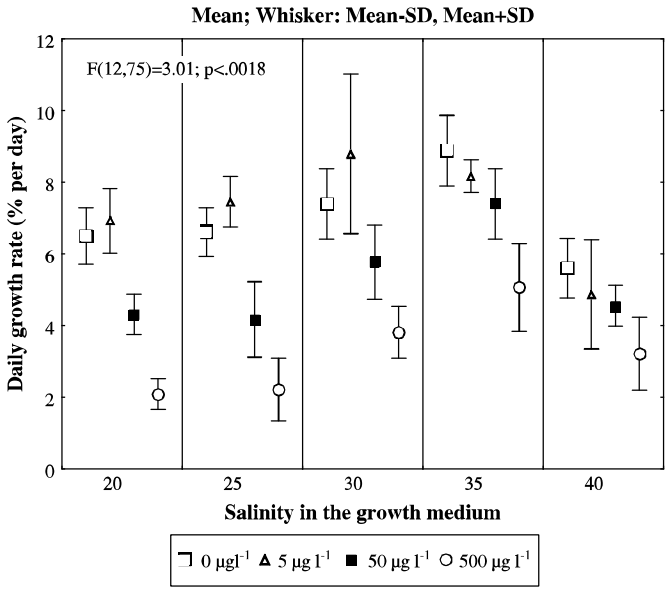


Fig. 3. Daily growth rate of *Ulva reticulata* exposed to different salinities and different concentrations of Cu ($n = 6$, mean ± standard deviation).

accumulated in algal thalli (Table 1) at all salinity experimental treatments.

Fig. 4 presents the growth dose-response diagram for *Ulva* exposed for 7 days at different concentrations of Cu, at a salinity of 25 as an example of how EC50 and NOEC values were determined. Plotted dose-response diagrams were used to determine EC50 and NOEC at the salinities displayed in Table 2; salinity affected both such that the lower the salinity, the lower the EC50 and NOEC concentrations (Table 2).

4. Discussion

4.1. Cu uptake at different salinities

This study demonstrates that decreased salinity can cause increased bioaccumulation of Cu in *Ulva reticulata*. The accumulation was approximately three times higher at salinity 20 than at a normal salinity of 35. This is similar to what has been previously reported for other *Ulva* species, were accumulation of Fe, Zn, and Cd in *Ulva rigida* and of Cr and Zn in *Ulva lactuca* increased with decreasing salinity (Favero et al., 1996; Wang and Dei, 1999). It was also observed in *Enteromorpha intestinalis* and *Scytosiphon lomentaria* that lower salinities enhanced the accumulation of Zn, Mn, and Co (Munda, 1984). On the other hand, other studies done on invertebrate species, such as *Atherinops affinis* and *Hediste deversicolor*, have found that low salinity enhanced the toxic effect of Cu, while leaving the accumulation of Cu constant (Anderson et al., 1995; Ozoh, 1994). Furthermore, it was found that exposing *Gracilaria tenuistipitata* and *E. intestinalis* for 3 days to Cu at reduced salinity (20) caused no change in tissue Cu levels (Elfwing and

Table 1
Correlation between heavy metal concentration in algal thalli and DGR of *Ulva reticulata* at different salinities.

Salinity	*N*	*r*	*p*
20	20	−0.930	<0.01
25	20	−0.909	<0.01
30	20	−0.756	<0.01
35	20	−0.823	<0.01
40	20	−0.669	<0.01

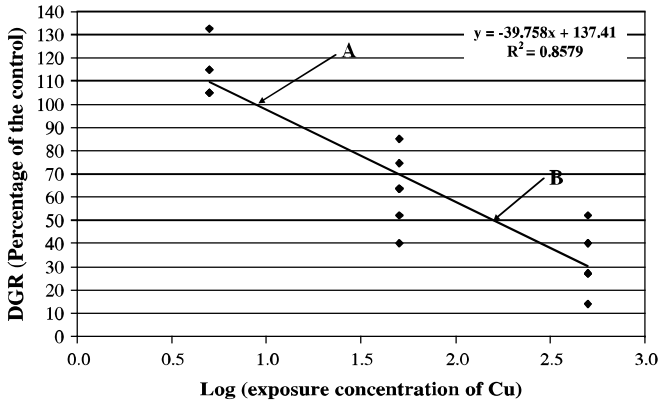


Fig. 4. Growth dose-response diagram for *Ulva reticulata* exposed to different concentrations of Cu for 7 days at a salinity of 25. (A) (NOEC) indicates the highest exposure concentration of Cu (*x*-axis) that will not produce any DGR inhibition in *Ulva*. (B) (EC50) indicates the point at which exposure concentration of Cu (*x*-axis) will produce a 50% inhibition of DGR when compared to a control.

