Chapter 1

INTRODUCTION

1. INTRODUCTION

1.1 THE STATE OF THE MALAYSIAN MARINE ENVIRONMENT

The Malaysian marine environment is currently experiencing a serious state of degradation due to overpopulation, agriculture, land degradation and pollution. Marine environmental quality data compiled by the Department of Environment of Malaysia (DOE) from 1992 to 1995 (Table 1.1) shows that in recent years, oil and grease, total suspended solids (TSS) and *Escherichia coli (E. coli)* have remained as the prevailing main contaminants of the coastal environment (DOE, 1996a,b).

Faecal coliform *(E. coli)* which indicates sewage contamination, arises from domestic as well as animal waste, affecting coastal waters, beaches and also river estuaries. TSS is another parameter which prevails abundantly in Malaysian coastal waters, being another land-originated product that results from earth-disrupting activities upstream such as large-scale land clearing for agricultural development. Monitoring results also indicate that heavy metal pollution is on the increase due largely to industrial activities (DOE, 1992).

1.1.1 Heavy metal pollution

Five metals which are regularly monitored by DOE are cadmium, chromium, mercury, lead and copper. Data compiled during the past five years shows a general widespread distribution of low concentrations of metals and with localised high pollution of certain metals.

Cadmium and chromium, though widely distributed, have remained relatively constant with concentrations below the Interim Standards (Table 1.2). However, the

Table 1.1
Malaysia : Status of Marine Water Quality 1992-1996
(DOE, 1996a,b)

Year	Measurement of average* by parameter								
	Total Suspended Solids **TSS** (mgL^{-1})	Oil and Grease **OG** (mgL^{-1})	*Escherichia coli* ***E.coli*** (MPN/100mL)	Cadmium **Cd** (mgL^{-1})	Chromium **Cr** (mgL^{-1})	Mercury **Hg** (mgL^{-1})	Lead **Pb** (mgL^{-1})	Arsenic **As** (mgL^{-1})	Copper **Cu** (mgL^{-1})
1992[a]	687# (60.13%)	4.2# (76.04%)	8,610# (40.32%)	0.010# (4.2%)	0.016# (0%)	0.058# (25.02%)	0.061# NA	0.071# NA	0.011# -
1993[a]	222 (57.77%)	3.1 (82.1%)	6,102 (24.41%)	0.008 (1.60%)	0.012 (0%)	0.018 (9.12%)	0.073 (21.38%)	0.144 (11.78%)	0.025 (1.32%)
1994[a]	296 (63.8%)	3.5 (93.5%)	1,818 (43.5%)	0.024 (3.4%)	0.044 (0%)	0.003 (17.6%)	0.139 (30.4%)	0.055 (0%)	0.056 (7.8%)
1995[a]	495 (59.5%)	3.3 (76.7%)	4,711 (34.1%)	0.013 (0.6%)	0.029 (0%)	0.001 (6.8%)	0.027 (6.0%)	0.039 (0%)	0.058 (8.7%)
1996[b]	NA (53.8%)	NA (72%)	NA (29.6%)	NA (0.3%)	NA (0%)	NA (9.4%)	NA (11.2%)	NA (0.2%)	NA (9.6%)

Note :
* Average based on 12 states of Malaysia (# Median based on 12 states)
( ) : percentage (%) of parameter exceeding Proposed Interim Standard
NA : Not available

Table 1.2
Malaysia : Interim Standards for Marine Water Quality
(DOE, 1992, 1996b)

Parameter	Unit	Interim Standard	Remarks
Escherichia coli (E. coli)	MPN/100 mL	100	*
Oil and grease (O&G)	mgL^{-1}	0	**
Total suspended soilds (TSS)	mgL^{-1}	50	* Type 1
Arsenic (As)	mgL^{-1}	0.1	* Type 2
Cadmium (Cd)	mgL^{-1}	0.1#	* Type 2
Chromium Cr)	mgL^{-1}	0.5	* Type 2
Copper (Cu)	mgL^{-1}	0.1	* Type 2
Lead (Pb)	mgL^{-1}	0.1	* Type 2
Mercury (Hg)	mgL^{-1}	0.001	* Type 2
Nickel (Ni)	mgL^{-1}	-	* Type 1

