Chapter 2

LITERATURE REVIEW

2. LITERATURE REVIEW

2.1 HEAVY METALS

2.1.1 Definitions

Metals with atomic number greater than iron (Passow *et al.*, 1961) or a specific gravity greater than 5 (Lapedes, 1974) have been termed *'heavy metals'*. This definition which is based solely on a physical parameter, is not a suitable criterion for the classification of heavy metals as it includes metals with widely different chemical and biological properties such as the lanthanides (atomic number Z=57-71) and actinides (Z=89-103), which are usually not considered to be heavy metals (Collins and Stotzky, 1989). Meanwhile, Bryan (1976) referred to heavy metals as all metals and metalloids with the exception of the alkali and alkaline earth elements. Nieboer and Richardson's (1980) classification of metals is based on the equilibrium constants that describe the formation of metal ion-ligand complexes, to A, B and borderline classes, where heavy metals are pedantically defined as metals with ions falling into categories B and borderline (Rainbow, 1993). The borderline ions, while consisting of the first row of transition metals in their common oxidation state (Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn), also include the metals, Ga, In, Cd, Sn, Pb and metalloids, As and Sb (Collins and Stotzky, 1989).

Most of the metal elements in the periodic tables were found typically at total concentrations well below 1 $mmolm^{-3}$ (in pristine fresh and saline waters of the hydrosphere), therefore referred to as trace metals (Sposito, 1986). The term *'trace metals'* may be used synonymously with heavy metals but alternatively be restricted to essential heavy metals only (Rainbow, 1993). Many trace metals are important to living

organisms, where as micronutrients, are required in small amounts (Eichenberger, 1986), they play an essential role in tissue metabolism and growth. However, at high concentrations most metal ions become *toxic metals*. *Essential metals*, are those when deficient result in impairment of functions in an organism which are relieved only by the administration of the substances while *non-essential metals* are not required in metabolism.

2.1.2 Natural sources and uses of heavy metals

Metals usually occur geologically as ores (Table 2.1) in mineral deposits (Collins and Stotzky, 1989) and are naturally recycled by geochemical processes of weathering, erosion and transportation (Andreae *et al.*, 1984) in the environment. Like other elements, metals are fundamental to the basic survival of man and to the functioning of traditional and modern societies (Manahan, 1992). Metals are physically or chemically processed to yield a purer form of the metal (Collins and Stotzky, 1989) and other by-product metals, which are used extensively by man (Table 2.1).

2.1.3 Essential heavy metals

Essential heavy metals include Fe, Cu, Zn, Mn, Co and Mo which always function in combination with organic molecules, most commonly proteins, either tightly bound in metalloproteins or more loosely bound in metal-protein complexes. A wide range of enzymes form more loosely bound metal-enzyme complexes and cannot achieve full catalytic activity in the absence of a specific metal or metals. Requirements of different plant and animal species vary substantially, but optimal concentration ranges for micronutrients are frequently narrow. Severe imbalances can cause death, whereas marginal imbalances contribute to poor health and retarded growth (Leland and

Table 2.1 Sources and various uses of heavy metals (Extracted and modified from Bryan, 1976).

Metal	Typical Ore	Uses
Ag	argentite	Ag_2S	Photography, electric conductors, sterling ware, solders, coinage, electroplating, catalyst, batteries
Al	bauxite	Al_2O_3	Binding and construction, transportation, electrical industry, packaging, consumer, durables, machinery, glass, pharmaceutical
As	arsenopyrite	FeAsS	Pb and Ca arsenate and sodium arsenite pesticides, glass and enamels, alloys, electronics
Cd	greenockite	CdS	Electroplating, pigments, thermoplastic stabilizer, batteries, low melting point, alloys
Co	cobaltite	CoAsS	Alloys, catalysts, pigments, enamels, glazes, electroplating
Cr	chromite	$FeO.Cr_2O_3$	Metallurgy-alloys, refractory bricks, electroplating, tanning, paint, algicide, wood preservative
Cu	chalcopyrite	$CuFeS_2$	Electrical industry, alloys(e.g. brass), chemical catalyst, antifouling paint, algicide, wood preservative
Fe	haematite	Fe_2O_3	Iron and steel industry
Hg	cinnabar	HgS	Chlor-alkali production, electrical apparatus, anti-mildew paint, instruments, catalysts, pesticides pharmaceuticals, antifouling paint
Mn	pyrolusite	MnO_2	Metallurgy-largely steel alloys, dry batteries, chemical industry (e.g. permanganate), glass, ceramic colouring
Mo	molybdenite	MoS_2	Metallurgy-steel alloys, catalyst, pigments, glass, lubricant and oil additive
Ni	pentlandite	$NiFeS_2$	Metallurgy-steel and other alloys, electroplating, catalyst
Pb	galena	PbS	Storage batteries, leaded petrol, pigments (e.g. red lead paint), ammunition, solder, cable covering anti-fouling paint
Sb	stibnite	Sb_2O_3	Antimonal lead (hardened lead), plastics, ceramics and glass, flame-proof chemicals, bearing metals, pigments, type metal
Se	selenides of Cu		Glass industry, electrical (e.g. rectifier), photocells, paints, rubber, insecticide-sodium selenite
Sn	cassiterite	SnO_2	Tinplate, solder, bronze, white metal, chemical reducing agent, fungicide-triphenyl tin acetate, anti-fouling paint,
V	vanadinite	$Pb_5(VO_4)_3Cl$	Metallurgy-steel and other alloys, catalyst, pigments
Zn	sphalerite	ZnS	Zn-based alloys, brass and bronze, galvanizing, rolled Zn, paints, batteries, rubber

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Kuwabara, 1985).

2.1.4 Non-essential heavy metals

Heavy metals such as Cd, Pb and Hg are non-essential where their metabolic or physiological requirements are not known (Rai *et al.*, 1981).

2.1.5 Toxic heavy metals

Consequently, most heavy metals, whether essential or not, are potentially toxic to living organisms as a result of their ability to denature protein molecules (Gadd and Griffiths, 1978). Ag, Hg, Cu, Cd, Pb and As are particularly toxic and usually inhibit enzymes by forming mercaptides with the sulphydril groups which are responsible for catalytic activity (Bryan, 1976).

2.1.6 Sources of heavy metal pollution

Human activities have modified the global cycles of the heavy metals where emissions from anthropogenic sources exceed contribution from natural sources by severalfold. Evidence shows a general elevation of pollutant metal burdens in many marine and land biota (Nriagu, 1984). The most important sources of heavy metal contamination in their order of significance are air pollution, river sediments, sewage sludges, town waste composts, agricultural chemicals, and industrial wastes (Kloke *et al.*, 1984).

2.1.7 Toxicity of heavy metals

The toxicity of heavy metals to plants, animals, and man has been known for centuries, though it is probable that toxic effects due to either industrial or environmental

exposure were frequently not recognised nor diagnosed correctly at the time (Thornton and Abrahams, 1984). Numerous reviews and references have been published on the toxicity of these metals to humans (Passow *et al.*,1961; Vallee and Ulmer, 1972), terrestrial and aquatic animals and plants (Hammond and Foulkes, 1986), and microorganisms such as fungi, bacteria and microalgae (Beveridge and Doyle, 1989).

2.1.8 Transport of metals through the aquatic food chains

The fact that heavy metals may be bioaccumulated through the food chain is of primary concern as they ultimately reach the highest trophic level and possibly cause hazards in humans. Industrial processes involving water are potential sources of metals in rivers and estuaries (Bryan, 1976). Mining, smelting and refining processes release a variety of metals into waterways while wastes such as sewage sludge which are often dumped at sea usually contain high concentrations of heavy metals. Atmospheric pollution as a result of burning of fossil fuels, smelting and refining of metals, the use of arsenical pesticides and leaded petrol vehicles also contribute significant amounts of heavy metals to the open ocean, coastal and semi-remote marine areas (Buat-Menard, 1984).

In solution, the heavy metals occur as inorganic cations or complexed species where they may be assimilated and concentrated by primary producers (Sorentino, 1978), namely seaweeds and phytoplankton, to be then incorporated in the food chain. Heavy metals are concentrated by all marine organisms but some species show exceptional powers of accumulation in certain tissues (Bryan, 1976). For most heavy metals, the first step from seawater to plants results in the highest enrichment of the entire food chain (Bernhard and Andreae, 1984) but for some, the levels in the biota can be lower than in seawater. The contribution of the toxic heavy metals to the human diet depends on the

last important step of the food chain, the step from seafoods to man (Bernhard and Andreae, 1984; Edmonds and Francesconi, 1993). Organisms in the higher trophic levels are often more developed, thus able to control their body concentration more efficiently by excreting or storing the metals in special organs. Although the ability to store and often detoxify heavy metals in certain organs is an excellent means of protection of the organism, it can lead to the heavy metal contamination of marine foodstuff (Bryan, 1976).

2.2 HEAVY METALS IN THE MARINE ENVIRONMENT

2.2.1 Introduction

Natural sources of heavy metals to the sea may be categorised to the coastal supply, which includes input from rivers and from erosion produced by wave action and glaciers; the deep sea supply which includes metals released by deep sea volcanism and those released from particles or sediments by chemical processes; the supply which by-passes the near-shore environment and includes metals transported in the atmosphere as dust particles and also material which is produced by glacial erosion in polar regions and is transported by floating ice (Bryan, 1976). The concentrations of metals in natural waters are so variable and available data are always subject to revision. Unlike other contaminants, heavy metals are normal constituents of the marine environment and traces at least are always found in marine organisms.

Two main systems which modify the distributions of metals in the sea are the many facets of metal introduction into inshore waters, and the seasonal contribution of plankton uptake and remineralisation. Copper, cadmium and manganese are among the

metals shown to be concentrated by plankton in coastal waters (Abdullah and Royle, 1974). Point sources of heavy metal pollution includes direct discharge of industrial and municipal effluents via pipes and sewers into waterways, while non-point sources include diffuse inputs such as landfills, atmospheric deposition, ground water, agricultural and urban runoff and storm sewers (Doust *et al.*, 1994). Heavy metals which enter the marine environment also adsorb to and accumulate in sediments. Concentrations of metals in ocean sediments vary considerably with geographical location where coastal metal levels are often significantly elevated due to nearby land-based pollution sources (Chongprasith *et al.*, 1995). Cu, Cd and Mn from wastewater discharges have been found to be loosely bound and accumulated on the surface of sediments (Tam and Wong, 1993) where they may be available to plants such as algae which may play an important role in mobilising sediment-bound metal ions (Laube *et al.*, 1979). Alternatively, water-borne heavy metals in mangrove sediments have been found to be immobilised and in non-bioavailable forms (Tam and Wong, 1995; Tam *et al.*, 1995).

2.2.2 Heavy metals in marine waters and sediments

2.2.2.1 Cadmium

Based on their study on 51 near-shore and 38 open-ocean surface seawaters from various regions of the world, Chester and Stoner (1974) once reported mean cadmium concentrations of 0.09 μgL^{-1} for nearshore waters and 0.07 μgL^{-1} for open-ocean waters, with none of the concentrations exceeding 0.3 μgL^{-1}. In a review Phillips (1980) gave the range of 0.01 to 85.0 μgL^{-1} for coastal and estuarine areas, and 0.01 to 0.41 μgL^{-1} for the open-ocean waters. Much more recent values have been presented by Ho (1995) (Table 2.2). Meanwhile, Maeda & Sakaguchi (1990) reported the total Cd concentration

of 0.04 μgL^{-1} in seawater, where 78% of it is in the organic form and the main dissolved species is $CdCl_2^0$. Cd is strongly associated with chloride ions over a wide pH range (Zirino and Yamamoto, 1972), where according to Sipos *et al.* (1980) the predominant dissolved species of Cd in seawater are labile chloro-complexes $CdCl_2$ (37%), $CdCl^+$(29%) and $CdCl_3^-$ (31%).   Uncomplexed Cd^{2+} constitutes about 2.5% of the total (Zirino and Yamamoto, 1972). The same species distribution is likely to be found in sediments. Helmers (1996) showed that more than 90% of the Cd in surface waters are localised in the dissolved state while the open-ocean surface particulate Cd consist mainly of biogenic material (phytoplankton).

Anthropogenic sources of Cd are predominantly industrial (Phillips, 1980). Other main sources contributing to seawater include waters from ore mines, sewage effluents and sludges, and contaminated agricultural soil run-offs.

Table 2.2 : Heavy metal contents of oceanic, coastal and estuarine waters

Metal	Concentration range		
	Oceanic (ngL^{-1})	Coastal (ngL^{-1})	Estuarine (μgL^{-1})
Ag	0.06-4	-	-
Cd	0.1-125	5-700	0.3-4.2
Cr	105-265	-	-
Cu	30-390	15-5800	0.2-176
Fe	6-145	-	-
Hg	0.4-2.1	1-80	0.002-16
Mn	11-170	-	-
Ni	120-720	10-4800	0.2-18
Pb	1-37	6-1230	0.35-16
Sn	0.1-1.5	-	-

Note : Extracted from Ho (1995)

2.2.2.2 Copper

Zirino and Yamamoto (1972) showed that at the average seawater pH of 8.1 the predominant chemical species of copper are $Cu(OH)_2^0$ (90%) and $CuCO_3^0$ (8%) where the

fractions of uncomplexed ion Cu^{2+} and the ion pair $CuOH^+$ are about 1%, while other species are negligible. Suspended particulate matter play an important role in processes related to trace metal mobility (Dassenakis *et al.*, 1997) and Cu is one of the metals readily removed from solution by absorption to particulates. Chester and Stoner (1974) reported an average of 0.9 μgL^{-1} for near-shore surface waters, and 0.8 μgL^{-1} for open-ocean waters comparable to the range reported recently by Ho (1995). Maeda & Sakaguchi (1990) stated that the total concentration of Cu in seawater is 0.6 μgL^{-1} where 78% is in the organic form and the main dissolved inorganic species are $CuCO_3$ and $CuOH^+$. Species such as $[CuHCO_3]^+$ and $CuSO_4$ would become more important in sediments as marine sediments are lower in pH than overlying waters (Zirino and Yamamoto, 1972.)

The main anthropogenic sources of Cu are the metal-plating and manufacturing industries (Mance *et al.*, 1984a). The use of Cu in paints as anti-fouling agents also introduce Cu into the marine environment.

2.2.2.3 Manganese

Chester and Stoner (1974) found the concentration of manganese in near-shore waters in the range of 0.12 to 1.2 μgL^{-1} with an average of 0.37 μgL^{-1}. They reported the range of 0.07 to 0.36 μgL^{-1} for open-ocean surface waters, with an average of 0.22 μgL^{-1}, which was within the range quoted by Ho (1995). They concluded that there were no evidence of any anthropogenic effect on the distribution of Mn in open-ocean surface waters. Meanwhile, Bender *et al.* (1977) concluded that the concentration of Mn in surface waters as typically 1 μgL^{-1} and that total dissolvable Mn in surface waters may originate from three possible sources : river run-offs, fallout of marine aerosol including

anthropogenic material, and input by diffusion out of nearshore reducing sediments. Dissolved Mn in seawater in the 2+ oxidation state tends to form relatively weak complexes with organic ligands and exists mainly as free hydrated Mn^{2+} (Bruland *et al.*, 1991) where Mn content of surface waters is higher than deep waters. The suspended loads of rivers sometimes have very high Mn concentrations. Most excess Mn in pelagic sediments is carried through the oceans to the sediments by terrigenous particles where Mn accumulates in association with fine-grained detritus.

2.2.2.4 Arsenic

The concentration of total As in clean coastal and ocean waters is 1-3 μgL^{-1}, with a mean of 1.7 μgL^{-1} (Neff, 1997). Human activities contribute little to the As budget of the open ocean, but may be of importance to estuaries and coastal waters where the concentrations may be higher than the open ocean, reflecting natural and anthropogenic inputs. Major sources of As in surface waters of the ocean are riverine inputs and upwelling of deep ocean water enriched with As (Waslenchuk, 1978) with little contribution from atmospheric As (Neff, 1997). Anthropogenic sources of As into the sea include industrial use, agricultural and deforestation activities, emission from coal and oil combustion, and loss during mining of metal ores (Sanders, 1985).

Arsenate (As(V)) and arsenite (As(III)) are the dominant forms of inorganic As where the most abundant forms for each at the normal pH of brackish and marine waters are anionic ($H_2AsO_4^{-1}$ and $HAsO_4^{2-}$) and neutral ($As[OH]_3^0$) respectively (Sadiq, 1990; Anderson and Bruland, 1991). In most coastal regions, more than 90% of the atmospheric and riverine inputs of As are in the form of As(V) (Sanders and Windom, 1980). As(V) is the thermodynamically most stable inorganic As while As(III) makes up 1.6 to 13.2% of total As in surface waters (Neff, 1997). Potential sources of thermodynamically

unstable As(III) in oxygenated brackish and marine waters are biotic and abiotic reduction of As(V), deposition from the atmosphere, upwelling from anoxic waters, and input from hypoxic river basins (Neff, 1997). Two organic forms of As, methylarsonic (MMA) and dimethylarsinic (DMA) acids, are also frequently found in seawater (Andreae, 1979). In aerobic seawater, As(III) is oxidised rapidly to As(V) both abiotically and by bacteria (Sanders and Windom, 1980). Seasonal increases in concentrations of As(III) and DMA in seawater are probably caused by reduction followed by oxidative methylation of As(V) accumulated by phytoplankton and other microbiota (Francesconi and Edmonds, 1994). Andreae (1978) observed that the diatom, *Thalassiosira pseudonana* can transform As(V) into As(III). These microbes excrete As(III) and methylarsenic compounds into ambient waters or the substances are released when they die (Andreae and Klumpp, 1979; Sanders and Windom, 1980; Wrench and Addison, 1981). The uptake and subsequent release of As in a reduced or methylated form which causes large changes in the speciation of As where 50% of As(V) may be reduced (Sanders and Windom, 1980), is a significant factor in determining the As biogeochemistry of productive systems. Sanders (1985) found that the presence of MMA was highly correlated with the abundance of *Chroomonas* spp., suggesting that the alga probably transformed As(V) to MMA.

Concentrations of total As in marine sediments range from 5 to 15 μgg^{-1} DW in uncontaminated nearshore marine and estuarine sediments to 40 μgg^{-1} DW in deep sea sediments (Neff, 1997) where As(V) is the most abundant form of As in oxidised sediments while As(III) is the dominant dissolved and solid species in reduced sediment layers. Marine sediments may also be an important source of MMA and DMA to overlying water column.

2.2.3 Heavy metals in marine organisms

Bruland *et al.* (1991) summarised the metal composition of marine phytoplankton representing natural oceanic assemblages of 'pure plankton' while Absil and van Scheppingen (1996) reported the concentrations of Cd, Cu, Zn and Pb in diatoms living in sediments. Much earlier, Riley and Roth (1971) reported the Cu contents in eight species of marine phytoplankton (See Table 2.3). Relatively little has been published on the concentration of total As in plankton.

Table 2.3 : Heavy metal composition of marine phytoplankton

Metal	Concentration range		
	Riley and Roth (1971) (μgg^{-1} DW)	Bruland *et al.* (1991) ($\mu molg^{-1}$ DW)	Absil and van Scheppingen (1996) (μgg^{-1} DW)
Cd	-	0.017 - 0.15	<0.1 - 2
Cu	25 - 210	0.05 - 0.15	3 - 29
Fe	-	1.3	-
Mn	3.8 - 118	0.096 - 0.10	-
Ni	2.8 - 48	0.05 - 0.24	-
Pb	<8 - 900	-	7.25
Zn	75 - 480	0.21 - 0.83	20 - 200

Reviews on the occurences of Cd in marine organisms have been extensively published by Mullin and Riley (1956) and while Cu has been covered by Eisler (1979) and Mance *et al.* (1984a). Folsom *et al.* (1963) reported the concentration of Mn-54 by marine organisms representing several phyla. The occurrences of As in marine organisms have also been extensively reviewed (Lunde, 1977; Sanders, 1979a; Mance *et al.*, 1984b; Phillips, 1990; Edmonds and Francesconi, 1993; Soto *et al.*, 1993; Neff, 1997). Marine organisms are able to accumulate dissolved As(V) from seawater and much of the

organic As in the tissues of marine animals may be derived directly or indirectly from consumption of marine algae (Neff, 1997).

2.2.4 Heavy metals in local and regional marine environment

Chester and Stoner (1974) once reported that the average concentrations of trace metals Cd, Cu, Fe, Mn, Ni and Zn in the Straits of Malacca were 0.10, 1.7, 0.7, 0.39, 1.0 and 1.2 µgL^{-1} respectively. Sivalingam (1978) considered the waters of Batu Ferringhi to be unpolluted where Cd, Cu and Fe were below detection limits and Mn, Cr and Pb were 0.35, 0.18 and 0.18 mgL^{-1} respectively. However the levels of metals in the sediments were fairly high. He later found Hg contamination in some marine macroalgal species in Penang (Sivalingam, 1980). Law and Singh (1986) found that the Kelang Estuary was polluted with Mn, Pb and Fe at levels of 27.1, 4.1 and 106.5 µgL^{-1} respectively while levels of Cu (10 µgL^{-1}) and Zn (17.9 µgL^{-1}) were similar to the world oceans.

Much more recent data on the heavy metal contents of local and regional marine waters based on research done by laboratories pariciparing in the ASEAN-Canada CPMS-II are summarised in Table 2.4. Ramachandran *et al.*(1995) stated that the average Cd (0.95 µgL^{-1}), Cr (0.45 µgL^{-1}), Cu (6.5 µgL^{-1}), Fe (192.0 µgL^{-1}) Mn (8.7 µgL^{-1}), Pb (57.0 µgL^{-1}) and Zn (44.0 µgL^{-1}) in local marine waters were all within Interim Standards.

Ismail *et al.* (1993) reported that the Cd and Cu in sediments collected from the west coast areas of Peninsular Malaysia were below 1 and 50 fgg^{-1} respectively, where overall, high concentrations were found only in the vicinity of river estuaries and ports. The mean range of Cd and Cu in mudflats of Batu Kawan has been recorded as 1.1 to 2.4 µgg^{-1} DW and 5.7 to 7.4 µgg^{-1} DW respectively while in Kuala Selangor the levels were 1.9 to 3.3 µgg^{-1} DW and 2.8-3.5 µgg^{-1} DW respectively, comparable to unpolluted sites

Table 2.4 : Heavy metal contents in the marine waters of ASEAN

AREA	Unit	As	Cd	Cu	Cr	Fe	Hg	Mn	Ni	Pb	Zn
[a] Estuaries of the Upper Gulf of Thailand :											
Chao Phraya Ya river mouth			0.2-1.0	2-31				1-2			3-5
Tha Chin river mouth	µgL⁻¹		0.1-0.6	2				1-2			5-9
Mae Klong river mouth			0.2-1.0	2-4				0.1-6.0			4-7
Bang Pa Kong river mouth			0.09-0.70	2				1-2			3-4
[b] Straits of Johor				1.4-3.9		8.5-171.1	0.3-1.9	1.1-9.0	0.3-3.2	0.1-1.4	0.5-2.8
Singapore, East Coast				0.2-0.8		1.1-19.0	2	0.1-3.7	0.2-0.5	1.4-2.1	0.3-1.3
Port of Singapore	µgL⁻¹			0.5-8.8		12.7-676.3	1.8-2.7	3.2-16.2	0.2-2.3	0.1-0.9	0.3-7.3
Tuas Bay				1.5		31.0	-	-	0.3		1.1
Jurong Fairway				6.8		503.9	2.3	11.9	1.7		4.0
[c] South of Johor	ngL⁻¹	0.38-1.89									
[d] Penang			1.9	3.6	0.5	347.0		12.0		61.0	40.0
Selangor			0.3	30.0	0.3	60.0		5.3		60.0	30.0
Negeri Sembilan	µgL⁻¹		1.0	12.0	0.4	100.0		3.5		65.0	28.0
Melaka			1.6	4.5	0.4	275.0		12.5		68.0	80.0
Johor			0.4	2.6	0.5	283.0		83.0		43.0	62.0
Terengganu			0.5	2.7	0.5	28.0		9.7		53.0	20.0
[e] Port of Singapore	µgL⁻¹		0.3-0.9	0.4-3.9		3-241		0.6-5	0.1-1.4	0.3-0.8	0.3-28
[f] Vietnam	µgL⁻¹	5.4	0.3-0.9	1.9-7.2			<0.1			1.0-6.8	0.3-28
[g] Semarang, Indonesia	µgL⁻¹		16.5-63.5	15.7-17.3	n d				79.4-83.3	72-75.9	149.5-151.6
[h] Vietnam coast	µgL⁻¹			9-15.2						120-210	21.3-29.5
[i] Upper Gulf Thailand	µgL⁻¹		0.013-0.144	0.13-11.62						0.13-12.18	
[j] Central Vietnam	µgL⁻¹			1.9-19.8						1.0-5.1	5.2-39.5

References :

(a) Sudharnuk et al. (1995); (b) Makjanic et al. (1995); (c) Mohd Yusof et al. (1995); (d) Ramachandran et al. (1995); (e) Tang et al. (1997); (f) Hoi et al. (1997); (g) Sunoko, 1997; (h). Khoa, 1997 (i) Chumchuchan and Suthanaruk, (1997); (j) Thorn and Tuan (1997)

(Mat and Maah, 1994).