Tedengren, 2002). In addition, lower salinities (10–28) had no significant influence on the accumulation of Cr or Zn (Wang and Dei, 1999).

The influence of salinity on heavy metal accumulation is probably due to the formation of complexes of metal ions and chloride ions at high salinity levels, as suggested by Greger et al. (1995), McLusky et al. (1986) and Erickson et al. (1996). In addition, macroalgae take up only dissolved metal ions (Luoma, 1983). Therefore, at higher salinity levels where more chloride ions are available, it is possible that there will be greater amounts of metals bound into complexes with chloride ions, hence reducing the chances of bioaccumulation occurring. This suggests that salinity is an important factor to be considered when comparing heavy metal accumulation in different areas subjected to different salinity regimes.

4.2. Growth at different salinities without Cu addition

Ulva reticulata grew well at all salinities tested, from 20 to 40, although growth was significantly higher at normal salinity (35) than at 20, 25 and 40 (Fig. 2). At low salinities the growth rate was reduced, possibly due to the low availability of nutrients and inorganic carbon, which is important for photosynthesis due to dilution, as has been suggested for *Ulva lactuca* (Gessner and Schramm, 1971) and *Zostera marina* at low salinities (Hellblom and Björk, 1999).

4.3. Effect of Cu on DGR at different salinities

Exposure of *Ulva reticulata* to Cu resulted in a decreasing DGR with increasing Cu concentration at all tested salinities, Cu having a more pronounced negative effect at the lower salinities. Previous studies have demonstrated that decreasing salinity enhances the toxicity of Cu to *Fucus vesiculosus* (Andersson and Kautsky, 1996). In the present study, salinity was found to have a significant influence on both Cu uptake and growth of *U. reticulata*. The decrease in growth at low salinity (30, 25 and 20) could possibly also be due to dilution that lowers the concentration of nutrients in the original medium.

Table 2
Results for determination of EC50 and NOEC values of Cu for *Ulva reticulata* at different salinities.

Salinity	EC50 (μg l^{-1})	NOEC (μg l^{-1})
20	513	8.9
25	125.9	7.9
30	398.1	18.0
35	1995.2	4.0
40	1584.9	1.1

The EC50 of other macroalgae, i.e. *Fucus spiralis* and *Pelvetia canaliculata*, has been reported to be at Cu concentrations ranging from 60 to 80 μg l^{-1}; EC50 and NOEC values increased with increasing salinities, suggesting that DGR inhibition was reduced with increased salinity. Similarly, in the case of *Gracilaria tenuistipitata* exposed to Cd, EC50 and NOEC values decreased with decreasing salinity (Haglund et al., 1996). Cu has also been reported to inhibit growth and photosynthesis in several other algal species, such as *Padina gymnospora* (Mamboya et al., 2007), *Chlamydomonas reinhardtii* (Macfie et al., 1994), and *Ascophyllum nodosum* (Strömgren, 1979, 1980).

5. Conclusion

The present study suggests that *Ulva reticulata* could be a potential bioaccumulator of heavy metals in areas with varying salinity, because of its ability to withstand such conditions while still accumulating high levels of Cu. However, since salinity drastically changes the accumulation level, this factor must be considered during sampling. In the field, *Ulva* growing in low salinity environments, due to dilution by freshwater water or in estuaries, will probably accumulate more heavy metals due to reduced chances of complex formation, than will algae growing in normal seawater or higher salinity environments. So when comparing levels of heavy metal pollution in two different areas using macroalgae as bioindicators, salinity measurements should be made and the possible effects of salinity should be considered when evaluating the data.

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