Chapter 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 INTRODUCTION

The procedures for conducting toxicity tests as described in this chapter were based on a number of relevant, established methodologies (ASTM, 1993; APHA, AWWA and WPCF, 1989; Reish and Oshida, 1986; Ward and Parrish, 1982), with some modifications.

3.2 QUALITY ASSURANCE AND QUALITY CONTROL

3.2.1 Negative Controls

All tests were conducted using well-established negative (clean) controls. The negative control used in this study was the dilution water, synthetic seawater. For each test series with a particular algae, a series of test chambers containing only dilution water was included. The complete test was repeated if the negative control fails to meet the acceptability criteria established for this test procedure.

3.2.2 Positive Controls (Reference Toxicant)

Reference toxicant tests were conducted on a regular basis and used to provide insight into changes in sensitivity of organisms which may occur as a result of acclimation or contamination. The tests were carried out using a well-established standard reference toxicant, cadmium, which had been prepared from the metallic salt cadmium chloride. Concurrent tests using the reference toxicant tests were carried out at regular intervals. Control charts were constructed for the reference toxicant and each test species. The cumulative mean value (recalculated with successive data points until

results were stable) and upper and lower control limits were plotted on each chart. If the results of a reference toxicant fell outside the control chart limits, the test procedure and health of the test organism was reviewed, and subject to the findings, the test was repeated.

3.2.3 Test Organisms

The phytoplankton cultures were obtained from a reliable source, their origin or collection site known. Taxonomic identifications of the cultures were confirmed prior to the study. Only unialgal and axenic cultures in the log phase growth were used as the inoculum at test initiation for the toxicity tests. The test species were cultured in the dilution water at least two weeks for acclimation before the test.

3.2.4 Replication

Sufficient number of replicates for each treatment were included to account for variability in test organisms. Each treatment in a test series began with the same number of replicates. In this study, for all experiments, each treatment was carried out in tripicates.

3.2.5 Water Quality Measurements and Maintenance

Sterile dilution water (See Section 3.8.2.2) was used in the toxicity tests and proper water quality conditions (pH, temperature, salinity) were maintained to ensure survival of organisms and that undue stress was not exerted on them, unrelated to the test material. The initial salinity and pH of the seawater prior to use were 30.0±2.0 gL^{-1} (ppt) and 8.0±0.5, respectively, with less than 10% fluctuations.

3.2.6 Standard Laboratory Procedures

Standard laboratory procedures were followed in all testing which included proper documentation, proper cleaning, avoidance of contamination and maintenance of appropriate test conditions.

3.3 TEST SYSTEM

3.3.1 Facilities and Equipment

Algal cultures were maintained in a separate area from where toxicity tests were conducted, stock solutions or test solutions prepared or equipment cleaned. All subculturing for algal maintenance purposes and the initiation of the tests were carried out in UV-sterilized inoculation chambers to avoid possible contamination. The algal toxicity tests were carried out in rooms and incubation chambers with constant temperatures and appropriate illumination. Equipment which came in contact with stock solutions or any water containing the test organisms were free from substances that could be leached or dissolved by aqueous solutions in amounts which adversely affect test organisms. Glass and high density polyethylene plastics were used to minimise dissolution, leaching and sorption (See Appendix 1 for List of Facilities and Equipment).

3.3.2 Water Supply

Apart from running water for general cleaning purposes, a supply of distilled and deionised water for cleaning glassware and equipment, and for preparation of chemical stock solution and dilution water were required.

The dilution water was used as the negative control, for preparing test solutions, and for holding and acclimating test organisms. As a source of natural seawater was not

readily available at all times, and as seasonal variation in quality makes natural waters unsuitable for comparable toxicity testing, synthetic seawater was used as the dilution water. Artificial seawater was prepared by mixing aquarium salt mix with distilled and deionised water to achieve the desired salinity (See Preparation of dilution water under Section 3.8.2.2). The same dilution water was used to culture the test species prior to and throughout the tests.

3.3.3 Lighting and Temperature

Specific needs for the test organism, as well as regional ambient conditions and the need to provide maximum growth, were considered in establishing the appropriate light intensity and photoperiod for acclimation and testing. The phytoplankton growth tests were conducted under continuous bright light provided by cool fluorescent lights. A constant temperature of 28.0±1.0°C was maintained in the incubation chamber throughout the toxicity tests.

3.3.4 Aeration

The phytoplankton tests did not require aeration during the tests.

