Durham E-Theses

Physiological studies on heavy metals and blue-green algae

Shehata, F. H. A.

How to cite:

Shehata, F. H. A. (1981) *Physiological studies on heavy metals and blue-green algae*, Durham theses, Durham University. Available at Durham E-Theses Online: http://etheses.dur.ac.uk/7508/

Use policy

The full-text may be used and/or reproduced, and given to third parties in any format or medium, without prior permission or charge, for personal research or study, educational, or not-for-profit purposes provided that:

- a full bibliographic reference is made to the original source
- a link is made to the metadata record in Durham E-Theses
- the full-text is not changed in any way

The full-text must not be sold in any format or medium without the formal permission of the copyright holders.

Please consult the full Durham E-Theses policy for further details.

Academic Support Office, The Palatine Centre, Durham University, Stockton Road, Durham, DH1 3LE
e-mail: e-theses.admin@durham.ac.uk Tel: +44 0191 334 6107
http://etheses.dur.ac.uk

PHYSIOLOGICAL STUDIES ON HEAVY METALS AND BLUE-GREEN ALGAE

by

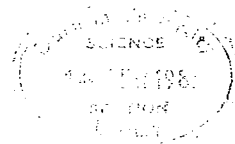
F. H. A. SHEHATA

(B.Sc. Riyadh - Saudi Arabia)

The copyright of this thesis rests with the author.
No quotation from it should be published without
his prior written consent and information derived
from it should be acknowledged.

A Thesis submitted for the degree of Doctor of Philosophy
in the University of Durham

Department of Botany, January 1981



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.

F. H. A. SHEHATA

TABLE OF CONTENTS

page

ABSTRACT 7

ACKNOWLEDGMENTS 8

ABBREVIATIONS 10

LIST OF TABLES 11

LIST OF FIGURES 17

CHAPTER 1 INTRODUCTION 21

1.1 Occurrence of blue-green algae in environments rich in heavy metals 21

1.2 Laboratory tolerance and toxicity 22

1.21 Is zinc essential for growth? 25
1.22 Effect of zinc on nitrogen fixation 26

1.3 Adaptation 27

1.31 Antibiotics 28
1.32 Morphological mutants 29
1.33 Metals 29

1.4 Factors influencing toxicity of metals 30

1.5 Accumulation of metals 34

1.6 Aims 35

CHAPTER 2 MATERIALS AND METHODS 37

2.1 Culture techniques 37

2.11 Cleaning of glassware 37
2.12 Culture vessels 37
2.13 Sterilization 37

2.2 Media 38

2.21 Mineral nutrients 38
2.22 Buffer 42
2.23 Chelating agent 44
2.24 Materials used for toxicity tests 44

2.3 Subculturing 47

2.31 Liquid 47
2.32 Solid 48
2.33 Incubation 48

2.4 Algal cultures 49

2.41 Origins 49
2.42 Isolation and purification 51

2.421 Physical
2.422 Antibiotics 52
2.423 Tests of purity of the cultures 52

page

2.5 Preparation of alga for assay 52

2.51 Estimation of growth 52

2.511 Unit counts and estimation of cell length 52
2.512 Extractions and estimation of pigments 53
2.513 Dry weight 56
2.514 Turbidity 58

2.52 Techniques used for comparison of toxicities 58

2.6 Production of resistant *Anacystis* strain 62

2.61 NTG 62
2.62 Serial subculture 63

2.7 Study of environmental factors 63

2.71 Factors influencing toxicity 63
2.72 Inoculum size 64
2.73 Factors influencing zinc solubility 64

2.8 Experimental procedure for accumulation studies 65

2.81 EDTA washing 65
2.82 Acid digestion 71

2.9 Special techniques 71

2.91 Filtration 71
2.92 Atomic absorption spectrophotometry 72
2.93 Acetylene reduction assay 72

CHAPTER 3 PRODUCTION OF RESISTANT STRAINS OF *ANACYSTIS NIDULANS* 75

3.1 Introduction 75

3.2 NTG 75

3.3 Serial subculturing................................. 75

3.31 Metals 75
3.32 Antibiotics 86

3.4 Morphology 86

CHAPTER 4 TOLERANCE AND TOXICITY STUDIES ON *ANACYSTIS NIDULANS* STRAINS 93

4.1 Metals 93

4.11 Cobalt 93
4.12 Nickel 93
4.13 Copper 106
4.14 Zinc 106
4.15 Cadmium 106
4.16 Mercury and lead 106

4.2 Antibiotics 130

4.3 NTG 130

4.4 Pigment composition 130

4.5 Zn requirement for growth 133

page

CHAPTER 5 EXPERIMENTAL FACTORS AFFECTING TOXICITY OF METALS TO *ANACYSTIS NIDULANS* 136

5.1 Introduction 136

5.2 Influence of complementary cations and anions 136

5.3 Influence of major cations 139

5.4 Influence of major anions 151

5.5 Influence of organic compounds 158

5.6 Influence of other heavy metals 166

5.7 Influence of pH 166

5.8 Influence of inoculum size 174

CHAPTER 6 ACCUMULATION OF ZINC BY *ANACYSTIS NIDULANS* 177

6.1 Introduction 177

6.2 Influence of culture density on removal of Zn from medium 179

6.3 Influence of environmental Zn concentrations on Zn accumulation by *Anacystis nidulans* 179

6.4 Release of Zn from *Anacystis* by washing with EDTA 188

CHAPTER 7 STUDIES ON ZINC RESISTANCE OF STRAINS ISOLATED FROM ZINC POLLUTED SITES 191

7.1 Introduction 191

7.2 Origins, isolation and culture 191

7.3 Toxicity of Zn and Cd under standard conditions 191

7.4 Factors influencing toxicity 196

7.5 Nitrogen fixation 204

CHAPTER 8 DISCUSSION 206

8.1 Production of resistant strains 206

8.2 Laboratory tolerance and toxicity 210

8.3 Environmental factors influencing metal-toxicity to *Anacystis nidulans* strains 214

8.31 Organic compounds in the medium 217

8.4 Accumulation of Zn by *Anacystis nidulans* 218

8.5 Tolerance and adaptation shown by field material.... 219

8.51 Nitrogen fixation 221

8.6 Concluding remarks 221

page

SUMMARY 223

REFERENCES 227

APPENDIX   Contents list A3.0 240
                           A4.0 241
                           A5.0 242
                           A6.0 243

Abstract

Mutants of *Anacystis nidulans* tolerant to high levels of Co, Ni, Cu, Zn and Cd were obtained by repeated subculturing at strongly inhibitory levels of metal. For instance, the level of Zn at which strong inhibition occurred was raised from 1.45 to 16.5 mg l^{-1} Zn after 75 subcultures. Isolates resistant to 5.0 mg l^{-1} Zn and 12.0 mg l^{-1} Zn maintained their resistance for at least 72 cell generations in the absence of Zn, though there was subsequently an increased lag during the first subculture back to high Zn levels. This and plating experiments indicate that the strains are mutants. Assays of cross-resistance of each of the five types of mutant were made to the other four metals. In most cases changes in cross-resistance were only slight, with about equal numbers of examples of increased and decreased resistance. Examples of marked changes were increased Co-resistance of a Cd-tolerant strain and decreased Cd-resistance of a Ni-tolerant strain. The environmental factors influencing toxicity were investigated for Cu, Zn and Cd. Increases in Ca, Mn, Fe and P reduced Zn toxicity to both wild-type and Zn-tolerant strains, but the two differed in their response to pH. Effects on morphology were evident at high metal levels with all strains. In most cases increased levels of metal led to the formation of filaments, but with Cu subspherical structures sometimes made up most of the population. Uptake from media enriched 0.1 and 1.0 mg l^{-1} Zn was similar in both wild-type and Zn-t12.0, whether judged by total Zn accumulated or by that remaining after EDTA washes.

Isolates from high Zn sites were found in general to tolerate considerably higher levels of Zn than laboratory research strains (presumably isolated from environments not enriched with Zn). A comparison of the influence of Zn on nitrogen fixation by a strain from low zinc site (*Anabaena cylindrica*) and one from a high Zn site (*Calothrix* D184) showed only a slight difference when Zn was first added, but a pronounced effect after 24 h.

ACKNOWLEDGMENTS

It is a pleasure to acknowledge everyone who has helped me during this work. I am extremely grateful to Dr B. A. Whitton for his supervision of my work and for his kind advice and criticism. It was his encouragement and inspiration more than anything else that enabled me to fulfil this work. I wish to thank the Ministry of Defence in Saudi Arabia for financial assistance throughout the course of the project. Thanks are due to Prince Sultan Ben-Abd-Alaziz who gave his permission for that financial support. In particular I should like to thank my parents and my wife for their help and constant encouragement in many ways.

Thanks are due to my colleagues in Durham, Dr M. Potts, Dr C. Sinclair, Dr A. Donaldson and Dr D. Livingstone who all gave generous help with gas chromatography for the acetylene reduction assay technique.
Dr D. Livingstone also gave advice and helpful discussion on chlorophyll *a* extraction. Dr P. J. Say, Dr J. P. C. Harding, Dr N. T. H. Holmes, Mr B. M. Diaz, Mr J. D. Wehr, Mr G. Patterson, Mr C. Rajendran, Mr I. G. Burrows, Mr Abdullah Al-Mousawi and Mr Abdul B. H. Zolaly gave many interesting discussions on heavy metals. Special thanks are due to Mr J. W. Simon for his helpful assistance in solving many technical problems and for valuable advice. I am grateful to Dr A. Yarwood and Mr G. H. Banbury for allowing me to attend their lectures. Mr T. Brett and Mr B. Coult advised me on the use of the atomic absorption spectrophotometer. I am also grateful to Dr N. Harris, Mr J. Gilroy, Mr G. Bainbridge, Mrs G. A. Walker, Mr A. Jamieson, Mr P. Masters, Mr J. H. Whittle for various types of technical advice; to the staff of Durham University Science Library for tracing my obscure references and for providing a favourable working environment during writing up the work.

I wish to thank Prof. D. Boulter for the provision of research facilities in the Botany Department. Finally very many thanks are offered to Mrs H. Winn who took on very efficiently the arduous task of typing this thesis.

ABBREVIATIONS

^{o}C	degrees Celcius
d	day
EDTA	ethylenediaminetetra-acetic acid (disodium salt)
g	gramme
h	hour
l	litre
M	molar
mg	milligramme
min	minute
ml	millilitre
µg	microgramme
µm	micrometer
n	number of measurements
nm	nanometer
s.d.	standard deviation
u.v.	ultra-violet
$\bar{x}$	mean value
filtrable	capable of passing through filter
non-filtrable	incapable of passing through filter
NTG	N-methyl-N'-nitro-N-nitrosoguanidine
T.I.C.	Tolerance Index Concentration (see p. 62)
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
P.A.R.	Photosynthetic active radiation between 400 - 700 nm
lux	Photometric measurements of illuminance (one lumen m^{-2})
Zn-t5.0	*Anacystis nidulans* tolerant to 5.0 mg l^{-1} Zn
Zn-t12.0	" " " " 12.0 mg l^{-1} Zn
Co-t1.8	" " " " 1.8 mg l^{-1} Co
Ni-t1.0	" " " " 1.0 mg l^{-1} Ni
Cu-t0.5	" " " " 0.5 mg l^{-1} Cu
Cd-t2.0	" " " " 2.0 mg l^{-1} Cd

LIST OF TABLES

Table		page
2.1	Composition of media (mg l^{-1} of salts)	39
2.2	Composition of media (mg l^{-1} of elements)	40
2.3	Amount of zinc as impurities in the stock (mg l^{-1})	41
2.4	Levels of EDTA and HEPES used for various experiments	43
2.5	Influence of EDTA on Zn solubility in ACM medium (pH 7.0; + HEPES: Table 2.2).	45
2.6	Factors influencing Zn toxicity	46
2.7	Sources of cultures.	50
2.8	Comparison between methods usedfor phycocyanin extraction from *Anacystis nidulans*.	55
2.9	Effect of incubation medium and time on the release and subsequent degradation of phycocyanin from *Anacystis nidulans*.	57
2.10a	Influence of Mn as $MnCl_2$ on turbidity of ACM medium	59
2.10b	Influence of Ca as $CaCl_2$ on turbidity of ACM medium	59
2.11	Influence of pH on Zn solubility in ACM medium (10 mg l^{-1} EDTA + HEPES).	66
2.12	Influence of Ca on Zn solubility in ACM medium (pH 7.0 + HEPES + 0.5 mg l^{-1} EDTA).	67
2.13	Influence of PO_4-P on Zn solubility in ACM medium (pH 7.0 + HEPES + 10 mg l^{-1} EDTA).	68
2.14	Influence of autoclaving and non-autoclaving on Zn solubility in ACM medium (pH 7.0 + HEPES + 10 mg l^{-1} EDTA).	69
2.15	Influence of EDTA (pH 5.4) on removal of Zn from *Anacystis nidulans*.	70

Table		page
3.1	Resistance obtained (so far) on repeated subculture ...... of *Anacystis nidulans* to progressively higher metal concentrations.	76
3.2	Tolerance of mutants used for experiments to the .... metals used in their isolation.	80
3.3	Influence of prior growth condition and Zn concen- .... trations on growth, lag shown by Zn-t5.0 and Zn-t12.0.	85
4.1	Influence of growth stage on colony-forming ability of ... wild-type *Anacystis* on 1% agar plates.	126
4.2	Influence of Zn on growth of wild-type *Anacystis nidulans* on 1% agar plates.	127
4.3	Changes (greater than a factor of two) in resistance .... to other metals of metal-tolerant strains of Table 8.1.	129
4.4	Comparison of response of wild-type and Zn-tolerant ..... strains to penicillin, polymixin and streptomycin.	131
4.5	Comparison of chl *a* content per unit length of alga ..... in Zn treated cultures.	131
5.1	Influence of environmental factors on toxicity of .... Cu, Zn and Cd to various strains of *Anacystis nidulans* Results based on turbidimetric measurements.	137
5.2	Key environmental factors changing toxicity of metals .... tested, based on entries in Table 5.1 differing by at least two scores.	138
5.3	Influence of Na on Zn toxicity to wild-type *Anacystis*....	140
5.4	Influence of Cl on Zn toxicity to wild-type *Anacystis*....	140
5.5	Influence of SO_4-S on Cd toxicity to wild-type *Anacystis*	141

Table		page
5.6	Influence of Mg on Zn toxicity to wild-type *Anacystis*; ... (high Mg le els).	141
5.7	Influence of Mg on Zn toxicity to Zn-t5.0 *Anacystis*......	142
5.8	Influence of Mg on Zn toxicity to Zn-t12.0 *Anacystis*.....	142
5.9	Influence of Mg on Zn toxicity to wild-type *Anacystis*; ... (low Mg levels).	143
5.10	Influence of Mg on Cu toxicity to wild-type *Anacystis*....	143
5.11	Influence of Mg on Cu toxicity to Cu-t0.5 *Anacystis*......	145
5.12	Influence of Mg on Cd toxicity to wild-type *Anacystis*....	145
5.13	Influence of Ca on Cu toxicity to wild-type *Anacystis*....	148
5.14	Influence of Ca on Cu toxicity to Cu-t0.5 *Anacystis*......	148
5.15	Influence of Mn on Zn toxicity to wild-type *Anacystis*....	149
5.16	Influence of Mn on Cd toxicity to wild-type *Anacystis*....	149
5.17	Influence of Fe on Zn toxicity to wild-type *Anacystis*....	152
5.18	Influence of Fe on Cd toxicity to wild-type *Anacystis*....	152
5.19a	Influence of PO_4-P on Zn toxicity to wild-type .... *Anacystis*; starved inoculum.	155
5.19b	Influence of PO_4-P on Zn toxicity to wild-type .... *Anacystis*; rich inoculum.	155
5.20	Influence of PO_4-P on Zn toxicity to wild-type .... *Anacystis*; (PO_4-P was introduced after algal inoculation and contact with Zn for 10 min.).	156
5.21	Influence of PO_4-P on Cu toxicity to wild-type .... *Anacystis*.	156
5.22	Influence of PO_4-P on Cu toxicity to Cu-t0.5 .... *Anacystis*.	157
5.23a	Influence of PO_4-P (as β-glycerophosphate) on Zn .... toxicity to wild-type *Anacystis*; units ml^{-1} was used as a growth criterion.	159

Table		page
5.23b	Influence of PO_4-P (as β -glycerophosphate) on Zn toxicity to wild-type *Anacystis*; chl *a* was used as a growth criterion.	160
5.24a	Influence of PO_4-P (α -D glucose-l-phosphate) on Zn toxicity to wild-type *Anacystis*; units ml^{-1} were used as a growth criterion.	161
5.24b	Influence of PO_4-P (α -D glucose-l-phosphate) on Zn toxicity to wild-type *Anacystis*; chl *a* was used as a growth criterion.	162
5.25	Influence of NO_3-N on Cd toxicity to wild-type *Anacystis*	163
5.26	Influence of HEPES on the growth of wild-type *Anacystis*; at two levels of Zn.	167
5.27	Influence of Ni on Zn toxicity to wild-type *Anacystis*	168
5.28	Influence of Cu on Zn toxicity to wild-type *Anacystis*	168
5.29	Influence of Hg on Zn toxicity to wild-type *Anacystis*	169
5.30	Influence of Pb on Zn toxicity to wild-type *Anacystis*	169
5.31	Influence of Ni on Cd toxicity to wild-type *Anacystis*	170
5.32	Influence of Cu on Cd toxicity to wild-type *Anacystis*	170
5.33	Influence of Hg on Cd toxicity to wild-type *Anacystis*	171
5.34	Influence of Pb on Cd toxicity to wild-type *Anacystis*	171
6.1	Influence of culture density on removal of Zn from culture medium over a short period of time.	180
6.2	Accumulation of Zn during batch culture growth of wild-type *Anacystis*. Results show values estimated when controls (Nuclepore filter through which 0.1 mg l^{-1} Zn had been passed) are subtracted from the measured values.	181

Table		page
6.3	Accumulation of Zn during batch culture growth of .... wild-type *Anacystis*. Results show values estimated when controls (Nuclepore filter through which 1.0 mg l^{-1} Zn had been passed) are subtracted from measured values.	182
6.4	Accumulation of Zn during batch culture growth of .... Zn-t12.0 *Anacystis* strain. Results show values estimated when controls (Nuclepore filter through which 0.1 mg l^{-1} Zn had been passed) are subtracted from measured values.	183
6.5	Accumulation of Zn during batch culture growth of........ Zn-t12.0 *Anacystis* strain. Results show values estimated when controls (Nuclepore filter through which 1.0 mg l^{-1} Zn had been passed) are subtracted from measured values.	184
6.6	Accumulation of Zn during batch cultures growth of....... Zn-t12.0 *Anacystis* strain. Results show values estimated when controls (Nuclepore filter through which 10.0 mg l^{-1} Zn had been passed) are subtracted from measured values.	185
7.1	Comparison of tolerance to zinc of organisms .... isolated from high zinc sites with tolerance strains taken from culture collection and almost certainly isolated initially from environment with low zinc levels.	192
7.2	Influence of Ca on Zn toxicity to *Phormidium autumnale* ... D476 as measured by chl *a*.	197

Table		page
7.3	Influence of Ca on Cd toxicity to *Phormidium autumnale* ... D476 as measured by chl *a*.	198
7.4	Influence of PO_4-P on Zn toxicity to *Phormidium autumnale* D476 as measured by chl *a*.	199
7.5	Influence of PO_4-P on Cd toxicity to *Phormidium autumnale* D476 as measured by chl *a*.	200
7.6	Influence of pH on Zn toxicity to *Phormidium autumnale*.... D476 as measured by chl *a*.	201
7.7	Influence of pH on Cd toxicity to *Phormidium autumnale* ... D476 as measured by chl *a*.	202
7.8	Influence of Zn on Cd toxicity to *Phormidium autumnale* ... D476 as measured by chl *a*.	203
7.9	Influence of Zn on acetylene reduction (nitrogen .... fixation) by Zn-resistant and Zn-sensitive heterocystous organisms.	205
8.1	Cross-resistance of strains tolerant to one .... particular metal to the other four metals. Strains are named according to strongly inhibitory level of metal at the time of isolation.	207
8.2	Tolerance to Cu and Zn of species studied .... by Rana and Kumar (1974).	211
8.3	Summaries of literature on Zn requirement by .... various organisms.	213

LIST OF FIGURES

page

Figure

2.1 Relationship between population density and turbidity 60
during growth of wild-type and Zn-tolerant strains of
Anacystis nidulans.

3.1 Increase in resistance to Zn on repeated subculturing 78
at progressively higher Zn concentration.

3.2 Influence of Co on growth of Co-t1.8. 81

3.3 Influence of Ni on growth of Ni-t1.0. 81

3.4 Influence of Cu on growth of Cu-t0.5. 82

3.5 Influence of Zn on growth of Zn-t5.0. 82

3.6 Influence of Zn on growth of Zn-t12.0. 83

3.7 Influence of Cd on growth of Cd-t2.0. 83

3.8 Influence of Zn on growth of Zn-t5.0, grown for 72 cell ... 84
generations in basal medium.

3.9 Influence of Zn on growth of Zn-t12.0 grown for 96 cell ... 84
generations in basal medium.

3.10 Diagrams showing representative forms of *Anacystis* 88
units in batch culture.

3.11 Influence of Zn and time of harvesting in batch 89
culture on length of *Anacystis*.

3.12 Influence of Zn and time of harvesting in batch 90
culture on length of Zn-t5.0; inoculum from
basal medium.

3.13 Influence of Zn and time of harvesting in batch 91
culture on length of Zn-t5.0; inoculum from
strongly inhibitory level of Zn.

Figure page

4.1 Influence of Co on growth of wild-type *Anacystis*. 94

4.2 Influence of Co on growth of Ni-t1.0. 95

4.3 Influence of Co on growth of Cu-t0.5. 96

4.4 Influence of Co on growth of Zn-t5.0. 97

4.5 Influence of Co on growth of Zn-t12.0. 98

4.6 Influence of Co on growth of Cd-t2.0. 99

4.7 Influence of Ni on growth of wild-type. 100

4.8 Influence of Ni on growth of Co-t1.8. 101

4.9 Influence of Ni on growth of Cu-t0.5. 102

4.10 Influence of Ni on growth of Zn-t5.0. 103

4.11 Influence of Ni on growth of Zn-t12.0. 104

4.12 Influence of Ni on growth of Cd-t0.2. 105

4.13 Influence of Cu on growth of wild-type *Anacystis*. 107

4.14 Influence of Cu on growth of Co-t1.8. 108

4.15 Influence of Cu on growth of Ni-t1.0. 109

4.16 Influence of Cu on growth of Zn-t5.0. 110

4.17 Influence of Cu on growth of Zn-t12.0. 111

4.18 Influence of Cu on growth of Cd-t2.0. 112

4.19 Influence of Zn on growth of wild-type *Anacystis*.......... 113

4.20 Influence of Zn on growth of Co-t1.8. 114

4.21 Influence of Zn on growth of Ni-t1.0. 115

4.22 Influence of Zn on growth of Cu-t0.5. 116

4.23 Influence of Zn on growth of Cd-t2.0. 117

4.24 Influence of Cd on growth of wild-type *Anacystis*.......... 118

4.25 Influence of Cd on growth of Co-t1.8. 119

4.26 Influence of Cd on growth of Ni-t1.0. 120

4.27 Influence of Cd on growth of Cu-t0.5. 121

4.28 Influence of Cd on growth of Zn-t5.0. 122

4.29 Influence of Cd on growth of Zn-t12.0. 123

Figure		page
4.30	Influence of Hg on growth of wild-type *Anacystis*.	124
4.31	Influence of Pb on growth of wild-type *Anacystis*.	124
4.32	Influence of Zn on growth of Zn-t12.0 in standing culture.	128
4.33	Influence of NTG on growth of wild-type *Anacystis*; population density and chl *a* were used as criteria of growth.	132
4.34	Pigment changes during growth of wild-type *Anacystis* at different Cd concentrations in batch culture.	134
4.35	Influence of minimum and maximum concentrations of Zn for the optimum growth of wild-type *Anacystis*.	135
5.1	Influence of Ca on Zn toxicity to wild-type *Anacystis*.	146
5.2	Influence of Ca on Zn toxicity to Zn-t5.0 *Anacystis*.	146
5.3	Influence of Ca on Zn toxicity to Zn-t12.0 *Anacystis*.	147
5.4	Influence of Ca on Cd toxicity to wild-type *Anacystis*.	147
5.5	Influence of Mn on Zn toxicity to Zn-t5.0 *Anacystis*.	150
5.6	Influence of Mn on Zn toxicity to Zn-t12.0 *Anacystis*.......	150
5.7	Influence of PO_4-P on Zn toxicity to wild-type *Anacystis*...	153
5.8	Influence of PO_4-P on Zn toxicity to Zn-t5.0 *Anacystis*. ...	153
5.9	Influence of PO_4-P on Zn toxicity to Zn-t12.0 *Anacystis*....	154
5.10	Influence of PO_4-P on Cd toxicity to wild-type *Anacystis*...	154
5.11	Influence of EDTA on Zn toxicity to wild-type *Anacystis*....	164
5.12	Influence of EDTA on Zn toxicity to Zn-t5.0 *Anacystis*.....	164
5.13	Influence of EDTA on Zn toxicity to Zn-t12.0 *Anacystis*.....	165
5.14	Influence of EDTA on Cd toxicity to wild-type *Anacystis*...	165
5.15	Influence of Zn on Cd toxicity to wild-type *Anacystis*......	172
5.16	Influence of Zn on Cd toxicity to Zn-t5.0 *Anacystis*........	172
5.17	Influence of pH on Zn toxicity to wild-type *Anacystis*......	173

Figure page

5.18 Influence of pH on Zn toxicity to Zn-t5.0 *Anacystis*. 173

5.19 Influence of pH on Zn toxicity to Zn-t12.0 *Anacystis*. 175

5.20 Influence of pH on Cd toxicity to wild-type *Anacystis*. 175

5.21 Influence of inoculum size on Zn toxicity to wild-type 176
Anacystis ; chl *a* used as growth criterion.

6.1 Influence of Zn on growth of wild-type *Anacystis*; 178
dry weight was used as a growth criterion.

6.2 Influence of Zn on growth of Zn-t12.0 *Anacystis*; 178
dry weight was used as a growth criterion.

6.3 Relationship between logarithm of Zn composition of 187
wild-type *Anacystis* and dry weight of alga during
growth of batch culture.

6.4 Relationship between logarithm of Zn composition of 187
Zn-t12.0 *Anacystis* and dry weight of alga during
growth of batch culture.

6.5 Relationship between logarithm of Zn composition of 190
wild-type *Anacystis* and dry weight of alga during
growth of batch culture.

6.6 Relationship between logarithm of Zn composition of 190
Zn-t12.0 *Anacystis* and dry weight of alga during
growth of batch culture.

7.1 Influence of Zn on growth of *Calothrix* D184, in the 194
presence and absence of combined nitrogen.

7.2 Influence of Zn on growth of *Phormidium autumnale* D475. ... 195

7.3 Influence of Cd on growth of *Phormidium autumnale* D475. ... 195

CHAPTER 1

INTRODUCTION

1.1 Occurrence of blue-green algae in environment rich in heavy metals

Blue-green algae are widespread and sometimes abundant in many different types of environment (Fogg 1973). They are also present in many habitats which are polluted with organic wastes (Palmer 1969) or heavy metals (Whitton 1980). Examples are widespread in Europe, U.S.A. and elsewhere of rivers and streams which are contaminated with zinc as a result of mining activities (Whitton 1980). For instance streams in north Wales draining old lead mine tips and carrying levels of zinc greater than about 1.5 mg l^{-1} Zn were reported by Whitton (1970b) to have a very reduced flora. At one silted site there were only three species of algae, among them the blue-green alga *Lyngbya* sp. Gale *et al.* (1973) reported that in some polluted streams in the New Lead Belt region of southeastern Missouri, algal growth developed to such an extent as to cause problems. Besides *Oscillatoria* the following were found to contribute to these problems: *Cladophora*, *Mougeotia*, *Zygnema*, *Spirogyra*. Whitton (1980) reported from the same area that in June 1977 among flocs in heavily contaminated streams below the smelter at Glover, *Phormidium* and sheathed, very narrow forms of Oscillatoriaceae were important. *Oscillatoria* was also found by Trollope and Evans (1976) in freshwater (1.96 mg l^{-1} Zn) near a zinc smelting waste in the lower Swansea valley, Wales. In some seepages and springs with very high zinc levels at Elvins Tailings, Old Lead Belt, Missouri, an especially characteristic community occurs in seepages below old tips with pH values within or near the 6.5 to 7.0 ranges. The dominants, *Plectonema* and the protonema of *Dicranella varia*,

were the same, and associated species quite similar to those at other sites with elevated zinc both in Europe and the U.S.A. (Whitton 1980). *Phormidium autumnale* has been recorded from the Riou Mort, France with 15.7 mg l^{-1} Zn at pH 6.7. In a stream of the Northern Pennine Orefield showing a gradient of zinc (30 to 1.5 mg l^{-1} filtrable Zn) *Plectonema gracillimum* was recorded for over two years among the algal species near the top of this gradient. While non-heterocystous blue-green algae are widespread in streams with high Zn levels, the only published record for a heterocystous one from such an environment involves *Nodularia spumigena* (Gopal *et al.* 1975); this covers moist soil near the effluent of a zinc smelter at Debari, India. Waste waters from the smelter were shown to be toxic to other heterocystous blue-green algae held in laboratory culture (*Tolypothrix tenuis*, *Calothrix brevissima*, *Anabaena doliolum*, *Fischerella muscicola*). The non-heterocystous blue-green alga *Plectonema boryanum* taken from the same site was extremely tolerant to Zn (Rana *et al.* 1971). *Anabaena*, *Nostoc* and *Scytonema* are abundant on high copper soils in Zimbabwe (Wild 1968).

1.2 Laboratory tolerance and toxicity

It has long been recognised that high concentrations of heavy metals such as Zn, Cu and Cd may prove toxic to aquatic plants and animals. A large body of literature exists concerning the toxicity of various metals to various organisms including algae (Whitton and Say 1975). The following brief review concentrates on the tolerance and toxicity of metals to algae, particularly blue-green, an aspect that has received less attention than for example, the toxicity of heavy metals to fish.

Many laboratory studies have been carried out on the toxicity of different heavy metals to different species of algae. A large number

of these studies have been concerned with the possible uses of algae as monitors of pollution, or with the toxic effects of metal-containing algicides (Whitton and Say 1975).

Several workers have used selected species of algae to assess the influence of one or more metals under controlled conditions in the laboratory. The species used, which are frequently obtained from culture collections, are therefore being used as bioassay organisms. For example Bartlett *et al.* (1974) found that algicidal concentrations of Cu, Zn and Cd for *Selenastrum capricornutum* were 0.3 mg l^{-1}, 0.7 mg l^{-1} and 0.65 mg l^{-1} respectively. Rachlin and Farran (1974) demonstrated that a concentration of 2 - 4 mg l^{-1} Zn brought about 50% reduction in the growth rate of *Chlorella vulgaris* within 96 hours. In a rather similar study, Malanchuk and Gruendling (1973) estimated the ED_{50} (median effective dose causing 50% reduction of $^{14}CO_2$ fixation) of Pb for four species of algae. This concentration lay within the range 15 - 18 mg l^{-1} Pb for species of *Anabaena*, *Chlamydomonas* and *Navicula*, but was only 5 mg l^{-1} for *Cosmarium* sp.

Only a few other workers have attempted to compare the sensitivity of different groups of algae to heavy metals. Whitton (1970b) carried out a laboratory study of the toxicity of Cu, Zn and Pb to 37 species of green algae. *Microspora* and *Ulothrix* tended to be resistant to all three metals, whilst *Oedogonium* spp. tended to be relatively sensitive. Zygnemales were on the whole intermediate in their resistance to Cu and Pb, but showed a wide range of resistance to Pb. On the basis of this study, he concluded the tolerance of algae to one heavy metal or another varies from species to species. Such variation is sometimes evident among algae belonging to the same class and even in the same family. This confirmed the results of Velichko (1968) who showed that 0.05 mg l^{-1} Zn had practically no effect on reducing the cell numbers

of *Microcystis aeruginosa*, while 0.1 and 0.2 mg l^{-1} Zn reduced the cell numbers by 2.3x and 25x respectively, while at the same time respiration and photosynthesis were inhibited completely. On the other hand, 0.2 mg l^{-1} Zn had approximately the same effect on *M. pulverea* as 0.5 mg l^{-1} Zn did on *M. aeruginosa*. A clear algicidal effect on the former was present only at 0.5 mg l^{-1} Zn and 1.0 mg l^{-1} Zn inhibits photosynthesis and respiration completely. Rana and Kumar (1974b) used bioassay techniques to evaluate the toxicity of a Zn-containing effluent (from a smelter) to ten species of algae. The results suggested that *Chlorella vulgaris*, *Scenedesmus* sp. and *Plectonema boryanum* were relatively tolerant to Zn, whilst *Anacystis nidulans*, *Oscillatoria* sp. and *Nodularia spumigena* were relatively sensitive. Overnell (1976) used the production of oxygen to measure the inhibitory effects of heavy metals on photosynthesis in seven species of marine algae. Of these, *Phaeodactylum tricornutum* and *Dunaliella tertiolecta* were found to be most sensitive to Zn,Cu, Cd and Hg.

It is not the purpose of the present section to consider the present knowledge of the biochemical effects of toxic or non-toxic concentrations of heavy metals. Say (1977) has reviewed such aspects for Zn. Say also reviewed the methods employed by different workers to assess the toxicity of metals, and noted that in several cases the effects of metal have been quantified by the degree of inhibition of a particular metabolic process. For algae, such processes include the production of chlorophyll *a* (Hargreaves and Whitton 1976), the rate of respiration (Velichko 1968), the rate of acetylene reduction or nitrogenase activity (Horne and Goldman 1974; Henriksson and Dasilva 1978; Delmotte 1980) and specific photosynthetic reactions (Overnell 1975; De Fillipis and Pallaghy 1976a; Delmotte 1980).

In addition to biochemical considerations, several workers have made observations on the effect of lethal or sub-lethal concentrations of heavy metals on the growth rate or morphology of different species of algae. Thus Bartlett *et al.* (1974) found that the most noticeable effect of sub-lethal concentrations of Zn, Cu, Cd on *Selenastrum capricornutum* was an extension of the lag phase of growth in culture. Similarly Rosko and Rachlin (1977) found that concentrations of 0.32 mg l^{-1} Cu, 2.4 mg l^{-1} Hg, 0.32 mg l^{-1} Cd, 7.5 mg l^{-1} Zn and 32.0 mg l^{-1} Pb extended the lag phase for *Chlorella vulgaris* to 28, 17, 11, 7 and 3 days respectively. In addition they observed that these concentrations led to decreases in the mean size of the control cells by about 84% after 33 days. Some studies have also demonstrated the adverse effects of metal toxicants on the cell division and morphology of algae. For instance Say *et al.* (1977) observed an increased frequency of geniculation in filaments of some populations of *Hormidium rivulare*. Recently Cu and Zn have been found to increase the chain length of the marine diatom *Thalassiosira aestivalis*, and to inhibit the normal separation of cells (Thomas *et al.* 1980).

1.21 Is zinc essential for growth?

A Zn requirement for normal plant growth was first recognised in the late ninteenth century, but acceptance of this element as an essential plant nutrient did not occur until the early 1930's. Relatively few studies have been conducted to determine the requirement of zinc to pure cultures of algae. The reason has been due largely to difficulty in developing satisfactory synthetic growth media low enough in Zn to demonstrate its requirement (Coleman *et al.* 1971). The amount of Zn required by most organisms is so small that it is frequently supplied in considerable part by the incidental impurities included in the culture medium.

112 years have passed since Raulin (1869) discovered the role of Zn as a nutrient for the fungus *Aspergillus niger*. The association of metals with proteins has been recognised and their essential function in the action of many enzymes has been repeatedly demonstrated (Vallee 1962). However no such critical research has been reported for Zn in algae. The earliest demonstration of a requirement in algae was probably for the green alga *Stichococcus bacillaris* (Eilers 1926). He gave no details of the experiment or critical level of Zn required. Arnon (1958) stated in a review article that *Anabaena cylindrica* requires a 'micro' level of zinc in the medium for growth; the research was carried out in his own laboratory, but no experimental details were given. Coleman *et al.* (1971) found that the optimum growth (dry weight) of *Pediastrum tetras* and *Euglena viridis* occurred at 4.2 mg l^{-1} Zn, while that of *Chlorella vulgaris* occurred at 18.03 mg l^{-1} Zn. Rana and Kumar (1974b) observed that increases in Zn in the medium initially increased the growth of *Plectonema boryanum*, but as the concentration exceeded 0.5 mg l^{-1} growth was retarded.

1.22 Effect of zinc on nitrogen fixation

Little seems to be known about the effects of metals on nitrogen fixation by blue-green algae. Observation at many field sites polluted by Zn have shown that most, if not all, blue-green algae are non-heterocystous types such as *Phormidium*, *Oscillatoria*, *Lyngbya* and *Plectonema*. The only heterocystous blue-green alga found at a Zn smelter site was *Nodularia spumigena* (Gopal *et al.* 1975). Acetylene reduction assays were carried out by Whitton (1980) both in the laboratory and field, on a *Plectonema* mat collected from the Harz. Although a strain of *Plectonema* was used for the first demonstration of nitrogen fixation by a non-heterocystous filamentous blue-green algae (Stewart and Lex 1970) there was no indication of nitrogenase activity

in the Harz material. The addition of Cu at 0.005 - 1 mg l^{-1} depressed nitrogen fixation by *Aphanizomenon* and *Anabaena* in Clear Lake, California (Horne and Goldman 1974; Elder and Horne 1978). However 0.002 mg l^{-1} Cu stimulated nitrogen fixation. Inhibition of nitrogen fixation might be a secondary effect, caused by a decrease in the energy supply to heterocysts, with subsequent breakdown in the removal of oxygen from heterocysts and inactivation of nitrogenase. A recent detailed laboratory study was carried out by Henriksson and Dasilva (1978), who found that 0.005 - 0.025 mg l^{-1} Zn stimulated nitrogen fixation by *Nostoc muscorum*, *Nostoc* sp., *Chlorogloea fritschii* and *Westiellopsis* sp. At concentrations above this, however, there was inhibition. This inhibition may be due to the particular sensitivity to the metal of the nitrogenase enzyme or some other feature of the nitrogen fixers.

1.3 Adaptation

An altered response to a heavy metal may occur through modification of the cells themselves, either phenotypic or genotypic. Where mutation and selection is involved it is assumed that mutant cells (i.e. mutants with a genetically determined increase in metal resistance) are always present in the cell population but in extremely low numbers. Upon addition of the appropriate metal the majority of cells are killed or inhibited while the mutant cells grow out, ultimately becoming the predominant cell type. In this kind of variation the modification is permanent i.e. resistance is maintained even when cells are passed through non-metal containing media. In most reported cases of the development of heavy metal resistance it is difficult to decide whether phenotypic adaptation or mutation is involved. This is particularly so where resistant strains have been obtained by

successive transfer into media containing increasing concentrations of the metal. Golomzik and Ivanov (1964) used this method to obtain cultures of *Thiobacillus ferrooxidans* which oxidized ferrous iron in the presence of very high concentrations of ferrous iron and copper more rapidly than cells of the original population. Similarly strains of *Proteus* have been obtained by successive transfer which are resistant to 150 mg l^{-1} cobalt, a concentration which completely inhibits the parent strains. It has already been reviewed by Ashida (1965) that, bacteria, fungi and yeast are capable of forming populations which are more tolerant to heavy metals after exposure for many passages through media with metal concentrates which partially inhibit growth of inoculants.

Blue-green algae are comparatively new to genetical research. Until about a decade and a half ago nothing was known about their mutagenicity, recombination or genetic systems. A few years later, research has not only established the existence of genetic recombination in some cyanophytes but also shown that these prokaryotes are no different from other living organisms in their response to mutational stimuli (Ladha and Kumar 1978).

1.31 Antibiotics

Although antibiotic and drug resistant mutants have been reported in blue-green algae, especially *Anacystis nidulans*, Kumar (1964) and Singh and Sinha (1965) selected strains by successive subcultures in liquid media containing increasing concentrations of the antibiotics penicillin and streptomycin. These antibiotic-resistant traits were fully stable even after several subcultures in antibiotic-free medium (Kumar 1964; Gupta and Kumar 1970). With the advent of plating techniques (Van Baalen 1965), successful clonal isolation has been

made both after spontaneous screening and after exposure to mutagens. However Ladha and Kumar (1978) pointed out that the efficacy of cloning on agar needs to be considered carefully; it may not be fully justified to regard all colonies developing on an agar plate as strictly clonal, for the chances are that at least some colonies would have developed from two or more cells, rather than single cells.

1.32 Morphological mutants

Many workers have isolated filamentous mutants of the unicellular *Anacystis nidulans* (see Ladha and Kumar 1978). These mutants are of two basic types: (i) filaments having cross-walls and (ii) filaments without cross-walls. Both types can be stable without having altered growth rate; however they show changes in colony morphology. In the second class of mutants, filaments may be quite long, up to 100 times longer than normal, without exhibiting any discontinuity in the photosynthetic lamellae throughout the length of the filament (Kunisawa and Cohen-Bazire 1970).

1.33 Metals

A relatively large number of mutant strains of blue-green algae have been isolated, but almost all those relating to mineral nutrition or toxicity involve nitrogen assimilation pathways (Ladha and Kumar 1978). A recent study by Singh *et al.* (1978) deals with the heavy metals W and Cr. Spontaneous mutants of *Nostoc muscorum* grown in the presence of these elements not only tolerated them but required them for growth with N_2 or NO_3 as the nitrogen source. In contrast, attempts by Sarma (1979) to produce spontaneous mutants of *Anacystis nidulans* resistant to Cu proved unsuccessful.

1.4 Factors influencing the toxicity of metals

The response of a particular organism to a particular metal may be altered in a number of ways. These include modification of the environment of the organism or of the organism itself by adaptation or mutation (Sadler and Trudinger 1967). Overnell (1976) noted that the addition of EDTA is usually necessary to prevent the precipitation of trace nutrients such as iron, but it may swamp the effects of small quantities of added heavy metals. Studies of the effect of EDTA on the Zn toxicity to blue-green algae are rare but Stokes and Hutchinson (1976) quoted from a study by Hall (1974) that EDTA increases Fe availability, while decreasing Zn toxicity to *Microcystis*. Say *et al.* (1977) found that raising the level of EDTA from 5 - 20 mg l^{-1} had a marked effect, increasing the resistance to Zn of *Hormidium rivulare*. There is also information on the effect of EDTA in reducing Cu toxicity, as a result of the fact that Cu is widely used for controlling algal blooms. Fogg and Westlake (1955) found extracellular "polypeptide" reduced Cu toxicity to *Anabaena cylindrica*. Studies with physiologically distinct populations of *Aphanizomenon* (Horne and Goldman 1974) have shown that the toxic effects of up to 70 $\mu g\ l^{-1}$ Cu were removed if EDTA is mixed with Cu a few minutes before addition to the lake water, but not when EDTA is added simultaneously. Feuillade and Feuillade (1977) reported that EDTA reduced the lethal action of Cu to *Oscillatoria rubescens*. Among the factors which have been mentioned as reducing Zn toxicity, the one quoted the most often is the hardness of the water. For instance, it seems widely accepted that Zn is almost always less toxic to fish in hard than in soft waters (Mount 1966). Laboratory studies of bacteria (Abelson and Aldous 1950) indicated that toxic

effects of Ni, Co, Cd, Zn and Mn to *Escherichia coli* were decreased with a high Mg content in the medium. Haavik (1976) demonstrated that amounts of Mn, Fe, Co, Ni and Cu inhibitory to *Bacillus licheniformis* could be antagonised by the addition of Mg (1 g l^{-1}) in the medium, although toxic concentrations of Zn and Cd were reduced less effectively. However Break *et al.* (1976) noted that Zn toxicity to four species of algae *Phaeodactylum tricornutum, Skeletonema costatum, Thalissiosira pseudonana, Amphidinium carteri* was reduced by elevated concentrations of Mg. They suggested that this might indicate a common route for divalent metal ions entering algal cells. In an investigation of the green alga *Hormidium,* Say and Whitton (1977) found that both Mg and Ca reduced the toxicity of Zn. This effect was marked with two Zn-tolerant populations of *Hormidium rivulare;* the effect of Mg was greater than that of Ca at lower concentrations but the influence of Ca increased over a much greater range of concentrations. The influence of Mg was relatively small to the Zn-sensitive population in agreement with the observations of Harding and Whitton (1976) on *Stigeoclonum tenue.* In contrast Gächter (1976) found that the concentration of Ca did not appear to affect the toxicity of Zn, Pb, Hg or Cu to natural populations of phytoplankton. Among algae the further factors which seem to have no clear antagonism to Zn toxicity are Na and Cl (*Hormidium rivulare*: Say and Whitton 1977; *Stigeoclonum tenue*: Harding and Whitton 1977). The situation is further complicated by the fact that antagonism can occur not only between heavy metals and essential elements as previously discussed, but also among heavy metals themselves. For instance, in the case of fish, both field and laboratory studies have shown that

the concentrations of other metals may influence the toxicity of any particular metal. Examples are known of antagonistic and synergistic interactions (Jones 1964). In laboratory studies of *Anabaena inaequalis*, Stratton and Corke (1979) found that the response towards combinations of Hg II and Cd or Ni and Cd resulting in either synergism or antagonism towards growth, depended upon the actual metal combinations used. When the pairs of metals Hg II and Cd or Ni and Cd were used at a sublethal concentration and incorporated simultaneously, they interacted synergistically and antagonistically in their effect on growth rate, respectively. Cd has also been found to reduce the inhibition of growth of *Selenastrum capricornutum* by Cu (Bartlett *et al.* 1974). Say and Whitton (1977) found that the toxic effects of Cd and Zn are synergistic toward the growth of *Hormidium rivulare*, thus resembling the response found by Hutchinson and Czyrska (1972) for *Lemna valdiviana*. Any level of Cd above 0.01 mg l^{-1} must be suspected of producing a significant increase in the toxicity to *Hormidium* of any Zn present. Cu and Ni interact synergistically towards the growth of some green algae, while Se antagonize Cd toxicity (Hutchinson 1973).

In a detailed laboratory study, Nakano *et al.* (1978) found antagonistic action between Zn and Cd to *Euglena gracilis*. In the presence of 20 mg l^{-1} Cd, the generation time in Zn-free medium of 57 h was reduced to 27 h after addition of 2 mg l^{-1} Zn. This is in agreement with Falchuk *et al.* (1975) who found Zn reduced Cd toxicity to *Euglena gracilis*; it also agrees with results by Pakalne *et al.* (1970) and Upitis *et al.* (1973), who found that Zn protects *Chlorella* against Cd toxicity.

Studies of vascular plants show that high concentrations of soil phosphate antagonize the toxicity of Zn. Direct experimental evidence

for this has been obtained for *Thlaspi al pestre* ssp. *calaminare* (Ernst 1974). Rana and Kumar (1974a) studied the influence of phosphate and nitrate on Zn toxicity to the blue-green alga *Plectonema boryanum* as well as the green alga *Chlorella vulgaris*. Relatively high concengration of phosphate, but not nitrate, improved the growth of both algae and protected them against Zn toxicity. These results are in apparent contrast to those of Green *et al.* (1975), who reported that Zn toxicity to the green alga *Selenastrum capricornutum* was not affected significantly by low PO_4-P in range of 0.047 mg l^{-1} to 0.93 mg l^{-1}. Phosphate also reduced Zn toxicity to green algae, *Hormidium rivulare* (Say *et al.* 1976; Say and Whitton 1977) and *Stigeoclonum tenue* (Harding 1978). Sulphate had no detectable influence in reducing Zn toxicity to *Hormidium rivulare* (Say and Whitton 1977).

Hydrogen ion concentrations may play a determinant role in affecting Zn toxicity to the algae. Most of the experimental studies reported in the literature on the influence of pH on Zn toxicity have so far been concentrated on green algae. For instance, Harding and Whitton (1977) reported an obvious decrease in Zn toxicity to a Zn-tolerant population of *Stigeoclonum tenue* with a rise in pH from 6.1 to 7.6, but a similar response was scarcely detectable with a Zn-sensitive population. The influence of pH on *Hormidium rivulare* contrasts with that on *Stigeoclonum*. With both the Zn-sensitive and Zn-tolerant populations of *Hormidium rivulare* isolated from a reach with a mean field pH of 4.4 and 6.8, respectively the toxicity of Zn decreased with a fall in pH between 8 - 3 (Say and Whitton 1977), agreeing with the observations of Hargreaves and Whitton (1977) who showed that the toxicity of Zn to *Hormidium rivulare* isolated from a stream at pH 3.1, increased over the range of pH 3.5 - pH 7.0. The toxicity of Cu was found to be pH-dependent; pH may also play a role in reducing Cd

toxicity. For instance, Hart and Scaife (1977) found that Cd inhibited the growth of the green alga *Chlorella pyrenoidosa*, the extent of inhibition being more pronounced at pH 7.0 than at pH 8.0.

1.5 Accumulation of metals

Freshwater algae exposed to Zn concentrations above "normal" background levels have the ability and tendency to accumulate Zn, as shown experimentally (Coleman *et al.* 1971) and in the field (Trollope and Evans 1976). It is clear that freshwater algae can be very significant environmental factors in the uptake and movement of Zn in freshwater systems. In water near Zn smelting wastes in the Lower Swansea Valley, with 1.96 mg l^{-1} Zn, a bloom of *Oscillatoria* accumulated 1.88 mg Zn g^{-1} dry weight (Trollope and Evans 1976). Studies in Strother Creek, Missouri, with 0.041 mg l^{-1} Zn (not filtered), 0.026 mg l^{-1} Zn (filtered), showed that two *Oscillatoria* samples found by Jennett *et al.* (1980) concentrated 3260 μg Zn g^{-1} dry weight and 4030 μg Zn g^{-1} dry weight, respectively. The authors reported a lower concentration factor for Cd by blue-green algae; three young cultures of *Nostoc muscorum*, *Nostoc* sp. and *Schizothrix calcicola* did not remove Cd significantly at any pH, while only one green alga *Mougeotia* showed such negative results. They suggested that green algae appear to be much more efficient at accumulating Cd than the blue-greens.

Laboratory studies on Zn uptake and accumulation have been made on vascular plants (Adams *et al.* 1973; Mathys 1980), bryophytes (Pickering and Puia 1969) and green algae (Coleman *et al.* 1971; Wixson and Gale 1975; Whitton and Say 1975). Few accounts for blue-green algae have been reported. Sparling (1968) found in laboratory studies that *Gloeocapsa* sp. *Nostoc muscorum*, *Anacystis nidulans* and *Merismopedia* sp. all took up a significant amount of Zn, Cu, Cd and Ni from solution, enough to

influence the metal balance in natural waters. In a study of metal uptake by *Anacystis nidulans*, Katagiri (1975) found that a logarithmically grown culture exposed to 0.5 mg l^{-1} Cd for 24 h accumulated 1.5 mg l^{-1} Cd. However Cd uptake was pH dependent, more Cd being accumulated at pH 7.0 than at pH 8.0. Jones *et al.* (1978) found a considerable variation in Zn and Cd accumulation between strains of blue-green algae, after they had been grown in medium containing 74 µg l^{-1} Zn and 20 µg l^{-1} Cd, as shown below:

	µg g^{-1} Zn	µg g^{-1} Cd
Anabaena cylindrica	93	1.5
Anabaena variablis	48	2.3
Anacystis nidulans	81	2.3
Chlorogloea fritschii	109	2.9
Nostoc muscorum	479	9.1

1.6 Aims

Blue-green algae are often the dominant organisms at moist sites combining high levels of Zn with high pH. Laboratory studies have confirmed that the strains present are highly resistant to Zn. The question arises as to how easy it is for strains lacking resistance to develop it? A relatively large number of mutant strains of blue-green algae have been isolated, but so far not ones resistant to heavy metals (1.33). On the other hand there are several reports in the literature showing a large number of *Anacystis nidulans* mutants resistant to drugs.

As a result of reviewing the above literature, it was planned to attempt to isolate strains of *A. nidulans* resistant to Co, Ni, Cu, Zn and Cd and make an account of the properties of these strains. It seemed logical prior to the start of these experiments to investigate the effect of these metals on the growth and morphology of this strain.

Many reports in the literature have shown that the behaviour of metals may be influenced by other factors (section 1.4) so experiments were also planned to determine the effects of such factors on metal toxicity to *A. nidulans*. Experiments were also planned to determine the extent to which *A. nidulans* can accumulate Zn.

CHAPTER 2

MATERIALS AND METHODS

2.1 Culture techniques

2.11 Cleaning of glassware

Glassware was washed with distilled water, soaked for at least 24 h in 10% Analar HCl (earlier experiments) or 2% Analar HNO_3 (later experiments), rinsed in distilled water, and dried at 105°C.

2.12 Culture vessels

The culture vessels for maintenance of stock cultures or preparing inocula in liquid medium were 100 ml and 250 ml Pyrex conical flasks. For toxicity experiments, 50 ml boiling tubes were used. Plastic disposable sterilised petri dishes were used for solid media.

Good quality cotton wool was used for plugging the conical flasks. Morton closures (Bellco stainless steel) or Axa closures (Axa Ltd) were used for routine toxicity tests for which many tubes were required.

2.13 Sterilization

All flasks and tubes containing medium were sterilized by autoclaving at 121°C (10 KN m^{-2}; 15 lb in^{-2}) for 15 min. The medium was allowed to stand overnight before inoculation to allow requilibration with the atmosphere; if the medium was to be stored longer than this it was kept in the dark in order to protect EDTA-metal chelates against photo-deterioration.

In media with high phosphate, calcium or zinc, these were autoclaved separately and added aseptically to each tube or flask, to avoid precipitation. In media containing certain organic compounds or antibiotics which might be heat sensitive the filter procedure in 2.91 (iii) was used.

2.2 Media

2.21 Mineral nutrients

Tables 2.1 (p. 39) and 2.2 (p. 40) show the composition of the media and the salts used in their preparation. The percentages of Zn present in these salts as impurities are given in Table 2.3 (p. 41). All salts were prepared in high concentrations, from Analar products in deionized distilled water and stored at 4°C in the dark. In studies of Zn toxicity to *Anacystis nidulans* a medium (ACM) was used derived from medium 'C' of Kratz and Myers (1955). The modification was performed as follows: the level of phosphate was reduced by a factor of 100 (to 1.78 mg l^{-1} P); Zn was omitted from the microelement stock leaving about 0.04 mg l^{-1} Zn derived as contaminants. Analysis of stock solution show that the amount of Zn as impurities of chemicals is 0.024 mg l^{-1}. The rest of Zn may perhaps come from glassware, and deionized water; the level of chelating agent was relatively low, with 0.5 mg l^{-1} (= 0.0017 mM) EDTA; pH was buffered at 7.0 ± 0.15 with HEPES, using NaOH to make the initial pH adjustment.

Two different media were used for culturing strains isolated from high Zn sites; both contain low levels of phosphate:

(i) Chu 10D + N medium is described by Sinclair and Whitton 1977: as Chu 10-D), but the version used here was buffered at a lower pH value. The nitrate-free version (Chu 10-N) was made by supplying Ca as $CaCl_2$.

(ii) ADM medium is a modification of the medium of Allen and Arnon (1955), with phosphate reduced to (0.445 mg l^{-1} P); the level of Fe and chelating agent was reduced by a factor of 2 (to 2 mg l^{-1} Fe; 10 mg l^{-1} (= 0.034 mM) EDTA).

Both media were buffered at pH 7.0 with 1200 mg l^{-1} (= 5.04 mM) HEPES.

Table 2.1 Composition of media (mg l^{-1} of salts)

salts	ADM	Allen and Arnon (1955)	Chu 10-D	ACM	Kratz and Myers (1955)
KNO_3	-	-	-	500	1000
$Ca(NO_3)_2.4H_2O$	-	-	40	-	25
K_2HPO_4	2.5	348.9	-	10	1000
KH_2PO_4	-	-	8.0	-	-
$MgSO_4.7H_2O$	200	246.5	25.0	250	250
$Na_2SiO_3.5H_2O$	-	-	11.0	-	-
NaCl	230	233.8	-	23	-
Na_2HCO_3	-	-	16.0	-	-
$CaCl_2.2H_2O$	66.2	73.5	-	19.86	-
$FeCl_3.6H_2O$	9.7	?	2.42	9.7	-
$Na_2EDTA.2H_2O$	25.4	?	3.17	0.635	-
Na citrate.$2H_2O$	-	-	-	-	165
$Fe_2(SO_4)_3.6H_2O$	-	-	-	-	4.0
$MnCl_2.4H_2O$	-	-	0.05	1.81	1.81
$MnSO_4.4H_2O$	0.5	2.03	-	-	-
$NaMoO_4.2H_2O$	0.19	0.15	0.007	0.027	0.017
$ZnSO_4.7H_2O$	0.05	0.22	0.056	-	0.222
$CuSO_4.5H_2O$	0.02	0.08	0.019	0.08	0.08
$CoCl_2.6H_2O$	0.01	-	-	-	-
$CoSO_4.7H_2O$	-	-	0.01	0.04	-
H_3BO_3	0.5	2.86	0.72	2.86	2.86
NH_4VO_3	-	0.02	-	-	-
$Na_2WO_4.2H_2O$	-	0.018	-	-	-
$NiSO_4.7H_2O$	0.01	0.045	-	-	-
$Cr(SO_4)_3.K_2SO_4 24H_2O$	-	0.96	-	-	-
pH	7.0	?	7.2-7.5	7.0	7.5-7.8

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

element	ADM(-N)	Allen and Arnon (1955)	Chu 10-D	ACM	Kratz and Myers (1955)
N	-	-	-	69.29	-
P	0.445	61.9	1.78	1.78	177.8
S	26.0	32.0	3.25	32.52	4.23
Cl	171.4	177.3	-	28.018	-
Na	90.5	92.0	8.44	10.62	-
K	112.2	156	2.24	-	835.6
Ca	18.1	20	9.77	5.45	-
Mg	19.7	24.3	2.47	24.65	24.65
Si	-	-	2.50	-	-
Fe	2.0	4.0	0.5	2.0	0.86
Mn	0.12	0.5	0.012	0.5	0.5
Mo	0.08	0.1	0.0025	0.01	0.01
Zn	-	0.05	0.012	-	0.05
Cu	0.005	0.02	0.005	0.02	0.02
Co	0.0005	0.01	0.002	0.008	0.008
B	0.09	0.05	0.125	0.50	0.50
V	0.004	0.01	-	-	-
W	0.005	0.01	-	-	-
Ni	0.002	0.01	-	-	-
Cr	0.001	0.01	-	-	-
EDTA	10.0	?	2.47	0.5	-

Table 2.3 Amount of zinc as impurities in the stock (mg l^{-1})

	Zn (mg l^{-1}) in stock	Zn (mg l^{-1}) in medium from stock
K_2HPO_4	0.18	0.000072
$MgSO_4.7H_2O$	0.08	0.0004
NaCl	0.072	0.000096
$CaCl_2.2H_2O$	0.08	0.0008
KNO_3	0.64	0.0168
Fe.EDTA	0.40	0.0002
EDTA	0.008	-
HEPES	0.003	0.0016
Trace-element	0.08	0.0008
NaOH	0.24	0.0024
Total		0.024

2.22 Buffer

Attempts were made to increase the buffering capacity of media. HEPES at 1200 mg l^{-1} (= 5.04 mM) and 600 mg l^{-1} (= 2.52 mM) were shown to restrict pH variations within 0.15 and 0.25 pH units, respectively, over a 10 day period without affecting the growth of *Anacystis nidulans* (Table 5.26). HEPES was chosen as a buffering agent on the basis of the results of Good *et al.* (1966). They reported that HEPES has a negligible binding capacity for the metals Mg, Ca, Mn and Cu. They calculated the approximate values for the metal-buffer binding constants, from the displacement of the pH titration curve in the presence of an equivalent of the chloride salt of the metal in question. Smith and Foy (1974) chose HEPES as a buffer for freshwater algal media because of its favourable PKa of 7.55 and the negligible metal binding capacity reported by Good *et al.*; no further experimental studies were made.

In a comparison of the effects of HEPES, EDTA and TES (N-Tris(hydroxymethyl)-methyl-2-aminomethanesulphonic acid) on the toxicity of Cd to *Daphnia*, Tevlin (1978) found that in contrast to EDTA and TES, 0.001 M and 0.002 M HEPES did not reduce toxicity. He related this to the absence of Cd complexing by HEPES.

It was realized at a late stage of the present studies that, in spite of the previous reports, HEPES is in fact likely to act as a chelating agent. The studies of Good *et al.* did not investigate all the possible ways this molecule can act as a chelating agent (M. Kilner, pers. comm.). The situation is complicated because both EDTA and HEPES were present simultaneously in the medium, with EDTA known to act as a strong chelating agent. The levels of EDTA and HEPES used for various experiments are given in Table 2.4.

HEPES was added to the medium before autoclaving and adjusting

Table 2.4 Levels of EDTA and HEPES used for various experiments.

experiment	medium	EDTA		HEPES		section
		mg l^{-1}	mM	mg l^{-1}	mM	
Production of resistant strains of *Anacystis*	ACM	0.5	0.0017	1200	5.04	3.0
tolerance and toxicity	ACM	0.5	0.0017	1200	5.04	4.1
(i) Zn				600	2.52	
(ii) Cd	ACM	0.5	0.0017	600	2.52	4.1
(iii) Co, Ni, Cu, Hg	ACM	0.5	0.0017	600	2.52	4.1
(iv) antibiotics	ACM	0.5	0.0017	600	2.52	4.2
factor affecting toxicity	ACM	0.5	0.0017	600	2.52	5.0
accumulation	ACM	0.5	0.0017	600	2.52	6.0
toxicity tests to:						
Phormidium autumnale, *Gloeothece* sp., *Phormidium* sp., *Synechococcus*	ACM	0.5	0.0017	600	2.52	7.0
Calothrix 184, *C. membranacea*, *C. parietina*	Chu10 + N, Chu10 - N	2.5	0.0085	1200	2.52	7.0
Aphaenothece castagnei	Chu10 + N	2.5	0.0085	1200	2.52	7.0

the pH. In some cases the pH of the medium was adjusted after autoclaving to its normal value by the aseptic addition of 0.2 N NaOH to each flask or tube. pH values were measured using a model 7050 (Electronic Instruments Ltd) pH meter.

2.23 Chelating agent

0.5 mg l^{-1} EDTA (ethylenediaminetetra-acetic acid, disodium salt) was added to the medium.

An experiment was carried out to measure the influence of EDTA on the solubility of Zn. In samples which had been passed through glassfibre filters, the level of Zn increased with increasing EDTA (Table 2.5).

2.24 Materials used for toxicity tests

Addition of metals

zinc: added to culture medium as $ZnSO_4.7H_2O$ from a 1000 mg l^{-1} Zn stock solution in deionized distilled water.

copper: added to culture medium as $CuSO_4.7H_2O$ from a 1000 mg l^{-1} stock solution in deionized water.

cadmium: added to the cultures as $CdSO_4.8H_2O$ from a 1000 mg l^{-1} stock solution in deionized water.

Addition of other selected cations and anions

A list of other metals whose toxicity was compared with that of Zn, together with the factors whose influence on Zn-toxicity was studied, is given in Table 2.6, together with the substances added to the medium to bring about required changes. The basal media lacking particular ions required as controls were obtained by substituting complementary salts.

Table 2.5 Influence of EDTA on Zn solubility in ACM medium (pH 7.0, + HEPES : Table 2.2); medium autoclaved and then left to stand for 24 h before experiment. See also tables 2.11, 2.12, 2.13, 2.14.

original Zn (mg l^{-1})	% Zn measured after filtration							% Zn measured before filtration						
	EDTA (mg l^{-1})							EDTA (mg l^{-1})						
	0	0.5	1.0	2.5	5.0	10.0	20.0	0	0.5	1.0	2.5	5.0	10.0	20.0
1.0	26	31	45	64	90	100	100	98	100	100.2	100	96	100	100
2.0	25	32	34	52	81	100	100	99	100	100.5	100	100	100	100
3.0	25	28	32	38	61	81	99	95	92	95	99	98	100	100
4.0	23	32	33	36	52	69	96	97	96	97	99	99	100	99
5.0	25	27	30	32	52	59	90	94	92	88	92	96	100	98
6.0	29	30	37	42	48	60	77	90	90	90	92	93	98	98
10.0	12	18	29	44	46	51	54	84	88	88	88	90	96	98

Table 2.6 Factors influencing Zn-toxicity

factors	salt used as a source for		complementary salt used if element omitted
	toxicity test	basal medium	
Na	NaCl	NaCl	-
Mg	$MgCl_2.2H_2O$ and $MgSO_4.7H_2O$	$MgSO_4.7H_2O$	Na_2SO_4
Cl	NaCl	NaCl	-
K	KCl	KNO_3 and K_2HPO_4	$NaNO_3$ and Na_2HPO_4
Ca	$CaCl_2.2H_2O$	$CaCl_2.2H_2O$	-
Mn	$MnCl_2.4H_2O$	$MnCl_2.4H_2O$	-
Fe	$FeCl_3.6H_2O$	Fe.EDTA	Na_2EDTA
Ni	$NiCl_2.6H_2O$	-	-
Co	$CoSO_4.7H_2O$	$CoSO_4.7H_2O$	
Cu	$CuSO_4.5H_2O$	$CuSO_4.5H_2O$	Na_2SO_4
Zn	$ZnSO_4.7H_2O$	$ZnSO_4.7H_2O$	Na_2SO_4
Cd	$CdSO_4.8H_2O$	-	
Hg	$HgCl_2$	-	
Pb	$Pb(NO_3)_2$	-	
NO_3-N	KNO_3	KNO_3	KCl
PO_4-P	K_2HPO_4	K_2HPO_4	KCl
SO_4-S	Na_2SO_4	$MgSO_4.7H_2O$	$MgCl_2.2H_2O$
EDTA	EDTA	Fe.EDTA	$FeCl_3.6H_2O$

Addition of antibiotics

penicillin-G: supplied as benzyl penicillin sodium salt (Sigma Products, 1669 units mg^{-1}).

polymixin B: supplied as the sulphate (Sigma Products, 7700 mg^{-1}).

streptomycin: supplied as sulphate with 3.0 H_2O per mole (Sigma Products 745 units mg^{-1}).

