Effects of heavy metals on freshwater chlorophyta.

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Frontispiece - Caplecleugh Low Level discharging mine drainage water to the River Nent at Nenthead, Cumbria

EFFECTS OF HEAVY METALS ON FRESHWATER CHLOROPHYTA

By

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This thesis is entirely the result of my own work. It has not been accepted for any other degree and is not being submitted for any other degree.

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ABSTRACT

A study was carried out on the chemistry and vegetation of two streams containing elevated levels of heavy metals. In neither stream had the presence of these metals prevented the development of an algal flora, though species numbers were low in comparison with uncontaminated streams. The algal biomass was high in both streams, probably due to the lack of invertebrate grazers. Green algae were dominant.

One of these streams, a highly calcareous mine effluent in Northern England (Durham code no. 0097), was studied throughout its annual cycle. Concentrations of heavy metals accumulated by the dominant algae (Mougeotia spp.) were equated with the physical and chemical properties of the water. Zn was supersaturated in the stream water (6.84 mg l^{-1} at pH 7.85); this was shown to be the major factor which influenced Zn accumulated by the algae.

Samples from the other stream, a smelter tip seepage in South-East France (Durham code no. 3026), were available from an earlier visit. This stream contained extremely high levels of heavy metals (Zn = 3840 mg l^{-1}; Cd = 345 mg l^{-1}) and was dominated by the green alga Hormidium rivulare.

Ten strains of green algae were isolated from these two streams and were shown to be resistant to Zn in the laboratory, probably as a result of genetic adaptation. Environmental factors which were likely to be affecting Zn toxicity were investigated for isolates of the dominant algae. Mg reduced Zn toxicity in both streams and may have an important role in the development of resistance by these algae. Field levels of Cd did not influence algal growth at field levels of Zn.

The role of carboxylic acids in algal Zn resistance was investigated but could not be established. Accumulation studies suggest that Zn resistance by green algae involves internal detoxification of the metal and not exclusion.

List of abbreviations

^{o}C	degrees Celcius
g	gramme
l	litre
m	metre
M	molar
h	hour
min	minute
s	second
lb	pound
in	inch
mg	milligramme
µg	microgramme
ml	millilitre
µl	microlitre
cm	centimetre
mm	millimetre
µm	micrometre
nm	nanometre
mM	millimolar
µM	micromolar
nM	nanomolar
P	photons
µE	microeinsteins
m-equiv	milli-equivalent
mV	millivolt
KN	kilonewton
rpm	revolutions per minute
λ	wavelength
v/v	volume for volume
n	number of samples
O.D.	optical density
chl a	chlorophyll a
chl b	chlorophyll b
DMGA	dimethyl glutaric acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
EDTA	ethylenediaminetetra-acetic acid (disodium salt)
zincon	o-[a-(2-hydroxy-5-sulphophenylazo)-benzilidenehydrazino] benzoic acid

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1 INTRODUCTION

1.1 General Introduction

In recent years there has been a great deal of interest directed towards the pollution of the environment by elevated levels of heavy metals. There have been two main reasons for this interest.

a) Though many heavy metals are essential to the growth of animals and plants (Bowen, 1966), higher concentrations may exert severe toxic effects. In extreme cases heavy metals may be lethal to man, as demonstrated by occurences of acute mercury and cadmium poisoning in Japan (Irukayama, 1967; Kobayashi, 1971). As human-induced mobilization of heavy metals in the environment continues to increase, scientifically established 'base-line' and 'pollutant' concentrations are required.

b) High concentrations of heavy metals in the environment may lead directly to a decrease in species diversity and hence an "extreme environment" in the sense of Brock (1969). This has stimulated interest in the responses of organisms to elevated levels of heavy metals, particularly the mechanisms by which organisms can withstand these levels.

The term "heavy metal", although not rigidly defined, is generally held to refer to those metals having a density greater than five, about forty elements in all (Passow et al., 1961). Nieboer and Richardson (1980) propose that the term heavy metal be abandoned as it has connotations of toxicity and therefore lighter elements are often included in this category. They suggest that metals be classified as oxygen seeking, nitrogen/sulphur seeking or intermediate; they demonstrate convincingly the biological relevance of this classification. Though the present study concentrates on the metal zinc, the general term 'heavy metal' is retained throughout, whilst it is realised that this is a somewhat artificial classification.

Zinc (atomic no.-30; atomic weight-65.38) is a white lustrous metal, almost invariably existing in nature in the +2 valence state. Zinc shares group IIb of the periodic table along with cadmium and mercury; the chemistry of zinc and cadmium is very similar, though mercury cannot be regarded as homologous (data from Cotton and Wilkinson, 1980).

Zinc has a wide variety of industrial uses including galvanizing, the production of zinc based alloys (e.g. brass, bronze), rubber production (as zinc oxide) and a wide range of other uses as various zinc compounds (Cammarota, 1980). According to Waldechuck and Woolhouse (1974), at present 3.9×10^6 tonnes of zinc per annum, are mobilized from geological deposits. This compares to the natural geological rate of 3.7×10^5 tonnes per annum. This fact emphasises the level of human-induced mobilization of zinc, being approximately ten times the natural geological rate. About 10% of the metallic zinc produced each year is transported to the ocean via fresh waters (Bowen, 1966).

1.2 Zinc in the aquatic environment

Zinc has been classified as a "scarce element" (Skinner, 1969), its crustal abundance being below 0.01%. Wedepohl (1972) has shown that the majority of this zinc is incorporated in certain structural positions of silicates and oxides, often substituting for magnesium and iron with which it has a similar ionic radius. Zinc is, however, concentrated in certain areas in the form of localized ore deposits, usually involving the crystallisation of metal-rich hydrothermal brines along existing fault planes. Wedepohl (1972) lists 102 naturally occurring zinc minerals, however sphalerite (ZnS; cubic) is by far the most important zinc mineral both economically and in distribution.

The concentrations of zinc in surface waters are generally low (< 10 μg l^{-1}: Hem, 1970; Florence, 1980) due to the resistance of silicates and

oxides to weathering. Aqueous oxidation of sphalerite, however, takes place readily, the reaction being:

$$ZnS + 2O_2 \longrightarrow ZnSO_4$$

under certain conditions microbes can oxidize sphalerite in the following way:

$$2ZnS + 4O_2 + H_2O \longrightarrow ZnSO_4 + ZnO + H_2SO_4$$

in this case the production of sulphuric acid will aid the mobilization of zinc ions (Zajic, 1969).

The patterns and processes of zinc entry into fresh water are reviewed by Weatherley et al. (1980). Elevated levels of zinc in freshwater can be expected in base-metal mining regions (derived from both groundwater drainage of mineral deposits and drainage of overburden of zinc or other ore bodies), in industrial regions (particularly those conducting the smelting of zinc or those containing zinc associated industries; Section 1.1) and urban regions (derived from domestic sewage). In addition acid mine drainages associated with coal mines (oxidation of pyrite (FeS) results in the production of sulphuric acid) may carry elevated levels of zinc, due to the increased mobilizing influence of acid waters (Hargreaves et al., 1975).

The chemistry of zinc in freshwater is reviewed by Hem (1972) and Florence (1980). pH is particularly important in controlling the solubility of zinc in aqueous environments. Zinc may be precipitated at higher pH values in the following way:

$$Zn^{2+} + H_2O \rightleftharpoons ZnOH^+ + H^+ \rightleftharpoons Zn(OH)_2 \downarrow + H^+$$

Based on thermodynamic calculations Hem (1972), however, showed that the solubility of zinc carbonate ($ZnCO_3$) and zinc silicate (Zn_2SiO_4) were both

less than that of zinc hydroxide and in the majority of freshwaters (where the carbonate and silicata ligands are available) these form the major control of zinc solubility. Florence (1980) states that both zinc carbonate and zinc silicate may exist as stable colloids in natural waters (particle diameter 1-2 nm). Other chemical forms of zinc may also occur in water, particularly organic complexes (e.g. Zn-humate, Zn-citrate) and as ions adsorbed onto colloids (e.g. Zn^{2+}- humic acid) or inorganic colloids (Zn^{2+} - clay minerals). (Clay mineral complexes are likely to lead to zinc deposition except during turbulent conditions, when they are maintained in suspension.) All complex forms of zinc in natural waters occur more readily at higher pH values and above approximately pH 7 zinc complexes are likely to be formed; below pH 6 zinc is likely to exist as the simple divalent ion or as the hydrated ion (Hem, 1972; Florence, 1980).

Many authors state the average level of zinc in unpolluted freshwater can suitably approximate to 10 $\mu g\ l^{-1}$ (Hem, 1970; Abdullah and Royle, 1972; Wedepohl, 1972; Florence, 1980). Levels of zinc tend to be elevated in thermal or acidic streams (Wedepohl, 1972); excepting these, levels of zinc above 0.1 mg l^{-1} in streams are indicative of human activity and those above 1 mg l^{-1} are usually associated with past or present mining activities (Harding, 1978). Say (1977) reported many streams containing >1 mg l^{-1} zinc, in his survey of streams draining the Northern Pennine Orefield; the maximum was 22.3 mg l^{-1} zinc. The maximum level reported in the literature is probably that for a site draining a smelter tip in southern France (Say and Whitton, 1982), where a small seepage was found to contain 3840 mg l^{-1} zinc. Current E.E.C. drinking water standards allow up to 3 mg l^{-1} zinc (quoted by Hargreaves, 1981).

In mineral veins sphalerite (ZnS) is frequently associated with galena (PbS), greenockite (CdS) and rarely chalcopyrite ($CuFeS_2$: Dunham, 1948). Effluent from mining regions would be expected to contain elevated levels

of cadmium, lead and possibly copper, as well as zinc. Lead is relatively insoluble in neutral-alkaline conditions (Brooks, 1972) and is also resistant to weathering (Dunham, 1981); its concentration in water, therefore, usually remains low. Cadmium is invariably associated with zinc minerals. The Zn:Cd ratio in the mineral veins of the Northern Pennines is calculated to be 230:1 (data from Dunham, 1948), which agrees closely with a mean ratio of Zn:Cd of 210:1 reported for the rivers and streams of Northern England (J.D. Wehr, pers. comm.).

1.3 Biological effects of zinc

1.31 Physiological and biochemical aspects

Since it was first shown by Raulin (1869) that zinc was essential for the growth of Aspergillus niger, its requirement has been shown for an increasing number of species and zinc is now regarded as an essential element for all living organisms (Bowen, 1966). Zajic (1969) has reviewed the importance of zinc in microbial metabolism; Vallee (1959) presents a more comprehensive review of the biochemistry of zinc. Zinc was first demonstrated to be an active component of carbonic anhydrase in both animals (Keilin and Mann, 1944) and in plants (Day and Franklin, 1946). Since then it has been shown to be an important structural component of many enzyme systems (Vallee, 1959).

The common feature of heavy metals, in relation to biological life, is that in excessive quantities they are poisonous and can cause death of most living organisms. The toxic effects of zinc to plants are, however, incompletely understood. It seems that an important mechanism of toxic action is through the alteration of enzymes and the resultant destruction of the relevant metabolic action (Passow et al., 1961). It has been found that the relative toxicities of a range of heavy metals can be correlated with the insolubility of their respective sulphide compounds, for both a

range of animals (Shaw, 1954) and an alga (Fisher and Jones, 1981). This suggests that binding onto the sulphydryl groups of proteins (including enzymes) and their resultant loss of function, accounts for a major component of the toxic effects of heavy metals. Zinc depresses photosynthesis in Chlorella (De Filippis and Pallaghy, 1976), this probably being due to impairment of enzyme function. In addition research by Rothstein (1959) indicates that heavy metals can cause disruption of membrane function. The resultant breakdown of the membrane as an efficient permeability barrier is likely to reduce or prevent many cellular functions. It has been shown for Dunaliella marina (Riisgård et al., 1980) and for red blood cells (Pritchard, 1979) that the main sympton of heavy metal toxicity is a loss of membrane integrity and the resultant uncontrollable uptake of sodium into the cells.

Much discussion has occurred as to whether or not zinc uptake by organisms is an active or a passive process. Previous studies indicated that it was an active process (see Giordano and Mortvedt, 1980); zinc uptake was found to increase in relation to increased repiration. Current evidence, however, suggests that this is an artefact. Changes in pH across cell membranes, caused by movement of respiratory metabolites, is probably indirectly enhancing zinc uptake (Bachmann, 1963; Gutnecht, 1963). Zinc uptake has now been shown to be essentially a passive process for a range of algae. These include Ulva lactuca (Gutnecht, 1961), Ankistrodesmus sp. and Golenkinia paucispina (Bachmann, 1963) and Dunaliella tertiolecta (Parry and Hayward, 1973).

The important first step in the uptake of zinc into plant tissue, is the adsorption of the metal onto the cation-exchange components of the cell wall (Gutnecht, 1963; Pickering and Puia, 1969). It is likely, therefore, that the only biologically available and, hence, toxic, species of many metals may be the free aqueo-metal ion (Sunda and Guillard, 1976; Mullins,

1977). This suggests that both toxicity and accumulation of zinc is likely to be strongly influenced by factors which compete with zinc for cation-binding sites (Section 1.321).

1.32 Growth of plants in zinc polluted environments

1.321 Environmental factors affecting zinc toxicity

The response of an organism to a pollutant *in situ* is likely to be strongly influenced by other environmental factors. Both field and laboratory observations have shown that many factors, particularly those which affect biological availability, are important in either increasing or decreasing the toxicity of zinc.

pH In general, in an acid pH zinc exists as free cations, but in an alkaline pH these may precipitate, particularly as hydroxide, carbonate or silicate compounds (Hem, 1972; see Section 1.2). Alkaline pH values are therefore likely to lead to a reduction in the toxic effects of zinc.

At lower pH values (c. pH 3.0) it has been noted (Hargreaves and Whitton, 1976) that the toxicity of zinc to *Hormidium rivulare* is reduced. It is likely that this is due to competition between H^+ ions and Zn^{2+} ions for cation-binding sites on the surface of the alga (Section 1.31); Bachmann (1963) was able to show that H^+ ions were the most effective of those he tested, in reducing zinc uptake by *Golenkinia paucispina* ($H^+ > Ca^{2+} > Mg^{2+} > Na^+ > K^+$). It is also noted that Patrick (1971) found that levels of accumulate zinc and cadmium were generally less in algal species living in a stream at pH 2.6 than those in a stream of pH 6.6. It is likely that this is also due to competition between H^+ and Zn^{2+} ions.

Eh Eh (redox potential) is the measure of the availability of electrons in an environment. High Eh values indicate oxidizing conditions and low Eh values indicate reducing conditions.

Zinc has only one naturally occuring valence state (+2) and therefore redox potential will not influence toxicity in the same way as has been shown by, for example, chromium; each valence state (Cr^{3+}, Cr^{6+}) having different toxic properties (Petrilli and de Flora, 1977).

Reducing conditions may lead to microbial conversion of sulphate (SO_4^{2-}) to sulphide (S^{2-}) (Zajic, 1969). This is likely to precipitate metal sulphides and therefore may indirectly affect the toxicity of zinc to plants (Hendricks, 1978).

Anionic components It is most likely that anions which affect zinc toxicity do so by causing it to precipitate and therefore become less toxic (Gadd and Griffiths, 1978). It is also possible, however, that increasing concentrations of an essential anion (e.g. phosphate) may reduce metal toxicity if inhibition of uptake of this anion is a direct toxic effect of that metal (Schulze and Brand, 1978). Phosphate has been shown to reduce zinc toxicity to a range of plant species (Ernst, 1974; Rana and Kumar, 1974; Say and Whitton, 1977) though the reason for this is not identified.

Cationic components Many studies have shown that the toxicity of zinc to plants may be influenced by environmental concentrations of other cations. Examples are known of antagonistic, additive and synergistic interactions (see Berry and Wallace (1981) for discussion of these terms).

Although it is clear that the toxicity of heavy metals to aquatic animals is usually less in hard than in soft water, few observations have been made on the effects of hardness components (such as magnesium and calcium) on the toxicity of heavy metals to plants. The liming of heavy metal tips has often been advocated as a possible method for restoring vegetation on mine soils (Antonovics et al., 1971); whether its effectiveness is due to the influence of calcium ions, or is due to precipitation of metal carbonates,

is not clear. Braek et al. (1976) noted that elevated magnesium in the medium reduced the toxicity of zinc to four algal species. Wong (1980) showed that zinc was less toxic to Chlorella pyrenoidosa in high concentrations of calcium ions. Due to competition for ionic binding sites, magnesium (Mg^{2+}) and calcium (Ca^{2+}) are both likely to antagonize the uptake of zinc. Keulder (1975) has shown that both ions antagonize the uptake of ^{65}Zn by Scenedesmus obliquus, similarly Bachmann (1963) has shown their ability to reduce the uptake of zinc by Golenkinia paucispina.

The protective role of calcium on membranes (Pooviah and Leopold, 1976) and protein structure (Gurd and Wilcox, 1956) probably explains part of its antagonism of the toxic action of zinc. The role of magnesium is less clear; Say and Whitton (1977) and Harding and Whitton (1977) showed, for the algae Hormidium and Stigeoclonium respectively, that magnesium only antagonizes zinc toxicity to strains shown to be resistant to zinc. This may implicate magnesium in a possible resistance mechanism in these algae.

Sodium ions (Na^{+}) and potassium ions (K^{+}) are also shown to antagonize zinc uptake to a slight degree (Bachmann, 1963) and may antagonize zinc toxicity to some plants. It is noted here, that treatment of cells with heavy metals may cause leakage of potassium ions from the cytoplasm (Overnell, 1975), suggesting a reduction of membrane integrity. It is possible that entry of sodium ions in these circumstances (Na^{+} ions are actively excluded by most living organisms; Bowen, 1966) may prove toxic (see Section 1.31).

Heavy metals are seldom present in the environment as single constituents (Section 1.2) and therefore their interactions may be important to assess when considering the effects of a single pollutant. The types of interactions which occur between heavy metals vary considerably (see review by Babich and Stotzky, 1980). As introduced in Section 1.2, cadmium invariably occurs at sites of elevated zinc and an examination of their toxic interactions to

plants serves to illustrate the variations which occurs in this type of study.

A review of the literature concerning the interaction of zinc and cadmium to plants, is presented in Table 1.1. It can be seen that this interaction varies from synergistic to antagonistic. The interaction of zinc and cadmium is synergistic when they are applied to isolated chloroplasts and the photosynthetic activity is measured (Hampp et al., 1976). The two elements, however, appear to compete in uptake reactions (Jarvis et al., 1976). These two types of interaction illustrate, respectively, examples of in vivo and in vitro interactions of metals (discussed by Wong and Beaver, 1981). Variation of interaction between zinc and cadmium (Table 1.1) may reflect the relative importance of in vitro uptake competition and in vivo metabolic synergism which is taking place in these plants.

Organic components Heavy metals that are dissolved in aquatic environments are often complexed to organic chelating agents including humic acids, amino acids, fulvic acids and phenols (Christman, 1970; Schnitzer, 1971). These substances are therefore likely to reduce metal ion activity and, hence, toxicity. Humic acid has been shown by Toledo et al. (1980) to reduce the toxicity of copper to Chlorella sp.

Surface adsorption It is noted by Babich and Stotzky (1980) that clay minerals, hydrous metal oxides and organic colloids, all possess surfaces which are negatively charged and to which charge-compensating cations (including heavy metals) may become adsorbed. This is likely to render them unavailable for uptake by plants (see Keulder, 1975).

1.322 Tolerance and resistance to zinc

There is an extensive literature on the tolerance and adaptation of higher plant species to heavy metals (reviewed by Antonovics et al., 1971). Many

organism	parameter measured	interaction	authors
Thalassiosira pseudonana	growth	S	Braek *et al.*, 1980
Skeletonema costatum (Skel-5)	growth	S	"
Phaeodactylum tricornutum	growth	A	"
Skeletonema costatum (Skel-0)	growth	A	"
Euglena gracilis	growth & morphology	A	Falchuck *et al.*, 1975
Euglena gracilis	growth & morphology	A	Nakano *et al.*, 1978
Selenastrum capricornutum	growth	+	Bartlett *et al.*, 1974
Chlorella sp.	growth	A	Upitis *et al.*, 1973
Salvinia natana	uptake & growth	S	Hutchinson & Czyrska, 1972
Lemna valdiviana	uptake & growth	S	"
Triticum sp.	growth	S	Roth & Oberländer, 1980

Table 1.1 The nature of the interactive effect of zinc and cadmium to plants

A, antagonistic; +, additive; S, synergistic

species growing in zinc rich soils have been shown to be genetically adapted to elevated zinc levels - though most studies have been on higher plants. There are many reports in the literature of algae growing at sites with elevated levels of zinc (Reese, 1937; Jones, 1958; Whitton 1970; McLean and Jones, 1975; Say et al., 1977); genetic tolerance to zinc, however, has only been clearly demonstrated for two freshwater genera, Hormidium and Stigeoclonium (Say et al., 1977; Harding and Whitton, 1976) though this phenomenon, if sought, is likely to be more widespread (Whitton, 1980).

Heavy metal tolerance in plants is likely to be extremely specific and if multiple tolerance does exist it is likely that a separate tolerance mechanism exists for each metal (Turner, 1969; Stokes, 1975). The various potential tolerance mechanisms are outlined by Antonovics et al. (1971), who state that there is no evidence to suggest that plants growing in zinc rich environments are able to exclude zinc. The tolerance mechanism to zinc must therefore be internal. (Exclusion mechanisms have been reported for other metals, particularly copper: Foster, 1977; Hall et al., 1979.)

The work of Mathys (1975) shows that, for higher plants, zinc-resistant strains do not possess enzyme systems which are adapted to function in the presence of elevated zinc ions. This applies to a range of zinc-resistant angiosperms (reviewed by Mathys, 1980) and it suggests that in these species, accumulated zinc is rendered less toxic and/or is stored away from sites of metabolic activity. Metal-tolerant organisms have been shown in some cases, to accumulate higher levels of metals than non-tolerant ones (Ashida, 1965; Antonovics et al., 1971; Stokes, 1975); this may be due to efficient internal binding of zinc by these organisms, therefore maintaining a zinc gradient across the membrane and allowing passive uptake to continue.

Zinc tolerance is therefore based on mechanisms which prevent a build-up of the metal in cytoplasm, either by complexation or by special transport. Mathys (1977, 1980) examined zinc-resistant strains of a number of higher plant species and was able to develop a comprehensive understanding of zinc tolerance in these species. He found that malate played a key role in binding and transporting zinc across the cell cytoplasm before releasing it into the vacuole where it was bound more firmly by oxalates or by precursors of mustard oil glucosides. Among those species studied by Mathys, Thlaspi alpestre has been shown to accumulate up to 16% of its dry weight as zinc (Zajic, 1969).

Other resistance mechanisms which have been demonstrated include the binding of zinc to cell wall material (possibly proteinaceous) in Agrostis tenuis (Wyn-Jones et al., 1971) and, at least for a fungus, the presence of specific metal-binding proteins (metallothioneins) has been demonstrated in zinc-resistant strains (Failla et al., 1976).

1.323 Plant communities and indicators

The extensive review of heavy metal tolerance in plants, carried out by Antonovics et al. (1971), contains a large section entitled "Ecology of Metal Tolerance". In this section they review the plant communities which may develop on metalliferous soils and have also compiled a list of plants which have been regarded as indicators ("a plant that in a given area or geographic region has been recognized as associated with a particular metal") when prospecting for metals. A considerable amount of the information presented by Antonovics et al. is based on the work of Ernst (1966, 1968, 1974). Antonovics et al. consider the plant communities which develop on metal-rich soils and show that in West and Central Europe, associations of species classified in the Alliance Thlaspion calaminariae (characterized by Thlaspi alpestre spp. calaminare) within the class Violetea calaminariae (characterized by Viola calaminaria) can be considered indicative of zinc contamination of

the soil. With regard to terrestrial indicator species only V. calaminaria can be regarded as an indicator of elevated zinc.

So far no aquatic parallels to V. calaminaria have been found that are confined to sites with high levels of zinc. If such a species should occur it would almost certainly prove to be too rare to be of value as an indicator (Whitton, 1980). Whitton (1970a) suggested that if the alga Stigeoclonium tenue is found alone in abundant growths in a clear, well illuminated, fast-flowing body of water, metal contamination should be suspected. Similarly studies by McLean and Jones (1975) suggest that abundant growths of Hormidium rivulare, found growing alone (or possibly with Scapania undulata) could be used as an indicator of high metal levels in the water. Besch et al. (1972) carried out a study to assess the possible use of benthic diatoms as indicators of the presence of metal contamination. They were unable to establish the presence of reliable indicator species, but obtained an indication of metal contamination by the dominance of species of a corresponding tolerance with the simultaneous absence or scarcity of all less tolerant forms. They regard this tentative indicator system, however, as being only applicable to the water conditions in their study area. A further point revealed in this study was that Mougeotia spp. dominated the most heavily polluted sites. Members of this genus are regarded by Whitton (1980) as usually dominant in highly calcareous (high pH), metal-polluted waters (probably superceding Hormidium rivulare).

1.4 Aspects of accumulation of metals by aquatic plants

1.41 Forms of metal available for uptake

The first stage in the uptake of metals by plants is shown to be the binding of cations to the surface of the cells (Section 1.31). It is therefore likely that the only forms of metals available for uptake by aquatic plants are those existing in solution as free cations or those

weakly adsorbed (Section 1.321).

1.42 Entrance of metals into the biosphere

Heavy metal accumulation by plants is of special concern to environmentalists because of the possibility that accumulated metals will move up the food chain and be 'biomagnified' in the same way as has been reported for halogenated hydrocarbons (Moriarty, 1975). It has often been assumed that this process will occur in aquatic environments (e.g. Mullins, 1977; Klotz, 1981) though there is evidence that concentrations of metals actually decrease in successive trophic levels (Hutchinson et al., 1976; Gächter and Geiger, 1979). Aquatic animals undoubtedly obtain heavy metals via food (Young, 1975; Stewart and Schulz-Baldes, 1976; Brown, 1977); the relative importance of ingestion versus direct absorption from the water has not been well studied and this is clearly important when evaluating trophic level effects. Movement of heavy metals up the aquatic food chain is not well documented and further studies are required.

1.43 Monitoring metal pollution

The ability of aquatic plants to take up heavy metals from the water, producing an internal concentration greater than their surroundings, is well documented (see review by Whitton, in press). As a consequence it has been suggested that chemical analysis of these submerged plants may give valuable information about contamination in the surrounding water. The potential value of using aquatic plants versus direct water analyses are as follows.

a) Plant "monitors" give an integrated picture of pollution within a particular system (Adams et al., 1973; Empain, 1976; Trollope and Evans, 1976). This may be particularly important where pollution is intermittent (Say et al., 1981).

b) Because of the high levels of accumulation found in most instances, measurement of the levels in plants increases the sensitivity of detection (Dietz, 1973).

c) It seems reasonable to assume that metal accumulation by a plant gives a better indication of the fraction of the metal in the environment which is likely to affect the aquatic ecosystem (Empain et al., 1980).

The use of algae in this context may not rival the potential use of bryophytes (Whitton, in press); pure stands of algae, in lotic environments, are infrequent and, at least in temperate latitudes, are usually absent in winter. In freshwater environments influenced by heavy metals, however, pure stands of algae tend to be quite common (Section 1.323) and possibly due to lack of grazing (Section 1.5) may persist throughout the year in temperate latitudes (Whitton, 1980). The use of algae to monitor heavy metal pollution in areas of local contamination may have important applications.

1.44 Water quality improvement

Practical systems have been developed which rely on the cation exchange capacity of algae to remove heavy metals from mine and industrial effluents (Jennett et al., 1977; Gale and Wixson, 1979; Filip et al., 1979). Filip et al. designed an alternate water/sand-filtration system in which the natural algal assemblage was able to remove up to 90% of copper and cadmium ions from the input water.

These systems have not been widely used, though as an inexpensive treatment system, preliminary results suggest they may be useful.

1.5 Chlorophyta in heavy metal polluted environments

As indicated in Section 1.323, green algae are commonly found in metal-polluted rivers and streams; for dissolved levels of zinc no upper limit (in otherwise clean water) has been established in which at least some

species of green algae cannot grow (Whitton, 1980). Metal polluted streams may develop an extremely high standing crop of green algae which is probably due to the absence of invertebrate grazers in these environments (Whitton, 1980; Klotz, 1981).

It seems likely that many green algae growing in heavy metal polluted environments have evolved metal tolerance (Section 1.322), though the only tolerance mechanism shown is that of a copper tolerant strain of Chlorella vulgaris (Foster, 1977; Section 1.322). This contrasts with the more general understanding of zinc tolerance in higher plants (Mathys, 1980). Work on terrestrial plants demonstrates clearly that metal tolerance is specific (i.e. resistance to one metal does not confer resistance to another; Antonovics et al. 1971) and this is shown to be the case for a nickel and copper tolerant strain of Scenedesmus acutiformis (Stokes, 1981).

The majority of laboratory studies of metal toxicity to algae rely on the use of strains obtained from culture collections. Though this work is useful, lack of any knowledge of the source of the algal material does not allow interpretations to be made concerning the environment. Similarly many laboratory studies which use algae isolated from defined field sites proceed to incorporate an algal growth medium which bears no relation to the prevailing field conditions. An example of this is the work by Stokes and co-workers (e.g. Stokes et al., 1973; Stokes, 1975; Stokes and Dreier, 1981). Their work considers various aspects of nickel and copper toxicity to green algae isolated from polluted and unpolluted lakes. The growth medium used, however, (Bolds basal medium; Bischoff and Bold, 1963) contained an extremely high level of phosphate (53.2 mg l^{-1} PO_4-P) and also, in many circumstances, bore no relation to the field pH. This renders interpretations invalid when referring back to nature, particularly when it is considered that both pH and phosphorus have major effects on the behaviour and toxicology

of metals in solution (Section 1.321).

An interesting study is that performed by Klotz (1981), who examined various aspects of copper tolerance of green algae. He combined field assays of cell growth rates (using dialysis bags incubated *in situ*), with laboratory assays using a growth medium specifically designed to duplicate field conditions (Klotz *et al.*, 1975). He showed that copper-tolerant strains had adapted to environmental levels of copper such that their growth rate was unaffected by these levels of copper (shown to be toxic to non-adapted strains). He notably was also able to show good correlation between laboratory and field results.

Rai *et al.* (1981) point out that there is a general lack of information concerning environmental factors which are influencing toxicity and accumulation of zinc to algae growing in the field. This, along with a general lack of information concerning growth rates and resistance mechanisms of green algae (particularly in the field), outline major areas where more information would be desirable.

Green algae occupy an important position in aquatic ecosystems. In light of increasing input of heavy metals into the environment, the genetics, physiology and biochemistry of algae in polluted environments may have important implications. For example; how rapidly can organisms adapt to colonise polluted environments? Can tolerant organisms be used to recolonise depopulated habitats? Can green algae be used to monitor (Section 1.43), or even reduce (Section 1.44), heavy metal contamination of aquatic systems?

1.6 Aims

The major areas in which knowledge is lacking, concerning the growth of green algae in heavy metal polluted environments, are outlined in Section 1.5. In addition Section 1.5 describes a number of reasons why an increased understanding of the ecology of these environments is desirable.

The aim of this study was to investigate the ecology of green algae in highly zinc-polluted environments. The project was planned to give particular emphasis to equating laboratory conditions with field conditions in an attempt to answer some of the following questions.

a) Can metal accumulation by green algae be related directly to water chemistry?

b) Which environmental factors are likely to have a major effect on the influence of zinc in the field?

c) By what mechanism do green algae tolerate elevated levels of zinc?

More specific aims, related to the experimental approach undertaken during the present study, will be outlined in the introduction to each chapter.

Investigation of a polluted environment may have a wider significance when considering the influence other pollutants. In particular investigations of green algae may have broader implications due to their similarity with higher plants.

2 MATERIALS AND METHODS

2.1 Durham coding systems

2.11 Streams

Each stream and river studied is allocated a four-digit number and a unique name. Nothing is implied by the particular number chosen. Distances along a stream are given from the upstream point; the left and right-hand banks are those looking downstream. For a fuller explanation see Holmes and Whitton (1981).

2.12 Plants

All species are given a unique six-digit number. The first two digits define the phylum, the second two the genus and the third two the species. Where recognition of individual species is impossible the genus is subdivided into a series of filament width categories. Details of this system are given in Whitton et al. (1978). A similar numerical classification has been developed by the Department of the Environment (D.o.E.) and is also included in this study for comparative purposes.

2.13 Algal cultures

Algal strains, when growing in uni-algal culture, are allocated three digit numbers; this system has no taxonomic significance nor does it correspond directly to the system of any other culture collection.

2.2 Environmental data

2.21 Sampling and analysis of stream water

All vessels used for the collection and analysis of water samples were previously soaked in dilute acid and rinsed five times in distilled water followed by two rinses in double-distilled deionised water. The dilute

acids used in the soaking stage were as follows: vessels used for cation analysis were soaked in 2% HNO_3 and those used for anion analyses in 6% HCl (excepting PO_4-P and Cl analyses where 2% H_2SO_4 was used).

Measurements were made in the field for current speed, temperature, pH, Eh, total alkalinity and dissolved oxygen; all other measurements were made in the laboratory. Water for the laboratory analyses was treated as follows.

Water was removed from the stream using a 2-l polypropylene beaker and this was left to stand for 5 min to allow sedimentation of large suspended particles. The water from this beaker provided four fractions for later analysis. (a) 25 ml water was decanted from the top of the water column into a 30-ml glass snap-top vial. One drop of atomic absorption grade HNO_3 (Fisons Ltd) was added to the sample; this fraction being referred to as 'total'. (b) A further 25 ml sample was collected by passing water through a Nuclepore membrane filter (pore size 0.2 μm). The technique involved mounting a 25 mm diameter filter in a Swinnex plastic holder and then washing the filter with 10 ml of the water to be sampled; a further 25 ml water was passed through the filter into a 30 ml glass snap-cap vial, this was acidified with 1 drop of atomic absorption grade HNO_3. This sample is referred to as the 'filtrable' fraction. The 'filtrable' and 'total' fractions were for subsequent metal analyses. (c) This water, used for subsequent anion analyses, was decanted from the two-litre beaker into a sintered glass funnel (Sinta No. 2) and was filtered into 250-ml polypropylene bottles (approximately 600 ml water was collected for anion analyses). (d) A 250-ml polypropylene bottle was filled underwater (excluding any air bubbles); this fraction was used to measure optical densities and conductivity of the water immediately on return to the laboratory.

All water samples were cooled immediately by immersion in a box of crushed ice and were transported in this manner back to the laboratory. On return

to the laboratory fractions (a) and (b) were stored in a refrigerator at $4^{o}C$; fraction (c) bottles were frozen and fraction (d) bottles were opened and used for optical density and conductivity measurements. Water used for anion chemistry was thawed immediately prior to analyses with the exception of filtrable reactive silicate in which the water was thawed 24 h prior to analysis.

Physical and chemical analyses of the water were carried out as follows.

(a) Current speed was measured using a calibrated Ott current meter. The impeller was positioned in the centre of the stream, 10 cm below the water surface and perpendicular to the direction of flow.

(b) Temperature was recorded as the mean value of three mercury thermometers.

(c) Optical density (O.D.) measurements were made on a Shimadzu double-beam spectrophotometer (UV-150-02) at wavelengths 240, 254 and 420 nm using a 1 cm path length Spectrosil cell, zeroed against a distilled water blank.

(d) Conductivity was recorded using a Lock Portable Transistorized Conductivity bridge, 3C1.

(e) pH was recorded in the field using a Pye Unicam portable meter No. 293 fitted with a Pye Unicam combined pH electrode No. 450.

(f) Eh (redox potential) was recorded using a Pye Unicam portable meter No. 293 fitted with a Pye Unicam redox probe No. 4805. The mV value obtained was adjusted to an $Eh_{7.0}$ value by adding 57.7 mV to the Eh value for every pH unit above 7.0 or fraction thereof: (Jacob, 1970).

(g) Total alkalinity was measured by performing the potentiometric titration described by Mackereth et al. (1978); values are calculated as m-equiv. l^{-1} CO_2.

(h) Dissolved oxygen was measured using a Simac dissolved oxygen electrode and meter No. 65.

(i) Cations were measured using a Perkin Elmer 403 atomic absorption spectrophotometer. The following cations were analyzed; Na, K, Mg, Ca, Mn, Fe, Zn, Cd and Pb for stream 0097 with the addition of Al, Ni, Co, Cu and Ag for stream 3026.

(j) Anions

PO_4-P	n-hexanol extraction method of Mackereth et al. (1978)	
NH_4-N	indophenol blue method	Stainton et al. (1977)
NO_2-N	azo dye method	
NO_3-N	Cd reduction method	
SO_4-S	turbidometric method	American Public Health Association (1981)
Si	hetero-polyblue method	
F	using an Orion specific ion electrode coupled to an EIL pH/pIon meter No. 7050.	
Cl	using an EIL specific ion electrode coupled to an EIL pH/pIon meter No. 7050	
H_2CO_3-C, HCO_3-C, CO_3-C	The dissolved carbonic dioxide forms were calculated based on the values of alkalinity and pH (Mackereth et al., 1978)	

(k) Total phosphorus was measured using the method described by Eisenreich et al. (1975). This method involved the conversion of organic phosphates and poly-phosphates to orthophosphate by digestion in a sulphuric acid/perchlorate solution. The resulting solutions were analyzed following the method of Mackereth et al. (1978).

All analyses were carried out in Pyrex glassware (with the exception of snap-cap vials which were boro-silicate glass); suitable standards and

blanks were used throughout.

2.22 Analysis of algal and sediment metal content

(a) Algae

Samples of algae were removed from the stream and placed in polypropylene bottles together with stream water; the bottles were cooled with ice for return to the laboratory. On return the algal material was divided into two approximately equal portions, one half being washed five times in double-distilled deionised water, the other half three times in a 40 mg l^{-1} EDTA solution, followed by two washes in double-distilled deionised water. Each of these portions were subdivided into five samples which were transferred to preweighed 30-ml glass vials. The glass vials were dried to a constant weight at 105°C and cooled, desiccated, before determining algal dry weight. 5 ml of atomic absorption grade nitric acid (Fisons Ltd) was added to each vial which was then heated to 100°C for 15 min (using a Tecam DB3H heating block) to allow digestion of the algal material and solubolisation of the metal component. The resultant solution was cooled to 20°C before being made up to a known volume (usually 25 ml) with double-distilled deionised water.

(b) Sediments

5 sediment samples were taken from within a 0.5 m radius of a predetermined site, using a trowel. They were then transferred to closeable, heat resistant, heavy-duty paper soil sample bags for transportation to the laboratory.

The sediment samples were dried to a constant weight at 105°C, cooled and, if necessary, ground using a mortar and pestle. The dried material was then passed through a 210 μm mesh nylon seive and collected in plastic vials. Sediment was stored in the vials until required for digestion,

determination of organic content (Section 2.23) or X-ray analysis (Section 2.24).

Prior to digestion sediment samples were redried at 105°C for 24 h and cooled in a desiccator. 50.0 mg portions of dried sediment were transferred to 18 mm diameter test tubes and acid digestion was carried out as described for algae (the only difference being a 1 h digestion time). The digest was then washed into a centrifuge tube using double-distilled deionised water and spun at 3500 rpm for 5 min. The supernatant was decanted into a 50-ml volumetric flask and made up to volume with double-distilled deionised water.

Both algal and sediment digests were stored in snap-cap vials at 4°C until analysis for metals was carried out using a Perkin Elmer 403 atomic absorption spectrophotometer. Elements analyzed were Ca, Zn, Cd and Pb for sediments and algae plus Na, Mg, K, Fe and Mn for algae.

2.23 Organic content of sediments

This was determined by placing approximately 1 g sieved sediment (Section 2.22) into a preweighed Vitreosil crucible. The crucible was then dried at 105°C for 24 h and cooled in a desiccator to determine the sediment dry weight. The crucible was then placed in a muffle furnace at 550°C for 48 h, removed, cooled and reweighed. Weight loss on ignition is assumed to be equal to the organic portion of the sediment (Ball, 1964).

2.24 X-ray analysis of dried algal and sediment material

2.241 X-ray diffraction analysis

This analysis allows the identification of the component minerals in a mixed (usually powdered) sample.

The dried algal and sediment material was passed through a 210 μm mesh nylon

sieve. A small amount of the dried material (approximately 50 mg) was slurried with acetone and then applied evenly to the glass plate in the centre of the goniometer (Phillips model no. PW 1050/25). The X-ray beam was produced by a Phillips PW 1130/00/60 X-ray generator supplying a copper targeted X-ray tube (Phillips model no. PW 2103/00; voltage 40 kV, current 20 mA). A nickel filter was placed in the primary X-ray beam to eliminate the Cu K_β emission line. This left the Cu K_α line (λ=0.1791 nm) as the incident X-ray emission line. The incident radiation was rotated from $2\theta=5^\circ$ to $2\theta=70^\circ$ at approximately 1° min^{-1}. Reflected X-ray energies were detected by a Phillips proportion detector probe (PW 1965/20) and recorded on a chart recorder.

Component minerals were 'fingerprinted' using the American Society for Testing and Materials Index (see references).

Principles of operation for X-ray diffraction are given by Jenkins and De Vries (1970).

2.242 X-ray fluorescence analysis

This relies on the specific wavelength emission of chemical elements when irradiated with a broad spectrum X-ray beam. Using the system described below all elements above atomic no. 19 (potassium) can be detected in a sample (excluding rhodium, cadmium and mercury).

The analyses were carried out using a Phillips wavelength dispersive X-ray flourescence spectrometer (PW 1400) fitted with a rhodium anode (voltage 60 kV; current 30 mA) and a LiF_{200} (lithium fluoride) analyzing crystal. A scintillation detector was used for wavelengths below 0.218 nm; a gas flow proportional counter was used for wavelengths above.

Powdered samples to be analyzed were spread on a specially designed Myalar sample holder and analyses were carried out under vacuum.

The emission wavelengths of cadmium and mercury are similar to that of rhodium (anode filament) and therefore their emission peaks are not detectable.

Principles of analysis are described by Jenkins and De Vries (1970). Applications of X-ray fluorimetry to plant tissue analysis are discussed by Knudsen et al. (1981).

2.3 Floristic records

The principle underlying the taxonomic coding system has been described by Whitton et al. (1978). For stream 0097 (Caplecleugh Low Level) emergent plant species are included in the records. Estimates of abundance for plant species within a portion of a stream were made using a simple scale of one to five, related to that used for terrestrial vegetation by Tansley and Crisp (1926):

abundance scale	description
1	rare
2	occasional
3	frequent
4	abundant
5	very abundant

These figures were subjectively applied to relative biomass of each species.

Details of the taxonomy of the main organisms in this study are given in Section 2.82. The taxonomic literature principally used were as follows:

General:	Bourrelly (1966, 1968, 1970); Prescott (1962)
Cyanophyta:	Geitler (1932)
Bacillariophyta:	Hustedt (1930)
Chlorophyta:	Ramanathan (1964); Fott & Nováková (1969)
Bryophyta:	Smith (1978)
Gramineae:	Hubbard (1968)

2.4 Estimation of variation in algal biomass

In stream 0097 (Caplecleugh Low Level) estimates of relative change in algal biomass of the stream were required. In order to avoid destruction of a significant portion of this short stream, observation of variation in the amount of algae present, were restricted (with one exception) to measurement of percentage cover over the boxed length of the stream (8-12 m downstream: see Fig. 3.6). Standing crop was measured on one occasion with the complete removal of algae from a 10 cm square chosen to represent a typical area within the boxed length of the stream. This algal sample was dried and weighed to give an estimate of standing crop (g m^{-2} dry weight).

2.5 Alkaline phosphatase activity of algal material

During the August visit five algal samples were removed from stream 0097 (Caplecleugh Low Level) and transported, on ice, back to the laboratory. Here they were washed in distilled water and suspended, each in a different buffer (Table 2.1).

Table 2.1 Buffers used for alkaline phosphatase assays

buffer	concentration	pH
glycine - NaOH	0.05 M	8.6
"	"	9.6
"	"	10.6
KCl - NaOH	0.2 M	11.0
"	"	11.6

p-Nitrophenyl phosphate substrate was then added (final concentration 5 mM) and the assay was incubated at 32°C for 30 min. After this period 0.05 M NaOH was added to the solution to stop any phosphatase activity

and the algal material was filtered off to be used for chl a estimation (see Section 2.925).

The optical density of the final solution was then measured at 410 nm (absorbance peak of p-nitrophenol) against a reagent blank (using a Shimadzu Digital Double-beam Spectrophotometer - UV-150-02). Results are given as μ moles p-nitrophenol h^{-1} μg Chl a^{-1}.

The above method is a modification of that described in the Sigma technical bulletin No. 104.

2.6 Sampling programme

2.61 Stream 0097 - Caplecleugh Low Level

The stream was visited monthly during 1980 (between the 15th and the 25th day of each month). Water physical and chemical variables were measured monthly (Section 2.21) with the exception of dissolved oxygen which was measured for the first five months, measurements then being abandoned due to equipment breakdown. Water was analyzed from two different positions in the stream; at the source (reach 01) and 12 m downstream (reach 02) (see Section 3.14 for description of site). Sediment collections were made in January, March, June, August, October and December of 1980.

Algal percentage cover was visually estimated monthly in the boxed section of the stream (8-12 m). Following this, if sufficient biomass was present, algae were removed for metal analysis. This was only possible in the months March-October.

A 10 x 10 cm area of algae was removed from the stream in July in order to estimate algal standing crop (Section 2.4). The dried algal material resulting from this estimate was subsequently used for X-ray analysis (Section 2.24).

In August an additional five replicate water samples, for anion analysis, were taken from the water source (reach 01). These were analyzed immediately on return to the laboratory for filtrable reactive phosphate and total phosphorus (Section 2.21). Concurrent with this five samples of algae, taken from the stream, were transported back to the laboratory where they were assayed for alkaline phosphatase activity (Section 2.5).

2.62 Stream 3026 - Le Crouzet Upper Slope Seepage

This site was visited on the 9th of July 1978 by Drs. B.A. Whitton and P.J. Say. Physical and chemical parameters of the water, measured, were limited to temperature, pH and cations ('total' and 'filtrable'). Algal material was collected and relative species abundances were estimated by B.A.W. and P.J.S., on a 1 - 5 scale (Section 2.3). A sediment sample was collected to be analyzed by X-ray diffraction and X-ray fluorescence (Section 2.24).

2.7 Culture techniques

2.71 Cleaning of glassware

Glassware was washed in detergent, rinsed in distilled water and then soaked for at least 24 h in 2% Analar HNO_3 solution. It was then rinsed thoroughly in distilled water and dried at 105°C.

2.72 Culture vessels

All cultures were grown in Pyrex glass vessels. For the growth of inocula 250-ml conical flasks were used; for experiments, 100-ml conical flasks or 50-ml boiling tubes were used.