3.3.5 Cleaning procedures

Test and holding chambers, instruments, and equipments used to store and prepare test and control materials were cleaned before use. Prior to use, and at the end of the tests, all the glassware and plasticware (to be used again in the toxicity tests) were cleaned with a non-phosphate detergent and rinsed with tap water. Then, they were rinsed once with 10% nitric acid (to remove metals), rinsed twice with deionised water, rinsed once with full-strength pesticide-grade acetone (to remove organics) and finally

rinsed again three times with deionised water.

3.3.6 Test Vessels

All containers used for a toxicity test were identical. Two types of vessels used in this study were 24-welled Nunclon multiwell plates and 250 mL Erlenmeyer flasks.

Sterile Erlenmeyer flasks of borosilicate glass were used as culture and test vessels. 18 such flasks were used in each single toxicity test with five test concentrations and a negative (clean) control, in triplicate.

The four columns and six rows of wells on the multiwell plate provided 24 units of 3mL capacity tests chambers. 18 of the wells (three columns and six rows of wells) were used to represent a complete set of a toxicity test consisting of 5 test concentrations and a negative control, in triplicate.

The chambers were covered to keep out extraneous contaminants, especially bacteria and undesirable algae, using covers which allowed the passage of air as algae consume CO_2. The plates came with loose fitting transparent covers while cotton or paper plugs were used to cover the shake flasks.

3.4 TEST MATERIALS

3.4.1. Introduction

The four heavy metals used for toxicity testing were cadmium, copper, manganese and arsenic. Cadmium served as a test material and the reference toxicant in this study.

3.4.2. Heavy metal main stock solutions

10,000 mgL^{-1} stock solutions of cadmium, copper, manganese and arsenic were prepared using the reagent-grade metallic salts $CdCl_2.H_2O$, $CuSO_4.5H_2O$, $MnCl_2.4H_2O$

and Na_2HAsO_4 respectively.

To obtain a stock solution of 10,000 mgL^{-1} Cd, 10 g of Cd was added to a litre of deionised distilled water.

The amount of cadmium chloride, $CdCl_2.H_2O$, containing 1 g of Cd

$$= \frac{\text{molecular weight of the metallic salt}}{\text{atomic weight of the metal}}$$

$$= \frac{\text{molecular weight of } CdCl_2.H2O}{\text{atomic weight of Cd}}$$

$$= \frac{201.32}{112.41} = 1.79094$$

Therefore the amount of $CdCl_2.H2O$, containing 10 g of Cd

$=10 \times 1.79094 = 17.9094g$

Using the same method, the amounts of the metallic salts of the other heavy metals required to prepare their respective stock solutions were determined. 39.2912g, 36.0242g and 41.6453g of the Cu, Mn, and As metallic salts respectively were used to prepare a litre of 10,000 mgL^{-1} stock solution for each metal.

The amount of each metallic salt was weighed using a fine balance. The salt was then dissolved in deionised and distilled water in a 1L volumetric flask and topped up to 1000mL volume, thoroughly stirred with a magnetic stirrer and stored in high density polyethylene (HDPE) bottles.

3.5 TEST ORGANISMS

3.5.1 Species

Four species of marine phytoplankton used as the test organisms were *Chaetoceros calcitrans* (Paulsen) Takano, *Isochrysis galbana* Parke, *Tetraselmis tetrahele* (West) Butcher, and *Tetraselmis* sp. (Plates 1, 2, 3 and 4 respectively).

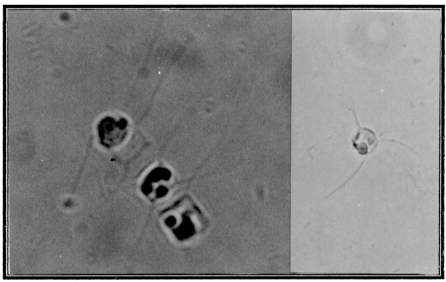

Plate 1 : *Chaetoceros calcitrans* (magnification : 200X)

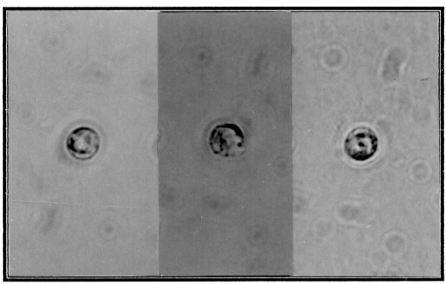

Plate 2 : *Isochrysis galbana* (magnification : 200X)

