CHAPTER 3

MATERIALS

AND

METHODS

3.0 Materials and Methods

3.1 Bioaccumulation studies

This study was conducted to characterise the heavy metal bioaccumulation of selected seaweed species. The effect of the following factors on heavy metal accumulation by the seaweeds was studied :

i) initial heavy metal concentration of test solution.

ii) salinity of test solution.

iii) pH of test solution.

Each factor was tested separetely.

3.1.1 Seaweed species. Seven species representing three classes of seaweeds were used in this study (as shown in Table 3.1 and Plate 1-7).

These species were selected based on the following criteria:

i) They had high contents of heavy metals as observed from a previous field survey (Sheila, 1993).

ii) They are found throughout the year; collecting material for study would not pose a problem.

iii) *Sargassum* and *Gracilaria* are species of great economic importance, being sources of phycocolloids. *Gracilaria* is also eaten fresh as a salad while *Sargassum* is used in herbal soups.

iv) These species are commonly found along much of the Malaysia coastline.

Table 3.1 : Seaweed species, collection sites and habitats

Seaweed	Collection site	Habitat
Chlorophyta (Green)		
Chaetomorpha linum (O.F. Mueller) Kuetzing	Port Dickson	Epiphytic on roots and trunks of mangrove tree
Phaeophyta (Brown)		
Padina tetrastomatica (Hauck)	Port Dickson	Silted muddy substrate, closer to shore
Sargassum siliquosum (J. Agardh)	Port Dickson	On reef flats
Sargassum baccularia (Mertens) C. Agardh	Port Dickson	On reef flats
Rhodophyta		
Gracilaria changii Xia & Abbott (Abbott, Zhang & Xia)	Morib,Carey Island	Muddy substratum at mangrove area
Gracilaria edulis (Gmelin) Silva	Morib	Muddy substratum at mangrove area
Gracilaria salicornia C. Agardh	Morib	Muddy substratum at mangrove area

3.1.2 Methods of seaweed sampling and storage. Seaweeds were collected from the wild populations. Seaweed samples collected were as far as possible of the same age (young full grown), size with a healthy appearance. Samples were handpicked at low tide as the water receded, and the entire plant was collected. Then they were placed in plastic bags and stored in the shade until they were brought back to the laboratory within 2-3 hours.