For solid media: isolation work was carried out in plastic petrie dishes; stock cultures were maintained, on slopes, in 12 mm diameter test tubes.

Boiling tubes were capped with Morton closures (Bellco Stainless Steel) or Axa closures (Axa Ltd). Other vessels were plugged with good quality cotton wool.

2.73 Sterilization

Sterilization of media was by autoclaving at 121°C (10.35 KN m^{-2}; 15 lb in^{-2}) for 15 min. The media were left to stand for 24 h after autoclaving to allow equilibration with the atmosphere; if the media were to be stored longer they were kept in the dark to prevent photo-breakdown of EDTA-metal chelates.

Precipitation of mineral components was found to occur in high pH media when they were autoclaved. For this reason a number of short term experiments were carried out in freshly made, non-sterilized, media.

2.74 Media

2.741 Mineral nutrients

All salts used in media preparation were Analar grade chemicals (except for $Na_2SiO_3.5H_2O$ for which Technical grade was the purest available) and were prepared as individual stocks in double-distilled deionised water. Stocks were stored at 4°C, in the dark, until required.

The basic algal growth medium used was Chu 10 E (Tables 2.2 and 2.3). This was modified originally from the number 10 formula of Chu (1942) as follows: carbonate and phosphate have both been reduced and EDTA has been added as a chelating agent. Trace element concentrations in the Chu 10 E medium were derived from the AC microelement stock of Kratz and Myers (1955), modified only by lowering the Mn concentration.

Algal material was grown in media without added Zn though a background level of Zn (c. 0.03 mg l^{-1}) was found in samples analyzed by atomic

absorption spectroscopy. This Zn is presumably derived from the impurities of the metal in the original Analar stocks. Attempts were made to remove this residual Zn using the cation-exchange resin Chelex 100 (Bio-rad). The resultant input of other cations, however, made this method impractical.

A second artificial medium was designed to resemble the physical and chemical characteristics of the water in stream 0097 (Caplecleugh Low Level). Details of the composition of this are given in Tables 2.2 and 2.3. Design of this medium, CAM (Caplecleugh Artificial Medium), was based on experiments in which 0097 stream-water was supplemented with various nutrient elements to allow the growth of 0097 algae in laboratory culture (see Section 6.2). From this a third medium made up of supplemented 0097 water was derived (CFM; Caplecleugh Field Medium).

2.742 Buffer

Droop (1959) emphasises the requirement for strict control of pH in artificial growth media. This point is made by Hargreaves and Whitton (1976), who demonstrate the important role of pH on the toxicology of Zn and Cu to Hormidium rivulare.

Many buffers have previously been included in algal growth media; however few have been shown to fulfil the following criteria:

1) the buffer should be neither toxic nor stimulatory to algae i.e. not be involved in cell metabolism;

2) the buffer should not act as a cation chelator;

3) the buffer should be able to maintain the pH of the growth medium to within 0.2 pH units of the required value.

Good et al. (1966) synthesised a series of organic buffers for use in biological research and of these hydroxyethylpiperazine-N^1-2-ethanesulphonic

Table 2.2 Composition of media (mg l^{-1} salts)

	Chu 10 E	CAM	CFM*
KH_2PO_4	3.91	4.5	-
NaH_2PO_4	-	-	3.87
$MgSO_4.7H_2O$	25.0	253.6	-
$Ca(NO_3).4H_2O$	57.6	-	-
$CaCl_2.2H_2O$	-	367.5	-
$NaHCO_3$	7.925	206.6	-
$NaNO_3$	-	41.45	41.45
$Na_2SiO_3.5H_2O$	10.87	10.87	-
$FeCl_3.6H_2O$	2.42	2.42	-
$Na_2EDTA.2H_2O$	3.17	3.17	-
$MnCl_2.4H_2O$	0.05	0.05	-
$NaMoO_4.2H_2O$	0.007	0.007	-
$ZnSO_4.7H_2O$	0.056	37.35	-
$CuSO_4.5H_2O$	0.019	0.019	-
$CoSO_4.7H_2O$	0.01	0.01	-
H_3BO_3	0.72	0.72	-
cyanocobalamin	-	1 nM	1 nM

*Figures refer to concentrations of salts added to stream water.

Table 2.3 Composition of media (mg l^{-1} elements)

Element	Chu 10 E	CAM	CFM*
N (excluding EDTA)	6.83	6.83	6.83
P	0.89	1.00	1.00
S	3.25	37.11	-
Cl	0.95	178.45	-
Na	4.92	70.68	11.97
K	1.12	1.29	-
Ca	9.76	100.0	-
Mg	2.47	25.0	-
Si	1.44	1.44	-
Fe	0.50	0.50	-
Mn	0.012	0.012	-
Mo	0.0025	0.0025	-
Zn	0.012	8.50	-
Cu	0.005	0.005	-
Co	0.002	0.002	-
B	0.125	0.125	-
EDTA	2.47	2.47	-
cyanocobalamin	-	0.00135	0.00135

*Figures refer to concentrations of elements added to stream water; for composition of unsupplemented stream water see Section 4.2

acid (HEPES) was subsequently recommended as a constituent of algal growth media by Smith and Foy (1974). Good et al. (1966) reported that this buffer did not significantly chelate Mg^{2+}, Ca^{2+}, Mn^{2+} or Cu^{2+} ions and also did not influence the in vitro bioassays of the Hill reaction, succinate oxidation or protein amino acid incorporation.

Smith and Foy (1974) tested HEPES with a wide range of algal strains. They found that HEPES, at a concentration of 20 mM, was efficient at maintaining the pH of algal growth media to within 0.2 pH units of pH 7.6. They also showed that HEPES was neither significantly toxic nor stimulatory (related to growth rate) for the range of algae they tested.

Tevlin (1978) reported the suitability of HEPES buffer for restricting pH variation in cultures of both Daphnia magna and Chlorella pyrenoidosa, grown at pH 7.50. He also observed that HEPES had no effect on the toxicity of Cd to D. magna and related this to the absence of cadmium complexation by HEPES.

The pKa value of HEPES is 7.55 at 20°C (Good et al., 1966). This allows HEPES to be useful in the pH range 6.5 - 8.5. The pH of the water at stream 3026 (from which many of the algal strains in the present study were isolated) was measured at 5.0 (Section 4.2). It was envisaged for experiments using these strains that the media would be buffered at the field pH (5.0).

Six potentially useful buffers (Table 2.4) were tested with strain 545 (Section 2.81). The buffers were chosen from those described by Malette (1967) which had pKa values close to 5.0 (Table 2.4).

Table 2.4 pH 5.0 buffers tested as potential constituents of algal growth media

Buffers tested	pKa values		
hemimellitic acid	2.98	4.25	5.87
trimesic acid	3.16	3.98	4.85
tricarbyllic acid	3.67	4.85	6.22
succinic acid dimethyl ester	4.11	6.29	
dimethyl glutaric acid	3.72	6.31	
trans-aconitic acid	3.04	4.25	5.89
malonic acid methyl ester	3.29	5.98	

Cultures of strain 545 were grown in a range of buffer concentrations adjusted to pH 5.0. Boiling tubes containing 10 ml of Chu 10 E medium were used and were inoculated with 2×10^4 cells of strain 545 (Section 2.922). After 7 days incubation, the pH values of the media were measured; in those cultures in which the pH had shifted less than 0.2 units, the cells were counted. The pH values of the media are given in Table 2.5.

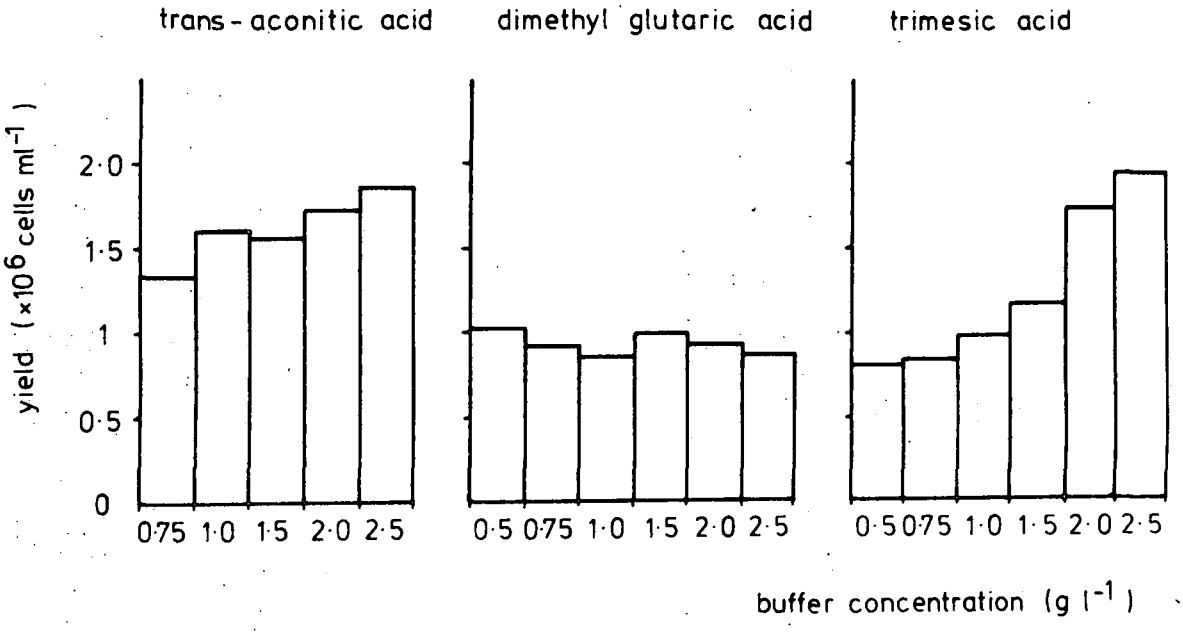
Table 2.5 pH values of media after growth of strain 545. (Values given are mean of four replicates)

	buffer concentration (g l^{-1})							
buffer	0.1	0.25	0.5	0.75	1	1.5	2	2.5
hemimellitic acid				toxic				
trimesic acid	5.43	5.26	5.17	5.17	5.14	5.11	5.09	5.07
tricarbyllic acid	4.64	4.44	4.40	4.35	4.36	4.38	4.36	4.38
succinic acid dimethyl ester				toxic				
dimethyl glutaric acid	5.86	5.45	5.19	5.13	5.13	5.08	5.08	5.08
trans-aconitic acid	5.90	5.58	5.36	5.20	5.20	5.15	5.13	5.12
malonic acid methyl ester				toxic				

Hemimellitic acid, succinic acid dimethyl ester and malonic acid methyl ester were obviously toxic to the alga at all concentrations. pH measurements

for these buffers were not made. Tricarbyllic acid and dimethyl glutaric acid, between concentrations of 0.5 - 2.5 g l^{-1}, and trans-aconitic acid, between concentrations 0.75 - 2.5 g l^{-1}, were able to maintain the medium pH to within 0.2 units of pH 5.0. Yield was estimated for these treatments; the results are presented in Fig. 2.1.

Fig. 2.1 Effect of buffers on yield of strain 545



Both trans-aconitic acid and trimesic acid caused increased growth of strain 545 at higher buffer concentrations. Doses of dimethyl glutaric acid, however, had no significant effect on the yield of strain 545. Dimethyl glutaric acid (DMGA) was chosen as a potentially useful buffer in algal growth media.

It was preliminarily decided to use 0.5 g l^{-1} (3.0 mM) of DMGA adjusted to pH 5.00 for the growth of algal strains isolated from stream 3026;

HEPES at a concentration of 1.20 g l^{-1} (5 mM) adjusted to pH 7.50 would be used for strains isolated from stream 0097.

The major emphasis of this project was the toxicology of Zn and it was felt that the possible chelation of Zn by HEPES and DMGA required further investigation. This was approached in three ways.

a) Good et al. (1966) titrated HEPES against NaOH, in the presence and absence of equimolar concentrations of one of the cations Mg^{2+}, Ca^{2+}, Mn^{2+} and Cu^{2+}. From the displacement of the titration curve, in the presence of a particular metal, it was possible to calculate the metal binding coefficient (Km) of the buffer. This method assumes that metal cations and protons compete only for the same sites on the buffer molecule. Good et al. (1966) reported negligible log Km values for the cations (Mg, Ca, Mn, Cu) they tested with HEPES.

It was decided to follow the titration method (originally devised by Bjerrum, 1941) to derive values of log Km for Zn with both HEPES and DMGA. These values were calculated to be 2.09 and 2.12 at 25°C respectively.

The log Km value for HEPES relied on extrapolation of the titration curve in the presence of Zn. This was due to precipitation of $Zn(OH)_2$ above approximately pH 6.7. Competition for hydroxyl ions invalidates the titration curve above this pH.

Although the log Km values given for HEPES and DMGA are low compared to those quoted for many buffers assayed by Good et al. (1966), they are greater than those value which they regard as negligible.

b) The second method of investigating the interaction of buffers and Zn was to use the colourimetric reagent for Zn O-[a-(2-hydroxy-5-sulphophenylazo)-benzilidenehydrazino] benzoic acid (zincon) first described by Yoe and Rush (1952). Collier (1979) used the interference of certain

buffers on the formation of the Zn-zincon complex as a measure of their Zn binding capacity. Details of the Zn/zincon reaction are given by Yoshimura et al. (1978). They describe the reaction of zincon (maximum absorbance at λ = 470 nm) with Zn, to give a Zn-zincon complex (maximum absorbance at λ = 650 nm). The optimum pH for this reaction is given as pH 9.0 when the Zn:zincon ratio in the reaction product is 1:1. Below this pH protons interfere and the reaction product (Zn-zincon complex) is reduced. Below pH 6.0 no reaction product is formed.

It was decided to investigate the influence of HEPES and DMGA on the Zn/zincon reaction. Interference of these compounds with Zn-zincon complex formation would indicate that they were chelating Zn (Collier, 1979). EDTA, a strong chelating buffer (Gardiner, 1976), was also tested for comparative purposes.

The Zn/zincon reaction was investigated at two pH values, 7.55 (pKa of HEPES) and pH 9.0, the pH optimum of the reaction (Yoe and Rush, 1952). The reaction mixture was buffered using inorganic buffers; for pH 7.55 1 mM phosphate buffer was used and for pH 9.0 1 mM borate buffer. 10 ml of reaction mixture was used, zincon reagent was added last to the mixture of Zn, inorganic buffer, $\pm$ the organic buffer to be tested. The pH of the organic buffers were adjusted to that of the reaction mixture prior to their addition. A range of Zn concentrations were used. The concentrations of the various reaction components in the final mixture were as follows:

Zn	-	0, 0.01, 0.02, 0.04, 0.06, 0.1 mM
inorganic buffer	-	10 mM
organic buffers		
HEPES	-	5 mM
DMGA	-	3 mM
EDTA	-	0.05 mM
zincon	-	0.05 mM

The reaction product (Zn-zincon complex) was formed immediately (Yoe & Rush, 1952), and its optical density read at 650 nm. The results of the Zn/zincon experiment are given in Fig. 2.2.

Fig. 2.2 shows that DMGA and HEPES have no detectable effect on the Zn/zincon reaction at either pH. EDTA, however, completely prevents the formation of any reaction product confirming its description as a strong chelating agent (Gardiner, 1976). The results of this experiment indicates that HEPES and DMGA have no observable chelating properties.

c) Both the pH titration and the zincon methods of assessing Zn chelation are indirect measures. It was felt that a more direct method of estimating the effect of HEPES and DMGA, on the free Zn ion concentration in solution, was required.

Specific ion electrodes measure only the concentration of free ions in solution (Hansen et al, 1972). Zn specific ion electrodes are not manufactured, and it was therefore decided to continue the investigation of chelation by HEPES and DMGA using the metal Cd. Cd occupies the same group as Zn (group IIb) in the periodic table and is chemically very similar to Zn (Cotton and Wilkinson, 1980). Free Cd^{2+} ions were measured in solution using a Cd specific ion electrode (Orion, model No. 94-48) attached to an EIL pH/pIon meter No. 7050.

The experimental procedure used, was similar to that given previously for the zincon reaction. 10 ml of reaction mixture was used at four different pH values - pH 5.0, 6.0, 7.5 (10 mM phosphate buffer) and pH 9.0 (10 mM borate buffer).

The concentrations of the components of the reaction mixture were:

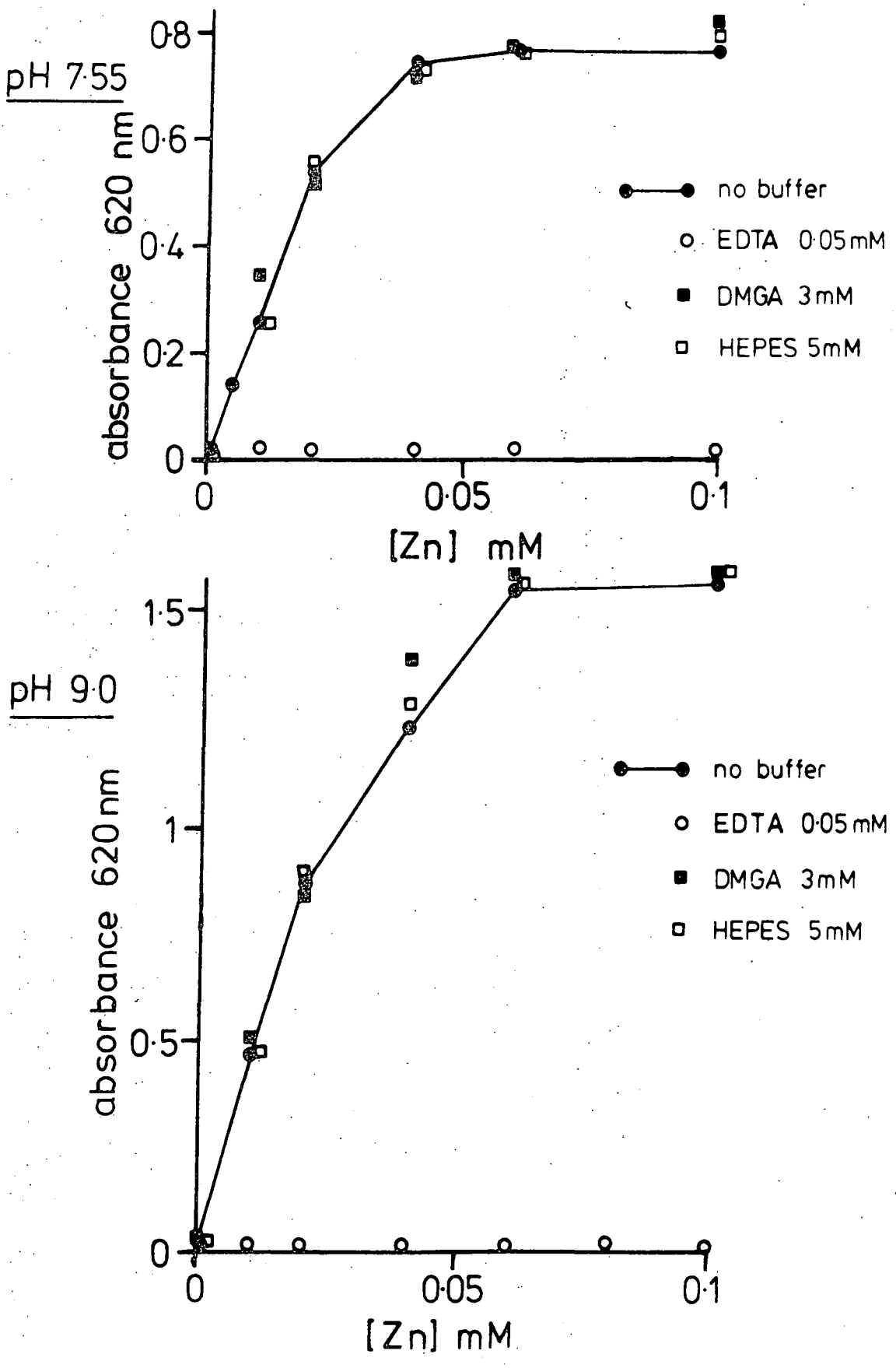


Fig. 2.2 Effect of HEPES, DMGA and EDTA on the Zn/zincon reaction at pH 7.55 and 9.0

Cd	-	0.01 and 0.1 mM
ionic strength adjuster*	-	0.1 M
inorganic buffer	-	10 mM
organic buffers		
HEPES	-	5 mM
DMGA	-	3 mM
EDTA	-	0.05 mM

* Ionic strength adjuster is added to maintain a high, constant background ionic strength. This is a requirement for the accurate use of the specific ion probe. $NaNO_3$ was used.

Results of Cd^{2+} concentrations measured, are summarised in Fig. 2.3. All Cd^{2+} values measured in the presence of EDTA were below the detection limit of 0.0005 mM. Fig. 2.3 shows that as the pH increases measurable Cd^{2+} ions decrease. The presence of the buffers HEPES and DMGA, however, does not appear to effect the concentration of free Cd^{2+} ions.

The results of sections a), b) and c) indicate that both HEPES and DMGA have only slight, if any, chelating properties. Subsequent to these experiments HEPES and DMGA were routinely added to laboratory growth media at concentrations of 5 mM and 3 mM respectively. Cultures of algae isolated from stream 0097 were grown in media containing HEPES adjusted to pH 7.50; algae from stream 3026 were grown in media containing DMGA adjusted to pH 5.0. In cases where media of other pH values were required, DMGA was used in the pH range 3.0 - 6.5; HEPES was used in the pH range 6.5 - 8.0.

Buffer was dissolved in distilled water and adjusted to the required pH of the medium using NaOH solution. Mineral nutrients were then added and the media autoclaved; no changes in pH were noted as a result of autoclaving.

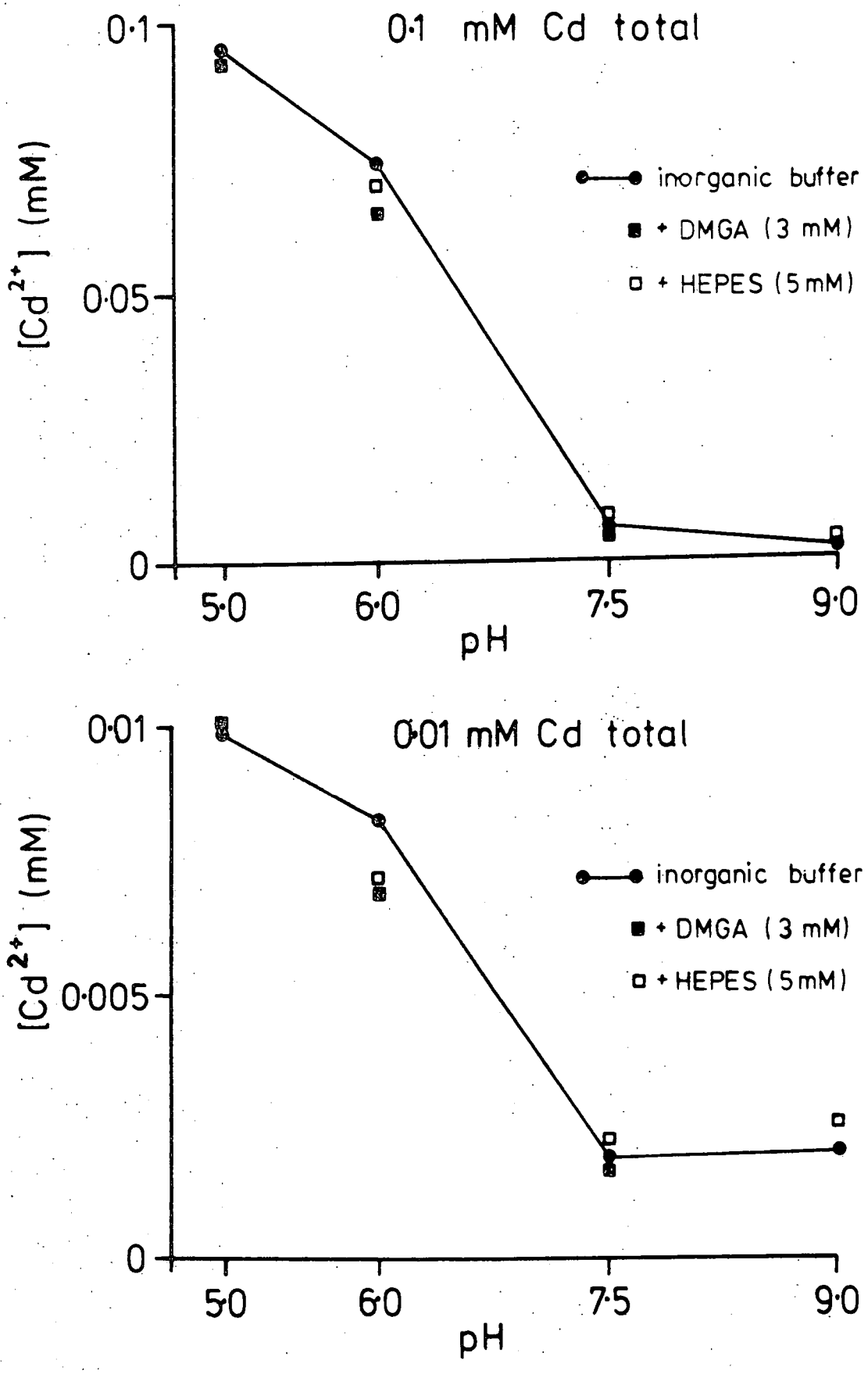


Fig. 2.3 Effect of HEPES and DMGA on Cd^{2+} concentration in solution

2.743 EDTA

The importance of having a metal chelator in an algal growth medium is stressed by Fogg (1975). Principally the presence of a chelator allows iron to remain in solution in an available form. In the absence of a chelator, iron would be expected to precipitate as a phosphate salt, particularly at high pH values. EDTA was first used by Waris (1953) for this reason, and is now used widely (Stein, 1973).

2.744 Addition of other selected cations and anions

A list of those ions whose influence in algal culture media were tested is given in Table 2.6 together with the substances added to the medium to bring about required changes. Complementary salts were used to maintain other ions at the basal medium concentration.

For variations in hydrogen ion concentration see Section 2.742.

2.75 Subculturing of algae

Subculturing of algal material was performed using standard aseptic techniques. The work was also carried out in a horizontal laminar flow cabinet (conforming to B.S. 5295 class 1). Inocula were tested regularly for contamination by both microscopic inspection and by subculturing onto a range of bacterial test media (Section 2.83). Subculturing algae for isolation and purification work, as well as routine subculturing of stock cultures, was performed by transferring algal material with a sterile needle or wire loop.

Uniform inocula for experimental work were subcultured from liquid media using a Gilson Adjustable Volume Pipetman with sterilised plastic tips, to dispense a known volume of suspension. Unicellular algal suspensions were pippeted directly; however to obtain a uniform inoculum of a filamentous alga the following routine was performed. Algal material was removed with a sterile wire loop and placed in a small volume of fresh liquid medium.

Table 2.6 Variations of Chu 10 E medium

species tested	salt in Chu 10 E medium	salt used as ion source	complementary salt for required absence of factor
NO_3^-	$Ca(NO_3)_2.4H_2O$	$NaNO_3$	$CaCl_2.2H_2O$
$PO_4^{3-}/HPO_4^{2-}/H_2PO_4^-$	KH_2PO_4	NaH_2PO_4-$2H_2O$	KCl
SiO_3^{2-}	$Na_2SiO_3.5H_2O$	$Na_2SiO_3.5H_2O$	-
HCO_3^{2-}	$NaHCO_3$	$NaHCO_3$	-
$EDTA^{2-}$	$Na_2EDTA.2H_2O$	$Na_2EDTA.2H_2O$	-
Na^+	-	Na_2SO_4	-
Mg^{2+}	$MgSO_4.7H_2O$	$MgSO_4.7H_2O$	-
Al^{3+}	-	$Al_2(SO_4)_3.16H_2O$	-
K^+	$KH_2PO_4.7H_2O$	K_2SO_4	$NaH_2PO_4.2H_2O$
Ca^{2+}	$Ca(NO_3)_2.4H_2O$	$CaCl_2.2H_2O$	$NaNO_3$
Mn^{2+}	$MnCl_2.4H_2O$	$MnCl_2.4H_2O$	-
Fe^{3+}	$FeCl_3.6H_2O$	$FeCl_3.6H_2O$	-
Ni^{2+}	-	$NiCl_2.6H_2O$	-
Cu^{2+}	$CuSO_4.5H_2O$	$CuSO_4.5H_2O$	-
Zn^{2+}	$ZnSO_4.7H_2O$	$ZnSO_4.7H_2O$	-
Cd^{2+}	-	$CdSO_4.8H_2O$	-
Pb^{3+}	-	$Pb(NO_3)_2$	-

This was sonicated for five seconds at maximum power using a Soniprep 150 sonicator (the sonicator tip was sterilized in ethanol). This gave a suspension of typically 3-6 celled lengths of algal filament which was inoculated into experimental containers using the Pipetman.

Experimental inocula were pre-incubated in experimental light and temperature conditions for 24 h prior to their use, whenever possible.

2.76 Incubation and light source

Algal isolation work and growth of algal inocula were carried out in a thermostatically controlled growth room, maintained at 25°C. Inoculum pre-incubation and experimental work were carried out in vessels suspended in a thermostatically controlled tank of distilled water, maintained at 20°C. A shaking mechanism moved the vessels through a horizontal distance of 35 mm approximately 80 times a minute. Continuous illumination was supplied from beneath by a series of warm white fluorescent tubes. Levels of illumination in the central part of the tank, used for experiments, were fairly constant.

Conical flasks were clipped onto the shaker; boiling tubes were suspended, at an angle, in a wire rack. The wire did cause some reduction in illumination (Table 2.7). Light was measured at the surface of the culture vessels.

Light measurements were made using a biospherical quantum scalar irradiance meter (Biospherical Instruments Inc.) No. QSP-170 with an attached laboratory sensor (QSL-100P). The sensor was designed to measure only photosynthetically active radiation between wavelengths 400-700 nm. Quantum values obtained were converted to μM photons m^{-2} s^{-1} (μE m^{-2} s^{-1}).

One experiment performed was incubated in a temperature gradient similar to that described by Van Baalen and Edwards (1973). Flasks were shaken by hand every day but otherwise were incubated standing.

All algal cultures were maintained in continuous light and all experiments (excepting the use of the temperature gradient) were performed at 20°C.

Table 2.7 Algal incubation conditions

growth chamber	type of fluorescent tube	irradiance (μM P m^{-2} s^{-1})
25°C growth room	white	40 - 50
shaking tank (flasks)	warm white	230 - 260
shaking tank with wire rack (tubes)	warm white	201 - 221
temperature gradient	white	120 - 130

2.8 Algal cultures

2.81 Origins

All algal strains used for experimental work (Table 2.8) were isolated from streams 0097 and 3026.

Table 2.8 Algal cultures and their origin

alga	Durham culture no.	Durham species no.	Stream	axenic	clonal
Hormidium sp.	532	152950	3026	✓	✓
Chlamydomonas sp.	533	130432	3026	✓	✓
Mougeotia sp.	535	121453*	0097	✗	✓
Mougeotia sp.	536	121451*	0097	✓	✓
Hormidium rivulare	537	152902	3026	✓	✓
Hormidium sp.	539	152950	3026	✓	✓
Hormidium rivulare	540	152902	0097	✓	✓
Chlamydomonas sp.	541	130432	3026	✓	✓
Stichococcus bacillaris	545	154456	3026	✓	✓
Chlorella zofingiensis	546	140549	3026	✓	✓

* These figures are based on width categories of cultured material and they differ from material observed directly from field collections (see Section 4.6).

2.82 Notes on taxonomy and morphology of isolated algal strains

A complete algal flora of the two streams studied in this thesis are given in Tables 4.7 and 5.2 for streams 0097 and 3026, respectively. Strains of green algae isolated from these streams, and whose growth in laboratory culture are investigated in the present study, are listed in Table 2.8. Observations concerning the taxonomy and morphology of these strains are included below.

Stream 0097. Three green algal species were identified from field material collected from stream 0097 (Table 4.7); these were isolated in laboratory culture as strains 535, 536 and 540.

Strains 535 and 536 - Mougeotia spp.

Two different Mougeotia spp. were identified from stream 0097 based on filament width categories (Section 2.3). The two filament widths recorded from the field were 8-10 μm and 5-6 μm. In culture (Chu 10 E, pH 7.5), however, both Mougeotia strains showed broader filaments of 12-13 μm and 6-7 μm for strains 535 and 536 respectively. A small portion of filament of strain 536 is shown in Fig. 2.4.

Conjugation of Mougeotia was not observed in the field nor did it prove possible to induce conjugation in strains 535 and 536, using methods described by Hoshaw (1968). These included incubation in the dark, in low nutrient medium and in a high CO_2 atmosphere. Identification of Mougeotia species is based on zygospore formation and morphology (Prescott, 1962) hence the two Mougeotia strains could not be further classified.

Strain 540 - Hormidium rivulare Kütz.

Use of the generic name Hormidium, for algae, is strictly invalid due to precedence of an identically named orchid genus. Silva et al. (1972) suggest that all members of this algal genus should be transferred to the genus Klebsormidium. It seems likely, however, that further nomenclatural changes will take place (see Pickett-Heaps, 1972), to reduce confusion,

therefore, the familiar name Hormidium has been retained in the present study, with the species recognized by Heering (1914).

A small portion of filament of field (stream 0097) H. rivulare is shown in Fig. 2.5; this shows a typical geniculation, or knee-joint, which is characteristic of this species (Ramanathan, 1964). Filaments of both field and cultured material of H. rivulare from stream 0097 showed no tendency to fragment.

Stream 3026. Two green algal species were identified from field material collected from stream 3026, H. rivulare and H. fluitans. Isolation of these algae (Section 2.83), however, revealed seven distinct green algal strains (Table 2.8); it is likely that many of these were too infrequent to be noted in field material. Algal strains isolated from stream 3026 are 532, 533, 537, 539, 541, 545 and 546.

Strains 532, 537 and 539 - Hormidium spp.

The main distinction between H. rivulare and H. fluitans is the ready dissociation of filaments of H. fluitans into unicells or small filament fragments (Ramanathan, 1964). Both species were recorded in stream 3026 (Table 5.2); in culture, however, three distinct Hormidium strains were isolated from the stream. These were morphologically very similar - filaments approximately 7 μm broad, parietal chloroplast typically small covering half or less the circumference of the cell and possessing an obvious pyrenoid. The distinction was made between the three strains based on the degree of fragmentation of the filaments in culture. When grown in Chu 10 E medium (pH 5.0) strain 537 consisted of filaments of indefinite length (no evidence of fragmentation), strain 539 of filaments of, on average, 30 cells in length and strain 532, which showed a high degree of fragmentation, consisting of many unicells with filaments rarely longer than five cells. Strain 537 was identified as H. rivulare, strains 532 and 539 were not classified to species level though both are probably strains of H. fluitans (based on Ramanathan, 1964).

Strains 533 and 541 - Chlamydomonas spp.

The distinction was made between these two Chlamydomonas strains based on a slight difference in cell size (strain 533, 13-14 μm length; strain 541, 10-12 μm length) and also strain 541 was occasionally seen to reproduce by forming endospores. Differences in experimental results for these two strains appeared to justify this distinction (e.g. Fig. 6.3).

Strain 545 - Stichococcus bacillaris Nageli

This strain exists in culture almost entirely of unicells (2-3 μm x 5-15 μm; Fig. 2.6) and was distinguished from the genus Hormidium by its lack of a pyrenoid (Ramanathan, 1964). A further distinction made by Ramanathan between these genera is the ability of Hormidium spp. to form zoospores; in the present study, however, even after incubation in the dark for prolonged periods (Cain et al., 1974), none of the Hormidium spp. were able to form zoospores. It is possible that zoosporogenesis is strongly inhibited by zinc and these algae (from zinc-polluted sites) have lost this ability.

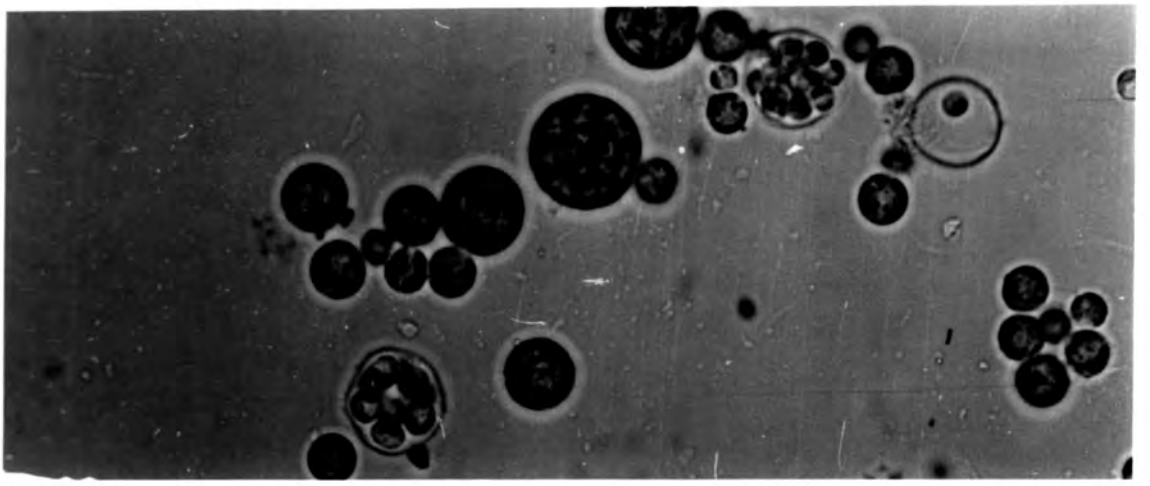
Strain 546 - Chlorella zofingiensis Donz

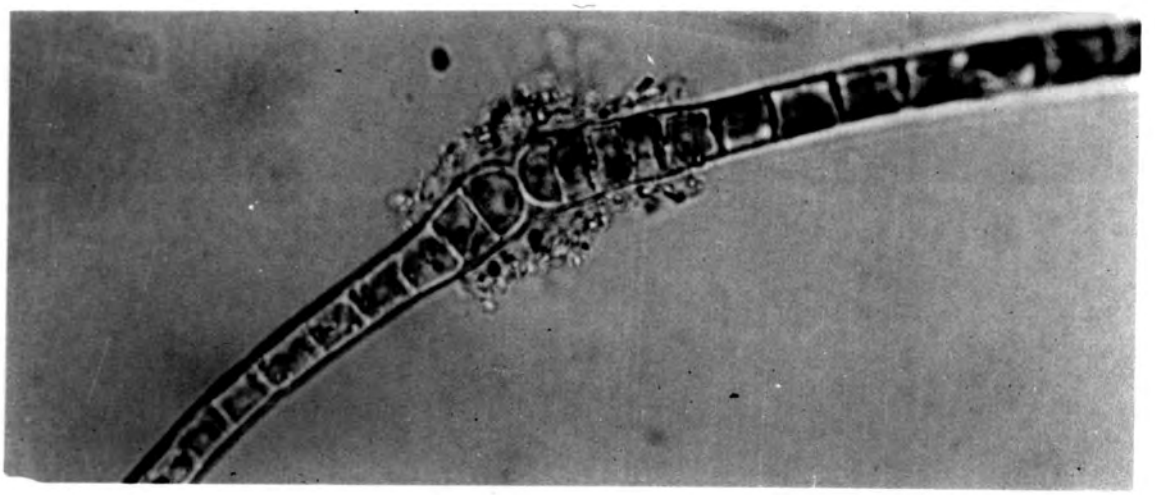
This species has rarely been recorded (Fott and Nováková, 1969) and therefore more detailed notes concerning its taxonomy are included. The following description of strain 546 was made.

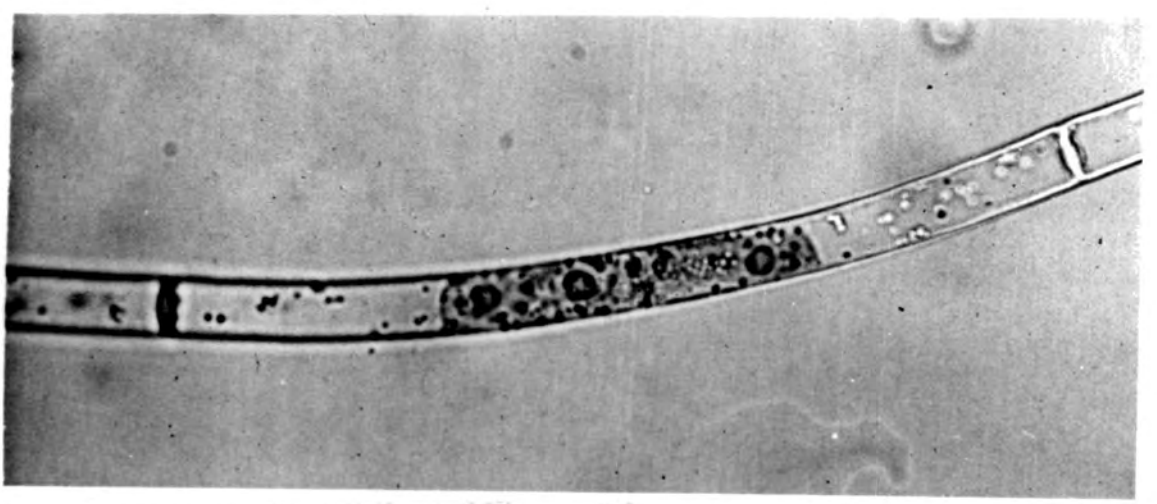
Obvious Chlorella sp. (Fig. 2.7).

Cells spherical, 3-13 μm diam. Chloroplast parietal filling complete cell; no apparent pyrenoid after staining with iodine. Autospores 8-64 (usually 32 or 64) which are apparently squeezed through a hole in the presistent cell wall. Some daughter cells seem to remain and grow within the old cell wall. Older cultures rapidly develop an orange colour which on microscopic examination is seen to be due to droplets, within the cell, of orange, probably carotenoid, pigments.

After consulting Fott and Nováková, (1969) this strain was identified as C. zofingiensis. The type culture of this species was obtained from the







Cambridge Culture Centre of Algae and Protozoa (strain CCAP 211/14) and the similarity between this and strain 546 was evident. The only notable morphological difference between these two strains was the distribution of carotenoid pigments which, in strain 546 formed distinct droplets whereas, in the CCAP strain the orange colour was diffuse throughout the cells. Donz (1934; who collected and classified the type culture), however, did describe reddish oil droplets within the cells of C. zofingiensis.

2.83 Isolation and purification

On return to the laboratory, fresh algal material was streaked onto Chu 10 E solid medium (+ 1% agar) in 50-ml, plastic, sterile petrie dishes. The agar plates were incubated in the 25°C growth room (Table 2.7) for approximately 1 week. After this period individual algal colonies were removed from the surface of the agar plate with a wire loop and were transferred to liquid medium (Chu 10 E) and were again incubated in the 25°C growth room for a one week period. Algal cultures grown in this way were examined under the microscope to ensure that they were unialgal.

A wide range of unialgal cultures were obtained from streams 0097 and 3026 in this way, however, all cultures contained bacterial, and some, fungal contaminants. As these algal strains were to be used for laboratory physiological studies it was essential that they be rendered axenic (Bold, 1942).

Many methods were tried in order to obtain axenic cultures; eventually all but one strain were rendered axenic (Table 2.8). The following procedure was used to obtain axenic cultures; it is based on a combination of published methods.

Cultures of algae growing in liquid medium were incubated for 24 h in the dark. After this period one drop of liquid nutrient broth (Section 2.84)

was added to the culture which was incubated in the dark for a further 3 h. The organic matter was added in order to induce cell division of the bacteria present, rendering them more susceptable to antibiotics, particularly penicillin (Droop 1967). Similarly algae were incubated in the dark to halt their growth and therefore render them less susceptable to antibiotics.

Following this dark incubation, a period of alternate sonication and centrifugation of the algal material was performed. The algal culture was sonicated in a Soniprep 150 for approximately five seconds before being centrifuged at 3500 rpm for two minutes. The supernatant was discarded and the alga resuspended in fresh sterile medium. This sonication/centrifugation process was repeated five times. The sonication step fulfilled two purposes; firstly bacterial cells adhering to the surface of the algal cells were likely to be dislodged, and secondly, algal filaments were broken into short lengths (see Section 2.922). The reason for the centrifugation step was as follows; bacterial particles being smaller than algal cells will sediment more slowly under the influence of gravity (Stoke's Law). By continually discarding the centrifuged supernatant the overall alga to bacterium ratio should increase.

Algal material was then incubated in one of three antibiotic solutions.

a) Antibiotic mixture

Application of an antibiotic mixture is recommended for algal purification by Jones et al. (1973). A short exposure to high levels of a range of antibiotics should be lethal to a wide range of possible bacterial contaminants, also, applying antibiotics in a mixture should prevent the selection of bacterial mutants resistant to a single antibiotic. An antibiotic mix similar to that of Droop (1967) was used at three overall concentrations (Table 2.9).

The antibiotic mixture was prepared as a dry mix and was dissolved in

distilled water just prior to use. The solution was sterilized by passing it through an autoclaved Nuclepore filter (0.2 μm) mounted in a plastic Swinnex filter holder.

Table 2.9 Antibiotic mixtures

antibiotics	antibiotic concentrations (μg ml^{-1})		
	I	II	III
benzyl penicillin-SO_4	400	200	80
streptomycin-SO_4	80	40	16
neomycin-SO_4	10	5	2
chloramphenicol	10	5	2
Total	500	250	100

Algae were exposed to the antibiotics for 12 h (in the dark) after which they were centrifuged and the supernatant was decanted off to be discarded. Sterile fresh medium was added and the algae were washed in fresh medium, by centifuging and discarding the supernatant, five times. The resultant suspension was used in the next stage of purification.

b) Phenol

A 1% (v/v) solution of phenol was added to the algal suspension and this was incubated in the dark for 1 h. The alga was then washed by centrifugation five times. The use of phenol as a bactericidal agent is recommended by McDaniel et al. (1962).

c) Methanol

Algal suspensions were incubated for 12 h in the dark in a 20% (v/v) solution of Analar methanol. After this period the alga was washed five times in fresh medium, before being used in the next stage of purification. Methanol proved to be a useful antifungal agent which was originally

incorporated as a solvent for griseofulvin (a specific antifungal antibiotic) the use of which was recommended by G.H. Banbury (pers. comm.). Griseofulvin proved to be more toxic to algae than their fungal contaminants; however, controls showed that algal survival in 20% methanol was quite high (c. 10%) but contaminants, particularly fungi, were much reduced.

The antibiotic stage was found to be important in allowing a favourably high alga to bacterium ratio for purification. It was possible to omit this stage for only one strain isolated (Table 2.10). Mutation of algae by the direct action of antibiotics or by selection of antibiotic resistant mutants is often cited as a danger with their use (Droop, 1967; Jones et al., 1973) so algal viability counts were made after treatment (Section 2.924); where greater than 90% fatality occurred, the algal material was not used subsequently. Viable counts of filamentous algae are less reliable than for unicellular algae. By regarding each length of filament (usually 3-10 cells) as a single unit (when counting in the haemacytometer), an overestimate of cell viability is likely. Mean filament lengths were estimated when haemacytometer counts were made. From this, minimum possible cell viabilities for filamentous algae were calculated; these were never less than 3%.