Stock solutions were prepared in deionized distilled water, sterilized by filtration (2.91 (iii)) and appropriate dilutions were added to cold, sterile medium. Sterile solutions of these antibiotics were stored at 4°C in the dark.

2.3 Subculturing

Subculturing was carried out with standard aseptic techniques, the subculturing process taking place in a horizontal laminar flow cabinet (confirming to B.S. 5295 class 1). This takes in air at the top through a filter which firstly removes the large particles and then passes the air through a high efficiency particulate air filter, out horizontally across the work surface in a laminar flow pattern. On the day of subculturing, the inoculum material was checked microscopically for contamination and in addition four petri dishes, each with a different bacterial test medium (2.423), were plated with a suspension of the *Anacystis nidulans* before being used as the inoculum for the experiment.

2.31 Liquid

No difficulty was found in obtaining a uniform inoculum of *Anacystis nidulans* due to the fact that typically the alga does not clump and suspends homogeneously. Experiments were commenced with a density of

(see P. 52)
2×10^5 units ml^{-1}. All toxicity tests were carried out in 10 ml of experimental sterilized medium in boiling tubes. In some cases when chlorophyll *a* was used as a growth criterion, or in the case of metal uptake, 50 ml medium was used in a 100 ml conical flask.

2.32 Solid

In order to obtain individual colonies, *Anacystis* was plated on to an agar surface. For this purpose, both low concentrations of agar (1% W/V: Allen 1968) and EDTA (instead of citrate as a chelating agent: Van Baalen 1965) were used.

The agar was mixed with the mineral medium before autoclaving. The inoculum was spread on the surface of the plates with an alcohol sterilised glass spreading rod.

2.33 Incubation and light source

Experiments were carried out either in a growth room or a tank of water with a shaking mechanism. Attempts to increase metal resistance and stock cultures were made in standing culture in the thermostatically controlled growth room maintained at 32°C. The cultures were shaken by hand once a day. Some other standing experiments were carried out in a 25°C room. Inocula for experiments and toxicity tests were grown in a thermostatically controlled tank of distilled water maintained at 32°C. A shaking mechanism moved the vessels through a horizontal distance of 33 mm about 72 times per minute. The flasks were illuminated continuously from beneath with warm white fluorescent tubes. The level of radiation at the surface of vessels was found to vary between different areas in the tank, particularly at the edges; it was however more or less constant in the middle of the tank, so to avoid these variations, no experimental vessel was incubated near the edges. In addition each

vessel was moved around daily. Tubes were held at a slanted angle in a wire cage in order to provide the circulation of media during shaking. Photosynthetic active radiation as measured by a Macam Quantum/Radiometer/Photometer Model Q 101 (Macam Photometrics Ltd) and light intensity as measured with an EEL lightmaster photometer (Evans Electroselenium Ltd) are summarized below:

growth chamber	source of fluorescent light	position of illumination	$\mu E\ m^{-2}\ s^{-1}$	lux
32°C growth room	white	above	25- 40	2000-2500
25°C growth room	white	above	25- 40	2000-2500
shaking tank	warm white	below	120-155	3000-4500
shaking tank with wire cage	warm white	below	75- 95	2500-3000

Light measurements by two methods not necessarily made at same time

2.4 Algal cultures

2.41 Origins

Anacystis nidulans was obtained from the Cambridge Culture Collection of Algae and Protozoa (1405/1); it has a Durham ref. no. 33A. It is the strain first characterized by Kratz and Myers (1955) and is considered identical to *Synechococcus* sp. PCC 6301, ATCC 27144 (Rippka *et al.* 1979). *Anacystis nidulans* was chosen because it is the blue green alga which has been most used both for experimental studies in general and studies of mutation in particular.

Details of other strains from culture collections and ones isolated from sites with elevated Zn are given in Table 2.7.

2.42 Isolation and purification

Algae were isolated by plating; the initial dried sample collected by Dr B. A. Whitton was sprinkled directly onto agar. ACM medium was used for *Synechococcus*, *Gloeothece* and *Phormidium*, while Chu10-D was

Table 2.7 Sources of cultures

organism	Durham culture no.	source	whether axenic	whether clonal
<u>low Zn</u>				
Anabaena cylindrica	2	Cambridge 1403/22	+	+
Aphanothece castagnei	551	G. A. Codd, Dundee		
Calothrix membranacea	179	Cambridge 1401/1	+	
C. parietina	550	Sand Sike, England (Zn, $\bar{x}$ 0.069 ± 0.006: Holmes and Whitton, in press)	+	+
<u>high Zn</u>				
Calothrix sp.	184	from Zn tank (Sinclair and Whitton 1977); subcultured at 8 mg l^{-1} Zn	+	+
Calothrix sp.	473	"La Croisette Ruisseau", Rhone Valley, France = Durham code 3027-50 (stream dry, but sediments with elevated zinc)	+	+
Gloeothece sp.	562	Elvins pond, Missouri (Zn, *c.* 8 mg l^{-1}: Whitton *et al.*, in press)		
Phormidium autumnale	475	Riou Mort, France = Durham code 3010-98 (Zn, *c.* 16 mg l^{-1})		+
Phormidium sp.	476	R. Etherow tributary England (Zn, *c.* 15 mg l^{-1}: Harding *et al.*, in press)		+
Synechococcus sp.	561	Elvins pond, Missouri (Zn, *c.* 8 mg l^{-1}: Whitton *et al.*, in press)		

used for all *Calothrix* strains, with the exception that for *Calothrix* D 473, 0.5 mg l^{-1} Ni was added (Ni apparently favoured healthy growth of *Calothrix* D 473 on initial isolation, though later experiments have failed to show this). Cycloheximide was added to the media to give a concentration of about 10 μg ml^{-1}. For agar media, the cycloheximide was dissolved in distilled water, and 0.1 ml of the solution pipetted on the top of the algal growth on the agar surface. Cycloheximide is active against a wide range of fungi, yeasts, and most eukaryotic algae, but is inactive against most bacteria (Kapoor and Sharma 1979). Blue-green algae are tolerant, at least in low concentrations.

2.421 Physical

Isolation was carried out by successive transfer on agar plates. This method had the advantage that gliding tended to separate new areas of growth from the original, contaminated inoculum. Areas containing the required algae could then be cut out and transferred for further subculture. It was normally possible to establish unialgal cultures after repeated sub-cultures. Clonal and bacteria-free cultures of three strains of *Calothrix* were successfully established after many transfers as follows. Algae from a young, vigorously growing culture were inoculated onto the middle of an agar plate, and incubated under normal growth conditions. After a week or so, there was usually a zone of hormogonia around the inoculum, sufficiently well separated for individuals to be picked off. A suitable area for hormogonia was located by using a binocular dissecting microscope, and a single hormogonium was picked off using a very fine needle, and transferred to a fresh agar plate. After successive transfers on agar, a hormogonium was transferred to fresh liquid medium. When visible growth was well established, the alga was tested for bacterial contamination.

2.422 Antibiotics

Attempts to obtain axenic cultures of *Phormidium* and *Calothrix* by means of exposure to various antibiotics mixtures based on some work by Droop (1967) were unsuccessful.

2.423 Tests of purity of the cultures

Tests have been made with the following media:

(i) beef peptone agar

(ii) malt extract agar

(iii) yeast extract agar

(iv) nutrient broth

(v) SST

The composition of those media was described by Hoshaw and Rosowski (1973). The most efficient medium found was the usual algal growth medium, supplemented with 0.02% casamino acid (Bacto-Difco) and 2% glucose and solidified by addition of 1% (W/V) agar.

2.5 Preparation of alga for assay

2.51 Estimation of growth

2.511 Unit counts and estimation of cell length

Most unit counts were carried out using a haemocytometer 0.1 mm deep (Improved Neubaur ruling) with cover glasses. After about 5 min settling time, nearly all the units were in focus. In most samples *Anacystis nidulans* did not exist as single cells, but as a mixture of rods, filaments and sometimes subspherical shapes. The term 'unit' is used here to cover the range of morphologies seen with the light microscope, including rods of various lengths, filaments apparently made up of closely attached rods, filaments without obvious cellular divisions and subspherical structures.

Most measurements of the units were made using an X40 objective lens and eye-piece of X10 magnification, with an X2 magnification lens in the light path. With this system the smallest eye-piece graticule unit of 0.01 mm was equivalent to 1.5 μm.

2.512 Extraction and estimation of pigments

(i) Chlorophyll *a*

The algal suspensions were made up to the original volume with distilled water, and were harvested by vacuum filtration through Whatman GF/F glass-fibre paper (2.91 (i)). The alga and the solvent (95% methanol) were then placed in 30 ml McCartney bottles and incubated for 10 min in a darkened water bath at 70°C, and then refiltered. The final filtrate was made up to a standard volume. The chlorophyll peaks were read immediately after extraction using an ultra violet-visible spectrophotometer (Perkin-Elmer 402). Absorption spectra were read at 665 nm and corrected for turbidity by subtracting the absorbance at 750 nm. Extracts were then acidified by adding one drop of 1N Analar HCl in the optical cell carefully mixed in with a pasteur pipette; the absorbance at 665 nm was then read again.

Neutralization of the extracts with magnesium carbonate was not used because neutralization by magnesium carbonate on absorption spectra shows no effect (Marker 1972).

Chlorophyll *a* was calculated from the formula given by Marker (1972), but here a different "acid factor" has been derived. This formula can be written as follows:

$$\text{Chl } a \ (\mu g/\text{sample}) = 2.61 \ (A_b - A_a) \times \frac{V}{l} \times 13.1$$

A_b = absorbance at 665 nm before acidification

A_a = absorbance at 665 nm after acidification

V = volume of extract (ml)

l = light path of optical cell (cm)

13.1 = constant, assuming a specific absorption coefficient of Chl *a* in 95% methanol of 76.07 l g^{-1} cm^{-1}

2.61 = constant derived from an acid factor of ($\bar{x}$ 1.60 $\pm$ 0.2)

The acid factor was calculated from 12 samples of the *Anacystis nidulans*. In the case of *Calothrix* D184 and *Anabaena cylindrica* an experimental maximum acid factor of 1.8 was determined. It was calculated according to the method of Marker (1972):

$$\frac{\text{absorbance at 665 nm before acidification}}{\text{absorbance at 665 nm after acidification}}$$

using the mean of acid factor, a constant of 2.61 and 2.28 were derived for use in the Chl *a* equation. This constant was derived as follows:

$$\text{constant} = \frac{\text{acid factor}}{\text{acid factor} - 1} = \frac{1.62}{1.62 - 1} = 2.61$$

(ii) Phycocyanin

The lysozyme technique to be described below was employed successfully. A range of physical techniques were tried: sonication, mortar and pestle with acid washed sand, rapid repeated freezing and thawing in the presence of 0.05 M phosphate, use of liquid nitrogen. All were not efficient in releasing phycocyanin completely. Comparisons are given in Table 2.8.

Lysozyme extraction was originally based on work by Crespi *et al.* (1962). The alga was collected from growing cultures by centrifugation for 30 min at 3000 x g. The supernatant was discarded; the residue was washed twice with distilled water. Prior to phycocyanin extraction, chl *a* was extracted first with 80% acetone and discarded (in this experiment only, because methanol was found to effect phycocyanin extraction). If chl *a* was required for comparison, the sample was divided into two 25 ml aliquots; one was used for chl *a* extraction (using 95% methanol), and the second was used for phycocyanin. The algal pellet, which was blue in colour, was incubated with 1 mg l^{-1}

Table 2.8 Comparison between methods used for phycocyanin extraction from *Anacystis nidulans*. Lysozyme experiments carried out in dark at 25°C; pH 6.8.

method	% control
1) lysozyme incubated for 96 h	100
2) lysozyme incubated for 96 h + 30 minutes sonication	100
3) lysozyme incubated for 48 h	96.7
4) lysozyme incubated for 36 h	81.2
5) lysozyme incubated for 24 h	70.9
6) lysozyme incubated for 12 h	35.4
7) sonication for 60 min	71.6
8) sonication for 45 min	62.0
9) sonication for 30 min	45.8
10) acid-washed sand extraction using mortar and pestle	14.6
11) freezing and thawing with liquid nitrogen	35.6
12) *in vivo* (no step for any extraction)	21.5

lysozyme (muramidase, mucopeptide N-acetylmuramoylhydrolase; E.C. No. 3.2.1.17) in 0.05 M phosphate buffer, at pH 6.8, incubated at 25°C in the dark. At intervals, the tubes were moved from the dark, centrifuged for about 10 min (to facilitate filtration), and then filtered through glass-fibre paper and the solution made up to a standard volume with 0.05 M phosphate buffer. It was found that phycocyanin started to degrade within 5 days after it had been released; it produced an odour resulting from lysozyme deterioration; addition of NaCl or incubation at 4°C helped to control this problem. Comparisons are given in Table 2.9.

The absorption peaks of phycocyanin were read directly at 618 nm. The concentration of phycocyanin was calculated from the following equation in which correction is made for chlorophyll absorption (Myers and Kratz, 1955):

$$OD\ phycocyanin = 1.016_{618} - 0.203\ OD_{677}$$

percent phycocyanin was then determined by dividing the correct absorption of 618 nm by 0.073, the extension coefficient of Craig and Carr (1968). These values were then normalised to a mg dry weight alga basis.

2.513 Dry weight

Algal material was separated from the growth medium by centrifugation for 30 min at 3000 x g, in acid washed centrifuged tubes, washed thrice with EDTA, for accumulation experiments. The liquid was decanted off and kept for further analysis. The washed algal pellet was transferred to acid-washed snap-top glass vials (previously dried at 105°C) and dried for 48 h at 105°C. On removal from the oven, the vials were placed immediately in a desiccator to prevent absorption of water as they cooled to ambient temperature. For accumulation studies (Chapter 6) the alga was weighed together with the Nuclepore filter (Filters without alga were shown to have a reasonably constant dry weight. After drying

Table 2.9 Effect of incubation medium and time on the release and subsequent degradation of phycocyanin from *Anacystis nidulans* by lysozyme. Experiments carried out in the dark.

incubation time (h)	phycocyanin (mg l^{-1})		
	+ NaCl, 25°C	- NaCl, 25°C	- NaCl, 4°C
24	2.1	2.1	2.0
48	2.7	2.7	2.6
72	2.8	2.7	2.7
96	2.8	2.7	2.8
120	2.8	2.4	2.8
144	2.7	2.0	2.8
168	2.8	1.4	2.7
182	2.8	0.8	2.8

at $105^{\circ}C$ for 48 h, $\bar{x}$ = 3.2 mg ± 0.068, n = 20.).

2.514 Turbidity

In applying this method it is necessary that the cultures are well shaken and evenly distributed. For this reason a Vortex stirrer (Griffin) was used. Factors such as precipitation of the medium, air bubbles, contamination of the algal suspension with bacteria can interfere with the results. Precautions were taken to avoid these factors. Clean tubes of uniform diameter were washed with distilled water after each single reading; blanks were used for both autoclaved and non-autoclaved media for individual sample. The deflection caused by the test tube and media, which was estimated before the culture started, was subtracted from the actual reading. High turbidity was found at higher concentrations of phosphate, calcium, manganese or zinc in autoclaved media. Comparisons are given in Tables 10a and 10b. The alga was checked at the end of each experiment to be sure no bacterial contamination had occurred. Measurements were made with a Unigalvo Nephelometer (Evans Electroselenium Ltd). The sample was illuminated with a light source, of the same colour as that of the sample by using a OGRI filter and photoelectric detector with a readout device to indicate the intensity of scattered light.

A standard reproducible calibration curve was prepared (Fig 2.1) showing the relationship between population density and turbidity scales on the nephelometer. The plot was linear up to 1.6×10^{8} units ml^{-1} for the wild-type and up to 7×10^{7} units ml^{-1} for the Zn-tolerant strains. In all cases of doubt a direct count was also made, using a haemocytometer.

2.52 Techniques used for comparison of toxicities

Estimates of toxicity to a particular strain were made in batch cultures on growth, lag and in some cases final yield.

Table 2.10b. Influence of Ca as $CaCl_2$ on turbidity of ACM medium (see 2.514).

Zn (mg l^{-1})	Ca (mg l^{-1})								
	0	2.5	5.0	10	20	40	80	160	320
0	2.0	2.0	2.2	2.5	2.5	2.6	2.8	3.4	3.5
1.0	2.0	2.0	2.5	2.5	2.5	2.5	2.8	3.4	3.5
1.5	2.0	2.2	2.6	2.8	2.5	2.8	2.8	3.6	3.6
2.0	4.0	4.0	4.0	4.0	4.5	4.6	4.5	4.5	4.8
2.5	4.5	4.7	4.8	4.6	4.7	4.8	4.8	6.0	6.0
3.0	5.0	5.5	5.6	5.8	5.7	5.7	5.8	6.2	6.2
3.5	5.0	6.0	5.8	6.0	6.2	6.2	6.2	6.4	6.6
4.0	6.0	7.0	7.2	7.0	7.0	7.0	7.5	7.5	7.5
4.5	6.2	7.5	7.5	7.6	7.6	7.8	7.6	8.1	8.2
5.0	6.5	8.0	8.2	8.1	8.5	8.6	8.5	9.0	9.5

Table 2.10a. Influence of Mn as $MnCl_2$ on turbidity of ACM medium (see 2.514).

Zn (mg l^{-1})	Mn (mg l^{-1})										
	0	0.05	0.10	0.5	1.0	1.5	2.5	5	10	15	20
0	1.2	2.0	2.0	2.0	2.0	3.0	3.0	4.0	5.0	5.0	6.0
1.0	1.2	2.0	2.0	2.0	2.5	3.0	3.0	4.0	5.0	5.0	6.0
1.5	1.5	2.0	2.5	3.0	3.0	3.0	4.0	4.0	5.0	5.0	6.0
2.0	1.6	4.0	4.0	4.0	4.0	4.0	5.0	5.0	6.0	6.0	7.0
2.5	1.8	4.5	4.0	5.0	4.0	4.0	5.0	5.0	6.0	7.0	7.0
3.0	2.5	5.0	5.2	5.6	5.6	5.6	6.0	6.0	7.0	7.4	7.5
3.5	3.0	5.0	5.0	6.2	6.5	6.5	6.5	6.6	7.0	7.4	7.4
4.0	3.5	6.0	6.0	6.2	6.6	6.6	6.5	6.7	7.4	7.6	7.8
4.5	4.0	6.2	6.5	6.5	6.6	6.6	6.5	7.2	7.8	8.0	8.0
5.0	4.6	6.5	6.5	6.5	6.6	6.6	6.5	8.0	8.0	8.0	8.0

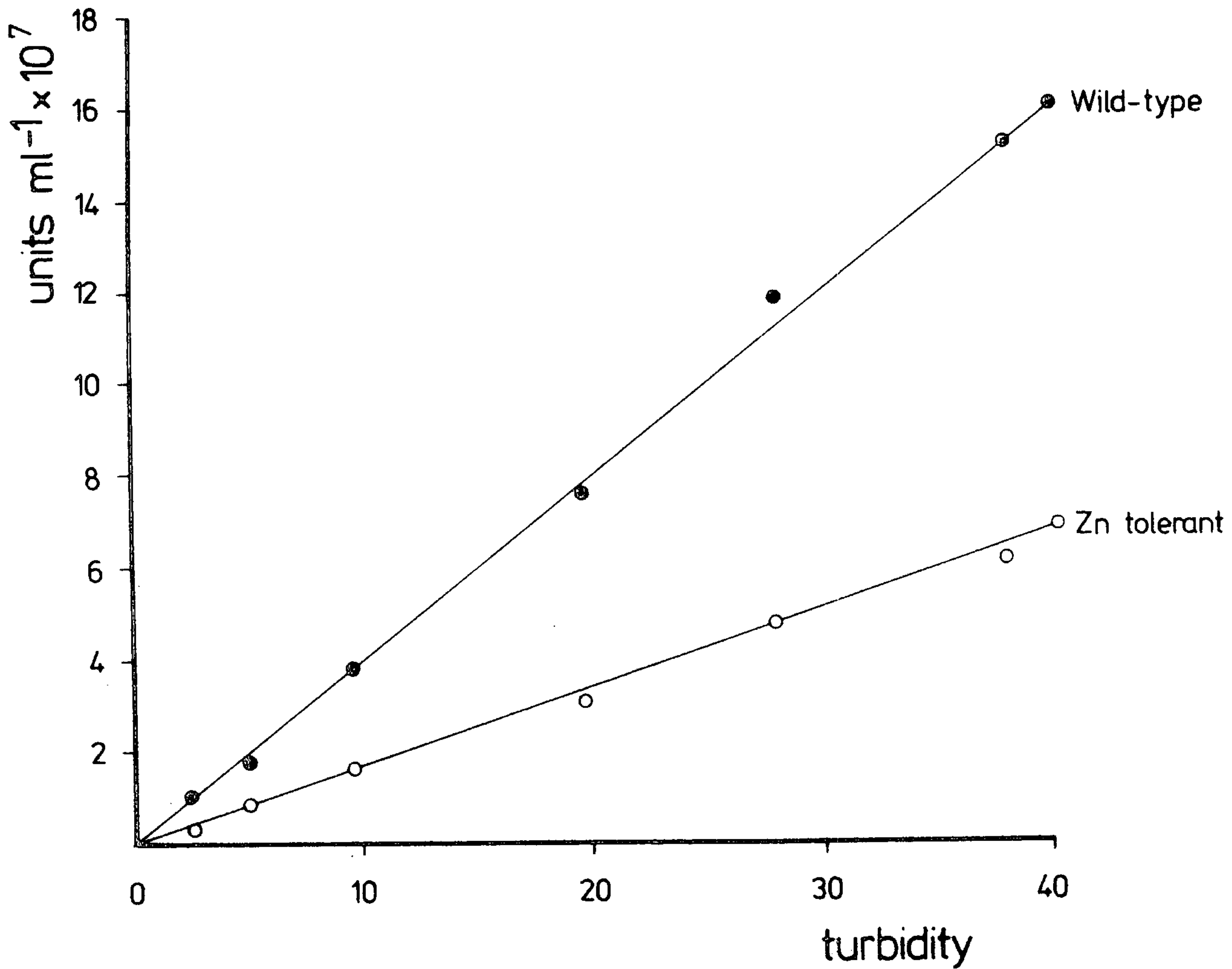


Fig. 2.1 Relationship between population density and turbidity during growth of wild-type and Zn-tolerant strains of *Anacystis nidulans*.

A series of growth curves was made for different metal levels and data from these were used to plot a further graph from which the various indices of toxicity could be estimated.

(i) Toxicity based on growth rate is expressed as that concentration of metal leading to a 50% reduction in rate of exponential growth as compared with a control grown in basal medium. In most cases part of the growth curve was in fact exponential, but when this was not so, an estimate of the fastest growth obtained was used for the present calculation.

(ii) Toxicity based on lag is expressed as the concentration of metal leading to an increased lag (as compared with that of the control) equal to the doubling time during exponential growth of the control.

(iii) Toxicity based on final yield is expressed as the concentration of metal permitting 12.5% of the yield obtained with the control. (In practice, yield was estimated as the number of units x mean length of units; this neglects possible changes in cell diameter.)

Other potentially ambiguous terms needed to summarize the results are defined as follows. A strongly inhibitory level of a toxic agent refers to that level which just permits detectable growth; a slightly higher level kills all cells unless mutation occurs. An estimate of the strongly inhibitory level can only be approximate, but provides a much quicker means of making a comparison than the time consuming methods described above. Resistant and tolerant are used in the manner adopted by many workers on higher plants. A resistant organism is one which can withstand relatively high levels of a potentially toxic agent, whatever the mechanism by which it does this;

a tolerant strain is one which survives higher levels due to genetic differences from other strains.

(iv) T.I.C. (Tolerance Index Concentration). The assay was a refinement of that described by Whitton (1970a). Growth in the flasks or tubes was compared visually on days 2, 4, 6, 8 both against preserved replicates of the original inocula and also with each tube one against the other. Observations were recorded on each occasion as follows:

I Maximum concentration of metal causing no lag in algal growth

II Maximum concentration of metal causing inhibition

III Maximum concentration of which alga is alive

IV Maximum concentration of metal required to kill alga

The Tolerance Index Concentration was calculated as = $(I.\ II.\ III.\ IV)^{1/4}$

2.6 Production of resistant *Anacystis* strains

2.61 NTG

The procedure was based on work of Kumar (1968), with antibiotics. In preparation for this experiment, exponential grown units were harvested by Millipore filtration, washed with M/20 Tris-maleic buffer of pH 8.0, and suspended in 20 ml of Tris-maleic buffer (mixture of Tris + maleic acid, pH 5.0). Aliquots of 0.2 ml from appropriately diluted suspensions were spread on agar plates, solidified by (1% W/V) agar, to serve as controls. Freshly prepared N-methyl-N'-nitro-N-nitrosoguanidine in Tris-maleic buffer (pH 5.0) was added into the remaining suspension to obtain a final NTG concentration of about 400 $\mu g\ ml^{-1}$. The reaction mixture was shaken for some time after mixing. One ml aliquots were withdrawn after time intervals between 0 - 60 minutes, then the units were washed twice with sterile

ACM (pH 7.0) on Millipore filters. The washed units were then suspended in ACM medium to obtain the same unit concentration as in untreated controls, and 0.2 ml aliquots spread on agar plates. All plates were sealed with parafilm and incubated at $32^{o}C$ for 10 days. They were then overlaid with a thin layer of agar (0.7 W/V) containing different Zn concentrations (2 up to 12 mg l^{-1}).

2.62 Serial subculture

see 3.2

2.7 Study of environmental factors

2.71 Factors influencing toxicity

During the studies outlined below, the addition of the salts of the ions, whose effect upon toxicity were being tested, caused simultaneous addition of varying levels of Na, Cl or SO_4. A series of toxicity tests were therefore performed upon *Anacystis nidulans* with varying levels of NaCl or Na_2SO_4 in the medium.

The initial pH in all experiments testing these factors was kept within limits 7.0 ± 0.1 pH units using NaOH to make the adjustment, except on study of effect of Na on toxicity, when KOH was used. For practical purposes the experimental procedure for investigation of each factor affecting metal toxicity was varied slightly.

(i) Zn toxicity: inocula for each factor tested were obtained from cultures incubated in a medium containing a very low level of that factor (i.e. if the factor to be tested was Mg, the algal stock was incubated in a medium with 0.25 mg l^{-1} Mg for 24 - 48 h prior to inoculation). For studies on the effect of phosphate, cultures to be used as inocula for the standard toxicity tests were incubated in

phosphate-free medium for four days prior to the experiment. Two other experiments were carried out to demonstrate the effect or organic phosphate on Zn-toxicity. Sterilized β-glycerophosphate and α-D glucose-1-phosphate (Sigma products) were added to sterile medium after autoclaving to provide the same level of P as in inorganic phosphate tested.

(ii) Cu toxicity: The procedure used for Cu closely followed that of Zn but due to the greater toxicity found with Cu a lower range of levels was used in the assay. Addition of Cu at the concentrations used caused no shift in the pH of ACM medium.

(iii) Cd toxicity: As with Cu, the procedure closely followed that used with Zn, but again a lower range of Cd concentrations was used. No pH shift was encountered.

2.72 Inoculum size

The size of the inoculum on Zn-toxicity was tested only for the wild-type. The investigations were carried out by using a series of dilutions of algal suspension, with sterilised ACM medium as diluent. Both chl *a* and unit counts were used as criteria for growth. The final units ml^{-1} in each vessel at the beginning of culture were 10^4, 10^5, 10^6, and 10^7 units ml^{-1}.

2.73 Factors influencing Zn solubility

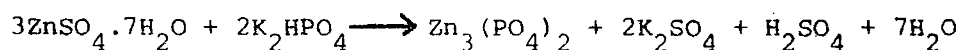
pH The experiment was carried out simultaneously to investigate the effects of various pH levels, on both the toxicity and the solubility of Zn. Four replicates in 100 ml flasks were set up at different pH levels, increasing by 0.5 units in the range 6.0 to 8.0, at six levels of Zn, range 1 - 10 mg l^{-1}. The medium was buffered and adjusted to the required pH, using 0.2 N NaOH or 0.2 N HCl. The flasks were incubated under the same conditions as used for the actual assay.

After a period of time each flask was filtered and the level of Zn was measured. The critical range of pH for significant precipitation of filtered Zn in ACM medium was pH 6.5 - 8.0. The results are given in Table 2.11.

EDTA: see (2.23)

Calcium The procedure was repeated with four Ca levels. Changes in the level of Ca in the range 5 - 100 mg l^{-1} has a very slight effect on the level of filtrable Zn in ACM medium (Table 2.12).

Phosphate The experiment was performed simultaneously with the investigation of the effect of different concentrations of phosphate on the Zn toxicity. A white to yellowish precipitate appeared in most high phosphate concentrations with Zn at above 5 mg l^{-1}; this precipitate may perhaps be due to the white Zn-phosphate precipitate $Zn_3(PO_4)_2$ which is insoluble in water:



$$3ZnSO_4.7H_2O + 2K_2HPO_4 \longrightarrow Zn_3(PO_4)_2 + 2K_2SO_4 + H_2SO_4 + 7H_2O$$

This precipitate was not visible if the phosphate was added after autoclaving. Comparisons of autoclaved and non-autoclaved media are given in Table 2.14. Samples were analysed for Zn, indicating that 70 - 75% of Zn was in solution after filtration in non-autoclaved medium in a comparison of that 46 - 50% in autoclaved medium.

2.8 Experimental procedure for accumulation studies

2.81 EDTA washing

In a preliminary experiment it was found that 40 mg l^{-1} EDTA was the most efficient level for removing all the absorbed Zn from the surface of the cells. Comparisons are given in Table 2.15.

An uptake experiment was carried out, in which *Anacystis nidulans*

Table 2.11 Influence of pH on zinc solubility on ACM medium ($10\ mg\ l^{-1}$ EDTA + HEPES : Table 2.2); medium autoclaved and then left to stand for 24 h before experiment. (See also Tables 2.5, 2.12, 2.13, 2.14).

original Zn ($mg\ l^{-1}$)	% Zn measured after filtration pH values				
	6.0	6.5	7.0	7.5	8.0
1.0	99	99	100	94	90
2.0	100	100	99	88	72
4.0	97	90	68	55	38
6.0	95	88	56	42	24
8.0	93	85	50	34	18
10.0	90	78	46	26	10

Table 2.12 Influence of Ca on zinc solubility in ACM medium (pH 7.0, + HEPES + 0.5 mg l^{-1} EDTA: Table 2.2); medium autoclaved and then left to stand for 24 h before experiment. (See also Tables 2.5, 2.11, 2.13, 2.14).

original Zn (mg l^{-1})	% Zn measured after filtration Ca (mg l^{-1})				% Zn measured before filtration Ca (mg l^{-1})			
	5	10	50	100	5	10	50	100
0.04	0.016	0.016	0.016	0.016	0.015	0.012	0.016	0.016
1.0	37	37	31	28	100	100	96	88
2.5	32	34	29	22	95	96	95	90
5.0	36	39	32	27	97	99	92	91
10.0	32	36	32	32	99	95	89	88
20.0	34	35	34	35	83	85	89	88

Table 2.13 Influence of PO_4-P on zinc solubility on ACM medium (pH 7.0 + HEPES + 10 mg l^{-1} EDTA: Table 2.2); medium autoclaved and then left to stand for 24 h before experiment. (See Tables 2.5, 2.11, 2.12, 2.14).

original Zn (mg l^{-1})	% Zn measured after filtration				% Zn measured before filtration			
	PO_4-P (mg l^{-1})				PO_4-P (mg l^{-1})			
	1.75	7.0	14.0	56.0	1.75	7.0	14.0	56.0
2 g	81	80	78	68	100.5	100	99	99
n	74	77	78	73				
3 g	77	75	73	73	100.3	99	98	98
n	75	73	72	71				
4 g	70	67	69	63	97	95	96	95
n	65	64	63	63				
5 g	60	58	55	52	98	95	94	95
n	58	57	55	53				
10 g	46	40	38	35	97	93	95	95
n	45	41	40	36				

Table 2.14 Influence of autoclaving and non-autoclaving on Zn solubility in ACD medium (pH 7.0 + HEPES + 10 mg l^{-1} EDTA: Table 2.2); medium autoclaved and left to stand for 24 h before experiment. (See also Tables 2.5, 2.11, 2.12, 2.13, 2.14).

original Zn (mg l^{-1})	% Zn measure after filtration		% Zn measure before filtration	
	autoclaved	non-autoclaved	autoclaved	non-autoclaved
1.0	100 ± 3.8	100 ± 2.6	100.1 ± 4.2	98 ± 8.2
2.0	99 ± 1.5	98 ± 2.6	100.5 ± 2.5	100 ± 1.6
3.0	80 ± 2.4	95 ± 3.7	100 ± 7.5	97 ± 4.4
4.0	68 ± 1.7	94 ± 2.5	100.3 ± 7.6	100 ± 2.5
5.0	57 ± 4.7	77 ± 5.6	100 ± 2.0	100 ± 9.6
6.0	58 ± 4.7	84 ± 1.3	99 ± 1.1	100 ± 1.4
7.0	51 ± 1.3	77 ± 8.2	99 ± 1.3	99 ± 1.5
8.0	50 ± 4.7	69 ± 8.2	99 ± 2.8	98 ± 1.3
9.0	50 ± 4.7	66 ± 1.4	99 ± 1.8	100 ± 1.3
10.0	46 ± 4.7	65 ± 8.2	98 ± 4.3	99 ± 9.6

Table 2.15 Influence of EDTA (pH 5.4) on removal of Zn from *Anacystis nidulans*; alga stirred for 5 min in contact with 10 ml of EDTA at 4°C, then collected by centrifugation.

time (d)	EDTA ($mg\ l^{-1}$)	Zn left in the media ($mg\ l^{-1}$)	Zn washed with EDTA ($mg\ l^{-1}$)	dry weight ($mg\ l^{-1}$)	μg Zn g^{-1} dry weight
0	0	0.41		30	19400
2		0.40		52	11360
4		0.45		96	6072.9
6		0.39		174	3030
0	0.5	0.41	0.12	36	12638
2		0.39	0.14	52	8884.6
4		0.39	0.09	94	5691.5
6		0.38	0.10	178	2520.2
0	5	0.38	0.33	32	11625
2		0.38	0.28	50	6720
4		0.36	0.16	100	4120
6		0.37	0.14	172	2427
0	10	0.38	0.37	32	8375
2		0.40	0.32	50	6320
4		0.40	0.26	98	3440
6		0.39	0.21	172	2213.5
0	20	0.38	0.40	32	6812.5
2		0.39	0.35	52	4923
4		0.40	0.26	100	3250
6		0.39	0.22	170	2026.3
0	40	0.40	0.58	30	400
2		0.37	0.60	56	446.43
4		0.38	0.54	96	645.8
6		0.38	0.48	174	701.03
0	80	0.40	0.58	30	366.6
2		0.38	0.56	50	480
4		0.38	0.60	98	489.8
6		0.37	0.56	176	494.74

cells which had taken up Zn were suspended in 40 mg l^{-1} EDTA (pH 5.4). The algal cells were stirred for 5 min in contact with measured volume of EDTA at 4°C, by using a vortex stirrer. The cells were collected by centrifugation with cooling using a MSE MISTRAL 4 l centrifuge. The procedure was repeated twice or sometimes thrice, the supernants being decanted each time into acid washed snap-top vials and stored in a refrigerator, with a drop of Aristar HNO_3 until analysis (usually within the period of the experiment).

2.82 Acid digestion

All samples of the alga were dried for 48 h at 105°C in acid washed snap-top glass vials and cooled in a desiccator. Digestion in 1 ml boiling Aristar HNO_3 was then carried out in the same vials for about 20 min, in which clear solution was nearly formed. Digest solutions were made up to 5 ml in volume in acid washed volumetric flask with deionized distilled water, stored in the same vials.

The reason for all the steps of digestion being in the same vial was to avoid any chance of cross contamination of Zn from one container to another. Three blanks were included with each new batch of digests:

(i) boiling Aristar HNO_3 only in snap-top vials;

(ii) deionised distilled water;

(iii) filter digested with boiling Aristar HNO_3.

2.9 <u>Specialized techniques</u>

2.91 Filtration

Three types of filtration were used:

(i) Filtration through Whatman GF/C or GF/F glass-fibre paper mounted into sintered glass discs of ultra-fine porosity, with a suitable holder, filtered, under pressure or partial vacuum. It is the simplest method and efficient for pigment extraction.

(ii) Filtration using a range of membrane pore sizes (0.22 μm, 0.45 μm Millipore filters, 0.2 μm Nuclepore filter) and Whatman GF/C glass-fibre paper was tested for metal analysis. Acid washed disposable plastic syringes were used to pass medium or sample through the filter, with 20 ml of deionized distilled water being through and discarded, before the collection of sample in acid washed snap-top vials.

(iii) Aseptic filtration technique was based on (ii). After mounting a filter with small pore size (usually 0.22 μm Millipore) in Swinnex plastic holder, the whole item was autoclaved. Sterilized disposable plastic syringes were used. The filtered sample was collected in a sterile empty flask.

2.92 Atomic absorption spectrophotometer

The analysis of metal ions in solution was determined by the flame Atomic Absorption Spectrophotometer (Perkin-Elmer 403). The aqueous samples are converted into their atomic vapour by aspiration into a flame through which is passed a beam of light energy of the same element being determined.

The atoms in the flame absorb energy from the beam and this absorption is related quantitatively to the concentration of the metal ions present in the sample.

Standard solutions were included at the beginning and end of a run and also periodically during longer runs. A blank was run normally between each sample or standard to verify baseline stability.

2.93 Acetylene reduction assays

Axenic cultures of *Calothrix* D 184 from the high zinc site and *Anabaena cylindrica* from an environment lacking Zn-enrichment were

used in acetylene reduction studies. The algae were maintained in liquid medium (ADM, nitrogen-free); 8.0 mg l^{-1} Zn was included in the medium for *Calothrix* D184. All experiments were incubated in a shaking tank maintained at 25°C and with an illumination about 120-155 $\mu Em^{-2}\ s^{-1}$/ 3000 - 4500 lux (see 2.33). Algae in the exponential growth phase were used in all experiments and Zn was added as $ZnSO_4.7H_2O$.

Nitrogenase activity was assayed using the acetylene reduction technique discussed by Hardy *et al.* (1973). Both algae were centrifuged under axenic conditions and the supernatant decanted; the algal pellet was then washed twice with ADM and the suspension homogenized by passing the algae gently two or three times through a sterilized syringe. A standard volume of each alga was re-suspended in fresh ADM medium with the required concentrations of zinc. The algae were incubated for 24 h, and sampled at 2 hourly intervals. Zero time and dark controls were included in all assays. Aliquots (2 ml) of algae were placed in 7 ml serum bottles and then sealed with perforated serum cap fitted with rubber liners. 1 ml acetylene (BOC) was injected through the serum liner with a syringe. Another syringe was used to equalize the pressure by venting through the liner. The serum bottles were shaken well to aid the dissolving of the gases prior to placing in a shaking tank maintained at 25°C with a 120-155 $\mu Em^{-2}\ s^{-1}$/ 3000 - 4500 lux. Five replicates were used in each case. The bottles were incubated for 90 minutes after the addition of acetylene. At the end of the experimental period, gas samples were removed with multiple-sample vacutainer needles (Becton and Dickinson Ltd) and stored in non-silicone coated, 5 ml draw vacutainers (Becton and Dickinson Ltd, A3206, formula 134).

A gas sample (1 ml) from the sealed vacutainer was injected into

a Varian Aerograph series 1200 gas chromatograph equipped with a hydrogen flame ionization detector, and a "Poropak" R (100/120 mesh) 1/8 inch by 6 feet (ca 3.0 mm x 1.7 m), stainless steel column. The operating conditions were as follows: detector temperature $150^{o}C$; column temperature $40^{o}C$; hydrogen flame rate 30 ml min^{-1}; air 300 ml min^{-1}; and nitrogen 30 ml min^{-1}. Ethylene peaks were identified on recorder traces by the retention time and quantified with standard curves. The chromatograph was calibrated using dilutions of high purity ethylene (99.9% Air Products Ltd) prepared using a Hamilton gas syringe. Aliquots of the standards including blanks, were injected into the serum bottles containing an equivalent liquid phase, and incubated along with the experiment. At the end of the experiment the standards were evacuated using vacutainers and then run through the gas chromatograph. In this way any deviation in the draw of a batch of vacutainers is eliminated.

The results of the acetylene reduction assays were expressed as the nMC_2H_4 produced μg chl a^{-1} min^{-1}.

CHAPTER 3

PRODUCTION OF RESISTANT STRAINS OF *ANACYSTIS NIDULANS*

3.1 Introduction

A relatively large number of antibiotic and drug-tolerant mutants have been isolated in blue-green algae, especially *Anacystis nidulans* (section 1.3). On the other hand no such metal tolerant strains have been reported.

3.2 NTG

The use of NTG as a mutagenic agent (section 2.6) failed to lead to any detectable increase in the rate of mutation for Zn tolerance. NTG also failed to lead to detectable mutation (1 in 5 x 10^8 units) in cultures lacking Zn enrichment.

3.3 Serial subculturing

3.31 Metals

It proved easy to increase the resistance of *Anacystis* to all five heavy metals studied (Co, Ni, Cu, Zn, Cd) by repeated subcultures being made from a strongly inhibitory level to a level just lethal to (wild-type) *Anacystis*. Four flasks were used for each subculture i.e. inoculum = 2 x 10^7 units. In any flask of the latter showing growth at least one flask each time was used as an inoculum for further serials subculture. In instances where more than one of the flasks showed growth, the culture was chosen which grew most rapidly. The process was repeated many times, leading to gradually increasing 'strongly inhibitory' levels. For instance, the level of Zn at which strong inhibition occurred was raised from 1.45 to 16.5 mg l^{-1} after 75 subcultures (Table 3.1). The lag period in most cases was very

Table 3.1 Resistance obtained (so far) on repeated subculture of *Anacystis nidulans* to progressively higher metal concentrations. (Four flasks used for each subculture i.e. inoculum = 2 x 10^7 units)

metal	no. subcultures	strongly inhibitory level of metal (mg l^{-1})	
		wild-type	most tolerant strain
Co	25	0.32	2.45
Zn	25	1.45	5.5
"	75	"	16.5
Ni	25	0.16	1.30
Cu	25	0.15	0.55
Cd	25	0.55	2.5

long, sometimes more than 48 h between each subculture, but it was shorted by repeated subculturing at least six times at that level which caused the lag.

Details of the maximum resistance obtained are given in Table 3.1 and the rate at which resistance to Zn was obtained is shown in Fig. 3.1. Colony formation on agar with different levels of Zn (Table 4.2) showed that the acquisition of increased resistance was due to the production of mutants.

The first mutants isolated from a wild-type population in response to strongly inhibitory Zn had an increase in tolerance, based on the criterion of exponential growth, of about 0.25 mg l^{-1} Zn. The mutants correspond to those regarded as spontaneous elsewhere in the blue-green algal literature, but critical experiments did not rule out the possibility that Zn itself has a role as a mutagenic agent. It proved impossible to demonstrate the presence of mutants when making subcultures from medium lacking Zn enrichment. Subcultures of 20 flasks each with 5×10^6 units from alga grown in Zn-free medium to a level just lethal to (wild-type) *Anacystis*, failed to lead to any increase in the rate of 'spontaneous' mutation (1 in 1×10^8 units) for Zn-tolerance.

All the metal-tolerant strains still grow well in basal medium. There was no indication that they now require higher levels of these metals for optimum growth. Only very slight changes were detectable in the lag, exponential growth rate and yield as compared with the wild-type. Two of the mutants with high tolerance for Zn

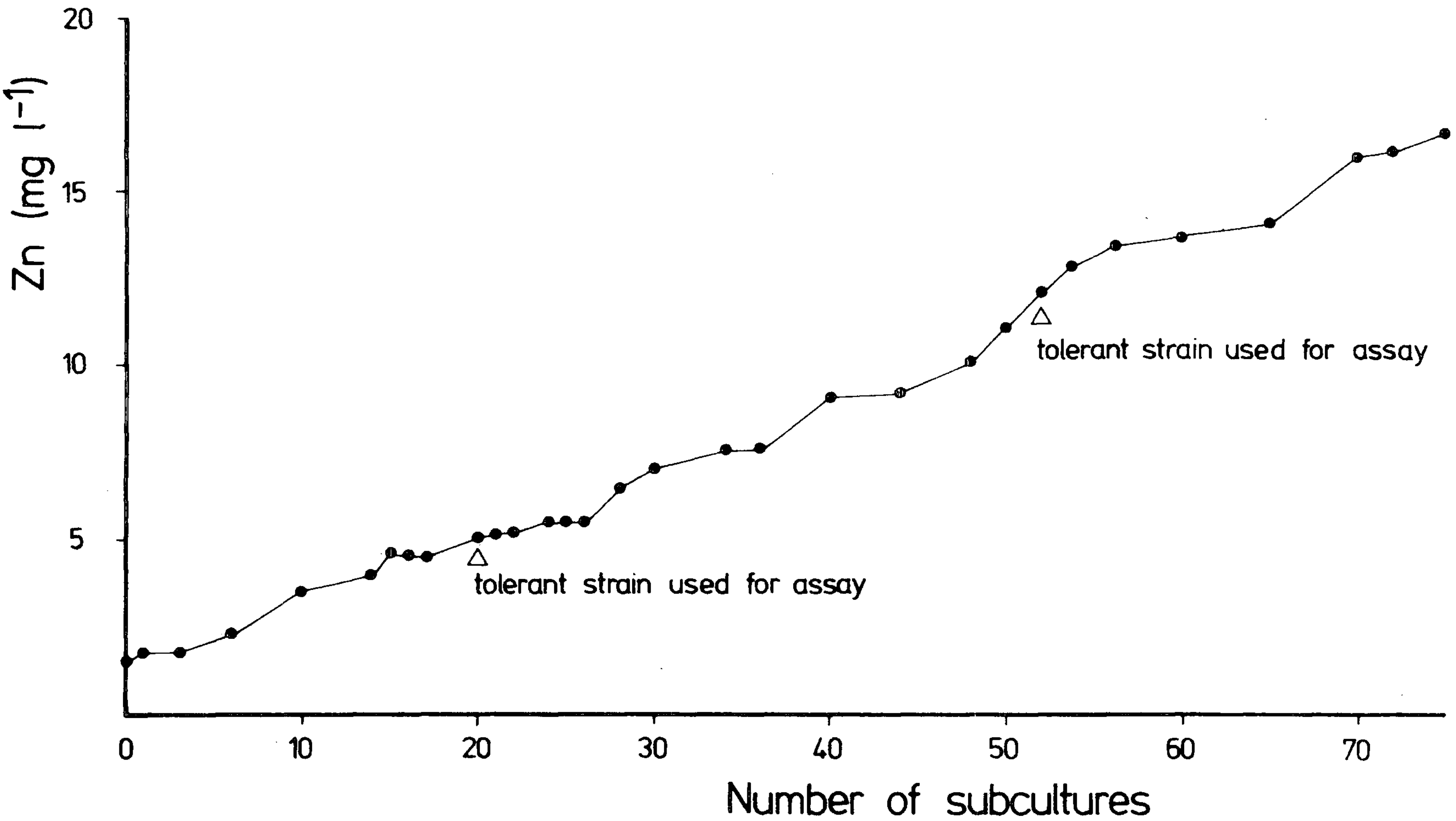


Fig. 3.1 Increase in resistance to Zn on repeated subculturing at progressively higher Zn-concentration (for detail see 3.31).

(Zn-t5.0, Zn-t12.0 mg l^{-1}) and one for each of the other metals were chosen for comparative studies.

The comparative response of the various strains to a particular metal was in general quite similar whether judged by growth rate, lag, or yield (Table 3.2); with the criteria used (2.52), the effect on yield was greatest. It can be seen from Table 3.2 that the partially subjective estimates of 'strongly inhibitory' growth are quite similar to the objective estimates of toxicity based on a 50% reduction in growth rate for four of the six mutants. Individual growth curves for tolerant strains are given below; those for wild-type *Anacystis* are included in Section 4.

Co-t1.8	Fig. 3.2 and	Table A3.1
Ni-t1.0	3.3	A3.2
Cu-t0.5	3.4	A3.3
Zn-t5.0	3.5	A3.4
Zn-t12.0	3.6	A3.5
Cd-t2.0	3.7	A3.6

Stability of Zn-resistance

Strains of *Anacystis* resistant to 5.0 and 12.0 mg l^{-1} Zn were subcultured in the absence of the metal for 72 and 96 generations respectively, and then inoculated into their respective Zn medium. Growth curves are shown in Figs 3.8 and 3.9, while the results are summarized in Table 3.3. These growth curves may be compared with those in Figs 3.5 and 3.6, in which algae were subcultured from a strongly inhibitory level of Zn. The Zn-t5.0 and Zn-t12.0 strains grew exponentially after a lag of about 24 and 48 h, respectively. This suggests that resistance to Zn is a stable trait.

Table 3.2 Tolerance of mutants used for experiments to the metals used in their isolation. (For details of assays, see Materials and Methods)

strain	toxic concentration of metal ($mg\ l^{-1}$) according to different criteria		
	lag	growth	yield
Co-t1.8	1.9	1.1	0.30
Bi-t-1.0	0.95	0.82	0.20
Cu-t0.5	0.35	0.45	0.12
Zn-t5.0	4.1	4.8	4.0
Zn-t-12.0	8.8	11.2	6.0
Cd-t2.0	1.5	1.35	0.50

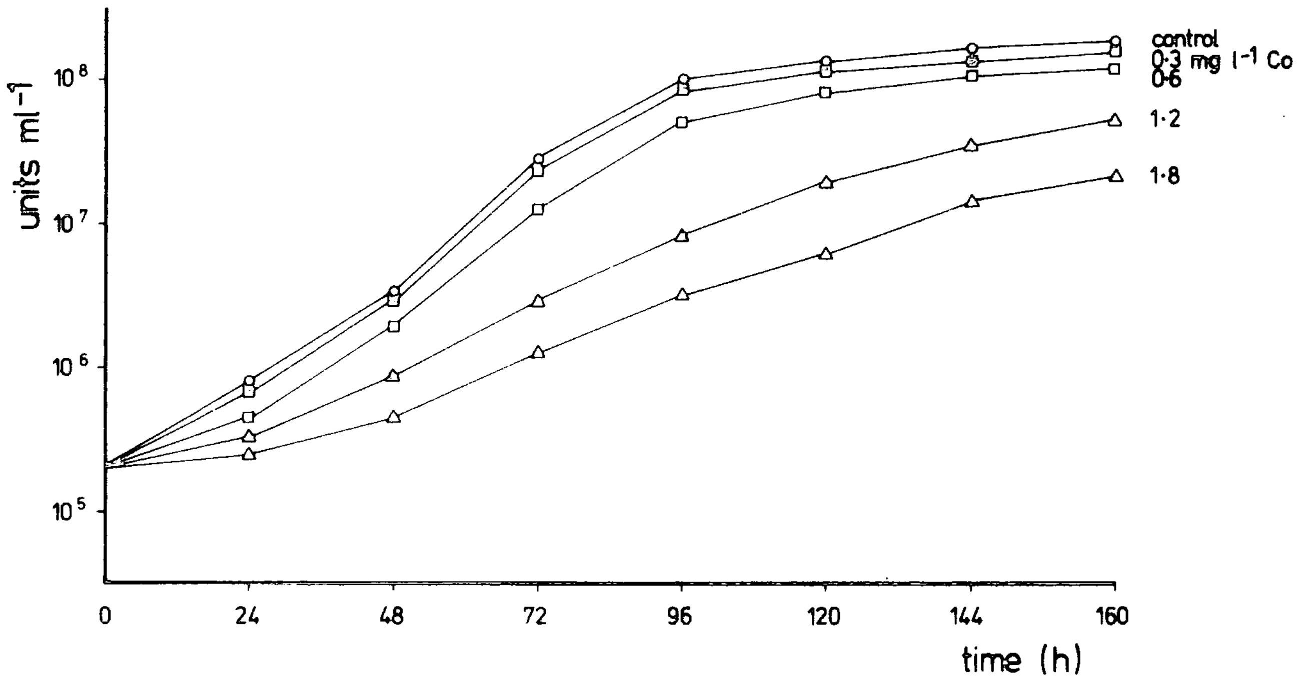


Fig. 3.2 Influence of Co on growth of Co-t1.8; inoculum was taken from strongly inhibitory levels of Co.

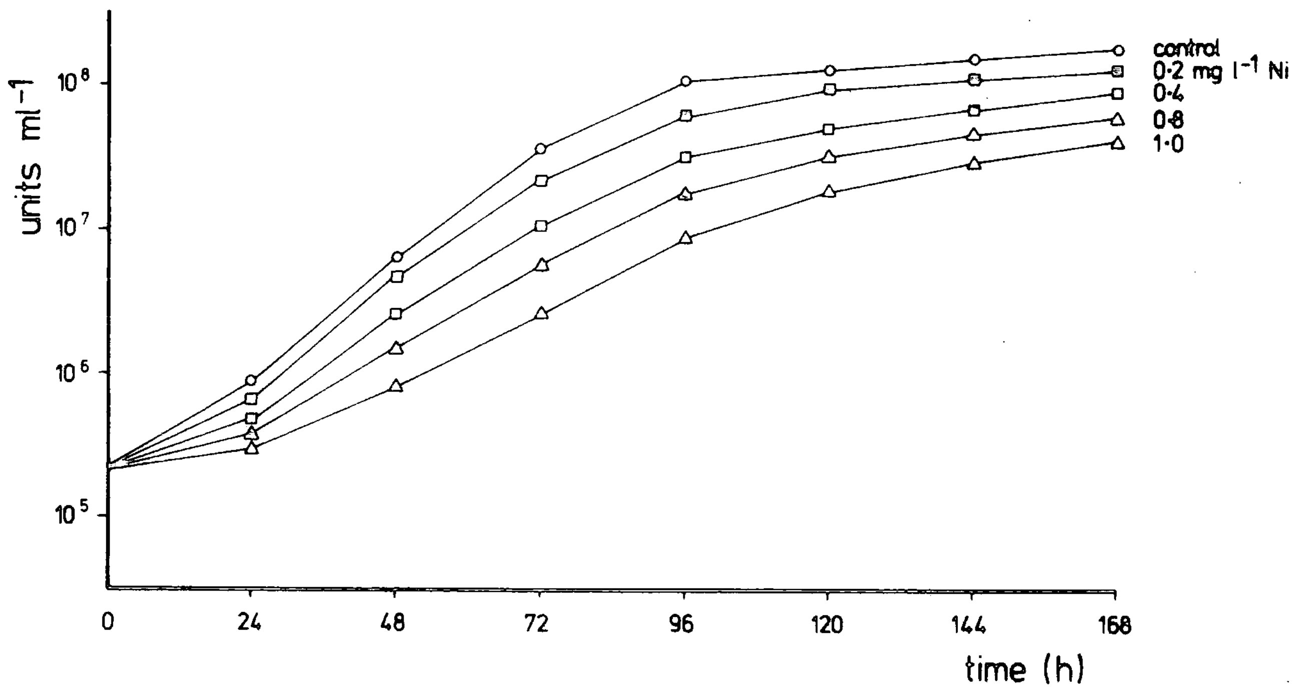


Fig. 3.3 Influence of Ni on growth of Ni-t1.0; inoculum was taken from strongly inhibitory levels of Ni.

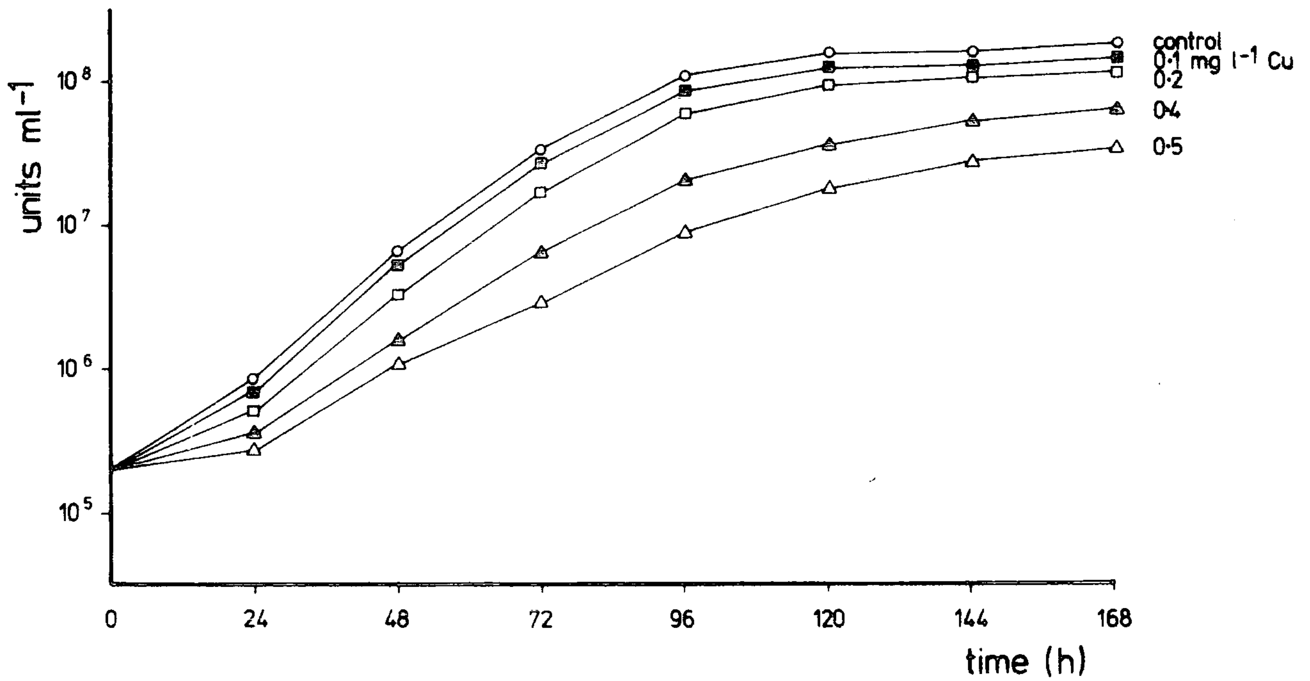


Fig. 3.4 Influence of Cu on growth of Cu-t0.5; inoculum was taken from strongly inhibitory levels of Cu.

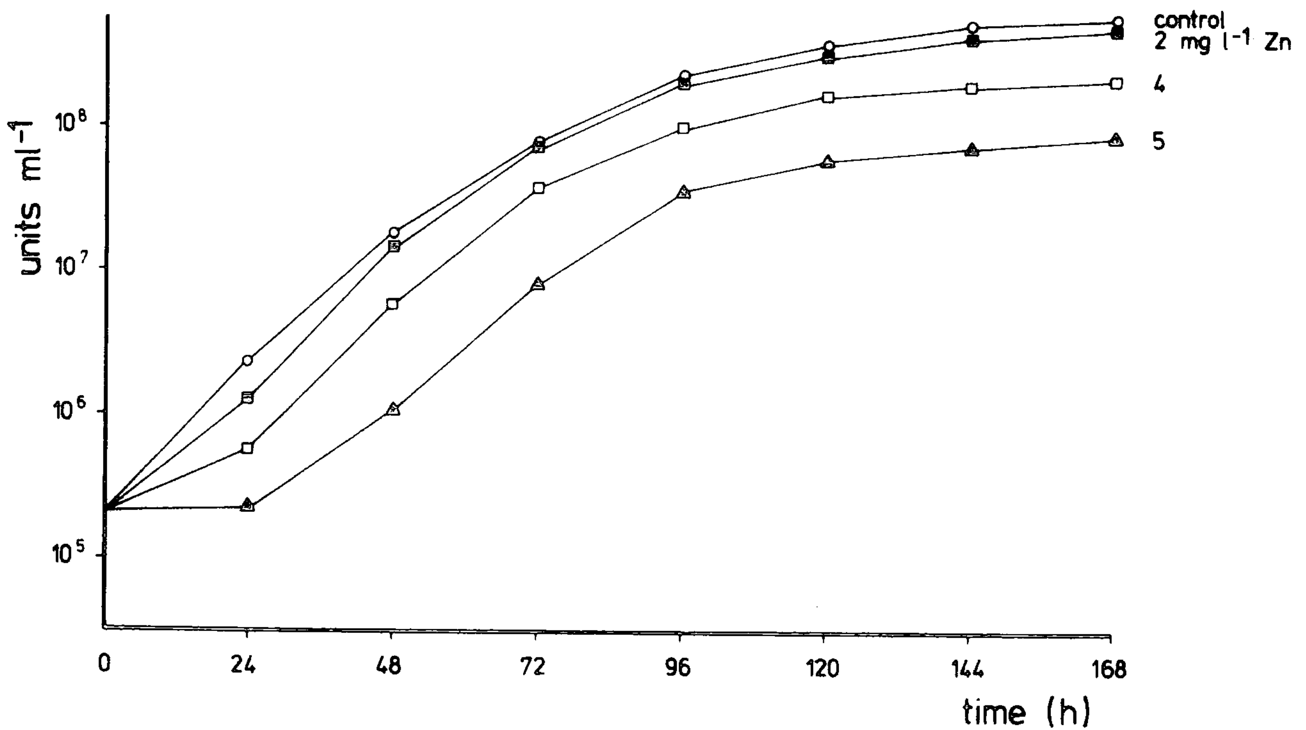


Fig. 3.5 Influence of Zn on growth of Zn-t5.0; inoculum was taken from strongly inhibitory level of Zn.

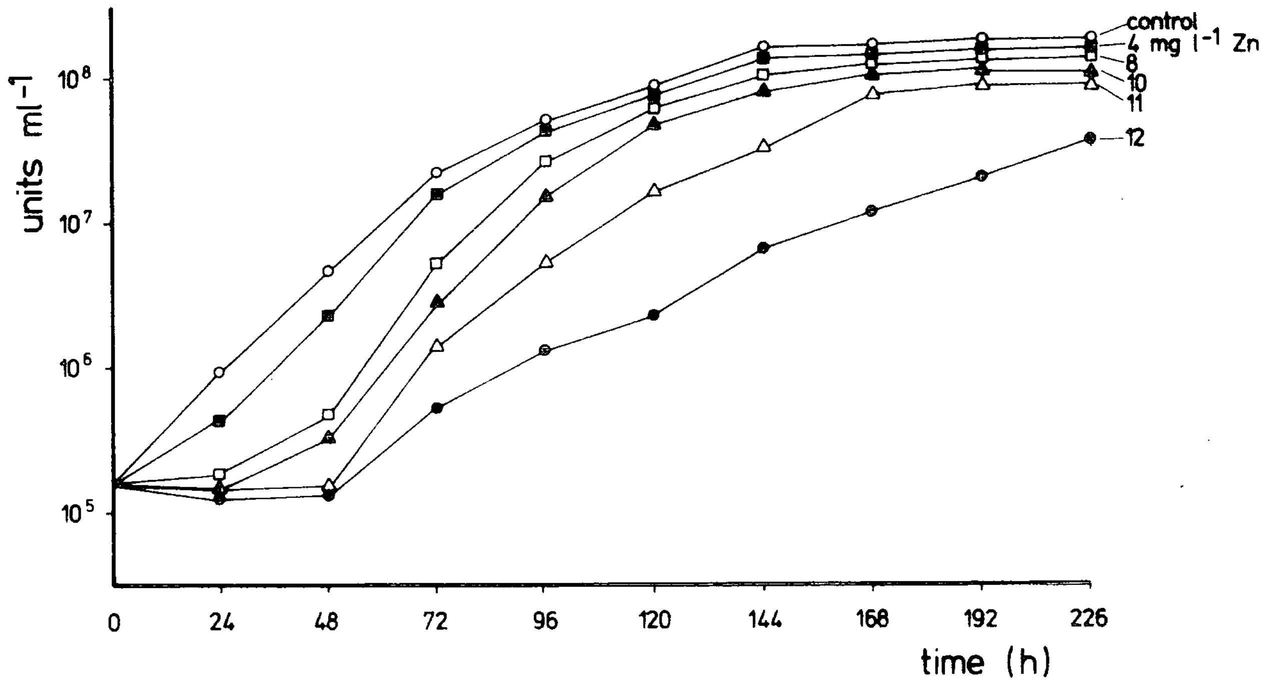


Fig. 3.6 Influence of Zn on growth of Zn-t12.0; inoculum was taken from strongly inhibitory level of Zn.

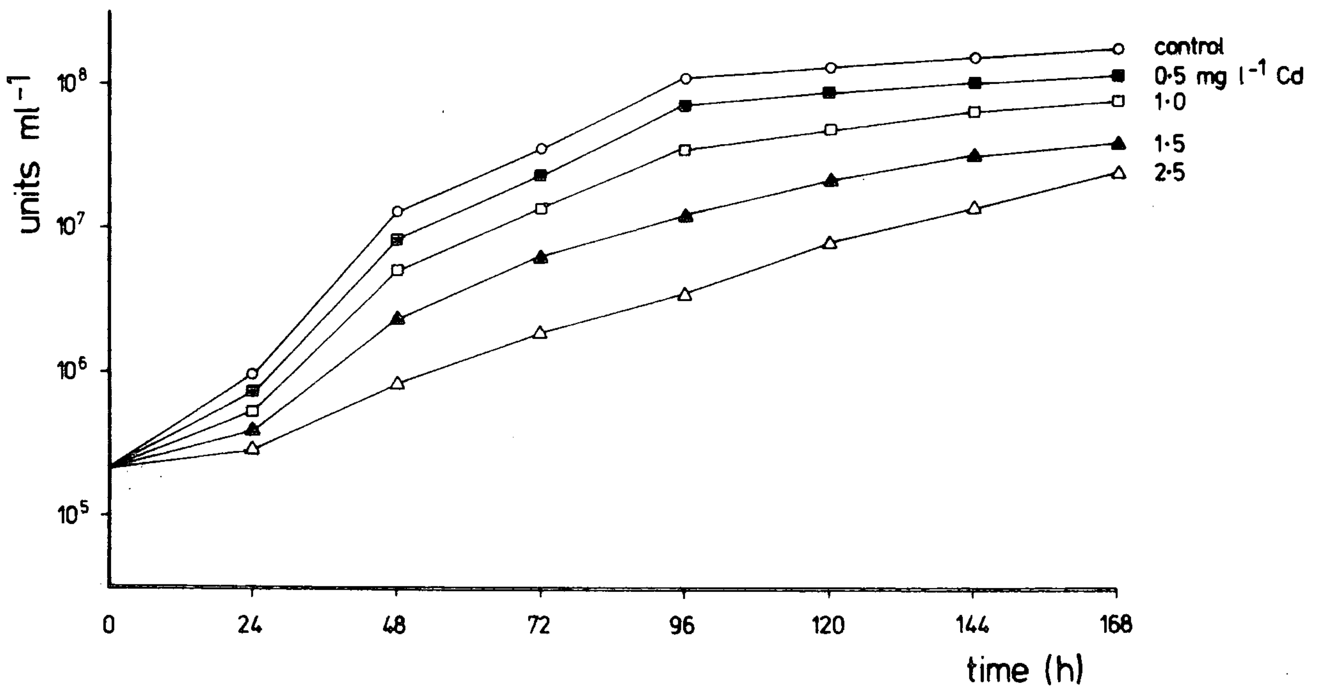


Fig. 3.7 Influence of Cd on growth of Cd-t2.0; inoculum was taken from strongly inhibitory level of Cd.

