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EFFECTS OF HEAVY METALS ON GROWING CULTURES OF *CHLORELLA EMERSONII.*

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ABSTRACT

This work evaluates the effect of some metals on a green alga *Chlorella emersonii,* under continuous and batch culture conditions with added metal and another, batch culture with no added metal but where organism had been exposed to metal for 18 hours prior to growth. It was found that *Chlorella* growth under continuous culture was reduced in the presence of silver at levels as low as 0.1 mg/l and ceased altogether at silver concentration of 1.0 mg/l. Under batch culture conditions no growth occurred with any of the silver concentrations tested. *Chlorella* that had been exposed to silver (1.0 mg/l) prior to cultivation without added metal showed growth. Above concentrations of 1.0 mg/l exposure their was no growth. Copper stimulated growth of *Chlorella* under continuous culture conditions up to levels of 0.5 mg/l but became severely toxic at levels of 1.0 mg/l and above. Under continuous culture conditions cadmium inhibited *Chlorella* growth at cadmium levels of 0.05 mg/l with no growth occurring at 0.5 mg/l. However under batch culture conditions *Chlorella* growth was not affected at levels below 1.0 mg/l.. *Chlorella* that had been exposed to cadmium up to 20 mg/l. prior to cultivation without added metal showed growth This study indicates that for short periods some microorganisms can tolerate elevated levels of heavy metal contamination, although environment can be seriously affected if contamination persists over long periods.

KEY WORDS: Heavy metals, Chlorella, continuous culture, batch culture, environment.

INTRODUCTION

Heavy metals are those elements with a density greater than 5g cm^{-3} (Rai, *et al.* 1981). Nieboer and Richardson (1980) suggested abandoning the term 'heavy metals' and reclassifying metals into a biologically and chemically significant metal-ion classification. However some chemists define heavy metals as the second and third row transition metal ions, and tin and lead. Others define heavy metal ions as metallic elements that are not essential for life and these exclude nickel, copper and zinc which are essential in trace amounts but toxic at higher concentrations (Borovik 1990). Reed and Gadd (1990) use the term more loosely and in a broad context state that about 65 elements exhibit metallic properties and may be termed 'heavy metals'. The latter include several metals and metalloids commonly encountered as environmental pollutants. Volesky (1990) uses a looser term and includes all metals of the periodic table except those in groups I and II.

These metals occur in mineralised rock, which can, due to weathering, be released into the environment. However mineralisation and weathering are a continuous process which if unperturbed maintains a balance on the free metal available in the environment. The activities of man invariably alter this balance dramatically. Mining activities (Denny and Welsh, 1979, Johnson and Eaton, 1980, Beyer *et al.*,1985) and manufacturing, (Ajmal and Khan, 1985) release large quantities of metals into the environment. For example discharge of cadmium into natural waters may be a consequence of electroplating activities (Higgins and Desher, 1986), nickel-cadmium battery manufacture and/or smelter operations (Butterworth, *et al.*, 1972). Much of the wastes from industry containing these metals finds their way into sewage treatment works (Cheng *et al.*, 1975, Tyagi *et al.*,1988). In many cases the sludge from these works containing high concentrations of metal is deposited on agricultural land (Schauer *et al.*, 1980, Dressler *et al.*, 1986). It may then become incorporated into food chains or be deposited in water courses through run-off. Freshwater streams may bear elevated levels of heavy metals due to the leaching and weathering of natural rocks or as a result of industrial activities. Such habitats often show a reduced algal flora (Reed and Gadd, 1990). In many coastal marine habitats the greatest heavy metal inputs are from rivers and freshwater run-off. Concentrations of heavy metals therefore usually decreases with distance from river mouths due to dilution and precipitation in the alkaline seawater.

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In some cases, pollutants in general, and metals in particular can be transported to areas far removed from their point source even in unpolluted areas such as the Arctic, as in the case of microbial radionuclide accumulation (Hanson, 1967).

Some metals such as copper and iron are required in trace amounts by organisms for metabolic processes. Others like lead, arsenic and cadmium may also be required (Schwarz 1977) and finally there are those such as mercury and silver that have no known metabolic role. However all metals in high enough concentrations can be toxic to organisms.

