Chapter 5

DISCUSSION

5. DISCUSSION

5.1 THE RAPID METHOD OF TOXICITY TESTING

5.1.1 Measurement of growth : Use of O.D. readings to determine cell counts

Growth is usually an understood measure in single species toxicity testing (Cairns and Pratt, 1989) where cell counts give the lowest IC_{50} values compared to other methods (Truhaut *et al.*, 1980). However direct cell counts are time-consuming and impractical especially when dealing with large number of samples. Direct cell counts conducted on random test samples in each experiment showed that the O.D readings correlated very well with actual cell counts (Tables 4.3 to 4.18), thus indicating that O.D. measurements may be employed as a rapid method of determining growth (cell counts). Cell counts may instead be calculated from the O.D. by using the linear regressions (Table 4.1) and eventually used to calculated the IC_{50} values. Trevors (1982) has also shown that OD correlates highly not only with cell counts but also dry weight, and chlorophyll content.

The use of the Elisa microplates and Multiskan machine to measure O.D. also provided a rapid method for measurement of growth by allowing the accomodation of small volumes of test samples and measurement of large number of samples in a single plate reading. Therefore, by combining the good correlations between O.D. and cell counts, and the use of the Multiskan machine, direct cell counts may be avoided.

As there is a good correlation between O.D. measured by the Multiskan and by the UV-VIS spectrophotometer (Table 4.2), both instruments may be used interchangeably for the same work.

5.1.2 The multiwell plates as test vessels

The multiwell plate offers an alternative vessel for toxicity testing in place of the conventional shake-flasks. A single multiwell plate (with 24 wells)is able to accommodate a complete range-finding or definitive test (5 test concentration and clean control, in triplicates or even 4 replicates) and consumes little space in the incubation chamber. The total volumes of test solutions and inoculum required for each experiment are small, thus economical in test material and culture. Postexperimental washing of glassware may be avoided as the plates are disposable. Alternatively, cleaning the plates requires less labour than flasks and reusing them only increases the economy of the method. Furthermore, several tests may be set simultaneously enabling more data to be generated in shorter time.

IC_{50} values obtained from tests conducted in multiwells were also similar to results in shake flasks (Tables 4.19 and 4.20), with generally good correlations (Table 5.1) between the IC_{50} values of both vessels, despite the relatively smaller test volumes used. Much smaller volumes have been used by other workers (Blaise *et al.*, 1986; Hassett *et al.*, 1981) in studying metals and phytoplankton. Therefore, the multiwell plate offers a simple, practical, economical and rapid method of toxicity testing with good reproducibility and results similar to shake flasks. The International Standards Organisation has stated in their guidelines that a method used on a routine basis has to be reproducible as well as simple and cheap (Madsen, 1984).

Table 5.1 : Correlations between results (IC_{50} values) of tests in multiwell plates and shake-flasks

(a) for each test species with all heavy metals, in the presences (+) and absence (-) of EDTA

	Chaetoceros calcitrans	*Isochrysis galbana*	*Tetraselmis tetrahele*	*Tetraselmis* sp.
+EDTA	1.0000[a]	1.0000[a]	0.9845[b]	0.9585[b]
-EDTA	1.0000[a]	1.0000[a]	0.9989[a]	0.9399

(b) for each heavy metal with all test species, in the presences (+) and absence (-) of EDTA

	Cadmium	Copper	Manganese	Arsenic
+EDTA	0.9159[c]	-0.4152	1.0000[a]	0.9810
-EDTA	0.9383	1.0000[a]	0.9704[c]	0.9996[b]

Note : Level of significance 0.01[a], 0.05[b], 0.1[c]

5.2 HEAVY METAL TOXICITY

Algal toxicity tests are usually conducted in media in which the algae is cultured (Gavis *et al.*, 1981) as they contain the essential micronutrients to support optimal growth for toxicity interpretations (Wong, 1995). These media contain substances such as phosphate, citrate, iron, silicate, EDTA and Tris buffer which can complex the heavy metal ion being tested for its toxicity (Lumsden and Florence, 1983). Chelating agents, therefore, should and usually are omitted from test media as their presence may render the heavy metals unavailable to the phytoplankton (Canterford and Canterford, 1980). Still, many investigators have included various chelating agents in their experimental culture media causing difficulty in interlaboratory comparisons of phytoplankton sensitivity to metals (Christensen *et al.*, 1979; Canterford and Canterford, 1980; Edding and Tala, 1996; Rosko and Rachlin, 1975).

In this study, toxicity tests for each metal and marine phytoplankton species were conducted in both the presence and absence of EDTA. Artificial rather than natural seawater was used as the dilution water to prepare the test solutions, in order to minimise biological effects and to provide a reproducible solution of known composition. Kester *et al.* (1967) has demonstrated the similarity in the composition of natural and artificial seawater.

The tests with EDTA were done to observe and demonstrate the effects of EDTA on the toxicity of the heavy metals at amounts usually present in the growth medium. As chelation is likely to be an important factor operating in polluted habitats (Morris and Russel, 1973), and as EDTA is resistant to biodegradation (Gardiner, 1976) and thus very likely to complex metals in natural waters, its effect on the toxicity of metals to marine phytoplankton should also be assessed.

It is of significance that the lowest inhibitory metal concentrations have been observed in cultures using either natural sea water or enriched sea water without added chelating agents (Davies, 1978). Therefore, another part of this study was carried out in the absence of EDTA, as recommended by CPMS-II (1995), in generating toxicity data for use in the derivation of a Marine Water Quality Criteria and Standards for ASEAN.

Variations in the experimental protocols are many, making it important to standardise procedures as much as possible. Therefore, in an effort to standardise toxicity test methods among laboratories of ASEAN, CPMS-II (1995) has recommended a set of test conditions to be used by these laboratories when performing toxicity tests with marine phytoplankton. With the exception of the use of the multiwell plates as test vessels in our aim to develop a rapid method of toxicity testing, the test conditions used in this study were similar to those used by other laboratories under the CPMS-II in

phytoplankton growth tests (Darmayati, 1997; Gonzales, 1995, 1997; Hindarti, 1997; Phang *et al.*, 1997; Thongra-ar *et al.*, 1995).

