CHAPTER 5

DISCUSSION

5.0 Discussion

Species of brown and green macroalgae have been employed most frequently as biomonitors or bioaccumulation indicators of heavy metal pollution in the marine environment. The red algae and other macrophytes such as seagrasses have also been used in this fashion in particular circumstances (Phillips, 1994).

Studies in both the laboratory and the field have provided valuable insights into the capacity of seaweeds to act as biomonitors of heavy metals. It was concluded that the analysis of macroalgae often provides useful information on the contamination of estuaries and coastal waters by heavy metals. However, certain enviromental variables may significantly affect accumulation of metal by algae, interfering with their use as biomonitors.

5.1 Effect of initial metal concentration on bioaccumulation in 24 h experiment

5.1.1 Metal accumulation patterns

The present study showed that the patterns of heavy metal accumulation in seaweeds are mainly dependent on the metal and seaweed species. Five patterns of heavy metal accummulation were observed;

Pattern 1 : An initial rapid uptake, followed by a gradual accumulation till 24 h.

Pattern 2 : A gradual accumulation for the entire 24 h.

Pattern 3 : An initial rapid uptake, followed by a release-uptake pattern before a steady state concentration or gradual accumulation continued till 24 h.

Pattern 4 : An initial net accumulative pattern, followed by a continuous regulatory discharge till 24 h.

Pattern 5 : An alternating uptake-release pattern throughout the 24 h.

Pattern 1, 2 and 3 are categorised as net accumulative patterns as suggested by Rainbow (1993). Pattern 4 is a clear continuous regulatory process. Pattern 5 seems to be a combination of the net accumulation pattern and pattern 4. The findings of the 28 sets of experiments carried out are summarised in Table 5.1.

The effect of metal concentration on accumulation patterns was only found in seaweeds with net accumulative patterns (as shown in Table 5.1).

5.1.2 Metal accumulation mechanisms

Initial rapid accumulation was evident in most of the patterns observed; the rapid uptake occurred within the first

Table 5.1 : Summary of the results of the 28 sets of experiments conducted in the study

Accumulation pattern	Seaweed species						
	C.l.	P.t.	S.s	S.b.	G.c.	G.e	G.s.
1	Cu(1 mgl^{-1})* Zn(10 mgl^{-1})* Mn	Mn Cd	Zn Mn(10 mgl^{-1})* Cd	Mn	-	Cd	Zn Cd
2	Zn	-	Zn(1 mgl^{-1})* Mn	-	-	-	-
3	Cu	Cu Zn Mn(10 mgl^{-1})*	Cu	Cu Zn Cd	-	-	-
4	-	-	-	-	Mn Cd	Mn	Mn
5	Cd	-	-	-	Cu Zn	Cu Zn	Cu

* : Concentration of metal specified
C.l. : *Chaetomorpha linum*
P.t. : *Padina tetrastomatica*
S.s : *Sargassum siliquosum*
S.b. : *Sargassum baccularia*
G.c. : *Gracilaria changii*
G.e. : *Gracilaria edulis*
G.s. : *Gracilaria salicornia*

hour of exposure. The uptake of Zn in *Padina gymnospora* (Karez *et al.*, 1994) and that of Cu, Mn, Ni, Pb and Zn in *Sargassum pallidum* (Tropin and Zolutukhina, 1994) has been shown to follow a similar trend. The rapid, reversible physicochemical uptake corresponds mainly to passive uptake involving cell surface adsorption, simple diffusion into cells or intracellular spaces (Karez *et al.*, 1994) and ion-exchange phenomena (Haug and Smidsrod, 1967); identified as first phase process. Reed and Gadd (1990) reported that the first phase process involves interactions between metal ions and reactive groups, followed by inorganic deposition of increased quantities of metal. This may lead to great amounts of metals being accumulated and means that such uptake cannot be interpreted solely in terms of ion-exchange phenomena. In many marine macroalgae the time period of passive sorption may be significantly extended (Reed and Gadd, 1990). An example, Cu accumulation in dead thalli of *Enteromorpha intestinalis* showed no evidence of equilibrium within 24 h (Reed and Darrig, 1983). As such, the period could vary among species and metal bound. Passive uptake is mostly unaffected by metabolic poisons, modest variations in temperature, and/or light-dark treatment (Gadd, 1988) because it doesn't require a catalyst or a living biological system to respond.

An initial rapid accumulation was followed by a slower uptake; identified as second phase process, or in some cases a continuous or non-continuous excretion (regulatory measures) in the seaweeds. The slower uptake could be due to metal ions penetrating into the cells via usage of energy. Reed and Gadd (1990) regarded this as intraprotoplast uptake, in contrast to rapid physical binding or biosorption. This process has been identified as the metabolically-dependent phase (Munda and Hudnik, 1991). However, in certain cases, algicidal concentrations of heavy metals may induce passive metal accumulation within the protoplast, due to permeabilization of the cellular membrane systems and the exposure of further sites for metal binding (Gadd, 1988). Skipnes *et al* (1975) and McLean and Williamson (1977) reported that the accumulation of Zn and Cd by macroalgae is generally considered to be due to active uptake. Consequently, metal content is greatly dependent both on external factors which affect metal interactions with the cell wall (pH, salinity, inorganic and organic complexing molecules) and on chemico-physical parameters which control metabolic rate (temperature, light, oxygen and nutrients) (Favero *et al.*, 1996). Since metal binding on the external wall is a continuous process during the life span of algae, older tissues usually contain higher amounts of many metals (Shimshock *et al.*, 1992; Barreiro *et al.*, 1993).