Metals may persist in the environment resulting in biomagnification especially in sharks and eagles, and other organisms at higher trophic levels. (Martin & Coughtrey, 1975, Gipps and Biro, 1978, Broda, 1972). *Homo sapiens* being at the top of many a food chain is highly vulnerable, as in the case of mercury poisoning in Minamata, Japan, where local inhabitants suffered from neurological illness after consuming sea fish and shellfish contaminated with methyl-mercury (Wood, 1983).

The oceans are frequently the ultimate recipients of heavy metal pollution and since algae have a major role in marine primary production, the effects of heavy metal toxicity, accumulation and transfer through food webs may be adverse.

The following investigations examine metal interactions with *Chlorella emersonii* under growing conditions. Organisms under growing conditions, in batch or continuous culture, are in a dynamic environment, with biomass levels continuously changing. Analysis of metal interaction with *Chlorella emersonii*, was examined from three different approaches, continuous culture and batch culture conditions, and exposure of the organism to metal for a specific time span.

MATERIALS AND METHODS

Source of organism

A stock culture of *Chlorella emersonii* (CCAP 211/11N) was obtained from Culture Collection of Algae and Protozoa, Freshwater Biological Association, The Ferry House, Ambleside, Cumbria LA22 OLP U.K.

Maintenance Of Cultures

JM (Jaworski's medium) algal growth medium described by Thompson *et al.* (1988) and JM agar which was prepared from JM medium solidified by the addition of 15g/l of Agar No. 3 (Oxoid) were used for the maintenance of algal cultures.

Cultures were stored in JM algal growth medium and on JM agar slopes at 5°C in a refrigerator. Fresh cultures were prepared by inoculating fresh media with a loopful of culture and by streaking a fresh agar slope every two months. These were then incubated, in natural daylight for two weeks at room temperature. Checks were made for purity using a light microscope. These were then stored in a refrigerator and used as required.

Solutions

All solutions were made using deionised water. Sterilization was achieved by autoclaving at 121°C for 15 minutes.

Metal solutions.

All metal solutions were made using deionised water. Stock solutions (1000 mg l^{-1}) were made every two months. Working solutions were made fresh from stock solution as required.

Silver, Cadmium and copper

Stock solutions of silver were prepared using Silver nitrate ANALAR, Hopkin and Williams, Chadwell Heath Essex. To avoid photoreduction, solutions were stored in brown bottles. Cadmium stock solutions were made using cadmium nitrate, or cadmium acetate (all ANALAR BDH Chemicals Ltd Poole England), while Copper stock solution was prepared using cupric sulphate {copper (II) sulphate pentahydrate} ANALAR BDH Chemicals Ltd. Poole England.

Atomic absorption spectrophotometry

Concentrations of copper and lead were determined using a Varian AA-1275 Series Atomic Absorption Spectrophotometer (Varian, 1979). Metal standards were made from stock solutions as described above.

Cell Count

Cell counts were carried out using a COULTER multisizer II (Coulter Electronics Ltd. Northwell Drive, Luton, Bed. England) in accordance with the manufacturer's handbook and instruction manuals. The electrolyte used was 0.9% sodium chloride solution filtered through a 0.22 micron filter.

Microbial Growth Methods

Batch Culture

Batch cultures were grown in 1l Erlenmeyer flasks (working volume 300 ml) containing JM medium. Medium was inoculated with 1% (v/v) of previously grown culture to give an initial concentration of about 10^5 cells per ml.

Stock cultures in 10l glass vessels were grown at room temperature in natural daylight. Air (600

ml/min) was pumped in using air pump, model B100 SEP (Charles Austen pumps Ltd.). A constant air flow rate was maintained and controlled using a Platon rotameter (Gap Basingstoke). On exiting rotameter air was passed through a 0.22 micron PTFE filter (Whatman MIDISART 2000) to prevent contamination. Vented air was also passed through a filter.

Continuous Culture

Chlorella emersonii was grown in 10 litre glass vessels (working volume 8 litres). Vessels were adapted using a length of glass tubing (diameter 1 cm) on the inside, exiting at a bung inserted in a port at the base of the vessel. This enabled a constant volume of 8 litres to be maintained.

The top bung was fitted with a number of stainless steel tubes (diameter 0.5 cm) for feed, sampling, aeration and ventilation.

