Heavy metal contamination and toxicity

Studies of Macroalgae from the Tanzanian Coast

Florence Alex Mamboya

Stockholm University
My father, Mzee Alex Roweta
My mother, Mama Ernester Ma-Rimoy
My lovely wife, Elizabeth, and
our beloved sons, Edwin and Collins
Abstract

Concentrations of various metals are elevated above background levels in several intertidal areas along the Tanzanian coasts. However, there is little available information concerning the toxicity of these metals and how the uptake of these metals by bioindicators are influenced by external factors, such as heavy rains and increased coastal eutrophication, which tend to fluctuate.

The present study focused on the uptake and toxicity of Cu and Zn in two common macroalgal species, *Padina gymnospora* (Phaeophyta) and *Ulva reticulata* (Chlorophyta). Laboratory studies were performed where metal content, growth (DGR), maximal quantum yields (Fv/Fm) and protein expression patterns (in *Ulva*) were measured as a response to exposure to Cu and Zn. The levels of metals accumulated in algal tissues correlated well to exposure concentrations and the longer the exposure time, the greater the uptake. However, an increased nutrient load (tested on *Padina*) or dilution of the seawater (tested on *Ulva*) affected both uptake of metals and their toxic effects. Here, DGR was more affected than Fv/Fm, suggesting DGR to be the more sensitive indicator of Cu and Zn toxicity. As shown by 2-D gel electrophoresis, more than ten proteins were up-regulated in *U. reticulata* after being exposed to Cu (1 μg/L), while at higher concentrations (10 and 100 μg/L) of Cu numerous proteins were down-regulated.

*P. gymnospora* was also used as a bioindicator to monitor long-term (1994–2005) and seasonal in-year variations in heavy metal concentrations in the Zanzibar Channel. No clear overall trends were revealed, but analysis of the combined dataset clearly pinpointed the most contaminated sites. It was concluded that seasonal and long-term variations, as well as environmental conditions need to be taken into consideration when using macroalgae as bioindicators.

**Key words:** Heavy metals, *Padina gymnospora*, *Ulva reticulata*, salinity, nutrients, proteins, uptake, growth, Fv/Fm, Zanzibar Channel
This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.


II. Mamboya, F.A., T.J. Lyimo, and M. Björk. 2007. Long-term and seasonal variations of heavy metal concentrations in a brown macroalga, the case of *Padina gymnospora* in the Zanzibar Channel (*Submitted*).

III. Mamboya, F.A., T.J. Lyimo, and M. Björk. 2007. Copper affects protein expression pattern and maximum quantum yield in the green macroalga *Ulva reticulata*. (*In manuscript*).


My contribution to the above papers:

**Paper I:** participated in planning and performed all laboratory work, except the heavy metal analysis; wrote most of the manuscript.

**Paper II:** participated in planning, did most of the fieldwork, and wrote most of the manuscript; performed all laboratory work and data analysis.

**Paper III:** did most of the planning and writing; performed all laboratory work and most of the sample and data analysis.

**Paper IV:** did most of the planning and writing, and performed all experiments and data analysis; participated in the Cu analysis.

*An additional relevant paper not included in the thesis:*

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<tr>
<td>2D PAGE</td>
<td>Two-dimensional gel electrophoresis</td>
</tr>
<tr>
<td>AAS</td>
<td>Atomic absorption spectrophotometer</td>
</tr>
<tr>
<td>DGR</td>
<td>Daily growth rate</td>
</tr>
<tr>
<td>DOM</td>
<td>Dissolved organic matter</td>
</tr>
<tr>
<td>EC50</td>
<td>Effect concentration of toxicant causing 50% inhibition</td>
</tr>
<tr>
<td>Fv/Fm</td>
<td>Maximal quantum yield</td>
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<tr>
<td>HN</td>
<td>High nutrient concentration</td>
</tr>
<tr>
<td>ICP-OES</td>
<td>Inductively Coupled Plasma - Optical Emission Spectrometer</td>
</tr>
<tr>
<td>IEF</td>
<td>Isoelectric focusing</td>
</tr>
<tr>
<td>IN</td>
<td>Intermediate nutrient concentration</td>
</tr>
<tr>
<td>LN</td>
<td>Low nutrient concentration</td>
</tr>
<tr>
<td>MALDI TOF</td>
<td>Mass-assisted laser desorption ionization time of flight</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometer</td>
</tr>
<tr>
<td>NN</td>
<td>No added nutrient</td>
</tr>
<tr>
<td>NOEC</td>
<td>Non-observable effect concentration</td>
</tr>
<tr>
<td>PAM</td>
<td>Pulse amplitude modulated</td>
</tr>
<tr>
<td>PE</td>
<td>Photosynthetic efficiency</td>
</tr>
<tr>
<td>PEA</td>
<td>Plant efficiency analyser</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>UEA</td>
<td><em>Ulex europaeus</em> agglutinin</td>
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</table>
1. Introduction

1.1. Heavy metals

Heavy metals are by definition metals having densities higher than 5 g mL$^{-1}$ (Sorentino, 1979), for example, Fe, Cu, Pb, Cd, Hg, Ni, Zn, and Mn. Approximately fifty three of the ninety naturally occurring elements are called heavy metals (Weast, 1984), and many of these, such as Cu, Mn, Fe, and Zn, are essential micronutrients, but can become toxic at concentrations higher than the amount required for normal growth (Nies, 1999). Other heavy metals, such as Cd, Hg, and Pb, have so far unknown roles in living organisms, and are toxic even at very low concentrations (Wood, 1974; Nies, 1999). Since many heavy metals can be very toxic and thus may threaten the health of organisms, studies have been conducted to investigate heavy metal levels in environmental samples, as well as heavy metal accumulation in and effects on organisms, and factors affecting heavy metal accumulation by various organisms. However, studies conducted in tropical environments are rare (Machiwa, 1992; Ferletta et al., 1996; Engdahl et al., 1998; Machiwa, 2000).

Several activities can contribute to heavy metal pollution in the marine environment, for example, seafloor and bedrock dredging, shipping activities, industrial and urban effluents, mining, agricultural fertilizer use, and burning of fossil fuels (Machiwa, 1992; UNEP, 1997; Lionetto et al., 2003). Natural weathering of rocks is yet another source (Pyle and Mather, 2003).