After antibiotic treatment algal material was inoculated onto sterile agar plates using an atomizer technique as described by Wiedman et al. (1964). A capillary tube was inserted into a centrifuge tube (containing the algal suspension) and held in place by a cotton wool plug. Compressed air was then passed through a Pasteur pipette the tip of which was positioned adjacent to, and perpendicular to, the upper tip of the capillary tube. The algal suspension was drawn up the tube and atomized in a fine spray (Fig. 2.8). Several sterile agar plates were uncovered briefly in the spray from each suspension. The solid medium used was Chu 10 E + agar (1%) + sucrose (0.05%) + soil extract (1%); soil extract was prepared as

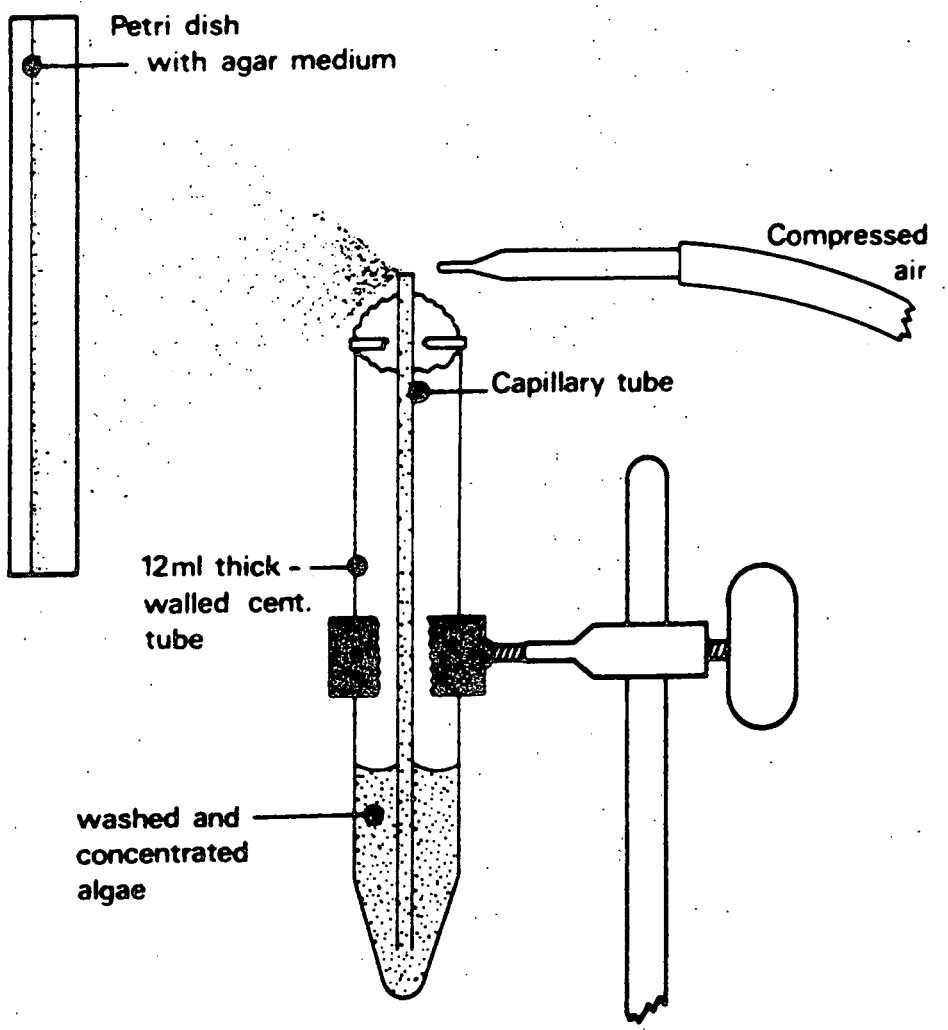
described by McLachlan (1973). Sucrose was added in order to encourage bacterial growth, soil extract was added to supplement the nutrients in the medium. The addition of soil extract circumvented the possible lack of a required algal nutrient, previously supplied by contaminating bacteria or fungi. The plates were incubated for a short period (2-3 days) in the 25°C growth room. It was important to keep the agar surface dry during incubation to prevent motile bacteria from swimming over the agar surface. After the incubation period the agar surface was examined under a binocular microscope contained in a laminar flow cabinet. Contaminated algal colonies could be distinguished from apparently non-contaminated colonies, the sucrose having led to very rapid growth of bacteria which often overgrew the algal colonies. Algal colonies which appeared to be free of bacterial contaminants were removed from the agar surface with a sterile needle and were streaked onto fresh (Chu 10 E agar +1% soil extract) plates. The resultant algal cultures were tested for contaminants (Section 2.84).

Eight algal strains were made axenic (Table 2.10).

Table 2.10 Axenic algal strains and the purification method used

alga	Durham culture no.	antibiotic used	total antibiotic
Hormidium sp.	532	mix	100 $\mu g\ ml^{-1}$
Chlamydomonas sp.	533	none	-
Mougeotia sp.	536	mix	100 $\mu g\ ml^{-1}$
Hormidium rivulare	537	methanol	20% (v/v)
Hormidium sp.	539	phenol	1% (v/v)
Chlamydomonas sp.	541	phenol	1% (v/v)
Stichococcus bacillaris	545	mix	500 $\mu g\ ml^{-1}$
Chlorella zofingiensis	546	methanol	20% (v/v)

Fig. 2.8 Atomizer apparatus used to obtain axenic cultures of algae



2.84 Tests for purity

Tests were made of all cultures after initial isolation and also at intervals throughout the period of experimentation. Algal material was inoculated onto agar plates of the following test media described by Hoshaw and Rosowski (1973).

beef peptone agar
malt extract agar
yeast extract agar
nutrient broth
SST

The plates were incubated at 32°C for 10 days. The algal material was also examined under the microscope using both normally transmitted light and phase contrast microscopy.

If the presence of contaminants could not be shown it was concluded that an algal strain was axenic.

2.85 Maintenance

Algal cultures were inoculated onto agar slopes in 18 mm diameter test tubes and incubated in the 25°C growth room. Algae were maintained as follows: from stream 0097 on Chu 10 E + 5 mg l^{-1} Zn; from stream 3026 on Chu 10 E + 10 mg l^{-1} Zn + 1 mg l^{-1} Cd. When sufficient algal biomass had developed the slopes were transferred to a cooler (10°C), where they were kept at a low light intensity (2.1 μM P m^{-2} s^{-1}). Algal material was subcultured regularly from these stocks into Zn/Cd-free medium to give a large biomass. This algal material was subcultured at least once more in Zn/Cd-free medium before being used as an experimental inoculum.

Algae were maintained in a way in which genetic change would be unlikely. The presence of heavy metals in the growth medium would prevent loss of algal resistance to the toxic effects of these metals; the low temperature, low light conditions would reduce the number of cell divisions and therefore the possibility of selection occurring. Individual slopes could be maintained in this way for approximately one year.

2.9 Laboratory culture studies

2.91 Algal assay

A simple procedure was performed, similar to that described by Whitton (1970b) which was designed to give a rapid estimate of algal performance in a wide range of treatments. A small amount of algal material was inoculated into 10 ml of medium in 50-ml boiling tubes (usually 50 µl of suspension in the case of unicellular algae). In the case of filamentous algae a small amount was teased apart using a dissecting needle and inoculated into the medium. Tubes were incubated in the growth tank (see Table 2.7) for six days after which the tubes were inspected visually. Algal biomass was estimated with respect to the tube showing maximum growth and to samples of preserved inocula. Biomass was scored on a 1 - 5 scale in the following way.

0 - dead

1 - no growth, alga still alive

2 - growth, biomass > inoculum < 1/3 maximum

3 - growth, biomass > 1/3 maximum < 2/3

4 - growth, biomass > 2/3 < maximum

5 - maximum biomass

Where the distinction between a score of 0 and 1 was not obvious, algal material was subcultured into normal Chu 10 E medium; failure to grow confirmed that the treatment had been lethal.

2.92 Quantitative experiments

2.921 Incubation conditions

Quantitative growth experiments were performed either in 100-ml conical flasks containing 25 ml of medium or in 50-ml boiling tubes containing 10 ml of medium. All quantitative experiments were incubated in the growth

tank at a temperature of 20°C (see Table 2.7) except for one experiment which was incubated in the temperature gradient.

2.22 Inoculum

This was performed as described in Section 2.75. The size of inoculum was typically 2 x 10^4 cells ml^{-1} for unicellular algae or 0.2 mg l^{-1} chl *a* for sonicated filamentous algae. Cell counts, or chl *a* measurements, were made just prior to inoculation in order to allow calculation of the dilution required. Sonication of filamentous algae undoubtedly caused disruption of some cells and therefore chl *a* values for inocula were an overestimate of viable biomass. In this case it can only be stated that the inoculum was constant (the inoculum was kept well mixed) and was $\leqslant$0.2 mg chl *a* l^{-1}. The inoculum was always less than 2% of the total experimental volume.

2.923 Cell counts

Growth estimates of unicellular algae were based on cell numbers ml^{-1}. Cells were counted in a haemacytometer counting chamber (depth 1 mm; Improved Neubar ruling). For each estimation at least 100 cells were counted which, using 95% confidence limits, ensures $<$ 20% error for each individual count assuming the cells are randomly distributed in suspension (i.e. a Poisson variable; Lund *et al.*, 1958).

2.924 Algal viability

A cell count was made of an algal culture using a haemacytometer (Section 2.923). Following this a dilution of the culture was made in fresh Chu 10 E medium. This was calculated to give a final concentration of 2 x 10^3 cells ml^{-1} or in the case of filamentous algae 2 x 10^3 filament lengths ml^{-1}. 0.1 ml of this dilution was spotted onto the surface of Chu 10 E + 1% agar medium in a 50-ml petri dish and spread over the surface using

a sterile glass rod. Petri dishes were incubated in the 25°C growth room for 7 days. After this period the number of algal colonies which had developed on the agar surface were counted. Percentage viability values were calculated as follows:

$$\% \text{ viability} = \frac{\text{no. of algal colonies x 100}}{\text{no. of cells inoculated (200)}}$$

Viable counts were used to monitor algal purification procedures (Section 2.83) and were not used as quantitative experimental measurements. They were therefore not replicated.

2.925 Extraction and estimation of chlorophyll a

Algae were harvested either by vacuum filtration through Whatman GF/C glass fibre paper or by centrifugation. The algae and the solvent (90% methanol) were sealed together in 30-ml universal bottles. They were then immediately placed in a 70°C water bath for 10 min to allow extraction of the pigment. The pigment extract was then filtered through GF/C glass fibre paper to remove cell debris. The O.D. of the resultant pigment extract was then measured at 665 nm against a solvent blank; subtraction of the optical density at 750 nm from this value compensated for the turbidity of the sample. The extraction procedure was carried out as much as possible in the dark to prevent photo-breakdown of the pigments.

Chl *a* was calculated using the following formula:

$C = K.\frac{V}{L}$ where

C = concentration of chl *a* in µg

K = 1000 x the reciprocal of the specific absorption coefficient of chl *a* + 4% (for chl *b*)

V = volume of solvent used to extract the sample in ml

L = path length of the cuvette in cm.

The specific absorption coefficient of chl *a* in 90% methanol is taken to be 77, as recommended by Marker *et al.* (1980). A certain proportion of absorbance at 665 nm, however, will be due to the presence of chl *b*. It is recommended by Marker *et al.* (1980) that in Chlorophyta this will approximate to a 4% increase in the specific absorption coefficient, which becomes 80.2. The equation for chl *a* calculation then becomes:

$$C = 12.47 \frac{V}{L}$$

Acidification of samples to allow calculation of phaeophytin *a* (Marker *et al.*, 1980) was found to be unnecessary. Phaeophytin *a* values were not high enough to interfere with chl *a* values in cultured algal material.

2.926 Dry weight

Algal material was separated from the growth medium by either centrifugation (unicellular algae) or by removal with a glass Pasteur pipette (filamentous algae). The algae were then washed briefly three times in distilled water before being transferred into pre-dried, acid washed, snap-top glass vials; these had been previously weighed. The vials were dried at 105°C for 48h. On removal from the oven they were placed immediately into a desiccator to prevent absorption of water as they cooled to ambient temperature. The vials were then re-weighed in order to determine algal dry weight.

2.927 Estimation of algal metal accumulation

Dried algal material, of known dry weight, was obtained as described in Section 2.926. The material was digested in the same way as field material (Section 2.22) and then analysed for metals. Accumulated metal was calculated using the following formula:

metal accumulated (mg g^{-1} dry weight) =

$$\frac{\text{concentration of metal in digest (mg } l^{-1}\text{) x volume of digest (ml)}}{\text{dry weight of digest (mg)}}$$

2.10 Analysis of algal material for carboxylic acid content

2.10.1 Growth and harvesting of algal material

All algal strains cultured for carboxylic acid analysis were isolated originally from stream 3026. It was found that a relatively high biomass of algal material was required for estimation of constituent carboxylic acids. Unicellular algae (533, 545, 546) were grown in 10-litre carboys containing 10 l of Chu 10 E medium, buffered at pH 5.0 with 0.3 mM DMGA, + and - Zn. Sterile air (passed through a 0.2 μm Nuclepore filter) was bubbled through the medium. These cultures were harvested in their exponential growth phase using a M.S.E. flow-through centrifuge to give an algal pellet. Filamentous algae (532, 537) were grown in 2-l conical flasks containing 1 l of Chu 10 E medium, pH 5.0 + 0.3 mM DMGA, + and - Zn. These algae were harvested by removing clumps of material with a Pasteur pipette tip.

The resultant algal material was washed three times in fresh Chu 10 E (-Zn) medium and was then transferred to predried, preweighed universal bottles. These bottles were dried to a constant weight at 60°C and algal dry weight was determined (Section 2.926).

In addition to cultured algal material a sample of Mougeotia collected from stream 0097 was also processed for carboxylic acid content. This was collected in August 1981 and was transported (cooled) back to the laboratory in a polypropylene bottle. This algal sample was washed and dried in the same way as laboratory filamentous algae. Table 2.11 shows the full range of samples prepared for extraction and analysis of carboxylic acids.

The level of DMGA used in the growth medium was 10% of that used in normal growth media (Section 2.742). This reduction was thought necessary to minimise contamination of the resultant carboxylic acid sample with the

buffer. pH drift of the cultures was monitored for this reason and where required, the pH was adjusted back to pH 5.0 with a sterile HCl solution.

Table 2.11 Samples used for carboxylic acid estimates

alga		Zn concn. in medium (mgl^{-1})		
		0	25	100
Hormidium sp.	532	✓	X	✓
Chlamydomonas sp.	533	✓	✓	X
Hormidium rivulare	537	✓	✓	✓
Stichococcus bacillaris	545	✓	✓	X
Chlorella zofingiensis	546	✓	✓	X
Mougeotia	-	field material		

total number of samples = 12

2.10.2 Extraction and purification of carboxylic acids

The procedure for extraction and purification of carboxylic acids follows closely that outlined by Redgwell (1980).

To the dried algal material (in Universal bottles) 20 ml of methanol/chloroform/water/formic acid (12/5/2/1, v/v) was added. The Universal bottles were sealed and placed in a water bath at 60°C for 2 h. The algal pellet was repeatedly macerated during this period to aid extraction. The residue was then separated from the extract by centrifugation and was re-extracted in the same way as above except this time in water/methanol/formic acid (39/10/1, v/v). To the first extract, in a separating funnel, 5 ml of chloroform and 7 ml of water were added to split the two phases. The chloroform phase (containing the pigments) was discarded and the aqueous phase was combined with the second extract. The combined extract was evaporated to dryness at 60°C using a rotary evaporator.

The carboxylic acids were further purified using ion-exchange resins. Two 5-ml plastic hypodermic barrels were used as columns. The two columns were mounted in series the uppermost containing 5 ml of cation-exchange resin (Dowex 50W-X8, 100-200 mesh size, H^+ form) the lower containing 5 ml of anion-exchange resin (Dowex 1-X4, 200 mesh size, formate form). The dried extract was dissolved in 20 ml of distilled water and applied to the upper, cation-exchange resin. The sample was allowed to elute onto the columns followed by 5 total bed volumes (50 ml) of distilled water.

Purified carboxylic acids were eluted from the anion-exchange column with 5 bed volumes of 4% (v/v) formic acid. This solution was dried at 60°C on a rotary evaporator. It was then further dried in a 60°C oven for 24 h to ensure complete dryness and also complete evaporation of the formic acid. This dried sample was stored desiccated at 4°C until analysis was performed. In order to test the efficiency of the ion-exchange system a 1 ml sample, containing 1 mg of malic acid and 1 mg of citric acid, was applied to the columns. Subsequent analysis of the eluted anion-exchange system allowed full recovery of these two carboxylic acids.

2.10.3 Analysis of carboxylic acids

Dried carboxylic acid samples were dissolved in 1 ml of anhydrous pyridine (sequencer grade; supplied by Rathburn Chemicals Ltd). This sample was stored in a dried, ground-glass stoppered, test tube. An aliquot of this sample was transferred to another glass-stoppered test tube and an equal volume of esterifying reagent was added to convert the carboxylic acids into their trimethylsilyl-derivatives. The esterifying reagent used was bis(trimethylsilyl)trifluoroacetamide (BSTFA). After 10 min of mixing the derived carboxylic acids were ready for analysis by gas-liquid chromatography.

The chromatograph used was a Varian Aerograph model 1400 fitted with a

flame ionization detector. The column was a 1m x 2mm glass column packed with 4% SE-30 on Chromosorb W, 100/200 mesh size (supplied by Phase Separations Ltd). Flame detector and injection port temperatures were 275°C and 225°C respectively; the gas flow rates were, nitrogen (carrier) 30 ml m^{-1}, hydrogen 30 ml m^{-1}, air 300 ml m^{-1}. The temperature of the chromatographic oven was programmed linearly from 85°C to 225°C at a rate of 8°C min^{-1}.

Liquid samples were injected onto the column using a microsyringe; sample volume ranged from 2 µl to 50 µl depending on the peak heights obtained.

The temperature programme was begun after the initial large peak, caused by the BSTFA and the pyridine, had emerged from the column. The trimethylsilyl derivatives of carboxylic acids were found to be stable for up to 56 h; they were, however, usually prepared fresh each day.

This method of carboxylic acid determination was first described by Horii et al. (1965) and was later refined by Phillips and Jennings (1976).

Mixed carboxylic acid standards were run on the chromatograph to give comparisons with extracts (Fig. 7.1). The temperature at which each peak emerged was noted and compared to a known acid (dimethyl glutaric acid) which was used as an internal standard. The chromatographic peak for the DMGA derivative, emerged from the column at approximately 140°C; any temperature deviations of this peak from 140°C were linearly applied to all other peaks in the same run. This allowed for improved reproducibility of retention data and follows the recommendations of Pierce (1968).

The standard carboxylic acid mixture used was:

lactic acid
oxalic acid
malonic acid
succinic acid
dimethyl glutaric acid (internal standard)
malic acid
∝ keto-glutaric acid
aconitic acid
citric acid

The concentration of each acid in the non-derived mixture was 1 mg ml^{-1}.

2.11 Electron microscopic studies of 0097 Mougeotia

Small portions of Mougeotia were removed from stream 0097 during August 1981. Some algal material was transported back to the laboratory for analysis of accumulated Zn levels (Section 2.22), the remainder was prepared for electron microscopic examination in the following way. Filaments, immediately on removal from the stream, were placed into 2.5% glutaraldehyde fixative (buffered at pH 6.8 with 0.1 M sodium cacodylate buffer) where they remained for 2 h. They were then washed in several changes of 0.1 M sodium cacdoylate buffer and post-fixed in 2% aqueous osmium tetroxide for 90 min. Following this the filaments were dehydrated through a graded ethanol series and placed for 24 h in a 50:50 mixture of 100% ethanol and Spurr resin (Spurr, 1969). After a further 24 h in fresh resin the algal material was embedded in gelatine capsules and polymerised at 80^{o}C for 12 h.

Ultrathin sections were cut on glass knives in an LKB ultrotome III ultramicrotome, collected on copper grids and stained for 10 min in each of uranyl acetate and alkaline lead citrate. They were then viewed in a Philips EM 400 electron microscope operating at 80 kV.

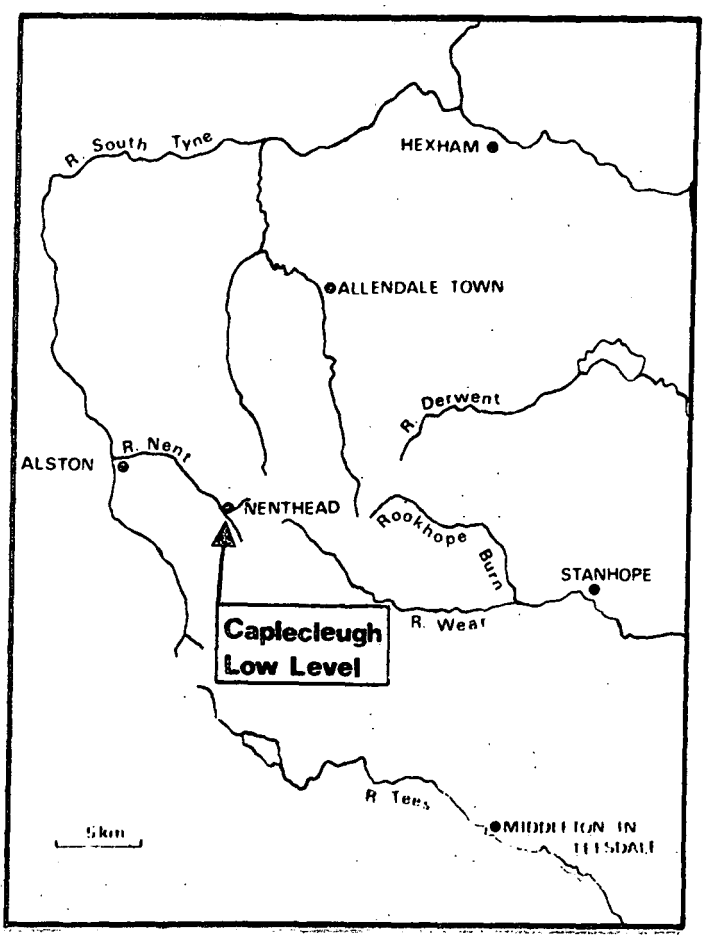
3 BACKGROUND TO AREAS OF FIELD STUDY

3.1 Stream 0097 - Caplecleugh Low Level

3.11 Introduction

Caplecleugh Low Level is a small stream draining from an adit of the Caplecleugh Mine in the village of Nenthead, Cumbria (54° 47' N, 2° 20' W; grid ref. NY 781434; elevation 434 m). Caplecleugh mine was previously a major source of lead and zinc concentrates in the Northern Pennine Orefield (Dunham 1948). Caplecleugh Low Level flows from one of the tunnels driven into the mineral complex; these acted as both drainage levels and access points into the mine. The stream flows for 14 m before draining into the River Nent which itself drains into the River South Tyne (Fig. 3.1).

Fig. 3.1 Upper catchment of South Tyne, showing location of Caplecleugh Low Level



3.12 Geological background

A thorough summary of the geology of the Northern Pennine Orefield is given by Dunham (1948). The Northern Pennines have been carved out of a double fault block. This is defined on the north and south boundaries by the Stublick and Craven faults respectively and on the western boundary by the Pennine and Dent faults.

The predominatnly carboniferous rocks of the Northern Pennines rest upon a basement of folded slates (Ordovician) into which cores of granite have been intruded. Dunham agrees with the suggestion of Goodchild (1889), that mineral deposits in the Northern Pennine Orefield derive from this granite and were emplaced along pre-existing channels by the crystallization of hydrothermal brines. Further evidence in support of this theory is given by Sawkins (1966). Two principle types of oreshoot exist: (1) vein oreshoots, developed by mineralisation along vertical fissures; (2) metasomatic flats, formed by the lateral replacement of limestone beds.

The majority of the productive mineral veins in the Northern Pennines follow the east-north-east tension fissures. The larger faults lying perpendicular to these productive veins are not strongly mineralized, being blocked by shale at the time of mineralization.

The age of primary mineralization in the Northern Pennines is not certain, though Dunham (1948) presents evidence for either a Hercynian or Tertiary date.

The Northern Pennine Orefield has been mined since Roman times (Section 3.13); since then an estimated total of 4.5×10^6 tonnes of lead concentrates and 3.0×10^5 tonnes of zinc concentrates have been produced (Dunham, 1981).

'Levels' were driven primarily to drain water from the mine workings and also the horizontal entrances allowed horses to be used for transportation

in the mines (often referred to as 'horse levels'). Caplecleugh Low Level was driven from Nenthead to give access to the Brigal Burn, Caplecleugh, Middle Cleugh and Black Ashgill veins (Fig. 3.2). Extensions to the level continued north from the Middlecleugh series of veins and linked with levels driven from the north-east side of the Nent valley. This gave connections with the Smallcleugh, Rampgill and Scaleburn veins. A branch of Caplecleugh Low Level also gave access to the Dowgang vein (Fig. 3.3), the majority of which had previously been excavated by the process of hushing. In the Nenthead area a total of 5×10^5 tonnes of lead concentrates and 2.5×10^5 tonnes of zinc concentrates have been won. The zinc total represents 83% of the value for the entire Northern Pennine Orefield. Caplecleugh Low Level was driven for the majority of its length beneath the 'Great Limestone', most lead and zinc being stoped from above the level, in and above the Great Limestone. The predominant minerals in the veins accessed by Caplecleugh Low Level were galena (PbS) and sphalerite (ZnS); in addition Middlecleugh N. vein contained high concentrations of fluorite (CaF_2).

3.13 Historical aspects

According to Raistrick and Jennings (1965), the first lead mining in the Northern Pennines probably began prior to the Roman invasion of Britain. Certainly during the Roman occupation lead mining became established in the area. Relatively small amounts of lead were mined until the late eighteenth century; this was usually by the digging of small shafts or by the process of hushing. Mine drainage was a major problem until this period, when technological and economic advances, under the guidance of the London Lead Company, allowed for the driving of major drainage levels. These opened up large mineral deposits to relatively safe access. According to Dunham (1948) Caplecleugh Low Level was driven in the late eighteenth century by the London Lead Company (holders of the Nent valley mineral rights). There followed a period of intense lead mining activity in the

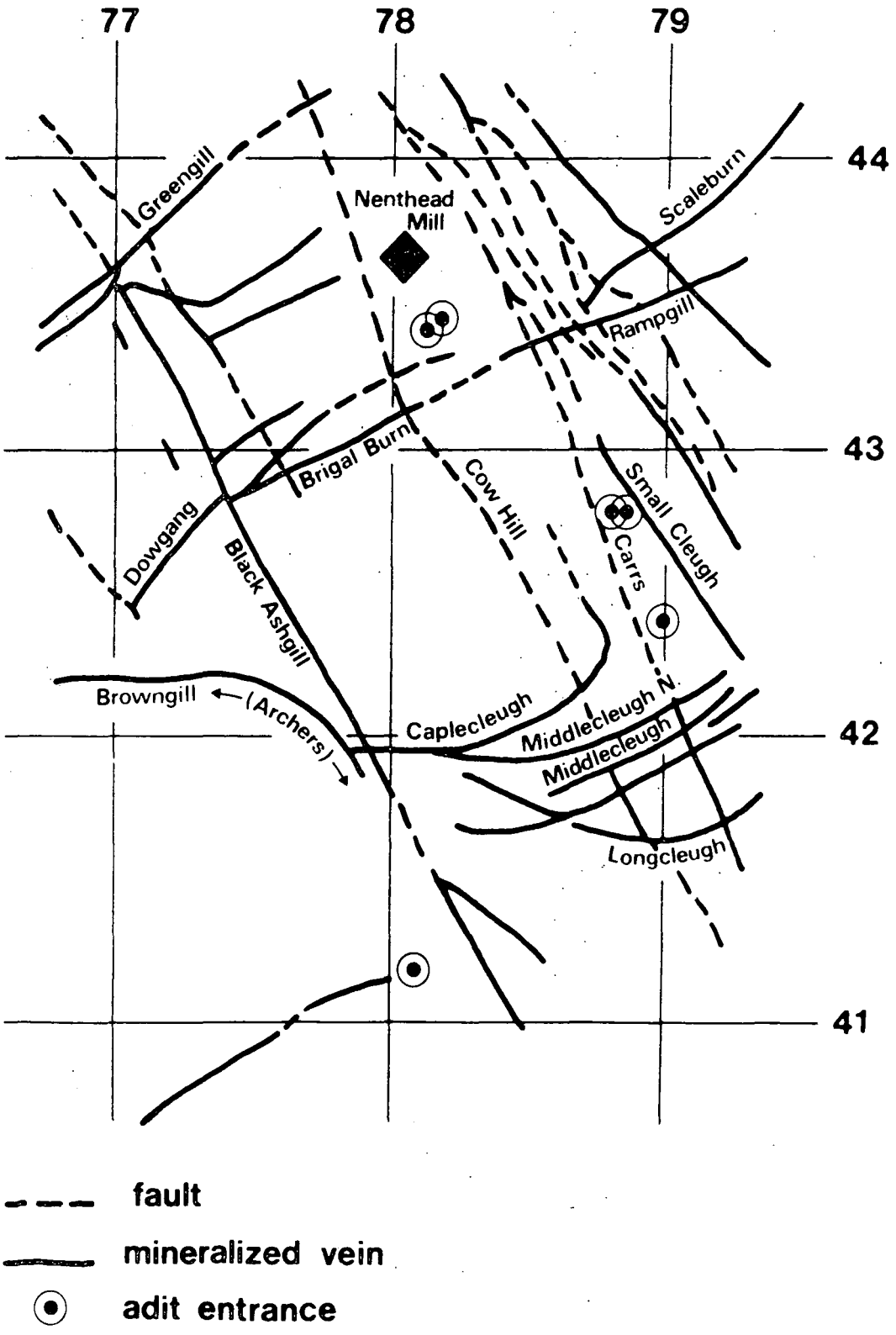


Fig. 3.2 Map of the mineral veins of the Nent valley. Data from Dunham (1948)

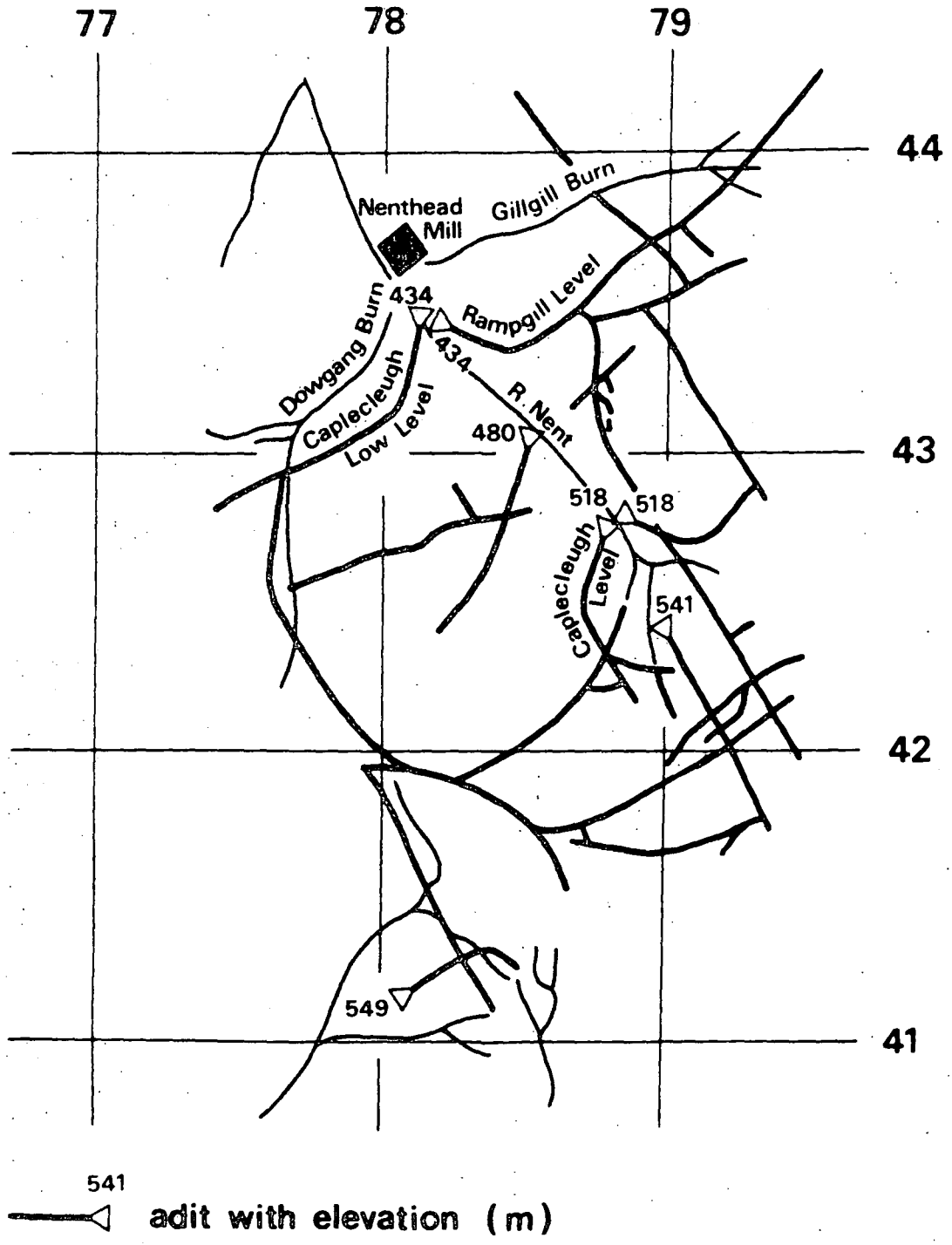


Fig. 3.3 Map of the network of levels in the Nent valley. Data from Raistrick and Jennings (1965)

Nent valley area. This continued until 1882 when, due to dwindling high grade reserves and a fall in the market price of lead, the London Lead Company surrendered its leases to the Nenthead and Tynedale Zinc Company. There followed a second burst in mining activity as the new company began to mine deposits of sphalerite (ZnS) presumably passed over by the London Lead Company as uneconomic. In 1906 the Nenthead and Tynedale Zinc Company sold its leases to the Vieille Montagne Zinc Company who continued to mine sphalerite in the south-west side of the Nent valley until approximately 1920. Since 1920 there has been no major mining activity in the area though some reworking of local, abandoned mine tailings did occur during the Second World War.

It is likely that water draining from Caplecleugh Low Level has flowed continuously since the level was driven i.e. 200 years; the main source of the water being the groundwater of the Great Limestone stratum. The level is one of the lowest levels in the Nenthead system (Fig. 3.3) and therefore is likely to drain water from the whole level complex.

3.14 Physical characteristics and geography

The water before emerging from Caplecleugh Low Level adit has flowed for a considerable distance underground along an open channel. The internal height of the level being approximately 1.5 m for at least the first 200 m. The part of the stream outside the adit is very short, flowing for only 14 m before entering the River Nent, with a total fall in height of 1.6 m. It descends 0.4 m over the first 8 m length, it next passes through a wooden box (4 m long, 0.9 m wide, 0.2 m fall over length); the stream bed inside the box consists of fine silt, together with a few boulders of millstone grit. The final 2 m length of stream descends a further 1 m over boulders of millstone grit before entering the River Nent (Fig. 3.4). A plan and elevation of Caplecleugh Low Level are given in Figs. 3.6 and 3.7.

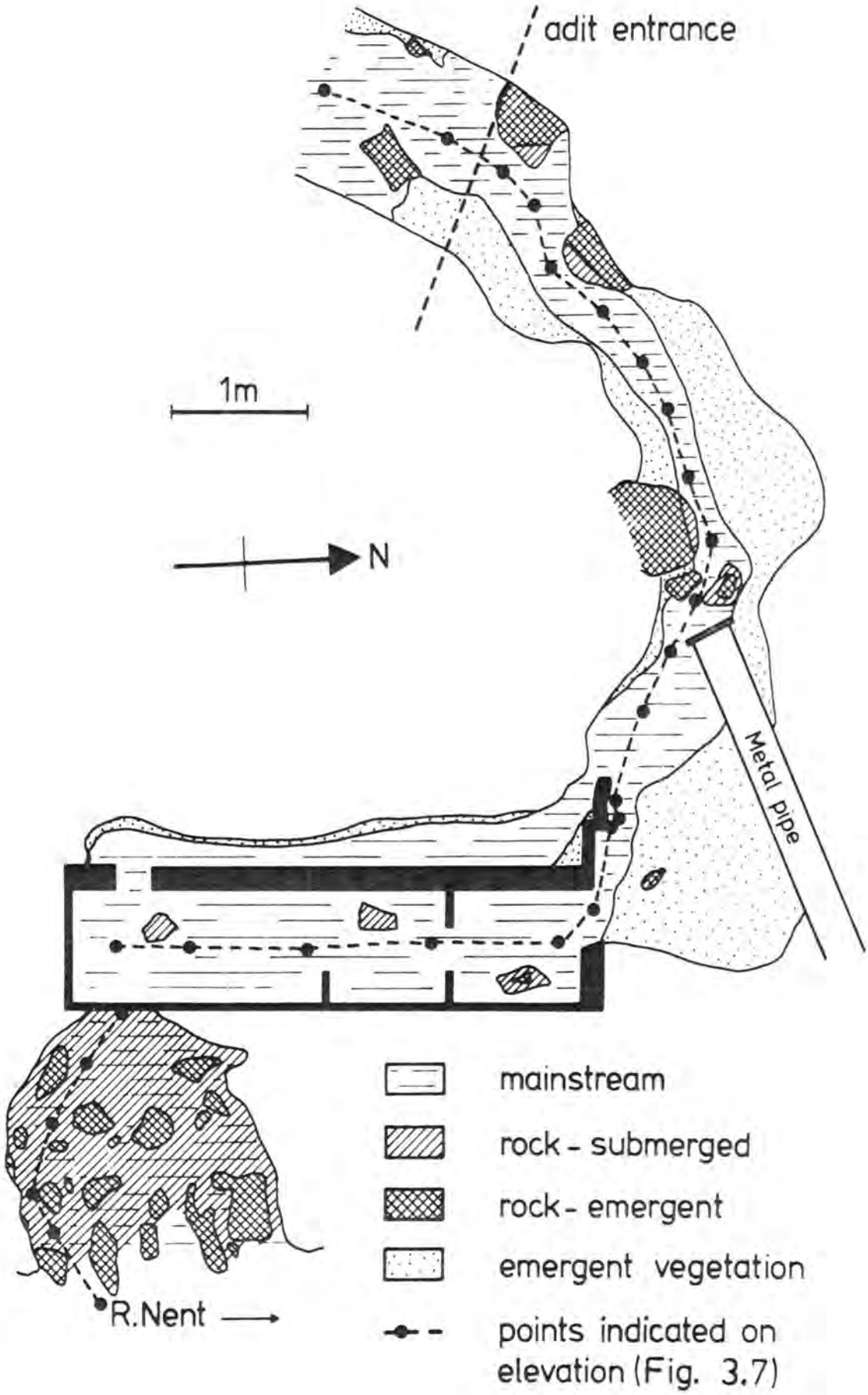


Fig. 3.6 Stream 0097; Caplecleugh Low Level (plan)

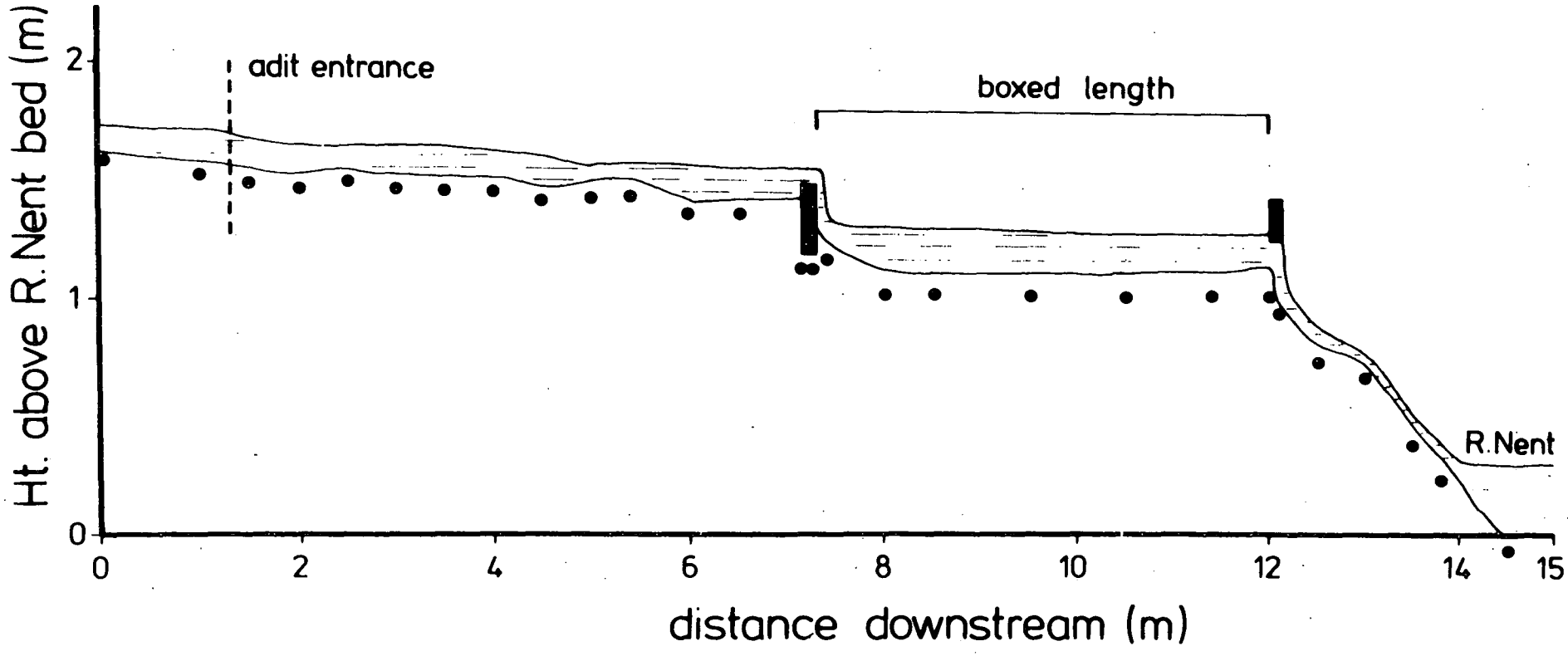


Fig. 3.7 Stream 0097; Caplecleugh Low Level (elevation)

The boxed length of the stream (see frontispiece), probably a remnant leat channel, delineates the area of the stream used for many of the environmental parameters measured during the present study (Section 2.61).

The stream has a north facing aspect and also a number of larch (Larix decidua) trees grow nearby; these factors together reduce light input to the stream. The aspect acts to totally exclude direct sunlight in the winter, probably exaggerating the seasonal pattern of irradiance. The larch trees appear to contribute little organic material to the stream.

The source of the water for Caplecleugh Low Level is that draining into the local hillsides, entering the groundwater system and ultimately draining into the level. The nearest upland meteorological station to Caplecleugh Low Level is on Widdybank Fell, 15 km to the South (54° 40', 2° 17'W; map ref. NY 817300; elevation 500 m). The mean annual rainfall distribution for Widdybank fell is given in Fig. 3.8; these data are based on the years 1971-1980. The annual rainfall distribution for 1980, the year of the present study (Section 2.61), is given in Fig. 3.9.

Fig. 3.8 Mean annual rainfall distribution for Widdybank Fell

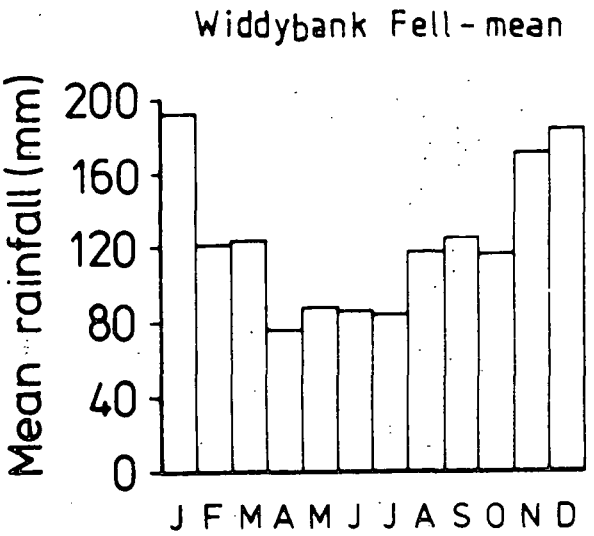
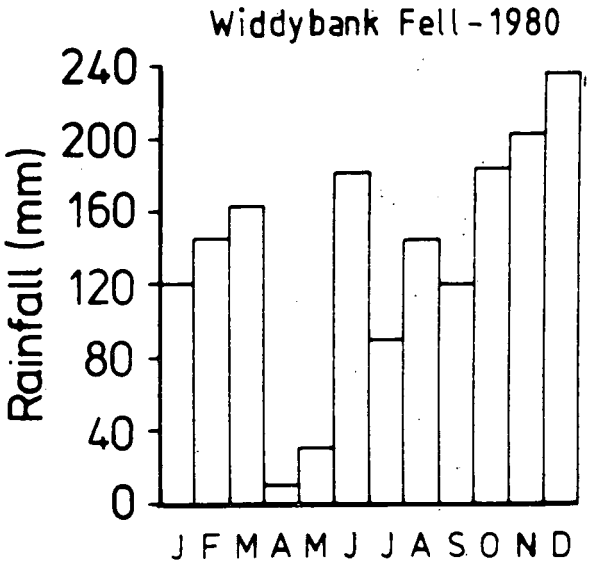


Fig. 3.9 Rainfall distribution for Widdybank Fell, 1980



3.15 Previous work on Caplecleugh Low Level

Say (1977) briefly described Caplecleugh Low Level in his ecological survey of metal pollution of the streams of the Northern Pennines. Armitage (1980) studied the fauna of Caplecleugh Low Level in his survey of benthic animals of the River Nent system. Previous authors have referred to Caplecleugh Low Level as Dowgang Level; the name of the stream was changed by the present author after the recommendations of K.C. Dunham (pers. comm.). According to Dunham a drainage level called Dowgang Level already exists close to the intersection of the Dowgang and Black Ashgill veins (Fig. 3.2).