Plate 1 : *Chaetomorpha linum.*

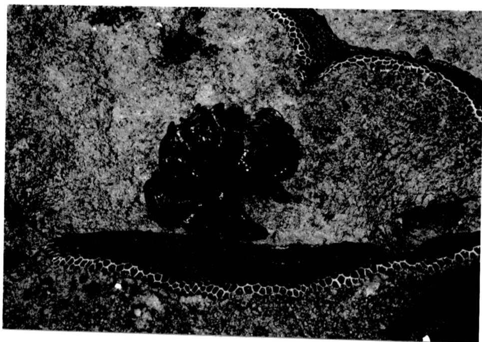

Plate 2 : *Padina tetrastomatica*

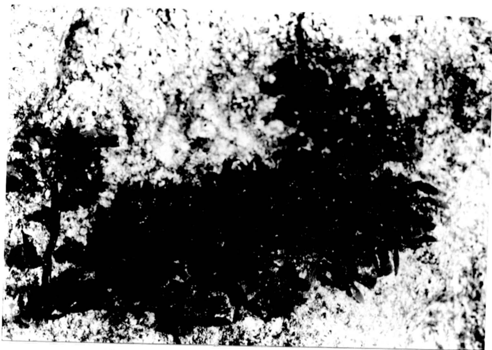

Plate 3 :*Sargassum siliquosum*

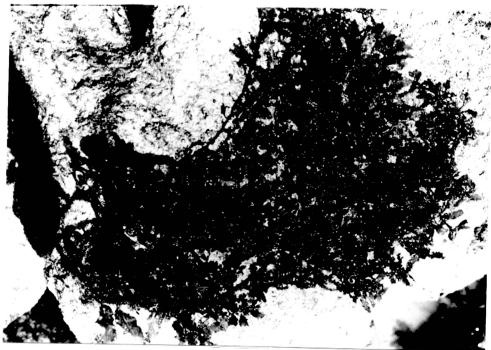

Plate 4 : *Sargassum baccularia*

Plate 5 : *Gracilaria changii*

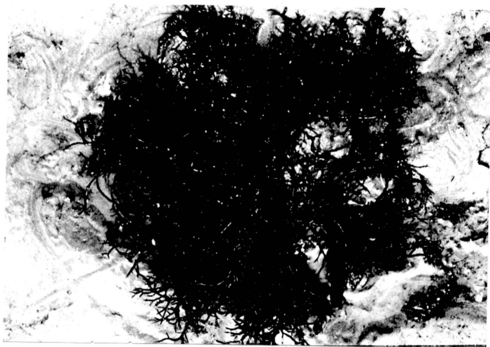

Plate 6 : *Gracilaria edulis*

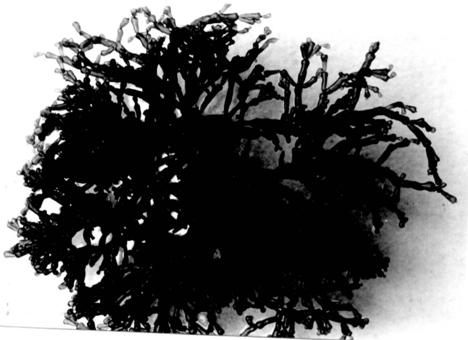

Plate 7 : *Gracilaria salicornia*

3.1.3 Preparation of seaweeds and seawater for experiments. In the laboratory, the seaweeds were cleaned thoroughly with seawater to wash off associated epiphytes and debris. The holdfasts were also removed. Natural seawater used for the experiments was collected from the Selangor Agriculture Development Corporation Hatchery seawater reserve at Jeram, Selangor. The seawater was filtered through 0.45 μm Millipore membrane filters. The concentration of the selected trace metal present naturally in the seawater are shown in Appendix XXIV. Cleaned seaweeds were conditioned in the filtered seawater in aerated plastic aquaria. The aquaria were placed on lighted shelves with an average light intensity of 33.65 μmol photon $m^{-2}s^{-1}$ with 12:12 h Light:Dark cycle, and average water temperature of $28^{o}C$. Each aquarium had been washed in 20% Nitric acid (Analytical Grade).

3.1.4 Cleaning of apparatus. Glassware and polyethylene containers used to prepare and store seawater, seaweeds, metal stock solutions and metal test solutions are cleaned before use. They were first washed with detergent (Teepol) and rinsed with tap water. Then all glasswares were soaked in 10% concentrated nitric acid overnight and rinsed twice with distilled deionised water the following day. Prior to use, all washed items were dried in the oven. At the end of experiments, all items that were to be used again were

immediately emptied, and subjected to the procedure that is mentioned above. Acid was used to remove mineral deposits.

3.2 Metal range determination studies

Prior to the metal bioaccumulation studies, seaweeds were exposed to a range of test metal concentrations in a 24 h static toxicity test. This test was conducted to find the suitable range of metal concentrations that did not kill or damage the seaweed tissues in a 24 h exposure experiment. This non-toxic range of heavy metal concentrations is important when conducting a bioaccumulation study in seaweeds because the bioaccumulation processes require living biological systems. Exposure to this range of metal concentrations would ensure that the bioaccumulation process observed would include both passive and active processes as observed in living plants and not just adsorption by dead or damaged plants.

3.2.1 Seaweed species. All seven seaweed species were subjected to this test (Table 3.1).

3.2.2 Metal tested. All four metal species, Cu^{2+}, Zn^{2+}, Mn^{2+} and Cd^{2+}, in a range of concentrations were tested individually for each seaweed species.

3.2.3 Range of metal concentrations. Increasing metal concentrations of 0.1, 1, 5, 10, 20, 50 and 100 mgL^{-1} were

prepared in filtered seawater (pH 8.2 ; salinity 30 ppt) using the respective metal stock solution (see Appendix I for preparation of stock solutions and metal concentrations). Metal stock solutions were prepared from metal salts such as $CuSO_4.5H_2O$, $ZnSO_4.7H_2O$, $MnCl_2.4H_2O$ and $CdCl_2.H_2O$ respectively. Any precipitation formed during preparation of test solutions in seawater were dissolved using concentrated nitric acid (Analar grade). The resulting pH of the test solutions at each concentrations for the respective metal species are shown in Table 3.2.

Table 3.2 : pH of test solutions at the initial hour (0 h).

Metal	pH							
	Concentrations (mgL^{-1})							
	0	0.1	1	5	10	20	50	100
Cu^{2+} *	8.2	8.0	8.0	7.5	6.5	6.3	6.1	6.1
Zn^{2+} *	8.2	8.1	8.0	8.0	7.8	7.8	7.8	7.5
Mn^{2+}	8.2	8.2	8.2	8.1	8.1	8.0	7.9	7.5
Cd^{2+}	8.2	8.2	8.2	7.9	7.8	7.8	7.5	7.3

* - where nitric acid was used to dissolve precipitation

3.2.4 Type of test. Static exposure system.

3.2.5 Duration of test. 24 h

3.2.6 Experimental procedure. Metal concentrations of 0.1, 1, 5, 10, 20, 50 and 100 mgL^{-1} respectively were used. The control consisted only of natural filtered seawater, without addition of any metal stock solution. 100 mL of each

concentration prepared was pipetted into 250 mL conical flasks. Fresh seaweeds kept in aquaria were blotted dry with absorbant tissue to remove excess moisture and 1 g portions were weighed. The portions were placed into the conical flasks containing the respective metal concentrations; 1 g in each flask. Then, cotton plugs were used to cover the mouths of the flasks. The experiment was conducted in triplicate.

