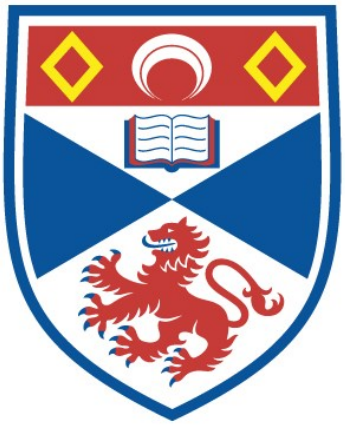
UPTAKE OF HEAVY METALS BY MARINE MICROALGAE, WITH A VIEW TOWARDS WASTEWATER DETOXIFICATION

Andrew James Whiston

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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Uptake of Heavy Metals by Marine Microalgae With a View Towards Wastewater Detoxification

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Submitted for the Degree of Doctor of Philosophy
in the University of St. Andrews

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for their encouragement and support

Abstract

Treatment of waste water by freshwater microalgae is rapidly becoming a popular sewage treatment practice throughout the world, but only a few studies have been made into the use of marine strains or of their potential for heavy metal removal. This study examines the heavy metal tolerance of a range of marine microalgal species and examines some of the biological processes involved in metal uptake. Initially over 350 marine microalgal species/isolates were screened for (a) growth in the presence of heavy metals (10 ppm), (b) growth in the presence of wastewater (1:1 sewage : seawater), and (c) heavy metal uptake. Of the microalgae examined, only one isolate, *Tetraselmis* sp. (TSAW92) was found to satisfy all of the screening conditions. Metal uptake was found to be a biphasic process, with an initial rapid saturable metabolism-independent stage followed by a slower non-saturable metabolism-dependent stage. Using a novel filtration technique metal uptake was measured at ten second intervals. The results show that most of the metal uptake occurs within one minute after exposure. The second stage of metal uptake was found to be associated with the extracellular release of up to three copper binding proteins of ca. 28, 30, 55 kDa. Protein release was specifically induced by the presence of heavy metals and was not due to metal mediated increases in cell membrane permeability.

Two practical applications of *Tetraselmis* sp. (TSAW92) metal uptake were investigated. First dried biomass was evaluated in a simple packed column. Second, live cells were grown on a wastewater treatment raceway (2m), upstream of a *Dunaliella salina* stocked nutrient removal raceway. Dried *Tetraselmis* sp. (TSAW92) was

capable of removing copper from a 1:1 seawater : sewage solution to a final loading of 30% at an efficiency of approaching 100%. Over four weeks live TSAW92 removed 100% of the applied copper to a final loading of 37%, and, after the second stage, the raceway was found to remove 95% of the applied nitrogen and 87% of the applied phosphorus.

This thesis shows that marine microalgae are capable of removing heavy metals from wastewater *in vitro*, on raceways, and as dried biomass. Live marine microalgae on raceways were further found to remove inorganic nutrients (nitrogen and phosphorous). In addition work is presented which suggests that copper uptake by the marine chlorophyte *Tetraselmis* sp. (TSAW92) is mediated through extracellular copper binding proteins.

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Abbreviations

The following abbreviations are used throughout this thesis:

AAS	Atomic Absorption Spectrophotometer
cm	centimetre(s)
^{o}C	degrees Centigrade
et al.	et alia (and others)
ES	modified Erd-Schreiber Medium
Fig.	figure
g	gram(s)
x g	times force of gravity (centrifugation)
h	hour(s)
kDa	thousands of Daltons
l	litre(s)
M	molar
m	metre(s)
mg	milligram(s)
mg/l	milligrams per litre (ppm)
min	minute(s)
ml	millilitre(s)
mM	millimolar
mm	millimetre(s)
µg	microgram(s)
µl	microlitre(s)
nm	nanometers
$N\text{-}NO_3^-$	nitrate-nitrogen
$N\text{-}NO_2^-$	nitrite-nitrogen
$N\text{-}NH_4^+$	ammonium-nitrogen
OD	optical density
ppm	parts per million (mg/l)
pers. comm.	personal communication
$P\text{-}PO_4$	ortho-phosphate-phosphorous
SD	standard deviation
sp.	species
Urea-N	urea-nitrogen
UV	ultraviolet light

Chemical formulae and element symbols have their conventional meanings.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 General Introduction

Heavy metal contamination of waste receiving waters are becoming a cause for concern throughout the world (Gay *et al.*, 1991; Clark, 1992; Vollenweider *et al.*, 1992). The main cause of this problem in marine environments is the traditional discharge of sewage effluent directly into the sea, often with little or no pre-treatment, by most countries with coastlines (Cooper & Lack, 1987; Figure 1.1). In addition to heavy metal contamination, sewage discharge also causes problems of eutrophication and may cause algal blooms. Historically, the natural processes of dilution and degradation in the marine environment were able to cope with this input, but with the increasing world population and greater industrialisation this is no longer the case (Harlin & Darley, 1988).

One of the major problems in sewage treatment is that in almost all countries domestic, industrial and surface water wastes are collected together in a mixed sewage system. Mixed sewage is approximately 95% water carrying a wide variety of industrial toxins, heavy metals, organic chemicals, pesticides, herbicides, bacteria, viral particles, and "rags" (e.g. sanitary towels, cotton buds, surgical dressings, condoms, and paper) (Table 1.1). This study focuses on the removal of heavy metals which are persistent, almost universally toxic to both plants and animals, and can be concentrated up the food chain by many orders of magnitude (Furr *et al.*, 1981; Clark, 1992).

Figure 1.1: The treatment steps applied to wastewater prior to discharge from a sample of 117 typical coastal outfalls around the UK, Gay *et al.*, 1991.

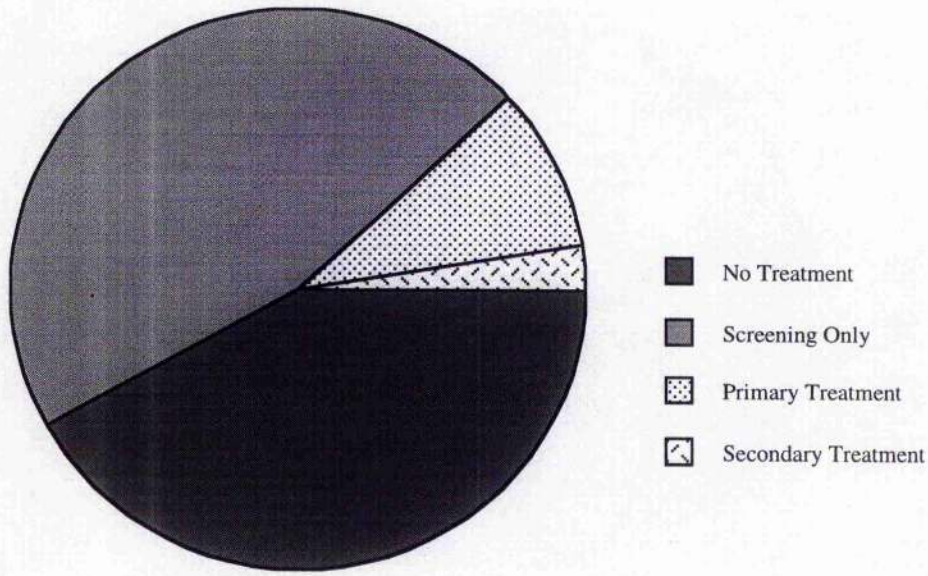


Table 1.1: The general composition of dried Scottish sewage sludge derived from data provided by the Scottish Association of Directors of Water and Sewerage Services (pers. comm.).

Component	Percentage by Weight
(Water)	(90-95)
Organic matter	50-70
Grease and fats	5-10
Minerals	20-45

1.2 Heavy Metals

The term "heavy metals" is somewhat imprecise. There are about 65 elements which are generally regarded as "heavy metals" (Duxbury, 1985). Some play a vital part in metabolic pathways (Co, Cu, Fe, Mn, Ni and Zn), and so with these metals there is the potential for confusion between their beneficial "trace metal" and toxic "heavy metal" roles. Other metals (Al, Cd, Hg, Pb and Sn) have no known metabolic functions, and therefore can be regarded purely as toxic heavy metals (Cooney, 1988; Duxbury, 1985). It is thought that whether a heavy metal has a metabolic function or not is determined by its relative solubility in water at physiological pH and its abundance in the Earth's crust (Gadd, 1990; Wood & Wang, 1983). There are two main sources of heavy metals; most are released as a result of natural weathering processes such as land run off and erosion, the remainder are released from industrial sources (Volesky, 1990). In the case of lead there are also significant inputs from road run off (Volesky, 1990). The toxic effects of heavy metals are due to their ability to form organic complexes and to enter cells where they inhibit the actions of many essential enzymes. Due to their stability and persistence, heavy metals can be concentrated by the food chain and are therefore a potentially serious health risk for top consumers, including humans (Volesky, 1990). The biochemical mechanisms underlying this toxicity are complex and are reviewed by Vallee & Ulmer (1972). The effects and sources of those heavy metals commonly found in sewage are summarised in the next section.

1.2.1 Cadmium

The world's average annual industrial release of cadmium is some 20,000 tonnes (Volesky, 1990), produced mainly as a by-product of zinc extraction. Industrial sources include: electroplating; mining; smelting; paint pigments; plastics; silver-cadmium and nickel-cadmium batteries; and refining (Volesky, 1990).

A disease known as "Itai-Itai" ("a cry of pain"), discovered in Japan in the 1950's, was specifically connected to cadmium poisoning. It resulted in: chronic multiple fractures caused by osteomalacia, kidney failure, and the death of over 100 people (Volesky, 1990). The source of the poisoning was traced to a mere 1 ppm of cadmium in the irrigation water of rice paddy fields. To date this contamination is still restricting the use of over 10% of paddy soils in Japan.

At birth, cadmium is almost absent from the body but it accumulates throughout life. The average American male has about 30 mg of cadmium in his body, with ca. 33% in the kidneys and ca. 14% in the liver (Volesky, 1990). Due its cumulative nature, the US safety limit for cadmium in drinking water has been set at just 10 ppb, and the daily "safe dose" at only 0.057 μg (Volesky, 1990).

1.2.2 Copper

Copper is one of the most commonly used metals in industry (Volesky, 1990) with some two million tonnes being released into the environment by human activity each year (Nriagu & Pacyna, 1988). Chronic copper intake in man results in a condition called "Wilson's disease", in which copper is deposited in the brain, liver, myocardium, and pancreas (Volesky, 1990). Unlike many heavy metals, copper also has metabolic functions in trace amounts, including participation in the electron transport chain in plants (South & Whittick, 1987).

In freshwaters, the most common form of copper is $CuCO_3$ (aq), with the remainder bound up in organic complexes. In seawater, copper is present in the form of particulate metallic copper, copper ions, and organic copper complexes, with cupric ions being the most toxic form (Volesky, 1990).

1.2.3 Iron

Iron is the most common industrial metal and is released in vast quantities by the steel industry. Iron is an essential mineral for all red blooded animals as it is required for the production of haemoglobin. It is not especially toxic in humans, but in large doses it may cause gastrointestinal problems (Volesky, 1990). In cyanobacteria, iron is an essential trace element involved in nitrogen fixation as a component of ferrodoxin which is required by

both nitrate and nitrite reductase as an electron donor (Hewitt *et al.*, 1976; Verateate *et al.*, 1980). Iron is also vital for the synthesis of the photosynthetic pigments chlorophyll-a and c-phycocyanin (South & Whittick, 1987)

1.2.4 Manganese

Manganese is mainly used in the battery making and steel industries. Long term chronic toxicity effects in humans have been observed, but these are very rare and so are poorly understood (Volesky, 1990). In microalgal photosynthesis manganese plays a vital role in the electron transfer chain (Richmond, 1986a), and is required for the maintenance of the chloroplast membrane (South & Whittick, 1987).

1.2.5 Zinc

Zinc is used in galvanising and in the production of brass and other alloys, such as gunmetal (Volesky, 1990). World production is approximately 7 million tonnes per year; with some 2.4 million tonnes being released annually (Volesky, 1990). Zinc is an important component of carbonic anhydrase, which carries out the hydration and dehydration of carbon dioxide in photosynthesis at low partial pressures of CO_2 (Raven & Glidewell, 1978). In higher plants zinc is also involved in the formulation of growth hormones and in protein synthesis. In animals zinc deficiency leads to alopecia, growth retardation, loss of appetite, and an induced lack of

vitamin A, whereas excess zinc leads to abortions and a disruption in iron metabolism (Conway & Pretty, 1991).

In natural seawaters zinc is present mainly as a chloro-complex, and in freshwaters as the hydrated ion, carbonate, sulphate, or hydroxyl species (Volesky, 1990).

1.3 Conventional Wastewater Treatment

Conventional wastewater treatment systems consist of several stages during which the waste is incrementally purified. Initially, the flow is mechanically screened to remove large objects, and in many coastal areas this is the only form of treatment applied (Figure 1.1). The next stage of treatment is to remove grit and coarse silt by the use of constant flow channels, after which the sewage is held in sedimentation tanks where the primary settlement of suspended solids occurs.

Primary settlement removes about two thirds of the suspended solids, one third of the Biological Oxygen Demand (BOD), and 30-90% of the pathogens present in the wastewater (Oswald, 1988c). However, primary effluent is still nutrient rich, with only 15-20% of the total nitrogen and phosphorus having been removed (Oswald, 1988c). From this point onwards each additional treatment step doubles the overall cost of the process (Oswald, 1988c) and hence most UK sewage is discharged at or prior to this stage.

During primary treatment the heavy metal content is shared in variable proportions between the liquid and solid phases, with

the majority in the solid phase. These solids are termed sewage sludge and are composed of 50-70% organic matter, 5-10% grease and fats, and 20-45% minerals, including heavy metals. Typical heavy metal contamination of sewage sludge is shown in Tables 1.2 (a) and 1.2 (b).

In common with many maritime nations, in the UK some 29% of this sludge is disposed of to sea either by outfall pipe or by ship (Fleming, 1987). However, due to recent legislation, this type of disposal is now regarded as a purely short term option. By December 1998 the UK will be required to adopt The European Community (now the European Union) urban waste water treatment directive of May 1991 which states that "*...the disposal of sludge to surface waters by dumping from ships, by discharge from pipelines or by other means must cease.*" (1991/271/EEC). Therefore, it is vital to rapidly develop new methods of sewage sludge disposal. At present there are two main alternatives, either landfill or incineration. However, both of these options treat sludge as a waste, whereas it could be regarded as a valuable resource.

Sewage sludge has potential value as an agricultural fertiliser (Bayes *et al.*, 1989), a soil conditioner (Hall *et al.*, 1986), a source of oil (Campbell & Bridle, 1985), and even as a building material (Bruce & Davis, 1989), but the presence of heavy metals prevents or restricts many of these uses (eg/ CEC, 1986; Table 1.2 (b)). For this reason heavy metals are widely regarded as a waste within a waste, whereas if recovered they could become a resource within a resource, valued at over £3 million per annum in the UK alone (Table 1.3).

Table 1.2 (a): Actual levels of metals present in sewage sludge from various countries, mg/kg of dry sludge.

Metal	Austria	Germany	Switzerland	USA	UK	**Mean**
Cd	3.5	21	9	30	20	**17**
Co	n/a	n/a	15	n/a	15	**(15)**
Cr	937	282	37	410	400	**413**
Cu	243	387	346	750	650	**475**
Hg	4	5	n/a	4	5	**(5)**
Ni	64	131	90	110	100	**99**
Mo	n/a	n/a	7	n/a	6	**(7)**
Pb	385	290	426	320	400	**364**
Zn	1537	2141	2828	n/a	1500	**(2002)**

Data modified from Tay River Purification Board 1992 (pers. comm.).
n/a data not available, () means with one or more data points missing

Table 1.2 (b): Mean legal requirements/guidelines in various countries for metal content in sewage sludge destined for agricultural use in mg/kg of dry sludge.

Metal	EEC	Germany	N'lands	Sweden	S'land	USA	**Means**
Cd	20	20	10	10	30	30	**20**
Co	n/a	n/a	n/a	n/a	100	n/a	**(100)**
Cr	750	1200	500	125	1000	1000	**763**
Cu	1000	1200	600	1000	1000	1000	**967**
Hg	16	25	10	6	10	10	**13**
Ni	300	200	100	63	200	200	**177**
Mo	n/a	n/a	n/a	n/a	20	n/a	**(20)**
Pb	750	1200	500	200	1000	1000	**775**
Zn	2500	3000	2000	2000	3000	2500	**2500**

Data modified from Tay river Purification Board 1992 (pers. comm.) n/a data not available, () means with one or more data points missing. N'lands = The Netherlands, S'land = Switzerland.

Table 1.3: The quantity and value (1986) of metals in sewage sludge in the UK per annum, modified from Fleming *et al.*, 1986.

Metal	Tonnes	Value in £'s
Cd	27	55,364
Cu	650	616,200
Pb	3900	1,384,500
Ni	195	500,760
Zn	1560	831,480
TOTALS	**6332**	**3,388,304**

The removal of these metals and their possible reuse would breakdown many of the barriers to the disposal of sludge to land, and is the ultimate aim of this project.

1.4 Metal Removal

Heavy metals may be removed chemically or by electrolysis (Jackson, 1972; Kammel & Lieber, 1981), but due to the large volumes of wastewater and the relatively low metal concentrations, these systems are both difficult and expensive to apply. Consequently, conventional metal removal systems are largely unknown at sewage treatment works.

A possible alternative is to make use of the metal removing abilities of microalgae (Volesky, 1990). It may even be profitable to recover the heavy metals from the microalgae, especially in the case of expensive metals such as uranium. Although not used in to date in the field of heavy metal removal, systems of this kind have been used on a small scale for the removal of radio-isotopes from waste waters (Brierley *et al.*, 1986; Hutchins *et al.*, 1986; Gadd, 1990; Kelly *et al.* , 1979; Tsezos, 1984). If different species of microalgae were found to take up different metals selectively, it may even be possible to use them to mine wastes biologically for useful metals.

1.5 Microalgae

Algae are non-vascular aquatic oxygen evolving photosynthetic protists which contain chlorophyll-a, and have

simple, unprotected, reproductive structures. They comprise some 1,800 genera with over 21,000 known species and range in size from unicells (0.2-10μm), through colonial forms (mm), to macroalgae (up to 80m).

Like higher plants, microalgae obtain their energy directly from the sun using photosynthesis to split water and build carbohydrates. Algae are extremely photosynthetically efficient and can convert up to 46% of the available light energy into biomass, an improvement of over 30% on the photosynthetic efficiency of higher plants (Aaronson & Dubinsky, 1982). Microalgae also exhibit very high specific growth rates of up to 5.5 divisions/day (Droop, 1974), and high net primary productivity (NPP) of 1,167 $gCm^{-2}\,yr^{-1}$, compared with 988 $gCm^{-2}\,yr^{-1}$ for tropical rain forest, and only 293 $gCm^{-2}\,yr^{-1}$ for cultivated land (Bott, 1983). In addition, microalgae can be grown in liquid culture, which allows for high surface area to volume ratios and easy waste handling by pumping. All of these factors give unicellular microalgae distinct advantages in biotechnological processes.

Records of algal biotechnology started in China in 2700 BC with the production of a curative herbal mixture. In Europe the Romans were known to use species of the division Rhodophycaea as a dye and cosmetic from 50 BC (Robinson *et al.*, 1989). Present day uses of microalgae are numerous, ranging from direct use as simple protein food supplements to the production of radio-labelled fine chemicals (Table 1.4).

Table 1.4: The potential uses of microalgal biomass.

Use	References
Animal feedstuffs	Shelef *et al.* , 1978; Lincoln & Hill, 1980; Mahadevaswamy & Venkataraman, 1986; Becker, 1988.
Aquaculture feeds	Paniagua-Michel *et al.* 1987; Pantastico, 1987; Herrero *et al.*. 1991; Benemann, 1992.
Fine chemicals	Borowitzka *et al.*, 1984; Hall & Rao, 1989.
Fuel (hydrocarbon)	Berkaloff *et al.*, 1984; Birch & Bachofen, 1988; Hall & Rao, 1989.
Fuel (indirect)	Benemann *et al.* , 1977
Glycerol production	Chen and Chi, 1981
Integrated aquaculture	Phang, 1990; Proulx & de la Noüe, 1985a, b; Ryther *et al.* , 1972.
Metal biosorption	Volesky, 1990
Oils	Borowitzka, 1988.
Organic fertilisers	Rodriguez-Lopez, 1983; Lembi & Waaland, 1988
Pharmaceuticals	Burton & Ingold, 1984; Metting & Pyne, 1986.
Pigments	Aasen *et al.*, 1969; Ben-Amotz & Avron, 1982
Poultry feeds	Becker, 1988.
Single cell protein (human food)	Becker, 1988; Cook, 1962; Moraine *et al.*, 1979
Swine feeds	Brune & Walz, 1978; Lincoln *et al.* ,1978.
Vitamins	Lem & Glick, 1985; Richmond, 1986b.

1.5.1 Microalgal wastewater treatment

Microalgal wastewater treatment is based on the principle of photosynthetic oxidation, in which microalgal oxygen production promotes the action of heterotrophic bacteria in breaking down the organic content of the waste, which in turn provides inorganic nutrients for the microalgae (Oswald *et al.*, 1988a). In natural systems, microalgae are known to play a significant part in the self-purification of aquatic environments (Adey & Loveland, 1991; Soeder, 1980). Microalgal systems have the unique advantage of turning wastewater directly into a resource, by recycling nutrients into usable microalgal biomass (Aaronson & Dubinsky, 1982; Becker, 1988). Coupled with very low running costs (1/10 to 1/100 less than conventional systems), this has led to the establishment of freshwater microalgal wastewater treatment technology in many areas of the world (Veber *et al.*, 1984; Belsare & Belsare, 1987; Oswald, 1972; de la Nöue & Bassères, 1989; Travieso *et al.*, 1992). However, in common with sludge, microalgal biomass produced in wastewater often contains high levels of heavy metals (Table 1.5) which limits its use in the human foodchain (Furr *et al.*, 1981; Richmond, 1988).

Microalgal wastewater treatment systems are generally based around shallow open ponds in which waste water is held for purification by exposure to microalgae, bacteria, and physical factors such as sunlight.

Table 1.5: Heavy metal contamination (mg/kg) in wastewater grown microalgae, data from Yannai *et al.*, 1980, and the World Health Organisation maximum recommended safe adult intake levels for selected heavy metals (*WHO, 1972).

Metal	*Chlorella*	*Micractinium*	*Scenedesmus*	*Max. Weekly Adult Intake (mg)
Aluminium	0.33	7.4	33.90	n/a
Arsenic	3.6	1.3	1.1	20.0
Cadmium	1.4	1.3	1.6	0.5
Copper	24.20	33.10	45.30	n/a
Lead	8.1	2.9	3.8	3.0
Mercury	0.26	0.64	0.30	0.3

Intuitively it is often thought that microalgal systems will only function in equatorial regions where temperatures are mild, days are long, and sunlight is plentiful. However, this is not the case, Oswald has estimated that on a purely climatalogical basis over 60% of the World's habitable surface is suitable for microalgal mass culture in outdoor ponds (Oswald, 1972).

In the context of this study, I shall refer to three main types of algal ponding systems: Waste Stabilisation Ponds (WSP); High-Rate Algal Ponds (HRAP); and Advanced Integrated Algal Ponds (AIAP). Confusingly, the terms waste stabilisation pond/lagoon, sewage lagoon, oxidation pond/lagoon, redox pond, maturation pond, facultative pond/lagoon, anaerobic pond/lagoon, aerobic stabilisation pond, mechanically-assisted oxidation pond, and terminal treatment pond/lagoon are used in a random and interchangeable manner throughout this field. The names of these ponds also tend to give no clues as to the type of waste which they are treating. I shall therefore prefix each term with the type of waste treated. Thus, a WSP treating primary or raw sewage will be termed a primary waste stabilisation pond. There are three main types of pond system, and one combined system:

(i) Primary Anaerobic Waste Stabilisation Ponds

These are the first and least technically demanding type of microalgal treatment system, and are often little more than holes in the ground. They can be thought of as direct replacements for the primary settlement stage of a conventional sewage treatment works

and are mainly concerned with the removal of suspended solids from raw sewage (Oswald, 1988c).

Anaerobic ponds are deep (2-5 m) and unmixed, their purpose is to cause the primary settlement of the wastewater into a thick sludge layer at the bottom of the pond. In this layer oxygen levels are very low and anaerobic bacteria flourish by breaking down complex organic compounds to simple mineral nutrients. This breakdown is a two step process with acid-producing facultative heterotrophs first degrading organic matter to fatty acids, aldehydes, alcohols, etc. Then anaerobic methane producing bacteria ferment these products to methane, ammonia, carbon dioxide, and nitrogen. By breaking down the complex organic compounds the bacteria convert the nutrients into a form which is readily available to the microalgae in subsequent treatments (Berner *et al.*, 1986).

Due to their anaerobic nature these ponds often provide a major source of malodour, in the form of hydrogen sulphide, methane, and other puterative gases. The other main disadvantage of this type of pond is that it produces significant amounts of sewage sludge which must be cleaned out and disposed of at regular intervals.

(ii) Facultative Ponds

Facultative ponds approximately two metres deep, unmixed, and combine both anaerobic and aerobic zones within the same pond. With a sunlight to heat conversion efficiency often approaching 90%, during the day a stable warm layer forms at the

surface of the pond. This layer is aerobic and supports the growth of a dense microalgal culture which utilises the inorganic nutrients released from the stagnant anoxic layer below (Oswald, 1988c). In the upper layer, algal photosynthesis rapidly uses up the available carbon sources, such as bicarbonate, causing a rise in alkalinity to as much as pH 11. At these high pH's dissolved ammonium is converted to ammonia which gasses off, and ions of calcium, magnesium and phosphorus form insoluble salts which precipitate out into the lower sludge layer (Arbiv & van Rijn, 1992; Oswald, 1988c).

Wastewater to be treated is slowly introduced into the cold lower layer where it settles to form a sludge. The anoxic sludge undergoes fermentation to methane and carbon dioxide, which escape into the atmosphere. As a result of bacterial anaerobic denitrification, organic nitrogen is removed as nitrogen gas (Verstrete & Alexander, 1973). Facultative ponds remove almost all of the suspended solids and produce an effluent with very little if any BOD (Oswald, 1988c).

Unfortunately, the controlling force behind a facultative pond is the weather, which is often far from predictable. On cold dull days, the thermocline may not be well established and thermal inversions can occur, thus mixing the pond from top to bottom. On hot bright days the algae may die off as a result of high temperature and pH in the surface layers. These problems can cause malodours, plagues of flies, and airborne foams and scums. The disadvantages of the facultative pond brought about the increased use of the High-Rate Algal Pond (HRAP).

(iii) High-Rate Algal Ponds (HRAP)

The use of HRAP's was first proposed almost forty years ago by Oswald & Gotaas, 1957, and they are little changed to the present day. Their main distinguishing features are lack of depth (typically 30 cm or less), and the high density of microalgae cultured in them.

HRAP are fully mixed and therefore have no anaerobic zones, this leads to the rapid aerobic growth of bacteria and prevents the production of sludges and malodours (Hendricks & Pote, 1974). At less than two days the retention times are shorter than in other types of ponds, allowing for a far higher throughput of wastewater. The operating principle is photosynthetic oxidation, where by oxygen produced by the microalgae stimulates the growth of aerobic bacteria which breakdown the organic matter in the wastewater releasing nutrients for more algal growth, and so on.

The main disadvantage of HRAP's is that due to the large amounts of algae, suspended solids and dissolved organics present the unmixed euphotic zone is very shallow (Oswald, 1988b). This means that significant energy inputs in terms of accurate level control and thorough mechanical mixing are vital in order to maintain acceptable levels of primary productivity. Having said that HRAP's are widely accepted as one of the best compromises to date for single pond microalgal wastewater treatment and are far more cost effective than aerobic bacteria only systems (Oswald, 1988c).

(iv) Advanced Integrated Ponding Systems

This technique takes the best features of all the various types of algal ponds and combines them into an integrated wastewater treatment system. It has been continually re-designed and developed by Oswald and his group in the US over the past forty years and represents the state of the art in microalgal wastewater treatment. The basic principle is to link the different types of algal ponds together in a manner appropriate to the character of the wastewater at each stage of treatment, rather than to try and achieve total treatment in one step (Oswald, 1991).

Wastewater enters the system via a primary pond (advanced facultative pond) which is constructed in the same way as a conventional facultative pond but with pits set into its base to form integral anaerobic ponds. In the primary pond the wastewater settles to produce a sludge which undergoes anaerobic fermentation to methane, nitrogen and carbon dioxide in the anaerobic pits.

The aerobic surface layers are inoculated with a dense algal culture by recirculation from the HRAP's in order to mop up the nutrients and carbon dioxide released from the sludge, and to "cap" the malodorous anaerobic lower layer (Green *et al.*, 1994). By careful design the risk of inversion is limited as sludge is only laid down in the pits and not across the whole pond which further reduces the possibility of malodour. The close proximity of the HRAP means that the microalgal surface layer can be easily replenished in unfavourable conditions, thus preventing a total die-off and subsequent whole pond anaerobic conditions.

The wastewater then passes into a conventional HRAP where photosynthetic oxidation encourages aerobic bacteria to mineralise the remaining organic matter for use by the microalgae. The waste then passes through unmixed settling ponds, where the algae and bacteria sediment out, and finally to holding or maturation ponds for subsequent re-use or discharge. The final effluent is microbiologically and chemically purified, has negligible BOD, low nutrient levels, and is safe for discharge into even sensitive receiving waters (Oswald, 1991).

1.5.2 The choice of marine versus freshwater microalgae

Freshwater microalgae have been extensively studied in wastewater treatment in preference to their marine counterparts, however this imbalance is not due to any superiority of freshwater microalgae but merely as a quirk of history (Borowitzka, 1988). In fact marine microalgae have several advantages over their freshwater counterparts, the most important of which is that their use enables culture media to be based on seawater. In many parts of the world freshwater is a costly and limiting resource, and its use in waste treatment could be regarded as wasteful (Myers, 1985; Wetzel, 1992). Seawater on the other hand is plentiful and is not regarded as a valuable resource, the sea is also generally the lowest point and so wastes can be gravity fed to a coastal treatment plant, thus avoiding the cost and difficulty of pumping effluents.

Seawater is naturally buffered and is far cheaper than complex freshwater based media (Dunstan & Menzel, 1971; Dunstan

& Tenore, 1972). In most freshwater microalgal wastewater treatment systems the main limiting nutrient is carbon (Oswald, 1970) which is usually replenished by expensive aeration. But in seawater the relatively high bicarbonate content (ca. 20 mmol m^{-3}) can be used by the microalgae as a supplementary carbon source (Burris, 1977; Rebello & Moreira, 1982). The use of bicarbonate by the microalgae tends to raise the pH to levels which favour the removal of ammonia by out-gassing (Arbiv & van Rijn, 1992), and are often lethal for many human enteric micro-organisms (Curtis *et al.*, 1992). It is also known that seawater itself is a potentially lethal environment for enteric bacteria and viruses (Fujioka *et al.*, 1981; Waldichuck, 1985).