3.2 Stream 3026 - Le Crouzet Upper Slope Seepage

Stream 3026 is a small seepage which emerges from the base of a zinc smelter waste tip. The smelter and the waste tip are adjacent to the town of Viviez in the Department of Puy de Dome, France. The seepage is situated at 44° 32' N, 2° 13' E (map ref. 59122498) at an altitude of 240 m. Stream 3026 flows for only 2.5 m before entering the Ruisseau de Creuzet.

Zinc smelting at Viviez was begun in 1871 by the Vieille Montagne Zinc Company. The discovery of large coal deposits in the area in 1825 resulted in the establishment of the smelting industry; coal was used to fire the smelting furnaces. In 1922 the smelting process at Viviez was changed to an electrolytic system. Zinc concentrates are transported to Viviez from throughout France.

Stream 3026 emerges from a depression in the base of a smelter tip to form a small pool (approximate diameter 400 mm, maximum depth 150 mm). Water from this pool seeps down a bank entering the Ruisseau de Creuzet (Fig. 3.5). A plan and vertical section of stream 3026 is given in Fig. 3.10. The bed of the seepage is similar to the nearby soil, mainly sand and pebbles. The algal mat growing over the seepage is probably important in consolidating the sediment.

Stream 3026 has a southerly aspect and therefore receives direct sunlight. The upper part of the seepage lies in a depression (Fig. 3.5) and is somewhat shaded; there are no trees nearby which could cause shading.

Meteorological observations for the area are based on measurements made at Clermont Ferrand 150 km N.-E. of Viviez (45° 48' N, 3° 9' E; elevation 329 m). Both Viviez and Clermont Ferrand are at similar altitudes in the Massif Central region of France. Meteorological data for Clermont Ferrand are from Arléry (1970) and are summarized in Fig. 3.11 and Table 3.1. Values are based on mean values from 1946-1960.

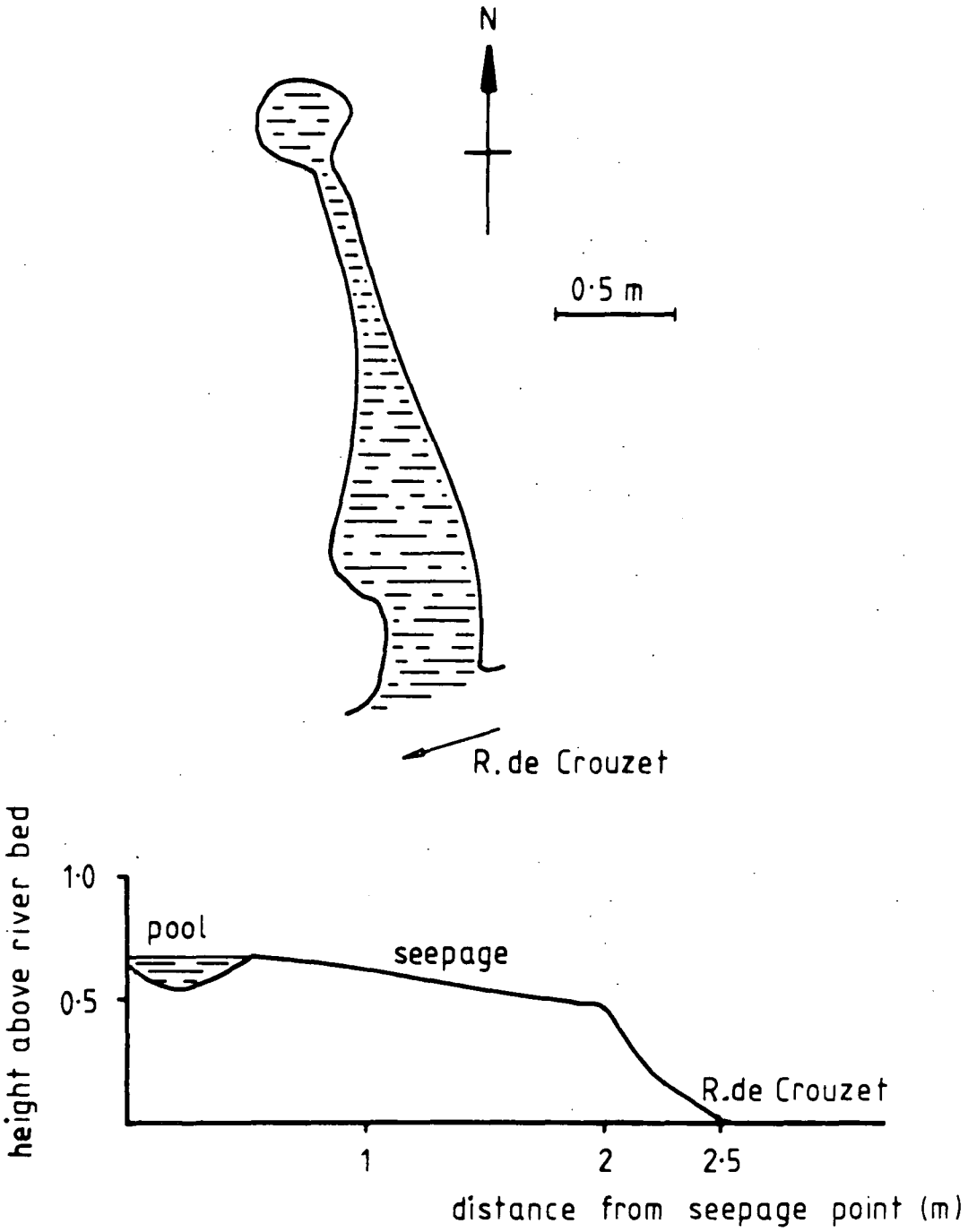


Fig. 3.10 Stream 3026; Le Crouzet Upper Slope Seepage (plan and elevation)

Fig. 3.11 Mean annual rainfall distribution for Clermont Ferrand

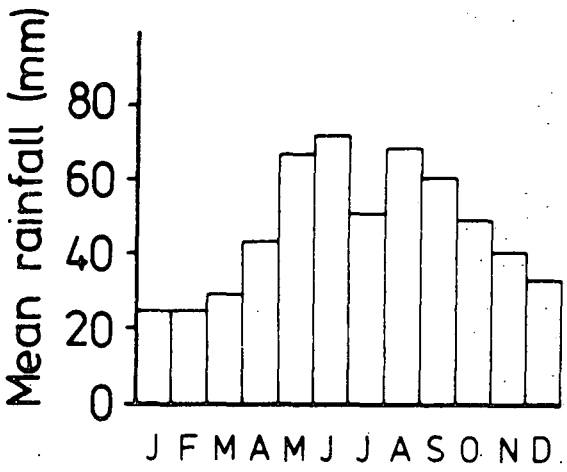


Table 3.1 Temperature data for Clermont Ferrand

month	mean daily temp.	mean daily temp. range
Jan	2.5	7.8
Feb	3.5	9.1
Mar	7.7	11.2
Apr	10.1	11.4
May	13.7	11.8
Jun	17.2	11.8
Jul	19.2	12.5
Aug	18.8	12.5
Sep	16.1	11.4
Oct	11.0	10.4
Nov	6.7	8.1
Dec	3.5	7.2

Stream 3026 was discovered and briefly described by Say and Whitton (1982). A further discussion of heavy metal pollution in rivers and streams in the general region of Viviez is given by Say (1978).

4 INTENSIVE STUDY OF STREAM 0097 - CAPLECLEUGH LOW LEVEL

4.1 Introduction

The general aim of the present study was to gain a more detailed understanding of the ability of certain members of the green algae (Chlorophyta) to survive in conditions of extreme heavy metal pollution (Section 1.6); two metal polluted sites were chosen to investigate this phenomenon. This chapter summarises the data collected at stream 0097; the following chapter summarises data collected at stream 3026.

Stream 0097 was chosen for investigation following observations made by Say (1977) and also because of its proximity, allowing ease of access at all times of the year.

Stream 0097 is a highly Zn-polluted stream which during part of the year contains luxuriant growths of the green alga Mougeotia (Say, 1977); a background description of the site is given in Section 3.1. An ecological investigation of stream 0097 was performed during 1980. The aims of the field study were as follows.

1) Analysis of algal metal content has often been advocated as a potential monitor of aquatic metal pollution (Section 1.43). Stream 0097 represents a suitable environment to test the validity of using Mougeotia to monitor changes in aquatic metal pollution. Phillips (1980) points out the importance of considering seasonal effects on the potential use of aquatic organisms as pollution monitors; monthly collections at stream 0097 were performed to isolate these effects.

2) The majority of laboratory studies concerning the effects of heavy metals on freshwater algae use cultures isolated from an undefined source (see Section 1.5). In the present study laboratory experiments were performed on an axenic culture of Mougeotia isolated from stream 0097.

The intensive study of stream 0097 allowed detailed background information about the environment from which this strain was isolated; this further allowed direct comparisons of the physiological effects of metal pollution on Mougeotia under both laboratory and field conditions (details of laboratory experiments are given in Chapter 6).

3) Stream 0097 forms an ideal study area to investigate the sinks of heavy metals in a polluted environment. It was felt that an understanding of these would be important when attempting to monitor, and possibly remedy, the effects of metal pollutants (see Section 1.4).

4) The study would allow a more detailed understanding of the ecology of a polluted environment.

This chapter summarises the results of the intensive study of stream 0097 performed during 1980 (Section 2.61). The major emphasis is placed on the distribution of metals within the stream (Sections 4.2 - 4.5); ecological observations made at the site are summarised in Sections 4.6 - 4.8.

4.2 Physical and chemical properties of the water

Results of the monthly water analyses for stream 0097 are given fully in Table 4.1. The upper collection point is designated as reach 01, the lower as reach 02 (Section 2.61). The filtrable metal vial for 0097-02 collected during September was damaged; analysis results are therefore not included in Table 4.1. Details of the analytical methods used are given in Section 2.21. It is noticeable from Table 4.1 that the water emerging from Caplecleugh Low Level adit remains constant with respect to many of the physical and chemical variables analyzed; water temperature in particular remains at 9.5^{o}C throughout the year. This indicates a deep groundwater source for the level which can be defined as a 'rheocrene', using the classification system of Bornhauser (1913).

stream	reach	year	month	day	current ($m\ s^{-1}$)	temperature (°C)	conductivity at 25°C ($\mu S\ cm^{-1}$)	pH (field)	pH (laboratory)	$Eh_{7.0}$ (field)	total alkalinity ($m\text{-}equiv\ l^{-1}$)	Na	
												T	F
0097	01	1980	1	17	0.145	9.5	775	8.00	8.15	217	3.60	9.1	9.5
"	02	"	1	17	-	"	765	7.90	8.10	212	3.48	9.0	9.7
"	01	"	2	13	0.175	"	670	8.10	7.30	213	2.72	7.4	8.1
"	02	"	2	13	-	"	705	7.85	7.64	169	2.76	7.4	7.6
"	01	"	3	26	0.155	"	760	8.20	7.64	185	3.40	8.9	9.3
"	02	"	3	26	-	"	790	8.00	7.68	160	3.24	9.3	9.2
"	01	"	4	23	0.169	"	820	8.10	8.20	162	3.64	9.8	9.4
"	02	"	4	23	-	"	730	8.10	8.20	153	3.60	9.5	9.6
"	01	"	5	20	0.160	"	520	8.10	8.14	178	3.72	9.9	10.2
"	02	"	5	20	-	"	525	8.00	7.96	132	3.80	9.4	9.4
"	01	"	6	24	0.183	"	810	7.80	7.98	136	3.24	8.6	8.8
"	02	"	6	24	-	"	810	7.90	8.10	131	3.24	8.6	8.4
"	01	"	7	24	0.171	"	910	7.60	7.50	115	3.68	9.0	8.7
"	02	"	7	24	-	"	860	7.60	7.30	118	3.72	8.6	8.8
"	01	"	8	19	0.208	"	650	8.00	8.00	202	3.52	8.4	8.4
"	02	"	8	19	-	"	640	8.65	8.05	235	3.36	8.5	8.6
"	01	"	9	16	0.213	"	660	7.80	7.94	267	3.32	7.8	8.2
"	02	"	9	16	-	"	670	7.70	7.60	292	3.16	8.2	-
"	01	"	10	19	0.220	"	412	7.30	7.90	159	3.72	7.7	8.0
"	02	"	10	19	-	"	389	7.30	7.90	137	3.00	7.5	7.7
"	01	"	11	19	0.251	"	465	7.60	7.90	204	2.96	6.5	6.6
"	02	"	11	19	-	"	452	7.40	7.68	183	3.04	6.4	6.5
"	01	"	12	10	0.248	"	540	7.75	7.82	143	3.52	7.6	7.6
"	02	"	12	10	-	"	500	7.70	8.04	135	3.52	7.6	7.6

Table 4.1 Physical and chemical properties of water for stream 0097 during 1980. Concentrations of elements expressed in mg l^{-1}

K		Mg		Ca		Mn		Fe		Zn		Cd	
T	F	T	F	T	F	T	F	T	F	T	F	T	F
6.8	6.6	28.4	27.7	105.1	103.3	0.15	0.14	0.08	0.04	7.53	7.14	0.013	0.012
6.8	6.9	27.9	27.5	104.7	102.8	0.13	0.13	0.04	0.04	7.30	6.90	0.019	0.013
5.9	6.3	23.4	23.8	87.7	87.8	0.18	0.18	0.02	0.05	8.70	8.65	0.010	<0.010
5.4	5.6	23.3	23.4	86.9	86.8	0.19	0.20	0.06	0.04	8.96	8.72	0.017	0.014
6.4	6.4	26.8	27.1	100.5	100.7	0.16	0.17	0.02	0.03	6.10	6.40	0.010	0.010
6.4	6.5	27.1	27.1	100.5	100.3	0.11	0.10	0.02	0.06	6.10	6.32	0.014	0.018
6.9	6.9	29.2	28.5	108.4	105.5	0.16	0.13	0.04	0.08	6.46	6.45	0.011	0.026
6.8	6.7	28.6	28.6	105.8	105.9	0.14	0.11	0.07	0.04	6.45	6.08	0.017	0.010
7.0	7.0	29.0	29.2	107.2	105.5	0.14	0.13	0.04	0.03	7.47	7.35	0.016	0.014
7.0	7.1	29.9	29.4	106.0	107.2	0.13	0.14	0.04	0.04	7.38	7.14	0.012	0.012
7.4	7.0	26.5	26.5	98.2	98.3	0.14	0.14	0.30	0.07	6.31	6.25	0.014	<0.010
13.0	12.9	24.8	25.3	97.7	98.2	0.14	0.15	0.13	0.02	6.30	6.30	0.019	0.011
6.7	6.7	26.8	26.6	98.9	99.0	0.15	0.14	0.08	0.05	8.20	8.27	0.017	0.019
6.5	6.6	26.6	26.6	98.8	99.7	0.15	0.14	0.08	0.04	8.34	8.24	<0.010	0.024
6.2	6.1	25.2	25.1	95.3	94.5	0.12	0.12	0.09	0.04	6.93	6.85	0.014	0.014
6.1	6.3	25.1	25.0	94.2	94.2	0.09	0.08	0.21	0.04	7.35	6.70	<0.010	0.010
5.8	5.9	24.0	23.9	90.4	91.4	0.11	0.13	0.08	0.06	6.67	6.70	0.014	<0.010
5.6	-	23.7	-	91.0	-	0.11	-	0.10	-	6.90	-	0.019	-
5.8	5.9	23.3	23.1	89.7	89.2	0.12	0.13	0.05	0.04	6.22	6.22	0.010	0.020
5.9	5.9	23.2	22.8	88.8	88.0	0.13	0.13	0.12	0.06	6.30	6.04	0.012	0.012
5.1	5.1	20.3	20.3	78.9	79.9	0.13	0.13	0.04	0.02	5.60	5.25	<0.010	0.014
5.1	5.2	20.4	20.3	80.0	80.4	0.13	0.14	0.06	0.04	5.65	5.70	0.013	0.010
6.2	5.8	24.6	24.5	95.2	94.7	0.14	0.13	0.05	0.04	6.54	6.53	<0.010	0.013
6.1	6.1	24.4	24.3	93.7	93.9	0.12	0.12	0.07	0.03	6.50	6.52	0.010	0.022

Table 4.1 (continued)

Pb													
T	F	PO_4-P	NH_4-N	NO_2-N	NO_3-N	SO_4-S	Si	F	Cl	total CO_2-C	CO_2-C	HCO_3-C	CO_3-C
0.003	0.002	<0.01	<0.05	<0.005	0.129	72.0	7.40	0.82	7.0	44.1	1.12	42.8	0.17
0.002	0.002	"	"	"	0.141	68.7	3.15	1.35	12.2	40.6	1.33	39.1	0.14
0.001	0.013	"	"	"	0.179	63.7	7.05	0.94	5.9	33.7	1.21	32.4	0.09
0.006	0.005	"	"	"	0.184	64.0	2.50	1.02	8.5	33.6	0.69	32.7	0.17
0.001	0.012	"	"	"	0.177	74.0	6.40	0.80	6.25	41.2	0.65	40.3	0.27
0.001	0.005	"	"	"	0.191	70.3	3.45	0.80	6.40	39.7	1.01	38.5	0.17
0.002	0.002	"	"	"	0.131	75.3	2.70	0.77	6.20	44.4	0.92	43.2	0.24
0.003	0.002	"	"	"	0.110	81.7	3.15	0.78	5.80	43.9	0.92	42.7	0.23
0.001	0.001	0.015	"	"	0.110	70.0	4.10	0.72	5.80	45.2	0.94	44.1	0.24
0.003	0.001	0.013	"	"	0.137	74.7	4.50	0.78	7.7	46.6	1.19	45.2	0.20
0.009	0.004	<0.01	"	"	0.190	72.7	3.20	0.85	6.7	40.4	1.63	38.7	0.10
0.006	0.004	"	"	"	0.137	68.3	2.00	0.87	6.5	37.8	1.28	36.4	0.13
0.004	0.004	"	"	"	0.119	69.0	2.35	0.82	6.4	47.0	2.92	44.0	0.08
0.005	0.011	"	"	"	0.124	70.7	2.70	0.82	6.4	47.5	2.95	44.5	0.08
0.008	0.007	"	"	"	0.154	66.0	5.05	0.84	6.2	44.9	2.79	41.9	0.18
0.010	0.005	"	"	"	0.132	64.7	2.65	0.89	6.2	39.8	0.21	38.8	0.77
0.007	0.010	"	"	"	0.130	59.3	2.45	0.92	6.2	41.4	1.67	39.6	0.11
0.011	-	"	"	"	0.162	63.0	2.05	0.97	6.2	39.9	1.97	37.8	0.08
0.008	0.007	"	"	"	0.123	63.0	2.40	0.95	5.9	43.4	1.47	41.8	0.15
0.013	0.009	"	"	"	0.141	63.3	3.90	0.97	7.9	35.1	1.19	33.8	0.12
0.004	0.003	"	"	"	0.164	73.0	3.65	1.18	7.0	37.8	2.35	35.4	0.06
0.004	0.003	"	"	"	0.165	63.3	5.40	1.08	6.6	40.3	3.82	36.4	0.06
0.011	0.011	"	"	"	0.112	60.6	2.65	0.88	6.8	44.1	2.00	42.0	0.10
0.009	0.011	"	"	"	0.105	65.0	6.75	0.88	7.4	44.4	2.20	42.1	0.09

Table 4.1 (continued)

All phosphorus analyses given in Table 4.1 were carried out on water which had previously been frozen and re-thawed (Section 2.21). It is possible that this process may interfere with the anlaysis of trace amounts of phosphorus. For this reason, to coincide with the alkaline phosphatase assay carried out on 0097 algal material (Section 2.5), it was decided to perform a more detailed analysis of phosphorus at stream 0097. This analysis was performed on freshly collected water during August, 1980; details of the analytical technique are given in Section 2.21. Replicate water samples were collected (Section 2.61); results are presented in Table 4.2.

Table 4.2 Phosphorus analysis of 0097 water, August 1980 (values in $\mu g\ l^{-1}$)

sample	filtrable P	total P
1	< 0.6	24.8
2	"	21.7
3	"	25.1
4	"	27.5
5	"	27.5
$\bar{x}$		25.32
S.D.		2.39

Measurements of dissolved oxygen were made from January to May. Values for dissolved oxygen were always close to 100% saturation; however due to the lack of precision of the instrument available at the time, oxygen measurements were abandoned.

All optical density measurements were below the detection limit (0.005).

4.3 Metal content of algae

The results of analyses of algal material for metal content are given in

Table 4.3. In general there were no significant differences between distilled water washed and EDTA washed algae for any of the metals analyzed. Changes in metal content of algae from March - October 1980 are plotted in Fig. 4.1. The mean values for distilled water washed material only are given in Fig. 4.1.

During the period March - October marked changes occurred in the metal content of the Mougeotia. The pattern of change is not obviously correlated with the seasonal pattern of light input. It is assumed that some variation in water chemistry is responsible for the distributions plotted in Fig. 4.1.

Dried algal material analyzed by X-ray diffraction (Section 2.241) indicated the presence of the following minerals:

quartz - SiO_2

hydrozincite - $Zn_5(CO_3)_2(OH)_6$

zinc ferrate - $ZnFe_2O_4$

The X-ray diffraction scan for the dried algal material had a very high background reading. For this reason it is likely that only the major mineral components of the dried sample could be identified.

The X-ray fluorimetry scan (Section 2.242) for the dried algal material is given in Fig. 4.2. The presence of the following elements is indicated: Ca, Ti, Mn, Fe, Zn, Sr, Ba, Pb.

	wash	Na	Mg	K	Ca	Mn	Fe	Zn	Cd	Pb
MAR	H_2O	6.65 (1.32)	3.27 (0.18)	12.60 (0.68)	3.17 (0.10)	2.18 (0.14)	8.90 (0.40)	237.3 (13.8)	89 (4.0)	0.75 (0.05)
	EDTA	4.66 (0.45)	-	14.00 (0.94)	2.25 (0.17)	2.07 (0.25)	8.37 (0.73)	257.5 (17.1)	99 (8.9)	0.79 (0.09)
APR	H_2O	1.71 (0.25)	2.32 (0.13)	10.90 (1.10)	2.72 (0.06)	2.77 (0.11)	12.40 (0.57)	297.4 (11.5)	131 (3.6)	1.22 (0.04)
	EDTA	1.46 (0.25)	-	10.04 (0.65)	2.60 (0.05)	2.67 (0.05)	11.73 (0.46)	316.5 (12.9)	132 (3.6)	1.22 (0.03)
MAY	H_2O	4.66 (0.63)	2.40 (0.07)	7.59 (0.21)	3.30 (0.15)	6.68 (0.13)	23.35 (1.01)	281.1 (13.0)	122 (4.5)	2.45 (0.09)
	EDTA	4.48 (0.43)	-	7.47 (0.27)	3.20 (0.08)	6.71 (0.17)	22.68 (0.99)	282.9 (6.8)	124 (1.3)	2.55 (0.05)
JUN	H_2O	2.95 (0.44)	2.06 (0.13)	2.21 (0.42)	4.22 (0.11)	10.18 (0.65)	38.30 (1.55)	308.6 (6.1)	130 (4.0)	4.46 (0.12)
	EDTA	2.32 (0.49)	-	1.25 (0.07)	3.61 (0.07)	10.63 (1.12)	40.20 (4.49)	293.0 (10.0)	125 (4.6)	4.58 (0.23)
JUL	H_2O	5.38 (0.84)	4.68 (0.68)	15.03 (1.08)	2.75 (0.29)	2.66 (0.24)	11.20 (0.89)	143.7 (10.7)	54 (6.3)	1.13 (0.11)
	EDTA	4.38 (0.40)	-	13.63 (0.86)	2.13 (0.19)	2.66 (0.16)	9.67 (0.50)	139.5 (10.1)	51 (5.8)	0.96 (0.05)
AUG	H_2O	7.76 (1.35)	2.13 (0.45)	5.48 (1.02)	8.72 (1.16)	4.46 (0.44)	14.55 (1.14)	125.2 (8.0)	60 (5.5)	1.62 (0.20)
	EDTA	5.04 (1.24)	-	2.34 (0.25)	6.54 (0.92)	3.85 (0.74)	14.16 (2.70)	125.1 (13.4)	55 (8.5)	1.58 (0.34)
SEP	H_2O	4.92 (0.51)	2.88 (0.07)	13.40 (1.11)	2.66 (0.08)	3.68 (0.13)	16.10 (1.29)	155.2 (2.2)	61 (3.1)	1.53 (0.06)
	EDTA	3.35 (0.36)	-	11.20 (0.78)	1.77 (0.10)	3.62 (0.28)	13.40 (0.61)	143.1 (8.7)	62 (4.0)	1.67 (0.08)
OCT	H_2O	3.44 (0.64)	1.82 (0.03)	7.64 (0.55)	2.40 (0.05)	5.52 (0.10)	17.20 (0.52)	202.2 (3.3)	95 (5.0)	2.30 (0.03)
	EDTA	4.86 (0.57)	-	9.49 (0.80)	2.26 (0.05)	4.43 (0.17)	15.50 (0.56)	177.4 (6.2)	65 (4.0)	2.02 (0.05)

Table 4.3 Metals in 0097 Mougeotia during 1980. Concentrations as mg g^{-1} excepting Cd ($\mu g\ g^{-1}$). Values in brackets refer to the standard error of the mean

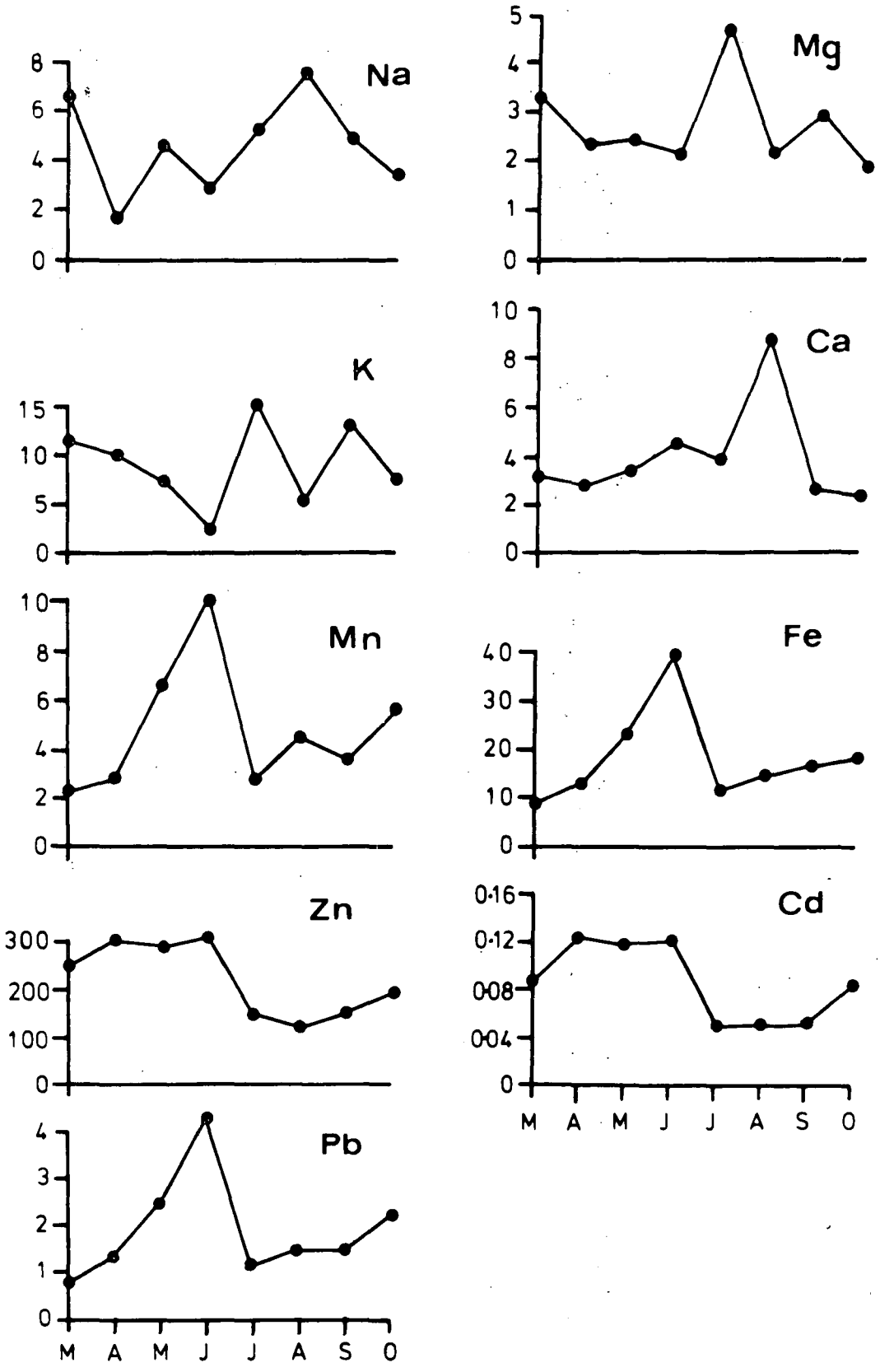
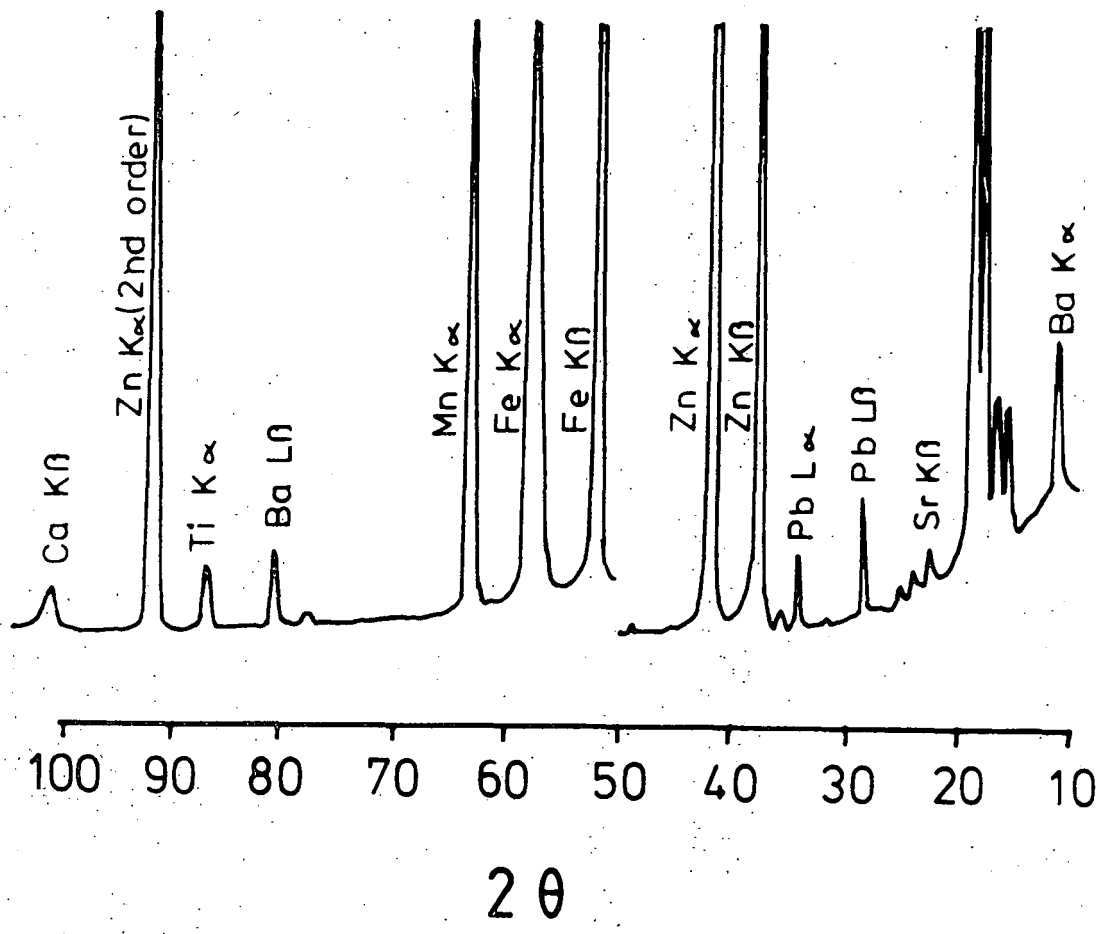


Fig. 4.1 Mean concentrations (mg g^{-1}) of accumulated metals in 0097 Mougeotia during 1980

Fig. 4.2 X-ray fluorescence scan of dried 0097 Mougeotia



4.4 Relationships between water and algal chemistry

Correlations of all water and algal chemical properties measured were made for the months March - October. r values were computed using the MIDAS statistical package (Fox & Guire, 1976). The important correlations noted are given in Table 4.4. All water chemistry values are for reach 02; all algal chemistry values are for the distilled water washed treatment.

For water chemistry Mg, Ca and SO_4-S form a positively cross-correlated group ($p < 0.01$).

For algal metal content there is a strong positive correlation between Mn, Fe and Pb ($p < 0.01$); there is also a positive correlation between algal

	pH.w	Mg.wf	Ca.wf	Zn.wf	SO_4.w	Si.w	Na.a	K.a	Mg.a	Ca.a	Mn.a	Fe.a	Zn.a	Cd.a	Pb.a	Si.wp
pH.w																
Mg.wf																
Ca.wf		+++														
Zn.wf																
SO_4.w		+++	+++													
Si.w																
Na.a																
K.a																
Mg.a				++				+								
Ca.a	++															
Mn.a								---								
Fe.a								--			+++					
Zn.a					+		-									
Cd.a							--						+++			
Pb.a								--			+++	+++				
Si.wp								-			+	++	++	++	++	

+++	+ve correlation	$p < 0.01$
++	"	$p < 0.05$
+	"	$p < 0.1$
---	-ve correlation	$p < 0.01$
--	"	$p < 0.05$
-	"	$p < 0.1$

w - water
f - filtrable (Nuclepore)
a - algae (digest)
wp - water (previous month)

Table 4.4 Intervariable correlation matrix for water chemistry and algal chemistry from eight monthly collections (March - October, 1980) on stream 0097

Zn and Cd ($p < 0.01$). Algal K shows a significant negative correlation with the Mn, Fe and Pb content of the algae ($p < 0.05$), similarly algal Na shows a negative correlation with accumulated Zn and Cd ($p < 0.01$).

Algal Ca is positively correlated with the stream pH ($p < 0.05$); algal Mg is positively correlated with filtrable Zn in the water ($p < 0.05$).

It was noted that when plotting monthly levels of filtrable reactive silicate, the shape of the curve was similar to that given for many of the accumulated metals in the algae. The curve was, however, a month out of phase, algal metal levels appearing to correspond a month later to levels of filtrable reactive silicate in the water. For this reason filtrable reactive silicate concentrations, obtained for the previous month to the collection of other parameters, were included in the correlation matrix. They proved to be positively correlated with the levels of accumulated Mn, Fe, Zn, Cd and Pb in the algae.

4.5 Stream sediment chemistry

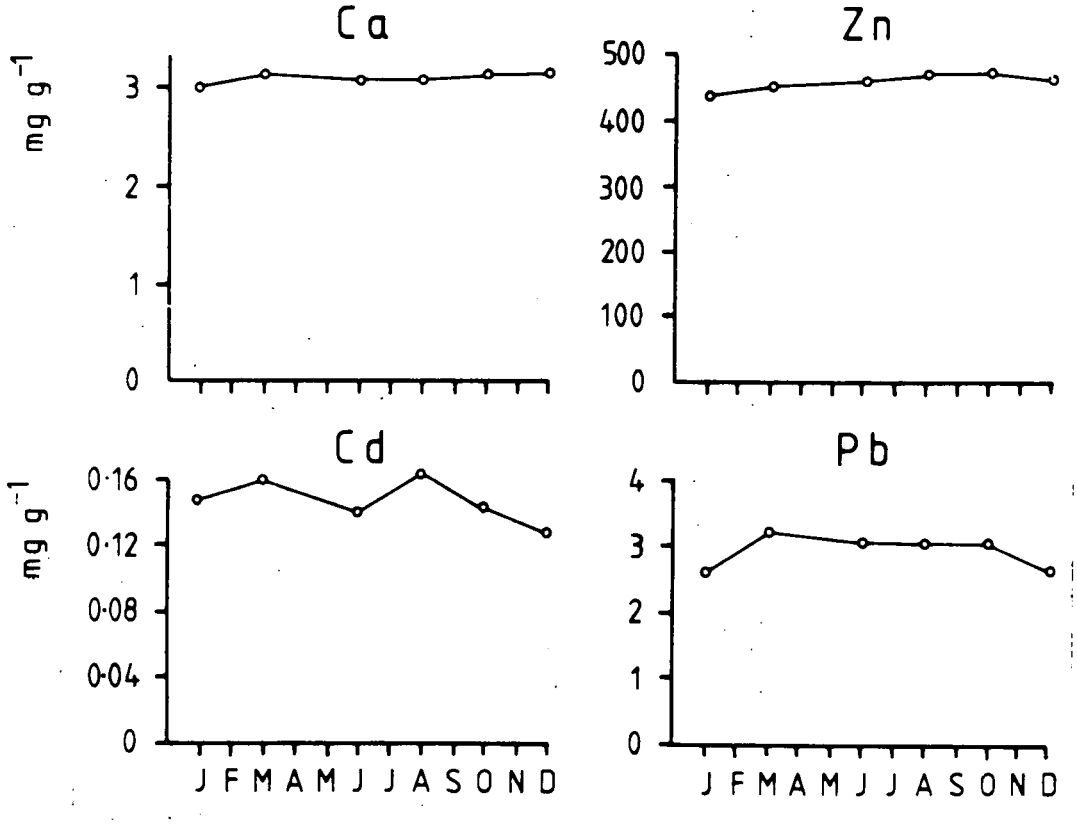
4.51 Metal content by digestion

The concentrations of Ca, Zn, Cd and Pb in 0097 sediments are given in Table 4.5 and presented graphically in Fig. 4.3 using data for reach 02. Sediment digestion and analysis was carried out as described in Section 2.22. The digestable metal component of reach 02 sediment is higher than that of reach 01 for Ca, Zn, Cd and Pb (Table 4.5). Metals in sediment remain at a relatively constant concentration throughout 1980 (Fig. 4.3); this does not correspond to the seasonal distribution of metals in the Mougeotia (Fig. 4.1).

Table 4.5 Metals (mg g^{-1}) in stream 0097 sediment during 1980. (Values in brackets refer to the standard error of the mean)

month	reach	Ca	Zn	Cd	Pb
Jan	01	2.06 (0.04)	380 (23)	0.098 (0.006)	2.30 (0.11)
	02	2.93 (0.12)	442 (14)	0.150 (0.007)	2.62 (0.12)
Mar	01	2.08 (0.07)	431 (13)	0.110 (0.003)	2.45 (0.09)
	02	2.74 (0.19)	450 (9)	0.160 (0.003)	3.26 (0.06)
Jun	01	2.13 (0.16)	406 (23)	0.120 (0.005)	2.50 (0.13)
	02	2.68 (0.09)	456 (8)	0.140 (0.005)	3.02 (0.94)
Aug	01	1.91 (0.14)	408 (22)	0.110 (0.009)	2.22 (0.19)
	02	2.71 (0.07)	471 (11)	0.163 (0.010)	2.98 (0.17)
Oct	01	2.00 (0.09)	400 (9)	0.110 (0.006)	2.33 (0.17)
	02	2.82 (0.02)	477 (17)	0.144 (0.003)	2.96 (0.08)
Dec	01	2.00 (0.04)	371 (13)	0.090 (0.003)	2.29 (0.08)
	02	2.89 (0.08)	465 (17)	0.127 (0.006)	2.60 (0.16)

Fig. 4.3 Changes in metal content of 0097 sediment during 1980



4.52 Total organic content

Values for sediment organic content are given in Table 4.6; details of the analytical method used, are described in Section 2.23. Reach 02 generally has a higher organic component than reach 01 sediment. It was noted that throughout stream 0097 there were many fragments of wood in the sediment. These occurred in stream sediment before it emerged from the adit and it would seem likely that they derive from mine construction material.

Table 4.6 Organic content (%) of sediments from stream 0097 in 1980

reach	month	mean % organic content*	S.E.
01	Jan	15.8	0.75
02	"	21.8	1.22
01	Mar	15.7	0.80
02	"	18.0	0.38
01	Jun	12.5	1.57
02	"	16.8	0.27

* method for estimation given in Section 2.23

4.53 X-ray analysis

X-ray diffraction analyses of sediment material from reaches 01 and 02 each gave very similar results. Sediments were made up predominantly of the minerals quartz (SiO_2), hydrozincite ($Zn_5(CO_3)_2(OH)_6$) and iron oxides (ɣFe.OH).

X-ray fluorescence analysis charts for reaches 01 and 02 are given in Fig. 4.4. Both traces are very similar, the detectable elements in both sediment samples being Ca, Ti, Mn, Fe, Zn, Sr, Zr, Ba and Pb.

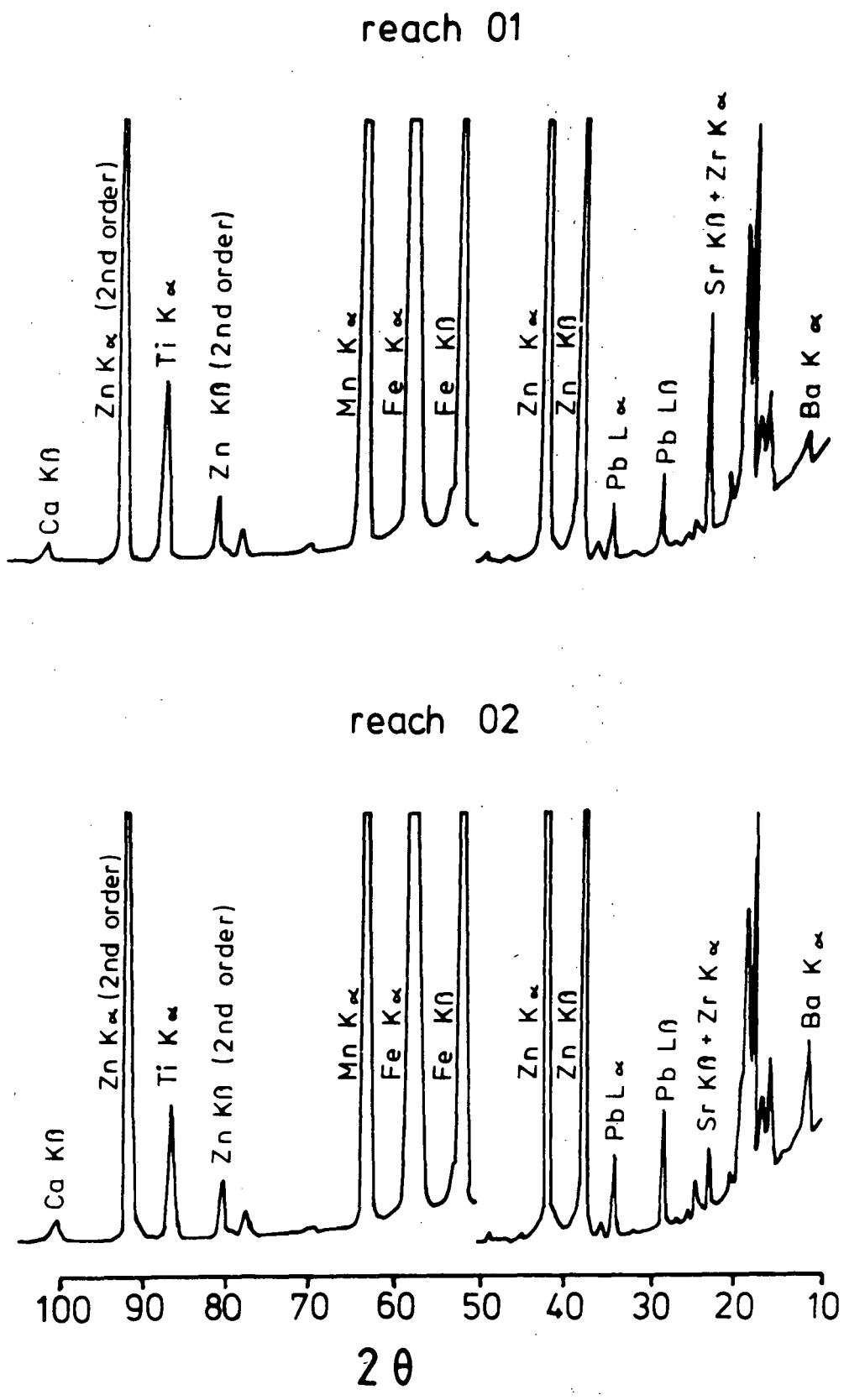


Fig. 4.4 X-ray fluorescence scans of dried 0097 sediment

4.6 Species composition

A summary of the plant species present in Caplecleugh Low Level is given in Table 4.7. Estimates of relative abundances of the species present are also given in Table 4.7.

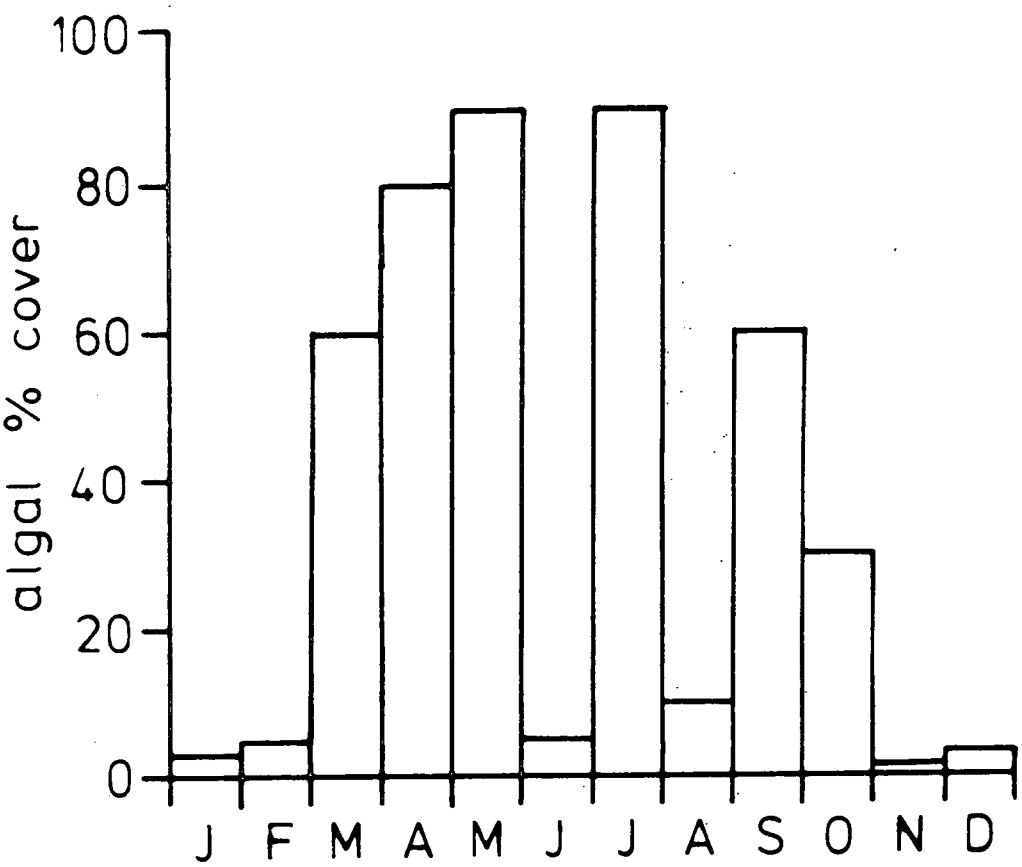
The two Mougeotia spp. were the dominant stream organisms forming conspicuous growths covering the stream bed at certain times of the year (see frontispiece). Sexual stages of the Mougeotia spp. life history could not be either found in the field or induced in the laboratory (Section 2.82); for this reason identification to the species level was impossible for these two algal strains.