The regulatory mechanism which may have played a part in this study could have been the development of energy driven efflux pumps that kept metal levels low in seaweed tissues (Wood and Wang, 1983). Decreased internal accumulation of a heavy metal has been proposed as a mechanism of tolerance in certain algae, e.g. nontolerant plants of *Ectocarpus siliculosus* contained up to seven times more Cu than their tolerant counterparts (Hall, 1981). This physical exclusion mechanism may account, in part, for the cotolerance of fouling isolates of *Ectocarpus siliculosus* to elevated levels of Co and Zn (Hall, 1980). Sometimes, the development of metal tolerance is not coupled to a decrease in metal uptake (Reed and Gadd, 1990).

5.2 Heavy metal accumulation patterns in individual species

5.2.1. *Chaetomorpha linum*

Four metal accumulation patterns were observed : i) An initial rapid uptake followed by increase throughout the 24 h ; ii) A rapid uptake followed by a release-uptake pattern before accumulation continued till 24 h ; iii) A continuous gradual uptake pattern during the entire 24 h ; iv) A net uptake followed by a continuous regulatory discharge pattern. The first 3 patterns are indicative of net accumulation. The net accumulative patterns were only observed for exposures to

Cu, Zn and Mn. These patterns in *C. linum* suggest the potential of high bioaccumulation and non-regulation of metal uptake. Ganesan *et al.* (1991) reported that *Chaetomorpha antennina* and other seaweeds readily accumulate Cu from seawater and could indicate the contamination levels. High metal accumulation is attributed to metal ions being irreversibly held within cell wall matrix of thallus (Kesava Rao, 1992).

After 24 h, at all concentrations tested, net metal accumulation in tissues of *C. linum* increased with increasing initial external metal concentrations. As a result, the correlation coefficients obtained for this relationship were also high (see Table 4.8). Wong *et al.* (1979), Ho (1987) and Rajendran *et al.* (1993) indicated that higher seawater metal levels lead to a greater bioaccumulation of metals in seaweeds while working with *Chaetomorpha* sp. Furthermore, the high positive correlation coefficient (see Table 4.10) between individual metal level in the seaweed tissues and exposure time, suggest that these three metals could be bound to compounds that are metal specific, thus making the uptake process irrevisible. Field surveys on *Blindingia minima*, *Enteromorpha linza*, *Ulva* sp., showed a good linear relationship between metal content in seaweeds and metal concentration in seawater (Seeliger and

Edwards, 1977). Similar strong finding was also evident in this study for *C. linum* when high correlation coefficients were obtained (see Table 4.9).

Continuous regulatory discharge patterns observed in this species when exposed to Cd, suggest a metal excretion mechanism which resulted in reducing bioaccumulated concentrations in the tissues (Phillips and Rainbow, 1989). This evidence was further highlighted by the poor correlation coefficient (ranging from -0.4517 - -0.0590, $p < 0.05$) (see Table 4.10) obtained between metal levels in the *C. linum* tissues and exposure time. Magalhaes *et al.* (1994) suggested that when metals are bound to compounds that are non-metal specific, eventual release of metals from seaweed tissues is inevitable. In some green algae, a subsequent release of metals has been postulated (Matzku and Broda, 1970).

5.2.2 *Padina tetrastomatica*

Two metal accumulation patterns were observed : i) A rapid uptake followed by gradual increase throughout the 24 h ; ii) An initial rapid uptake followed by a release-uptake pattern, before accumulation continued till 24 h. The two patterns are indicative of net accumulation. After 24 h, net metal accumulation in the seaweed tissues increased with increasing initial external metal concentrations. High correlation coefficients (see Table 4.8) obtained for this relationship proves the above statement.

Furthermore, correlation between metal accumulated in *P. tetrastomatica* and time of exposure revealed a good linear relationship (see Table 4.10). A similar study was carried out by Karez *et al.*(1994) using *P. gymnospora* exposed to Zn for 48 h. The study showed over the time of exposure, metal could be strongly bound to cellular sites with no subsequent release. This resulted in the net accumulation pattern obtained for this species as well as in *P. tetrastomatica* in the present study. Correlation between metal content in *P. tetrastomatica* and final metal concentration in seawater revealed a positive relationship (see Table 4.9). This was also evident in *P. gymnospora* (Karez *et al.*, 1994) and *Laminaria digitata* (Bryan, 1969). Field survey by Sheila (1993) (see Table 2.6) showed that *P. tetrastomatica* had positive relationship for Cd ($r=0.8593$, $p < 0.05$) only, while this 24 h study revealed that the relationship was positive for all the metals tested.

5.2.3 *Sargassum* species

Both the *Sargassum* species in the present study showed a net metal accumulation pattern for all the metals and metal concentrations tested. Three forms of net metal accumulation patterns were observed : i) An initial rapid uptake followed by gradual increase throughout the 24 h ; ii) A continuous gradual uptake during the entire 24 h ; iii) A rapid uptake followed by a release-uptake pattern before a steady state concentration or gradual accumulation continued.

Net accumulation patterns have also been shown in *Sargassum pallidum* exposed to Cd, Cu, Mn, Ni, Pb and Zn (Tropin and Zolutukhina, 1994). Such accumulation patterns may be due to the strong polyanionic groups of the sulphated polysaccharides and alginic acid in brown seaweeds. Metal ions bind strongly to the polyanionic groups and therefore, subsequent release is not possible (Bryan, 1969), which complies with the results obtained for *P. tetrastomatica* (see Section 5.2.2.)

After 24 h, metal contents in the seaweeds increased with increasing initial external metal concentrations. High correlation coefficients (see Table 4.8) obtained for this relationship proves the above statement. Furthermore, correlation analysis carried out between metal accumulated in both the *Sargassum* species and time of exposure, revealed a good linear relationship (see Table 4.10). Such non-regulative characteristic in *Sargassum* in response to metal ions suggest that *Sargassum* is a potential indicator of heavy metal pollution, especially in tropical waters. In comparison, *Fucus, Laminaria digitata* and *Ascophyllum nodosum* are brown seaweeds that are commonly used in temperate regions in heavy metal studies and have been shown to be good indicators of the bioavailable forms of metals in seawater (Bryan, 1969; Munda, 1982; Ho, 1984).