In the marine environment, heavy metals may occur as dissolved free metal ions or as complex ions, chelated with certain inorganic ligands such as Cl$^-$, OH$^-$, CO$_3^{2-}$, and NO$_3^-$; and sometimes heavy metals can form complexes with organic ligands such as fulvic acid, amines, humic acids, and proteins (Beijer and Jerenlöv, 1979; Batley et al., 2004). Heavy metals can be present in various particulate forms: as colloids or aggregates, bound into particles, precipitated as metal coat-
ings on particles, incorporated into organic matter such as algae, and
held in crystalline detrital particles (Beijer and Jerenlöv, 1979). The
physical and chemical forms of heavy metals in the marine environ-
ment are governed by environmental variables such as salinity, tem-
perature, pH, redox potential, organic and particulate matter, biologi-
cal activities, and metal properties (Lobban and Harrison, 1994).

1.2. Bioaccumulation of heavy metals

Unlike many other pollutants in the environment, heavy metals are
non biodegradable (Kaewsarn and Yu, 2001). Remediation processes
for heavy metal-polluted ecosystems are difficult, and expensive.
Heavy metals can also be accumulated by some organisms either di-
rectly (e.g., in the case of macroalgae) or through the food chain,
eventually posing a serious health risk to inhabitants of an ecosystem,
including humans (Galloway et al., 1982; Angelone and Bini, 1992;
Chan et al., 2003). The bioaccumulation of toxicants, such as heavy
metals, by living organisms is often a good integrative indicator of expo-
sure, and has been extensively used to assess contamination levels of
heavy metals in polluted ecosystems (Phillips and Rainbow, 1994).

Macroalgae are major primary producers in the marine environment and
play an important role in food chains. Since marine pollution is most
serious in coastal waters adjacent to major pollutant sources, macroalgae
from marine environment are particularly suitable for pollution studies.
Additionally, they have the ability to accumulate high levels of various
metals in their cell walls (Burdin and Bird, 1994; Salgado et al., 2005).
Macroalgae, especially from the Phaeophyceae, have, besides negatively
charged polysaccharides, special compartments (physodes) that enhance
their ability to accumulate high concentrations of heavy metals (Salgado
et al., 2005, Fig. 1).

The accumulation of heavy metals by macroalgae can take place either
passively or actively (Eide et al., 1980). Macroalgae have been used in
studying the contamination status of coastal ecosystems, due to their
ability to accumulate and tolerate high metal concentrations (Bryan,
1983; Wekwe et al., 1989; Ferletta et al., 1996; Amado Filho et al.,
1999; Ho, 1990; Muse et al., 1999). Unlike several other bioindicators
of heavy metal contamination (such as filter-feeding animals), macro-
algae accumulate only metal ions that are dissolved in the seawater
(Luoma, 1983; Luoma et al., 1982).
1.3. Factors affecting heavy metal accumulation by macroalgae

In marine environments, the concentration of heavy metals is largely governed by the biological, chemical, and physical characteristics of the surrounding seawater (Wangersky, 1986). For example, Rice and Lapointe (1981) found that light and nitrogen availability positively affected rates of uptake of Fe, Mn, Zn, Cd, and Rb in *Ulva fasciata*, and it has been demonstrated that uptake of Cd in *Ulva fasciata* increased with increased ambient concentration of nitrate in the growth medium (Lee and Wang, 2001). However, in the freshwater plant *Ipomoea aquatica* (water spinach), heavy metal accumulation was negatively affected by increased nutrient levels, the uptake of Hg, Cd and Pb decreasing when the nutrient levels were higher (Göthberg et al., 2004). Phytoplankton studies of the influence of major nutrients on heavy metal bioaccumulation demonstrated that nutrient enrichment increased concentrations of Cd and Zn uptake (Wang and Dei, 2001). In environments with high nutrient levels, metal uptake can be inhibited because of complex formation between nutrient and metal ions (Göthberg et al., 2004; Haglund et al., 1996; Paper I).

Growth rate is another factor that reportedly affects heavy metal accumulation in macroalgae, Cd and Rb levels decreasing and Mn levels increasing as the specific growth rate increases. This probably indicates the metabolic regulation of these metals (Rice, 1984), or possibly the presence of a “dilution factor” (Greger et al., 1991; Wang and...
Dei, 1999; Göthberg et al., 2004) as a result of an increase in the heavy metal-to-biomass ratio.

Salinity is yet another factor reported to affect heavy metal bioavailability. However, most studies of salinity have described its effects on heavy metal accumulation by animals; information concerning the effects on macroalgae is scarce (Munda 1986; Nugegoda and Rainbow, 1989; Anderson et al., 1995; Shazili, 1995; Ozoh, 1994; Lee et al., 1998; Wang and Dei, 1999). Paper IV describes the influence of salinity on Cu uptake by a tropical marine macroalga, *Ulva reticulata*.

The pH and redox potential affects the bioavailability of metals in solution: at high pH elements are present as cations, while at low pH the bioavailability of metals ions is enhanced (Peterson et al., 1984). It is known, however, that metals in seawater may exist in either particulate, or dissolved form mainly determined by the properties of a particular metal and other factors, such as pH, salinity, redox potential, ionic strength, alkalinity, persistent organic and particulate organic matter, and biological activity (Stokes, 1983).

Humic substances in the aquatic environment may influence the accumulation of metal ions (Koukal et al., 2003). It has been demonstrated that the bioavailability and toxicity of heavy metals are reduced through complex formation with dissolved organic matter (DOM) hence, reduce the concentration of free ionic metals in the aquatic environment (Tubbing et al., 1994; Kim et al., 1999; Guo et al., 2001). DOM may also block the accumulation of some heavy metals by blocking the algal surface sites (Campbell et al., 1997; Guo et al., 2001). Temperature affects the metabolic rate of organisms, and hence also their heavy metal uptake (Lemus and Chung, 1999). Indeed, temperature also affects the water chemistry hence the distribution of organisms in an ecosystem (Countant, 1987). On the other hand according to Zumdahl (1992), seasonal variation in temperature does not affect heavy metal accumulation. Sometimes, heavy metal bioaccumulation has been regarded as both species specific and metal specific (Lee et al., 1998).