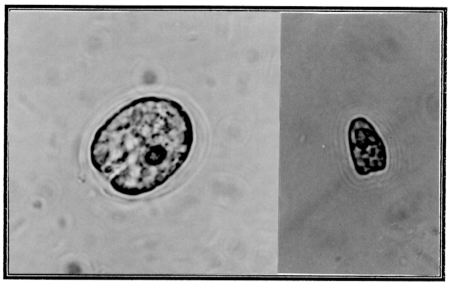

Plate 3 : *Tetraselmis tetrahele* (magnification : 200X)

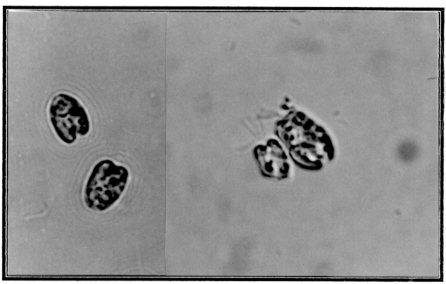

Plate 4 : *Tetraselmis* sp. (magnification : 200X)

3.5.2 Source and Origin

Chaetoceros calcitrans (147) was obtained from the Fisheries Research Institute, Penang. *Isochrysis galbana* (141) was locally isolated from the coastal waters of the National Prawn Fry Production and Research Centre (NAPFPRE) Pulau Sayak, Kedah, while *Tetraselmis tetrahele* (144) and *Tetraselmis* sp. (146) originated from Japan. All three latter species were obtained from NAPFPRE.

3.5.3 Isolation of pure algal strain

Small amounts of the newly received algal cultures were aseptically dispensed into 250mL Erlenmeyer flasks containing fresh maintenance medium (see Section 3.5.4.1) and allowed to grow to a bloom under growth conditions as described in Section 3.5.4.2.

After each culture had increased in density, the algae was streaked onto fresh plates of solid maintenance medium, by means of a sterile inoculation loop, using the 'dilution streak' method. The plates were then incubated for a few days, upside down, on an illuminated shelf.

After many colonies had appeared the best colony was selected and transfered using a sterile loop into a test tube containing fresh liquid maintenance medium, agitated and allowed to grow. After a colour change is observed in the tube, a drop is taken and examined under the microscope. A loopful of the same culture was dilution-streaked onto a plate of solid maintenance medium and left to grow again to check its purity and ensure it was pure from other microbial contamination. Further subculturing was done until a pure algal strain had been obtained. This purified algal culture formed the initial stock culture for use in toxicity tests throughout the whole

study.

3.5.4 Maintenance

Method for maintenance of culture was adopted from those of NAPFPRE (Palanisamy *et al.*, 1991) with some modifications. Culture maintenance was done to retain a unialgal culture for a long period of time and also to ensure a (continuous) supply of pure culture for production of algae for use in the experiments.

For long term maintenance, a loopful of the purified strain (from Section 3.5.3) was streaked on fresh plates and slunts of solid maintenance medium and allowed to grow under growth conditions as described in 3.5.4.2. Subculturing to new medium was done once a month.

Strain maintenance for production was achieved by inoculating 1 mL of the purified strain into several test tubes containing 9 mL sterile liquid maintenance medium. Subculturing was done often enough to provide a continuous supply of algal cells at the appropriate age and growth phase for initiating toxicity tests. A larger amount of algal culture was obtained by aseptically inoculating a tube of the culture to a flask containing 90 mL of sterile liquid maintenance medium and allowed to grow.

3.5.4.1 Maintenance and Growth Medium

Conway media (AQUACOP, 1984) was used to maintain and grow the algal cultures. The media was prepared by adding several nutrient medium to synthetic seawater. The nutrient medium consisted of a main mineral solution, a vitamin solution, a silicate solution and a nitrate solution. Each nutrient media stock solution was prepared by mixing the necessary chemicals (as shown by Appendix 2) with deionised and distilled water, over a hot-plate with a magnetic stirrer, and sterilised by autoclaving (except the

vitamin). They were then stored in the refrigerator at 4°C.

The synthetic seawater was prepared by mixing 33g of Marine Environment sea salt (See Appendix 3 for the chemical constituents) with 1000mL deionised and distilled water using a magnetic stirrer. The sea salt solution was then filtered through a 0.45μ membrane filter via a vacuum filtering apparatus.

To prepare the liquid maintenance and growth medium, 1mL each of the main mineral solution, the silicate solution and the nitrate solution and 0.1mL of the vitamin solution were added to 1000mL of the filtered synthetic seawater. The pH was adjusted to 8.0 with hydrochloric acid (3N HCl) and the salinity checked (to ensure a salinity of 30 ppt). The medium was then dispensed into appropriate containers (test tubes or flasks) and autoclaved at 121 p.s.i. for 15 minutes. The artificial seawater was allowed to age for several days after preparation.