Sivalingam (1978) reported a range of 3.7 to 16.3, trace levels to 49.7, and 23.7 to 316.0 μgg^{-1} DW of Cd, Cu and Mn respectively in seaweeds collected from Batu Ferringhi, Penang, while Sheila *et al.* (1994) reported the contents of Cd, Cr, Cu, Fe, Mn, Pb and Zn in tissues of local seaweeds ranging from 1.0-5.0, 0.5-21.5, 1.0-12.5, 100-5225, 8.5-1200, 2.0-21.5 and 4.5-135 μgg^{-1} DW respectively.

Heavy metal contents ôf some local species of shellfish and fish have also been studied (Sivalingam and Baskaran, 1980; Shahunthala, 1986).

2.3 AQUATIC TOXICITY TESTS

Chemical analyses are insufficient to predict environmental risks and therefore should be complemented by toxicological studies (Lambolez *et al.*, 1994). As pollution affects living organisms, it is only logical that it should be measured biologically (Rana and Jameson, 1995). A wide range of toxicity tests has been developed in recent years to provide rapid and accurate screening of chemicals for toxic effects (Capuzzo *et al.*, 1988). For the purpose of literature review, the term 'bioassay' has been retained as mentioned by the respective authors although in most cases it is more appropriate to replace the term with 'toxicity tests' (Krajnovic-Ozretic and Ozretic, 1986).

Even though the conceptual framework of toxicity testing has changed little since it was first used to assess water quality, modifications in design have expanded its utility in biomonitoring (Capuzzo *et al.*, 1988). Testing procedures have been developed further for application of methods to specific waste management problems such as ocean dumping, ocean inceneration, disposal of dredged materials and characterisation of complex industrial mixtures. There has also been a tendency to shift from the use of

macrobiotests, which use organisms such as fish, to microbiotests which involve exposing unicellular or small multicellular organisms to the toxicants (Blaise *et al.*, 1988). Caquet *et al.* (1996) presented the methodologies and complementary approaches used in environmental toxicology and ecotoxicology. Laboratory approaches include mono- or plurispecific toxicity tests, environmental trophic chains and microcosms while field approaches are the mesocosms, enclosures and *in situ* studies.

2.3.1 Test Types

The typical aquatic toxicity test consists of subjecting a species to serial dilutions of a toxicant, usually for a 96h period of exposure (Reish, 1988). The effect measured is death, and the concentration at which 50% live and 50% die at the end of the test is then defined as the 50% lethal concentration (LC_{50}). However, toxicity tests may be classified according to various other types of responses, exposure systems and experimental designs (Rand and Petrocelli, 1985; Reish and Oshida, 1987; Ward and Parrish, 1982).

2.3.2 Types of responses

The responses demonstrated by the test organisms exposed to toxicants fall into four categories (CPMS-II, 1997). Toxicity tests are designed to measure one or more of the following responses.

2.3.2.1 Acute toxicity

Acute toxicity involves a stimulus severe enough to induce a response within a short period of time (less than 96h), ultimately mortality.

2.3.2.2 Chronic toxicity

Chronic toxicity is defined as the adverse effects manifested over at least one full life-cycle of the organism. Many 'chronic' tests methods use partial life cycles, ideally with the most sensitive life stages.

2.3.2.3 Lethal toxicity

Lethal toxicity involves a stimulus sufficient to cause death.

2.3.2.4 Sublethal toxicity

Sublethal toxicity involves a stimulus which causes adverse effects other than death, with less obvious effects on behaviour, biochemical, or physiological function, and histology of organisms which includes changes in growth, reproduction, feeding, and behaviour.

Apart from the acute, chronic, lethal and sublethal tests, bioaccumulation tests are also performed to demonstrate cumulative toxicity to an organism (Rand and Petrocelli, 1985).

2.3.3 Test endpoints

An endpoint is the variable which indicates the termination of a test (time, effect on organism, etc.) and also means the measurement or value derived that characterises the results of the test (CPMS-II, 1997). These endpoints vary according to the type of test conducted and species used.

2.3.3.1 Biological endpoints

2.3.3.1.1 Lethality

Lethality means the endpoint measured is death.

2.3.3.1.2 Sublethality

Sublethal endpoints include growth (weight or length), reproduction (number of offspring), abnormal larval development, gamete viability, avoidance and swimming performance.

2.3.3.2 Statistical endpoints

2.3.3.2.1 Endpoints derived from hypothesis testing

The Lowest Observed Effect Concentration (LOEC) is the lowest concentration of a material in a toxicity test which has a statistically significant adverse effect on the population of test organisms as compared to the control.

The No Observed Effect Concentration (NOEC) is the highest concentration of a material in a toxicity test which has no statistically significant adverse effect on the population of test organisms as compared to the control.

2.3.3.2.2 Endpoints derived from point estimation

LC_{50} is the median lethal concentration which is the concentration of the material in a toxicity test estimated to be lethal to 50% of the test organisms, usually expressed as a time-dependent value (e.g. 24 h or 96 h LC_{50}).

EC_{50} is the concentration of a substance that causes a specified effect, lethal or non-lethal, in 50% of the test organisms in a test of specified time. The effect as well as the exposure must be specified (e.g. 96h EC_{50} for inhibition of growth). EC_{50} is limited to quantal measurements i.e. the number of individuals showing a particular effect.

ICp is a specific point estimate of the concentration of test material that causes a specified percentage (p) impairment in a biological function such as reproduction or

growth, which are 'continuous' data, not 'quantal', where the responses are measured as "either or".

2.3.4 Types of exposure system

2.3.4.1 Static tests

This is a simple and inexpensive system which requires a minimum of test solution, no renewal and generates minimal waste for disposal (Reish and Oshida, 1987). They are suitable for short exposures and testing substances whose concentration remains relatively stable throughout the exposure period. Their disadvantages are changes in sample toxicity due to volatility, degradation and adsorption, buildup of metabolic waste products and maintenance of acceptable dissolved oxygen levels.

2.3.4.2 Static renewal tests

This system is a simple modification of the static tests where the test solutions are renewed periodically during the test (CPMS-II, 1997). They are suitable for testing substances which do not remain stable over time but cannot be used for testing very small organisms such as phytoplankton. The tests require more test solution volumes and generates more waste. They are also known as batch replacement, static replacement or semi-static.

2.3.4.3 Flowthrough tests

This system involves continuous replacement of the test solution by a constant inflow of fresh test solutions or a frequent intermittent inflow (Reish and Oshida, 1987). They are more representative of true conditions and reduce problems of dissolved oxygen and metabolic products. However they are more expensive and more complex to maintain and operate, require large amounts of test solution and correspondingly generate large

amounts of waste material and require a reliable power supply.

2.3.4.4 Other toxicity tests

2.3.4.4.1 *In situ* tests

Toxicity tests are almost exclusively carried out under controlled laboratory conditions but in recent years field toxicity experiments have been conducted. *In situ* tests involve placing the test organisms in cages and exposing them directly to the test materials (CPMS-II, 1997).

2.3.5 Experimental design

Marine toxicity tests consist of water column tests and sediment tests, which differ in their experimental design (CPMS-II, 1997). Water column tests may be divided into three types : rangefinding, screening, and definitive tests. Sediment tests normally do not use dilutions. A specific proportion of sediment and dilution water is added to each test container, and comparisons are made between the responses to the controls and responses to the test material.

2.3.5.1 Range-finding test

The range-finding test is a test recommended when there is no information available on the toxicity of a substance. Its purpose is to determine the lower and the upper limits of concentrations to test in the definitive toxicity test.

2.3.5.2 Screening test

A screening test is where a sample is only tested at a single concentration such as 100% (v/v) for an industrial effluent. No more testing is usually required if the response falls within limits.

2.3.5.3 Definitive test

A definitive test usually involves testing a series of at least 5 concentrations of the test material to measure and calculate specific biological and statistical endpoints (EC_{50}, LC_{50}, IC_{25}, IC_{50}, NOEC and LOEC).

2.3.6. Test method development

Due to the variability in toxicity testing in the past, efforts have been made to standardise toxicity tests (Reish, 1988). Standardised methods have been developed for several types of toxicity tests and among those in use have been published by organisations such as U.S. Environmental Protection Agency (U.S. EPA), American Society for Testing of Materials (ASTM), Organisation for Economic Cooperation and Development (OECD), Food Agriculture Organisation of the United Nations (FAO) and Environment Canada (CPMS-II, 1997). Some of these and also other protocols provide general guidance while the rest have strict performance requirements for regulatory purposes. Individual have also written manuals to standardise techniques for regional areas (Ward and Parrish, 1982; Reish and Oshida, 1987). Although these procedures are well-established, new techniques are being developed.

2.3.7 Test Materials

Procedures have been published for conducting toxicity tests with selected toxicants such as metals, petrochemicals, pesticides, liquid effluents (APHA, AWWA and WPCF, 1989; Reish and Oshida, 1987) and contaminated sediments (PSEP, 1995).

2.3.8 Test organisms

2.3.8.1 Criteria for selecting test species

Several factors should be taken into consideration when selecting a test species (Rand and Petrocelli, 1985; Reish, 1988; Reish and Oshida, 1987; Walsh, 1988). The species should be of commercial, recreational or ecological importance, and of wide availability with wide geographic distribution. It should be easy to maintain in the laboratory, tolerant to handling and genetically stable. The biology should be known and the desired life stage should be available whenever required. The species should be tolerant to a wide range of water quality characteristics but also sensitive to a range of contaminants. It should also be able to produce consistent, reproducible results. However, it is unlikely that one species can satisfy all these criteria (Reish and Oshida, 1987).

The actual selection of the species itself, however, may depend on several other factors. Realistically, the important factors have been the interest, experience and specialisation of the researcher (Reish, 1988). A well-designed water column toxicity testing programme should include a battery of tests and species from three taxa consisting of fish, invertebrates and algae (CPMS-II, 1997). This, when performed by a single experienced personnel in a single facility would facilitate comparative toxicity studies, but has not been a common practice. Other important factors which influence the selection are availability of space, facilities, funds and very importantly the test objective. Naturally, no universal species or group of species can satisfy all geographical areas.

2.3.8.2 Marine tests species

The "Standard Methods" (APHA, AWWA and WPCF, 1989) listed the different orders of marine representatives from algae, corals, annelides, crustaceans and fish. Reish

and Oshida (1987) also recommended similar groups of organisms including zooplankton, echinoderms, polychaetes and molluscs. Seaweeds, heterotrophic microorganisms, coelentrate and nematodes, have also been used in marine toxicity testing. Reish (1988) recommended five species from at least four major animal groups, comprising of two crustaceans (a shrimp and and/or an amphipod), a species of polychaete (to represent the infaunal environment), a pelecypod (for their economic value and ability to bioaccumulate) and a fish (for its economical or recreational importance).

2.3.9 Toxicity testing with tropical marine organisms

In North America, standard toxicity test procedures have been developed for a variety of different test organisms and endpoints (McPherson, 1995). For water column tests, these procedures include tests with algae, invertebrates and fish so that a range of taxonomic groups and sensitivities are used. However, most manuals or standard methods of toxicity testing which have been written recommend species for temperate regions (Reish and Oshida, 1987; Ward and Parrish, 1982). Relevantly, selection of species to be utilised must be based on local conditions and needs (Reish, 1988).

Generally, much data is available on the toxicity of metals and other toxicants to temperate marine species. Not only is data on tropical species lacking, established protocols specifically designed for conducting toxicity tests with tropical marine species are also lacking (McPherson, 1995).

2.3.9.1 The ASEAN-Canada Cooperative Programme on Marine Science-Phase II

Toxicity testing is relatively new to the Association of Southeast Asian Nations (ASEAN) (McPherson, 1995). Under the action plan for conservation of nature in ASEAN, the seven-year ASEAN-Canada Cooperative Programme on Marine

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Science-Phase II (CPMS-II) (1991-1998) focuses on establishing environmental criteria for development and management of living marine resources (Peters *et al.*, 1997). An objective of the Environmental Criteria (EC) technical study under the ASEAN-Canada Cooperative Programme on Marine Science-Phase II (CPMS-II, 1997), is the development of standardised toxicity testing procedures with tropical marine organisms, and this has been occuring at the same time that ASEAN laboratories are setting up their toxicity testing programmes. Methods for acute and sublethal test are based on procedures already established in North America, but adapted for use with tropical species. Approximately ten chemicals are being tested with more than 11 laboratories from the ASEAN region participating in this programme (Peters *et al.*, 1997).

2.3.9.2 The ASEAN test methods

The first method to be developed was a static or static-renewal acute toxicity test for fish and invertebrates (CPMS-II, 1993). As acute tests provide limited information about the effects of exposure on an organism, sublethal or short-term chronic toxicity test methods were developed in the next phase (CPMS-II, 1995). The chronic tests developed included a 96 h static, non-renewal, phytoplankton growth test, the static echinoderm fertilisation and larval development tests, a 24 h static, bivalve larval development test and a 7-day static-renewal larval fish survival and growth test.

The subsequent phase focused on the development of sediment tests (CPMS-II, 1997) which included a 10-d static, acute or sublethal amphipod sediments test, a 48-h static, lethal or sublethal bivalve larval development test, a sublethal bivalve fertilisation test and a 20-d static-renewal, acute polychaete worm sediment test.

2.4 MARINE PHYTOPLANKTON

2.4.1 Definitions

The word 'plankton' which was taken from a Greek verb meaning to wander (Tait, 1981), refers to organisms which live in the water column, and are too small to be able to swim counter to typical ocean currents (Levinton, 1995). 'Phytoplankton' or microalgae are photosynthetic planktonic protists and plants, usually consisting of single-celled organisms or chains of cells. Some may have locomotory organs such as flagella, but phytoplankton movements in the water column are nearly completely controlled by water turbulence and currents and by the bulk density of the organisms.

2.4.2. Classification

The major groups of algae have been classified traditionally, to divisions and classes, based on pigmentation, cell wall composition, flagellar characteristics, storage products (Chapman and Chapman, 1981; Moestrup, 1982; Bold and Whyne, 1985) and more recently, a variety of ultrastructural characteristics (Harlin and Darley, 1990). The phytoplankton consists mainly of Bacillariophyceae (diatoms), Chlorophyta (green algae including Chlorophyceae and Prasinophyceae), Chrysophyceae and Haptophyceae (golden brown algae including silicoflagellates and coccolithophores), Cryptophyceae (cryptomonad flagellates), Cyanophyceae (cyanobacteria or blue-green algae) and Phyrrophyta (class Dinophyceae or dinoflagellates) (Chapman and Chapman, 1981).

2.4.3 Primary production

In nature, the phytoplankton plays a vital function in ecology mainly in primary

productivity. Although individually very small in size, they form the basis of the food web and account for most of the organic matter production in the sea (Boney, 1975). Photosynthetic phytoplankton utilise light for the synthesis of organic compounds from inorganic sources . These primary producers are consumed by the secondary producers, the zooplankton, in the lower trophic levels, which in turn will be eaten by other organisms in higher trophic levels. Production from one trophic level is transferred to the next where the potential production at the top of the food chain depends on the the food-chain efficiency (Levinton, 1995).

2.4.4 Marine aquaculture feed

Increasing attention has been given to marine aquaculture which includes intensive production of commercially and feed important organisms (Shamsuddin, 1992) such as fish, shrimps and shellfish, where large-scale algal cultures are grown and maintained for the aquaculture feed. The biochemical fractions in microalgae that are important for animal nutrition are proteins, lipids, carbohydrates, vitamins and minerals as well as specific nutrients such as essential amino acids and fatty acids, sterols sugars and vitamins (Brown *et al.*, 1993). The major lipid classes in microalgae includes the polar lipids, mostly phospholipids and glycolipids which are common membrane components, and the triacylglycerols which are a reserve of fatty acids for cellular division, metabolic energy, membrane maintenance, synthesis and a variety of physiological uses. Polyunsaturated fatty acids (PUFAs), in particular eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids are essential for growth and development of aquaculture organisms. Selected strains of microalgae serve as the preferred food for bivalve larvae, seed and adults and fish (Benemann, 1992). Larval and juvenile stages are more restricted in their requirements while adults are able to feed on a greater variety of

species and strains. Marine fish also require feeds containing high amounts of high value proteins and lipids.

Phytoplankton species commonly used as aquaculture feed are mainly from the groups of diatoms, chrysophytes and chlorophytes. Microalgae considered highly desirable include *Isochrysis galbana*, *Tetraselmis suecica*, *Chaetoceros gracilis*, *Thalassiosira pseudonana*, *Dunaliella salina* and related species in these genera, which are generally characterised by the lack of a heavy cell wall and elevated contents of omega-3 fatty acids.

2.4.4.1 *Chaetoceros* (Diatom; class Bacillariophyceae, division Bacillariophyta)

Diatoms dominate the phytoplankton in many diverse habitats. They are ubiquitious on intertidal rocks and soft bottoms, and common on the shallow-water sea bed. These unicellular algae occur as single-cells or form chains, in the size range of nanoplankton (2-20μm) and microplankton (20-200μm) (Levinton, 1995). The photosynthetic pigments include chlorophylls *a* and *c*, xanthophyll and fucoxanthin while their food storage products include fats and chrysolaminarin (Morris, 1971b). A main feature of the diatom is the cell wall (frustules) which is silicified and consists of two valves (the outer lid or epitheca, and the inner part or hypotheca) that fit together much like a pill box (Morris, 1971b). The shell may be covered with spines or ornamented with a complex series of ridges and pores which connect the cell and its external environment. Diatoms usually reproduce by binary cell division where the valves of the parent separate and the new silica valves formed becomes the hypotheca of each daughter cell.

Radially symmetrical planktonic diatoms are known as centric diatoms (order Centrales), in contrast to pennate diatoms (order Pennales) which are bilaterally symmetrical and usually live in sediments (Levinton, 1995). In the Centrales, the more

detailed classification of the diatoms depends on the structure of the siliceous skeleton such as cell shape and the presence or absence of particular processes (Morris, 1971b).

The centric diatom genus *Chaetoceros* consists mainly of planktonic marine taxa with approximately 170 species (Kaczmarska *et al.*, 1985) and less than twenty species reported from inland, usually brackish, waters (Reinke, 1984). The diatom cells in valve view are oval, while in girdle view, long horn-like projections extend from the four corners (Chapman and Chapman, 1981). In some *Chaetoceros* species the spines which are forms of prominent siliceous setae projecting from valves of the cell frustule, serve to link the unicellular cells together to form colonies (Morris, 1971b; Rogerson *et al.*, 1986). Sugar accumulation points on diatom surfaces which are species-specific may be responsible in cell aggregation (Waite *et al.*, 1997).

The membrane composition of *Chaetoceros* consists of a variety of sterols and polyunsaturated fatty acids (PUFA) (Lehmal and Kirst, 1997). In *Chaetoceros calcitrans*, like most diatoms, nitrogen deficiency results in the storage of carbohydrates while silica deficiency stimulates lipid production (Wickfors *et al.*, 1989). Detailed fatty acid composition changes in *C. calcitrans* and related species due to irradiance (Thompson *et al.*, 1990) and variation in temperature (Thompson *et al.*, 1992) have been reported. *C. calcitrans* has been used intensively and proven as good unialgal culture for aquaculture feed (Shamsuddin, 1992). Recently, the potential use of *C. calcitrans* paste as an alternative feed has been assessed based on the ascorbic acid contents of the alga (Brown, 1995).

In many species of *Chaetoceros*, sporulation occurs in response to nutrient changes (Kaczmarska *et al.*, 1985). Spermatogenesis has been well described particularly in this genus (Furnas, 1985).

2.4.4.2 *Isochrysis* (Chrysophyte; class Haptophyceae, division Chrysophyta)

The division Chyrsophyta consists mainly of unicellular flagellates. Photosythetic pigments include chlorophyll *a*, and carotenoids, fucoxanthin and diadinoxanthin. The main reserve products are oil and chrysolaminarin (Morris, 1971b).

On the basis of the flagella form, it can be divided into two classes, Chrysophyceae and Haptophyceae, the former possessing a pantonematic flagellum (longitudal rows of fine hair arranged along the axis of the flagellum), with or without a second acronematic (smooth and whip-like) one, while the latter possessing two acronematic flagella. Some species of Haptophyceae possess a third filiform appendage, haptonema, which is used for temporary anchorage of the cell but does not have a locomotory function.

Both classes have 'naked' species where the cells are bounded only by the cytoplasmic membrane, and also species which are covered with 'scales'. Haptophyceae have delicate and uniform scales which do not contain silica but appear to consist solely of organic material. Most Haptophyceae are predominantly marine, comprising two orders, the Isochrysidales in which the motile cells lack the haptonema and the Prymnesiales which possess the haptonema.

An important member of the Isochrysidales is the genus *Isochrysis* (a microflagellate; 1-20μm) (Chapman and Chapman, 1981) which possesses two equal flagella (isokont). Individual cells are naked, capable of metabolic movements, pyriform in shape, with two typical chloroplasts placed laterally on each side and a red stigma at the anterior end. They are characterised by their golden brown colour. Chlorophyll for Haptophytes can be grouped into six different pigment types. *Isochrysis galbana* is a chlorophyll *a*/*c* alga. Zapata and Garrido (1997) reported the

occurrence of phytylated chlorophyll c in *I. galbana* which lacked chlorophyll c_3.

Mass cultures of *I. galbana* have been used successfully for rearing commercial shellfish (Boney, 1975). *I. galbana* have been widely used as a mariculture feed due to its high contents of fatty acids (Dunstan *et al.*, 1993a) especially polyunsaturated fatty acids including the eicosapentaenoic and docosahexaenoic acids and amino acids (Brown *et al.*, 1993) which are nutritionally required by bivalve larval stages. However, the fatty acid profiles and lipid content vary between strains of *Isochrysis* (Zhu *et al.*, 1997) depending on the environmental factors (Molina Grima *et al.*, 1995). Dunstan *et al.* (1993b) stated the lipid contents in *Isochrysis* sp. to be between 2.6-7 pgcell^{-1}. It also produces hydrocarbons and methyl and ethyl alkeones.

Reproduction takes place by the formation of an endogenous cyst which, on germination gives rise to zoospores or palmelloid phase. There appears to be some form of primitive sexuality, in which two non-motile cells fuse, after which one nucleus disappears and divisions take place giving four daughter cells. Each of these cells produces a cyst and from this a new motile cell eventually emerges.

2.4.4.3 *Tetraselmis* (Green algae; class Prasinophyceae, division Chlorophyta)

The division Chlorophyta encompasses a morphological range from simple unicells to rather complicated multicellular plants (Chapman and Chapman, 1981). The pigments are characteristically those of the higher plants, chlorophyll a, b and b-carotene, xanthophylls (violaxanthin and neoxanthin). The cell wall is composed commonly of cellulose, plus mannans and xylans with sitosterol as the predominant sterol. Starch accumulates as a reserve product. Sexual reproduction is common and ranges from isogamy to anisogamy and oogamy (Chapman and Chapman, 1981). Asexual reproduction normally takes place by means of motile zoospores, but a variety of

non-motile spores exist.

Green algae can be flagellated or non-motile. The class Prasinophyceae possess flagella which feature minute scales, lateral hairs, terminal turfs, or bipartite arrangement with a narrower terminal extremity (Chapman and Chapman, 1981), a distinct feature which distinguishes this class to the Chlorophyceae, in which the motile cells normally possess two or four acronematic flagella (Morris, 1971b).

Tetraselmis is the most speciose genus and advanced member of the heterogenous class Prasinophyceae of green flagellates. While it has been synonymised with the generas *Platymonas*, *Prasinoclodus* and *Aulachlamys* (Huber and Lewin, 1986), the placement of *Tetraselmis* is controversial where it was reassigned to its own order with the class Pleurastrophyceae (Mattox and Stewart, 1984). However, it is still often referred to as a prasinophyte in literature.