Apart from the wide range of techniques and conditions used in heavy metal toxicity studies, inconsistency in the selection of toxicity criteria also causes difficulty in analyses and comparison of toxicity data. Various parameters have been used as indices of the toxicity of metals including cell division, cell counts, chlorophyl, ATP, doubling time, dryweight, turbidity, volume, growth rate, oxygen evolution and many more (Stratton, 1987). Due to the lack of reported IC_{50} values based on cell counts in the literature, IC_{50} values based on cell counts obtained from this research were compared to IC_{50}, EC_{50} and LC_{50} values based on cell counts and other indices available for the purpose of comparison and discussion.

As our prime interest was to determine the toxicity of the metals on the marine phytoplankton, this discussion has been approached with priority or emphasis on their toxicity in the absence of the chelator, EDTA, as toxicity is a function of free metal concentrations (Erickson *et al.*, 1970; Steemann-Nielsen and Kamp-Nielsen, 1970; Steemann-Nielsen and Wium-Anderson, 1970; Davey *et al.*, 1973; Anderson and Morel, 1978), and then later touch on the effects of chelation on the toxicity.

5.2.1 Comparative toxicity of heavy metals

The toxicity data from Table 4.21 (a-d) may be simplified according to the toxicity of the heavy metals to the test algae, in the presence (+EDTA) and absence (-EDTA) of EDTA.

The metals ranked according to the decrease in toxicity :

+EDTA :

C. calcitrans : Cu > Cd > Mn > As

I. galbana : Cd > Cu > Mn > As

T. tetrahele : Cu > Cd > As > Mn

T. sp : Cu > Cd > Mn > As

-EDTA :

C. calcitrans : Cu = Cd > Mn > As

I. galbana : Cu > Cd > Mn > As

T. tetrahele : Cu > Cd > Mn > As

T. sp : Cu > Cd > Mn > As

The sequences of heavy metal toxicity to the marine phytoplankton species were generally similar in the presence and absence of EDTA, although toxicity was more pronounced in the absence of the chelating agent as demonstrated by the lower IC_{50} values. In their complex form heavy metals are rendered unavailable to algae and their toxicity is decreased (Rai *et al.*, 1981). In both cases Cu was the most toxic heavy metal to all test species. In aquatic organisms only mercury has consistently been more toxic than copper (Manahan and Smith, 1973). There were some exceptions with *I. galbana*, where Cd was more toxic in the presence of EDTA, and *C. calcitrans*, where Cd was as toxic as Cu, in the absence of EDTA. The order of Cd and Cu in the general sequence of toxicity is species dependent (Babich and Stotzky, 1978; Sorentino, 1978; Rai *et al.*, 1981) and there are equal number of studies supporting the two orders of Cd>Cu and Cu>Cd (Trevors *et al.*, 1986). Meanwhile Mn was relatively less toxic to all species

while As was the least toxic, both in the presence and absence of EDTA.

5.2.2. Cadmium

Cd is one of the most toxic heavy metals with no described biological functions (Trevors *et al.*, 1986). Most studies on Cd and phytoplankton have emphasised comparative toxicity sequences of heavy metals (Rai *et al.*, 1981; Sorentino, 1978). Fewer Cd specific toxicity data are available for marine phytoplankton.

Cadmium effects are dependent both upon the organism used and toxicity criterion employed (Stratton, 1987). It is an established trend that marine algae are usually less sensitive to Cd than are freshwater species (Berland *et al.*, 1976) probably due to differences in Cd speciation (Kuiper, 1981) as Cd is less available in seawater due to its complexation with chlorides and other anions (Rebhun and Ben-Amotz, 1984). However, the test algae *Chaetoceros calcitrans* and *Isochrysis galbana*, which were the most sensitive to Cd in the absence of EDTA, both with IC_{50} values of 0.06 mgL^{-1}, were as sensitive to Cd as some freshwater species including *Chlorella vulgaris* (Rosko and Rachlin, 1977), and *Selenastrum capricornutum* (Laegreid *et al.*, 1983) which have exhibited EC_{50} values of 0.06 mgL^{-1} (cell counts) and 0.08 mgL^{-1} (photosynthesis) respectively. *Skeletonema costatum* and *Chlorella saccharophila* have been described in literature as among the most sensitive marine phytoplankton to Cd with their growth inhibited at 0.05 and 0.11 mgL^{-1}, respectively (Berland *et al.*, 1977; Rachlin *et al.*, 1982). Therefore the test species used in this study, *C. calcitrans* and *I. galbana*, were as sensitive as the former and twice as sensitive as the latter species. Canterford and Canterford (1980) observed a similar range of EC_{50} values for inhibition of growth (cell counts) for *Ditylum brightwellii* between 0.06 to 1.2 mgL^{-1} Cu. Hollibaugh *et al.* (1980)

found that natural marine phytoplankton were generally less sensitive and required 0.11 mgL^{-1} Cd^{2+} for significant inhibition although much lower concentrations (0.06 mgL^{-1}) were required for noticeable growth inhibition with *Thalassiosira aestivalis*.

Growth of other diatoms of the same genus, *Chaetoceros gracilis* and *Chaetoceros ceratosporum* have been found by another ASEAN laboratory to be 50% inhibited by higher concentrations ranging from 0.3 to <1.0 and 0.6 to 1.0 mgL^{-1} Cd respectively (Hindarti, 1997). Both species have also been reported to be more sensitive to cadmium than to chromium (Darmayati, 1997). The genus *Chaetoceros* has also been shown to be the most sensitive organism in studies with other metals. Hannan and Patouillet (1972) observed that inhibition of *Chaetoceros galvestonensis* by 0.1 mgL^{-1} mercury was more pronounced than to *Phaeodactylum* and *Cyclotella*. Cd has not only been found to inhibit growth rate of *Chaetoceros debile* but also the formation of its resting spores (Sanders and Cibik, 1985a). Diatoms have an absolute requirement for silica (O'Kelley, 1968), using SiO_2 to construct cell walls. A second requirement for silicon in the vegetative division cycle is involved with net DNA synthesis which has been observed in the diatom *Cylindrotheca fusiformis* (Darley and Volcani, 1969). Silicon uptake has been shown to decline in the presence of -SH binders like $CdCl_2$ (Lewin, 1954).