Legend :
* : Based upon The People's Republic of China Standard
** : Based upon Japan's Standard
Types of water are in accordance to use :
Type 1 : For the conservation of marine aquatic resources and safe utilization by humans (includes salt field, food processing, desalination, fisheries, aquaculture and marine parks (conservation area)
Type 2 : For recreation
Type 3 : For industrial processing, harbour, port and oceanic exploitation and development

Value was previously 0.01 mgL^{-1} (DOE, 1992)

past five years have seen an increase in chromium pollution. The average levels of mercury have decreased in recent years but examination of the detailed data (DOE, 1996a) shows high levels in coastal areas of highly industrialised states. Past monitoring by DOE showed an increase in the average lead and arsenic concentrations in the local marine environment but recent results show an average decrease in both metal levels. However, as in the case of mercury, both metals have been found in levels higher than the Standards in certain states. Pollution by low levels of copper have been observed with occasional levels exceeding the Interim Standards, but the average copper levels have gradually increased throughout the years.

1.2 THE MARINE ENVIRONMENTAL CRITERIA AND STANDARDS

Comprehensive discharge standards now exist for the local rivers but not for the Malaysian marine waters. At present, DOE depends on the Interim Marine Environmental Quality Criteria and Standards (Table 1.2) for the management of coastal environment based on the Standards of the People's Republic of China and Japan (DOE, 1992; DOE, 1996b). There is a need to establish a local Marine Environmental Quality Criteria and Standards as a guideline for the safe discharge of these pollutants into the marine environment and this may be achieved with the assistance of toxicity data generated from toxicity tests.

1.3 TOXICITY TESTING

A "toxicant" is an agent which can produce an adverse response (effect) in a biological system, seriously damaging its structure or function or producing death (Rand and Petrocelli,1985).

"Toxicity" is a relative property of a chemical which refers to its potential to have a harmful effect on a living organism. A toxic effect is a result of the concentration of the toxicant and the duration of exposure.

The term "toxicity test" is frequently used interchangably, and often erroneously, with the term "bioassay", although they are not equivalent terms (Reish, 1988), especially the aquatic toxicity test. More often than not, vast literature and even laboratory manuals use "bioassay" although they are really referring to "toxicity test".

While a bioassay is a test to evaluate the relative potency or strength of a chemical (e.g. vitamin or other pharmacologically active compound) by comparing its effect on a living organism with that of a standard preparation (Rand and Petrocelli, 1985), a toxicity test is used to evaluate the adverse effects of a chemical on a living organism under standardised, reproducible conditions which permit a comparison with other chemicals tested. In other words, a toxicity test is performed to measure the degree of response produced by a specific level of stimulus (test concentration) while a bioassay is performed to determine the strength of the chemical from the degree of response elicited in the test organisms, not to estimate the concentration of the chemical that is toxic to those organisms.

Aquatic toxicity tests are used to detect and evaluate the potential toxicological effects of toxicants on aquatic organisms. Marine toxicity tests, in which the scientific protocol of freshwater tests were adopted (Reish, 1988), were initiated much later than the freshwater tests and have been expanded greatly in the past 25 years.

The general purpose of performing toxicity tests is to measure the effect of one or more substances on one or more species of organisms. Aquatic toxicity data have a variety of applications (Reish, 1988). It may be utilised in research on the toxic effects of

a substance on an organism. The data may also assist in the assessment of the danger of a newly manufactured chemical. It may help to determine the toxic components of a waste discharge. The data may also be useful in monitoring an existing discharge or environment. It is also valuable in the identification of acceptable limits for the safe discharge of a potential toxicant, and can help in the establishment of a water quality criteria.