The genus *Tetraselmis* are euryhaline and common in the littoral habitats around the world, rarely occuring in freshwater (Marin *et al.*, 1993) and easily isolated and cultured (Huber and Lewin, 1986). The genus is characterised by large oval, walled cells (10-15 μm) and a single cup-shaped chloroplast occupying the bulk of the cell volume (Mattox and Stewart, 1984). The cell wall contains crystalline subunits. *Tetraselmis* are also characterised by flagellar insertion in a depression and the presence of cell-surface associated organic structures of distinct size and shape called scales mainly made of acidic polysaccharides containing 2-keto sugar acids (Becker *et al.*, 1995) on the cell body and flagella, which are assembled in the Golgi apparatus (Marin *et al.*, 1993). The scales are widely used for taxonomic purposes, most recently that of hair scales. Ultrastructure variation in flagellar hair between strains are sufficient to distinguish most strains from one another (Marin *et al.*, 1993). The flagella of *Tetraselmis*

tend to be shed under diverse circumstances including in response to a variety of irritants (Zmarly and Lewin, 1986).

Species of *Tetraselmis* have been investigated for a variety of industrial purposes, ranging from a potential source of biochemical to reducing air pollution (De Jesus *et al.*, 1995). The species *Tetraselmis tetrahele* has been primarily used as feed for filter-feeding animals, either alone or in combination with other microalgae such as *I. galbana*.

Many strains of *Tetraselmis* are able to produce carotenoids even in darkness but not chlorophyll, when supplied with glucose (Lewin, 1997). They also contain neutral polysaccharides (Gooday, 1971) as well as proteinaceous elements (Lewin, 1958). *Tetraselmis* could accumulate amino acids from very dilute solutions and much higher rates of uptake have been found in nitrogen deficient cells (Ricketts, 1990). Their biochemical composition (carbohydrates, protein and lipids) are affected and can be altered by manipulating the carbon sources (Cid *et al*, 1992) supplied during cultivation. Recently, dried *Tetraselmis* has been served with live algal feeds (Benemann, 1992).

Tetraselmis is rich in chlorophyll *b* and exhibit several unique features in the pigment-protein composition of their synthetic apparatus which includes prasinoxanthin which enhances light harvesting in the PSII (Smith and Albert, 1991). Most strains of *Tetraselmis* are completely autotrophic, but a few require thiamine and cobalamin for growth. Vegetative reproduction is by binary fission within the parental wall (Lewin, 1997). Sexual reproduction is not known in this class (Huber and Lewin, 1986).

2.4.4.4 Phytoplankton used as aquaculture feed in Malaysia

Numerous species of marine microalgae are maintained by the Department of Fisheries (Palanisamy *et al.*, 1991) for use as aquaculture feed. *Chlorella* sp. and

Tetraselmis sp. are cultured as feed for the rotifers which in turn serve as food for the sea

bass, *Lates calcarifer*. The early zoea larval stage of the tropical tiger prawn, *Penaeus*

monodon, are fed with diatoms such as *Chaetoceros* sp. while the shrimp *Penaeus*

japonica protozoean larvae also require microalgae as food. The matured, D-shape

trocophore larvae of the shellfish *Anadara granosa* which are reared at the Peninsula

coastal mud flats are fed with *Isochrysis* sp. Various microalgae are added to tanks

containing the veliger larvae of the mangrove species of oyster *Crassostrea belcherii*,

now commercially reared, including *Chaetoceros calcitrans*, *Isochrysis galbana* and

Tetraselmis suecica.

2.4.5 Phytoplankton as biological indicators of marine pollution

Because of their nutritional needs, their position at the base of aquatic foodwebs,

their small size, and consequent sensitivity to environmental changes at small spatial

scales, algal indicators provide relatively unique information concerning ecosystem

conditions compared with commonly used animals (McCormick and Cairns, 1994).

Algae respond rapidly and predictably to a wide range of pollutants, providing potentially

useful early warning signals of deterioration and possible causes. Algae are sensitive

indicators of water quality where changes in their population can provide information

about water chemistry as the chemical determination themselves (Joy and Joseph, 1995;

Rana, 1995).

Numerous algal parameters have been proposed for assessing environmental

quality such as functional properties (algal metabolism, productivity, biomass), and

structural indicators (taxonomic analyses such as community composition, % sensitive or

tolerant species, and species richness). Algal assemblages exhibit excellent continuity

through time and with changes in water quality. Changes in phytoplankton species

composition and loss of sensitive species from these assemblages provide the earliest reliable indicators of ecosystem stress. Algal pollution indices based on genus and species for use in rating water samples for high and low organic pollution have also been developed (Palmer, 1969). Diatoms assemblages have been recommended as advantageous indicators due to their importance, and well understood and widely accepted taxonomic identification (Joy and Joseph, 1995; Pan *et al.*, 1996).

Algal toxicity tests or 'bioassays' under *in situ* or laboratory conditions, complement biological indices by providing quantitative data expressing the relationship between pollution load and biological responses.

2.5 TOXICITY TESTS AND STUDIES INVOLVING HEAVY METALS AND ALGAE

Bioassays first introduced to investigate fish contamination by industrial effluents were only adapted for algal studies in the 1970s (Wong, 1995). Algal assays measure chronic effects of toxicants over a span of several algal generations (Wong, 1995). Single- and multi-species freshwater and marine algal assays are useful in the assessment of the toxicity of municipal, industrial, and agricultural wastewater effluents (Greene *et al.*, 1975; Walsh and Alexander, 1980). Not only have they been used to determine the toxicity of single and mixtures of metals (Wong and Beaver, 1980) and other toxicants, but also to determine algal productivity in natural waters (Miller *et al.*, 1974). The common and conventional method recommended for toxicity testing with microalgae is the batch culture method using shake-flasks (APHA, AWWA and WPCF, 1989; ASTM, 1993) However literature shows that a multitude of methods, test conditions and parameters of inhibition have been employed by various researchers in

toxicity tests and studies with algae.

Munawar *et al.* (1989) presented an overview on the various procedures using algae in toxicity testing including laboratory tests with laboratory cultures and natural assemblages, with an emphasis on field and *in situ* techniques. Bioassays using algal cultures include batch cultures, continuous (turbidostat or chemostat) cultures, microplate algal assays, the *Selenastrum capricornutum* ^{14}C uptake assay, and the *Ankistrodesmus bibrianus* two-chamber device while tests employing natural assemblages include the Algal Fractionation Bioassays (AFBs), the autotrophic picoplankton assay and biosensors.

To bridge the gap between laboratory results and the actual environment, researchers have also used complex systems such as laboratory and field microcosms (Krajnovic-Ozretic and Ozretic, 1986) and mesocosms (Caquet *et al.*, 1996) which are considered to approximate field conditions.

2.5.1 Laboratory approaches

The standard algal assay procedure, the EPA Algal Assay Procedure Bottle Test, which employs *S. capricornutum* has been proven to be a reproducible and reliable assay for assessing freshwater algal growth potential and in metal toxicity testing (Turbak *et al.*, 1986). A short-term (24-h) algal bioassay method, the Oxygen Evolution Assay, has also been found to be useful for the rapid preliminary screening of waterborne herbicides and metals. Another test is the Algal Growth Inhibition Test (AGIT) developed by the OECD (Rana and Jameson, 1995). The Algal Growth Potential (AGP) test in enriched and unenriched seawater, and *in vitro* and *in situ* are among the common approaches used in marine algal bioassays (Rana, 1995). Wong *et al.* (1995a) has combined algal assays, electron microscopy and EDX-ray microanalysis to determine total and metal

toxicity in full strength effluent water.

Even though protocols employing free algal cells have been widely used for toxicity assessment, in recent years interest in using immobilised cell technology has also grown rapidly (Duncan *et al.*,1997; Lau *et al.*, 1998; Tam *et al.*, 1998). This system could expand the utility of algal toxicity assays (Bozeman *et al.*, 1989) as the test alga may be encapsulated and exposed to the toxic substance under field conditions and then retrieved for laboratory analysis, and possibly reused (Garnham, 1992). Endpoints of the tests may be determined directly by chlorophyll extraction or physiological measurements such as oxygen evolution or ^{14}C uptake.

Development of microbiotests and their application in the field of ecotoxicological monitoring have increased during the recent years (Benhra *et al.*, 1997). The microalgal microplate method has conferred a number of advantages over the classical flask test (Blaise *et al.*, 1986). Benhra *et al.* (1997) who argued that the method was labour-intensive due to the need for regular maintenance of pure algal cultures for use, has described another method, cryoalgotox, using cryopreserved alga *S. capricornutum*, to determine 72-h $EC_{50}s$ with Cd^{2+}, Cu^{2+} and Cr^{6+}, which was more sensitive, repeatable and reproducible than results obtained with the classical microplate tests. Apart from the microplate tests, two other techniques used to study water pollution are the dynamic and turbidostat culture tests (Wong, 1995).

Hassett *et al.* (1981) developed a microplate technique to determine the conditions under which pure cultures of algae removed heavy metals from aqueous solutions. Plastic, U-bottomed microtiter plates were used in conjunction with heavy metal radionuclides to determine concentration factors (CF) for metal-alga combinations. The technique developed was rapid, statistically reliable, and economical of materials

and cells.

The Algal Fractionation Bioassays (AFBs), simulated dredging or disposal impact, picoplankton bioassays, microcomputer-based toxicity testing, navigational impact, and *in situ* plankton cages (I.P.C.) have also been used for sediment testing with algae (Munawar and Munawar, 1987). Schmidt (1986) described two bioassays for algizide substances; the algae-fluorescence bioassay and the *Phormidium* inhibition test, which were almost completely controlled by a microcomputer system. Other techniques used in algal bioassays are the VAS - fluorescence technique, Limited Sample Bioassays (LSBs) and flow cytometry (Munawar *et al.*, 1989).

Microcosms which have been employed to approximate field conditions (Krajnovic-Ozretic and Ozretic, 1986; Rai and Mallick, 1993) is different from the real environment in the sense that the systems are partially or entirely closed or isolated, with limited dimensions, reduced number of species, and the number of individuals involved unbalanced. The systems which may range from a simple shake flask with a mixed population culture to larger enclosures along coastal waters have been useful tools in the development of toxicity models (Swartzman *et al.*, 1990). Cairns (1983) mentioned the development of a sealed microcosm which had stable characteristics and species composition for over a year. Meanwhile Austen and Somerfield (1997) recently applied a community level sediment bioassay, a simple laboratory microcosm system, to an estuarine heavy metal gradient.

2.5.2 Field approaches

Phytoplankton bioassays conducted in the field or *in situ* include the use of bottles or moorings, flow-through bottles, periphyton enclosures, dialysis bags, microcosms or

mesocosms, and *in situ* plankton cages (Munawar *et al.*, 1989). Effluent impact, dredging or disposal impact and navigational impact assessments are also applied in field or *in situ* situations.

Mesocosms are small-scale artificial ecosystems placed under natural conditions or outdoor experimental ponds usually bigger than 15m³ (Caquet *et al.*, 1996). They are considered as an intermediate level study between laboratory and field, which offer more realistic ecological conditions than laboratory tests.

Incubation of cultures *in situ*, in the sea or lakes may be carried out using a variety of chambers (Rana and Jameson, 1995). While the use of dialysis sacs is a less expensive method of incubation (Jensen *et al.*, 1976), more advanced are the Controlled Ecosystem Pollution Experiments (CEPEX) which are mostly restricted to marine ecosystems where natural assemblages of algae and associated organisms are enclosed in huge polythene enclosures (Menzel and Case, 1977; Takahashi *et al.*, 1977; Thomas *et al.*, 1977a,b; Kremling *et al.*, 1978) which are moored in the natural water body. To determine the influence of low concentrations of pollutants on the structure and function of a marine plankton system, Kuiper (1977; 1981) conducted experiments in large plastic bags housing the plankton communities with the bags being suspended in natural water. Meanwhile Eide *et al.* (1979) developed a simple apparatus for *in situ* use of cage culture technique to monitor the growth and metal uptake of three diatoms in two metal-polluted Norwegian fjords.

2.5.3   Other tests with heavy metals

There have been few attempts at toxicity testing with macroalgae or seaweeds (Murugadas, 1998) even though they dominate sheltered shore communities and are involved in different food chains compared to the more usually tested phytoplankton.

Wilkinson and Tefler (1992) suggested a variety of lethal and sublethal measures for seaweeds including cellular viability, regeneration potential, spore survival and germination, growth rates of germlings to adults, reproductive ability, spermatozoid motility, physiological activity and ion leakage. Dahl and Blanck (1996) has recently presented a new ecotoxicological test system employing sand-living microalgal communities (epipsammon).

Much recently many tests on metal ions have been done using bacteria by assays such as the Microtox® bioluminescence assay (McCloskey *et al.*, 1996; Wong *et al.*, 1995b) and SOS Chromotest Microplate assay (Legault *et al.*, 1996). Wong *et al.* (1995b) demonstrated that an algal bioassay was more sensitive than the Microtox in evaluating the toxicity of sediment elutriates. Wittekindt *et al.* (1996) described a microtiter-plate urease inhibition assay for the screening of heavy metals in water, which mwas based on the incubation of samples with the urease of selected bacteria and subsequent determination of the released ammonia and background ammonia in the water samples. Another interesting test involving metals was evaluated by Rumbold and Snedaker (1997); the sea-surface microlayer (SSML) toxicity test involving the microscopic embryos of marine organisms.

2.5.4 Toxicity testing using marine phytoplankton

As the phytoplankton species and communities are sensitive to environmental changes, their growth may either be inhibited or stimulated by the exposure to various substances. Therefore the response of these algae should be considered when assessing the potential effects of chemicals and toxic substances on the marine environment (Parrish, 1985).

Phytoplankton are convenient for toxicity testing because of their small size, requirement of minimal laboratory space, often known nutritional requirements, short life cycle, and suitability for bioaccumulation tests. The disadvantages include their small size, which requires microscopic and specialised equipment for identification and counting (Reish and Oshida, 1987).

2.5.4.1 Protocols for conducting toxicity tests with microalgae

Standard protocols exist for conducting static toxicity tests with phytoplankton. While some are general (OECD, 1984), others are quite elaborate (ASTM, 1993; APHA, AWWA and WPCF, 1989; Reish and Oshida, 1987; Ward and Parrish, 1982). A guideline for conducting toxicity tests with marine phytoplankton for ASEAN has been developed under the ASEAN-Canada Cooperative Programme on Marine Science Phase II (CPMS-II, 1995).

2.5.4.2 General principles of toxicity testing with microalgae

The principles of toxicity testing with marine phytoplankton has been outlined by Walsh (1988). Phytoplankton toxicity tests are conducted using the same principles as fish and invertebrates testing but may differ in the test duration, type of response measured, the endpoints seeked, and the type of exposure system used. They also require the application of the principles of phycology and microbiology to culturing, handling and exposing the organisms.

Toxicity to algae may be expressed by a broad range of responses, whether inhibitory (reduction in cell numbers at specific time intervals), algistatic (halting of cell division) or algicidal (cell death) (Parrish, 1985), which can be determined by algal toxicity tests. The response usually measured is inhibition of growth. The tests endpoints

may also vary depending on the responses and are usually reported as EC_{50} or IC_{50} values. Other endpoints are the NOEC and LOEC values. Algal toxicity tests are usually static, non-renewal type as the size of the organism is unsuitable for renewal and flow-through tests.

Synchronous culture are not necessary in toxicity testing because the exponential growth phase, in which cell number increases at a high rate, is the phase at which the algae are tested (Walsh, 1988). Toxicants and growth-stimulating substances cause deviations from the normal population growth pattern which may include increased lag phase and reduced or increased growth rate and yield. To minimise the length of the lag phase, cultures must be initiated with the proper amounts of cells from algal stocks in the early exponential phase. Algal tests should be conducted for as short a period as possible to avoid problems with the fate of the test chemicals in the test chamber which may decline in concentration due to photodegradation, molecular instability, adsorption to test vessel walls and volatilisation from surface of medium. A typical phytoplankton test lasts for 96h, though bioaccumulation tests may continue longer. A sublethal 96 h toxicity test conducted with phytoplankton is considered more of a 'chronic' test where a 'short-term' exposure represents a long-term exposure of the culture to the test material due to the rapid growth of the culture, thus involving the life cycle of the algae (Reish and Oshida, 1987).

Walsh (1988) recommended continuous lighting at a constant temperature to ensure large enough population densities by the end of the test which allows statistical evaluation of the toxicity data. Unless a relatively unchanging source is available, toxicity tests with media prepared from natural seawater may not yield comparable data and therefore may be substituted with seawater prepared from commercial sea salt mixtures

and enriched with nutrients. pH of growth media and natural seawater at test initiation is generally around 8.0 but measurement of pH after a test is of little value as algal activities may change it by as much as one pH unit even in a highly buffered medium.

2.5.4.3 Criteria for selection of test species

The principle criteria for the selection of test species includes economical and ecological value, wide geograpical distribution, well-known nutrient requirements, ease of culture and tolerance to handling, high growth rate, good taxonomical characterisation, small genetic and phenotypic variability, consistent performance and sensitivity (Reish, 1988; Walsh, 1988). Test species may be conveniently obtained from several laboratories (Reish and Oshida, 1987) which have collections of pure and established microalgal cultures. Various marine phytoplankton species have been recommended as test organisms for testing (Table 2.5).

Table 2.5 : Marine phytoplankton recommended as test species for toxicity tests

Marine phytoplankton	Reference
Chlorophyta	
Chlorella sp.	Parrish, 1985
Chlorococcum sp.	Parrish, 1985
Dunaliella tertiolecta	Parrish, 1985; Reish and Oshida, 1987
Chrysophyta	
Isochrysis galbana	Parrish, 1985
Bacillariophyta	
Minutocellus polyformis	Walsh *et al.*, 1988
Skeletonema costatum	Walsh and Alexander, 1980;
	Ward and Parrish, 1982;
	Parrish, 1985; Reish and Oshida, 1987
Thalassiosira pseudonana	Parrish, 1985; Reish and Oshida, 1987
Rhodophyta	
Porphyridium cruentum	Parrish, 1985

However, it may be more relevant to use locally isolated or species indigenous to a region for testing. The test species should occur naturally in the area that may be affected by the toxicant and isolated from a non-polluted environment. Testing several species of phytoplankton enables a comparative toxicity study.

2. 6 HEAVY METAL TOXICITY TO PHYTOPLANKTON

2.6.1 Introduction

Heavy metals such as iron, nickel, copper, cobalt, manganese and zinc are essential for the growth of phytoplankton (Eichenberger, 1986; Morel *et al.*, 1991) where extremely low concentrations in seawater may limit their productivity. These metals are involved in all general metabolic processes of phytoplankton including photosynthesis, respiration, and major nutrient assimilation. Even cadmium at very low concentrations have been shown to be beneficial under conditions of moderate Zn-limitation (Lee and Morel, 1995). However, all heavy metals have been shown to exhibit inhibitory and deleterious effects on phytoplankton with increasing concentrations. Among metals in general, the toxicity of essential metals rank high (Eichenberger, 1986).

Numerous studies have been done, and extensive reviews written, on the effects of heavy metals on phytoplankton (Davies, 1978; Sorentino, 1978; Hollibaugh *et al.*, 1980; Rai *et al.*, 1981; Stratton, 1987). Pollution problems usually become apparent at the ecosystem or community level, but all effects start at the level of individual organisms (De Kruijf, 1988). Initial interest in studying toxicity centered on lethal effects (Eicherberger, 1986). However, to understand the long-term consequences of pollution in ecosystems, it is essential to recognise sublethal effects.

Growth inhibition of phytoplankton by heavy metals, usually measured by reduction in dry weight, cell counts and O.D., have been commonly observed and reported (Davies, 1978). Algae accomplish primary productivity by a balance between two metabolic processes, photosynthesis and respiration (De Filippis *et al.*, 1981b). Heavy metals have been observed to disrupt physiological and metabolic processes including photosynthesis (Steemann-Nielsen and Wium-Anderson, 1971; Wu and Lorenzen, 1984) and respiration (De Filippis *et al.*, 1981b). Other physiological processes inhibited by toxic metals also include nutrient uptake (NH_4^+ and NO_3^-), carbon fixation, and enzymatic activities of urease, glutamine synthetase, nitrate reductase (Mallick and Rai, 1989) and nitrogenase (Delmotte, 1980). Another physiological parameter affected by metals is cell movement (De Filippis *et al.*, 1981a).

Short-term effects of heavy metals also include inhibition of cell division (Fisher *et al.*, 1981) and reduction in cell volume (Christensen *et al.*, 1979). They can also alter various metabolic pathways (Bischoff, 1982), and metabolic activites like protein synthesis (Hart and Scaife, 1977), carbon assimilation, and chlorophyll (De Filippis and Pallaghy, 1976b; Rebhun and Ben-Amotz, 1984) and ATP content (Mallick and Rai, 1989). They also induce changes in biochemical composition of microalgae (Prevot and Soyer-Gobillard, 1986; Thompson and Couture, 1991).

Morphological abberations (Thomas *et al.*, 1980), intracellular structural change (Silverberg, 1976), or ultrastructural damage by metals (Rachlin *et al.*, 1984), and genetic modifications (Eichenberger, 1986) have also been found.

The ecological implications of heavy metal pollution have also been described (Gibson, 1972). Long-term effects of the above may result in changes in algal species composition and ultimately alter the overall community balance.

2.6.2 Physiological Effects

2.6.2.1 Cadmium

2.6.2.1.1 Growth

Many species of freshwater and marine phytoplankton have been shown to be sensitive to cadmium (Bartlet *et al.*, 1974; Berland *et al.*, 1977; Rosko and Rachlin, 1977; Conway, 1978; Rachlin *et al.*, 1982). A Cd concentration as low as 1.2 μgL^{-1} caused serious growth inhibition during the exponential stage of *Prorocentrum micans* (Kayser and Sperling, 1980). Berland *et al.* (1976) reported sublethal toxicity thresholds with *Amphidinium carteri* and *Exuviella mariae-lebouriae* at levels of 25 and 50 μgL^{-1} Cd, respectively.

Lawrence *et al.* (1989) observed a reduction in the cell number of *Chlamydomonas reinhardii* when the Cd concentration was increased from 7.5 to 20 μgL^{-1} Cd. Test concentrations between 10 to 500 μgL^{-1} Cd caused a 71-81% reduction inthe cell number of the marine phytoflagellate *Olisthodiscus luteus* (Fernandez-Leborans and Novillo, 1996).

Cd also have also been found to cause a decrease in the normal growth rate of *Dunaliella minuta* (Visviki and Rachlin, 1991), probably due to linkage with sulphydryl groups. It also reduced the maximum population density and specific growth rate of *Thalassiosira weissflogii* (Li, 1984) where it has been proposed that the toxicity of Cd in the alga involves an impairment of iron nutrition (Foster and Morel, 1982).

Some studies on the inhibitory effect of Cd on the growth of diatom *Skeletonema costatum* (Berland *et al.*, 1976) and *Selenastrum capricornutum* (Thompson and Couture, 1991) have shown an increase in the growth rate in the first hours which may be attributed to a transitory physiological compensation in response to chemical stress, as

part of the process of species acclimatisation. The response known as hormesis, can be observed as a consequence of natural pressures. *S. capricornutum* was strongly inhibited by exposure to 30-100 μgL^{-1}, causing a reduction in the range of 37-74% with respect to the control (Thompson and Couture, 1991).

   Scenedesmus obliquus, Ankistrodesmus falcalutus, Chlorococcum sp., and *Navicula incerta* are more tolerant to Cd than others, with their growth inhibited at 2.5-3.0, 2.0-2.5, 3.01 and 18.0 mgL^{-1} respectively (Cain *et al.*, 1980; Devi Prasad and Devi Prasad, 1982; Rachlin *et al.*, 1982).

2.6.2.1.2 Photosynthesis

   Cd^{2+} concentrations as low as 0.01 μgL^{-1} decreased photosynthetic rates in a marine phytoplanktonic assemblage (Zingmark, 1972). It has also been observed that primary productivity and chlorophyll *a* levels of natural, freshwater phytoplankton communities were significantly reduced by Cd^{2+} levels as low as 3 μgL^{-1} (Marshall *et al.*, 1983). 35.85% and 86% reduction in chlorophyll levels of *Olisthodiscus luteus* have been observed in 10 and 500 μgL^{-1} Cd treatments respectively (Fernandez-Leborans and Novillo, 1996). The pronounced differences in the levels of chlorophyll may be a result of the increased internal concentrations of Cd, which is highest in the 500 μgL^{-1} treatment. Cd^{2+} also affected the components of photosystem I and II in freshwater alga *Euglena gracilis*, with NADP oxidoreductase being the most sensitive target (De Filippis *et al.*, 1981a).