Aeration was carried out as mentioned for batch culture above. Illumination was provided by 6 R80 Sungro-lite natural light lamp (75 watts, light intensity 330 lux). Lights were approximately 40 cm from vessel. Medium was delivered from a vessel (20 l) using a peristaltic pump (Watson Marlow 501U Camlab ltd.). The pump flow rate was determined using a measuring cylinder (100ml) and stop watch. This was verified by measuring the rate of flow from the harvest outlet. Chemostats were inoculated with 200 ml of a growing culture of *C. emersonii.* They were operated in batch mode for 3 days before continuous mode was initiated. Samples were taken into sterile sample bottles connected to a harvest tube. Samples (20 ml) were collected twice daily for cell counts.

In a continuous culture system, changes in the concentration of organisms depend upon the relationship between growth and washout. Thus:

dx/dt = □.x - D.x (Pritchard and Tempest, 1982).

x = biomass concentration

t = time

D = Dilution rate (flow rate of the medium divided by the total volume of culture).

□ = growth rate.

Doubling time (t_d) = ln2/μ

Chemostats were operated at dilution rates higher than the critical dilution rate. Any variation from the calculated [illegible] was due to growth.

Determination of Washout Kinetics

This was determined by operating chemostats at dilution rates higher than the critical dilution rate. Cell numbers were monitored using a Coulter counter. Acquisition of such data enables theoretical cell numbers at a given time to be calculated. Any variation from the theoretical value is due to growth. Algal cultures at various concentrations of metal were compared.

RESULTS AND DISCUSSION

The effect of silver on the growth of *Chlorella emersonii* during washout under continuous culture conditions is shown in table 1. The results show that 40% reduction in growth rate was caused by about 0.1 mg/l of silver, while growth ceased completely with a concentration of 1.0 mg/l. Copper on the other hand appears to stimulate growth at low concentrations, resulting in a 60% reduction in doubling time at a concentration of 0.5 mg/l as shown in table 2. However at higher concentrations there is strong inhibition to growth, with growth stopping altogether at 5.0 mg/l. Table 3 shows inhibition of growth when cadmium is present at levels as low as 0.05 mg/l, with growth ceasing altogether at a concentration of 0.5 mg/l.

Table 1: Effect of Silver on the growth of *Chlorella emersonii* during washout, under continuous culture conditions.

Silver Conc. (mg/l)	μSpecific Growth Rate. (h^{-1})	Doubling Time Hours
0	0.018	38.5
0.1	0.013	53.3
0.5	0.008	86.6
1.0	-0.09	No Growth

Table 2: The effect of Copper on the growth of *Chlorella emersonii* during washout, under continuous culture conditions.

Copper Conc. (mg/l)	μSpecific growth Rate (h^{-1})	Doubling Time Hours
0	0.016	43.3
0.1	0.022	31.5
0.5	0.044	15.8
1.0	0.003	231.04
5.0	-0.012	No Growth

Table 3: Effect of Cadmium on the growth of *Chlorella emersonii* during washout, under continuous culture conditions.

Cadmium Conc. (mg/l)	? Specific growth rate(h^{-1})	Doubling time (Hours)
0	0.044	19.1
0.05	0.024	28.8
0.1	0.009	77
0.5	- 0.11	No Growth

Table 4: Effect of growing *Chlorella emersonii* in the presence of silver (10 day batch culture)

Silver Conc. (mg/l)	Comments
0	Growth
0.1	No Growth
4	No Growth
13	No Growth
27	No Growth

Table 5: Effect of growing *Chlorella emersonii* in the presence of Cadmium (10 day batch culture)

Cadmium Conc. (mg/l)	Comments.
0	Growth
0.1	Growth
1.0	Growth slight
5.0	No Growth
17.0	No Growth

Under batch conditions no growth occurred if silver was present as shown in table 4. Growth of C. emersonii under batch culture conditions was inhibited at a concentration of 1 mg/l cadmium as shown in table 5.

Chlorella cells that were exposed to silver for 18 hours showed a discernable colour change at levels of 1.0 mg/l and above (see Table 6) However cells that had been exposed to levels of up to 1.0 mg/l silver were able to grow when cultured in fresh medium. At levels above this no growth was detectable The colour change that occurred at levels of silver of about 1.0 mg/l and above, was fast, occurring in the first minute of adding the silver. This colour change only occurred with silver. Neither cadmium, or copper caused any colour change at concentrations up to 1000 mg/l. Exposure to cadmium up to 20 mg/l for 18 hours prior to growth in metal free medium showed no inhibition to growth of *Chlorella* (Table 7).