Knowledge of the factors affecting heavy metal bioaccumulation by macroalgae enhances our understanding of the usefulness and limitations of using macroalgae as bioindicators of heavy metals in the marine environment. Thus, Papers I and IV report on how two macronu-
trients (phosphates and nitrates) and salinity affect the bioaccumulation of selected metals. This might provide insight into what regulates uptake in the field, since the inflow of pollutants to the environment is not as isolated compounds, but rather as combinations of several.

1.4. Toxicity of heavy metals

Several studies have reported on the toxic effects of heavy metals on various species of macroalgae (Markham et al., 1980; Amado Filho et al., 1993, 1996; Kangwe, 1999). Most studies of macroalgae have been done in temperate regions, while information regarding tropical environments, especially the Western Indian Ocean is scarce. As well, it has been found that different species may respond differently when exposed to different heavy metals (Carreras and Pignata, in press). It has previously been reported that even the same species growing in different areas subject to different environmental parameters may respond differently to heavy metal contamination (Hall et al., 1979). Furthermore, several other factors, such as concentration of dissolved metal, pH, salinity, temperature and nutrients, are known to influence the toxicity of certain metals to macroalgae (Rai et al., 1981; Florence et al., 1984; Munda and Hudnik, 1988; Langston, 1990).

Thus, the mechanism of heavy metal toxicity to plants is not yet well understood. Research into how various species from different areas respond to various heavy metals at different levels could help improve our understanding of the action of heavy metals on, and the tolerance of heavy metals by, macroalgae. Papers I, III, and IV describe the hitherto unexamined toxic effects of selected metals on tropical macroalgae from the Zanzibar Channel in the Western Indian Ocean.

The toxicity of heavy metals in macroalgae has been reported to follow the general order of Zn < Pb < Ag < Cd < Cu < Hg, which may vary depending on experimental conditions and macroalgal species (Rai et al., 1981, Kangwe, 1999). Heavy metals are among the major environmental hazards due to their affinity for metal sensitive groups, such as thiol groups. Heavy metals block functional groups of proteins, displace and/or substitute essential metals, induce conformational changes, denature enzymes and disrupt cells and organelle integrity (Hall, 2002). Different heavy metals have been reported to affect macroalgae by interacting with enzymes and inhibiting their normal functions (Van Assche and Clijsters, 1990).
Heavy metal toxicity is often linked to the formation of free reactive oxygen radicals causing the inhibition of macroalgae development (Collén et al., 2003; Pinto et al., 2003). Molecular oxygen is un-reactive with organic molecules because it has two unpaired valence shell electrons in outer shell. However, when activated through reduction it forms reactive oxygen species (ROS) such as superoxide radical (O$_2^-$), singlet oxygen ($^1$O$_2$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH$^-$) and finally water (H$_2$O) (Fig. 2). Reactive oxygen species are toxic because they have ability to interact rapidly with biological molecules (proteins, lipids, DNA) causing oxidative stress which can result into cell death via apoptosis or necrosis (Kannan and Jain, 2000).

Oxidative stress occurs as a result of imbalance between the production of reactive oxygen and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage. Damage as a result of oxidative stress can occur in biological molecules such as DNA, proteins and lipids (Fig. 2). Oxidative attack on proteins results in site-specific amino acid modifications, fragmentation of the peptide chain, aggregation of cross-linked reaction products, altered electrical charge and increased susceptibility to proteolysis. The oxidative degradation of protein is enhanced in the presence of metal cofactors that are capable of redox cycling, such as iron. In these cases, the metal binds to a divalent cation binding site on the protein. The metal then reacts with hydrogen peroxide in a Fenton reaction to form a hydroxyl radical that rapidly oxidises an amino acid residue at or near the cation binding site of the protein (Stadtman, 1986). Detoxifications of ROS are mainly by production of antioxidants such as enzymes (superoxide dismutase, catalase, peroxidase), thioredoxin superfamiliy, glutathione and vitamin E (Dowling and Sheen, 2006).
Figure 2. Oxidative stress is elicited by ROS derived by a univalent reduction of $O_2$. ROS can be induced by endogenous or external sources such as heavy metals, PAHs, polyaromatic hydrocarbons. *(Source: Dowling and Sheehan, 2006).*

More specifically in macroalgae, heavy metal toxicity is known, for example, to inhibit growth and photosynthesis, reduce chlorophyll content, affect reproduction, interfere with cell permeability, cause the loss of K ions, affect protein synthesis and degradation, and cause oxidation and lipid peroxidation (Sorentino, 1979; Strömgren, 1980; Rai et al., 1981; Kremer and Markham, 1982).

### 1.5. External factors affecting metal toxicity

Toxicity of heavy metals in macroalgae in marine environments largely depends on the biological availability of the heavy metals (Campbell, 1995; Sunda and Huntsman, 1998), which is determined by both their physical and chemical states (Langston, 1990). For example, the toxicity of heavy metals may be reduced when they are adsorbed to suspended organic matter, thus reducing their ionic fraction in the water column. Both pH and redox potential affect the toxicity of heavy metals by
limiting their availability (Peterson et al., 1984). At low pH, metals generally exist as free cations; at alkaline pH, however, they tend to precipitate as insoluble hydroxides, oxides, carbonates, or phosphates. Thus, measurements of total heavy metal concentrations in the water column sometimes may not correlate with toxicity in macroalgae (Florence et al., 1984); this may explain a situation in which two studies examining the effect of a metal at the same concentration may obtain different results.

Little is known of how salinity and temperature influence the toxicity of metals in macroalgae. Munda (1984) noted the effect of salinity on the bioaccumulation of Mn, Zn, and Co by Enteromorpha intestinalis and Scytosiphon. Andersson and Kautsky (1996) reported that the addition of 20 µg Cu/L of water caused an approximately 70–80% decline in the germination of Fucus vesiculosus zygotes at 6 psu and also at 20 psu (the latter being higher than optimum). At a salinity close to optimum (14 psu), no negative effects on germination were noted when 20 µg Cu/L of water was added. These results suggest that the degree of salinity stress acting on the zygotes is a more important determinant of their response to Cu than is the influence of salinity on metal availability.