2.6.2.1.3 Cell division

   Anikeeva *et al.* (1975) noted that Cd disturbed the cell division *of Chlorella pyrenoidosa* while Singh and Pandey (1981) found that 0.02 mgL^{-1} Cd caused

approximately 10% of *Nostoc calcicola* cells to undergo abnormal division probably as a result from binding of Cd with the cell genome regulating cell division. They suggested that longer contact with the metal probably leads to more uptake than the instant killing at higher concentrations, and thus to loss in precision of cell division. The overall effects of Cd in this cyanobacterium were similar to those obtained with manganese (Singh and Kashyap, 1978).

2.6.2.1.4 Cell size and motility

A Cd^{2+} concentration of 0.32 mgL^{-1} caused an increase in the cell size and unequal partitioning of dividing cells in *Chlorella vulgaris* (Rosko and Rachlin, 1977). Cellular swelling has been observed in *Chlorella ellipsoidea* when exposed to Cd^{2+} levels of 0.56 to 5.6 mgL^{-1} (Lue-Kim *et al.*, 1980). Fernandez-Leborans and Novillo (1996) found that 10 - 500 µgL^{-1} Cd caused 21-27% reduction in biovolume (cell size) of *Olisthodiscus luteus*. Reduction in biovolume has also been observed by Lawrence *et al.* (1989) and explained as a metabolic cost of the detoxification process.

With *Euglena gracilis*, Cd^{2+} causes a loss of cell motility (Nakano *et al.*, 1978; De Fillipis *et al.*, 1981a), cellular swelling, and cell clumping (Nakano *et al.*, 1978). The effects have been attributed to Cd^{2+}-induced alterations in normal cell division processes (Nakano *et al.*, 1978; De Filippis *et al.*, 1981a).

2.6.2.1.5 Metabolism

Li (1978) suggested Cd caused alterations in NO$_3$$^-$ metabolism by *Thalassiosira fluviatis*. Author also found elevated N content in Cd-stressed *T. fluviatis* grown at the same rate as Cd-free control cells. Li (1979) noted that in Cd-adapted *T. fluviatis* cells which grew at the same growth rates but were larger than control cells, many cellular

constituents occurred in greater amounts. Accomodation of Cd stress was manifested most apparently in altered metabolism of protein. Protein : DNA, protein : RNA, protein nitrogen : total cell nitrogen and protein : carbohydrate ratios were all higher in Cd-adapted cells, although the ratio of protein to total carbohydrate plus lipid was not affected by Cd. These findings and those of De Filippis and Pallaghy (1976b) and Conway (1978) indicate that heavy metals may induce changes in the carotenoid : chlorophyll ratios.

2.6.2.1.6 Other effects

Quantitive changes in cellular composition of phytoplankton stressed by heavy metals in batch culture (De Filippis and Pallaghy, 1976a; Berland *et al.*, 1977; Rivkin, 1979) and non-steady state continuous culture (Conway, 1978) have been reported. Fernandez-Pinas *et al.* (1997) noted that Cd caused significant decreases in iron (40%) and magnesium (43%) content of *Nostoc* sp. after 24h exposure, and cell potassium (60%) after 96h exposure.

Kogan *et al.* (1975) showed that the presence of Cd increased ultraviolet-induced mutation in *Chlorella pyrenoidosa*. Cd^{2+} also caused irregular sporulation in *C. ellipsoidea* and *C. pyrenoidosa* (Anikeeva *et al.*, 1975; Silverberg, 1976; Lue-Kim *et al.*, 1980). Cd induced abnormal colonial patterns in *Tabellaria flocculosa* (Adshead-Simonsen *et al.*, 1981). A documented biological damage caused by Cd is the destruction of photosynthetic pigments (Conway, 1978). Cell lysis and and production of aggregates of heterocysts were reported by Stratton and Corke (1979) in blue-green algae, apart from inhibition of photosynthesis and nitrogen fixation by Cd. Li (1979) observed the decreased amount of DOC excreted in response to increasing Cd in previously unstressed *Thalassiosira fluviatis* and suggested that since membrane permeability is an

important factor in determining characteristics of extracellular release (Hellebust, 1974), the change may be in part due to metal-induced alterations of membrane properties by increased binding by Cd.

Okamoto *et al.* (1996) reported that Cd significantly inhibited the growth of *Tetraselmis gracilis* at low concentrations and promoted the induction of superoxide dismutase (SOD) activity, suggestive of an oxidative stress state.

2.6.2.2 Copper

Not only is Cu an essential micronutrient for growth, metabolism and enzyme activities of algae (Manahan and Smith, 1973), but it is also a proven inhibitor of algal growth at high concentrations (Erickson, 1972). The range of deficiency and toxicity may be separated by about 1.5-2 orders of magnitude. In the dinoflagellate *Gonyaulax tamarensis* Cu limitation occurs at 5×10^{-13}M Cu activity, with an optimum growth around 10^{-12}M and an abrupt drop to no growth at 10^{-11} M (Eichenberger, 1986).

2.6.2.2.1 Growth

Steemann-Nielsen and Wium-Anderson (1970) who found that concentrations of ionic Cu as low as 1 to 2 μgL^{-1} inhibited growth of unicellular algae concluded that the fact that Cu, ordinarily in natural waters does not effect algae, is due to the formation of complexes with various organic substances whereby the toxicity is lost. They also observed that increased Cu decreased the growth rate of *Nitzschia palea* during exponential growth while the lag time of *Chlorella pyrenoidosa* was extended before the onset of exponential growth.

Others have also shown Cu at concentrations as low as 1 μgL^{-1} to be toxic to a number of marine microalgae (Mandelli, 1969; Erickson, 1972; Lumsden and Florence,

1983) while some have suggested that the growth of marine phytoplankton is inhibited by Cu concentrations between 17 - 30 ngmL^{-1} (Ibragim and Patin, 1975; Saward *et al*, 1975; Goering *et al*, 1977). Sorentino (1978) stated that algal media contain no more than 30 ngmL^{-1} Cu and considerable amounts of chelating agents where above this concentration, the metal gradually becomes inhibitory to growth.

Using a 96-hour bioassay method, Erickson (1972) found that effects of Cu increased with time and concentration. Population growth and ^{14}C uptake by *Thalassiosira pseudonana* displayed inhibition over the entire range of Cu added (5 - 30 µgL^{-1} Cu) where inhibition of cell division was greatest at all Cu concentrations after 72-hour exposure. Growth rate constant (k) of the alga decreased with increasing Cu concentration and during the course of growth at each concentration.

Amphidinium carterae and *Prorocentrum micans* exposed to concentrations of labile Cu of, respectively 605 nM (40 µgL^{-1}) and 55 nM (3 µgL^{-1}), which resulted from the addition of 1.58 µM (100 µgL^{-1}) of total Cu, also showed decreases in the growth of the cultures (Lage *et al.*, 1994). Sunda and Guillard (1976) found that growth of *Nannochloris atomus* was totally inhibited by 0.1 µgL^{-1} Cu while growth of *T. pseudonana* ceased above 0.32 µgL^{-1}. The range 1.6 to 1.8 µgL^{-1} Cu caused total growth inhibition in the planktonic diatom *Ditylum brightwellii* (Canterford and Canterford, 1980).

In a study, while growth rate of *Nitzschia closterium* was halved by 20 µgL^{-1} Cu, photosynthesis was only affected above 100 µgL^{-1} Cu (Florence and Stauber, 1986). Growth has been observed to be more susceptible to Cu than photosynthesis in *N. closterium* (Lumsden and Florence, 1983) and also in *Phaeodactylum tricornutum* (Cid *et al.*, 1995).

2.6.2.2.2 Photosynthesis

Fitzgerald and Faust (1963) reported that photosynthesis of *Chlorella* sp. was inhibited at a concentration of a few parts per million (mgL^{-1}) of Cu while some, but not all blue green algae and a diatom were sensitive to concentrations as low as 25 μgL^{-1}. Other workers found that *Chlorella* sp. photosynthesis were inhibited at much lower levels, at approximately 1 μgL^{-1} (Steemann-Nielsen *et al.*, 1969; Steemann-Nielsen and Kamp-Nielsen, 1970). Photosynthesis in *Nitzschia palea*, was also depressed at 1 μgL^{-1} (Steemann-Nielsen and Wium-Anderson, 1971) but growth was less affected by Cu. Meanwhile, Wong and Chang (1991) have observed that 0.1 mgL^{-1} Cu produced only a slight inhibition of photosynthesis in *C. pyrenoidosa* while 0.25 mgL^{-1} was required for total inhibition.

Inhibitory levels of Cu for cultures of marine phytoplankton have been ascertained by several workers (Mandelli, 1969; Erickson *et al.*, 1970; Erickson, 1972; Hannan and Patouillet, 1972). Such levels were mostly less than 100 μgL^{-1}, but varied with the species tested. Davies and Sleep (1980) observed that the lowest Cu concentrations causing detectable inhibition of photosynthesis in a coastal phytoplankton community lay in the range of 1-2.5 μgL^{-1}, well below levels reported to be present in some seas, thus metal pollution may be influencing primary production in these polluted regions.

Thomas and Seibert (1977) found that 10 to 50 μgL^{-1} Cu initially reduced crop levels and photosynthesis (and growth rate) of centric diatoms, comparable to results of Erickson *et al.* (1970) who showed that *Cyclotella nana* was inhibited at 5 mgL^{-1} Cu. Results of Thomas *et al.* (1980) also suggested that the immediate effect of Cu was a reduction in photosynthesis, leading to reduced algal crops. In a Controlled

Ecosystem Pollution Experiment (CEPEX), levels of Cu required to inhibit short-term photosynthesis of phytoplankton in control enclosures were lower than the levels required to inhibit algae from treated enclosures, indicating that algae which grew under stress were truly more resistant to Cu (Harrison *et al.*, 1977). Cid *et al.* (1995) also reported that Cu also interfered with photosynthesis of *Phaeodactylum tricornutum*. Photosynthesis inhibition by copper has been observed previously in other diatoms (Erickson, 1972; Overnell, 1976; Stauber and Florence, 1987).

2.6.2.2.3 Cell Division

Cell division in diatoms may be depressed by metal interference with silica deposition (Fisher *et al.*, 1981; Rueter and Morel, 1981). Fisher *et al.* (1981) found that when exposed to elevated levels of Cu, the diatom *Asterionella japonica* showed a reduced cell division rate and a marked increase in cell size. Cu-treated cells had greater cell volumes, dry weights, carbon, nitrogen, chlorophyll, and DNA contents. Two protoplasts often appeared to be contained within one frustule. Metal-treated cell photosynthesised at near-normal rates on a per chlorophyll basis and above normal rates on a per cell basis. Excretion of photosynthetically fixed carbon was depressed where less than 1% of fixed carbon was excreted in treated cells as compared to 10-22% in control cells. Thus metal-treated cells showed an uncoupling of photosynthesis from cell division and continued to enlarge when fixed carbon could not be excreted or utilised in cell division. This uncoupling can be due to Cu inhibiting the process of cell division independently of any effect on the production of new cell material (Stauber and Florence, 1987).

Lack of inhibition of internal metabolic processes but inhibition of the cytokinetic process have also been observed in copper-treated *Chlorella* (Kanazawa and Kanazawa,

1969), lead-treated *Platymonas* (Hessler, 1974) and in other studies with Cu (Bentley-Mowat and Reid, 1977; Erickson, 1972; Foster, 1977). Sunda and Lewis (1978) observed that 6.4 μgL^{-1} Cu^{2+} caused a total inhibition in the cell division of *Monochrysis lutheri*. Cu^{2+} has also been reported to reversibly inhibit cell division in *C. pyrenoidosa*, *Gonyaulax tamarensis*, and *Skeletonema costatum* (Steemann-Nielsen and Kamp-Nielsen, 1970; Anderson and Morel, 1978; and Morel *et al.*, 1978).

2.6.2.2.4 Cell size and motility

Giant cells have been reported in response to heavy metal toxicity in *Chlorella* (Rosko and Rachlin, 1977), *Isochrysis*, and *Dunaliella* (Davies, 1974, 1976). Lumsden and Florence (1983) reported that a highly toxic Cu complex, copper-oxine, caused *Nitzschia closterium* cells to become thinner and transparent, and increase three-fold in volume, suggesting that both growth and photosynthesis are affected by the complex.

The naked marine flagellate *Dunaliella marina* responds to hypotonic media in the same way as animal cells but Riisgard (1979) observed that Cu at 2 mgL^{-1} and higher concentrations inhibited its volume regulation in the medium and suggested this was achieved by reducing permeability of the cell membrane to K^+ and/or Cl^-. Meanwhile Riisgard *et al.* (1980) suggested that Cu inhibited volume regulation in hypertonic media by increasing the permeability of the cell membrane to Na^+ which enters the cell and makes it stay swollen. That the instantaneous shrinkage becomes smaller with increasing Cu contamination may reflect reduced cell membrane elasticity due to denaturation of membrane-bound proteins by copper. Overnell (1975a) found that several heavy metals including Cu, resulted in leakage of potassium from the marine flagellate *Dunaliella tertiolecta*.

Bentley-Mowatt and Reid (1977) discovered that growth of *Phaeodactylum tricornutum*, *Dunaliella primolecta* and *Cricosphaera elongata* in batch culture were not arrested in addition of Cu and Cd below a concentration of 6.4 mgL^{-1} while *Ditylum brightwelli* underwent osmotic disturbances in 0.64 mgL^{-1} and 6.4 mgL^{-1} Cu, with swelling of the cell contents, suggesting that Cu injury is to the semipermeable cell membrane in the alga.

Erickson (1972) noted that mean cell volume *Thalassiosira pseudonana* increased correspondingly at Cu concentrations of 10-30 μgL^{-1}. Most severe changes were in cells exposed to 30 μgL^{-1} Cu for 72 hours when the mean cell volume increased to 165% of control. Microscopic examination revealed these cells to be aberrant with cytoplasm extruding from them. Fisher and Frood (1980) indicated that *Asterionella japonica*, showed marked swelling when exposed to Cu. Dry weight of these cells increased to 400% of control values.

Anderson and Morel (1978) reported cells of the red-tide producing dinoflagellate, *Gonyaulax tamarensis* were 100% nonmotile and did not divide or increase in size at a calculated cupric ion activity of $10^{-9.7}$ M [pCu* 9.7 and below]. In *Prorocentrum micans*, irreversible loss of cell motility, growth inhibition, a large reduction in protein, and eventually lethality was achieved with a concentration of labile Cu of about 3.16 μM (406 fmol dissolved labile Cu per cell) [resulting from 15.8μM (1000 μgL^{-1} of total Cu)] (Lage *et al.*, 1994).

2.6.2.2.5 Metabolism

Diatoms require SiO_2 to construct cell walls (O'Kelley, 1968). Uptake of $Si(OH)_4$ by cultures from the growth medium is apparently limited to the cell wall formation stage of the division cycle and once taken up it is rapidly incorporated into the growing shell

(Darley and Volcani, 1969). Research with natural plankton assemblages (Goering *et al.*, 1977) and the diatom, *Skeletonema costatum* (Morel *et al.*, 1978) indicated that Cu may interfere with silicate metabolism in diatoms. Since enzymes and sulphydryl groups have been implicated with Si(OH)$_4$ uptake, Cu which interacts with them would be expected to to inhibit Si(OH)$_4$ uptake. In short-term exposures of marine phytoplankton to 2.5 - 25 μgL^{-1} Cu, silicic acid uptake was found to be inhibited by Cu (Goering *et al.*, 1977). Morel *et al.* (1978) suggested modifications of silicon metabolism under Cu stress to account for copper-silicate interaction in lag phase prolongation of *S. costatum*. Cu has also been found to affect nitrate and amino acid metabolism in phytoplankton (Fisher *et al.*, 1981).

2.6.2.2.6 Other effects

Apart from inhibition of nitrate uptake, photosynthetic carbon assimilation and synthesis of nitrate reductase by Cu, Harrison *et al.* (1977) also observed cell disruption and loss of accumulated ammonium in *Noctiluca* sp., a free living flagellate. Reduced photosynthetic carbon fixation is another short-term response of *Gonyaulax tamarensis* to Cu (Anderson and Morel, 1978). Carbon fixation rates decreased linearly as the Cu contents of phytoplankton, measured as the Cu/chlorophyll *a* ratios in the plant cells, increased (Davies and Sleep, 1980).

Gillan *et al.* (1983) observed that Cu interfered with carotenoid biosynthesis and affected the fatty acid distribution in the diatom, *Asterionella japonica*. Cu may also exert its toxicity in subcellular organelles, interfering with photosynthesis in the chloroplasts (Overnell, 1975a,b) and ATP production. 0.05 mgL^{-1} Cu caused a significant decrease in the cellular pool of ATP of *Phaeodactylum tricornutum* cells (Cid *et al.*, 1995) but had no effect on photosynthesis and growth due to the higher consumption of

ATP in the cell to avoid the toxic effects of Cu, maintaining cellular integrity and activating metabolic processes related with cell growth.

Pigment analysis by HPLC showed that Cu affected the pigment pattern of *P. tricornutum* (Cid *et al.*, 1995); chlorophyll *a* proportion decreased while its allomer increased with the Cu concentration, being maximum at 1 mgL^{-1} Cu.

A decrease in the protein content of *Prorocentrum micans* was observed at labile Cu concentrations of 0.163μM (32.7 fmol dissolved labile Cu $cell^{-1}$) and 3.16 μM (406 fmol dissolved labile Cu $cell^{-1}$) (Lage *et al.*, 1994), probably due to Cu inhibition of photosynthesis and respiration. The sensitivity of photosynthetic and mitochondrial membranes to heavy metals, especially Cu has been described by Sicko-Goad (1982).

For the planktonic diatom *Ditylum brightwelli*, exposure to Cu induced oxidative stress, as indicated by a decrease of gluthathione reductase and increase of SOD activity (Rijstenbil *et al.*, 1994).

2.6.2.3 Manganese

Relatively much less study has been done on the toxicity of manganese to microalgae. Instead, more work has been focussed on its function as a growth limiting factor (Brand *et al.*, 1983) and on the effects of its interaction with other metals on toxicity to algae (Kazumi *et al.*, 1987). Mn-deficient cells of *Chlamydomonas reinhardtii* have been found to be unable to maintain chloroplast structure and to carry out at normal rates, photosynthetic reactions involving system II of the photosynthetic electron transport chain (Teichler-Zallen, 1969).

2.6.2.3.1 Photosynthesis

Studies on the potential impacts of deep-sea mining of Mn nodules on marine

phytoplankton in the North Pacific Ocean have indicated a significant short-term reduction in the photosynthesis and primary production in the plume of the mining discharge due to metal inhibition (Chan and Anderson, 1981).

2.6.2.3.2 Growth and cell division

Mn chloride has been found to induce assymmetric cell division in *Chroococcus limneticus*, a coccoid blue-green alga, leading to the formation of abnormal cells whose proportion was concentration dependent (Singh and Kashyap, 1978). 0.05 μgL^{-1} of Mn which caused less than 50% lethality were most efficient at inducing the formation of abnormal cells. However, similar observations were not obtained with *Plectonema boryanum*, a filamentous blue-green algae. Instead, mutagenic effects were found with the latter species.

2.6.2.3.3 Cell volume

Christensen *et al.* (1979) observed that Mn had little effect on *Selenastrum capricornutum* but decreased the average cell volume of *Chlorella stigmatophora* slightly. 3.1 mgL^{-1} and 50 mgL^{-1} Mn caused 50% reductions in cell volume of *S. capricornutum and C. stigmatophora* respectively.

2.6.2.4 Arsenic

2.6.2.4.1 Growth

Eisler (1988) in his review cited that 0.13 mgL^{-1} arsenate (As(V)) caused growth inhibition in *Skeletonema costatum*, and reduced the biomass of a marine phytoplankton population within four days. Growth of *Dunaliella* sp. was found to be inhibited by a three-day exposure to 0.1 mgL^{-1} and higher concentrations of As(V), but gained recovery after continued exposure after 12 days, surviving concentrations up to 2 mgL^{-1} (Yamaoko

and Takimura, 1986).

Bottino *et al.* (1978) found that 10 mgL^{-1} or more As(V) in modified natural seawater (MSW) caused a lag phase in the growth of *Tetraselmis chuii* which increased linearly with the concentration of As(V) in the medium. However, the alga did not exhibit a lag period when exposed to arsenite (As(III)). 20 mgL^{-1} or more As(V) shortened the normally four-day exponential phase to two days. The cells also continued to multiply at a slower rate without achieving a well-defined stationary phase. Meanwhile As(V) in enriched artificial seawater (ASW) caused a much longer lag phase in growth of *T. chuii* but not a decrease in the exponential phase. *T. chuii* exposed to 10, 20, and 30 mgL^{-1} As(III) in MSW decreased in cell density with increasing As(III) concentrations during the stationary phase but did not vary in 10 mgL^{-1} As(III) in ASW. As(V) and As(III) had very little effect on the growth of *Hymenomonas carterae* in both media.

Sanders (1979b) observed that growth of *S. costatum* was significantly inhibited by 167nM (0.02 mgL^{-1}) of As(V). Several species of marine phytoplankton in single species cultures exposed to 0-100 µgL^{-1} As(V) (0-20 pgcell^{-1}) varied in their sensitivity to dissolved As(V) (1-10X ambient) (Sanders and Vermersch, 1982). As(V) terminated the growth of *Amphidinium carterae* while growth rates of *Thalassiosira pseudonana* and *S. costatum* were depressed by 27% and 60% respectively. Meanwhile, in natural phytoplankton assemblages, biomass (cell number) decreased by 60% of controls. 67nM (9.3 µgL^{-1}) of As has been found to cause a significant deviation in growth rate of centric diatom *Rhizosolenia fragilissima* (Sanders and Cibik, 1985b). Other species inhibited by As were flagellates *Pyramimonas* and *Chroomonas* sp.

2.6.2.4.2 Photosynthesis

Sanders (1979b) was the first to demonstrate As inhibition of marine

phytoplankton at near ambient levels. As(V) additions of 67nM or greater significantly inhibited photosynthesis and carbon uptake in *Skeletonema costatum* during the log and stationary phase. Small amounts of As(III) also inhibited carbon uptake. Sanders and Vermersch (1982) observed that in natural phytoplankton assemblages, chlorophyll *a* and carbon were reduced to 15-30% of controls in the presence of 0-100 μgL^{-1} As(V) while Planas and Lamarche (1983) noted that As slightly decreased the chlorophyll *a* and biomass of algae, the lack of effect probably due to the As^{5+}/P ratio. Meanwhile, 0.075 mgL^{-1} As(V) caused a reduction of chlorophyll *a* in *Thalassiosira aestivalis* (Eisler, 1988)

2.6.2.4.3 Cell motility

Bottino *et al*. (1978) reported that *Tetraselmis chuii* exposed to 1 and 10 mgL^{-1} of As(V) in artificial seawater (ASW), and 10, 20 and 30 mgL^{-1} of As(III) in modified natural seawater (MSW), respectively, withdrew or cast-off their flagela, rounded up, sank to the bottom of the culture vessel, and encysted while 10 mgL^{-1} of As(III) in ASW caused loss of cell motility. The cells in the media containing As(V) also exhibited erratic movements. Permanent loss of motility was also observed in *Hymenomonas carterae* exposed to 30 mgL^{-1} of As(V) in MSW although the cells continued to reproduce, while a reversible of loss of motility occurred in more than 1 mgL^{-1} As(V) in ASW.

2.6.3 Morphological effects

Abnormal cell morphology observed in samples from nature, should be used with caution as an indicator of heavy metal pollution, as the abnormalities may also be caused by nutrient limitation. However, in nutrient rich water these abnormalities may serve as an indicator of heavy metal pollution (Thomas *et al*., 1980). Change in morphology is one of the most commonly observed effects of heavy metal toxicity

(Thomas *et al.*, 1980) which has been observed in representatives from the Chlorophyceae (Rosko and Rachlin, 1977), Chrysophyceae (Davies, 1974) and Bacillariophyceae (Nuzzi, 1972; Sunda and Guillard, 1976; Berland *et al.*, 1977; Morel *et al.*, 1978). The uncoupling of cell growth and cell division has been suggested as an explaination of this phenomenon (Davies, 1976).

2.6.3.1 Cadmium

Thomas *et al.* (1980) observed with *Thalassiosira aestivalis* that cadmium disrupted cell separation, causing cells to clump, and induced a decrease in chain length. However, the main effect was the formation of elongated, bent, out-of-column cells. Cd also induced cell elongation in *Cyclotella meneghiniana*, *Nitzschia palea* and *Navicula confervacea* (Rao and Subramaniam, 1982).