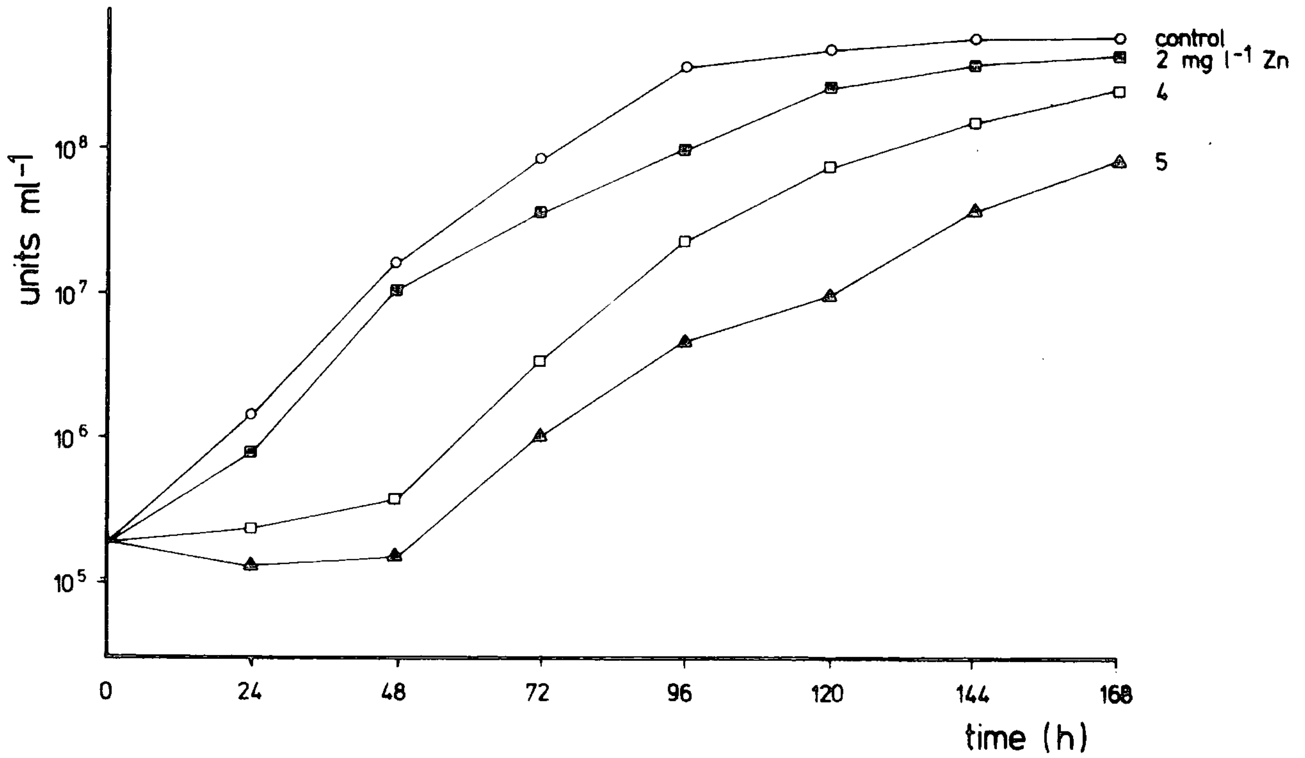


Fig. 3.8 Influence of Zn on growth of Zn-t5.0, grown for 72 cell generations in basal medium.

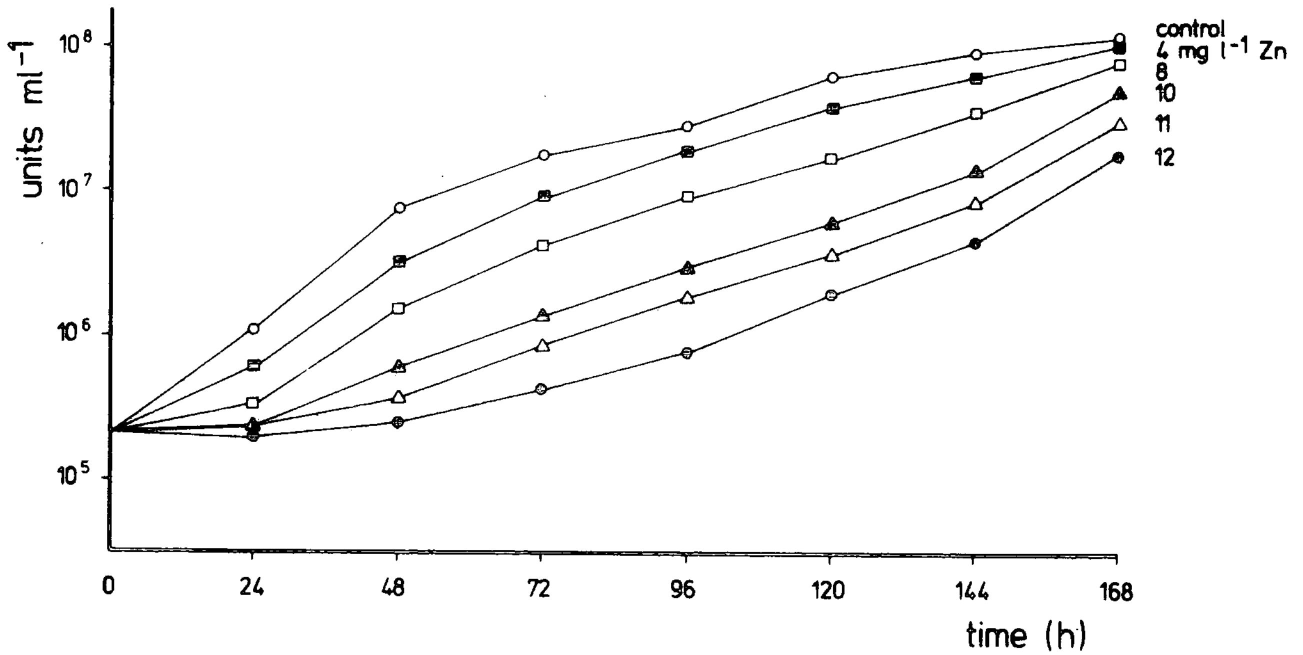


Fig. 3.9 Influence of Zn on growth of Zn-t12.0, grown for 96 cell generations in basal medium.

Table 3.3 Influence of prior growth conditions and Zn concentrations on growth lag shown by Zn-t5.0 and Zn-t12.0. Growth measured as units m l^{-1}.

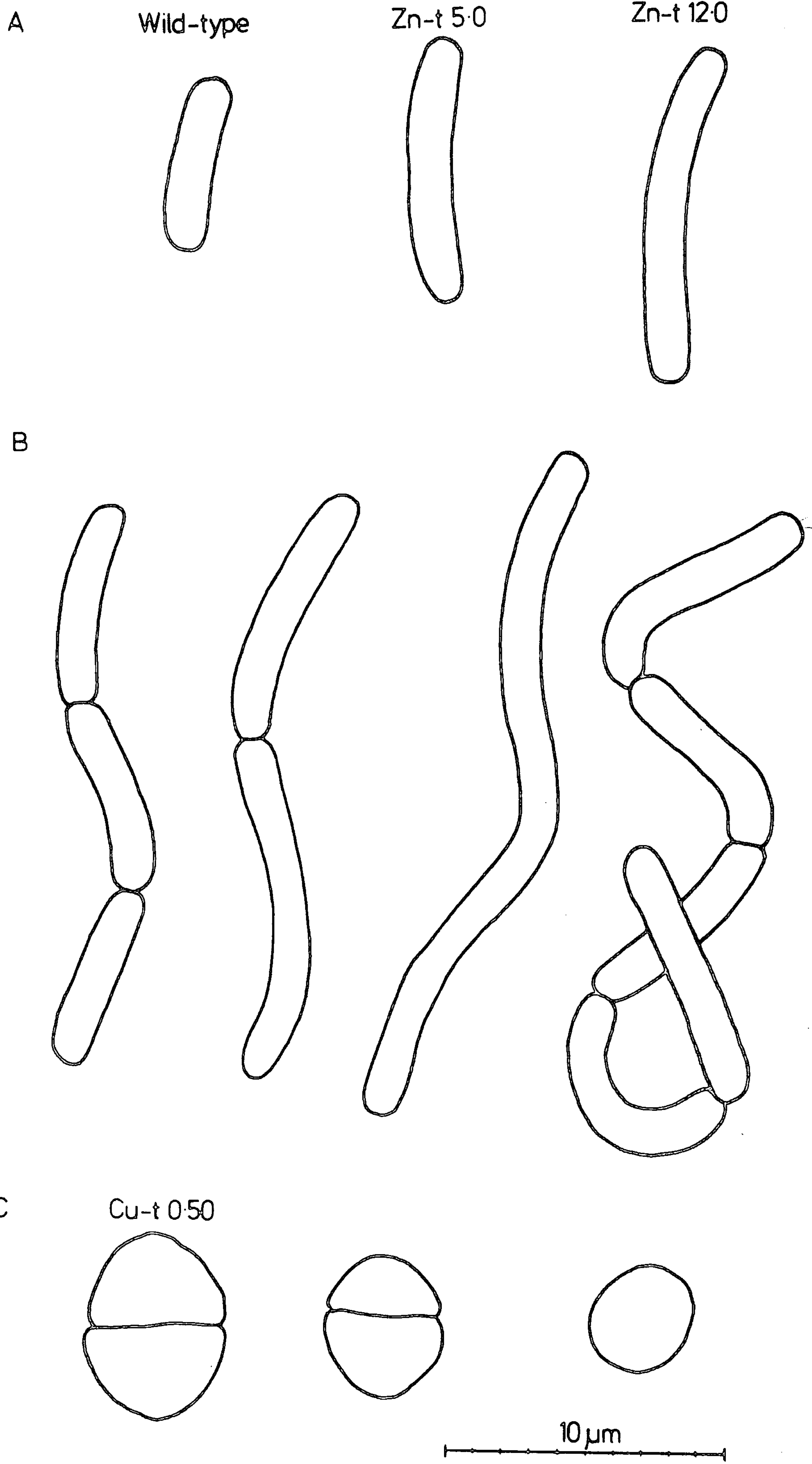
strain	source of inoculum	lag (h) Zn (mg l^{-1})						morphology of inoculum
		0	2	4	5	8	12	
Zn-t 5.0	In basal medium:							
	for 64 generations	2.8	3.6	16	22	-	-	slightly longer than normal
	for 72 generations	3.2	4.1	20	24	-	-	" " " "
	From 5.0 mg l^{-1} Zn	1.5	1.2	7.6	10	-	-	> 10 x longer
Zn-t 12.0	In basal medium:							
	for 88 generations	2.6	2.5	3.8	4.0	16.4	37	slightly longer than normal
	for 96 generations	3.4	3.5	3.8	4.4	28.6	45	" " " "
	From 12.0 mg l^{-1} Zn	1.6	1.4	1.2	2.0	7.2	16	> 25 x longer

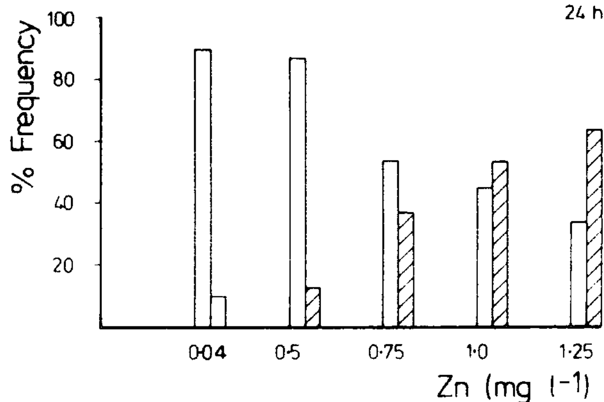
3.32 Antibiotics

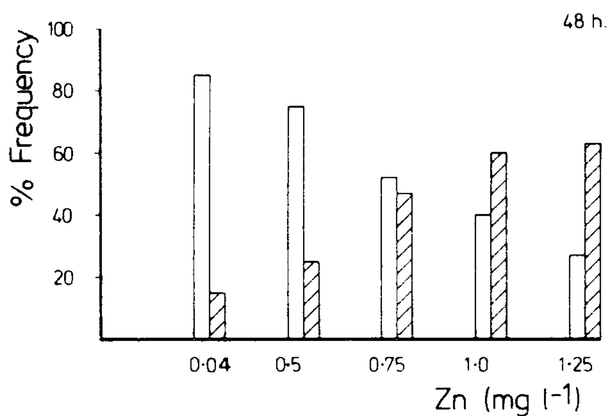
A similar procedure was used to isolate strains tolerant to 3 mg l^{-1} streptomycin, and 1.25 mg l^{-1} penicillin; this took about 4 and 11 subcultures respectively. At these concentrations the resistant strains did however show a 50% reduction in growth rate. The presence of Zn at a sub-inhibitory level did not lead to any increase in the rate of 'spontaneous' mutation (1 in 1 x 10^8 units) for resistance to either antibiotic.

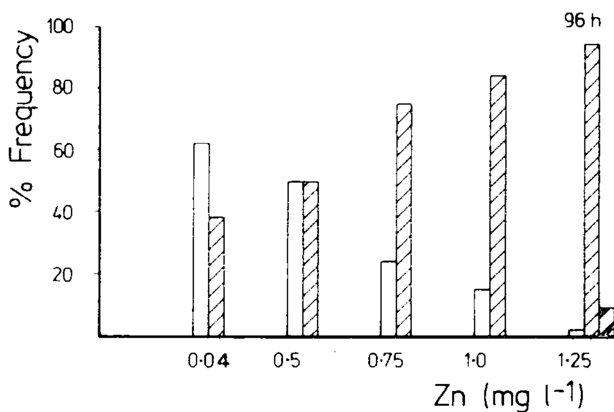
3.4 Morphology

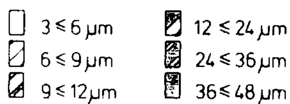
Morphological changes were noted at the higher concentrations of metals and in some cases there was considerable diversity within a single flask. The changes were not followed in detail but representative forms are shown in Fig. 3.10. No marked difference in cell length were observed in wild-type with any metal up to the sixth day after inoculation. In older cultures, however, the average cell length of metal-treated alga was slightly greater than that of the control, and at the strongly inhibitory level of Cu, subspherical units were often formed. The Co, Ni, Zn and Cd-tolerant strains in general showed a similar behaviour when grown in the absence of metal, although the average length of the units of both Zn-t 5.0 and Zn-t 12.0 was greater. At the levels of metals sub-inhibitory for each of these strains, filaments were produced, occasionally reaching 100 x original length in Zn-t 5.0 and Zn-t 12.0. Cytological studies were not made to establish the extent to which these were truly multicellular or only coenocytic like the mutants described by Kunisawa and Cohen-Bazire (1970). The distribution of these units in the flasks at various intervals during growth (in batch culture) are given in Figs 3.11, 3.12, 3.13 and Tables A3.7, A3.8, A3.9.


A
Wild-type
Zn-t 5·0
Zn-t 12·0
B
C
Cu-t 0·50
10 μm









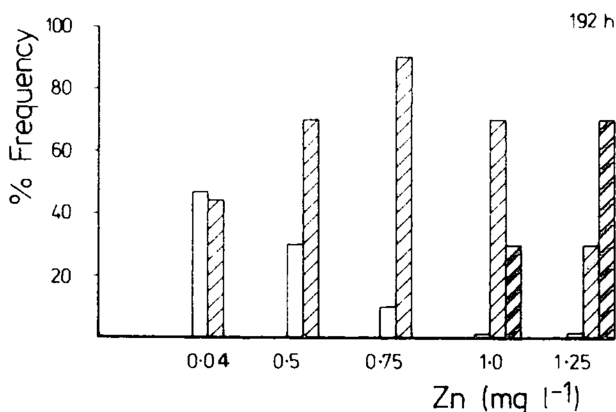
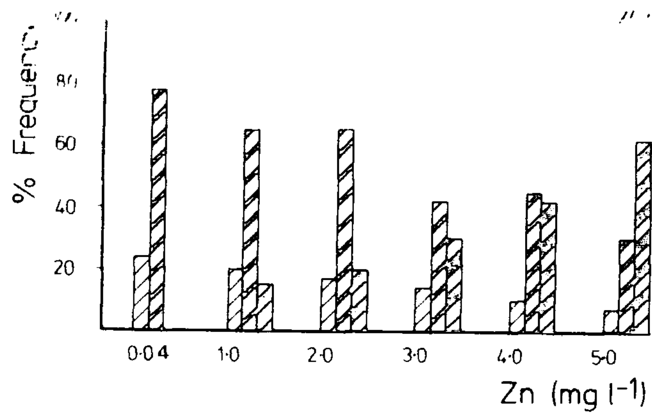
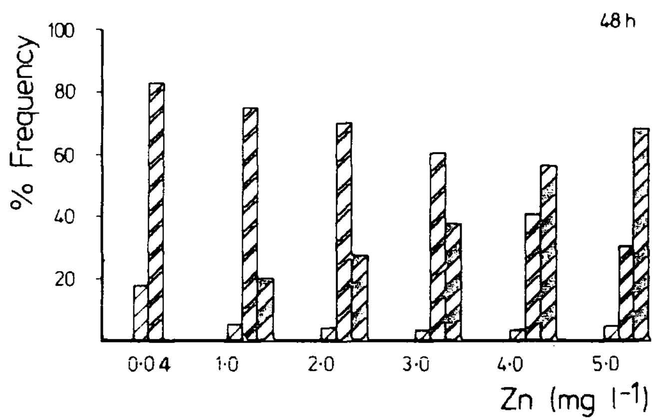
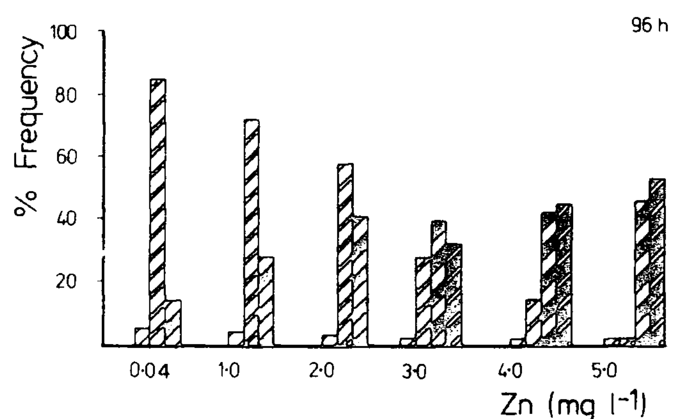
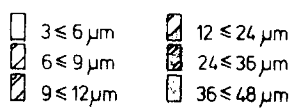


Fig. 3.11 Influence of Zn and time of harvesting in batch culture on length of *Anacystis*.









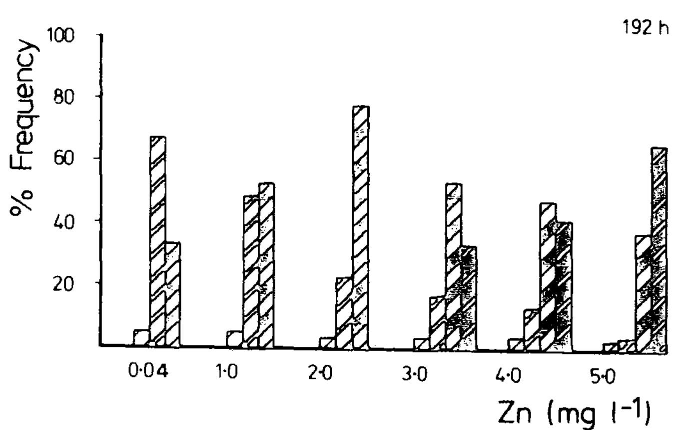
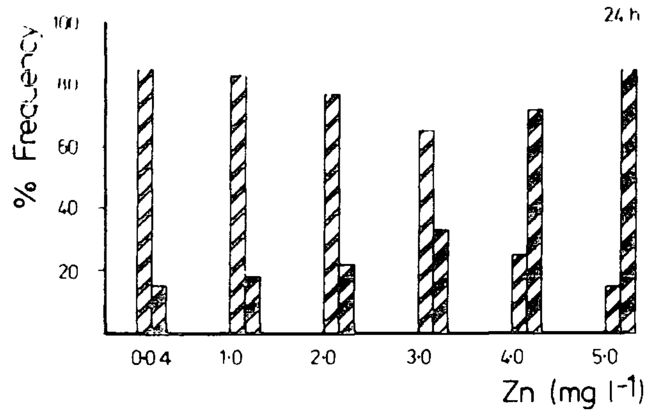
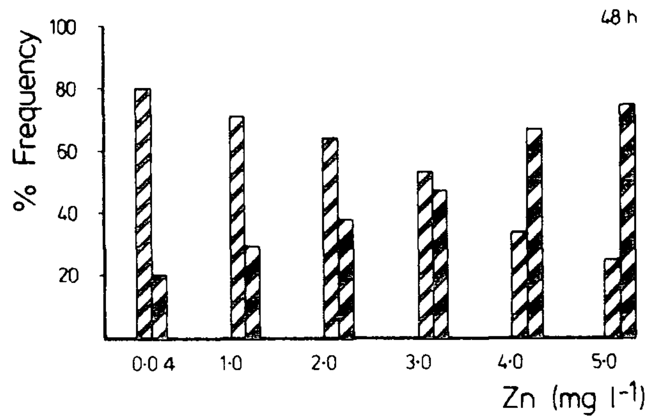
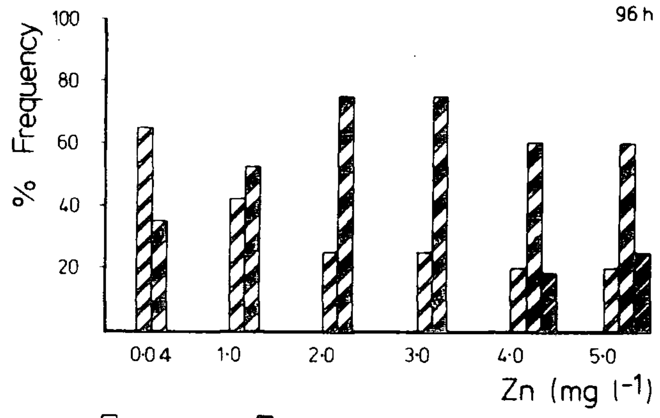
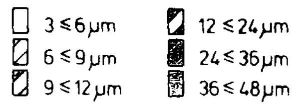


Fig. 3.12 Influence of Zn and time of harvesting in batch culture on length of Zn-t5.0; inoculum from basal medium.









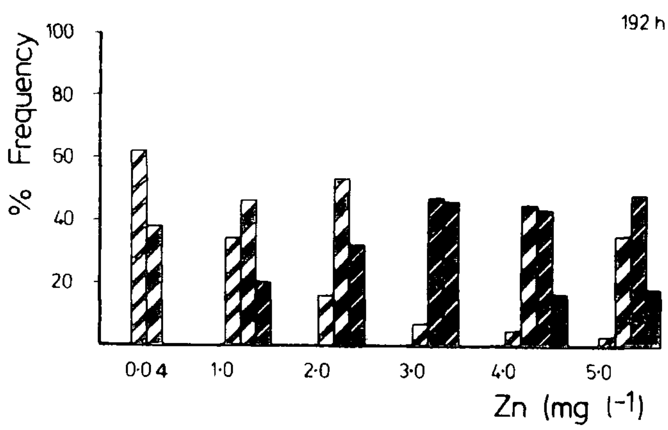


Fig. 3.13 Influence of Zn and time of harvesting in batch culture on length of Zn-t5.0; inoculum from strongly inhibitory level of Zn.

The morphological response to Cu was different. Inhibitory levels of Cu, the wild-type, Cu-t 0.5 and both Zn-tolerant strains all formed many subspherical units, quite different in shape from the normal rods. In most cases the populations were markedly heterogeneous, with rods about 3.2 μm to 6.3 μm, subspherical units, and filaments about 30 μm to 100 μm, but at least in the case of Cu-t 0.5 the subspherical units often formed the bulk of the population. Although unequivocal evidence was not obtained, it is almost certain that the units were capable of growth in this form. A subspherical form similar to those found at high levels of Cu was noted also with Co-t 1.8 grown at high Co levels, but here these units formed less than 1% of the population.

CHAPTER 4

TOLERANCE AND TOXICITY STUDIES ON *ANACYSTIS NIDULANS* STRAINS

4.1 Metals

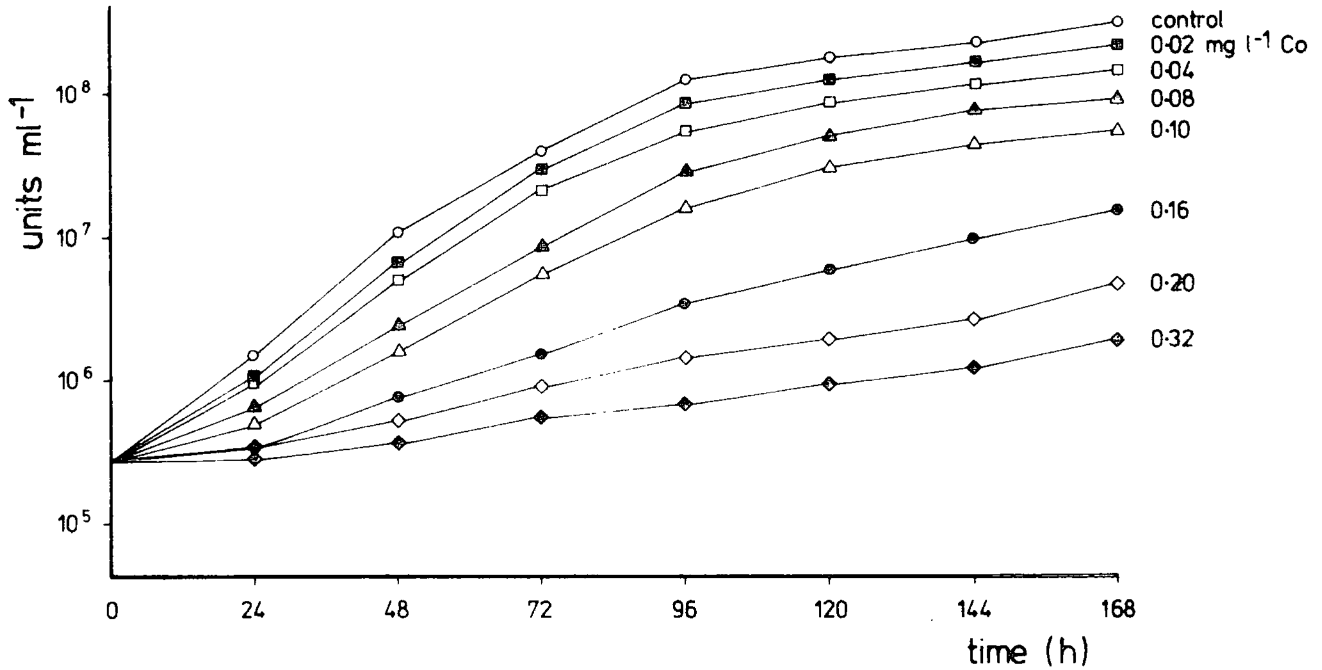
Comparative studies were made of the relative toxicity of Co, Ni, Cu, Zn, Cd, Hg and Pb to *Anacystis nidulans* strains, namely wild-type, Co-t1.8, Ni-t1.0, Cu-t0.5, Zn-t5.0, Zn-t12.0 and Cd-t2.0. Toxicity tests were performed under controlled laboratory conditions using growth rate and sometimes also lag (section 2.52) as indication of responses.

The influence of each metal on the growth of all various strains (wild-type, Co-t1.8, Ni-t1.0, Cu-t0.5, Zn-t5.0, Zn-t12.0 and Cd-t2.0) is shown in a series of figures.

Two sources of inoculum were used for each metal investigated: a) from basal medium; b) from strongly inhibitory level of metal at which strain was adapted.

4.11 Cobalt The influence of variation in level of environmental Co on the growth of

	Strain	Fig.	Table
4.11 Cobalt	wild-type	Fig. 4.1 a, b	Table A4.1
	Ni-t1.0	4.2 a, b	" A4.2
	Cu-t0.5	4.3 a, b	" A4.3
	Zn-t5.0	4.4 a, b	" A4.4
	Zn-t12.0	4.5 a, b	" A4.5
	Cd-t2.0	4.6 a, b	" A4.6
4.12 Nickel	wild-type	4.7 a, b	" A4.7
	Co-t1.8	4.8 a, b	" A4.8
	Cu-t0.5	4.9 a, b	" A4.9
	Zn-t5.0	4.10 a, b	" A4.10
	Zn-t12.0	4.11 a, b	" A4.11
	Cd-t2.0	4.12 a, b	" A4.12



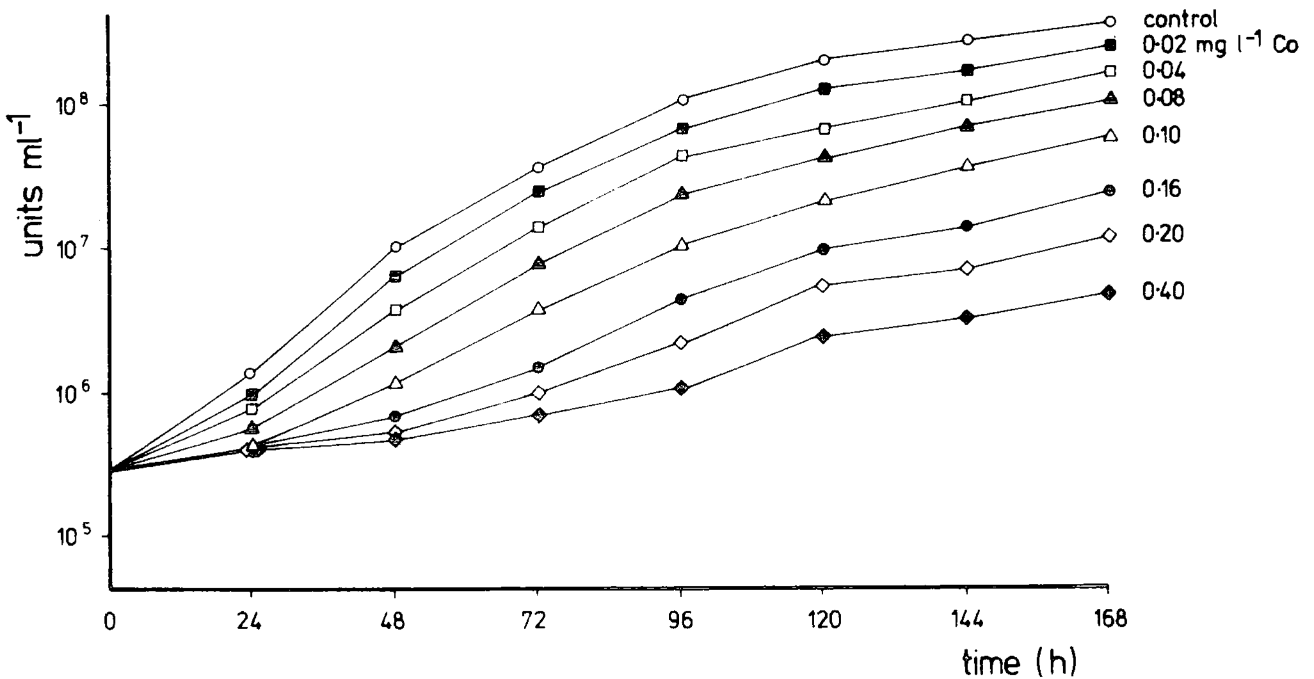
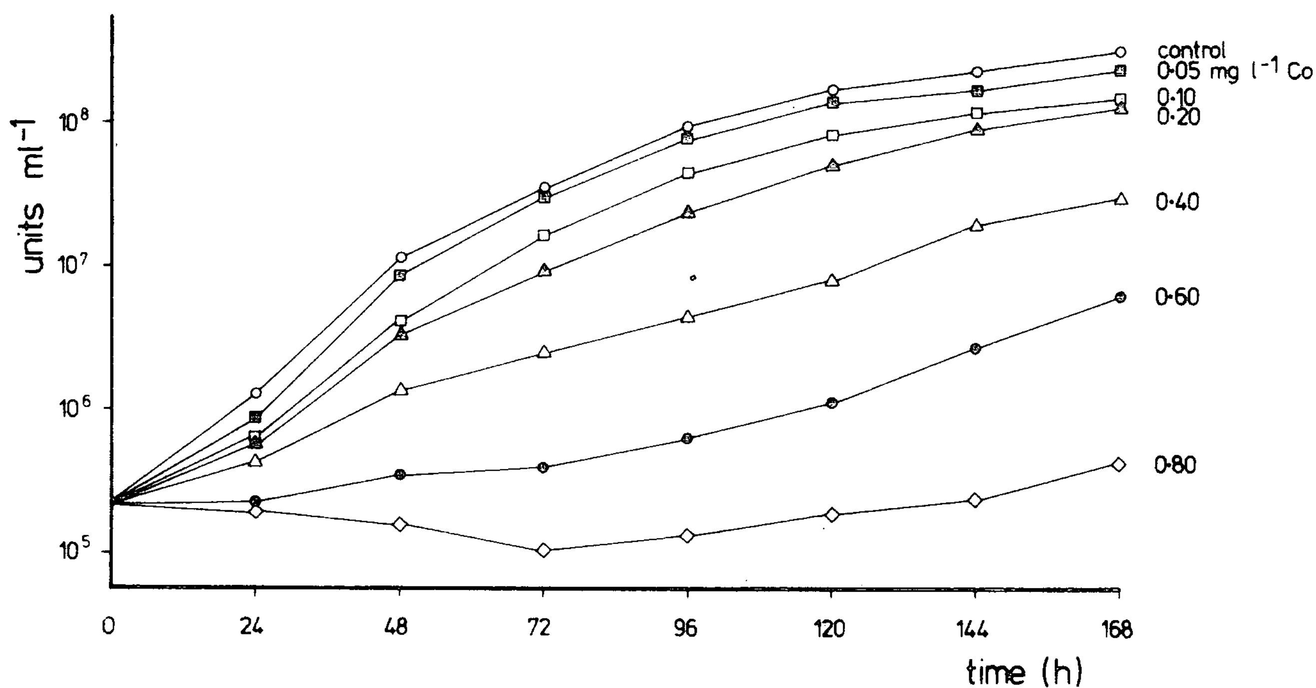


Fig. 4.1 Influence of Co on growth of wild-type *Anacystis*;

a) inoculum from basal medium

b) inoculum from strongly inhibitory level of Co



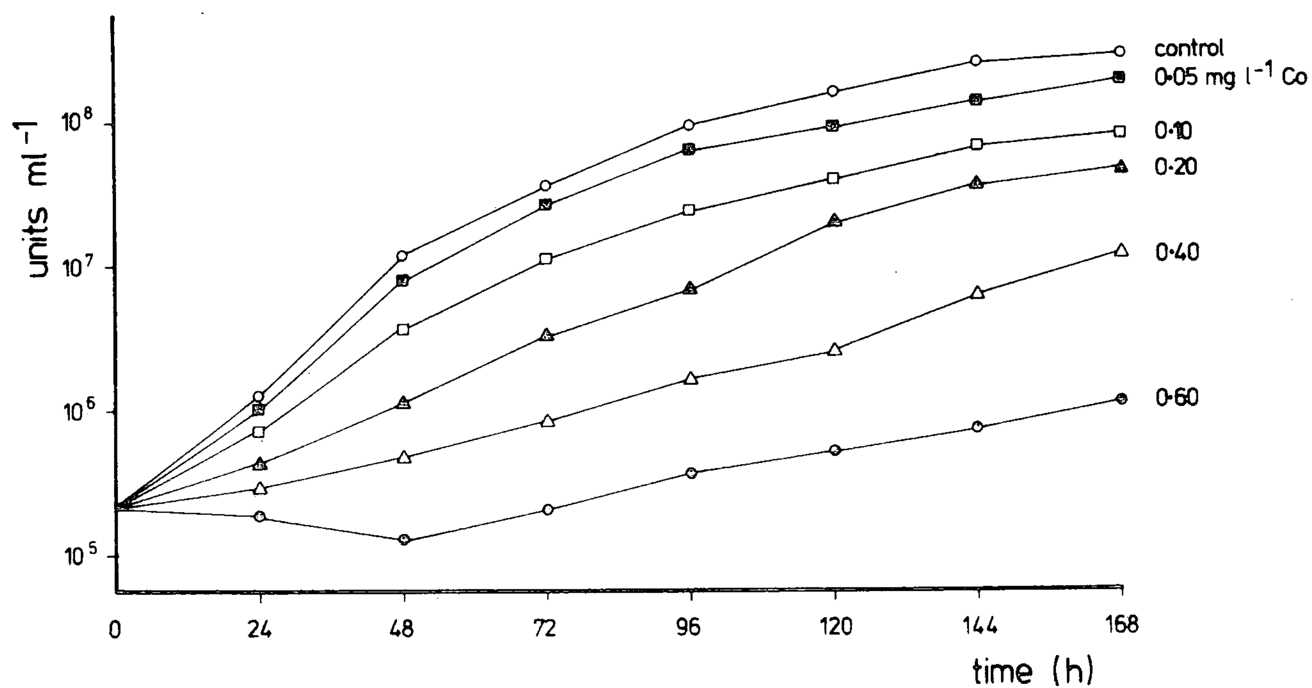
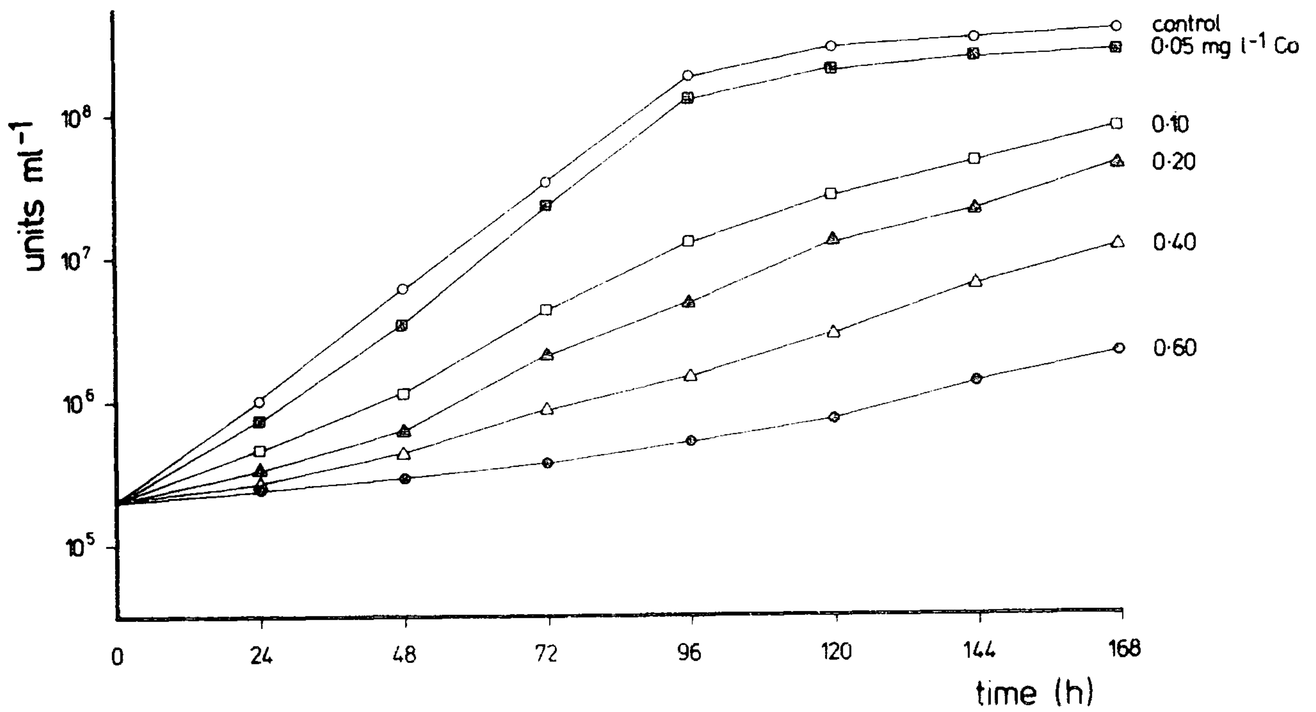


Fig. 4.2 Influence of Co on growth of Ni-t1.0;

a) inoculum from basal medium

b) inoculum from strongly inhibitory level of metal at which adapted



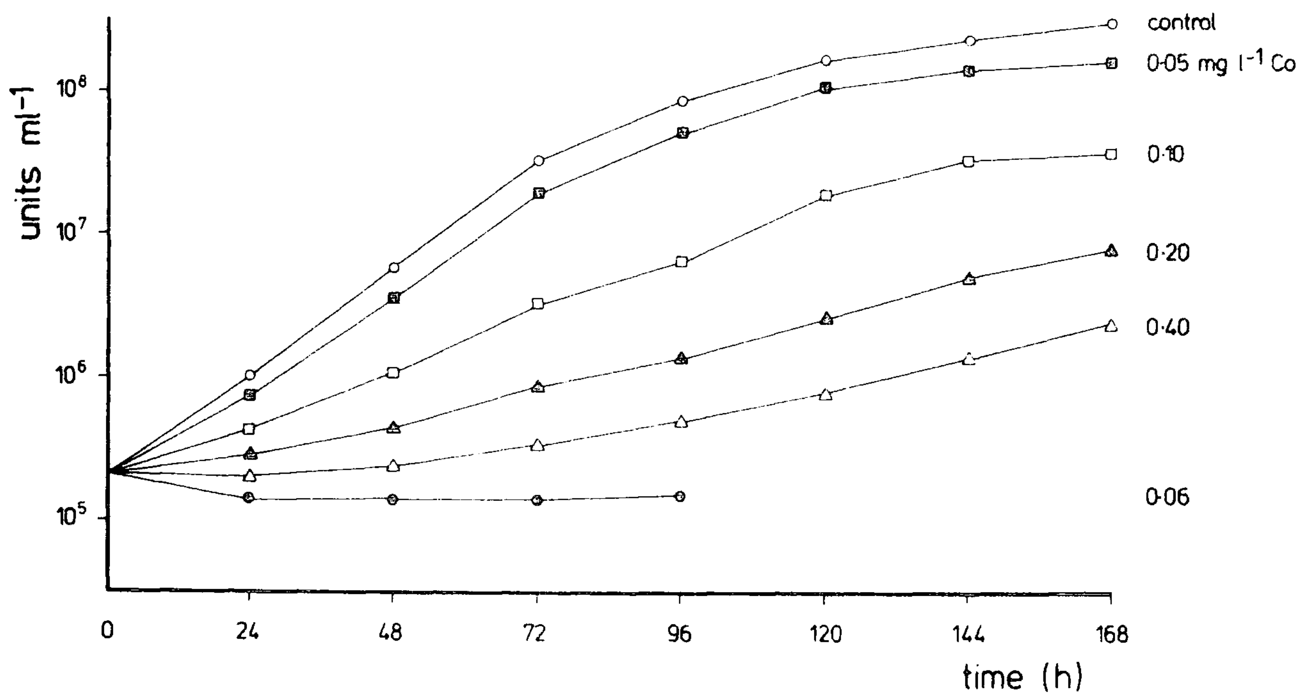
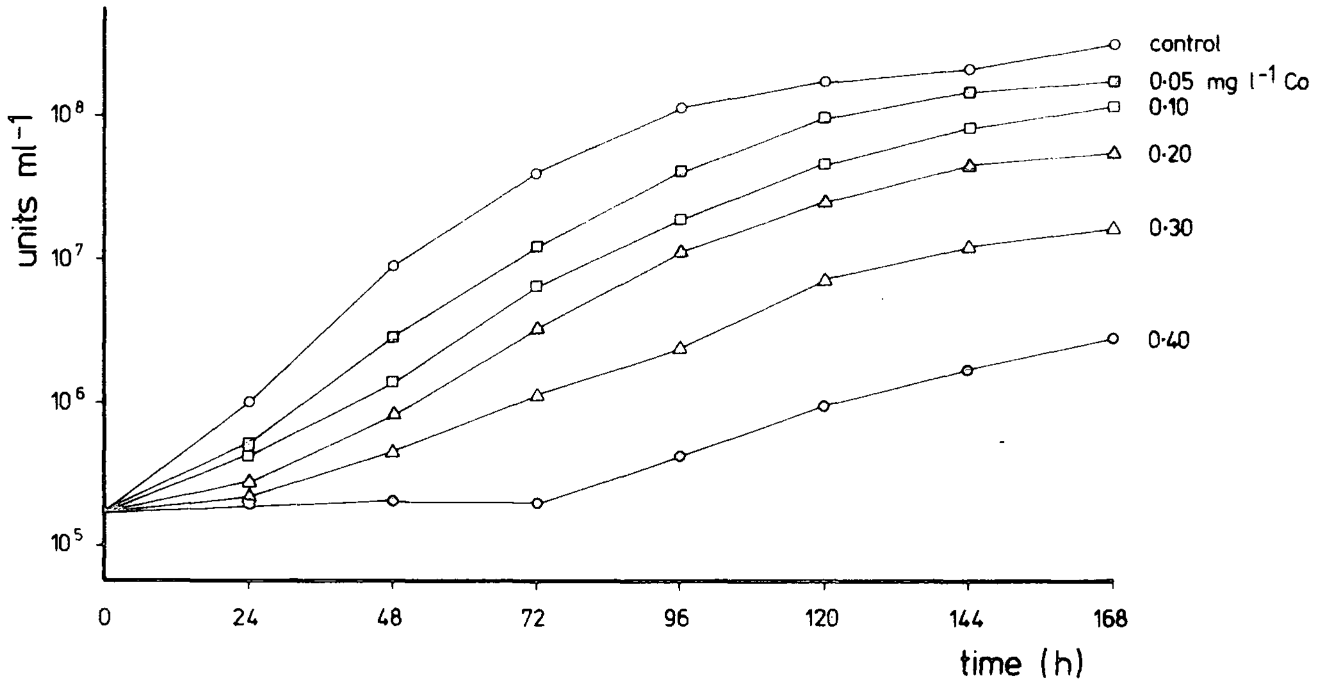


Fig. 4.3 Influence of Co on growth of Cu-t0.5;

a) inoculum from basal medium

b) inoculum from strongly inhibitory level of metal at which adapted



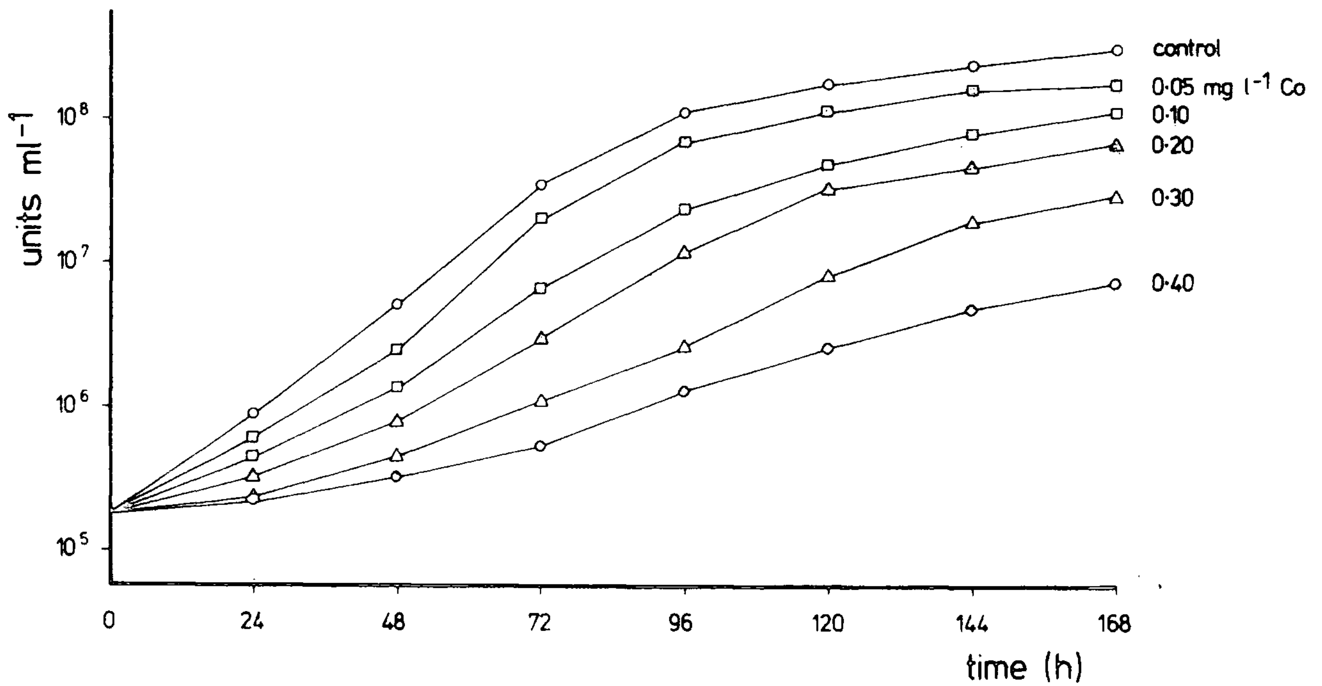
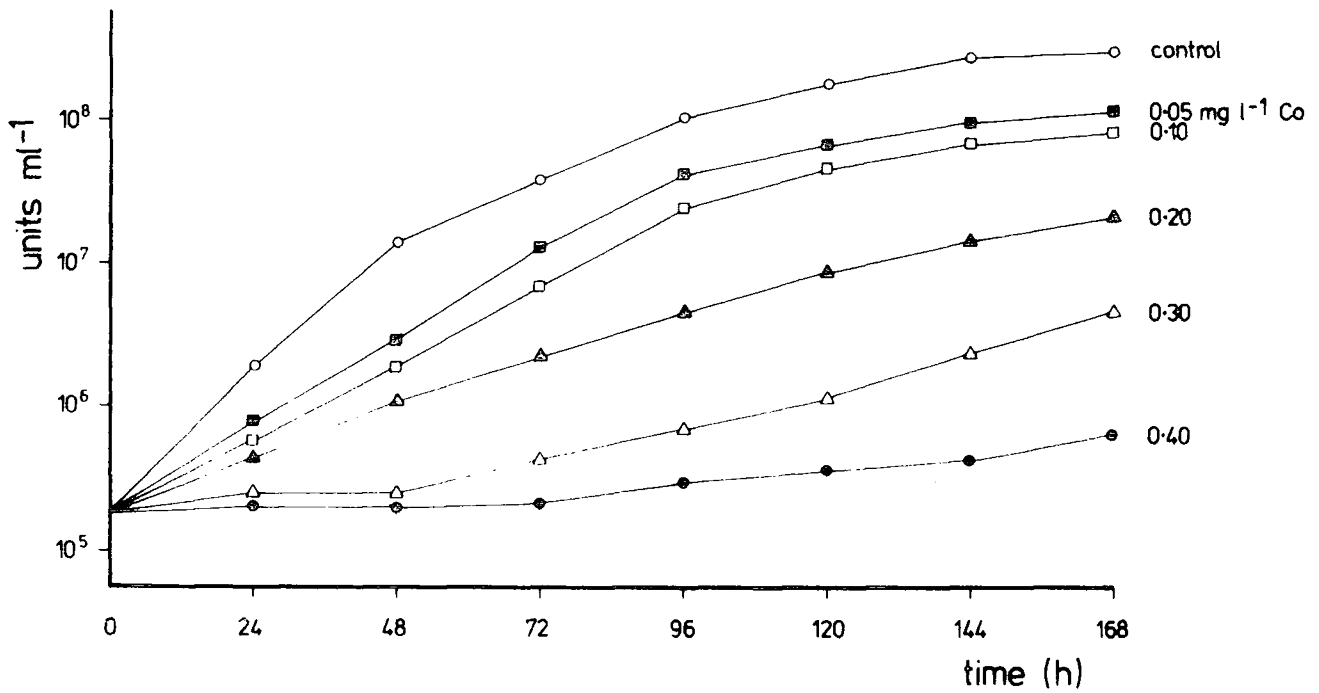


Fig. 4.4 Influence of Co on growth of Zn-t5.0;

a) inoculum from basal medium

b) inoculum from strongly inhibitory level of metal at which adapted



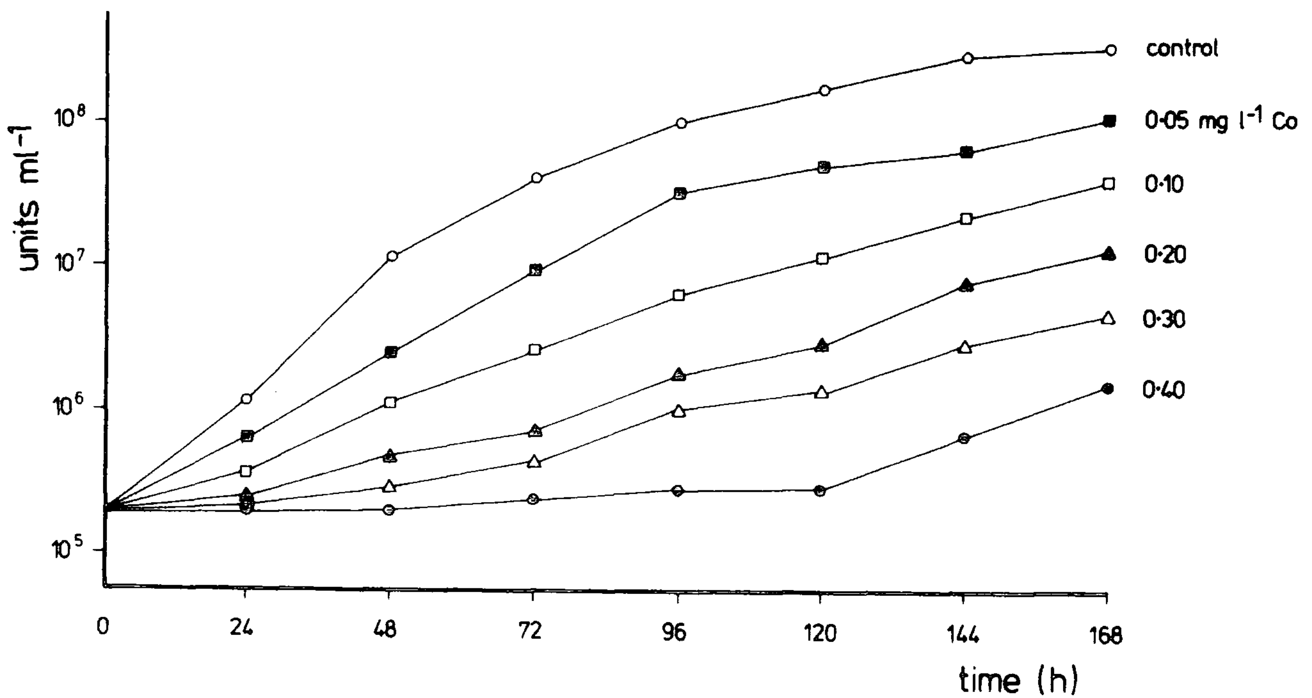
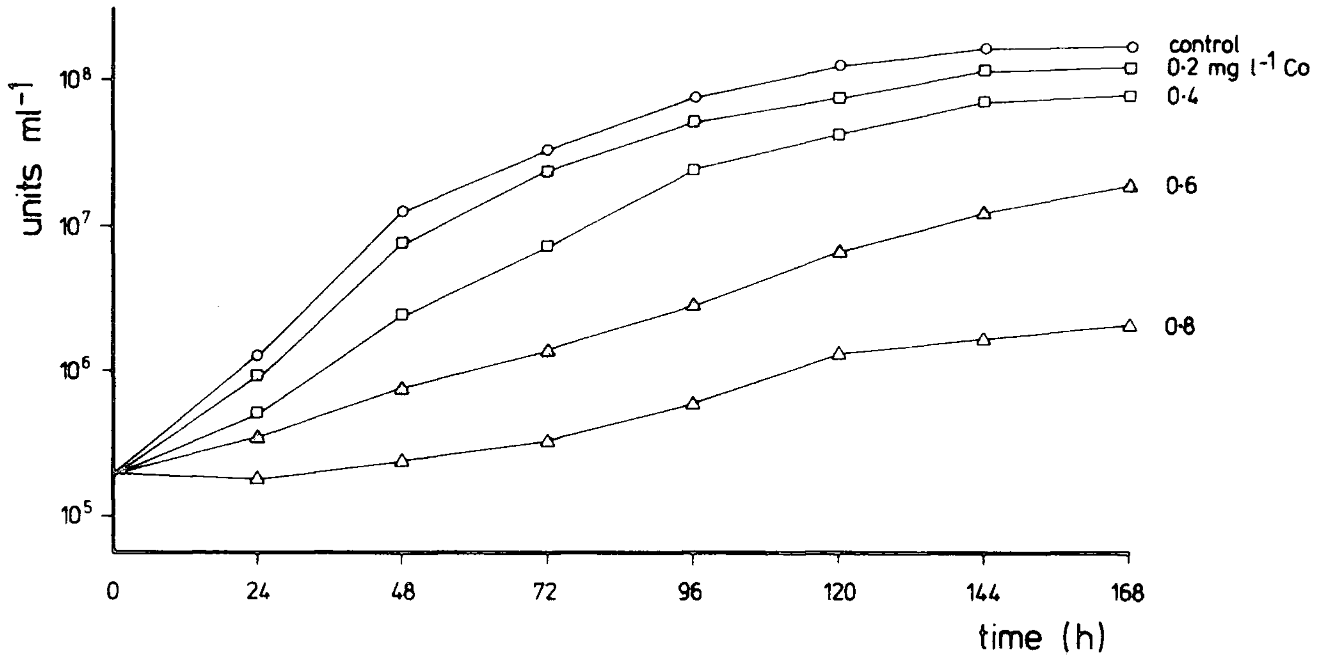


Fig. 4.5 Influence of Co on growth of Zn-t12.0;

a) inoculum from basal medium

b) inoculum from strongly inhibitory level of metal at which adapted



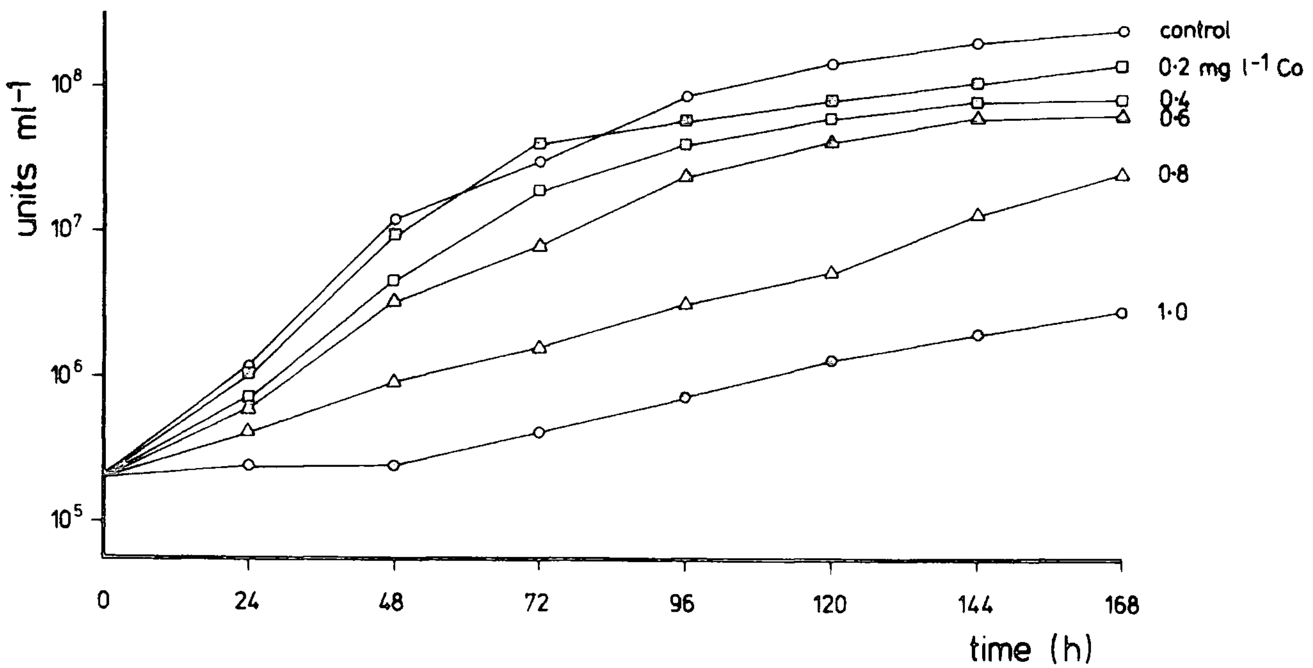
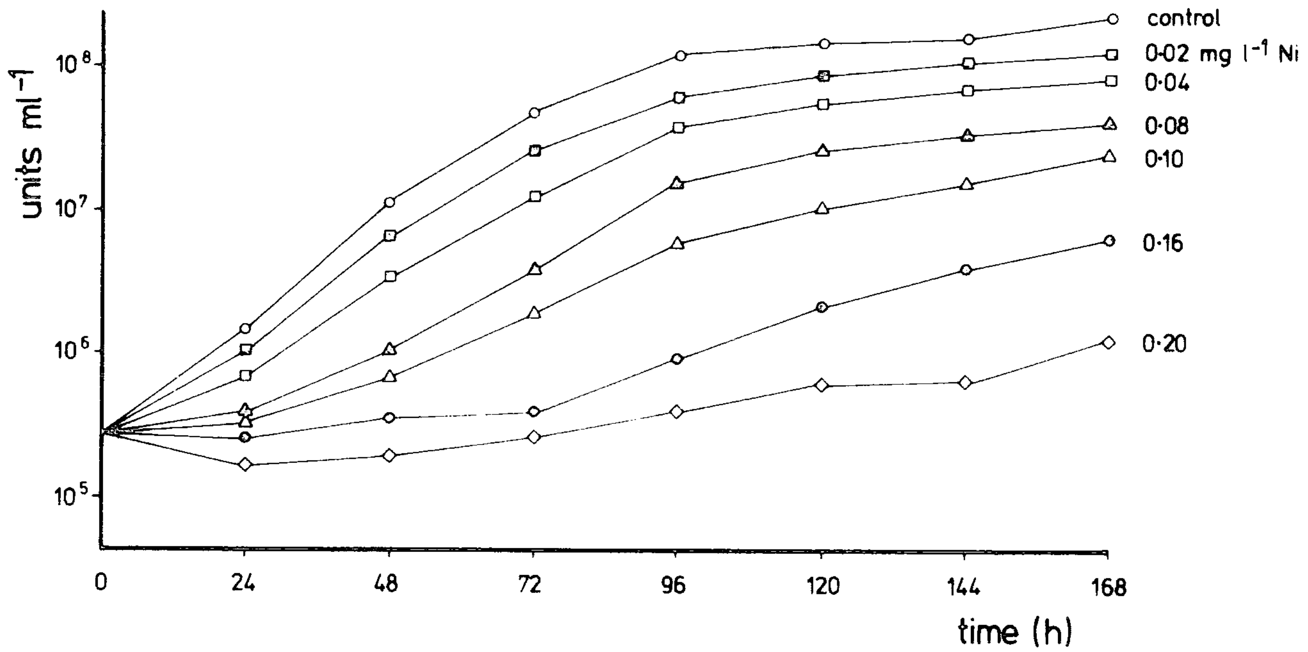