Meanwhile, the test species *Tetraselmis tetrahele* and *Tetraselmis* sp. were much more tolerant to Cd in the absence of EDTA, with an average IC_{50} value of 5.6 mgL^{-1}, comparable to a recent work by Okamoto *et al.* (1996) who described another marine prasinophyte, *Tetraselmis gracilis*, as one of the most tolerant algal species to Cd with its cell growth significantly inhibited only at concentrations of 5.0 mgL^{-1} or higher. Okamoto *et al.* (1996) showed that Cd promoted the induction of superoxide dismutase (SOD)

activities in *T. gracilis*, which is suggestive of an oxidative stress state, and perhaps the mode of toxicity operative in the test species. Bentley-Mowatt and Reid (1977) reported that growth of *Tetraselmis* sp. in batch culture were not arrested by the addition of Cd and Cu below a concentration of 6.4 mgL^{-1}. Meanwhile more tolerant strains have been observed by Gonzales (1997) who reported the average Cd IC_{50} values of 8.8 mgL^{-1} and > 9.7 mgL^{-1} for *T. tetrahele* and *T.* sp. respectively. Earlier, Gonzales (1995) had reported an IC_{50} range of 3.9 to >10 mgL^{-1} for *T.* sp. Similiar range of IC_{50} values have been observed in another green algae, *Dunaliella tertiolecta* (1.96-7.73 mgL^{-1}), by Thongra-ar *et al.* (1995).

5.2.3 Copper

Based on the experiments carried out in the absence of EDTA, Cu was the most toxic metal inhibiting 50% of growth of the test species at concentrations 0.04-0.37 mgL^{-1}. Toxicity studies carried out by others also showed that Cu as one of the most toxic metals. Cu, an essential micronutrient, becomes toxic to algae at higher concentrations where the toxicity of Cu to phytoplankton is a function of its free metal concentrations or Cu^{2+} ion activity (Davey *et al.*, 1973; Sunda and Morel, 1976; Anderson and Morel, 1978; Hollibaugh *et al.*, 1980). The flagellate, *Isochrysis galbana*, was the most sensitive test species to Cu with the average IC_{50} value of 0.04 mgL^{-1} comparable to EC_{50} values reported by some researchers. Rosko and Rachlin (1975) observed that 0.03 mgL^{-1} Cu reduced the growth rate of the marine diatom *Nitzschia closterium* by 50%. Meanwhile, Lumsden and Florence (1983) noted that 0.02 mgL^{-1} Cu caused 50% reduction in the growth rate of *Nitzschia* although 0.05 mgL^{-1} had no measurable effects on photosynthesis. Saifullah (1978) has reported an EC_{50} of 0.02 mgL^{-1} (cell number) for the

dinoflagellate, *Gymnodium splendens*. In his review, Stratton (1987) listed some specific Cu^{2+} toxicity data (EC_{50} values), showing the considerable variations among algae in Cu sensitivity, where most are inhibited by levels much lower than observed in this study. Some very sensitive algae are significantly affected by Cu concentrations of less than 1 μgL^{-1}. Anderson and Morel (1978) reported that < 0.1 μgL^{-1} Cu^{2+} inhibited 50% of the motility of *Gonyaulax tamarensis* while Sunda and Lewis (1978) discovered that 0.2 μgL^{-1} inhibited 50% cell division of *Monochrysis lutheri*. The range 0.2 - 0.6 μgL^{-1} caused 50% inhibition in growth of the planktonic diatom *Ditylum brightwellii* (Canterford and Canterford, 1980).

The centric diatom *C. calcitrans* was also sensitive to Cu with IC_{50} value 0.07 mgL^{-1} although relatively less than *I. galbana*. Previous studies have shown that diatoms appeared to be the most sensitive organisms to Cu (Bentley-Mowatt and Reid, 1977; Berland *et al.* 1976; Overnell, 1976). Thomas and Seibert (1977) have also shown that centric diatoms particularly *Chaetoceros* sp. were the algae first inhibited in the presence of copper. Zhang *et al.* (1992) observed that growth rate, μ, of *Chaetoceros* sp. decreased from 0.79 in the control to 0.22 when Cu concentration was increased to 0.2 mgL^{-1}. The lack of tolerance in diatoms may be explained by the observation of Goering *et al.* (1977) that Cu^{2+} ions inhibit the uptake of silicic acid. Morel *et al.* (1978) also suggested that one of the targets of Cu in diatoms is silicon metabolism. Copper has been shown to interfere directly or indirectly, with silicon uptake in diatoms where this interference may lead to depressed cell division rates, as the cell must regulate its silicon uptake for frustule formation and cell division (Fisher *et al.*, 1981). Centric diatoms have been shown to be more sensitive than pennate diatoms to copper, perhaps due to a more sensitive $Si(OH)_2$ uptake mechanism (Thomas and Seibert, 1977). Gavis *et al.* (1981)

showed that Cu caused total inhibition of growth in *Skeletonema costatum*, *Phaeodactylum tricornutum* and *Thalassiosira pseudonona* at ≤ 0.2, < 0.2 and 0.1 μgL^{-1} respectively.

EC_{50} values similar to the range observed in this study have also been reported. Hawkins and Griffiths (1982) quoted an EC_{50} of 0.05 mgL^{-1} (growth rate) for *P. tricornutum* while Davey *et al.* (1973) reported the value of 0.03 mgL^{-1} with *T. pseudonana*. Goering *et al.* (1977) found an EC_{50} of 0.03 mgL^{-1} for silicic acid uptake in a marine phytoplankton community.

However, in the natural environment, Thomas and Seibert (1977) reported that dinoflagellates were the most sensitive phototrophs to Cu followed by centric diatoms, while microflagellate and pennate diatoms were quite resistant to copper and green algae intermediate in their sensitivity to Cu. In this study *T. tetrahele* and *T.* sp. were also moderate in their sensitivity to Cu with IC_{50} values 0.13 and 0.37 mgL^{-1} respectively. Craigie *et al.* (1966) has reported the production of dimethyl-β-propiothetin by *Tetraselmis* spp. which seemed to confer mercury but not copper tolerance upon this genus. Some species of phytoplankton have the ability to excrete organic compounds which have the capacity to complex and ameliorate Cu toxicity. This been observed with *Skeletonema costatum* (Stolzberg and Rosin, 1977), *Gleocystis gigas* (Swallow *et al.*, 1978) and *Cricosphaera elongata* (Gnassia-Barelli *et al.*, 1978). Recently, another ASEAN laboratory found that 0.22 mgL^{-1} Cu caused 50% inhibition of growth in *T. tetrahele*, slightly higher than the values obtained in this study (Gonzales, 1997). Earlier Gonzales (1995) also had reported the average IC_{50} value of 0.16 mgL^{-1} Cu for *T.* sp., lower than our values. Marine phytoplankton have also been reported to be inhibited at

relatively higher concentrations of copper in the absence of EDTA. Overnell (1976) found that photosynthesis in *Brachiomonas submarina*, *Skeletonema costatum*, *Attheya decora*, *Dunaliella tertiolecta*, *Navicula incerta* and *Monochrysis lutheri* were inhibited by 50% by 1.3-3.2, 3.2, 4.5, 4.5, 10.5 and 12.7 mgL^{-1} Cu^{2+} respectively.