The flasks were placed in a controlled environment incubator shaker (Model G25, New Brunswick Scientific Co. Inc. and Model 718, HOTECH Instruments Corp.). The cultures were continuously shaken at 150 rpm with the temperature set at 28 ± ^{o}C. Illumination was provided by Gro-Lux fluorescent lamps with 33.65 μmol photon m^{-2} s^{-1}, with 12:12 Light:Dark cycle.

After 24 h, the flasks, were removed from the shaker. The seaweeds were removed for measurement of chlorophyll-a content or dry weight. pH of the respective metal solutions were again determined (see Table 3.3).

Table 3.3 : pH of test solutions after 24 h

Metal species	pH							
	Concentrations (mgL^{-1})							
	0	0.1	1	5	10	20	50	100
Cu^{2+}	8.2	8.0	7.5	7.3	6.2	6.0	5.8	5.5
Zn^{2+}	8.2	8.1	7.8	7.5	7.5	7.3	7.1	7.0
Mn^{2+}	8.2	8.2	8.0	8.0	7.8	7.4	7.2	7.0
Cd^{2+}	8.2	8.0	8.0	7.4	7.1	7.0	6.7	6.2

3.2.7 End point measurements in seaweeds. The toxic effect of heavy metals on the tissue was assessed by growth inhibition or damage to tissues, as observed through determination of chloropyll-a content and dry weight (as shown by Table 3.4).

Table 3.4 : End point measurement of the respective seaweed species.

Seaweed species	End point mesurement
Chaetomorpha linum	Chlorophyll-a content (mgg^{-1})
Padina tetrastomatica	Dry weight (g)
Sargassum siliquosum	Dry weight (g)
Sargassum baccularia	Dry weight (g)
Gracilaria changii	Dry weight (g)
Gracilaria edulis	Dry weight (g)
Gracilaria salicornia	Dry weight (g)

3.2.7.1 Determination of Chlorophyll-a content. The extraction and analysis procedure employed was based on the Strickland and Parsons method (1968).

Chaetomorpha linum was removed from the flasks and rinsed quickly in distilled-deionised water to remove any trace of the incubation medium. They were then pressed between two absorbant tissues to remove excess water. The seaweed portion was ground with a washed pestle and mortar to a fine paste and washed into a centrifuge tube with 90% acetone (Analar grade). The volume was made up to 10 mL with acetone in the tube.

For complete extraction, the centrifuge tubes were sealed and placed in a refrigerator for 24 h. After the extraction period, samples were removed from the refrigerator and allowed to attain room temperature. Samples and blank (90% acetone) were centrifuged for 5 minutes at 2000 rpm.

The absorbance of the samples were determined with the Shimadzu UV-Visible Recording Spectrophotometer UV-160A at the wavelength of 630, 645 and 655 nm. Standardisation against the acetone blank was performed at all wavelengths.

The Strickland and Parsons (1968) equation for chlorophyll-a analysis was used :

$$C_a = 11.6D_{665} - 1.31D_{645} - 0.14D_{630}$$

Where C_a is the concentration of chlorophyll-a in mgL^{-1} and D is the absorbance.

The chlorophyll-a content was calculated as amount of pigment (mg) in per gram of sample with the following

equation :

$$\textbf{Chlorophyll-a (mgg}^{-1}\textbf{)} = C_a/V_c\ (V_a)$$

Where V_c is the amount of seaweed used (in gram) and V_a is the volume of acetone (mL).

3.2.7.2 Determination of dry weight. *Padina tetrastomatica, Sargassum* and *Gracilaria*, were removed from the flasks and rinsed quickly in distilled-deionised water to remove any trace of the incubation medium.

The seaweeds were then pressed between two absorbant tissues to remove excess water. Then they were placed in glass petri dishes and oven-dried at 60°C to constant weight. Dry weight (in gram) was measured using the Analytical Balance (Mettler AJ 100).

3.2.7.3 Calculation of % inhibition after 24 h. The % inhibition was calculated using the following equation:

$$\text{\% inhibition} = \frac{\text{Growth in control} - \text{Growth in treatment sample}}{\text{Growth in control}} \times 100$$

3.3 Effect of initial heavy metal concentration on bioaccumulation in 24 h exposure experiment

3.3.1. Seaweed species and metal tested. All seven seaweed species (as shown in Table 3.1) were exposed to the four metal solutions individually.