Kayser and Sperling (1980) observed morphological abberations in the form of brittleness of the cell walls in *Prorocentrum micans* which seems to be a general effect of Cd. Cd ionic concentrations of 5-100 µg L^{-1} caused morphological alterations in *Euglena gracilis* where the most abnormal shaped cells were starfish-shaped (Nakano *et al.*, 1980).

Apart from the morphological effects, Cd also causes ultrastructural damages in microalgae. Cd and other heavy metals are known to bind to the mitochondrial membranes of various eukaryotic cells (Byczkowski and Sorenson, 1984), but mitochondrial damage seems to be a Cd^{2+} specific effect in algae (Soyer and Prevot, 1981). Silverberg (1976) reported Cd-induced ultratructural changes in mitochondria of freshwater green algae, *Ankistrodesmus falcatus*, *Chlorella pyrenoidosa* and *Scenedesmus quadricauda*, and considered the mitochondria as the primary target for Cd-associated cytotoxicity.

Ultramicroscopic examination of thin sections of the free-living marine dinoflagellate *P. micans* which had been exposed to 10-100 μgL^{-1} of Cd revealed heightened vacuolation and increased number of lysosomes, which was probably related to accumulation and detoxification properties; two general or non-specific response to Cd toxicity (Soyer and Prevot, 1981). The organelles visibly most affected in Cd-treated *P. micans* cells were the mitochondria, a specific response to toxicity of Cd, where the damage caused by Cd was sufficient to affect the cell metabolism, particularly respiration.

Soyer and Prevot (1981) found that chloroplasts were not very sensitive to Cd where the chloroplasts of *P.micans* were only affected by Cd after 2 months at 40 μgL^{-1}. Changes in the levels of the photoynthetic pigments may reflect the disorganisation of the membrane in chloroplasts, caused by the internal presence of Cd. Soyer and Prevot (1981) also reported that even though the nuclear apparatus was not structurally affected by the presence of Cd, the nuclear division in *P. micans* was inhibited.

Other celullar abnormalities such as the loss of cross walls (Rao and Subramaniam, 1982), swollen chloroplasts membranes (Smith, 1983), and alterations in cytoplasmic vacuolisation and granulation (Thomas *et al.*, 1980; Smith, 1983) have been observed in various diatoms.

2.6.3.2 Copper

Thomas *et al.* (1980) found that with the centric diatom *Thalassiosira aestivalis* the main effects were granular, yellowed cytoplasm; a disruption of chloroplast integrity and dispersion; and more delicate spines extruded from the marginal processes. Copper increased the chain length and prevented normal separation of the cells, although complete frustules were formed. Cu also caused the inability to form the central

chitinous threads that separate normal cells. Very similar morphological abberations were observed in *Thalassiosira polychorda*. Some of the effects on the cell morphology of *T. aestivalis* were reversible.

Thomas *et al*. (1980) found that centric diatoms, *Chaetoceros danicus*, *C. curvisetus*, and *Skeletonema costatum* elongated in the pervalvar axis in the presence of Cu but no abberant chains of the *Thalassiosira* type were observed. The pennate diatom, *Nitzchia delicatissima;* was occasionally observed in a twinned state while *Cylindrotheca closterium* sometimes formed cells that were abnormally swollen in the central portion of the cell, or had twisted, corkscrew spindles. *Stephanopyxis palmeriana* formed bulbuous protrusions in the girdle region where the cell normally constricts prior to division, that eventually lysed, in response to Cu while the silicoflagellate, *Distephanus speculum*, formed abnormal spicules.

Berland *et al*. (1977) and Morel *et al*. (1978) have also described the elongation of *Skeletonema* cells in response to metals while Menzel and Case (1977) observed abberant *Coscinodiscus* sp. cells in a controlled ecosystem pollution experiment (CEPEX).

2.6.3.3 Arsenic

Bottino *et al*. (1978) observed the deposition of mucilaginous substance of unknown composition on the theca of *Tetraselmis chuii* exposed to more than 20 mgL^{-1} arsenate in modified natural seawater but not in artificial seawater. They also found the occurrence of sloughing and loss of coccoliths of *Hymenomonas carterae* in 30 and 1000 mgL^{-1} As(V) respectively. Loss of flagella and coccoliths were also observed in *H. carterae* grown in arsenite (As(III)).

2.6.4 Ecological Effects

'Toxicity' in an ecological context refers to the effects of a substance which reduce the fitness of single populations and alter the interactions between populations (Eichenberger, 1986). Observations have been done on the reactions towards metals in complex seminatural communities. Many studies have demonstrated differential sensitivities of various phytoplankton species to heavy metals (Davies, 1978, Sanders and Cibik, 1985b; Tadros *et al.*, 1990) and the influence of pollutants on the course of competition (Dayton and Lewin, 1975; Kayser, 1977) and resume of population growth after the period of decline due to the metals (Davies, 1974; Kayser, 1976; Li, 1980).

A study by Li (1984) clearly indicate that Cd^{2+} is a potentially important factor influencing algal competition, where there may be more than one simple mechanism. Thomas *et al.* (1977a) investigated the effects of copper in a Controlled Ecosystem Pollution Experiment (CEPEX). The addition of 10, 20 and 50 μgL^{-1} of Cu to marine enclosures at an inlet caused initial inhibition of phytoplankton crop, photosynthesis, and growth rates, followed by a recovery. However the recovery was accompanied by a selection of Cu resistant taxa, where the initially present diatom *Chaetoceros* sp. and dinoflagellates were replaced by microflagellates and some other diatoms such as pennate diatoms *Nitzschia delicatissima* and *Navicula distans*.

In another experiment by Thomas *et al.* (1977a), 5 and 10 μgL^{-1} Cu seemed to increase the algal standing crop but not the rate of photosynthesis, over the levels in the control enclosures. The increase in the crop may be due to inhibition of grazers in treated enclosures but not in the control, or, since Cu was added late in the experiment, a natural selection for resistant algal species may have already occurred. These results suggests that an immediate effect of Cu is a reduction in photosynthesis that leads to

reduced algal crops, but that recovery, with selection for resistant taxa, may occur as pollutant stress continues.

Thomas and Seibert (1977) also pointed out that the definite reduction of taxonomic diversity due to Cu, without reduction of phytoplankton biomass, may have important implications for food chain dynamics through selective grazing preferences by zooplankton. Microflagellates, because of their small size, might not be available to all zooplankton or to larval fish. On the other hand, Cu treatment, by inducing the dominance of flagellates, might be useful in oyster aquaculture, since oysters feed mainly on these organisms.

The change in species diversity and succession of dominance due to arsenic has also been observed (Sanders and Cibik, 1985b; Sanders and Vermersch, 1982) where at low As concentrations large centric diatoms were replaced by smaller species. Austin (1995) pointed out that resistance of phytoplankton to contaminants and their ability to concentrate toxic metals may also result in magnified concentrations in higher trophic levels.

2.6.5 Quantitative toxicity data

One of the most striking aspects about the compilation of toxicity data is the wide range of values reported (Davies, 1978; Rai *et al.*, 1981; Sorentino, 1978; Stratton, 1987). Tables 2.6, 2.7, 2.8 and 2.9 summarise the toxicity data of cadmium, copper, manganese and arsenic respectively, which have been produced by various researchers with numerous species of marine phytoplankton, by various methods of bioassay or toxicity testing under varying test conditions, using numerous biological variables and endpoints.

Table 2.6 : Summary of cadmium toxicity data from previous studies with marine phytoplankton

Microalgal species	Metal	Endpoint	Value	Parameter of inhibition	Reference
Asterionella japonica	Cd	EC_{50}	2.1 μM *(0.24 mgL⁻¹)*	growth rate	Fisher and Jones, 1981
Chaetoceros gracilis	Cd	NOEC	0.3- <1.0 mgL⁻¹ (96h)	cell number	Hindarti, 1997
		LOEC	0.7-0.9 mgL⁻¹ (96h)		
		IC_{25}	0.5-1.1 mgL⁻¹ (96h)		
		IC_{50}	0.9-1.7 mgL⁻¹ (96h)		
Chaetoceros ceratosporum	Cd	NOEC	0.6-1.0 mgL⁻¹ (96h)	cell number	Hindarti, 1997
		LOEC	≤0.9-1.3 mgL⁻¹ (96h)		
		IC_{25}	1.1-2.1 mgL⁻¹ (96h)		
		IC_{50}	2.5-4.2 mgL⁻¹ (96h)		
Chlorella saccharophilla	Cd	EC_{50}	0.105 mgL⁻¹ (96h)	population growth	Rachlin *et al*., 1982
Ditylum brightwellii	Cd	EC_{50}ᵃ	0.06-1.2 mgL⁻¹ total Cd 2.2-8.5 μgL⁻¹ free metal	growth (cell counts)	Canterford and Canterford, 1980
Dunaliella minuta	Cd	EC_{50}ᵇ	0.34 μM *(0.04 mgL⁻¹)*	population growth	Visviki and Rachlin, 1991
Dunaliella tertiolecta	Cd	NOEC	0.30-1.02 mgL⁻¹ (96h)	cell growth	Thongra-ar *et al*., 1995
		LOEC	0.91-3.22 mgL⁻¹ (96h)		
		IC_{25}	0.17-1.74 mgL⁻¹ (96h)		
		IC_{50}	1.96-6.64 mgL⁻¹ (96h)		
Dunaliella tertiolecta	Cd	NOEC	0.91-1.02 mgL⁻¹ (96h)	Chlorophyll *a*	Thongra-ar *et al*., 1995
		LOEC	2.38-4.02 mgL⁻¹ (96h)		
		IC_{25}	1.83-2.92 mgL⁻¹ (96h)		
		IC_{50}	4.52-7.73 mgL⁻¹ (96h)		

Note : All values based on studies without EDTA unless stated otherwise

<u>With EDTA</u> : a (6.7X10⁻⁷ - 1.0X10⁻⁶M)　　<u>With Tris</u> : b; c (2mM)

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...cont.

Table 2.6 : Summary of cadmium toxicity data from previous studies with marine phytoplankton

Microalgal species	Metal	Endpoint	Value	Parameter of inhibition	Reference
Navicula incerta	Cd	EC_{50}	26.8 μM *(3.01mg L⁻¹)* (96h)	population growth	Rachlin et al., 1983
Navicula incerta	Cd	EC_{50}	3.008 mgL⁻¹ (96h)	population growth	Rachlin et al., 1982
Nitzschia closterium	Cd	EC_{50}	0.476 mgL⁻¹ (96h)	population growth	Rachlin et al., 1982
Skeletonema costatum	Cd	EC_{50}ᶜ	0.05mgL⁻¹	growth (cell yield)	Berland et al., 1977
Skeletonema costatum	Cdᵇ	EC_{50}ᶜ	1.15 mgL⁻¹	cell number	Gowrinathan and Rao, 1989
Tetraselmis gracilis	Cd	NOEC	1.0 mgL⁻¹ (48h and 96h)	growth	Okamoto et al., 1996
Tetraselmis gracilis	Cd	LC_{50}	3.2 mgL⁻¹ (48h) 1.8 mgL⁻¹ (96h)	growth	Okamoto et al., 1996
Tetraselmis sp.	Cd	NOEC LOEC IC_{25} IC_{50}	1.4-1.8 mgL⁻¹ (96h) 3.1-3.2 mgL⁻¹ (96h) 1.8-7.8 mgL⁻¹ (96h) 3.9->10.0 mgL⁻¹ (96h)	Cell number	Gonzales, 1995
Tetraselmis sp.	Cd	NOEC LOEC IC_{25} IC_{50}	3.1 mgL⁻¹ (96h) 9.7 mgL⁻¹ (96h) 4.1 mgL⁻¹ (96h) > 9.7 mgL⁻¹ (96h)	Cell number	Gonzales, 1997
Tetraselmis tetrahele	Cd	NOEC LOEC IC_{25} IC_{50}	1.2-5.4 mgL⁻¹ (96h) 3.6-9.5 mgL⁻¹ (96h) 4.0-5.1 mgL⁻¹ (96h) 8.1-9.4 mgL⁻¹ (96h)	Cell number	Gonzales, 1997

Note : All values based on studies without EDTA unless stated otherwise

With EDTA : a (6.7X10⁻⁷ - 1.0X10⁻⁶ M)   With Tris : b; c (2mM)

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Table 2.7 : Summary of copper toxicity data from previous studies with marine phytoplankton

Microalgal species	Metal	Endpoint	Value	Parameter of inhibition	Reference
Amphidinium carterae	Cu	EC$_{50}$	50 mgL⁻¹ (14d)	growth rate	Erickson et al., 1970
Amphidinium carterae	pCu*	EC$_{50}$ [a]	10.1 (0.005 μgL⁻¹)	growth rate	Hawkins and Griffiths, 1982
Asterionella japonica	Cu	EC$_{50}$	0.2 μM (0.013 mgL⁻¹)	growth rate	Fisher and Jones, 1981
Attheya decora	Cu²⁺	IC$_{50}$	7 X 10⁻⁸ M (4.45 mgL⁻¹)	photosynthesis	Overnell, 1976
Brachiomonas submarina	Cu²⁺	IC$_{50}$	2-5 X 10⁻⁵ M (1.27-3.18 mgL⁻¹)	photosynthesis	Overnell, 1976
Chlorella stigmatophora	Cu²⁺	EC$_{50}$ [b]	0.07 mgL⁻¹	cell volume	Christensen et al., 1979
Ditylum brightwellii	Cu	EC$_{50}$ [c]	0.015-0.58 mgL⁻¹ total Cu 0.23-0.65 μgL⁻¹ free metal	growth (cell counts)	Canterford and Canterford, 1980
Dunaliella minuta	Cu	EC$_{50}$ [d]	7.57 μM (0.48 mgL⁻¹)	population growth	Visviki and Rachlin, 1991
Dunaliella tertiolecta	Cu	EC$_{50}$ [e]	4X10⁻⁶ M (2.54 mgL⁻¹) 2X10⁻⁶ M (1.27 mgL⁻¹)	oxygen evolution K+ loss	Overnell, 1975a
Dunaliella tertiolecta	Cu²⁺	EC$_{50}$	10⁻⁵ M (0.64 mgL⁻¹) 5X10⁻⁶ M (0.32 mgL⁻¹)	oxygen evolution K+ loss	Overnell, 1975a
Dunaliella tertiolecta	Cu²⁺	IC$_{50}$	7 X 10⁻⁸ M (4.45 mgL⁻¹)	photosynthesis	Overnell, 1976
Dunaliella tertiolecta	pCu*	EC$_{50}$ [a]	7 (0.006 mgL⁻¹)	growth rate	Hawkins and Griffiths, 1982
Dunaliella tertiolecta	Cu	EC$_{50}$ [f]	58.93 mgL⁻¹ (24h) 44.76 mgL⁻¹ (48h) 38.80 mgL⁻¹ (72h)	growth rate	Edding and Tala, 1996
Dunaliella tertiolecta	Cu	NOEC [f]	12.5 mgL⁻¹	growth rate	Edding and Tala, 1996
Gonyaulax tamarensis	pCu*	EC$_{50}$ [a]	10.4 (0.003 μgL⁻¹) (2&24H)	cell motility	Anderson and Morel (1978)

Note: All values based on studies without EDTA unless stated otherwise

With EDTA : b (300μgL⁻¹ Na₂EDTA); c (6.7X10⁻⁷ - 1.0X10⁻⁵M); f; g (5X10⁻⁷ - 5X10⁻⁵ M EDTA plus 10⁻³ M Tris used to regulate ionic activity)

With other chelators : a (10mM Tris used to regulate ionic activity); d (Tris); e (0.01 MOPS and 0.01M HEPPS); h (10⁻³ M NTA); i (1μM Tris plus organic ligands); j (amino acids)

...cont.

Table 2.7 : Summary of copper toxicity data from previous studies with marine phytoplankton

Microalgal species	Metal	Endpoint	Value	Parameter of inhibition	Reference
Gymnodinium splendens	Cu	EC$_{50}$	0.02 mgL^{-1} (48h)	cell number	Saifullah, 1978
Isochrysis galbana	Cu	EC$_{50}$ [f]	5.88 mgL^{-1} (24h); 2.38 mgL^{-1} (48h); 1.58 mgL^{-1} (72	growth rate	Edding and Tala, 1996
Isochrysis galbana	Cu	NOEC [f]	1.0 mgL^{-1}	growth rate	Edding and Tala, 1996
Marine phytoplankton	Cu	EC$_{50}$	0.025 mgL^{-1}	silicic acid uptake	Goering *et al*, 1977
Marine phytoplankton	Cu	IC$_{50}$ [h]	10^{-11} and 10^{-10} M *(0.0006 and 0.006 µgL^{-1})*	reproduction rate	Brand *et al*, 1986
Monochrysis lutheri	Cu^{2+}	IC$_{50}$	2 X 10^{-4} M *(12.7 mgL^{-1})*	photosynthesis	Overnell, 1976
Monochrysis lutheri	Cu^{2+}	EC$_{50}$ [i]	4 X10^{-9} M *(0.25 µgL^{-1})*	cell division	Sunda and Lewis, 1978
Nannochloris atomus	pCu*	EC$_{50}$*	9.3 *(0.03 µgL^{-1})*	growth rate	Sunda and Guillard, 1976
Navicula incerta	Cu	EC$_{50}$	164.5 µM, *(10.5 mg L^{-1})* (96h)	population growth	Rachlin *et al*, 1983
Nitzschia closterium	Cu	EC$_{50}$ [j]	0.237 mgL^{-1} (96h)	growth rate	Rosko and Rachlin, 1975
Nitzschia closterium	Cu	EC$_{50}$	0.033 mgL^{-1} (96h)	growth rate	Rosko and Rachlin, 1975
Nitzschia closterium	Cu	EC$_{50}$	0.02 mgL^{-1}	growth rate	Lumsden and Florence, 1983 Florence and Stauber, 1986
Olisthodiscus luteus	Cu	EC$_{50}$	50 mgL^{-1} (14-d)	growth rate	Erickson *et al.*, 1970
Oscillatoria (Trichodesmium) thelbautii	Cu	EC$_{50}$ [d]	10^{-10} M *(0.006 µgL^{-1})*	[14C] CO2 fixation	Rueter and McCarthy, 1979
Phaeodactylum tricornutum	Cu	EC$_{50}$	0.1 mg/L; 0.5 mg/L	growth photosynthesis rate	Cid *et al*, 1995
Phaeodactylum tricornutum	pCu*	EC$_{50}$*	9.1 *(0.05 µgL^{-1})*	growth rate	Hawkins and Griffiths, 1982

continued...

Note : All values based on studies without EDTA unless stated otherwise
With EDTA : b (300µgL^{-1} Na$_2$EDTA); e (6.7X10^{-7} - 1.0X10^{-6}M); f; g (5X10^{-7} - 5X10^{-6}M EDTA plus 10^{-3}M Tris used to regulate ionic activity)
With other chelators : a (10mM Tris used to regulate ionic activity); d (Tris); e (0.01 MOPS and 0.01M HEPPS); h (10^{-4}M NTA);
i (1µM Tris plus organic ligands); j (amino acids)

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Table 2.7 : Summary of copper toxicity data from previous studies with marine phytoplankton

...cont.

Microalgal species	Metal	Endpoint	Value	Parameter of inhibition	Reference
Prorocentrum micans	Cu	EC$_{50}$	0.02 mgL^{-1} (48h) 0.01 mgL^{-1} (72h) 0.005 mgL^{-1} (8 days)	cell number	Saifullah, 1978
Scrippsiella faeroense	Cu	EC$_{50}$	0.02 mgL^{-1} (48h) 0.01 mgL^{-1} (72h) 0.005 mgL^{-1} (5-6 days)	cell number	Saifullah, 1978
Skeletonema costatum	Cu	EC$_{50}$	50 mgL^{-1} (14-d)	growth rate	Erickson et al., 1970
Skeletonema costatum	Cu^{2+}	IC$_{50}$	5 X 10^{-9} M (3.18 mgL^{-1})	photosynthesis	Overnell, 1976
Skeletonema costatum	Cu^{2+}	EC$_{50}$	1.45 mgL^{-1}	cell number	Gowrinathan and Rao, 1989
Stichococcus bacillaris	pCu*	EC$_{50}$*	7 (0.006 mgL^{-1})	growth rate	Hawkins and Griffiths, 1982
Tetraselmis sp.	Cu	NOEC LOEC IC$_{25}$ IC$_{50}$	0.1 mgL^{-1} (96h) 0.18 mgL^{-1} (96h) 0.09-0.12 mgL^{-1} (96h) 0.15-0.16 mgL^{-1} (96h)	Cell number	Gonzales, 1995
Tetraselmis tetrahele	Cu	NOEC LOEC IC$_{25}$ IC$_{50}$	<0.10 mgL^{-1} (96h) ≤0.10 mgL^{-1} (96h) <0.10 mgL^{-1} (96h) 0.22 mgL^{-1} (96h)	Cell number	Gonzales, 1997
Thalassiosira pseudonana	Cu	EC$_{50}$	0.02->0.03 mgL^{-1} (0-24h) 0.007-> 0.03mgL^{-1} (24-48h) 0.003- 0.012mgL^{-1} (48-72h)	growth rate	Erickson, 1972
Thalassiosira pseudonana	pCu*	EC$_{50}$	9.3 (0.03 µgL^{-1})	growth	Davey et al., 1973
Thalassiosira pseudonana	pCu*	EC$_{50}$*	8.6 (0.16 µgL^{-1})	growth rate	Sunda and Guillard, 1976

Note : All values based on studies without EDTA unless stated otherwise

With EDTA : b (300µgL^{-1} Na$_2$EDTA); e (6.7X10^{-7} - 1.0X10^{-5}M); f ; g (5X10^{-7} - 5X10^{-5}M EDTA plus 10^{-3}M Tris used to regulate ionic activity)

With other chelators : a (10mM Tris used to regulate ionic activity; d (Tris); e (0.01 MOPS and 0.01M HEPPS); h (10^{-4} M NTA);
i (1µM Tris plus organic ligands); j (amino acids)

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Table 2.8 : Summary of manganese toxicity data from previous studies with marine phytoplankton

Microalgal species	Metal	Endpoint	Value	Parameter of inhibition	Reference
Asterionella japonica	Mn	EC_{50}	88.3 μM *(4.85mgL⁻¹)*	growth rate	Fisher and Jones, 1981
Chlorella stigmatophora	Mn[a]	EC_{50} *	50mgL⁻¹	cell volume	Christensen *et al*, 1979
Ditylum brightwellii	Mn	EC_{50} [b]	1.5 mgL⁻¹	growth (cell counts)	Canterford and Canterford, 1980
Nitzschia closterium	Mn	EC_{50} *	53.8 mgL⁻¹ (96h)	growth rate	Rosko and Rachlin, 1975
Nitzchia closterium	Mn	EC_{50}	25.7 mgL⁻¹ (96h)	growth rate	Rosko and Rachlin, 1975

Note : All values based on studies without EDTA unless stated otherwise;

With EDTA : a (300μgL⁻¹ Na₂EDTA); b (6.7X10⁻⁶M); Other chelators : c (amino acids)

Table 2.9 : Summary of arsenic toxicity data from previous studies with marine phytoplankton

Microalgal species	Metal	Endpoint	Value	Parameter of inhibition	Reference
Amphidinium carterae	As(V)	EC_{50} *	2 pgcell⁻¹	growth rate	Sanders and Vermersch, 1982
Chaetoceros pseudocrinitum	As(V)	EC_{50} *	4 pgcell⁻¹	growth rate	Sanders and Vermersch, 1982
Cylindrotheca closterium	As(V)	EC_{50} *	> 20 pgcell⁻¹	growth rate	Sanders and Vermersch, 1982
Isochrysis galbana	As(V)	EC_{50} *	0.4 pgcell⁻¹	growth rate	Sanders and Vermersch, 1982
Peridinium trochoidium	As(V)	EC_{50}	18 pgcell⁻¹	growth rate	Sanders, 1978*
Skeletonema costatum	As(V)	EC_{50}	1 pgcell⁻¹	growth rate	Sanders, 1979b
Skeletonema costatum	As(V)	EC_{50} *	3 pgcell⁻¹	growth rate	Sanders and Vermersch, 1982
Tetraselmis contracta	As(V)	EC_{50} *	> 20 pgcell⁻¹	growth rate	Sanders and Vermersch, 1982
Thalassiosira pseudonana	As(V)	EC_{50} *	> 20 pgcell⁻¹	growth rate	Sanders and Vermersch, 1982

Note : All values based on studies without EDTA unless stated otherwise;

a - with 0.5μM phosphate; 0-20 pgcell⁻¹ = 0-100μgL⁻¹; i.e pg of added As(V)/initial cell density ; * mentioned by Sanders and Vermersch, 1982

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2.7 FACTORS AFFECTING HEAVY METAL TOXICITY

Factors that regulate availability, uptake, and effects of heavy metals are of varied nature and include chemical, physical and biological factors (Eichenberger, 1986) which interact with each other in determining the toxicity to algae. The main physico-chemical factors affecting the response of organisms to toxicants have been extensively reviewed (Gadd and Griffiths, 1978; Rai *et al.*, 1981). These include metal speciation, temperature, dissolved oxygen, pH, hardness, alkalinity, and the presence of chelating agents and other pollutants (Eichenberger, 1986). The toxicity of a metal can be influenced by the concentrations of other metals (Braek *et al.*, 1976, 1980) and algal nutrients (Morel *et al.*, 1978; Rueter and Morel, 1981). Heavy metal toxicity towards phytoplankton is also influenced by the phytoplankton species, composition of seawater, cell population, changes in the metal tolerance of the algae or in the chemical state of the metal during the period of growth, and the concentration of the metal (Davies, 1978).