5.2.4 Manganese

Relatively less toxicity data is available on the seemingly less toxic metals rendering it neccessary to conduct more research. Although Mn is an essential element required for algal growth, higher concentrations have been reported to affect their growth (Rai *et al.*, 1981). In our study, growth of *I. galbana*, the species most sensitive to Mn, was 50% inhibited by 7.2 mgL^{-1} in the absence of EDTA. The other three test species were relatively less sensitive to Mn. An average IC_{50} value of 18.9 mgL^{-1} Mn was observed with the centrate *C. calcitrans* comparable to the observation of Rosko and Rachlin (1975) who found that 25.7 mgL^{-1} Mn reduced the growth rate of the pennate diatom, *N. closterium*, by 50%. Meanwhile Fisher and Jones (1981) has reported a much lower EC_{50} value of 4.85 mgL^{-1} with another pennate diatom *Asterionella japonica*. The relative tolerance to Mn may possibly be due to the fact that a number of phytoplankton species are capable of maintaining constant intracellular concentrations of metals (including Mn) over a range covering several orders of magnitude of extracellular free ion activities, via a homeostatic mechanism of control (Mason and Jenkins, 1995). Growth of test species *T. tetrahele* and *T.* sp. were inhibited by 50% by the concentrations 20.2 and 21.4 mgL^{-1} respectively. No value has been reported by other laboratories involved in the CPMS-II .

5.2.5 Arsenic

Generally, there is a lack of specific toxicity data on arsenic. Previous research have indicated that phytoplankton are differentially sensitive to arsenate when grown in single-species cultures (Bottino *et al.*, 1978; Planas and Healey, 1978) but, as demonstrated by results of this study, generalisation concerning taxonomic groups cannot be made (Sanders and Vermersch, 1982). Chrysophytes, dinoflagellates and prasinophytes have shown marked differences in their relative sensitivity to As(V) stress (Bottino *et al.*, 1978; Planas and Healey, 1978; Sanders and Vermersch, 1982).

In experiments conducted in the absence of the chelating agent EDTA, *Tetraselmis tetrahele* and *T.* sp were the most sensitive test species to As with IC_{50} values of 35.6 and 33.9 mgL^{-1} respectively. Irgolic *et al.* (1977) found that *Tetraselmis chuii* was able to grow well even at 50 mgL^{-1} As(V) while Sanders and Vermersch (1982) have observed the depression of the growth of *Tetraselmis contracta* by < 100 μgL^{-1}. Meanwhile, Irgolic *et al.* (1977) and Bottino *et al.* (1978) have observed higher tolerance in *T. chuii* which was able to thrive at 1000 mgL^{-1} As(V) after repeated transfer into increasingly higher concentrations. Bottino *et al.* (1978) also reported that *Hymenomonas carterae* were able to grow even when added without adaptation to medium with 1000 mgL^{-1} As(V).

The chrysophyte, *I. galbana*, which was the most sensitive species to Cd, Cu and Mn, and the diatom, *C. calcitrans*, were found to be quite tolerant to As with average IC_{50} values of 319.3 and 300.4 mgL^{-1} As respectively. Sanders and Vermersch (1982), however, have shown single-species cultures of *I. galbana* and *Chaetoceros pseudocrinitum* to be much more sensitive to As(V) where their growth were terminated

by <100 μgL^{-1}. On the other hand, these workers have also observed that a natural phytoplankton assemblage exposed to 5-25 μgL^{-1} As(V) was dominated by *Chaetoceros* spp. while growth rates of microflagellates including *I. galbana*, were largely unaffected. Meanwhile, with *Chaetoceros subtile*, Sanders and Cibik (1985b) has found that 9.3 μgL^{-1} As(V) caused a significant deviation in growth rates.

Insensitivity to arsenic does not imply its non-toxicity to the test species or other marine phytoplankton species. Sanders (1979) has attributed the higher values obtained by others (Bottino *et al.*, 1978; Irgolic *et al.*,1977) to the higher phosphate levels in their test medium, and this may be the reason for the observed tolerance in the test species. Arsenate which is analogous to phosphate, is taken up indiscriminately by phytoplankton (Sanders and Windom, 1980) probably by the same transport system (Blum, 1966). Arsenate and phosphate compete for uptake by algal cells, thus the external phosphate concentration may be important in determining the toxicity of As(V) where inhibition of growth may be greatest where phosphate concentrations are lowest (Sanders, 1979). Sanders and Windom (1980) have demonstrated the effect of external As(V) concentration and phosphate concentration on the rate of As(V) uptake in the diatom, *Skeletonema costatum*. Working with relatively lower levels of As(V) and phosphate, they showed that over the range of As(V) concentrations studied (5-25 μgL^{-1}), increasing phosphate concentration (0.07-3.4μM) decreased the As(V) uptake by the algae. Hollibaugh *et al.* (1980) reported that As(V) at > 300nM in low-phosphate medium repressed growth while As(V) was not toxic at 1000nM in high-phosphate medium. Pilson (1974) has also suggested that in regions where the arsenate : phosphate ratio is high, significant amounts of unwanted arsenate may be transported in along with phosphate. Phosphate enrichment (approximately 0.1mM) in the test solution probably

reduced the uptake of As(V) by the test species, thus reducing the toxicity of the metalloid and resulting in high IC_{50} values as observed in this study. The even much higher levels of phosphate in their medium (approximately 0.2mM) may also explain the higher tolerance reported by Bottino *et al.* (1978) and Irgolic *et al.* (1977). Sanders (1985) has observed that species which exhibit affinity for nutrients appear to be better able to discriminate between necessary nutrient and competing ions than do species exhibiting lower nutrient affinity where in the case of arsenate and phosphate, varying degress of discrimination have been found for both natural phytoplankton species and individual species, which may account for the varying degrees of sensitivity or tolerance observed among the test species.