Fig. 4.6 Influence of Co on growth of Cd-t2.0

a) inoculum from basal medium

b) inoculum from strongly inhibitory level of metal at which adapted



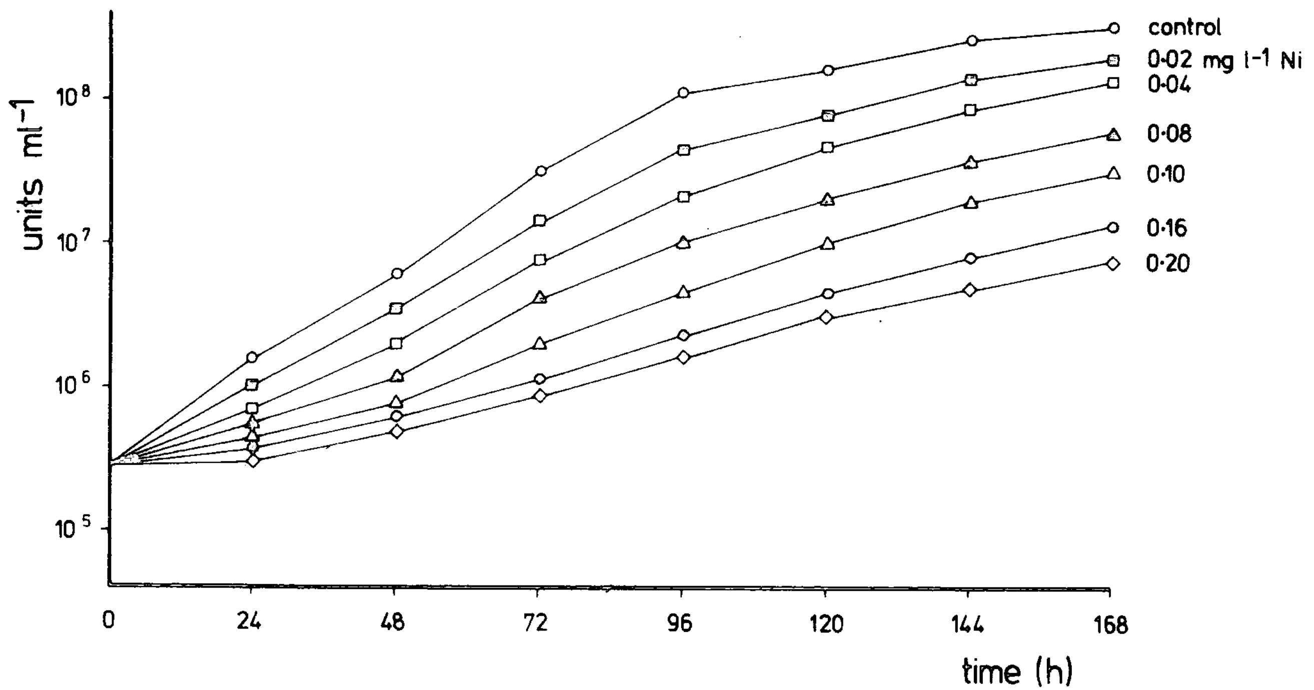
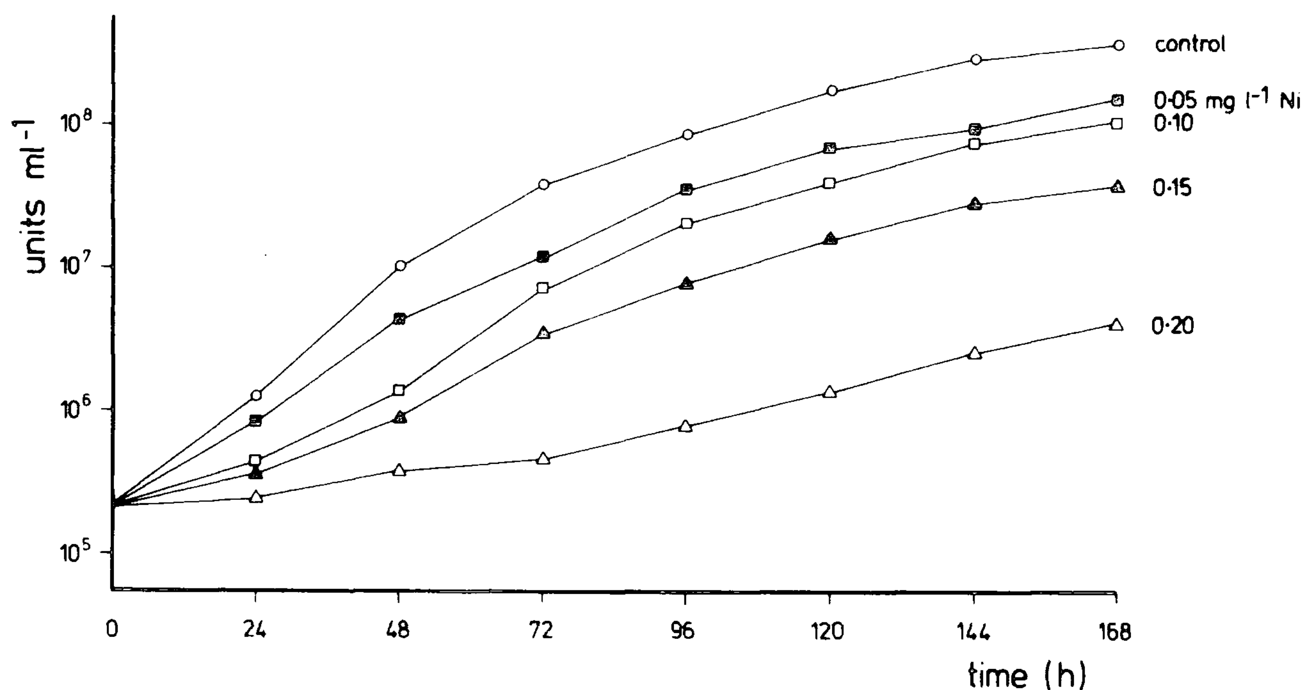


Fig. 4.7 Influence of Ni on growth of wild-type

a) inoculum from basal medium

b) inoculum from strongly inhibitory level of Ni



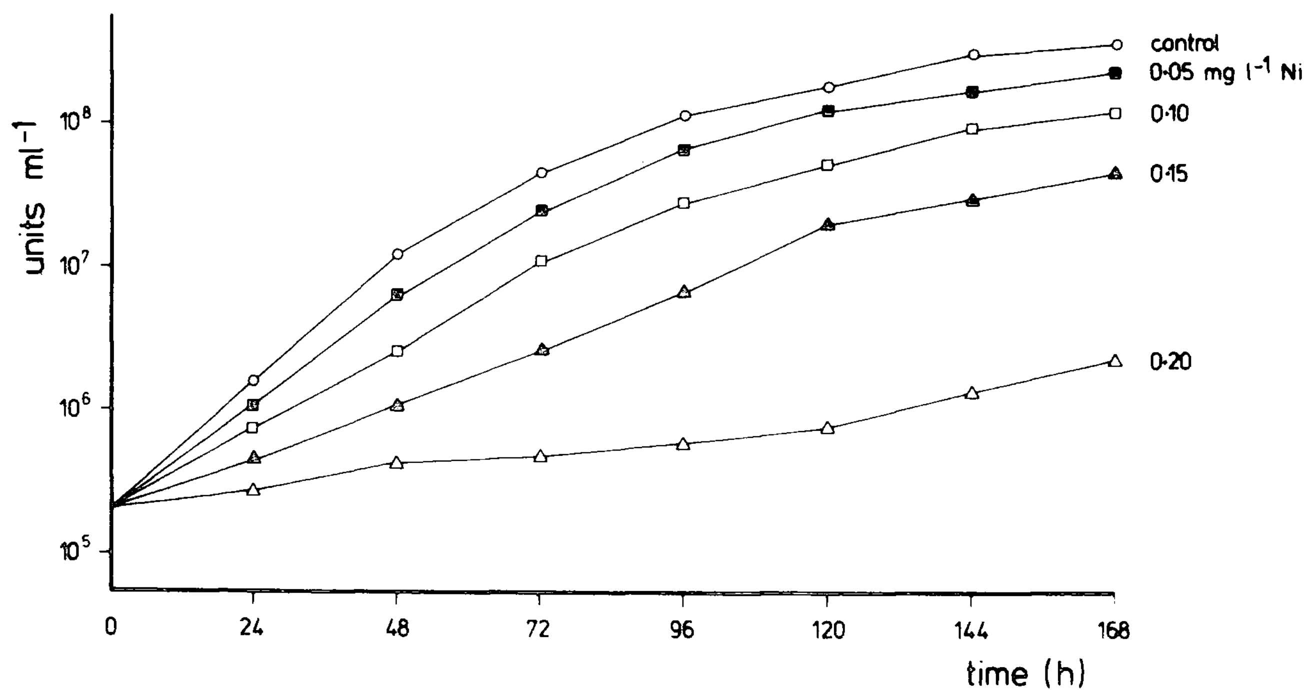
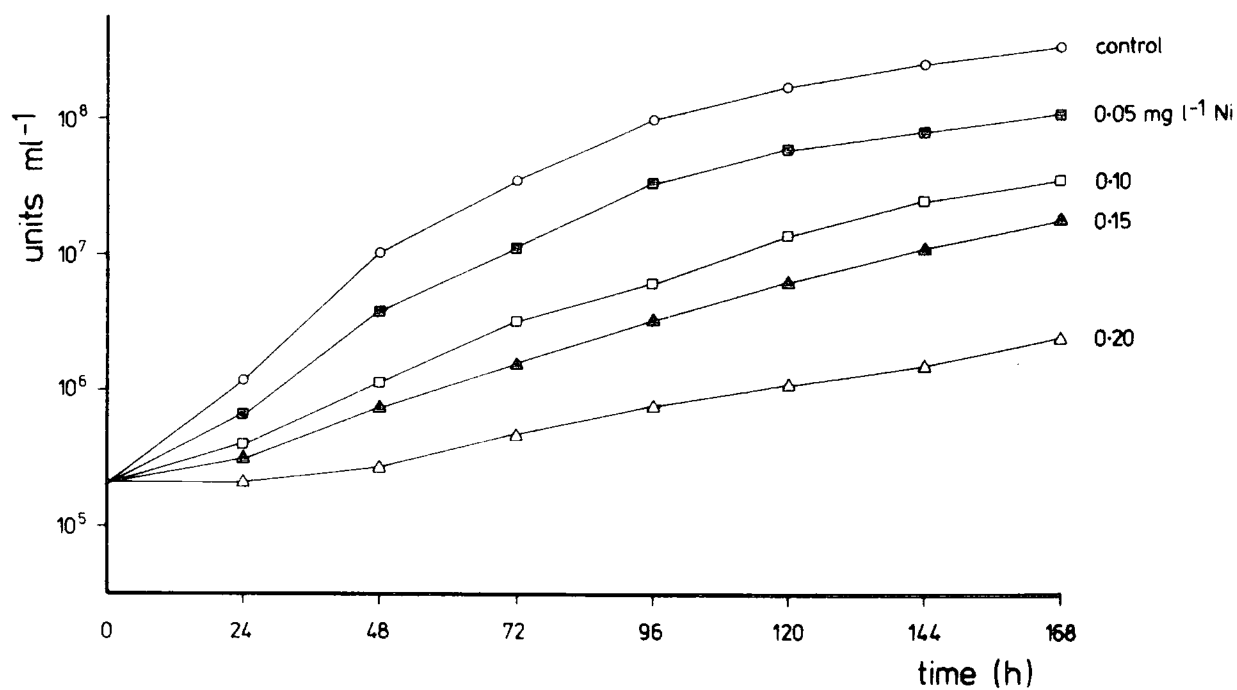


Fig. 4.8 Influence of Ni on growth of Co-t1.8

a) inoculum from basal medium

b) inoculum from strongly inhibitory level of metal at which adapted



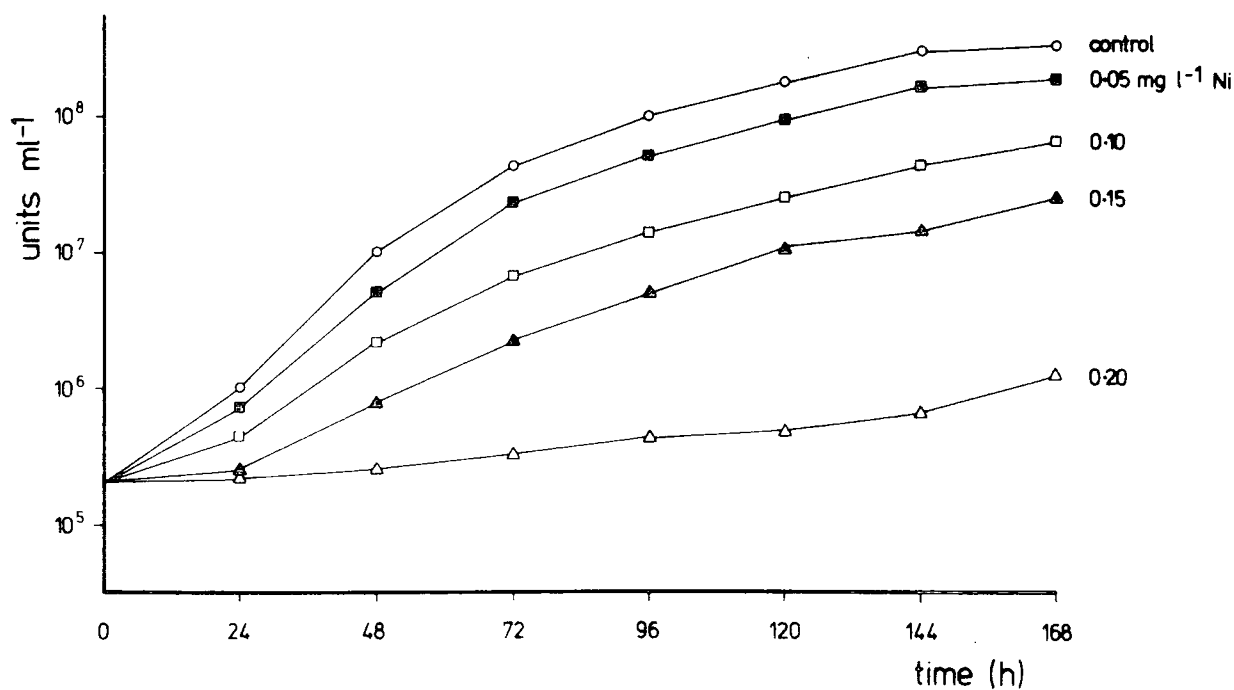
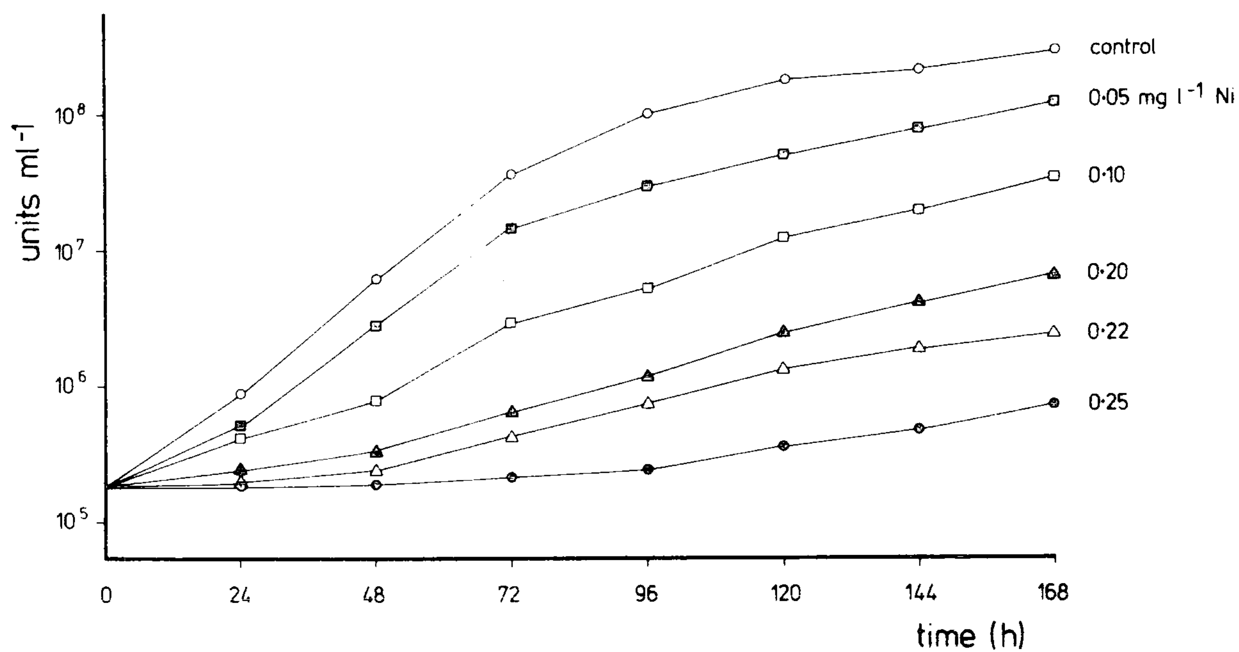


Fig. 4.9 Influence of Ni on growth of Cu-t0.5

a) inoculum from basal medium

b) inoculum from strongly inhibitory level of metal at which adapted



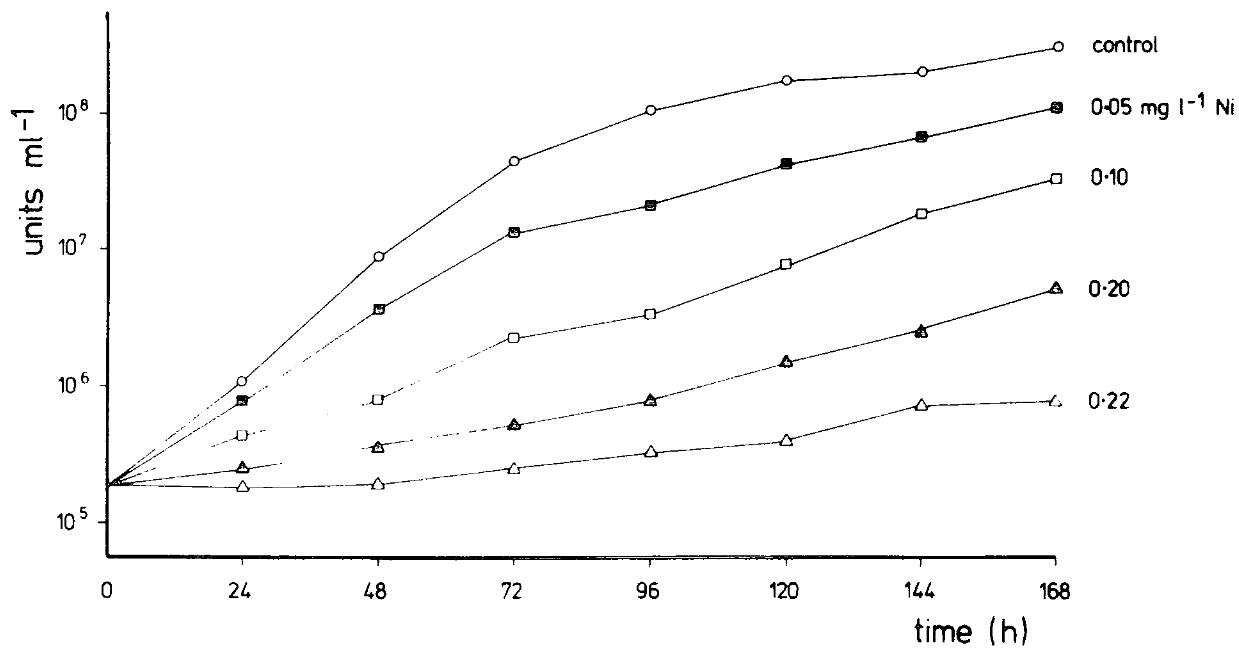
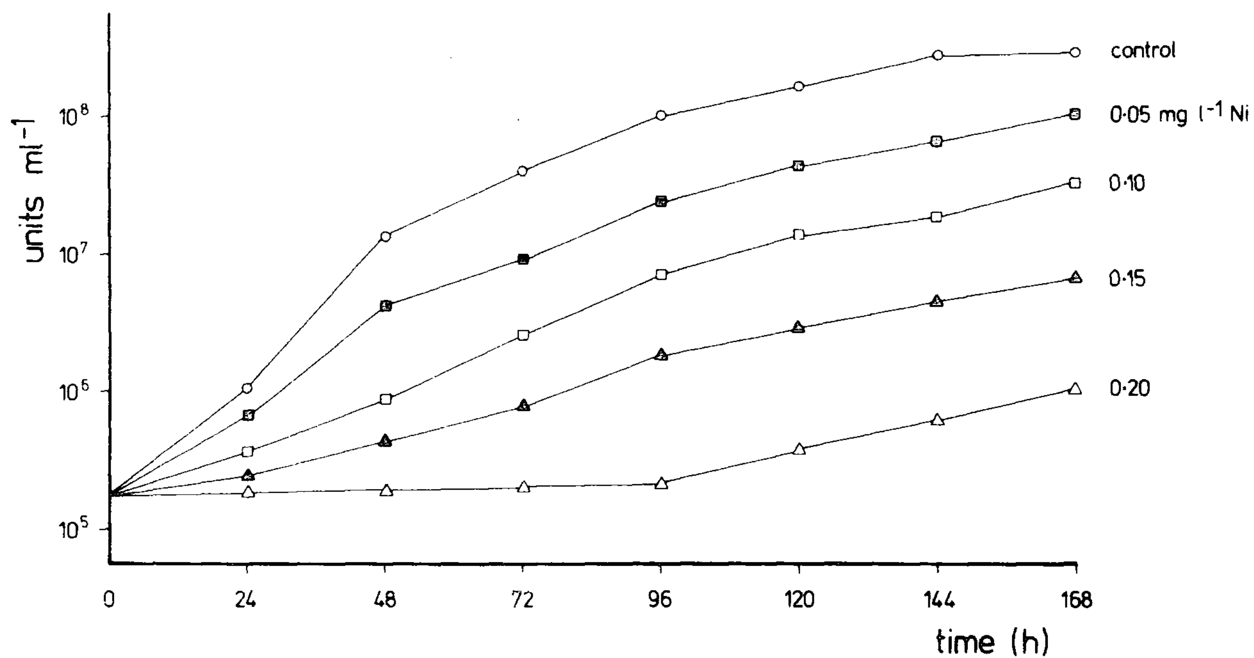


Fig. 4.10 Influence of Ni on growth of Zn-t5.0

a) inoculum from basal medium

b) inoculum from strongly inhibitory level of metal at which adapted



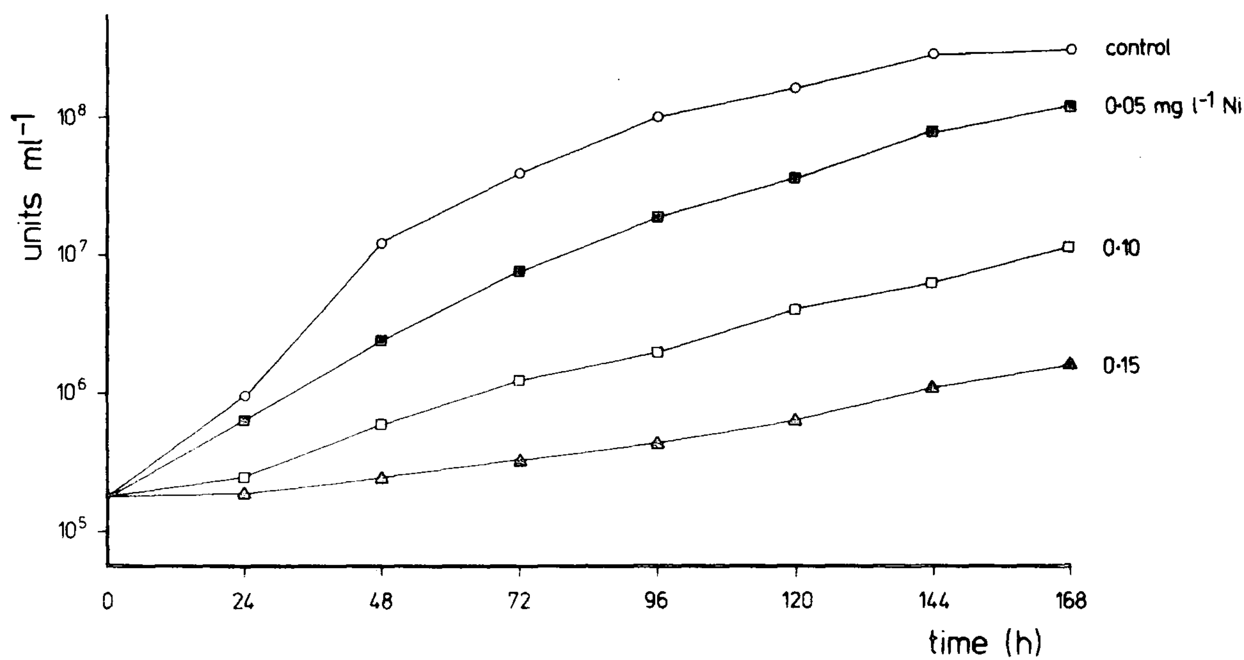
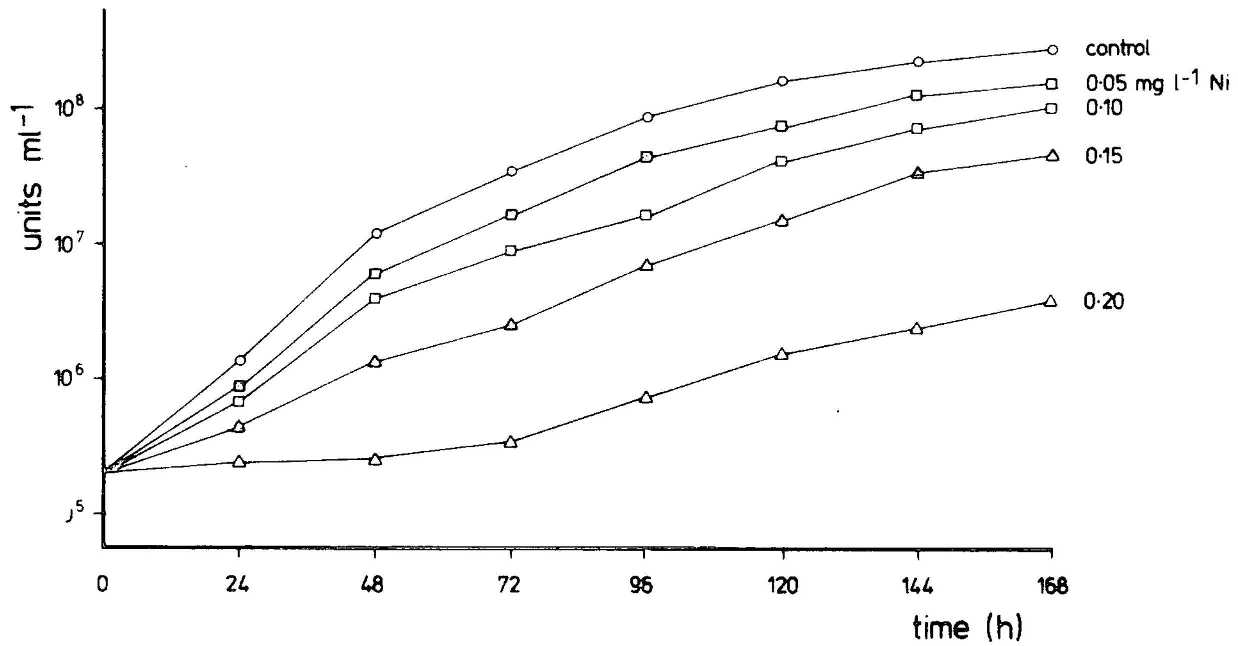


Fig. 4.11 Influence of Ni on growth of Zn-t12.0

a) inoculum from basal medium

b) inoculum from strongly inhibitory level of metal at which adapted



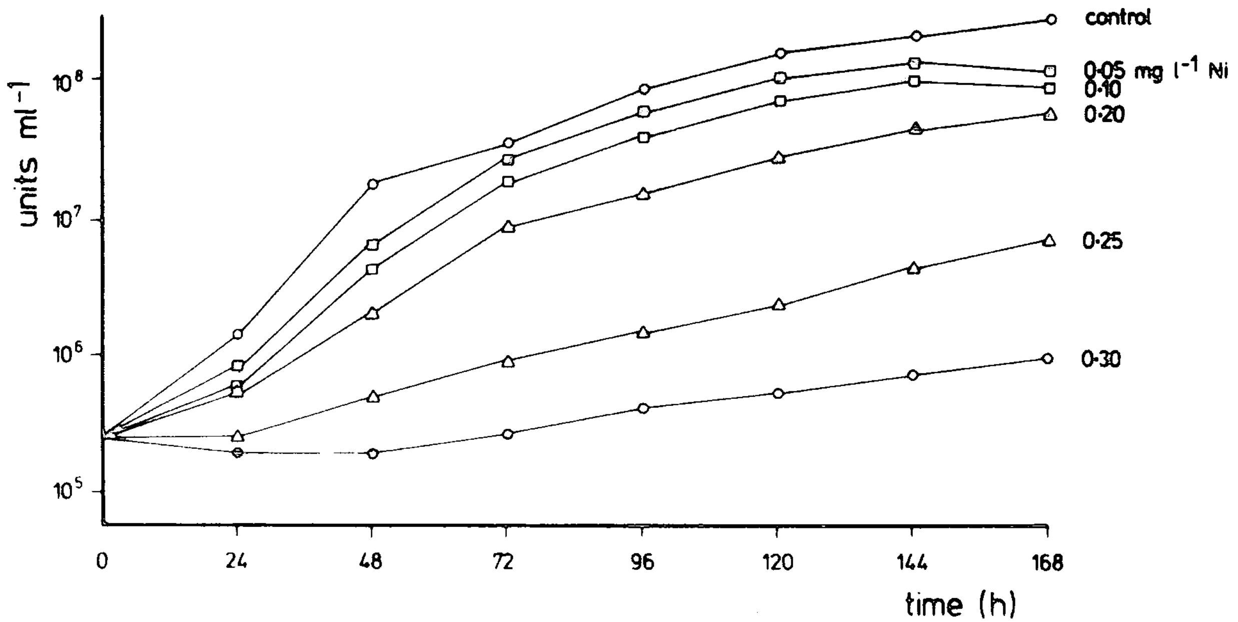


Fig. 4.12 Influence of Ni on growth of Cd-t0.2

a) inoculum from basal medium

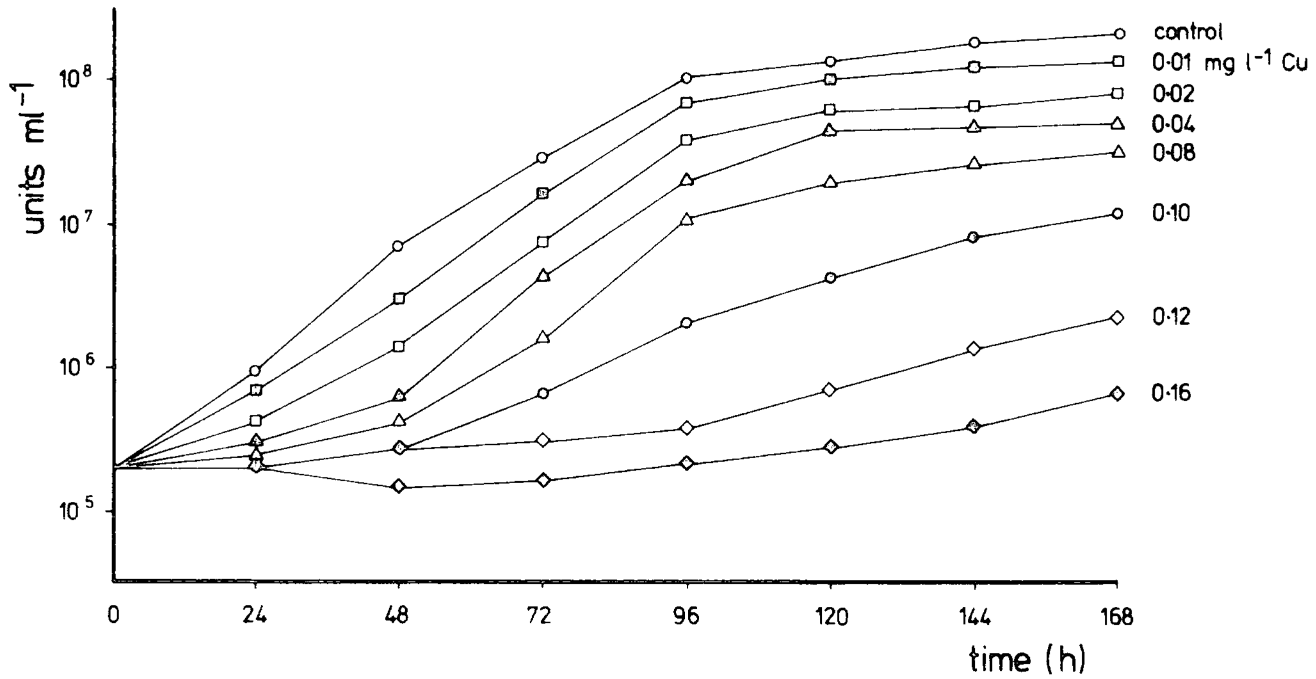
b) inoculum from strongly inhibitory level of metal at which adapted

4.13	Copper	wild-type	Fig. 4.13 a, b	Table	A4.13
		Co-t1.8	4.14 a, b	"	A4.14
		Ni-t1.0	4.15 a, b	"	A4.15
		Zn-t5.0	4.16 a, b	"	A4.16
		Zn-t12.0	4.17 a, b	"	A4.17
		Cd-t5.0	4.18 a, b	"	A4.18
4.14	Zinc	wild-type	4.19 a, b	"	A4.19
		Co-t1.8	4.20 a, b	"	A4.20
		Ni-t1.0	4.21 a, b	"	A4.21
		Cu-t0.5	4.22 a, b	"	A4.22
		Cd-t2.0	4.23 a, b	"	A4.23
4.15	Cadmium	wild-type	4.24 a, b	"	A4.24
		Co-t1.8	4.25 a, b	"	A4.25
		Ni-t1.0	4.26 a, b	"	A4.26
		Cu-t0.5	4.27 a, b	"	A4.27
		Zn-t5.0	4.28 a, b	"	A4.28
		Zn-t12.0	4.29 a, b	"	A4.29

Influence of Zn on growth of wild-type and Zn-t12.0 are given later in Figs 6.1 and 6.2 respectively; dry weight was used as a growth criterion.

4.16 Mercury and lead Two further metals, Hg and Pb, although not used for the majority of studies were tested for their toxicity to the wild-type (Figs 4.30, 4.31: Table A4.30, A4.31) respectively. A marked influence of Hg was noted at very low concentrations; for instance the alga was killed at 0.06 mg l^{-1} Hg; in contrast Pb was not toxic at levels up to 30 mg l^{-1} Pb.

The data for cross-resistance of strains tolerant to one particular metal to the other four metals are summarized later (Table 8.1).



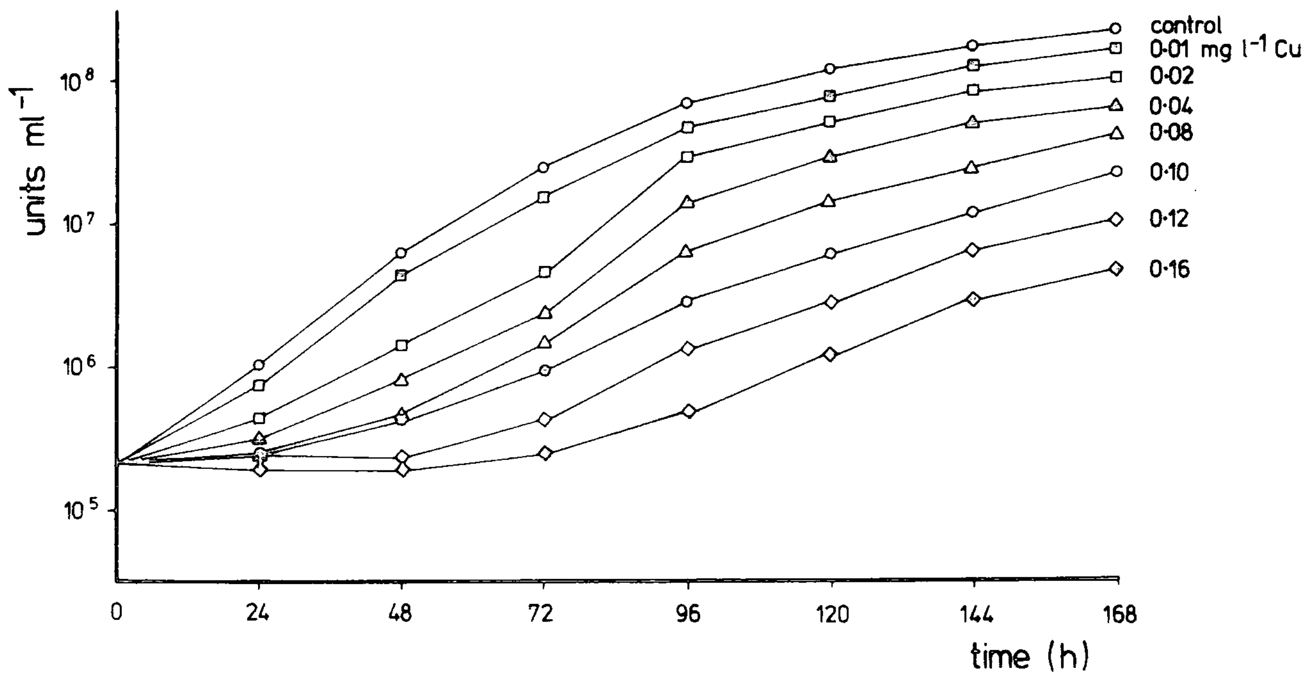
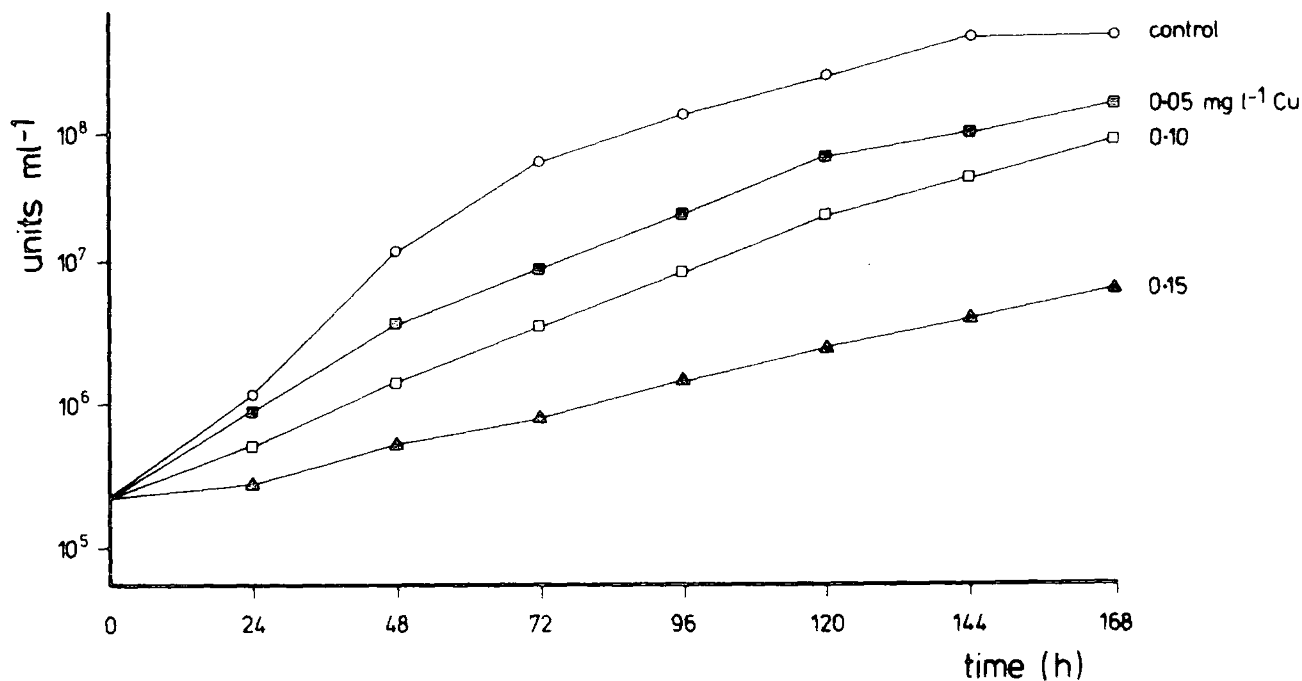


Fig. 4.13 Influence of Cu on growth of wild-type *Anacystis*

a) inoculum from basal medium

b) inoculum from strongly inhibitory level of Cu



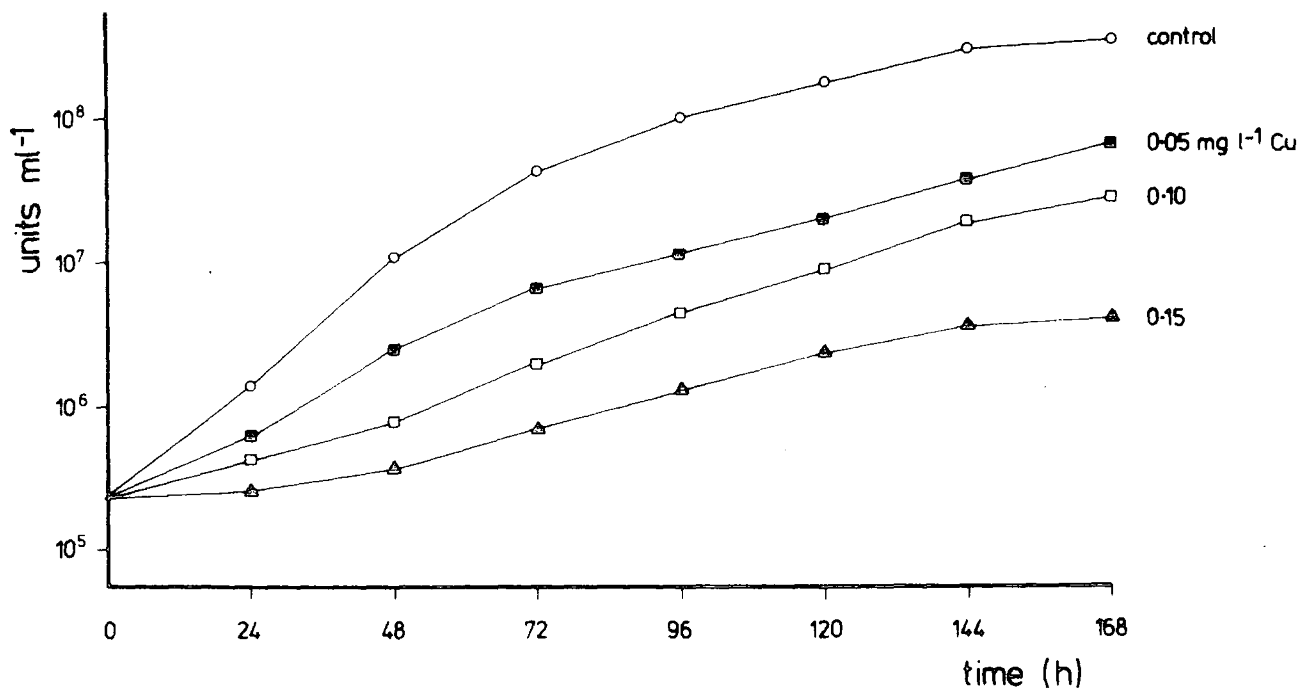
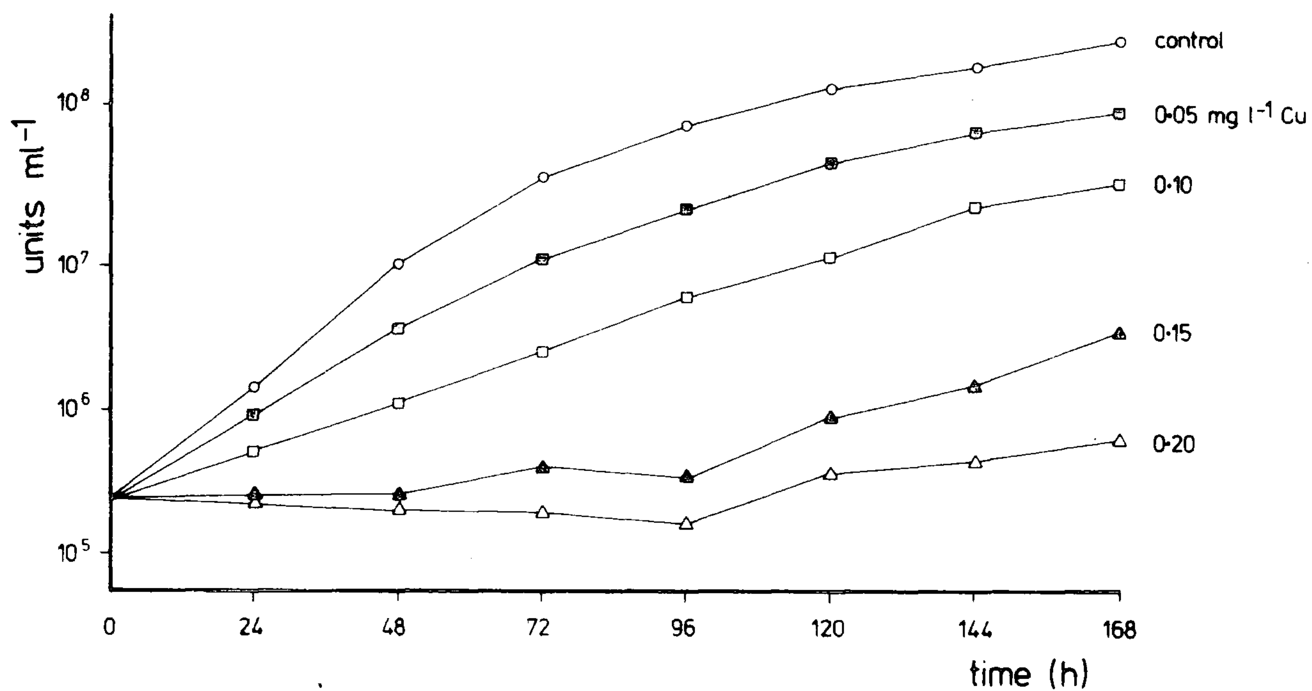


Fig. 4.14 Influence of Cu on growth of Co-t1.8

a) inoculum from basal medium

b) inoculum from strongly inhibitory level of metal at which adapted



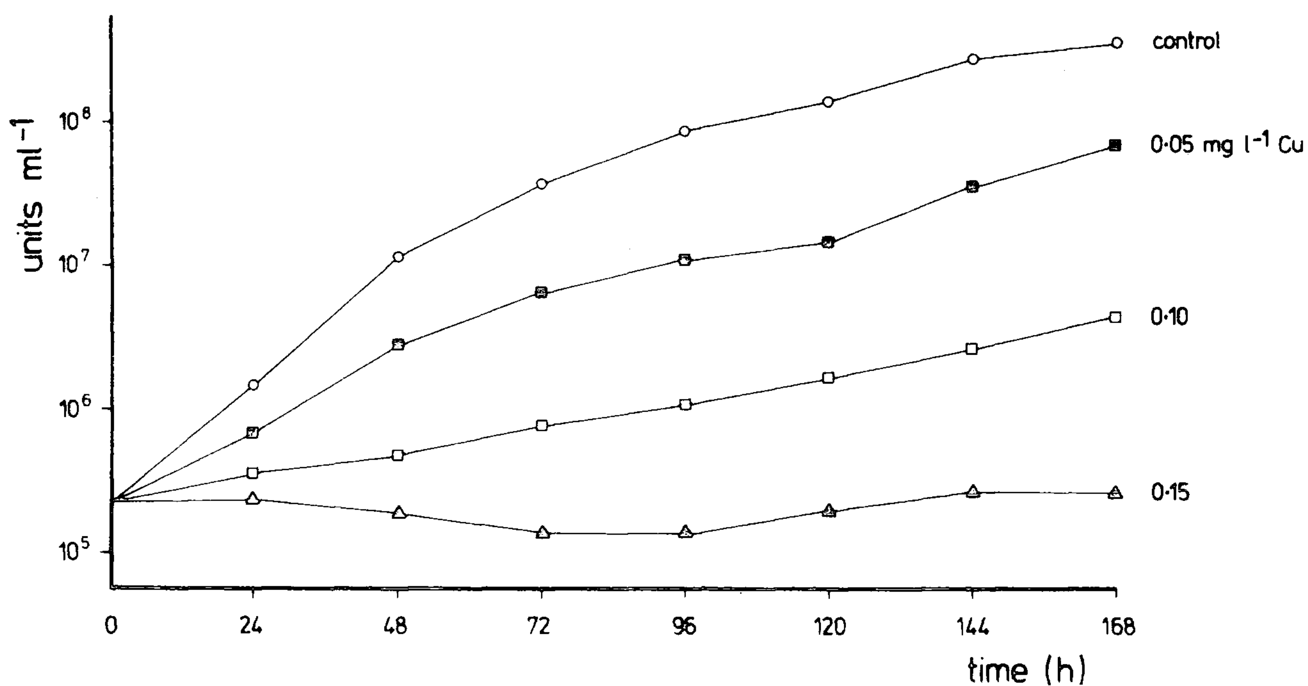
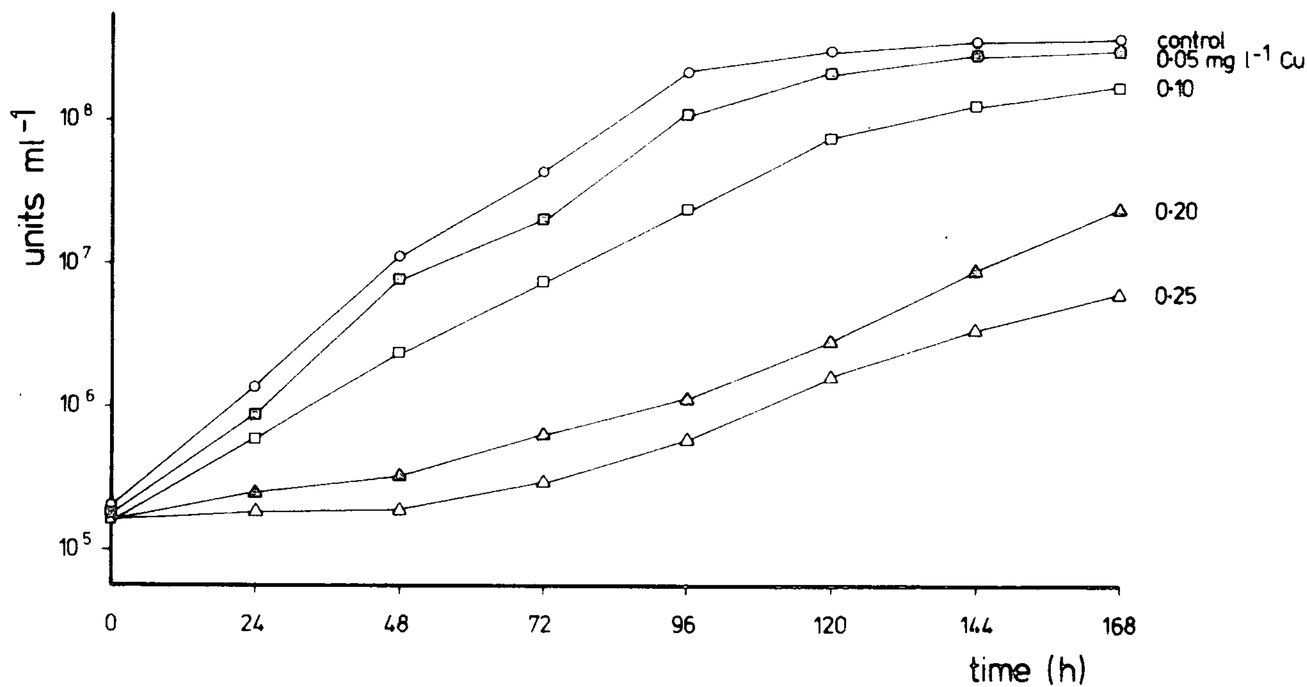


Fig. 4.15 Influence of Cu on growth of Ni-t1.0

a) inoculum from basal medium

b) inoculum from strongly inhibitory level of metal at which adapted



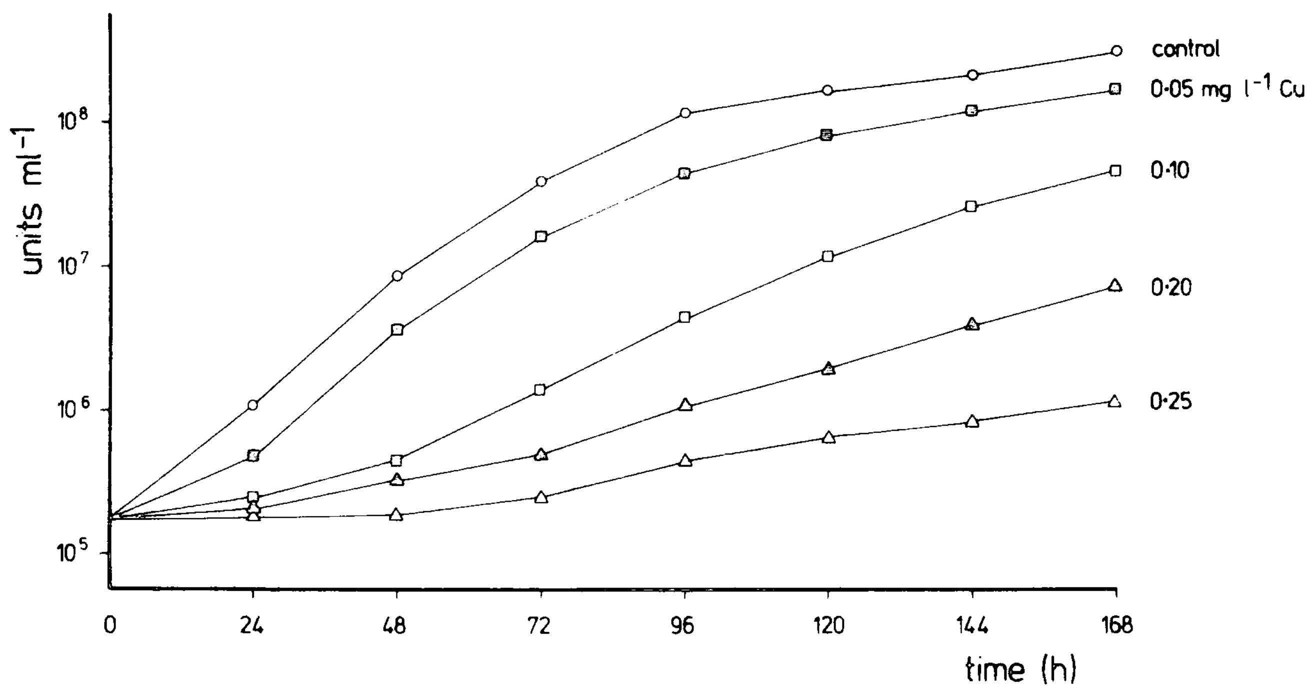
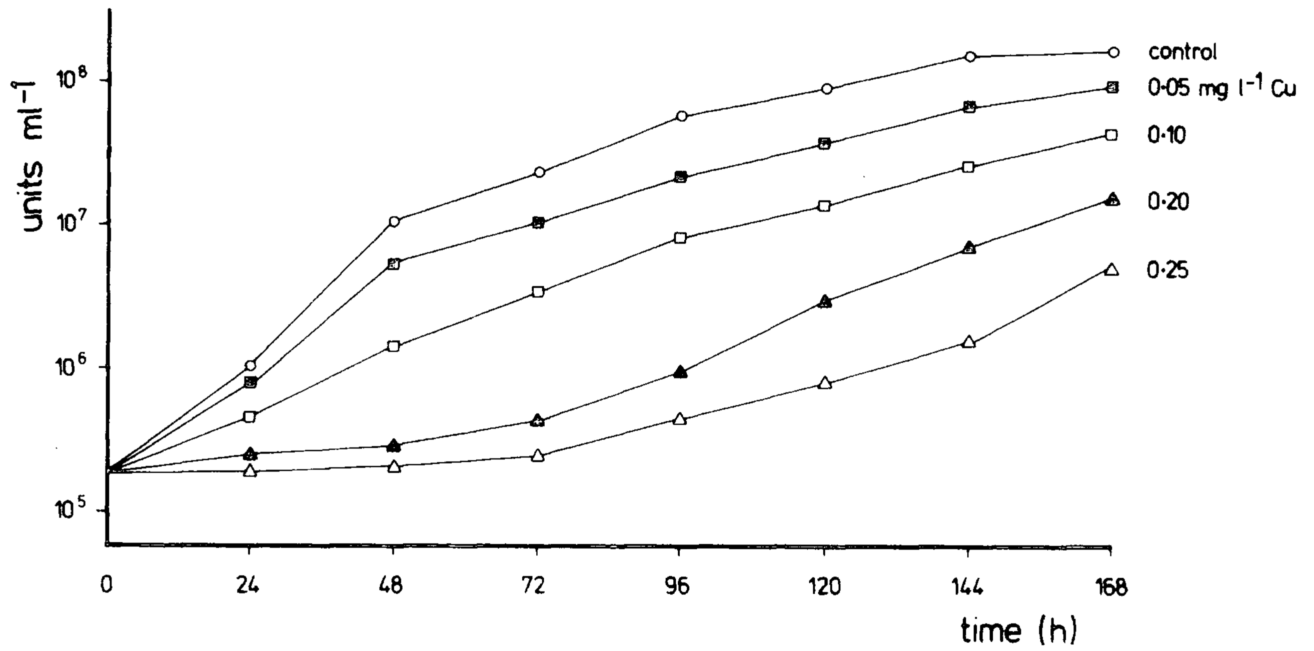


Fig. 4.16 Influence of Cu on growth of Zn-t5.0

a) inoculum from basal medium

b) inoculum from strongly inhibitory level of metal at which adapted



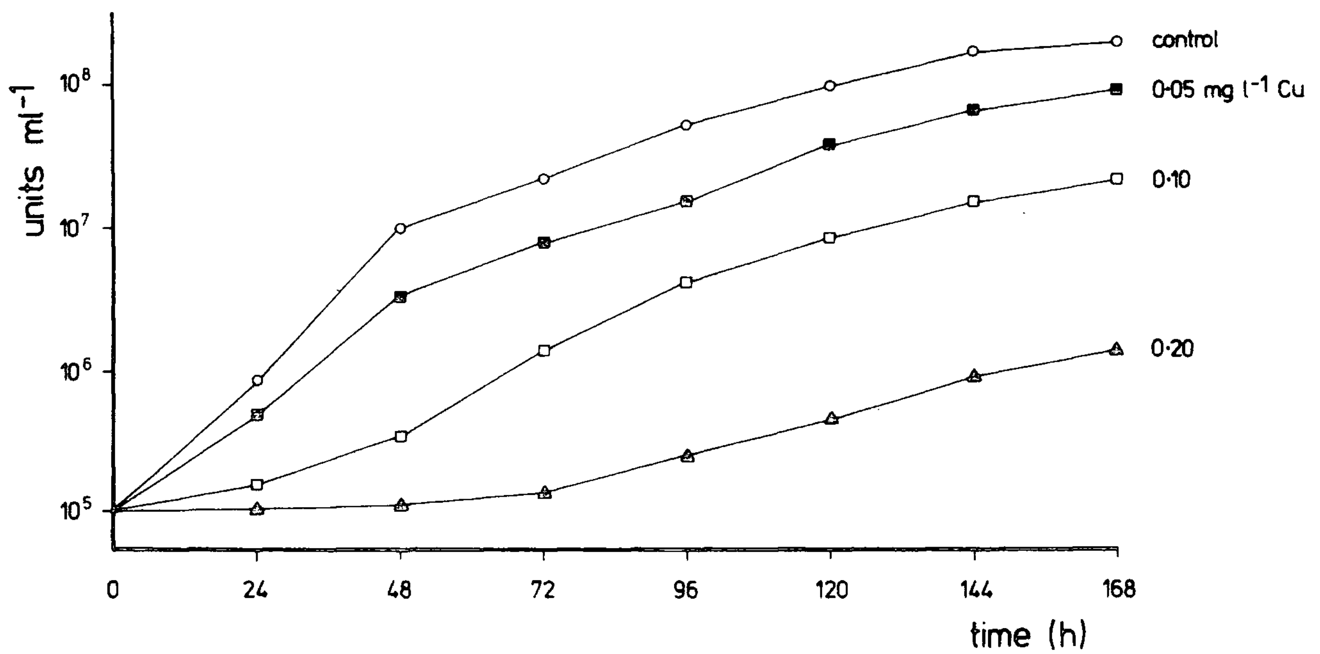
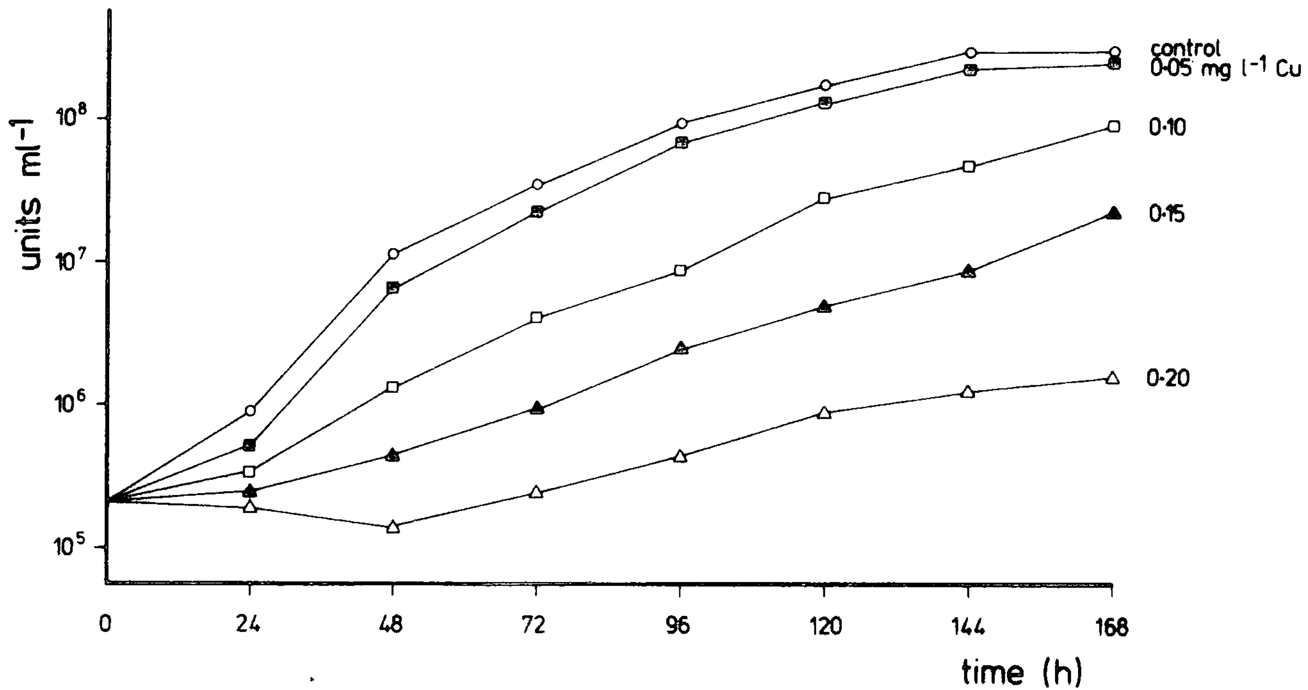


Fig. 4.17 Influence of Cu on growth of Zn-t12.0

a) inoculum from basal medium

b) inoculum from strongly inhibitory level of metal at which adapted



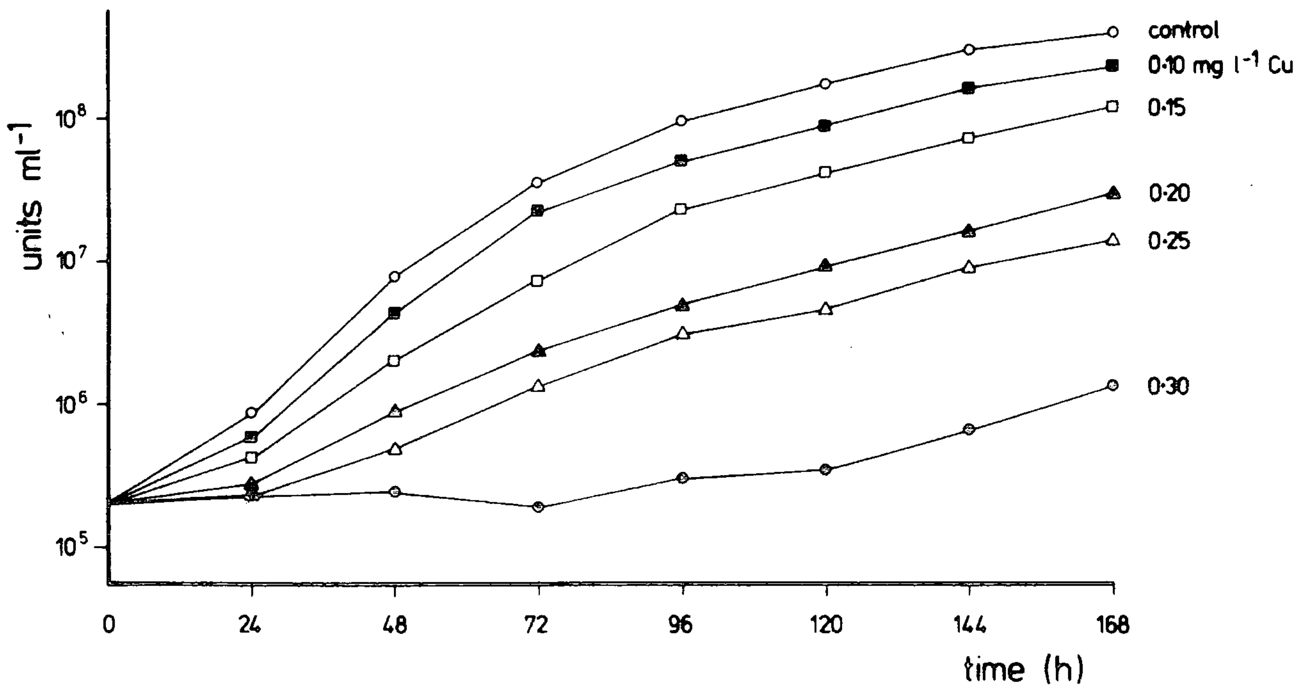
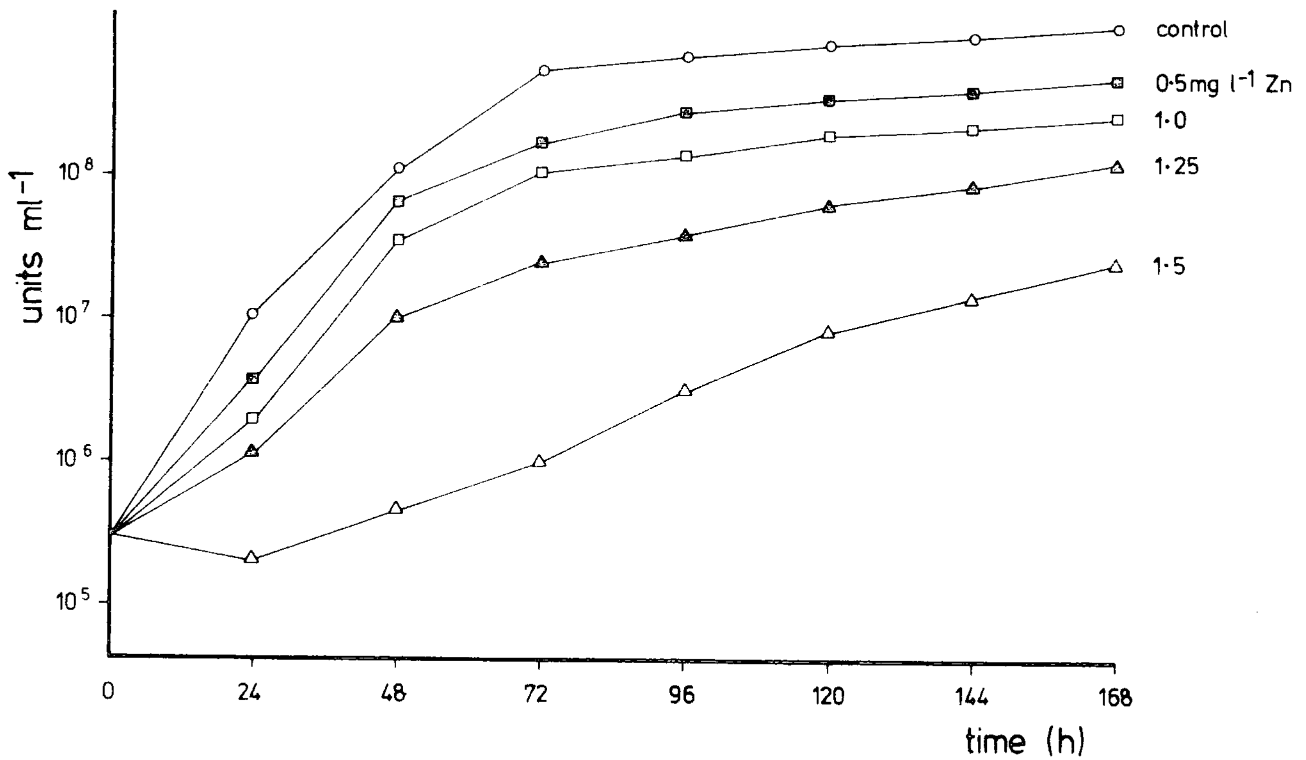