Unlike their freshwater counterparts marine microalgae inhabit a hyperosmotic environment and many species therefore produce osmoticants, some of which are of direct commercial value. For example *Dunaliella salina* can consist of up to 50% dry weight of glycerol (Ben-Amotz & Avron, 1982), which has numerous industrial applications from pharmaceuticals to dynamite production.

The growth of marine microalgal biomass could also ease our requirements for fossil fuels in two main ways; firstly, by direct use of the alga or its components as a fuel, and secondly by the production of chemicals and feed-stocks from the algae which would normally have been sourced from fossil fuels (Birch & Bachofen, 1988). Certainly, *D. salina*, is hydrocarbon rich and has the potential for direct use as a fuel (Berkaloff *et al.*, 1984).

In marginal areas, such as arid regions or areas with saline groundwaters marine microalgae can be grown as a crop utilising water which would be unsuitable for conventional agriculture (Hall *et al.*, 1986; Richmond, 1986a). Their biomass could then be sold as a cash crop, or used locally as a single cell protein food for both Man and animals. Marine microalgal biomass is also of unique value as a live food in the integrated marine aquaculture of high unit value organisms such as clams and oysters (Nelson *et al.*, 1992).

1.6 Microbial Heavy Metal Uptake

A number of workers have identified microalgae which are able to remove heavy metals, including cadmium, copper, iron, gold, lead, uranium and zinc, from wastewater by bioaccumulation (Table 1.6). Microalgae and bacteria appear to bioaccumulate metals by one of three mechanisms (Volesky, 1990). First, by binding metals to sites within the cell wall (Xue *et al.*, 1988). Second, by release of metal-binding polysaccharides or small peptides (Daniel *et al.*, 1987; Gadd, 1990). Third, by actively taking up metals and sequestering them within compartments inside the cell (Gadd, 1990). The first mechanism does not require living cells; the second and third mechanisms require metabolically competent cells. To prevent this uptake many microbes can release chelating agents. These often take the form of anionic extracellular polysaccharides which are able to complex with, and effectively lower the level of "free" metal reaching the cell membrane (Lester *et al.*, 1984). In biological sewage treatment plants which are infected with

Table 1.6: The maximum uptake of metals observed in selected microalgae, on a percentage dry weight basis. Unless otherwise stated all experiments were carried out with free cells in "freshwater" ([I] immobilised cells). For details of the specific experimental conditions please see the original work.

Alga	Metal(s)	% Metal (dry wt.)	References
Ankistrodesmus sp.	U	1	Gadd, 1990
Chlorella regularis	Mn	≤0.8	Nakajima *et al.*, 1979
Chlorella regularis	Mo	1.32	Nakajima *et al.*, 1979
[I]*Chlorella regularis*	U	15	Nakajima *et al.*, 1979
Chlorella sp.	Hg	<0.01	De Fillippis & Pallaghy, 1976
Chlorella vulgaris	Cu	≤0.15	Butler *et al.*, 1980
Chlorella vulgaris	Cu	≤1.55	Butler *et al.*, 1980
[I]*Chlorella vulgaris*	Au	10	Darnall *et al.*, 1986
Euglena sp.	Al	1.8	Gadd, 1990
	Ba	0.013	Gadd, 1990
	Cu	0.010	Gadd, 1990
	Pb	0.037	Gadd, 1990
	Mn	0.041	Gadd, 1990
	Ni	0.006	Gadd, 1990
	Th	0.004	Gadd, 1990
	U	0.001	Gadd, 1990
	Zn	0.021	Gadd, 1990
Scenedesmus sp.	Cd	0.3	Gadd, 1988

Zoogloea sp.; these extracellular polysaccharides are thought to be responsible for much of the observed metal removal (Brown & Lester, 1979). The release of organic chelating agents has also been demonstrated in microalgae such as *Chlorella stigmatophora* and *Chlorella salina* (Kaplan *et al.*, 1987b). These chealating agents were found to be polysaccharides containing high proportions of sulphate and urionic acids. The presence of these groups would give the molecule a high negative charge which is consistent with a metal binding capability. However, it was noted that the polysaccharides were different in both structure and function with the *Chlorella salina* polysaccharide having no demonstrable *in vitro* metal binding ability. It is therefore clear that there is more than one type of polysaccharide released by microalgae, even in closely related species, and that these polysaccharides have markedly different metal complexing abilities.

1.6.2 Adsorption onto the cell surface

Microalgal and bacterial cells differ from those of animals in that they possess a rigid cell wall which forms a definite barrier between the external solution and the cell cytoplasm. In cyanobacteria the cell envelope is double walled with the inner layer being composed of cellulose microfibrillae and the outer layer being mucilage (Frey-Wyssling & Stechner, 1974). The eukaryotic Chlorophyceae generally possess a cell wall made up from a multi-layered latticework of cellulose microfibrils interspersed with amorphous material, which mainly comprises of glycoproteins.

Their walls may also contain mannan and xylan based microfibrils in addition to, or with the exclusion of cellulose. In brown algae, the cell wall cellulose is joined by algin and fucoidin, which also have the potential to bind metals. Red algae possess agar, carrageenan, pectin and xylans in their cell walls, which give further possibilities for metal binding. In diatoms, the cell wall matrix is heavily coated and impregnated with silicon, which may have implications in terms of their metal uptake ability.

The outer surface of the microalgal cell wall is often covered with a mucilaginous layer, which is rich in uronic acids and polysaccharides and may be involved in heavy metal binding (Ford & Mitchell, 1992; Kaplan *et al.*, 1987b). In industrial terms the microalgal cell wall may be regarded as a complex, and very high capacity, ion-exchange resin. From the point of view of the microalga the cell wall forms a barrier to the entry of heavy metal ions by transferring them from a "free" to a "bound" state in three main ways:

1) Adsorption and sequestration by cell wall cations (Crist, *et al.*, 1981; Volesky, 1990). Heavy metals may also bind to cell wall components as a result of specific functional groups exchanging protons for divalent cations (Ford & Mitchell, 1992). These groups are thought to include amino, carboxylic, hydroxo, and hydroxyl-carboxylic groups (Xue *et al.*, 1988) which are able to form co-ordinate bonds with heavy metals. Unlike micro-precipitation these methods rely on relatively weak bonding rather than on salt formation, and hence they are reversible by such things as pH changes and the presence of competing ions (Gadd, 1990). In

industrial applications the reversible nature of this bonding could well prove to be of critical importance in the non-destructive recovery of bound metals.

2) Micro-precipitation of the metals as insoluble phosphates, sulphides, carbonates, oxides, and oxalates at the cell surface (Sollins *et al.*, 1981; Murphy & Levy, 1983; Wood & Wang, 1983). The formation of insoluble salts effectively removes metals from solution either as a coating around the cell, or as a conventional precipitate. This method of metal removal tends to be the most permanent as the salts formed are usually very stable and insoluble.

3) Chelating agents similar to bacterial iron siderophores on the cell wall may bind strongly to both iron and other metals such as copper, molybdenum and cadmium (Ford & Mitchell, 1992). Bacterial siderophores occur in two main types; the hydroxamate and catecholate siderophores. The hydroxamate siderophores are known to bind iron using hydroxymate groups, this function is due to the size and charge of the iron ions (Davis & Byres, 1971). It is therefore thought likely that iron analogues such as aluminium, gallium and chromium may also be bound in this way (Raymond, *et al.*, 1984).

In cyanobacteria a hydroxamate siderophore has been implicated in copper binding to the cell wall as a copper toxicity resistance mechanism (McKnight & Morel, 1980). It would therefore seem reasonable to suggest this as a possible mechanism in microalgae, in fact "siderophore-like" compounds have been

tentatively identified in cultures of *Chlorella* sp. grown in the presence of copper (Butler *et al.*, 1980).

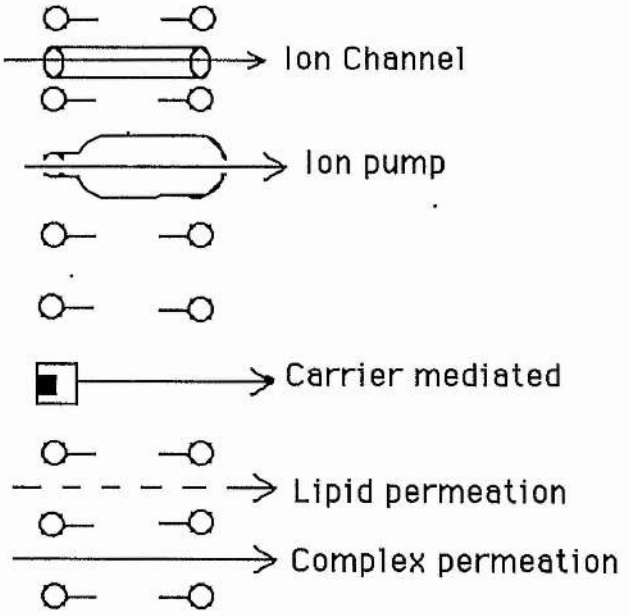
The initial uptake of metal ions by the microalgal cell wall is thought to be independent of metabolism as it takes place at the same rate in both living and dead cells (Volesky, 1990). It is a rapid process which is reported to be complete within five to ten minutes (Gadd, 1990), and is unaffected by metabolic inhibitors and modest ranges of temperature (0-30°C) (Trevors *et al.*, 1986). These factors could allow the use of dead microalgal biomass for metal removal, which would avoid the problems associated with the toxicity and unsuitable conditions for microalgal growth often encountered in industrial processes.

The proportion of metals bound to the cell and located within the cell is highly variable. In the case of *Chlorella vulgaris* approximately 80% of the observed cadmium uptake was due to cell wall binding , however, in the case of *Eremospharea viridis* the majority of uptake was reported to be intracellular (Gadd, 1988).

1.6.3 Intracellular uptake

Secondly, there is a metabolism-dependent stage which continues for some time, and relies on the accumulation of the metals within the cell. In bacteria metals are initially rapidly adsorbed by complex formation with cell surface ligands. These ligands then transport the metal complex through the cell membrane for release within the cell (Ford & Mitchell, 1992) (Figure 1.2). In bacteria lipopolysaccharide (LPS) is thought to be responsible

Figure 1.2: Proposed methods of trans-membrane heavy metal transport in microalgae.



Taken from Ford and Mitchell 1992

for the initial binding of heavy metals (Ferris *et al.*, 1989). LPS is a complex compound with a core of oligosaccharide, a hydrophobic phosphorylated section (Lipid A), and a number of side chains composed of unusual sugars (Ford & Mitchell, 1992). The construction of LPS results in the presence of many neighbouring electronegative binding sites giving this molecule the ability to bind polyvalent metal ions (Ferris *et al.*, 1989). Microalgae do not possess LPS and so this method of uptake is not possible, however, other carbohydrate compounds may perform a similar role.

In algae the transport step is known to be a slow process which is depressed or prevented by metabolic inhibitors and low temperatures (Gadd, 1990). However, the specific mechanism for this process in microalgae is largely unknown, although it is thought to involve membrane potentials and ionic gradients of H^+ and/or K^+ (Trevors *et al.*, 1986). Several other methods relying on proposed carriers and channels have also been suggested (Figure 1.2). Further complications occur when metal toxicity effects cause the microalgal cell membrane to become leaky thus revealing a host of additional intracellular metal binding sites which are not normally available in intact cells (Gadd, 1988). In diatoms such as *Nitzschia palea* copper toxicity has been found to cause a shedding of organic compounds from the cell surface and a loosening of the cell membrane further complicating the observed kinetics of copper uptake (Crist *et al.*, 1981).

1.6.4 Intracellular detoxification

Once the metal is within the cell it must be rapidly detoxified in order to avoid adverse metabolic effects and cell death. This may be achieved by; compartmentalisation, conversion, precipitation, or binding.

(a) Compartmentalisation

Removing the free metal ions from the cytoplasm and either expelling them or packaging them into vacuoles is a common method of metal detoxification in bacteria and fungi (Okorokov *et al.*, 1980). In algae, polyphosphate containing electron dense granules have been seen after metal exposure and are thought to be a major site of intracellular metal deposition (Gadd, 1990). In the alga *Stigeoclonium tenue* similar electron dense bodies were seen after exposure to lead (Silverberg, 1975). These bodies were found to be concentrated cytoplasmic vacuoles and were thus reasoned to be an intracellular method of lead detoxification. In *Scenedesmus* sp., Silverberg found copper rich nuclear inclusions after copper exposure. In tolerant cells the copper inclusions were found to be concentrated in pores in the nuclear envelope, and were totally absent from the contents of the nucleolus. However in copper-sensitive cells the inclusions were also found in the nucleolus. This indicated that the tolerant cells were able to closely control the intracellular distribution of copper in a way that the sensitive cells were not.

(b) Conversion

In the case of volatile metals such as mercury it is possible for microalgae to expel the metal in the form of a vapour (Wilkinson *et al.*, 1989). In order to volatilize mercury the microalga must carry out a bio-transformation process such as the demethylation of organic mercury complexes (Ben-Bassat & Mayer, 1977). Whilst the results of this mechanism have been demonstrated experimentally the details of the underlying system remain unclear in algae. In bacteria, such as *E. coli*, it is well known that mercuric reductase is responsible for the reduction of Hg^{2+} ions to Hg^{0} vapour (Schottel, 1978), and it is likely that microalgae use a similar system.

(c) Precipitation and Binding

A very common response to metal toxicity in many micro-organisms is the production of metal binding proteins (metallothioneins) which are able to bind to and detoxify intracellular heavy metals (Gadd, 1990). Metallothioneins have been found in animals (Anderson *et al.*, 1978; Imber *et al.*, 1987; Marghoshes & Valle, 1957), fungi and yeasts (Brenes-Pomales *et al.*, 1955; Butt & Ecker, 1987), higher plants (Casterline & Barnett, 1982; Fujita, 1985, Grill, 1985), and cyanobacteria (McLean *et al.*, 1972), and more recently in the chlorophyceae (Gekeler *et al.*, 1988).

Metallothioneins are small heat-stable proteins with several identifying characteristics (Reddy & Prasad, 1989):

(i) High cysteine (approximately 30%) and the absence of aromatic amino acids. With the typical optical properties of thiolate complexes, *viz.* a high 254 nm : 280 nm absorbance ratio.

(ii) High heavy metal content, often including cadmium, copper, and zinc.

(iii) Low molecular weight (typically ca. 6 kDa) and highly heat stable.

(iv) High affinity to anion exchangers.

Any metal inducible proteins which fit the above description are termed metallothioneins (MT's). Metallothionein synthesis and biochemistry is reviewed in Kägi & Schäffer, 1988. The group of MT proteins has been sub-divided into three main classes (Fowler *et al.*, 1987) of which Class III are of most relevance to this study.

(i) Class I MT's: Classical mammalian metallothioneins and metallothionein like polypeptides in animals.

(ii) Class II MT's: Metallothioneins which do not show a close relationship to Class I or Class III. eg yeast and sea urchin MT's. Frequently found in "simple" animals and prokaryotes.

(iii) Class III Mt's: Classical plant metallothioneins (phytochelatins) comprising of repeated gamma-glutamyl cysteinyl units. To date these proteins have been identified in the cells of eight algae: *Chlorella ellipsoida, Chlorella pyrinoidosa, Dunaliella bioculata, Euglena gracilis, Scenedesmus acutiformis , Scenedesmus quadricauda,* and *Synechococcus* sp. (Nagano *et al.*, 1982; Hart & Bertram, 1980; Heullet *et al.*, 1988; Gingrich *et al.*, 1986; Stokes *et al.*, 1977; Reddy & Prasad, 1989; & Olafson *et al.*, 1979; respectively.).

1.7 Aims

The aims of this study are as follows:

(i) Screening

To screen a large number and varied range of microalgae for their ability to grow in the presence of heavy metals. To re-screen those species which are able to grow in the presence of heavy metals for their ability to take up metals and/or remove them from solution.

(ii) Investigation of heavy metal uptake mechanisms in microalgae

To investigate the kinetics and underlying mechanisms of heavy metal uptake in microalgae. To measure the metal uptake capacity of microalgae and to asses its specificity.

(iii) Applications

To investigate the potential application of the identified microalgae in metal removal systems.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Marine Microalgal Culture

Modified ethylenediaminetetra-acetic acid (EDTA) free sterile Erd-Schreiber (ES) was used as the standard culture medium for this study (after Schreiber, 1927). ES was selected for ease of preparation and cost effectiveness, it is also a complete culture medium which is known to support the growth of a wide range of marine microalgal species (Stein, 1979). ES was made up in artificial sea water (Instant Ocean; Aquarium Systems Inc., USA), with potting compost (Levington M2; Fisons, UK) substituting for soil, and adjusted to pH 8.0 with dilute hydrochloric acid (AnalaR, BDH, UK).

To reduce the risk of heavy metal and nutrient contamination all glassware was thoroughly cleaned and acid-washed (10% hydrochloric acid) prior to use. All reagents were of AnalaR grade (BDH, UK) or better. Aseptic technique was used throughout.

The growth characteristics of marine microalgae are highly dependent on the conditions under which they are cultured. Conditions which affect microalgal growth include physical factors such as light intensity, photoperiod, temperature, and turbulence; as well as chemical factors such as pH, salinity, nutrient quality and level (Fabregas *et al.*, 1987; Oswald, 1988b). Preliminary physical culture conditions were chosen from those known to be optimal for native marine microalgae, being 10-15°C at ca. 25 $\mu E\ m^{-2}\ s^{-1}$ PAR (Starr, 1964; Guillard, 1979; Starr, 1979).

Three types of controlled environment incubators were used, lighting was on a 12 : 12 diurnal cycle, and temperature was

regulated to within 0.5°C of 15°C . A Fisons Mk II growth cabinet (model 140G2/TC with Philips TLD 36 w white fluorescent lamps) and a Conviron growth cabinet (model S10h, Conviron, UK, fitted with Sylvania 38 w cool white fluorescent lamps) were used for growth experiments, and a Foster Modular Refrigerated room (CF refrigeration, Scotland, fitted with Sylvania 38 w cool white fluorescent lamps) was used for the maintenance of 200 ml stock cultures of all marine microalgal isolates tested.

2.2 Sources and Isolation of Marine Microalgae

Over 350 types of marine microalgae were screened for heavy metal tolerance (Table 2.1). Of these, 103 were kindly gifted by Dr Rupert Craggs, including 54 species from culture collections (Craggs *et al.*, 1994). The remainder were endemic isolates from around the primary sewage outfall in St Andrews Bay, Fife, Scotland and from various other polluted shore sites around the UK (Appendix 1; Map). .Microalgae for isolation were collected by scraping biofilms from the rocks, hand tows (50 μm bottom-trap) were taken from the same sites at slack water during the spring bloom of 1993 and the autumn bloom of 1994. Marine microalgae were isolated from the samples by pipette isolation or by enrichment in ES followed by serial dilution.

Table 2.1: Marine microalgae which exhibited photoautotrophic growth or biomass maintenance in the presence of a 10 ppm mixture of the heavy metals cadmium, copper, iron, manganese, and zinc. All of these microalgae grew in sewage effluent diluted 1:1 with seawater and remained in pure culture (as in Craggs *et al.*, 1994).

CULTURE COLLECTION SPECIES	PRASINOPHYCEAE
	Tetraselmis rubens (4)+
BACILLARIOPHYCEAE	*Tetraselmis sp.* (TSAW'92)++
Chaetoceros calcitrans (4)+	*Tetraselmis suecica* (5)+
Phaeodactylum tricornutum (5)++	*Tetraselmis verrucosa* (5)+
Skeletonema costatum (5)+	
	PRYMNESIOPHYCEAE
CHLOROPHYCEAE	*Coccolithus sp.* (4)+
Chlorella salina (5)+	*Pavlova lutheri* (5)+
Chlorella stigmatophora (5)+	
Dunaliella salina (1)+	RHODOPHYCEAE
Dunaliella tertiolecta (5)++	*Porphyridium purpureum* (5)+
Nannochloropsis oculata (7)+	
Stichococcus bacillaris (5)+	**UNIDENTIFIED ENDEMIC ISOLATES**
DINOPHYCEAE	
Oxyrrhis marina (5)+	Bacillariophyceae (6) 10 isolates + 1 isolate ++
CRYPTOPHYCEAE	Chlorophyceae (6) 1 isolate +
Rhodomonas marina (2)+	
Rhodomonas sp. (3)+	Rhodophyceae (6) 1 isolate +

Numbers in () refer to isolate origin: (1) Biobred Ltd, (2) CCAP, (3) Gatty Marine Laboratory, (4) Millport Marine Biological Station, (5) Plymouth Culture Collection, (6) Endemic isolates. Species marked + showed some growth, and species marked ++ log growth, under the screening conditions. For non-successful algae see Appendix 3.

2.2.1 Pipette isolation

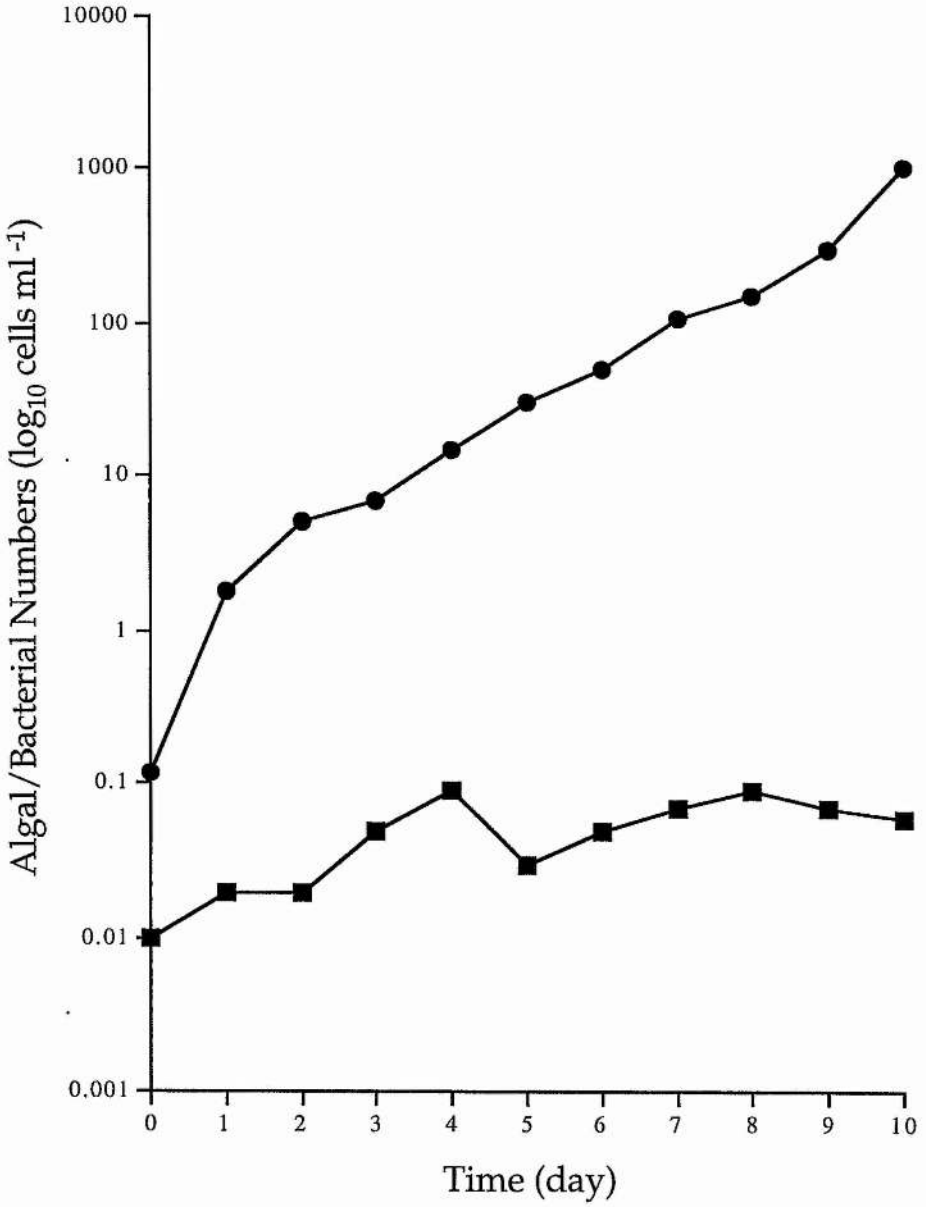
Tow and scrape samples were resuspended in sterile ES medium, and serially diluted with sterile ES to enable individual cells or colonies to be isolated (Hoshaw & Rosowski, 1979). The individual cells were separated using fire drawn and polished Pasteur pipettes under a dissecting microscope (Zeiss Stereomicroscope, SR D7082 Oberkochen) with a x50 objective. The isolated cells were inoculated into test-tubes containing 5 ml of sterile ES culture medium and incubated in the Conviron incubator for three weeks (15°C, 12:12 light : dark, 25μEm^{-2} s^{-1} PAR).

2.2.2 Raw enrichment

Tow and scrape samples were resuspended in sterile ES and incubated for three weeks in the Conviron Incubator, under standard conditions (15°C, 12:12 light : dark, 25μEm^{-2} s^{-1} PAR). The mean cell density of each sample was calculated by direct counts (Improved Neubauer Haemocytometer, BDH, UK), and serial dilutions were made using ES medium to obtain cultures statistically containing only a single cell or colony (Guillard, 1979). Replicate samples (10 ml) of each dilution were incubated in inclined test-tubes for up to three months in the Fisons cabinet (15°C, 12:12 light : dark, 25μEm^{-2} s^{-1} PAR). The purity of the marine microalgal isolates was checked, and species identified, using optical microscopy (Nikon Labophot II x400 phase-contrast, Nikon, Japan). Unialgal cultures were sub-cultured and grown up as 200 ml stock

cultures under standard conditions. In common with other studies, these isolates were unialgal but not necessarily axenic (Borowitzka, 1988, Oswald, 1988b). However, spread plate counts on marine agar (Marine Agar 2216; Difco Inc., Detroit, USA, made up to the manufacturer's directions) gave consistently low bacterial numbers of between 1 and 6 orders of magnitude less than those of the microalgae, for the duration of the experiments (Figure 2.1).

Figure 2.1: Mean bacterial numbers (■—■) in marine microalgal cultures (●—●). Bacteria were enumerated by spread plate culture of 0.5 ml of algal culture on marine agar, incubated in the dark at 15°C for 72 hours.



2.3 Biomass Determination

The efficiency of four biomass determination methods (haemocytometer cell counts, optical density (OD), chlorophyll *a* and protein) were initially compared using serial dilutions of cultures of two representative marine microalgae, *Chlorella salina*, and *Phaeodactylum tricornutum*. Efficiency was judged in terms of the reproducibility, cost, and the speed of the method.

The cultures were shaken to ensure homogeneity before sampling, and spectrophotometric and protein analyses were made in 96-well microtitre plates read on a Dynatech MR5000 plate reader (Dynatech, Billinghurst, UK) (see below).

2.3.1 Haemocytometer cell counts

Cell densities were estimated from the means of ten replicate counts per sample using an improved Neubauer haemocytometer (Stein, 1979). Samples containing motile cells, such as *Tetraselmis* sp., were fixed with 0.01% (v/v) Lugol's iodine prior to counting.

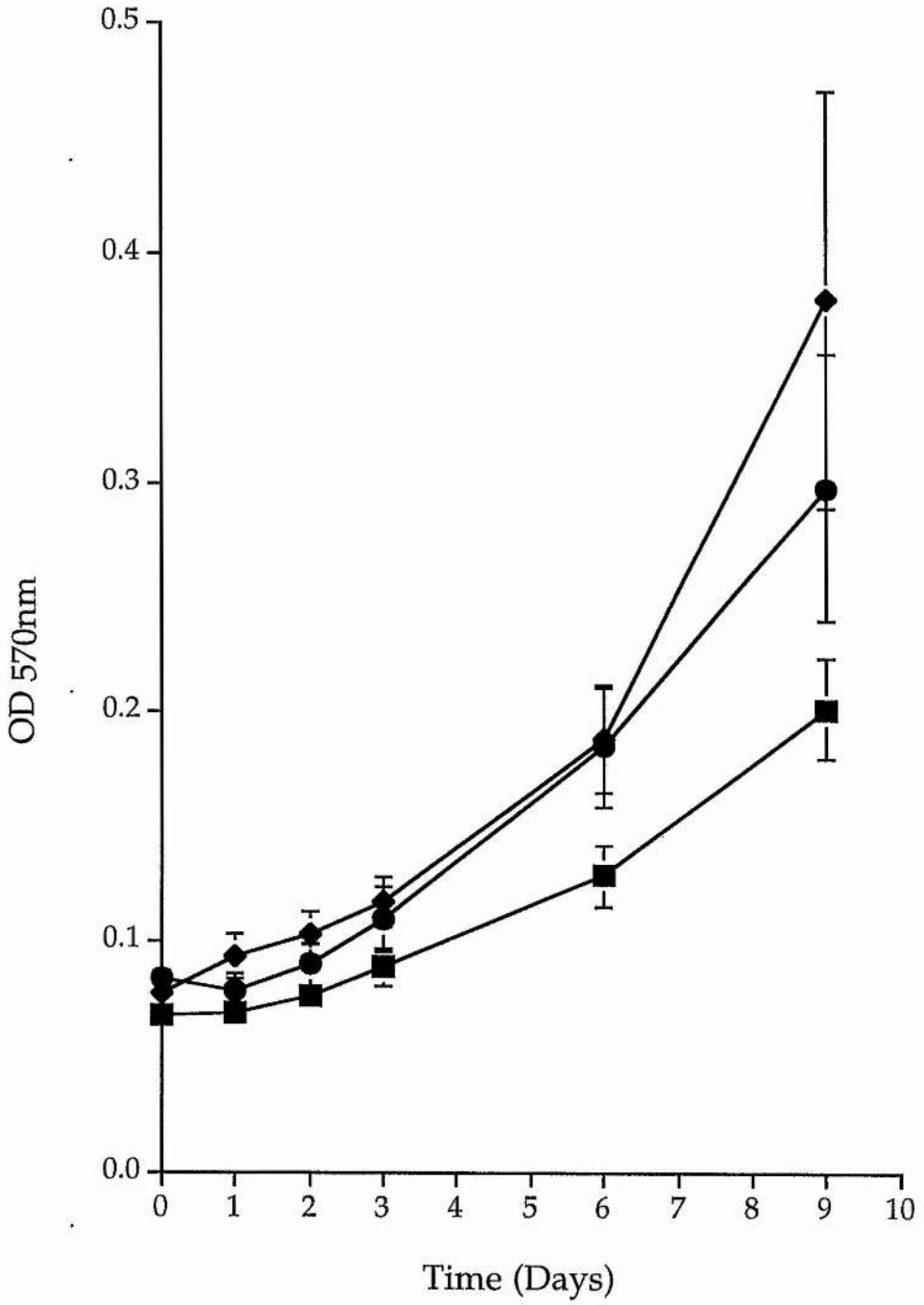
2.3.2 Optical density (OD) at 570 nm

Optical density based biomass measurements (Sorokin, 1979) were made directly from 3 ml aliquots of each microalgal culture in disposable polystyrene cuvettes at 570 nm against a blank of ES medium. Measurements on 250 µl aliquots, in 96 well microtitre

plates (of V, U, and flat profiles), were also made, using the MR5000 plate reader.