Availability of metal in seawater after 24 h correlated strongly with metal content in *Sargassum* (see Table 4.9). Similar laboratory based work on temperate species, such as *Fucus vesiculosus*, *Ascophyllum nodosum* (Bryan *at al.*, 1985) and *Fucus* (Gutknecht, 1965) have shown similar strong relationship. Field surveys for this correlation relationship, also proved the worthiness of brown alga as indicators of heavy metals. Seeliger and Edwards (1977) and Johansen *et al.* (1991) demonstrated strong correlation relationship while working with *Fucus vesiculosus* in the field. Whereas Sheila (1993) reported that the correlation coefficients were variable over the range of metal tested in *Sargassum siliquosum* in Malaysian (see Table 2.6).

5.2.4 *Gracilaria* species

In *Gracilaria*, the following metal accumulation patterns were shown: i) An initial rapid accumulation was followed by a gradual increase throughout 24 h; ii) An alternating uptake-release or vice versa patterns for the entire 24 h; iii) An initial net accumulation pattern was followed by a continuous regulatory discharge throughout 24 h. The first pattern is indicative of a net accumulation.

After 24 h, the Mn contents in *Gracilaria* increased with decreasing initial external Mn concentrations giving a negative or reverse correlation (see Table 4.8). Such a relationship was not observed in other metals tested. The reason for this trend is not known but observation indicate a good regulation mechanism that keeps Mn content low in seaweed tissues at higher external Mn concentrations or a higher affinity of *Gracilaria* to lower metal concentration (Sheila, 1993). As such, this trend may not be ideal for a good bioaccumulative indicator. Munda and Hudnik (1991) also reported that Mn content in *Fucus virsoides* proved to be the main distinguishing factor, exhibiting the widest variations over time of exposure, compared to other metals. Availability of metal in seawater after 24 h correlated with metal content in *Gracilaria* strongly for Cu, Zn and Cd exposure (see Table 4.9). Field results reported by Sheila (1993) showed that the best correlation was obtained only for Cd ($r=0.4731$, $p < 0.05$) (see Table 2.6) in *G. edulis*.

The alternating uptake-release pattern observed in all three *Gracilaria* species (Mn) and *G. changii* (Cd) could be due to a non-continuous regulatory mechanism that serves to maintain the ambient tissue metal concentration (Rainbow *et al.*, 1990). On the other hand, continuous regulatory patterns were observed with Cu (all three species) and Zn (only in *G. changii* and *G. edulis*). The poor correlation between metal accumulated in the *Gracilaria* species and time of exposure, reveals the existence of the regulatory mechanism (see Table 4.10) in the above mentioned species and metal tested, respectively. The continuous regulatory patterns may be due to excretion of excess metals to reduce the bioaccumulated metal concentrations in seaweeds; acting as a defence mechanism.

In *G. edulis* (Cd) and *G. salicornia* (Zn and Cd), metal uptake was probably not regulated and this contributed to the net accumulation patterns observed. Such accumulation patterns are advantageous if the seaweed is to be used as a metal indicator as more metals can be absorbed. The high affinities for these heavy metals (Zn and Cd) in *Gracilaria* is attributed to the high cellular content of the polysaccharide that exist in red seaweeds (Munda & Hudnik, 1991).

5.3 Comparison between species

The net accumulation patterns were observed for the four metals tested (as shown in Table 5.1), only in *P.*

tetrastomatica, *S. siliquosum* and *S. baccularia*. This observation suggests an accumulation strategy that is similar within the three brown seaweeds. The browns are known to produce polyphenols extensively which plays an important part in metal regulation in this seaweed class. Based on the net accumulation patterns obtained for these browns, it is suggested that regulation of metal uptake via exudation of metal binding polyphenols might not have taken place in this 24 h time course study. Excretion of metal has been reported for brown alga, *Padina gymnospora* by Magalhaes *et al.* (1994). The author reported that when metal are bound to non metal-specific compounds, eventual release of metal from these compounds is possible.

C. linum showed net accumulation patterns for the metals exposed except for Cd. Similarity of accumulation strategy is indicated for this Chlorophyta and the Phaeophyta exposed to Cu, Zn and Mn. The *Gracilaria* species failed to show any clear net accumulation patterns for Mn and Cu. This suggests a similar accumulation strategy within the *Gracilaria* genus. The actual regulation mechanism involved is unclear.

All the seaweed species except for *Gracilaria* showed the best affinity for Mn or Zn and the least affinity for Cd (see Fig. 4.8a-g). This is related to the preference shown by the binding compounds (intra and extra celullar), type of binding mechanisms (Geddie and Sutherland, 1994) and its availability to receive metal ions. Ion exchange or displacement mechanism is a mode of preferential binding which is very much related to metal affinity. Competition of metal ions are negligible due to the nature of these experiments which are conducted as single metal bioaccumulation studies. Introduction of elevated concentration of single metal compound minimises any competition from other metal ions in the existing natural seawater. Another reason to ponder would be, Mn is classified as essential metal unlike Cd. The metabolic requirements, hence active accumulation, for Mn could have influenced the high affinity shown by these seaweed species towards Mn (Favero *et al.*, 1996). As for *Gracilaria* species, the variation seen within this genus (see Fig. 4.8e-g) for metal affinity could be attributed to the levels of sulfation of the agar and carrageenan (Munda and Hudnik, 1991) and possibly existence of regulatory mechanisms which are quite diversified within the genus. Increasing net accumulation according to order of seaweeds species showed that *C. linum* and *P. tetrastomatica* generally had high accumulation relatively, for many metal concentrations. Whereas the *Gracilaria* species showed otherwise (an exception can be seen