For solid medium, 2g of Bacto agar was added to 100mL of the liquid medium (2%) and mixed with a magnetic stirrer over a hot plate until all the agar was well dissolved. The medium was then dispensed into test tubes, autoclaved, and later slunted to make agar slunts. Autoclaved agar media was poured into sterile plastic petri dishes by sterile technique to make agar plates. The slunts and plates were left to solidify and cool before use.

3.5.4.2 Growth conditions

All test tube, flask and plate cultures of the marine phytoplankton were maintained and grown in a culture room under a 12 h:12 h light : dark period, at light intensity of 50.4 μmol photon $m^{-2}s^{-1}$ provided by cool fluorescent lights, and temperature of 28±1.0°C.

3.5.5 Acclimation

The species to be used in a toxicity test was acclimated to the test conditions at least two weeks prior to the tests. The microalgae was cultured under normal growth conditions (Section 3.5.4.2 above) in the appropriate dilution water, but under continuous illumination of 50.4 μmol photon $m^{-2}s^{-1}$ provided by cool fluorescent lights.

3.6 EXPERIMENTAL DESIGN

3.6.1 Correlation between OD and cell counts

Prior to the toxicity tests a separate experiment was conducted to determine the correlation and regression between optical density (OD) and cell counts of each microalgal culture. The good correlations would enable the use of O.D. measurements to determine cell counts via the regression equations obtained.

3.6.2 Range-Finding Tests

Initially, for each heavy metal and test species, at least one range-finding test was conducted to determine the upper and lower limits of concentration to test in the definitive tests. All range-finding tests were conducted in multiwell plates. The tests consisted of 5 test concentrations, each differing by a factor of 10 (0.01, 0.1, 1.0, 10.0 and 100.0 mgL^{-1}) and a clean control (negative control), in triplicate.

3.6.3 Definitive Tests

Based on the range-finding tests, definitive tests were carried out using the suitable series of test concentrations. Each definitive test included a minimum of 5 test concentrations and a clean control, in triplicate. The definitive tests were carried out in

the multiwell plates and 250mL Erlenmeyer flasks. The following series of concentrations were used in this study :

i) 0.10, 0.18, 0.32, 0.56 and 1.0 mgL^{-1}

ii) 1.0, 1.8, 3.2, 5.6 and 10.0 mgL^{-1}

iii) 6.25, 12.5, 25.0, 50.0 and 100.0 mgL^{-1}

iv) 100.0, 200.0, 400.0, 800.0 and 1600.0 mgL^{-1}

3.7. TEST CONDITIONS

The general test conditions employed for the phytoplankton growth tests were as recommended by CPMS-II (1995) with minor modifications and are summarised in Table 3.1.

3.8 EXPERIMENTAL PROCEDURES

All test procedures were conducted under sterile conditions.

3.8.1 Determination of correlation and regression between O.D and cell counts

The microalgal cultures were grown until dense. Each culture was then serially diluted by a factor of 0.5. The OD of each diluted sample was then measured at 620nm, by the Multiskan MCC/340 MKII and the UV-VIS Spectrophotometer, using Elisa microplates and a glass cuvette respectively. Cell number of the diluted samples were determined by direct cell count using the Neubauer Brightline haemocytometer.

After obtaining the values of the two variables, the correlation coeficient, r, and regression between the OD and cell count for each microalgae were determined. The correlation and regression between OD measurements of the Multiskan and UV-VIS Spectrophotometer were also determined.

Table 3.1 : Summary of recommended test conditions for phytoplankton growth tests (CPMS-II,1995) with some modifications

1. Test type : static, non-renewal.

2. Test conditions :

Temperature	:	28±1.0°C
Light quality	:	"cool white" fluorescent lighting
Photoperiod	:	continuous illumination
Light intensity	:	50.4 μmol photon $m^{-2}s^{-1}$
pH	:	8.0±0.5
Salinity	:	30±2.0 gL^{-1}.