Although animals were not included in the present study, it may be helpful to summarize the data of Armstrong (1979) who examined the fauna of Caplecleugh Low Level. He found only two taxa to be present in the stream (Orthocladiinae and lumbricid worms) at a very low density.

4.7 Estimation of variation in algal biomass

Monthly estimates of Mougeotia percentage cover are given in Fig. 4.5. The cover of Mougeotia was generally much greater in summer than in winter though there are two marked drops in percentage cover during summer (June and August). The single direct measurement of algal standing crop (Section 2.4) gave a value of 200 g dry weight m^{-2}. This measure was made in July, corresponding to a Mougeotia percentage cover of 90% (Fig. 4.5).

Fig. 4.5 Changes in percentage cover of Mougeotia in Stream 0097 during 1980.



4.8 Alkaline phosphatase activity of algae

The enzyme assay was performed as described in Section 2.5 using five replicate samples of mixed Mougeotia material. Results are given in Table 4.8.

Table 4.7 Composition and relative abundance of taxa in stream 0097. Data based on records for all 1980, relative abundance on scale of 1-5 (see Section 2.3)

plants	Durham code no.	D.o.E. code no.*	relative abundance reach 01	02	01+02
BLUE-GREEN ALGAE					
Lyngbya sp. (1.2-2.0 μm)	014232	012590	1	1	1
Phormidium sp. (1.0-1.5 μm)	015732	013390	1	1	1
Pseudanabaena catenata Lauterborn	016101	013601	3	3	3
DIATOMS					
Achnanthes minutissima Kütz	100171	120114	1	2	1
Amphora veneta Kütz	100269	120403	3	3	3
Pinnularia subcapitata Gregory	102073	123300	2	1	2
Surirella ovata Kütz	102370	123804	0	1	1
GREEN ALGAE					
Hormidium rivulare Kütz	152902	171903	2	1	2
Mougeotia sp. (5-6 μm)	121451	211592	4	4	4
Mougeotia sp. (8-10 μm)	121452	211593	5	5	5
BRYOPHYTES					
Bryum pallens Sw.	231005	321211	4	0	3
Dicranella varia (Hedw.) Schimp.	232106	322608	1	1	1
ANGIOSPERMS					
Deschampsia caespitosa (L.) Beauv.	-	381801	4	0	3

* see Section 2.12

Table 4.8 Alkaline phosphatase activity of 0097 Mougeotia at 32°C, collected during August 1980

pH	enzyme activity (μM p-nitrophenol h^{-1} μg chl a)
8.6	1.03×10^{-2}
9.6	1.74×10^{-2}
10.6	1.86×10^{-2}
11.0	1.77×10^{-2}
11.6	0.77×10^{-2}

Alkaline phosphatase activity was measured using algal material collected on the same day as the water samples which were analyzed for total and filtrable reactive phosphorus (Section 4.2).

5 OBSERVATIONS MADE AT STREAM 3026 - LE CROUZET UPPER SLOPE SEEPAGE

Stream 3026 was originally discovered in 1978 by Say and Whitton (1982); a background description of the site is given in Section 3.2.

Levels of heavy metals in stream 3026 are extremely high (see Table 5.1); there is no evidence in the literature of photosynthetic organisms being recorded at higher concentrations of Zn and Cd (see Section 1.5).

Table 5.1 Physical and chemical properties of water measured at stream 3026. (Elements are in mg l^{-1})

collected July 1978

temperature (^{o}C) 22.0

pH (field) 5.00

metals	total	filtrable (Nuclepore)
Na	71.3	64.4
K	85.0	74.5
Mg	445	500
Ca	163.2	163.4
Al	68.0	0.03
Mn	325	294
Fe	3.37	0.07
Ni	252.0	240.0
Co	3.64	3.36
Cu	5.00	1.85
Zn	3840	3610
Ag	0.015	0.025
Cd	345	300
Pb	0.68	0.32

Due to the difficulty of access the amount of data collected at stream 3026 is limited (Section 2.62); however due to the extreme values of heavy metals found at the site it was felt that algae isolated from stream 3026

would prove worthy of further laboratory investigation. Aspects of the ability of these organisms to survive such extreme metal pollution were investigated in laboratory culture; details of experiments using axenic algal strains from stream 3026 are given in Chapter 6.

This chapter summarises the data collected at stream 3026; they may be important when considering the performance of isolated algal strains in culture.

The physical and chemical properties of the water in stream 3026 are given in Table 5.1. Water was not collected for anion chemistry; however P.J. Say (pers. comm.) was able to supply analyses for the Ruisseau de Creuzet of which stream 3026 is a tributary.

The following anion concentrations were found in the Ruisseau de Creuzet; n = 6.

SO_4-S	1492 mg l^{-1}
Si	12.6 mg l^{-1}
Cl	0.52 mg l^{-1}
NH_4-N	1.84 mg l^{-1}
PO_4-P	1.96 µg l^{-1}

3026 sediment was analyzed by X-ray diffraction and X-ray fluorescence (Section 2.24).

X-ray diffraction analysis identified the following minerals in general order of abundance

1	quartz	SiO_2
2	zinc ferrate	$ZnFe_2O_4$
3	plagioclase feldspar	
4	beudanite	$PbFe_3(AsO_4)(SO_4)(OH)_6$
5	illite and kaolinite	
6	cadmium sulphate	$CdSO_4$

X-ray fluorescence analysis of 3026 sediment indicated the presence of the elements Fe, Zn, As, Rb, Sr, Sn, Ba and Pb in the sediment.

Total stream flora and estimates of relative abundance of species made on fresh field material are given in Table 5.2. The algae formed a conspicuous cover at the site (see Fig. 3.5).

Table 5.2 Flora of stream 3026

plants	Durham code no.	estimate of abundance
Hormidium rivulare Kütz	152902	5
Hormidium fluitans A. Braun	152904	2
Pinnularia subcapitata Gregory	102073	2

Culture studies on algal material from stream 3026 revealed a richer flora for the site than is given in Table 5.2. Details are given in Section 2.82.

6 LABORATORY STUDIES ON ALGAL STRAINS ISOLATED FROM STREAMS 0097 AND 3026

6.1 Introduction

Chapters 4 and 5 describe the ecology of two highly Zn-polluted streams in which green algal species are the dominant organisms present. The overall aim of the present study was to examine aspects of the biology of these algae, in relation to the extreme environment in which they were growing (Section 1.6). This chapter summarises the results of laboratory studies on green algal strains isolated from streams 0097 and 3026. The aims of the culture studies were:

a) to assess the toxicity of Zn to these algal strains in conditions which are controlled and yet resemble the field;

b) to investigate the role of other environmental factors which may prove important when assessing the effects of Zn;

c) to consider the accumulation of Zn in the laboratory in relation to accumulation in the field.

Particular emphasis was placed on relating experimental conditions to those found in the field; this allows direct comparisons of field and laboratory data.

6.2 Preliminary observations

With the exception of the two Mougeotia strains (535, 536) all axenic algal strains (Section 2.81) showed satisfactory growth in liquid Chu 10 E medium buffered, at pH 7.5 for stream 0097 algae, and pH 5.0 for stream 3026 algae (Section 2.74). Strains 535 and 536 grew satisfactorily on solid Chu 10 E medium (+ agar; Section 2.82) but would not grow when inoculated into liquid Chu 10 E medium. Batches of media were supplemented with suspected additional growth requirements including Ba, Ni, Al, vit. B_{12} and soil extract. Satisfactory growth of both 535 and 536 was found to

occur only in media supplemented with vit. B_{12} or soil extract. It is apparent that both Mougeotia strains have a requirement for vit. B_{12}, which was a constituent of the soil extract and also probably occured as a contaminant in the agar. 1 nM vit. B_{12} was routinely included in all media in which these two strains were grown (Section 2.741).

Due to the constancy of the water in stream 0097 (Section 4.2) it was envisaged that stream water could be used as the culture medium for stream 0097 algae. This would aid correlations between laboratory experiments and field analyses.

10 litres of water were collected from stream 0097 during August 1981 and a limited analysis of the water was performed (Section 2.21):

pH	7.80
temperature	9.5°C
Zn-t	8.50 mg l^{-1}
Zn-f	8.30 mg l^{-1}

this water was passed through a Sinta glass filter (Section 2.21) before being stored at 4°C in the dark. Aliquots of this stock were used throughout the present study for all laboratory experiments where 0097 water was included as a component of the growth media.

Growth of strain 536 was tested in batch culture using stream water, buffered at pH 7.5, as the growth medium. No growth was found to occur. Nevertheless, this strain had been found to grow well, in Chu 10 E + B_{12} medium, also in batch culture. Comparison of the chemistry of Chu 10 E (Section 2.741) and stream 0097 (Section 4.2) indicates that 0097 water is particularly low in nitrogen and phosphorus (and possibly vit. B_{12}). 10 ml aliquots of buffered stream water were placed in 50-ml boiling tubes and supplemented with various combinations of N, P and vit. B_{12} at the

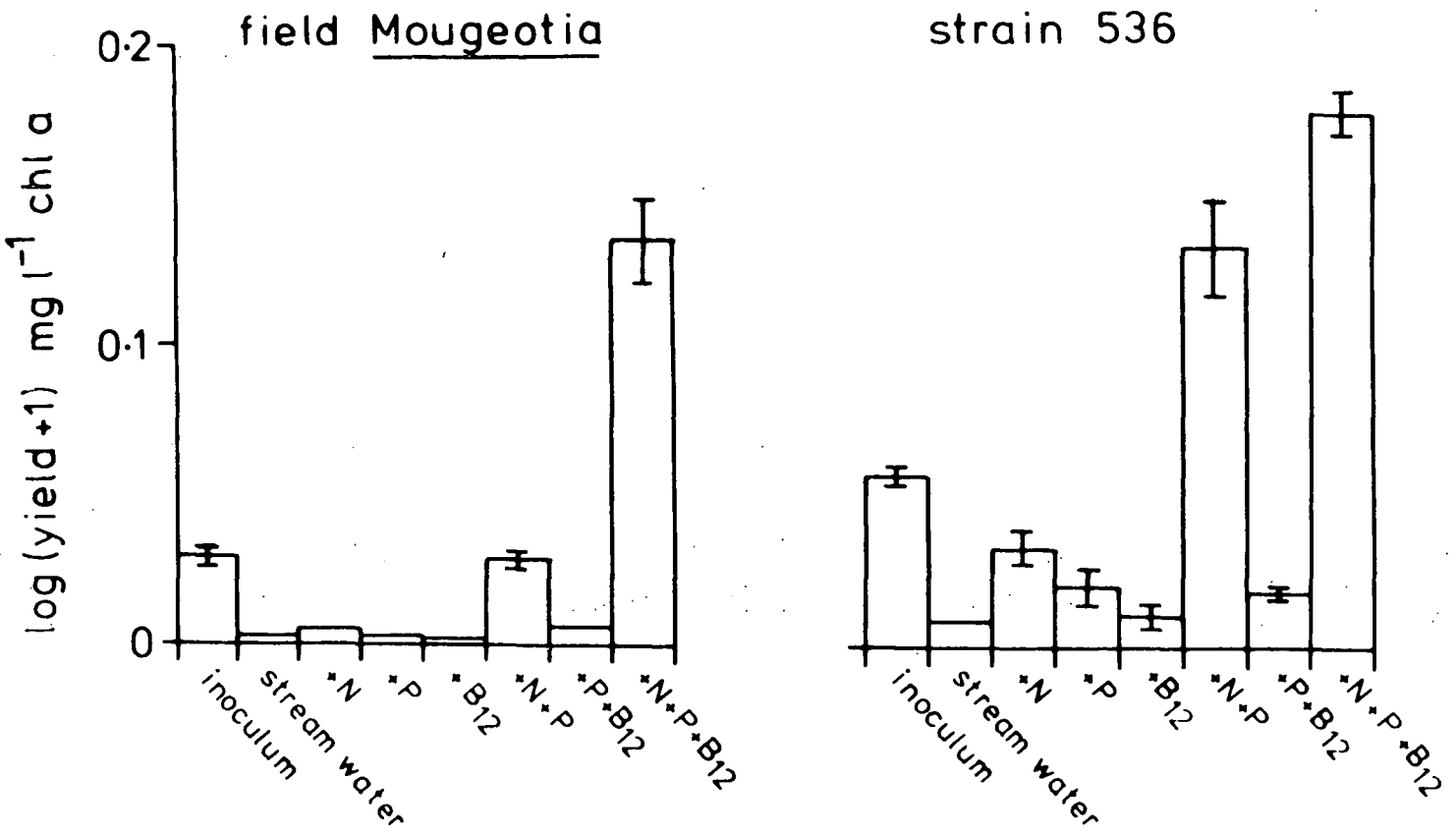


Fig. 6.1 Growth of 0097 Mougeotia in stream water and supplemented stream water; error bars indicate the standard deviation of the mean; n = 4

following concentrations:

N 6.83 mg l^{-1} as $NaNO_3$

P 100 mg l^{-1} as NaH_2PO_4

vit. B_{12} 1 nM.

The combinations tested are given in Fig. 6.1. Each series of treatments was tested (with four replicates) for both strain 536 and field Mougeotia. Strain 536 had been previously grown in Chu 10 E + B_{12}; the mixed Mougeotia sample had been transported, on ice, directly from stream 0097. A standard sonicated inoculum of both was used (Section 2.922).

Chl a was measured (Section 2.925) after a six-day incubation period (Section 2.76). Results are given in Fig. 6.1: growth is expressed as log (yield + 1) mg l^{-1} chl a; this value is shown to be directly related to algal growth rate (Section 6.32). Where error bars are not given (Fig. 6.1), replicates were pooled to give a measurable chl a value. Inocula sizes are also given in Fig. 6.1, though it is unlikely that these inocula are totally viable after sonication (for explanation see Section 6.32).

Maximum yield for both strain 536 and field Mougeotia occurred in stream water plus N, P and vit. B_{12}. This combination was used subsequently throughout the study as Caplecleugh Field Medium (CFM; see Section 2.741). Growth of both 536 and field Mougeotia is similar in CFM (Fig. 6.1). Growth is also similar in all other combinations of treatments except for the N + P treatment where growth of strain 536 is much greater than that of field Mougeotia. This is probably due to carryover of vit. B_{12} in the 536 inoculum (previously grown in Chu 10 E + B_{12}), sufficient to meet algal requirements.

The use of CFM made it possible to represent closely field chemical conditions for 0097 algae. There was no facility readily available, however, whereby

algal cultures could be incubated both illuminated and at the field temperature (9.5°C; Section 4.2). The temperature response of Mougeotia strain 536 was tested using a temperature gradient (Section 2.76).

100-ml conical flasks were positioned to give a range of temperatures from 15°C - 25°C. Five replicates were placed at each of six temperatures, including the two extremes. The flasks contained 25 ml of Chu 10 E + B_{12} medium (pH 7.5) and were inoculated with a standard sonicated inoculum of strain 536 (Section 2.922). The algae were harvested after six days incubation and chl a concentrations were estimated (Section 2.925). The effect of temperature on the growth of strain 536 is given in Fig. 6.2; the optimum growth temperature of strain 536 is shown to be close to 23°C. Extrapolation of the curve to 9.5°C (field temperature) suggests a very slow growth rate for strain 536 at this temperature.

During the present study it was intended to grow isolated algal strains at the field pH. To check the validity of this, the influence of pH on the growth of these strains was tested in the laboratory.

Cultures were incubated in 25 ml of Chu 10 E contained in 100-ml conical flasks (Section 2.76). Growth media were buffered as described in Section 2.742. Strains tested for the effect of medium pH on growth were 532, 533, 536, 537, 539, 541, 545 and 546 (Table 2.8). They were inoculated as described in Section 2.91; treatments were not replicated. Results of these pH/growth assays are presented in Fig. 6.3.

Strain 536 showed increasing growth above pH 5.0; pH 7.5 was chosen for quantitative work on this strain. The mean field pH is slightly above pH 7.5 for this alga (Section 4.2); problems of media precipitation, however, increased markedly above pH 7.5.

The remaining algal strains isolated from stream 3026 all grew at pH 5.0 (field pH). Growth of the two Chlamydomonas strains, 533 and 541, showed

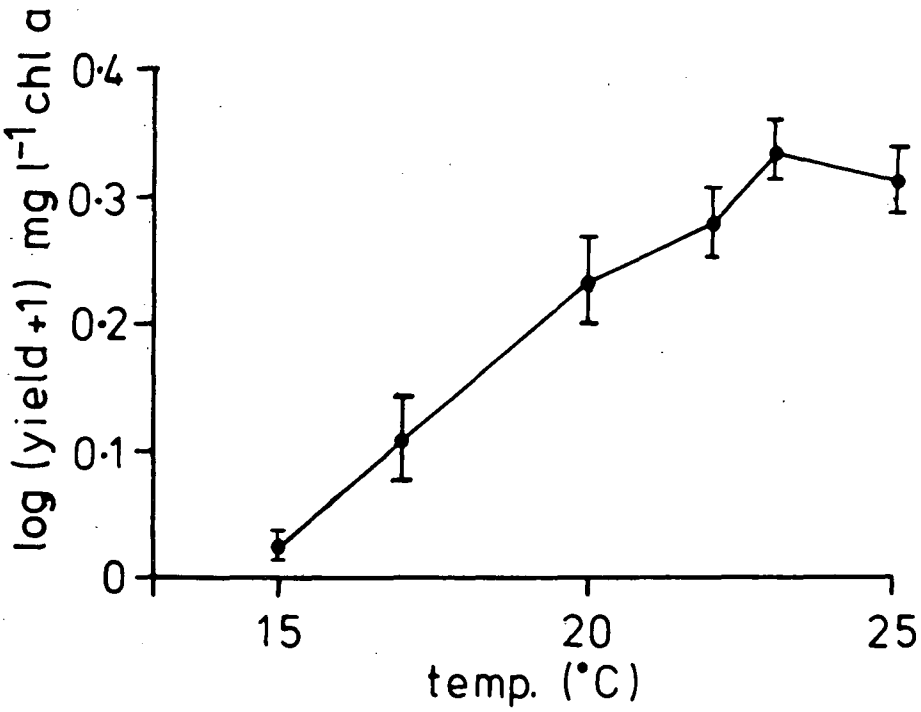


Fig. 6.2 Effect of temperature on growth of strain 536 in Chu 10 E medium; error bars indicate the standard error of the mean; n = 4

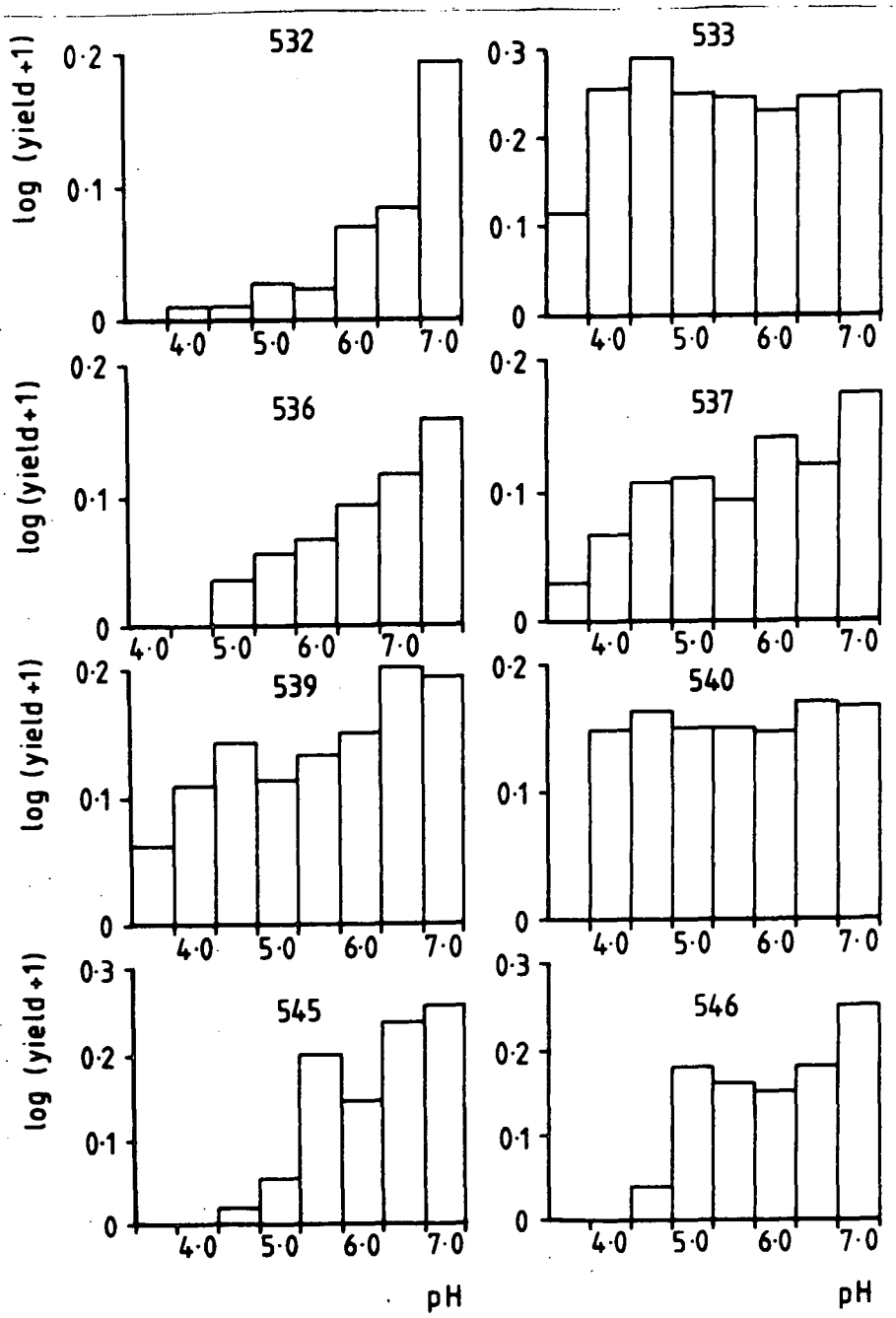


Fig. 6.3 Influence of pH on growth of strains isolated from streams 0097 and 3026

little change above pH 4.0. The remaining 3026 strains showed increased growth in higher pH media (above pH 5.0); the field pH, however, was used for further quantitative work with these strains.

6.3 Zinc toxicity

6.31 Preliminary assay

Assays were performed following the routine described in Section 2.91. Strains isolated from stream 0097 were assayed in Chu 10 E medium buffered at pH 7.5 + 1 nM vit. B_{12}; the remaining strains, isolated from stream 3026, were assayed in Chu 10 E medium buffered at pH 5.0. Table 6.1 gives the biomass estimates (Section 2.91) for all algal strains isolated from streams 0097 and 3026 (Table 2.8); Zn concentrations range from zero to lethal for each strain tested.

Strains 536 and 537, which represent the dominant algae at their respective field sites (streams 0097, 3026), were chosen for more detailed study. No changes of Zn tolerance were observed for these two strains throughout the period of the study.

6.32 Effect of zinc on growth

Three strains (536, 537, 545; Table 2.8) were chosen for more detailed study of the influence of Zn on growth. The reasons for choosing strains 536 and 537 are outlined in Section 6.31; strain 545 was chosen because, as a unicellular alga, analysis of growth parameters was simple and rapid.

Strain 536. The effect of Zn on growth was investigated in two media, Chu 10 E and CFM (Section 2.74). Both media were buffered at pH 7.5 and 1 nM vit. B_{12} was added. The experiment was performed in boiling tubes, each containing 10 ml of growth medium. Zn as $ZnSO_4$ was added to each tube from a highly concentrated stock so as not to alter significantly the overall mineral concentrations of the media. Twenty one replicates

Table 6.1 Preliminary assay of Zn tolerance of algal strains isolated from streams 0097 and 3026

	532	533	535	536	537	539	540	541	545	546
0	5	5	5	5	5	5	5	5	5	5
1		5	5	5			5	5		
5	5	5	5	5			5	5	5	5
10	5	4	4	4	5	5	4		5	5
15				4			3			5
20		4	4	3			2	5		
25	5		2	1	5	5	1		5	5
30							0	5		
40		3	0	0				4		
50	4	2			4	4		4	4	5
75		1						2		5
100	4	1			4	4		1	2	4
150		0						0	1	
250	4				4	4			0	3
500	3				3	3				1
750	2				2	2				0
1000	2				2	1				
2000	2				1	1				
3000	1				1	0				
5000	0				0	0				

n_1^{-1})

were prepared for each Zn concentration and medium. One replicate was set aside, while the remainder were inoculated with a standard sonicated inoculum of strain 536. Tubes were then incubated in the 20°C growth tank (Section 2.76). At intervals of 1, 3, 5, 7 and 9 days, four tubes of each treatment were removed, and chl a measured.

Precipitation was seen to occur in these high pH media; the replicate tube, which was not inoculated with algae, was analyzed for soluble Zn at the end of the experiment. Samples of medium were filtered through G.F.C. glass fibre filter paper and the filtrable component was analyzed for Zn, by atomic absorption spectroscopy (Section 2.21). The differences between total and filtrable Zn concentrations for both Chu 10 E and CFM at pH 7.5 are given in Table 6.2.

Table 6.2 Zinc precipitation in pH 7.5 media; values in mg l^{-1}

Chu 10 E medium		CFM	
total zinc	filtrable zinc	total zinc	filtrable zinc
0	0.1	8.5	1.5
5	5.2	15	2.1
10	9.8	25	2.7
15	13.9	40	4.5
20	19.7	100	15.7
25	25.3	200	65.4
40	39.4		

The effects of varying Zn concentration on the growth of strain 536 are given in Fig. 6.4 (Chu 10 E medium) and Fig. 6.5 (CFM); due to the precipitation in CFM, growth is related to filtrable and not total Zn (Table 6.2). Values, together with their standard deviations (n = 4), are given fully in Tables 6.3 (Chu 10 E medium) and 6.4 (CFM).

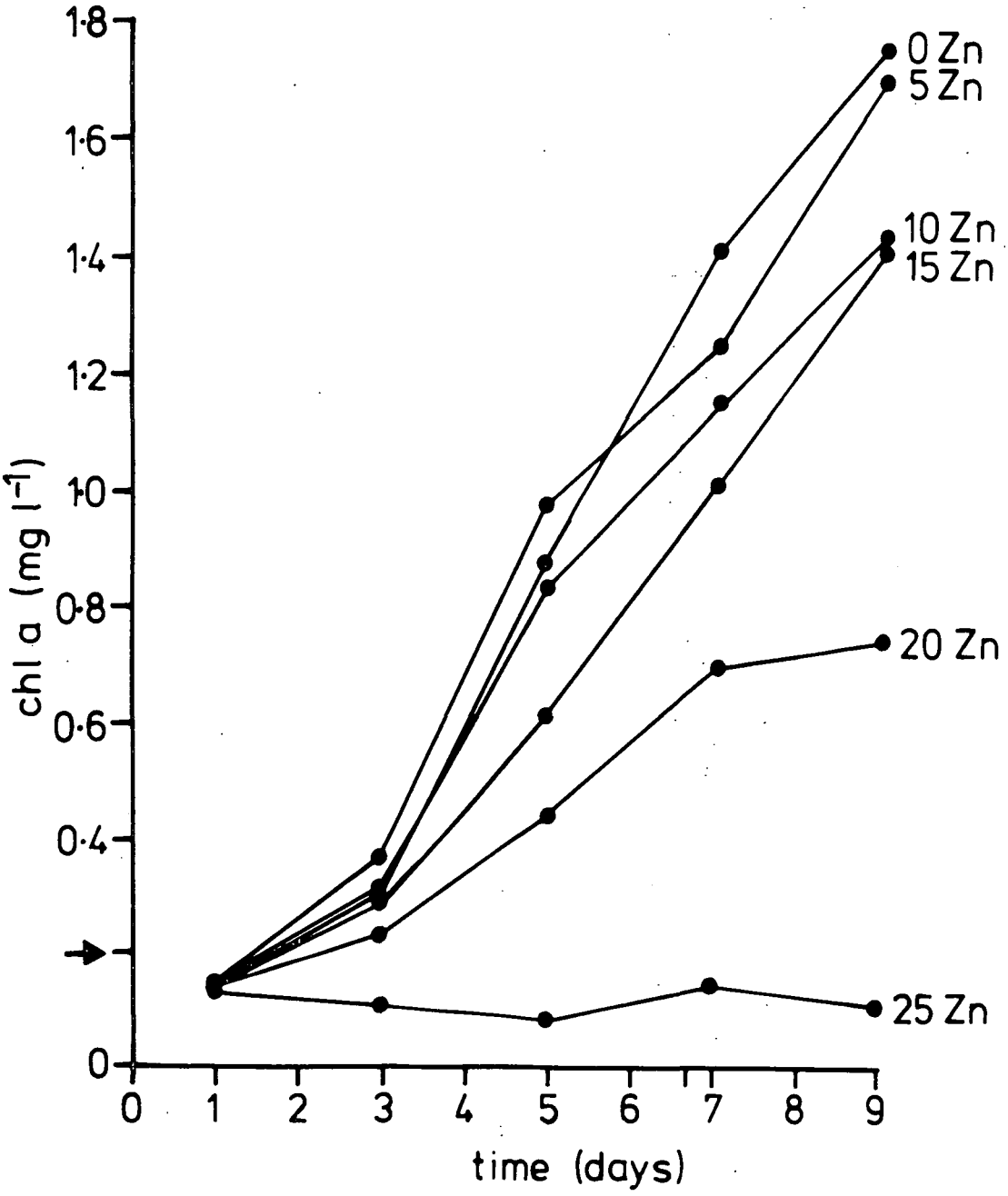


Fig. 6.4 Effect of Zn (mg l^{-1}) on growth of strain 536 in Chu 10 E (pH 7.5); arrow indicates concentration of inoculum

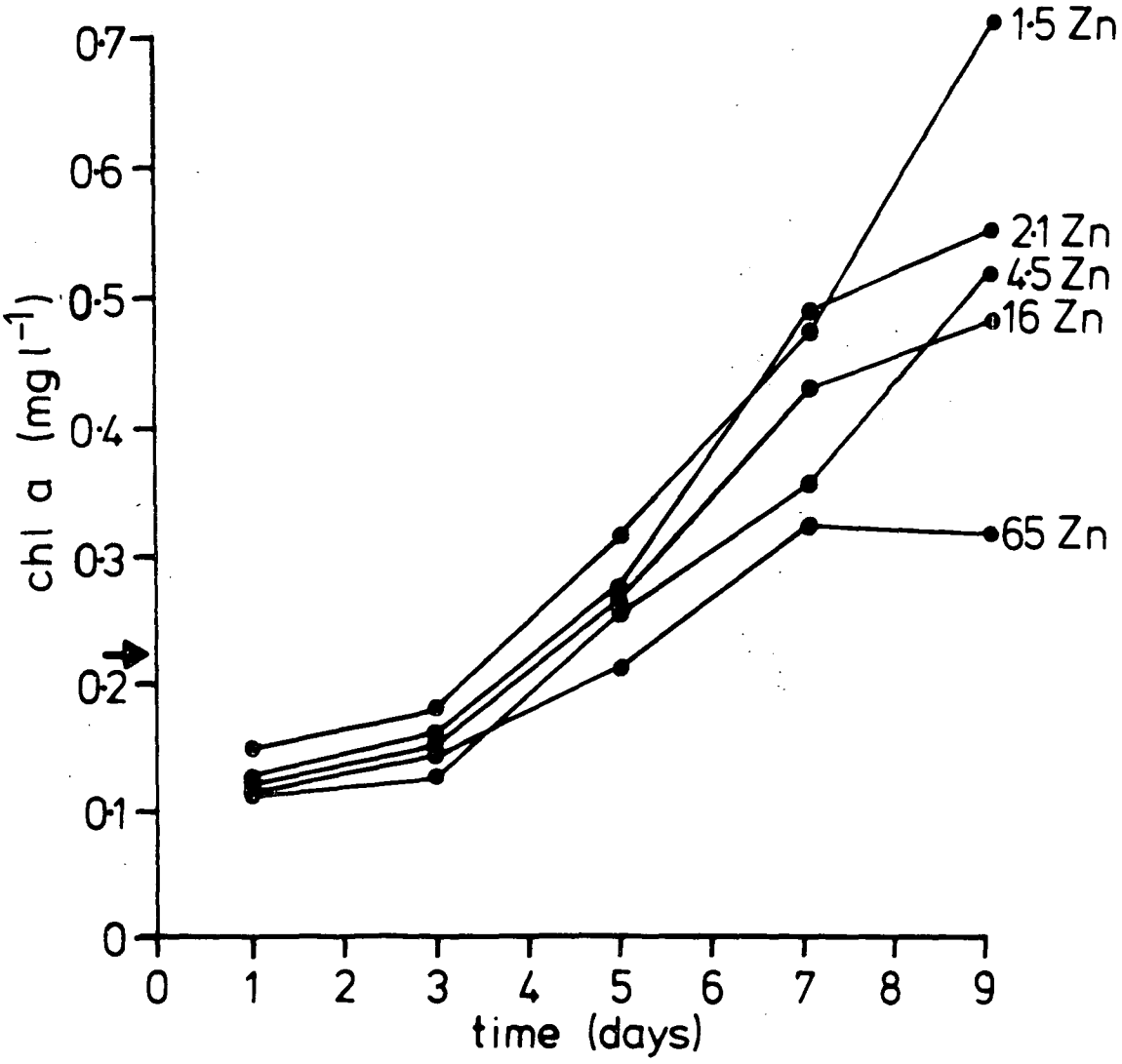


Fig. 6.5 Effect of Zn (mg l^{-1}) on growth of strain 536 in CFM (pH 7.5); arrow indicates concentration of inoculum

[Zn] (mg l^{-1})	time (days) 1	3	5	7	9
0	0.152(0.012)	0.301(0.081)	0.882(0.093)	1.422(0.108)	1.762(0.364)
5	0.163(0.009)	0.385(0.067)	0.982(0.054)	1.248(0.033)	1.700(0.186)
10	0.170(0.021)	0.322(0.043)	0.839(0.051)	1.159(0.149)	1.414(0.141)
15	0.165(0.021)	0.316(0.023)	0.626(0.083)	1.022(0.059)	1.414(0.059)
20	0.168(0.020)	0.245(0.030)	0.450(0.061)	0.702(0.090)	0.748(0.061)
25	0.171(0.014)	0.135(0.030)	0.086(0.048)	0.169(0.096)	0.120(0.051)
40	0.166(0.018)	0.084(0.032)	0.015(0.003)	0.049(0.018)	0.053(0.019)

Table 6.3 Effect of Zn on growth of strain 536 in Chu 10 E medium at pH 7.5; values (mg l^{-1} chl *a*) are the mean of four replicates; figures in parentheses are the standard deviation of the mean; inoculum = 0.20 mg l^{-1} chl *a*

[Zn] (mg l^{-1})	time (days)				
	1	3	5	7	9
1.5	0.150(0.028)	0.187(0.042)	0.317(0.070)	0.474(0.057)	0.711(0.072)
2.1	0.115(0.018)	0.158(0.018)	0.264(0.008)	0.494(0.088)	0.547(0.06)
4.5	0.113(0.007)	0.132(0.008)	0.259(0.034)	0.357(0.071)	0.514(0.055)
15.7	0.124(0.024)	0.166(0.022)	0.275(0.018)	0.430(0.056)	0.478(0.044)
65.4	0.121(0.021)	0.153(0.016)	0.217(0.017)	0.325(0.053)	0.312(0.096)

Table 6.4 Effect of Zn on growth of strain 536 in CFM at pH 7.5; values (mg l^{-1} chl *a*) are the mean of four replicates; figures in parentheses are the standard deviation of the mean; inoculum = 0.20 mg l^{-1} chl *a*

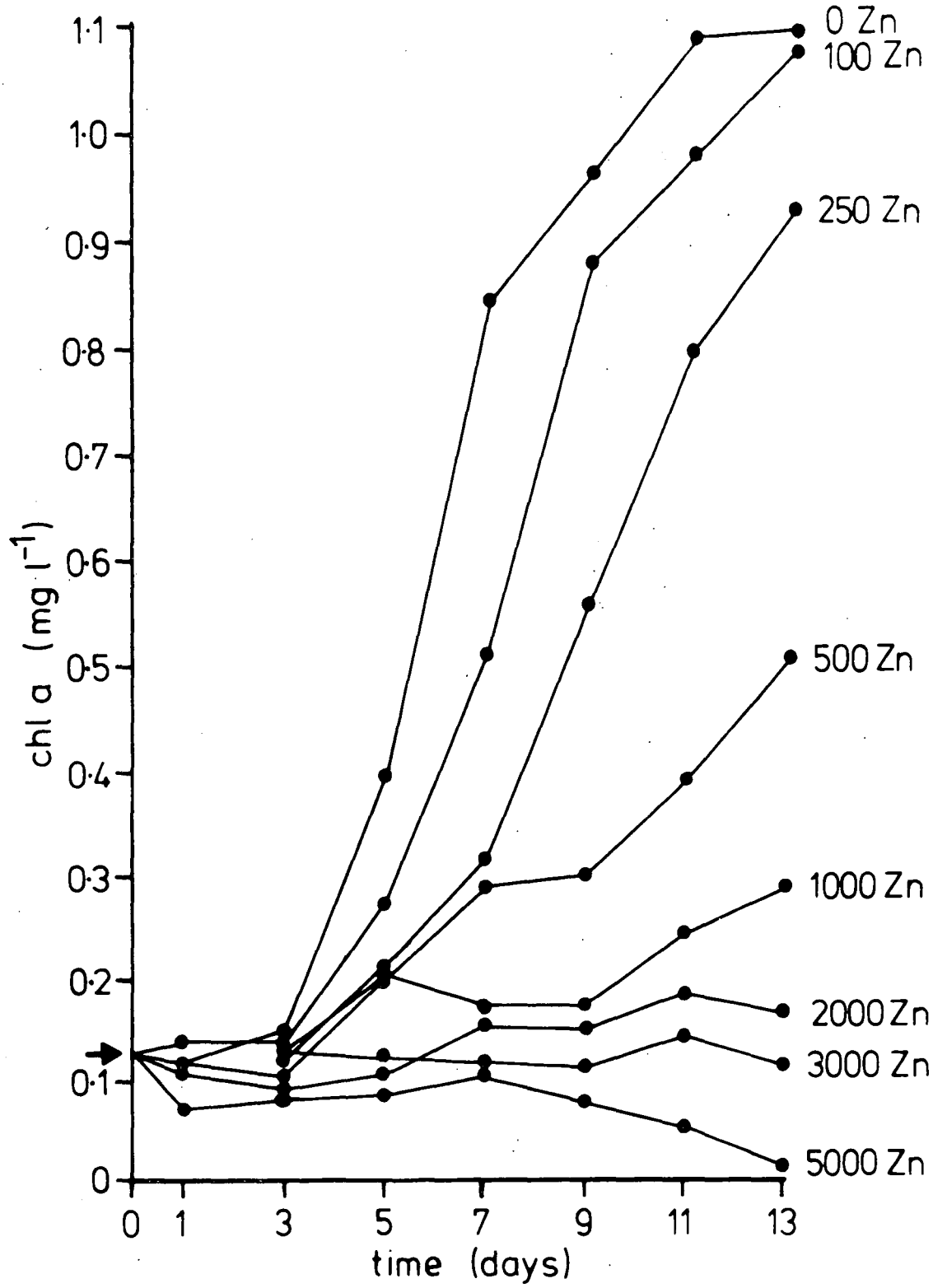


Fig. 6.6 Effect of Zn (mg l^{-1}) on growth of strain 537 in Chu 10 E (pH 5.0); arrow indicates concentration of inoculum

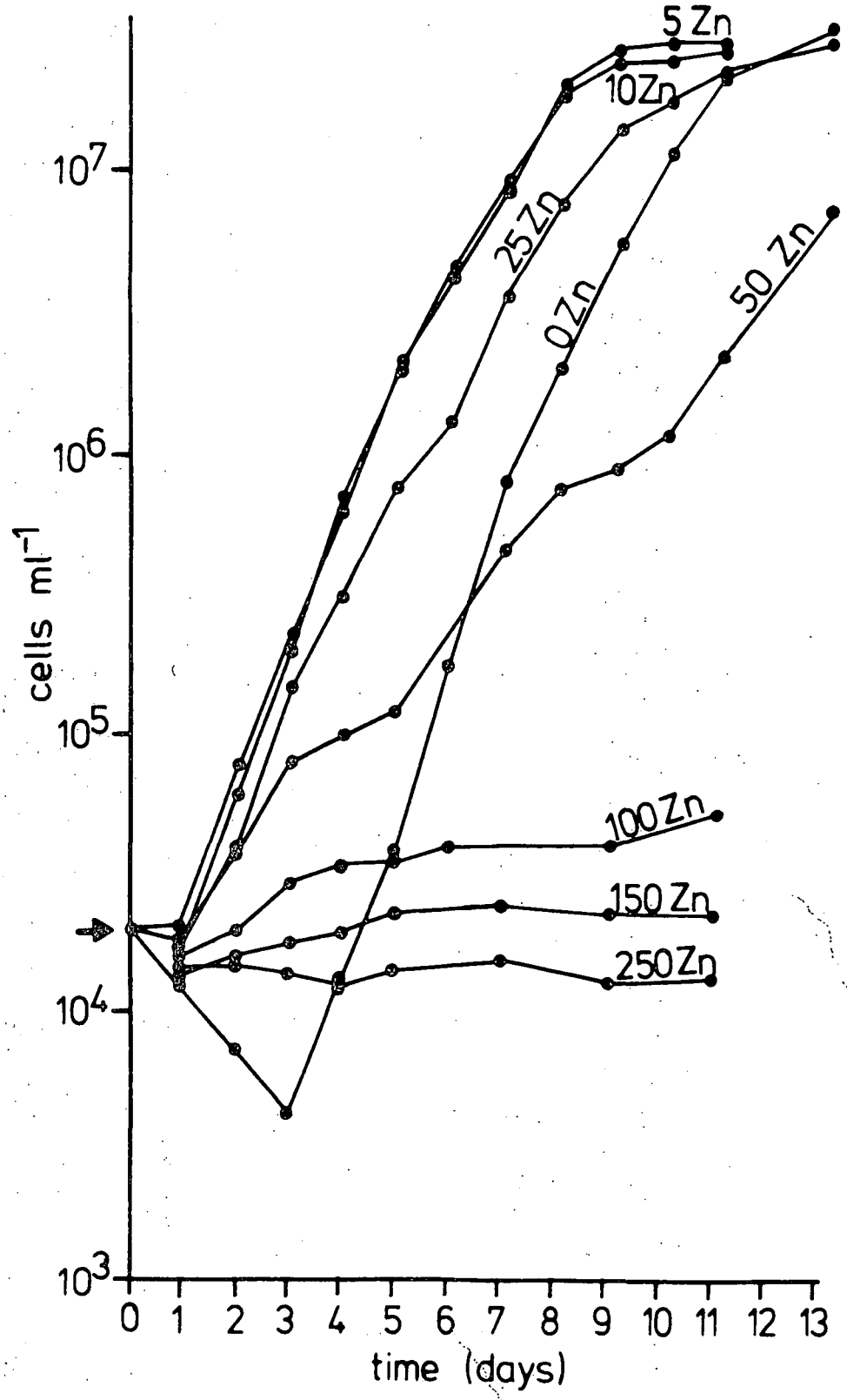


Fig. 6.7 Effect of Zn (mg l^{-1}) on growth of strain 545 in Chu 10 E (pH 5.0); arrow indicates concentration of inoculum

[Zn] (mg l^{-1})	time (days) 1	3	5	7	9	11	13
0	0.119(0.011)	0.150(0.032)	0.398(0.021)	0.846(0.012)	0.964(0.076)	1.090(0.055)	1.097(0.136)
10	0.130(0.015)	0.140(0.023)	0.475(0.061)	0.888(0.215)	1.070(0.146)	1.111(0.021)	1.055(0.032)
50	0.115(0.009)	0.116(0.023)	0.405(0.074)	0.657(0.067)	0.978(0.032)	1.094(0.078)	1.048(0.042)
100	0.134(0.019)	0.139(0.011)	0.273(0.021)	0.510(0.116)	0.878(0.075)	0.978(0.135)	1.076(0.064)
250	0.131(0.001)	0.119(0.013)	0.210(0.021)	0.314(0.021)	0.552(0.032)	0.797(0.055)	0.929(0.053)
500	0.112(0.018)	0.105(0.007)	0.212(0.030)	0.294(0.055)	0.301(0.064)	0.391(0.024)	0.517(0.106)
1000	0.106(0.018)	0.081(0.005)	0.137(0.011)	0.217(0.032)	0.168(0.021)	0.245(0.068)	0.294(0.042)
2000	0.115(0.013)	0.091(0.016)	0.109(0.011)	0.166(0.014)	0.158(0.022)	0.189(0.033)	0.168(0.008)
3000	0.099(0.034)	0.084(0.008)	0.131(0.039)	0.127(0.023)	0.112(0.013)	0.148(0.034)	0.120(0.013)
5000	0.071(0.011)	0.085(0.011)	0.087(0.023)	0.108(0.013)	0.080(0.015)	0.056(0.009)	0.017(0.004)

Table 6.5 Effect of Zn on growth of strain 537 in Chu 10 E medium at pH 5.0; values (mg l^{-1} chl *a*) are the mean of four replicates; figures in parentheses are the standard deviation of the mean; inoculum = 0.13 mg l^{-1} chl *a*

[Zn] (mg l^{-1})	time (days) 1	2	3	4	5	6	7	9	11	13
0	1.22(0.34)	0.72(0.15)	0.42(0.09)	1.26(0.30)	3.75(0.25)	17.1(2.53)	79.8(26.0)	547 (25.3)	2230(345)	3362(480)
5	2.04(0.28)	7.70(0.71)	22.5(2.91)	61.3(9.10)	198 (15.3)	411 (46.5)	850 (48.6)	2720(287)	2750(271)	-
10	1.80(0.15)	6.08(0.29)	19.8(2.70)	69.0(6.96)	208 (42.6)	456 (16.3)	930 (140)	2430(247)	2730(253)	-
25	1.55(0.15)	3.88(0.24)	14.6(2.40)	30.9(4.85)	75.5(15.2)	136 (24.3)	365 (62.7)	1410(276)	2290(320)	2810(475)
50	1.81(0.11)	3.88(0.89)	6.93(2.02)	9.68(1.81)	11.9(1.07)	-	46.3(3.71)	88.2(11.3)	224 (36.0)	743 (343)
100	1.60(0.35)	1.91(0.12)	2.81(0.81)	3.30(1.61)	3.34(0.82)	3.90(0.65)	-	3.84(0.67)	5.15(1.72)	-
150	1.39(0.20)	1.59(0.17)	1.75(0.39)	1.90(0.24)	2.26(0.11)	-	2.46(0.54)	2.28(0.74)	2.24(0.60)	-
200	1.21(0.12)	1.48(0.42)	1.24(0.41)	1.38(0.44)	1.22(0.06)	-	1.00(0.28)	1.40(0.41)	1.50(0.44)	-
250	1.43(0.14)	1.45(0.45)	1.38(0.14)	1.22(0.17)	1.41(0.58)	-	1.54(0.44)	1.25(0.19)	1.33(0.21)	-

Table 6.6 Effect of Zn on growth of strain 545 in Chu 10 E medium at pH 5.0; values (mg l^{-1} chl *a*) are the mean of four replicates; figures in parentheses are the standard deviation of the mean; inoculum = 2×10^4 cells ml^{-1}

Strain 537. The effect of Zn on the growth of strain 537 was investigated in Chu 10 E medium buffered at pH 5.0 (Section 2.742). The design of the experiment was similar to the previous growth experiment on strain 536. Twenty eight replicates of each Zn treatment were prepared, allowing four to be removed for chl a analysis after days 1, 3, 5, 7, 9, 11 and 13. The range of Zn concentrations used, were based on those shown previously to cover the toxicity range for strain 537 (Table 6.1); no precipitation occurred at pH 5.0, even at the highest Zn concentrations. The results of the effect of Zn on the growth of strain 537 are plotted in Fig. 6.6. Mean chl a values corresponding to the points on Fig. 6.6 are given in Table 6.5 along with the standard deviation of each point. Curves for 10 and 50 mg l^{-1} Zn (values in Table 6.5) are not given in Fig. 6.6 as they run very closely with the 0 mg l^{-1} curve.