2.7.1 Speciation

Trace metals are present in natural waters in different physico-chemical forms (Ruzic, 1996) or chemical 'species', upon which their bioavailability and toxicity to different aquatic organisms including phytoplankton strongly depend (Anderson and Morel, 1978; Sunda & Guillard, 1976). Chemical species of heavy metals (Kramer, 1988) result from the process of speciation, which refers to the set of all concentration values for the various chemical species present in the system (Pankow, 1991).

The speciation of metals in natural waters and in culture media is controlled by major chemical processes such as precipitation, adsorption by particulate matter, and inorganic and organic complexation. By releasing metabolites that complex metals, algae

could modify metal speciation in the medium and effectively control metal availability or toxicity (Jackson and Morgan, 1978). These processes can be affected by the metal concentration, pH, alkalinity, and concentration of metal-binding sites associated with dissolved organic material and suspended particulates (McKnight *et al.*, 1983). Detailed chemical speciation of cadmium, copper, and other metals has been elaborated by numerous authors (Kramer, 1988; Pagenkopf, 1986; Pankow, 1991; Sylva, 1976).

It has been accepted for most, if not all trace metals, that availability and toxicity to phytoplankton are determined by the metal's ionic activity. Based on the bioassays done by Davey *et al.*(1973), Jackson and Morgan (1978) confirmed that free Cu ions are toxic in natural seawater over a wide range of concentrations. Although ionic Cu has been highly correlated with toxicity (Steemann-Nielsen and Wium-Anderson, 1970; Sunda and Guillard, 1976), some Cu hydroxyl species have also been correlated with toxicity (Meador, 1991).

Until more is known about the possibility of uptake of the different species of dissolved Cu by algae, it is assumed that algae can only take up free Cu (Jackson and Morgan, 1978; Sunda and Guillard, 1976). However, the definition of free Cu is not always the same (Gerringa *et al.*, 1995). Sometimes ionic Cu is meant, whereas inorganic complexes of Cu and labile organic complexes of Cu may also be included (Brand *et al.*, 1986). Recently, Lage *et al.* (1996) mentioned that evidence have been found that the labile fraction, which includes the hydrated metal and its inorganic or organic complexes from which it can be released quickly, may be bioavailable, and thus, toxic. The presence of adsorption and complexing materials in natural waters makes the correlation between total metal and toxic metal species difficult (Pagenkopf, 1986).

Precisely which chemical species enters the phytoplankton is not known, but a lot

of evidence suggests that organically-bound metals are not taken up. Indirect evidence that organic metal complexes are not assimilated by phytoplankton has been shown by Myers *et al.* (1975) who found that the presence of humic acids decreased the amount of Hg being taken up by *Cyclotella meneghiniana* and by Cossa (1976) who showed that *Phaeodactylum tricornutum* accumulated less Cd in the presence of EDTA than in its absence.

Algae may influence arsenic speciation in natural waters (Sanders and Windom, 1980) where As and many other trace elements participate in a biological cycle of speciation changes and transformations by means of oxidative and reductive reactions within organisms. The reduced form, arsenite, As(III), is more toxic to phytoplankton than arsenate, As(V) (Planas and Lamarche, 1983).

2.7.2 pH

pH and redox potential are two important physico-chemical factors influencing mobilisation and immobilisation of heavy metals including Zn, Fe, Mn, and Cu in aquatic systems (Rai *et al.*, 1981; Byrne *et al.*, 1988). Most surface waters have been found to range from moderately acidic to moderately alkaline (pH 5.0 - 8.5) (Rai *et al.*, 1981).Valency state, speciation and bioavailability of metals such as Cu and Hg are highly governed by pH and redox potential of the medium where a metal is present (Hem, 1972). Relevant studies have indicated that heavy metals exert more toxic effects in acidic conditions and that toxicity of all the metals decreases at alkaline pH. This is due to the fact that at acidic pH metals exist in free cationic forms which are more available to algae whereas at alkaline pH they tend to precipitate as insoluble carbonates, phosphates, sulphides, oxides or hydroxides (Forstner and Prosi, 1979) thereby exerting less toxicity on the algae. Exceptional cases do exist, however, where an increase in pH

towards the alkaline range has been found to increase the toxicity of Cu and Zn to natural populations of algae, including *Chlorella* sp. and *Hormidium rivulare* (Steemann-Nielsen and Kamp-Nielsen, 1970; Hargreaves and Whitton, 1976).

The precipitation potential of various metals differ with pH. Under acidic, reducing conditions, Fe and Mn exist in relatively soluble ferrous (Fe^{2+}) and manganous (Mn^{2+}) compounds, whereas under alkaline or near neutral and oxidising conditions, formation of insoluble ferric (Fe^{3+}) and manganic (Mn^{3+}) oxides and hydroxides occur. Lowering of pH causes metal carbonates and hydroxides to dissolve, leading to an increased availability of metal cations (Rai *et al.*, 1981). Ionic Cu concentration decreases about one order of magnitude for every 0.5 increase in pH above 6 (Meador, 1991).

Biological studies on metal-polluted acid mine streams where the low pH can release metals such as Pb, Mn, Fe and Zn into solution, show evidents that in spite of metal toxicity, there is still much microbiological life such as algae, bacteria, yeasts, protozoa and fungi (Say and Whitton, 1978; Whitton and Say, 1975). The mechanism determining their increased tolerance to such extreme environments is not understood but may possibly be partly due to genetic adaptations, or the algae endemic to such environments may require acid for their metabolism.

2.7.3 Hardness

Hardness is generally caused by carbonate, bicarbonates or hydroxides of metals namely calcium and magnesium (Rai *et al.*, 1981) but its possible influence on the toxicity of heavy metals to algae has not received much attention. Supplementation of Ca and Mg were found to considerably relieve the toxicity of various heavy metals including Cu, Zn, Cd, Hg and methylmercury, where Ca salts were more effective than Mg in

mitigating metal toxicity to algae, bacteria and fungi (Rai *et al.*, 1981; Gadd and Griffiths, 1978). Reduction of toxicity is perhaps due to precipitation and co-precipitation, or formation of insoluble, complex carbonate and hydroxy compounds of heavy metals with Ca and Mg which eliminates or inactivates the metals, thus reducing their bioavailibility and toxicity. The possible inability of the complex forms to cross the biological membrane may also reduce their availibility and hence their toxicity to the phytoplankton.

2.7.4 Salinity

Salinity is generally constant over a range for a given kind of water and has been found to play an insignificant role in heavy metal toxicity to fresh water algae (Rai *et al.*, 1981). Estuaries, where intermixing of salt water and fresh water occurs, are characterised by high salt concentrations and salinity does have some role in such systems (Forstner and Prosi, 1979). Here, the decrease in heavy metal toxicity results from intermixing of rivers which contain large amounts of particulate substances with the cleaner sea water. Mandelli (1969) found a negative correlation of salinity with the log ratio of Cu uptake and phytoplanktonic (dinoflagellates and diatoms) biomass. Forstner and Prosi (1979) suggested that an increase in salinity leads to competition between adsorbed heavy metals and dissolved cations, the latter partially replacing the heavy metals thereby decreasing their bioavailability.

Reduction in toxicity to algae and other biota in saline waters, especially sea and estuaries, may be due to the alkaline pH of such waters. Eisler and Gardner (1973) demonstrated the role of salinity in decreasing heavy metal toxicity by showing that in seawater with 20 ppt salinity, precipitation as chlorides caused 17% loss of Cu^{2+} after one hour followed by 95% loss after 8 hours and 99.8% loss after 24 hours from a system

containing 30 mgL^{-1} Cu^{2+}.

2.7.5 Temperature and light

Little is known about the impact of temperature in either increasing or decreasing heavy metal toxicity to phytoplankton. The toxicity of zinc to *Nitzschia linearis* and *Cyclotella meneghiniana* increased with increasing temperature from 22°C to 30°C while contradictory effects were found with *Scenedesmus quadricauda* and *Chlamydomonas* sp. (Rai *et al.*, 1981). The mechanism governing the increased toxicity of heavy metals at high temperature may be due to enhanced respiratory activities (Forstner and Prosi, 1979). Other processes affecting toxicity are adsorption and desorption of heavy metals due to the increase or decrease in temperature (Rai *et al.*, 1981). However, decreased toxicity at high temperatures has not been satisfactorily explained.

Light has been shown to influence the inhibitory effects of heavy metals on chlorophyll metabolism and photosynthetic activities (Azeez and Banerjee, 1987, Wu and Lorenzen, 1984).

2.7.6 Seawater and culture media composition

An aspect of the culture media used to study heavy metal toxicity which is often overlooked is the high concentrations of the nutrient ions, nitrate and phosphate, which are frequently present. Interaction of nutrients with metal toxicity have been demonstrated (Say and Whitton, 1978; Say *et al.*, 1977; Zarnowski, 1972). Hannan and Patouillet (1972) have suggested that the toxicity of metals towards phytoplankton may be inversely related to the available nutrient levels. Even though it is not feasible to carry out extended growth studies at the nutrient levels normally present in seawater, the enrichments made to culture media have often been considerably in excess of those

necessary to allow the cultures to develop for the minimal period required. Keeping the nutrient level as low as possible, reduces the density of the cell populations, allowing a more realistic appraisal of the toxicity of a metal to be made.

Synthetic chelating agents and organic pH buffers such as TRIS and glycylglycine which are also metal chelators are routinely added to marine culture media. The relationships between trace metals, chelators, and phytoplankton can be complex. Jackson and Morgan (1978) advanced two major explainations for the effects of chelators on phytoplankton growth; that a chelator detoxifies the water by binding certain toxic metal ions in an unavailable form, and that chelation increases the availability of essential metal ions in forms that are biologically active. The former argument would apply to Cu ion which is toxic to algae at very low total concentrations where chelating agents such as EDTA can detoxify normally deleterious total Cu concentrations. The second would hold for iron, an essential nutrient for algal growth which is present in seawater predominantly as a colloidal precipitate, where the high affinity of EDTA for ferric ion may act to transfer iron from a solid to a soluble form that is available to the cell.

2.7.6.1 Nitrate and phosphate

Very little work has been done on the role of nitrate in antagonising heavy metal toxicity in algae. Rana and Kumar (1974) concluded that nitrate played an insignificant role in relieving Zn toxicity to *Plectonema boryanum* and *Chlorella vulgaris*. Hall *et al.* (1989) also showed that decrease in cell number of algae due to toxicity was less pronounced under N-limitation than under P-limitation. However, Li (1978) found reduction in Cd toxicity by nitrate in the marine diatom *Thalassiosira fluviatilis*.

Data from various workers on the factors influencing heavy metal toxicity

suggests that phosphorus plays a very important role in decreasing toxicity of zinc, copper, mercury, methylmercury, iron and nickel in different algae belonging to the Chlorophyceae, Cyanophyceae and Bacillariophyceae (Rai *et al.*, 1981). The role of phosphate in antagonising metal toxicity is widespread, if not universal. Increase in phosphate concentration of the ambient medium was found to decrease their toxicity, and the more toxic the metals, the more efficient was antagonisation by phosphate supplementation (Rai *et al.*, 1981).

   With the majority of heavy metals, phosphate forms an insoluble precipitate or co-precipitate, reducing their bioavailability and complete or partial detoxification. However Skaar *et al.* (1974) suggested that the addition of phosphate increased the nickel-binding capacity of the marine diatom *Phaedactylum tricornutum*, thus the bioavailability and toxicity of nickel is decreased. Toxicity amelioration by phosphate may also be attributed to its role as a nutrient (Rai *et al.*, 1981). It is also likely that in stressed conditions algae may require large amounts of various nutrients such as nitrogen, phosphorus and carbon, thus phosphorus could be acting simply as a nutrient and so facilitate better growth of algae. Meanwhile, concentration of phosphate which is a chemical analogue of arsenate may be important in determining the toxicity of As(V) to marine phytoplankton (Sanders, 1979b).

2.7.6.2 EDTA (Ethylenediaminetetraacetic acid)

   EDTA have long been used in small quantities in detergent preparations to complex trace metals which catalyse the decomposition of the added perborate (Gardiner, 1976). It is non-toxic to humans and has a wide variety of applications. Calculations by Spencer (1958) indicated that even at EDTA concentrations as low as 10 μM (Guillard and Rhyter, 1962), metals like Cu and Zn are present largely in chelated form. The data

of Sunda and Guillard (1976) similarly showed that in a culture medium containing 2mM TRIS, as used by Berland *et al.* (1976), only about 0.03% of the Cu added to give a concentration of 1000 μgL^{-1} would be present as uncomplexed cupric ion (assuming an activity coefficient of 0.2).

Zhang *et al.* (1992) showed that EDTA decreased the toxicity of Cu to algae in artificial seawater and natural seawater, and that EDTA in excess of that needed to complex metal ions also stimulated growth, but excess EDTA concentrations > 1000 μM was harmful. Spencer (1957) found that even in the presence of Cu concentration two times higher than normal lethal concentration, Cu^{2+} was not toxic to *Phaedactylum tricornutum* if EDTA was added to the seawater. The fact that toxicity of Cu is moderated by the presence of chelating agents can be explained as being due to the lower cellular burdens of the metal accumulated by the phytoplankton in the presence of the chelators (Davey *et al*, 1973; Steemann-Nielsen and Wium-Anderson, 1970).

2.7.7 Complex formation

Complexation of trace metals has long been implicated as the dominant control on their biovailability and toxicity to phytoplankton (Anderson and Morel, 1978; Jackson and Morgan, 1978). Existing theories of trace metal complexation in aquatic systems has been presented in a critical review by Ruzic (1996). In most aquatic systems inorganic and organic complexation, adsorption, and precipitation are capable of reducing free Cu levels to very low values even in the presence of high levels of total Cu (Sylva, 1976). Zirino and Yamamoto (1972) showed that Cu, Cd, Zn and Pb are complexed to a certain extent in seawater. Coale and Bruland (1988) found that Cu complexation gave rise to extremely low cupric ion activities in surface waters ($\{Cu^{2+}\}$ =

1.4 X 10^{-14} M) to higher values at middepth ($\{Cu^{2+}\} = 10^{-11}$M).

2.7.7.1 Binding to environmental constituents

One of the most important factors which determines the biological availability of

a metal in a system is its binding to other environmental constituents (Gadd and Griffiths,

1978). Strickland (1972) stated that although the addition of chelating agents to seawater

often improved the growth of phytoplankton, there was little evidence that the function

of dissolved organic matter in the ocean was to complex metals so as to increase or

decrease their bioavailability to phytoplankton. He argued that even if all dissolved

organic carbon were present as a compound of strong complexing ability it would not be

able to compete for most metals with inorganic ligands such as chloride, sulphate and

hydroxide. However, much data indicate that metals in natural waters may exist in

chelated forms with dissolved organic matter (Davey *et al.*, 1973).

Natural waters are frequently contaminated with dissolved organic matter and

thus harbour various metal ligands such as $-SO_4$, $-PO_4$, $-CO_3$, $-OH$, NTA

(nitrilotriacetate), NOC (nocardamine), SAL (salicylate), CIT (citrate), GLY (glycine),

GLU (glutamate), organic substances such as glycollic acid, humic acid, amino acids,

saccharides, and also various functional groups ($-COOH$, $-SH$, $-OH$, $-NH_2$) (Rai *et al.*,

1981). The mechanism by which these substances antagonise heavy metal toxicity seems

to be based on the formation of complexed heavy metal compounds.

In aquatic habitats, metals such as Zn and Cu can be bound and removed from the

water by organic sediments, which effectively reduces the total metal ion concentration

in solution. Certain oxidised sediments can bind up to 96% of added Zn (Whitton and

Say, 1975). Because of such binding in aquatic systems, it has been shown that toxic

effects of certain metals on microbes can be decreased (Gadd and Griffiths, 1978). Batley

and Gardner (1978) noted that 40-60% of total Cu and 15-35% total Cd was associated with colloidal matter. Although binding to environmental constituents usually reduces toxicity, certain metal complexes such as methylated derivatives of Hg are more toxic than the free metal, but they are often volatile and may disappear from an environment (Ramamoorthy and Kushner, 1975).

2.7.7.2 Humic acids (HAs)

Humic acids are known to play a significant role in phytoplankton productivity by regulating the trace metals required for plant growth (Prakash and Rashid, 1968). Aquatic fulvic acid, the acid soluble fraction of humic substances, is the major fraction of the dissolved organic material in natural waters (McKnight *et al.*, 1983). Mantoura *et al.* (1978) found that in seawater > 99% of the humic material were complexed by Ca and Mg due to their relatively high concentrations, and metal chelation was only appreciable for Cu (approx. 10%). The toxicity of Cu, Cd and Pb, on a blue-green algae, *Synechosystis aquatilis*, was significantly reduced in the presence of 0.01 mgL^{-1} HA (Shanmukhappa and Neelakantan, 1990) and in controls, the addition of HA caused a 10% increase of chlorophyll under light but not under dark, in agreement with Prakash and Rashid (1968) who found that HA enhanced chlorophyll synthesis (growth) of marine algae and diatoms.

2.7.7.3 Algal extracellular products and exudates

Much of the complexing ability of marine waters that occur naturally is believed to come from organic matter excreted by phytoplankton and zooplankton (Jackson and Morgan, 1978). Seritti *et al.* (1986) suggested that the contribution of phytoplankton to dissolved organic matter (DOM) in the sea is not neglible and that their exudates form

electrochemically non-labile copper complexes with a high affinity. Fogg and Westlake (1955) observed reduction in Cu toxicity to *Anabaena cylindrica* due to complexation with extracellular polypeptides. Steemann-Nielsen and Wium-Anderson (1971) had also indicated the possibility of complexation of metals with algal extracellular products.

Amino acids are among the few marine organic compounds which are known, where histidine has shown a substantial ability to bind Cu (Jackson and Morgan, 1978), but concentrations of dissolved free amino acids are too small to account for Cu complexation properties of seawater indicating that other classes of substances must be involved in chelating Cu in seawater. By releasing metabolites that complex metals, algae could, in principle, modify metal speciation and effectively control metal availability or toxicity in the medium (McKnight and Morel, 1979). Hydroxamic acids may be the only examples of strong metal-complexing agents that are known to be released by algae and to be present in natural waters (McKnight and Morel, 1979). It has been suggested that blue-green algae produce hydroxamate siderophores which are strong Cu-complexing agents (McKnight & Morel, 1980) under conditions of iron limitation (Simpson and Nielands, 1976; Murphy *et al.*, 1976; McKnight and Morel, 1979, 1980).

Cultures of *Anabaena cylindrica, Anacystis nidulans, Lyngbya* sp., *Microcystis aeruginosa, Nostoc muscorum,* and *Phormidium foveolarum* produced their own chelators and grew as well as the controls with artificial chelating agents (Lange, 1974) while in the absence of artificial chelating agents, non-chelator-forming species grew in the filtrates of the chelator-forming algae the same as in the presence of artificial agents. Among 21 microalgal species, McKnight and Morel (1979) found that most of the eucaryotic algae in the stationary phase contain micromolar concentrations of extracellular weak organic acids which complex Cu while blue-green algae produce

similar weak organic acids and also strong Cu complexing agents during the later growth of batch culture. Exudates produced by *Anabaena cylindrica*, *Navicula pelliculosa* and *Scenedesmus quadricauda* (6.73, 2.86 and 0.66 µgL[-1] respectively) were able to ameliorate Cu toxicity towards the primary production of *Chlorella vulgaris* where *Anabaena* filtrates had the most efficient complexing capacity (Van den Berg *et al.*, 1979). Thus, algal extracellular products might produce the amelioration effect on metal toxicity in natural aquatic environments. Although waters harbouring sparse populations are unaffected, dense algal blooms can significantly lower metal toxicities to algae (Rai *et al.*, 1981).

2.7.7.4 Sulphur compounds and amino acids

Lipids and polysaccharides which are important constituents of cell wall and membranes of various algae seem to be the prefered sites of heavy-metal interaction (Rai *et al.*, 1981). They may contain many ligands which not only maintain the integrity of a membrane by acting as a diffusion barrier, but also provide suitable sites for binding of metal ions. Therefore, metals may interact by binding onto cell walls and membranes with reactive groups including hydroxyl, phosphoryl, amino, carboxyl, sulphydryl and thiol groups, interfering with their activities and disrupting physiological processes. The role of sulphur-containing compounds and amino acids in mitigating heavy metal toxicity are known. The destruction and breakdown of the pemeability barrier of cells may depend upon the interaction of heavy metal ions with sulphydryl groups where the stability of a membrane depends upon its affinity for metal ions (De Filippis, 1979a). Amelioration of toxicity by sulphur compounds and sulphur-containing amino acids as observed by De Filippis (1979b) may be due to binding of mercury with sulphydryl compounds. The larger the number of sulphydryl groups available in the system, the

greater the Hg binding, eventually leading to amelioration of its toxicity.

2.7.8 Phytoplankton species

By using the same culturing conditions and medium in studying the effects of a metal upon a range of phytoplankton species, it is possible to place the species in order of their metal sensitivity or tolerance (Davies, 1978). Based on the work done by Berland *et al*. (1976), Davies (1974), Erickson *et al*. (1970), Kayser (1976), Mandelli (1969), and Overnell (1976) on Hg and Cu with marine phytoplankton, Davies (1978) concluded that green algae are generally more Hg tolerant than brown algae and diatoms, and that the situation, although less clear cut, is largely reversed with Cu. Saward *et al*. (1975) who provided a historical review, including references on the different sensitivities to Cu among various taxonomic groups of algae also concluded that diatoms seem most sensitive, green algae least sensitive, with dinoflagelates and blue-green algae intermediate. Maloney and Palmer (1956) provided evidence for species differences in Cu sensitivity among freshwater phytoplankton. Apparent differences in sensitivity between freshwater and marine algae can be pronounced (Overnell, 1975a,b).

2.7.9 Population density

Like most aquatic organisms, algae too influence metal toxicity to themselves but this influence is not merely dependent upon the large mass of algae but the cellular activity which is a critical factor determining the impact (Rai *et al*., 1981). Williams (1960) found that the bigger cells or denser populations of *Euglena* and *Chlorella* were able to absorb greater amounts of ^{137}Cs than actively dividing low populations. Morris (1971a) observed that the Mn concentration in seawater was decreased to about half its initial value by thick blooms of the marine flagellate *Phaeocystis*. Murphy *et al*. (1976)

suggested that blooming blue-green algae excreted iron-selective chelators which chelated a large amount of Fe from the water, thus decreasing its bioavailability and toxicity. Delcourt and Mestre (1978) observed that the increase in population of *Chlamydomonas variabilis*, was followed by a decrease in toxicity of phenylmercuric acetate and reasoned that the toxicity of phenylmercuric acetate was due to binding of the metal to a definite number of cellular sites available, thus leading to a decreased toxicity.

It has not been uncommon for extremely high cell densities to be used and this can give rise to an underestimate of the toxicity of a metal when determining the concentration which causes growth inhibition in culture (Davies, 1978). This is due to the fact that it is the quantity of metal taken up by the cells which ultimately determines its effect upon their growth and, in dense cultures, more metal is required to produce a given cellular burden.

2.7.10 Ionic Interactions

The biological activity of heavy metal ions can be markedly affected by the presence of other ions. Toxicity of ionic Cu is ameliorated by trivalent metal ions or divalent metals that can be oxidised by algae in the growth medium, including those of Mn, Co, Al, Fe and Cr which form a layer of metal (III) hydroxide around the algal cells, adsorb Cu and reduce its penetration into the cells (Stauber and Florence, 1987). In addition, Mn and Co catalytically scavenge damaging H_2O_2 and superoxide radicals, respectively, produced by the cell. This reaction was also observed by Stauber and Florence (1985a, 1985b) between Cu, Mn and Fe, with *Nitzschia closterium*. The addition of anions such as phosphate, thiosulphate, carbonate, and bicarbonate which can form precipitates with heavy metals depending on their concentration and pH, to growth media, often reduces metal toxicity (Gadd and Griffiths, 1978).