Table 5.2 : Summary of results of the bioaccumulation study by varying the external initial metal concentration

Seaweed species	Metal	Accumulation pattern	Correlation between metal accumulated vs. time of exposure	Correlation between metal accumulated vs. available metal concentration in seawater
C. linum	Cu	Net	Strongly positive	Strongly positive
	Zn	Net	Strongly positive	Strongly positive
	Mn	Net	Strongly positive	Strongly positive
	Cd	Regulatory	Negative	Strongly positive
P. tetrastomatica	Cu	Net	Strongly positive	Strongly positive
	Zn	Net	Farely positive	Strongly positive
	Mn	Net	Strongly positive	Strongly positive
	Cd	Net	Farely positive	Strongly positive
S. siliquosum	Cu	Net	Strongly positive	Strongly positive
	Zn	Net	Strongly positive	Strongly positive
	Mn	Net	Strongly positive	Strongly positive
	Cd	Net	Strongly positive	Strongly positive
S. baccularia	Cu	Net	Strongly positive	Strongly positive
	Zn	Net	Strongly positive	Strongly positive
	Mn	Net	Strongly positive	Strongly positive
	Cd	Net	Strongly positive	Strongly positive
G. changii	Cu	Regulatory	Weakly positive	Strongly positive
	Zn	Regulatory	Negative	Strongly positive
	Mn	Regulatory	Indefinite	Negative
	Cd	Regulatory	Weakly positive	Strongly positive

G. edulis	Cu	Regulatory	Indefinite	Strongly positive
	Zn	Regulatory	Negative	Strongly positive
	Mn	Regulatory	Strongly positive	Strongly positive
	Cd	Net	Strongly positive	Strongly positive
G. salicornia	Cu	Regulatory	Indefinite	Strongly positive
	Zn	Net	Strongly positive	Strongly positive
	Mn	Regulatory	Farely positive	Negative
	Cd	Net	Strongly positive	Strongly positive

for certain *Gracilaria* species exposed to Mn).

Table 5.2 summarises results from this section. Seaweeds as potential bioaccumulative indicators for heavy metal pollution, should fulfill the following criteria, observed in this 24 h study; i) they should possess a net accumulation pattern; ii) correlation coefficient between metal accumulated in seaweeds and time of exposure should be strongly positive; iii) correlation coefficient between metal accumulated in seaweeds and available metal concentration in seawater should be strongly positive.

Table 5.2 also indicates that the brown seaweeds, *P.tetrastomatica* and the *Sargassum* species, exhibited these criteria satisfactorily for all the metals tested, followed by *C. linum* and *Gracilaria* species, thus proving that the browns are suitable organisms for heavy metal pollution monitoring studies as reported by many authors (Bryan, 1969; Bryan and Hummerstone, 1973; Bryan, 1983; Bryan *etal.*, 1985; Cullinane *et al.*, 1987; Foster, 1976; Fuge and James, 1973; Ho, 1995; Jayasekera and Rossbach, 1996; Johansen *et al.*, 1991; Kangas and Autio, 1986; Melhuus *et al.*, 1978; Molloy and Hills, 1996).

5.4 Effect of salinity on bioaccumulation in 2 h experiment

Salinity is a complex abiotic factor which affects the physicochemical characteristics of trace metals in estuaries as well as the organism/environmental complex, resulting in changes in the metal uptake (Munda, 1984). In the present study, exposure at lowered salinities increased uptake of metals in all seaweed species after 2 h of exposure for all the metal tested, except in *C. linum* exposed to Cd. This is further proven by the strong negative correlation coefficent (see Table 4.11) obtained between salinity and metal accumulated in seaweeds after 2 h. Previous experiments have already proven that metal accumulation in algal tissues is enhanced at lowered salinities (Munda, 1984; Bryan *et al.*, 1985; Munda and Hudnik, 1988).

The dilution enhanced accumulation can be tentatively explained by the decreased competition with other divalent ions in diluted media as well as by the selective interactions with alginates of the membranes (Munda and Hudnik, 1988). Favero *et al.* (1996) also reported that *Ulva rigida* stores larger amounts of Fe, Zn and Cd where salinity of the site was lower. Deviations from the general trend of salinity-dependent accumulation in *C. linum* exposed to Cd, may be due to interactions of the ions with components of the membranes or protein complexes within the cells (Munda and

Hudnik, 1988). Munda (1984) also reported similar deviation when exposing *Enteromorpha intestinalis* and *Scytosiphon lomentaria* to Co concentration of 4.9 mgL^{-1}. Result indicate that increasing net uptake of Co into these seaweed species decreased with the following sequence of salinities : 19.2ppt>7.7ppt>38.6ppt. Bryan *et al*. (1985) noted that the accumulation of Zn by macroalgae was not enhanced by lower salinities. Klumpp (1980) also recorded the similar observation on the uptake of As by *Fucus spiralis*.

Results confirmed the general trend of enhanced metal accumulation by marine algae under estuarine conditions; they also point to the necessity of taking environmental variables into consideration when interpreting metal accumulation in indicator organisms. Nevertheless, correlation coefficients between metal accumulated in seaweed tissues and time of exposure over the salinity range (see Table 4.13) indicate good relationship basically for all species, though exceptions can be made for *C. linum* exposed to Cd at salinity of 20 ppt ($r=0.2490$, $p < 0.05$) and *G. changii* exposed to Mn at 35 ppt ($r=-0.1653$, $p < 0.05$). These exceptions can be related to the regulative nature of metal accumulation which has been discussed previously in Section 5.2. Donard *et al.* (1987) reported that salinity influences the uptake and excretion of tin by species of the green algal genus

Enteromorpha, which was demonstrated to be active in the methylation and demethylation of tin. However, correlation coefficients between metal accumulated in seaweeds and final metal concentration in seawater after 2 h exposure, over the salinity range, showed strong relationship (see Table 4.12).