Strain 545. The effect of Zn on the growth of strain 545 was investigated in Chu 10 E medium buffered at pH 5.0. The cultures were incubated in 100-ml conical flasks in 25 ml of growth medium. Four replicates of each Zn concentration were inoculated with 2×10^4 cells ml^{-1} of strain 545. 0.1 ml samples of the cell suspension were removed aseptically at daily intervals to be counted using a haemacytometer (Section 2.923). The effects of varying Zn concentration on growth of strain 545 are plotted on Fig. 6.7. The values on which Fig. 6.7 is based are given in Table 6.6.

The sensitivity of haemacytometer counting of unicellular algae, compared to estimation of chl a for filamentous algae, allowed a relatively lower inoculum to be used with strain 545. The increase of biomass of strain 545 over approximately three orders of magnitude made it more convenient to plot log biomass versus time for this strain.

The growth constant k was calculated for each algal strain at each Zn concentration (Table 6.7). k is derived from the equation below:

$$k = \frac{\log_2(N_1/N_2)}{t_1 - t_2}$$

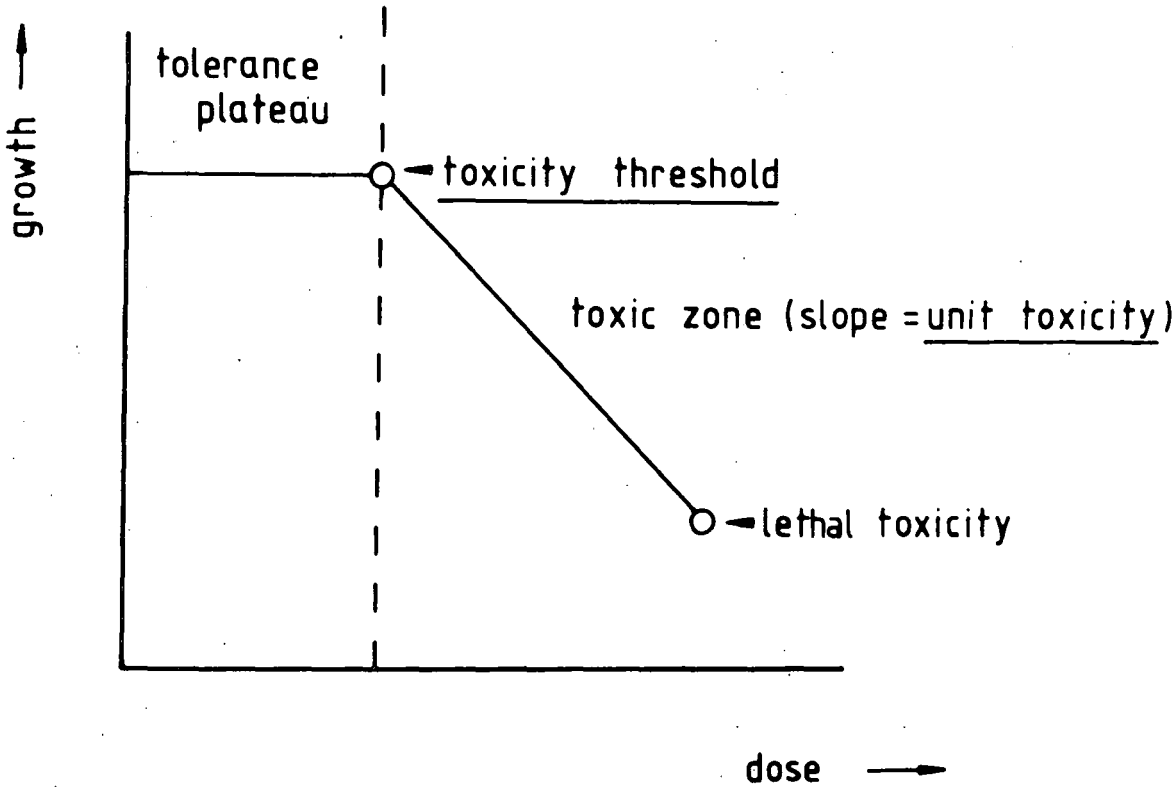
were N = concentration of algae in the culture and t = time (days). The value k corresponds to the number of biomass doubling times per day. k was calculated by fitting a straight line, using a least squares regression, to a semi-log plot of log biomass versus time (days). The exponential part of the growth curve (straight line on semi-log plot) is estimated by eye. If $\log_{10}$ values are plotted then k = 3.322K where K = the slope of the line.

Table 6.7 Influence of Zn on growth rates of strain 536 (Chu 10 E, CFM), strain 537 (Chu 10 E) and strain 545 (Chu 10 E); Zn values in mg l^{-1}

536 (Chu 10 E)		536 (CFM)		537 (Chu 10 E)		545 (Chu 10 E)	
Zn	k	Zn	k	Zn	k	Zn	k
0	0.392	1.5	0.321	0	0.643	0	1.71
5	0.342	2.1	0.322	10	0.683	5	1.41
10	0.319	4.5	0.324	50	0.500	10	1.39
15	0.236	15.7	0.260	100	0.440	25	1.28
20	0.130	65.4	0.193	250	0.330	50	0.72
25	0.037			500	0.152	100	0.15
40	-0.014			1000	0.131	150	0.14
				2000	0.120	250	-0.01
				3000	0.006		
				5000	-0.058		

A generalized toxicant dose response curve, as described by Berry and Wallace (1981), is given in Fig. 6.8:

Fig. 6.8 Generalized toxicant dose response curve



where; toxicity threshold is the lowest concentration of a toxicant at which an additional dose will cause a growth decrease; unit toxicity is the amount of response (growth decrease) per unit of toxicant after the threshold toxicity is reached. The slope of the dose response curve in the toxic zone (Fig. 6.8) represents the unit toxicity i.e. the steeper the curve, the greater the unit toxicity.

Commonly in toxicant dose response curves the relationship between a toxicant and the response of an organism can be linearised by plotting response versus the logarithm of the toxicant concentration (Finney, 1947; Kjellstrom, 1976). Fig. 6.9 shows the growth constant k plotted against

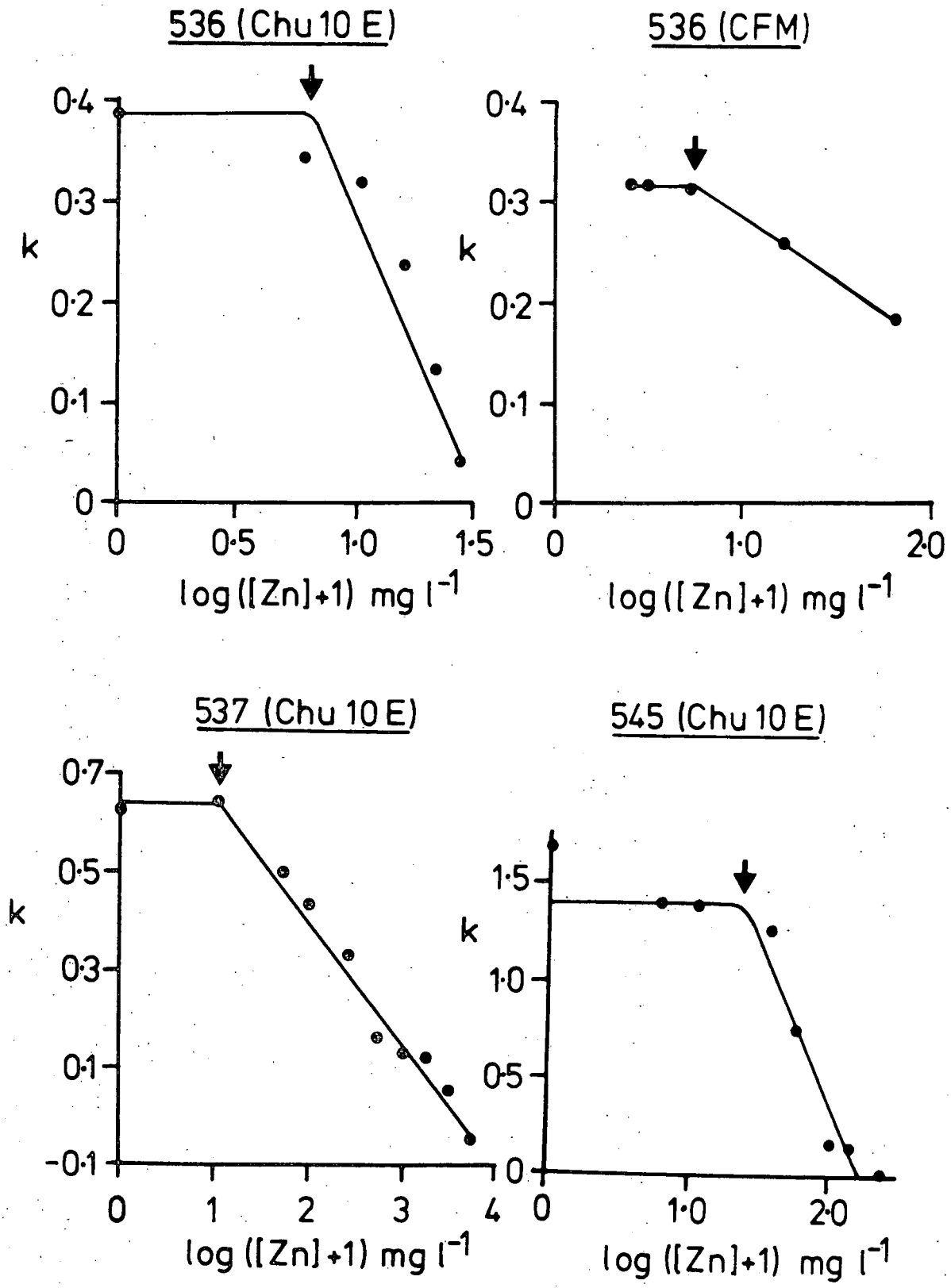


Fig. 6.9 Effect of Zn on growth rate of strains 536 (Chu 10 E, CFM), 537 (Chu 10 E) and 545 (Chu 10 E); arrows indicate the toxicity threshold value

$\log_{10}(Zn + 1)$ for the four growth curves given in Figs. 6.4 to 6.7. To allow control values of Zn (zero values) to be plotted, one unit is added to each Zn concentration (mg l^{-1}) before the logarithm is calculated; this is a routine procedure where zero values are included in logarithmic transformations (Steel and Torrie, 1960).

A curve corresponding to that given in Fig. 6.8 can be fitted to each scatter (Fig. 6.9). Values for toxicity threshold and unit toxicity, for each dose response curve (Fig. 6.9), are given in Table 6.8. For the strain 545 dose response curve, the zero Zn value is not used in the construction of the curve, the reason for this is discussed in Section 9.41.

Table 6.8 Parameters of the dose response curves for strains 536, 537 and 545

strain	536	536	537	545
medium	Chu 10 E	CFM	Chu 10 E	Chu 10 E
Zn toxicity threshold	5.5 mg l^{-1}	5.3 mg l^{-1}	11.0 mg l^{-1}	20.4 mg l^{-1}
Zn unit toxicity	-0.47	-0.12	-0.27	-1.34

Many experiments in the present study rely on single measurements of algal yield sampled prior to the end of the exponential growth phase. It proved impossible to measure the growth constant (k) by plotting the slope of the $\log_2$ curve between the inoculum value and the yield value; due to sonication of the inocula (Section 2.922) the values quoted for inocula concentrations include both living and dead material. Figs. 6.4, 6.5 and 6.6 show that for strains 536 and 537, after 1 days growth, the chl a concentration falls below the inoculum value. This probably represents the breakdown of cells rendered inviable by sonication. The growth curves given in Figs. 6.4 to 6.7 were used to test the validity of quoting log yield to represent the growth rate (k). For each growth curve a time was chosen prior to the end of the exponential growth phase and log yield was correlated

with k (Table 6.7), for each Zn concentration, using the least squares regression technique. Results were as follows:

strain	536	536	537	545
medium	Chu 10 E	CFM	Chu 10 E	Chu 10 E
time (days)	7	9	7	7
correlation coefficient	0.991	0.905	0.980	0.998

again for alga 545 the zero zinc concentration was not included in this calculation (Section 9.41).

The high correlation values indicate that log yield values can be used to represent growth rate. In experimental results where log yield figures are quoted, these can be assumed to be directly proportional to growth rate for strains 536, 537 and 545.

In order to allow the logarithmic transformation of values of zero yield (i.e. cell death), one unit is added to the yield value before transformation (Steel and Torrie, 1960). This is represented on figures as log (yield + 1) e.g. Fig. 6.1.

6.4 Environmental factors affecting zinc toxicity

As described in Section 1.321, when relating laboratory toxicity studies to the likely effects of these toxicants in the field, the influence of other environmental variables on toxicity must be considered. Isolates of the two dominant algae at streams 0097 and 3026 were chosen to study the influence of a number of chemical factors likely to affect Zn toxicity in the field. The algal strains used were strain 536 and strain 537 (isolated from stream 0097 and 3026 respectively).

Basic Chu 10 E medium was adjusted with respect to both Zn concentration and the chemical factor to be tested. Zn levels ranged from zero to toxic

(based on values derived previously; Table 6.1); the concentration of the chemical factor was based on field analysis (Section 4.2 and Chapter 5) and for each tested, a range of concentrations was chosen to cover field conditions.

Small portions of strains 536 and 537 were inoculated into 10 ml of Chu 10 E medium (inoculation procedure described in Section 2.91) contained in 50-ml boiling tubes. Media were buffered at appropriate pH designed to reproduce field conditions (Section 6.2); 536 media contained 1 nM vit. B_{12}.

Various combinations of Zn and the chemical factor under investigation were tested in a matrix format (for addition of selected ions to Chu 10 E medium see Section 2.744). Analysis of algal growth was carried out after 6 days incubation in the 20°C growth tank (Section 2.76); growth estimates were based on the simple 1-5 scale described in Section 2.91.

The various interactions tested are given in Fig. 6.10; the effect of EDTA on zinc toxicity was included in this series of experiments, (1.25 mg l^{-1} EDTA corresponds to levels used in Chu 10 E medium; Section 2.743).

Though detailed interpretations cannot be made on the semi-quantitative data presented in Fig. 6.10, the following factors can be seen to interact with zinc toxicity.

For strain 536 both Mg and Ca strongly reduced Zn toxicity. Bicarbonate also appears to have an amelioratory affect on Zn toxicity though, at higher concentrations of bicarbonate, there was precipitation in the media; it is likely that Zn levels in the media are reduced due to this. It is also evident that K and P show an amelioratory effect on toxicity, to a lesser degree. None of the other factors tested appear to influence Zn toxicity to strain 536. Cd at the highest concentration tested (1 mg l^{-1}) is significantly toxic, though there is no indication of anything other

strain 536

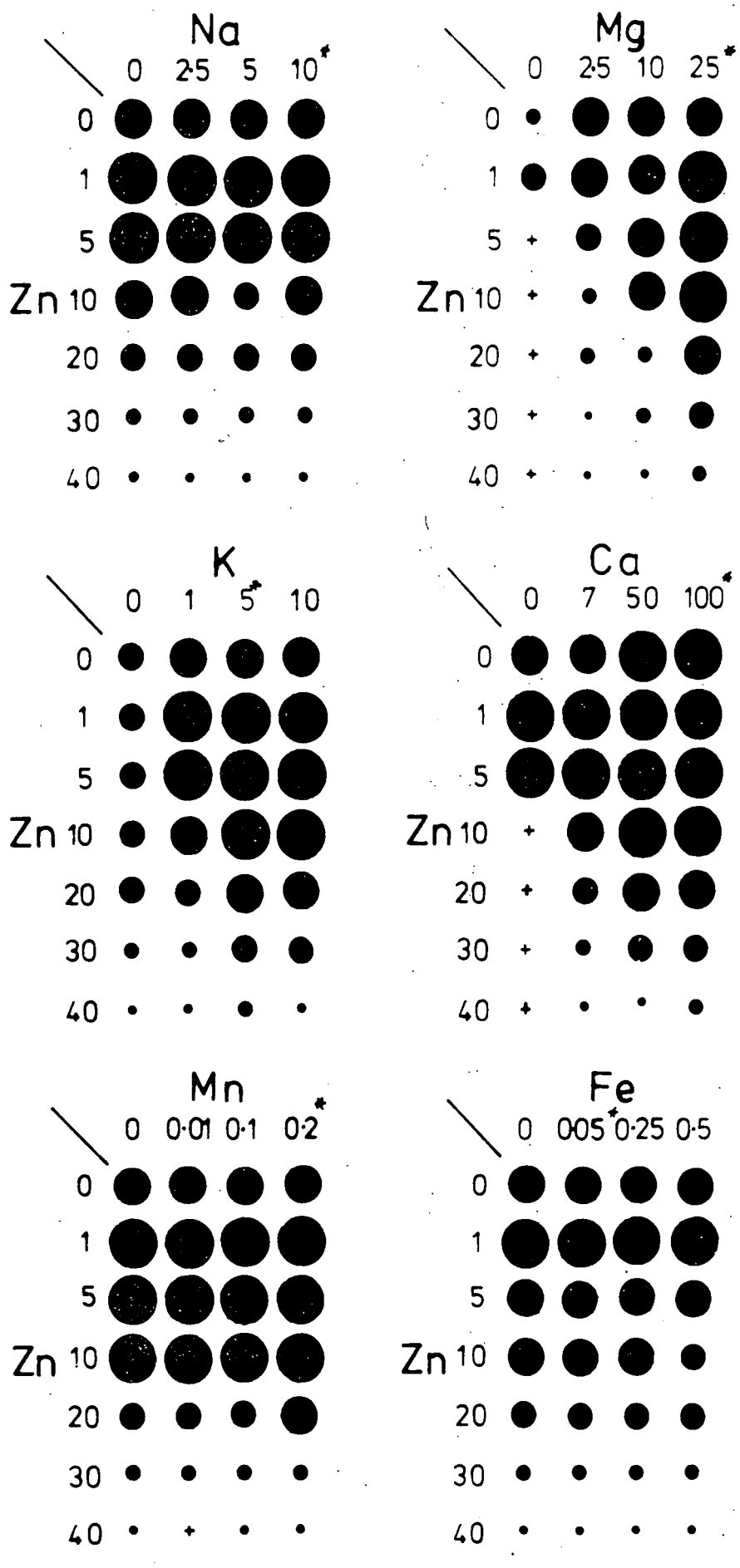
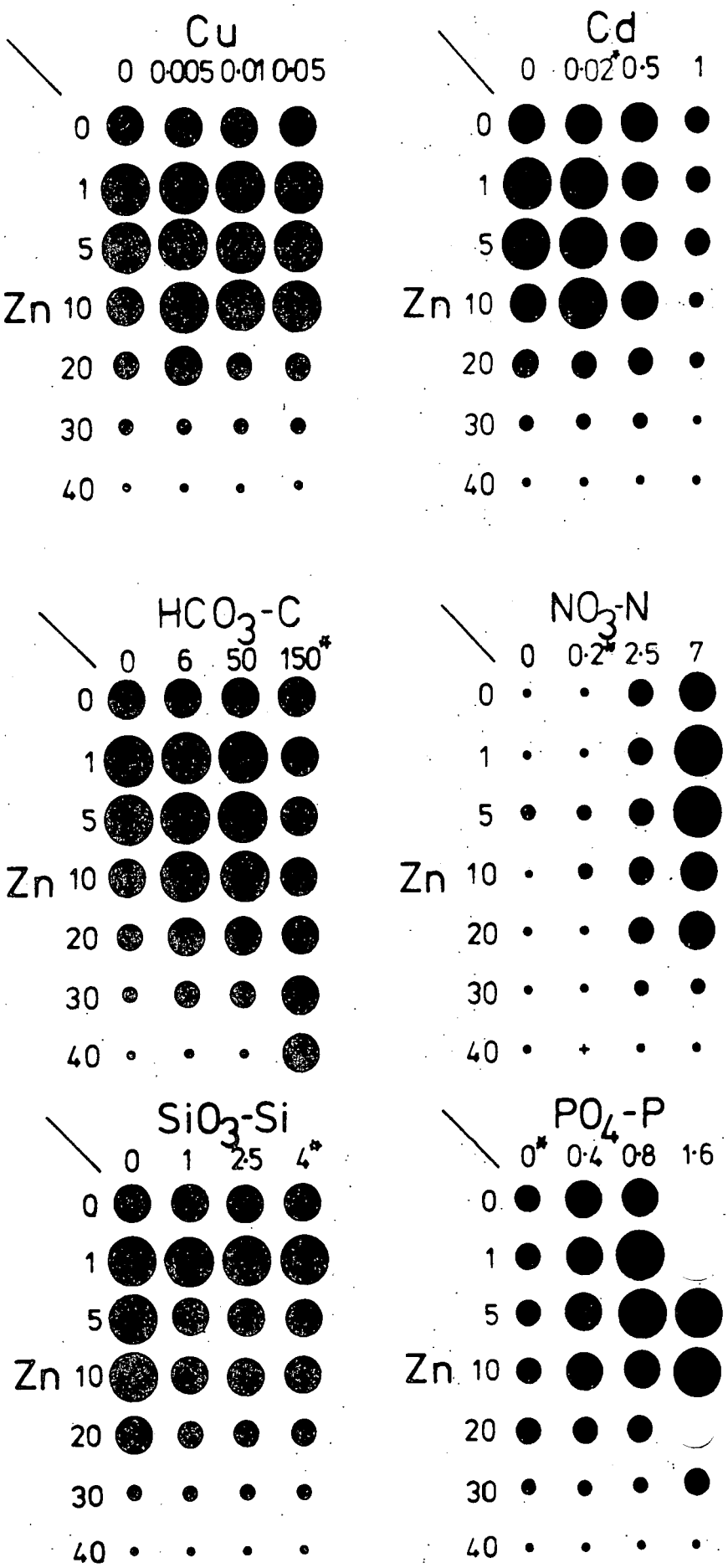


Fig. 6.10 (continued)



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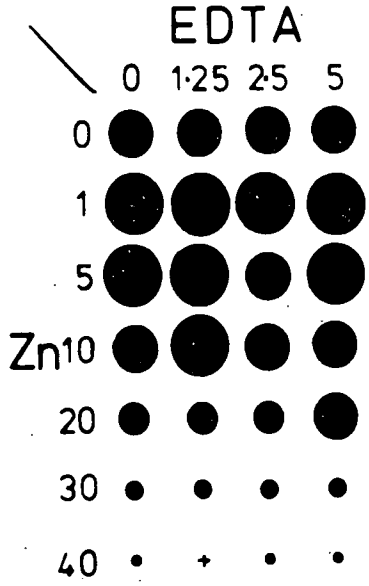
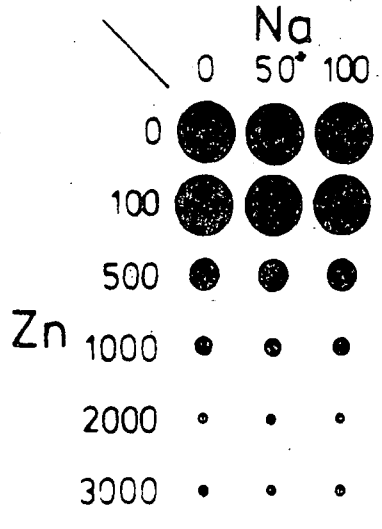
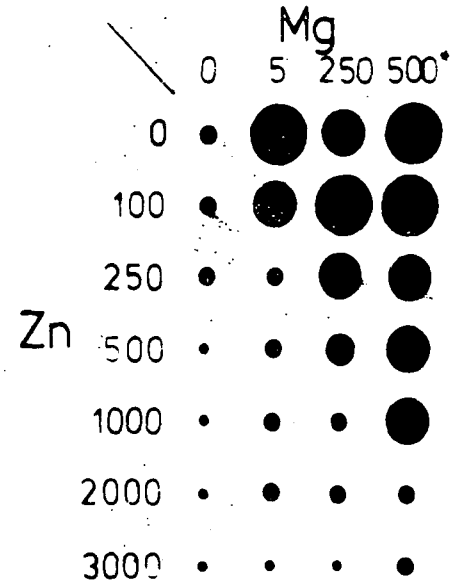
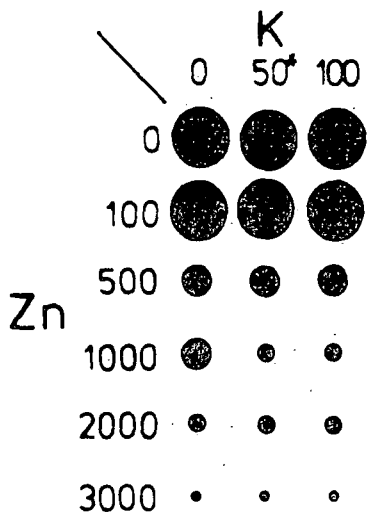


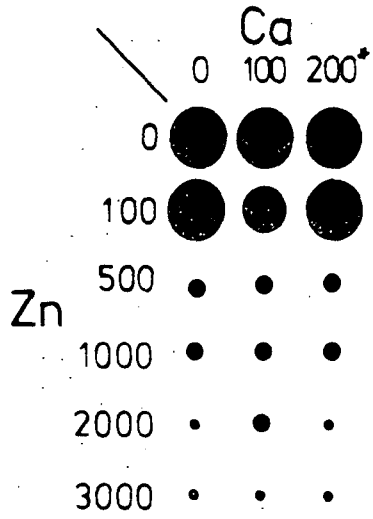
Fig. 6.10 (continued)

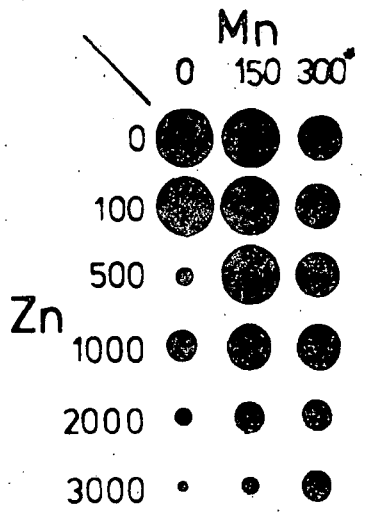
strain 537











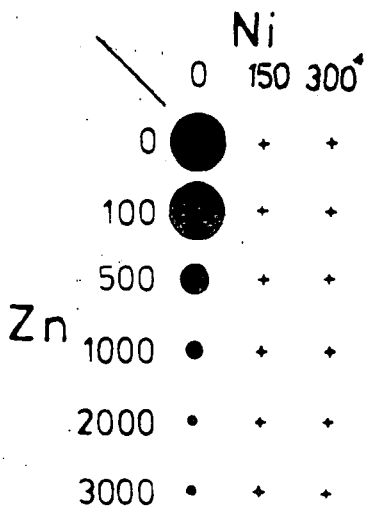
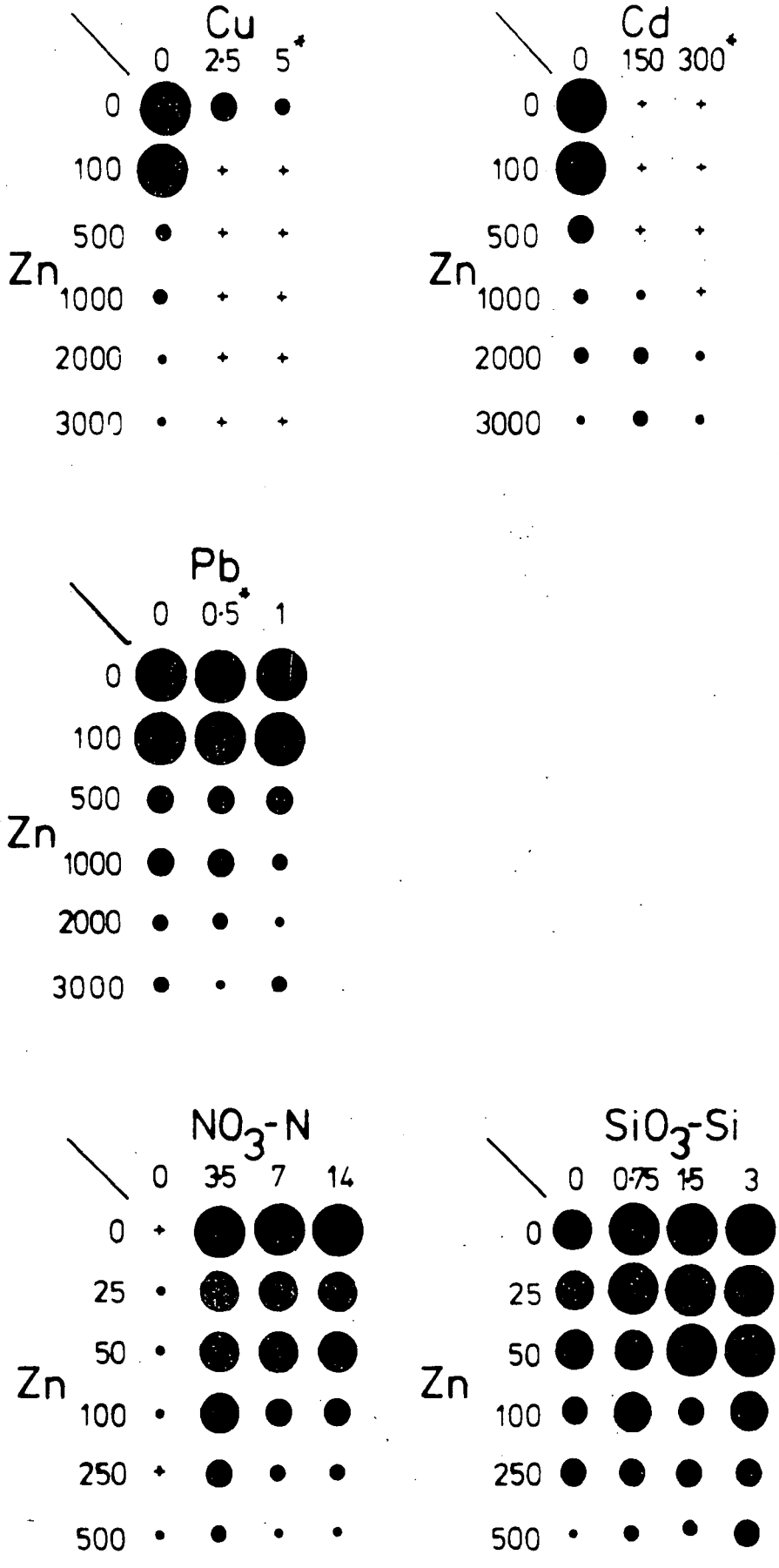


Fig. 6.10 (continued)



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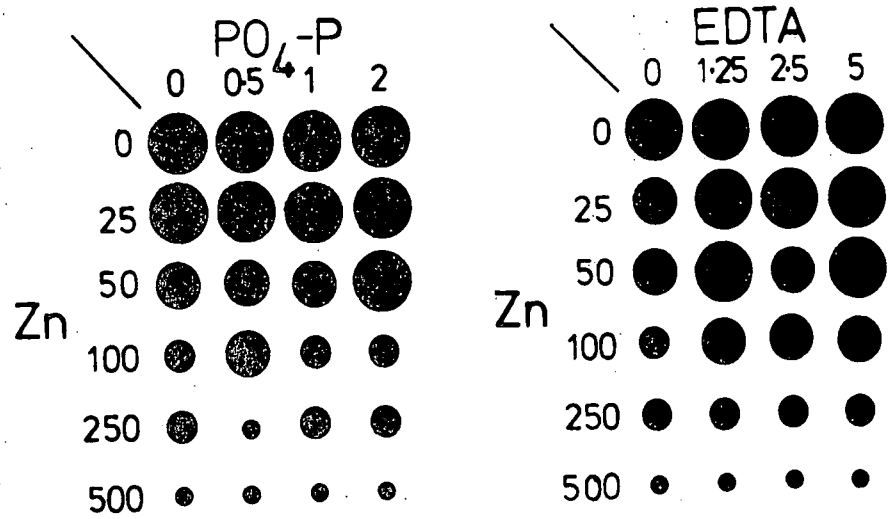


Fig. 6.10 (continued)

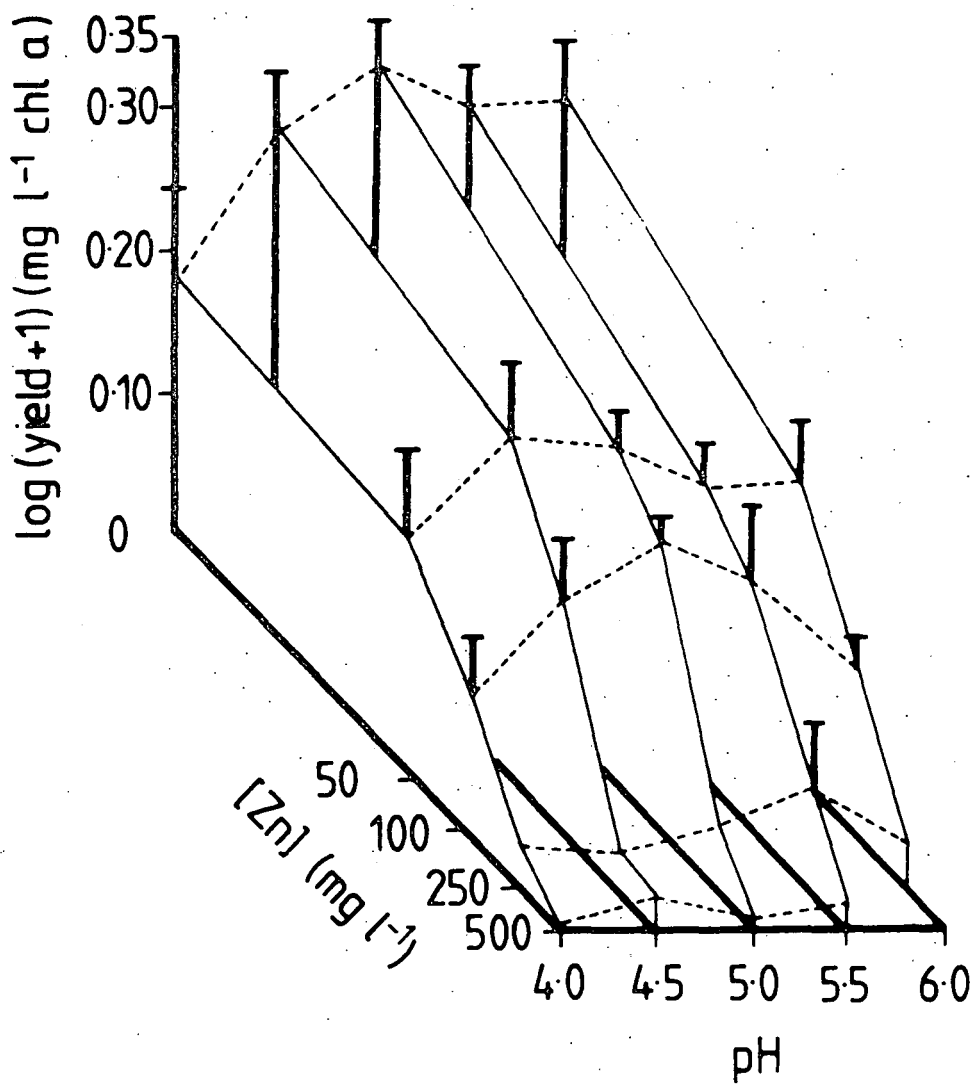
than an additive interaction with Zn toxicity. The requirement for the addition of N and P above field levels, in batch culture, is also demonstrated (Section 6.2).

For strain 537 Mg has a major amelioratory effect on Zn toxicity though Ca does not appear to act similarly. Mn also acts to reduce Zn toxicity at field concentrations; Ni is highly toxic and therefore no interaction with Zn toxicity is revealed at these Ni concentrations. Zn and Cu act synergistically in combination; the interaction of Zn and Cd is studied in more detail later in this Section for strain 537. Si appears to have a slight amelioratory effect on Zn toxicity.

The influence of pH on Zn toxicity was investigated in the pH range 4.0 - 6.0 for strain 537, which had originally been isolated from a field pH of 5.0 (Section 4.2). (The response of alga 537 to pH, in Zn-free medium, is given in Section 6.2.) Boiling tubes containing 10 ml of Chu 10 E medium were inoculated with a standard sonicated inoculum of strain 537 (Section 2.922). Four replicates of each combination of Zn and pH, indicated on the horizontal axes of Fig. 6.11, were incubated for six days in the 20°C growth tank (Section 2.76). After this period, chl *a* values were calculated (Section 2.925), where these were very low the replicates were pooled. Fig. 6.11 shows algal growth (plotted on vertical axis as log (yield + 1) as explained in Section 6.3) versus both Zn and pH. Standard deviation bars are indicated for treatments where it was not necessary to pool the replicates for chl *a* analysis.

Fig. 6.11 shows a similar response in Zn-free medium for strain 537 as was shown previously (Fig. 6.3). The toxicity curves of Zn at pH 4.0 - 5.5 are very similar; at pH 6.0, however, the toxic action of Zn appears to be slightly greater. This is particularly evident at 500 mg l^{-1} Zn which proved lethal at this pH.

Fig. 6.11 Interactive effects of Zn and pH on the growth of strain 537 in Chu 10 E medium; error bars indicate the standard deviation of the mean; n = 4



The ubiquity of Cd in Zn-polluted environments is introduced in Section 1.2; interactions of Zn and Cd toxicity, to plants, are found to range from ameliora-tory to synergistic (Section 1.321). The interaction of Zn and Cd was further investigated for strains from stream 3026 where the Cd:Zn ratio is particularly high (approximately 1:10; Chapter 5) compared to many Zn polluted sites (Section 1.2).

Fig. 6.10 indicates that high levels of Zn reduce Cd toxicity for strain 537, though at the highest levels of Zn and Cd (corresponding to field levels;

Section 4.2) there is no detectable growth. This interaction was studied in more detail for strain 537. A matrix of Zn and Cd concentrations was set up in 10 ml aliquots of Chu 10 E medium buffered at pH 5.0. Four replicates of each Zn/Cd combination shown on the horizontal axes of Fig. 6.12, were inoculated with a standard sonicated inoculum of strain 537. After a six day incubation period (for incubation conditions see Section 2.76) chl a values were calculated, though where these were very low the replicates were pooled. Fig. 6.12 shows algal growth (vertical axis) versus Zn and Cd concentrations. Standard deviation bars are indicated for treatments where it was not necessary to pool the replicates for chl a analysis.

Fig. 6.13 shows the results of a similar experiment using strain 545. A matrix of Zn/Cd combinations (indicated on Fig. 6.13) was set up in 25 ml volumes of Chu 10 E (pH 5.0) contained in 100-ml conical flasks. Each flask was inoculated with 10^5 cells ml^{-1} of strain 545. Cell concentrations were counted after six days incubation (Section 2.76) using a haemacytometer (Section 2.923). Treatments were not replicated; log cell concentrations (vertical axis) are plotted against Zn and Cd concentrations (horizontal axes).

The Zn and Cd interaction experiments for strains 537 and 545 both show that zinc has a significant amelioratory effect on cadmium toxicity when both are present together in the algal growth medium. At higher Zn concentrations Cd appears to have no converse effect on Zn toxicity.

Fig. 6.12 Interactive effects of Zn and Cd on growth of strain 537 in Chu 10 E medium (pH 5.0); error bars indicate the standard deviation of the mean; n = 4

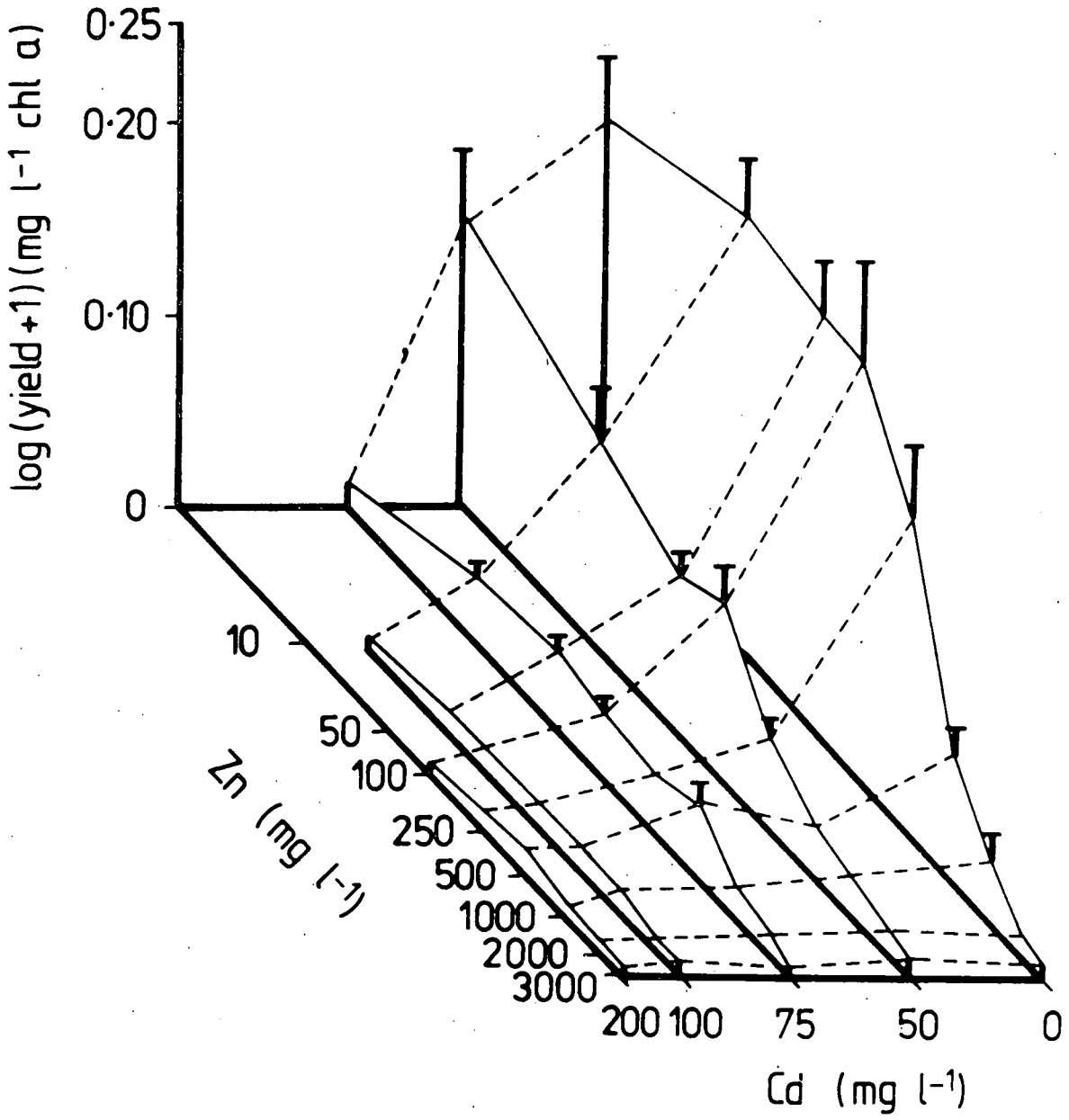
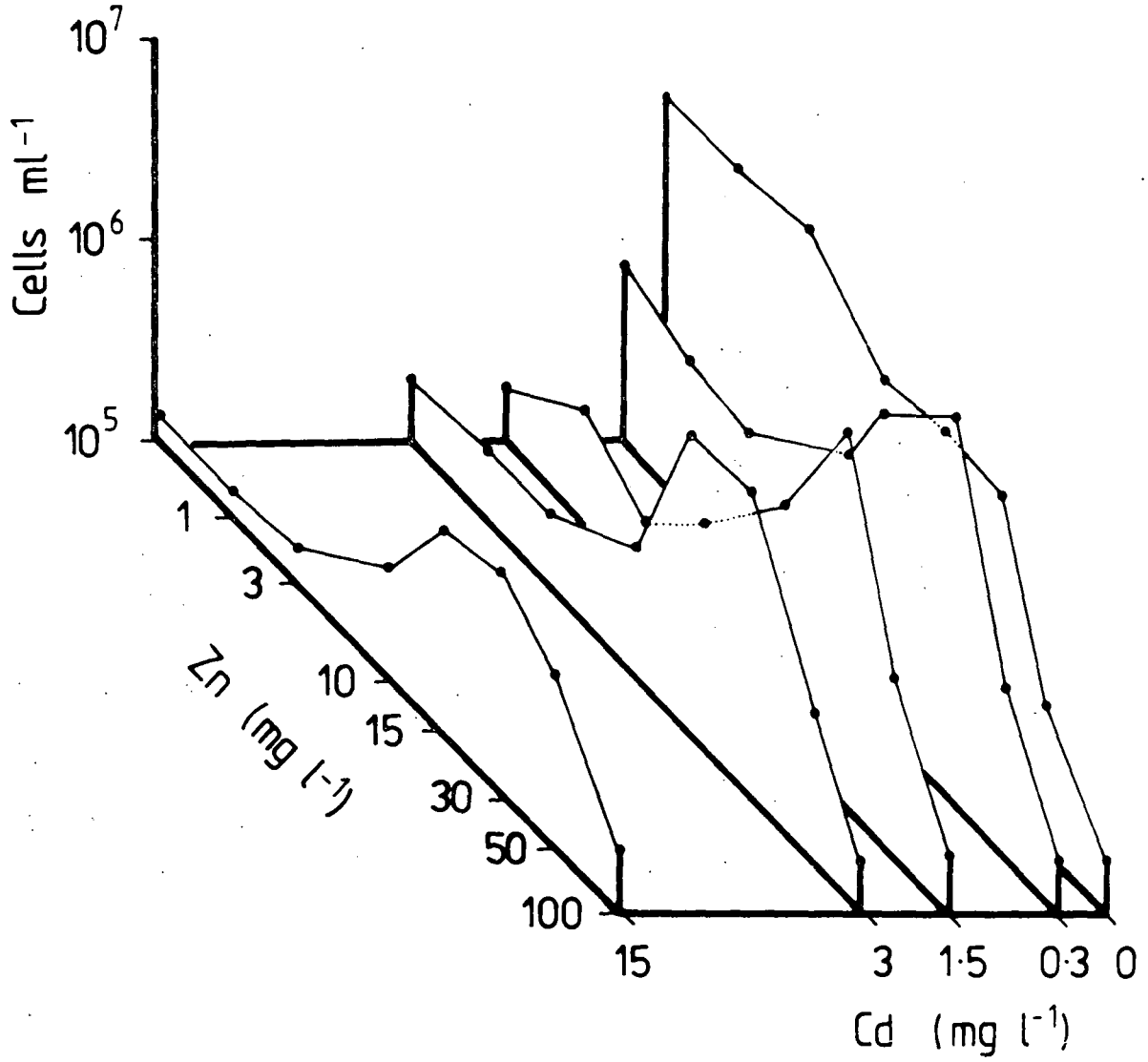


Fig. 6.13 Interactive effects of Zn and Cd on growth of strain 545 in Chu 10 E medium (pH 5.0)



As indicated by Wong and Beaver (1981), by presenting an algal culture with a combination of toxicants in the external growth medium, the interaction observed may be due to coagulation or co-precipitation in the medium. In order to study the in vivo interaction of Zn and Cd, cultures of strain 537 were pre-incubated for four days in Chu 10 E medium (control), Chu 10 E + Zn (100 mg l^{-1}) or Chu 10 E + Cd (10 mg l^{-1}). The algae were then washed in normal Chu 10 E medium before being sonicated and reinoculated into boiling tubes containing Chu 10 E medium supplemented with a range of Cd concentrations. The metal pretreatment stage introduces metal into the algal

cells and it is possible to assay the effect of cellular metal levels on Cd toxicity. The cultures were incubated for six days after which chl a analyses were carried out (Section 2.925). Algal growth is plotted against Cd concentration for the three preincubation treatments (Fig. 6.14). The toxicity threshold (Section 6.3) is found to change as follows:

preincubation treatment	toxicity threshold of Cd
normal Chu 10 E	2.4 mg l^{-1}
Chu 10 E + Zn (100 mg l^{-1})	4.3 mg l^{-1}
Chu 10 E + Cd (10 mg l^{-1})	5.6 mg l^{-1}

6.5 Zinc accumulation

Zn accumulation was studied in strains 536 and 537, isolated as the two dominant algae from streams 0097 and 3026 respectively.