1.4 AIM AND SCOPE OF STUDY

1.4.1 Reasons for the study

1.4.1.1 Lack of toxicity data

In brief, the local and regional marine environment is being contaminated by heavy metal pollution which is hazardous to aquatic organisms and humans. Therefore there is a need to establish a local marine environmental quality criteria and standard for heavy metals. Toxicity tests are important tools which can generate useful data for the identification of acceptable limits for the safe discharge of potential toxicants (Reish, 1988). The toxicity data can assist in the establishment of a water quality criteria. Formulation of the local or regional environmental criteria should be based on tropical toxicity data (McPherson, 1995). However, there is a general lack of data on the toxicity of these substances on local and tropical marine organisms, including phytoplankton.

1.4.1.2 The need of a rapid toxicity test method

While the standard methods (APHA, AWWA and WPCF, 1989; ASTM, 1993) recommend the use of the 250mL Erlenmeyer shake flasks as test vessels, direct cells counts are usually used to determine growth at the end of the tests. Considering the amount of data needed to be generated, there was the need to employ a more rapid

method of toxicity testing which was also simple, practical and economical, yet with good reproducibility and results similar to the shake flask.

1.4.2 Elements and assumptions

As primary producers the marine phytoplankton form the basis of the marine food chain. They are essential to the normal function of the marine ecosystem. A drastic increase or decrease in their growth due to a substance may influence higher trophic levels. In this respect, the effect of a substance on the algal growth is a highly relevant for the assessment of potential effects in the environment (OECD, 1984).

Algal toxicity tests using the conventional flasks require large incubation space, large volumes of test solutions and culture, and are labourious. If toxicity tests conducted in multiwell plates produce results similar to the conventional shake-flasks, then these plates could be the alternative, convenient vessels for toxicity testing in place of the flasks.

The effect measured in the phytoplankton growth tests is inhibition of growth while the common endpoint sought is the IC_{50}. It is known that cell counts give the lowest IC_{50} values. However, direct cell counting is very time-consuming and impractical when there are large numbers of samples to count. O.D. is often used in assessing cell growth. If there is a good correlation between the O.D. of the culture and the cell counts, O.D. measurements can be used to determine the cell number by calculation via the regression between O.D. and cell counts. Furthermore, using Elisa plates which are capable to accomodate many samples at a time for O.D. reading by the Multiskan MCC/340 MKII machine would enhance the rapidity of measurement of growth.

A toxicity test method which combines the uses of multiwell plates as test

vessels, O.D. measurements to determine cell number in the test samples, and the Elisa plates machine for O.D. determination, would provide a rapid method of toxicity testing.

Chelators added to seawater can significantly reduce metal toxicity to algae (Sunda and Guillard, 1976), thus they are usually omitted from the test media. However, chelation may play an important role in polluted aquatic environments (Canterford and Canterford, 1980). Therefore, as well as determining the toxicity of a metal to phytoplankton in the absence of a chelator, it may be just as important as to establish the extent to which chelation affects this toxicity.

1.4.3 Objectives of study

1.4.3.1 Generation of heavy metal toxicity data

The main aim of this study was to determine the chronic toxicity of cadmium, copper and arsenic which are commonly found in the local marine waters, and manganese, which has received relatively less attention and lacks in specific toxicity data, to four species of local and tropical marine phytoplankton, *Chaetoceros calcitrans*, *Isochrysis galbana*, *Tetraselmis tetrahele* and *Tetraselmis* sp., which are economically important as aquaculture feed.

Heavy metal toxicity tests were carried out in the absence of chelating agent EDTA, as recommended by CPMS-II (1995), and also in the presence of EDTA to observe the effects of the normal level of the chelator in the media on heavy metal toxicity to the marine phytoplankton.

Species suitable for toxicity testing were also determined based on the sensitivity of the test species to the heavy metals.

1.4.3.2 Development of a rapid toxicity test method

Another objective of the study was to examine a novel toxicity test method. The method employed Nunclon multiwell plates which are usually used in tissue culture studies, as test vessels for toxicity testing of heavy metals with marine phytoplankton. Another feature of the method was the use of O.D. to determine cell numbers in the test samples, based on the good correlations and via the regressions between O.D. and cell counts. In addition, Elisa plates and Multiskan machine which are normally used for immunological studies, were used for measuring O.D. At the end of the study, the test method was compared to the tests in shake-flasks, and observed for its simplicity, practicality, economy, rapidity and reproducibity of results.