Tolerance of the test species to As(V) may also be attributed to their ability to metabolise As(V) to less toxic As species (Andreae, 1978; Wangberg, 1995). Marine phytoplankton are able to actively take up arsenate at natural concentrations and regulate cellular arsenic levels (Andreae and Klumpp, 1979). Arsenate is converted into monoethyl arsonic acid, dimethyl arsonic acid and arsenolipids (Wrench and Addison, 1980) where dimethyl arsonic acid is found to be non-toxic (Sanders, 1979a). Some species are very efficient in reducing As(V) to As(III) in their environment (Andreae and Klumpp, 1979) where the As(III) formed may possibly diffuse back out again more easily than would arsenate, and thus avoid building up significant cellular contents of arsenic (Pilson, 1974). Sanders and Vermersch (1982) however, have shown that As speciation did not vary greatly during their 15-day study where only 6-8% of the added As(V) were reduced to As(III) and approximately 5% were methylated within two days of the first addition while the remaining 85-89% were present as arsenate throughout the experiment, where As(V) may be as toxic as, or more toxic than As(III) due to its

inhibition of phosphate uptake (Scudlark and Johnson, 1982).

5.3 EFFECTS OF CHELATION

The presence of chelating agents in cultural medium play an essential role in reducing metal toxicity (Chiaudani and Vighi, 1978). Not taking this fact into consideration many workers assayed the toxicity of heavy metals using cultural media supplemented with high concentrations of chelating agents, thus explaining the large variability in toxicity data (Bartlet *et al.*, 1974; Fitzgerald and Faust 1963; Manahan and Smith, 1972; Young and Lisk, 1972). In media without chelating agents, toxicity of metal to culture may be pronounced (Davies, 1978). Reduction in metal toxicity in the presence of chelating agents have been attributed to decrease in the concentration of free metal ions (Spencer, 1957; Morris and Russel, 1973; Sunda and Guillard, 1976) simply due to the chelation of the metals in the medium (Morel, 1983) and not the physiological effects of chelators.

EDTA is the most widely used chelating agent in synthetic cultural media and complexes most of the important trace metals to a considerable extent, normally more than other common complexing substances (Chiaudani and Vighi, 1978). However, according to Florence (1982), a ligand as powerful as EDTA in seawater would not complex significant amounts of metals including Cd and Cu unless present at concentrations unrealistically high for natural seawater. He calculated that between 0.002 μM to 0.2μM EDTA, the fraction of Cu and Cd which exist as EDTA complex were between 0.3-35 % and <0.1 - 6.8 % respectively. Therefore, the normal and higher level of EDTA present in our test medium (120 μM) would not only swamp out any naturally occuring organic ligands present in the seawater used to prepare the medium (Canterford

and Canterford, 1980) but also chelate a much higher precentage of Cu and Cd in the test solutions. A reduction of the toxic effect is evident for all the metals in this study except As.

5.3.1 Cadmium

Relatively less work has been done on the effects of chelation on Cd toxicity in comparison to Cu (Erickson *et al.*, 1970; Steemann-Nielsen and Kamp-Nielsen, 1970; Davey *et al.*, 1973; Riisgard *et al.*, 1980; Lage *et al.*, 1994; Edding and Tala, 1996). In this study, toxicity of Cd to *C. calcitrans*, *I. galbana*, *T. tetrahele* and *T.* sp. was reduced approximately 200, 100, four and three times respectively by the presence of EDTA. *C. calcitrans* and *I. galbana* which had been quite sensitive to Cd in the absence of EDTA were notably tolerant to Cd at concentrations two degrees of magnitude higher. Cadmium toxicity is a function of free metal ion concentration, not the total metal ion level (Foster and Morel, 1982). Canterford and Canterford (1980) showed that a range of 0.06 - 1.2 mgL^{-1} of total Cd concentrations in the presence of $6.7X10^{-7}$-$1.0X10^{-5}$ M EDTA, only 2.3 - 8.5 μgL^{-1} of the Cd were available as free ions. These workers also demonstrated that for a given EDTA concentration, the toxicity effects of Cd (and also Cu) on growth of diatom *Ditylum brightwellii* generally decreased with increasing metal concentration. Effects of chelation on Cd toxicity to diatom *Skeletonema costatum* (Berland, 1977) and chlorophyte *Dunaliella minuta* (Visviki and Rachlin, 1991) have been observed with Tris though the effects were not as strong as observed in our study, where the EC_{50} values were not much different than the range observed in this study in the absence of EDTA with the two former species. Meanwhile, Singh and Pandey (1981) observed that 0.05mM EDTA rendered the cyanobacterium *Nostoc calcicola* immune to Cd up to 4 mgL^{-1} with

no significant difference from the control while in the presence of 0.014mM EDTA, the growth in 10 mgL^{-1} Cd was equal to growth in 2 μgL^{-1} Cd.

5.3.2 Copper

In the presence of EDTA, growth of all test species were 50% inhibited at Cu concentrations one to two degrees of magnitude higher than in the absence of EDTA, with similar IC_{50} values averaging at 6.3 mgL^{-1}. Sunda and Guillard (1976) have shown that in highly chelated seawater media, the growth rate and the copper content of algal cells are related to cupric ion activity and not the total copper concentration. Several workers have also shown that the effective toxicity of copper to some unicellular algae is considerably reduced in the presence of chelating agents (Davey *et al.*, 1973; Erickson *et al.*, 1970; Steemann-Nielsen and Kamp-Nielsen, 1970) and that the stronger the metal complex, the lower the toxicity of a given concentration of total metal (Steemann-Nielsen and Wium-Anderson, 1970). EDTA has a stronger affinity to free cupric ions than most natural chelators including 'binding sites' on membranes (Riisgard *et al.*,1980) and in solution, copper-EDTA complex is also thermodynamically more stable than other main metal-EDTA complexes (Anderson and Morel, 1978), hence a significant fraction of the copper when bound by the EDTA produces an inert complex, causing a dramatic reduction in bioavailable copper (Lage *et al.*, 1994). Canterford and Canterford (1980) showed in their work that 0.015-0.58 mgL^{-1} of total Cu concentration in the presence of $6.7X10^{-7}$-$1.0X10^{-5}$ M EDTA were available as 0.23-0.65 μgL^{-1}. Lee (1973) has also stated that copper (II)-EDTA is a soluble complex of Cu(II) which shows little or no toxicity to aquatic organisms such as algae. Biological membranes are also impermeable to $Cu\text{-}EDTA^{2-}$ (Davies, 1970). The protective mechanism, competitive binding, has also

been found to be operative in chelating medium (Sunda and Huntsman, 1983) instead of the usual formation of the hydrated metal oxides which adsorp copper and reduce its penetration into the cell, as the strong ligands would complex trivalent metal ions (Stauber and Florence, 1987)