3.3.2 Test solutions. Results (see Section 4.1) of the

metal range determination indicate that seaweed species exposed to metal concentrations ranging from 0.1 to 10 mgL^{-1} for 24 h, show no inhibition in their respective end point measurements. Thus, proving that this range would be suitable for the 24 h heavy metal bioaccumulation studies. Based on this result, the working metal concentrations for the bioaccumulation studies were set as 1, 5 and 10 mgL^{-1}. Increasing concentrations of Cu, Zn, Mn and Cd from 1, 5, 10 mgL^{-1} were prepared in filtered seawater (salinity of 30 ppt and pH of 8.2) from the metal stock solutions (Appendix I) respectively.

3.3.3. Acidification. Concentrated nitric acid (Analar grade) was used to disssolve any precipitation formed, thus reducing the pH of some test solutions. Table 3.5 shows the pH of the test solutions after addition of nitric acid.

Table 3.5 : pH of test solutions after addition of acid.

Metal solution	pH			
	Concentration (mgL^{-1})			
	0	1	5	10
Cu^{2+}	8.2	8.0*	7.2*	6.5*
Zn^{2+}	8.2	8.1	8.1	7.8*
Mn^{2+}	8.2	8.2	8.1	8.0
Cd^{2+}	8.2	8.2	8.0	7.8

* where nitric acid was used to dissolve precipitation.

3.3.4 Adjustment of pH of test solutions. pH of each

set of the test solution was adjusted. This was to ensure that the single metal accumulation study in seaweeds was not influenced by the varying pH at each concentrations of the test solution. It is known that chemical speciation of metals varies accordingly to pH. The adjusting process would enable only one varying parameter in this study, that is the metal concentration.

The pH value selected for the test solution was based on the reduced pH produced for the highest concentration (10 mgL^{-1}) for each metal solutions. In the subsequent concentration, acid was added until a similar pH was attained. Table 3.6 shows the final pH of the respective metal solutions prior to the bioaccumulation studies.

Table 3.6 : pH of test solutions in bioaccumulation tests.

Metal solution	pH
Cu^{2+}	6.5
Zn^{2+}	7.8
Mn^{2+}	8.0
Cd^{2+}	7.8

3.3.5. Experimental procedure. 28 static tests were conducted. 1.8 L of the respective metal solution (prepared in seawater - Appendix I) at concentrations of 1, 5, 10 mgL^{-1} were measured into the acid-washed 2 L beakers. The control medium was just filtered seawater without addition of metal stock. Every concentration was carried out in duplicate.

48 g of total fresh weight of seaweed biomass, comprising 12 separately compiled 4 g portions of seaweed thalli, were incubated in each beaker over an incubation period of 24 h. The separate 4 g portions seaweed were meant to make sampling easier and to prevent excess sampling of seaweed biomass, while allowing removal of approximately similar sample biomass throughout the sampling process. Preparation of the 4 g portions of fresh biomass of seaweeds varied between species. For branching plants such as *Gracilaria* and *Sargassum*, the whole cleaned plant (excluding the holdfast) was first removed from the plastic aquaria and pressed between two absorbent tissues to remove excess water. Then, the plants were excised into small fragments weighing each 4 g. Then, they are ready for incubation in the test solution. Excision technique was not used for *P. tetrastomatica* and *C. linum* because they are non branching plants. The designated weight of 4 g was measured for these species respectively after removing excess moisture. Then a thread was tied around each of the 4 g biomass to keep this biomass intact over the 24 h exposure and also to allow easy sampling at intervals.

Beakers were placed on shelves and illuminated with 33.65 μmol photon m^{-2} s^{-1} light by 12:12 h Light:Dark cycle. Air temperature was 28±1°C while the water temperature was

27±1°C. Test solutions in beakers were aerated continuously throughout the 24 h.

Time intervals for sampling of seaweeds and seawater from the beaker were fixed at 0.25, 0.50, 0.75, 1.0, 1.50, 2, 3, 4, 6, 8, 16 and 24 h. The initial metal levels in seaweeds which is known as the background content were derived from triplicate samples.

3.3.6. Sampling of seaweeds and seawater. At each interval, 4 g seaweed biomass were removed as dry as possible from each duplicate beaker. Water samples of 5 mL were also taken to monitor metal levels in test solutions. Water samples were acidified and stored in closed plastic vials at 10°C. pH of the medium was measured after every sampling interval and adjusted back to the initial value in each beaker. This was to ensure that variation in accumulation of metal into seaweed tissues caused by changes in pH are reduced. The pH of test solutions after 24 h are shown in Table 3.7.