It was found that many of the test species and isolates had a strong tendency to form films on the walls of the vessels in which they were cultured, especially in the presence of heavy metals. This resulted in a lack of homogeneity even in well-mixed cultures, causing potential inaccuracies in all enumeration methods relying on sub-sampling. By screening the algae in flat-bottomed 96 well microtitre plates, growth could measured *in situ* by using the Dynatech MR 5000 plate reader. This removed the risk of sampling error, and allowed the rapid measurement of microalgal growth in many replicate cultures in a small space. Various types of microtitre plate were investigated for their suitability for marine microalgal growth. Both 'U' and 'V' type wells were found to cause the formation of a dense microalgal pellet at the lowest point of the well, which coincides with the optical axis of the plate reader. This effect produces inflated and highly variable results, and may give transmittance values of approaching zero even at fairly modest overall cell densities. In Figure 2.2 it can be seen that even a motile alga like *Tetraselmis* sp. (TSAW) gives a higher variation in optical density data when grown in V and U profiled wells. For this reason

Figure 2.2: The effect of microtitre plate profile: V(◆—◆), U(●—●), and flat (■—■) on microalgal biomass determination by optical density measurement on *Tetraselmis* sp. (TSAW). Values given are means of 96 samples ± 1 standard deviation.



flat-bottomed microtitre plates were used, and a software package which enabled the plate reader to average up to 32 readings of optical density across each of the wells (TCG, Dynatech, Bilingshurst, UK).

2.3.3 Chlorophyll-a

A 10 ml sub-sample was centrifuged (1500 g, 10 min), the supernatant was discarded and the pellet mixed with a few drops of saturated $MgCO_3$ solution and 5 ml of 90 % acetone (v/v in Milli-Q water). The tube was capped, and the sample extracted at 4°C for 24 h in darkness. After extraction, the sample was shaken and centrifuged (1500 g, 10 min) to remove cell debris. The OD of the resulting supernatant was measured as described in Parsons *et al.* (1984) against a Milli-Q reagent blank.

The chlorophyll-a concentration of the sample was calculated from the following equation:

$$\text{Chlorophyll-a } (\mu g\ ml^{-1}) = C = 11.85\ OD_{664} - 1.54\ OD_{647} - 0.08\ OD_{630}$$

Then:

$$\text{Chlorophyll-a } (\mu g\ l^{-1}) = \frac{C \times v}{V}$$

Where: v: the volume of extraction agent (90% Acetone) (ml)

V: the volume of sample centrifuged (l)

(Parsons *et al.* 1984)

2.3.4 Determination of protein

Total cell protein was measured using Coomassie blue as an indicator of biomass. Cells were harvested by centrifugation (1500 xg, 10 min) from a 10 ml sub-sample of microalgal culture. The pellet was resuspended in 0.5 ml of 0.1 M NaOH and extracted at room temperature for 12 h. Following centrifugation (1500 xg, 10 min) to remove cell debris, triplicate 100 µl aliquots were mixed with 100 µl of Coomassie Brilliant Blue reagent (G-250, Pierce, Rockford, Illinois, USA) in 96-well microtitre plates. Loaded plates were incubated at room temperature for 30 min, and absorbance was read at 595 nm against triplicate Milli-Q reagent blanks on the MR5000 plate reader. Absorbance values were compared to a standard curve generated from serially diluted bovine serum albumin (0.1-100 µg ml^{-1}; Sigma, UK).

2.3.5 Choice of biomass determination method

A dilution series was produced from a culture of TSAW and the biomass determined separately using each of the above methods. The results from these biomass determinations were compared to the known dilution series using protected t-tests. It was found that all four methods produced values for cell numbers which were not significantly different from the dilution series (Table 2.2). However, both the chlorophyll-a and total protein content methods produce far lower values ($p=0.051$ and 0.058 respectively) than the direct counting ($p=0.14$) and the optical

Table 2.2: Comparison of four marine microalgal biomass determination methods. Cell densities given by each method were compared to a dilution series of TSAW using protected t-testing, df=6. Values given are p values from the protected t-tests at the 95% confidence limit.

Biomass Determination Method	p Value
Haemocytometer Counts	0.086
Optical Density	0.180
Total Protein	0.058
Chlorophyll-a	0.051

density ($p=0.18$) methods. It could therefore be argued that in terms of accuracy the optical density technique was the best. This technique also proved to be the easiest and fastest to apply, and it was therefore used throughout the study.

2.4 Microalgal Growth Characteristics

The growth characteristics of the main microalgal test isolates were established by inoculating triplicate flasks containing 90 ml of sterile ES medium with 10 ml of actively growing cell culture. Triplicate control flasks were also set up containing 100 ml of sterile ES medium. For two weeks, at daily intervals, the flasks were shaken to homogeneity and the optical density of triplicate 250 µl aliquots was read at 570 nm in microtitre plates using the MR5000. Increase in optical density was calculated from the means of the triplicate cultures against the means of the controls.

The duration of the exponential growth phase and exponential growth rate of each microalgal species was calculated from a semi-log (base 2) plot of microalgal OD_{570} against time. All subsequent inoculations physiological and biochemical experiments were carried out using mid-log phase cultures. In every case the volume of the initial inoculum was 10% of the total volume of the culture (Liao *et al.*, 1983).

2.5 Analytical Methods

Due to the very large number of measurements required during this study and the need for direct comparisons to be made over the whole three year duration of the work, analytical methods were selected primarily on the basis of reproducibility. Because of the high number of samples required it was also necessary to select techniques which were both rapid and cost effective.

2.5.1 Physical factors

Routine temperature measurements were carried out in the growth chambers using self-contained recording thermometers (Castella Mk II Charting Temperature Recorder 838012, Castella Ltd., London, UK). The temperature of media was measured using mercury in glass thermometers, and digital thermistor meters (Model DTM 2, Tandy Ltd., Walsall, UK). The pH of the media was determined using a Philips digital pH meter (Type PW9409), equipped with a combination pH electrode (Type CWL, Russell, Auchtermuchty, Fife), and calibrated at pH 7.00 and pH 10.00. For online pH measurements in cultures, the pH electrode was protected from fouling using a dialysis membrane cell which was renewed on alternate days. Salinity (‰) was measured using a glass hydrometer (Seareadrometer, Waterlife Ltd., West Drayton, UK) at 25°C.

2.5.2 Inorganic nutrients

Dissolved nutrients (nitrogen + phosphorus) were measured in triplicate samples against Milli-Q water reagent blanks by colourmetric methods, described below (2.5.2), scaled down and modified for use in 96 well microtitre plates. Particulate free samples were routinely produced by centrifugation, and in the case of short time course studies (>10 min), by filtration (Whatman, GFC). Ninety six well microtitre plates were pre-loaded with reagents using a multi-dispenser pipette (Eppendorf, Hamburg, Germany). Triplicate aliquots of the samples were then transferred to the plates by Gilson pipette, and through mixing of the reagents and samples was effected by repeated loading and unloading of the Gilson pipette within the well.

2.5.2 (a) Urea-Nitrogen

N-$CO(NH_2)_2$ was broken down to ammonium by urease using a Sigma Diagnostics (Sigma Diagnostics, St Louis, USA) urea nitrogen kit (procedure No. 640). The resulting ammonium was then determined by the method described below.

2.5.2 (b) Ammonium-Nitrogen

N-NH_4^+ was measured by a scaled down version of the method described in Parsons *et al.* (1984). Microplates were loaded with 10 µl of both phenol and ammonium nitroprusside, 30 µl of

oxidising reagent, and 250 µl of sample, plates were read at 630 nm after a 30 min incubation in the dark, at room temperature. Samples were diluted 1:4 with Milli-Q water prior to the addition of the reagents, and a standard curve was constructed using a 293.8 mmol N m^{-3} Sigma standard of $(NH_4)_2 SO_4$ serially diluted with ammonia free 25% artificial seawater.

2.5.2 (c) Nitrite-Nitrogen

$N\text{-}NO_2^-$ was analysed by the method of Snell (1981) scaled down for use in microplates. Samples were compared to 99.9 mmol N m^{-3} Sigma standards of $NaNO_2$, serially diluted with nitrite free artificial seawater (Craggs *et al.*, 1994).

2.5.2 (d) Nitrate-Nitrogen

$N\text{-}NO_3^-$ was determined using a NAS Szechrome reagent (Diphenylamine Sulphonic Acid Chromogene) kit scaled down for use in microtitre plates (Park Scientific Ltd, Northampton, UK). Samples were compared to a standard curve obtained using a solution of KNO_3 (BDH, UK) in nitrate free artificial seawater (Craggs *et al.*, 1994).

2.5.2 (e) Ortho-Phosphate

P-PO_4^{3-} was analysed by the malachite green method, modified by Craggs *et al.* (1994) from a method originally developed for the microplate determination of ATPase modified by Henkel *et al.*, (1988).

2.6 Sources and Characteristics of Wastewater and Seawater

2.6.1 Seawater

For large-scale cultures natural seawater was obtained via the seawater intake line at the Gatty Marine Laboratory, University of St Andrews, Fife, Scotland, UK. This seawater intake system comprised of a sand trap filter followed by a 5 μm filter and UV steriliser. The physical (pH, temperature, and salinity) and chemical properties (nitrogen and phosphate) of this seawater were analysed at each collection, and compared to the mean values from previous collections (Table 2.3). For small-scale experiments and media preparation Instant Ocean (Aquarium Systems Inc., USA), made up to the same average salinity and pH as St Andrews Bay seawater, was used in place of natural seawater.

Table 2.3: Chemical and physical properties of primary sewage effluent and seawater at the East Sands, St Andrews Bay, Scotland, for the period October 1992 to September 1995.

Factor	Primary Effluent		Seawater	
	Mean ± s.d.	Range	Mean ± s.d.	Range
Temp.(°C)	14.5 ± 1.8	9.0-22.1	12.0 ± 2.6	4.0-15.5
pH @ 15°C	7.6± 0.1	6.9-7.8	8.0± 0.5	6.8-8.3
Salinity (‰) @15°C	0.31 ± 0.20	0.12-0.40	33.09 ± 2.80	21.9-37.0
*BOD (mg l^{-1})	172.1 ± 41.3	86.7-320.2	n.a.	n.a.
*COD (mg l^{-1})	389.7 ± 121.4	140.5-767.4	n.a.	n.a.
*S. Solids (mg l^{-1})	101.8 ± 28.3	43.1-168.4	<0.1	0-0.1
Urea-N (mmol m^{-3})	20.9 ± 12.0	0.2-42.3	0.6 ± 2.1	<0.01-4.3
N-NH_4^+ (mmol m^{-3})	964.2 ± 193.5	281.0-2328.2	1.5 ± 1.1	<0.01-4.5
N-NO_2^- (mmol m^{-3})	3.2 ± 1.1	1.1-7.9	0.2 ± 0.3	<0.01- 0.9
N-NO_3^- (mmol m^{-3})	19.6 ± 13.2	<0.01-37.4	9.4 ± 10.9	<0.01-18.9
P-PO_4^{3-} (mmol m^{-3})	117.9 ± 23.7	61.6-218.4 6	1.7 ± 1.1	<0.01-3.0

*Includes data from Tay River Purification Board 1994 (pers. comm.)

2.6.2 Wastewater

Primary sewage effluent (wastewater) was collected from the treatment works at St Andrews, Fife, Scotland. This works receives approximately 6.8 million litres of wastewater per day mainly from domestic and rural sources. For this study wastewater was taken after the screening and primary sedimentation processes. The physical factors (temperature, pH, and salinity), nutrient levels (nitrogen and phosphate), and BOD/COD content of this effluent were measured at each collection and the mean and range values were calculated (Table 2.3).

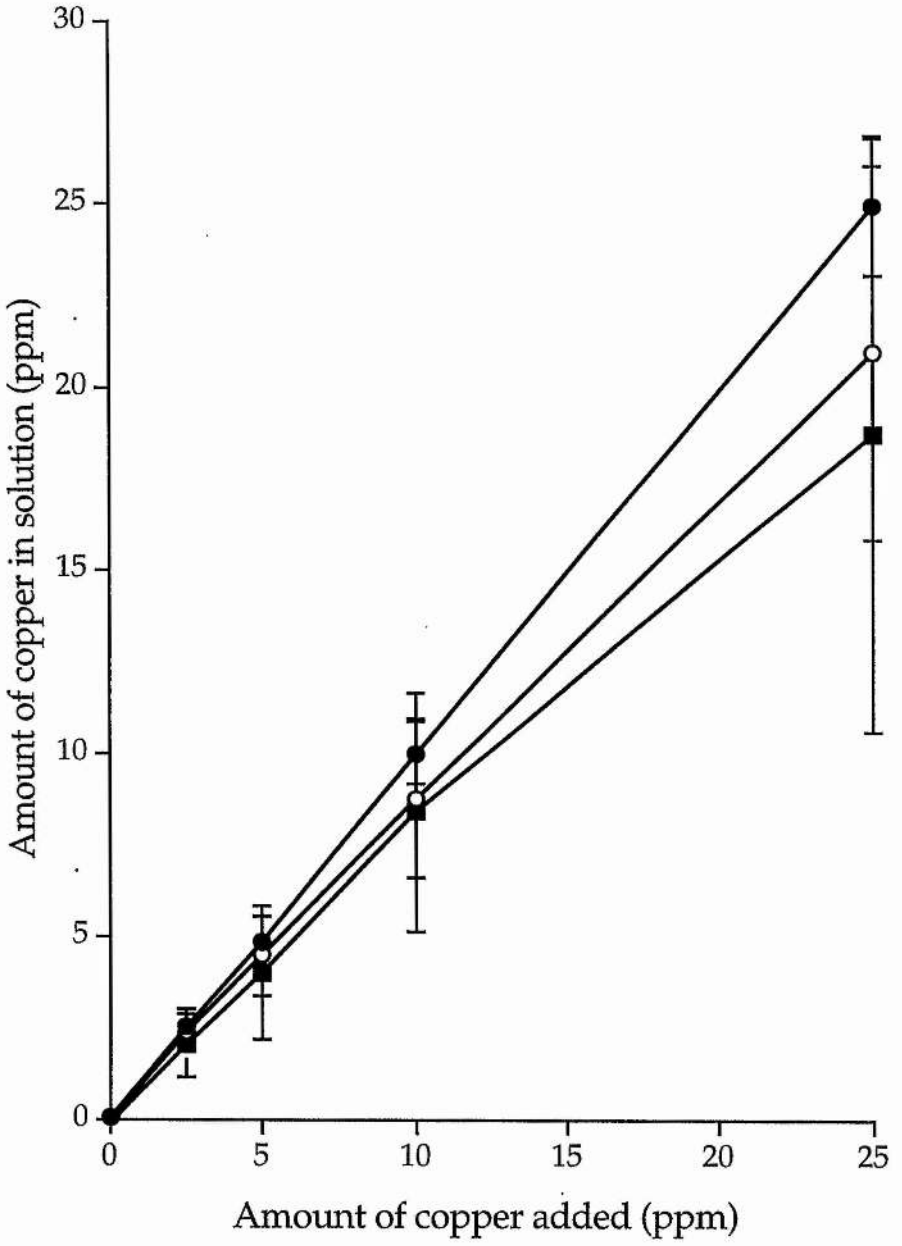
2.7 Determination of Metal Levels by Atomic Absorption Spectrophotometry

A Pye Unicam PU9000 Flame Atomic Absorption Spectrophotometer (AAS) (Pye Unicam Ltd, Cambridge, UK) with background correction, a high solids burner, and platinum fittings was used for the determination of metal levels during this study (Plate 2.1). The use of platinum fittings and background correction allowed the accurate measurement of heavy metals even in an acidified seawater matrix. Automatic calibration was achieved using the maximum five standards made up in the matrix to be analysed. Metals were added in the form of chlorides and were of analytical quality (AnalaR, BDH, UK). Periodically the absolute accuracy of the machine was checked using Sigma certified metal standards (AAS Metal Standard Solutions Kit C6042, Sigma, UK).

Plate 2.1: Pye Unicam PU9000 Flame Atomic Absorption Spectrophotometer (AAS).

Machine noise was corrected by using an internal standard both at the start and finish of each analytical run. Aliquots of the same internal standard were used for the duration of study. It was noticed that by adding the correct theoretical weight of copper chloride to a seawater or ES solution the resulting measured level of copper in solution was somewhat lower than the expected value (Figure 2.3). This was assumed to be due to non-specific binding and precipitation by substances such as phosphate. In order to correct for these factors the soluble metal levels in all solutions were routinely measured by AAS, and adjusted where necessary. Measurement of metal levels in solids was achieved by ashing a known amount of solid in a ceramic crucible using an electric muffle furnace (Carbolite, Sheffield, UK). The ashed sample was then resuspended in 1 ml of Milli-Q water, centrifuged to remove particulates, and assayed for metal content by AAS. Metal levels in solutions were measured by AAS after centrifugation or filtration to remove particulates.

Figure 2.3: The effect of matrix type on metal levels measured by AAS. Copper chloride was added to 100 ml of either distilled water (●—●), sea water (○—○), and ES culture medium (■—■) to provide theoretical copper levels of 2, 5, 10, and 25 ppm. After a ten minute incubation at room temperature actual copper levels were measured by AAS. Error bars are one standard deviation.



CHAPTER THREE

SCREENING

3.1 Introduction

In order to investigate heavy metal uptake in marine microalgae it was first necessary to obtain and screen a wide and representative sample of endemic strains. Isolation was carried out as described in Chapter 2.

The first series of screening experiments was designed to select for those microalgae which were able to grow under conditions of acute metal contamination. Successful microalgae from the preliminary screening were then re-screened for their ability to grow on a 1:1 mixture of wastewater and seawater. Wastewater diluted in this way is less saline (approximately half) than ES medium and supplies nitrogen in the form of ammonium rather than as nitrate/nitrite. Finally, the remaining algae were screened for their metal uptake ability. In this way it was possible to identify those isolates able to grow in the presence of heavy metals and wastewater, and to remove these metals from solution.

3.2 Materials and Methods

3.2.1 Microtitre plate and flask screening for growth in the presence of heavy metals

The three hundred and fifty types of microalgae described in Chapter 2 were screened for their ability to grow in the presence of heavy metals (Table 2.1; Appendix 3).

One hundred and fifty millilitre stock cultures of the test isolates were grown up, in sterile ES medium in 250 ml conical flasks, in the Foster Room (15°C, 12:12 light : dark, 25μEm^{-2} s^{-1} PAR). One hundred millilitres of mid-log phase stock culture were sub-cultured into 200 ml of ES medium supplemented either with a mixture of cadmium, copper, iron, manganese, and zinc chlorides (final soluble metal concentrations of 10.00 ppm for each metal), or with an osmotically equivalent amount of sodium chloride. Final soluble metal levels, in the medium, were adjusted by atomic absorption spectrophotometry (Section 2.7) of triplicate filtered (0.2 μm GS membrane filter; Millipore, France) sub-samples.

From these experimental cultures, 200 μl sub-samples were pipetted into each of the 96 wells of flat bottomed microtitre plates (Dynatech, Bilingshurst, UK). As a control, similar plates and flasks were also set up using only ES medium. Experimental cultures in flasks and in microtitre plates were incubated for nine days under standard conditions. The microtitre plates were placed in separate airtight transparent acrylic boxes lined with damp paper towels to prevent dehydration. After incubation, microscopic examination of the culture medium blanks failed to find any examples of contamination (x400 phase contrast Nikon Labophot 2; Nikon, Japan.).

Growth in flask grown experimental cultures was measured daily by counting on a haemocytometer (Section 2.3.1) and by

increase in optical density at 570 nm (Section 2.3.2). Growth of cells in microtitre plates was determined by *in situ* optical density measurement at 570 nm (Section 2.3.1). Growth rates (*k*) were calculated according to Hoogenhout & Amesz (1965).

3.2.2 Microtitre plate screening for growth in the presence of wastewater

One hundred and fifty millilitre stock cultures of the test isolates were grown up, in sterile ES medium in 250 ml conical flasks, in the Foster Room (15°C, 12:12 light : dark, 25μEm^{-2} s^{-1} PAR). One hundred millilitres of mid-log phase stock culture were sub-cultured into a mixture of 100 ml of sterile ES medium and 100 ml of fresh wastewater. Experimental cultures were shaken and 200 μl aliquots pipetted into each of the 96 wells of a microtitre plate. As controls, two sets of similar plates were set up: using only a 1:1 mixture of sterile ES medium and wastewater; and using sterile ES medium inoculated with the test isolates. Microtitre plates were incubated for nine days under standard conditions (15°C, 12:12 light : dark, 25μEm^{-2} s^{-1} PAR), in separate airtight transparent acrylic boxes lined with damp paper towels to prevent dehydration. Growth was measured daily by *in situ* optical density measurement at 570 nm (Section 2.3.1) using the 1:1 ES medium and wastewater plates as blanks. Growth rates (*k*) were calculated according to Hoogenhout & Amesz (1965).

3.2.3 Screening for metal uptake by marine microalgae

One hundred millilitres of mid-log phase stock culture were sub-cultured into triplicate 250 ml conical flasks containing 200 ml of ES medium supplemented either with cadmium chloride (final soluble metal concentrations of 10.00 ppm), or with an osmotically equivalent amount of sodium chloride (AnalaR, BDH, UK). The final soluble cadmium level, in the medium, was adjusted by atomic absorption spectrophotometry (Section 2.7).

Cultures were incubated for nine days in the Conviron incubator (15°C, 12:12 light : dark, 25$\mu Em^{-2}\ s^{-1}$ PAR). Cells were harvested and washed three times by centrifugation in ES medium, at 15°C with a pH of 8.00. Cadmium levels in the supernatant from the final washings were directly measured by AAS, in the case of cadmium being detected the final result was discarded. The washed cells were then adjusted to a standard of 10^6 cells ml^{-1} using ES medium, and triplicate 10 ml aliquots were freeze dried overnight, at < -40°C, in weighed tubes. After freeze drying the tubes were re-weighed (to four places) and sealed. To convert any metals present to the soluble chloride form the samples were digested with 1 ml of concentrated hydrochloric acid for two hours at 80°C in sealed tubes. Following resuspension in 10 ml of Milli-Q water the acidified samples were centrifuged to remove undissolved particulates and cadmium levels measured by AAS. This process was then repeated for the metals copper, iron, and manganese. Samples containing ES medium alone were used as a controls for precipitation, non-specific binding, and external

metal contamination. In all cases the measured metal content of the ES samples was found to be below the detection threshold of the technique (≤0.001% metal by dry weight).

3.2.4 The effect on microalgal growth of single heavy metals

In order to get a clearer view of the effect of heavy metals on the growth of marine microalgae the best microalga identified in the three stage screening program was grown in the presence of single metals at different concentrations in ES medium. One hundred millilitres of mid-log phase stock culture were sub-cultured into a series of triplicate 250 ml conical flasks. These flasks contained 200 ml of sterile ES medium with or without the addition of metals. Control flasks were inoculated with sufficient sodium chloride (AnalaR, BDH, UK) to give the same osmotic potential as zero ppm, 1.00 ppm, 5.00 ppm, 10.00 ppm, 50.00 ppm, and 100.00 ppm metal chloride. Experimental flasks were supplemented with sufficient metal chloride to give a final soluble metal levels of 1.00 ppm, 5.00 ppm, 10.00 ppm, 50.00 ppm, and 100.00 ppm. As before the final soluble metal levels, in the medium, were adjusted by atomic absorption spectrophotometry (Section 2.7). Similar cultures were set up containing the metals copper, iron, manganese. 200 µl aliquots of the experimental cultures were pipetted out into a separate row of a 96 well microtitre plate (i.e. 12 replicates). Cultures were incubated for eight days in the Conviron incubator at 15°C under 100µEm^{-2}S^{-1}

PAR 12:12 light : dark. Growth was measured daily by the *in situ* optical density method.

3.3 Results

3.3.1 Microtitre plate screening for growth in the presence of heavy metals

Out of over 350 marine microalgae screened only ca. 1%, *Tetraselmis* sp. (isolate TSAW92), *Dunaliella tertiolecta* (isolate of Butcher 1959), and *Phaeodactylum tricornutum* (isolate PTAW92) were found to have the ability to sustain logarithmic growth under the metal rich screening conditions (Table 2.1).

A further 10% of the microalgal isolates screened showed either slight growth or biomass maintenance under the screening conditions for at least nine days (Table 2.1), and when sub-cultured into metal free ES medium resumed growth after a long lag phase.

The remaining 89% of the microalgal isolates screened showed a rapid decrease in cell numbers, often accompanied by bleaching, and died within nine days of metal exposure. The most metal tolerant group identified by this mass screening study were the Bacillariophyceae, representing 11 out of the 32 isolates found to show some heavy metal tolerance. The genus *Tetraselmis* is also somewhat over-represented, containing four out of the 32 isolates found to show some heavy metal tolerance. Conversely, the Dinophyceae and Rhodophyceae, each contained only one isolate showing some heavy metal tolerance.

Table 3.1: Growth rate (*k*: divisions day^{-1}) of *Tetraselmis sp.* (= isolate TSAW'92), *Dunaliella tertiolecta, Phaeodactylum tricornutum,* and *Botryococcus braunii* . Twelve replicate cultures were grown in ES medium at 15°C ±0.5°C; 150 $\mu Em^{-2}s^{-1}$ PAR, in 96 well microtitre plates either with and without the addition of the standard metal cocktail (final soluble metal concentration of 10 ppm each cadmium, copper, iron, manganese, and zinc); or in 250 ml conical flasks in the absence of heavy metals. For the microtitre plate growth experiments values are means of twelve replicates ± 1 sd, in the case of flask growth experiments values are means of three replicates ± 1 sd. Figures in brackets are % of control growth rates in microtitre plates without metals.

	Microtitre Plates		Flasks
Microalgal Isolate	+ Metal	- Metal	- Metal
Tetraselmis sp. (*TSAW'92)	0.60 ±0.02 (37.5)	1.60 ±0.01 (100)	0.47 ±0.01 (29.4)
Phaeodactylum tricornutum	0.14 ±0.01 (9.3)	1.50 ±0.01 (100)	0.38 ±0.01 (25.3)
Dunaliella tertiolecta	0.31 ±0.02 (28.2)	1.10 ±0.02 (100)	0.61 ±0.02 (55.5)
Botryococcus braunii	No Growth (0)	0.85 ±0.01 (100)	0.43 ±0.02 (50.6)

* See Table 2.1 and Appendixes 1 and 2

In Table 3.1, detailed growth data from the preliminary screening experiments has been given for the three highly metal tolerant isolates, and a metal sensitive isolate *Botryococcus braunii.* All of these microalgae exhibited an increase in growth rate of between 49 and 75% when grown in microwell plates instead of the more conventional flask based systems (Table 3.1). Light intensity measurements have shown there to be 30% more light available to a microwell plate grown culture than to a culture grown in 250 ml conical flasks under the same conditions. In the presence of heavy metals all of the isolates screened showed a variable decline in growth rate of between 62.5 and 100%. Of all the microalgae screened the highest growth rate in the presence of heavy metals (0.6 divisions/day) was exhibited by TSAW 92. This isolate was identified as an atypically metal tolerant strain of the cyst forming, thick-walled, naked flagellated Prasinophyte *Tetraselmis* sp.

3.3.2 Microtitre plate screening for growth in the presence of wastewater

Of the 31 species identified as being metal tolerant in the preliminary screening experiments all were found able to tolerate 1:1 seawater diluted wastewater. One isolate, *Phaeodactylum tricornutum* (isolate PTAW92), was actually found to grow better in the presence of wastewater than in the ES medium control cultures. Microscopic analysis of the cultures showed that none of the endemic microalgal species present in the wastewater were able to contaminate the test algae. However, it was noted that a yeast was present in the *Tetraselmis* sp. (Isolate TSAW92) cultures, this

was subsequently identified as a laboratory contaminant and eliminated.

3.3.3 Screening for metal uptake by marine microalgae

Of the 4 species tested all showed some metal uptake (Table 3.2). *Tetraselmis* sp. (Isolate TSAW92) showed the highest level of metal uptake in every case. In comparison, the diatom *Phaeodactylum tricornutum* (Isolate PTAW92) took up very little of the test metals. The non-metal tolerant isolate, *Chlorella vulgaris* (NC64A), was included as a control and was found to undergo a high level of metal uptake, which was subsequently fatal. Iron was taken up to a greater extent than the other metals tested by all the test isolates. Manganese was not taken up in large amounts by any isolate. It should be noted that all of the microalgae had less than 0.01 ppm of Cd, Cu, Fe, or Mn prior to metal exposure.

3.4 Discussion

The high microalgal growth rates and short lag phases obtained in the microwell plates, coupled with the advantages of *in situ* growth measurement, resulted in this method being favoured over flask based formats. All subsequent growth and screening investigations were based in microtitre plates. The use of the microtitre plate technique enabled the rapid and accurate screening of far more isolates than would have been possible by conventional means.

Table 3.2: Heavy metal content of selected marine microalgae exposed to 10 ppm metal for nine days. Values shown are means of three replicates ± 1 sd, units are ppm.

Metal	*Tetraselmis* sp. (TSAW92)	*Dunaliella tertiolecta*	*Chlorella vulgaris* (NC64A)	*Phaeodactylum tricornutum* (PTAW92)
Cd	2.75±0.08	0.02±0.00	0.82±0.00	0.01±0.00
Cu	37.16±0.2	1.31±0.04	1.42±0.05	0.07±0.01
Fe	38.41±0.4	2.69±0.1	2.79±0.08	0.05±0.00
Mn	0.78±0.01	0.03±0.00	≤0.001	≤0.001

Microtitre plate screening identified only three highly metal tolerant isolates out of over 350 screened. The purpose of this study was to identify microalgae capable of use in an industrial environment. The screening conditions were therefore far more demanding than those employed by other workers, both in terms of the specific concentrations and the numbers of heavy metals in simultaneous use (eg *Lemanea fluvatilis* 0.1-5 ppm zinc only, Harding & Whitton, 1980; *Chlamydomonas reinhardtii* 0.2 mM Cadmium only, Collard & Matagne, 1990). This coupled with the stringent pass criteria of sustained logarithmic growth explains the low identification rate, and emphasised the need for a broad ranging study covering many hundreds of species.

The preliminary screening shows that some marine microalgae have the ability to tolerate heavy metals, but that this ability is neither common nor identical in capacity between even closely related isolates. In the case of *Tetraselmis* sp. it was found that one isolate (TSAW92) was highly metal tolerant, but that isolates of *Tetraselmis rubens*, *Tetraselmis suecica*, and *Tetraselmis verrucosa* were far less metal tolerant (Table 2.1).

The observation that several isolates were able to maintain their biomass during metal contamination could imply that, for some marine phytoplankton, an acute short-lived heavy metal contamination event may not have a catastrophic effect at the population level. A possible explanation for this is the formation of resistant cysts which has been observed in *Tetraselmis* sp. (Regan, 1988).