3. Test vessels : 24-welled Nunclon multiwell plates, 250mL Erlenmeyer flasks

4. Test volumes : 2 mL, 100mL respectively

5. Inoculum :

Age	: 4-7 days old (log phase culture)
Cell density	: 1 X 10^6 cells mL^{-1}

6. Dilution water : algal growth medium; with and without EDTA

7. Concentrations : 5 test concentrations + negative control
   Dilution factor : approximately 0.5
   Number of replicates : 3

8. Initial cell density : 1 X 10^4 cells mL^{-1}

9. Shaking rate : handshaken twice daily

10. Test duration : 96h

11. Effect measured : growth (cell counts)

12. Endpoints seeked : IC25, IC50, LOEC and NOEC for inhibition of growth

13 The test acceptability criteria : mean increase in growth of control by a factor of 20

Plate 5 : The Multiskan MCC/340 MKII used for measurement of O.D.

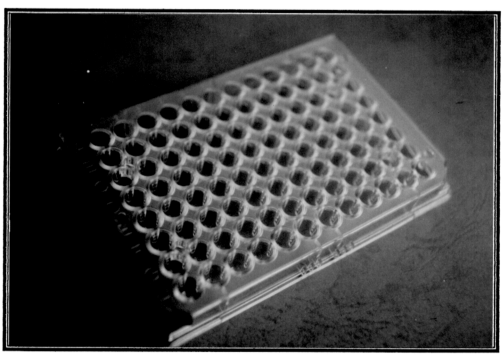

Plate 6 : An Elisa microplate which is able to hold a large number of samples during O.D. measurement by the Multiskan

3.8.2 Toxicity testing

The same procedures were applied to both range-finding and definitive tests. However, the range-finding tests were conducted using the multiwell plates only.

3.8.2.1 Glassware

All glassware used in this study were clearly labelled and kept separate from the main laboratory glassware to reduce the chance of contamination. The glassware to be used for preparing algal cultures and in toxicity testing were cleaned as previously described (Section 3.3.5). After cleaning, the flasks were covered with cotton plugs and aluminium foil, autoclaved at 121 p.s.i. for 15 minutes. They were then allowed to cool, dried in the oven and kept in a dry cabinet prior to use. Other glassware including volumetric flasks, disposable glass pipettes and beakers which were used in initiating the tests were also sterilised by autoclaving.

3.8.2.2 Preparation of dilution water

Two types of dilution water were used in this study as the heavy metal toxicity tests were conducted in the presence and in the absence of EDTA. For experiments using EDTA, the dilution water used was the same as the liquid maintenance medium which was sterile synthetic seawater (pH 8.0±0.5, salinity 30±2.0 gL^{-1}) enriched with the four nutrient solutions (Section 3.5.4.1). For experiments performed without EDTA, the dilution water was made up by the same procedure as making the maintenance media, with the exception of the main mineral stock solution which was separately prepared with the omission of EDTA.

3.8.2.3 Inoculum

Microalgal cultures to be used as the inoculum were acclimated to the laboratory

test conditions at least two weeks prior to the test. The inoculum was prepared in a sterile inoculation chamber within 2-3 hours of the test initiation. 4 to 7 day cultures in the log phase of growth were examined under the microscope and counted using an improved Neubauer brightline haemocytometer. A duplicate count was done and the average cell number in the inoculum noted.

If there were more cells than the minimum density required in the stock culture for use as inoculum, the stock would be diluted by adding the dilution water and recounted. The initial culture density and any adjustment done were recorded. The culture was adjusted using dilution water until an inoculum of approximately 1 X 10^6 cells mL^{-1} was achieved.

3.8.2.4 Test solution preparation

Test solutions were prepared immediately prior to the initiation of the tests. The desired concentration range was selected, using at least 5 test concentrations and a negative (clean) control (See Section 3.6.2 and 3.6.3). Extra solution was prepared for each treatment for heavy metal analyses whenever possible.

The test solutions were prepared on a volumetric basis. The following equation was used to determine the amount of heavy metal stock solution and dilution water to add in preparing each treatment concentration :

$$C_1V_1=C_2V_2$$

where, C_1=concentration of the heavy metal stock solution

V_1=the volume of stock solution to add

C_2=the desired test solution concentration

V_2=the test solution volume

As an example, to prepare a 500 mL volume of 100 mgL^{-1} Cd solution the volume of the 10,000 mgL^{-1} Cd stock solution required was :

$$V_1 = \frac{(100\ mgL^{-1})(500\ mL)}{(10{,}000\ mgL^{-1})} = 5\ mL$$

5 mL of the 10,000 mgL^{-1} Cd stock solution was mixed with 495 mL dilution water or topped up to 500mL in a volumetric flasks to produce a 500mL treatment of 10 mgL^{-1} Cd.