Rai et al. (1981) reported both a reduction and increase in metal toxicity in algae with increased temperature. The increase in toxicity with increased temperature is due to higher energy demand, which causes a higher respiration rate in the organism (Rai et al., 1981). In addition, temperature is might affect the chemistry of water (Fritioff et al., 2005) which might influence heavy metal toxicity. However, the reasons for the decrease in toxicity with increased temperature are not well understood (Förstner and Wittmann, 1979).

High concentrations of nutrients, such as phosphates and nitrates, have been reported by several authors to reduce the toxicity of heavy metals (Paper I; Haglund et al., 1996). The presence of other pollutants in the growth medium can also affect the toxicity of heavy metals. Whereas the presence of 2,4-dichlorophenoxy acetic acid (2,4-D) was found to reduce the toxicity of both nickel and aluminium in marine phytoplankton, Cu decreased the toxicity of the herbicide paraquat to freshwater phytoplankton (Rai et al., 1981). Interactions between metals occurring together in the environment, for example, via metal–metal antagonistic or synergistic effects, have also been reported (Strömgren,

The Tanzanian coast has many estuaries, sewage outfall areas, and intertidal areas with freshwater intrusion where fluctuations of nutrients and salinity are frequent. In this work the effects of nutrient levels on the accumulation and toxicity of Cu and Zn are investigated for *P. gymnospora* in Paper I, while the influence of salinity on the toxicity of Cu to *Ulva* is examined in Paper IV.

1.6. **Heavy metals along the Tanzanian coast**

Tanzania borders on the Indian Ocean on its eastern side, with an extended coastline of approximately 800 km. Highly populous Dar es Salaam, the biggest city in the country, lies on the Indian Ocean. Disposal of untreated industrial waste and raw sewage is common in the city, due to a lack of proper sanitary facilities. The Zanzibar Islands are

situated to the east of Dar es Salaam, separated from it by the Zanzibar Channel. Tanzania’s population is growing rapidly and almost linearly (Fig. 3), the current population coming to approximately 37 million people (Census, 2003). Many people are now migrating to coastal urban areas increasing the coastal population; this population growth is associated with environmental degradation, wastes being disposed untreated into the environment. Industrial emissions and effluents are increasing due to an increase in industrial activities, especially in Dar es Salaam. Mining, shipping, and agricultural activities are also increasing in line with population growth. All these activities pose a serious threat to the environmental, especially the aquatic environment. Amongst the pollutants that are expected to increase in amount are heavy metals and nutrients from industrial and sewage effluents.

Despite the increased exposure of the Tanzanian coast to pollution, few studies have investigated the levels and effects of pollution in the area’s marine biota (Machiwa, 1992; Ferletta et al., 1996; Engdahl et al., 1998; Mremi and Machiwa, 2003). The available studies are limited to short-term sampling and only a few examine toxicity effects. Hence, no long-term or seasonal studies have been conducted to evaluate variations in pollutant levels. Few studies have examined heavy metal levels in sediments or biota, and the effects of heavy metals and factors that might affect their uptake and bioaccumulation are poorly understood. No information is available as to whether pollution is increasing or decreasing along the Tanzanian coast.

The present study attempts to determine seasonal and long-term fluctuations of the levels of various metals in the Zanzibar Channel, using the previously recommended macroalga *P. gymnospora* as a bioindicator. It was also of interest to determine the effects on selected macroalgae species of heavy metals that were found in high concentrations in the environment. Papers I, III, and IV examine the toxicity of heavy metals and factors that affect their uptake and toxicity in macroalgae. Paper II demonstrates the use of *P. gymnospora* for the seasonal and long-term monitoring of heavy metal contamination, and its potential as a pollution bioindicator in the Zanzibar Channel.
2. Objectives

I. To map heavy metal contaminations along the coasts of Tanzania and to determine variations in concentrations over time (Paper II)

II. To evaluate how surrounding factors, such as increased nutrient levels and fluctuations in salinity, affect metal accumulation patterns in selected macroalga (Papers I and IV)

III. To determine physiological responses induced by the heavy metals found to have increased in Zanzibar Channel (Papers I, III, and IV).

IV. To evaluate the potential of macroalgae as natural bioindicators for heavy metal pollutions.
3. Comments on materials and methods

The following is a summary description of the materials and methods used; detailed information is provided in Papers I–IV.

3.1. Description of the study site

The Zanzibar Islands are separated from the Tanzanian mainland by the Zanzibar Channel (Fig. 4). The Channel is mostly dominated by the East African Coastal Current, which has a net northward flow. Of the study sites selected, some are subject to human environmental degradation pressures near Dar es Salaam and the town of Zanzibar, while others are located away from anthropogenic input. Approximately six study sites in Dar es Salaam (the Mbudya Island, Ocean Road, Kunduchi, Yacht Club, and Oyster Bay sites) and five in the Zanzibar Islands (the Bawe, Chapwani, Maruhubi, and Nungwi sites) (Fig. 4) were selected for this study.

3.2. Collection of macroalgae

Fresh submerged *P. gymnospora* samples (Fig. 5) were collected for laboratory experiments from Bawe Island (one of the Zanzibar Islands), at low tide (Paper I), while *U. reticulata* samples (Fig. 6) were collected from the intertidal area in Oyster Bay, Dar es Salaam (Papers III and IV, Fig. 4). *Padina* was selected because previous studies showed that is suitable as bioindicators of heavy metals compared to other macroalgae investigated (Ferletta et al., 1996). *U. reticulata* was used in this study because it is available in many intertidal areas including polluted and areas with high variability of salinities. *U. reticulata* samples were transported to Stockholm University and cultured under tropical conditions in a climate chamber using natural seawater. To allow acclimatization, the macroalgae were cultured in Plexiglas cylinders and left for at least two weeks before starting experiments. The field study involved collecting *P. gymnospora* samples for the seasonal and long-term monitoring (Paper II) of heavy metals in the Zanzibar Channel. This was done at various study sites in 1994, 1997,
1998, 2002, 2003, and 2005 (Fig. 4). Samples for seasonal study were collected in 1998 (Paper II).