In the presence of heavy metals all of the isolates screened showed a variable decline in growth rate of between 62.5 and 100% (Table 3.1), which reinforces their widely known toxicity (Furr *et al.*, 1981). Of all the microalgae screened the highest growth rate in the presence of heavy metals (0.6 divisions/day) was exhibited by *Tetraselmis* sp. (TSAW 92) (Table 3.1). This isolate also showed the highest level of metal uptake (Table 3.2), and was therefore selected for further investigation.

In general it is accepted that a highly metal tolerant micro-organism is likely to exhibit reduced levels of metal uptake, where as a similar micro-organism showing high levels of metal uptake is likely to be metal sensitive (Gadd, 1988). However, in this study it was found that the characteristics of heavy metal tolerance and heavy metal uptake in marine microalgae are often independent, and are by no means always mutually exclusive.

All the metal tolerant isolates took up at least some metal, and therefore did not tolerate the screening conditions by a simple exclusion strategy. This apparent contradiction was also noted in the study of Stokes (1981) where a copper tolerant strain of *Scenedesmus acutiformis* was found to take up almost an order of magnitude more copper than a non-tolerant strain. In order to survive these high levels of metal uptake it is likely that these microalgae possesses an enhanced internal sequestration and detoxification system. Various mechanisms of this type have been proposed varying form metallothioneins to cell wall binding (see Section 1.6.4). The fact that the non-tolerant control *Chlorella vulgaris* (strain NC64A) also took up metal, but then died,

reinforces the argument for efficient intracellular metal detoxification in the tolerant isolates.

The percentage by dry weight of metal taken up was found to be highly variable; both with the same metal in different algae, and with different metals in the same alga. The isolate *Tetraselmis* sp. (TSAW92) showed high levels of uptake with each metal tested. Whereas the diatom *Phaeodactylum tricornutum* (PTAW92) was found to take up the least metal in every case. The main gross difference between *Phaeodactylum tricornutum* and the other metal tolerant isolates is the fact that it is a diatom and therefore possesses a silica impregnated cell wall. It would seem reasonable to suggest that this wall may in some way prevent metal uptake and hence cytotoxicity. Some diatoms are also known to secrete extracellular polysaccharides (EPS) which have been implicated in extracellular metal chelation (Daniel *et al.*, 1987). The Bacillariophyceae were the most metal tolerant group identified by this mass screening and represented 11 out of the 32 isolates found to show some heavy metal tolerance. *Phaeodactylum tricornutum* has been shown to be a robust and highly dominant microalga which is well suited to growth in dense cultures, being able to grow under low light intensities and to tolerate a wide range of pH's (Nelson *et al.*, 1979; Goldman *et al.*, 1982). Other workers have also commented on the ability of another diatom *Skeletonema* sp. to grow in the presence of metals (Sanders *et al.*, 1981).

The genus *Tetraselmis* was also somewhat over-represented, containing four out of the 31 isolates found to show some heavy metal tolerance. This genus was also identified in a

screening programme for growth in a 1:1 mixture of seawater and sewage (Craggs *et al.*, 1994). *Tetraselmis* species are well known for their wide temperature and salinity tolerance and have the ability to dominate even in hostile environments (Regan, 1988).

The Dinophyceae and the Rhodophyceae on the other hand, each contained only one isolate showing some heavy metal tolerance. The lack of metal tolerance in the Rhodophyceae, and in particular in *Porhyridium* sp., was unexpected. The mucilage surrounding *Porhyridium* sp., contains large amounts of poly-anionic, sulphated polysaccharides, and could therefore be expected to be an efficient metal chelator (Ramus, 1972).

Not all metals were taken up to the same extent, even by the same microalga. This indicates that microalgal metal uptake systems are somewhat selective in their action. In every case manganese gave the lowest level of uptake, and iron the highest. The physical characteristics of the metals studied are relatively similar (Table 3.3). Manganese and iron have similar relative atomic masses, and both form a divalent aqueous ion of similar radius and electronegativity. The metal selection mechanism is therefore highly discerning and does not appear to function on the basis of a simple character such as ionic charge. The observation that the level of microalgal uptake encountered for both copper and cadmium is very similar reinforces this argument (Table 3.2). These observations suggest the presence of a specific and selective metal uptake system in at least some marine microalgae. In the next chapter I shall discuss the steps taken to further characterise this system by the analysis of microalgal metal uptake kinetics.

Table 3.3: Selected physical characteristics of the metal ions investigated in this study. Taken from the Handbook of Chemistry and Physics, Chemical Rubber Company, 1982.

Metal Ion	Relative Atomic Mass	Metallic Radius (nm)	Ionic Radius (nm)	Electro-negativity (Paulings)
Cd^{2+}	112	n/a	n/a	n/a
Cu^{2+}	64	0.128	0.072	1.9
Fe^{2+}	56	0.124	0.074	1.8
Mn^{2+}	55	0.129	0.080	1.5
Zn^{2+}	65	0.133	0.074	1.6

CHAPTER FOUR

METAL UPTAKE KINETICS

4.1 Introduction

Initially, the kinetics of copper, cadmium, iron, and manganese uptake were investigated in the live cells of a range of isolates. Copper and iron uptake in both live and killed *Tetraselmis* sp. (TSAW92) was compared to determine the effect of metabolic activity on metal uptake. The relative importance of these two steps was investigated by attempting to remove copper bound to the cell walls by acidic EDTA extraction the effect of the ratio of cells to metal was examined in a series of dose response experiments. Finally, the effects of environmental variables such as temperature, pH, and salinity on metal uptake were investigated.

4.2 Materials and Methods

4.2.1 Metal removal by various live marine microalgal isolates

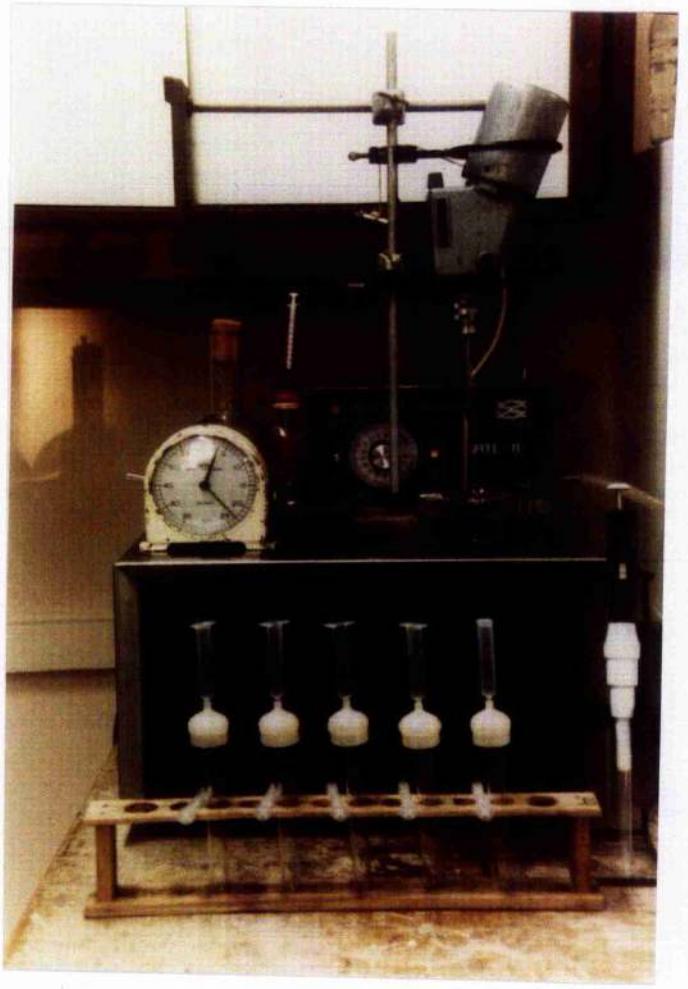
Triplicate batch cultures of *Botryococcus braunii, Chlorella vulgaris* (NC64A), *Dunaliella tertiolecta, Phaeodactylum tricornutum,* and *Tetraselmis* sp. (TSAW92) were grown under standard conditions (15°C, 12:12 light : dark, 25μEm^{-2} s^{-1} PAR) to mid-log phase in 250 ml conical flasks containing 200 ml of metal free sterile ES medium. Cells were harvested, washed three times by centrifugation in sterile ES medium, and cell numbers were adjusted to a standard density of 10^6 cells ml^{-1} by dilution with ES medium. Ninety nine ml sub-samples were allowed to equilibrate

for 15 minutes at 15°C ±0.1°C in 100 ml Pyrex beakers. A low-shear fire polished glass paddle powered by an overhead stirrer motor was used to gently mix the microalgal suspensions throughout the experiment (Plate 4.1).

At time zero, one ml of either copper, cadmium, iron, or manganese chloride stock solution was added to the microalgal suspensions to give an initial soluble metal concentration of 10 ppm. For each batch of ES medium, and each set of experimental apparatus, the strength of the metal chloride stock solution was carefully adjusted to allow for losses caused by non-specific binding and phosphate precipitation. At intervals after metal addition, five ml samples were withdrawn from the microalgal suspensions and rapidly filtered into test tubes (Swinnex-25 syringe filter; Millipore, France) containing a pre-wetted 1.2 µm GF/C filter (Whatman, Maidstone, UK). These filtrates were then immediately assayed for metal content using the AAS. Aliquots of each filtrate were also checked for the absence of cells, using light microscopy, and the results from any filtrates found to contain cells were discarded.

To investigate the effect of temperature on metal uptake by *Tetraselmis* sp. (TSAW92) the above experiment was repeated, using iron only, at the additional equilibration temperatures of five and 30°C. Iron was selected as the test metal due to its relatively high second stage uptake (Figure 4.1).

Plate 4.1: Metal Removal Time Course Measurement Apparatus.



4.2.2 Metal removal by killed cells of marine microalgal isolates

Cells of *Tetraselmis* sp. (TSAW92) and *Chlorella salina* were grown up and washed as before. In the case of live cell controls, all subsequent procedures were as above, killed cells were pasteurised for five minutes at 50°C at the final washing stage. Ninety nine ml standardised aliquots of either killed or live cells were allowed to equilibrate for 15 minutes at 15°C in the Pyrex beaker. At time zero one ml of either copper or iron chloride solution was added to give an initial soluble metal level of 10 ppm. At intervals after metal addition, 5 ml samples were withdrawn filtered and analysed.

4.2.3 Estimation of the proportion of copper bound to the cell wall and taken into the cells of *Tetraselmis* sp. (TSAW92)

A standard suspension of 10^6 cells ml^{-1} of *Tetraselmis* sp. (TSAW92) was prepared in the usual way and allowed to equilibrate to 15°C. At time zero one ml of a copper stock solution was added to the medium to give an initial copper level of 10 ppm. After 15 seconds a five ml sub-sample was withdrawn and filtered to remove cells. Copper levels in the filtrate were measured by AAS , and the filter paper was placed in five ml of 0.1 M di-sodium ethylenediaminetetra-acetic acid (EDTA) in ES medium at pH 6.0 and stirred for one minute. The solution was then re-filtered and the level of copper in this filtrate was measured by AAS. The filter paper was washed with four ml of deionised water, and one ml of

concentrated hydrochloric acid (AnalaR, BDH, UK) was added to the washings to release any copper present within the cells. As a control the same procedures were carried out with sodium chloride substituting for the copper stock solution, copper levels were found to be below the detection threshold of the technique ≤0.01 ppm.

4.2.4 Copper removal by *Tetraselmis* sp. (TSAW92) from different initial copper concentrations

Live cells of *Tetraselmis* sp. (TSAW92) were prepared as above, and a 99 ml aliquot was placed in the Pyrex beaker to equilibrate to 15°C. At time zero, one ml of a copper chloride stock solution was added to the microalgal suspensions to give soluble copper concentrations of either 1, 5, or 10 ppm. As before the strength of this copper chloride stock solution had previously been adjusted, using the AAS, to give the correct initial copper concentration. At intervals after metal addition, five ml samples were withdrawn filtered and analysed in the usual way. For this experiment the usual suspension of 10^6 cells was diluted by half with ES medium, in order to reduce the rate of the initial metal uptake.

4.2.5 The effect of pH and salinity on copper removal by live *Tetraselmis* sp. (TSAW92) in ES medium

Three one litre batches of sterile ES medium were prepared, and adjusted to either pH 3.00, 8.00, or 10.00 by the addition of 2 M HCl and 1 M NaOH (AnalaR, BDH, UK). An additional two batches of sterile ES medium were prepared with half and double the normal amounts of distilled water. For each of these pH and salinity levels a standard copper removal experiment was carried out as above.

4.2.6 Statistical analysis of experimental data

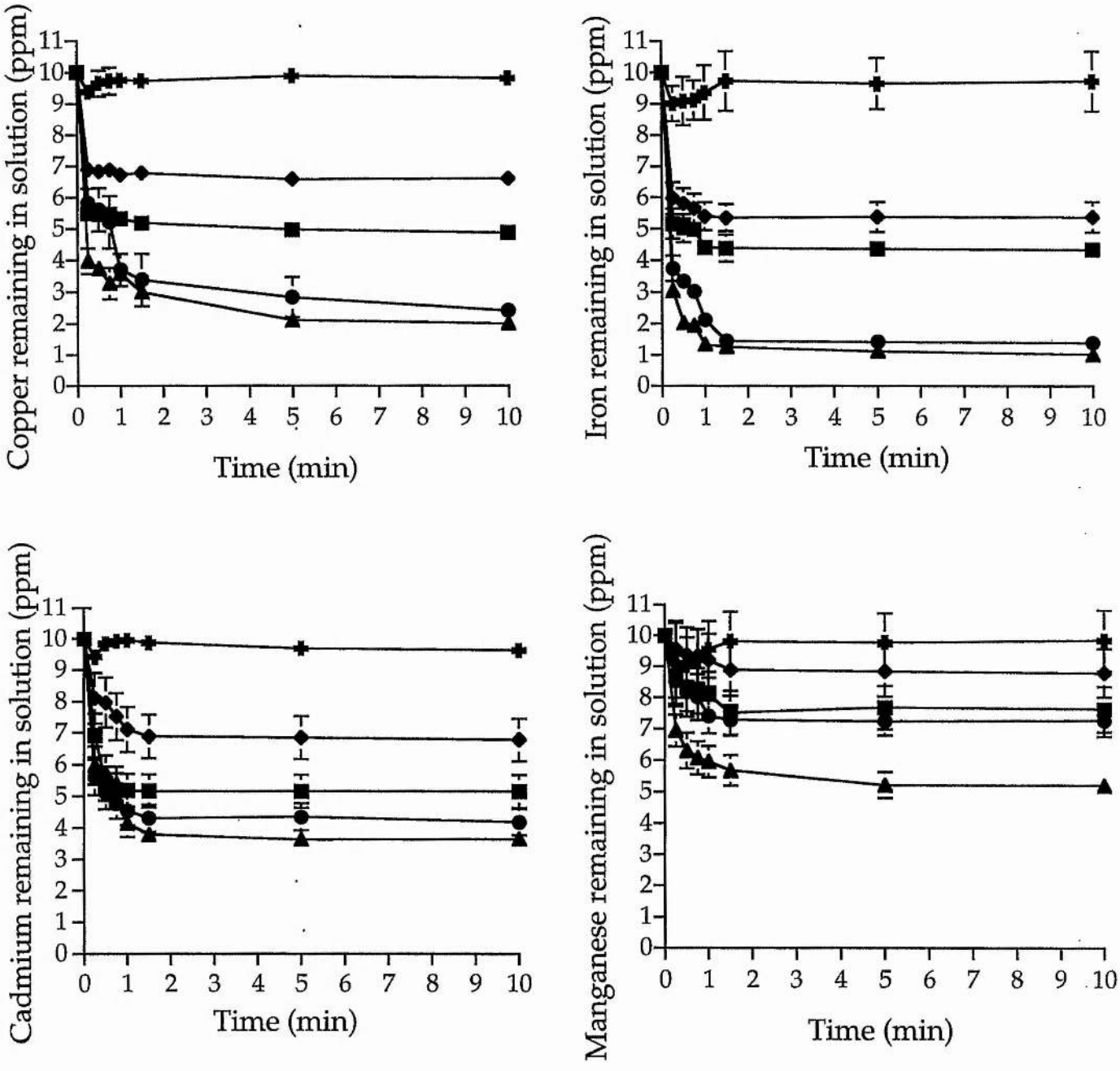
Prior to the application of parametric statistics, all data were checked for normality by the simplified correlation to normal scores method (Cramer, 1946), no abnormally distributed data were identified. Second stage metal removal rates were established using the gradients of metal removal graphs from the last three data points by microcomputer analysis (Cricket Graph III version 1.5, 1992-93, Computer Associates International Inc., New York, USA). To enhance accuracy gradients were calculated for each replicate and tested separately. Potential differences in metal removal rates were tested using the product-moment correlation coefficient (r) (Cramer, 1946). All statistical tests were performed by microcomputer using the Minitab computer package (Minitab version 8.2, 1991, Minitab Inc., Washington, USA).

4.3 Results

4.3.1 Metal removal by various live marine microalgal isolates

Time courses for metal removal by live cells of various marine microalgal isolates are presented in Figure 4.1. In all cases, metal uptake was found to be a two stage process, with an initial rapid stage during the first one to two minutes, followed by a slower but continuous stage. Of the isolates tested, *Tetraselmis* sp. (TSAW92) proved to have the greatest metal removal ability. This ability was closely matched by the non-metal tolerant isolate *Chlorella vulgaris* (NC64A). In *Chlorella vulgaris*, it can be seen that the second stage of copper uptake remains at a high level (0.01 ppm cu min^{-1}, r=0.861) for at least five minutes longer than that of *Tetraselmis* sp. (TSAW92). Lower capacities of metal removal were shown by *Dunaliella tertiolecta* and *Botryococcus braunii*, while the diatom *Phaeodactylum tricornutum* showed no significant metal removal. Copper and cadmium removal were found to be similar for each of the algae tested. In every case, the removal of iron was found to be faster and more complete than the removal of any other metal tested. On the other hand, manganese removal proceeded far more slowly and was less complete than that of any other metal tested.

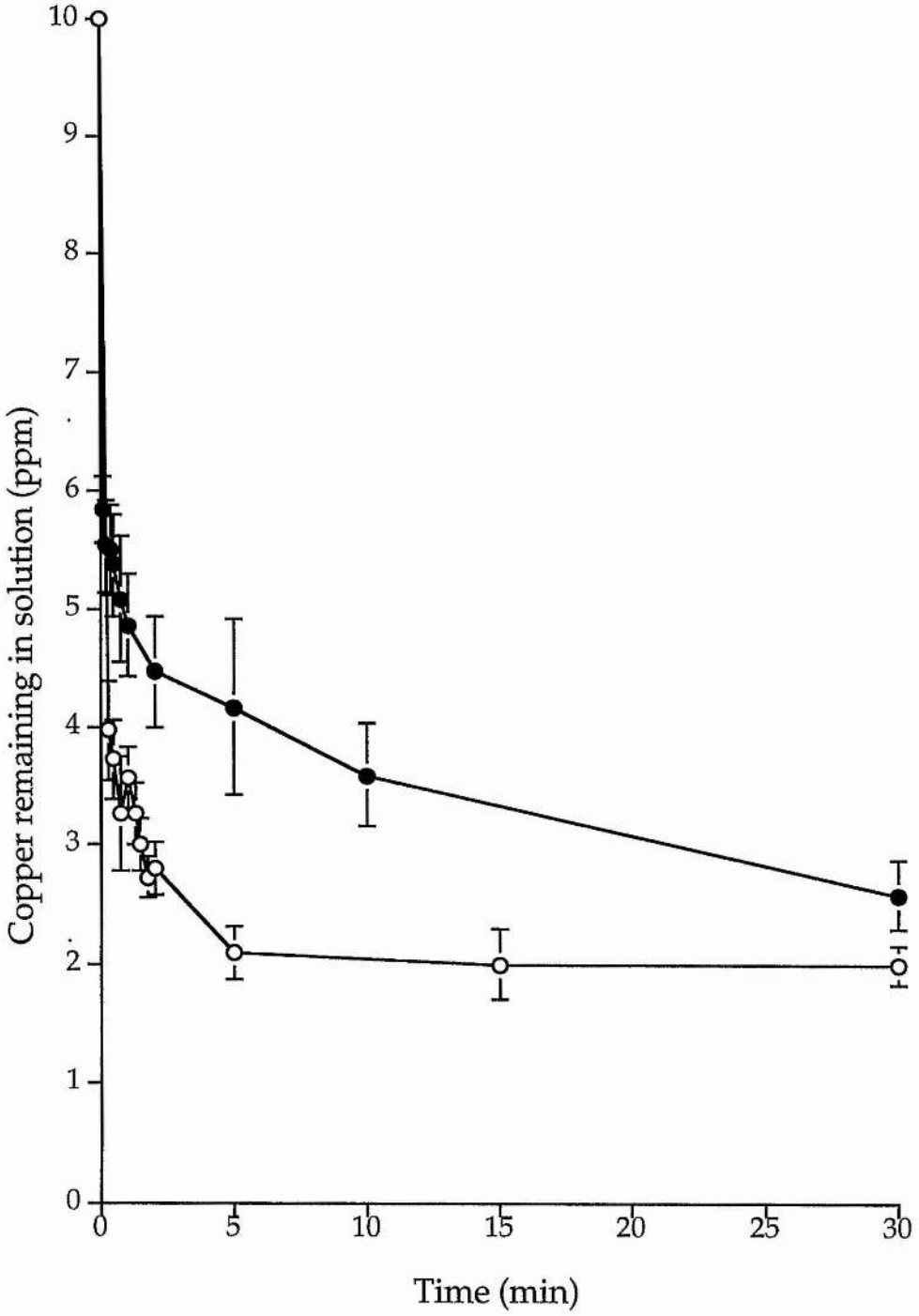
Figure 4.1: The removal of metals from a 10 ppm solution in ES medium under standard conditions by 10^6 live cells of: *Tetraselmis sp.* (TSAW92) (▲—▲); *Chlorella vulgaris* (NC64A) (●—●); *Dunaliella tertiolecta* (■—■); *Botryococcus braunii* (◆—◆); and *Phaeodactylum tricornutum* (✚—✚).



4.3.2 Metal removal by killed cells of marine microalgal isolates

Figure 4.2 shows the removal of copper from solution by equivalent numbers of live or killed *Tetraselmis* sp. (TSAW92) cells. In the case of live cells, a rapid copper uptake step occurred during the first two minutes after copper exposure. This rapid step then tailed off into a far slower uptake step, which continued for at least the next 28 minutes. In killed cells, only the initial rapid stage of uptake was exhibited. This rapid uptake step was found to be more efficient in terms of both speed and capacity in killed cells than in live cells, and removed copper down to almost half the level found with live cells (Figure 4.2).

Figure 4.2: The removal of copper from a 10 ppm solution, in ES medium under standard conditions, by 10^6 cells of live (●—●) and killed (○—○) *Tetraselmis sp.* (TSAW92). Values given are means of three replicates, ±1 sd.



4.3.3 Estimation of the proportion of copper bound to the cell wall and taken into live cells of *Tetraselmis* sp. (TSAW92).

In order to localise the copper which had been removed from solution it was necessary to differentiate between copper within microalgal cells and copper on microalgal cells. This was achieved by measuring the total amount of copper removed from solution, then applying EDTA to remove copper from the cell surface, and finally measuring the level of copper released from within the cells by acid digestion.

After 15 seconds, 6.4 ppm of copper had been removed from the initial 10 ppm solution by cell wall binding and almost no copper had entered the cells (Table 4.1). After ten minutes 9.1 ppm of copper had been removed form solution; with 77% on the cell wall and 23% within the cell. In both cases the total amount of copper initially added closely matched that recovered from the cell contents and cell wall (Table 4.1).

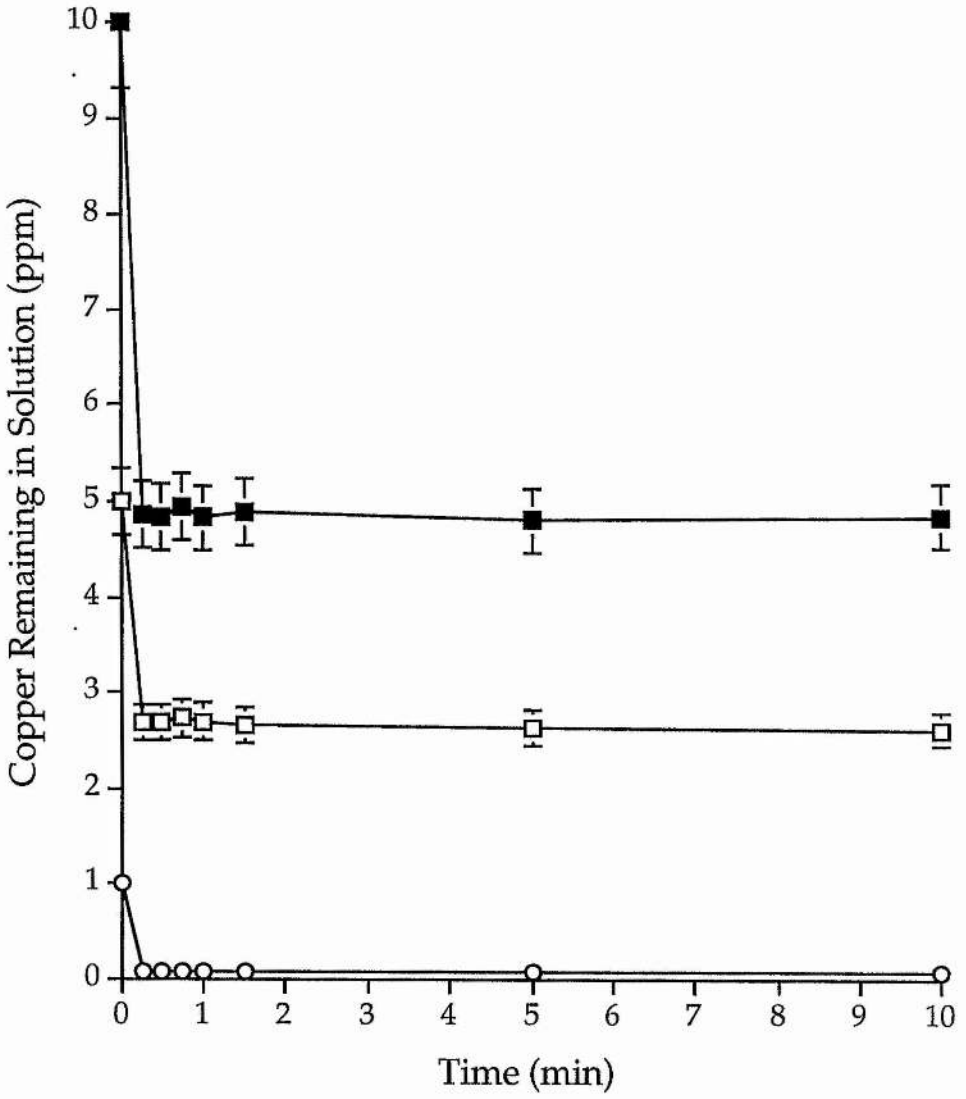
4.3.4 Copper removal by *Tetraselmis* sp. (TSAW92) from different initial copper concentrations.

The effect of altering the initial copper concentration on uptake live *Tetraselmis* sp. (TSAW92) cells is shown in Figure 4.3. The initial rapid uptake step removed approximately half of the

Table 4.1: Estimation of the proportion of copper bound to the cell wall and taken into live cells of *Tetraselmis* sp. (TSAW92).

Initial Copper Incubation Time (s)	Copper Remaining in Solution (ppm)	Copper on Cell Wall (ppm)	Copper Inside Cells (ppm)
15	3.6±0.21	6.2±0.39	≤0.01
600	1.9±0.17	7.0±0.45	2.1±0.20

Figure 4.3: The removal of copper from 10 (■—■), 5 (□—□), and 1 (○—○) ppm solutions, in ES medium under standard conditions, by 10^6 cells of live *Tetraselmis* sp. (TSAW92). Values given are means of three replicates, ±1 sd. Note some error bars are too small to be visible.



starting metal concentration in each case. The rate of this removal increased as the starting metal concentration was reduced.

4.3.5 The effect of pH on copper removal by live *Tetraselmis* sp. (TSAW92) in ES medium.

At both pH 3.0 and pH 10.0, the amount of copper removed during the first stage of uptake was considerably reduced (Figure 4.4). There was no apparent pH effect on the second stage of copper uptake.

4.3.6 The effect of temperature on iron removal by live *Tetraselmis* sp. (TSAW92) in ES medium.

The first stage of iron uptake in live *Tetraselmis* sp. (TSAW92) was unaffected by increasing and decreasing the incubation temperature. However, at both 5 and 30°C the second stage of iron uptake was reduced (Figure 4.5). In the case of copper no differences were seen, hence the use of iron. No temperature effects were seen with copper.

4.3.7 The effect of salinity on copper removal by live TSAW92

Halving the salinity of the ES medium caused a very slight reduction in both the first and second stages of copper removal by *Tetraselmis* sp. (TSAW92) (Figure 4.6). A two fold increase in salinity caused a reduction in both the rate and capacity of the first

Figure 4.4: Copper removal from a 10 ppm solution in ES medium under standard conditions by 10^8 cells of live Tetraselmis sp. (TSAW92) at pH 10 (▲—▲), 8 (●—●), and 3 (▼—▼). Values given are means of three replicates ±1 s.d.