Fig. 4.18 Influence of Cu on growth of Cd-t2.0

a) inoculum from basal medium

b) inoculum from strongly inhibitory level of metal at which adapted



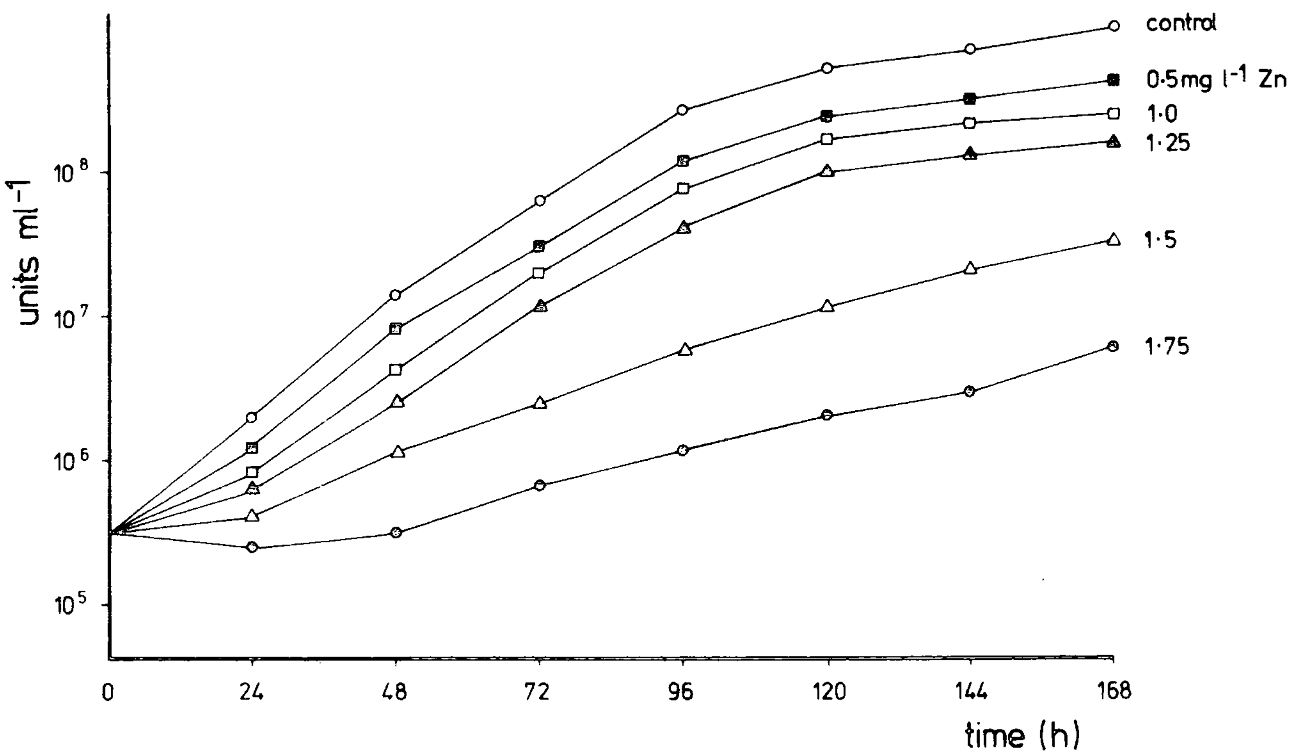
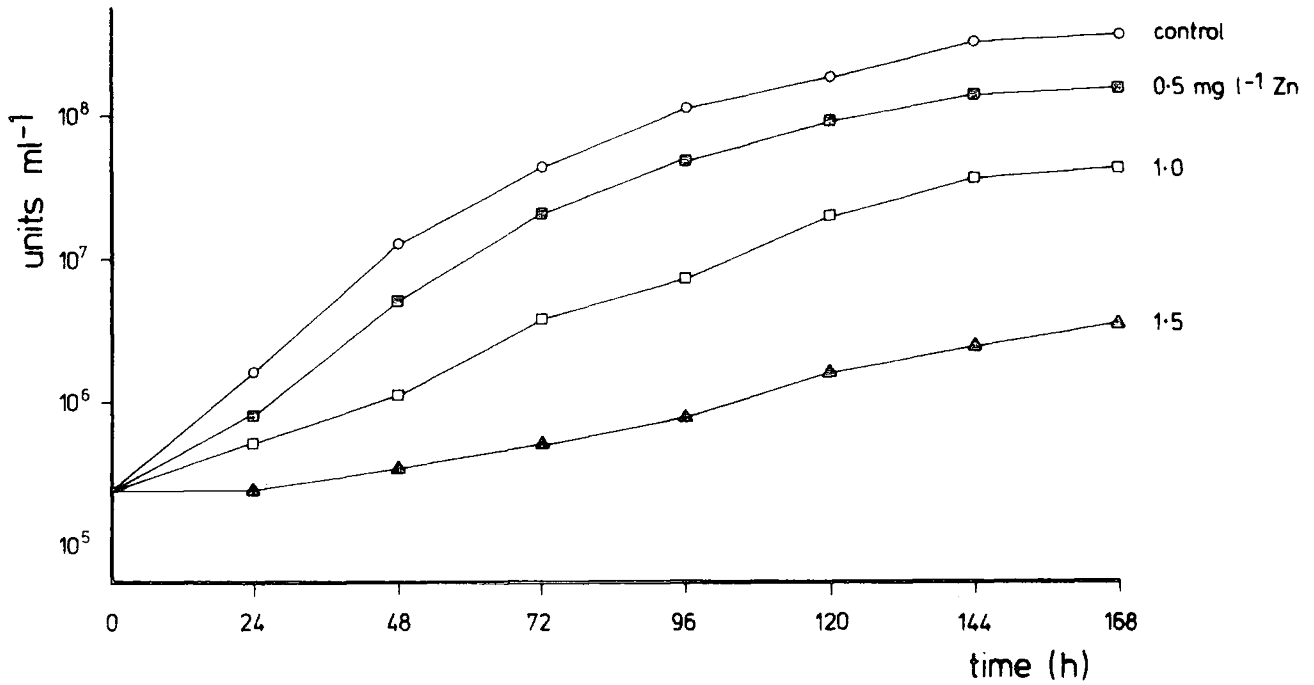


Fig. 4.19 Influence of Zn on growth of wild-type *Anacystis*

a) inoculum from basal medium

b) inoculum from strongly inhibitory level of Zn



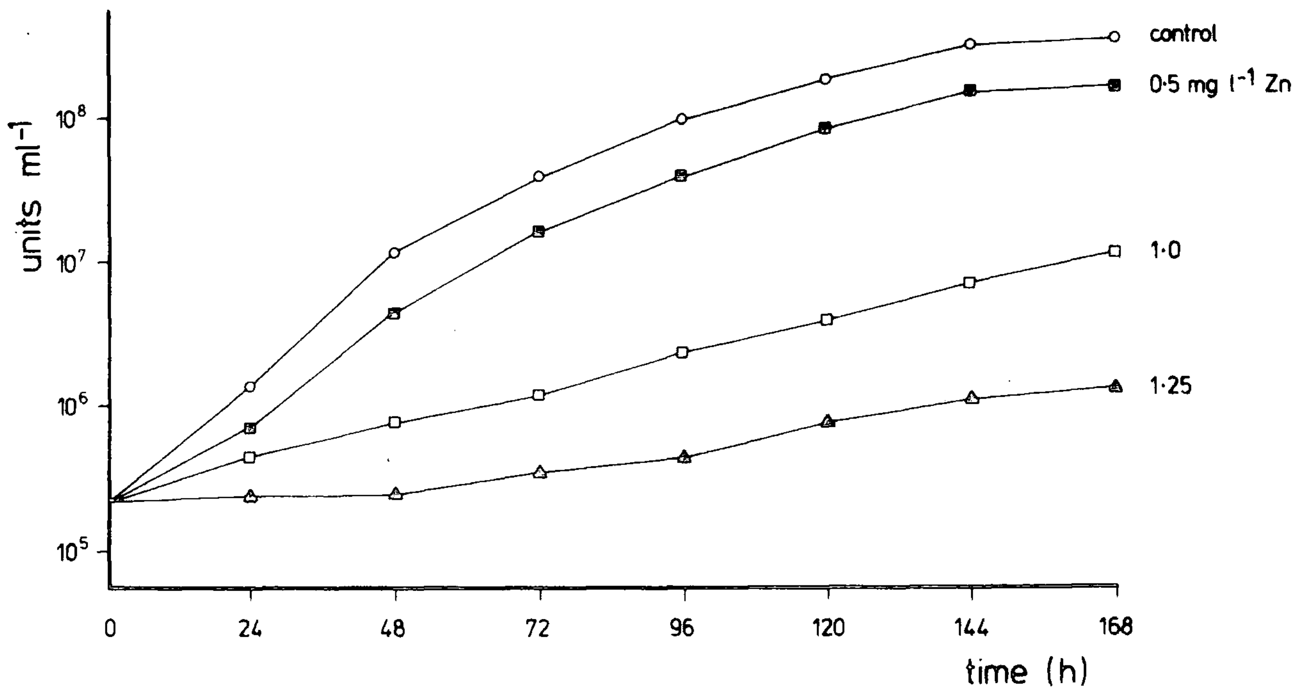
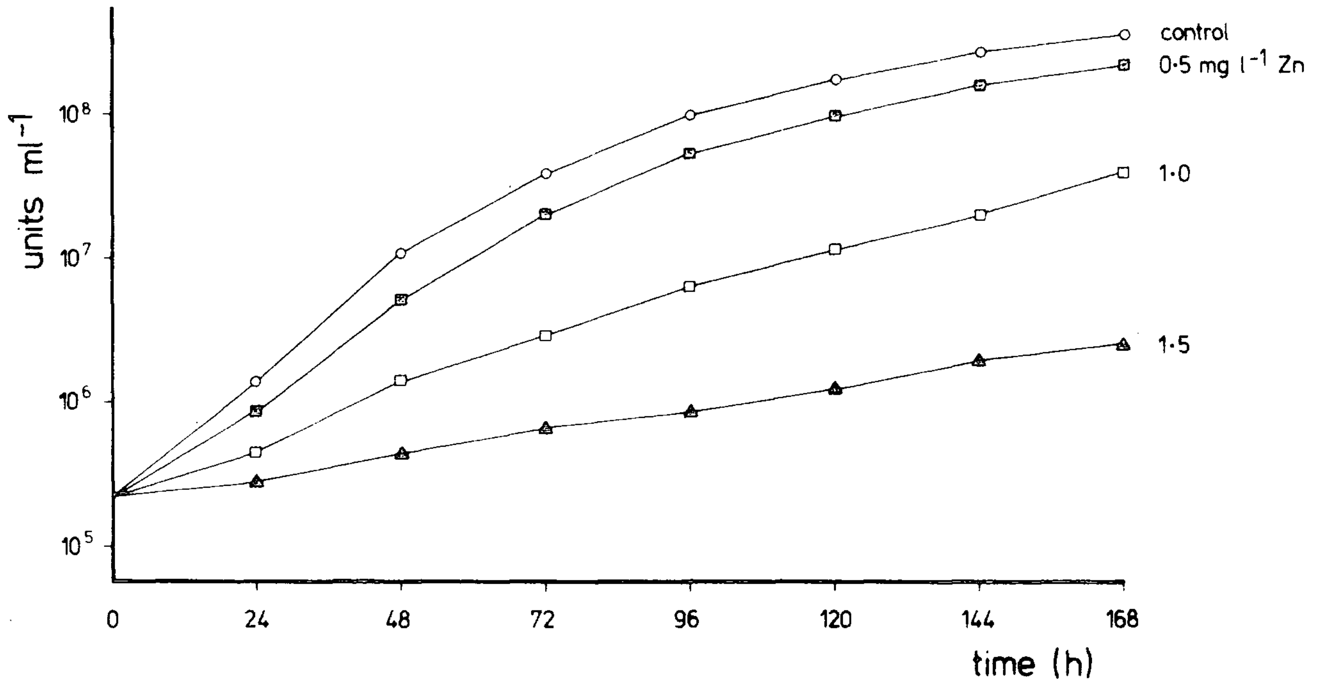


Fig. 4.20 Influence of Zn on growth of Co-t1.8

a) inoculum from basal medium

b) inoculum from strongly inhibitory level of metal at which adapted



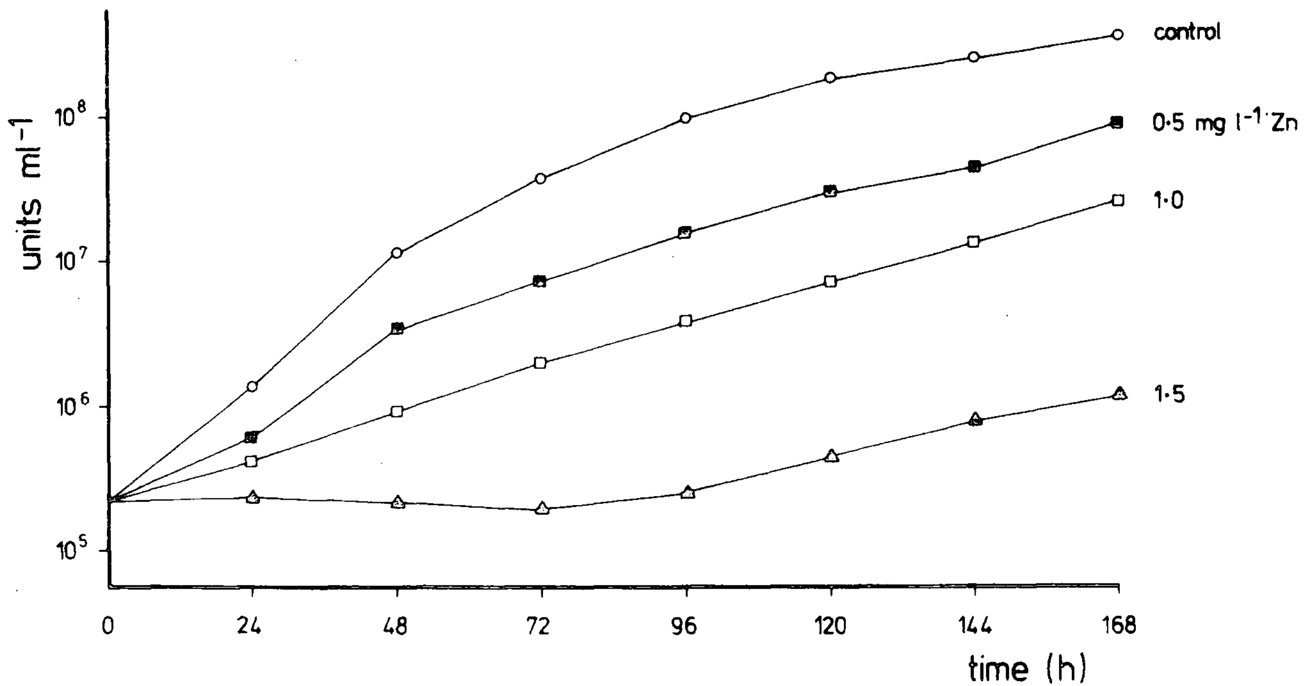
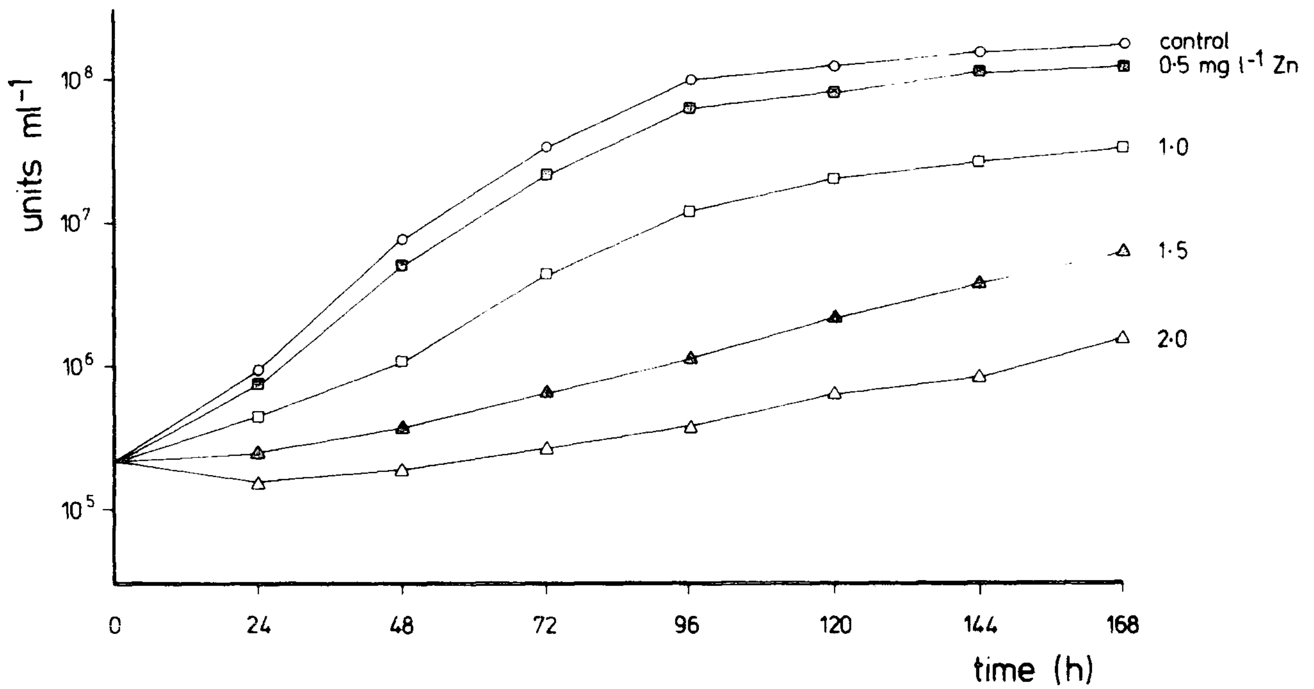


Fig. 4.21 Influence of Zn on growth of Ni-t1.0

a) inoculum from basal medium

b) inoculum from strongly inhibitory level of metal at which adapted



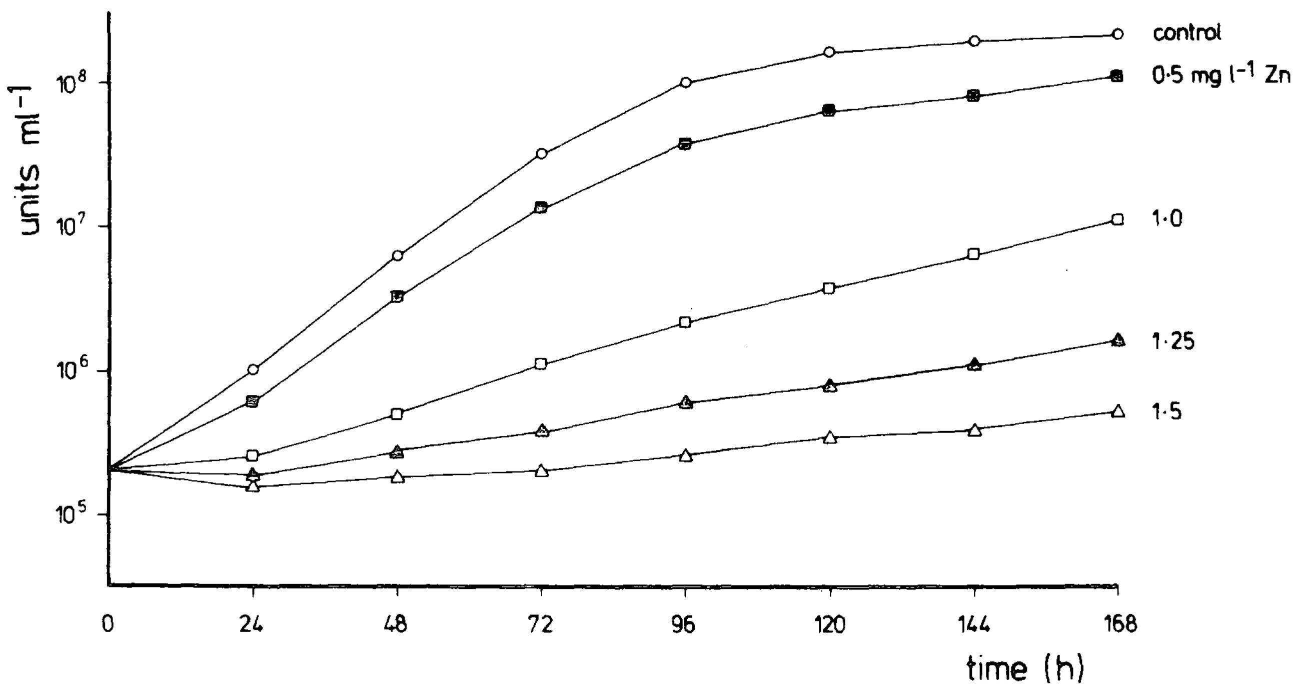
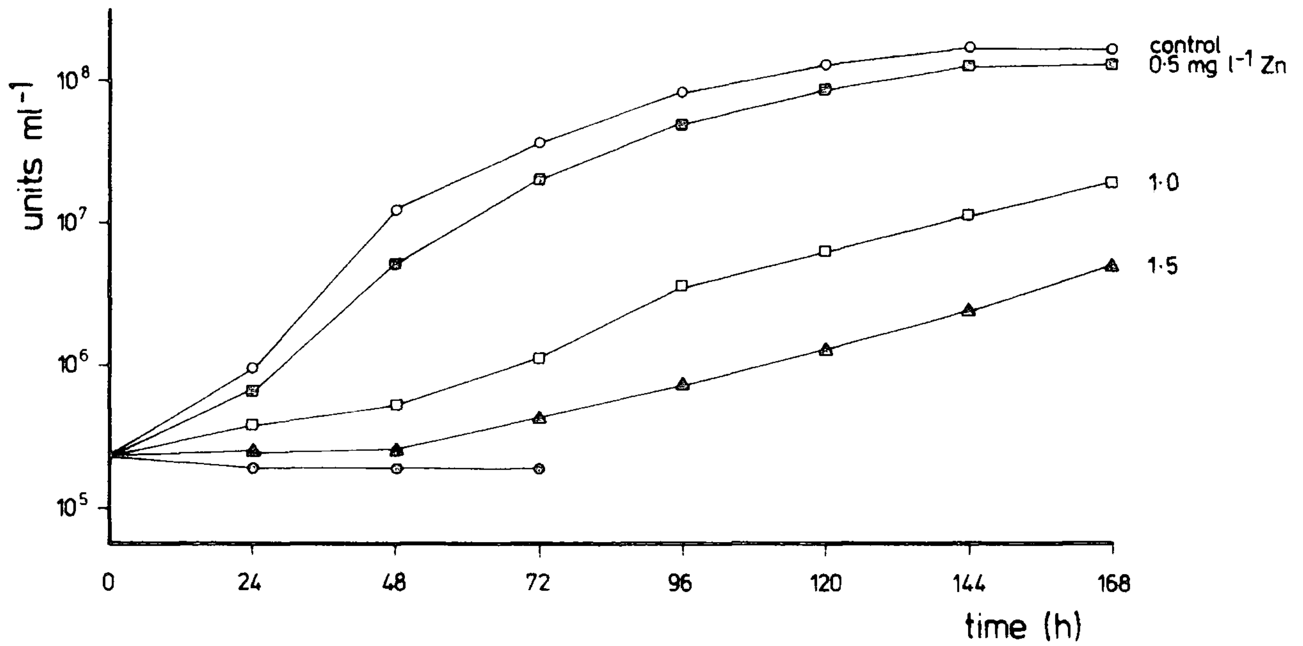


Fig. 4.22 Influence of Zn on growth of Cu-t0.5

a) inoculum from basal medium

b) inoculum from strongly inhibitory level of metal at which adapted



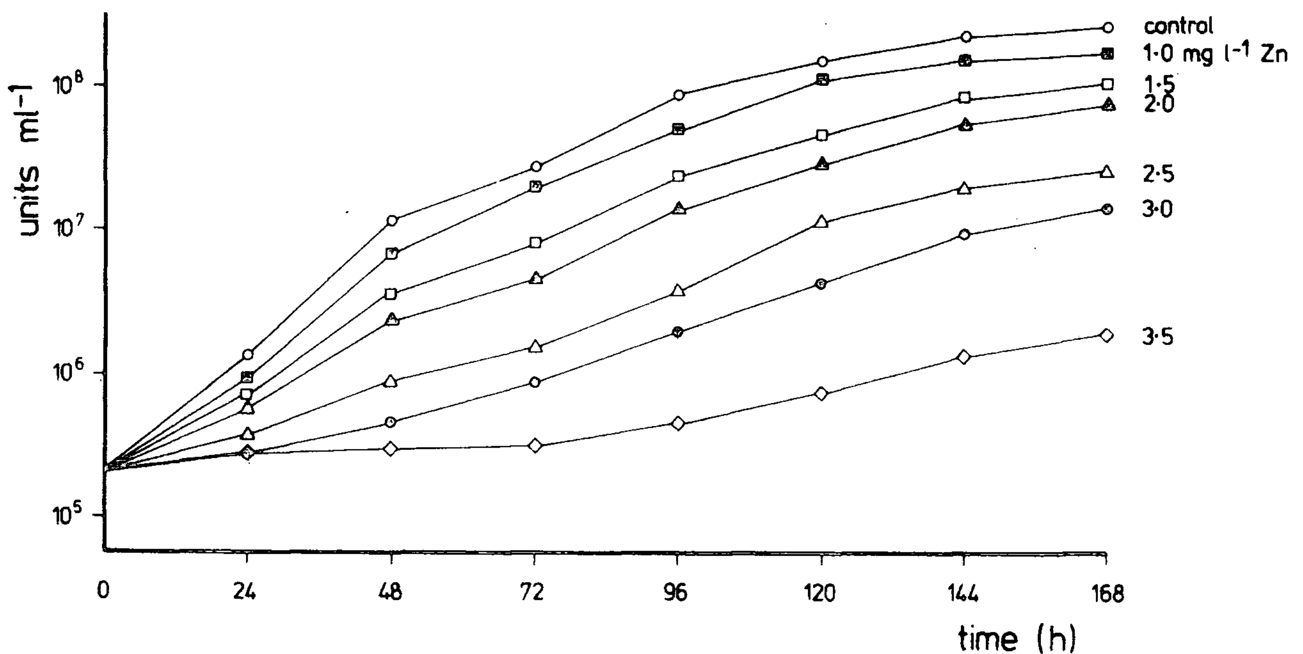
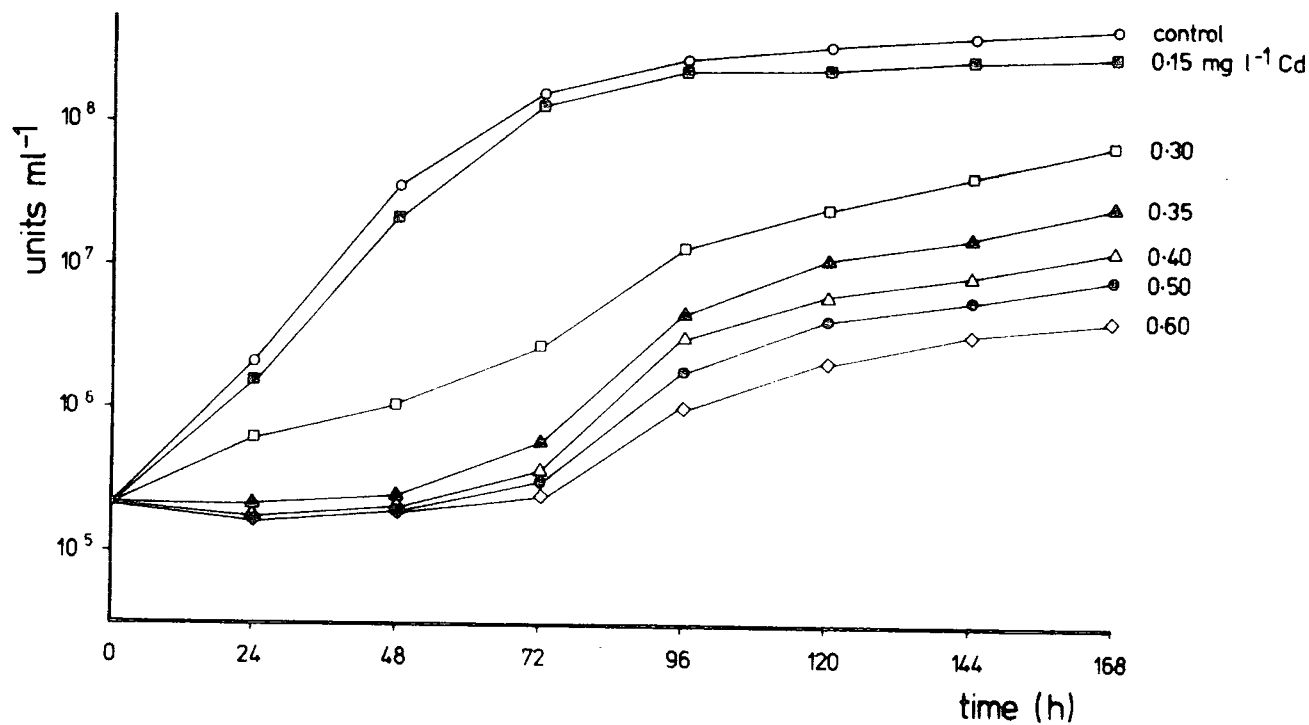


Fig. 4.23 Influence of Zn on growth of Cd-t2.0

a) inoculum from basal medium

b) inoculum from strongly inhibitory level of metal at which adapted



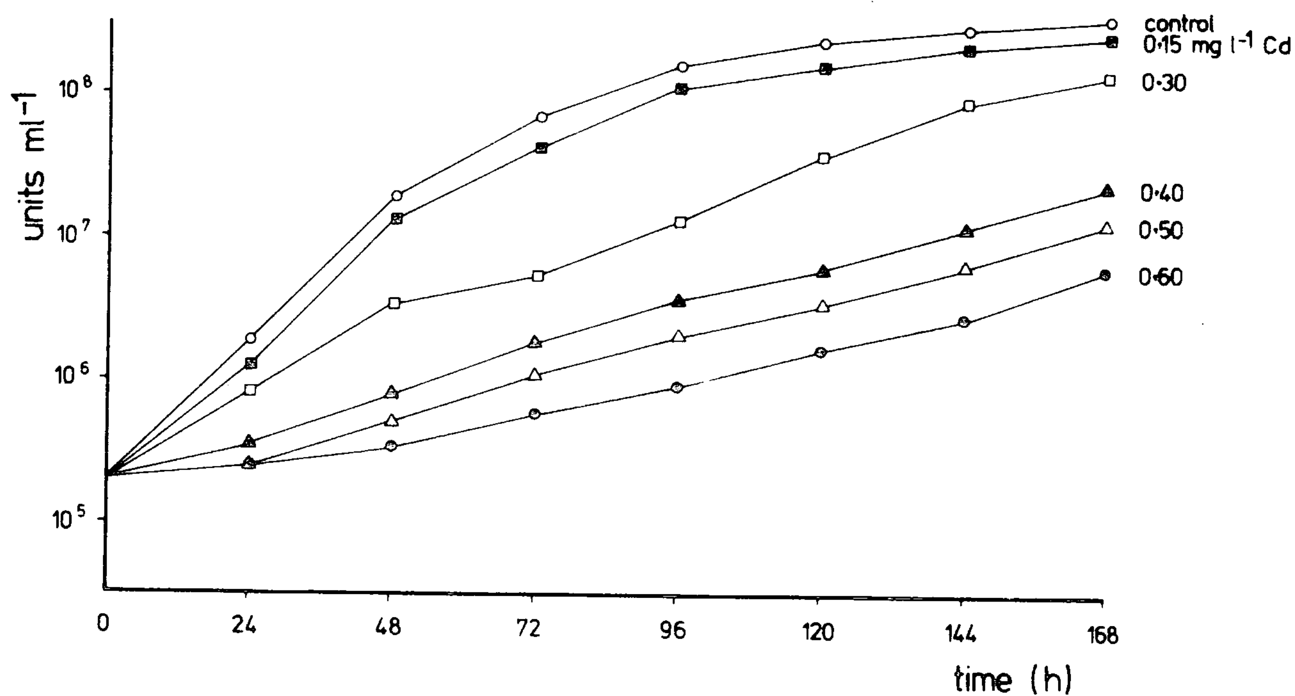
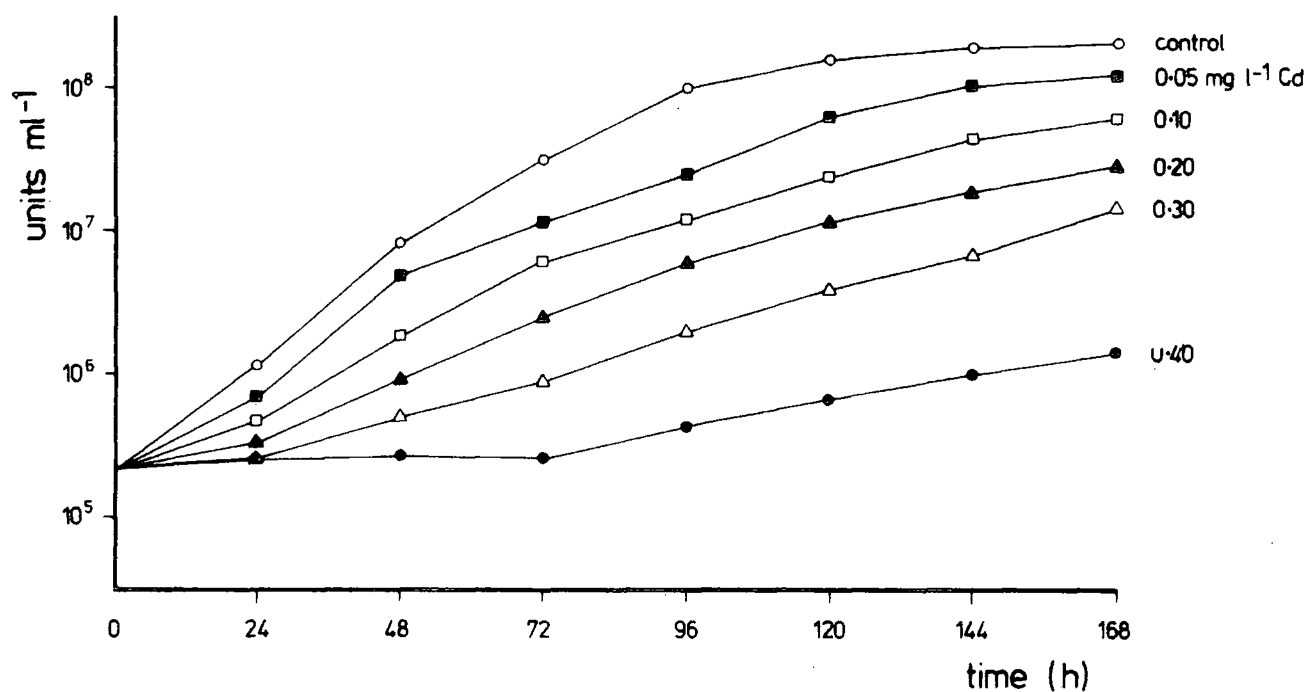


Fig. 4.24 Influence of Cd on growth of wild-type *Anacystis*

a) inoculum from basal medium

b) inoculum from strongly inhibitory level of Cd



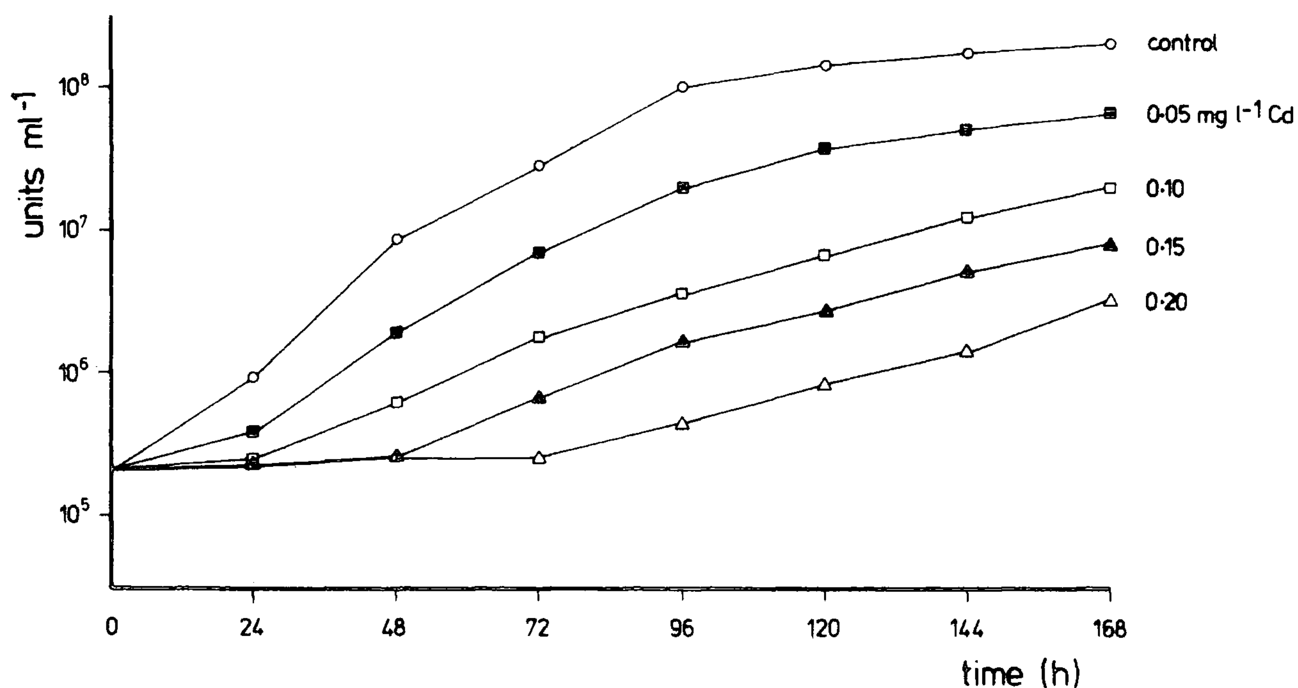
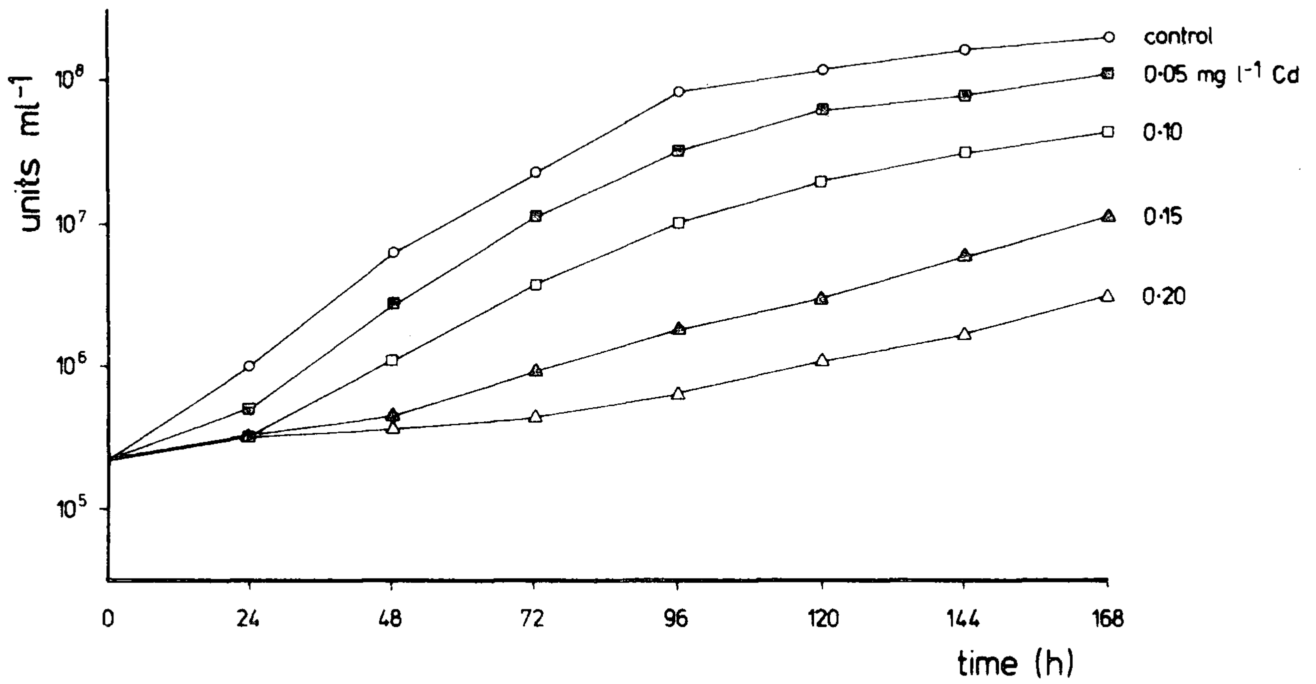


Fig. 4.25 Influence of Cd on growth of Co-t1.8

a) inoculum from basal medium

b) inoculum from strongly inhibitory level of metal at which adapted



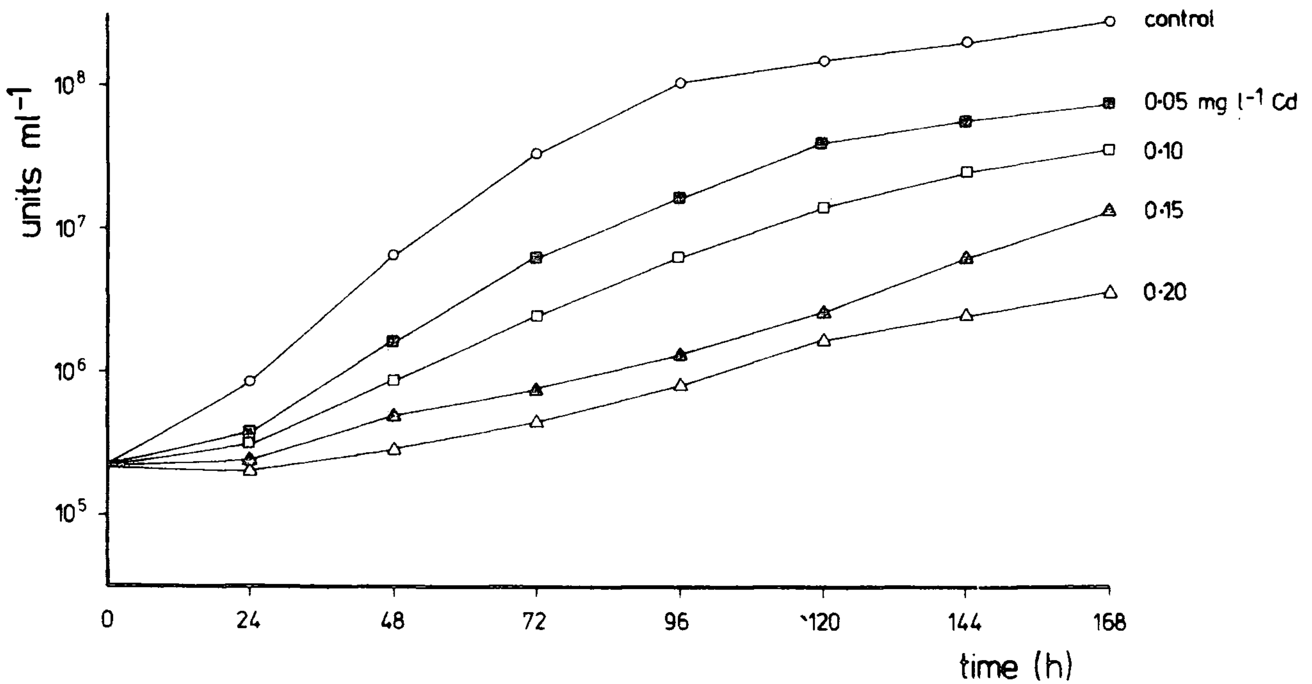
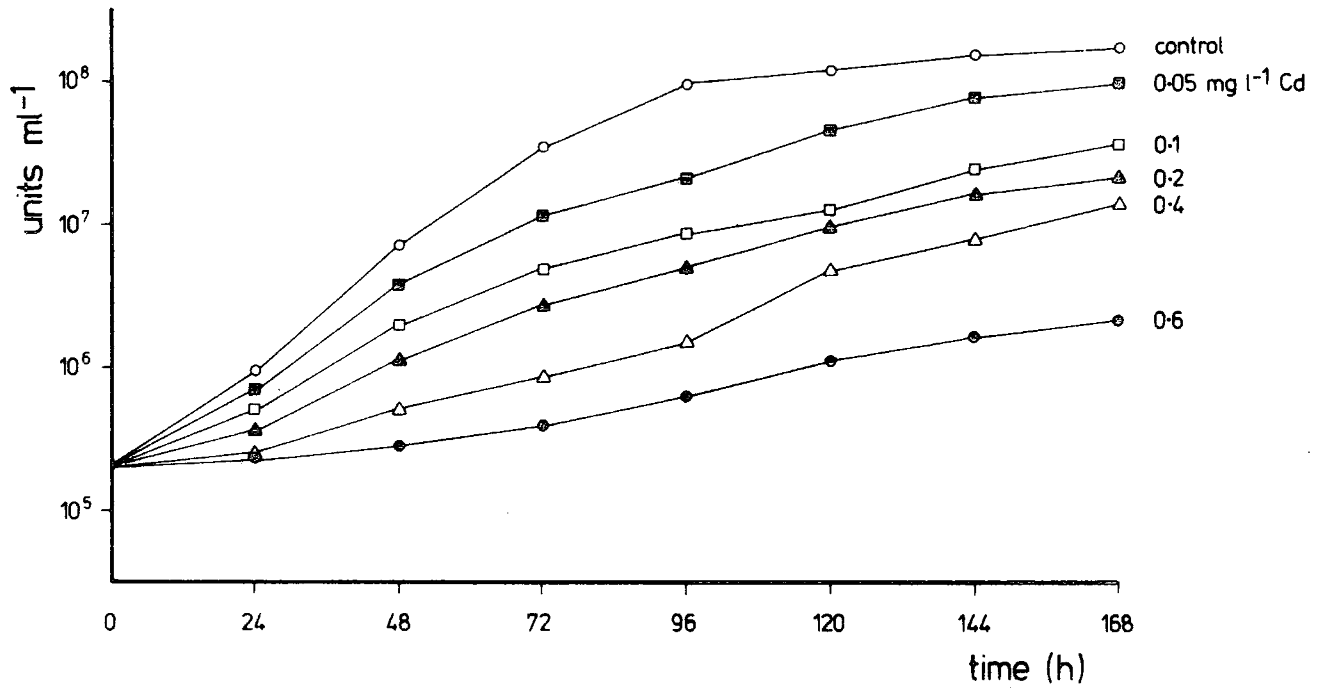


Fig. 4.26 Influence of Cd on growth of Ni-t1.0

a) inoculum from basal medium

b) inoculum from strongly inhibitory level of metal at which adapted



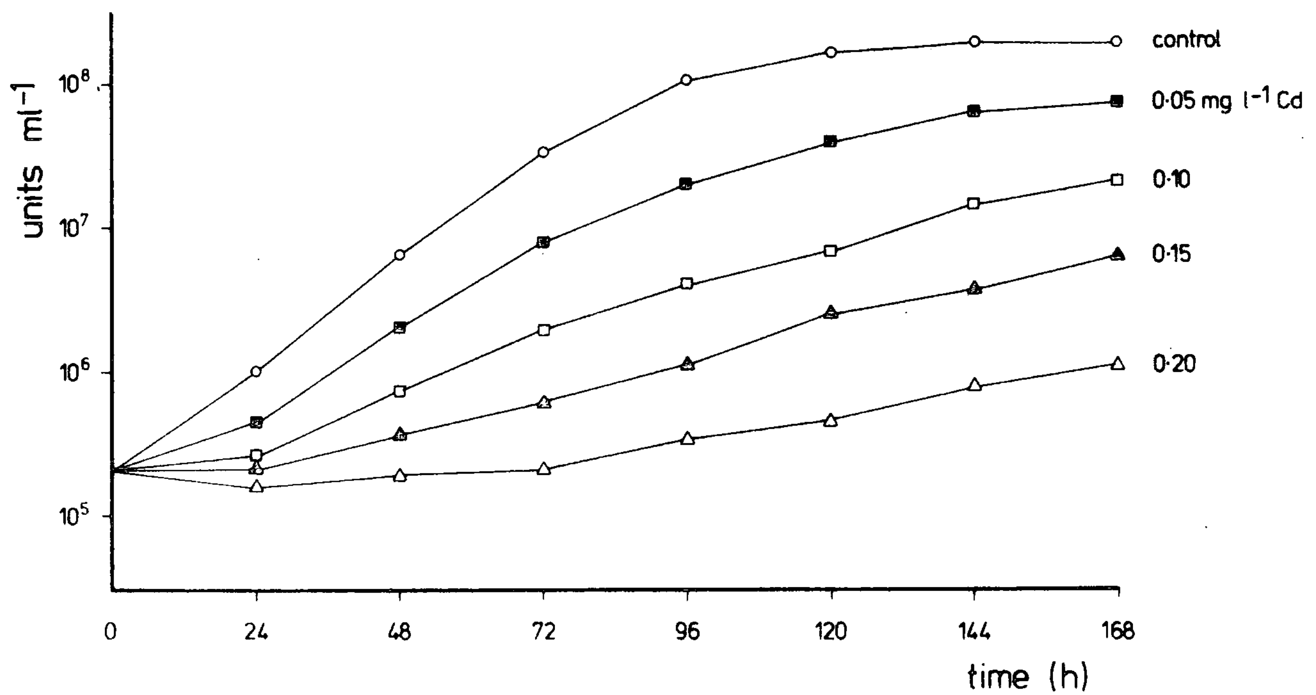
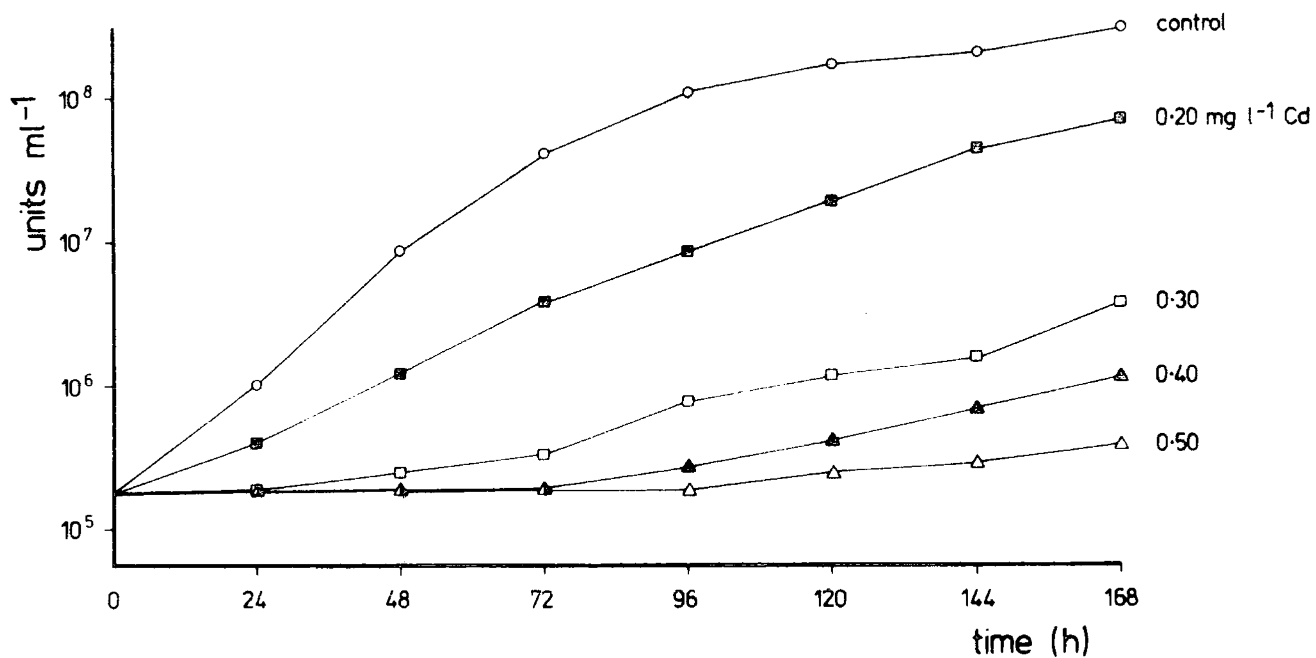


Fig. 4.27 Influence of Cd on growth of Cu-t0.5

a) inoculum from basal medium

b) inoculum from strongly inhibitory level of metal at which adapted



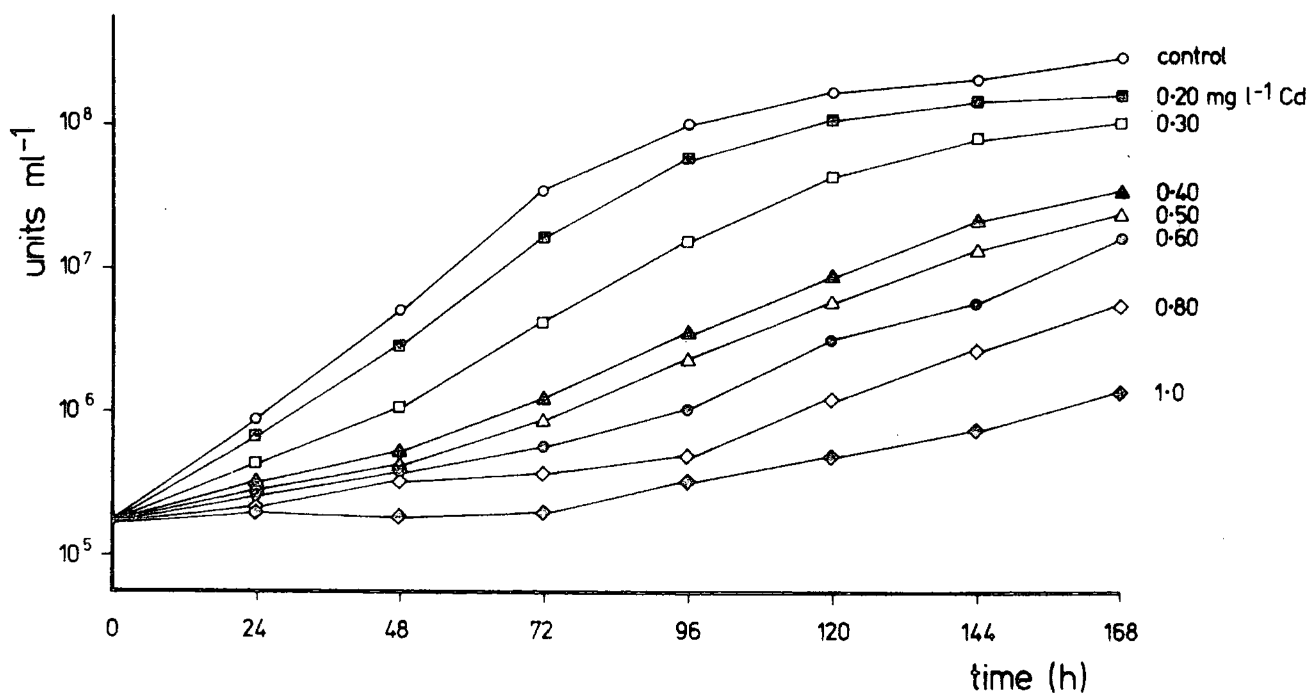
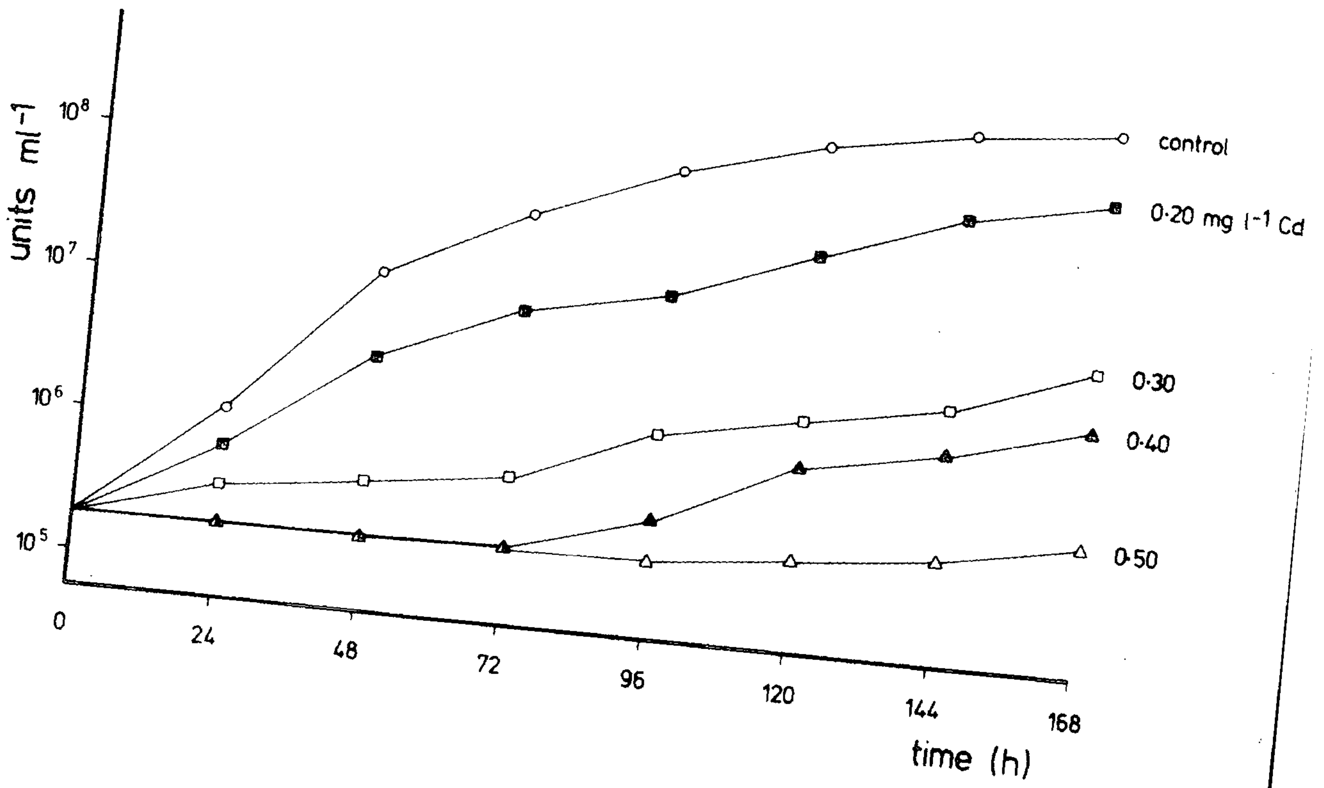


Fig. 4.28 Influence of Cd on growth of Zn-t5.0

a) inoculum from basal medium

b) inoculum from strongly inhibitory level of metal at which adapted



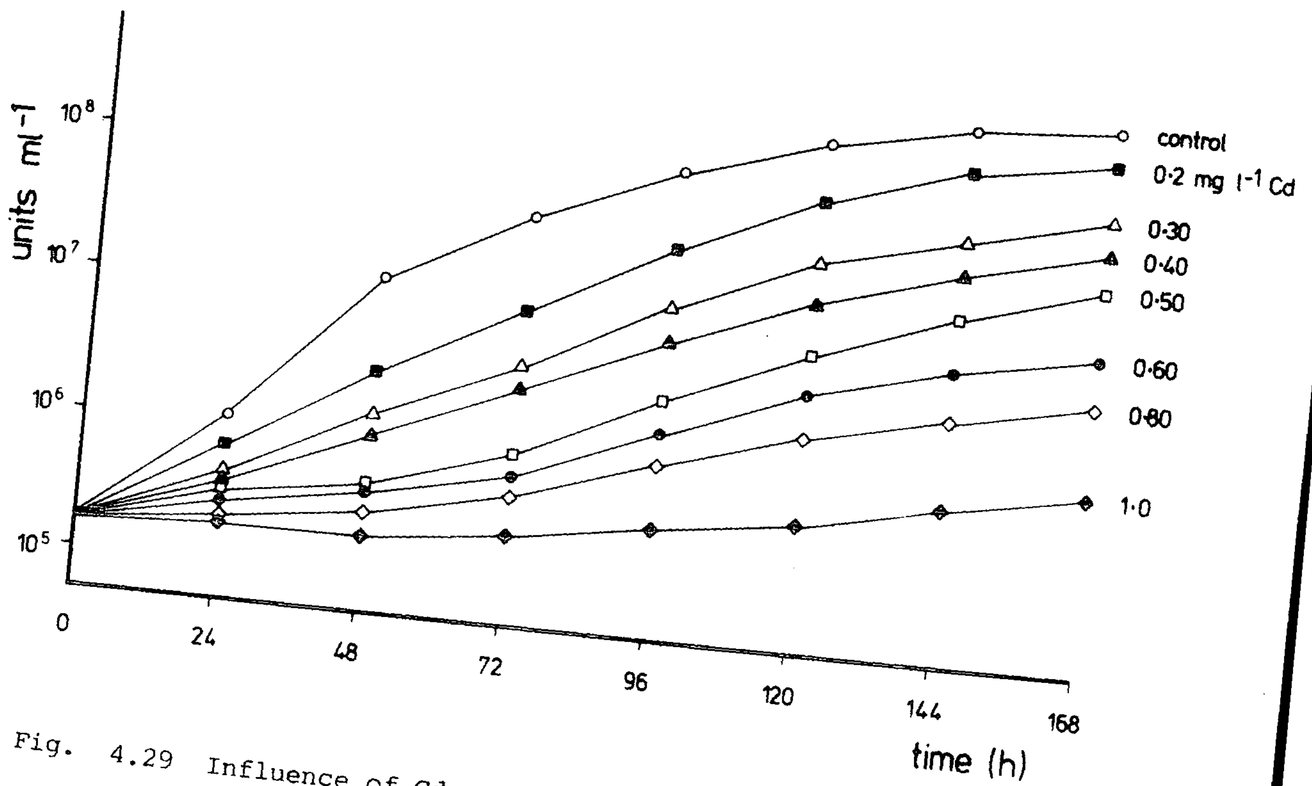


Fig. 4.29 Influence of Cd on growth of Zn-t12.0

a) inoculum from basal medium

b) inoculum from strongly inhibitory level of metal at which adapted

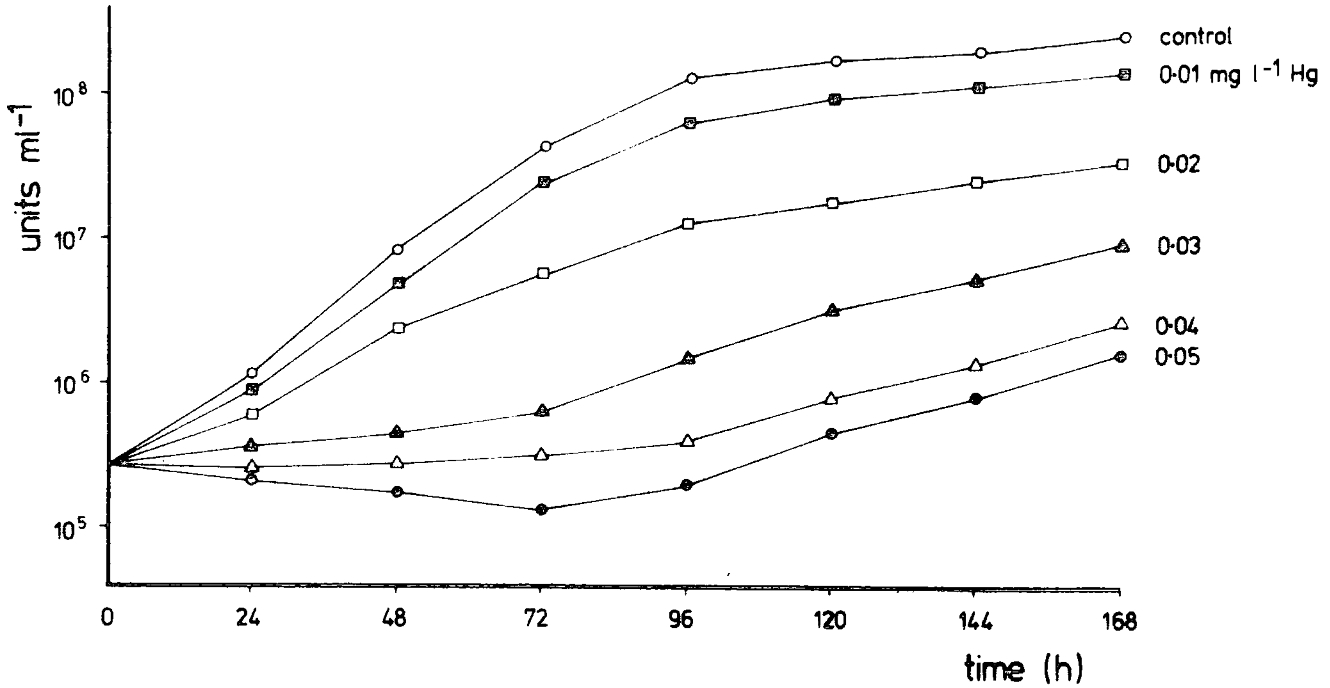


Fig. 4.30 Influence of Hg on growth of wild-type *Anacystis*.