3.8.2.5 Test initiation

As soon as the inoculum had been adjusted and the test solutions had been prepared, the 24-welled Nunclon multiwell plates or the 250 ml Erlenmeyer flasks were assembled in the sterile inoculation chamber. The plates and flasks were accordingly labelled (date, experiment number). The test solutions were then dispensed into the test vessels and inoculated with the inoculum to result in an intial cell density of 1×10^4 cells mL^{-1} in each test vessel. Multiwell plate and flask experiments were not neccessarily carried out at the same time.

3.8.2.5.1 Multiwell plate experiments

Using an Eppendorf calibrated pipettor and sterile disposable pipette tips, 2 mL of each treatment was dispensed into the the appropriate well, in triplicate. Each well was then inoculated with 0.02 mL of inoculum.

3.8.2.5.2 Flask experiments

Using a sterile 100 mL volumetric flask, 100 mL of each test concentration was dispensed accordingly into the respective flasks, in triplicate. Each flask was then inoculated with 1mL of inoculum.

Plate 7 : A 24-welled Nunclon Multiwell plate

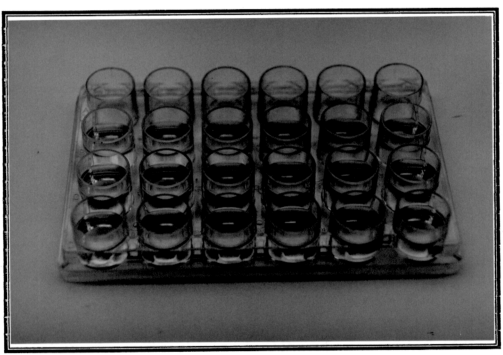

Plate 8 : A toxicity test in a multiwell plate at 96 h

3.8.2.5.3 Cell density verification

At least three subsamples of the inoculum were taken and preserved in 4% formalin. These were later subjected to cell counts to verify the inoculation density.

3.8.2.5.4 Initial pH

The initial pH of the test solutions were measured.

3.8.2.6 Test incubation

Multiwell plates which had been inoculated were covered and place in the incubation chamber. The inoculated flasks were arranged randomly in the chamber. The test vessels were then incubated for 96 hours at 28±1.0°C, under continuous illumination of 50.4 μmol photon $m^{-2}s^{-1}$ provided by the chamber cool fluorescent lights. The flasks were hand-shaken twice daily to resuspend any settled algae, and their position rearranged once a day.

3.8.2.7 Test termination

3.8.2.7.1 Growth measurements

At the end of the 96 h toxicity tests, growth (cell counts) in each treatment was determined. The culture in the wells were gently resuspended using an Eppendorf pipette while those in the flasks were gently swirled to obtain a homogenous sample for measurement of growth. Test samples of 0.25mL volumes from each well or flask were pipetted and transferred to an Elisa plate and the O.D. readings were taken at 620nm with the Multiskan MCC/340 MK II machine.

The cell number in each test sample was calculated from the O.D. measurements by Multiskan, via the linear regression between O.D. and cell counts which had been

determined previously (Section 3.8.1).

3.8.2.7.2 Random cell counts

For quality control purposes, actual cells counts using the haemocytometer were also done on randomly selected test samples, at least one replicate of each treatment. At least two cell counts were done for each selected sample and the average recorded. The correlation between these actual cell counts and the O.D. readings by the multiskan were determined.

3.8.2.7.3 Test acceptability criteria

The test was considered acceptable if the algal cell density in the control vessels had increased by a factor of 20 at the end of the test. It was also ensured that the cell density in the controls did not vary by more than 20% between replicates.

3.8.2.7.4 Final pH

The pH of each test sample in the shake-flasks experiments were measured at the end of the 96h incubation period.

3.8.3 Heavy Metal Analyses

Heavy metal analyses by the ICP-AES (Inductively-Coupled Plasma - Atomic Emission Spectrophotometer) were done to determine the actual initial concentrations of the test solutions.

3.8.3.1 Preparation of samples

3.8.3.1.1 Dilution technique

Dilution technique using a dilution factor of 50 was employed based on the fact

that the dilution minimises the interference by other dominant ions in the seawater such as Na^+, SO_4^{2-}, Mg^{2+}, Ca^{2+}, K^+ and Cl^-, and that the metal concentration was within detectable limits of the ICP-AES after the dilution.

The test solutions were diluted 50 times with ultra-high quality water (UHQ) and acidified with acid nitric to result in a 0.5 M solution, and then stored in HDPE bottles prior to the heavy metal analyses.