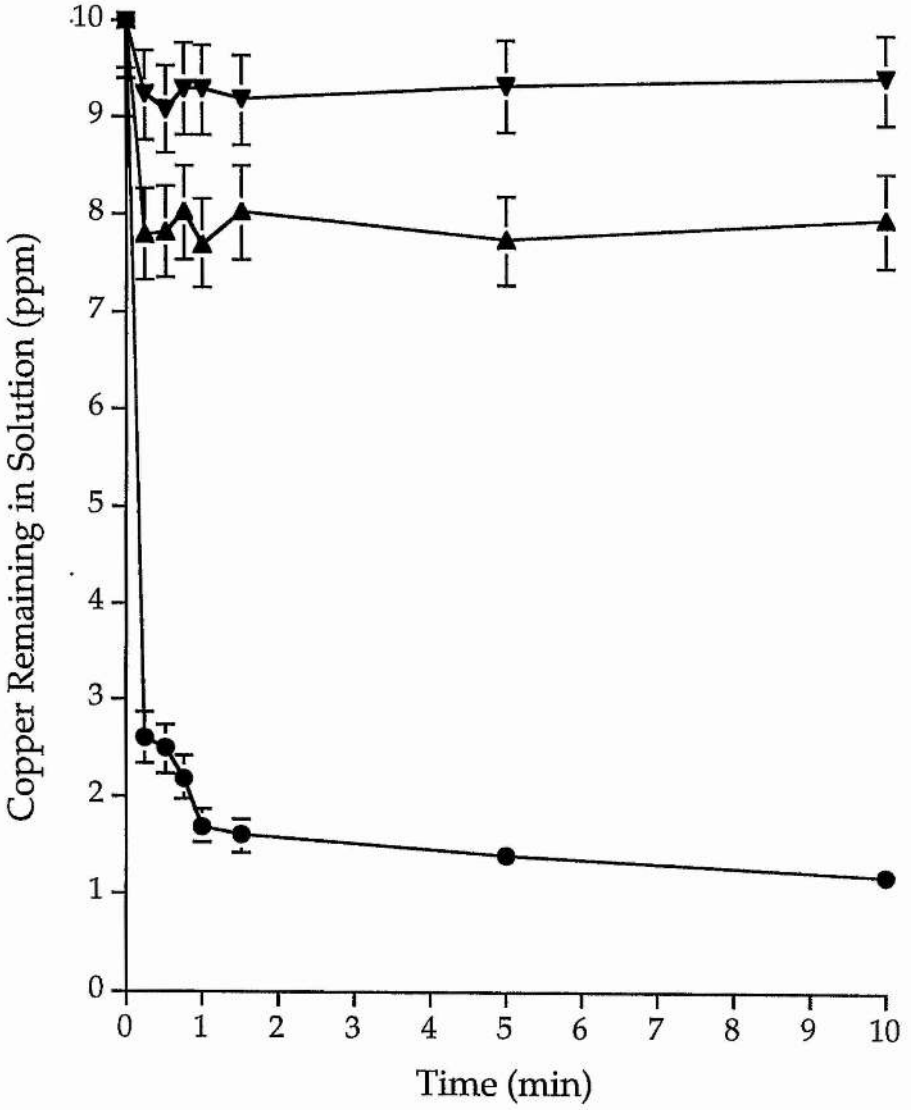


Figure 4.5: Iron removal from a 10 ppm solution in ES medium by 10^5 cells of live *Tetraselmis sp.* (TSAW92) under standard conditions at 5 (✳—✳), 15 (■—■), and 30°C (○—○). Values given are means of three replicates ±1 s.d.

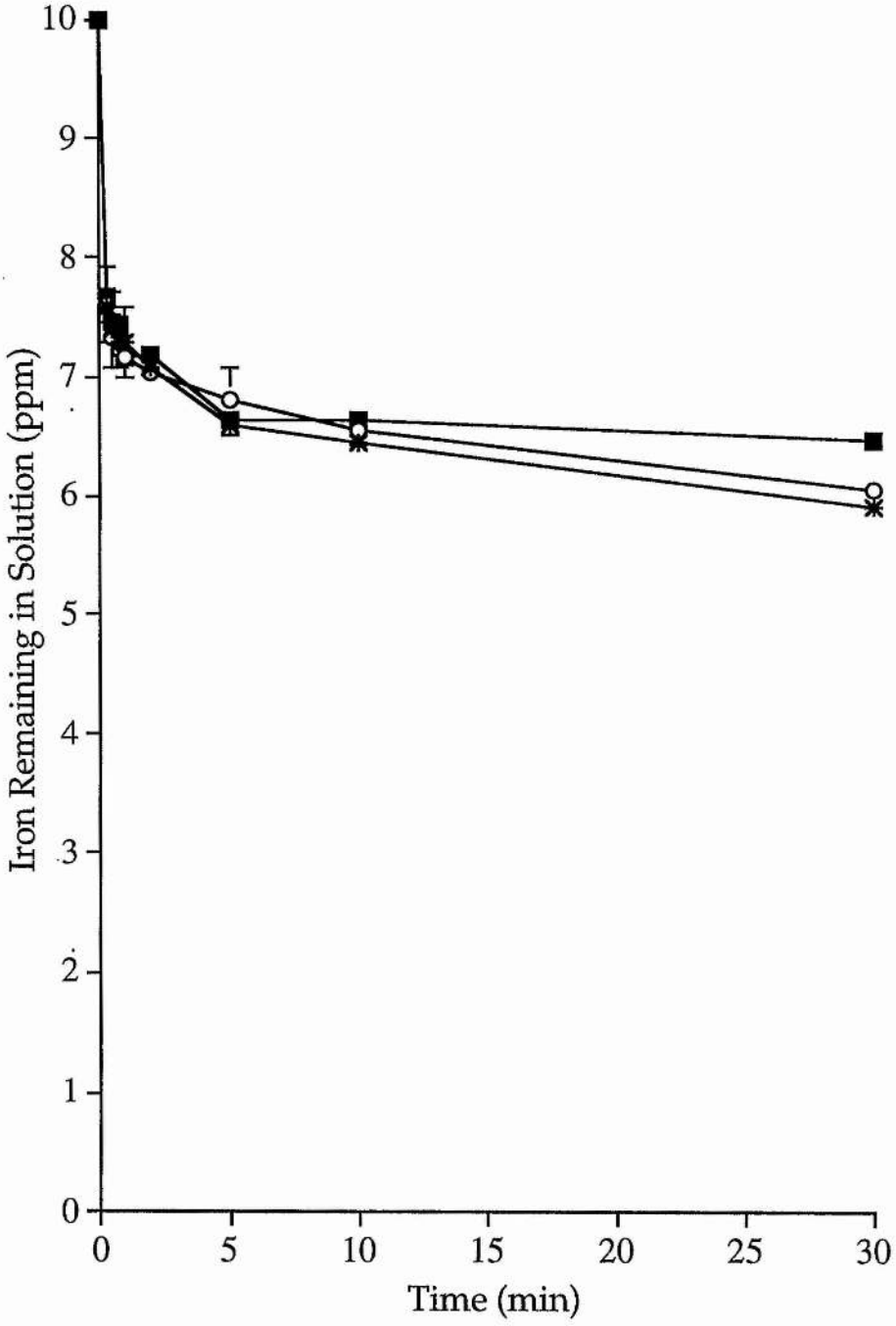
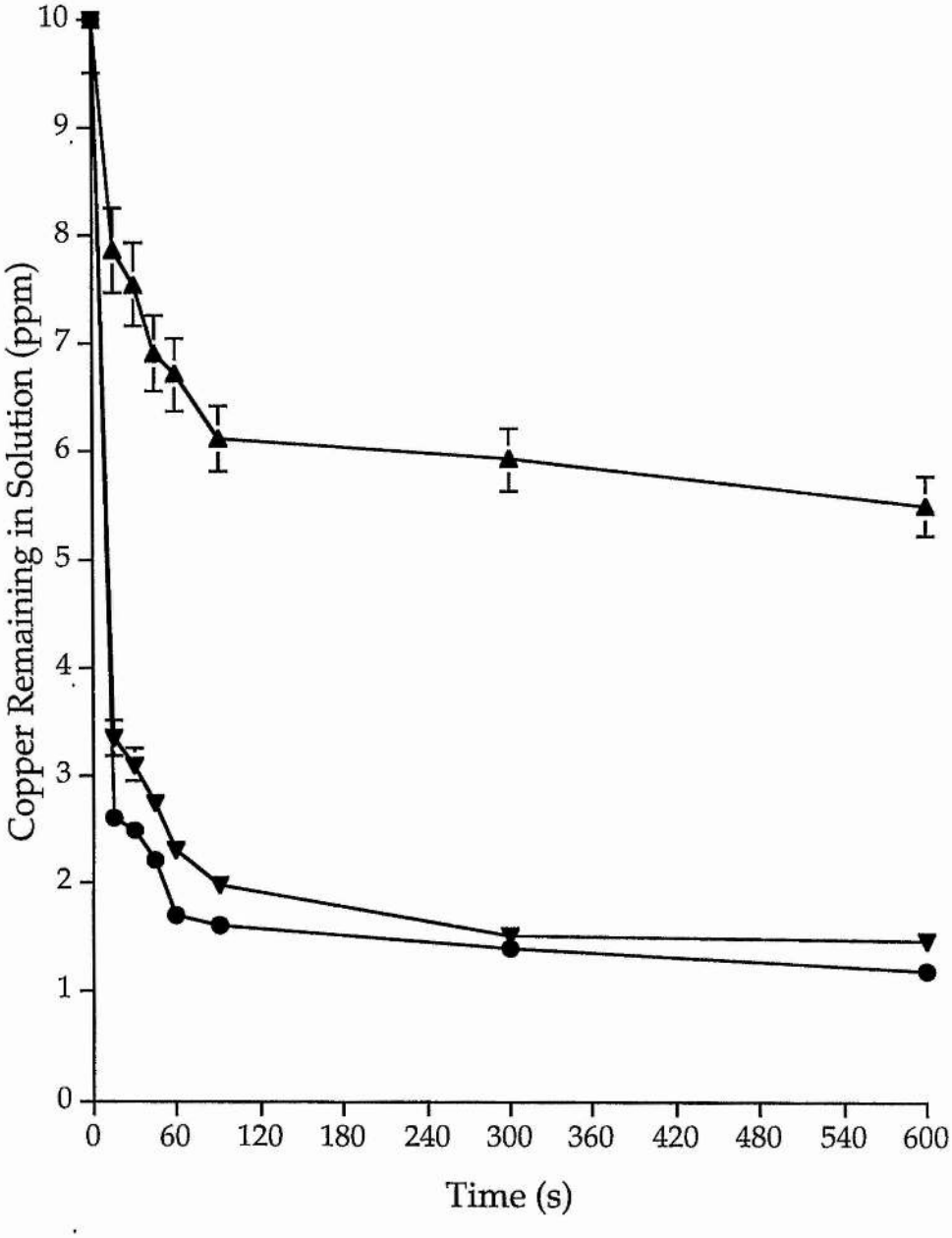


Figure 4.6: Copper removal from a 10 ppm solution in normal (●—●), double (▲—▲), and half (▼—▼) strength ES medium under standard conditions by 10^8 cells of live Tetraselmis sp. Values given are means of three replicates ±1 s.d.



stage of copper uptake, but caused a very slight increase in copper removal by the second stage of uptake.

4.4 Discussion

4.4.1 Biphasic metal uptake

It is clear that heavy metal uptake by the marine microalgal isolates tested follows the same general pattern both with different algae and with different metals. Metal uptake was a two phase process with an initial rapid stage followed by a slower second stage. The initial step saturated very rapidly and was effectively complete within two minutes. However, the exact point of saturation is unclear as the two stages of uptake are concurrent rather than sequential (Figure 4.1). The first stage of microalgal metal uptake is thought to rely on simple adsorption by charged groups on the cell wall (Ford & Mitchell, 1992; Gadd, 1988), and as such it occurs independently of metabolism. This metabolism independence has been confirmed in *Chlorella emersonii* (211 8b) by the use of 3,3,4-dichlorophenyl-1,1-dimethylurea (DCMU), a metabolic inhibitor (Garnham *et al.*, 1992). In the present study, it can be seen that the first stage of metal uptake occurs in both living and killed *Tetraselmis* sp. (TSAW92) (Figure 4.2). Oddly, in killed cells the initial uptake is more rapid and of higher capacity in comparison to that seen in live cells. A similar effect has been reported for strontium uptake in a variety of marine phytoplankton, for copper uptake in scalded *Chlorella vulgaris* , and for zinc uptake by the

thalli of *Ulva lactuca* (Rice, 1956; Hassall, 1963). One possible explanation for this is that when the cells are heat killed mucilage and debris is removed from the outer surface of the cell wall, leaving more available sites for subsequent metal binding. This is consistent with reduction in first stage binding rate and capacity which is seen when the cells are killed by freeze drying rather than by heating (Chapter 5). Gadd (1990) reported that work by Glooschenco suggested that the maximum possible mercury uptake by the marine diatom *Chaetoceros costatum* , was obtained by killing with formalin. It is therefore important to pay attention to the method of preparation when considering the use of killed microalgal biomass as a metal biosorbent.

4.4.2 Metal uptake by live marine microalgae

The metal uptake kinetics of five different isolates of marine microalgae (Figure 4.1) show a strong correlation with the final metal loading levels measured in Chapter 3 (Table 3.2). *Tetraselmis* sp. (TSAW92) shows both the most rapid and complete removal of metals, and the highest final metal loading of all the isolates tested. The diatom *Phaeodactylum tricornutum* showed almost no metal loading and exhibited no significant metal removal during the time-course experiments, reinforcing its status as a metal tolerant excluder. By comparing cadmium toxicity in normal and cell wall lacking strains of *Chlamydomonas reinhardtii,* this exclusion strategy has been shown to be due to the cell wall (Collard & Matagne, 1990). The lack of even the initial stage of metal uptake

indicates that there is some fundamental difference between the cell walls of *Phaeodactylum tricornutum* and the other isolates tested. It is highly unlikely that the cell wall of this microalga contains no charged groups suitable for metal binding as it has been shown to contain polymers of xylose, mannose, fucose, and galactose (Lewin *et al.*, 1958). However, the cell walls of diatoms, such as *Phaeodactylum tricornutum*, are often coated with a layer of polysaccharide containing mucilage (Daniel *et al.*, 1987). It is possible that this mucilage obscures and blocks potential metal binding sites on the cell wall. It is also possible that metals bind to polysaccharides in the mucilage rather than to the cell wall, and that when the sample is pressure filtered this mucilage is stripped off and wrongly included with the filtrate, giving an artificially low reading for metal removal. It would be possible to verify this directly by SEM (scanning electron microscope) examination of the cells both before and after filtration, or indirectly by measuring the polysaccharide levels in the filtrate.

The non-metal tolerant isolate *Chlorella vulgaris* (NC64A) showed a rapid and complete level of metal uptake second only to that of *Tetraselmis* sp. (TSAW92). Interestingly, the second metabolism-dependent phase of copper uptake in *Chlorella vulgaris* (NC64A) was greater than that of *Tetraselmis* sp. (TSAW92) (Figure 4.1). It is possible that this enhanced second stage of uptake overwhelms the copper detoxification systems of this microalga and so causes cell death. In *Tetraselmis* sp. (TSAW92), the second stage of copper uptake is far slower and would result in a lower demand on the intracellular detoxification systems of this microalga.

However, it must be noted that at least one worker (Gadd, 1988) has suggested that one symptom of microalgal metal toxicity is an increase in cell wall permeability to metals. This could mean that *Chlorella vulgaris* (NC64A) is caught in a feedback loop with copper uptake leading to cytotoxicity and cell wall permeability, causing enhanced copper uptake, and so on. This hypothesis will be examined in more detail in Chapter 5.

When comparing the uptake of various metals by *Tetraselmis* sp. (TSAW92) it can be seen that there are few major differences in terms of both uptake rate and capacity (Figure 4.1). However, iron uptake appears to be more rapid and more complete than the uptake of any other metal tested. It is known that bacteria possess specific iron transport mechanisms of high affinity and capacity, often based on carrier molecules called siderophores (Davis & Byres, 1971). It is possible that microalgae possess a similar or equivalent system, as iron is a common limiting nutrient for marine microalgae (Scoy & Coale, 1994). This hypothesis is supported by the fact that the rate and capacity of the second metabolism-dependent stage of iron uptake was higher than that of the other metals (Figure 4.1). The implication of this observation is that the microalgal cell wall is not acting as a simple non-specific metal binder, but that there is some degree of selectivity in the metal binding.

By stripping bound copper from the cell wall using acidic EDTA it was possible to differentiate between copper within and copper on *Tetraselmis* sp. (TSAW92) cells (Table 4.1). In the period from zero to 15 seconds, 6.4 ppm of copper had been removed from

solution by cell wall binding alone with almost no copper within the cells. This suggests that intracellular metal uptake proceeds only after initial binding to the cell wall and is partially reversible for at least 15 seconds. After ten minutes 77% of the removed copper was on the cell wall and 23% was within the cell, this ratio is very similar to that reported by Gadd (1988) for cadmium uptake by *Chlorella vulgaris* (80% : 20%). However, bias in favour of cell wall metal uptake is not always the case, in *Eremosphaera viridis* for example almost all of the bound metal is located within the cells (Gadd, 1990).

4.4.3 First stage binding

Gadd (1988) suggested that microalgal metabolism-independent metal uptake is usually complete within five to ten minutes. In most studies of this type the microalgal cells are separated from the metal containing solution by centrifugation which places severe limits on the maximum temporal resolution available (>10 min Avery *et al.*, 1993a; >5 min Garnham *et al.*, 1992). Metal removal by the microalgae begins at the moment when the metal is added, and continues while the centrifuge is loaded, spun to speed, run, braked, and unloaded. In fact metal removal is only halted when the supernatant is finally separated from the cells, which places a lower limit of approximately five minutes on the first measurement of metal removal. In this study total and virtually instant separation of cells and supernatant was achieved by micro-filtration, allowing for temporal resolution down to tens

of seconds. This technique showed that the initial stage of metal uptake was all but complete within one minute and that most of the metal removal actually occurs in less than ten seconds (Figure 4.1). Measurements of metal removal between zero and ten seconds were found to be highly variable and inconsistent in nature. Addition of metal chloride solutions to deionised water showed that this high variability was due to inadequate mixing of the injected metal with the bulk solution. Attempts to improve mixing by vortexing and high -speed stirring were found to result in damage to and rupturing of the microalgal cells. The speed of first stage removal has very important implications for the industrial use of a microalgal based metal remover. The bulk of the microalgal metal removal capacity could be saturated during a far shorter exposure than was previously suggested by conventional techniques of metal uptake analysis. This would allow for even higher contaminant flow rates and for more limited retention times, hence cutting both capital and operating costs.

The initial stage of metal uptake is highly predictable in nature and follows relatively simple dose response kinetics (Figure 4.3). Which is consistent with the idea that the microalgal cell wall contains a finite and relatively fixed number of potential metal binding sites and that when these are filled no more metal may be removed from solution. The fact that the rate of the initial uptake step was found to drop as the starting concentration of copper was increased also supports this hypothesis by indicating that as saturation nears it becomes less and less likely that a metal ion will encounter an unoccupied binding site. The predictable nature of

this initial stage of metal uptake would allow the application of standard biotechnology and engineering equations (Khummongkol *et al.*, 1982). This would be of enormous value in process design and scaling up, it would for example allow one to predict the amount of algae required to remove a given amount of metal from a given solution at a given flow rate, and to predict when the microalgal absorbent would reach saturation and need to be replaced.

4.4.4 The effects of environmental variables on microalgal metal uptake

Iron was selected to study the effect of temperature on metal uptake by *Tetraselmis* sp. (TSAW92), as it had previously been shown to have a high rate of second stage metabolism-dependent uptake. However, even at the extremes of the microalga's tolerance temperature (Regan, 1988) little effect was seen on iron uptake (Figure 4.5). The initial stage of metal uptake was not significantly altered by either extreme of temperature. Trevors *et al.* (1986) report that initial microbial cadmium uptake was largely unaffected by temperatures of 0 to 30°C. In *Tetraselmis* sp. (TSAW92) this observation holds true for the initial stage of copper uptake, but a negligible reduction was seen in the second stage of uptake at both 0 and 30°C.

The first stage of metal uptake was adversely affected by changes in pH, presumably due to its reliance on charged groups (Figure 4.4). As the pH moves away from the normal (pH 8.0) it is

likely that the surface charges on the microalgal cell wall alter, and that any carriers or specific binding loci begin to lose their shape and tertiary structure, hence reducing metal binding and uptake. Both of these phenomena would severely hamper the binding of metals and could have critical implications in industrial processes. Fortunately seawater is a well buffered alkaline medium and would strongly resist such pH changes.

In contrast to this study, in *Chlorella salina* it is reported that caesium accumulation after two hours was "similar" over a range of pH from pH 7.0 to pH 10.0 (Avery *et al.*, 1993a). However, no data is given and the authors state that caesium accumulation was approximately 16% greater at pH 8.0 than at pH 7.0, which is consistent with the observed effect in *Tetraselmis* sp. (TSAW92). In common with the present study copper uptake in *Scenedesmus* sp. has been shown to be highly pH dependent with acidic conditions having a marked inhibitory effect (Mirele & Stokes, 1976).

In the marine macroalga, *Ascophyllum nodosum,* the initial step of cobalt uptake is known to be a result of ion exchange with Ca^{2+} and H^{+} ions (Kuyucak & Volesky, 1988), it is therefore reasonable to assume that an increase in external H^{+} ion concentration would have an inhibitory effect on this uptake system. In a seawater matrix, acidification would also lead to the conversion of insoluble calcium salts (eg calcium phosphate) to soluble Ca^{2+} ions. Indirect evidence of this pH effect was also seen in *Hormidium rivulare* by Hargreaves & Whitton (1976), where an increase in zinc toxicity was observed as the pH was increased.

Conversely, in one case acidic conditions (pH 2) were found to encourage gold and silver uptake by *Chlorella vulgaris* (Darnall *et al.*, 1986). This observation is not as contradictory as it may first seem, the initial stage of metal uptake is dependent upon the charge and shape of various cell wall sites and it is possible that a lowering of pH could just as easily improve as impair the metal binding ability of these sites. In fact it could be argued that microalgae under normal conditions would be less likely to take up large amounts of toxic metals than those under abnormal conditions.

Dilution of the seawater based culture medium by half caused only a slight reduction in both stages of copper uptake. However, a doubling of salinity was found to cause more than a 50% reduction in the first stage of uptake. This cannot be explained by increased chelation and precipitation in the double strength medium, as these factors were taken into account by adjustment of the strength of the initial copper stock solution. Therefore, it is likely that the reduction in copper removal is due to competition for binding sites between copper and the other ions present in the ES medium. In *Scenedesmus accuminatus* calcium ions (Ca^{2+}) have been shown to have a strong inhibitory effect on copper uptake, thought to be caused by direct competition for binding sites (Mirele & Stokes, 1976). This ionic competition effect is commonly reported in the case of conventional ion-exchange resins (pers. comm. Pharmacia technical support). Gimmler *et al.* (1981) found that increased salt stress reduced the toxicity of copper to the microalga *Dunaliella parva.* .In *Chlorella salina* salt stress was found to increase caesium removal by a factor of 28. The explanation given for this is that

caesium uptake is mediated by potassium transport systems (Avery *et al.*, 1993) and that a common halotolerance mechanism in micro-organisms is potassium accumulation (Pick *et al.*, 1986 *Dunaliella salina*; Walderhaug *et al.*, 1987 *Escherichia coli* ; and Gilmour, 1990)

4.4.5 Summary

Marine microalgal metal uptake is a two step process, with an initial rapid, saturable, binding stage followed by a slower, non-saturable, continuous uptake step. Both the first and second steps appear to be capable of responding differently to different metals, with iron being taken up at high rates and manganese at low rates. Altered pH and increased salinity had dramatic effects on the first uptake step, but little effect on the second stage. Alterations in temperature, to the limits of microalgal tolerance, had little effect on the first stage and a slight effect on the second stage of metal uptake. Both of these observations are consistent with a metabolism-independent first stage, followed by a metabolism-dependant second stage. The characterisation of the second stage of microalgal metal uptake will be examined in detail in the next chapter.

CHAPTER FIVE

METAL BINDING PROTEINS

5.1 Introduction

It was noticed that a dense foam formed on the surface of aerated cultures of *Tetraselmis* sp. (TSAW92) when they were grown in the presence of heavy metals. A standard protein assay using Coomassie blue showed that this foam was protein rich. Higher plants possess proteins called phytochelatins which are involved in the regulation and detoxification of heavy metals (Grill*et al.*, 1985), these proteins are also present in green algae (Gekeler *et al.*, 1988). In both cases the proteins in question were located in, and thought to act within, the cells. The presence of the protein rich foam on metal treated *Tetraselmis* sp. (TSAW92) cultures suggested that in this instance there may be phytochelatin proteins outwith the cells. The purpose of the work described in this chapter was to identify this protein and to determine if it plays a role in heavy metal detoxification.

5.2 Materials and Methods

5.2.1 The effect of copper on the extracellular protein concentration in *Tetraselmis* sp. (TSAW92) cultures

Two sets (A & B) of triplicate *Tetraselmis* sp. (TSAW92) cultures were grown to mid-log phase under standard conditions in the Conviron incubator (15°C, 12:12 light : dark, 25$\mu Em^{-2}\ s^{-1}$ PAR). At time zero, the experimental set of cultures (A) were inoculated with sufficient copper chloride to give a 10 ppm solution. The

control cultures (B) were inoculated with an osmotically equivalent amount of sodium chloride. After 72 hours both sets of cultures (A & B) were spun free of cells by centrifugation (4°C, at 5,000 xg for ten minutes), and the level of protein in the supernatants was measured using Coomassie reagent (Chapter 2). Samples of centrifuged cells were examined by light microscopy to check that they were intact.

5.2.2 SDS Polyacrylamide gel electrophoresis (SDS PAGE) of *Tetraselmis* sp. (TSAW 92) extracts

Triplicate sets of *Tetraselmis* sp. (TSAW 92) cultures were grown in ES medium under standard conditions (15°C, 12:12 light : dark, 25$\mu Em^{-2}\ s^{-1}$ PAR) to mid log phase. Copper, cadmium, manganese, iron, nickel, or zinc chloride was added to give a dissolved metal concentration of 10 ppm. Control cultures were spiked with an osmotically equivalent amount of sodium chloride. Heat shocked cultures were treated as control cultures but were incubated at 35°C for 24 hours. The media used for osmotically shocked cultures was either double or half strength ES medium.

After 24 hours, 100 ml samples were withdrawn from each culture and filtered thorough low protein binding capacity membrane filters (Cat. TSW/PVDF 0.2 µm, Millipore, France) with a glassfibre pre-filter (GF(C), Whatman, UK). The filtrate was desalted by dialysis, freeze-dried, and re-suspended in 1 ml of 0.1 M NaCl buffered to pH 8.0 with 20 mM phosphate. Samples were centrifuged (13,000 xg, for five minutes) to sediment particulates,

and the supernatant was decanted into fresh tubes. The samples of supernatant were then run on discontinuous SDS PAGE gels: 4% pH 6.8 stacking gel, 12% pH 8.8 separating gel (Laemmli, 1970). Gels were run at room temperature on a BioRad Mini Protean II gel rig (BioRad, UK) at 200v for 1 hour. After rinsing, the gel was stained in Coomassie brilliant blue G250 for two hours and de-stained overnight. Where necessary, gels were stored in the dark at 4°C under de-stain prior to photography.

5.2.3 The effect of heat and osmotic shock on copper toxicity in *Tetraselmis* sp. (TSAW 92)

On day zero, three sets of triplicate cultures of *Tetraselmis* sp. (TSAW 92) were inoculated into 250 ml conical flasks containing 200 ml of sterile ES medium (sets A, E, and F). On day 3, a further five similar sets of triplicate cultures were started (sets B, C, and D). At mid-log phase, cultures from Sets A, E and F were transferred to a 35°C water bath in the Conviron Incubator. Seventy two hours later all cultures were harvested by centrifugation (4°C, at 5,000 xg for ten minutes). Set A cultures were resuspended in fresh sterile ES medium containing one of 10, 50, 100, 150, 200, 250, 300, 350, 400, 450, or 500 ppm of copper. The controls (set B cultures) were resuspended in normal strength sterile ES medium containing no copper. Sets C and D were resuspended in half strength sterile ES medium, and Sets E and F were resuspended in double strength sterile ES medium, in both cases in the range of copper concentrations shown above. All cultures were then returned to

the Conviron incubator (15°C, 12:12 light : dark, 25μEm^{-2} s^{-1} PAR). After a further week culture health was scored by the presence or absence of a green coloration as determined by eye (Section 3.2.4).

5.2.4 The effect of copper on the free amino acid content of *Tetraselmis* sp. (TSAW 92) cells

Two sets of triplicate 250 ml cultures of *Tetraselmis* sp. (TSAW 92) were grown to mid log phase under standard conditions in the Conviron incubator (15°C, 12:12 light:dark, 25μEm^{-2} s^{-1} PAR). The experimental set of cultures was inoculated with sufficient copper chloride to give a 50 ppm solution, and the control set was inoculated with an osmotically equivalent amount of sodium chloride.

After 72 hours incubation, both sets of cultures were washed three times by centrifugation (4°C, at 5,000 xg for five minutes) in metal free sterile artificial seawater (ASW: 35 ‰ Instant Ocean, Aquarium Systems Inc., USA), and 10^8 cells from each were resuspended in 10 ml of sterile ASW. After shaking, triplicate 100 μl samples were withdrawn, added to 400 μl of absolute ethanol (HPLC Grade, BDH, UK) and freeze dried overnight, at below -50°C. The freeze dried samples were resuspended in 100 μl of 125 μmol alpha-amino butyric acid (ABBA) in a 1.5 ml Eppendorf sample tube and centrifuged to remove particulates (4°C, at 13,000 xg for five minutes).

Free amino acids were extracted and measured by pre-column derivitisation with *o*-phthaldialdehyde (Jones *et al.*, 1981), coupled

with post column detection and quantification by a Milton Roy Fluoromoniter III connected to a Spectra Physics SP42900 Integrator (McAuley, 1988). Separation was carried out on a Waters Resolve C18 column using a stepped gradient of methanol: sodium acetate (McAuley, 1988). Absolute amounts of free amino acids were calculated by reference to 12.5 μM standards, corrected for variation between samples using a 25 μM ABBA internal standard. Absolute amounts of free amino acids were then expressed on a per cell basis.

5.2.5 The effect of cysteine and glycerol on copper toxicity in *Tetraselmis* sp. (TSAW 92)

Both glycerol and cysteine are precursors involved in phytochelatin production (Kägi & Schäffer, 1988, Gekeler *et al.*,, 1988) and were added to the medium in a free and bio-available form. A five litre batch of ES medium was made up and three one litre aliquots were removed. One of the aliquots was spiked with 5 mM cysteine (HPLC Grade, BDH), and one with 5 mM glycerol (AnalaR, BDH). The other aliquot was used as a control, and was spiked with 5 mM sodium chloride (AnalaR, BDH). Each of these one litre aliquots was then divided into seven 100 ml sub-samples. Sufficient copper chloride stock solution was added to each sample to give: 0, 250, 300, 350, 400, 450, or 500 ppm. It is possible that copper could bind to the free cysteine and/or glycerol in the medium. Copper levels were therefore checked, and adjusted when needed, by AAS before and after cysteine or glycerol addition. Each of the 100 ml sub-samples was then inoculated with 100 ml of mid-

log phase *Tetraselmis* sp. (TSAW 92) culture, and incubated under standard conditions in the Conviron incubator for one week (15°C, 12:12 light : dark, 25μEm^{-2} s^{-1} PAR). At this time culture viability was assessed by eye, the criteria being the presence or absence of a green colouration.

5.2.6 The effect of copper on the permeability of *Tetraselmis* sp. (TSAW 92) cells

Each day for five days the same clonal *Tetraselmis* sp. (TSAW 92) parent culture was sub-cultured (10% inoculum) into 250 ml of metal free sterile ES medium from the same batch as that used for the parent cultures, and incubated under standard conditions in the Conviron Incubator (15°C, 12:12 light:dark, 25μEm^{-2} s^{-1} PAR). In this way a series of five clonal daughter cultures, reaching mid log phase at daily intervals, was produced.