Data are presented for accumulation of metals by Mougeotia spp. growing in stream 0097 in Section 4.3.

A culture of strain 536, which had previously been subcultured several times in Chu 10 E medium minus Zn, was divided and inoculated into Zn supplemented media. These cultures, contained in 100-ml conical flasks (25 ml of medium), were incubated for four days (Section 2.76). Two media were employed, Chu 10 E (pH 7.5) and CFM (pH 7.5). As discussed in Section 6.3 precipitation occurs in CFM when additional Zn is added and therefore results are quoted with respect to filtrable Zn and not total Zn. After the four day incubation period, alga were removed and algal Zn levels were estimated for each Zn treatment (Section 2.927). Fig. 6.15 gives the relationship between Zn concentration in the medium and accumulated levels of Zn in the alga for the two media used.

Accumulation of Zn by strain 536 is much greater in CFM than in Chu 10 E. By projection (based on the CFM curve) the mean field Zn concentration would

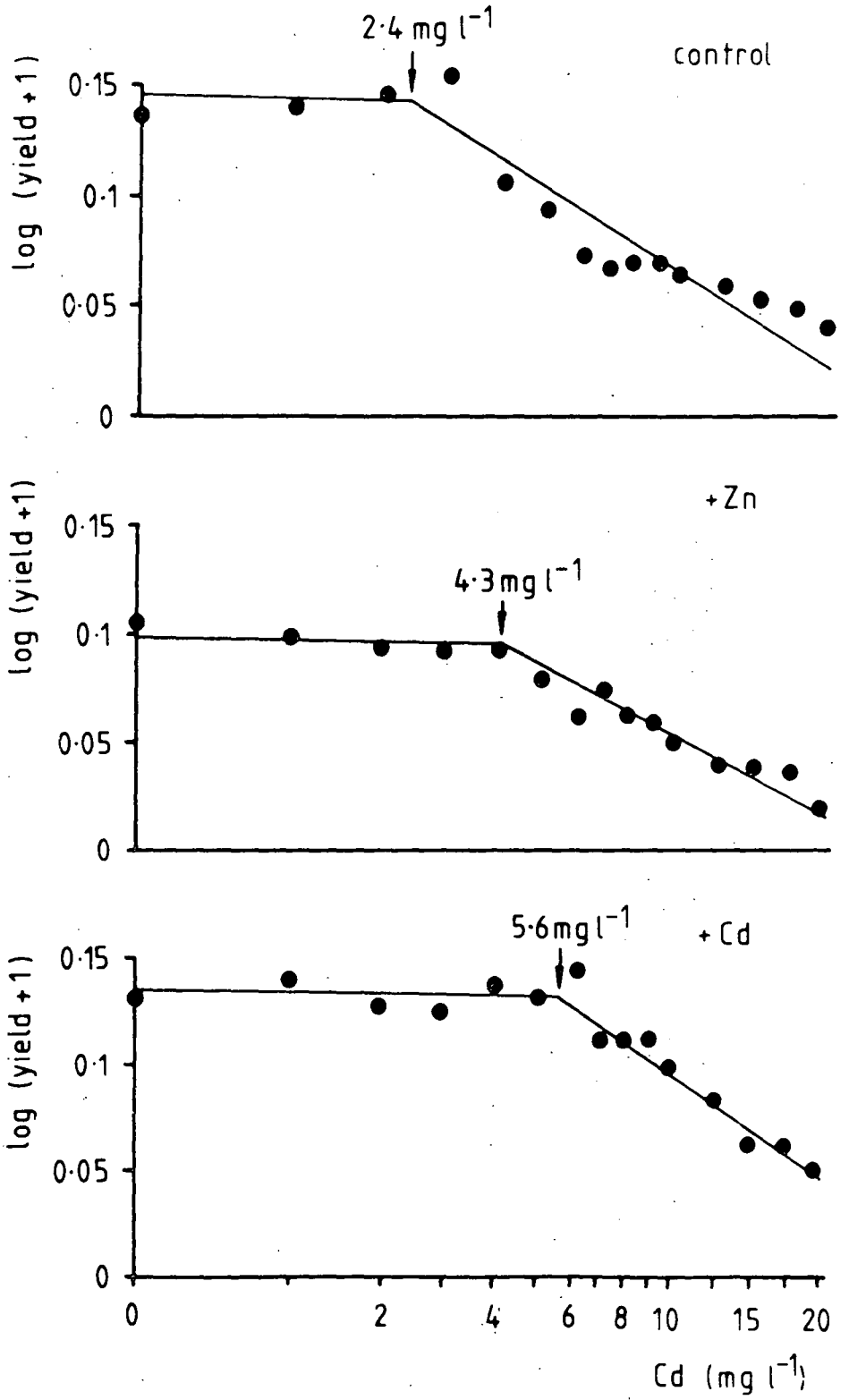


Fig. 6.14 Effect of Cd on growth of strain 537 after three different preincubation treatments (control, + Zn, + Cd)

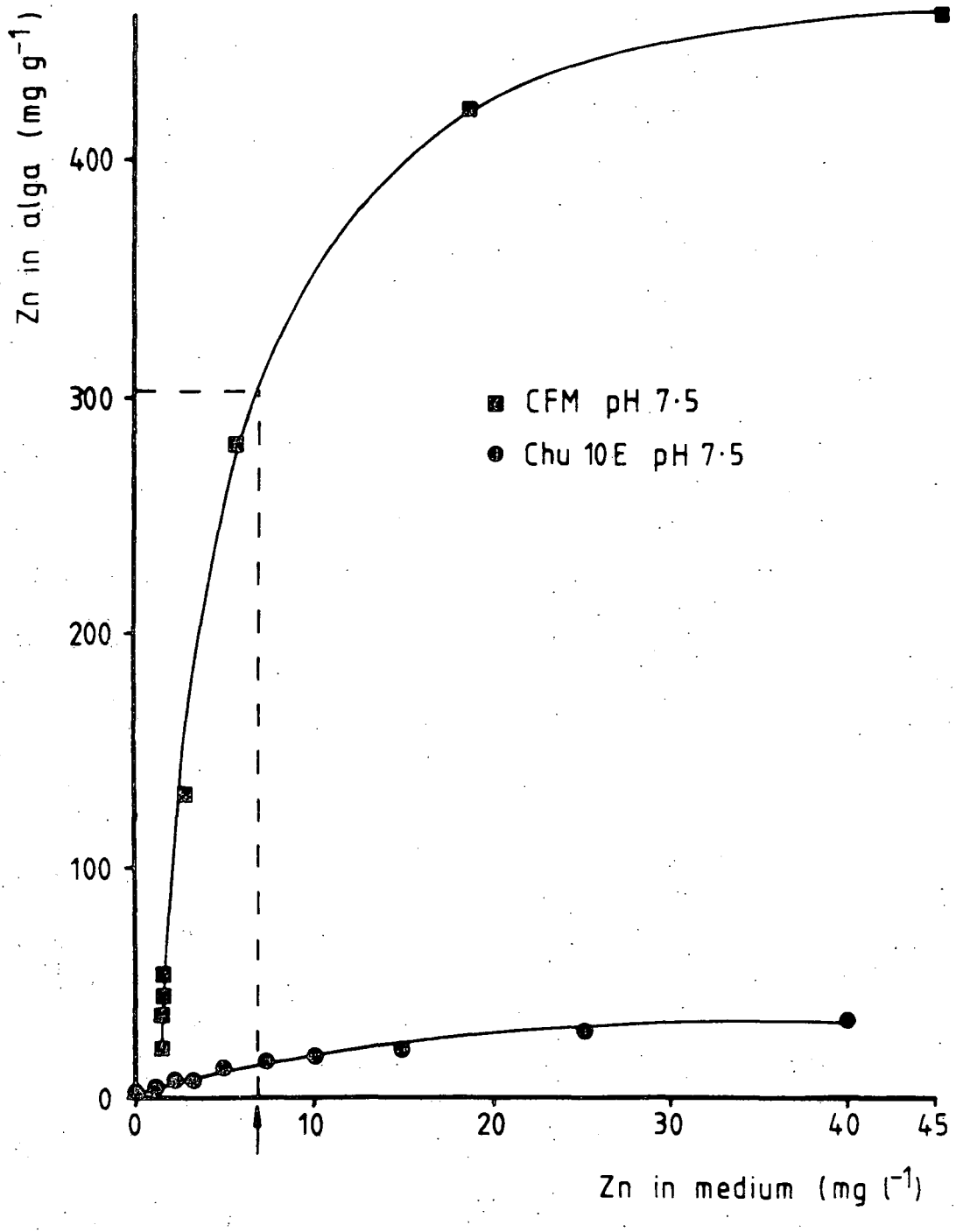
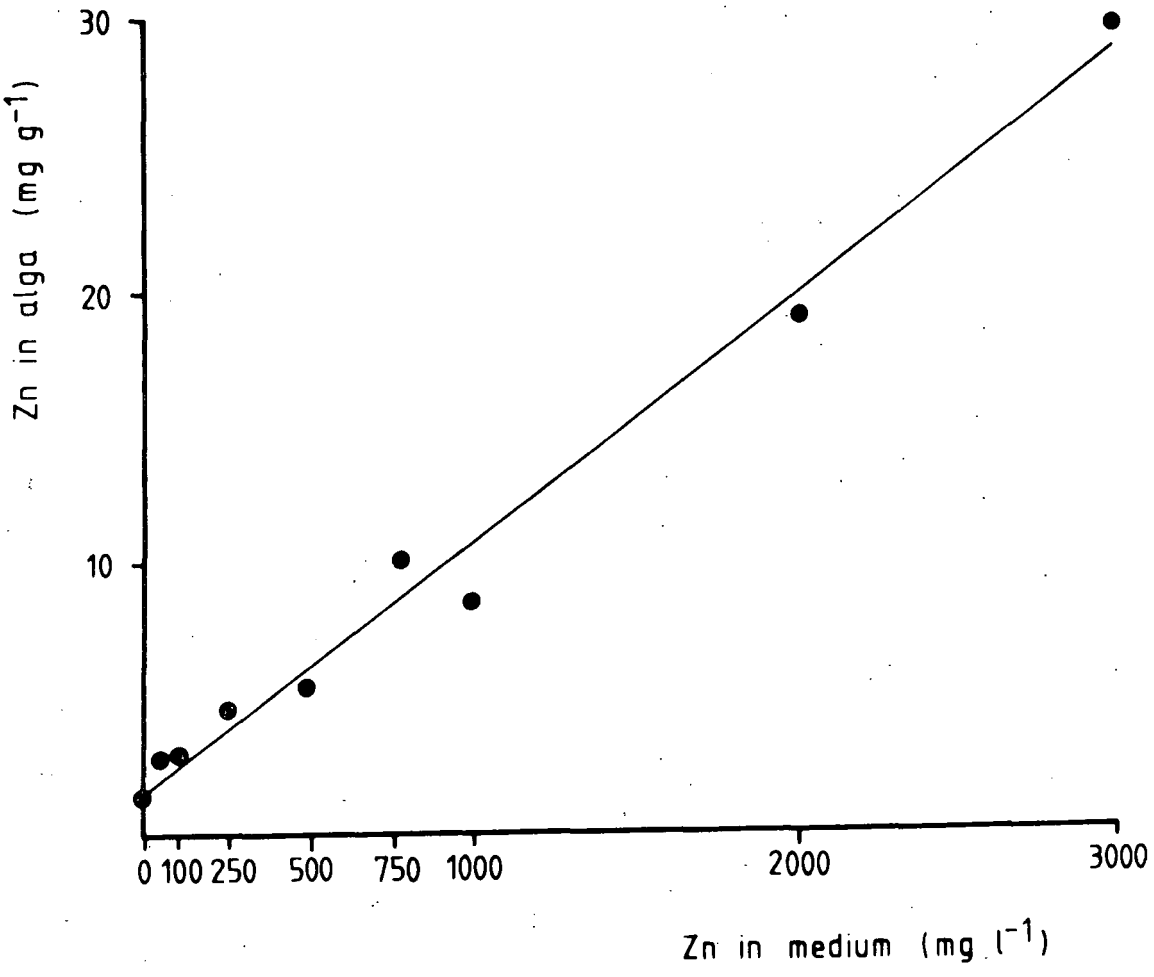


Fig. 6.15 Accumulation of Zn by strain 536 in laboratory culture; arrow indicates mean field Zn concentration at stream 0097 (6.84 mg l^{-1})

give a predicted Zn concentration in the alga of 304 mg g^{-1}. This corresponds closely with the maximum value reported for this alga in the field (Table 4.3).

Zn accumulation was studied in strain 537. Again algae grown in Zn-free Chu 10 E medium were transferred to a range of Zn concentrations, in Chu 10 E medium (pH 5.0), and incubated for four days. After this period the Zn content of the algae was estimated (Section 2.927). Fig. 6.16 shows the relationship between the Zn concentration in the medium and accumulated Zn in the algae; the straight line plot is calculated using a least squares regression of the points indicated.

Fig. 6.16 Accumulation of Zn by strain 537 in laboratory culture



7 CARBOXYLIC ACID CONTENT OF ALGAL MATERIAL

7.1 Introduction

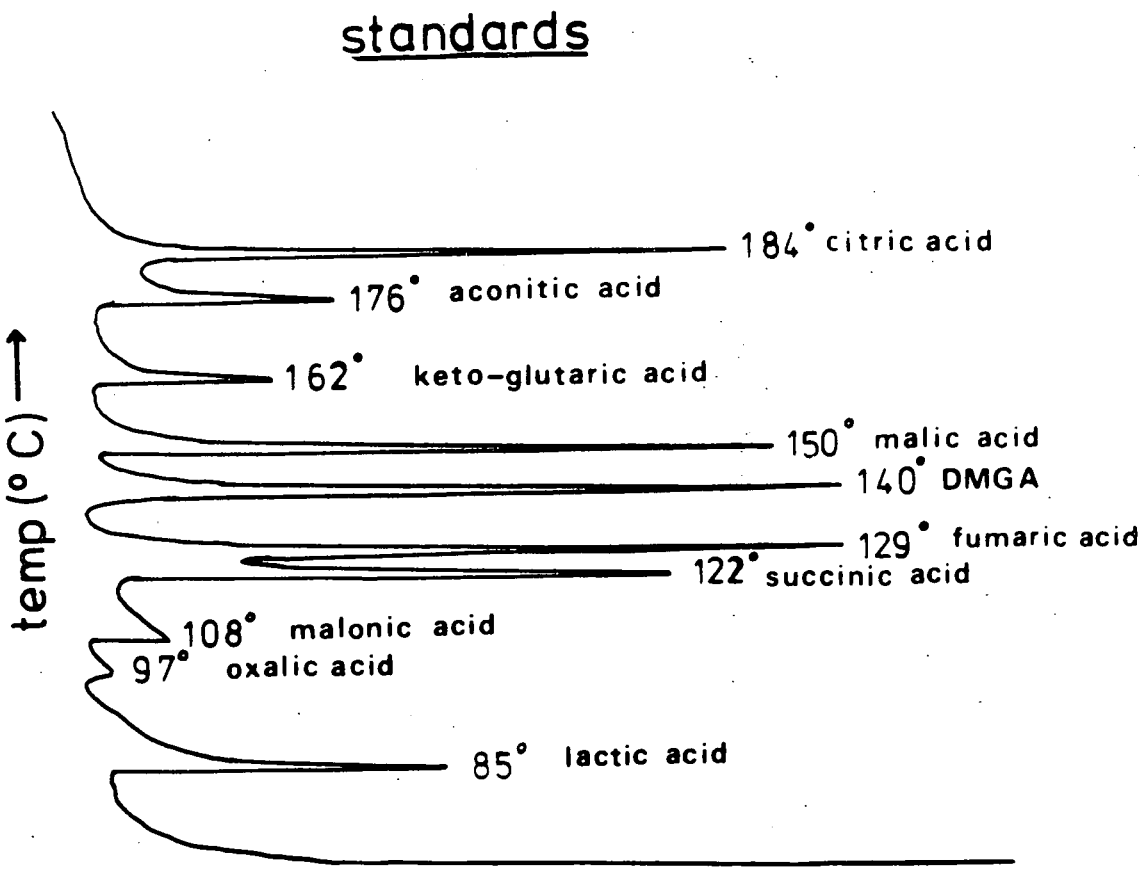
The present study is an investigation of the ability of green algae (Chlorophyta) isolated from two zinc-polluted streams (streams 0097 and 3026) to tolerate extreme levels of zinc. The previous Chapter has shown that these algae are able to grow, in laboratory culture, at levels of Zn well above those which are normally toxic to green algae (Section 9.41). As summarized in Section 1.322, many mechanisms have been suggested by which plants develop resistance to elevated levels of heavy metals. The experiments summarised in the present Chapter were planned as an attempt to find out the mechanism(s) by which algae from streams 0097 and 3026 achieved their resistance. Mathys (1980) presents evidence that in certain higher plants, carboxylic acids are involved in a process of detoxification of Zn. In view of this, it seemed worthwhile to investigate the effect of Zn on the carboxylic acid levels in algal material.

The methodology on which this chapter is based is presented in Section 2.10.

7.2 Carboxylic acid analyses

As described in Section 2.10.3, a standard mixture of 10 carboxylic acids was prepared. The chromatogram given in Fig. 7.1 is produced by injecting onto the chromatographic column 1 µg of each of the acid derivatives indicated. The temperature at which each acid emerges from the column is first obtained separately; this enables identification of each peak when the acid mixture is analyzed. It is evident that, though each of the acids is present in equal quantities (1 µg), the peak heights vary considerably.

Fig. 7.1 Gas chromatogram of trimethylsilyl derivatives of a mixture of standard carboxylic acids



Figs. 7.2 to 7.6 show the gas chromatograms of each of the treatments given in Section 2.10.1 (Table 2.11). The chromatogram for the field Mougeotia sample is not included as it shows no peaks indicating the presence of carboxylic acids.

Table 7.1 gives the equivalent dry weight of alga (prior to carboxylic acid extraction), injected onto the column, for each chromatogram (Figs. 7.2 to 7.6); this value is calculated from both the total algal dry weight and the volume of injection (Section 2.10.3).

Table 7.1 Column loadings of gas chromatograms

alga	Zn (mg l^{-1})	equivalent algal dry weight injected (mg)	Fig.
532	0	0.95	7.2
532	25	3.50	"
533	0	1.14	7.3
533	25	1.16	"
537	0	0.15	7.4
537	25	0.17	"
537	100	0.36	"
545	0	1.88	7.5
545	25	1.97	"
546	0	1.23	7.6
546	25	1.39	"
field Mougeotia	-	6.25	-

In Fig. 7.2 the two chromatograms for strain 532 both show carboxylic acid peaks emerging at 161°C; there are no other significant peaks on these chromatograms. The peak on the 532 + Zn chromatogram is substantially larger than that on the 532 - Zn chromatogram; it is likely, however, that this is due to the higher injection dry weight of the + Zn treatment (Table 7.1). The peak on each of the two chromatograms (Fig. 7.2) emerges at a very similar temperature to that of ∝keto-glutaric acid indicated on Fig. 7.1.

Fig. 7.3 shows the chromatograms of carboxylic acid extracts of strain 533. The injection dry weight of the two Zn treatments are very similar for this strain (Table 7.1). Both chromatograms show peaks emerging at temperatures 117, 132, 140, 208 and 222°C. The - Zn treatment shows a peak emerging at 166°C; this does not appear on the + Zn chromatogram. Conversely the + Zn

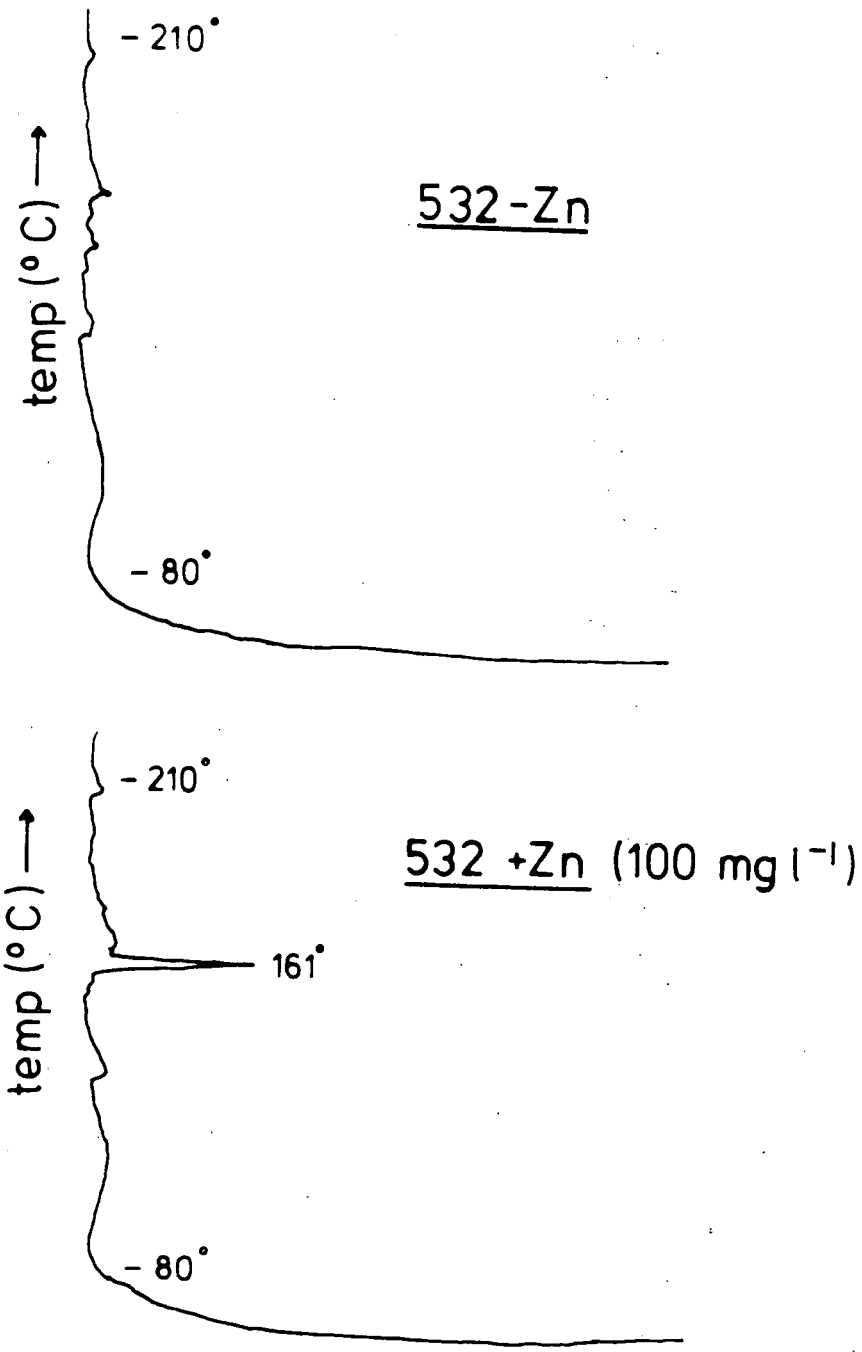


Fig. 7.2 Gas chromatograms of trimethylsilyl derivatives of carboxylic acids extracted from strain 532

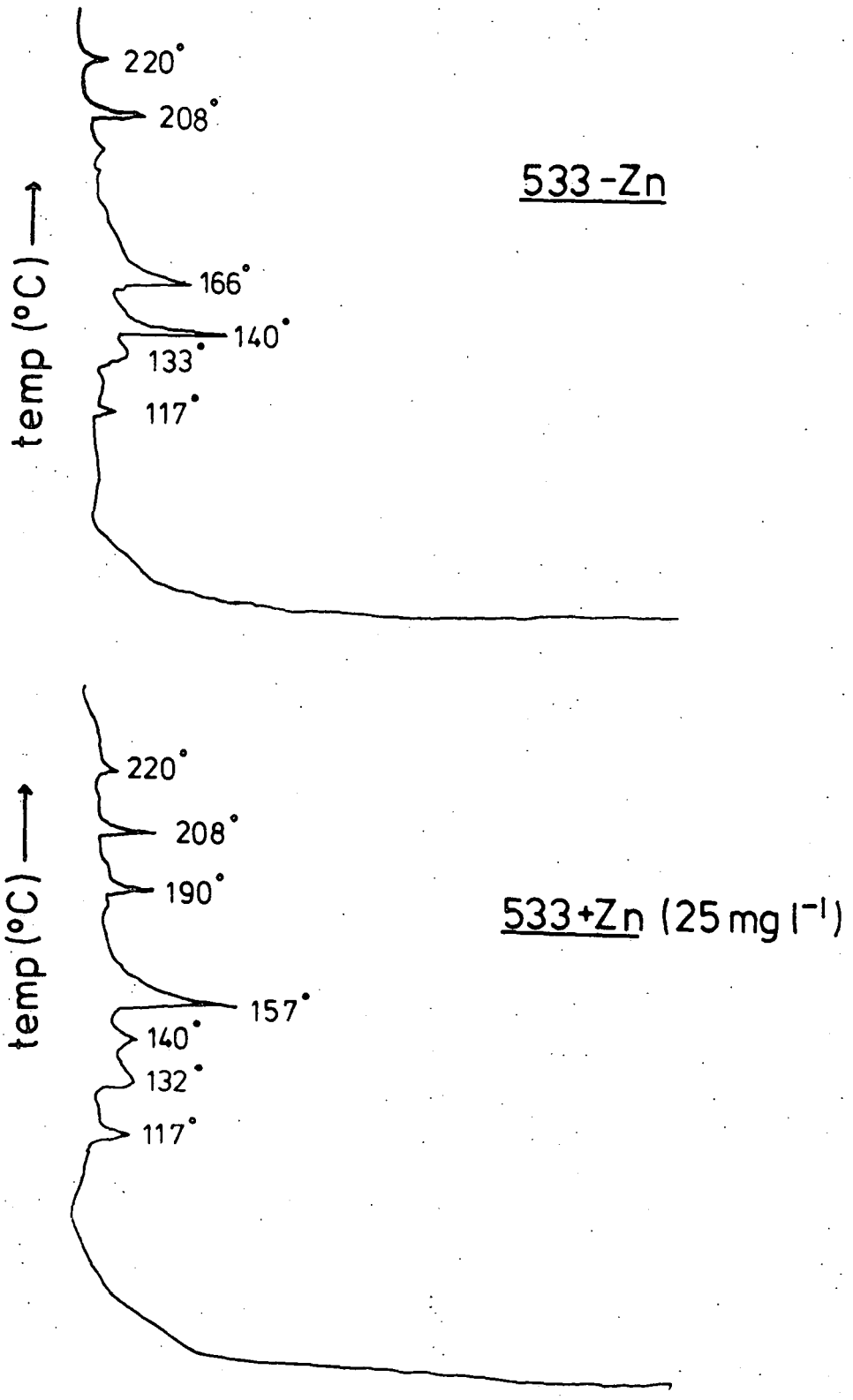


Fig. 7.3 Gas chromatograms of trimethylsilyl derivatives of carboxylic acids extracted from strain 533

treatment shows peaks at 157 and 190°C which do not appear on the - Zn chromatogram. The 140°C peak on both chromatograms (Fig. 7.3) probably corresponds to DMGA buffer carried over from the algal growth medium (Section 2.10.1); none of the other peaks in Fig. 7.3 correspond to standards given in Fig. 7.1.

The chromatograms for strain 537 (Fig. 7.4) each show a peak emerging from the column at 140°C. It is likely that this represents DMGA which is a contaminant derived from the algal growth medium (Section 2.10.1). The variation in size of this DMGA peak is probably due to the difference in the injection dry weight equivalent (Table 7.1) of each treatment and is not a product of different Zn treatments. Acid peaks emerging at 200 and 225°C appear to develop in higher Zn treatments; these do not correspond to any of the standard acids tested (Fig. 7.1). The acid peak emerging around 84°C, in each of the chromatograms in Fig. 7.4, possibly represents lactic acid (Fig. 7.1).

Fig. 7.5 shows the two chromatograms for strain 545 (Section 2.10.1). Both chromatograms show carboxylic acid peaks emerging from the column at 83, 129, 133, 140, 147, 160, 173, 181, 190, 200 and 218°C. The heights of these peaks are similar in both chromatograms; the injection dry weight equivalent for each chromatogram is also very similar (Table 7.1). The - Zn treatment shows a very large peak emerging at 157°C; at the equivalent temperature in the + Zn treatment a small peak occurs. Conversely the + Zn chromatogram shows a large peak emerging at 194°C; the - Zn treatment shows no corresponding acid peak at this temperature. In addition the + Zn treatment shows carboxylic acid peaks emerging at temperatures of 127 and 184°C which do not occur in the - Zn treatment. Except for the peaks emerging at 129°C, which correspond to fumaric acid in the standard run (Fig. 7.1), none of the peaks given in Fig. 7.5 correspond closely to any of the 10 standard acids (Fig. 7.1).

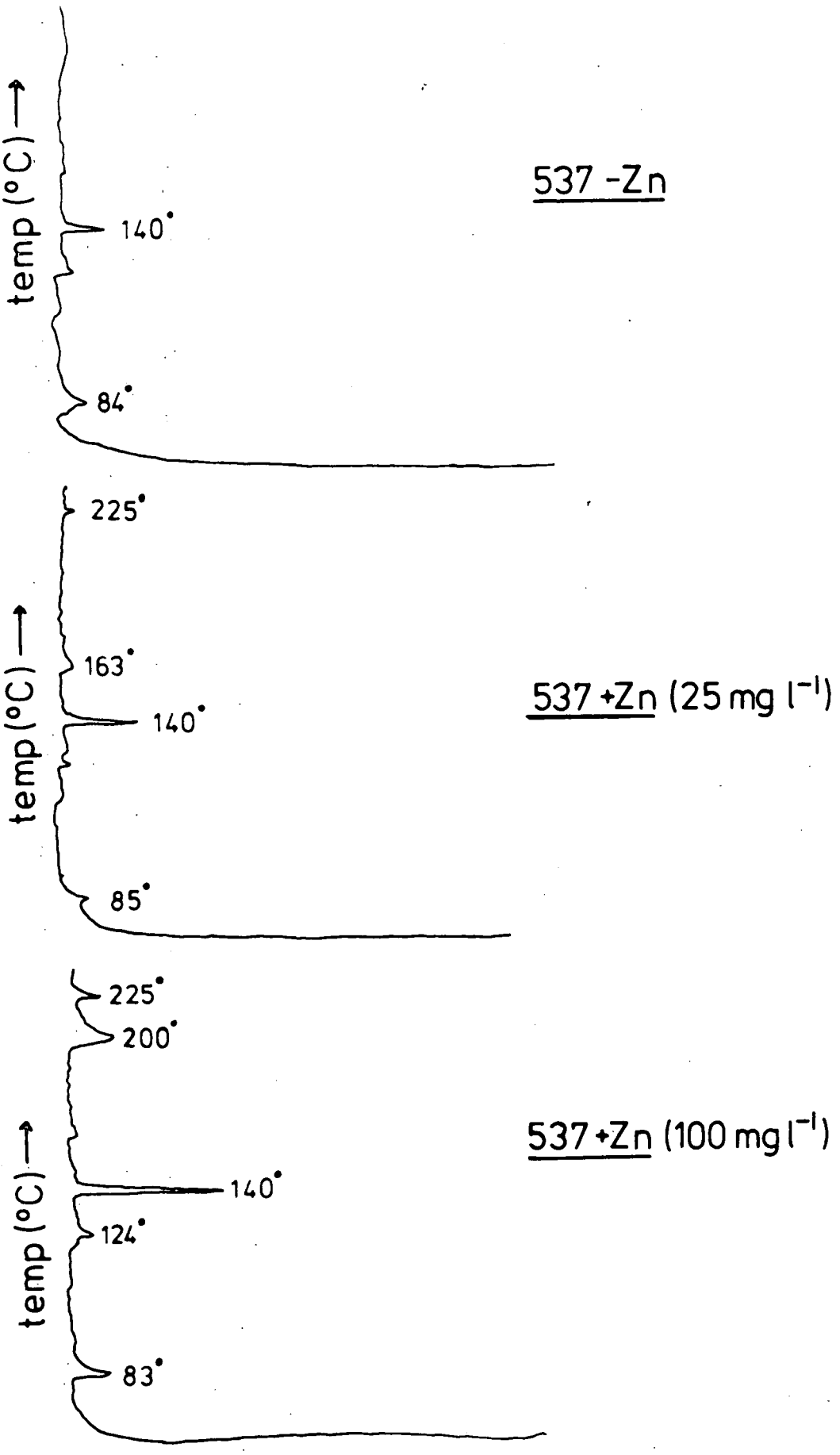


Fig. 7.4 Gas chromatograms of trimethylsilyl derivatives of carboxylic acids extracted from strain 537

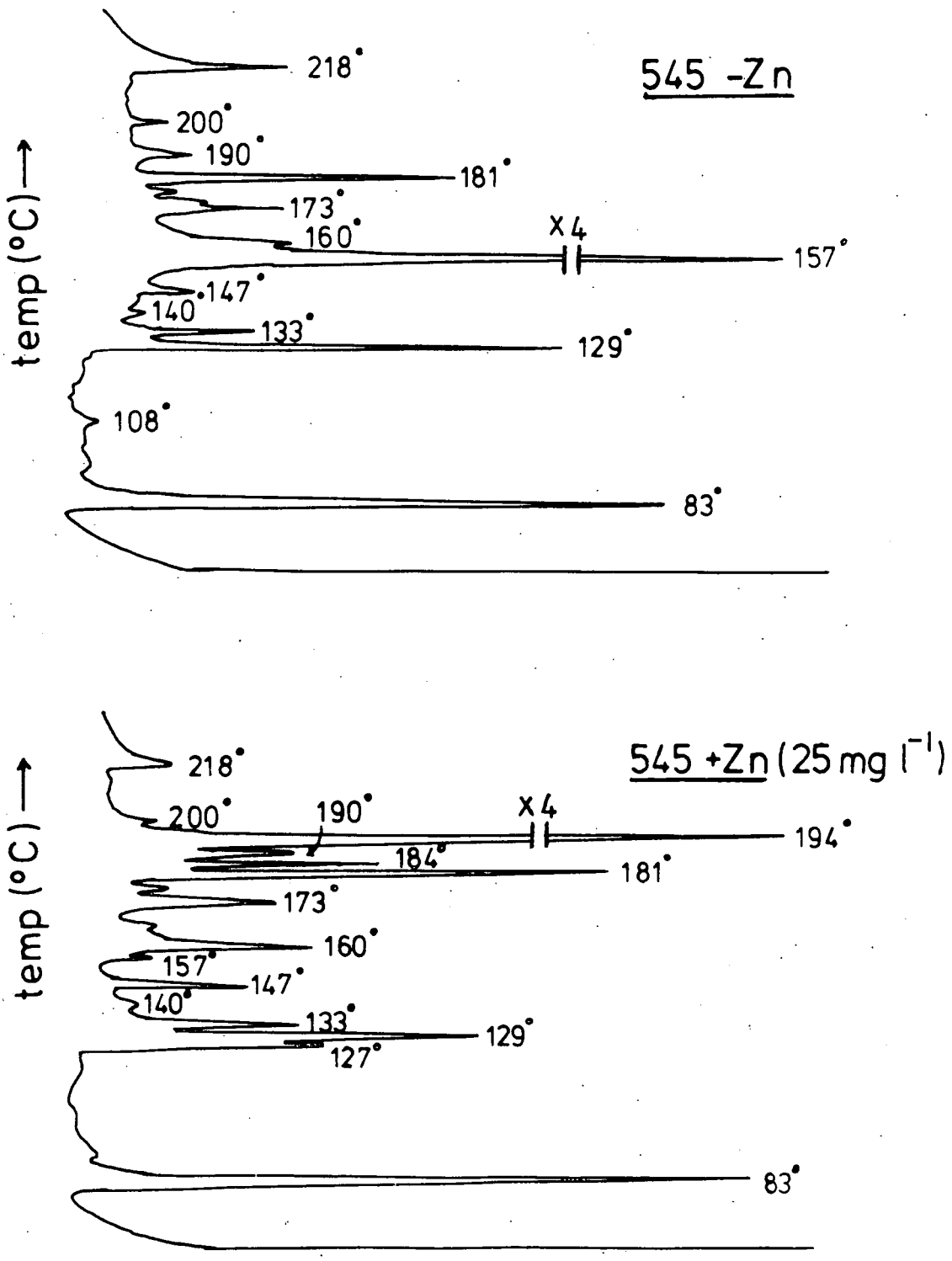


Fig. 7.5 Gas chromatograms of trimethylsilyl derivatives of carboxylic acids extracted from strain 545

The two chromatograms for strain 546 are given in Fig. 7.6; the dry weight equivalent of the injection is similar for both Zn treatments (Table 7.1). The - Zn treatment shows two chromatographic peaks emerging at 119 and 152°C; the + Zn treatment shows a single carboxylic acid peak emerging at 153°C. By injecting onto the chromatographic column a mixture of the extracts of the two Zn treatments, it was found that the peaks emerging at 152 and 153°C for each treatment emerged as a single peak. This strongly suggests that the two peaks indicate the presence of the same carboxylic acid. Neither of the carboxylic acids demonstrated in Fig. 7.6 corresponds to any of those in the standard run (Fig. 7.1).

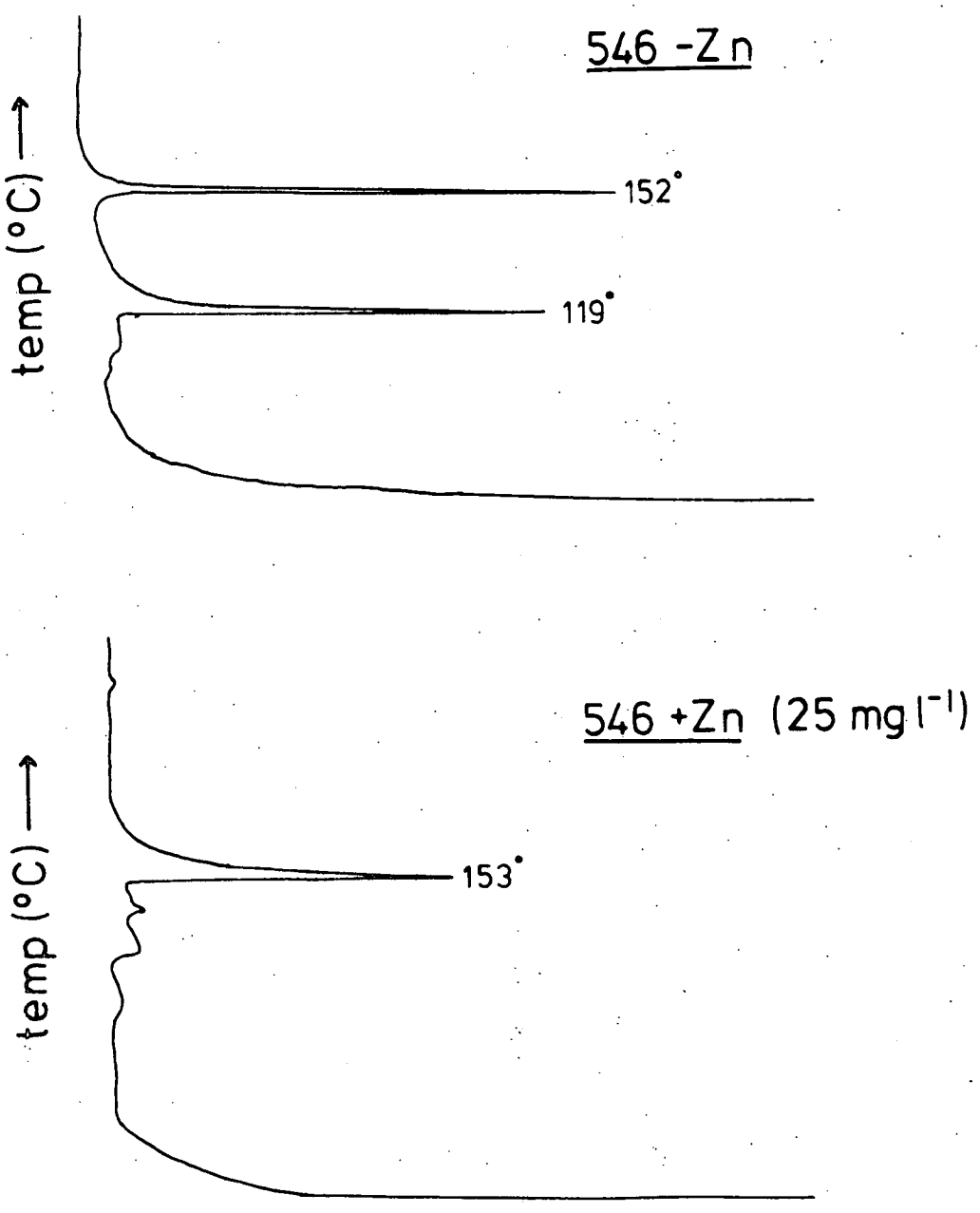


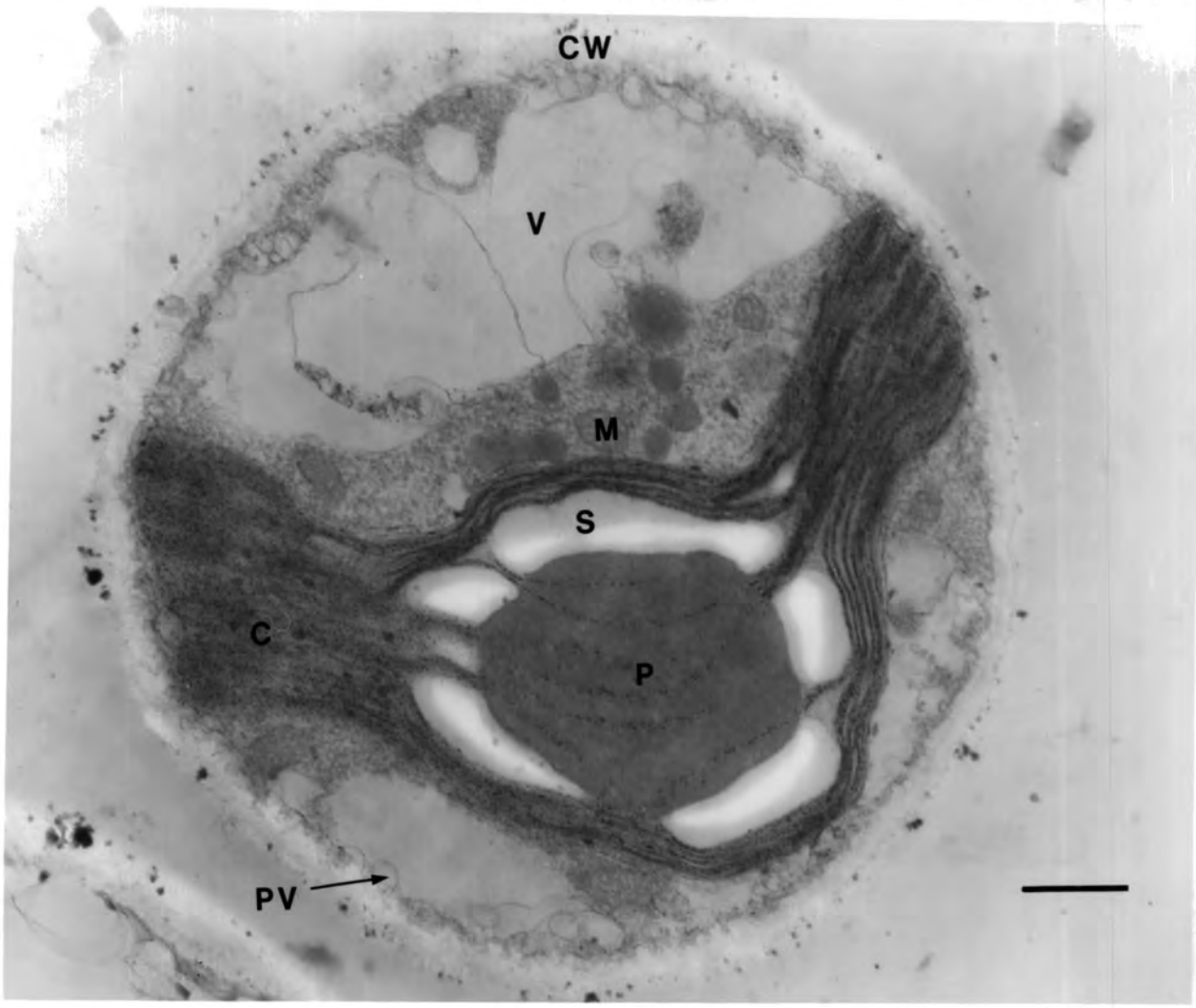
Fig. 7.6 Gas chromatograms of trimethylsilyl derivatives of carboxylic acids extracted from strain 546

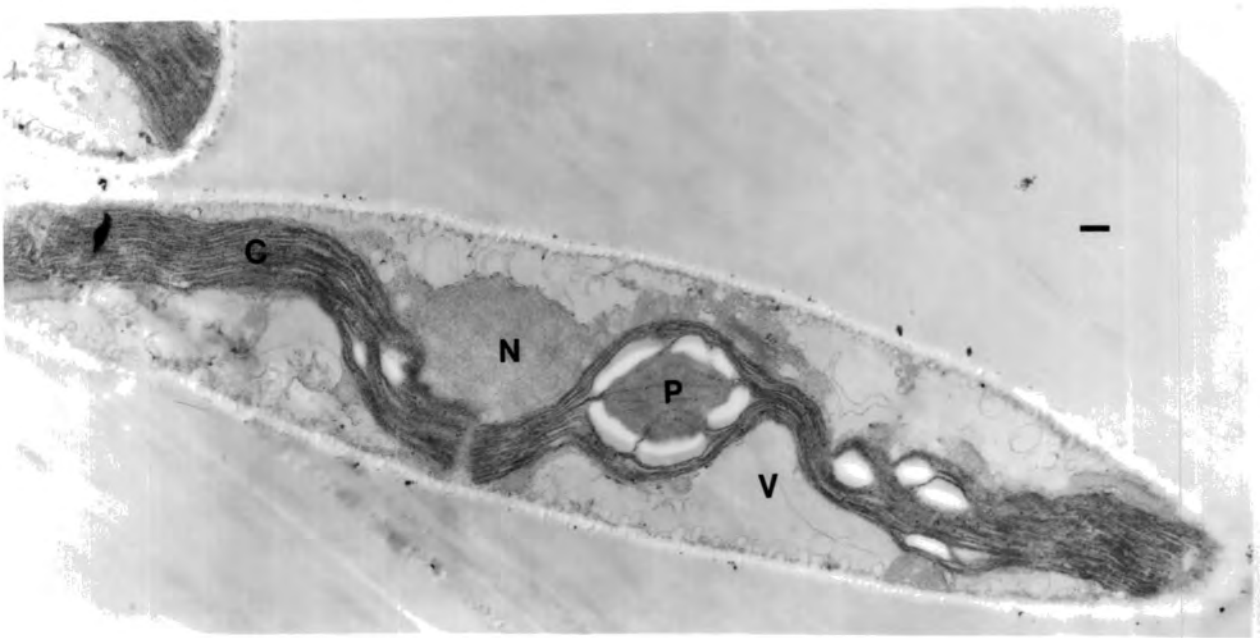
8 ULTRASTRUCTURE 0097 *MOUGEOTIA*

In an attempt to elucidate the resistance mechanism(s) of stream 0097 *Mougeotia* it was intended to examine the alga's fine structure and augment this with energy dispersive x-ray microanalysis (see Silverberg, 1976). It was envisaged that this would identify sites of metal deposition within the cell. Although algae were prepared for fine-structural examination, the facility for x-ray microanalysis became unavailable for use. Preliminary results on the ultrastructure of 0097 *Mougeotia* are presented in this Chapter.