Edding and Tala (1996) has reported an EC_{50} value of 1.6 mgL^{-1} for *I. galbana* in the presence of EDTA, approximately six times lower than the value obtained in this study. Meanwhile, much lower values have been reported by other workers. Canterford and Canterford (1980) has reported an EC_{50} range of 0.02-0.58 mgL^{-1} (cell counts) with *Ditylum brightwellii* while Christensen *et al.* (1979) reported that 0.07 mgL^{-1} Cu caused a 50% reduction in the total cell volume of the *Chlorella stigmatophora*. Overnell (1975a) has shown with *Dunaliella tertiolecta*, the increase in EC_{50} values from 0.64 mgL^{-1} (oxygen evolution) and 0.32 mgL^{-1} (potassium loss) in the absence of EDTA to 2.5 mgL^{-1} and 1.3 mgL^{-1} respectively, in the presence of EDTA. Values much higher than those obtained in this study have also been observed. Edding and Tala (1996) has reported an EC_{50} of 38.8 mgL^{-1} with *Dunaliella tertiolecta* while Erickson *et al.* (1970) have shown the extreme reduction in the toxicity of Cu to *Amphidinium carterae, Olisthodiscus luteus* and *Skeletonema costatum*, all with EC_{50} values of 50 mgL^{-1}, due to chelation of Cu. The variation observed may be attributed to the variation in levels of EDTA and also sensitivity of the algae to the Cu- EDTA complex. According to Morris and Russell (1973) , complex formation could occur between one Cu^{2+} ion and 1.0-1.5 molecules of EDTA and assuming that EDTA will also combine with other ions in the medium (for example Fe^{2+}) it is likely that the reduced toxicity of copper is directly related to EDTA content of the medium. Once the EDTA is fully complexed, toxicity again becomes

related to the amount of copper or in other words, copper toxicity will be observed only when the concentration of total copper is close to or in excess of the chelator concentration in the medium (Morel *et al.*, 1978). Canterford and Canterford (1980) demonstrated that 0.05 to 0.1 mgL^{-1} Cu in culture medium with $6.7X10^{-7}M$ EDTA was sufficient to completely inhibit growth of *Ditylum brightwellii* while in 0.1 mgL^{-1} Cu with $1.0X10^{-5}M$ EDTA growth was similar to the control culture.

5.3.3 Manganese

Tolerance of the test species to manganese were much greater in experiments containing EDTA due to chelation of the metal. As it has been demonstrated that the toxicity of Cu to various phytoplankton is a function of Cu^{2+} ion activity, it is therefore reasonable to extrapolate the idea that ion activity rather than concentration controls metal toxicity, to metals other than Cu, at least to those that are toxic as divalent cations (Hollibaugh *et al.*, 1980). Sunda and Huntsman (1985) have also shown with an estuarine species of *Chlamydomonas* that cellular manganese is related to the concentration of free ion rather to the concentration of total manganese. Toxicity of manganese to the four test algae was reduced three to five times in the presence of EDTA. Relatively weaker responses of Mn^{2+} to EDTA is consistent with the lower stability constant (log K) of its metal-EDTA complex (Mn^{2+}, 13.8; Cu^{2+}, 18.7; Cd^{2+}, 16.4) (Hockett and Mount, 1996) which has also been demonstrated by Sorvari and Sillanpaa (1996) with *Daphnia magna*.

In the presence of EDTA, 50 mgL^{-1} Mn caused 50% reduction in cell volume in the green algae *C. stigmatophora* (Christensen *et al.*, 1979) within the range which caused inhibition of growth in the chlorophytes *T. tetrahele* and *T.* sp. Rosko and Rachlin (1975) had also observed that the toxicity of Mn was two times lower to the

diatom *Nitzschia closterium* due to chelation by amino acids with 96h EC_{50} value of 53.8 mgL^{-1}. Meanwhile Canterford and Canterford (1980) who used a much lower level of EDTA (0.67 μM) observed a much higher toxicity to *D. brightwelli* due to Mn with EC_{50} of 1.5 mgL^{-1}.

5.3.4 Arsenic

Toxicity of arsenic to *C. calcitrans* and *I. galbana* was not reduced in the presence of EDTA but remained similar as in the absence of the chelator. As(V) and As(III) which are present as anions ($HAsO_4^{2-}$) are not expected to form stable complexes with EDTA which is also also negatively charged ($EDTA^{4-}$) (Hockett and Mount,1996). This has also been demonstrated by Hockett and Mount (1996) where the addition of EDTA did not alter the toxicity of As(III) and As(V) to a cladoceran *Ceriodaphnia dubia*. However observation with *T. tetrahele* and *T.* sp cannot be explained by the same phenomena as the toxicity of As was reduced approximately by half in experiments containing EDTA. The phenomenon of resistance to heavy metal toxicity allows no simple explanation due to the multiplicity of interactions that can occur between phytoplanktonic cells, heavy metal ions, and other environmental constituents. In some instances, where components of the environment which may not be a direct result of their activity are responsible for detoxification, neither "resistance" nor "tolerance" are appropriate terms for describing the persistence of these organisms in the presence of high metal concentrations (Gadd and Griffiths, 1978).

5.4 SUITABILITY AS TEST ORGANISMS

Based on the results, the test species may be ranked according to their sensitivity to the heavy metals, in the presence (+EDTA) and absence (-EDTA) of EDTA.

The test species ranked according to decrease in sensitivity (or increase in tolerance) :

+ EDTA :

Cd : *I. galbana* > *C. calcitrans* = *T.* sp. > *T. tetrahele*

Cu : *I. galbana* = *C. calcitrans* = *T. tetrahele* = *T.* sp.