At mid log phase, after the morning cell division, a 200 ml sub-sample was withdrawn and cell numbers were adjusted to 10^6 cells ml^{-1} by dilution with sterile ES medium. At time zero this standardised sample was spiked with sufficient copper stock solution to give: 0, 10, 50, 100, or 500 ppm. For one hour, five 5 ml sub-samples were withdrawn at ten minute intervals, added to 0.25 ml of Evan's blue dye solution (final dye concentration 5 x 10^{-7}g ml^{-1}), and placed in Sedgwick Rafter counting chambers under a pre-focused microscope. After staining for ten minutes the microscope lamp was turned on and the number of stained and non-stained cells in three fields of view was scored.

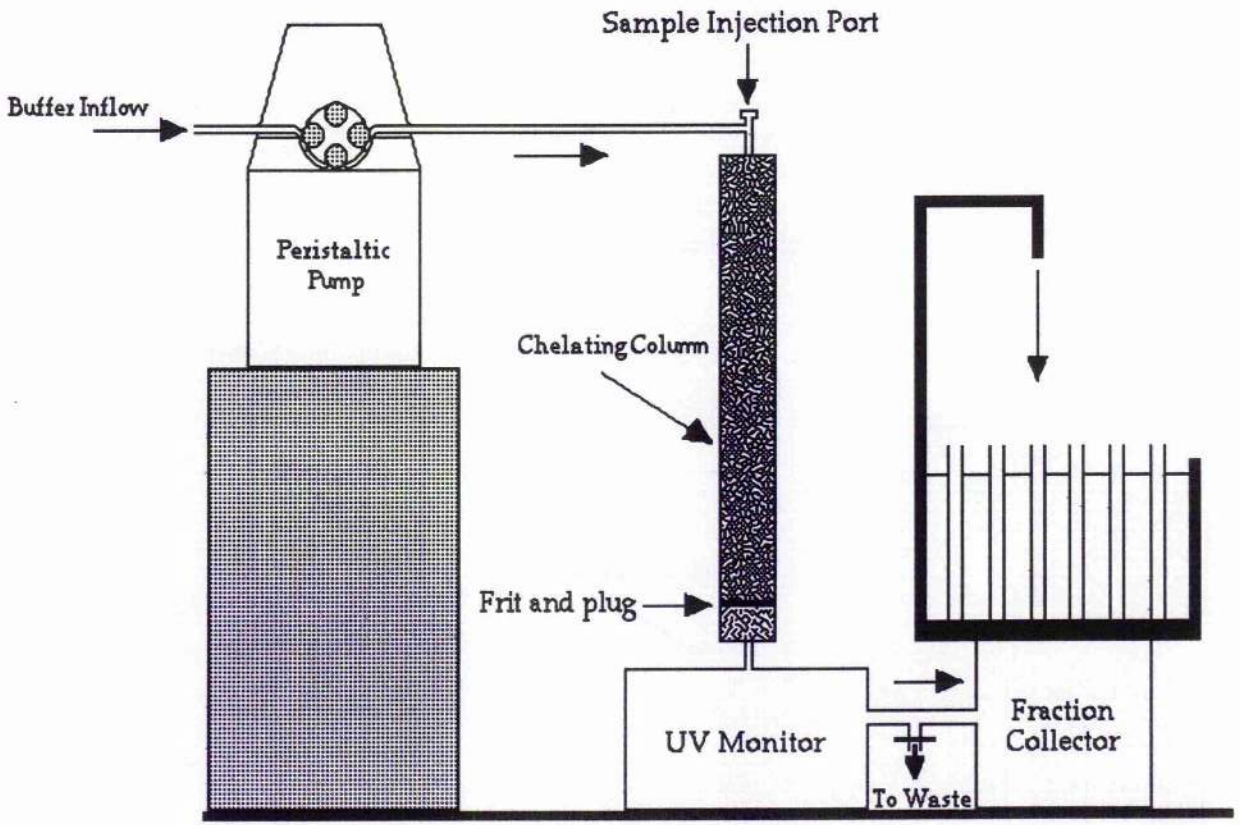
Over the course of a day the experiment was repeated for each copper concentration. The next day the whole experiment was repeated again using the next time-delayed daughter culture. In order to reduce the risk of temporal effects on microalgal metabolism and metal uptake the copper concentrations were used in a different order each day (eg. on day one the order was 0, 10, 50, 100, 500; on day two 10, 50, 100, 500, 0; and so on).

5.2.7 Immobilised Metal Ion Affinity Chromatography (IMAC)

Triplicate 250 ml cultures of *Tetraselmis* sp. (TSAW92) in sterile ES medium containing either zero or 50 ppm copper were grown to mid log phase in the Conviron incubator (15°C, 12:12 light : dark, 25$\mu Em^{-2}\ s^{-1}$ PAR). Cell density was established by direct counting and a volume of culture containing 1.0×10^8 cells was withdrawn from each culture. To minimise damage, the cells were gently spun out of the medium by chilled (5°C) centrifugation at 5000 xg for fifteen minutes. The supernatant was freeze dried overnight and resuspended in 10 ml of sterile washing buffer (0.02 M phosphate buffer made up to pH 7.5 in ASW). Extracts from the cell fraction were produced by dissolution in 10 ml of absolute ethanol (HPLC Grade, BDH, UK) followed by freeze drying and re-suspension in 10 ml of washing buffer. Prior to application to the IMAC column, samples were subjected to high speed chilled centrifugation to remove all particulates (4°C, at 13,000 xg for five minutes).

A 5 ml Hi-Trap Chelating Column (Pharmacia LBK Biotechnology, Sweden) was charged using 1 M copper sulphate in pH 9.0, 0.05 M Tris-Acetate buffer made up in metal free artificial seawater. Unlike conventional IMAC, the whole column was loaded with copper to saturation. At saturation a blue colouration could be seen in the outflow and the pump was switched off. The column was rinsed free of unbound copper by the application of ten column volumes of 0.02 M phosphate buffer made up to pH 7.5 in metal free artificial seawater; the copper level in the final washing buffer was measured by AAS and washing was halted only when this fell to below 1 ppm. This left the column only partially charged, but with all the copper present being very strongly bound. The chosen sample was then injected into the washing buffer flow and allowed to bind to the column. Elution of bound proteins was achieved using 0.5 M NaCl in 0.1 M sodium acetate at pH 4.0. When sufficient time had passed for 90% of the column volume to have been displaced, fractions were collected from the outflow at 30 second intervals. Fractions were analysed for protein and metal content using absorbance at 280 nm and AAS respectively. Real time protein levels in the outflow were estimated using an LKB UV Monitor II (Pharmacia LBK Biotechnology, Sweden) connected directly to the column, this measurement was used for guidance and fraction labelling. Quantitative protein analysis was carried out on the fractions themselves by measuring their absorbance at 280 nm on a uv/vis spectrophotometer (SP6-450 UV/VIS Spectrophotometer, Pye Unicam, Cambridge, UK) (Figure 5.1).

Figure 5.1: Immobilised Metal Ion Affinity Chromatography (IMAC) Apparatus.



5.3 Results

5.3.1 The effect of copper on the extracellular protein concentration in *Tetraselmis* sp. (TSAW92) cultures

In cultures exposed to 10 ppm copper for 72 hours the protein concentration in the cell free culture medium was approximately a factor of ten greater than in similar cultures not exposed to copper (21.7 ± 0.1 and 3.4 ± 0.03 $\mu g\ ml^{-1}$ respectively).

5.3.2 SDS Polyacrylamide gel electrophoresis (SDS PAGE) of *Tetraselmis* sp. (TSAW 92) extracts

SDS PAGE of culture medium samples from *Tetraselmis* sp. (TSAW 92), which had been exposed to heavy metals for 24 hours, produced three protein bands (a single band at ca. 55 kDa and a doublet at ca. 28-30 kDa) (Plate 5.1: Lanes B to G; Plate 5.2: Lane A) which were not observed in extracts from cultures which had not been treated with metals (Plate 5.2: Lanes C & D). The pattern of protein bands was largely identical, irrespective of the heavy metal used. On the original gels iron appeared to produce less dense bands than any of the other metals, but this effect is not as obvious in the photographs (Plate 5.1: Lane E).

Heat shock alone produced a dark background to the lane with numerous very small indistinct bands, but with no strong banding pattern of the type observed after metal shock (Plate 5.2:

Lane C). A combination of heat and Cu shock produced a faint additive pattern with the dark background of heat shock alone coupled with the typical metal banding pattern (Plate 5.2: Lane B). Hypo-osmotic shock produced no detectable bands and gave a clear background to the lane (Plate 5.2: Lane D). Hyper-osmotic shock produced a dark background to the lane (Plate 5.2: Lane E). Interestingly there was also a diffuse band centred on ca. 30 kDa, which is the location of the metal shock doublet, however the heavy metal single band normally found at ca. 55 kDa is absent. With hyper-osmotic and Cu shock combined the banding pattern is much the same (Plate 5.2: Lane F).

Plate 5.1: SDS PAGE of *Tetraselmis* sp. (TSAW 92) culture medium extracts. Lane A: BioRad Broad Range Molecular Mass Markers (BioRad, UK); Lane B: Cd shock; Lane C: Cu shock; Lane D: Mn shock; Lane E: Fe shock; Lane F: Ni shock; Lane G: Zn shock.

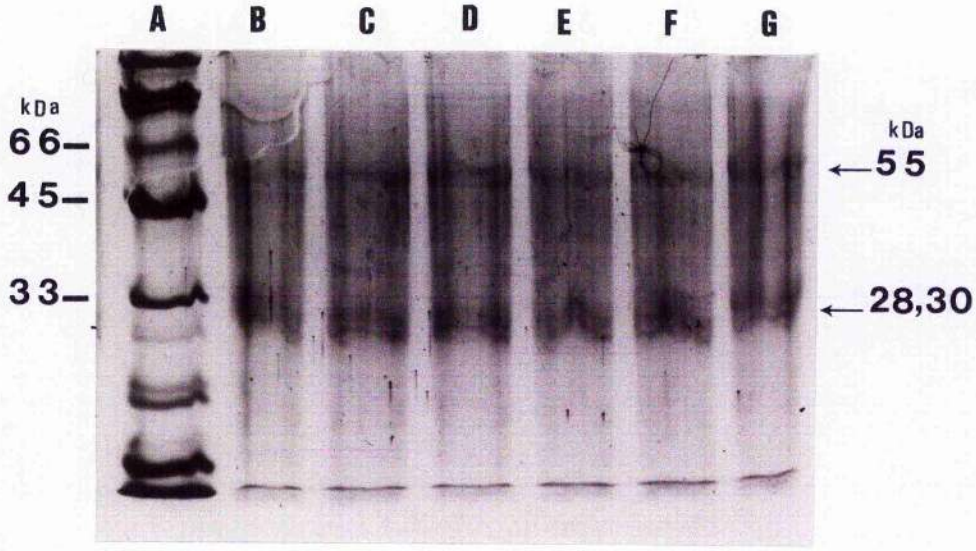
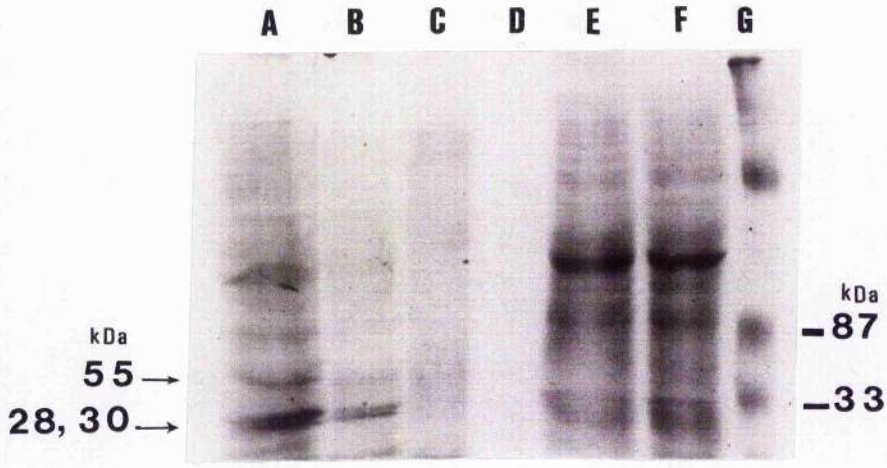


Plate 5.2: SDS PAGE of *Tetraselmis* sp. (TSAW 92) culture medium extracts. Lane A: Cu shock; Lane B: combined heat and Cu shock; Lane C: heat shock; Lane D: hypo-osmotic shock; Lane E: hyper-osmotic shock; Lane F: combined hyper-osmotic and Cu shock; Lane G: BioRad Pre-Stained Molecular Mass Markers (BioRad, UK).



5.3.3 The effect of heat and osmotic shock on copper toxicity in *Tetraselmis* sp. (TSAW 92)

When *Tetraselmis* sp. (TSAW 92) cultures were incubated at 30°C, their copper tolerance was enhanced. The upper lethal limit for *Tetraselmis* sp. (TSAW 92) was raised from the normal 200-250 ppm (Section 3.3.4) to ≤ 500 ppm copper at 30°C. Halving the osmotic potential of the medium led to enhanced copper toxicity with the upper lethal limit being reduced to 100-150 ppm. However, when the salinity of the medium was doubled no effect on copper toxicity was observed.

5.3.4 The effect of copper on the free amino acid content of *Tetraselmis* sp. (TSAW 92)

The most noticeable difference between the levels of the five major free amino acids (aspartate, glutamate, glycine, serine, and methionine) in metal and non metal shocked *Tetraselmis* sp. (TSAW 92) was that the overall levels of amino acids were far lower (by a factor of approximately three) in the metal shocked cells (Table 5.1). Free glutamate levels were less depressed in metal shocked *Tetraselmis* sp. (TSAW 92) cells than the levels of the other amino acids.

Table 5.1: Levels of major free amino acids in *Tetraselmis sp.* (TSAW 92) cells (pg cell^{-1}) which have been exposed to 10 ppm copper. Values given are the means of three replicates, ± 1 standard deviation.

Amino Acid	Control	Metal Treated	C:M Ratio
Aspartate	0.71±0.03	0.3±0.02	2.4±0.03
Glutamate	7.34±0.94	4.42±0.51	1.7±0.73
Glycine	1.38±0.23	0.46±0.02	3.0±0.13
Methionine	0.42±0.01	0.17±0.00	2.5±0.00
Serine	1.03±0.10	0.37±0.01	2.8±0.06

5.3.5 The effect of cysteine and glycerol on copper toxicity in *Tetraselmis* sp. (TSAW 92)

The addition of 5 mM cysteine to the culture medium increased the upper lethal limit of copper in *Tetraselmis* sp. (TSAW 92) from 200-250 ppm to 400 ppm. The addition of 5 mM glycerol had a similar but less pronounced effect and raised the upper lethal limit to 300 ppm.

5.3.6 Immobilised Metal Ion Affinity Chromatography (IMAC)

The two large peaks of protein elution coincide closely with two similar peaks of copper elution (Figure 5.2). Background copper elution is very low (typically <1 ppm) throughout the experiment. This suggests that the protein of interest binds copper *in vitro.*

5.3.7 The effect of copper on the permeability of *Tetraselmis* sp. (TSAW92) cells

At the usual experimental level of 10 ppm copper no increase in cell permeability could be detected for at least one hour (Figure 5.3). At the 50 ppm level a time threshold effect was observed, up to 30 minutes no increase in cell permeability was observed. After 30 minutes cell permeability rapidly increased until approximately 40% of the cells were permeable after one hour. At 100 ppm copper permeability increased from time zero to a peak of almost 50%, permeability then appeared to fall, however, this observed fall is

Figure 5.2: Protein (●—●) and copper (■—■) elution from an IMAC column loaded with an extract of TSAW92 cells pre-exposed to 50 ppm Cu.

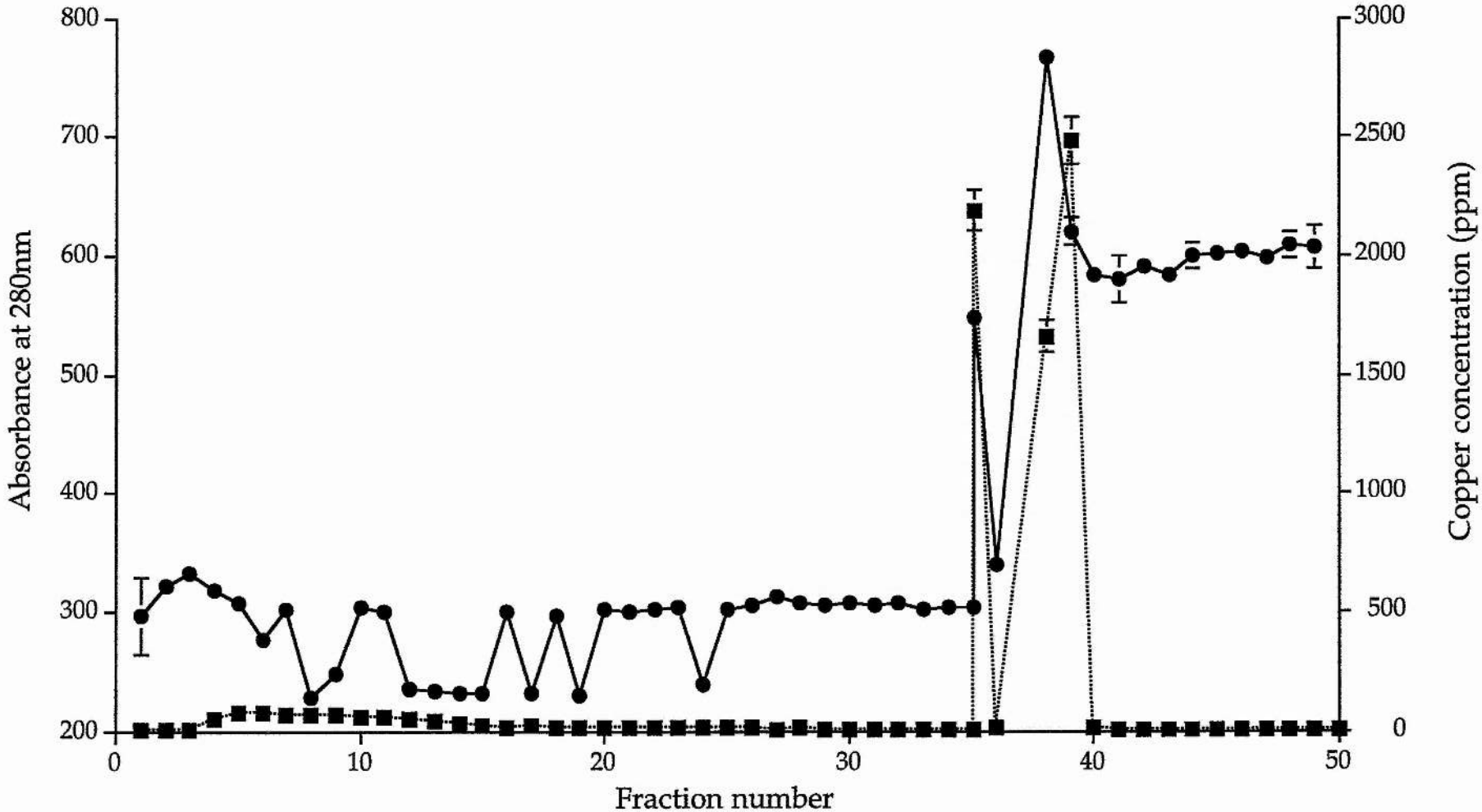
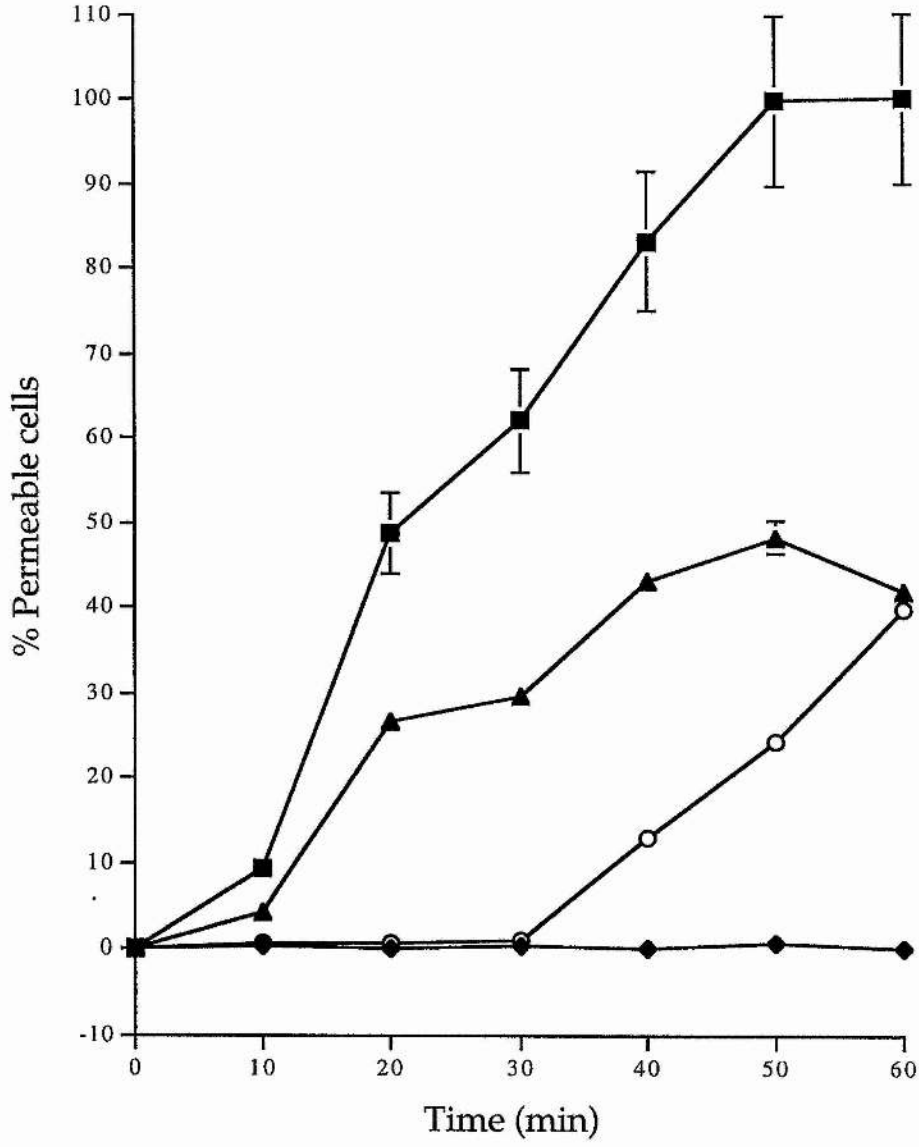


Figure 5.3 : Copper induced permeability in *Tetraselmis sp.* (TSAW92) cells as indicated by the uptake of Evan's Blue dye. Values given are expressed as a percentage of permeable cells, and are the means of five replicates. Error bars are ± 1 s.d., and are sometimes too small to be visible. Copper concentration applied: ◆—◆ 10 ppm; ○—○ 50 ppm; ▲—▲ 100 ppm; and ■—■ 500 ppm. For clarity control data are not shown, in the non-metal exposed controls the percentage of permeable cells was ≤ 0.3 at all times.



dependent on the validity of one data point. At high levels of copper (500 ppm) a rapid rise in cell permeability was observed with 100% permeability being reached after 50 minutes (Figure 5.3).

5.4 Discussion

When exposed to copper, *Tetraselmis* sp. (TSAW92) releases protein into the culture medium. Initially it was thought that this protein release was non-specific and simply a result of an increase in cell permeability brought about by the toxic effects of copper. Gadd (1988 & 1993) reported that heavy metal toxicity has been implicated in increasing cell permeability. With this in mind, the effect of copper toxicity on *Tetraselmis* sp. (TSAW92) cell permeability was investigated, and it was found that at 10 ppm copper caused no significant additional permeability (Figure 5.3). As 10 ppm was the normal level used for the experiments in this study it is unlikely that cell permeability plays any significant role. At a copper level of 50 ppm *Tetraselmis* sp. (TSAW 92) remained impermeable for approximately 30 minutes; after this a rapid increase in permeability was observed, and after one hour approximately 40% of the cells were permeable (Figure 5.3). The 30 minute time threshold was somewhat unexpected, since it was anticipated that cell permeability would rise in a continuous fashion from time zero onwards. One possible explanation for this is that the extracellular copper exclusion mechanisms are overwhelmed after 30 minutes, causing an in rush of copper and subsequent toxicity induced cell permeability. The fact that

increasing the initial copper concentration to 100 or 500 ppm reduced the time taken for the cells to become permeable supports this idea.

The metal dependent nature of protein release and the lack of evidence of cell permeability suggests that these proteins are actively released as a response to metal shock, and are likely to be involved in metal resistance. One possible role for these proteins is that of extracellular chelators. It is well known that higher plants posses a class of proteins called phytochelatins which are thought to play a role in intracellular metal resistance (Grill *et al.*, 1985). Likewise, extracellular metal chelation by algae is known, although it is due to polysaccharides and not proteins (Kaplan *et al.*, 1987a, Zhou & Wangersky, 1985). McLean *et al.* (1972) first reported the occurrence of a cadmium binding component of unknown composition within the cells of *Anacystis nidulans*, a freshwater cyanobacterium. Latterly, these internal components have been identified as phytochelatins and have been found in many freshwater microalgae (page 35). However, there appear to be no reports of a protein based extracellular metal chelation system in the algae, or indeed of phytochelatins in marine microalgae.

In *Tetraselmis* sp. (TSAW92) three proteins bands, of masses ca. 55, 28, and 30 kDa, are seen as a result of metal shock (Plate 5.1). The presence of the three protein bands could indicate that there is in fact only one protein which is made up of two sub-units. Several metal binding proteins are known to be highly refractory and readily split along well defined planes (Prof. E. Vierling, University of Arizona, Dept. of Biology, USA, pers. comm.). If this were the

case then the protein of interest would have a mass of ca. 55-60 kDa and would be comprised of two approximately equal sub-units. However, this structure is not proven and it could be argued that there are three separate proteins.

Whether or not there is one protein or three, none of the indicated masses are appropriate for any of the known class III algal metallothioneins, which tend to have weights in the 6-10 kDa range (Reddy & Prasad, 1989). It is possible that the weight of the *Tetraselmis* sp. (TSAW92) proteins was slightly over estimated due to the use of the detergent sodium dodecyl sulphate (SDS), which may cause such over-estimates by its variable binding to non-typical proteins (Harris & Angal, 1989). During purification, it was found that the proteins of interest bound very strongly to glassware, plastics, and many filters, and it is therefore highly probable that they also bind atypically to SDS. However, it is highly unlikely that this binding could be responsible for such a large difference. The most reasonable conclusion is that these proteins are not typical metallothioneins. The use of mass spectrometry would have resolved this uncertainty, however, this facility was not available in St Andrews.

Native PAGE was therefore attempted to confirm both the structure and weight of the proteins, but in their native and biologically active form the proteins of interest bound to the gel matrix at the base of the sample wells and failed to migrate. Gel filtration was also attempted, but again the protein bound to the gel column and could only be eluted by destructive means.

However, this strong binding ability was used to advantage in the design of an experiment to show that the protein was able to bind copper *in vitro*. A chelating Sephadex column was fully saturated with copper, thus blocking all the available binding sites. When the protein sample was applied, the proposed metal binding protein bound to the copper which was attached to the column, and subsequently by lowering the pH the copper-column bond was broken causing the protein to co-elute from the column with the copper (Figure 5.2). Interestingly, there were only two peaks of coincidental copper and protein release (Figure 5.2), since three bands of protein were seen on the gels this observation was unexpected. There are two likely explanations for this. Firstly, that there is a single protein of ca. 55 kDa which splits into two sub-units during IMAC, or second, that one of the peaks is the ca. 55 kDa protein and the other is composed of both of the two smaller proteins. An attempt was made to clarify this situation by running the fractions containing the peaks out on electrophoresis gels, but unfortunately none of the samples would migrate correctly due to the presence of bound copper.

The fact that the proteins from metal shocked *Tetraselmis* sp. (TSAW92) did not have the correct mass to be conventional phytochelatins raised the possibility that they were some other type of stress protein, such as heat shock proteins. In order to test this hypothesis, protein extracts from *Tetraselmis* sp. (TSAW92) cells which had been subjected to heat and osmotic shock were run out on electrophoresis gels. Neither of these treatments produced the banding pattern seen after metal shock (Plates 5.1 and 5.2).

However, it was also noticed that heat shocked cells appeared to grow far better in the presence of copper than non-heat shocked cells. This was tested experimentally, and it was found that a 35°C heat shock increased the upper lethal limit for copper in *Tetraselmis* sp. (TSAW92) from 200-250 ppm to ≥500 ppm.

An attempt was made to dot-blot extracts from *Tetraselmis* sp. (TSAW92) cells which had been metal shocked with a monoclonal HSP 70 antibody from mung beans (kindly gifted by Zeneca, UK). Unfortunately, once again the extreme non-specific binding ability of the native protein(s) prevented this technique from working.

In plants, heat shock proteins are produced by a similar biochemical pathway as that which is thought to produce phytochelatins (Kägi & Schäffer, 1988). Therefore, it is possible that under heat shock this pathway is up regulated, thereby making more precursors available for the production of phytochelatins allowing for enhanced metal resistance. This hypothesis was tested by measuring the levels of free amino acids in metal and non-metal shocked *Tetraselmis* sp. (TSAW92) cells. Unfortunately, no large increase in the pools of known phytochelatin precursors was observed, in fact the concentration of all the measured amino acids fell by a factor of approximately three (Table 5.1). This suggests that metal shock may inhibit protein synthesis, although given that *Tetraselmis* sp. (TSAW92) cells seem to produce a metal tolerance protein this is somewhat counter intuitive. However, these measurements are only of pool size, not turnover, and it is possible that the amino acid pools were being used faster than new amino

acid synthesis. This would give the appearance of reduced synthesis even if the opposite was true. The experiments described here do not differentiate between these two possibilities. Further work using radio-labelled amino acids and their precursors may produce a clearer picture as it would be possible to follow the radio-label all the way through to the final product. Using this technique it would be possible to determine whether the proteins expressed by metal shocked *Tetraselmis* sp. (TSAW92) cells are produced by the classical phytochelatin pathway in the same way as those of higher plants.

To further test the hypothesis that the observed protein is a type of phytochelatin, glycerol and cysteine, which are known precursors of phytochelatins, were added to the culture media. It was found that the addition of free cysteine enhanced the copper resistance of *Tetraselmis* sp. (TSAW92) to give an upper lethal limit of 400 ppm, as opposed to the normal 200-250 ppm. The addition of glycerol had a similar but far less pronounced effect on copper toxicity, raising the upper lethal limit to 300 ppm. This is consistent with the precursor theory, as glycerol is further downstream from cysteine on the metabolic pathway used to produce phytochelatins. The possibility that either glycerol or cysteine were binding metal in the medium was ruled out by the careful use of controls and by measuring the level of free copper in the medium after their addition.