The toxicity of a metal may also increase in the presence of other ions. An asymmetric respiratory response was observed in *Chlorella vulgaris* when fluoride and Cu ions were applied jointly; respiration was completely inhibited by the mixture, but individually the ions had little effect (Hassall, 1967). Synergism and antagonism between heavy metals have been widely studied (Eichenberger, 1986). Pronounced effect of mixtures of heavy metals on algae has been observed with Cu and Ag ions (Young and Lisk, 1972) but this can be accounted for by simple additive effects (Whitton and Say, 1975).

2.7.11 Other pollutants

The presence of 2,4-D in a system inhibited the toxicity of Ni and Al to *Dunaliella tertiolecta* (Walsh, 1978). Rai *et al.* (1981) mentioned contradictory finding which suggested that the presence of Cu ions decreased the toxicity of the insecticide Paraquat (1,1-dimethyl-4,4'bipyridium) to *Chlorella pyrenoidosa*. De Filippis (1979b) found mercuric chloride and phenyl mercuric acetate was less toxic to *Chlorella* sp. after supplementation with sodium selenite. The alleviating effect was noticed, however, only after prior exposure of the algal cells to the selenite. On the other hand, prior exposure to sodium selenite did not relieve toxicity to Zn in the same alga. Detoxification of Hg probably resulted from it binding to selenium compounds.

2.7.12 Metal concentration

Davies (1974) and Sunda and Guillard (1976) demonstrated that growth rate is related to the heavy metal content of the phytoplankton cells with Hg and *Isochrysis galbana* and with Cu and *Thalassiosira pseudonana* respectively. Meanwhile, Kayser and Sperling (1980) found that the uptake of Cd was highly dependent on its concentration in

the water; this typical of weak ion-exchange behaviour where saturation depends strongly on the concentration of the metal available.

2.7.13 Changes in algal tolerance and chemical state of metal

Stockner and Antia (1976) have suggested that short term investigations lasting only a few hours or days of the effects of pollutants upon marine phytoplankton take insufficient account of the possibility that the cell populations being studied might, given the time, adapt to the hostile conditions created by the presence of the substance in the culture. While the existence of higher plants with acquired metal tolerance is well documented (Antonovics *et al.*, 1971), marine phytoplankton may be trained to withstand levels of metals which would normally be lethal (Davies, 1978). The recovery of growth in polluted cultures may not always be of biological origin, as the detoxification of the contaminant due to its transformation chemically can also produce a similar result (Stockner and Antia, 1976).

2.8 MODE OF HEAVY METAL TOXICITY TO PHYTOPLANKTON

2.8.1 Cadmium

Although cadmium is known to be a potent toxicant to algae, little information is available on its specific mode of action (Okamoto *et al.*, 1996). Cd may either express toxicity effects indirectly, by adhering to cell wall sites and preventing the transport of nutrients, or directly by becoming localised intracellularly and replacing essential divalent metal ions in enzymes (Rebhun and Ben-Amotz, 1984). Cd must first be sorbed onto cell surfaces before being translocated to various cellular target sites and eliciting toxicity (Trevors *et al.*, 1986).

The most important mechanism of Cd toxic action in microalgae and cyanobacteria operates through damage to specific enzymes (Fernandez-Leborans and Novillo, 1996). Due to a great affinity with sulphydryl groups, Cd can inactivate many important enzymes resulting in inhibition of photosynthesis, respiratory rate, growth and other cell processes (Hart and Scaife, 1977; Gipps and Coller, 1982).

Harrison and Morel (1983) considered competitive interactions between essential toxic metals to be the most probable and the most general mode of metal toxicity at low free metal concentrations that predominate in nature. Hart *et al.* (1979) who observed competitive inhibition between Cd and Mn in *Chlorella pyrenoidosa* concluded that Cd^{2+} and Mn^{2+} share a common transport system in *C. pyrenoidosa* and that Cd^{2+} toxicity may be related to this, in that it could lead to a cellular deficiency in Mn^{2+} which would seriously inhibit photosystem II in which Mn participates. Based on their observation with *Thalassiosira weissflogii*, Harrison and Morel (1983) concluded that the mechanism of Cd toxicity is based on the inhibition of Fe transport and utilisation resulting in an effective Fe deficiency although it does not mean that under other conditions, Cd toxicity may not be mediated through other mechanisms. Cd also shows a similar behaviour to that of Zn, which has a known biological function and is displaced by Cd (Fernandez-Leborans and Novillo, 1996).

Changes in size of unicellular algae subject to heavy metal stress can be caused by uncoupling of growth and cell division (Davies, 1976). However, Li (1978) found that growth did not uncouple from division although there was a change in size of *T. fluviatis*. The change in cell size and morphology may also possibly be due to pathological processes (Bentley-Mowat and Reid, 1977; Erickson, 1972) or alterations in cellular chemical composition (De Filippis and Pallaghy, 1976a; Berland *et al.*, 1977; Rivkin,

1979). The observation by Fernandez-Pinas *et al.* (1997) on the increase in cellular content of Ca, Mg, Fe, Zn, Mn and K in *Nostoc* sp. after a short exposure to Cd, indicated that the cell membrane is probably the primary site of toxicity of cadmium. The direct binding of Cd to specific sites in the membrane by changing the membrane structure may modulate the permeability of the membrane to specific ions, resulting in an unbalanced flux of vital ions into the cells.

The induction of superoxide dismutase (SOD) found in cells chronically exposed to cadmium (Okamoto *et al.*,1996) may be related to the cellular mechanism of antioxidant defense, in particular against O_2^-, thus suggesting Cd produces a cellular oxidative stress state. Toxicological studies have suggested that Cd could affect the antioxidant protection system of different tissues of some algae (Reed and Gadd, 1990). In the presence of Cd, there is an increase in cell production of reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydroxyl radicals (OH.), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$), which are known to cause deleterious effects. To combat high levels of ROS, cell are able to induce the activity of SODs. Generally, in prokaryotes, algae and other protisits the Mn-SOD and Fe-SOD predominates. In algae, observations have been made on the increase in SOD levels in response to ROS (reactive oxygen species) production by different substances and environmental factors such as sulfur dioxide, paraquat and Cu exposure, high oxygen pressure, changes in temperture, salinity, and UV radiation (Rijstenbil *et al.*, 1994).

2.8.2 Copper

Although the mechanisms of growth inhibition are not well understood, important metabolic processes like photosynthesis and respiration have been shown to be affected and thus likely to be involved (De Filippis *et al.*, 1981b). Steemann-Nielsen *et al.* (1969)

suggested that Cu ions are adsorbed onto the cytoplasmic membrane of algal cells, thereby preventing their division. They found giant cells of *Chlorella pyrenoidosa* which they interpreted as cells with arrested division due to the adsorption of Cu onto the cytoplasmic membrane. According to Steemann-Nielsen *et al.* (1969) the major part of Cu ions was bound to the cell wall and slime envelopes of *C. pyrenoidosa*. Their findings were supported by the observations of Saifullah (1978) who reported the formation and removal of slime envelopes from the body of the protoplast of *Scripsiella faeroense*, probably as an adaptive mechanism of this species to overcome the stress caused by Cu.

Another mode of action of Cu is direct where it penetrates into the cell and affects its metabolism (McBrien and Hassall, 1967; Steemann-Nielsen *et al.*, 1969). In the study by Saifullah (1978), the protoplasts of the cells all contracted and shrank to the centre, most probably due to the excretion of dissolved organic matter by the cells in response to the penetration of Cu. Steeman-Nielsen and Wium-Anderson (1971) also observed a simultaneous loss of organic matter by *Nitzschia palea* in response to uptake of Cu.

Fisher and Jones (1981) found that the relative toxicities of metals including Cu, Cd and Mn, correlated with the solubility products of the corresponding metal sulphides, and suggested a common action for these heavy metals with toxicity resulting from metal binding to sulphydryl-containing compounds in the cell. Based on their observations on the decoupling of cell division from other metabolic processes due to Cu, and extrapolation to work by others (Davies, 1976; De Filippis and Pallaghy, 1976a,b; Goering *et al.*, 1977; Harrison *et al.*, 1977; Lewin, 1954; Morel *et al.*, 1978) on Cu and other metals, Fisher *et al.* (1981) speculated that Cu, or other toxic heavy metals, may bind to sulphydryl (-SH) groups of enzymes, inactivating a biochemical process such as

the GSH/GSSG cycle which controls the rate of mitosis and also interfere with a number of associated metabolic pathways, one or more of which are essential for maintaining normal cell division rates. They also suggested that toxic metals may bind to groups on cell membranes, impairing normal membrane function and reducing silicic acid uptake and amino acid synthesis, thereby resulting in depressed cell division rates.

Thomas *et al.* (1980) observed similar morphological effects of Cu, Zn and Ge in the diatom *Thalassiosira aestevalis* and suggested that at least one biochemical pathway of Si metabolism was affected by these metals. Germanic acid, an analogue of silicic acid, is thought to act as a competitive inhibitor of Si metabolism. Although the effect of Cu did not appear to be competitive with respect to silicate, the similarity between the effects of Cu and Ge on morphology suggests that Cu may also act as an inhibitor of Si metabolism. As it is reasonable to assume that there is a physiological basis for the morphology of diatoms, a site where Ge, heavy metals and silicate limitation could have a common effect on cell morphology would be an enzyme, which either has silicate as a substrate or allosteric effector and that is sensitive to heavy metals.

Meanwhile, Rueter *et al.* (1981) disproved the possibility of the indirect inhibition of nutrient uptake as a mechanism of growth inhibition by Cu. They showed that the long-term inhibition of growth rate in *T. pseudonana* by Cu was not a result of Si deficiency caused by the inhibition of $Si(OH)_4$ uptake. They observed a high quota of Si in the copper-inhibited cells and presence of aberrant frustules which suggested that Cu may inhibit growth by interfering with Si metabolism but argued that the causality was difficult to establish as several unrelated effects of Cu could be taking place simultaneously. Rueter *et al.* (1981) observed a contrast in the time course of $Si(OH)_4$ uptake inhibition (which is very rapid) and that of growth rate inhibition (which develops

over days) and suggested that inhibition of $Si(OH)_4$ uptake is a surface process and that intracellular Cu is the causative agent of the decrease in growth rate through the inhibition of intracellular functions.

Rueter *et al*. (1981) also added that the effect of Cu on the size and shape of the cells which appear to be a common response among diatoms (Morel *et al*., 1978; Thomas *et al*., 1980) suggests a particularly simple mechanism of toxicity by cellular Cu. Cu may be deposited in the frustule resulting in gross deformation and, ultimately in an inability of the cell to divide.

Evidence for the mechanism of toxicity of ionic Cu has also been indirect (Stauber and Florence, 1987). Gavis (1983) proposed that initial Cu-binding to the cell may be to membrane protein carboxylic and amino residues, rather than to thiols groups because the algae-Cu stability constant is orders of magnitude lower than the thiol-Cu binding constant. At the cell membrane, Cu may interfere with cell permeability or the binding of essential metals (Sunda and Huntsman, 1983). Following Cu transport into the cytosol, Cu may react with -SH enzyme groups and free thiols (eg. glutathione), disrupting enzyme-active sites and leading to a lowering of the GSH:GSSG ratio and suppression of mitosis and cell division (Stauber and Florence, 1985b; Florence and Stauber, 1986). Cu may also exert its toxicity in subcellular organelles, interfering with mitochondrial electron transport, respiration and photosynthesis in the chloroplast (Overnell, 1975a,b). Stauber and Florence (1987) then concluded that effect of Cu ions on cell division, photosynthesis, respiration, ATP production, elextron transport and cellular ultrastructure of *Nitzschia closterium* suggests that the main toxic effect of Cu is to act within the cytosol, possibly by lowering the intracellular thiol concentration, thus inhibiting cell division but not affecting the other cellular functions. Contributory toxicity

may result from the ability of Cu to inhibit the enzymes catalase and gluthathione reductase. They concluded that a different mechanism of toxicity was operative in *Asterionella gracilis* and *Chlorella pyrenoidosa* as in these algae cell division was not uncoupled from photosynthesis when exposed to Cu.

2.8.3 Manganese

Observations of abnormalities after exposure of *Chroococcus limneticus* to manganese indicated interference with cell division, probably at level of genome replication and distribution (Singh and Kashyap, 1978). However the absence of significant effect on cell size of *Plectonema boryanum* after being exposed to Mn indicated that the inhibition of genome replication is not the most important factor for inhibiting cell division in this alga. It was suggested that Mn exerts its mutagenic action through decreasing fidelity of DNA polymerases.

2.8.4 Arsenic

Arsenite (As(III)) and arsenate (As(V)) inhibit cellular functions at low levels but in different ways. As(III) probably reacts with the -SH groups of proteins (Lewin, 1954). As(V) competes with phosphate, a chemical analogue, for transport into the algal cell (Blum, 1966) where in *Euglena*, the affinity of the phosphate transport system for As(V) is close to that for phosphate. It inactivates the phosphate active transport system, and may inhibit glucose metabolism (Rothstein, 1963). It may also compete with phosphate in oxidative phosphorylation and esterification reactions (Da Costa, 1972). Since As(V) competes with phosphate, the external phosphate concentration may be important in determining the toxicity of As(V) and inhibition of cellular growth may be greatest when phosphate concentrations are lowest (Sanders, 1979b). Wangberg (1995) has attributed

the mechanism of toxicity of As to microalgae to competition with phosphate on the chloroplastic ATP-synthetase.

2.9 MICROALGAL TOLERANCE TO HEAVY METALS

The toxic effects of heavy metals on algae differ markedly with algal species, chemical species of the element and aquatic conditions. An algae may be very sensitive to one chemical species of an element, but have high resistance to another of the same element, or to other toxic metals (Maeda and Sakaguchi, 1990).

The ability of unicellular algae to adapt to high concentrations of heavy metals has been widely reported. Antonovic *et al.* (1971) mentioned that vigorous growth of filamentous green algae and crusts of blue-green algae, diatoms and green algae were readily observable on Zn and Cu mine soils, and pools heavily contaminated with metals, where some of them able to withstand metal concentrations which were toxic even to the most tolerant higher plants.

In marine ecosystems, diatoms appear to be the most sensitive organisms (Bently-Mowatt and Reid, 1977; Berland *et al.*, 1976; Overnell, 1976; Thomas and Seibert, 1977). Resistance does not depend solely on size since Mandelli (1969) has shown that small dinoflagellates are more sensitive than diatoms. Bentley-Mowat and Reid (1977) showed that nanoplankton species were the most resistant to Cu while *Cricosphaera* was slightly more sensitive and *Ditylum*, a large diatom, was more sensitive still. It has been observed that Cu ions inhibit the uptake of silicic acid (Goering *et al.*, 1977), perhaps explaining their relatively low tolerance to the metal. The greater sensitivity of the dinoflagellates and the strong resistance of the green algae, were also reported by Mandelli (1969) and Hawkins and Griffiths (1982).

Nakajima *et al.* (1979) reported that Mn in the range of 1.8-202 mgL^{-1} had no effect on growth of *Chlorella regularis*. Conway (1978) found no detrimental effects on growth or micronutrient utilization of freshwater diatom, *Asterionella formosa* exposed to a range of As concentrations <160 µgL^{-1}. Bottino *et al.* (1978) reported that *T. chuii* could survive concentrations of As(V) as high as 1000 mgL^{-1} when added without adaptation to the medium containing the metalloid while *Hymenomonas carterae* was able to grow in both situations though much slower.

Some physiological and morphological cases of increased tolerance may also be of importance; Say *et al.* (1977) observed geniculation in *Hormidium* sp. but Stokes *et al.* (1973a) who noticed that the isolates of *Chlorella* and *Scenedesmus* from metal-enriched lakes took up more Cu from the medium compared with their respective laboratories strains, could not find any change in growth characteristics.

2.9.1 Mechanisms of tolerance

The biochemical basis of heavy metal tolerance is generally, poorly understood, but emerging. It is complicated by the different resistance mechanisms (Wood and Wang, 1983). The mechanisms of heavy metal resistance appears to depend on both the algal species and metal involved (Bariaud *et al.*, 1985). The main mechanisms suggested to account for tolerance include internal detoxifying mechanisms (Stokes *et al.*, 1973b) and exclusion mechanisms (Eichenberger, 1986).

2.9.1.1 Surface binding of metal ions

Non-specific binding of the metal to cell surfaces, slime layers and extracellular matrices can be important since most heavy metals can be adsorbed onto the cell surface of both living and dead algae (Gadd and Griffiths, 1978). Such is the essentially

irreversible binding of Cu^{2+} ions to extracellular polysaccharides, mainly uronic acid polymers. Wood and Wang (1983) found that green algae and cyanobacteria could concentrate Ni primarily at the cell surfaces to 3000 times over the concentration in the culture medium.

2.9.1.2 Internal detoxification

Metabolism-dependent intracellular uptake removes significant amounts of metals than surface binding (Gadd and Griffiths, 1978). At higher concentrations, intracellular precipitation of a metal may occur after uptake. This itself can be a means of detoxicification since the metal is compartmentalised and may be converted to another more innocuous form. Electron microscopical examination of *Cyanidium caldarium*, which has the ability to grow in acidic conditions and high temperature, revealed microcrystals of metal sulfides (Cu, Ni and Cr) adhering to the external cell membrane, suggesting that *C. caldarium* possesses a membrane-associated sulphate reductase system, and sulfide precipitation of metals may be a cellular detoxification mechanism (Wood and Wang,1983). Cupric sulphide, an insoluble product renders the cupric ions innocuous, thereby protecting the cell cytoplasm from heavy metal poisoning.

Silverberg *et al.* (1976) found accumulation of Cu as an insoluble intracellular complex in Cu-tolerant *Scenedesmus* B4 which probably resulted through attachment of Cu to protein ligands. Tolerance of *Chlorella pyrenoidosa* to high intracellular Cd levels have been suggested to be due to its ability to synthesise a Cd-binding metallothionine protein (Hart and Betram, 1980).

Amphora and *Navicula* species colonising cuprous oxide paints and exposed to increasing concentrations of $CuCl_2$ revealed that Cu had entered the cells and was located in either polyphosphate or "copper" bodies (Daniel and Chamberlain, 1981). The

spherical polyphosphate bodies, normally located within the cell vacuoles presumably offered a convenient site for the indiscriminant binding of Cu which may not be a metabolic reaction to remove high concentrations from the cytoplasm. The Cu bodies, irregular in outline and usually assosiated with membranes, were developed in a relatively short period of time, indicating an important mechanism for the removal of high concentrations from the cytoplasm. These bodies are important structures in maintaining low concentrations of free Cu within the cell where such traps have also been described for Cd, Ca and Ni (Wood and Wang, 1983). Apart from intracellular immobilisation, these diatoms are likely to be protected by large quantities of the extracellular mucilages in the film. The accumulation of heavy metals in the nuclei may also decrease metal content in the cytoplasm, masking the toxic effects of heavy metals (Gadd and Griffiths, 1978).

2.9.1.3 Exclusion mechanisms

In copper- and zinc-tolerant strains of *Chlorella vulgaris*, the existence of exclusion mechanisms have been demonstrated (De Filippis and Pallaghy, 1976a,b; Foster, 1977). Reduction in number of Zn-binding sites and inhibition of a temperature sensitive component of Zn uptake after the gradual development of tolerance in *Chlorella* (De Filippis and Pallaghy, 1976c) suggests the physiological development of an exclusion mechanism.

Hall *et al.* (1979) found that tolerant strains of the ship-fouling alga *Ectocarpus siliculosus*, grown on metal plates coated with a copper-based paint accumulated less Cu compared to intolerant strains, indicating exclusion as a means for tolerating high Cu concentrations. They, however, observed some insignificant difference between the metal uptake and content of tolerant and intolerant strains, and concluded that in addition

to the exclusion mechanism operating, probably as a result of some changes in cell membrane, an internal detoxifying mechanism may also be involved.

Wood and Wang (1983) mentioned the development of energy-driven efflux pumps which keep the levels of toxic elements such as Cd^{2+} and As^{5+} low in the interior of the cell. Meanwhile, Bariaud *et al.* (1985) concluded that Cd^{2+} resistance of *E. gracilis* appeared to be a definitively acquired character, associated with a lower accumulation of Cd^{2+} and decreased affinity for Cd^{2+}, resulting from a membrane transport mechanism; inhibition of the membrane Cd^{2+} transporter or increase of the Cd^{2+} exclusion mechanism. Even though other studies do suggest the existence of such a mechanism (Foster, 1977), Silverberg *et al.* (1976) and Stokes *et al.* (1973b) did not consider 'exclusion' as the mechanism for the high metal tolerance of organisms from metal-contaminated waters.

Exclusion mechanisms also include production and release of organic material which chelates the metal thus rendering it non-toxic (Visviki and Rachlin, 1991). Some microalgae are capable of producing such substances which may reduce toxicity (Gadd and Griffiths, 1978), a mechanism determining the resistance of *Chlorella* to Cu (Butler *et al.*, 1980). Diatoms subjected to high concentrations of heavy metals have also been shown to release large quantities of organics which interact with metal ions (Steemann-Nielsen and Kamp-Nielsen, 1970). Mandelli (1969) noted that the uptake of Cu was accompanied by excretion of organic matter by algal cells which, according to him and to Steeman-Nielsen and Wium-Anderson (1971), chelates ionic Cu. Thus, excretion of organic matter by algal cells is another adaptation of algae to overcome the stress of Cu. However, Hall *et al.* (1979) discounted the possibility of any role of algal extracellular products in decreasing metal toxicity as they observed that the tolerant strain did not always produce greater amounts of extracellular products and, moreover, the

extracellular products of the tolerant strain did not confer tolerance upon the intolerant strains.

When exposed to higher sublethal Cu levels, some algae may lose their thecal plates and probably, as a consequence of the initial Cu impact, some cells die and release substances and organelles into the culture medium which could bind the metal and therefore contribute to the decrease in the labile Cu fraction (Lage *et al.*, 1994).

Cd induces the production of high levels of the metal-binding polipeptide, phytochelatin. Lee *et al.* (1996) showed the efflux of Cd and phytochelatin from the cells of *Thalassiosira weissflogii* at high inorganic Cd concentrations, where over half of the Cd taken up was returned to the medium. They then hypothesised that the alga exports the Cd-phytochelatin as a detoxification mechanism. Pistocchi *et al.* (1997) who observed the production of carbohydrate in *Cylindrotheca fusiformis* and *Gymnodium* sp. when exposed to Cu, indicated carbohydrate extrusion as one of the mechanisms against Cu toxicity.

2.9.1.4 Metal transformation

Metals cannot be broken down into other products but may, as a result of a biological action undergo changes in valency and conversion into organometallic compounds. Both processes can be considered to be detoxification mechanisms since volatisation and removal of the metal may result. Biomethylation is a widely occuring phenomenon, thus metal resistance resulting from this ability will also be common (Rai *et al.*, 1981). It has been found in response to Hg, Pb, Cd, and Sn (De Filippis and Pallaghy, 1976a, b). The role of biomethylation is also considered generally to be a mechanism of detoxification of inorganic As (Edmonds and Francesconi, 1977; Andreae, 1978; Wangberg, 1995). Although products of methylation may be more toxic than free

metal, they are often volatile and can be released into the atmosphere. Biomethylation is followed by transport through cell membranes by diffusion-controlled processes. Oxidation (e.g. AsO_2^{-3} to AsO_3^{-4}) or reduction (Hg^{2+} to Hg^0) can enzymatically and intracellularly convert a more toxic form of an element to a less toxic form (Wood and Wang, 1983).

2.9.1.5 Genetically-determined metal resistance

Say *et al.* (1977) in their study on *Hormidium* sp. failed to increase its Zn tolerance even after repeated subculturing and they inferred that Zn tolerance in their alga had a genetic basis. Some researcher have indicated that Cd resistance in *Euglena gracilis* is due to its ability to control Cd^{2+} uptake, possibly via the involvement of plasmids (Bariaud and Mestre, 1984). Further research is required in all aspects of heavy metal transport in phototrophic microorganisms before specific biochemical and genetic mechanisms can be elucidated (Trevors *et al.*, 1986).