Metal ions are essential for the function of all microbial organisms. Potassium and magnesium are bulk intracellular species, while sodium, calcium, zinc and copper, amongst others, are required in trace amounts. Some of these trace elements are toxic at higher concentrations, (Hughes and Poole (1989).

The *Chlorella* cell envelope can be described as being anionic, that is, possessing an overall negative charge (Arikpo 1994). The anionic charge on the bacterial cell wall is described as being 'wettable', that is, being anionic in nature, consisting of an abundance of chemical functional groups (Beveridge 1988, 1989). Thus it is essential, as the bacteria must depend on diffusion for nutrition and waste removal. (Beveridge 1988; Beveridge 1989). Hence it can be regarded that the anionic nature of the *Chlorella* cell wall is necessary for the binding and transport of essential chemicals, including metals, required for growth. Therefore when silver, cadmium and other heavy metals bind to the anionic surface, a blockage arises preventing the binding and entry into the cell of essential chemicals, thereby impairing proper functioning of the cell. These metals silver and cadmium are toxic.

Many of the heavy metals including silver and cadmium are readily polarised and may be classed as 'soft' in a chemical sense (Hughes & Poole 1989). Many of the metal ions of microbiological

Table 6: Effect of exposing *Chlorella emersonii* to silver for 18 hours, prior to cultivation under normal conditions.

Silver conc. (mg/l)	Cell appearance at end of 18hr exposure	End of 10 day batch culture.
0	Dark green	Growth
0.1	Dark green	Growth
0.5	Dark green	Growth
1.0	Light green	Growth
5.0	Yellowish brown	No growth
10.0	Yellowish brown	No growth
20.0	Yellowish brown	No growth

Table 7: Effect of exposing *Chlorella emersonii* to Cadmium for 18 hours prior to cultivation under normal conditions.

Cadmium conc. (mg/l)	Cell appearance at end of 18 hr exposure	End of 10 day culture.
0	Dark green	Growth
0.1	"	"
0.5	"	"
1.0	"	"
2.0	"	"
10.0	"	"
20.0	"	"

importance are described as being 'hard' or borderline hard. Hard metals cannot compete with the soft metals for binding sites, and so are displaced from their sites by soft cations. Hard metal ions such as Na^+ and K^+ interact weakly with ligands, due to their poor polarizing power (charge/radius ratio of the ion). Hard cations like K^+ are small, usually have a high charge and are not easily polarised (Remacle 1990). Hence toxic metals are said to exert toxic effects in several ways, including displacing native metals from their normal binding sites or by binding to proteins and nucleic acids and altering their conformation (Hughes & Poole 1989).

In this study it was found that at low levels of heavy metal, where only some of the anionic sites are occupied by the heavy metal, some growth does occur, but as metal levels increased, more sites became occupied and the equilibrium within the system prevented further binding of ions including necessary ones. During continuous addition of metal as in continuous culture, metal was continuously deposited onto the cell, which prevented binding by essential chemicals. This is seen when cadmium or silver is used. Copper behaves differently, having caused an increase in growth at low concentrations. This is because copper is classed as an essential metal and is required in small amounts for growth (Hughes & Poole 1989).

When placed into metal free medium after exposure to heavy metals, the equilibrium was changed and essential chemicals could once more enter and bind to available sites and some growth could resume. As no fresh metal was added, as cells grew, more and more sites then became available for normal binding and normal growth resumed.

In this study it was observed that silver was more toxic than cadmium and this appeared to be linked to the colour change observed in *Chlorella* cells at concentrations of silver of about 1.0 mg/l and above. The colour change observed may be due to oxidation of bound silver. So not only does silver bind and block sites but may affect light getting to the organism. As *Chlorella* is a photosynthetic organism, light is an important part of its requirement for growth and survival. This is likely to be the reason why silver is more toxic than other heavy metals. However this is an area that requires further investigation.

These investigations showed that there was a difference between growing an organism in the presence of heavy metal and growing organisms that have been exposed to and removed from heavy metals. This has important implications for environmental heavy metal pollution. It indicates that for short periods some microorganisms can tolerate elevated levels of chemical contaminants but if this contaminant is persistent over long periods then environments can be seriously affected.

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