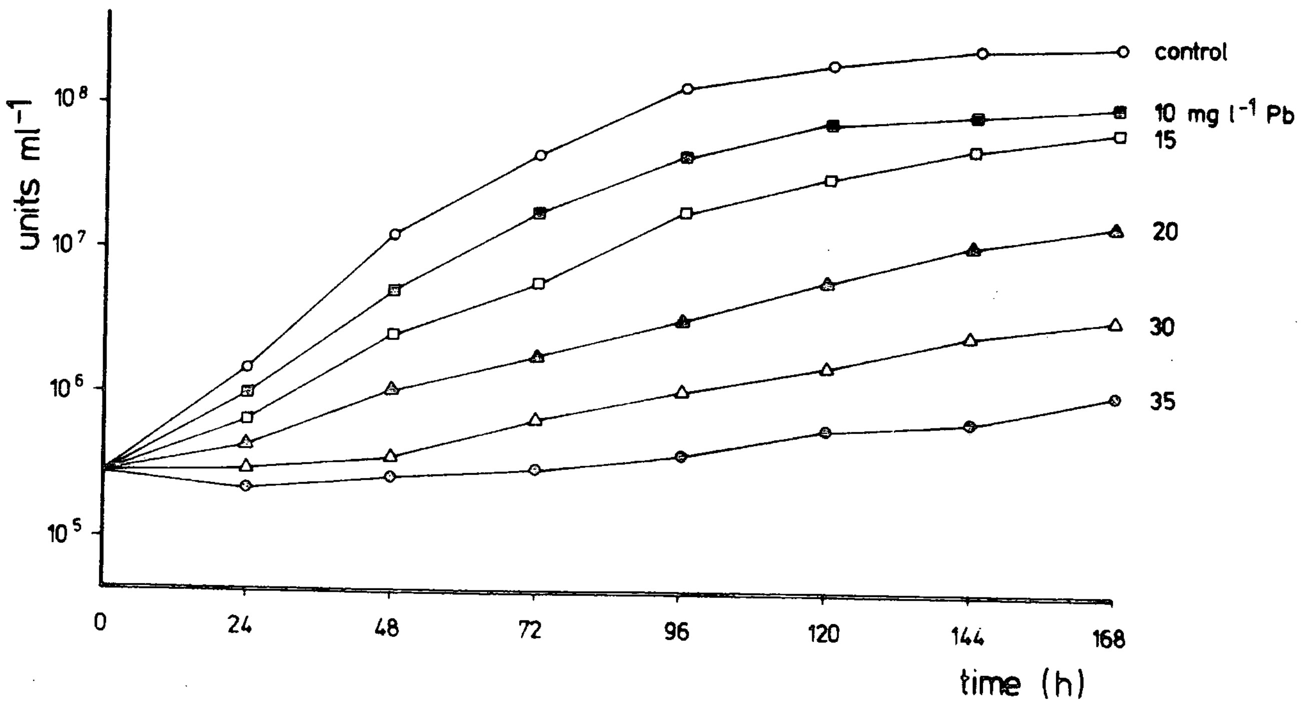


Fig. 4.31 Influence of Pb on growth of wild-type *Anacystis*.

A comparison was made of the toxicity of Zn to *Anacystis* in liquid and in agar. As a preliminary experiment showed that the colony formation on plates was dependent on the growth stage of the alga (Table 4.1), units were taken from growth stage B. The alga was slightly less tolerant to Zn in liquid than with agar. For instance, in liquid the alga was inhibited strongly at 1.5 mg l^{-1} Zn and died at 1.75 mg l^{-1} Zn; in solid medium, the comparative values were 2.25 mg l^{-1} Zn and 2.5 mg l^{-1} Zn, respectively. The full results are given in Table 4.2.

A comparison was also made of the toxicity of Zn to *Anacystis* in liquid shaking (Fig. 3.6) and standing cultures (Fig. 4.32). The alga had mainly the same tolerance to Zn in liquid in both conditions, except that, shaking culture was faster than standing culture. The generation time of shaking culture was 9.7 h in comparison to that of 16.5 h in standing culture.

<u>Summary to 4.1</u>

The results present thus far indicate marked differences in the toxicity of metals to the test *Anacystis* strains (Table 4.3). Exposure of a culture to a particular metal often led to a slight increase in tolerance to that metal during the next subculture, even without mutation. In the case of wild-type slight decreases in lag were noted with all seven metals and increases in growth rate with Co and Zn. All metals exhibited similar inhibitory effects with increasing concentrations. These included the depression of growth rate, increasing lag, some morphological changes in units, and eventually death. In general the order of metal toxicity for each strain from the previous results are:

Table 4.1 Influence of growth stage on colony-forming ability of wild-type *Anacystis nidulans* on 1% agar plates; n = 5.

	growth stage	population density of original cultures (units ml^{-1})	units per plate	colonies per plate	s.d.	% recovery
A	young alga (lag phase)	2.0×10^5	20	19.5	± 1.3	97.5
B	increasing growth rate	5.2×10^6	50	51.5	± 3.4	103
C	exponential phase	9.6×10^7	100	88.7	± 5.6	88.7
D	old culture	1.8×10^8	180	125	± 4.6	69.4

Table 4.2 Influence of zinc on growth of wild-type *Anacystis nidulans* on 1% agar plates; initial inoculum, 520 units per plate; results assessed after growth for 2 weeks; counts based on 10 colonies.

	colonies developing 520 units			units per colony		total units per plate		
	$\bar{x}$	s.d.	% of control	$\bar{x}$	s.d.	$\bar{x}$	% of control	
0.04	473.8	± 16.7		3.5×10^5	± 679	1.6×10^8		
0.5	477.0	± 8.5	100.7	3.5×10^5	± 585	1.7×10^8	106	as wild-type
1.0	458	± 26.4	96.7	3.5×10^5	± 683	1.6×10^8	100	"
1.25	339.8	± 24.8	71.7	3.5×10^5	± 683	1.2×10^8	75	"
1.5	268.5	± 18.7	56.7	2.8×10^5	± 564	7.6×10^7	47.5	"
1.75	107.5	± 8.4	22.8	2.5×10^4	± 564	3.6×10^6	2.3	long units
2.0	60.0	± 6.3	12.6	2.5×10^4	± 420	1.5×10^6	1.1	"
2.25	18.3	± 3.5	3.9	2.5×10^4	± 436	4.6×10^5	0.3	"
2.5	0	0	0	0	0	0	0	

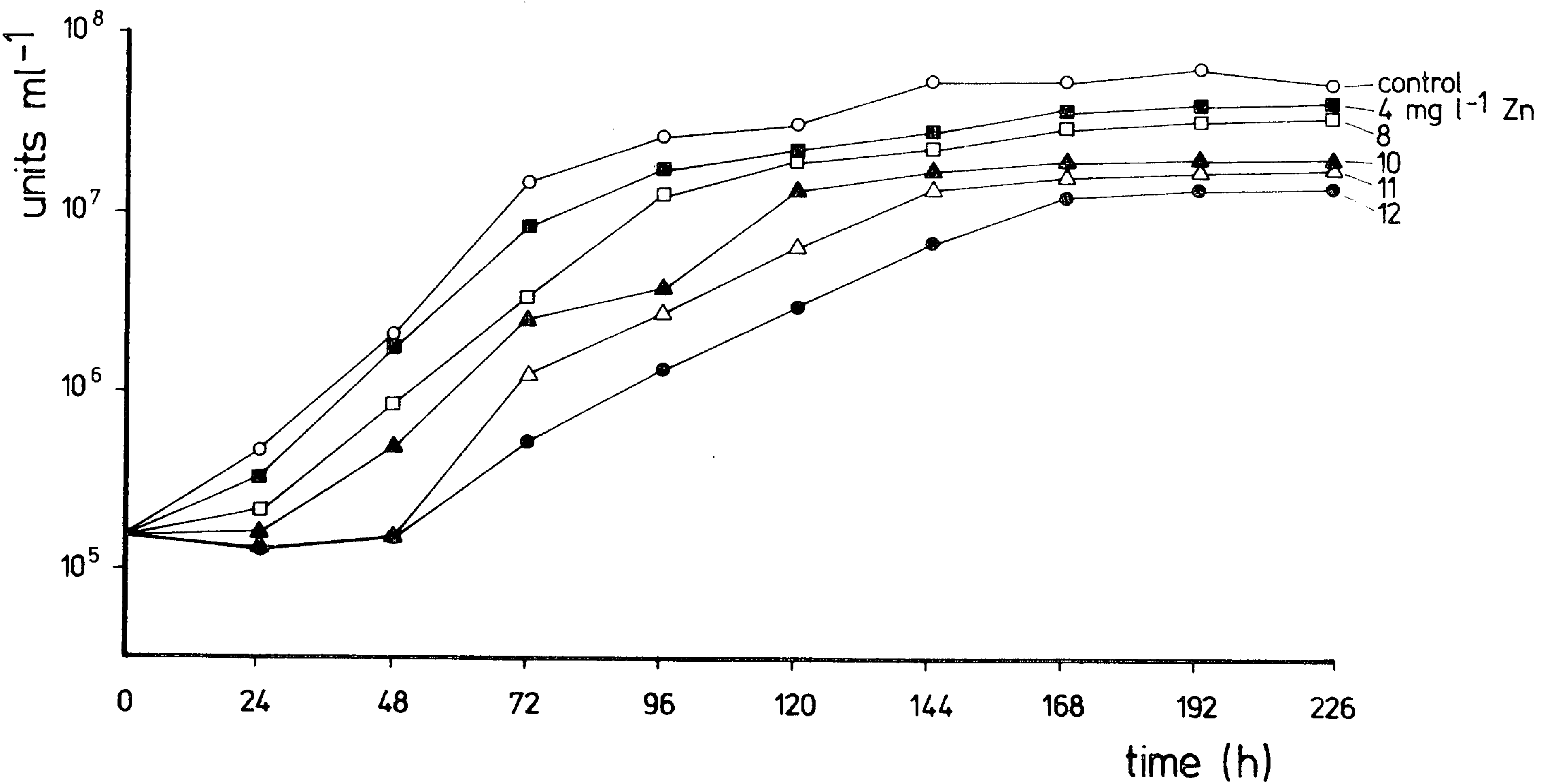


Fig. 4.32 Influence of Zn on growth of Zn-t12.0 in standing culture.

Table 4.3 Changes (greater than a factor of two) in resistance to other metals of metal-tolerant strains *cf* Table 8.1.

strain	change in resistance			
	increase		decrease	
medium used for growth of inoculum:	basal	strongly inhibitory	basal	strongly inhibitory
Co-t1.8				Zn Cd
Ni-t1.0	Co		Cd	Zn Cd
Cu-t0.5		Ni		Co Cd
Zn-t12.0				
Cd-t2.0	Co	Co Ni Cu		

Wild-type	Hg > Ni > Cu > Co > Cd > Zn > Pb
Co-t1.8	Cu > Ni > Cd > Zn
Ni-t1.0	Cu > Cd > Co > Zn
Cu-t0.5	Ni > Cd > Co > Zn
Zn-t5.0	Ni = Cu > Co > Cd
Zn-t12.0	Ni > Cu > Co > Cd
Cd-t2.0	Ni > Cu > Co > Zn

4.2 Antibiotics

A brief attempt was made to compare the toxic effects of three antibiotics, penicillin, polymyxin and streptomycin on the growth of wild-type, Zn-t5.0 and Zn-t12.0 *Anacystis* strains. It was clear from the results (Table 4.4) that relatively low concentrations of antibiotics were toxic to all the *Anacystis* strains tested. The wild-type was however in each case more sensitive.

4.3 NTG

The growth response of wild-type *Anacystis* to NTG (Fig 4.33) differed according to whether population density or Chl *a* was used as a criterion of growth. The reduction in growth was more pronounced in the latter; for instance at 2.0 mg l^{-1} NTG, the cells still divided, and gave a slightly longer unit (4 x 10^6 unit ml) than untreated alga, while chl *a* was reduced to about 0.5 mg l^{-1}. The colour of the algal suspension at higher NTG levels was pale yellow.

4.4 Pigment composition

During the routine experimental studies, no shifts in the absorption maxima of the methanol extractions (430 nm, 665 nm) were observed as a result of treatment with the heavy metals. There was

Table 4.4 Comparison of response of wild-type and Zn-tolerant strains to penicillin, polymixin and streptomycin. Toxicity estimated as concentration leading to a 50% reduction in growth rate.

strain	toxic agent		
	penicillin ($\mu g\ ml^{-1}$)	polymixin ($\mu g\ ml^{-1}$)	streptomycin ($\mu g\ ml^{-1}$)
wild-type	0.01	0.01	0.01
Zn-t5.0	0.06	0.065	0.06
Zn-t12.0	0.08	0.058	0.06

Table 4.5 Comparison of Chl *a* content per unit length of alga in Zn-treated cultures; n = 8.

strains	Chl *a* ($mg\ l^{-1}$)	
	$\bar{x}$	s.d.
wild-type	3.41	± 0.65
Zn-t5.0	2.86	± 0.52
Zn-t12.0	2.65	± 0.48

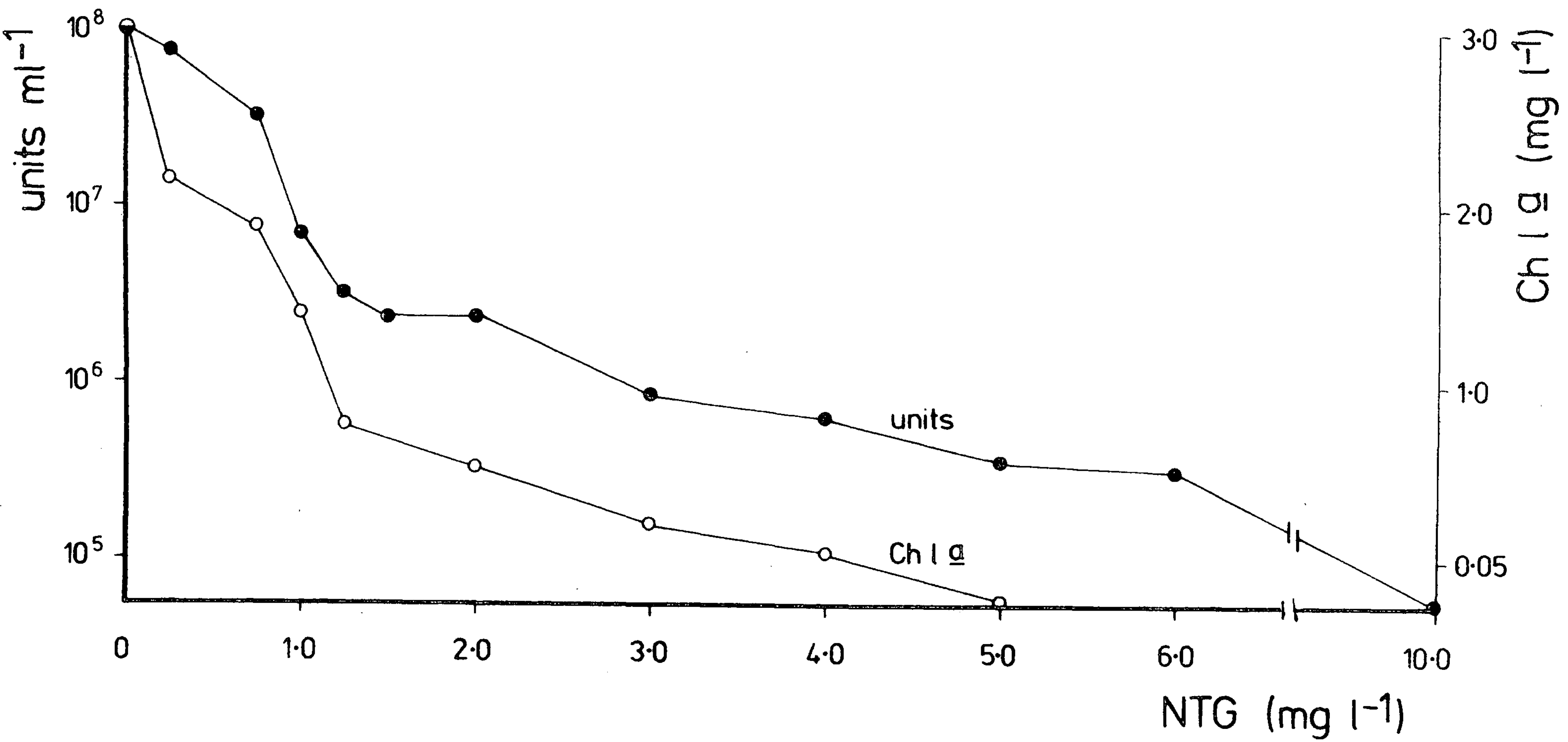


Fig. 4.33 Influence of NTG on growth of wild-type *Anacystis*; population density and chl *a* were used as criteria of growth.

however apparently a decrease in chl *a* content per unit length of alga in Zn-treated cultures (Table 4.5). In addition no changes in phyco: chl *a* ratio were observed as a result of Zn-treatment. In Cd-treated alga, however, visual changes in the colour of the alga were obvious in earlier stages of growth. The cultures became an intense blue colour; these later reverted to green. This sequence took place immediately at lower Cd levels (0.005 mg l^{-1}) but at higher levels (0.25 mg l^{-1}) there was an initial period when they remained green (i.e. green → blue → green). Pigment extracts (Fig. 4.34; Table A4.32) confirmed that these changes were the result of marked differences in phyco:chl *a* ratio.

4.5 Zn requirement for growth

An experiment was designed to determine the minimum and maximum concentrations of Zn for the optimum growth of wild-type and two Zn-tolerant strains of *Anacystis* (Fig. 4.35). The lowest possible level of Zn obtained in ACM medium was 0.04 mg l^{-1}, derived as contaminants (section 2.2). This experiment failed to demonstrate any stimulation of growth either at that minimum concentration tested, 0.04 mg l^{-1} Zn, or at maximum concentrations up to 1.0 mg l^{-1} Zn. Increasing toxicity however developed for wild-type at increasing concentrations of Zn above 0.5 mg l^{-1}.

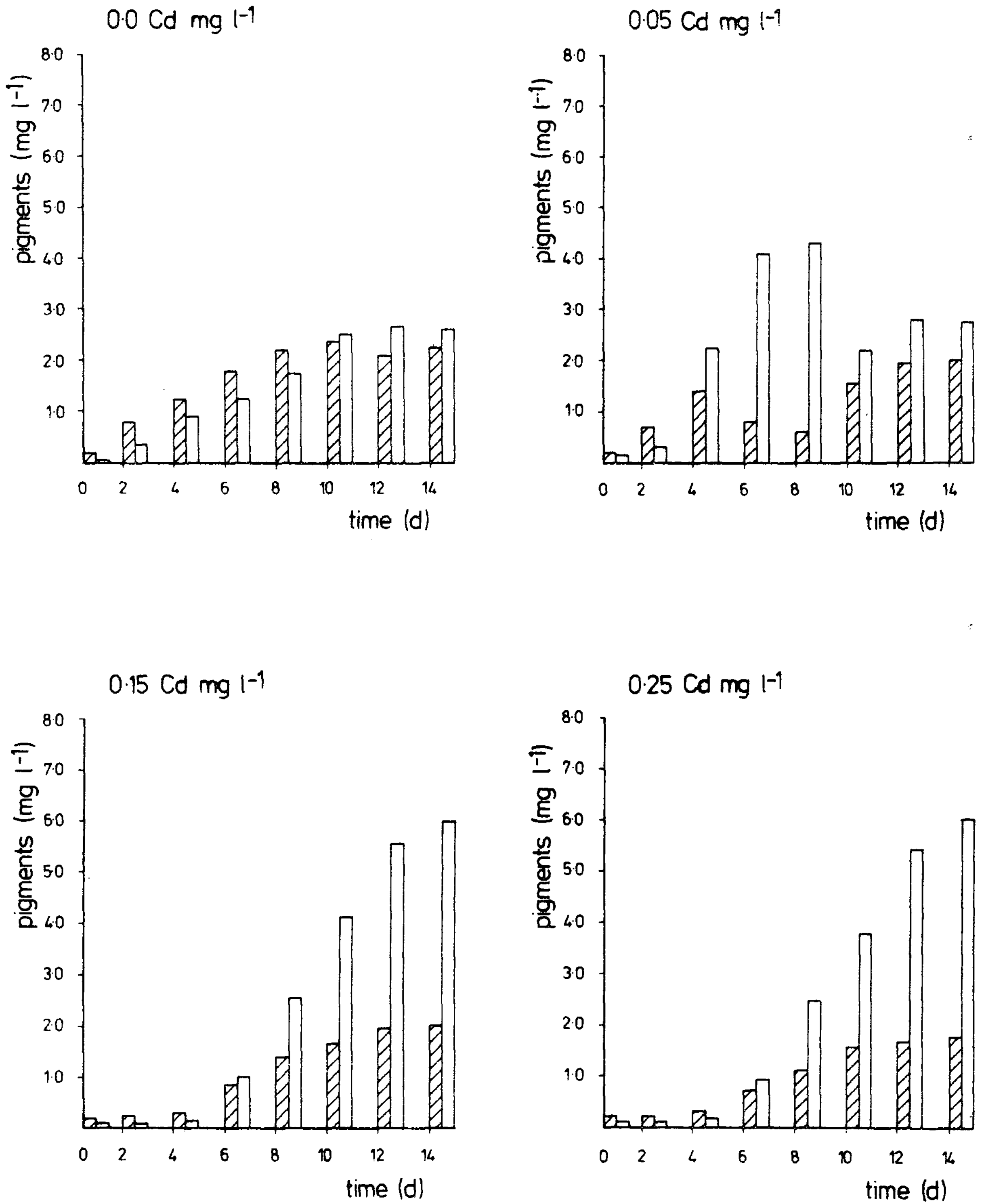


Fig. 4.34 Pigment changes during growth of wild-type *Anacystis* at different Cd concentrations in batch culture.

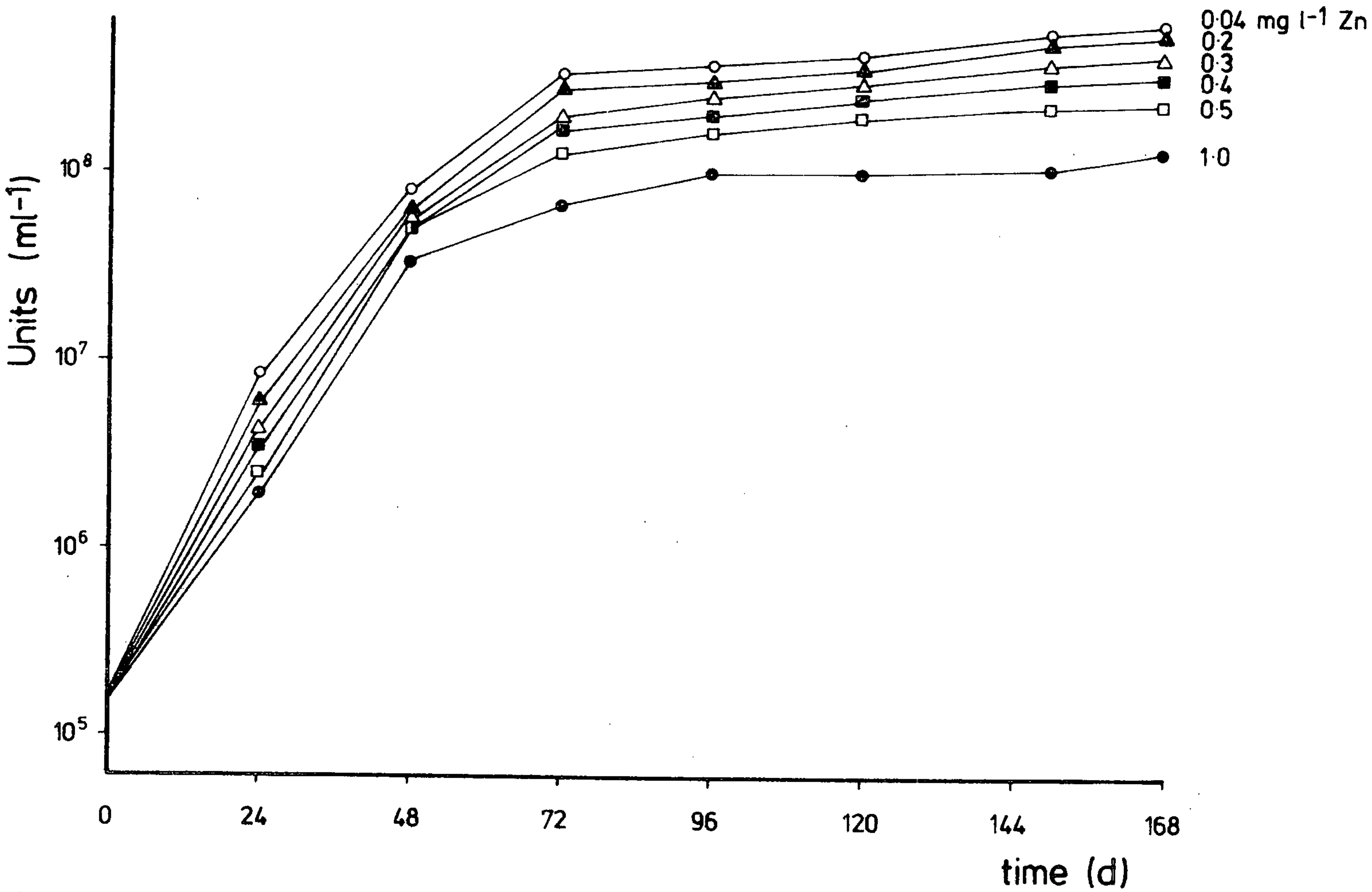


Fig. 4.35 Influence of minimum and maximum concentrations of Zn for the optimum growth of wild-type *Anacystis*.

CHAPTER 5

ENVIRONMENTAL FACTORS AFFECTING TOXICITY OF METALS TO *ANACYSTIS* STRAINS

5.1 Introduction

It seemed probable from a knowledge of the literature (see 1.3) that the toxicity of metals in the field and laboratory may be reduced by the concentration of other substances present. In order to establish clearly what influence, if any, other factors had upon the toxicity of Zn, Cu and Cd in the laboratory, a series of experiments were performed upon *Anacystis*. In order to investigate whether the effects differed between strains with varying genetic tolerances to metals, all the experiments were performed simultaneously on cultured material of wild-type and two Zn-tolerant strains (Zn-t5.0; Zn-t12.0), and on Cu-tolerant strain (Cu-t.0.5). Most of the results are summarized in Tables 5.1 and 5.2; data indicating no detectable influence on growth or toxicity are given in Appendix A5. Assays were carried out both in basal medium and in the sub-inhibitory level of the metal under test. The results are based on turbidimetric measurements, converted to units $ml^{-1} \times 10^{7}$ (section 2.514); other experimental methods are given in sections 2.2, 2.3

5.2 Influence of complementary cations and anions

In order to test the effects of other element addition to basal medium on metal toxicity, it is necessary to add salts, which lead to the addition of further ion. As far as possible Na^{+}, K^{+}, Cl^{-} and SO_4^{-} were used as complementary ions. Preliminary tests were therefore carried out on the influence of NaCl, KCl and Na_2SO_4 on metal toxicity.

Table 5.1 Influence of environmental factors on toxicity of Cu, Zn and Cd to various strains of *Anacystis nidulans*. Results based on turbidimetric measurements and summarized here as follows: +++ marked increase in growth; ++ moderate increase; + slight increase; 0 negligible effect; - slight decrease in growth; -- moderate decrease; --- marked decrease Assays carried out both in basal medium and in sub-inhibitory level of metal under test; influence of a factor on metal toxicity is based on a comparison of the two *e.g.* if the response is +++ in the presence of test metal, but only + in basal medium, then the environmental factor is clearly decreasing toxicity: see Table 5.2.

variable	range tested (mg l^{-1})	response of wild-type in absence of metal	Cu toxicity			Zn toxicity					Cd toxicity
			wild-type	Cu-t0.5		wild-type	Zn-t5.0		Zn-t12.0		wild-type
			+Cu	-Cu	+Cu	+Zn	-Zn	+Zn	-Zn	+Zn	+Cd
Na	2.5 - 300	+	0	+	0	++	0	0	0	0	0
Mg	0.25 - 200[a]	0	0	0	0	+[b]	0	++	+	++	+[b]
Ca	2.5 - 200	+	+	+	+	+++	+	+++	0	++	+++
Mn	0.05 - 20	0	0	0	0	++	+	++[c]	-	+	-
Fe	0.05 - 20	-	+	-	+	++	-	+	-	+	+[d]
Co	0 - 0.24	-	--	-	--	--	-	-	--	---	--
Ni	0 - 0.24	-	---	--	---	--	-	---	-	---	--
Zn	0 - 2.0	--	---	--	---						+++
Pb	0 - 40	-	---	--	---	--	--	---	--	---	---
NO_3-N	10 - 400	++	0	0	+	+	+	0	+	0	++
PO_4-P	0.88 - 112	++	+	+	+	+++	+	+++	+	+++	+++
SO_4-S	2.5 - 320	0	0	0	0	0	0	0	0	0	-
pH(+)	6.0 - 8.0	++	0	++	0	+++	+	--	+	--	+++

a in all cases, toxic above 160 mg l^{-1}

b up to 2.5 mg l^{-1}

c between 1 - 5 mg l^{-1}

d up to 10 mg l^{-1}

Table 5.2 Key environmental factors changing toxicity of metals tested, based on entries in Table 5.1 differing by at least two scores.

Reduced toxicity

Cu on wild-type					Fe		pH (-)
Cu on Cu-t0.5					Fe		pH (-)
Zn on wild-type		Ca		Mn	Fe	P	
Zn on Zn-t5.0	Mg	Ca		Mn	Fe	P	pH (-)
Zn on Zn-t12.0	Mg	Ca		Mn	Fe	P	pH (-)
Cd on wild-type		Ca	Zn		Fe	P	

Increased toxicity

Zn on Zn-t5.0		Ni					
Zn on Zn-t12.0		Ni					
Cd on wild-type				Pb			

Na (2.5 - 300 mg l^{-1}) led to no detectable change in the toxicity of Cu or Cd to wild-type (Table 5.1), of Zn to Zn-tolerant or of Cu to Cu-tolerant strain. Na at higher concentrations did however decrease the toxicity of Zn to wild-type strain (Table 5.3). This influence can not be due to increasing Cl levels in the medium, since addition of similar levels of Cl as KCl proved no response (Table 5.4).

Variation in the levels of K (5 - 500 mg l^{-1}) as KCl had no detectable effect upon any of the strains tested; examples are given (Tables A5.2, A5.3). SO_4-S (2.5 - 320 mg l^{-1}) had no detectable effect on growth of any strain tested in metal-free medium. SO_4-S had also no detectable effect on the toxicity of Zn to either wild-type or the two Zn-tolerant strains, of Cu to wild-type or Cu-t0.5 (Table 5.1). On the other hand there was a slight increase in the toxicity of Cd to wild-type (Table 5.5) when SO_4-S concentrations were increased from 2.5 - 320 mg l^{-1}.

5.3 Influence of major cations

Magnesium

This inhibited growth of the wild-type and Cu-t0.5 *Anacystis* slightly in basal medium at concentrations above 160 mg l^{-1} Mg, although the two Zn-tolerant strains were not affected (Table 5.1). The influence of Mg (0.25 - 200 mg l^{-1}) on the toxicity of Zn to wild-type and the two Zn-tolerant strains is shown in Tables 5.6, 5.7, 5.8. There was a detectable increase in tolerance of the wild-type up to 2.5 mg l^{-1} Mg (Table 5.9), but little change took place at higher concentrations. In the two Zn-tolerant strains, the toxicity was slightly lower at higher concentrations. Mg had no detectable effect on Cu toxicity to either wild-type (Table 5.10) or Cu-t0.5

Table 5.3 Influence of Na on Zn toxicity to wild-type *Anacystis*; the results based on units $ml^{-1} \times 10^{7}$; age of the alga 5 days; d = died.

Zn ($mg\ l^{-1}$)	Na ($mg\ l^{-1}$)									
	2.5	5	10	20	30	40	50	100	200	300
0.04	7.2	7.2	7.3	7.5	7.5	8.2	8.5	8.6	8.8	8.8
0.25	7.0	7.0	7.2	7.4	7.4	8.2	8.4	8.6	8.8	8.9
0.50	6.4	6.5	7.0	7.5	7.4	8.1	8.0	8.4	8.8	8.8
0.75	5.2	5.5	5.8	6.6	6.8	7.7	7.8	8.2	8.6	8.7
1.0	3.4	4.1	5.4	5.7	6.0	7.5	7.8	8.2	8.4	8.6
1.25	2.5	2.4	3.2	3.6	5.2	7.2	7.5	7.8	7.9	8.1
1.5	d	d	d	d	0.77	0.45	4.2	6.9	7.1	7.4
2.0	d	d	d	d	d	d	2.5	3.2	3.6	4.6

Table 5.4 Influence of Cl as KCl on Zn toxicity to wild-type *Anacystis*; the results based on units $ml^{-1} \times 10^{7}$; age of the alga 5 days; d = died.

Zn ($mg\ l^{-1}$)	Cl ($mg\ l^{-1}$)				
	10	20	40	100	200
0.04	9.2	9.6	9.4	9.6	9.4
0.10	9.6	9.5	9.5	9.8	9.4
0.25	9.4	9.6	9.5	9.7	9.4
0.50	8.5	8.5	8.4	8.7	8.4
0.75	7.8	7.6	7.8	7.5	7.7
1.0	7.7	7.4	7.4	7.4	7.6
1.25	7.5	7.2	7.2	7.2	7.5
1.50	0.05	0.02	0.03	0.04	0.04
2.0	d	d	d	d	d

Table 5.5 Influence of SO_4-S on Cd toxicity to wild-type *Anacystis*; the results based on units $ml^{-1} \times 10^7$; age of the alga 5 days; d = died.

Cd (mg l^{-1})	SO_4-S (mg l^{-1})							
	2.5	5.0	10	20	40	80	160	320
0.04	8.6	8.6	8.2	8.6	8.4	8.8	8.4	8.2
0.15	7.0	6.8	7.0	7.0	6.5	5.6	4.4	4.2
0.30	4.8	4.8	4.6	4.4	4.5	3.8	3.0	3.2
0.45	4.2	4.4	4.2	3.8	3.4	3.2	2.8	2.6
0.60	2.5	2.8	3.4	2.0	1.2	1.4	1.3	1.2
0.90	d	d	d	d	d	d	d	d
1.20	d	d	d	d	d	d	d	d

Table 5.6 Influence of Mg on Zn toxicity to wild-type *Anacystis*; (high Mg levels); the results based on units $ml^{-1} \times 10^7$; age of the alga 5 days; d = died.

Zn (mg l^{-1})	Mg (mg l^{-1})							
	2.5	5	10	20	40	80	100	200
0.04	9.1	9.3	9.7	9.5	9.5	9.3	8.4	0.05
0.25	8.9	9.4	9.3	9.5	9.8	9.2	7.8	0.05
0.50	8.8	9.2	9.3	9.4	9.6	9.2	7.8	0.05
0.75	8.7	9.2	8.8	9.2	9.4	9.1	7.6	5.0
1.0	8.7	8.8	8.7	8.2	9.2	8.8	7.4	4.6
1.25	7.6	8.2	8.12	8.1	8.6	8.6	7.0	4.6
1.50	d	0.26	d	0.13	d	d	0.07	d
2.0	d	d	d	d	d	d	d	d

Table 5.7 Influence of Mg on Zn toxicity to Zn-t5.0 *Anacystis*; the results based on units $ml^{-1} \times 10^7$; age of the alga 5 days; d = died.

Zn (mg l^{-1})	Mg (mg l^{-1})								
	1.0	2.5	5	10	20	40	80	160	200
0.04	8.2	8.0	8.3	8.0	8.2	8.3	8.4	8.2	8.2
2	7.6	7.6	7.8	7.8	7.8	7.8	8.0	7.8	7.6
4	5.4	5.2	5.4	5.4	5.5	5.4	5.6	5.5	5.4
5	1.4	1.8	2.0	1.8	2.2	2.8	3.4	4.2	4.4
6	d	d	d	d	d	d	0.05	3.2	3.2
8	d	d	d	d	d	d	d	0.05	0.05
10	d	d	d	d	d	d	d	d	d

Table 5.8 Influence of Mg on Zn toxicity to Zn-t12.0 *Anacystis*; the results based on units $ml^{-1} \times 10^7$; age of the alga 5 days; d = died.

Zn (mg l^{-1})	Mg (mg l^{-1})							
	2.5	5	10	20	40	80	160	200
0.04	7.8	8.3	8.0	8.2	8.3	8.4	8.2	8.2
4.0	7.6	7.8	7.8	7.8	8.0	8.0	7.8	7.6
8.0	5.2	6.4	6.5	6.6	6.4	6.5	6.6	6.8
10.0	4.8	5.4	5.8	5.4	5.8	5.4	5.8	6.0
12.0	2.2	2.3	2.6	3.2	3.6	3.8	4.5	4.6
16.0	d	d	d	d	d	0.5	3.2	3.2
20.0	d	d	d	d	d	d	0.5	0.5
24.0	d	d	d	d	d	d	d	d

Table 5.9 Influence of Mg on Zn toxicity to wild-type *Anacystis*; (low Mg levels); the results based on units $ml^{-1} \times 10^{7}$; age of the alga 5 days; d = died.

Zn (mg l^{-1})	Mg (mg l^{-1})							
	0.25	0.5	0.75	1.0	1.25	1.5	1.75	2.0
0.04	8.5	8.8	9.4	9.5	9.2	9.4	9.5	9.4
0.25	8.5	8.6	9.0	9.2	8.8	9.0	9.3	9.4
0.5	7.5	8.8	8.8	8.5	8.6	8.6	8.6	8.8
0.75	7.4	7.6	7.5	7.8	8.5	8.6	8.4	8.5
1.0	6.4	7.2	7.1	7.6	7.2	7.8	7.8	8.7
1.25	0.4	0.6	0.8	1.6	3.4	4.5	6.5	7.8
1.5	0.05	0.05	0.05	0.05	0.06	0.05	0.04	0.05
2.0	d	d	d	d	d	d	d	d

Table 5.10 Influence of Mg on Cu toxicity to wild-type *Anacystis*; the results based on units $ml^{-1} \times 10^{7}$; age of the alga 5 days; d = died.

Cu (mg l^{-1})	Mg (mg l^{-1})								
	0.25	2.5	5	10	20	40	80	160	200
0	7.5	8.0	8.0	10	10	6.5	7.0	4.0	2
0.02	6.3	7.2	6.8	7.0	6.3	5.8	4.6	1.6	1.4
0.04	5.0	6.0	6.0	5.4	5.0	3.6	2.1	0.65	d
0.08	2.3	2.4	2.6	2.4	2.3	1.5	0.8	0.03	d
0.10	0.62	0.72	0.71	0.65	0.71	0.32	0.06	d	d
0.16	0.05	0.06	0.04	0.05	0.06	0.04	d	d	d
0.20	0.02	d	d	d	d	d	d	d	d
0.24	d	d	d	d	d	d	d	d	d
0.30	d	d	d	d	d	d	d	d	d

strain (Table 5.11). The influence of Mg on Cd toxicity to wild-type was similar to that of Zn, i.e. a slight increase in tolerance up to 2.5 mg l^{-1} Mg (Table 5.12).

Calcium

An increase in Ca from 2.5 to 200 mg l^{-1} brought about a slight increase in the growth of *Anacystis* strains in basal medium. The influence of Ca on Zn toxicity to wild-type and the two Zn-tolerant strains is shown in Figs 5.1, 5.2, 5.3; Tables A5.4, A5.5, A5.6. A marked ameliorating effect was evident in all strains up to the highest level (200 mg l^{-1} Ca) investigated, even allowing for the fact that Ca has some effect in basal medium. The response of wild-type and Zn-tolerant strains was similar. Increasing Ca caused a slight increase in tolerance of Cu to wild-type and Cu-t0.5. It was more pronounced to wild-type (Table 5.13) than Cu-t0.5 (Table 5.14). The influence of increasing Ca on the tolerance of Cd to wild-type is shown in Fig. 5.4; Table A5.7. A marked increase in tolerance of alga was found as the Ca was increased from 2.5 to 200 mg l^{-1}.

Manganese

Mn (0.05 - 20 mg l^{-1}) had no detectable effect upon the growth of wild-type in basal medium, while a slight decrease in the growth of the two Zn-tolerant strains occurred. The influence of increasing Mn on Zn toxicity to the wild-type and Zn-tolerant strains differed. In the case of wild-type increasing Mn up to 20 mg l^{-1} reduced the toxicity of Zn (Table 5.15), while in the case of the Zn-tolerant strains, increase in tolerance was found as Mn was increased from 0.05 to 5.0 mg l^{-1} (Figs 5.5, 5.6; Tables A5.8, A5.9). The effect was more pronounced on Zn-t5.0 than the Zn-t12.0 strain. Mn had negligible effect on Cu toxicity to either wild-type or Cu-t0.5. On the other hand increased Mn caused a very slight decrease in Cd toxicity to wild-type (Table 5.16).

Table 5.11 Influence of Mg on Cu toxicity to Cu-t0.5 *Anacystis*; the results based on units $ml^{-1} \times 10^7$; age of the alga 5 days; d = died.

Cu ($mg\ l^{-1}$)	Mg ($mg\ l^{-1}$)							
	0.25	2.5	5	10	20	40	80	160
0	6.2	5.8	5.0	6.3	5.8	6.0	6.2	3.0
0.2	6.0	3.6	4.1	5.0	3.0	4.6	5.0	1.0
0.4	3.6	2.5	3.5	4.8	4.6	4.8	4.8	0.6
0.5	0.4	0.4	0.3	0.3	0.88	1.0	3.6	0.02
0.6	d	d	d	d	d	0.08	0.2	d
0.8	d	d	d	d	d	d	0.04	d
1.0	d	d	d	d	d	d	d	d
1.5	d	d	d	d	d	d	d	d

Table 5.12 Influence of Mg on Cd toxicity to wild-type *Anacystis*; the results based on units $ml^{-1} \times 10^7$; age of the alga 5 days; d = died.

Cd ($mg\ l^{-1}$)	Mg ($mg\ l^{-1}$)								
	0.25	2.5	5	10	20	40	80	100	200
0	9.8	9.6	9.2	9.6	9.0	0.4	7.5	4.0	0.05
0.15	7.8	7.6	7.8	7.4	6.4	6.6	5.0	3.2	1.8
0.30	5.0	4.8	4.9	4.6	4.8	4.8	4.1	0.03	d
0.45	4.5	4.4	4.5	4.0	4.2	4.0	3.2	0.04	d
0.60	3.8	3.6	3.2	2.8	3.0	3.2	2.3	0.05	d
0.9	d	d	d	d	d	d	d	d	d
1.2	d	d	d	d	d	d	d	d	d

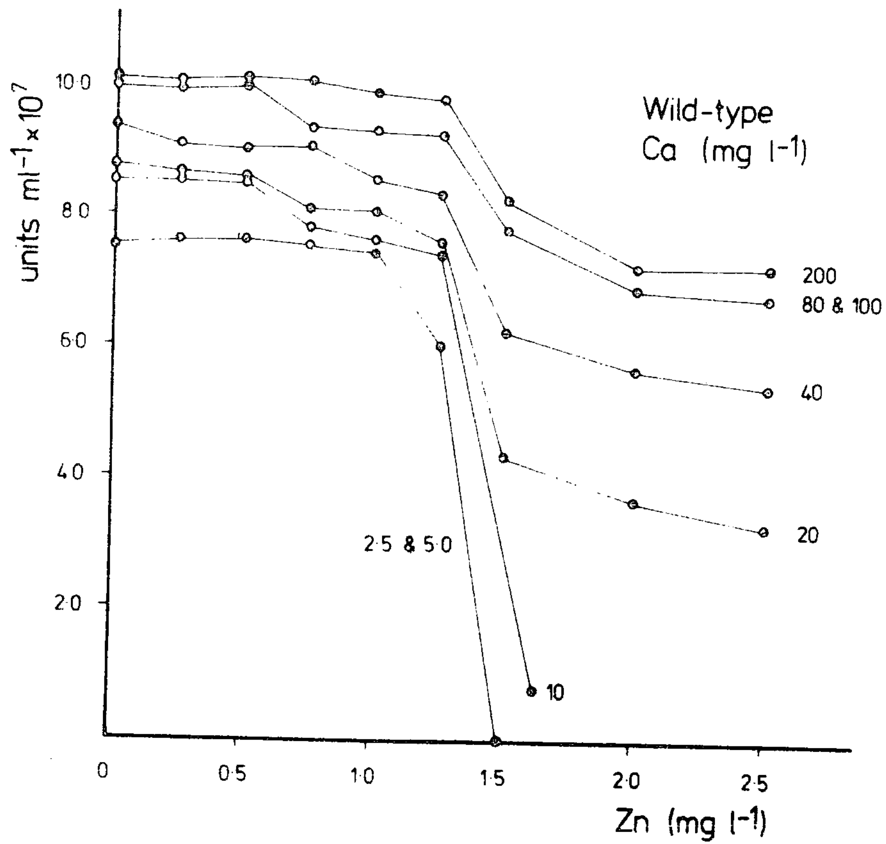


Fig. 5.1 Influence of Ca on Zn toxicity to wild-type *Anacystis*.

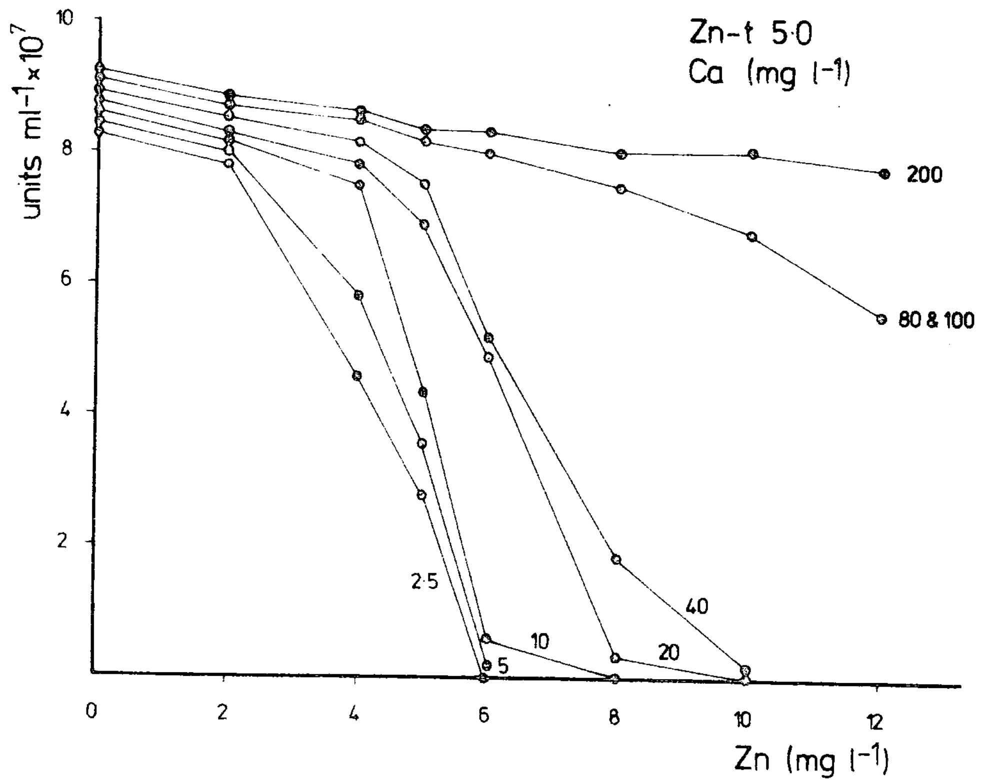


Fig. 5.2 Influence of Ca on Zn toxicity to Zn-t5.0 *Anacystis* strain.

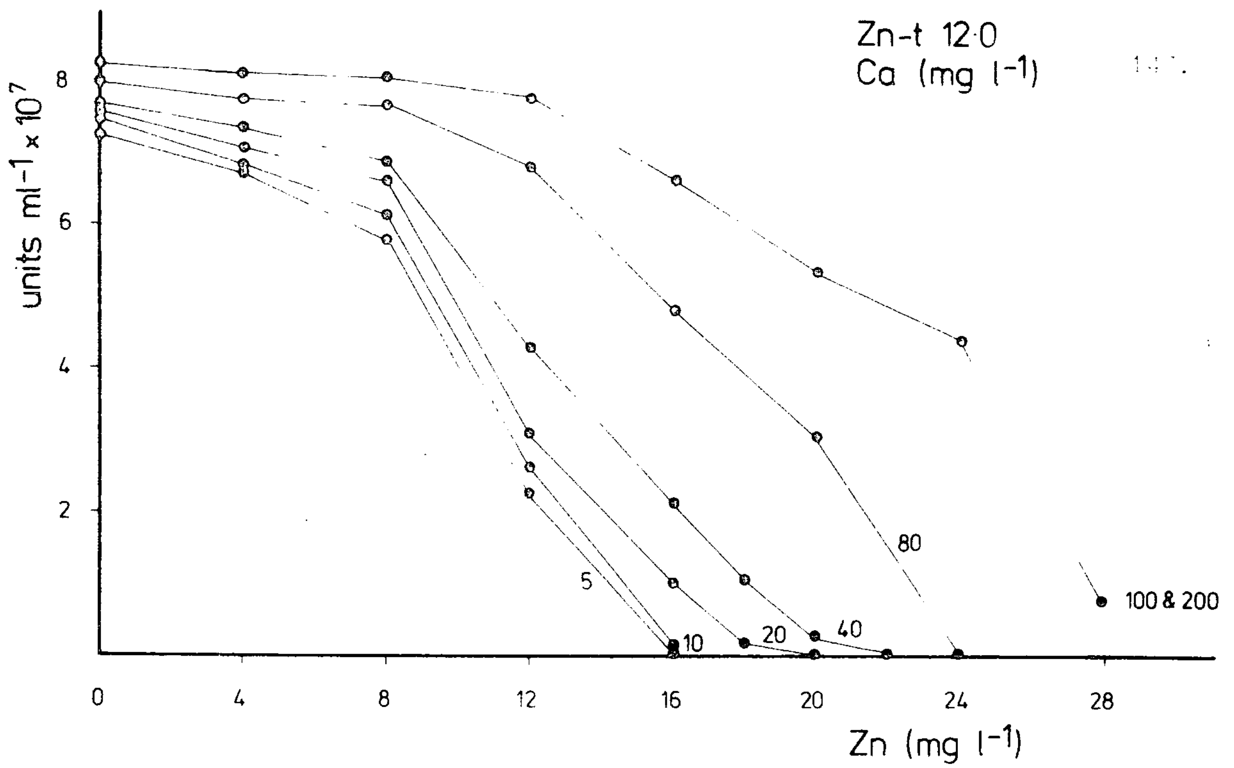


Fig. 5.3 Influence of Ca on Zn toxicity to Zn-t12.0 *Anacystis* strain.

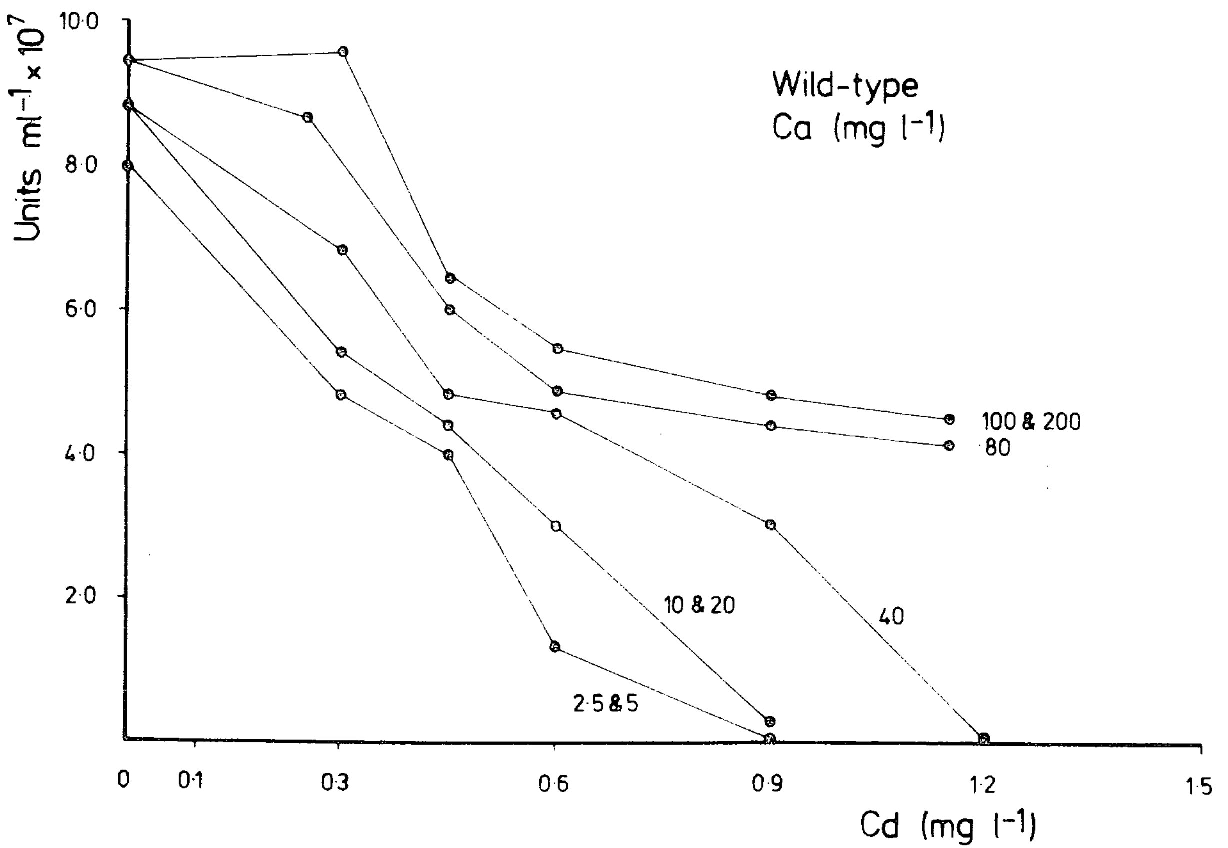


Fig. 5.4 Influence of Ca on Cd toxicity to wild-type *Anacystis*.

Table 5.13 Influence of Ca on Cu toxicity to wild-type *Anacystis*; the results based on units $ml^{-1} \times 10^{7}$; age of the alga 5 days; d = died.

Cu ($mg\ l^{-1}$)	Ca ($mg\ l^{-1}$)							
	2.5	5	10	20	40	80	160	200
0.0	7.2	7.4	8.1	9.0	9.3	10.2	10.4	10.6
0.02	2.4	6.3	7.0	7.2	8.0	10	8.8	8.8
0.04	1.3	5.0	5.8	6.0	7.3	8.0	8.0	8.1
0.08	0.6	2.3	2.3	4.2	6.8	8.0	7.5	7.4
0.10	0.42	0.71	0.72	1.3	6.2	6.8	7.1	7.0
0.16	d	0.06	0.06	0.86	2.0	6.0	7.0	6.8
0.20	d	d	d	0.06	1.0	6.1	5.8	6.1
0.24	d	d	d	d	0.45	3.4	4.6	5.2
0.30	d	d	d	d	d	0.03	0.02	0.4

Table 5.14 Influence of Ca on Cu toxicity to Cu-t0.5 *Anacystis*; the results based on units $ml^{-1} \times 10^{7}$; age of the alga 5 days; d = died.

Cu ($mg\ l^{-1}$)	Ca ($mg\ l^{-1}$)							
	2.5	5	10	20	40	80	160	200
0.0	4.0	4.0	5.2	5.6	6.0	6.3	8.8	9.4
0.2	2.2	2.3	3.4	3.8	3.6	4.4	6.5	8.6
0.4	0.8	1.0	2.6	3.2	4.2	4.6	5.2	6.3
0.5	0.07	0.85	2.1	3.4	3.6	3.0	6.0	6.2
0.6	d	d	0.03	0.38	1.8	0.8	0.45	0.50
0.8	d	d	d	0.02	0.66	d	d	d
1.0	d	d	d	d	0.52	d	d	d
1.5	d	d	d	d	0.45	d	d	d

Table 5.15 Influence of Mn on Zn toxicity to wild-type *Anacystis*; the results based on units $ml^{-1} \times 10^{7}$; age of the alga 5 days; d = died.

Zn ($mg\ l^{-1}$)	Mn ($mg\ l^{-1}$)									
	0.05	0.10	0.5	1.0	1.5	2.5	5	10	15	20
0.04	8.8	8.9	8.8	8.9	8.8	8.4	8.4	8.6	8.0	8.6
0.25	8.9	8.8	8.6	8.7	8.8	8.3	8.2	8.4	8.3	8.8
0.50	8.7	8.8	8.5	8.6	8.7	8.2	8.0	8.5	8.4	8.8
0.75	8.0	7.9	8.0	8.3	8.5	8.0	8.2	8.6	8.4	8.6
1.0	8.2	8.3	7.9	8.3	8.0	8.1	7.8	8.5	8.3	8.5
1.25	7.6	7.1	7.2	8.0	8.0	7.7	7.8	7.6	7.9	7.5
1.5	0.05	0.05	0.06	0.04	0.05	0.40	7.7	7.6	7.6	7.8
2.0	d	d	d	d	d	0.60	7.6	7.7	7.5	7.5

Table 5.16 Influence of Mn on Cd toxicity to wild-type *Anacystis*; the results based on units $ml^{-1} \times 10^{7}$; age of the alga 5 days; d = died.

Cd ($mg\ l^{-1}$)	Mn ($mg\ l^{-1}$)							
	0.05	0.10	0.50	1.0	2.5	5.0	10	20
0.0	9.8	9.0	8.8	8.8	8.6	8.4	8.6	8.2
0.15	8.4	8.0	7.6	7.5	6.6	6.2	5.6	5.1
0.30	5.6	5.4	5.4	5.5	5.2	4.8	4.5	3.5
0.45	4.8	4.6	4.5	4.4	4.2	3.8	3.5	3.2
0.60	1.2	1.8	1.5	1.6	1.2	1.3	1.4	1.5
0.90	d	d	d	d	d	d	d	d
1.20	d	d	d	d	d	d	d	d

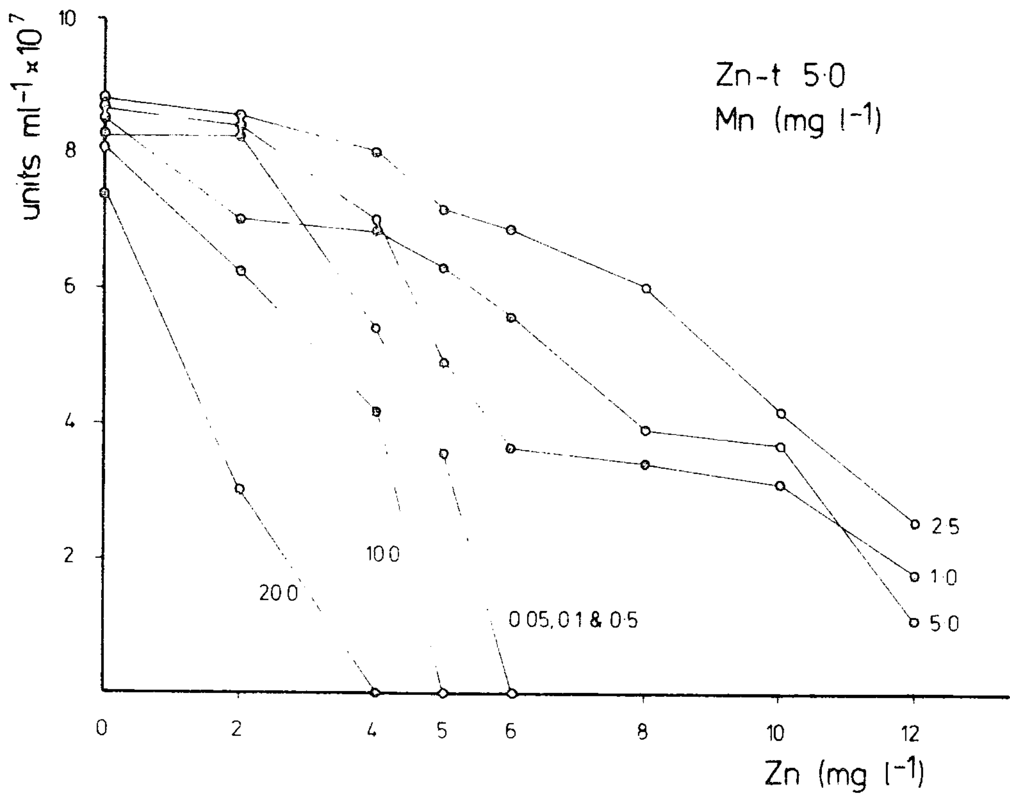


Fig. 5.5 Influence of Mn on Zn toxicity to Zn-t5.0 *Anacystis* strain.

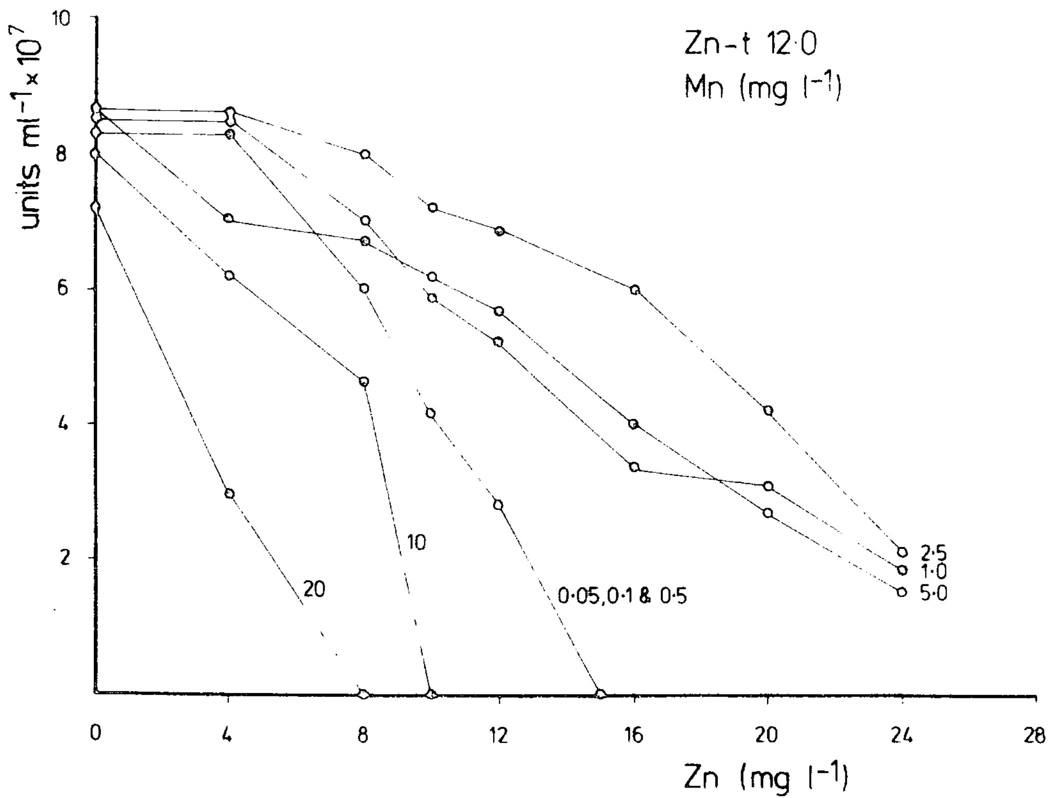


Fig. 5.6 Influence of Mn on Zn toxicity to Zn-t12.0 *Anacystis* strain.

Iron

This caused a slight decrease in the growth of *Anacystis* strains in basal medium with increasing concentrations from 0.05 to 20 mg l^{-1} (Table 5.1). The influence of Fe (0.05 - 20 mg l^{-1}) on Zn toxicity to wild-type and Zn-tolerant strains is summarized in Table 5.2. Slight decreases in the toxicity were evident, the effect being more pronounced with the wild-type (Table 5.17). Fe also brought about a slight decrease in Cu toxicity to both wild-type (Table A5.12) and Cu-t0.5 strains (Table A5.13), and decrease in Cd toxicity to wild-type (Table 5.18).