3.3. Experimental set-up for heavy metal exposure

The set-ups for Papers I, II, and IV involved three replicates and for Paper I, six replicates, of controls and each treatment, several repetitions being made of the experiments. Before exposure to any toxicant, set-ups were left for 24 hr to acclimatize. The macroalgae were exposed to selected concentrations of Cu or Zn; Cu was added in the form of CuCl$_2$ (Paper I, Merck, Darmstadt, F.R. Germany; Paper III and IV, Sigma Aldrich, Steinhelm, Germany) while Zn was added in the form of ZnCl$_2$ (Merck, Darmstadt, F.R. Germany) in natural filtered seawater. Measurements of toxicity (Fv/Fm and daily growth rate – DGR) were made for certain durations presented in Papers I, II, and IV. Samples were taken and processed/preserved for heavy metal analysis (Papers I,
III, and IV) and protein extraction (Paper III).

3.4. Influence of nutrients on accumulation and toxicity of Cu and Zn (Paper II)

*P. gymnospora* specimens growing in a medium containing either 500 µg Cu l⁻¹ or 1000 µg Zn l⁻¹ were exposed to three different concentrations of nitrate and phosphate. The sources of the nitrate and phosphate nutrients were sodium nitrate (NaNO₃, Merck, Darmstadt, F.R. Germany) and hydrous sodium hydrogen phosphate (NaHPO₄·12H₂O, Merck, Darmstadt, F.R. Germany), respectively. Three different nutrient concentrations were defined as (i) high nutrient (HN), containing 20 mg/L nitrate and 2 mg/L phosphate; (ii) intermediate nutrient (IN), containing 10 mg/L nitrate and 1 mg/L of phosphate; and (iii) low nutrient (LN), containing 1 mg/L nitrate and 0.1 mg/L phosphate. While measuring for toxicity (Fv/Fm and DGR), samples for heavy metal analysis were taken as described in Paper I.

3.5. Influence of salinity on accumulation and toxicity of Cu (Paper IV)

Tests of the influence of salinity were made at 20, 25, 30, 35, and 40 psu S at different concentrations of copper; the controls contained no added
Cu. *U. reticulata* samples were exposed to different levels of salinity at different concentrations of Cu for 7 d; the exposure concentrations of Cu used were 0, 5, 50 and 500 μg Cu/L. The measurement of wet weight for DGR determination was done after 7 d. After the experiments, samples were washed and analysed for Cu concentration.

### 3.6. Preparation for heavy metal analysis

Field samples (Paper II) and laboratory samples (Papers I, II, and IV) were ground in a porcelain mortar; the homogenous algal powder (approximately 0.1–0.5 g) was digested using concentrated nitric acid and perchloric acid. After dilution with double distilled water, the resulting solutions were filtered and analysed for heavy metal concentrations using Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES, Spectro, Marlborough, MA, USA) (*Paper I*) or atomic absorption spectrophotometer (AAS, Paper III and IV, Spectra AA-100 and GTA 100, Varian, Springvalve, Australia; Paper II, AnalytikJena novAA 400, Jena, Germany). Both ICP-OES and AAS were used in this study because they are sensitive instruments able to detect low concentrations of heavy metals in environmental samples and they do not require a big sample size.

### 3.7. Determination of daily growth rate (DGR; Papers I and IV)

Daily growth rate (DGR), expressed as a percentage, was estimated according to Lignell et al. (1987), Mtolera et al. (1995), and Haglund et al. (1996) using the following formula:

\[
DGR = [(Wt/Wo)^{1/t} - 1] \times 100
\]

where Wt represents fresh weight at time t, Wo represents initial fresh weight, and t is time in days.

### 3.8. Measurements of maximum quantum yield (Fv/Fm; Papers I and III)

The maximum quantum yield (Fv/Fm) has been shown to be a suitable measure of stress in plants caused by various stressors (Kangwe, 2006; Beer and Björk, 2003). Measurements were made using a plant efficiency analyser (PEA, Hansatech Instruments Ltd, Lynn, Norfolk, UK) (*Paper I*) and a Diving Pulse Amplitude Modulation Fluorometer, (Diving PAM, Walz, Effeltrich, Germany) (*Paper III*). For more details
about the techniques, see Beer and Björk (2000) and Beer et al. (2001). Before measurement, plants were dark adapted for 15 min.

3.9. Protein expression profiling (Paper III)
Proteomics is the new technique applied by toxicologist to achieve high-throughput analysis of effects of toxicants on protein populations and sub-populations with the potential to identify novel biomarkers or toxicity targets (Dowlig and Sheehan 2006; Jamers et al., 2006; Lay et al., 2006; Gianazza et al., 2007). Limited studies are available on the use of proteomic tools in the marine environmental studies. Therefore in this study, 2D gel electrophoresis, PdQuest software programme and Mass Spectrometry, MALDI TOF (Voyager-DE STR mass spectrometer, Applied Biosystems, Foster City, CA USA) were employed as tools to investigate the effect of different concentrations of Cu in *U. reticulata* as a baseline.

3.10. Statistical data analysis
Two-way ANOVA testing was used to test the effects of two variables on growth (Fv/Fm) or on the uptake of metals. For multiple comparisons testing, the post-hoc HSD test was used to test the level of significant and non-significant effects after ANOVA testing (Siegel and Castellan, 1988). Cluster analysis was employed in assessing homogenous groups (sampling stations) for overall levels of heavy metal concentrations from 1994 to 2005 (Paper III). Spearman rank correlation tests were performed to determine the correlation between effects (e.g., growth and accumulated Cu in algal tissue). Student’s t-test was used to compare the measurable effects between different treatments. All statistical tests were performed using the Statistica, version ’99, software package (StatSoft, Tulsa, OK, USA), at a 95% significance level.
4. Results and discussion

4.1. Heavy metal uptake

The uptake of two metals, Cu and Zn, by the brown alga *P. gymnospora* was investigated in Paper I; the uptake of Cu by *U. reticulata* was investigated in Papers III and IV. It was found that both macroalgae accumulated metals proportionally to the concentration in the growth medium ($p < 0.05$); the higher the exposure concentration of heavy metals in the growth medium, the greater the accumulation of metal. It was also found that the exposure time substantially influenced the accumulation (Papers I and IV). Many studies have previously reported the ability of various macroalgae to accumulate metals from surrounding media. This ability to accumulate is usually attributed to the presence of charged polysaccharides in the cell walls. However, brown macroalgae have been reported to have higher accumulation ability than green algae do. This is explained by the presence in their cell wall material of polysaccharides (alginites and sulphated fucans) with a higher affinity for cations, and by the presence of so-called physodes – bodies containing phenolic compounds, which are known to accumulate large amounts of heavy metals (Salgado et al., 2005; Andrade et al., 2002; Farina et al., 2003).