In terms of metal affinity at different salinities, collective findings sugggest that Mn showed the best affinities for the three seaweed species which again could be related to metabolic requirements. In contrast, Munda (1984) reported that Zn had the best affinity towards tissues of *Enteromorpha intestinalis* and *Scytosiphon lomentaria* at differing salinities, compared to Mn and Co.

5.5. Effect of pH on bioaccumulation in 2 h experiment

One of the important environmental variables is pH; in acidic conditions metals tend to exist as more toxic free, hydrated ions, whereas in alkaline media (including seawater) they may precipitate as insoluble complexes (Sunda and Guillard, 1976). Thus in many cases, an increase in pH will reduce bioaccumulation of metals and vice versa. However, in other instances, toxicity may be reduced at low pH; decreased uptake of metals at low pH was attributed to decreased transplasmalemma electrical potential, reducing the driving force for metal accumulation (Reed and Gadd, 1990).

Additionally, H^+ may compete with free metal ions for uptake sites and so a decrease in pH may lead to a decrease in heavy metal toxicity (Peterson *et al.*, 1984).

Exposure at increasing pH, increases net accumulation of metals, only in *C. linum* exposed to Cu and *S. siliquosum* exposed to Cu and Zn respectively (see Table 4.14). Correlation coefficient between pH and metal accumulated in seaweeds after 2 h showed strong positive relationship (see Table 4.15) overall, indicating that there was no antagonistic relationship between pH and metal accumulation in seaweeds, unlike that seen in Section 5.4. However, Table 5.3 did indicate that generally net accumulation of certain metals was not significantly different ($p>0.05$) in seaweed species, especially at pH ranging from 5-8 , which could suggests that pH-dependent uptake of metal is quite constant at this pH range. In *Ulva lactuca* (Gutknecht, 1963), increasing the pH from 7.3 to 8.6 promoted Zn^{65} uptake and retarded Zn^{65} loss. In *Chlamydomonas variabilis* (Harrison *et al.*, 1986), a constituent of phytoplankton, a decrease in pH from 7 to 5 led to lower values of Zn, which may be due both to a pH-induced change in algal surface potentials or to competition between H^+ and Zn^{2+} for specific binding sites on the cell surface. Furthermore, studies to evaluate biomass for metal-binding ability (Kuyucak and Volesky, 1989) have

Table 5.3 : Summary of the LSD multiple range analysis conducted for the effect of pH in correspond to net accumulation of metal in seaweeds after 2 h of exposure

Seaweed species	Metal type	pH range with no significant difference ($p>0.05$) in net metal accumulation
C. linum	Cu	Between pH 5,6,7 and 8
	Zn	Between pH 5 & 6 ; pH 7 & 8
	Cd	Between pH 5 &6 ; pH 6 & 7
S. siliquosum	Cu	Between pH 5 & 6 ; pH 6 & 7 ; pH 7 & 8
	Zn	Between pH 5, 6 & 7
	Cd	Between pH 5, 6 & 7
G. changii	Cu	Between pH 5, 6, 7 & 8
	Mn	-
	Cd	Between pH 4, 5, 6 & 7 ; pH 5, 6, 7 & 8

Note : This table is a summary from Appendix XVIII

pointed out that metal binding is pH-dependent and that this response differs with algal species, according to the chemical composition of the cell walls. Favero *et al.* (1996) postulated that variations in pH may be effective in the first phase of metal uptake in seaweeds; by changing the number of metal binding sites on the cell wall.

However, one clear indication in the present study is the significantly ($p<0.05$) lowered uptake of metal at pH 4, which may be due to a decreased electrical potential at the membrane, thus reducing bioaccumulation process (Reed and Gadd, 1990). This is further shown by the correlation coefficients between metal accumulated in seaweeds and time of exposure over the pH range (see Table 4.17). The Table show poor correlation at pH 4 relatively, for *C. linum*, *S. siliquosum* and *G. changii*. However, correlation was relatively poor at all pH for Cd exposure to *C. linum* and Mn and Cd exposure to *G. changii*. Whether regulation of metal uptake took place in *C. linum* and *G. changii* for these metal exposures, is not known. However, based on results from Section 5.2., possibilities are evident. Metal affinity differed among seaweed species, metal type and pH, unlike in Section 5.3.

5.6 Toxicity testing of heavy metals using seaweeds as test species

Algae are the primary producers of the marine environment and the most abundant in estuaries and coastal waters, regions that are most likely to be affected by the input of of toxic metals (Rai *et al.*, 1981), so any adverse effects of toxic metals on algae could have a significant effect on primary production. An example, 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 *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 severe problems to the Baltic Sea (Andersson and Kautsky, 1996). The *F. vesiculosus* belt offers a place for foraging and shelter for many organisms, including fish, and the plants also function as a substratum for epiphytes. A decline in population size, and even disappearance at some localities, has been noticed during recent years, and chemical pollution is one of the presumed possible reasons (Andersson *et al.*, 1992). As such, to use seaweeds as possible test species for toxicity testing of heavy metals would be of great importance.

The use of young growing seaweed plants for toxicity studies has its limitation due to being less sensitive compared to reproductive stages. Nevertheless it gives first hand knowledge on the state of toxicity of pollutants to these growing organisms. An example to show the discrepancy of results of toxicity is shown in a study of localization of Cd in *F. vesiculosus* by Lignell *et al.* (1982). The author found the highest metal content in the physodes and in the cell walls. The cell walls of brown algae contain alginate and fucoidan, polysaccharides that can bind cations. However, the effect of Cd is more detrimental to zygotes which are exposed to metals before the development of a protecting cell wall that can adsorb some of the Cd and thereby reduce the amount of metal taken up into the cells.