5.4 Influence of major anions

Phosphate-P

It was evident that increasing PO_4-P from 1.75 mg l^{-1} to 112 mg l^{-1} had a slight influence on the growth rate of all strains tested in basal medium. The influence of PO_4-P on Zn toxicity on wild-type and the two Zn-tolerant strains was very similar. Both showed a marked Zn tolerance at higher P concentrations (Figs 5.7, 5.8, 5.9; Tables A5.14, A5.15, A5.16). An investigation on the influence of PO_4-P on the toxicity of Zn to wild-type was carried out using both PO_4-P-starved inocula (Table 5.19a) and PO_4-P-rich inocula (Table 5.19b); a very slight difference in toxicity occurred. The influence of PO_4-P was tested also by introducing the PO_4-P later, i.e. 10 min after contact of the inoculum with Zn in the medium. The results (Table 5.20) show that PO_4-P introduced after algal inoculations had much less effect in reducing Zn toxicity, in comparison to phosphate introduced before inoculation (Table 5.19). The influence of PO_4-P on the toxicity of Cu to wild-type and Cu-t0.5 was moderate (Tables 5.21, 5.22) while it had a marked effect on

Table 5.17 Influence of Fe on Zn toxicity to wild-type *Anacystis*; the results based on units $ml^{-1} \times 10^{7}$; age of the alga 5 days; d = died.

Zn (mg l^{-1})	Fe (mg l^{-1})									
	0.05	0.10	0.50	1.0	1.5	2.5	5	10	15	20
0.04	5.5	7.6	7.8	7.8	7.8	7.8	6.9	6.5	5.2	4.8
0.25	5.8	7.6	8.0	7.8	7.8	7.9	6.8	6.3	5.4	5.2
0.50	5.6	7.5	7.6	7.5	7.6	7.6	6.8	6.5	5.3	5.1
0.75	5.4	7.2	7.5	7.4	7.5	7.7	6.9	6.0	5.0	4.6
1.0	5.2	6.4	7.3	7.3	7.4	7.5	6.8	5.8	4.8	4.3
1.25	d	0.3	0.40	4.2	6.5	6.8	4.01	5.6	4.6	4.2
1.5	d	d	d	d	0.05	0.06	0.05	5.2	3.8	4.1
2.0	d	d	d	d	d	d	d	4.6	3.5	3.8

Table 5.18 Influence of Fe on Cd toxicity to wild-type *Anacystis*; the results based on units $ml^{-1} \times 10^{7}$; age of the alga 5 days; d = died.

Cd (mg l^{-1})	Fe (mg l^{-1})							
	0.05	0.10	0.50	1.0	2.5	5.0	10	20
0.0	8.5	8.8	8.8	8.9	9.2	9.4	7.4	6.8
0.15	6.6	7.8	7.6	7.6	7.8	7.5	6.0	1.4
0.30	3.8	4.2	4.5	4.5	4.8	4.5	4.2	0.05
0.45	2.1	3.8	4.4	4.5	4.2	4.4	3.0	0.04
0.60	0.05	1.4	2.5	2.6	2.8	3.2	1.5	0.05
0.90	d	d	d	d	d	d	d	d
1.2	d	d	d	d	d	d	d	d

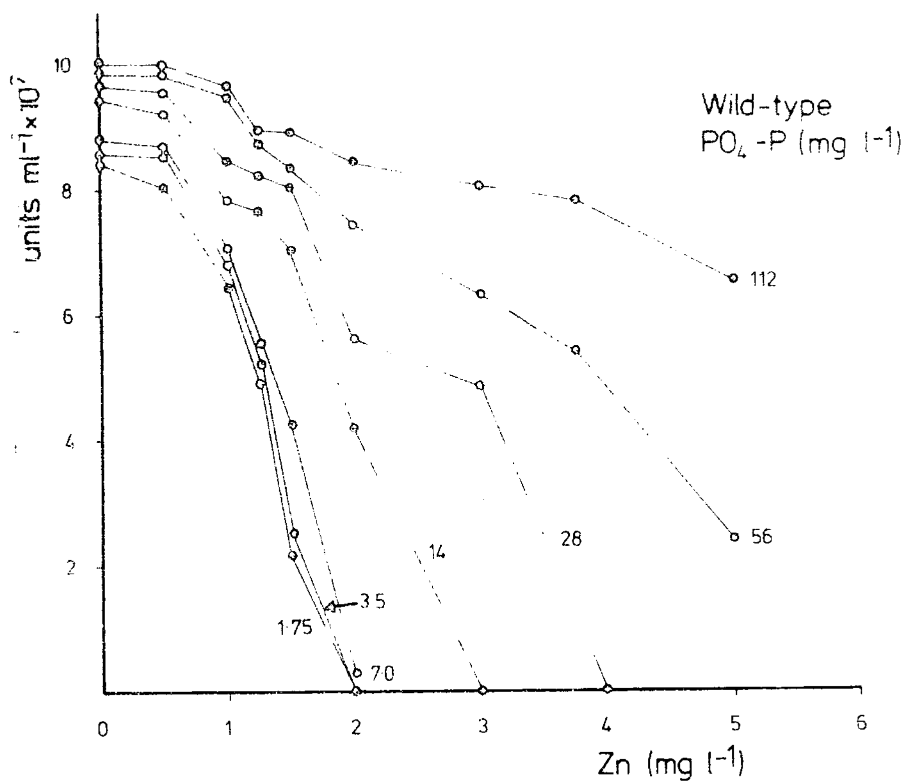


Fig. 5.7 Influence of PO_4-P on Zn toxicity to wild-type *Anacystis*.

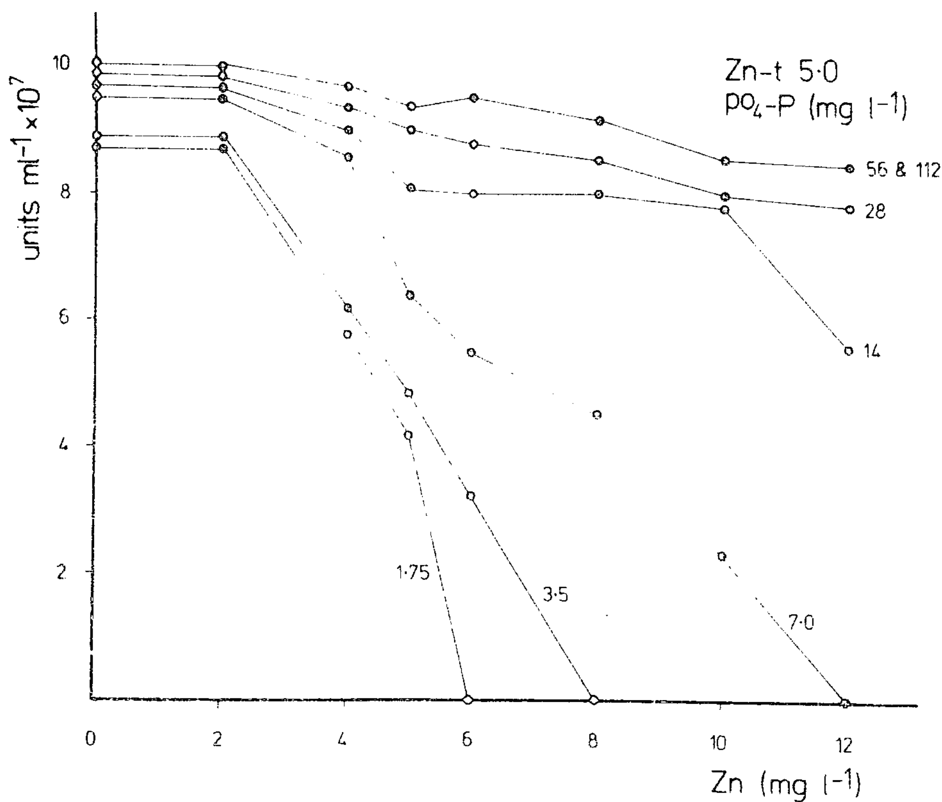


Fig. 5.8 Influence of PO_4-P on Zn toxicity to Zn-t5.0 *Anacystis* strain.

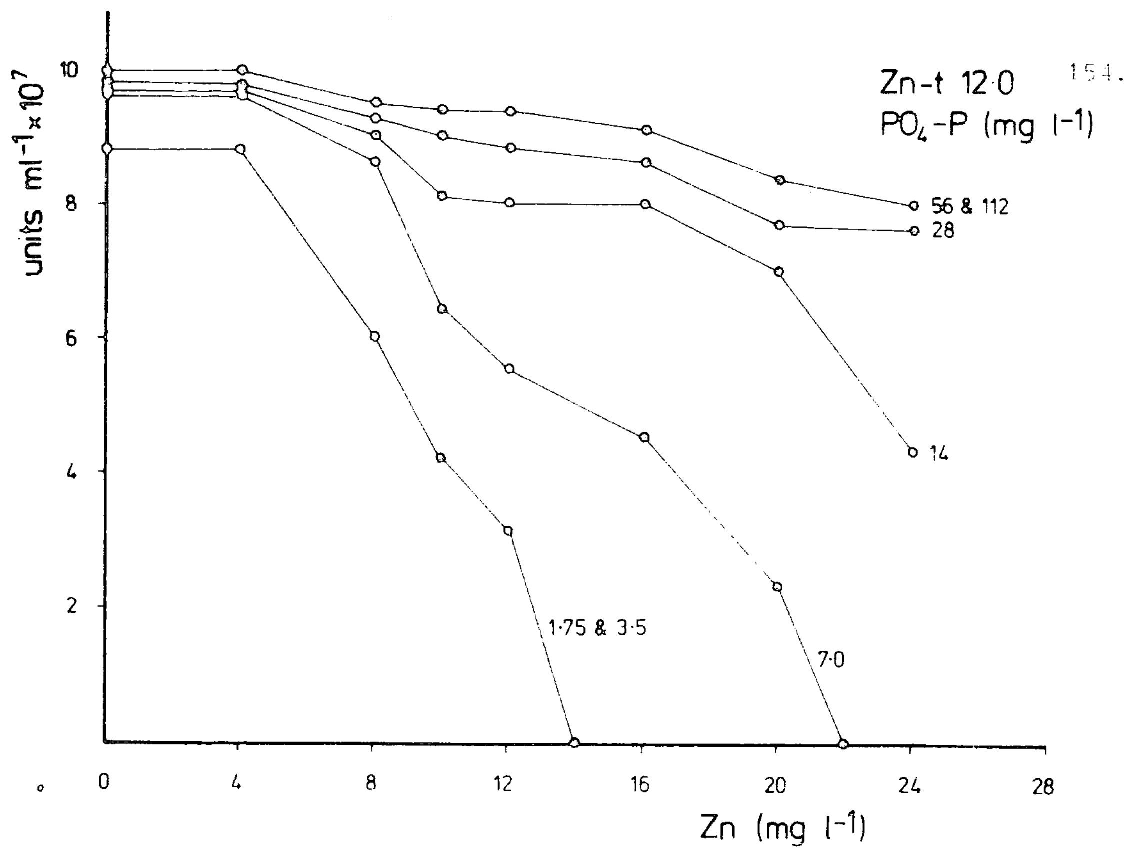


Fig. 5.9 Influence of PO_4-P on Zn toxicity to Zn-t12.0 *Anacystis* strain.

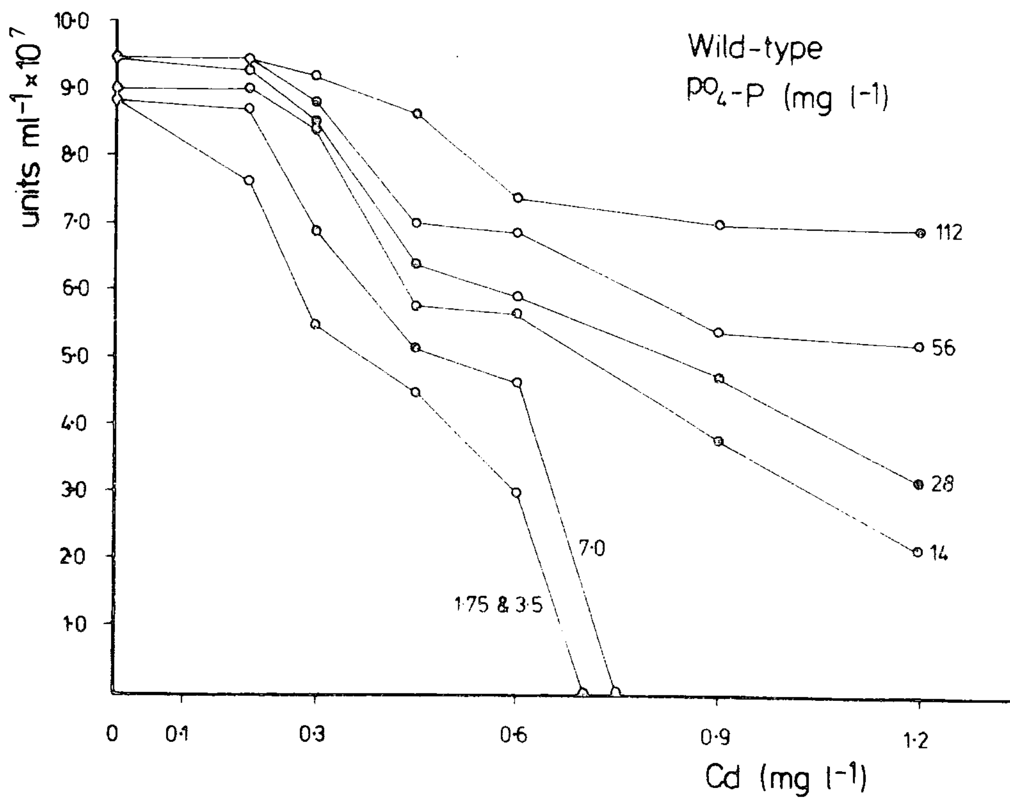


Fig. 5.10 Influence of PO_4-P on Cd toxicity to wild-type *Anacystis*.

Table 5.19(a) Influence of PO_4-P on Zn toxicity to wild-type *Anacystis*; starved inoculum; the results based on units $ml^{-1} \times 10^7$; age of the alga 5 days; d = died.

Zn ($mg\ l^{-1}$)	PO_4-P ($mg\ l^{-1}$)						
	1.75	3.5	7	14	28	56	112
0.04	8.4	8.6	8.8	9.6	10.0	9.8	9.6
0.5	8.2	8.3	8.4	9.2	9.4	9.3	9.4
1.0	6.4	6.8	7.0	8.0	8.2	8.5	8.6
1.25	4.4	4.8	5.5	7.6	7.6	8.1	8.0
1.5	0.06	0.32	2.1	4.6	8.0	7.8	7.8
2.0	d	d	d	1.4	5.6	7.2	7.8
3.0	d	d	d	d	4.6	6.8	7.0
4.0	d	d	d	d	1.4	4.8	6.8

Table 5.19(b) Influence of PO_4-P on Zn toxicity to wild-type *Anacystis*; rich inoculum; the results based on units $ml^{-1} \times 10^7$; age of the alga 5 days; d = died.

Zn ($mg\ l^{-1}$)	PO_4-P ($mg\ l^{-1}$)						
	1.75	3.5	7	14	28	56	112
0.04	8.4	8.6	8.8	8.4	9.2	11.0	10.0
0.5	8.0	8.5	8.6	9.2	8.5	9.8	9.6
1.0	6.4	6.8	7.0	7.8	8.4	8.8	9.2
1.25	4.8	5.0	5.5	7.6	8.2	8.6	9.0
1.5	0.50	2.4	4.2	7.0	8.0	8.3	8.8
2.0	d	d	d	4.2	5.6	7.4	8.4
3.0	d	d	d	d	4.8	6.2	8.0
3.5	d	d	d	d	d	5.4	8.0
4.0	d	d	d	d	d	d	7.5

Table 5.20 Influence of PO_4-P on Zn toxicity to wild-type *Anacystis*; (PO_4-P was introduced after algal inoculation and contact with Zn for 10 min.); the results based on units $ml^{-1} \times 10^7$; age of the alga 5 days; d = died.

Zn (mg l^{-1})	PO_4-P (mg l^{-1})						
	1.75	3.5	7.0	14	28	56	112
0.04	8.0	9.6	9.6	9.8	9.0	9.8	9.8
0.5	4.5	5.2	6.0	6.5	6.8	7.2	7.8
0.75	1.8	1.8	4.8	4.8	5.2	7.2	7.8
1.0	d	d	d	d	2.4	7.3	8.0
1.25	d	d	d	d	1.8	7.1	7.2
1.5	d	d	d	d	d	6.2	7.2
2.0	d	d	d	d	d	5.4	6.8
3.0	d	d	d	d	d	2.6	7.0

Table 5.21 Influence of PO_4-P on Cu toxicity to wild-type *Anacystis*; the results based on units $ml^{-1} \times 10^7$; age of the alga 5 days; d = died.

Cu (mg l^{-1})	PO_4-P (mg l^{-1})						
	1.75	3.5	7	14	28	56	112
0	7.2	7.4	8.1	9.0	9.3	10	10
0.02	6.4	6.5	7.2	7.8	8.1	8.6	9.0
0.04	4.8	5.0	6.0	6.8	8.0	8.2	8.8
0.08	2.2	2.4	2.5	5.0	7.3	8.2	8.0
0.10	0.72	0.85	0.70	1.6	7.0	7.8	7.5
0.16	0.06	0.05	0.06	0.7	6.8	7.1	7.6
0.20	d	d	d	0.06	6.2	7.0	5.6
0.24	d	d	d	0.05	2.0	2.6	3.1
0.30	d	d	d	d	d	0.02	0.02

Table 5.22 Influence of PO_4-P on Cu toxicity to Cu-t0.5 *Anacystis*; the results based on units $ml^{-1} \times 10^7$; age of the alga 5 days; d = died.

Cu (mg l^{-1})	PO_4-P (mg l^{-1})						
	1.75	3.5	7	14	28	56	112
0.0	5.0	5.2	6.2	6.3	6.0	8.0	3.6
0.2	3.0	3.4	4.5	6.8	6.2	6.4	2.5
0.4	1.5	2.0	3.6	4.2	5.4	6.0	0.7
0.5	0.52	1.2	5.5	3.1	3.2	1.5	0.02
0.6	d	d	d	d	0.64	0.38	d
0.8	d	d	d	d	0.24	0.05	d
1.0	d	d	d	d	d	d	d
1.5	d	d	d	d	d	d	d

reducing Cd toxicity to wild-type (Fig 5.10; Table A5.17).

Two quantitative experiments (Based on Chl *a* and units ml^{-1}) were carried out to demonstrate the effect of organic phosphate on Zn toxicity. Firstly it was shown that β-glycerophosphate (Tables 5.23 a, b) and α-D-glucose-1-phosphate (Tables 5.24 a, b) can act as a P source for *Anacystis*. At lower levels of P ($\leqslant$ 14 mg l^{-1}) these sources of phosphate led to slightly slower growth rates than inorganic phosphate, but at $\geqslant$ 28 mg l^{-1} P there was no detectable difference in growth rate. In contrast however the two organic phosphate had much less effect in reducing Zn toxicity (Tables 5.23 a, b;5.24 a, b).

Nitrate-N

A slight effect upon growth of differing NO_3-N levels was evident in metal-free controls The influence of increasing NO_3-N concentrations (from 10 to 400 mg l^{-1}) on the tolerance of Zn to the wild-type (Table A5.18) and two Zn-tolerant strains and Cu to wild-type and Cu-t0.5 was very similar. A slight decrease in the toxicity was found in all cases. NO_3-N caused a slight reduction in toxicity of Cd to wild-type (Table 5.25).

5.5 Influence of organic compounds

Experiments were carried out to investigate the influence of EDTA on Zn, Cu and Cd toxicity to all *Anacystis* strains and of HEPES on Zn toxicity to wild-type.

EDTA

EDTA (0.5 - 32 mg l^{-1} = 0.0017 - 0.11 mM) led to marked reduction in the toxicity in all cases (Figs 5.11, 5.12, 5.13, 5.14; Tables A5.19, A5. 20, A5.21 , A5.22). For instance in the case of Zn or Cd toxicity to wild-type *Anacystis*, the presence of 32 mg l^{-1} EDTA, led the alga to grow at 2.0 mg l^{-1} Zn or 0.45 mg l^{-1} Cd, similarly to the control.

Table 5.23a Influence of PO_4-P (as β-glycerophosphate) on Zn toxicity to wild-type *Anacystis*; units ml^{-1} was used as a growth criterion.

Zn ($mg\ l^{-1}$)	PO_4-P ($mg\ l^{-1}$)						
	1.75	3.5	7	14	28	56	112
0.04	6.2	7.6	7.6	8.2	9.4	9.8	9.5
0.5	d	d	a	a	4.2	4.8	7.8
1.0	d	d	d	d	d	d	d
1.25	d	d	d	d	d	d	d
1.5	d	d	d	d	d	d	d
2.0	d	d	d	d	d	d	d
3.0	d	d	d	d	d	d	d

Table 5.23b Influence of PO_4-P (as β-glycerophosphate) on Zn toxicity to wild-type *Anacystis*; Chl *a* was used as a growth criterion.

Zn ($mg\ l^{-1}$)	PO_4-P ($mg\ l^{-1}$)						
	1.75	3.5	7	14	28	56	112
0.04	0.96 ± 0.04	1.18 ± 0.08	1.18 ± 0.10	1.42 ± 0.06	1.63 ± 0.15	1.84 ± 0.12	1.88 ± 0.08
0.5	d	d	still alive	still alive	0.96 ± 0.8	1.18 ± 0.06	1.3 ± 0.11
1.0	d	d	d	d	d	d	a
1.25	d	d	d	d	d	d	d
1.5	d	d	d	d	d	d	d
2.0	d	d	d	d	d	d	d
3.0	d	d	d	d	d	d	d

Table 5.24a Influence of PO_4-P (α -D glucose-1-phosphate) on Zn toxicity to wild-type *Anacystis*; units ml^{-1} was used as a growth criterion.

Zn ($mg\ l^{-1}$)	PO_4-P ($mg\ l^{-1}$)						
	1.75	3.5	7	14	28	56	112
0.04	6.0	7.4	7.6	8.2	10.0	9.8	9.6
0.5	d	4.0	5.3	6.4	7.4	7.8	8.0
1.0	d	d	d	d	d	d	a
1.25	d	d	d	d	d	d	d
1.5	d	d	d	d	d	d	d
2.0	d	d	d	d	d	d	d
3.0	d	d	d	d	d	d	d

Table 5.24b Influence of PO_4-P (α -D glucose-1-phosphate) on Zn toxicity to wild-type *Anacystis*; Chl *a* was used as a growth criterion.

Zn (mg l^{-1})	PO_4-P (mg l^{-1})						
	1.75	3.5	7	14	28	56	112
0·04	0.88 ± 0.02	1.14 ± 0.12	1.18 ± 0.08	1.42 ± 0.13	1.63 ± 0.14	1.67 ± 0.10	1.96 ± 0.08
0.5	d	0.86 ± 0.08	0.96 ± 0.05	1.14 ± 0.06	1.18 ± 0.07	1.52 ± 0.12	1.67 ± 0.13
1.0	d	d	d	d	d	d	still alive
1.25	d	d	d	d	d	d	still alive
1.5	d	d	d	d	d	d	still alive
2.0	d	d	d	d	d	d	still alive
3.0	d	d	d	d	d	d	still alive

Table 2.25 Influence of NO_3-N on Cd toxicity to wild-type *Anacystis*; the results based on units $ml^{-1} \times 10^7$; age of the alga 5 days; d = died.

Cd (mg l^{-1})	NO_3-N (mg l^{-1})							
	5	10	20	40	50	100	200	400
0	3.8	4.0	4.2	7.8	8.6	8.8	8.8	8.8
0.15	2.4	2.4	2.8	6.5	7.4	7.8	7.8	7.5
0.30	0.05	2.2	2.4	4.2	6.1	5.4	5.4	5.9
0.45	d	d	d	3.4	4.2	3.8	3.6	4.2
0.6	d	d	d	d	1.6	1.4	1.5	1.5
0.9	d	d	d	d	d	d	d	d

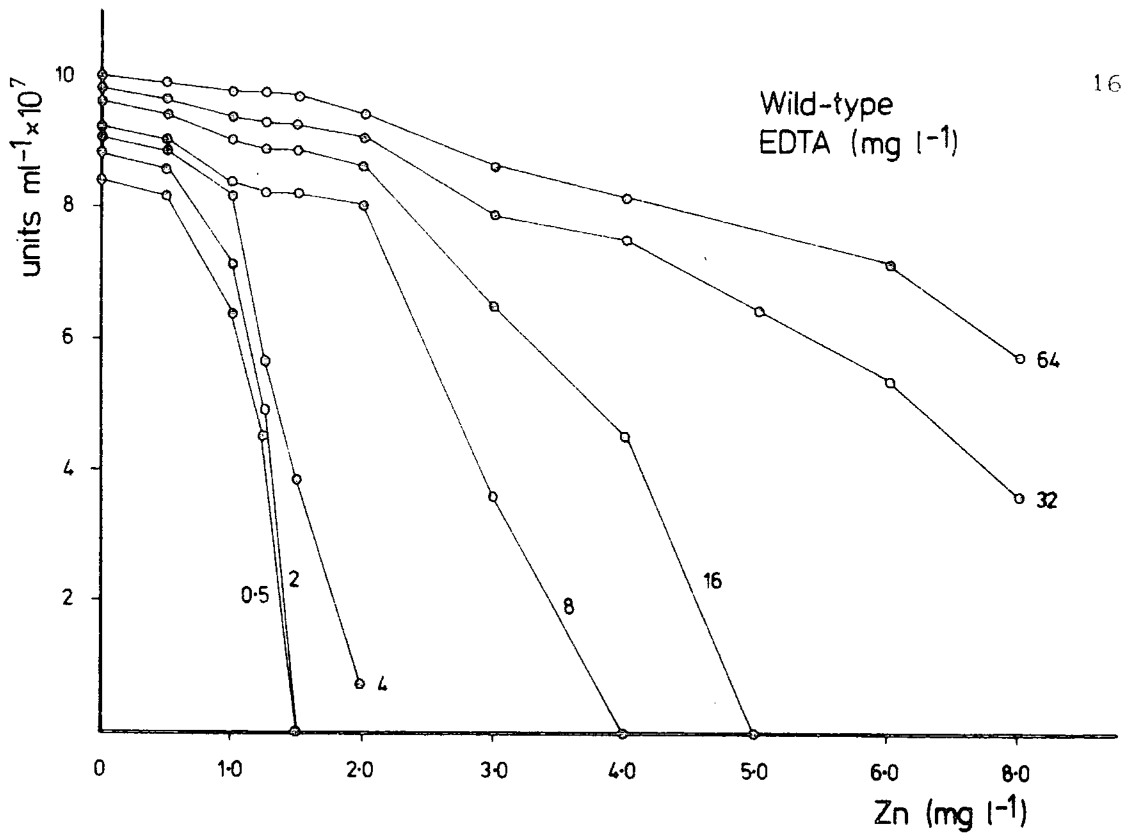


Fig. 5.11 Influence of EDTA on Zn toxicity to wild-type *Anacystis*.

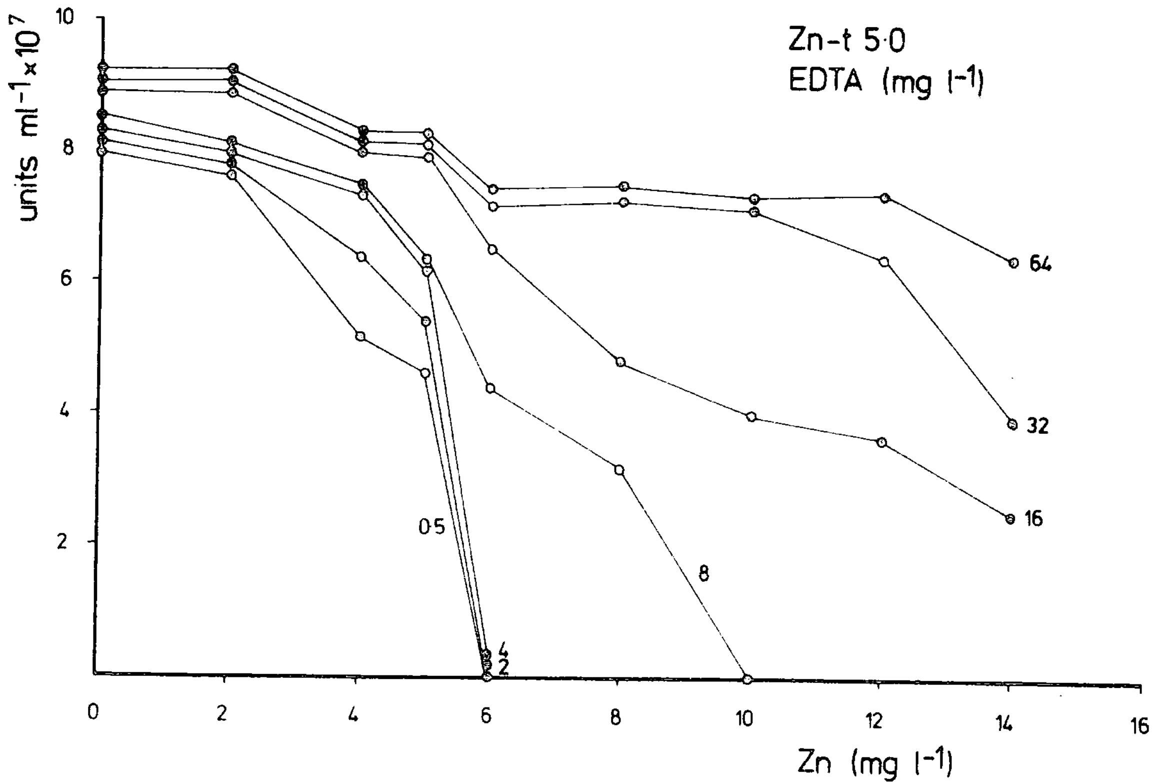


Fig. 5.12 Influence of EDTA on Zn toxicity to Zn-t5.0 *Anacystis* strain.

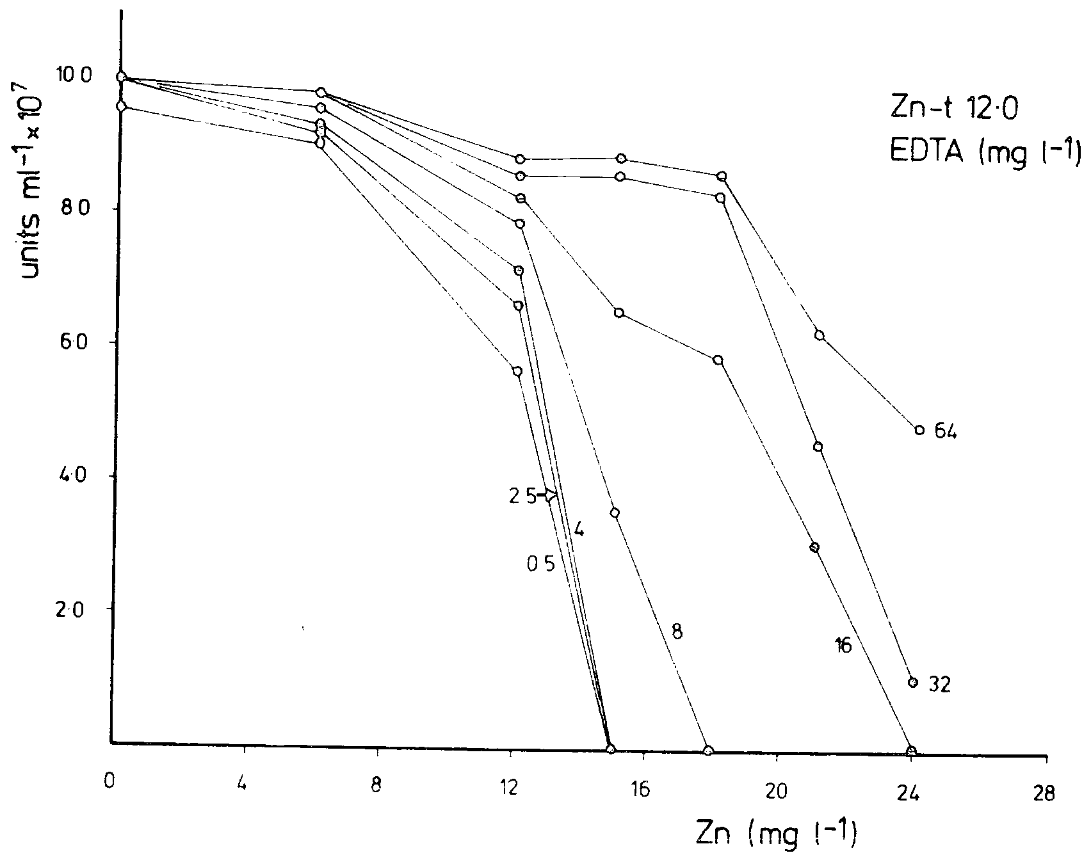


Fig. 5.13 Influence of EDTA on Zn toxicity to Zn-t12.0 *Anacystis* strain.

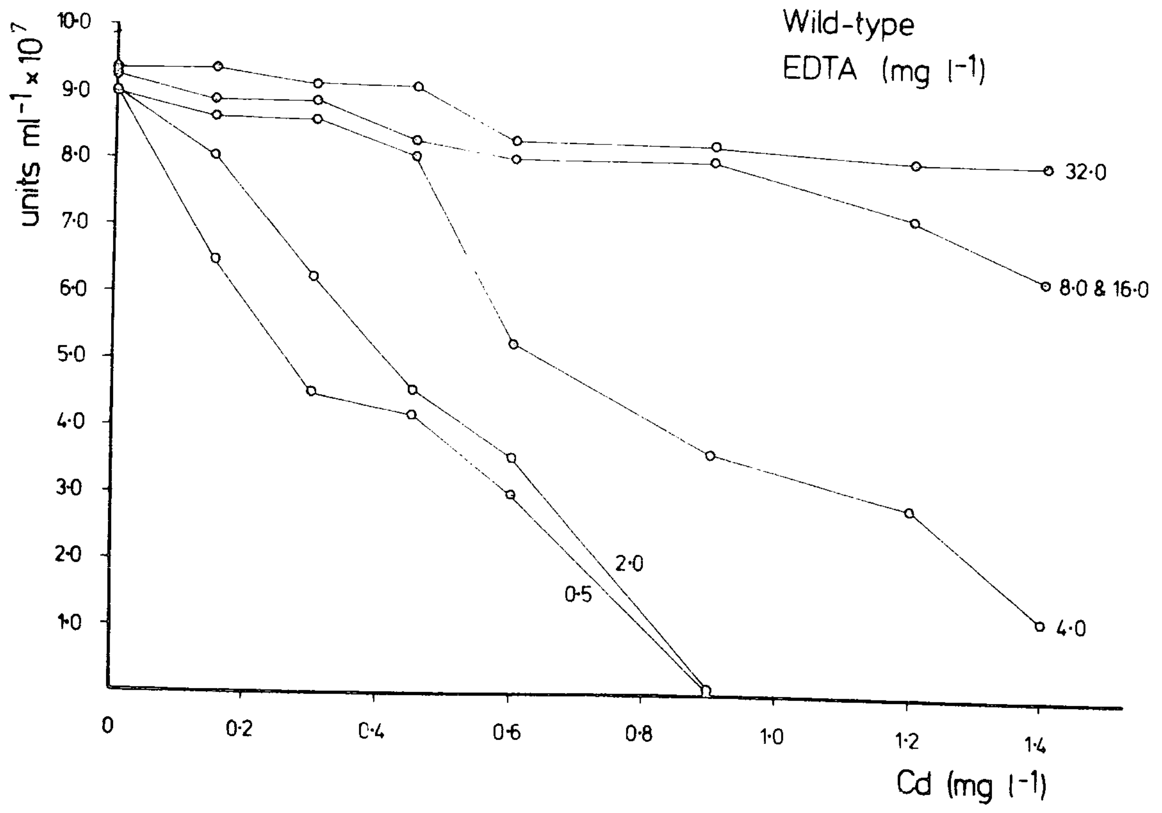


Fig. 5.14 Influence of EDTA on Cd toxicity to wild-type *Anacystis*.

HEPES

HEPES (0 - 1200 mg l^{-1} = 0 - 5.04 mM) led to a slight decrease in the toxicity of Zn to wild-type *Anacystis* (Table 5.26).

In the absence of HEPES there were marked rises in the pH of the medium and at 300 mg l^{-1} HEPES, rises of up to 0.8 units. As a rise in pH leads to a decrease in Zn toxicity to wild-type (section 5.7), increased buffering would be expected to lead to increased toxicity. The slight changes in toxicity observed with increasing HEPES can therefore not be explained by the poor buffering at lower pH values and it is concluded that they are a direct effect of the HEPES molecule, presumably due to chelation (section 8.31).

5.6 Influence of other heavy metals

Increasing levels of other heavy metals such as Ni, Cu, Hg and Pb all led to increased toxicity of Zn (Tables 5.27, 5.28, 5.29, 5.30) and of Cd to wild-type (Tables 5.31, 5.32, 5.33, 5.34). The influence of Zn on the toxicity of Cd was tested using either Chl *a* or population density as criteria of growth. A marked increase in tolerance of Cd by the wild-type with increasing Zn from 0.04 to 1.0 mg l^{-1} (Fig. 5.15; Tables A5.23, A5.24) and by the Zn-t5.0 strain (Fig. 5.16; Tables A5.25, A5.26). In contrast Cd brought about no detectable change in the toxicity of Zn to either strain.

5.7 Influence of pH

In basal medium growth of all three strains was less at pH 6.0 and 6.5 than 7.0, 7.5 and 8.0; this effect was more pronounced for the wild-type. The influence of pH on the toxicity of Zn to wild-type (Fig. 5.17; Table A5.27) shows that a marked decrease in toxicity occurred when the pH was raised from 6.5 to 8.0. In contrast a similar

Table 2.26 Influence of HEPES on the growth of wild-type *Anacystis*; at two levels of Zn; the results based on units $ml^{-1} \times 10^{7}$; age of the alga 5 days; d = died.

time (h)	0.04 mg l^{-1} Zn, HEPES (mg l^{-1}) 0.0		300		600		1200		1.0 mg l^{-1} Zn, HEPES (mg l^{-1}) 0.0		300		600		1200	
	chl *a* (mg l^{-1})	pH	chl *a* (mg l^{-1})	pH	chl *a* (mg l^{-1})	pH	chl *a* (mg l^{-1})	pH	chl *a* (mg l^{-1})	pH	chl *a* (mg l^{-1})	pH	chl *a* (mg l^{-1})	pH	chl *a* (mg l^{-1})	pH
0	ND	6.8	ND	7.1	ND	7.10	ND	7.1	ND	6.8	ND	7.1	ND	7.1	ND	7.1
24	0.07	7.4	0.07	7.2	0.06	7.10	0.04	7.1	0.04	7.1	0.02	7.2	0.02	7.1	0.05	7.1
48	0.57	9.0	0.43	7.4	0.36	7.20	0.43	7.2	0.28	7.3	0.28	7.4	0.31	7.2	0.43	7.2
72	1.14	10.8	0.85	7.5	0.85	7.30	0.85	7.2	0.57	8.95	0.57	7.45	0.67	7.25	0.85	7.2
96	0.71	11.3	1.42	7.7	1.29	7.35	1.47	7.2	0.89	10.0	0.92	7.5	1.07	7.2	1.24	7.2
120	1.42	12.5	1.95	7.8	1.78	7.4	1.78	7.25	1.07	10.2	1.42	7.70	1.37	7.25	1.42	7.2
144	2.13	12.8	1.95	7.9	2.13	7.4	1.95	7.25	1.24	10.4	1.48	7.75	1.67	7.3	1.95	7.25

Table 5.27 Influence of Ni on Zn toxicity to wild-type *Anacystis*; the results based on units $ml^{-1} \times 10^{7}$; age of the alga 5 days; d = died.

Zn ($mg\ l^{-1}$)	Ni ($mg\ l^{-1}$)						
	0	0.02	0.04	0.08	0.10	0.2	0.24
0 .04	9.6	9.2	9.5	9.4	9.1	7.3	4.2
0.25	9.4	9.0	9.2	9.2	8.8	6.2	3.4
0.50	9.4	9.1	8.8	8.6	8.8	5.8	2.3
0.75	9.2	8.8	8.6	7.2	6.3	4.4	d
1.0	9.0	7.9	6.4	4.8	3.6	2.8	d
1.25	8.0	6.8	5.2	2.8	2.6	1.8	d
1.50	3.5	2.4	1.6	d	d	d	d

Table 5.28 Influence of Cu on Zn toxicity to wild-type *Anacystis*; the results based on units $ml^{-1} \times 10^{7}$; age of the alga 5 days; d = died.

Zn ($mg\ l^{-1}$)	Cu ($mg\ l^{-1}$)							
	0	0.02	0.04	0.06	0.08	0.10	0.16	0.20
0 .04	9.6	9.2	9.5	9.2	8.8	8.8	4.6	2.5
0.25	9.2	9.0	9.4	9.6	8.6	7.8	1.8	d
0.50	9.2	8.6	8.2	8.2	8.0	4.4	d	d
0.75	9.4	8.2	7.8	4.8	2.8	2.1	d	d
1.0	9.0	8.8	5.8	2.2	d	d	d	d
1.25	8.0	8.0	3.6	d	d	d	d	d
1.50	3.2	2.5	1.8	0.8	d	d	d	d

Table 5.29 Influence of Hg on Zn toxicity to wild-type *Anacystis*; the results based on units $ml^{-1} \times 10^{7}$; age of the alga 5 days; d = died.

Zn ($mg\ l^{-1}$)	Hg ($mg\ l^{-1}$)							
	0	0.02	0.04	0.08	0.10	0.20	0.22	0.24
0 .04	9.2	8.8	8.4	7.5	6.2	1.6	0.10	d
0.25	9.5	8.8	8.0	7.4	6.5	1.8	d	d
0.50	9.4	9.0	7.8	7.0	5.2	1.5	d	d
0.75	9.0	8.8	6.8	6.8	4.6	d	d	d
1.0	8.8	7.8	6.5	5.5	4.8	d	d	d
1.25	7.8	7.2	6.0	4.2	2.6	d	d	d
1.50	3.1	2.7	1.4	d	d	d	d	d

Table 5.30 Influence of Pb on Zn toxicity to wild-type *Anacystis*; the results based on units $ml^{-1} \times 10^{7}$; age of the alga 5 days; d = died.

Zn ($mg\ l^{-1}$)	Pb ($mg\ l^{-1}$)						
	0	1	2	5	10	20	40
0.04	9.3	9.5	9.4	9.3	9.2	8.5	1.2
0.25	9.5	9.4	9.3	9.2	8.8	8.6	0.70
0.50	9.5	9.3	9.2	9.3	8.0	6.5	d
0.75	9.0	9.0	9.1	9.2	7.8	5.2	d
1.0	9.1	8.8	8.8	8.4	6.8	4.3	d
1.25	7.8	7.5	7.1	6.4	4.2	d	d
1.50	3.2	2.6	1.8	1.5	d	d	d

Table 5.31 Influence of Ni on Cd toxicity to wild-type *Anacystis*; the results based on units ml^{-1} x 10^7; age of the alga 5 days; d = died.

Cd ($mg\ l^{-1}$)	Ni ($mg\ l^{-1}$)						
	0	0.02	0.04	0.08	0.10	0.20	0.24
0	9.6	9.3	9.5	9.4	8.8	7.5	4.2
0.05	9.4	9.2	9.2	9.0	8.8	3.2	1.2
0.10	9.3	8.8	8.8	7.6	4.5	2.1	d
0.20	8.8	8.6	6.4	5.2	3.1	1.6	d
0.40	6.8	5.8	3.4	2.2	d	d	d
0.60	0.1	0.06	d	d	d	d	d

Table 5.32 Influence of Cu on Cd toxicity to wild-type *Anacystis*; the results based on units ml^{-1} x 10^7; age of the alga 5 days; d = died.

Cd ($mg\ l^{-1}$)	Cu ($mg\ l^{-1}$)							
	0	0.02	0.04	0.06	0.08	0.10	0.16	0.20
0	9.5	9.2	9.5	9.0	8.8	8.6	4.2	2.4
0.05	9.3	9.1	9.2	9.3	8.5	7.8	1.8	d
0.10*	9.2	8.6	8.3	8.2	7.8	6.8	d	d
0.20	9.0	8.8	6.4	3.2	d	d	d	d
0.40	6.6	3.4	d	d	d	d	d	d
0.60	0.08	d	d	d	d	d	d	d

Table 5.33 Influence of Hg on Cd toxicity to wild-type *Anacystis*; the results based on units $ml^{-1} \times 10^7$; age of the alga 5 days; d = died.

Cd ($mg\ l^{-1}$)	Hg ($mg\ l^{-1}$)							
	0	0.02	0.04	0.10	0.15	0.20	0.22	0.24
0	9.3	8.7	8.5	7.6	5.8	1.8	0.2	d
0.05	9.4	8.2	8.2	7.4	5.8	2.1	0.1	d
0.10	8.7	7.4	6.8	5.5	4.2	1.5	0.08	d
0.20	8.8	7.6	6.2	5.2	3.1	2.2	d	d
0.40	6.8	5.8	5.6	3.4	2.2	1.5	d	d
0.60	0.08	d	d	d	d	d	d	d

Table 5.34 Influence of Pb on Cd toxicity to wild-type *Anacystis*; the results based on units $ml^{-1} \times 10^7$; age of the alga 5 days; d = died.

Cd ($mg\ l^{-1}$)	Pb ($mg\ l^{-1}$)						
	0	1	2	5	10	20	40
0	9.3	9.4	9.4	9.3	9.3	8.6	1.3
0.05	9.5	9.2	9.0	9.2	8.8	7.2	d
0.10	8.8	8.8	7.8	7.8	6.2	4.4	d
0.20	8.7	8.6	7.8	7.2	5.8	3.5	d
0.40	7.2	6.8	5.2	3.8	2.3	d	d
0.60	0.06	d	d	d	d	d	d
0.80	d	d	d	d	d	d	d

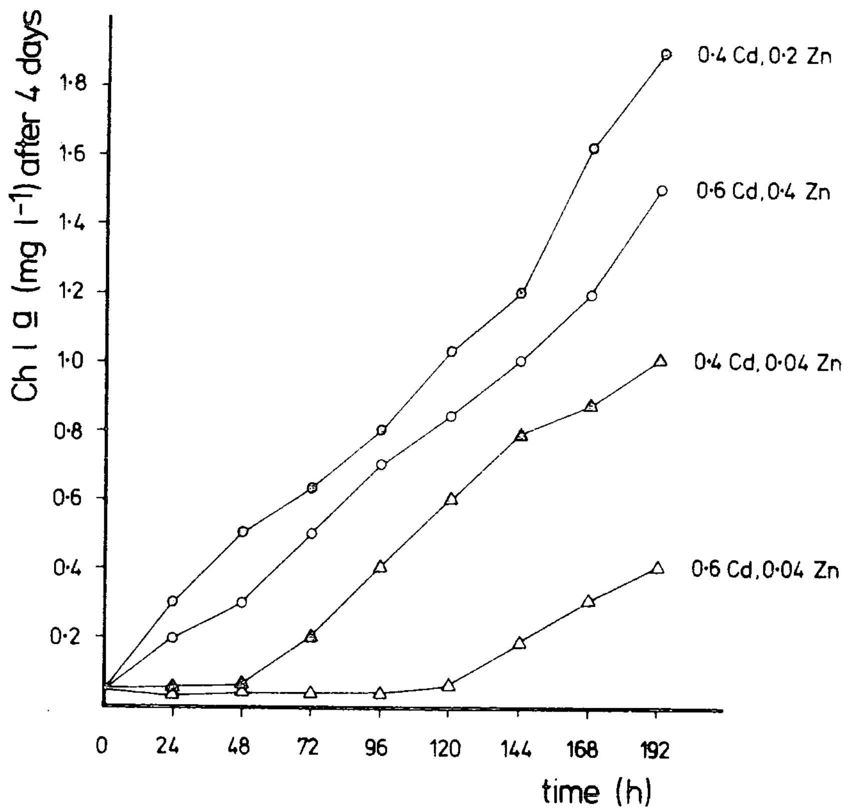


Fig. 5.15 Influence of Zn on Cd toxicity to wild-type *Anacystis*.

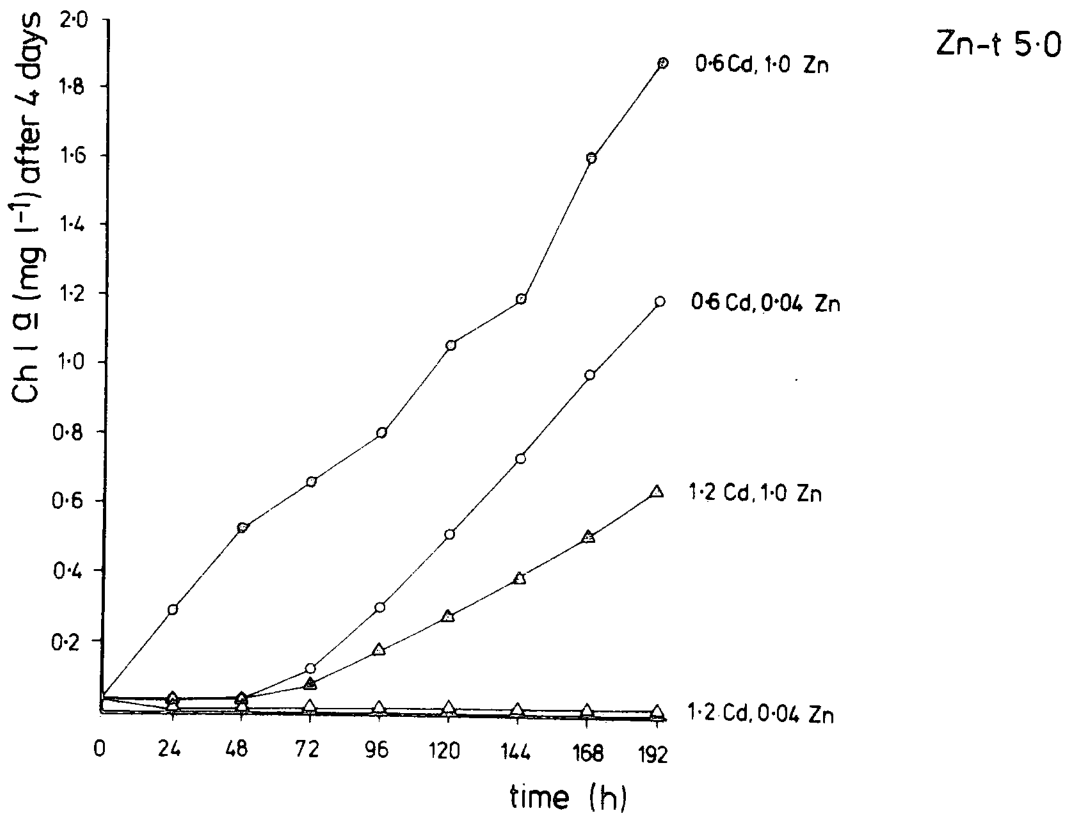


Fig. 5.16 Influence of Zn on Cd toxicity to Zn-t5.0 *Anacystis* strain.

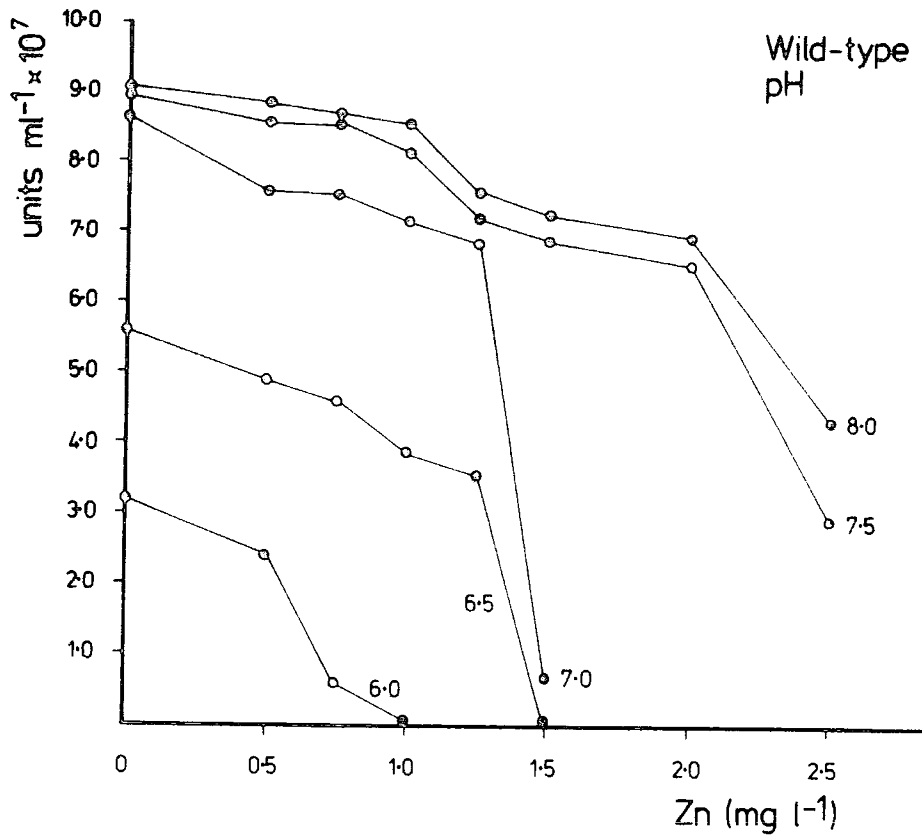


Fig. 5.17 Influence of pH on Zn toxicity to wild-type *Anacystis*.

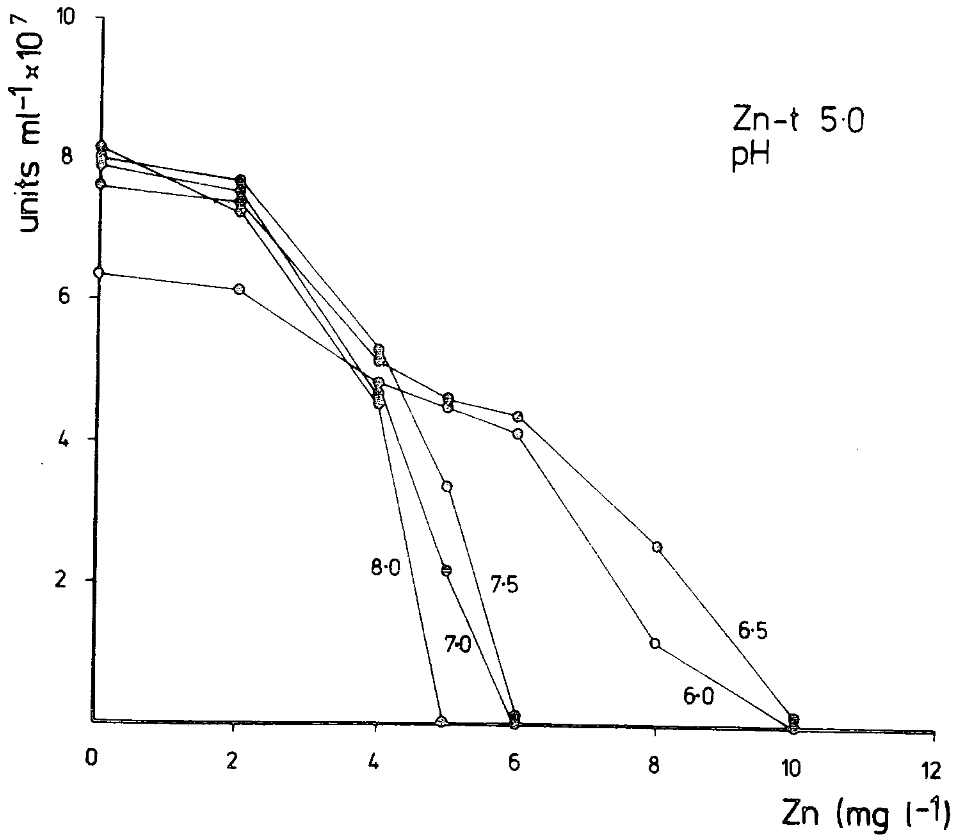


Fig. 5.18 Influence of pH on Zn toxicity to Zn-t5.0 *Anacystis* strain.

rise in pH led to increased Zn toxicity to the two Zn-tolerant strains (Figs 5.18, 5.19; Table A5.28, A5.29). In the case of both wild-type and Cu-t0.5 strains, pH had no detectable influence on the Cu toxicity. An increasing pH value between 6.0 and 8.0 caused a marked decrease in the toxicity of Cd to the wild-type (Fig. 5.20; Table A5.30).

5.8 Influence of inoculum size

The inoculum size was increased from 2×10^4 units ml^{-1} to 2×10^7 units for wild-type *Anacystis* to see what is the influence of inoculum size on Zn toxicity. The results (Fig. 5.21; Table A5.31, A5.32) confirm that the size of inoculum chosen (2×10^5 units ml^{-1}) is the most suitable. Use of a larger inoculum led to reduced toxicity and erratic results.

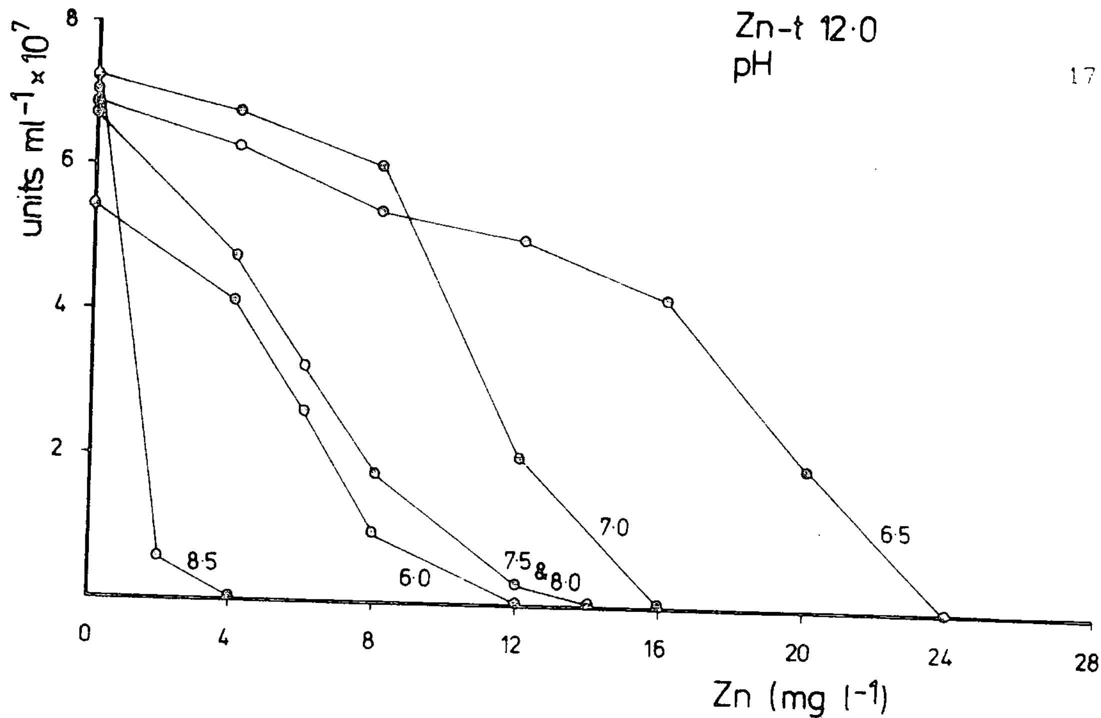


Fig. 5.19 Influence of pH on Zn toxicity to Zn-t12.0 *Anacystis* strain.

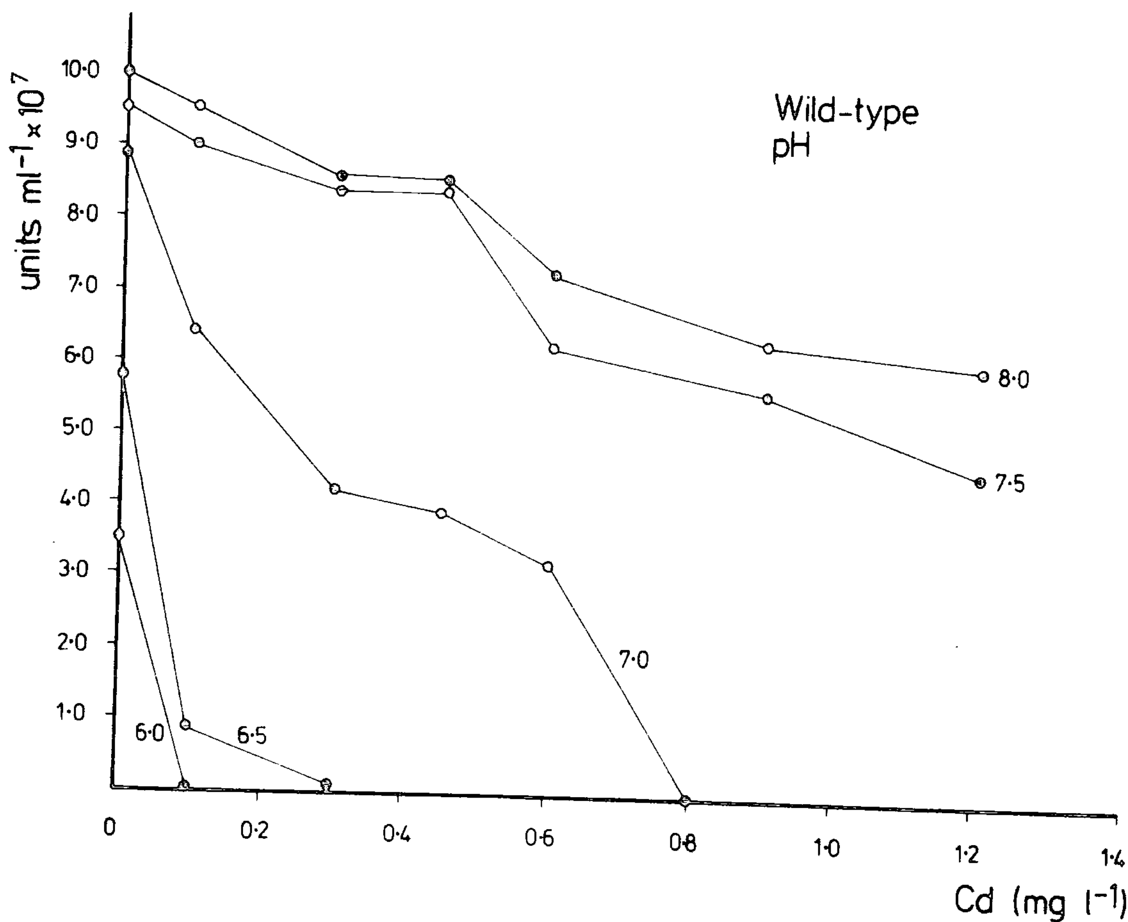


Fig. 5.20 Influence of pH on Cd toxicity to wild-type *Anacystis*.

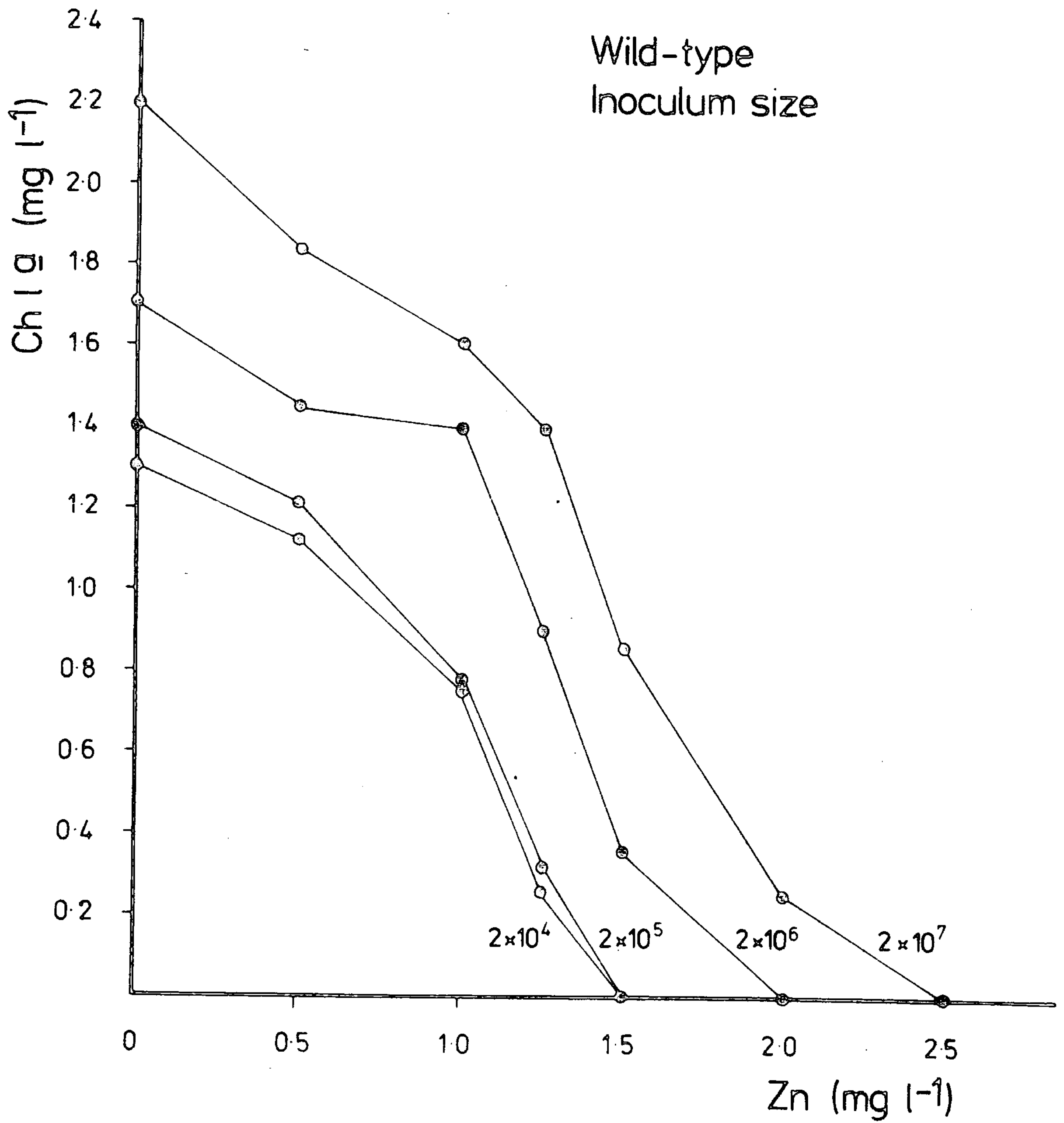


Fig. 5.21 Influence of inoculum size on Zn toxicity to wild-type *Anacystis*; chl *a* used as growth criterion.