5.5 Summary

When exposed to heavy metals, *Tetraselmis* sp. (TSAW92) cells produced proteins which were able to bind copper *in vitro.* This protein production was specific to heavy metal shock and could not be induced by heat or by osmotic shock. SDS PAGE indicated that the proteins produced fell into three weight bands at ca. 55, 30 and 28 kDa. Without the use of gel filtration or native PAGE it is impossible to say whether or not these weights are gross overestimates caused by atypical SDS binding, or whether there are three proteins or one protein composed of two sub-units. Unfortunately, no effective protocol for either of these two techniques could be derived as the proteins of interest showed a very high degree of non-specific binding. Heat shock and the addition of known phytochelatin precursors improved the tolerance of *Tetraselmis* sp. (TSAW92) to copper and it is therefore likely that the protein(s) of interest are some form of phytochelatin-like protein.

CHAPTER SIX

METAL UPTAKE APPLICATIONS

6.1 Introduction

In the preceding chapters I examined the physiology and biochemistry of marine microalgal heavy metal uptake, and in this chapter preliminary tests of possible practical applications of this uptake will be discussed. When using microalgae as a metal uptake material there is a fundamental choice to be made between the use of live and dead cells. One of the main arguments against the use of live micro-organisms in industrial processes is that they require relatively moderate conditions in terms of temperature, pressure, pH, turbulence, and the requirement for nutrients (Gadd, 1993). In addition, live microalgae require the presence of photosynthetically active radiation and a suitable carbon source. However, in terms of cost the use of dried microalgal biomass is relatively expensive, since at the minimum, a large scale culture must be grown up, and the cells must then be harvested and dried. Due to their small size, neutral buoyancy and low culture density it is widely accepted that harvesting is the most technically difficult and consequently expensive process in mass microalgal culture (Soeder, 1984). One novel harvesting technique for microfungi exploits the metal binding capacity of the organism itself by binding magnetite to the cells and subsequently using a strong magnetic field to collect them (Wainwright *et al.*, 1990; Dauer & Dunlop, 1991). This technique could also be applied to a metal accumulating microalga making harvesting relatively simple.

The harvested biomass must then be dried to prevent degradation and spoiling, which is very energy intensive (Mohn,

1980). To produce prills (extruded pellets) and pellets suitable for use in columns would require further costly processing and the use of binding agents. Having said that, dead biomass has several advantages over its live counterpart. Dead biomass is largely immune to the harsh conditions often encountered in industrial processes, pellets of dried biomass could be handled and used in an almost identical way to conventional ion-exchange resins (Tsezoz & Deutschmann, 1990). Dried biomass is also far easier to store than live biomass, and could be transported for use in areas where the climate is unsuitable for mass microalgal culture. In the first set of experiments killed *Tetraselmis* sp. (TSAW92) was evaluated as a metal removal medium.

Despite the stated drawbacks, described above, in certain circumstances the use of live microalgal biomass has several advantages over dead biomass. All microalgal biomass is derived from live cultures and the direct use of these cultures eliminates the additional costs of processing and transporting the biomass to where it is needed. In other words, if the algae is grown and used *in situ*, as part of a self-sustaining process the only running costs are the supply of nutrients and of power for mixing. In addition to cost savings, the use of live microalgae has advantages over dead microalgae from a purely biological point of view. Being live they are potentially open to genetic manipulation techniques (Craig *et al.*, 1988). Live microalgae could also bring a measure of homeostasis to the system, for example in the conditions of increasing nutrient concentrations, live microalgae could grow in numbers and absorb the excess. It is also known that in some cases

the uptake of metals is far greater in the live alga than in the same alga when dried (Gadd, 1988). Finally and importantly, nutrient removal is only possible with live cells.

Similar conclusions are reached in a review of fungal metal uptake by Gadd (1993), with dead biomass appearing to have advantages over live biomass in terms of a greater tolerance to industrial conditions and the relative ease of harvesting and metal recovery (Kelly *et al.*, 1979; Brierley *et al.*, 1985). However, he also points out the fact that live fungi are capable of intracellular metal uptake, extracellular precipitation and complexation, and the production of metal binding proteins. In short the choice between live and dead biomass is far from simple and depends largely on the nature of each individual application.

In the second set of experiments live microalgal cells were seeded onto raceways to investigate the feasibility of simultaneous biomass growth and metal removal. The small-scale pilot raceway system described in this chapter is based on that of Craggs (Craggs *et al.* 1994), however, four important refinements were made. Firstly, the waste stream containing metals was mixed with one containing nutrients (domestic sewage), thus removing the cost of nutrient supply. Second, by careful design and the use of gravity feed the requirement for powered mixing was removed. Third, metal removal and nutrient removal were combined within the same system. Finally, the use of a glass bead bed at the bottom of the raceway provided a refuge for seed algae during raceway cleaning. The beads also increased the turbulence of the flow hence assisting

in the gassing off of ammonia and in the provision of carbon dioxide to the growing culture.

6.2 Materials and Methods

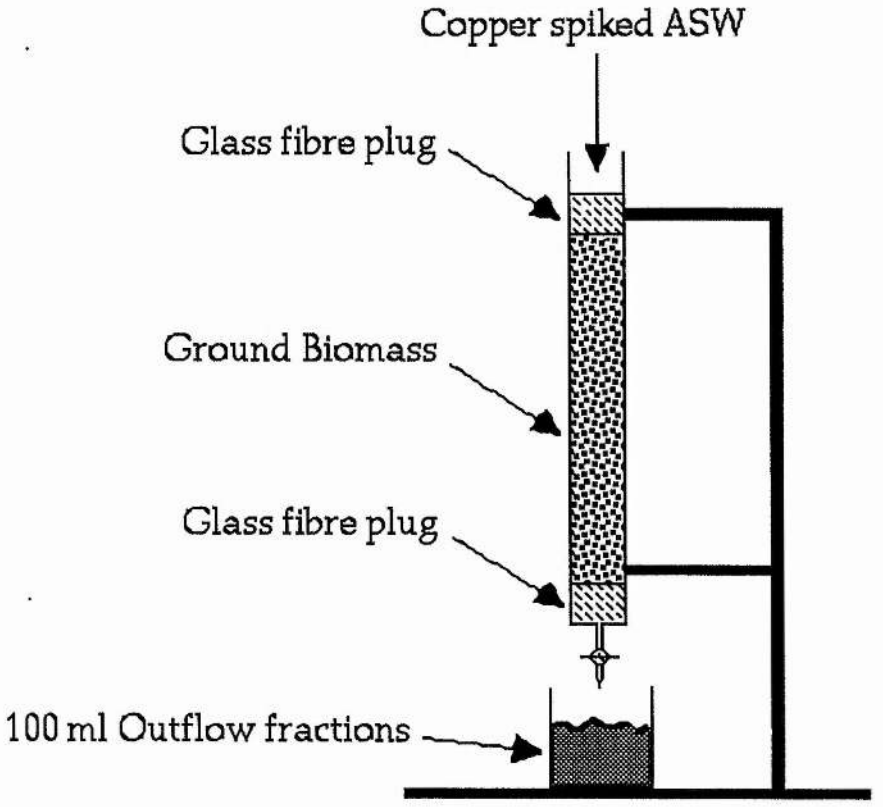
6.2.1 Preparation of dried *Tetraselmis* sp. (TSAW92) biomass for metal removal

A ten litre culture of *Tetraselmis* sp. (TSAW92) was grown up to mid-log phase in sterile ES medium under standard conditions in the Conviron incubator (15°C, 12:12 light:dark, 25$\mu Em^{-2} s^{-1}$ PAR). Cells were harvested by centrifugation (10,000 xg for 10 minutes) and freeze dried overnight in an open evaporating basin. The dried biomass was gently ground through a 2 mm mesh sieve to produce a coarse powder. This powder was stored in sealed glass sample tubes in a desiccator in the dark at -20°C until required.

6.2.2 Copper uptake by dried microalgal biomass and selected materials

A simple column was constructed in order to assess the copper removal ability of dried microalgal biomass. A glass burette was cut down to a length of approximately 10 cm and mounted vertically in a retort stand, and the column was plugged with a 1 cm deep layer of glass fibre wool at the lower (tap) end (Figure 6.1). The column was gravity filled with 5 g of dried powdered material, and flushed with 100 ml of metal free sterile ASW. Once the biomass

Figure 6.1: Column apparatus for measuring copper uptake by dried microalgal biomass and selected materials.



had taken up water and swelled to its full size the column was completed by the addition of a 1 cm thick glass fibre wool plug to the top surface of the biomass.

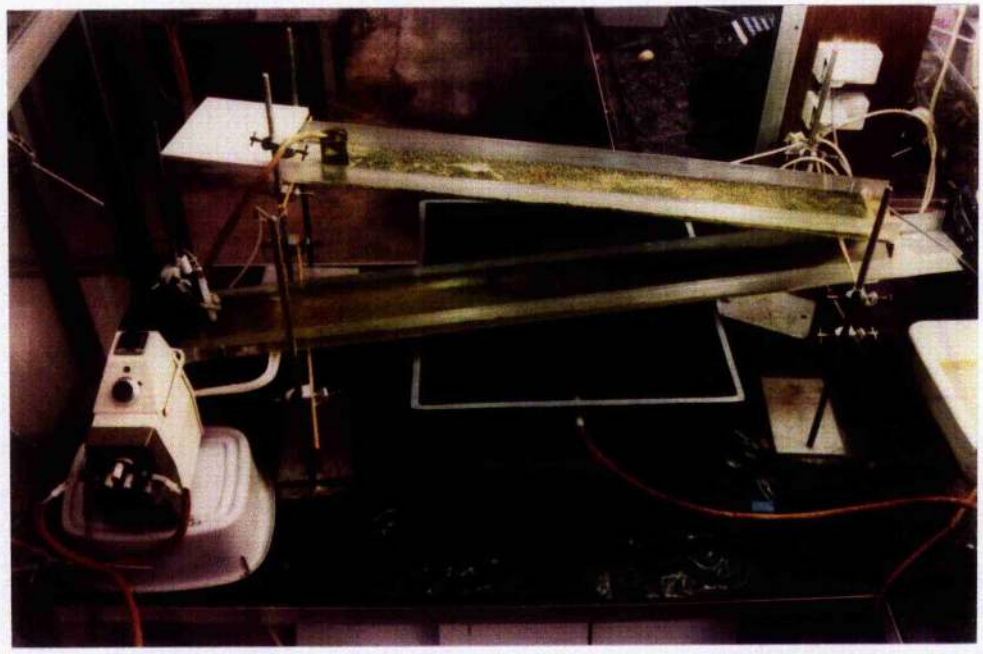
One litre of 1000 ppm copper solution in ASW was allowed to percolate through the column under gravity. The outflow was collected in 100 ml fractions and copper levels in each of the fractions were measured by AAS. At the end of each run the column was rinsed with two volumes of metal free sterile ASW and allowed to drip dry. The column was then inverted over a ceramic crucible and the biomass removed using careful pressure from an air-filled 50 ml syringe connected to the column tap. In this way the biomass was forced out of the column almost in one piece. The last traces of biomass were removed from the column using a piece of filter paper moistened with the minimum amount of de-ionised water threaded onto a length of thin stiff stainless steel wire. This filter paper was then added to the crucible, and the biomass ashed in a muffle furnace overnight at approximately 900°C. The copper in the ash was dissolved by the addition of 10 ml of concentrated hydrochloric acid (AnalaR, BDH, UK). To produce a measurable level of copper and to avoid corrosion damage to the AAS the acid extract was diluted by the addition of 990 ml of Milli-Q water prior to the measurement of copper content. The materials with which the column was packed included *Tetraselmis* sp. (TSAW92), *Fucus vesiculosis*, *Macrocystis* sp., ground wood chips, and Chelex 100 resin (BioRad, UK).

6.2.3 Metal and nutrient removing microalgal raceways

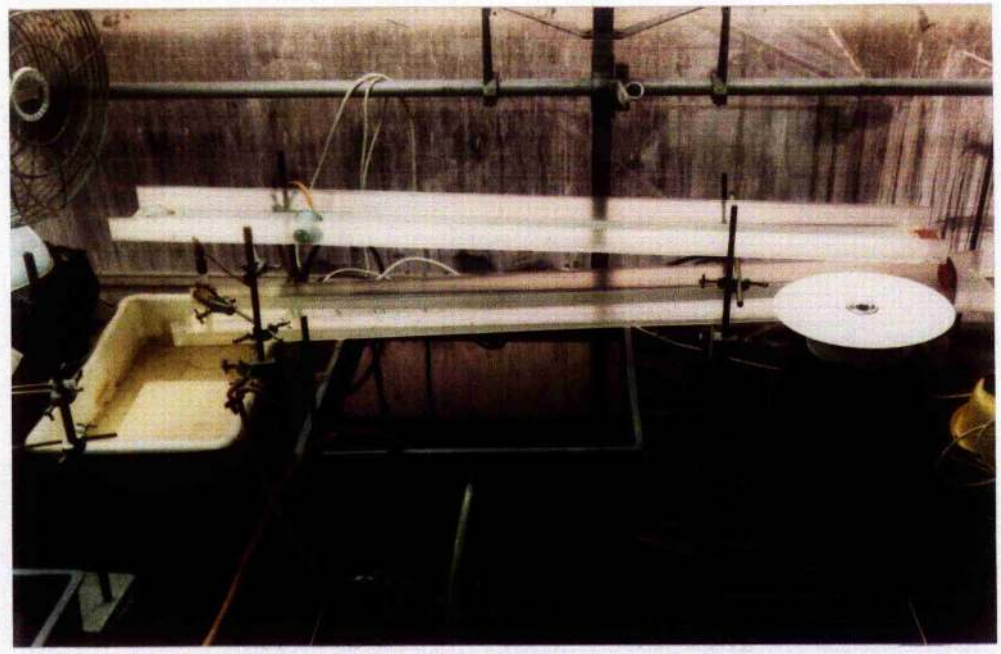
The raceways were constructed from 2 m long plastic fluorescent lamp diffusers filled with a single layer of clear glass balls (4 mm diameter) and angled at 10° to the horizontal (Plate 6.1). The raceways were housed under glass, at ambient levels of light and temperature, culture temperature was monitored using a Casella charting thermometer (Casella, London, UK) sealed in a plastic bag and placed in the raceway outflow. Culture pH was continuously measured at the system outflow using a pH meter (Model 290 pH, WG Pye, Cambridge, UK fitted with a Combination GP electrode, Russel, Fife, UK) linked to a chart recorder (CR650S Recorder, J. J. Instruments, Southampton, UK), and averaged over each 24 hour period. Incident light was measured using a radiometer (Solar Integrator II, Kipp & Zonen, Holland) set to integrate total sunlight on a 24 hourly basis. The growth medium was settled primary sewage (St Andrews sewage works, Fife, UK) mixed 1:1 with un-filtered natural sea water (St Andrews Bay, Fife, UK) (Chapter 2). During metal removal experiments the medium was spiked with copper, adjusted after AAS analysis until a level of 5 ppm was reached. Substantial non-specific binding and precipitation of copper occurred, presumably due to the organic matter present in the sewage and to phosphate precipitation. To ensure constant nutrient levels in the inflow the same batch of medium was used throughout the series of experiments. This 600 litre batch was stored in the dark at 4°C in ten sealed 60 litre

Plate 6.1: Dual Microalgal Raceways

(a) Experimental Raceway



(b) Control Raceway



aspirators. Each day fresh medium was transferred from the cold-room aspirators to the glasshouse supply tank, a 60 litre aspirator painted black and kept under cover outside the glasshouse. To prevent heterogeneity, the medium in the current cold-room aspirator and in the glasshouse supply tank, was continuously mixed using a small submersible aquarium water pump (Aquaclear 201, Hagen, UK). The inflows and outflows of these pumps were set below the surface to avoid the entraining of air and the subsequent oxidation of the medium. All pipe-work was shielded with opaque hose-pipe and black polythene from the supply tank up to the point of raceway entry. Due to the unusually hot weather during the course of these experiments the air temperature inside the glass-house approached 50°C on several occasions, effectively killing the raceway algae. In order to avoid this, the strictly ambient approach was partially sacrificed by the installation of a forced air cooling system in the glasshouse roof which was thermostatically triggered at 35°C. During the course of the experiments this system operated only very occasionally and can be regarded as a safety device rather than a temperature control mechanism.

A set of two control raceways which remained algae free throughout, were also set up (Plate 6.1). Ammonia, phosphate, and copper levels were measured on a twice daily basis at ten am and three thirty pm. These measurements were made at the inflow to the system, at the outflow from the first (upper) raceway, and at the outflow of the system.

Initially, the first set of two experimental raceways was set up in parallel and a medium recirculated through each. One was

seeded with *Phaeodactylum tricornutum*, a marine diatom which is known to form films, to tolerate sewage (Craggs *et al.*, 1994), and to resist 10 ppm copper without accumulating it (Chapter 3). The other was seeded with the green marine microalga *Botryococcus braunii*, which is known to tolerate sewage (Craggs *et al.*, 1994), but not 5 ppm copper (Chapter 3). After two weeks, the raceways were placed in series, with the diatoms upstream, and the growth medium spiked with 5 ppm copper as described above. The flow rate was adjusted to give a four hour residence time in the unit, i.e. two hours per raceway. The health of the green microalgal film was monitored by eye on a twice daily basis for one week.

A second set of two experimental raceways was erected, one raceway was seeded with *Tetraselmis* sp. (TSAW92) a marine chlorophyte known to resist and accumulate copper (Chapter 3), and the other with *Dunaliella tertiolecta* a green marine chlorophyte known to remove nutrients from sewage when grown in this way (Craggs *et al.*, 1994). As before the raceways were first run in parallel for two weeks, prior to being placed in series , with *Tetraselmis* sp. (TSAW92) upstream, and the medium was spiked with 5 ppm copper. Nutrient, metal, and pH measurements were made as previously described (Section 6.2.3).

6.3 Results

6.3.1 Copper uptake by dried biomass

The greatest final copper loading was found to be 38 ±1.9% in the commercial metal removal resin Chelex 100 (Table 6.1). For *Tetraselmis* sp. (TSAW92) copper loading was found to be maximal with live cells (37 ±0.2%, Chapter 4), fresh killed cells performed slightly less well (30 ±0.9%, Chapter 4), and least with dried cells (28 ±0.3%, Table 6.1). The dried biomass of the marine macroalgae *Fucus vesiculosis* and *Macrocystis* sp. were found to be a poor copper removers with loadings of 4.2 ±0.04 and 1.6 ±0.07% respectively. Wood chips were included as an example of a simple cellulose medium and were found to perform very poorly with a copper loading at the limit of detection (<0.002%) (Table 6.1).

6.3.2 Metal removing microalgal raceways

In the first experiment, with the metal excluding diatom *Phaeodactylum tricornutum* on the upper raceway, it was found that very little metal removal had occurred (< 2% ± 0.7). The metal sensitive microalga *Botryococcus braunii* on the lower raceway bleached and died out within three days, suggesting that the high pH induced by microalgal photosynthesis and the release of organic compounds by the algae had not converted the copper into the form of a less toxic salt.

Table 6.1.: Copper loadings at saturation for various materials. Copper was taken up from a 100 ppm solution in pH 8.0 seawater at 15°C. Copper loading was determined by AAS analysis of a hot acid digest of the washed material.

Material	% Copper by Dry Weight at Saturation
Chelex 100 Resin	38 ±1.9
Live TSAW'92	37 ±0.2
Fresh killed TSAW'92	30 ±0.9
Dried TSAW'92	28 ±0.3
Dried *Fucus vesiculosus*	4.2 ±0.04
Dried *Macrocystis sp.*	1.6 ±0.07
Dried wood chips	<0.002
Live *Chlorella vulgaris*	1.55±0.03

In the second experiment, with the metal removing microalga *Tetraselmis* sp. (TSAW92) on the upper raceway, copper removal was total after the first raceway from day zero (Figure 6.1). AAS analysis of samples of the nutrient removing microalgal biomass (*Dunaliella tertiolecta* , Craggs *et al.* 1994) scraped from the lower raceway failed to detect any measurable copper (detection limit $\leq$ 0.01 ppm). Ammonia removal increased gradually over the first week to approaching 70% after the first raceway and 100% for the system as a whole (Figures 6.1 and 6.2 respectively). Maximum phosphate removal increased somewhat more slowly and was less complete at approximately 28% after the first raceway and 87% after the second (Figures 6.1 and 6.2 respectively). Microalgal washout into the outflow was relatively low, with the outflow tank remaining clear of microalgal blooms for the duration of the experiment. At the end of the experiment, microalgal film formation was complete in the upper raceway, but gradually faded from complete coverage at the proximal end to absence at the distal end of the lower raceway. No evidence of *Tetraselmis* sp. growth was seen in the lower raceway and no contaminants grew up.

From the temperature data (Figure 6.3) it can be seen that these algae are highly temperature tolerant, with a daytime peak of approaching 40°C on day two failing to kill them. Culture pH rapidly rose from the raw medium level of 8.20 to 9.78 within the first 24 hours, and subsequently remained above pH 9.68 for the duration of the experiment.

Figure 6.1: Nutrient and copper removal by the first stage raceway over a one week period. The white column is ammonia removal, the light shaded column is phosphate removal, and the dark shaded column is copper removal. Values given are means of triplicate measurements, error bars represent one standard deviation, and are too small to be visible.

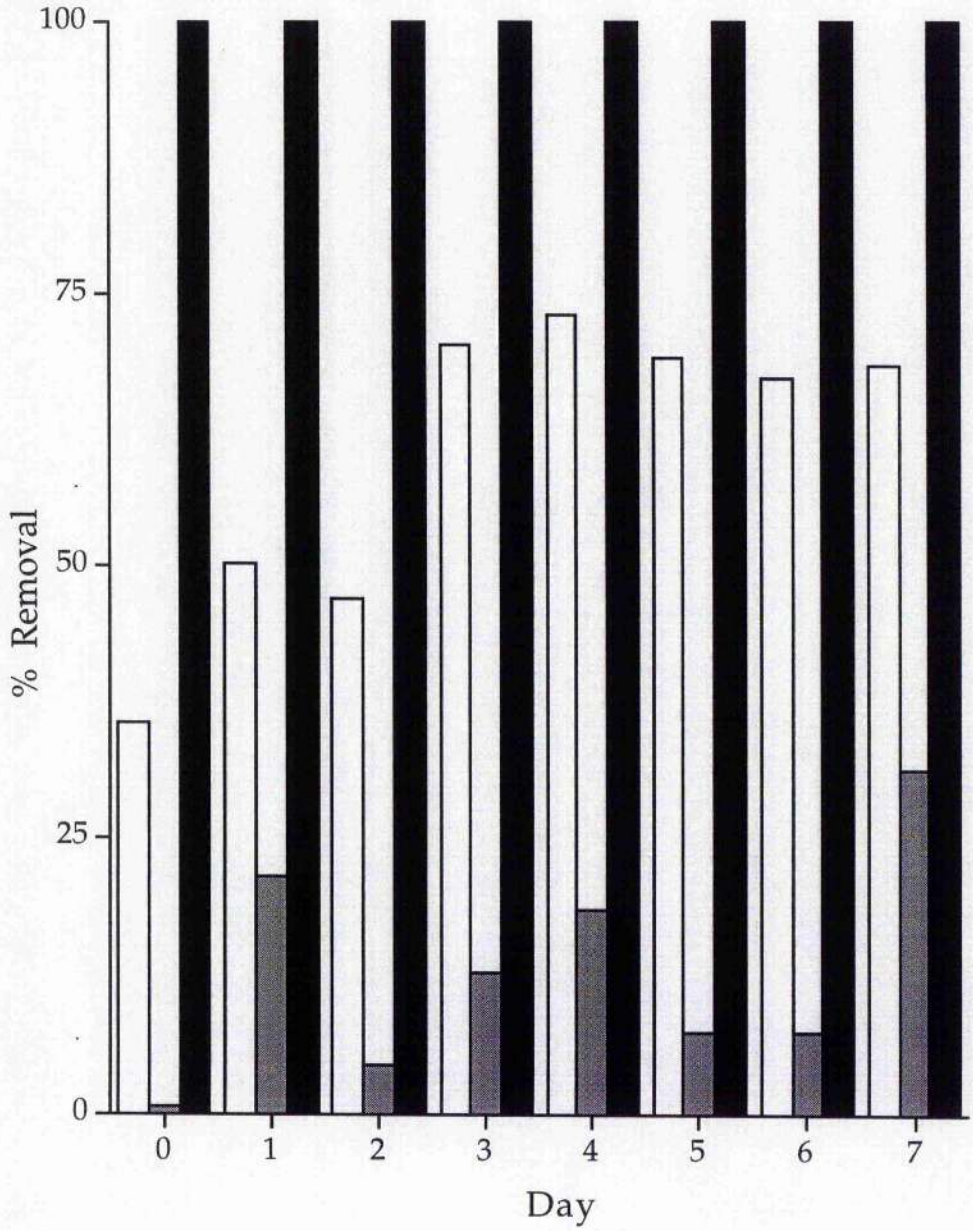


Figure 6.2: Nutrient and copper removal by the first and second stage raceways over a one week period. The white column is phosphate removal, the light shaded column is ammonia removal, and the dark shaded column is copper removal. Values given are means of triplicate measurements, error bars represent one standard deviation, and are too small to be visible.

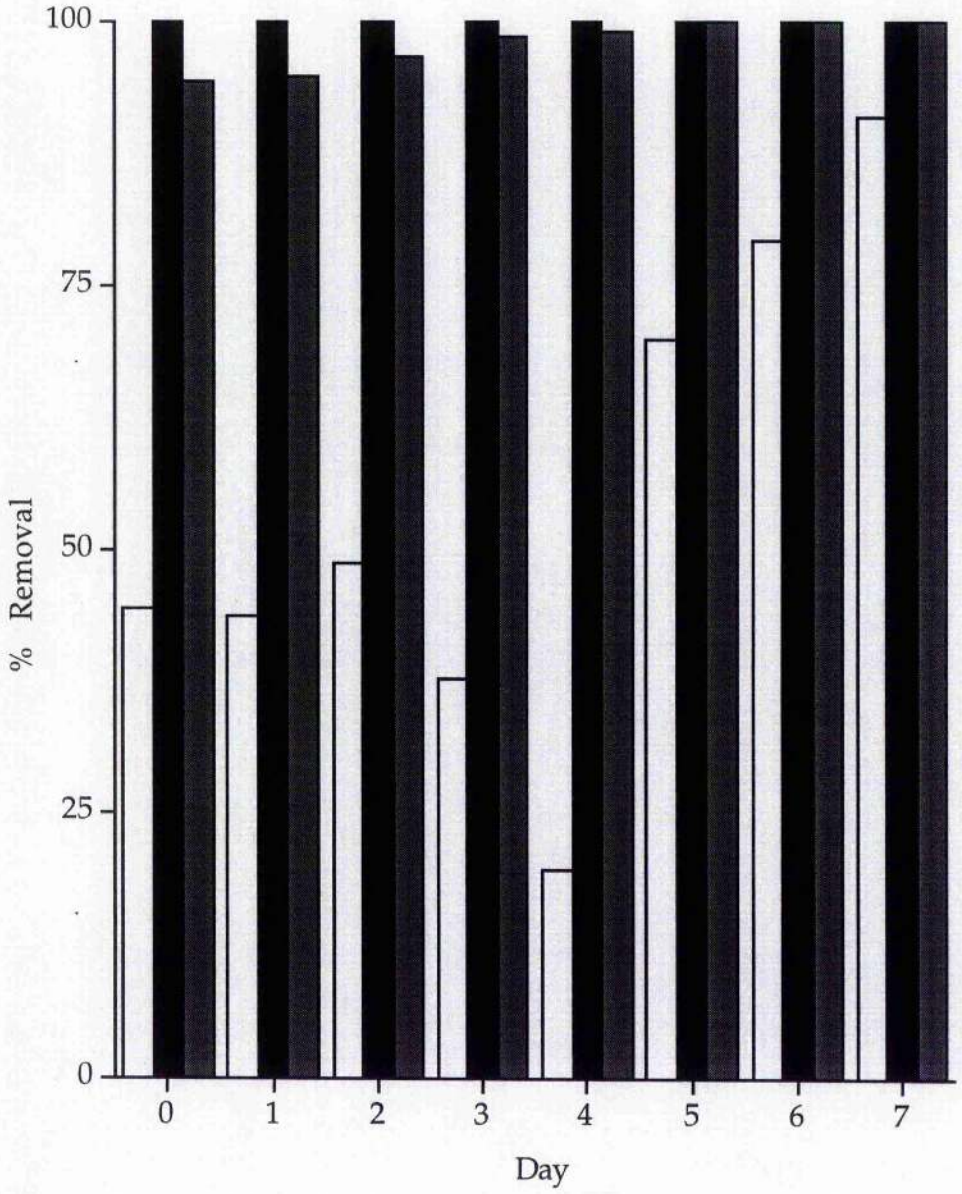
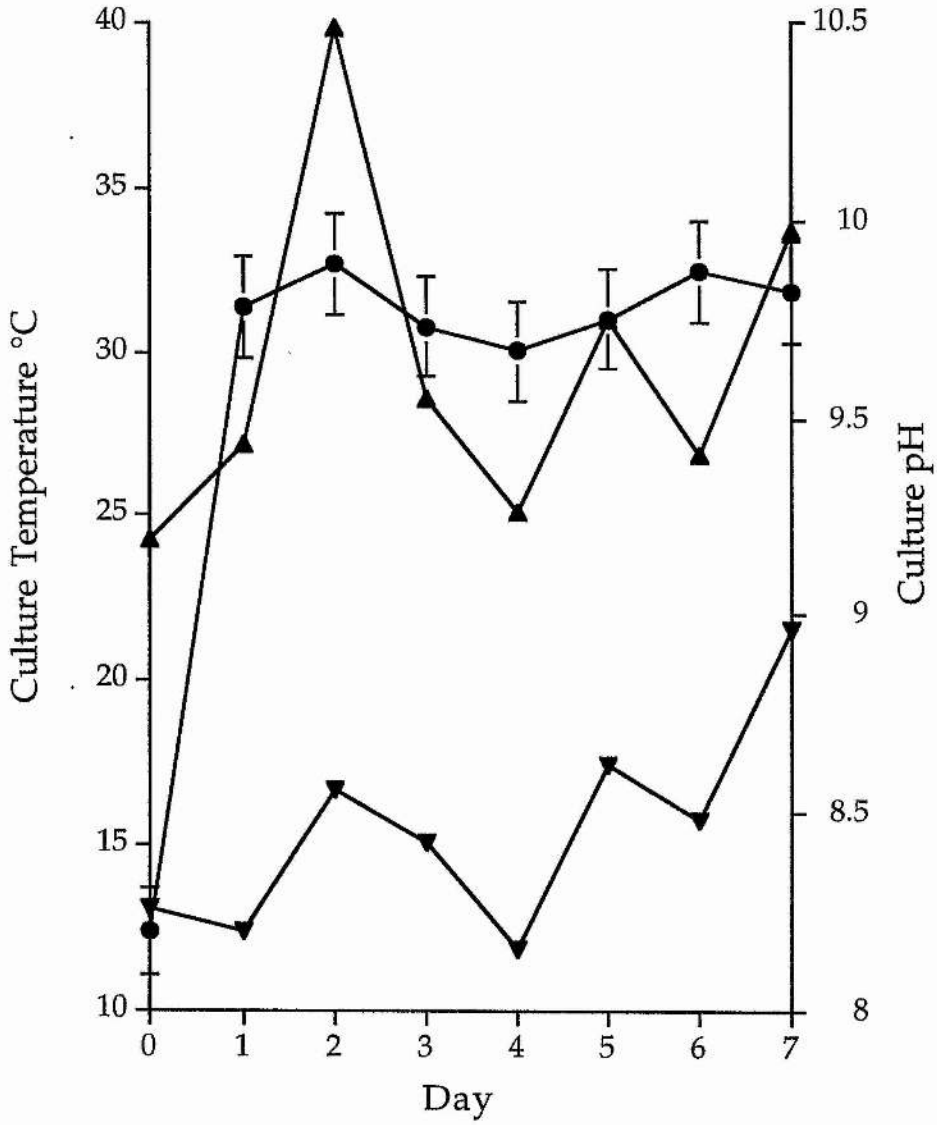


Figure 6.3: The climatic conditions experienced by raceway cultures. Peak daytime temperature (▲—▲), minimum night-time temperature (▼—▼), and culture pH (●—●). Measurements were taken at the outflow of the final raceway and are means of three samples. Error bars represent one standard deviation and are too small to be visible for the temperature data. Note that both y-axis scales are different and do not commence at zero.