In the present study, *G. changii* was found to be the most sensitive species to Cd since this species exhibited a 50% inhibition in dry weight after 96 h in the range finding test (see Table 4.20a). Toxicity results for Cd exposure in 96 h definitive test conducted on *G. changii* : IC_{50} = 31.0714 $mgCdL^{-1}$, LOEC = 15 $mgCdL^{-1}$, NOEC < 15 $mgCdL^{-1}$ (see Table 4.34). In contrast, exposure of *C. linum* and *S. baccularia* to Cd in 96 h range finding test did not produce 50% inhibition in chlorophyll-a content and dry weight respectively (see Table 4.18a and 4.19a). Further exposure to Cd for 7 and 10 days showed that *G. changii* was still the most sensitive species (see Table 4.34). However, the inhibitory concentrations decreased with the incubation period used. The order of decreasing sensitivity to Cd exposure according to species is as follows : *G. changii*>*S. baccularia*>*C. linum*.

A similar experiment by Haglund *et al.* (1996) on *Gracilaria tenuistipitata* at two different salinities (6 and 25 ppt) for 96 h Cd^{2+} toxicity, showed EC_{50} values at 0.8 and 0.63 $mgCdL^{-1}$, respectively (see Table 5.4). Dry weight was also used as end point measurement in the study. By comparing the result with the present study (see Table 4.34), it is clearly seen that there is a big contrast in the respective inhibitory/effective concentration, which may be attributed to many environmental and physical factors. Nyholm (1985) reported that between laboratories, EC_{50} values varied by a factor of 1000. It appears that a method of quality control has to be developed (Wong, 1995). Table 5.4 shows the $EC_{50}(mgL^{-1})$ and NOEC (mgL^{-1}) values obtained for *G. tenuistipitata* exposed to metals at different salinities (Haglund *et al.*, 1996). The NOEC values between the two studies could not be compared due to values in the present study being below range.

C. linum was generally tolerant to both Cd and Cu (see Table 4.34). *S. baccularia* showed higher tolerance to Cd rather than Zn at day 7 of exposure while the opposite occurred at day 10. Whereas *G. changii* showed better tolerance to Mn than Cd at day 7 and 10, which again suggests the importance of Mn as essential metal in seaweeds.

Table 5.4 : Growth inhibition of *Gracilaria tenuistipitata* by different metals at varying salinities in a 96 h static test regime (Haglund *et al.*, 1996).

Metal	Salinity (ppt)	EC_{50} (mgL^{-1})	NOEC (mgL^{-1})
Cu(II)	6	0.05	0.013
Cu(II)	17	0.10	0.036
Cu(II)	25	0.12	0.042
Ni(II)	17	17	2.2
Pb(II)	17	4	0.45
Cr(VI)	6	0.6	0.04
Cr(VI)	17	2.2	0.26
Cr(VI)	25	23	0.95
Cd(II)	6	0.8	0.06
Cd(II)	25	0.63	0.01
Hg(II)	25	0.18	0.06

As for the use of young growing seaweeds in bioassay or toxicity tests, 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, Stromgren (1980 a and b) 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. When different metals are compared after 10 d exposure, a

50% reduction of growth rate occurs as shown in Table 5.5. Haglund *et al.* (1996) used maturing *Gracilaria tenuistipitata* as test species for toxicity assessment in marine and brackish environment. Growth in the form of dry weight was used as test variable.

Table 5.5 : Comparison between results obtained by Stromgren (1980a,b) with the present study by means of using maturing seaweed plants.

Seaweed species	Metal	Time of exposure	EC/IC_{50} value/range (mgL^{-1})
Pelvetia canaliculata *Fucus spiralis* *Fucus serratus* *Ascophyllum nodosum*	Cu	10 d	0.06-0.08
Fucus vesiculosus	Cu	10 d	0.05
Pelvetia canaliculata *Fucus spiralis* *Fucus serratus* *Ascophyllum nodosum*	Zn	10 d	5-10
Pelvetia canaliculata *Fucus spiralis* *Fucus serratus* *Ascophyllum nodosum*	Hg	10 d	0.1-0.2
Fucus spiralis *Fucus serratus* *Ascophyllum nodosum*	Pb	10 d	>2.6
Present study			
Chaetomorpha linum	Cd	10 d	3.1075
	Cu	10 d	8.2051
Sargassum baccularia	Cd	10 d	0.7487
	Zn	10 d	0.1342
Gracilaria changii	Cd	10 d	0.1345
	Mn	10 d	0.5931

Comparison of toxicity can be made between the Fucales and *Sargassum baccularia* in the present study, especially for Zn exposure, since both represent the class Phaeophytha (see Table 5.5). The table indicates that generally the Fucales species are more tolerant to Zn exposure, compared to *S. baccularia*. Zn is an essential micronutrient for algae and is necessary for efficient functioning of certain enzymes (Webster and Gadd, 1996). However at elevated concentrations, Zn can be toxic to algae. Differing tolerance of the brown seaweeds towards Zn exposure may relate to internal detoxification or metal transformations (Reed and Gadd, 1990). Another mechanism to ponder would be the stimulation of growth via respiration by high concentrations of Zn through an alternative respiratory pathway, which has been discussed for *Ulva lactuca* (Webster and Gadd, 1996).