1.4.3.3 Establishment of a Marine Water Quality Criteria and Standard

As the toxicity data being generated under the ASEAN Canada CPMS-II project are primarily for use in formulation of marine environmental criteria (McPherson, 1995), the ultimate goal of the study is to produce high quality toxicity data (comparable with other ASEAN laboratories) which will be useful for the identification of acceptable limits for the safe discharge of the selected metals, thus assisting in the development of a Marine Water Quality Criteria and Standards for heavy metals, for Malaysia and the ASEAN region. While toxicity data on marine phytoplankton are being generated in our laboratory, tests on other marine organisms are also being carried out in other participating laboratories in this country.

1.4.4 Study plan

1.4.4.1 O.D. vs cell counts

Prior to the main study, separate experiments were conducted for each of the four

species of algae, to determine the correlation between their O.D. and cell counts. This was done by growing the cultures until dense, serially diluting them, and then measuring their O.D. The O.D. measurements were done by the Elisa plate via the Mutiskan MCC/340 MKII and the UV-spectrophotometer. The correlations and regressions between the O.D. and cell counts were then determined.

1.4.4.2 The toxicity tests

Based on the standard methods and tests conditions recommended by CPMS-II (1995) for the toxicity testing of tropical marine phytoplankton under the ASEAN-Canada CPMS-II, 96 h chronic toxicity tests were conducted using cadmium, copper, manganese and arsenic as test materials, and *Chaetoceros calcitrans*, *Isochrysis galbana*, *Tetraselmis tetrahele* and *Tetraselmis* sp. as test organisms. A quality assurance and quality control program as recommended in the guidelines (CPMS-II, 1995) was also observed.

1.4.4.2.1 Experimental design

Initially, for each heavy metal and each test species, a rangefinding test was carried out in the multiwell plates to determine the range to test in the definitive tests. The definitive tests were then conducted both in multiwell plates and shake-flasks to determine the LOEC, NOEC, IC_{25} and IC_{50} values. All toxicity tests were conducted in the presence and absence of EDTA.

1.4.4.2.2 Measurements of growth and quality control

The O.D. of the test samples were measured by the Multiskan MCC/340 MKII, in Elisa plates, and the cell number was calculated via the regression equations. For quality control purposes, actual cell counts were also done and the correlation between

the O.D. and actual cell counts were determined.

1.4.4.2.3 The test system

The type of test conducted was static, non-renewal. All tests were carried out at 28 ±1.0°C, under continuous illumination of 50.4 μmol photon $m^{-2}s^{-1}$. The negative control and the dilution water for preparing test solutions were synthetic seawater (pH 8.0±0.5; salinity 30±2.0gL^{-1}) which had been prepared from commercial sea salt (Marine Environment) and enriched with nutrients as in the maintenance medium. EDTA was omitted from the medium used for tests without EDTA. The test solution were analysed for actual heavy metal concentrations whenever possible.

Tests were conducted in 24-welled Nunclon multiwell plates and 250mL Erlenmeyer shake-flasks with test solution volumes of 2 mL and 100 mL in each test vessel respectively. 4 to 7- day old phytoplankton stock cultures in the log-phase were used as the inoculum. The initial cell density for each treatment was $1X10^{-4}$ cells mL^{-1}. Each test consisted of at least five test concentrations and a clean control, each in triplicate. Flasks were hand-shaken twice daily and their positions rearranged during the 96 h incubation period. Cadmium was used as the reference toxicant through out the whole study, as recommended for CPMS-II.

The effects measured were inhibition of growth based on cell counts and the endpoints sought were the LOEC, NOEC, IC_{25} and IC_{50} values.

1.4.4.3 Statistical Analyses

Statistical analyses to determine the NOEC and LOEC values were performed using the TOXSTAT program (Gulley *et al.*, 1990) while the IC_{25} and IC_{50} values were determined using the ICPIN program (Norberg-King, 1993).