In the control raceway with no microalgae, no significant copper or phosphate removal occurred (copper < 1.6% ± 0.5, phosphate < 0.8% ± 0.1,), although between 5 and 20% of the ammonia was removed. The ammonia removal did not show any trend of increase or decrease over time and followed no obvious pattern. However, it appeared to be temperature related, increasing on hotter days and reducing on cooler ones.

6.4 Discussion

6.4.1 Copper removal by dried biomass

Dried *Tetraselmis* sp. (TSAW92) biomass removed copper from solution to a final loading of 28 ±0.3% by weight (Table 6.1). There is little published work in this area, in some cases due to commercial interests. A small scale industrial metal removal process based on dried algal biomass has been developed by B.V. Sorbex Inc. of Montreal, however no hard data on its performance is available, a similar process called Algasorb has also been developed (Anon.). It is therefore very difficult to say how this system compares to others in the field, although the favourable comparability with Chelex 100, a commercial metal removal resin, is encouraging. The results for macroalgal biomass were somewhat disappointing with very low levels of copper loading being achieved (Table 6.1). One possible explanation for this result is that the dried microalgal biomass had a far higher specific surface area than the dried macroalgal biomass. The microalgal biomass was

initially composed of individual cells which were dried to a cake and then ground to uniform grain size, but the macroalgal biomass was dried tissue, subsequently ground to the same size. This potential difference could be checked by the use of microscopy, or by repeating the experiment with macroalgal biomass which had been ground very finely prior to caking.

The final grinding of the dried biomass also had a major impact on the practicality of the column method. If the biomass was ground too finely it packed down hard and blocked the flow of liquid through the column. On the other hand, if the biomass was left too coarse the liquid poured rapidly through the column and was found to contain high levels of copper.

It is clear that a careful choice must be made between live and dead biomass for each individual application, and that great care must be taken in selecting the appropriate grain size for the dried biomass. It is possible that the column method with its inherent risk of blockage is not the best technique for metal removal using dried biomass. One possible alternative would be the use of a fluidised bed in which the biomass granules were suspended in solution by balancing their negative buoyancy with an up-flowing current of waste water. Using this system it would be possible to prevent blockages and to fine tune the flow rate/ residence time in response to the outflow quality during metal removal. Preliminary experiments were carried out using home-made equipment of this type but proved to require design and construction techniques out with our abilities.

6.4.2 Copper removal by live *Tetraselmis* sp. (TSAW92) biomass

There is a clear gradient in copper loading ability running down from live to dry *Tetraselmis* sp. (TSAW92) cells (Table 6.1). Although, the total reduction in copper loading from live to dried *Tetraselmis* sp. (TSAW92) cells is only 9 ± 0.5%, and even at the lower levels it compares very well to commercial resins (eg 38 ± 1.9% for Chelex 100). Therefore, the compromise between the robustness of dried microalgal biomass and the higher metal loading potential of live biomass may not be of vital importance, especially if the production costs of the biomass are low.

The use of live microalgae on the raceway system removed the need for potentially expensive biomass drying and processing, and enabled the growth of biomass to be coupled with continuous metal removal. The use of live algae also provided the opportunity to remove nutrients from the waste stream (Oswald, 1988c). Initially a dual raceway was set up using a non-metal accumulating alga *Phaeodactylum tricornutum* followed by the non-metal tolerant alga *Botryococcus braunii*. This system was used as a control for the presence of non-specific algae or for some type of bacterial or physical metal removal process. No significant metal removal was observed (< 2% ± 0.7) and the metal sensitive alga died out completely within the week. This suggests that a generic microalgal presence, with associated high pH and leached organic compounds, does not cause any significant metal removal or state change. In other words, a specific metal removing alga is required for effective treatment. However, it also presents the possibility of

including *Botryococcus braunii.* prior to the final outflow as a bio-indicator of metal removal efficiency.

A dual raceway was then set up with the metal accumulating green alga *Tetraselmis* sp. (TSAW92) on the upper level followed by the nutrient removing green microalga *Dunaliella tertiolecta* on the lower level. This system was found to remove 100% of the applied copper and ammonia and 87% of the applied phosphate. In another study an artificial algal meander system was reported to remove 99% of the heavy metals from a mine waste stream, although specific details of the running conditions were not given (Hassett *et al.*, 1979). A laboratory scale batch culture wastewater treatment system based on *Scenedesmus* sp. was capable of removing 52.3% cadmium present in the wastewater (Hammouda *et al.*, 1994). Conventional wastewater treatment plants are unable to achieve these levels of metal removal (Oswald, 1988c), and do not concentrate the metals in the same way as the dual raceway. This lack of concentration is a major drawback in terms of the final disposal of the contaminated material. In the case of the activated sludge process the metals are actually concentrated into the sludge, which is the only potentially useful by-product of the system (Lester *et al.*, 1984). In terms of nutrient removal this system compared very well to the expected optimum nitrogen removal (up to 80%) obtainable from high-rate algal ponds (HRAP) . It is also worth noting that the dual raceway achieves these levels of nitrogen removal with a residence time of four hours and not several days as in the HRAP method (Oswald, 1988c). This huge reduction in residence time would reduce the costs of both building and

operating a sewage treatment plant, it would also allow for the use of a physically smaller plant for a given population.

Finally a completely bare dual raceway was set up and run in the usual way, as a control for purely physical processes which could be responsible for metal or nutrient removal. Only ammonia was removed by this system, probably as a result of gassing off (Hammouda *et al.*, 1994).

6.4.3 Summary

Both dried and live *Tetraselmis* sp. (TSAW92) biomass were found to be able to remove copper from solution in small scale pilot studies. Overall copper loading is better in the live (37 ± 0.2%) rather than the dead systems (28 ± 0.3% (Table 6.1). It is possible to achieve continuous 100% copper and almost 95% nitrogen removal from a 5 ppm copper contaminated 1:1 mixture of sewage and seawater using a purely microalgal based system, with no artificial power input.

CHAPTER SEVEN

GENERAL DISCUSSION

7.1 General Discussion

The aims of this study were to screen a wide range of marine microalgal isolates for their ability to grow in the presence of heavy metals and to remove heavy metals from solution. Subsequently, these mechanisms of metal uptake were characterised and potential applications in the bioremediation of waste waters were investigated (Section 1.7).

The initial screening experiments were very wide ranging, with over 350 marine microalgal isolates from seven algal classes being screened. Previous screening investigations in the field of microalgal wastewater treatment have concentrated mainly on freshwater microalgae, and each study usually dealt with only a few species (Pouliot & de la Noüe, 1985; Strain *et al.*, 1986; Tam & Wong, 1989). Workers in this field are mainly interested in nutrient removal, and regard any microalgal heavy metal uptake as a hindrance which may severely limit the use of any biomass produced (Furr *et al.*, 1981). The work described in this thesis is unique, both in regarding this "problem" as a potential advantage, and in the mass screening of marine microalgae for heavy metal tolerance and removal.

Some ecological studies on the effects of heavy metal pollution on microalgal populations have been carried out: Gale *et al.* (1973) examined algal abundance and species distribution in polluted streams in Missouri's lead belt; Say & Whitton (1979) recorded changes in endemic freshwater flora downstream of a zinc/lead pollution point source. The general conclusion of these

studies were that heavy metals cause a decrease in diversity and productivity, with cyanobacteria and diatoms being most severely affected. The most tolerant algae were found to be the Chlorophycae, with the filamentous forms being the most resistant. In the present study, the Chlorophyceae were also found to be metal tolerant, whereas only one highly metal tolerant diatom was identified.

These ecological studies could be regarded as a type of natural mass screening experiment for microalgal heavy metal tolerance, but, they were all made in freshwater environments, and presented no evidence for a selective effect of a metal on a particular class (Whitton, 1983). These field studies were based on the assumption that the metal gradient downstream of the point source was linear, no attention was paid to potential differences in the physio-chemical environment along the gradient, nor to the possibility of atypical niches and habitats. Brock (1978) reported that gradients of this type were rarely simple or linear in nature due to possibility of additional factors such as a thermal gradients overlying the metal gradient. In the present study the advantages of a wide ranging field survey were combined with the specificity and controlled nature of a laboratory study.

As with freshwater microalgae, very little work has been done with regard to the ability of marine microalgae to take up heavy metals and their potential uses in the field of bioremediation. Most of this work is ecological in nature, concentrating on the effects of metal and sewage pollution on the growth and species composition of endemic microalgal. For example, there has been an

exhaustive series of over 11 studies on the toxic effects of metals on endemic strains *Amphidinium carterae* and *Prorocentrum micans* (Antia & Klut, 1981; Berland *et al.* 1976; Hawkins & Griffiths, 1982; Kayser, 1976; Klut *et al.*, 1981; Prevot, 1978; Prevot & Soyer, 1985; Prevot & Soyer-Gobillard, 1986; Matthys-Rochon, 1980; Soyer & Prevot, 1981). However, in common with other investigations these studies concentrate purely on the effects of metals on microalgae and pay no regard to the converse.

The culture of marine microalgae in a sewage/seawater mixture is somewhat better understood, and has been investigated both from the point of view of a cheap nutrient source (Dunstan & Menzel, 1971; Dunstan & Tenore, 1972; Goldman & Ryther, 1976, and as a potential method of wastewater nutrient removal (Craggs *et al.*, 1994; De Pauw & DeLeenheer, 1979; Witt *et al.*, 1981). The studies of Dunstan and co-workers conclude that marine microalgal growth is stimulated by sewage enrichment of the medium, but that there are sporadic toxic effects, presumably due to the presence of metals. Craggs *et al.* (1994) also noted opposing toxic and stimulatory effects of sewage on the growth of various marine microalgal isolates. It is interesting to note that none of the metal tolerant isolates identified in the present study were adversely affected by the presence of sewage in the culture medium.

Until the present study, research into marine microalgal metal uptake and research into microalgal wastewater treatment have been carried out separately. Here, it was found that a high degree of heavy metal tolerance in UK endemic marine microalgae was relatively rare, with only 23 out of over 350 isolates tested being

able to survive under the screening conditions (Table 3.1). Further only three isolates were found to be highly tolerant of heavy metals and able to sustain logarithmic growth under the screening conditions. The occurrence of tolerant isolates was patchy, both in terms of location and algal class. Some sites yielded no tolerant isolates, whilst two of the three highly tolerant isolates (TSAW92 and *Dunaliella tertiolecta*) were cultured from the same site in the Clyde estuary in Strathclyde. This relatively low and patchy occurrence of metal resistance emphasises the need for a wide-ranging study covering many different isolates from a range of locations and preferably from a variety of algal classes.

This study was also unusual in the fact that the majority of the microalgae screened were directly isolated from the environment and were not simply obtained from culture collections. Craggs (pers. comm.) noted that culture collection species were in general far less robust than their "wild" counter parts. In particular, culture collection samples of *Phaeodactylum tricornutum* and *Oscillatoria* sp. were unable to survive in 1:1 sewage : seawater culture and yet their directly isolated partners were among the best candidates identified in his study (Craggs *et al.*, 1994). It is well known that microalgae which have been routinely maintained in laboratory culture for many generations show many physiological differences to their "wild" equivalents (Craig *et al.*, 1988).

One possible explanation for the low occurrence of heavy metal tolerant isolates is that in the "natural", high levels of heavy metals such as those used in this study, are very uncommon (Gadd,

1990). It could be argued that there is no significant evolutionary pressure on marine microalgae to develop mechanisms of metal tolerance, and thus the ability is uncommon. In fact in many cases the converse is true, with active trace metal uptake being required just to meet the organism's metabolic needs. In bacteria, beneficial iron uptake systems based on siderophores have also been shown to be involved in cadmium, copper, and molybdenum uptake (Ford & Mitchell, 1992). It is possible that microalgal metal tolerance is due, at least in part, to some modification or even reversal of these uptake systems. A modification of this type has been reported in the bacterium *Staphylococcus aureus* , with cadmium being exported from the cell using a specialised Cd^{2+}/H^{+} antiport (Gadd, 1990). In *Chlorella* sp. a similar reversed siderophore system appears to be involved in copper resistance (Butler *et al.*, 1980). Therefore, it could be of value to search for metal tolerant strains both in atypically heavy metal polluted sites, and in those in which there are very low levels of trace elements. In habitats with a low level of available trace elements the endemic microalgae may have developed more efficient metal uptake systems which could be exploited, particularly if they were coupled with a high degree of metal tolerance. This study examined only the former, as an attempt to harvest pre-selected metal tolerant isolates.

To avoid the high costs associated with artificial media and controlled environmental conditions, it would be beneficial to select local endemic algae at each proposed treatment site. At present this type of survey would be impractical, both in terms of labour and time. Using the techniques employed in the present

study it took approximately one year to identify just three highly metal tolerant microalgal isolates. It may be possible to speed this process up by the use of molecular tools, such as antibodies. Monoclonal antibodies to bacterial and fungal siderophores have been made (pers. comm. Dr A. Siwak, Dept of Molecular Biology, University of Shanghai) and it could be of value to investigate the possibility of using them, or their microalgal equivalents, as the basis of a more rapid screening technique. If a pure sample of the metal binding protein from *Tetraselmis* sp. (TSAW92) could be obtained it may be possible to produce a monoclonal antibody based assay capable of the rapid screening of microalgae for the possession of metal binding proteins.

In common with other studies in fungi (Gadd, 1988; Huang *et al.*, 1990; Tsezos, 1986) and in unicellular algae (Gadd, 1988; Gadd, 1990; Garnham *et al.*, 1992; Khummongkol *et al.*, 1982; Wilkinson *et al.*, 1990) this study has shown that marine microalgal metal uptake is a two stage process with an initial rapid, metabolism-independent stage, followed by a slower metabolism-dependent stage (Chapter 4). However, this study is unique in its use of a novel technique for the examination of microalgal metal uptake over tens of seconds rather than minutes as was previously the case. It is clear that marine microalgae are capable of far more rapid metal uptake than has been reported to date (Table 7.1).

In the case of *Tetraselmis* sp. (TSAW92), this thesis has shown that the bulk of the initial uptake is complete within the first thirty seconds.

Table 7.1: The temporal resolution of algal metal uptake studies

Minimum time interval used (min)	Organism	Metal	Reference
60	*Chlorella salina*	Cs	Avery *et al.*, 1993 (a & b)
20	*Chlorella emersonii* *Chlamydomonas reinhardtii* *Scenedesmus obliquus*	Co, Mn, & Zn	Garnham *et al* , 1992
20	*Scenedesmus obliquus*	Cs	Garnham *et al* , 1992
10	*Scenedesmus sp.*	Cu	Mirele, 1982

This observed difference is not necessarily at odds with other studies, as the techniques which they used were only able to measure uptake over relatively long time intervals. In general, the form of time versus uptake graphs produced in these studies is identical to the latter parts of those produced by this study (see Table 7.1 for references). In industrial terms, characterising heavy metal uptake over such small time intervals is of great importance, as it would allow for the use of faster flow rates and hence give a potential cost saving.

Intact live marine microalgae have even more potential as metal removers than their dried biomass. In addition to simple metabolism-independent biosorption, live marine microalgae are capable of intracellular metal accumulation, and intra and extra-cellular precipitation. These additional mechanisms frequently allow them to take up more metal than dead biomass (Gadd, 1989; Gadd, 1990; Kelly *et al.*, 1979). Live cells are also potentially susceptible to genetic, physiological, and morphological manipulation (Gadd, 1990). Genetic manipulation has already been attempted in yeasts, in which it was proposed that different forms of metal-binding proteins could be engineered to produce enhanced and selective metal uptake (Butt & Ecker, 1987). The physiological and morphological manipulation of a living metal biosorbent could provide enhanced metal removal (Gadd, 1990).

In general terms, certain forms and types of cells are better accumulators than others, and it is therefore feasible to select for these cell types by altering the culture conditions (Gadd, 1988). More specifically, the nutritional status of the microalga is known to have

an effect on its ability to remove metals from solution, thus presenting another opportunity for control and enhancement in a live cell system (Avery *et al.*, 1993(a); Garnham *et al.*, 1992). In some cases even a single physical factor such as salinity can have an effect on the level of metal uptake in a live cell system. For instance, the addition of 0.5M sodium chloride increased the caesium uptake ability of *Chlorella salina* by a factor of 28 (Avery *et al.*, 1993b).

This study presents solutions to the four main problems encountered in the industrial use of live microalgal biomass as a metal removal/recovery material, namely: toxicity; harvesting of the metal loaded biomass; recovery of the trapped metals; and the risk of contamination of the biomass culture (Gadd, 1990).

First, due to the extreme metal tolerance of the screened isolates the problems associated with the inherent toxicity of the target heavy metals are avoided.

Second, the use of the raceway system effectively immobilises the microalgal cells in a way which is to date unsurpassed in terms of its low cost and ease of application (Table 7.2). Immobilisation avoids the problems often associated with a free cell suspension, such as small particle size, low density, and low mechanical strength/poor resistance to agitation (Gadd, 1990). These characteristics often severely limit the choice of the reactor system and provide special problems in the areas of biomass/effluent separation (Tsezos, 1986). The raceway/biofilm method of immobilisation has the further advantage that it allows the microalgal cells to remain in direct contact with the effluent, avoiding any diffusion associated problems which may affect other

Table 7.2 A summary of previously applied cell immobilisation techniques used for heavy metal removal.

Immobilisation technique	Organism	Reference
Absorption to reticulated foam particles	*A. oryzae & R. arrhizus*	Kiff & Little, 1986 Lewis & Kiff, 1988
Molochite packing	*Trichoderma viride*	Townsley *et al.*, 1986
Sand filled column	*S. cervisiae*	Huang *et al.*, 1990
Manipulation of growth conditions to promote pellet formation	*Aspergillus niger*	Yakubu & Dudeney, 1986
Entrapment in alginate beads	*Chlorella emersonii*	Wilkinson *et al.*, 1990
Entrapment in alginate beads	*Chlorella salina*	Avery *et al.*, 1993 (b)
Mixing with textile and paper fibres to form a filter pad	Unidentified microfungi	Wales & Sager, 1990

immobilisation techniques. It is also pertinent to point out that in at least two studies (Garnham *et al.*, 1992; Wilkinson *et al.*, 1990) the un-seeded immobilisation matrix was found to be capable of greater metal removal than either the algae alone or any algae/matrix combination. In the study of Avery *et al.* (1993a), 46% of the observed caesium removal was found to be due to the presence of the calcium-alginate matrix. In the present study no metal removal was observed in the un-seeded control raceway (Chapter 6).

In addition to heavy metal removal, the dual raceway system is also capable of the simultaneous removal of inorganic nutrients, which is only possible in a living system. The application of this strategy to a microalgal based system is apparently novel, although a similar concept was used in a bacterial system designed to incorporate bacterial de-nitrification and uranium removal in a sophisticated anthracite based fluidised bed reactor (Hollo *et al.*, 1979). By using two or more raceways seeded with different types of microalgae it is possible to tailor the system to fulfil a variety of separate functions. For example, in the present study the first raceway was able to remove 100% of the applied heavy metals without reducing the nutrient concentration to levels unable to support subsequent microalgal growth (Chapter 6). This feature enabled a second raceway to be used to grow a metal sensitive microalga which was found to be free from heavy metal contamination, this alga would be a potential source of safe single cell protein (SCP). At present the metal contamination of, conventionally produced, waste water grown microalgae is a major obstacle to its use as a source of SCP (Furr *et al.*, 1981).

The third problem is the recovery of the metal from the absorptive material, in the case of dead biomass this is seldom a problem as the metals are loosely held at the cell surface. Metal removal can be achieved by the use of mild non-destructive techniques such as pH and salinity changes, which can allow the dead biomass to be recycled several times (Avery *et al.*, 1993(b); Tsezos, 1984). With live microalgal biomass the metals are tightly bound to intracellular sites and their removal often requires the total destruction of the cell (Gadd, 1989). In the case of costly metal removal materials such as ion-exchange resins the multiple use and recycling of the material can represent a cost saving. However, by its very nature waste-grown microalgal biomass is self-replenishing and of very low cost, and therefore its destruction is of little consequence and may even be of economic value. For instance, the biomass may have potential as a bio-ore. One mining company is regularly smelting copper from ores of just 5% purity, which compares very poorly to a microalgal "ore" of some 30% purity (pers. comm. Rio Tito Zinc Inc.). Destructive techniques maybe inherently cheaper, simpler, and safer than some of the instinctively more attractive so called non-destructive techniques. For instance Darnall *et al.*, 1986, proposed the use of polyacrylamide entrapped live *Chlorella* sp. to reversibly remove copper, zinc, mercury, silver and gold from solution. In this scheme, the absorbed copper and zinc would be removed by lowering the pH to 2 using a strong mineral acid, the mercury, silver, and gold would then be removed by the use of mercaptoethanol. A scheme of this

type could hardly be described as cheap, simple, or safe, and even more importantly it may be of no net environmental benefit.

A final problem with large scale mass microalgal culture systems is the constant threat of contamination by naturally occurring microalgal species and their grazers (Becker & Venkataraman, 1982; De Pauw *et al.*, 1984; Richmond, 1986b). It should be noted that this threat cannot be avoided by the use of dead microalgal biomass, as this too must be originally derived from a large scale culture. Fortunately, due to the simultaneous growth and *in situ* use of the microalgae biomass this threat proved not to be a problem in the system used here. The presence of the heavy metals was found to totally inhibit the growth of both grazers and endemic microalgal species. Microscopic examination of the raceways (Chapter 6) revealed no contamination over a period of at least one month.

Possibly the most interesting avenue for further research opened by this study is the presence of copper binding protein(s) in the culture medium of *Tetraselmis* sp. (TSAW'92) when grown in the presence of copper (Chapter 5). *Tetraselmis* sp. (TSAW'92) released three specific proteins (molecular masses ca. 28, 30 and 55 kDa respectively) in response to the presence of heavy metals only, there was no production in response to osmotic or thermal shock (Chapter 5). An *in vitro* binding study showed that these proteins were capable of strongly binding to copper, and it can therefore be assumed that they play some part in the atypically high degree of copper tolerance and accumulation shown by this microalga.

The molecular masses indicated for these proteins by SDS PAGE are higher than those suggested for intracellular microalgal metal binding proteins, which are typically ca. 6 to 10 kDa (Reddy & Prasad, 1989). There are two possible explanations for this difference. Either the proteins are indeed heavier than those currently recorded, or the indicated masses are inaccurate. Due to its high capacity for non-specific binding, it is possible that the protein binds far more SDS than would normally be expected. However, this is unlikely to fully account for the three to five fold difference in molecular mass between that of general metallothioneins and that of the proteins of interest. This situation could have been clarified by the use of mass spectrometry, however this facility was not available at St Andrews. Therefore, the techniques of native PAGE and native size exclusion chromatography were attempted. However, no suitable protocol could be developed to allow the application of either of these techniques. In the former, the protein binds to the gel wells and will not migrate. In the latter, the protein could not be eluted from the column without resorting to extreme and destructive measures.

It is likely that these proteins are functional analogues of intracellular microalgal phytochelatins (see Introduction for review), although they differ from conventional phytochelatins in that they are relatively large and are found outside the cell. While this extracellular location has not been previously reported for metal binding proteins, it is well known that metal binding polysaccharides, which act as a first line of metal defence at a distance from the cell and are located externally (Gadd, 1990). It is

proposed that the observed protein release by *Tetraselmis* sp. (TSAW92) is analogous to the release of extracellular polysaccharides as a metal defence system operating at a distance from the cell. The observed extracellular location of these proteins may not be as a result of deliberate secretion, but could be due to enhanced cell permeability brought about by metal toxicity (Gadd, 1990). However, this explanation has been discounted on the grounds that the levels of metals used in this study were not found to cause an increase in *Tetraselmis* sp. (TSAW92) cell permeability (Chapter 6). In addition, if the cell membrane was compromised to the extent of allowing unrestricted protein release it would be reasonable to expect cell death to follow, but in the case of *Tetraselmis* sp. (TSAW92) the culture maintained logarithmic growth. It would also be reasonable to expect that a protein leakage of this type would be non-specific with many types of proteins being released, but again this is not the case (Chapter 5).

The presence of microalgal metal binding proteins raises the possibility of genetic manipulation. In yeasts similar metal binding proteins have been identified and have already become the subjects of genetic manipulation, both to enhance the overall levels of these proteins and to produce a degree of metal selectivity (Butt *et al.*, 1984). Using the Cu-MT (copper metallothionein gene) from a metal tolerant strain of *S. cervisiae* , it has also been possible to transform non-metal tolerant *S. cervisiae* brewing yeasts into highly copper tolerant strains (Butt *et al.*, 1984; Butt & Ecker, 1987).

It is not unreasonable to suggest that these techniques could be applied to microalgae to produce different strains which could

selectively sequester different metals, providing the opportunity to "mine" even mixed wastes for useful metals. Alternatively, the general metal sequestering ability of a microalga could be enhanced by the amplification or up-regulation of its metal defence proteins. Gadd (1990) reported that metallothionein production in *S. pombe* gave added protection against the anti-fungal agents tetra-methythiuram disulphide (TMTD) and dimetthyl dithiocarbamate (DMDTC). They also proposed that metallothioneins may be involved in the scavenging of free radicals and in the detoxification of xenobiotics. It is therefore possible that up-regulation of metallothionein genes in marine microalgae could also produce organisms capable of resisting a wide variety of pollutants. An ability of this type would be of great value in any industrial metal removal system relying on live microalgae, as it would increase the range of effluents amenable to this type of treatment.

However, in order to pursue any of these avenues the gene or genes responsible for the production of microalgal metallothioneins must be identified. This was outwith the scope of this study, but would present an interesting area for future work.

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Appendix 1 UK Microalgal Sampling Sites

Key:

1 St Andrews Bay sewage outfall
2 Faslane Naval Base outfall pipe Firth of Clyde
3 Port Talbot car plant outfall pipe Swansea bay
4 Wick Ferry Terminal anitifouling dock
5 Tilbury Docks
6 Cornwall tin mine discharge

Appendix 2 Properties of *Tetraselmis* sp. (TSAW92)

A small green unicellular marine/estuarine microalaga isolated from a heavy metal polluted shore site on the River Clyde Estuary, Scotland, UK.

The physical appearence is that of any typical *Tetraselmis* sp. being a small green coccoid of diameter 5-15μm. This particular isolate is characterised by having absent or sessile flagellae, extreme metal tolerance, and high culture dominance.

pH tolerance pH 4-11, optimal at pH 8

Temperature tolerance 0-38°C, optimal at 15°C

Appendix 3 Algae which failed Screening (Chapter 3)

KNOWN SPECIES	UNIDENTIFIED ENDEMIC ISOLATES	
BACILLARIOPHYCEAE	**BACILLARIOPHYCEAE**	**CHLOROPHYCEAE**
Nitzschia longissima (1)	SA90B1 (*)	•SA90C1 (*)
Nitzschia ovalis (3)	•SA90B2 (*)	•SA90C2 (*)
Thalassiosira weissflogii (5)	•SA90B3 (*)	•SA90C3 (*)
	SA90B3a (*)	SA90C4 (*)
CHLOROPHYCEAE	•SA90B4 (*)	SA90C5 (*)
Botryococcus braunii (2)	•SA90B5 (*)	•SA91C6 (*)
Chlamydomonas reginae (5)	SA90B6 (*)	SA91C7 (*)
	•SA90B7 (*)	SA91C8 (*)
CHRYPTOPHYCEAE	SA90B8 (*)	SA91C9 (*)
• *Rhodomonas baltica* (1)	SA90B9 (*)	•SA91C10 (*)
• *Rhodomonas marina* (5)	SA90B10 (*)	SA91C11 (*)
• *Rhodomonas* sp. (3)	SA90B9/10 (*)	SA91C12 (*)
	SA90B11 (*)	78 Isolates
CYANOPHYCEAE	•SA91B12 (*)	
Oscillatoria animalis (1)	SA91B13 (*)	**RHODOPHYCEAE**
Spirulina platensis (1)	SA91B14 (*)	•SA90R1 (*)
	SA91B15 (*)	7 Isolates
DINOPHYCEAE	SA91B16 (*)	
Amphidinium carterae (5)	SA91B17 (*)	**DINOPHYCEAE**
	SA91B18 (*)	4 Isolates
PRASINOPHYCEAE	SA91B19 (*)	
Micromonas pusilla (5)	•SA91B20 (*)	**OTHER ISOLATES**
• *Tetraselmis rubens* (3)	SA91B21 (*)	SA91B36 (*)
Tetraselmis sp. (1)	SA91B22 (*)	•SA91B37 (*)
• *Tetraselmis* sp. (4)	SA91B23 (*)	SA91B38 (*)
• *Tetraselmis tetrathele* (5)	SA91B24 (*)	•SA91B39 (*)
	•SA91B25 (*)	SA91B40 (*)
PRYMNESIOPHYCEAE	SA91B26 (*)	SA91B41 (*)
Chrysochromulina chiton (5)	•SA91B27 (*)	SA91B42 (*)
Coccolithophora sp. (1)	SA91B27a (*)	•SA91B43 (*)
Isochrysis galbana (5)	SA91B28 (*)	SA91B44 (*)
Phaeocystis poucheti (5)	SA91B29 (*)	SA91B45 (*)
• *Prymnesium parvum* (5)	SA91B30 (*)	SA91B46 (*)
	SA91B31 (*)	SA91B47 (*)
RHODOPHYCEAE	•SA91B32 (*)	
• *Porphyridium* sp. (1)	•SA91B33 (*)	•SA91C13 (*)
	SA91B34 (*)	SA91C14 (*)
	SA91B35 (*)	SA91C15 (*)
	91 Isolates	82 Isolates

Numbers in () refer to origin: (1) Biobred, (2) CCAP, (3) Gatty Marine Laboratory, (4) Millport Marine Biological Station, (5) Plymouth Culture Collection, (*) Craggs *et. al.* 1994