Mougeotia material analyzed for zinc content was found to contain 120 mg g^{-1} dry weight Zn. A portion of this *Mougeotia* was prepared for electron microscopic examination.

Fig. 8.1 is a transverse section of a *Mougeotia* filament through a pyrenoid; Fig. 8.2 shows a longitudinal section of the same alga.


CW
V
M
S
C
P
PV


C
N
P
V

9 DISCUSSION

9.1 Introduction

Both streams 0097 and 3026 can be regarded as "extreme environments" in the sense of Brock (1969). He defines an extreme environment broadly as one in which species numbers and diversity are reduced as a direct consequence of the inability of organisms to adapt to this environment, and it has been shown for streams 0097 and 3026 that many potential colonisers are excluded due to elevated levels of metals in the stream water. A limited number of species have been able to colonise both streams and of these, green algae are able to grow abundantly.

This discussion considers aspects of the biology of these green algae in order to gain a more detailed understanding of their ability to survive in the presence of elevated concentrations of heavy metals.

9.2 Stream 0097

9.21 Environmental chemistry

The water emerging from Caplecleugh Low Level remained at a constant temperature of 9.5°C throughout 1980 (Table 4.1), close to the mean annual air temperature for the region (9°C: Smith, 1970).

Stream 0097 can be regarded as a man-made spring and fits closely the description of a "limestone spring" using the classification system of Odum (1971). Such springs are typically highly buffered, have a pH greater than 7.0, contain relatively high concentrations of dissolved calcium and bicarbonate ions and are typically oxygen saturated. Water entering the groundwater system normally becomes de-oxygenated as it passes through soil and peat; in limestone groundwater systems, however, the open channels, which usually occur in limestone strata, allow the water to re-equilibrate with the atmosphere before emerging at the surface (Davis and De Wiest,

1966). Stream 0097 fulfils the criteria given above, the source of the relatively high concentrations of dissolved calcium and bicarbonate ions in the water (Table 4.1) being the "Great Limestone" (Section 3.12) directly under which Caplecleugh Low Level was driven originally. (Livingstone (1963) quotes the mean concentrations of calcium and carbon (as bicarbonate) in river-water as 15 and 11 mg l^{-1}, respectively.) Open channels, both natural and man-made, exist for the re-equilibration of the water with the atmosphere, resulting in the effluent being fully oxygenated (Section 4.2).

Hem (1972) and Florence (1980) both regard the background concentration of dissolved zinc in uncontaminated waters, as less than 10 µg l^{-1}. 0097 water contained considerably higher concentrations of dissolved zinc (Table 4.1), the source of which was undoubtedly the local mineral veins to which Caplecleugh Low Level offers access (Section 3.11). Elevated levels of dissolved zinc derived from mineralized areas, normally result from the oxidation of sphalerite (ZnS) to soluble zinc sulphate ($ZnSO_4$), as described in Section 1.2. Although galena (PbS) is a common mineral in the underground drainage system of stream 0097 (Section 3.12), values for filtrable lead in the water (Table 4.1) were little above the median level found by Durum (1963) in his survey of rivers of the U.S.A. According to Dunham (1981), at higher pH values oxidation of galena (PbS) generally produces cerussite ($PbCO_3$) which forms an insoluble skin over the sulphide, preventing further oxidation; in this way galena can persist in the zone of oxygenated ground water.

Apart from the elevated zinc concentrations, the most noticeable feature of 0097 water was the constancy of its chemistry throughout 1980 (Table 4.1). The major ions Ca, Mg and SO_4 showed only a slight decrease through the year; this was probably linked with the increase in the water input during the latter part of the year, indicated by the increased current speed (Table 4.1).

This in turn may have been due to a higher than average rainfall in the region during this period (Figs. 3.8, 3.9).

Examination of Table 4.1 shows that a number of physical and chemical properties of 0097 water did vary throughout 1980. Conductivity, in particular, varied from a minimum of 389 $\mu S\ cm^{-1}$ to a maximum of 910 $\mu S\ cm^{-1}$. Hem (1970) states that in aqueous solutions, conductivity is very closely related to the total ionic concentration of the solution; he goes on to point out, however, that "even in relatively simple solutions, the relationships that affect conductance may be complicated". The conductivity results presented in Table 4.1 suggest that, though chemical analyses do not reveal any significant temporal variation in water quality, there was variation in the ionic properties of the water. Using data from a typical water analysis (June; Table 4.1), assuming that all chemical analyses made on the filtrable component of the water refer to dissociated ions, this would give a calculated conductivity of approximately 870 $\mu S\ cm^{-1}$ at 25°C (using a simple conversion of the individual ions given by the American Public Health Association, 1981). Overall chemical concentrations in 0097 water did not vary significantly from the June values (Table 4.1) and therefore it must be assumed that when the conductivity measured was below the calculated value, the chemical components of the water were not completely dissociated into free ions.

For the major cation present in 0097 water, calcium, data presented by Hem (1970) suggest that calcium was supersaturated in the water and would be expected to precipitate as calcite ($CaCO_3$). Again using values from the June water analysis (Table 4.1), based on Hem's equilibrium data the saturated dissolved calcium level in 0097 water is calculated to be 18 $mg\ l^{-1}$. This is considerably less than the filtrable calcium concentration for June of 98 $mg\ l^{-1}$ (Table 4.1). It is possible that calcite may have existed in undissociated form (i.e. colloidal), the differing degrees to

which calcium and bicarbonate ions were dissociated being responsible for the variations in conductivity. Groundwaters supersaturated with respect to calcite are not uncommon according to Hem (1970) and it would seem that equilibrium is slow to be reached; Weber and Posselt (1974) point out that "slow attainment of equilibrium is probably the most significant limiting factor in many applications of thermodynamic solubility relationships, particularly precipitation reactions". Potentially of more importance to the present study, equilibrium data suggest that 0097 water was supersaturated with respect to zinc. Based on figures presented by Hem (1972), zinc would be expected to precipitate both as zinc carbonate (calamine) and zinc silicate (willemite). If the zinc/silicate equilibrium is examined, at saturation the dissolved zinc concentration is calculated to be only 0.02 mg l^{-1} in 0097 water (June data; Table 4.1). Possibly a component of the filtrable zinc values given in Table 4.1 may be due to colloidal zinc. Florence (1980) stated that $ZnCO_3$ and Zn_2SiO_4 can both exist in natural waters as a stable colloidal suspension with an average particle diameter of 1 - 2 nm. This is sufficiently small to pass through the 0.2 μm pore-size filter, used to sample the filtrable fraction for water analysis (Section 2.21).

Zinc silicate is the most likely form in which zinc would precipitate from 0097 water (Hem, 1972). Examining the silicate analyses given in Table 4.1 it can be seen that silicate values vary considerably throughout the year from a minimum of 2.05 to a maximum of 7.40 mg l^{-1} silicon (as silicate). The hetero-polyblue analysis used for measuring silicate in the present study (Section 2.21) measures only filtrable reactive silicate and would not respond to colloidal forms (American Public Health Association, 1981). Possibly variation in the observed values of filtrable reactive silicate reflect variations in the relative amounts of zinc present in colloidal form in 0097 water. (Further discussion of the zinc/silicate interaction,

in 0097 water, is given in Section 9.23.)

Concentrations of filtrable reactive phosphorus in 0097 water were below the detection limits of the analytical technique throughout the year (contamination of the filtration equipment is suspected for the May analysis; Table 4.1). Phosphate released into solution by weathering tends to recombine rapidly with either clay minerals or (as is probably more important in stream 0097) with metal oxides (Hem, 1970). Data presented in Table 4.1 indicate that much the larger phosphorus component of 0097 water was present in the non-reactive fraction, probably either as polyphosphate or as organic phosphate (Stumm & Morgan, 1981).

Based on various equilibrium data, there seems little doubt that 0097 water was supersaturated with respect to $CaCO_3$ (calcite), $ZnCO_3$ (calamine) and Zn_2SiO_4 (willemite). Indirect evidence, including variations in conductivity and filtrable reactive silicate concentrations, suggest that a variable chemical component of 0097 water was in a non-ionic or colloidal form.

X-ray fluorescence analyses of 0097 sediments (Fig. 4.4) indicate the presence of a number of elements. Titanium and zirconium can be regarded as clastic components of the sediment i.e. rock particles resulting from the weathering and removal of more mobile elements (Andrew-Jones, 1968). Manganese, iron and lead can also be considered as being immobile under neutral-alkaline conditions (Andrew-Jones, 1968); calcium, zinc, strontium, cadmium and barium, are the more mobile elements of the sediment. Strontium and barium probably derive from the barium minerals, barite and witherite, which are known to occur as a component of the mineralized veins in the region; these are reported to contain approximately 1% amorphous strontium (Dunham, 1948).

The calcium, zinc, cadmium and lead content of 0097 sediment increased from reach 01 to reach 02 at all times of the year (Table 4.5). The organic component of the sediment increased similarly (Table 4.6) and it seems likely

that these factors are directly related. Förstner and Wittmann (1979) and Burrows (1981) showed that an increase in the organic component of a sediment increases its capacity for metal accumulation.

9.22 Biology

Odum (1971), in his ecological treatise, states that "springs are the aquatic ecologist's natural constant temperature laboratory". Stream 0097 forms an ideal stable environment in which aspects of the biology of a zinc-polluted stream can be studied (Section 4.1).

The complete photosynthetic flora for stream 0097 (Table 4.7) shows a total of 13 species of which 10 are algae. The concentration of zinc in which these algal species were growing is well above that toxic to most algal strains isolated from uncontaminated streams (Say, 1977; Whitton, 1970b). It is likely that zinc is a major selection pressure accounting for the very low numbers of species present in stream 0097. Factors causing environmental stress (including heavy metal pollution) have often been cited as causing a reduction of species numbers in a given environment (Brock, 1969; May, 1974). Whitton and Diaz (1980) discussed the effect of elevated zinc on species numbers, for photosynthetic plants in rivers and streams; they demonstrated, by comparing examples from a wide range of sites (n = 424), that increasing zinc causes a reduction in the number of species present. This is presumably due to selection against those species which are unable to evolve metal tolerance or whose metal tolerance mechanisms are inefficient. Comparison of the present algal species total of 10 (for stream 0097; Table 4.7), with results for the same level of zinc in the scattergram shown by Whitton and Diaz indicates that, even allowing for the high zinc concentration, the species total is relatively low (up to a maximum of 30 species would be expected). In view of the two century long history of stream 0097 (Section 3.13) it seems unlikely that this is due to lack of inocula. Teal (1957)

comments that species numbers in groundwater effluents are usually low. Odum (1971) points out that environments which possess a strong physical stability show a decreased species diversity and similarly a reduction in species numbers; this would apply readily to stream 0097.

Of the photosynthetic species which dominate stream 0097 (Table 4.7) many are characteristic of heavy metal polluted waters and form a community which suggests the influence of heavy metals. Though there are no known algal species restricted to sites of heavy metal pollution, communities at these sites are recognized (Section 1.323). Say (1977), in his survey of zinc-polluted environments throughout Northern England, showed that the majority of species found during the present study in stream 0097 also occurred in the more heavily contaminated of his sites. Mougeotia spp., the dominant organisms of stream 0097, are said by Whitton (1980) to be usually dominant in calcareous, heavy metal polluted streams. Besch et al. (1972) confirm this: they found that the more zinc-polluted sites in a large river system were dominated by Mougeotia (see Section 1.323).

The most visually obvious feature of stream 0097 at certain times of the year was the extremely high standing crop of Mougeotia (see frontispiece); the July standing crop of 200 g m^{-2} dry weight compares closely with values for Cladophora glomerata forming a very dense crop in the River Wear (Whitton, 1970c). A high algal crop is a common feature of heavy metal polluted streams (Whitton, 1980). This phenomenon has also been noted by Klotz (1981) whose river survey showed that the most copper-polluted site, had the highest standing crop of algae. Klotz confirmed that this was not due to an increased algal growth rate but rather, due to a lack of invertebrate grazers removing algae from this site. Stream 0097 similarly had a very sparse invertebrate fauna (Section 3.15) and it is likely that this was the direct cause of the high Mougeotia standing crop observed (Section 4.7); the levels of heavy metals in the Mougeotia filaments (Section 4.3)

presumably rendered them toxic to invertebrates.

Phosphorus deficiency is commonly a major factor in freshwater systems limiting algal growth (Provasoli, 1958). Examination of chemical data for 0097 water shows that levels of filtrable reactive phosphate were particularly low (see Section 9.2). 0097 Mougeotia, however, were shown to possess alkaline phosphatase enzymes (Section 4.8) and were probably able to utilise the (presumed) organic phosphorus (total less filtrable reactive) which formed the major phosphorus component of the water (Table 4.1).

The percentage cover of Mougeotia in stream 0097 was much greater in summer than in winter (Fig. 4.5). This was presumably a reflection of the seasonal pattern of light input since no other environmental factors showed a seasonal pattern. The reason for the marked drop in cover which occurred twice during the summer (Fig. 4.5) has not been identified. One possibility is that the high standing crop may have caused sufficient self-shading of the attached basal parts of the Mougeotia filaments to lead to their detachment and loss downstream. Odum (1957), in his study of a constant temperature spring, showed that photosynthesis of the dominant primary producer (the perennial macrophyte Sagittaria lorata) was correlated with the radiant energy input. It is assumed that during the winter the photosynthetic rate and hence growth rate of 0097 Mougeotia was not sufficient to replace algal material lost downstream. The growth rate of Mougeotia in the field was not measured in the present study; the extreme difficulties of measuring production accurately in lotic environments are outlined by Bombowna (1972). Klotz (1981) approached this problem by culturing algae in dialysis bags attached to the river bed. He found that algae isolated from a copper-polluted reach were sufficiently adapted to copper that their growth rates were not significantly reduced compared to those growing in unpolluted sites; (the concentration of copper at the polluted site was sufficient to severely reduce the growth rate of algae isolated from unpolluted sites). Laboratory

studies indicate that the situation for 0097 algae may be similar. The mean zinc concentration in the field was only slightly higher than the "toxicity threshold" (Section 6.32) for strain 536 growing in CFM; this suggests that the elevated zinc concentration in stream 0097 had little effect on Mougeotia growth rate. The field temperature of 9.5°C was likely to be a major factor in causing a low growth rate. Laboratory studies on strain 536 (Fig. 6.2) suggest a very low growth rate at this temperature; the possibility exists, however, that strain 536 had adapted to laboratory temperatures. Between the June and July collection at stream 0097 the percentage cover of Mougeotia increased from 4 to 90% (Fig. 4.5). This suggests that the Mougeotia biomass increase during this period would require a doubling time of approximately five days (k = 0.2; Section 6.32). This growth rate is based solely on a subjective estimate by the author but is likely, if anything, to be an underestimate as it takes no account of algal material lost downstream during the period. The growth rate given above represents that during the summer - at other times of the year it is likely to be somewhat lower.

No significant differences in water quality were apparent between reaches 01 and 02 (Table 4.1) even when the algal biomass was at its highest. The stream is too short for the biota to influence significantly water quality.

9.23 Accumulation of metals by algae

Plants which have adapted to growing in regions of elevated heavy metals usually do not restrict the uptake of metals into their tissue; the majority of heavy metal tolerance mechanisms so far resolved, involve isolation of metals from cellular sites of metabolic activity rather than exclusion from cells (Section 1.322). Mougeotia spp. growing in stream 0097 were capable of accumulating extremely high concentrations of zinc; values of zinc accumulated, for the period March-June (Table 4.2), are probably the highest

recorded levels of metal in plant tissue (Section 1.322). It is noted that washing Mougeotia filaments in EDTA solution, prior to analysis of tissue levels of metals, had no effect on these levels (Table 4.2). This strongly implies that metals were not bound to the external cell wall (where they would be removed by the strong chelator), but rather they had passed through the cell membrane and were accumulated within the cell.

The form in which metals existed within the algal cells is not clear. X-ray diffraction analysis of dried Mougeotia material would be expected to identify any major crystalline forms of the metal compounds (Section 2.241). Results of X-ray analysis do not reveal an abundant mineral in the dried algal material, suggesting a non-crystalline form of accumulated metals (for further discussion see Section 9.43).

The chemistry of 0097 water throughout 1980 was relatively constant (Section 9.21); the seasonal patterns of individual metal concentrations accumulated by 0097 Mougeotia, however, varied considerably throughout the year (Table 4.2). No significant correlations between levels of metals in water and algae are found (Table 4.4). The seasonal pattern of light input (causing changes in algal growth rate; Section 9.22) also shows no correlation with metal concentrations accumulated by the algae.

Concentrations of the heavy metals, manganese, iron, zinc, cadmium and lead, in 0097 Mougeotia, were found to be correlated positively with concentrations of filtrable reactive silica in the water, recorded for the previous month (Section 4.4). As indicated in Section 9.21, it is believed that high levels of filtrable reactive silica in 0097 water coincided with a reduction in colloidal zinc silicate, therefore releasing more free zinc ions which were available for algal accumulation. Many authors have shown that only free metal ions are available for uptake and accumulation (Section 1.41) and this may explain the pattern of zinc uptake in 0097 Mougeotia; the one month lag

probably represents the period over which the algae were able to equilibrate with the ambient metal ion concentrations. Laboratory study of zinc accumulation by strain 536 growing in CFM (Fig. 6.15) indicate that reduction of zinc below the mean field zinc concentration, causes a marked reduction of zinc concentrations in the algae, corresponding to the variation shown in field material (Section 4.3). The pattern of cadmium accumulation by 0097 Mougeotia (Fig. 4.3) follows closely that of zinc (an element with which it is chemically very similar: Cotton and Wilkinson, 1980); cadmium is probably behaving as a chemical analogue of zinc. The seasonal patterns of uptake of manganese, iron and lead by Mougeotia, form a highly significant positively correlated group (Table 4.4). Similarly to zinc, the pattern of uptake of these metals during 1980 was correlated with the behaviour of silica in the water (Table 4.4); it is unclear whether this was a direct effect of water chemistry or was related to uptake of the predominant metal (zinc).

Table 4.4 indicates an inverse relationship between the heavy metal concentrations, and the sodium and potassium concentrations in Mougeotia digests. Sodium and potassium play a major role in balancing ionic equilibria in plant cell physiology (MacRobbie, 1971) and it is possible that when cell metal levels were elevated in Mougeotia, sodium and potassium ion concentrations were reduced to balance ionic charges.

9.3 Stream 3026

Concentrations of heavy metals in the water of stream 3026 are extremely high. For zinc and cadmium (Table 5.1), a thorough survey of the literature did not reveal values approaching those of stream 3026 (Section 1.2); these metals were not, however, sufficient to prevent growth of photosynthetic organisms in the stream (Table 5.2).

Zinc and cadmium are likely to be present in their divalent ionic forms at pH 5.0 (Hem, 1972). Examination of the equilibrium data summarised by Hem (1970) suggests that none of the dissolved fractions of 3026 water were supersaturated. 3026 water was slightly acid (pH 5.0; Table 5.1); bacterial oxidation of metallic sulphides is probably taking place in the soil leachate, releasing free metal ions and sulphuric acid (Zajic, 1969). The major anionic species in stream 3026 is therefore likely to be sulphate (based on oxidation of sulphide ores and also analysis of the Ruisseau de Creuzet; Chapter 5).

The number of algal species in stream 3026 was low. It is likely that elevated metal concentrations are playing a major role in eliminating many potential colonisers (see Section 9.22). Three algal species were recorded from direct examination of material collected in the field; laboratory isolation however, yielded a total of 7 species and, including Pinnularia subcapitata (recorded in the field), this gives a total of eight species for the stream. The dominant organism in the field was Hormidium rivulare (Table 5.2), a species which is typically dominant in highly zinc-polluted aquatic environments (Section 1.323). H. rivulare formed a visually obvious algal mat (Fig. 3.5) and it is likely that this high biomass resulted from a lack of invertebrate grazing (c.f. the situation for Mougeotia in stream 0097; Section 9.22). H. rivulare isolated from stream 3026 (strain 537) shows a very slow growth rate at field zinc concentrations in the laboratory (Fig. 6.9), though the growth medium does not resemble closely stream water. The complex series of interactions which are likely to exist between ionic species in the stream water (Section 6.4) may allow for a faster growth rate. In addition stream 3026 has a very low flow (seepage; Section 3.2) and during periods of rainfall it is likely that rainwater will dilute the streamwater, allowing a more rapid growth of the H. rivulare mat.

9.4 Studies on isolated strains of green algae

9.41 Zinc toxicity

Toxicity studies indicate a high degree of zinc resistance by all green-algal strains isolated from streams 0097 and 3026 (Table 6.1). Say et al. (1977) assayed the zinc tolerance of a range of Hormidium strains isolated from sites of varying zinc concentration. They showed that tolerance in the laboratory (in Chu 10 E medium) was highly correlated with the logarithm of the field zinc concentration; strains subcultured in the absence of zinc showed no change in their tolerance to the metal. It is probable that all strains assayed in the present study are genetically adapted to elevated zinc levels. Strains of Hormidium isolated from stream 3026 show a much higher resistance to zinc than any of those assayed by Say et al.; levels of zinc in stream 3026 were, however, almost two orders of magnitude higher than any examined by Say et al.

The lethal zinc concentration for many of the stream 3026 strains (Table 6.1) is lower than that found in the field (Table 5.1). These data, however, do not consider other factors which may be important in affecting the toxicity of zinc in the field (see Section 9.42).

All algal strains tested during the present study showed that in minimal zinc medium (0.03 mg l^{-1} Zn occurs as contaminant; Section 2.741), there was no evidence of reduced growth due to zinc deficiency (Table 6.1). It seems unlikely, therefore, that there is an increased requirement for zinc in these tolerant strains, as has been shown for some zinc - tolerant higher plants (Mathys, 1980).

The zinc dose response curves for strains 536, 537 and 545 (Fig. 6.9) all fit closely the theoretical curve indicated by Berry and Wallace (1981; see Fig. 6.8) for the response of an organism to a single toxicant. Comparison

of the two curves for strains 537 and 545 show that at lower zinc concentrations strain 545 shows a higher degree of resistance than strain 537, at higher zinc concentrations the situation is reversed; this illustrates the importance of representing the complete dose response curve when comparing the effect of a toxicant on a range of organisms.

Strain 536, in both Chu 10 E medium and CFM, shows almost maximal growth in zinc concentrations corresponding to the mean field zinc concentration (6.84 mg l^{-1}; Fig. 6.9). It would seem that the growth of Mougeotia in stream 0097 is not reduced by the elevated zinc levels in the water, rather the algae have been able to adapt completely to this. The slope of the dose response curve for strain 536 in the "toxic zone" is much less in CFM than in Chu 10 E; it is likely that many of the ionic species present at higher concentrations in CFM are able to ameliorate zinc toxicity (see Section 9.42). Factors which reduce metal uptake are often regarded as important in reducing metal toxicity. It is noted, however, that accumulation of zinc by strain 536 is higher in CFM than in Chu 10 E though the toxicity of zinc is decreased. A possible explanation for this phenomenon is discussed in Sections 9.43 and 9.44.

The 0 mg l^{-1} zinc growth curve for strain 545 (Fig. 6.7) shows a high degree of cell mortality over the first three days of growth. If the curve is projected back to the x-axis this gives an estimated viable inoculum of approximately 10^2 cells ml^{-1}. The reason for the shape of this curve is not clear. It is possible that, for this strain, selection for an increased zinc requirement had taken place and the growth curve represents the growth of a small proportion of cells which had not developed this requirement. The fact that the 0 mg l^{-1} zinc value of growth rate does not fit the standard dose response curve for strain 545 (Fig. 6.9) suggests that the growth curve for this treatment may represent a transformation of the strain described by

the other treatment curves in Fig. 6.9.

It is noted that the toxic zone for each dose response curve is linear with respect to the logarithm of the toxicant concentration (Fig. 6.9). This relationship has often been noted in toxicological studies (Bliss, 1935; Sprague, 1969) and many texts advocate the use of a logarithmic scale of a toxicant in toxicological studies on an a priori basis (Finney, 1947; Kjellstrom, 1976). Few studies of the effects of heavy metals on algae present sufficient data to construct an accurate dose response curve, though Sunda and Guillard (1976) present data for the toxicity of copper to Thalassiosira pseudonana (diatom) and Nannochloris atomus (green alga) which clearly fit the standard dose response curve (Berry and Wallace, 1981; Section 6.32) when growth rate is related to the logarithm of the copper ion concentration. In the many reports given in the standard toxicological literature (reviewed by Sprague, 1969) no explanation is offered as to why a curved relationship between a toxicants concentration and the target organisms response, should exist. The present author believes that it is reasonable to assume that toxicity can be related linearly to the chemical potential of a species. The chemical potential can be related to the activity of a chemical species by the following formula:

$$u_1 = u_1^o + RT \ln \frac{a}{a_o}$$

where u_1 = the chemical potential and RT is constant in a constant pressure/temperature atmosphere. The activity (a) = γC where γ is the activity coefficient and C is the concentration of the species. In solutions of low ionic strength γ tends to unity, thus a = C. This allows

$$u_1 = u_1^o + RT \ln \frac{C}{C_o}$$

which shows that the chemical potential of a species is related logarithmically to the ionic concentration (formulae from Stumm and Morgan, 1981). In support of this Say et al. (1977) and Harding and Whitton (1976)

showed, for a range of algal strains of Hormidium and Stigeoclonium respectively, that zinc resistance was more directly correlated with the logarithm of the field zinc concentration (and therefore the chemical potential) than directly to the field zinc concentration.

9.42 Influence of environmental factors on zinc toxicity

As described in Section 6.4 most studies of environmental toxicology deal simply with the response of a target organism to a single toxicant, usually under artifical laboratory conditions. Section 6.4 presents the results of experiments which were designed to assay the importance of certain physio-chemical properties of streams 0097 and 3026, which may influence the toxicity of zinc to the indigenous algal strains.

Water hardness properties (magnesium, calcium) are generally regarded as competitive inhibitors of zinc uptake and therefore lead to a reduction of zinc toxicity (Section 1.321). Magnesium is seen to reduce the toxicity of zinc to both strains 536 and 537; calcium, however, is only important in this respect for strain 536 (Fig. 6.10). The ratio of both magnesium and calcium concentrations to zinc concentration in stream 0097 was high and both are likely to act as important competitive inhibitors of zinc activity, therefore reducing toxicity. This does not, however, appear to be linked to a reduction of zinc uptake. As shown in Fig. 6.15, strain 536, incubated in CFM, shows both an increased resistance to zinc coincidental with an increased uptake of the metal (in comparison to incubation in Chu 10 E). It is likely that the presence of relatively higher concentrations of magnesium and calcium in CFM are factors causing the reduction of zinc toxicity though uptake is increased (see Section 9.43). Calcium is known to be important in stabilising membranes (Poovaiah and Leopold, 1976) and it is likely that its presence in algal media aids in the protection of cellular membranes; membrane function is known to be particularly impaired by toxic metals

(Pritchard, 1979). Environmental levels of magnesium and calcium in stream 3026 were much lower than those of zinc. For this reason it is unlikely that either metal could be significantly active as a zinc competitor. Calcium, at the field concentration, does not appear to influence zinc toxicity to strain 537 (Fig. 6.10); magnesium, however, remains important in reducing zinc toxicity to this strain. Say et al. (1977) and Harding & Whitton (1977) performed laboratory studies showing that, for Hormidium and Stigeoclonium respectively, magnesium was important in reducing zinc toxicity in zinc-resistant strains but had much less influence on the toxicity of zinc to zinc-sensitive strains. It is apparent that magnesium is important in reducing the influence of zinc to zinc-resistant algae though evidence suggests that this is not due to competition with zinc ions (see Section 9.44).

Many studies have shown that phosphate is able to antagonize zinc toxicity (Section 1.321) though, as indicated in Section 9.21, levels of free phosphate ions in heavy metal polluted environments are usually low. Phosphate ions showed the ability to reduce zinc toxicity to strain 536 (Fig. 6.10); phosphate levels in stream 0097 are extremely low compared to those in this experiment and it is unlikely that phosphate is able to influence zinc toxicity in the field.

During the present study special emphasis was placed on the interaction of zinc and cadmium in studies of metal toxicity; the reasons for this are outlined in Section 6.4. For strains 537 and 545 Figs. 6.12 and 6.13 respectively, demonstrate that zinc was able to ameliorate cadmium toxicity; at higher zinc concentrations cadmium had no significant influence on growth of these strains. The in vivo interaction of zinc and cadmium also leads to a reduction of the toxicity of cadmium to strain 537 (Fig. 6.14: Section 6.4).

A zinc/cadmium antagonism is generally regarded as being due to the less toxic metal (zinc) being able to uncouple cadmium from metalloenzyme systems and this has been shown for both plants and animals (Falchuck et al., 1975). The existence of a synergistic interaction between these metals, however, has been demonstrated for a number of plant species (Section 1.321); though field levels of cadmium do not influence the effect of zinc on strains 537 and 545, this type of interaction cannot be assumed for other algal strains growing in the presence of both metals.

No marked interaction of zinc and cadmium is noted for strain 536; there is no evidence that field levels of cadmium influence growth of strain 536 at any levels of zinc tested (Fig. 6.10).

Environmental pH has previously been shown to be an important factor affecting the toxicology of metals (Section 1.321). Stream 0097 showed a limited variation of pH throughout 1980 (Table 4.1) and it is likely also, due to the high levels of solutes, that stream 3026 is sufficiently buffered to prevent large pH changes. Data presented in Fig. 6.11 show that pH variation of up to one pH unit each side of the field pH, has little effect on the toxicity of zinc to strain 537. Though wider pH variation might prove to influence the toxicity of zinc to strain 537 it is unlikely that these conditions will occur in the field.

Acidophilic organisms tend to be able to tolerate high levels of heavy metals, probably due to binding of hydrogen ions in competition with metallic ions (Section 1.321). Though stream 3026 was acid (pH 5.0), the above role of hydrogen ions is unlikely to be important. The extreme levels of zinc in this stream (Section 9.3) gave a Zn^{2+} :H^{+} molar ratio of 6000:1; in competition reactions, therefore the influence of hydrogen ions are likely to be negligible.

9.43 Accumulation of zinc

Concentrations of zinc accumulated by 0097 Mougeotia probably represent the highest levels of metals in plant tissue recorded in the literature. Zinc constitutes up to 30% of the filament dry weight in 0097 Mougeotia, presumably as an insoluble non-toxic compound. The form in which zinc exists in the Mougeotia tissue has not been identified in the present study; there are a number of indications, however, as to its likely nature. As shown in Table 4.2, an EDTA wash does not significantly reduce metal levels accumulated by 0097 Mougeotia (in comparison to distilled water washed material) and this indicates that zinc is not adsorbed to the external cell surfaces. X-ray diffraction analysis of dried 0097 Mougeotia shows a very high background of reflected X-ray energy (Section 4.3), suggesting a mainly amorphous structure of the component metal salts. This situation is supported by electron microscopic evidence (Chapter 8) which indicates no crystalline metal deposits within the filaments.

The majority of studies in which the form of deposited metal in plant tissue has been identified, show that metals are either deposited as insoluble organic salts (e.g. oxalates, glucosides) or as metallo-proteins (see Section 1.322). In the case of 0097 Mougeotia, however, large organic deposits within the cells are unlikely from both an energetics point of view (large zinc deposits in the cells do not appear to have a significant effect on algal growth rate; comparison of Figs. 6.5 and 6.15) and also an anionic ligand of a large ionic weight would not allow the deposition of zinc at levels of up to 30% of the cell dry weight. Zinc is therefore probably deposited as an inorganic salt; the salts $Zn(OH)_2$, $ZnCO_3$ and Zn_2SiO_4 are all readily precipitated in slightly alkaline conditions (Hem, 1972) and these are the three most likely forms of zinc in 0097 Mougeotia (all are known to form amorphous colloids; Florence, 1980). Though magnesium is thought to be directly involved in zinc tolerance (Section 9.42), Fig. 4.3

shows that magnesium and zinc levels in 0097 Mougeotia are not correlated and therefore the mode of action of magnesium does not involve co-precipitation with zinc. As described in Section 9.42, accumulation of zinc by strain 536 is much greater in CFM than in Chu 10 E medium. With respect to anionic species available for co-accumulation with zinc, the major difference between the two media is the greater levels of bicarbonate ions in CFM (Section 2.741); zinc carbonate is suggested as the likely form in which zinc is deposited in 0097 Mougeotia (for further discussion see Section 9.44).

Levels of accumulated zinc in strain 537 appear relatively low; even in 3000 mg l^{-1} zinc, only 3% of the cell dry weight consists of zinc (Fig. 6.16). These values are low when compared to those shown for strain 536, even when growing in considerably lower levels of zinc (Section 4.3). Zinc concentrations found in Hormidium collected from the field, were, however, lacking (Section 2.62). As discussed above for 0097 Mougeotia, levels of zinc in the alga when grown in artificial medium were considerably lower than those of Mougeotia growing in the field. This suggests that values derived from zinc accumulation studies using strain 537 (growing in Chu 10 E medium) should be treated with caution (see Section 9.44).

9.44 Resistance mechanisms

Analysis of the carboxylic content of algae (Chapter 7) reveals little concerning their resistance mechanisms. Mathys (1980) was able to show for a number of higher plant species, that malic acid was produced in response to external zinc levels; this was active in binding, and therefore detoxifying, the zinc. Carboxylic acid analyses presented in Section 7.2, do not indicate the presence of malic acid in any of the algal strains tested; it is thought likely, however, that interference may have rendered carboxylic acid analyses inaccurate. (Metals are known to interfere with

the analysis of carboxylic acids (Redgewell, 1980) and though the use of cation exchange columns should prevent this, it is possible that zinc levels were too high for these to be completely effective.)

Probably the only satisfactory carboxylic acid results are those for strain 545 (Fig. 7.5) which show many similar acid peaks for both zinc treatments. This suggests that any differences between the two chromatograms are not due to zinc interference in the analysis. Exposure of strain 545 to zinc appears to change the dominant carboxylic acid present, from one emerging from the column at 157°C, to one emerging at 194°C. These acids were, however, not identified and therefore it is not possible to comment on their specific roles.

The potential importance of magnesium in the development of zinc resistance is discussed in Section 9.42. Magnesium has been shown to ameliorate the toxic effects of many heavy metals (Section 1.322) and this has generally been assumed to be due to competition for uptake sites between magnesium and the metal concerned (Braek et al., 1976). As seen for strain 536, however, increased zinc resistance in CFM (compared to Chu 10 E medium) is related to increased zinc uptake (Section 9.42). It is noted that electron micrographs of 0097 Mougeotia (Figs. 8.1 and 8.2) show a high number of pinocytotic vesicles deriving from the external cell membrane; these suggest that material is being transported into the cell from the external medium (Bennet, 1956). It is probable that controlled transport of zinc into 0097 Mougeotia is important in conferring resistance (magnesium may act to maintain the integrity of transport vesicles). It is postulated in Section 9.43, that zinc is deposited within the Mougeotia cells as zinc carbonate. The reduction of both zinc resistance and accumulation which occurs when strain 536 is grown in Chu 10 E medium may be due to a deficiency of bicarbonate ions in this medium; these would be required to combine with the zinc to allow

uptake and accumulation of high levels of zinc carbonate within the cells. (Free zinc ions are likely to be much more toxic than a combined form of the metal; Section 1.31.)

As discussed in Section 9.43 concentrations of zinc accumulated by strain 537, are extremely low. Whether this is due to an exclusion mechanism (as has been shown for some copper resistant algal strains; Section 1.322) is unclear. It has been demonstrated for strain 536 that when incubated in Chu 10 E medium, accumulated zinc levels bear no relation to those found in the same alga growing in the field. The absence of field accumulation data from stream 3026, therefore, prevents further interpretation of laboratory data for strain 537.

9.5 Concluding remarks

During the present study the ecology of the two zinc-polluted streams has been described. Zinc was shown to be important in reducing the number of species able to colonise these streams, though a number of organisms (particularly green algae) had been able to adapt to the elevated zinc levels.

Mougeotia spp. growing in stream 0097 were shown to accumulate extremely high concentrations of zinc from the stream-water. Variation in accumulated concentrations of zinc were not correlated with variations of the filtrable zinc concentration in the water. Indirect evidence suggested that variation in the zinc speciation was the important factor affecting its biological uptake. This indicates that to gain a more detailed understanding of the behaviour of zinc in aquatic systems a more elaborate analysis of zinc is required, than was performed during the present study. Florence (1980) presents a review of the various methods used to measure the different chemical forms of zinc in natural waters and it is considered that some

differentiation of the various zinc species would be highly advantageous when studying its behaviour in freshwater (particularly in waters with a pH of greater than 7 where complexation occurs more readily; Hem, 1972). The metal composition of field populations of algae has often been recommended as a useful monitor of contamination of the surrounding water (Section 1.43). The metal composition of 0097 Mougeotia appeared to be responding primarily to variations in the speciation of zinc. This phenomenon probably explains some of the variation found when previous workers (investigating the use of plants as monitors) have sought for a direct relationship between levels of metals in plants and their natural medium (e.g. Whitton et al., 1982). As pointed out by Empain et al. (1980), however, metal accumulation by a plant gives a better indication of the fraction of the metal in the environment which is likely to affect the aquatic ecosystem. 0097 Mougeotia are reflecting the variation of this available metal and therefore may prove a more reliable pollution monitor than simple water analyses.

Practical systems have been suggested for the removal of heavy metals from mine and industrial effluents by encouraging the growth of algae (see Section 1.44). The high metal capacity of 0097 Mougeotia and also the high biomass of algae allowed by the absence of grazers, suggest that systems of this type may be feasible. Simple meander systems built into potentially hazardous metal effluents could lead to a substantial reduction of free metal ions, via algal accumulation. Filip et al. (1979) were able to show that an algal/sand-filtration system was able to effectively remove 70 - 90% of the copper and cadmium from the input water in a pilot plant.

This study has centred upon equating the performance of algae growing in the field, with that in the laboratory. It has been shown that use of artificial media may lead to completely misleading results (Section 9.43), which bear no relation to the field situation for a given alga.

By designing a specific growth medium related to field conditions this problem has been part overcome during the present study (Section 6.2), allowing legitimate comparisons between laboratory and field data. The use of continuous culture would further facilitate these comparisons, though the method of culturing algae in the water body from which they were isolated (in sealed dialysis bags; Klotz, 1981), could prove even more valuable.

SUMMARY

a) The chemistry and flora were studied in two streams containing elevated concentrations of heavy metals in the water; this had not prevented, however, the development of a plant community in neither case. Green algae in particular were able to grow abundantly in both streams.

b) One stream (Durham code no. 0097), a mine effluent, was sampled monthly throughout 1980. This stream drained from an abandoned Zn/Pb mine in the Northern Pennine Orefield and lay in the catchment area of the River Tyne, England. Water analyses revealed that the physical and chemical properties of the water were relatively constant throughout the year (e.g. temperature remained at a constant 9.5°C) suggesting a deep groundwater source for the effluent. The water was calcareous (mean Ca = 95.8 mg l^{-1}), had a high pH (mean = 7.85) and contained a mean Zn concentration of 6.84 mg l^{-1}. Both Ca and Zn were supersaturated in the water.

The flora was dominated by Mougeotia spp. which were able to accumulate extremely high concentrations of heavy metals, particularly Zn (maximum of 316.5 mg g^{-1} Zn dry weight), from the stream water. A large degree of temporal variation occurred in these concentrations of accumulated metals which did not appear to reflect changes in the overall concentrations of metals in the stream water. Indirect evidence, however, suggests that this variation is due to differing amounts of colloidal zinc silicate in the water - the colloidal form of Zn being unavailable for uptake by the algae.

Concentrations of Na and K in 0097 algae were shown to vary inversely with those of heavy metals in the algae (throughout 1980). It is

possible that Na and K are active in balancing ionic equilibria in the Mougeotia cells, in response to varying levels of heavy metals.

c) A second stream (Durham code no. 3026) was sampled once and was found to contain extremely high concentrations of heavy metals particularly Zn and Cd (Zn = 3840 mg l^{-1}; Cd = 345 mg l^{-1}). Stream 3026 drained from a Zn smelter tip in the River Lot catchment area, S.E. France. Three algal species were reported to be growing in the stream; of these Hormidium rivulare was dominant.

d) Both streams 0097 and 3026 contained a high algal biomass. It is suggested that this was due to the lack of invertebrate grazers in these metal-contaminated streams. Similarly the number of algal species found in these streams was relatively low, presumably in part due to elevated levels of heavy metals (particularly Zn) selecting against those species unable to evolve metal resistance.

e) Nine strains of green algae were isolated into axenic culture from streams 0097 and 3026. Culture studies strongly suggested that all were genetically resistant to elevated Zn concentrations. The growth rate of isolated algae was found to decrease linearly when plotted against the logarithm of the Zn concentration in the growth medium.

f) The toxicity of Zn to strain 536 (Mougeotia sp. isolated from stream 0097) was markedly reduced by field concentrations of Mg and Ca. For strain 537 (H. rivulare isolated from stream 3026) Zn toxicity was reduced by field concentrations of Mg, though Ca had no similar effect. It is suggested that Mg is an important factor in the Zn resistance mechanism developed by these green algae.

g) Field concentrations of Cd had no influence on the growth of isolated algal strains in the presence of field concentrations of Zn. It is likely that Zn is competing with the more toxic metal, Cd.

h) For 0097 Mougeotia resistance to Zn seems to involve controlled accumulation of high concentrations of an insoluble Zn salt (perhaps zinc carbonate). Reduced concentrations of carbonate in the algal growth medium led to reduced levels of accumulated Zn. Zn, however, becomes more toxic in this circumstance, possibly due to the prevention of compartmentation of the metal in the algal cells.

i) The role of carboxylic acids in the Zn resistance mechanism of green algae was studied but remains unclear. Evidence for strain 545 (Stichococcus bacillaris isolated from stream 3026), however, shows that Zn causes a major shift in cellular carboxylic acids. Whether this is a component of the resistance mechanism in this alga, is not certain.

j) The potential use of algae to both monitor heavy metals, and also to remove heavy metals from polluted effluents, is discussed.

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