Mn: *I. galbana* > *C. calcitrans* = *T.* sp. > *T. tetrahele*

As : *T. tetrahele* = *T.* sp. > *C. calcitrans* = *I. galbana*

– EDTA :

Cd : *I. galbana* = *C. calcitrans* > *T. tetrahele* = *T.* sp.

Cu : *I. galbana* > *C. calcitrans* > *T. tetrahele* > *T.* sp.

Mn : *I. galbana* > *C. calcitrans* = *T. tetrahele* = *T.* sp.

As : *T. tetrahele* = *T.* sp. > *C. calcitrans* = *I. galbana*

One of the main criteria when selecting test organisms for toxicity testing is their sensitivity towards the toxicant (Walsh, 1988). Monitoring results showed that the range of cadmium, copper and arsenic in the Malaysian marine environment between 1992-1995 were 0.008-0.024, 0.011-0.058 and 0.039-0.144 mgL^{-1} respectively (DOE, 1996a). Based on the IC_{50} values obtained from tests without EDTA, and the ambient metal concentrations mentioned above, the marine phytoplankton used in the tests may be classified according to their suitability as test organisms for toxicity testing. Results of the reference toxicant tests also indicate the sensitivity of the test species and the

reproducibility of results using the organisms (CPMS-II, 1997). The most sensitive test algae to Cd, *I. galbana* and *C. calcitrans* had an average IC_{50} value of 0.06 mgL^{-1}, higher than the range observed in the natural environment. Even though the two species may not be suitable for toxicity testing of Cd, *I. galbana* and *C. calcitrans* may still be used for monitoring of Cd in the event of a pollution as their Cd IC_{50} values were of the same degrees of magnitude with the natural levels. Although the levels of heavy metals in the open sea are usually low, high concentrations of these metals can sometimes occur in coastal waters close to sewage systems (Rebhun and Ben-Amotz, 1984; Wickfors and Ukeles, 1982). Test species used in this study are also more sensitive than species and strains used by other ASEAN laboratories (Table 2.6). *C. calcitrans* is more sensitive to Cd than *Chaetoceros ceratosporum* and *Chaetoceros gracilis* which have been used by Hindarti (1997). In fact, *T. tetrahele* and *T.* sp., though not as sensitive as the diatom, are more sensitive to Cd than strains used by Gonzales (1997).

This study shows that *I. galbana* which was the most sensitive to Cu is also suitable for the toxicity testing of Cu as its IC_{50} value (0.04 mgL^{-1}) was within the range of the ambient levels. It is assumed that if an organism produces good results with the reference toxicant (Cd), it should do so with other toxicants. The lower IC_{50} values and narrower 95% confidence limits observed in the reference toxicant control chart (Figure 4.6) also show the sensitivity and lower variability in the results produced by *I. galbana* compared to *C. calcitrans*. In fact, *I. galbana* is an established species of phytoplankton for toxicity tests and has been used for toxicity testing of various chemicals including those from oil refinery industry (Roseth *et al.*, 1996). As the Cu IC_{50} value observed for *C. calcitrans* (0.07mgL^{-1}) was almost within the mentioned range, the diatom may also be

used in toxicity testing and monitoring of Cu. Meanwhile *T. tetrahele*, though relatively less sensitive to Cu than the two former species, is almost twice as sensitive to Cu as the strain used by Gonzales (1997) and may still be used for monitoring Cu. It should be noted that *T.* sp. performed better than *T. tetrahele* in the reference toxicant tests (Figures 4.4 and 4.8), keeping within the 95% confidence limits, thus indicating its stability and reproducibility of results although it was not as sensitive to Cu as the other test species. *T.* sp was also slightly more tolerant than the strain used by Gonzales (1995) (See Table 2.7). Differences in metal sensitivity among strains or clones of the same species may be attributable to differential penetration of toxic metals to sensitive sites within the cells (Davies, 1976; Daniel and Chamberlain, 1981).

Despite the fact that Mn is an essential nutrient for all algae (Sorentino, 1978) elevated amounts in the environment may be detrimental as shown by the results of tests with Mn in the absence of EDTA. The species most sensitive to Mn was *I. galbana* which exhibited IC_{50} value of 7.2 mgL^{-1}, a concentration which may be considered much higher above the local ambient levels which have been reported to be in the range of 0.001-0.08 mgL^{-1} (Makjanic *et al.*, 1995; Ramachandran *et al.*, 1995). Thus, this species is not suitable for use in toxicity tests with Mn but perhaps may be used for monitoring. A more sensitive local species should be sought for use in toxicity tests with Mn in generating toxicity data for the derivation of a marine water quality criteria.

Predictions can be made complicated when considering a species' general sensitivity or tolerance to stress. *S. costatum* which has been shown to be consistently sensitive to arsenate (Sanders and Vermersch, 1982) is not always sensitive to other types of stress where it has been found to be highly resistant to copper (Morel *et al.*, 1978). Meanwhile *T. pseudonana* has shown an opposite sensitivity to copper (Sunda and

Guillard, 1976), and relative insensitivity to arsenate. Similarly, in this study, *I. galbana* which is the most sensitive species to Cd, Cu, and Mn, is however quite tolerant to As, and thus not suitable for toxicity testing and also monitoring of As contamination. Despite their relative sensitivity to As, *T. tetrahele* and *T.* sp. are suitable for monitoring only large increases in As concentrations in the ambient water as the IC_{50} values obtained were 4 degrees of magnitude higher than concentrations reported in the natural environment. Therefore, the screening of other local species which are more sensitive to As and suitable as toxicity test species is needed. Highly resistant species or strains should neither be used to established standards, nor for bioassay of toxicity effects in natural waters (Gavis *et al.*, 1981). The possibility of obtaining reproducible results from toxicity studies will be enhanced not only by the use of standard methodologies but also by the use of selected strains of test organisms. The choice of test organisms should also be restricted to the most sensitive species which could easily be maintained under standard laboratory conditions (Chiaudani and Vighi, 1978).