Table 3.7 : pH of test solutions after 24 h

Metal solution	pH			
	Concentration (mgL^{-1})			
	0	1	5	10
Cu^{2+}	8.2	7.5	7.5	7.6
Zn^{2+}	8.2	7.6	8.3	7.3
Mn^{2+}	8.2	8.0	7.5	7.5
Cd^{2+}	8.2	8.0	7.2	7.2

Seaweeds sampled were quickly rinsed in distilled-deionised water to remove the effects of the test solution and non-accumulating metal adhering to plant surfaces. Then they were passed between two absorbant tissues to remove excess water and placed in plastic petri dishes in an incubator. They were air-dried prior to oven-drying at $60^{o}C$.

Sampling of 5 mL of seawater at intervals were carried out at all concentrations except the control. Such loss of seawater volume to sampling is negligible due to the high volume of existing total volume. Metal levels in control test solutions were only determined at initial hour (0 h) and after 24 h. This is due to the anticipated low levels of metal concentration in natural seawater which require, standard extraction and preconcentration treatment (Appendix II) prior to metal determination in the ICP-AES. This requirement needs a larger volume of seawater to be sampled at intervals (100 mL). By allowing such large volumes to be

sampled at all intervals in the control, the total volume of test solution would be significantly reduced.

Dilution technique was used to determine metal levels at elevated concentrations. Dilution factor of 50 (using distilled-deionised water) was used. This technique was based on the fact that the introduced metal concentration was high. Therefore, by diluting the seawater it is still within the detectable limit of the ICP-AES. The dilution also further minimises the interference by other dominating ions in the natural seawater such as Na, SO_4, Mg, Ca, K and Cl.

3.3.7. Analytical procedure. Each replicate of the air-dried seaweed samples were further oven-dried at $60^{o}C$ for 4 h in glass petri dishes to give sufficient material of at least 200 mg dry weight. The seaweed samples were then ground to a powder using a mortar and pestle. An aliquot of 200 mg powder of each seaweed sample was digested with 15 mL concentrated nitric acid (Analar Grade) in 50 mL Kjeldhal flask, a modification of the technique by Say *et al.* (1990). A digester unit with 6 heating wells was used for the digestion of the seaweed samples, in a fume-chamber.

Digestion was carried out until there were no more brown fumes and a clear yellow liquid remained. This was then digested further until the volume was reduced to about 5 mL. The digest was filtered through a Whatman fliter paper (No.

4) and diluted to 50 mL in a volumetric flask. Samples were stored in 50 mL polyethylene bottles at 10^oC prior to ICP-AES determination of heavy metal content in the seaweeds.

3.3.8 Quality assurance procedures. Replicates and analysis of the certified reference material NIES No. 9 *Sargasso* were used as quality assurance procedure for seaweeds. Results of the analysis of the NIES No. 9 *Sargasso* are shown in Table 3.8. Quality assurance procedure for seawater includes the standard addition test (see Appendix III) and replicates.

Table 3.8 : Analysis of the NIES No. 9 *Sargasso*

Element	Metal content (μgg^{-1} dry wt. of seaweed)	
	Triplicate samples (Mean ± S.D.)	Certified value
Zn	15.007 ± 0.037	15.60 ± 1.20
Cd	0.140 ± 0.008	0.15 ± 0.02
Cu	5.053 ± 0.031	4.90 ± 0.20
Mn	20.127 ± 0.042	21.20 ± 1.00

3.3.8.1 Analysis of the NIES No. 9 *Sargasso*. This certified reference material of seaweed was obtained from the National Institute for Environmental Studies, Japan. The purpose of this material is to verify the metal determination technique used. The validity of this technique are expressed in results shown in Table 3.8. The result shows that the method used for metal determination in seaweeds are valid because the upper and lower limits of Cu, Zn, Mn and Cd

contents derived are within the certified limits.

3.3.8.2 Standard addition test for seawater analysis. This test was carried out as mentioned in Appendix III. Known single metal standard concentrations (5 mgL^{-1}) is spiked into seawater samples prior to solvent extraction technique as stated in Appendix II. The recovery efficiency (%) of the extraction technique used is calculated as follows:

$$\text{Recovery Efficiency (\%)} = \frac{\text{Tabulated value of the spiked concentration}}{\text{Expected value of the spiked concentration}} \times 100$$

This test was done in every batch of sample testing. The results of the test are shown in Table 3.9. Results suggest that the solvent extraction techniques used are satisfactory, taking into account of metals lost to glassware and contamination.

Table 3.9 : Efficiency of trace metal solvent extraction technique for seawater using the standard addition test.

Element	Recovery Efficiency (%)
Cu	82.5-85.6
Zn	92.3-94.8
Mn	88.5-92.3
Cd	87.4-102.5

3.4 Effect of salinity on bioaccumulation in 2 h exposure experiment

3.4.1 Seaweed species. *Chaetomorpha linum, Sargassum*

by the varying salinities of the test solution. The pH selected for the test solution was based on the reduced pH produced for the highest salinity (35 ppt) for each metal solutions. In the subsequent salinity, acid was added until a similar pH was attained. Table 3.11 shows the final pH of the respective metal solutions prior to the study.