2.9.1.6 Physiological and genetic adaptation

Fisher (1977) who observed that the physiological responses of oceanic phytoplankton differed greatly from those of estuarine algae with each clone reflecting the characteristics of its isolation site, suggested that the physiological adaptations of estuarine organisms to a particular set of pressures may enable them to tolerate a multitude of other stresses. The data indicate that phytoplankton which have evolved in and adapted to physically variable environments would, because of their adaptations, be better able to tolerate any toxic compound than would morphologially similar organisms adapted to stable environments. It was further suggested that the degree of phytoplankton sensitivity to pollutants may be correlated with certain adaptations of their membrane

systems; open-ocean cells, whose membrane system permit nutrient exploitation and maximal growth at low ambient concentrations, have lower resistance to perturbation than estuarine algae, whose membranes are capable of tolerating rapidly fluctuating environmental conditions. Harrison *et al*. (1977) and Azam *et al*. (1977) found that phytoplankton assemblages and bacterioplankton dominant in Cu-treated enclosures were more resistant to Cu than assemblages that developed in control enclosures.

Except for the studies by Jensen *et al*. (1974) and Murphy and Belastock (1980), little evidence exist on the ability of marine phytoplankton to evolve enhanced tolerance of chemical toxicants (Fisher, 1981). Murphy and Belastock (1980) argued that most experiments conducted do not distinguish the development of physiological adaptation state from genetic adaptation by selection of resistant genotypes and proposed that clones compared under identical conditions can delineate genetically based differences. They found similar results with Jensen *et al*. (1974) who compared two necritic clones of *Skeletonema costatum* and showed that the clone isolated from a Zn-polluted fjord was several magnitude less sensitive to Zn than was another clone of the species isolated from a nearby but polluted fjord, the two fjords were otherwise similar, thus the resistance appeared to be a genetic adaptation to a specific stress. Therefore, short term pollution history of the original environment is at least as important in determining sensitivity of the clone as is the long term stability and predictability of that environment.

2.10 UPTAKE AND BIOACCUMULATION OF HEAVY METALS IN PHYTOPLANKTON

2.10.1 Introduction

Bioaccumulation and biomagnification are terms frequently used to describe biological uptake of a compound from the environment. While bioaccumulation is used to indicate how extensively an organism accumulates a compound from its surrounding environment by all processes including absorption, adsorption and ingestion, biomagnification indicates that a compound is concentrated through the consumption of lower by higher food chain organisms with a net increase in tissue concentration (Isensee *et al.*, 1973). It has been emphasised that bioaccumulation is not reflective of toxicity but merely informs us of the activity of the chemical and how it acts kinetically; whether or not the chemical is highly bioaccumulable says nothing about its toxic potency (McKim and Schmieder, 1991).

Marine phytoplankton act as efficient bioaccumulators of dissolved heavy metals in seawater and being the principal food source for most marine herbivores, these plants provide one of the significant entrance stages for the introduction of toxic heavy metals into marine foods webs (Dongmann and Nurnberg, 1982; Fisher and Reinfelder, 1995). This transfer depends, in part, on their ability to accumulate and tolerate to a large extent, high concentrations of the metals, before they themselves become obviously affected (Mandelli, 1969; Jensen *et al.*, 1976). In a study on the trophic transfer from phytoplankton to herbivores, Fisher and Reinfelder (1995) showed that the assimilation efficiencies in bivalve larvae fed with *Isochrysis galbana* were directly proportional to the distribution of elements in the cytoplasm of the algae.

An aspect of metal bioaccumulation which is of special interest is its possible

usefulness as a method of metal recovery (Greene and Bedell, 1990), and bioremoval and reduction of metal concentrations in polluted wastewaters (Phang, 1993), particularly in the case of freshwater algae (Nakajima *et al.*, 1981a; Phang, 1990; Wilde and Benemann, 1993; Wong and Tam, 1998).

Some algae accumulate toxic metals at high levels while others do not. Maeda and Sakaguchi (1990) described an As-tolerant algae, *Chlorella vulgaris*, which survived at 10,000 mgL^{-1} As and accumulated As up to 50,000 mgkg^{-1} dry cell weight, much higher than non-tolerant strains, while with the same algae, the non-tolerant strain was four times more sensitive to Cu but accumulated five to ten times more than the tolerant strain, the tolerance attributed to Cu-exclusion by the algae (Foster, 1977). Most toxic elements such as Cd are also accumulated by dead algal cells (Chu *et al.*, 1997; Hashim *et al.*, 1997). In this case, uptake of the metal by the algae is not mediated directly by any metabolic process, but is dependent upon physical and chemical adsorption by cell compounds. Glooschenko (1969) found that formalin-treated *Chaetoceros costatum* cells adsorbed significantly more Hg than untreated cells. On the other hand As is accumulated only by living *Chlorella* and *Nostoc* (Maeda and Sakaguchi, 1990).

The accumulation of trace metals by phytoplankton in culture has been the object of numerous studies. A noticeable feature of the data is that uptake depends on many different factors including species composition, variation in culture conditions, and chemical state of the metal in the culture medium (Hardstedt-Romeo and Gnassia-Barelli, 1980). Concentration factor (CF) values depend on the surface : volume ratios of the cells, with smaller cells displaying greater concentration factors (Fisher and Reinfelder, 1995). They are also dependent on the speciation of the metal, with metal accumulation increasing inversely with dissolved organic matter concentration.

2.10.1.1 Cadmium

There is considerable variation in the amounts of Cd bioaccumulated by algae (Hassett *et al.*, 1981). Trevors *et al.* (1986) listed the concentration factors (CF) of Cd for various species of microalgae which ranged between 3500 to 24,000 and 86 to 32,000 for freshwater diatoms and green algae respectively. The data indicates that Cd accumulation is dependent upon both the algal species, the Cd concentration in the medium and other variables that affect cellular physiology. Meanwhile Conway (1978) showed that the bioaccumulation ratio (BR) of Cd for *Asterionella formosa* ranged between 4220 to 17,000. Table 2.10 demonstrates the capability of some marine phytoplankton to bioaccumulate cadmium and other selected heavy metals.

The use of concentration factors or accumulation ratios have been criticised by Geisweid and Urbach (1983) and Drbal *et al.* (1985). Geisweid and Urbach (1983) noted that Cd uptake is better described by the coefficients of the Langmuir equation, rather than by accumulation ratios, the latter being too dependent upon free concentration of the sorbable ion in the medium. This dependence of the Cd^{2+} accumulation on Cd^{2+} activity in the medium, however, is well established (Hart and Scaife, 1977; Hart *et al.*, 1979; Gipps and Coller, 1980; Truhaut *et al.*, 1980).

Kayser and Sperling (1980) observed that Cd content in *Prorocentrum micans* increased from 2.7 μgg^{-1} DW in controls to 500 μgCd g^{-1} DW in media containing 100 μg L^{-1} Cd corresponding to data of Bryan (1976) who reported a mean of 2 μgg^{-1} DW in phytoplankton. Dongmann and Nurnberg (1982) found that Cd applied in the range 0.4-27 $\mu molL^{-1}$ to a *Thalassiosira rotula* culture were found to accumulate in the dry biomass from 0.1 to 2.5 $mmolkg^{-1}$ Cd where the average maximum uptake was 600 μgCd g^{-1} DW.

Table 2.10 : Concentration factors for selected heavy metals in marine phytoplankton

Metal	Phytoplankton species	Concentration factor
Cadmium	*Chlorella salina* [a]	1420-3900
	Dunaliella tertiolecta [b]	1,000
	Diatoms [g]	<1.0 - 7.9
	Emiliania huxleyi [b]	3,700
	Green algae [h]	6,700
	Phaeodactylum tricornutum [c]	10,000
	Thalassiosira pseudonana [b]	3,100
	Oscillatoria woronichinii [b]	1,000
Copper	*Asterionella japonica* [d]	309
	Chlamydomonas sp. [d]	135
	Chlorella salina [d]	74
	Diatoms [g]	1.2 - 96.3
	Dunaliella primolecta [d]	153
	Dunaliella tertiolecta [d]	168
	Hemiselmis virescens [d]	273
	Hemiselmis brunescens [d]	553
	Heteromastix longifilis [d]	617
	Micromonas squamata [d]	279
	Monochrysis lutheri [d]	138
	Olisthodiscus luteus [d]	182
	Phaeodactylum tricornutum [d]	323
	Pseudopedinella pyriformis [d]	85
	Phytoplankton [e]	2,800
	Stichococcus bacillaris [d]	156
	Tetraselmis tetrahele [d]	265
Manganese	*Gonyaulax polyedra* [f]	2500-6300
	Phytoplankton [e]	3,800
Arsenic	Phytoplankton [i]	710-2900

Note :
a : Trevors *et al.* (1986); μgg^{-1} DW/μgL^{-1} ; b : Fisher and Reinfelder (1995); volume/volume
c: Cossa (1976); bioaccumulation ratio
d : Riley and Roth (1971); dry weight to wet weight conversion; e : Hawker (1990)
f : Folsom *et al.* (1963); g : Absil and van Scheppingen (1996); metal in diatom/metal in sediments;
h : Kerfoot and Jacob (1976); i : Lunde (1973)

It is known that Cd is transported internally into phototrophic algal cells. Laube *et al.* (1980) found that 29-34% and 66-71% of the Cd bound by *Ankistrodesmus braunii* was associated with the cell wall - cell membrane fraction and the cytoplasm, respectively. Nakajima *et al.* (1981) found that 74, 22, and 4% of the Cd^{2+} accumulated by the whole cells was associated with intracellular soluble fraction, the intracellular particulate fraction, and the cell wall fraction, respectively. Within the soluble fraction, Cd was associated only with high molecular weight compounds, probably irreversibly bound to metalloenzymes and complexed with amino acids, peptides and proteins (Conway and Williams, 1979). McLean and Williamson (1977) suggested a specific intracellular location for bound Cd, the nucleus. Okamura and Aoyama (1994) found that the amounts of Cd accumulated in the soluble fraction and the membrane fraction of *C. ellipsoidea* cells were 50% and 20% respectively.

Nagano *et al.* (1977) observed that the relative ability of a range of green microalgae to absorb Cd was : *Chlamydomonas reinhardtii > Chlorella regularis > Scenedesmus bijuga > S. obliquus > C. angulosa > S. chlorelloides.* The differing amounts of Cd absorbed by species of the green algae may have significant ecological consequences (Maeda and Sakaguchi, 1990). Fernandez-Leborans and Novillo (1996) found that in 500 µgL^{-1} Cd, *Olithodiscus luteus* bioaccumulated Cd 0.90 to 56.42 fgcell^{-1}. The bioaccumulation was in clear relation to the concentration of the metal dissolved in the culture, until a level at which the toxic effects determined a reduction in the uptake.

Bioaccumulation may decrease due to a dimunation of permeability, active accumulation and absorption surfaces, while active excretion also may play an important role (Albergoni *et al.*, 1980). In addition, intracellular chelators may be observed in algae

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in the presence of the metal. Albergoni *et al.*(1980) have established that *Euglena gracilis* is able to produce two glycoproteins, one accumulating Cd and the other, Cu. *Dunaliella* and *Chlorella pyrenoidosa* produce metallothioneins when exposed to high concentrations of Cd (Gipps and Coller, 1980). Cossa (1976) reported that with *Phaeodactylum tricornutum* adsorption of Cd onto the cell walls was followed by a gradual elution (desorption) by external metabolites.

Prahalad and Seenayya.(1986) observed in food chains involving nanoplankton, phytoplankton and zooplankton, that most organisms had higher concentrations of Cd and Cu than the background levels but the concentration decreased with increasing trophic levels. Hardy *et al.* (1984) introduced Cd in a *P. tricornutum - Crassostrea viginica* food chain and observed that 59% of Cd accumulated in oyster tissues were from the phytoplankton.

2.10.1.2 Copper

Bentley-Mowatt and Reid (1977) noted that at 10^{-3} M, uptake of Cu increased from 3 μgL^{-1} to 89 μgL^{-1} in 24 h and proved to be lethal, suggesting that dead cells take up more Cu than living cells. The amount of Cu taken up by the cells were proportional to the concentration in the medium for both *Phaeodactylum tricornutum* and *Cricosphaera elongata* in agreement with Riley and Roth (1971), who both, however concluded that the phytoplankton is restricted in the amounts of trace elements which it can take up. It is unlikely that all the Cu taken up by the cells is entering the cells and taking part in metabolism, since there is virtually no effect on long-term growth of the cultures Bentley-Mowatt and Reid (1977). Various workers have demonstrated that diatoms under stress, including *Phaeodactylum*, secrete mucilages which could possibly absorb and detoxify Cu. This mechanism may be a factor in the greater resistance of

Phaeodactylum to Cu as compared with *Cricosphaera*.

Jensen *et al.* (1976) reported that cells of *Phaeodactylum* accumulate more Cu when cultured in batch compared to dialysis cultures. Hardstedt-Romeo and Gnassia-Barelli (1980) found a linear relationship between Cu sorption and external Cu concentration. Eide *et al.* (1979) studying 3 species of diatoms growing in *in situ* cages, observed that uptake of Zn and Cu increased generally with increasing heavy metal content in sea water.

2.10.1.3 Manganese

Folsom *et al.* (1963) found that certain dinoflagellates (predominantly *Gonyaulax polyedra*) can concentrate Mn-54 several fold. Nakajima *et al.* (1979) who examined the uptake of Mn by *Chlorella regularis* found that scalded *Chlorella* cells in a solution of 200 mgL^{-1} accumulated 5100 μgg^{-1} DW, far larger than by living cells. Garnham *et al.* (1992) observed that immobilised live cells of *Chlorella salina* accumulated greater amounts of Mn^{2+} than free cells where 6 nmol 10^{6}cells^{-1} of Mn^{2+} was taken up within a 15 minute initial phase, and 8 nmol 10^6 cells^{-1} in a slower phase of 5 h.

2.10.1.4 Arsenic

Algae seem to be able to accumulate arsenic more efficiently than the higher members in the food chain (Woolson, 1975). An As-tolerant strain of *Chlorella vulgaris* which had been cultured in medium containing 1000 mgL^{-1} of As(V) was found to accumulate 8.7 mgg^{-1} DW As (Maeda and Ohki, 1998). Lunde (1973) observed that three freshwater algae (*Chlorella* sp., *Phaeodactylum* sp. and *Oscillatoria* sp.) and three marine algae (*Chlorella* sp., *Phaeodactylum* sp. and *Skeletonema* sp.) accumulated As from an aqueous phase containing 1-30 mgL^{-1} of As and concentrated it by factors of

240-2800 and 710-2900, respectively. Lunde (1973) reported that As compounds taken up by the phytoplankton were all in organic forms, where 60% were lipid soluble and 40% water soluble. Andreae and Klumpp (1979) who observed nine species of phytoplankton showed that marine phytoplankton is able to actively take up As(V) (85%) at natural concentrations from its environment, and to regulate cellular As levels independently of phosphate concentrations over a large concentration range.

The question of whether As is accumulated up the food chain is controversial. Giddings and Eddlemon (1977) studied an experimental ecosystem consisting of freshwater phytoplankton, zooplankton and snails for their As accumulation and found concentration factors of 965, 192 and 11, respectively, indicating that the lower organisms accumulate As more effectively than the higher organisms. A similar conclusion was drawn from the experimental results on a marine ecosystem (seaweed-herbivorous snail-carnivorous snail) (Klummp, 1980; Klumpp and Peterson, 1981). That As does not accumulate up the food chain has also been stated by Ferguson and Gavis (1972) and Isensee *et al.* (1973).

Meanwhile Wrench *et al.* (1979) studied the As metabolism in three trophic levels of marine organisms (phytoplankton-zooplankton-shrimp) and concluded that organic forms of As in marine food webs are derived from an *in vivo* synthesis by primary producers and are efficiently tranferred along a marine food chain. The algae, *Dunaliella tertiolecta* was capable of immobilising As into the lipid fraction. The shrimp, the highest trophic level in this food chain could not itself form organic As, thus As(V) taken up from water is converted largely to As(III). Similar conclusions were derived from experimental results on a phytoplankton-lobster system (Cooney and Benson, 1980) and a phytoplankton-crab system (Unlu, 1979). In a food chain involving *Thalassiosira*

pseudonana, S. costatum and *D. tertiolecta,* with brine shrimp and glass shrimp, Lindsay and Sanders (1990) observed an overall decrease in tissue As concentrations.

According to Sanders and Windom (1980), phytoplankton take up As(V) readily, and incorporate a small percentage of the metalloid into the cell. The majority of the As(V) is reduced, methylated, and released into the surrounding media. Algae have been found to methylate inorganic As to monomethyl arsenic (MMA), and dimethyl arsenic (DMA) (Andreae, 1977, 1978, 1983; Andreae and Klumpp, 1979). The methylated As compounds exist in the algal cells in the form of arseno-lipids (Wrench and Addison, 1981) such as phosphatidyl arsenocholine (Irgolic *et al.*, 1977; Bottino *et al.*, 1978) and 0-phosphatidyl trimethylarsonium lactic acid (Cooney and Benson, 1980). Since algae may contain As in concentrations more than 1000 times greater than the surrounding water, they may be a significant reservoir for this element in the marine environment (Sanders and Windom, 1980).

2.10.2 Mechanisms and kinetics of uptake and bioaccumulation

Passive and active transport processes have been proposed to account for heavy metal bioaccumulation. The passive mechanisms do not require the involvement of metabolic energy, where the metals are simply adsorbed onto cell surfaces, slime layers, and cellular matrices (Gadd and Griffiths, 1978; Rai *et al.*, 1981). Phytoplankton populations present large surface areas to the seawater or culture medium in which they are growing, thus adsorption onto the outside cells represents an important aspect of metal uptake by phytoplankton (Davies, 1978). The amount of metals taken up by active processes far exceeds those bound by cell surfaces (Gadd and Griffiths, 1978) but existing data suggests that metal sorption is related mainly to chemical and physical

change rather than physiological activity (Bollag and Duszota, 1984).

Studies on the changes in the Cd concentration in cultures of *Phaeodactylum tricornutum* and *Skeletonema costatum* (Motohashi and Tsuchida, 1974; Cossa, 1976) indicate the uptake of the metal during the growth phase followed by loss in the stationary phase. Diatoms form morphologically complex shells composed of hydrated amorphous silica which are enclosed by a three-layered organic membrane (Dongmann and Nurnberg, 1982). Thus it can be expected that a complex mechanism of metal uptake by algae depending on the algal species, their physiological and environmental conditions as well as the chemical forms of the metals in the adjacent medium. Andreae and Klumpp (1979) while studying the biosynthesis of products of *S. costatum*, *Platymonas* cf. *suecica*, *Gonyaulax polyedra*, and *Cricosphaera carteri*, observed a complex short-term As uptake behaviour suggesting that arsenate is taken up by more than one mechanism.

The kinetics of heavy metal uptake by algae which involves two stages (Khummongkol *et al.*, 1982) has been discussed by Trevors *et al.* (1986) with emphasis on Cd. The first phase is rather rapid, short-lived, and occurs immediately after initial contact with the metal, usually lasting for less than 5 to 10 minutes (Conway and Williams, 1979; Gipps and Coller, 1980; Khummongkol *et al.*, 1982; Geisweid and Urbach, 1983; Les and Walker, 1984). This initial phase is considered to be passive, involving physical sorption or ion exchange phenomena at cell surfaces. The second phase which is slow and extended, has been followed up to 600 hours in algae (McLean and Williamson, 1977) and may be separated from the fast phase by a lag period (Khummongkol *et al.*, 1982; Sakaguchi *et al.*, 1979). It may be linear (McLean and Williamson, 1977) or hyperbolic (Conway and Williams, 1979) in nature. This slow stage

is possibly active, involving some type of metabolic activity of the cell. The relative importance of these two stages is species-dependent (Trevors *et al.*, 1986). Geisweid and Urbach (1983) observed that *Ankistrodesmus braunii* and *Chlorella vulgaris* showed very little slow uptake of Cd^{2+} with more than 80% of the sorbed metal being accumulated in the first 5 minutes of the fast phase, while in *Eremosphaera viridis*, significant amounts of Cd^{2+} were accumulated by the cells during the slow phase, where within 3 hours, 85% of the Cd was probably taken up internally.

Geisweid and Urbach (1983) observed that the 5-minute rapid Cd uptake in *A. braunii*, *C. vulgaris*, and *E. viridis*, was described by Freundlich or Langmuir sorption isotherm and resulted in a rapid equilibrium between sorbed Cd and that remaining in the solution. Meanwhile, Khummongkol *et al.* (1982) developed a model for Cd^{2+} sorption onto *C. vulgaris* based upon a linear, equilibrium relation between concentrations of Cd^{2+} in solution and that sorbed on cell surfaces. The model accurately predicted Cd^{2+} uptake during the fast phase but could not predict Cd^{2+} accumulation during the slow phase, indicating that it involved mechanism other than physical adsorption, such as intracellular uptake (Trevors *et al.*, 1986).

Gonzales-Davilla *et al.* (1995) who studied the binding of Cu^{2+} to the surface and exudates of the marine alga *Dunaliella tertiolecta* demonstrated that the adsorption process of metal ions on the surface of living alga involved three components; the algal surface, the exudates which affect the complexed labile and non-labile metal concentration, and the inorganic metal. The adsorption process was affected by changes in factors such as pH, temperature, and salinity. Trevors *et al.* (1986) stated that sorption of Cd onto algal cell surfaces does not involve van der Waal forces at the cellulose network of algal cell walls, but probably acid group binding sites (Geisweid and

Urbach, 1983). Crist *et al.* (1981) concluded that both ionic charge bonding and covalent bonding play important roles in heavy metal uptake; proteins and polysaccharides were postulated to be involved where covalent bonding can be expected with amino and carbonyl groups, and ionic charge bonding with carboxyl and sulphate groups. Studies by Stary and Kratzer (1982) on *S. obliquus* indicated that algal cell walls behaved like a weakly acidic cation exchanger containing various cell wall ligands with different exchange capacity, whereas Stary *et al.* (1983) suggested that Cd is also accumulated on the surface as neutral complexes, rather than as charged ions. As most of the dissolved Cd in the sea occur as chlorides complexes, (Rebhun and Ben-Amotz, 1984), anion binding may be important in some algae (Trevors *et al.*, 1986).

Little information is available on the physico-chemical nature of the surfaces of marine phytoplankton. Myers *et al.* (1975) found that estuarine species *Nannochloris oculata*, *Pavlova (Monochrysis) lutheri* and *Cyclotella meneghiniana* had negatively charged surfaces due probably to the ionisation of groups in the polysaccharide-glyco-protein matrices exposed to the seawater. On the basis and by anology with more intensely studied cellular systems, Davies (1978) tentatively pictured the physico-chemical nature of the surfaces of phytoplankton as consisting of a mosaic of interspersed cationic and anionic exchange sites, provided by carboxylic, sulphydryl, phosphatidic, amino and other groups, the net charge on the surface being related to the degree to which the sites are occupied by protons and other ions present in the sea, therefore being a function of pH and salinity. The intial uptake of a positively charged heavy metal ion was then envisaged as occuring by the displacement of the cations already occupying the binding sites, the amount of metal finally bound onto the surface at equilibrium being determined by the relative affinities of the sites for the metal and

the seawater cations and also the concentrations of each remaining in solution, in accord with the principle of ion exchange. The metal once bound to the surface, would be suitably placed for being transported, actively or passively, through the diffusion barrier presented by the membrane into the cytoplasm.

McLean and Williamson (1977) showed that Cd accumulation in the slow phase was dependent on illumination and inhibited by the protein synthesis inhibitor, cycloheximide, and that Cd uptake was the result of an on-going anabolic process and not a consequence of a pH gradient provided by photosynthesis. It was also suggested that *de novo* protein synthesis was a prerequisite for Cd accumulation but may be species dependent (Conway and Williams, 1979) Although it has been suggested that Cd uptake is via a passive diffusion along a concentration gradient (Mang and Tromballa, 1978), it is generally considered, at least partially, an energy-dependent process (Skowronski, 1984a, 1984b). Hart *et al.* (1979) found that Cd^{2+} accumulation in *Chlorella pyrenoidosa* was light and temperature dependent, indicating an active process.

Geisweid and Urbach (1983) found that the relative importance of Cd uptake in the slow phase was species dependent and involved a slow, probably active, uptake of Cd into the cell interior, removing it from the equilibrium at the cell surface during the fast phase, thus encouraging more Cd sorption from solution and a slow, steady bioaccumulation of the metal. Competitive uptake studies, have indicated that Cd^{2+} may share an active system with Fe^{3+} (Gipps and Coller, 1982; Harrison and Morel, 1983). With some marine algae, it has been suggested that Cd uptake involves an anion transport mechanism utilising $CdCl_3^-$ (Rebhun and Ben-Amotz, 1984).