4.2. Effects of nutrient levels on bioaccumulation of metals

Although the accumulation of metals by *P. gymnospora* from the surrounding medium was proportional to the available concentration of metals, the addition of nutrients, such as nitrate and phosphate, significantly inhibited the accumulation (Paper I). It was found that the higher the concentration of nutrients in the growth medium, the lower the accumulation of both Cu and Zn (Paper I). It is impossible, from this data, to explain exactly why the accumulation decreased with nutrient additions. However, when a similar effect was found in the red alga *Gracilaria tenuistipitata*, it was suggested to be due to complex
formation, competition for the binding sites in the cell wall during accumulation, or simply the improving overall nutrient status of the alga (Haglund et al., 1996). Macroc algae accumulate only the available dissolved metal ions from the growth medium (Luoma, 1983).

If this decrease in accumulation due to nutrient levels also exists in the field, it would have implications when macroalgae are used as indicators of heavy metals: if high levels of both metals and nutrients are present together in the environment, the estimated environmental levels of heavy metals could be underestimated. The experimental nutrient concentrations used in this study, however, are higher than would normally be found in the field, though such levels could well be found near raw sewage outfalls, which could directly raise nutrient concentrations in the surrounding seawater. Reported nutrient levels in coastal waters near different parts of Zanzibar and Dar es Salaam are quite low (Lugomela et al., 2002; Björk et al., 1995; Hamisi et al., 2004), and are unlikely to affect bioaccumulation significantly. However, in an area such as Ocean Road (Paper II), which is indeed close to a major sewage outlet area, the metal levels might have been underestimated. In another case, nutrient enrichment has been reported to enhance the accumulation of various heavy metals in phytoplankton (Wang and Dei, 2001). In the present study, the uptake of Cd and Zn was enhanced by the addition of nitrate but not of phosphate.

4.3. Effects of salinity on bioaccumulation of heavy metals

Salinity significantly influenced the accumulation of Cu in *U. reticulata*. Just as decreased nutrients caused an increase in metal accumulation, decreased salinity enhanced the accumulation of Cu in the algal thallus (Fig. 7, Paper IV). Previously reported results (mostly for animals) regarding heavy metal accumulation at varying salinities, display a similar pattern, high salinity inhibiting accumulation (Wright, 1977; Amiard-Triquet et al., 1991). However, still other studies found that salinity had no influence on the accumulation of copper in *Atherinops affinis* and the Ragworm (Anderson et al., 1995; Ozoh, 1994).

In tropical regions with estuaries, large river mouths, and freshwater intrusion into intertidal areas, *U. reticulata* might be used to study metal accumulation, because it can endure in these areas better than many other macroalgae can. In Tanzania, for example, at the mouth of the River Msimbazi, which might be highly polluted because it drains urban
and industrial areas (including former waste dumping sites), it is impossible to find \textit{P. gymnospora}, which has been recommended as a bioindicator; hence, alternative algae that thrive in this area, such as \textit{Ulva}, could be used instead.

Figure 7. Effect of salinity on the accumulation of Cu in \textit{Ulva reticulata} exposed to different Cu concentrations for 7 day (N = 6).

4.4. Effects of heavy metals on DGR and maximum quantum yield

Cu is ranked among the most toxic metals to plants (Rai et al., 1981). It was evident in this study that Cu inhibited both DGR (Papers I and IV) and Fv/Fm. Cu toxicity displayed a dependency on exposure time: the longer the exposure time, the greater the inhibition (Papers I and III). We observed a strong negative correlation between DGR in \textit{U. reticulata} and both the level of Cu in the medium and the accumulated Cu in the algal tissue; this indicates that the inhibition of DGR was due to Cu (Table 1, Paper IV). Along with the decrease in both DGR and Fv/Fm, high concentrations of Cu were found to induce chlorosis. Toxicity of heavy metals such as Cu has been linked with the production of free oxygen radicals, which are known to induce the peroxidation of membrane lipids (Halliwell and Guttereridge, 1984; Vavilin et al., 1998; Monnet et al., 2006). Despite Cu being an important micronutrient, at
elevated concentrations it has been found to inhibit growth by replacing cofactors in key enzymes (e.g., Mg, an essential element in chlorophyll molecules), disrupting photosynthetic activity and other important cellular processes (Küpper et al., 1996; Strömgren, 1980). Cu was demonstrated to affect the growth and photosynthesis performance of *Gracilaria tenuistipitata* exposed to different Cu concentrations; it was concluded that Cu might have inhibited the electron transport chain of the light reaction of photosynthesis at the site of the secondary quinine acceptor in photosystem II (Haglund et al., 1996).

**Table 1.** Correlation between Cu concentration in thalli and DGR of *U. reticulata* exposed to different concentrations of Cu at different salinities. (Significant correlation at *P*<0.05)

<table>
<thead>
<tr>
<th>N</th>
<th>r</th>
<th><em>r</em>²</th>
<th><em>p</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>20  psu</td>
<td>20</td>
<td>-0.930</td>
<td>0.865</td>
</tr>
<tr>
<td>25  psu</td>
<td>20</td>
<td>-0.909</td>
<td>0.827</td>
</tr>
<tr>
<td>30  psu</td>
<td>20</td>
<td>-0.756</td>
<td>0.571</td>
</tr>
<tr>
<td>35  psu</td>
<td>20</td>
<td>-0.823</td>
<td>0.676</td>
</tr>
<tr>
<td>40  psu</td>
<td>20</td>
<td>-0.669</td>
<td>0.448</td>
</tr>
</tbody>
</table>