5.5 RELEVANCE OF TOXICITY DATA TO THE MALAYSIAN MARINE WATER QUALITY STANDARDS

It should be noted that the Cd and Cu IC_{50} values observed for the most sensitive tests species, *I. galbana* (0.06 and 0.04 mgL^{-1} respectively), and also *C. calcitrans* (0.06 and 0.07 mgL^{-1} respectively) were all lower than the Malaysian Interim Standards for Marine Water Quality which is 0.1 mgL^{-1} for both metals (DOE, 1996b). If their growth are inhibited by 50% at these concentrations (the IC_{50} values), it is clear that in the natural local marine environment, the higher levels of Cd and Cu permitted by the Interim Standards will undoubtedly be detrimental to these two naturally occuring

species. In addition, other local species which are much more sensitive than the two former species would thus be much more affected by these levels. In fact, the Interim Standards are within the range of the average LOEC values (0.09-0.11 mgL^{-1}) of both heavy metals for both *I. galbana* and *C. calcitrans* which shows that at the lowest concentrations tested, growth of the tests species were significantly inhibited compared to the controls. Therefore it is recommended that the existing Standards (DOE, 1996b) for Cd and Cu be reviewed. The term "criteria" has been defined by the CCME (Canadian Council of Ministers of the Environment) as numerical concentrations or narrative statements recommended to support and maintain a designated resource use while "standards" are the numerical concentrations or narrative statements that are recognised in enforcable environmental control laws of the relevant authorities such as the government (Keenleyside *et al.*, 1995). The Marine Water Quality Standards for heavy metals, may be developed based on the Marine Environmental Quality Criteria (Tong, 1995) which basically represents concentrations of the metal, which if not exceeded, will protect aquatic organisms with an adequate degree of safety (CPMS-II, 1997). By applying the methodology developed by the ASEAN Marine Environmental Quality Criteria (AMEQC) Working Group (Tong, 1995), chronic toxicity data generated from toxicity tests may be used to derive a local or ASEAN Marine Water Quality Criteria. The Cd and Cu LOEC values of the most sensitive test species when multiplied by a safety factor of 0.1 to account for differences in sensitivity to a chemical variable due to differences in species, laboratory versus field conditions, and test endpoints, produce a local marine environmental quality criteria of approximately 0.01 mgL^{-1} for both Cd and Cu. It should be emphasised that the derivation of the local or ASEAN Marine Environmental Quality Criteria is beyond the scope of this work as the

derivation process involves more than the generation and compilation of toxicity data. The minimum data set requirements must include at least three studies on three or more tropical marine fish species, at least two chronic studies on two or more tropical marine invertebrates from different classes, and at least one study on a tropical marine vascular plant or algal species. More chronic toxicity data covering a sufficient range of sensitive marine species are required before a final ASEAN Marine Water Quality Criteria for these metals can be established. The toxicity data on algae generated from this study if compiled together with results of other local and ASEAN toxicology laboratories working with other marine organisms may contribute towards the formulation of a Malaysian and ASEAN Marine Environmental Quality Criteria for heavy metals and eventually the Marine Water Quality Standards. However it is recommended that an Interim Water Quality Criteria of 0.01 mgL^{-1} for Cd and Cu be adopted and taken into consideration when reviewing the existing Interim Standards which should be lowered for the protection of marine aquatic organisms. It is also recommended that monitoring be carried out in the future on Mn in the local marine environment as this metal may pose potential hazards at elevated levels.

Chapter 6

CONCLUSION

6. CONCLUSION

The following conclusion has been drawn based on the findings of this research.

1. Generally, copper was the most toxic heavy metal to the marine phytoplankton both in the absence and presence of the chelating agent, EDTA. Cadmium was as toxic as Cu, or slightly less toxic than Cu while manganese was relatively less toxic than Cd and Cu. Meanwhile, arsenic was the least toxic heavy metal to the marine phytoplankton. Cd, Cu and Mn were less toxic, while there were generally no differences in the toxicity of As to the test species in the presence of EDTA. In the absence of EDTA, the 96h Cu IC_{50} ranged between 0.04 - 0.37 mgL^{-1} while in the presence of EDTA, the 96h IC_{50} ranged between 6.0 - 6.6 mgL^{-1}. The 96h Cd IC_{50} ranged between 0.06 - 5.7 mgL^{-1} in the absence of EDTA and 4.7 - 18.1 mgL^{-1} in the presence of EDTA while the 96h Mn IC_{50} ranged between 7.2 - 21.4 mgL^{-1} in the absence of EDTA and 38.1 - 80.1 mgL^{-1} in the presence of EDTA. Meanwhile, the 96h As IC_{50} ranged between 33.9 - 319.3 mgL^{-1} in the absence of EDTA and 74.4 - 307.1 mgL^{-1} in the presence of EDTA.

2. The test species ranked according to decrease in sensitivity to the heavy metals in the presence (+) and absence (-) of EDTA :

   +EDTA : Cd : *I. galbana* > *C. calcitrans* = *T.* sp. > *T. tetrahele*

   Cu : *I. galbana* = *C. calcitrans* = *T. tetrahele* = *T.* sp.

   Mn: *I. galbana* > *C. calcitrans* = *T.* sp. > *T. tetrahele*

   As : *T. tetrahele* = *T.* sp. > *C. calcitrans* = *I. galbana*

- EDTA : Cd : *I. galbana* = *C. calcitrans* > *T. tetrahele* = *T.* sp.

Cu : *I. galbana* > *C. calcitrans* > *T. tetrahele* > *T.* sp.

Mn : *I. galbana* > *C. calcitrans* = *T. tetrahele* = *T.* sp.

As : *T. tetrahele* = *T.* sp. > *C. calcitrans* = *I. galbana*

Isochrysis galbana which is the most sensitive test species to Cd, Cu and Mn is suitable for the toxicity testing of Cu while the other species will only be useful for the long-term monitoring of these metals in the marine environment or the monitoring of elevated levels of the heavy metals in the event of a pollution. There is a need to seek for more sensitive local species of marine phytoplankton for use as test species in the toxicity testing of heavy metals especially Mn and As.

3. The method of toxicity testing combining the use of the Multiwell plates as the test vessels, the Elisa microplate and Multiskan machine for the quick measurement of O.D., and the good correlation between O.D. measurements and cell counts which enabled the determination of growth (cell counts) via the linear regressions between O.D. and cell counts, provides a rapid method of toxicity testing of heavy metals with marine phytoplankton, which is also simple, economical, and practical with reproducible results which are similar to the conventional shake-flasks.

4. The IC_{50} values of Cd and Cu obtained with the most sensitive test species were lower than the Malaysian Interim Marine Water Quality Standards (0.1 mgL^{-1}). Therefore it is recommended that the existing Interim Standards be reviewed and lowered to 0.01 mgL^{-1} for both Cd and Cu, for the protection of marine aquatic life.