Use of the reproductive phase of seaweeds to evoke early response of toxicity is gaining importance. Reproduction is a sensitive phase in the life cycle of an organism. It is crucial that it is effective if a population is to continue to survive in its environment (Eklund, 1995). There are very few studies on the effect of metals on seaweed reproduction. Chung and Brinkhuis (1986) reported that 0.01-0.05 $mgCuL^{-1}$, added to natural seawater , inhibits development of *Laminaria saccharina* gametophytes and young sporophytes. The work further showed that settlement and germination of meiospores were not affected up to 0.5 $mgCuL^{-1}$. North and James (1987) reported the use of *Cystoseira* and *Sargassum* embryonic sporophytes for testing toxicity effects of hydrazine. The sensitivity of the reproductive stages of *Fucus edentatus* and *F. saccharina* to hydrocarbons may be used to test for oil pollution (Steele and Hanisak, 1979). On the other hand, members of the Rhodophyta have been much less frequently used as toxicity or assay organisms (Staples *et al.*, 1995). Carcinogenic compounds isolated from marine muds were detected using cultured *Porphyra tenera* (Ishio *et al.*, 1972), and a wound repair hormone was demonstrated with *Griffithsia pacifica* (Waaland, 1975) and *Antithamnion sparsum* (Kim and Fritz, 1993). The measurement of growth and reproductive activity in *Champia parvula* formed the basis of a toxicity test (Steele and Thursby, 1983) that has been evaluated using arsenite, arsenate, several heavy metals and organic compounds (Thursby and Steele 1984,1986; Thursby *et al.*, 1985). *Chondrus crispus* and other seaweeds were used to evaluate the effects of pulp mill effluent in a marine ecosystem (Hellenbrand, 1979).

Andersson and Kautsky (1996) reported the effects of Cu on egg volume, fertilization, germination and development of apical hairs of Baltic Sea *Fucus vesiculosus*. Germination was found to be the most sensitive stage. Low concentration of Cu, 0.0025 $mgCuL^{-1}$, added to natural brackish water before fertilization, adversely affected germination at ambient conditions. An addition of 0.02 $mgCuL^{-1}$ caused about 70 to 80% decline in germination at ambient conditions. Cu(II) ions cause a loss of K^+, and changes in cell volume. When transported to the chloroplasts, Cu^{2+} inhibits electron transport to $NADP^+$. As reviewed by Chung and Brinkhuis (1986), Cu is believed to affect the Fe transportation into plastids or the incorporation of Fe into Fe-binding enzymes and may also be responsible for peroxidative degradation of chloroplast membrane lipids.

Eklund (1995) described a method to appraise toxicity of metals in terms of inhibition of fertilisation frequency of the marine red alga *Ceramium strictum* after 24 h exposure period to the toxicant and total test time of 7 days. Table 5.6 shows the EC_{50} obtained from this test.

Table 5.6 : EC_{50} values for single compounds using the reproduction test method with *Ceramium strictum* (Eklund, 1995).

Single metal compound	EC_{50} value (mgL^{-1})
Cu^{2+}	0.016
Hg^{2+}	0.015
Cd^{2+}	3.3
Zn^{2+}	0.099
Cr^{6+}	4.6

By comparing results obtained from Table 5.6 with Table 4.34, it is evident that reproduction test methods evoke an earlier response to metal toxicity rather than adult seaweed plants. Similarly, the growth rate response of *Gracilaria tenuistipitata* to Cu (EC_{50} = 0.1 mgL^{-1} at 25 ppt) (Haglund *et al.*, 1996) may be compared with the cystocarp development response at 20 ppt salinity of *Ceramium strictum* to the same substance (EC_{50} = 0.016 mgL^{-1}) (Eklund, 1995). The latter test showed more sensitivity to Cu, which may be due to the involvement of reproductive stages. Reproductive stages of seaweeds will experience the effects of toxic compounds released into aquatic media sooner than adult plants. In this respect, they may be able to act as an "early warning signal" of impending contaminant impacts of aquatic environment (Doust *et al.*, 1994).

The single species toxicity test has provided the great majority of data used in evaluating the hazard of waste materials and is an unsurpassed tool for studies of relative sensitivity of organisms, relative toxicity of chemicals or effluents, or effects on population level responses such as growth or reproduction (Cairns, Jr. and Niederlehner, 1987). However, these tests are also widely used to derive limits of exposure to protect entire ecosystems, which play great importance when formulating Criteria of Marine Water Quality for Marine Life Conservation in the respective countries.

5.7 Linking biavailability of metals in seawater with bioaccumulation of metals in seaweeds and toxicity

Knowing the fact that seaweeds only respond to dissolved metals (bioavailable metals) in seawater, it may be relatively simpler to simulate the integrated exposures that occur in complex, dynamic ecosystems and relate those to toxicity. However, the multifactorial ecosystem, namely, biological and abiotic factors regulate the availability, uptake and retention of metals in algae. These include : the form of metal in seawater, the presence of other metals or substances in solution, including inorganic ligands and organic matter, in dissolved colloidal, and particulate fractions; the physicochemical regime of seawater including temperature, salinity, light, pH, water hardness, redox potential and dissolved gasses; biological factors such as the age, metabolism, condition, nutritional status of the seaweeds and other factors, namely species variation (Phillips, 1977; Bryan, 1976; Rai *et al.*, 1981).

In Section 5.2, 5.4, 5.5, three different environmental parameters of the seawater were studied separately. The results sugggest the influence of each parameter on metal accumulation in seaweeds, respectively. But to simulate the actual conditions in the environment, simultaneous effects of different combination of chemicophysical parameters should be studied eventually. Munda and Veber (1996) studied effects of trace metals and excess nutrients on the Adriatic seaweed *Fucus virsoides*, which revealed metal accumulation of a totally different nature compared to studies with single parameter effect. Synergistic interrelationships were present between metals when Favero *et al.* (1996) studied metal accumulation in *Ulva rigida*.