Similarly, Zn also inhibited both DGR and Fv/Fm in *P. gymnospora* (Paper I); however, it was found to be less toxic than Cu was, producing less inhibition of both DGR and Fv/Fm. In addition, the concentration of Zn was far higher than that of Cu, yet Cu proved to be the more toxic. Similar findings regarding the possible effect of Zn on growth were reported previously for *Padina* spp., in which growth inhibition occurred with exposure to 20 μg/L of Zn (Amado Filho et al., 1996, 1997). In both cases, i.e., Cu and Zn exposures (Paper I), the inhibitory effect was higher in terms of DGR than of Fv/Fm, indicating that growth is the more sensitive indicator of heavy metal toxicity. This may be because the toxicity of these metals did not only target photosynthetic pathways, but also inhibited other metabolic processes. This has also been reported in the related green macroalgae *Enteromorpha intestinalis*, in which growth was more inhibited by Cu than photosynthesis was (Lewis et al., 1998).

### 4.5. Effects of nutrient levels and salinity on toxicity of metals

The toxicity of heavy metals to algae is reportedly influenced by several factors. In this work, both nutrient addition to the growth medium and changes in salinity were found to affect the toxicity of heavy metals
significantly. Nutrient additions to the growth medium increased growth in *P. gymnospora* (Paper I). Similarly, increases of salinity of up to 35 psu enhanced the DGR in *U. reticulata* (Paper IV), after which the DGR dropped. Both increased nutrient levels (Paper I) and increased salinity (Paper IV) negatively affected metal accumulation, and as a result, the degree of inhibition was significantly lower (Fig. 8, Paper IV). It would be interesting in the future to test the effects of the two variables together, because in an estuarine environment low salinity would enhance accumulation while concurrent high anthropogenic nutrient input would have a decreasing effect on heavy metal accumulation.

**Figure 8.** Effect of salinity on Cu toxicity, expressed as effect on DGR of *U. reticulata* exposed to different concentrations of Cu at varying salinities.

4.6. *Effects of heavy metals on protein expression pattern*

Recently, there has been interest in studying the effects of environmental parameters and toxicants on gene and protein expression in various organisms (Aina et al., 2007; Silvestre et al., 2006; Lay et al., 2006; Jamers et al., 2006; Gianazza et al., 2007). However, limited attention has been paid to the effects of heavy metals on marine organisms (Jamers et al., 2006). In addition, the protein expression pattern arising from Cu exposure is poorly understood in plants. The present study (Paper III) presents some findings regarding the protein expression
pattern of *U. reticulata* exposed to Cu, as determined using two-dimensional gel electrophoresis. It appears that the protein expression pattern can be at least as sensitive as other sensitive toxicological testing methods reported for assessing Cu toxicity in macroalgae.

Exposure of *U. reticulata* to the lowest experimental concentration of Cu, i.e., 1 μg/L, for 24 h, caused drastic changes in the protein expression pattern (Fig. 9). Several newly induced proteins appeared after the exposure, and although they have not yet been identified, they could be part of a defence mechanism against Cu toxicity. At higher levels of exposure to Cu, more changes were evident, in which a large numbers of protein spots were down-regulated and a few up-regulated. The up-regulated proteins are possibly proteins responsible for counteracting Cu toxicity, while the down-regulation of proteins could represent a toxicity mechanism. Cu toxicity is largely linked to the production of oxygen free radicals (reactive oxygen species, ROS), which are known to induce proteotoxicity and other major protein expression changes in living organisms (Cu has been reported to increase the protein expression of defence proteins against oxidative stress (Collén et al., 2003; Weber et al., 1991). However, when the concentration of Cu is high, it has been reported to suppress the expression of proteins, including stress and anti-oxidative proteins (Okamoto et al., 1996; Pinto et al., 2003). Also, the tissue protein content has been shown to decrease with Cu exposure (Weber et al., 1991; Collén et al., 2003; Malea et al., 2006). In the macroalgae *Gracilaria* and in some higher plants, for example, rice, Cu exposure can result in a decrease of protein content of up to 50% after 24 h (Collén et al., 2003).

The down-regulation of five successfully identified proteins, namely, actin, lectin, protein synthesis elongation factor, ATP synthase beta subunit, and ATP binding proteins, occurred with increased Cu concentration. As mentioned earlier, this could be due to proteotoxicity as a result of Cu exposure. Information regarding the effect of Cu on protein levels is lacking, making it difficult to pinpoint the mechanism underlying the observed down-regulation and upregulation of various proteins. However, a similar study of *Mytilus edulis* exposed to Cu found that actin expression decreased significantly (Manduzio et al., 2005). In some organisms, Cu has been found to induce highly conserved stress proteins, especially the well-known HSP70 (Parsell and Lingist, 1993; Tedengren et al., 1999); however, a study by Lewis et al.,
2001 on effect of Cu on induction of HSP70 in *Enteromorpha intestinalis* (now renamed *Ulva*) found no significant changes in HSP70 levels due to Cu exposure. Consequently, response to toxicants at the protein level might be species and protein specific.

The present study has demonstrated that Cu and Zn induce toxic effects in the studied algae; however, this finding does not disqualify these algae for use as bioindicators of heavy metals in the Zanzibar Channel, since they were able to tolerate even the highest levels tested. In the natural environment, it is rare to find concentrations close to the highest concentrations tested in this study, except in highly polluted environments. For example, concentrations of approximately 454 µg Cu/L and 329 µg Zn/L were reported along sewage-impacted shoreline of Mauritius (Daby, 2006). What is probably more important is the consideration of factors that influence bioaccumulation; this would allow the better interpretation of results from two different sites, or from one site but obtained on two different sampling occasions subject to dissimilar factors (e.g., salinity and nutrient concentrations).