Table 3.11 : pH of test solutions prior to experiment

Metal solution	pH
Cu^{2+}	7.6
Zn^{2+}	8.0
Mn^{2+}	8.0
Cd^{2+}	8.0

3.4.5 Experimental procedure. Duration of test was 2 h. Batch experiments were conducted. 100 mL of the metal solution (1 mgL^{-1}) at the respective salinity was measured into the acid-washed 250 mL borosilicate Erlenmeyer flasks. The control medium was just filtered seawater at the respective salinities (without addition of stock metal concentration). Every batch experiment was carried out in duplicate.

2 g of fresh seaweed thalli was placed into each flask. For every metal and salinity, there were twelve flasks because six sampling times were used. The flasks were then covered with cotton plugs. Then the flasks were shaken in a controlled environment incubator shaker (Model G25, New

Brunswick Scientific Co. Inc. and Model 718, HOTECH Instruments Corp.) at 150 rpm. Illumination was provided by Gro-Lux fluorescent lamps 33.65 μmol photon m^{-2} s^{-1} with 12:12 h Light:Dark cycle. Air temperature was 28±1°C while the water temperature was 27±1°C.

Seaweed and seawater samples were removed from each flask were at 0.25, 0.50, 0.75, 1.0, 1.50, 2 h. The initial metal levels (0 h) in seaweeds which is known as the background content were derived from triplicate samples.

3.4.6 Sampling of seaweeds and seawater. At each time interval, the whole 2 g seaweed biomass were removed from each duplicate batch flasks. Water samples were acidified and stored in closed plastic vials at 10°C. pH of the test solutions in each flask was measured after each interval (as shown in Table 3.12) .

Table 3.12 : pH of test solutions after 2 h

Metal solution	pH range
Cu^{2+}	7.5-8.0
Zn^{2+}	7.2-7.6
Mn^{2+}	7.4-7.6
Cd^{2+}	7.6-8.1

Seaweeds sampled were quickly rinsed in distilled-deionised water to remove the effects of the test solution. Then they were passed between two absorbant tissues to remove excess water and placed in plastic petri dishes on closed

shelves. They were air-dried prior to oven-dry. Metal levels in seaweeds and seawater were determined as stated in Section 3.3.7.

3.5 Effect of pH on bioaccumulation in 2 h exposure experiment

3.5.1 Seaweed species and metals tested. *Chaetomorpha linum* (tested for Cu, Zn and Cd), *Sargassum baccularia* (tested for Cu, Zn and Cd) and *Gracilaria changii* (tested for Cu, Mn and Cd) were used in this study.

3.5.2 Test solutions. Metals at a concentration of 1 mgL^{-1} were used. Salinity of filtered seawater was adjusted to 30 ppt. Then, the appropriate amount of metal stock solution was added to give a metal concentration of 1 mgL^{-1}.

3.5.3 Range of pH tested. This study was designed to show the effect of varying pH of the metal solution on accumulation of heavy metal by seaweeds. Seaweeds were exposed to pH of 4, 5, 6, 7 and 8. Concentrated nitric acid (Analar grade) was used to adjust the pH of test solutions to the designated value.

3.5.4 Experimental procedure. Duration of test was 2 h. Batch experiments were conducted. 100 mL of the metal solution (1 mgL^{-1}) at the respective pH was measured into the acid-washed 250 mL borosilicate Erlenmeyer flasks. The

control medium was just filtered seawater at the respective pH (without addition of stock metal concentration). Every batch experiment was carried out in duplicate. The rest of the procedures are as stated in 3.4.6, 3.4.7 and 3.4.8. Final pH of test solution after 2 h of exposure is shown in Table 3.13.

Table 3.13 : pH of test solutions after 2 h

Metal	Initial pH				
	4	5	6	7	8
Cu^{2+}	3.8-4.2	4.9-5.2	5.9-6.1	7.2-7.5	7.8-8.2
Zn^{2+}	3.5-4.1	5.0-5.3	5.8-6.3	7.1-7.4	8.0-8.2
Mn^{2+}	4.0-4.2	5.2-5.5	6.0-6.2	7.3-7.5	7.9-8.1
Cd^{2+}	3.5-4.1	5.2-5.4	5.8-6.5	6.8-7.5	7.9-8.2

3.6 Statistical analysis.

Statsgraphic Version 5.0 was used. Tests such as analysis of variance (ANOVA) followed by multiple range test (using the 95% LSD method) and simple correlation were carried out where required.

3.7 Instrument Operation for the Determination of Metal Levels in Seaweeds and Seawater.

Metal levels in the digested seaweeds and in the seawater were analysed using an Inductively Coupled Plasma-Atomic Emmission Spectrometer (ICP-AES) Model 2000 (BAIRD). The operational involved centering of the polychromator,

running of blanks, measuring of standards, performing the calibration routine, and running of the samples.