The ability of algae to survive and reproduce in metal-polluted habitats may depend on tolerance over extended time periods (Reed and Gadd, 1990). It is possible that populations undergo genetic adaptation to long term exposure to elevated metal levels (Gledhill *et al.*, 1997). High bioavailability of metals in seawater leads to high bioaccumulation of metals in seaweeds and may, finally, due to breakdown of regulation mechanisms, leads to toxicity and eventual death. Results of toxicity tests (as shown in Table 4.34) to Cd exposure showed that *Chaetomorpha linum* was the most tolerant species. This tolerance may relate to regulatory measures in the seaweed species, which has already been discussed in Section 5.2. Due to the existence of such mechanism, *C. linum* may be more tolerant to Cd toxicity, comparatively to other species. Hence, a link is formed between metal availability, accumulation, regulation and toxicity. The effects of heavy metal toxicity in algae may include (Reed and Gadd, 1990) : (1) an irreversible increase in plasmalemma permeability, leading to the loss of cell solutes and changes in cell volume; (2) a reduction in photosynthetic electron trasport and photosynthetic carbon fixation; (3) the inhibition of respiratory oxygen consumption; (4) the disruption of nutrient uptake processes; (5) enzyme inhibition, due to displacement of essential metal ions; (6) inhibition of protein synthesis; (7) abnormal morphological development and ultrastructural changes (including mitichondrial swelling, granulation, multinucleation, and alterations in vacuolar and chloroplast size); and (8) the degradation of photosynthetic pigments, coupled with reductions in growth and in extreme case, cell mortality.

Though tolerance via regulation of uptake is evident in seaweeds, continuous exposure at elevated metal concentration may prove too toxic eventually, especially for plants at reproductive stages. However, work by Andersson and Kautsky on *Fucus vesiculosus* (1996) showed that at a salinity close to optimum (14 ppt) , no negative effect was noticed on germination when 0.02 CumgL^{-1} was added to seawater. At the salinity of 6 and 20 ppt, there was about 70 to 80% decline in germination. This result suggested that the degree of salinity stress acting upon the zygotes of *F. vesiculosus* is a more important factor than the influence of salinity on Cu bioavailability. When 0.0025 to 0.06 $mgCuL^{-1}$ was added to the medium 24 h after fertilization, the zygotes were more resistant, resembling the response of adult marine fucoid tissue.

Inherent abilities for seaweeds to withstand pollution in the field have been sited for brown (*Fucus* spp. and *Laminaria digitata*) and green (*Ulva* spp. and *Enteromorpha* spp.) seaweeds (Bryan, 1983; Seeliger and Cordazzo, 1982; Ho, 1990). Swanson *et al.* (1991) reviewed the use of algae in toxicity tests and concluded that, in general rooted aquatic macrophytes are more sensitive to most toxicants than are algae. However, these observations likely result from the fact that rooted macrophytes are exposed to both sediment-associated toxicants and waterborne pollutants, whereas, algae are only exposed to the latter (Doust *et al.*, 1994). So waterborne pollutants are best detected, monitored and assessed of their toxicities using algae or any other free floating plants like *Lemna minor*.

5.8 Appraisal of study and areas for future research

This study formed part of an ongoing project on the use of seaweeds as biomonitors of heavy metal pollution in the estuarine and coastal environment of Malaysia. As this was the first proper preliminary laboratory based bioaccumulation study in the project, not many species were used, due to difficulty in collecting samples over the study period. In fact, *Chaetomorpha linum*, *Padina tetrastomatica* and the *Gracilaria* species were found throughout the year in the localities and they may represent the changing environment effectively in the field compared to other seasonally arising species. Representation of seaweed species from the same genus such as *Sargassum* and *Gracilaria* were used to observe whether similarity arises in the metal bioaccumulation processes within a genus.

Laboratory analysis involves demanding sample treatments. The acquirement of NIES No. 9 *Sargasso* as the certified reference material for seaweed digestion technique has validified the technique used in the study (see Section 3.3.8.1). The incorporation of standard addition test as quality control measurements for extraction of low metal levels in seawater, is essential in every batch of sample testing (see 3.3.8.2).

Problem of precipitation was encountered during preparation of metal solution in seawater. This was overcome by acidifying the media which resulted in pH levels different from the actual conditions (the changes were generally small). However, the controls were always adjusted to the same pH as the test media. Errors arising from the preparation of fixed test media metal concentrations could be minimised with careful measurements and metal contamination free environment.

Diffferent elevated levels of external metal concentrations, were used in the study, to show pronounced metal uptake. However, the 24 h duration study did not clearly reveal a plateau phase, as such, bioconcentration factors could not be calculated.

Seaweed plants used for studies were sampled from the same locality throughout the study period. Healthy and clean appearance, young growing plants were chosen for studies. Selection was done visually. Background content of metals in seaweeds were derived from triplicate samples of a whole plant (without the holdfast). All this procedures were meant to minimise experimental errors.

Cd was given preference in this studies as it could be used as a reference toxicant, hereafter. Toxicity tests for each seaweed species were conducted concurrently for both metal exposure to minimise experimental errors caused by species conditions and variations. Use of dry weight as growth parameter in seaweeds is acceptable if number of replicates per treatment is increased to more than three. In order to obtain a more detailed results for NOEC, the working concentration range has to be changed to a lower range.

The results achieved in this study, warrants for further research, which include :

i) a longer period of metal exposure to seaweeds at levels not toxic to the organisms and preferably till the accumulation levels reach plateau stage.

ii) use of other potentially toxic metals such as Hg and As in both bioaccumulation and toxicity studies.

iii) effects of other abiotic and biotic factors on metal accumulation process in seaweeds. Single, dual or multi combination effects of these factors on metal accumulation is necessary.

iv) the effects of metal on other growth parameters such as increase in thallus length, apical segments etc. should be studied.

v) effects of metal on physiological changes in seaweeds such as cell viability, germination etc should be emphasised.

vi) ultrastructural changes in seaweeds caused by metal toxicity, using the electron microscopy should be studied.

vii) the use of reproductive stages of seaweeds as toxicity test material should be stressed.

viii) development of uniformal seaweed clones for metal accumulation and toxicity studies in the laboratory should be carried out.