### 4.7. Seasonal accumulation of heavy metals by macroalgae

Macroalgae have been used in studying the seasonal and long-term variations of heavy metal levels in the marine environment (Villares et al., 2002; Ho et al., 1990; Haritonidis and Malea, 1999; Malea and Haritonidis, 1999a). Most of these studies were conducted in temperate areas, and a few studies observed that some heavy metals fluctuated seasonally (Malea and Haritonidis, 1999a), while others found no clear seasonal patterns but rather monthly irregular fluctuations (O’Leary and Breen, 1998; Malea and Haritonidis, 1999b). In the present study, heavy metal levels were found to vary with no clear seasonal pattern. A few metals at certain study sites displayed a tendency to increase during rainfall, but this was not significant. Concentrations of various metals were higher at those sites located near anthropogenic inputs than at sites located away from such inputs. The observed fluctuation of heavy metal levels could be influenced by factors that affect heavy metal bioavailability. However, a known major factor affecting heavy metal bioavailability is the heavy metal concentration itself in the environment. Hence, sources of contamination at local study sites could have had an influence on the observed fluctuations.
Figure 9. Protein expression pattern revealed in the 2D PAGE of *Ulva reticulata*. Immobiline dry strip pI range 4–7, SDS Gel 12.5%. Gel stained with SYPRO Ruby. White arrows indicate spots excised for identification with MALDI TOF mass spectrometer (Paper III).

The heavy metal concentrations in the Zanzibar Channel fluctuated significantly (Two Way ANOVA, p<0.05) between both years and sites (Paper II). Even though some sites were close to each other, for example, the Oyster Bay and Ocean Road or the Chapwani and Maruhubi sites, the long-term heavy metal concentration patterns at them could differ completely. These differences were likely caused by localized sources of metals influencing the long-term trends of heavy metal levels at the study sites. Environmental factors affecting bioavailability might also have influenced the long-term fluctuation of metal levels at the study sites.

The Ocean Road site was found to have higher levels of heavy metals than the other sites did, most likely because of its proximity to a trunk sewer pipe and to the Dar es Salaam harbour. A similar study site in Greece was also found to receive a large amount of heavy metals from a sewer pipe and harbour (Haritonidis and Malea, 1995). Compared to the Dar es Salaam study sites, the Zanzibar sites were found to have lower concentrations of heavy metals. This is not surprising, because Dar es Salaam has more anthropogenic pollution than Zanzibar does, due to its higher number of industries and larger population. An overall similarity analysis of heavy metal levels at the various study sites for all years between 1994 and 2005 grouped sites according to close similarity in
heavy metal concentration patterns (Fig. 10, Paper II). Ocean Road was singled out as the most contaminated study site. Study sites such as Nungwi and Maruhubi, which were found to have lower concentrations of various metals, were placed into a single group. Comparison with the results of other studies indicates that the Zanzibar Channel is less contaminated with various metals than many other such areas.

**Figure. 10.** Similarity classification (cluster analysis) of the sampling stations for different concentrations of heavy metals in *P. gymnospora* collected from the Zanzibar Channel from 1994 to 2005.
5. General conclusions

In summary, the present study found that, even though macroalgal accumulation of heavy metals largely depends on the available concentration in the growth medium, factors such as salinity and nutrient concentration can affect the accumulation significantly. This is important to consider when using these algae as indicators of heavy metal loads in field environments where there are high salinity fluctuations or high nutrient inputs.

Seasonal and long-term studies of heavy metals in the Zanzibar Channel revealed significant monthly and yearly fluctuations of concentrations, probably due to variations in local sources of heavy metals in the study areas and/or the influence of abiotic and biotic factors on heavy metal accumulation. The Ocean Road and Chapwani study sites were found to be the most contaminated sites in Dar es Salaam and Zanzibar, respectively, because they were close to harbours and raw sewage inputs. It is recommended that the high fluctuations in metal content of bioindicators is considered in future studies involving macroalgae as indicators of heavy metals, since the choice of sampling time might affect the accumulation levels of the alga. To determine accurately whether heavy metal contamination is increasing or decreasing in a certain area, one should follow the trends for a number of years.

The level of DGR and Fv/Fm inhibition caused by the metals depended on the metal concentration in the growth media. The DGR of *P. gymnospora* was thus a more sensitive indicator of stress than was Fv/Fm. However, high salinity and high nutrient concentrations in the growth medium reduced the toxicity of the metals. The protein expression pattern changed drastically in *U. reticulata*, even at 1µg Cu /l; in the future, this phenomenon could possibly be developed into a useful indicator of heavy metal stress in macroalgae.
6. Future perspectives

1. It will be important to examine in the future, seasonal and long term studies to monitor the status of heavy metal concentrations in order to evaluate increasing or decreasing of heavy metals.

2. Other environmental factors, such as temperature and light, and their effects on the accumulation and toxicity of heavy metals are poorly understood, especially in combination with other factors, and hence merit research efforts.

3. Separating proteins by fractionation in the future would be more reproducible, allowing for more comprehensive analysis of the expression of many proteins via 2D PAGE.

4. The identification of proteins was limited in this study by limited database resources. However, by employing other identification techniques, for example, using a tandem mass spectrometer along with a sequence similarity search, it might be possible to identify more proteins, which could help explain the mechanisms involved in Cu toxicity responses. In addition, not all the proteins spots were excised for identification, so there is still a chance in the future to identify other more conserved proteins using MALDI TOF.

5. Protein database resources are updated frequently, and new protein sequence information is added as available. This creates another opportunity to identify more as-yet-unidentified proteins in the future, using the peptide mass data we already possess.
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Carreras, H.A. and M.L. Pignata. Effects of the heavy metals Cu(2+), Ni(2+), Pb(2+), and Zn(2+) on some physiological parameters of the lichen Usnea amblyocladá. Ecotoxicol. and Environ. Safety. In press.
411–418.
Haritonidis, S. and P. Malea (1995). Seasonal and local variation of Cr, Ni and Co concentrations in Ulva rigida C. Agardh and Enteromorpha linza Linnaeus from Thermaikos Gulf, Greece. En-


Munda, I. (1986). The effects of Zn, Mn and Co accumulation on growth and chemical composition of *Fucus vesiculosus* under different temperature and salinity conditions. Mar. Ecol. 9:


Pinto, E., T.C.S. Sigaud-Kutner, M.A.S. Leitão, O.K. Okamoto, D. Morse, and P. Colepicolo (2003). Heavy metal-induced oxida-


Villares, R., X. Puente and A. Carballeira (2002). Seasonal variation and background levels of heavy metals in two green seaweeds. Env. Poll. 119: 79–90.