The instrument is equipped with a 46.68 MHz RF generator. The RF power was set at 1100 watts. The coolant gas flow rate was set at 10 L min^{-1} and the auxillary gas flow rate was 1.0 L min $^{-1}$. The carrier gas flow rate was 0.6 L min^{-1}. The vacuum pressure to evacuate the polychromator was set at 5 millitor.

Ultrapure water (Elgastat UHQPS) was aspirated into the chamber to check the formation of mist in the spray chamber and to clear the plasma torch. Centering of polychromater was performed at the initial stage of the run in order to optimise the optical alignment of the spectrometer so that the spectral lines are exactly centered on their exit slits.

0.5 M nitric acid (spectrosol grade) was used as a blank. For the baseline correction the intensity of the metals in the blank were determined. No detectable amounts of Cu, Zn, Mn and Cd were found in the reagent blanks. Single element standards which cover the expected range in samples were used to calibrate the spectrometer (see Appendix IV for standard preparation). This was due to the bioaccumulation studies being carried out as single metal exposure experiments. Integration time chosen for the measurement was 5 seconds and the number of integration was 3. In the

collection of calibration data, intensity of the prepared standard of each metal was measured and the average value calculated. Curve set files were prepared for the elements of interest, using the polynomial calculation routine. Curve coefficients were calculated automatically for each element using the standard data. Standard calibration plots were obtained for each element. The content of metal in seaweeds were expressed as μg metal g^{-1} dry weight of seaweed, while the metal concentration in the seawater was expressed as mg metal L^{-1} of seawater.

3.8 Toxicity testing of heavy metals with seaweeds

This test was conducted to obtain laboratory data concerning the adverse effects of heavy metals added to dilution water (natural seawater) to seaweed species in a static renewal exposure system.

Results of tests are reported in terms of IC_{50}, based on reduction (inhibition) in growth. IC_{50} is a statistically or graphically estimated concentration that is expected to cause a 50% inhibition of one or more specified biological processes (such as growth or reproduction) for which the data are not dichotomous, under specified conditions. LOEC is the lowest observable effect concentration while NOEC is an abbreviation for no observable effect concentration (ASTM,

1993).

Since prediction based on the result of a similar test on seaweed species was not available, a range finding test in which the seaweed species are exposed to a control and five concentrations of metal that differ by a factor of ten is conducted. This was followed by a definite test using the streamlined range obtained from the above mentioned range finding test.

3.8.1 Test organism and test material. Toxicity tests were conducted on the seaweed species for the selected metals as shown in Table 3.14. The endpoint measurements (biological data) for the tests are shown in Table 3.14.

Table 3.14 : List of seaweed species, metals and end point measurement used in the toxicity tests

Seaweed species	Metal	End point measurement
Chaetomorpha linum	Cu and Cd	Chlorophyll-a content (mgg^{-1})
Sargassum baccularia	Zn and Cd	Dry weight (g)
Gracilaria changii	Mn and Cd	Dry weight (g)

3.8.2 Toxicity test facilities. Seaweed toxicity tests were conducted in a controlled environment incubator shaker with constant temperature and illumination as in previous experiments. Aeration was not provided. In a toxicity test with aquatic organisms, test chambers are defined as the smallest physical units between which there are no water connections (ASTM, 1993). Sterile 250 mL Erlenmeyer flasks of

borosilicate glass are used as test chambers. Volume of test solution used was 100 mL, which adhered to the ASTM standard for conducting tests on a shaker (ASTM, 1993). The standard stated that the test solution volume should not exceed 50% of the flask volume for tests conducted on a shaker.

3.8.3 Dilution water. Dilution water for tests with seaweeds is prepared by adding appropriate amounts of specified heavy metal stock solution to natural filtered seawater. Prior to this, seawater was filtered with 0.22 μm membrane filter to remove endogenous organisms. Then, salinity was adjusted to 30 ppt by adding distilled-deionised water.

3.9 Range finding test

3.9.1 Test organism. Seaweed species tested are as stated in Table 3.14. Young growing plants were used in this test. They were collected from the wild, cleaned and conditioned as stated in Section 3.1.2 and 3.1.3. The fresh cleaned plants were excised into portions of 2 g fresh weight, prior to placing them into the test solution.

3.9.2 Test concentrations/medium. Metals selected are as stated in Table 3.14. Five concentrations 0.01, 0.1, 1.0, 10 and 100 mgL^{-1} of metal test solutions were prepared with filtered seawater. Precipitation of metals formed during

preparation of test solution was treated with acid as stated in Section 3.2.3. pH of metal concentrations prior to test are shown in Table 3.15. Seawater without addition of any metal stock was treated as control. Every concentration was carried out in triplicate.