3.8.3.1.2 Heavy metal extraction

Heavy metal extraction was done mainly on the negative control solutions (seawater only) due to the anticipated very low levels of metal concentrations in the synthetic seawater which require extraction and preconcentration prior to the ICP-AES analyses (See Appendices 4(1) and 4(2) for heavy metal extraction methods). The standard addition test was also done for the seawater samples as a quality assurance procedure (See Appendix 4 (3)) where a metal standard of known concentration was spiked into one or two seawater samples prior to the solvent extractions and then recovery efficiency of the extraction technique was calculated (See Appendix 4(3)).

3.8.3.2 Preparation of standard solutions for ICP-AES analyses

A mixed stock standard solution containing 1000 mgL^{-1} of each metal was prepared. Individual standard solutions (0.1563, 0.3215, 0.6250, 1.25, 2.5, 5.0 mgL^{-1}) were then prepared from this stock solution.

3.8.3.3 Instrument operation and sample analyses

The ICP-AES (Inductively-Coupled Plasma - Atomic Emission Spectrophotometer) Model 2000 (BAIRD) was used to analyse the prepared samples. The operational steps included centering the polychromater, running the blanks,

measuring standards and calibrating, and running the samples.

The RF power was set at 1100 watts, the coolant glass flow rate at 10 $Lmin^{-1}$, the auxillary gas flow rate at 1.0 $Lmin^{-1}$, and the carrier gas flow rate at 0.6 $Lmin^{-1}$. The vacuum pressure to evacuate the polychromator was set at 5 millitor.

UHQ water (Elgastat UHQPS) was aspirated into the chamber to check the formation of mist in the spray chamber and to clear the plasma torch. The polychromator was centered to optimise the optical alignment of the spectrometer so that the spectral lines were exactly centered on their exit slits.

Prior to the analyses of test samples, the instrument was calibrated. 0.5 M nitric acid (spectrosol grade) was used as the blank. The blank was used to perform the baseline correction. The standard solutions (see 3.8.3.2) covering the expected range of elements of interest in the seawater samples were then used to calibrate the spectrophotometer. The selected intergration time for measurement was 5 seconds while the number of intergration was 3. A collection of calibration data was produced for the blank and each standard preparation, listing the calibrated concentration and intensity of each element. Curve set files were prepared for each element using the polynomial calculation routine and the curve coefficients calculated automatically for each element using the standard data. Standard calibration plots were obtained for each element.

The prepared test samples were then analysed. A standard preparation was ran again between every several test samples to check on the performance of the machine. The metal concentrations in the test samples were expressed using the unit mgL^{-1}.

3.9 DATA ANALYSES

3.9.1 Range-finding tests

The percentage of growth inhibition (and/or stimulation) in each treatment relative to the clean (negative) control was determined using the following equations :

$$\text{percentage of inhibition (\%)} = \frac{\text{control response - treatment response}}{\text{control response}} \times 100$$

$$\text{percentage of stimulation(\%)} = \frac{\text{control response - treatment response}}{\text{control response}} \times 100$$

where the response refers to the growth (cell number) of the phytoplankton in the sample at 96h.

3.9.2 Definitive tests

3.9.2.1 Determination of NOEC and LOEC values

NOEC is the highest concentration tested which is not significantly different form the control while LOEC is the lowest concentration tested which is significantly different from the control.

3.9.2.1.1 Statistical analyses

Statistical analyses to determine the NOEC and LOEC values were performed using the TOXSTAT (Release 3.2) software programme (Gulley *et al.*, 1990). TOXSTAT calculates summary statistics, performs data transformations, tests data for normality and homogeneity of variance, and performs parametric or non-parametric tests on the data (See Appendix 5(1) on Using the TOXSTAT programme). As toxicity tests are designed to measure adverse effects relative to a control (or reference), one-sided (one-tailed)

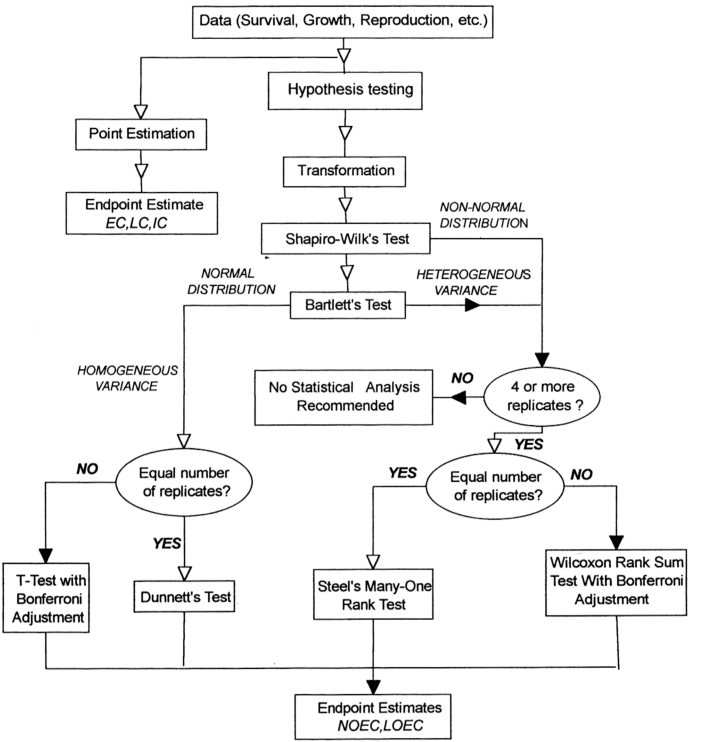

Figure 3.1 : Flowchart for Hypothesis Testing Methods

statistical comparisons using a probability of $\alpha = 0.05$ were done.

Data (cell counts) from the phytoplankton tests were transformed using a $\log_{10}$ transformation. Then, Shapiro-Wilks test was used to determine normality while Bartlett's test was used to determine homogeneity of variance.

If the data passed both tests, parametric statistics were used to analyse the data. The ANOVA and Dunnett's test were used if the replicates were equal while the ANOVA and Bonferroni's test were used if the the replicates were not equal.

On the other hand if the data failed one or both of the normality and homogeneity of variance tests, non-parametric tests (Steel's Many-One Rank or Wilcoxon Rank Sum tests) were used. Figure 3.1 presents a flow-chart on the statistical analyses.

3.9.2.1.2 Interpreting Output

For each statistical test, TOXSTAT gave a table of calculated values (See Appendix 5(2)) and compared them to tabulated critical values for a given probability (in this study =0.05) and degrees of freedom. Treatments which were significantly different from the control (the LOEC) were identified by an asterisk "*" (See Appendix 5(2)). In a normal data set, the NOEC and LOEC values were adjacent test concentrations. The most conservative or lowest NOEC and LOEC were always reported.

3.9.2.2 Determination of IC_{25} and IC_{50} values

IC_p is the concentration of the test material which results in a specific percentage inhibition ("p") of the endpoint being measured. IC_{25} and IC_{50} which are concentrations of the test material resulting in 25% and 50% inhibitory effects, are the standard endpoints. These values provide a specific point estimate of the effect being measured.

The IC_{25} and IC_{50} values were determined using ICPIN software programme

(Norberg-King, 1993). The ICPIN programme which was developed by the U.S. EPA and uses a bootstrap method of calculation is used to calculate ICp values for sublethal test data such as growth and reproduction endpoints.

3.9.2.2.1 Data Analyses

Data (cell counts) from the toxicity tests and the concentrations tested were entered into the programme (See Appendix 6(1) on Using the ICPIN programme). Actual initial test concentrations as determined by the ICP-AES were used when available. Otherwise the nominal concentrations were used. The desired IC_p (IC_{25} or IC_{50}) was chosen and the programme executed.

3.9.2.2.2 Interpreting the Output

The IC_p value (IC_{25} or IC_{50}) was shown as "The Linear Interpolation Estimate" in the printouts (Appendix 6(2)) while the chosen "p" as "Entered P Value". As there were ≤ 6 replicates for each treatment, the "Expanded Confidence Limits" were reported.

3.10 REFERENCE TOXICANT CONTROL CHART

3.10.1 Introduction

Reference toxicant testing was carried out to monitor the relative health and sensitivity of the test organisms, and to measure the reproducibility of the test results. Ideally, a reference toxicant should be tested concurrently with each toxicity tests. However, this is not always practical. The tests should then be performed at least once a month for species cultured in the laboratory.

3.10.2 Constucting the control chart

The results of each reference toxicant (cadmium) test for each species were plotted on control charts. The data for a particular species were plotted against the x-axis, which represented the test number, and the y-axis, which represented the IC_{50} values. The cumulative mean and standard deviation (SD) were used to define the range of "normal" or acceptable variability. A range of ±2SD represented 95% confidence limits (upper and lower warning limits) for the mean while a range of ±3SD represented 99.7% confidence limits (upper and lower confidence limits).

The reference toxicant results were considered acceptable if they were within the 95% confidence limits. A coefficient of variation (CV=100XSD/Mean) of 30% or less was used as a guideline as there is no standard width of the 95% confidence limits.

Data for each species were plotted and analysed separately.