Table 3.15 : pH of test solutions prior to toxicity test

Metal species	pH					
	Concentrations (mgL^{-1})					
	Control	0.01	0.1	1	10	100
Cu^{2+}*	8.2	8.0	8.0	7.9	7.2	6.0
Zn^{2+}*	8.2	8.1	8.0	8.0	7.5	7.3
Mn^{2+}	8.2	8.2	8.0	8.0	7.8	7.5
Cd^{2+}	8.2	8.0	8.0	7.8	7.8	7.2

* - where acid was used to dissolve precipitation.

3.9.4 Type of test. Static renewal system was used in this test. This was to determine the toxicity of selected heavy metals to the seaweeds. Medium was renewed once every three days.

3.9.5 Duration of test. Biological data (endpoint measurement) was collected at 24 h, 96 h, 7 d and 10 d of exposure.

3.9.6 Biological data. Growth parameters used for the respective seaweed species are stated in Table 3.14.

3.9.7 Experimental procedure. 100 mL of the metal solution at the respective concentration was measured into

the acid-washed 250 mL borosilicate Erlenmeyer flask. The control medium was just filtered seawater (without addition of stock metal concentration). Every concentration was carried out in triplicate.

2 g of seaweed thalli was placed into the respective concentration and the flasks were covered with cotton plugs and placed in a controlled environment incubator shaker (model 718, HOTECH Instruments Corp. and Model G25, New Brunswick, Scientific Co. Inc.) set at 120 rpm. The air temperature was set at 28±1°C and illumination was provided by Gro-Lux fluorescent lamps (33.65 μmol photon m^{-2} s^{-1}, with 12:12 Light-Dark cycle).

The seawater media were changed once every three days during the course of the experiment. Biological data for growth was derived at 24 h, 96 h, 7 d and 10 d.

3.9.8 Other measurements. At 24 h, 96 h, 7 d and 10 d, the following measurements were also taken : pH of test solution, air temperature, water temperature, light intensity and salinity. Data are shown in Appendix V.

3.9.9 Calculation of toxicity. Mean inhibition of growth (endpoint measurement) in % was calculated at day 24 h, 96 h, 7 d and 10 d using the equation as shown in Section 3.2.7.3.

3.10 Definitive tests

This test was conducted based on results obtained from the range finding tests (see Section 4.5.1). The test protocols were followed as in 3.8.4. and 3.8.7., except for changes which are highlighted below.

3.10.1 Test concentrations. Range of concentrations used now for this test were based on the range which caused the 50 % inhibition of growth in the range finding tests (see Section 4.5.1). Table 3.16 summarises the range of concentrations used for this test.

3.10.2 Test medium renewal. Test mediums were renewed at 96 h and 7 d for tests stretching more then 96 h.

3.10.3 Duration of test. Biological data was collected at day 7 and 10 for all the seaweed species tested. *G. changii* was also tested at 96 h for Cd exposure.

3.10.4 Calculation of toxicity. The Linear Interpolation Method for Sublethal Toxicity : The inhibition Concentration (IC_p) Approach (Version 2.0) by Norbeg-King (1993) was used to tabulate IC_{50} values, while, Toxstat Version 3.2 was used to conduct : i. Shapiro Wilks test for Normality; ii. Bartletts test for homogeneity of variance, and iii. ANOVA. A schematic diagram of how the IC_{50} was tabulated using the method is shown in Appendix XIX. As comparison, the graphic demonstration of the dose-response

curves are also incorporated for visual impact.

3.10.5 Other measurements. Data of the following measurements : pH of test solution, air temperature, water temperature, light intensity and salinity are shown in Appendix V.

Table 3.16 : Series of concentration used for the definitive tests

Species	Metal	Results of range finding test (Occurrence of 50 % inhibition in growth was obtained at the following range of metal concentration (mgL^{-1}))			Concentration used for definitive test (mgL^{-1})		
		96 h	7 d	10 d	96 h	7 d	10 d
C. linum	Cd		10 - 100	1 - 10		15,30,45,50,75	3,6,9,12,15
	Cu		10 - 100	1 - 10		20,40,60,80,100	4,8,12,16,20
S. baccularia	Cd		1 - 10	0.1 - 1		2,4,6,8,10	0.4,0.8,1.2,1.6,2.0
	Zn		0.1 - 1	0.01 - 0.1		0.4,0.8,1.2, 1.6,2.0	0.05,0.10,0.15,0.20 0.25
G. changii	Cd	10 - 100	0.1 - 1	0.01 - 0.1	15,30,45,50,75	0.2,0.4,0.6, 0.8,1.0	0.04,0.08,0.12, 0.16,0.20
	Mn		1 - 10	0.01 - 0.1		3,6,9,12,15	0.2,0.4,0.6, 0.8,1.0