CHAPTER 6

Accumulation of zinc by *Anacystis nidulans*

6.1 Introduction

An investigation was carried out to compare Zn accumulation by wild-type and Zn-tolerant *Anacystis* strains. These algae were subjected to various levels of Zn (0.1, 1.0 mg l^{-1} for wild-type; 0.1, 1.0 and 10.0 mg l^{-1} for Zn-t12.0) for periods up to 192 h, but otherwise standard growth conditions were used (section 2.33; p. 48). Growth curves for both algae are shown in Figs 6.1 and 6.2, with dry weight used as a growth criterion. These curves may be compared with Figs 4.19 and 3.9 in which population density was used as a growth criterion. The experiments were repeated twice. Comparisons were made first to study the ability of wild-type and Zn-t5.0 *Anacystis* strains to accumulate environmental Zn, using centrifugation (section 2.513) to separate the alga from the medium. The second experiment was quite similar, but compared wild-type and Zn-t12.0 strains and used the filtration (section 2.9(ii)) to harvest the alga. Because both experiments led to similar results, only the latter will be discussed here. Inocula were grown with as low a level of Zn as possible in the medium (0.04 mg l^{-1}; see 2.2). The inocula for wild-type and Zn-t12.0 had 7.5 and 12.5 µg Zn g^{-1} dry weight, respectively. There was no satisfactory technique to separate the actual level of Zn associated with alga from that due to precipitation. (In addition to removal of Zn by the alga and consequent lowering of the overall Zn level, changes in CO_2 during the growth of alga may influence this precipitation.) The results are summarized in two ways:

(i) Zn in alga plus filter.

(ii) As (i) less value obtained from filter used as a control.

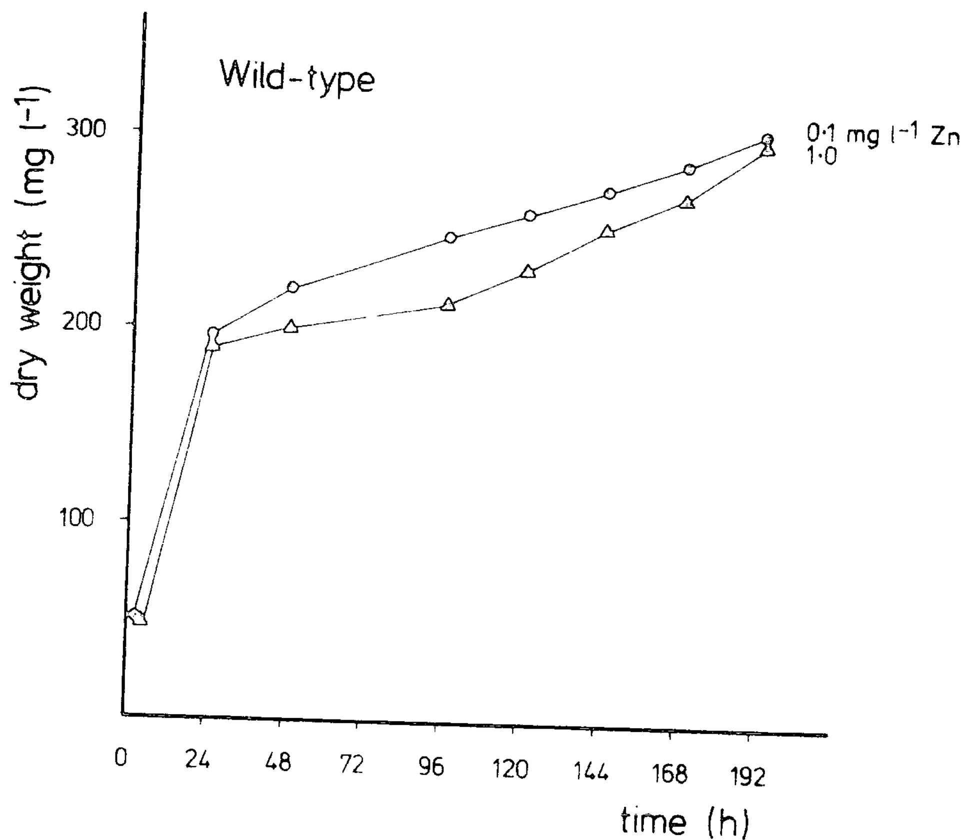


Fig. 6.1 Influence of Zn on growth of wild-type *Anacystis*; dry weight was used as a growth criterion.

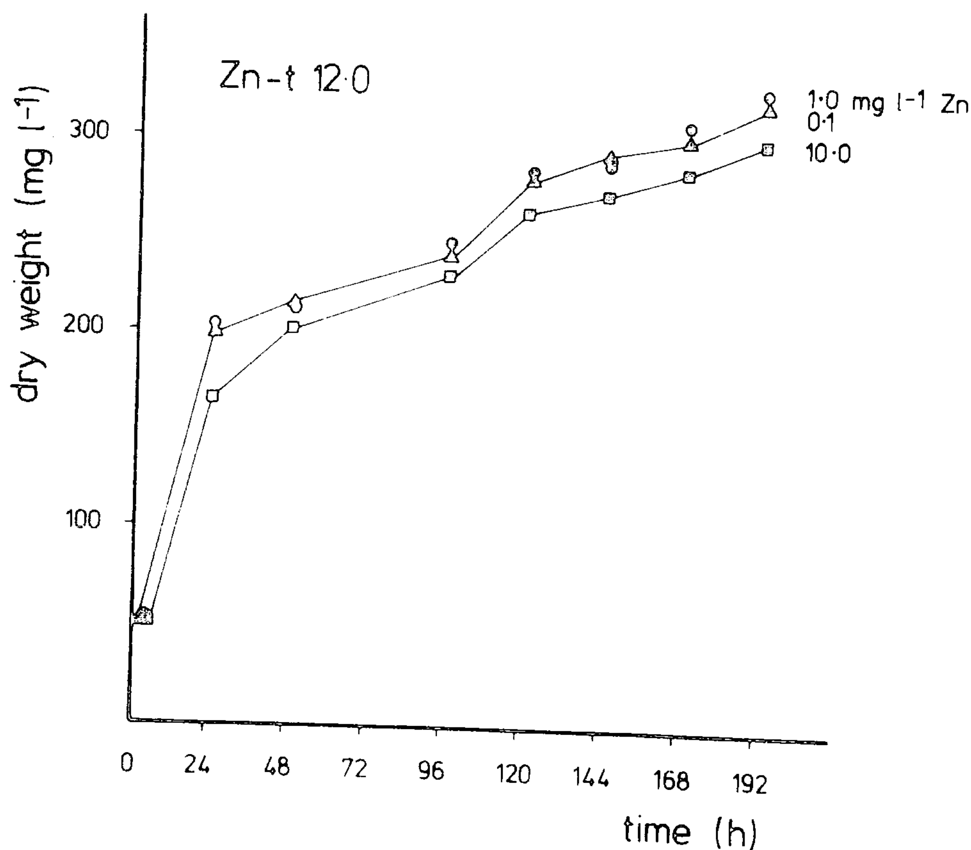


Fig. 6.2 Influence of Zn on growth of Zn-t12.0 *Anacystis*; dry weight was used as a growth criterion.

The "true" value of Zn associated with alga must lie between these two extremes. The level of Zn associated with a Nuclepore filter through which 0.1, 1.0 and 10.0 mg l^{-1} Zn, followed by EDTA washes, had been passed were 0.008, 0.008 and 0.022 mg l^{-1} Zn, respectively; unwashed filters were 0.016, 0.026 and 0.89 mg l^{-1}, respectively. The physical adsorption of Zn onto the cell surface, was examined by washing with 40 mg l^{-1} EDTA (= 0.138 mM; Table 2.15). At the beginning of the experiments it was thought that 3x EDTA washes might be enough to release all the Zn adsorbed. It was later realized that these were not enough for the higher Zn concentrations.

6.2 Influence of culture density on removal of Zn from medium

An experiment was initiated to determine whether the initial culture density affected the amount of Zn removed from the medium over a short period of time (30 minutes). Cultures in which initial units ml^{-1} were 0, 4 x 10^6, 6 x 10^6, 8 x 10^6, 10 x 10^6, 20 x 10^6, 30 x 10^6, 40 x 10^6 and 50 x 10^6 were exposed to 0.1, 0.5, 1.0 and 1.5 mg l^{-1} Zn. The amounts of Zn remaining in the medium are given in Table 6.1. The percentage of Zn removed from the medium rises slightly with increased population density. For instance at 1.5 mg l^{-1} Zn, 12% Zn removed by 4 x 10^6 units ml^{-1} while 22.6% was removed by 50 x 10^6 units ml^{-1}.

6.3 Influence of environmental Zn concentration on Zn accumulation by *Anacystis nidulans*

The total Zn accumulation and accumulation ratio by two *Anacystis nidulans* strains subjected to various environmental Zn concentrations is given in Tables 6.2, 6.3, 6.4, 6.5, 6.6, A6.1 and A6.2. The growth of both strains in medium containing 0.1 and 1.0 mg l^{-1} Zn for wild-type and 0.1, 1.0 and 10.0 mg l^{-1} Zn for Zn-t12.0 are shown in Figs 6.1

Table 6.1 Influence of culture density on removal of Zn from culture medium over a short period of time (30 minutes)

initial Zn (mg l^{-1})	population density units ml^{-1}	Zn left in medium (mg l^{-1})
0.10	0.0	0.10
0.50	0.0	0.48
1.0	0.0	0.84
1.5	0.0	1.32
0.10	4×10^6	0.093
0.50	4×10^6	0.48
1.0	4×10^6	0.85
1.5	4×10^6	1.30
0.10	6×10^6	0.09
0.50	6×10^6	0.46
1.0	6×10^6	0.88
1.5	6×10^6	1.26
0.10	8×10^6	0.09
0.50	8×10^6	0.48
1.0	8×10^6	0.86
1.5	8×10^6	1.27
0.10	10×10^6	0.083
0.50	10×10^6	0.45
1.0	10×10^6	0.87
1.5	10×10^6	1.28
0.10	20×10^6	0.080
0.50	20×10^6	0.45
1.0	20×10^6	0.86
1.5	20×10^6	1.25
0.10	30×10^6	0.080
0.50	30×10^6	0.46
1.0	30×10^6	0.84
1.5	30×10^6	1.22
0.10	40×10^6	0.080
0.50	40×10^6	0.45
1.0	40×10^6	0.84
1.5	40×10^6	1.20
0.10	50×10^6	0.072
0.50	50×10^6	0.45
1.0	50×10^6	0.85
1.5	50×10^6	1.18

Table 6.2 Accumulation of Zn during batch culture growth of wild-type *Anacystis*. Results show values estimated when controls (Nuclepore filter through which 0.1 mg l^{-1} Zn had been passed) are subtracted from the measured values. Compare with Table A6.1.

				washed - filter			unwashed - filter		
initial Zn (mg l^{-1})	time (h)	dry weight (mg l^{-1})	Zn left in medium (mg l^{-1})	Zn in alga	µg Zn g^{-1} dry weight	Zn in alga after washes / Zn left in medium	Zn in alga	µg Zn g^{-1} dry weight	total Zn / Zn left in medium
0.1	0.166	50	0.076	0.002	40	526.3	0.008	160	2105.3
	24	196	0.067	0.004	43.5	649.2	0.017	184.8	2758.2
	48	220	0.062	0.007	63.6	1026	0.020	181.8	2932
	96	248	0.054	0.017	137.1	2539	0.030	242	4481.5
	120	260	0.050	0.023	176.9	3538	0.034	261.5	5230
	144	272	0.044	0.030	220.6	5013.6	0.039	286.8	6518.2
	168	288	0.035	0.034	236.1	6745.7	0.044	305.6	8731
	192	310	0.026	0.037	238.7	9180.8	0.052	335.5	12903

Table 6.3 Accumulation of Zn during batch culture growth of wild-type *Anacystis*. Results show values estimated when controls (Nuclepore filter through which 1.0 mg l^{-1} Zn had been passed) are subtracted from measured values. Compare with Table A6.1.

initial Zn (mg l^{-1})	time (h)	dry weight (mg l^{-1})	Zn left in medium (mg l^{-1})	washed - filter: Zn in alga	washed - filter: µg Zn g^{-1} dry weight	washed - filter: Zn in alga after washes / Zn left in medium	unwashed - filter: Zn in alga	unwashed - filter: µg Zn g^{-1} dry weight	unwashed - filter: total Zn / Zn left in medium
1.0	0.166	50	0.96	0.002	40	41.6	0.014	280	291.6
	24	195	0.95	0.004	43.5	45.8	0.022	231.6	243.8
	48	200	0.90	0.027	270	300	0.069	690	766.6
	96	212	0.85	0.052	490.6	577.2	0.122	1151	1354
	120	232	0.76	0.097	836.2	1100.3	0.206	1775.8	2336.6
	144	256	0.70	0.124	968.8	1384	0.258	2015.6	2879.4
	168	272	0.68	0.144	1058.8	1557.1	0.276	2029.4	2984.4
	192	304	0.56	0.244	1605.3	2866.6	0.394	2592.1	4628.8

Table 6.4 Accumulation of Zn during batch culture growth of Zn-t12.0 *Anacystis* strain. Results show values estimated when controls (Nuclepore filter through which 0.1 mg l^{-1} Zn had been passed) are subtracted from measured values. Compare with Table A6.2

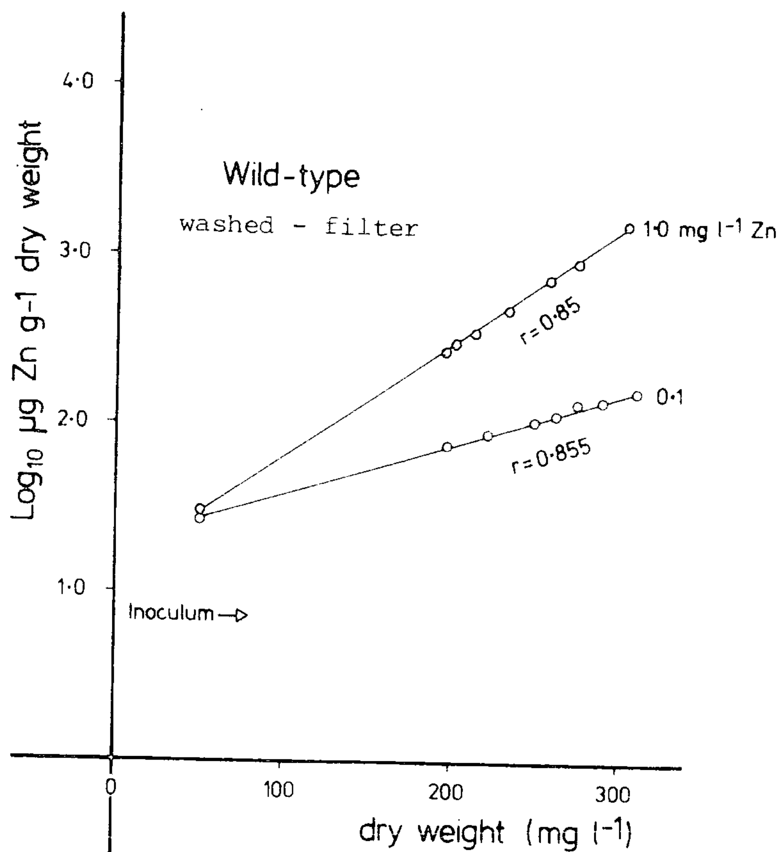
initial Zn (mg l^{-1})	time (h)	dry weight (mg l^{-1})	Zn left in medium (mg l^{-1})	washed - filter			unwashed - filter		
				Zn in alga	μg Zn g^{-1} dry weight	Zn in alga after washes / Zn left in medium	Zn in alga	μg Zn g^{-1} dry weight	total Zn / Zn left in medium
0.1	0.166	50	0.071	0.002	40	563.4	0.012	240	3380
	24	200	0.065	0.008	80	1231	0.017	170	2615.4
	48	216	0.060	0.020	185.2	3086.6	0.022	203.7	3395
	96	240	0.052	0.024	200	3846.2	0.030	250	4807.7
	120	280	0.046	0.032	228.6	4970	0.035	265	5434.8
	144	292	0.040	0.034	232.8	5820	0.042	287.6	7190
	168	300	0.032	0.037	246.6	7706.3	0.049	326.6	10206
	192	320	0.022	0.048	300	13636.4	0.058	362.5	16477.3

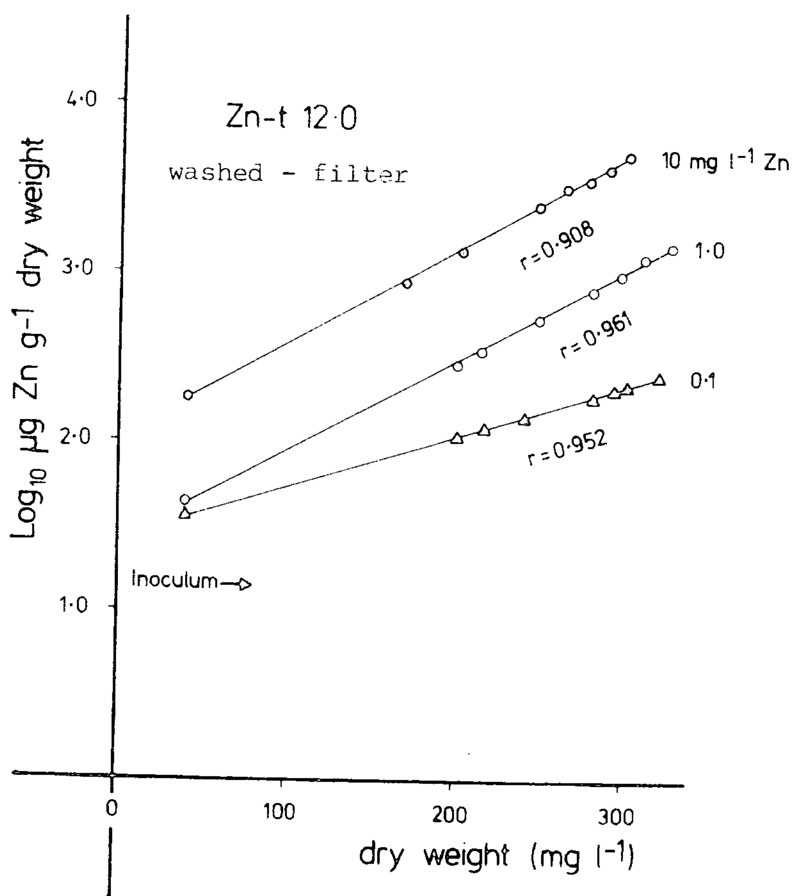
Table 6.5 Accumulation of Zn during batch culture growth of Zn-t12.0 *Anacystis* strain. Results show values estimated when controls (Nuclepore filter through which 1.0 mg l^{-1} Zn had been passed) are subtracted from measured values. Compare with Table A6.2.

initial Zn (mg l^{-1})	time (h)	dry weight (mg l^{-1})	Zn left in medium (mg l^{-1})	washed - filter			unwashed - filter		
				Zn in alga	µg Zn g^{-1} dry weight	Zn in alga after washes / Zn left in medium	Zn in alga	µg Zn g^{-1} dry weight	total Zn / Zn left in medium
1.0	0.166	50	0.96	0.003	60	62.5	0.014	280	292
	24	202	0.92	0.015	150	163	0.062	620	674
	48	216	0.90	0.037	342.6	380.6	0.072	666.6	740.6
	96	248	0.80	0.052	419.4	524.3	0.162	1306.5	1633
	120	280	0.71	0.118	842.8	1187	0.252	1800	2500
	144	296	0.60	0.178	1202.7	2004.5	0.369	2493.2	4155.3
	168	310	0.52	0.207	1335.5	2568.3	0.444	2864	5508
	192	328	0.44	0.294	1792.6	4074	0.516	3146.4	7151

Table 6.6 Accumulation of Zn during batch cultures growth of Zn-t12.0 *Anacystis* strain. Results show values estimated when controls (Nuclepore filter through which 10.0 mg l^{-1} Zn had been passed) are subtracted from measured values. Compare with Table A6.2.

initial Zn (mg l^{-1})	time (h)	dry weight (mg l^{-1})	Zn left in medium (mg l^{-1})	washed - filter			unwashed - filter		
				Zn in alga	µg Zn g^{-1} dry weight	Zn in alga after washes / Zn left in medium	Zn in alga	µg Zn g^{-1} dry weight	total Zn / Zn left in medium
10.0	0.166	50	8.55	0.014	280	32.7	0.56	11200	1310
	24	168	8.08	0.023	274	34.0	1.03	12262	1517.5
	48	202	7.20	0.218	2180	302.8	1.89	18900	2625
	96	248	7.0	0.296	2387.1	341	2.09	16855	2408
	120	264	6.77	0.385	2916.6	430.8	2.32	17561	2594
	144	272	6.77	0.493	3625	535.5	2.31	16985	2509
	168	288	6.45	0.623	4326.4	670.8	2.62	18264	2832
	192	300	6.0	1.028	6453.3	1142.2	3.06	20400	3400


Wild-type
washed - filter
1·0 mg l-1 Zn
r=0·85
0·1
r=0·855
Log10 µg Zn g-1 dry weight
4·0
3·0
2·0
1·0
Inoculum
0
100
200
300
dry weight (mg l-1)

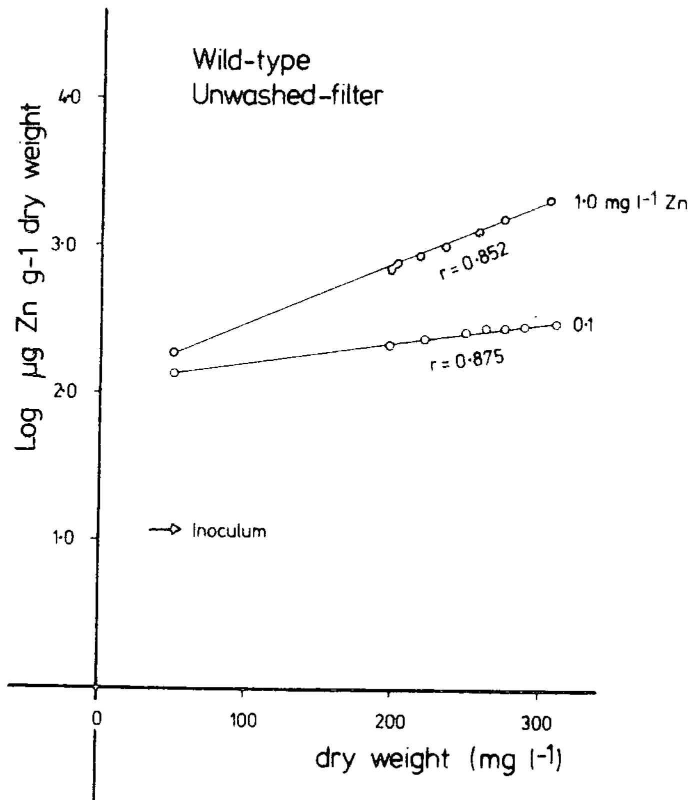

Zn-t 12·0
washed - filter
10 mg l-1 Zn
r=0·908
1·0
r=0·961
0·1
r=0·952
Log10 µg Zn g-1 dry weight
4·0
3·0
2·0
1·0
Inoculum
0
100
200
300
dry weight (mg l-1)

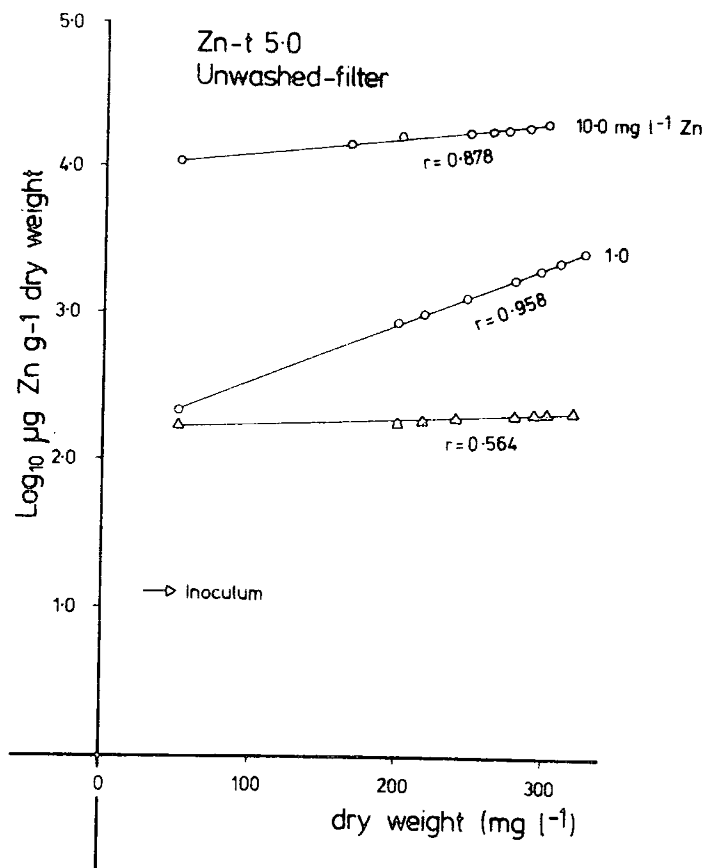
and 6.2, respectively. These show a similar growth pattern for each alga; the maximum growth rate occurred during the first few days of inoculation, then growth rate levelled-off with increased incubation time. There was a linear relationship between the logarithm of the Zn composition of the alga and the dry weight of the culture over the whole 192 h periods (Figs 6.3, 6.4). The amount of Zn in the alga increases with increasing Zn in the medium (Figs 6.3, 6.4). The ratio between that taken up at (initial) 10 mg l^{-1} Zn and that at (initial) 1 mg l^{-1} Zn is about 5.0 over the whole growth period (Fig. 6.4). The ratio between that taken up at (initial) 1 mg l^{-1} Zn and (initial) 0.1 mg l^{-1} Zn changes during growth, presumably at least partly because there is a greater change in environmental Zn during batch culture starting with 0.1 mg l^{-1} Zn (Tables 6.2, 6.3).

Correlation coefficients were calculated for both strains with y = logarithm µg Zn g^{-1} dry weight; x = dry weight (mg l^{-1}), the results are included with Figs 6.3, 6.4, 6.5 and 6.6. Anomalous correlation coefficients were obtained when no allowance was made for the presence of Zn in the filter.

6.4 Release of Zn from *Anacystis* by washing with EDTA

The total amount of Zn associated with wild-type and Zn-t12.0 *Anacystis*, without EDTA washes during the growth in batch culture are shown in Figs 6.5 and 6.6. These Figs may be compared with those in Figs 6.3 and 6.4, in which alga were washed with EDTA, and details of the results are given in Tables A6.1 andA6.2. These results suggest that the initial high Zn uptake observed, may be due to Zn only loosely associated with the cells.


Wild-type
Unwashed-filter
Log µg Zn g-1 dry weight
4·0
3·0
2·0
1·0
1·0 mg l-1 Zn
r = 0·852
0·1
r = 0·875
Inoculum
0
100
200
300
dry weight (mg l-1)


Zn-t 5·0
Unwashed-filter
Log10 µg Zn g-1 dry weight
5·0
4·0
3·0
2·0
1·0
10·0 mg l-1 Zn
r = 0·878
1·0
r = 0·958
r = 0·564
Inoculum
0
100
200
300
dry weight (mg l-1)

CHAPTER 7

STUDIES ON ZINC RESISTANCE OF STRAINS ISOLATED FROM ZINC POLLUTED SITES

7.1 Introduction

Blue-green algae are the dominants of some sites with elevated zinc, the organisms present usually being narrow forms of *Plectonema* (section 1.1). The purpose of the present chapter is to summarize the features of examples of species which are tolerant to high levels of zinc.

7.2 Origins, isolation and culture

Clonal strains isolated from sites with elevated zinc, in England, France and U.S.A. (Table 2.7) were obtained by plating. Two different media were required for culture (see 2.21).

7.3 Toxicity of Zn and Cd under standard conditions

The studies reported in this section were planned to determine the laboratory responses to Zn of isolates from sites with elevated zinc. Strains from presumed low zinc sites were included as "controls". Isolates were first tested for sensitivity to Zn using the assay whose results summarized as a Tolerance Index Concentration (T.I.C. see section 2.52) . Isolates from high Zn sites, in general, tolerated considerably higher levels of Zn than the "control" strains (Table 7.1). The situation is however not completely clear cut:

(i) *Calothrix* D 473 was isolated from a dry stream with sediments relatively rich in Zn ($<$210 µm fraction : 540 $\mu g\ g^{-1}$ Zn), yet was relatively sensitive in laboratory assay.

(ii) The results obtained with *Synechococcus* D 561 were variable but in all cases the strain was much more sensitive than *Gloeothece* D 562

Table 7.1 Comparison of tolerance to zinc of organisms isolated from high zinc sites with tolerance of strains taken from culture collection and almost certainly isolated initially from environments with low zinc levels. Toxicity of zinc to an organism is indicated by the Tolerance Index Concentration (section 2.52).

organism	Durham culture no.	level of zinc at source (mg l^{-1})	assay medium	Tolerance Index Concentration (mg l^{-1} Zn)
low Zn				
Anabaena cylindrica	2		ACM	1.5
			Chu10-D	0.71
Anacystis nidulans	33		ACM	0.94
Aphanothece castagnei	551		Chu10+N	*c.* 0.70
Calothrix membranacea	179		Chu10+N	1.07
			Chu10-N	0.89
C. parietina	550	$\bar{x}$ = 0.069 ± 0.006	Chu10+N	3.8
			Chu10-N	3.3
high Zn				
Calothrix sp.	184	8.0	Chu10+N	15
			Chu10-N	*c.* 15
Calothrix sp.	473	sediment with elevated Zn	Chu10-N	1.4
Gloeothece sp.	562	8.0	ACM	20
Phormidium autumnale	475	*c.* 16.0	ACM	10.2
Phormidium sp.	476	*c.* 15.0	ACM	*c.* 15
Synechococcus	561	*c.* 8.0	ACM	can survive 5.0, but variable

isolated from the same sample.

(iii) *Calothrix* D 550 was relatively Zn-resistant although it comes from a site with low Zn levels. The alga had been isolated by physical means and subcultured in a medium with 0.04 mg l^{-1} Zn until the time of assay, so presumably Zn-resistance can not have been enhanced during isolation of the strain.

In all four instances where the alga was assayed in the presence and absence of combined nitrogen, it was more sensitive under the latter conditions. The difference in response was due not only to a decreased growth rate, but at least for *Anabaena cylindrica* and *Calothrix membranacea* also to the alga being killed at lower concentrations of Zn. The influence of Zn on the growth rate of *Calothrix* D 184, both in the presence and absence of combined nitrogen is shown in Fig. 7.1. The alga was more sensitive under the latter conditions.

One strain, *Phormidium autumnale* D 475, was chosen for detailed investigation. It was selected because of easy growth in ACM medium, the one used for all major experiments, and because of a slightly faster growth rates than other strains i.e. *Calothrix* D 184, *Calothrix membranacea* D179, *Calothrix* D 473, and *Phormidium* sp. D 476. The alga was tested for its sensitivity both to Zn and Cd, the two metals to which all the algal populations had been exposed in the field to some extent. The influence of Zn on the growth of *Phormidium* D 475 using chl *a* as a growth criterion is shown in Fig. 7.2. The alga tolerated up to 25 mg l^{-1} Zn. The influence of Cd on growth is shown in Fig. 7.3. The alga tolerated up to 0.8 mg l^{-1} Cd. At these concentrations of Zn and Cd, there was a lag of about 6 days.

Fig. 7.1 Influence of Zn on growth of *Calothrix* D184, in the presence and absence of combined nitrogen.

Fig. 7.2 Influence of Zn on growth of *Phormidium autumnale* D475.

Fig. 7.3 Influence of Cd on growth of *Phormidium autumnale* D475.

7.4 Factors influencing toxicity

A comparison was made of the influence of environmental factors upon Zn and Cd toxicity to a strain isolated from a high Zn site (*Phormidium autumnale* D 475) and a "control" strain (*Anacystis nidulans*):

(i) Ca A moderate effect upon the growth of alga at different Ca levels (5 - 200 mg l^{-1}) was evident in the metal-free controls. The influence of Ca on the toxicity of Zn is given in Table 7.2. A marked ameliorating effect occurred up to the highest level (200 mg l^{-1} Ca) investigated. Increasing Ca caused a similar increase in tolerance to Cd (Table 7.3).

(ii) EDTA caused marked decreases in toxicity in all cases.

(iii) PO_4-P A slight decrease upon the growth of alga occurred in basal medium at $>$ 56 mg l^{-1} PO_4-P. The influence of PO_4-P on the toxicity of both Zn and Cd is shown in Tables 7.4, 7.5. A similar decrease on the toxicity of Zn and Cd was evident in all cases up to 28 mg l^{-1} PO_4-P.

(iv) pH Only very slight growth of the alga occurred at pH 7.0 in controls in Zn-free medium. A moderate decrease in the toxicity of Zn occurred when the pH was raised from 6.5 to 8.0, (Table 7.6), but Cd-toxicity was slightly affected by a similar rise in pH (Table 7.7).

(v) Zn and Cd interaction The influence of Zn on the toxicity of Cd, using chl *a* as a criterion of growth is shown in Table 7.8. A marked increase in the tolerance of Cd, occurred with increasing Zn concentrations. In contrast Cd brought about no detectable change in the toxicity of Zn.

Table 7.2 Influence of Ca on the toxicity of Zn to *Phormidium autumnale* D 476 as measured by chl *a* (mg l^{-1}); n = 5; d = died; inoculum size was 0.35 mg l^{-1} chl *a*; age of the culture 16 days.

Ca (mg l^{-1})	Zn (mg l^{-1})									
	0.04		10		20		30		40	
	$\bar{x}$	s.d.	$\bar{x}$	s.d.	$\bar{x}$	s.d.	$\bar{x}$	s.d.	$\bar{x}$	s.d.
5	7.6	± 0.94	5.2	± 0.68	2.6	± 0.05	0.14	± 0.04	d	d
10	7.5	± 0.83	5.4	± 0.52	3.2	± 0.48	2.5	± 0.22	d	d
25	7.4	± 0.76	5.5	± 0.65	3.8	± 0.26	2.8	± 0.32	0.36	± 0.02
50	7.8	± 0.53	6.2	± 0.56	5.6	± 0.87	4.7	± 0.85	2.3	± 0.36
100	8.2	± 1.6	7.0	± 0.95	6.0	± 1.2	5.2	± 0.56	4.2	± 82
200	8.3	± 1.8	7.3	± 1.5	6.8	± 1.3	7.0	± 0.68	5.8	± 1.5

Table 7.3 Influence of Ca on the toxicity of Cd to *Phormidium autumnale* D 476 as measured by chl *a* (mg l^{-1}) n = 5; d = died; inoculum size was 0.35 mg l^{-1} chl *a*; age of the culture 16 days.

Ca (mg l^{-1})	Cd (mg l^{-1})									
	0		0.40		0.80		1.0		1.2	
	$\bar{x}$	s.d.	$\bar{x}$	s.d.	$\bar{x}$	s.d.	$\bar{x}$	s.d.	$\bar{x}$	s.d.
5	7.6	± 0.65	5.2	± 0.64	2.5	± 0.32	d	d	d	d
10	7.8	± 0.72	6.2	± 0.52	3.2	± 0.48	2.5	± 0.22	d	d
25	7.8	± 1.1	6.4	± 0.63	5.2	± 0.43	5.4	± 0.6	2.3	± 0.38
50	8.0	± 1.3	6.6	± 0.95	5.6	± 0.78	5.7	± 0.58	3.4	± 0.36
100	8.2	± 1.4	7.2	± 1.6	6.6	± 1.1	6.6	± 0.65	5.6	± 0.82
200	8.0	± 1.5	7.3	± 1.6	6.8	± 1.0	7.0	± 0.86	6.3	± 1.3

Table 7.4 Influence of PO_4-P on the toxicity of Zn to *Phormidium autumnale* D 476 as measured by chl *a* (mg l^{-1}); n = 5; d = died; inoculum size was 0.35 mg l^{-1} chl *a*; age of the culture 16 days.

PO_4-P (mg l^{-1})	Zn (mg l^{-1})									
	0.04		10		20		30		40	
	$\bar{x}$	s.d.	$\bar{x}$	s.d.	$\bar{x}$	s.d.	$\bar{x}$	s.d.	$\bar{x}$	s.d.
1.78	7.5	± 0.52	4.0	± 0.62	2.6	± 0.10	0.14	± 0.01	d	d
3.5	7.4	± 0.38	4.2	± 0.80	3.4	± 0.61	2.0	± 0.60	d	d
7.0	7.4	± 0.72	4.5	± 0.56	3.8	± 0.52	2.8	± 0.15	0.36	± 0.05
14.0	6.8	± 0.35	4.8	± 1.3	4.8	± 0.51	3.2	± 0.28	1.4	± 0.15
28.0	6.8	± 0.36	5.6	± 0.58	5.2	± 0.82	5.0	± 0.45	2.3	± 0.38
56.0	6.5	± 0.45	6.4	± 0.62	5.4	± 0.42	5.2	± 0.60	3.4	± 0.32

Table 7.5 Influence of PO_4-P on the toxicity of Cd to *Phormidium autumnale* D 476 as measured by chl *a* (mg l^{-1}); n = 5; d = died; inoculum size was 0.35 mg l^{-1} chl *a*; age of the culture 16 days.

PO_4-P (mg l^{-1})	Cd (mg l^{-1})									
	0		10		20		30		40	
	$\bar{x}$	s.d.	$\bar{x}$	s.d.	$\bar{x}$	s.d.	$\bar{x}$	s.d.	$\bar{x}$	s.d.
1.78	7.5	± 0.68	5.2	± 0.45	2.5	± 0.21	d	d	d	d
3.5	7.4	± 0.35	5.2	± 0.72	2.8	± 0.36	d	d	d	d
7.0	7.2	± 0.65	5.5	± 0.63	3.2	± 0.42	2.8	± 0.32	0.36	± 0.06
14.0	7.0	± 1.1	5.8	± 0.42	4.6	± 0.51	3.2	± 0.18	1.14	± 0.15
28.0	6.8	± 0.36	6.0	± 0.80	5.2	± 1.0	5.4	± 0.45	2.30	± 0.38
56.0	6.5	± 0.45	6.4	± 0.42	5.4	± 0.65	5.5	± 0.72	3.4	± 0.32

Table 7.6 Influence of pH on the toxicity of Zn to *Phormidium autumnale* D 476 as measured by chl *a* (mg l^{-1}); n = 5; d = died; inoculum size was 0.35 mg l^{-1} chl *a*; age of the culture 16 days.

pH	Zn (mg l^{-1})									
	0.04		10		20		30		40	
	$\bar{x}$	s.d.	$\bar{x}$	s.d.	$\bar{x}$	s.d.	$\bar{x}$	s.d.	$\bar{x}$	s.d.
6	d	d	d	d	d	d	d	d	d	d
6.5	2.6	± 0.45	1.2	± 0.08	d	d	d	d	d	d
7.0	7.4	± 0.72	4.2	± 0.56	2.6	± 0.05	0.14	± 0.01	d	d
7.5	7.5	± 0.84	4.6	± 0.38	3.8	± 0.44	2.8	± 0.26	0.36	± 0.05
8.0	7.4	± 0.25	5.6	± 0.55	5.5	± 0.67	4.8	± 0.63	3.4	± 0.38

Table 7.7 Influence of pH on the toxicity of Cd to *Phormidium autumnale* D 476 as measured by chl *a* (mg l^{-1}); n = 5; d = died; inoculum size was 0.35 mg l^{-1} chl *a*; age of the culture 16 days.

pH	Cd (mg l^{-1})									
	0		0.40		0.8		1.0		1.2	
	$\bar{x}$	s.d.	$\bar{x}$	s.d.	$\bar{x}$	s.d.	$\bar{x}$	s.d.	$\bar{x}$	s.d.
6.0	d	d	d	d	d	d	d	d	d	d
6.5	2.8	± 0.4	1.6	± 0.05	d	d	d	d	d	d
7.0	7.5	± 0.72	5.2	± 0.46	2.5	0.22	d	d	d	d
7.5	7.8	± 1.2	6.3	± 0.52	5.2	± 0.4	5.4	± 0.6	2.3	± 0.38
8.0	7.0	± 0.52	6.4	± 0.55	5.5	± 0.72	5.4	± 0.58	3.4	± 0.32

Table 7.8 Influence of Zn on toxicity of Cd to *Phormidium autumnale* D 476 as measured by chl *a* (mg l^{-1}); n = 5; d = died; inoculum size was 0.35 mg l^{-1} chl *a*; age of the culture 16 days.

Cd (mg l^{-1})	Zn (mg l^{-1})							
	0.04		0.25		0.5		1.0	
	$\bar{x}$	s.d.	$\bar{x}$	s.d.	$\bar{x}$	s.d.	$\bar{x}$	s.d.
0	6.2	± 0.25	6.4	± 0.31	6.2	± 0.41	5.8	± 0.26
0.4	5.4	± 0.21	6.0	± 0.28	6.4	± 0.40	6.4	± 0.24
0.6	4.0	± 0.10	5.1	± 0.10	5.6	± 0.22	5.4	± 0.18
0.8	2.3	± 0.20	4.2	± 0.0	4.8	± 0.28	5.0	± 0.14
1.0	0.0	± 0.0	2.8	± 0.34	3.5	± 0.11	4.6	± 0.21

7.5 Nitrogen fixation

In view of the fact that non-heterocystous forms occurred widely at higher levels of Zn in the field (section 1.2), while heterocystous forms were rare, it seemed possible that Zn might interfere with nitrogen fixation. On the basis of sensitivity of the algae to growth in combined nitrogen-free medium at different levels of Zn, a laboratory assay was carried out using two heterocystous forms, *Anabaena cylindrica* and *Calothrix* D 184 to establish the effect of Zn on nitrogenase activity, using the acetylene reduction assay (section 2.9) as indicator. The comparison is made on a strain from a presumed low Zn site (*Anabaena cylindrica*) and from a high Zn site (*Calothrix* D 184).

The results are summarized in Table 7.9. It can be seen that while *Anabaena cylindrica* is only slightly more sensitive than *Calothrix* D 184 when Zn is first added, the effect is pronounced 24 h after the addition of the Zn. For instance, at 5 mg l^{-1} Zn, the rate of acetylene reduction was 0.003 and 0.016 nmol C_2H_4 µg chl a^{-1} min.$^{-1}$ for *Anabaena* and *Calothrix* respectively.

Table 7.9 Influence of zinc on acetylene reduction (nitrogen fixation) by Zn-resistant and Zn-sensitive heterocystous organisms. (Acetylene reduction assay was carried out over 90 min; n = 5)

organism	routine subculture prior to expt	length of treatment with test Zn (h)	Zn (mg l^{-1}) used for expt.	nmol C_2H_4 µg chl a^{-1} min^{-1} $\bar{x}$		s.d.
Anabaena cylindrica	0.04 mg l^{-1} Zn	0	0	0.035	±	0.002
			1	0.016		0.004
			5	0.015		0.003
			10	0.014		0.001
		24	0	0.028		0.008
			1	0.010		0.001
			5	0.003		0.0002
			10	0.0014		0.0008
Calothrix D 184	8 mg l^{-1} Zn	0	0	0.022		0.010
			5	0.017		0.008
			10	0.012		0.003
			20	0.012		0.004
		24	0	0.028		0.004
			5	0.016		0.002
			10	0.007		0.001
			20	0.005		0.0006

CHAPTER 8

DISCUSSION

8.1 Production of resistant strains

It is evident that for *Anacystis nidulans* the development of tolerance to any one particular heavy metal takes place easily, but that this does not confer general resistance to all metals. Marked changes in resistance to metals other than the one used to 'develop' tolerance were few and there were as many examples of a decrease as of an increase in resistance (Table 8.1). Only in the case of the Cd-tolerant strain taken from a Cd-rich medium was there increased resistance to all the other four metals (Table 8.1). Most field sites associated with heavy metal mining activities are contaminated simultaneously by several metals, so any blue-green alga behaving in a similar manner to *A. nidulans* would need to acquire separate mechanisms of tolerance for each metal. Presumably this makes evolution of tolerant populations considerably more difficult than if only one metal was present at elevated levels. The conditions required in culture of gradually increasing strongly inhibitory levels are however likely to be fulfilled at many field sites, such as the surroundings of mine wastes. Typically, a wide range of micro-habitats with differing metal levels occurs at such sites. At least under laboratory conditions, strains of *A. nidulans* can grow at comparatively high levels of metals with only slight changes in lag, exponential growth rate and final yield (Table 3.2). The resistant strains obtained by training with these metals do not lose their resistance by subculturing without metal. When culturing in the presence of a metal is repeated, the resistance of the culture becomes more stable. Repetition of training

Table 8.1 Cross-resistance of strains tolerant to one particular metal to the other four metals. Strains are named according to strongly inhibitory level of metal (mg l^{-1}) at the time of isolation. Comparison of toxicity is indicated by the level of metal which reduces growth rate to 50% that in basal medium.

strain used for assay	toxic level of metal (mg l^{-1}): inoculum from basal medium					inoculum from strongly inhibitory level of metal to which adapted				
	Co	Ni	Cu	Zn	Cd	Co	Ni	Cu	Zn	Cd
wild-type	0.12	0.09	1.10	1.05	0.28	0.16	0.09	0.10	1.45	0.32
Co-t1.8	0.85	0.11	0.10	0.70	0.20	1.1	0.14	0.07	0.60	0.11
Ni-t1.0	0.38	0.78	0.08	0.75	0.12	0.24	0.82	0.06	0.55	0.12
Cu-t0.5	0.22	0.12	0.35	1.25	0.14	0.07	0.16	0.45	0.85	0.04
Zn-t5.0	0.20	0.12	0.12	4.5	0.21	0.27	0.08	0.14	4.8	0.32
Zn-t12.0	0.15	0.09	0.10	8.5	0.24	0.12	0.07	0.08	11.2	0.35
Cd-t2.0	0.46	0.12	0.14	0.90	1.20	0.65	0.25	0.21	2.1	1.35

cultures presumably eliminates sensitive units and thus renders the resistant population stable. The level of resistance to Zn of both Zn-t5.0 and Zn-t12.0 tolerant strains did not change during long term subculturing, 72 and 96 generations respectively (Table 3.3) at a low concentration of Zn (0.04 mg l^{-1}). The fact that resistance is not lost even after several subcultures of the resistant strains in metal-free media strongly suggests that these strains are mutant strains (section 1.3).

The uses of strongly inhibitory levels of metal was suggested earlier for producing Cu-resistant strains of fungi and yeasts by (Ashida 1965), who concluded that "in successful laboratory training for adaptation to metal toxicants fungi are usually exposed for many passages to the toxicants at concentrations which partially inhibit the growth of inoculants". In addition DE Fillippis and Pallaghy (1976b) were able to develop Zn and Hg II resistance in the Emerson strain of *Chlorella* under laboratory culturing conditions by maintaining cells in the exponential phase of growth while exposing them to sub-lethal concentration of either Zn or Hg II. The lack of mutants tolerant to Cu reported previously (Sarma 1979) may perhaps have been due to cultures not being made from strongly inhibitory levels of metals. The reason why such levels of metals are needed to isolate metal tolerant mutants are not clear. As mentioned above for Zn, a possible specific role of the metal cannot be excluded, but a more likely explanation is that the mutation rate is greatly increased in the slower growing cultures containing many filaments or sub-spherical structures. Nevertheless there was apparently no increased rate of mutation for antibiotic resistance (Section 3.32). A further explanation may be that units growing in a strongly inhibitory level of metal have already undergone phenotypic adaptation for partial resistance to that metal. In basal medium any mutant, which is potentially capable of growing at a higher level of metal than that possible for the wild-type, will not have undergone such phenotypic

adaptation. Only a mutant acquiring in one step a particularly large increase in resistance will grow on transfer from basal medium to a potentially inhibitory level of metal. This last seems plausible for an element like Zn where there is a distinct difference in the lag shown by the wild-type according to whether or not the culture was grown previously at a strongly inhibitory level.

The present results fit in with Ashida's (1965) conclusions for bacteria, filamentous fungi and yeasts that resistance to some organic toxicants and antibiotics may develop more readily than to metal toxicants. In the present study with *A. nidulans*, it was found that none of the metal-resistant strains isolated after at least 25 subcultures had increased in tolerance by a factor greater than 10 times that of the wild-type (Table 3.1). In contrast, strains of *A. nidulans* resistant to 3 mg l^{-1} streptomycin (300 x wild-type), and 1.25 mg l^{-1} penicillin (125 x wild-type) were isolated after 4 and 7 subcultures into a medium with strongly inhibitory levels of streptomycin and penicillin respectively. These results suggest a similar behaviour to that recorded by Kumar (1964) who isolated *A. nidulans* tolerant to 50 mg l^{-1} streptomycin (50,000 x wild-type) and 8 mg l^{-1} penicillin (400 x wild-type) after 15 and 10 serial subcultures, respectively.

The production of filamentous forms following treatment of *A. nidulans* with heavy metals, was similar to that reported with mutagenic or other toxic agents (section 1.32). The metal-induced resistant strains of *A. nidulans* showed profuse filamentation during growth in this metal supplemented medium, no such effect was obtained in the wild-type strain. Cytological studies were not made to establish the extent to which these filaments were truly multicellular or only coenocytic like the mutants described in Kunisawa and Cohn-Bazire (1970). Sarma (1979) suggested that formation of long

filamentous cells by *A. nidulans* after exposure to Cu might be due to the Cu sorbed to the cell walls interfering in some way with cell division.

In the present study an initial morphological response to Cu was the formation of sub-spherical units quite different in shape from the normal rods (Fig. 3.10). This observation is similar to that reported earlier by Sadler and Trudinger (1967) for *Pseudomonas*. At sub-lethal concentrations of Cu, morphological changes took place by conversion of bacterial rods into spherical forms. In the case of *A. nidulans*, filamentous structures did eventually dominate in cultures of the Cu-resistant strain.

The possibility that metal treatment, in addition to leading to the selection of mutations of the wild-type for metal-resistance, might also have selected for other mutations from rods to filaments can not be ruled out. However the filamentation process was reversible, i.e. all the metal-tolerant strains showed normal rod shaped units when grown in the absence of metal, although the average length of the units of Zn-t12.0 strain was greater (Fig. 3.10 , 3.11).

8.2 Laboratory tolerance and toxicity

All the heavy metals whose influence was tested on the growth of *A. nidulans* were found to be more toxic than Zn to all the strains tested (section 4.1); Cu was found in general to be the most toxic to both wild-type and metal-tolerant strains. These results confirm the observations on filamentous green algae made by Whitton (1970a) where Cu was demonstrated to be more toxic than Zn. The results of Sparling (1968) contrast with the observations of other workers in that Zn and Cu each had the same effect on the total growth of *A. nidulans*. Up to 5.0 mg l^{-1} of both metals stimulated growth. In addition the present

results for Pb agree with those of Whitton (1970a) in that when Pb is added as ($Pb(NO_3)_2$),it is much less toxic than Zn; however under these circumstances little of Pb is in solution. Davies *et al.* (1976) found that Pb was highly toxic to rainbow trout and the toxicity was more pronounced in soft than hard water.

The tolerance of *A. nidulans* to various metals appears in general to be considerably less than exhibited by the green algae (section 1.2). Rana and Kumar (1974b) demonstrated that *A. nidulans*, *Oscillatoria* sp. and *Arthrospira jenneri* were all very sensitive to Zn, while *Chlorella vulgaris* and *Scenedesmus* sp. were highly tolerant, growing in 25 and 30 mg l^{-1} Zn, respectively. Katagiri (1975) found that 0.10 mg l^{-1} Cd caused a 50% reduction in the growth rate of *A. nidulans*. In contrast, *Chlorella* sp. required more than 0.5 mg l^{-1} Cd to inhibit the growth rate by a similar amount (Hart and Scaife 1977). The maximum and median tolerance limits of some blue and green algae to Cu and Zn found by Rana and Kumar (1974b) (calculated from copper sulphate quoted by the authors) are given below (Table 8.2):

Table 8.2 Tolerance to Cu and Zn of species studied by Rana and Kumar (1974).

	maximum tolerance (mg l^{-1})		median tolerance (mg l^{-1})	
	Cu	Zn	Cu	Zn
Anacystis nidulans	0.4	1.5	0.16	1.0
Oscillatoria sp.	0.4	1.0	0.2	0.5
Arthrospira jenneri	0.4	1.5	0.16	0.4
Plectonema boryanum	3.98	30	0.16	10
Nodularia spumigena	0.279	2.0	0.2	0.7
Anabaena doliolum	0.2	1.0	-	-
Fischerella muscicola	0.2	1.0	-	-
Chlorella vulgaris	2.0	25	1.0	5.0
Scenedesmus sp.	3.2	30	1.6	5.0

Sparling's (1968) results are surprising in that *A. nidulans* was reported to require 5 mg l^{-1} Zn for 50% reduction of the total growth. His results contrast with the much greater sensitivity found in the present study (Table 8.1). Rama and Kumar (1974b) reported that *A. nidulans* is very sensitive to high concentrations of Zn, any increase in the concentrations beyond that of basal medium (presumably about 0.05 mg l^{-1} Zn) had a growth retarding effect. Their results are similar to those of Katagiri (1975) who found that *A. nidulans* cultures with 0.001 and 0.01 mg l^{-1} Zn had doubling times of 8 h, whereas in the presence of 0.1 and 1.0 mg l^{-1} Zn, they had doubling times of 10 and 15 h, respectively. In comparison the present study found 0.04 mg l^{-1} Zn led to a doubling time of 8.2 h, and 0.5 and 1.0 mg l^{-1} Zn to doubling times of 11.6 h and 15 h, respectively.

Zinc requirement The failure to demonstrate a requirement for Zn in the growth of either wild-type or Zn-tolerant *A. nidulans* strains may be due to the fact that the level of Zn in the medium was not reduced below 0.04 mg l^{-1}, the level derived as contaminants from other chemicals (Table 2.3), beside glassware and deionized water. A survey of the literature (section 1.21) summarized in Table 8.3 shows that the amount of Zn required by most organisms for their optimum growth is very small. This contrasts with the results of Coleman *et al.* (1971) where *Chlorella vulgaris* required 18.03 mg l^{-1} Zn for optimum growth. The results of Lange (1971) are anomalous. Although most workers who have investigated micronutrient requirements have found it impossible to detect levels of Zn $\leqslant$ 1 µg l^{-1}, Lange reported addition of 0.08 µg l^{-1} Zn to filtered Lake Erie water enhanced the growth of 15 cultures of *Anabaena cirinalis*, and *Nostoc muscorum* in at least 3 and 5 instances respectively.

Table 8.3 Zinc requirement by various organisms in literature

organism	level (mg l^{-1})	function	References
Bacillus subtilis	trace	stimulated growth	Feeney *et al.* (1974)
Bacillus subtilis *Pseudomonas* sp.	0.016	optimum growth and essential for activated alkaline phosphatase	Pickett and Dean (1978)
Escherichia coli	trace	increased activity of alkaline phosphatase	Mitrae *et al.* (1975)
Rhizobium meliloti " *phaseoli*	0.007	maximum growth	Wilson and Reisenauer (1970)
Aspergillus niger	0.18	maximum growth	Steinberg (1939)
" "	0.10	maximum sporulation	Nicholas and Fielding (1951)
" "	0.065 - 0.13	maintaining growth phase	Wold and Suzuki (1976)
Neocosmospora visinfecta	0.13	stimulated growth	Paten and Budd (1972)
Neurospora crassa	trace	activated, alcohol dehydrogenase, and tryptophan synthetase	Nason *et al.* (1951)
Penicillum ochro-chloron	trace	increased final yield	Okamoto *et al.* (1977)
Ustilago sphaerogena	2.0	synehtsis of cytochrome *c*	Grimm and Allen (1954)
Euglena viridis and *Pediastrum tetras*	1.88 - 4.2	stimulated the growth to its optimum	Coleman *et al.* (1971)
Euglena gracilis	trace	maintaining configuration of RNA molecules	Wacker and Vallee (1959)
" "	1.0	Structural integrity of the cytoplasmid ribosomes	Prask and Plocke (1971)
Chlorella vulgaris	18.03	stimulated growth	Coleman (1971)
Anacystis nidulans	0.04	optimum growth	present work
Porphyra tenera	0.03	optimum growth	Iwasaki (1962)
Lycopersicum esculentum (tomato plants)	trace	synthesis of molecule tryptophan which auxin is produced, regulate stem elongation and cell enlargement	Tsui (1948)
Zea mays	trace	stimulated growth	Mazé (1914)

8.3 Environmental factors influencing metal-toxicity to *Anacystis nidulans* strains

The results presented in Chapter 5 show clearly that several environmental factors have a marked influence on the toxicity of the three metals tested (Zn, Cu, Cd), but in each case the factors differ (Table 5.2). In view of the large literature on environmental effects on metal toxicity (section 1.4) only a few comparative comments will be made. Many of the effects are similar to those reported for other organisms. For instance, the marked influence of EDTA and PO_4-P in reducing Zn-toxicity is found also in many eukaryotic plants (Whitton 1980). Phosphate also reduced the toxicity of Zn to *Plectonema boryanum* (Rana and Kumar 1974b), while nitrate had little effect on reducing Zn-toxicity, thus resembling the present results for *A. nidulans*. A possible explanation of these results may be that Zn and phosphate, but not nitrate, enter the cells at the same site thus interfering with each other during uptake by algae. This interference of phosphate and Zn with the entry of each other across the cell membrane previously suggested by Rana and Kumar (1974a) may be supported by the results in Table 5.21. When phosphate was introduced after inoculation, it had much less effect in reducing Zn-toxicity than if introduced together with Zn. Recently Rigby *et al.* (1980) found that Zn inhibited phosphate uptake by *Synechococcus leopoliensis*, while other cations (except Mg) stimulated it. It is therefore possible that some of the observed effects of metals on Zn toxicity reported in Chapter 5 may be due to indirect effects on phosphate uptake in addition to direct cation competition. Both Ca and Mg reduced the toxicity of Zn and Cd to *A. nidulans* in most cases. With the two Zn-tolerant strains, the effect of Mg was greater at several lower concentrations than that of Ca, but the influence of Ca increased over a much greater range of

concentrations. The greater influence of Mg in reducing Zn toxicity to tolerant rather than sensitive strains has been reported for the green algae, *Hormidium rivulare* (Say and Whitton 1977) and *Stigeoclonum tenue* (Harding and Whitton 1977). The effectiveness of Ca at higher levels in reducing the toxicity of Zn and Cd to both wild-type and tolerant strains would fit the hypothesis that a mechanism exists whereby the Zn is initially bound passively. With such a mechanism, Ca might compete with Zn for these sites. It is well documented that the presence of Ca around roots may greatly reduce heavy metal toxicity (Wyn-Jones and Lunt 1967). The effect of Mg could be by way of the same process, particularly since this element shares some similar ionic properties with Zn. The lack of any influence of Mg and the only slight effect of Ca on Cu-toxicity is surprising. The explanation may perhaps lie in the release of a strong Cu complexing agent, such as has been shown for *Anabaena flos-aquae* (McKnight and Morel 1979). In contrast to *Anacystis nidulans*, Mg antagonizes Cu-toxicity to *Bacillus licheniforme* more than Zn or Cd-toxicity (Haavik 1976).

Comparison of the results of the toxicity tests with observations on the growth of *A. nidulans* in a metal-free medium, but with a similar concentration of ions, showed that Mg, Ca, Fe and PO_4-P had quite a different influence on antagonism than on growth. The difference is most obvious for Mg and Fe, where raising the concentrations from 0.25 to 160 mg l^{-1} and 0.5 to 20 mg l^{-1} respectively, brought about an increased antagonism to the toxicity of Zn, Tables 5.6, 5.17, at the same time causing a marked reduction in total growth of wild-type. This is similar to the results of Harding and Whitton (1977) for *Stigeoclonum tenue* in which Mg had a quite different influence on antagonism than on growth. The present results on the influence of Fe on the toxicity of Cu to *A. nidulans* contrast with those of Steeman Nielsen and Kamp-Nielsen (1970) on *Chlorella pyrenoidosa*. In the

latter Fe detoxified the effect of Cu at the concentrations used in growth medium (6 µg l^{-1} Fe), but it had no effect on Cu toxicity to *Anacystis nidulans*. Steemann Nielsen and Kamp-Nielsen suggested that their results might be due to the presence of ferrous chloride, which in alkaline solution forms negatively charged colloids capable of adsorbing cations or positively charged complexes. Greene *et al.* (1975) suggested that the protection of *Selenastrum capricornutum* from the toxic effects of Zn ions by Na, Mg, Ca and P was due largely to increases in ionic strength. They further suggested that the formation of an 'ion pair' between Zn and such ions might lower the availability of Zn to the alga. This does not fit with the present results for *Anacystis nidulans* (Tables 5.1; A5.2, A5.4, A5.6) or for *Stigeoclonum tenue* (Harding and Whitton 1977); in both cases some ions (e.g. K^+, Cl^-, SO_4^{2-} and to some extent Na^+) had no detectable effect on toxicity even at very high concentrations. The behaviour of *Anacystis nidulans* in mixed solutions of Zn and Cd also differs markedly from some other organisms. For *Bacillus subtilis* ssp. *niger*, *Pseudomonas* sp. (Pickett and Dean 1979) and *Hormidium rivulare* (Say and Whitton 1977) Zn and Cd are synergistic in their toxic effects. On the other hand Zn reduces markedly Cd toxicity to wild-type *Anacystis nidulans* (Fig. 5.15, Table A5.25) and probably also the two Zn-tolerant strains (Fig. 5.16; Table A5.26); similarly the presence of Cd appears to reduce Zn toxicity to the Cd-tolerant mutant (Table 8.1) thus resembling the results of Nakano *et al.* (1979) for *Euglena gracilis* and of Pickett and Dean (1976) for *Klebsiella* (*Aerobacter*) *aerogenes*, in which an antagonistic interaction occurred between Zn and Cd. The remainder of the data in Table 5.1 indicate that the toxic effect of Zn with other heavy metals was additive or perhaps sometimes synergistic (e.g. Cu) in their action (Tables 5.28, 5.32).

The influence of pH on Zn toxicity differed between wild-type and the two Zn-tolerant strains (Figs 5.17, 5.18, 5.19). A rise in pH however brought about a marked reduction in Zn and Cd toxicity to Wild-type (Figs 5.17, 5.20) while the same rise in pH caused increase in Zn toxicity to the Zn-tolerant strains (Figs 5.16, 5.19). An increase in pH over the range 6.5 to 8.0 leads to increased precipitation, so this alone might perhaps explain the decreased toxicity found with the wild-type; it obviously can not explain the increased toxicity found with the other two strains. The observation that a rise in pH over the range pH 6.5 to 8.0 led to a decrease in the toxicity of Zn contrasts with some observations in the literature (section 1.4). Mount (1966) showed that a rise in pH led to an increase in Zn toxicity to fathead minnow. Hargreaves and Whitton (1976) demonstrated that rises in pH above 3.0 led to an increase in the toxicity of Zn to a population of *Hormidium rivulare* isolated from a stream at pH 3.1. Say and Whitton (1977) confirmed that rises in pH from pH 3.0 to pH 8.0 led to an increase in the toxicity of Zn to Zn-sensitive and Zn-tolerant populations of *Hormidium rivulare*. On the other hand the present results are confirmed by the results of Katagiri (1975) for *Anacystis nidulans* and Hart and Scaife (1977) for *Chlorella pyrenoidosa*; in both cases a rise in pH between 7 and 8 reduced the toxicity of Cd. Rises in pH also reduced the toxicity of Zn to both Zn-sensitive and Zn-tolerant populations of *Stigeoclonum tenue*, but this effect was more marked with the latter (Harding and Whitton 1977). There was no influence of pH on the toxicity of Cu to either wild-type or Cu-t5.0 tolerant strains of *A. nidulans*.

8.31 Organic compounds in the medium

A major problem in any heavy metal toxicity investigation is to understand the influence of factors other than the particular one

under study. This is particularly so when the metal levels are low and toxic effects could easily be masked. Of the inorganic synthetic media available in the literature ACM has been formulated specifically for *Anacystis nidulans*. This medium includes EDTA as a chelating agent. Since EDTA is known to form complexes with (presumably all) metal ions, ion toxicity is likely to be reduced. It was not possible to exclude EDTA completely from the medium, but its level was kept as low as possible.

The lower level of phosphate in the medium led to increased need to buffer the pH. Of the common buffers used in freshwater cultures most are known to possess some complexing ability, or to be unsuitable for some other reason such as being toxic, precipitation on autoclaving, or being used up by algae as essential nutrients (Smith and Foy 1974). HEPES was chosen as a buffering agent before it was realized that this too can act as a chelating agent (section 2.22). The present work showed slight changes in the toxicity of Zn to wild-type *A. nidulans* with increasing levels of HEPES (Table 5.26) which can not be explained by the poor buffering with the lower levels of HEPES; they are presumably a direct effect of chelating by the HEPES molecule. The situation is slightly complicated because both EDTA and HEPES were present simultaneously in the medium, with EDTA known to act as a strong chelating agent. The influence of HEPES in the absence of EDTA has not so far been studied. However the levels of EDTA and HEPES used for various experiments were given in Table 2.4.

8.4 Accumulation of Zn by *Anacystis nidulans*

Anacystis nidulans cultures are capable of accumulating high concentrations of Zn and there is little indication that resistant strains accumulate different amounts of zinc compared with the wild-type.

For instance wild-type and Zn-t12.0 strains grown in batch culture with 1.0 mg l^{-1} Zn eventually accumulate 1605 and 1792 µg Zn g^{-1} dry weight, respectively (Tables 6.2 and 6.4); respective accumulation ratios are 2866 and 4074. The initial high Zn uptake is mostly removed by EDTA washes (section 6.3), suggesting that this Zn is only loosely associated with the cells. At later stages in growth only a small percentage of the Zn is removable with EDTA, indicating that its accumulation is associated with growth of the alga. With increasing Zn in the medium, increased Zn is accumulated by the alga. With the Zn-t12.0 strain the proportional increase in old cultures is about the same between 0.1 and 1 mg l^{-1} Zn as between 1 and 10 mg l^{-1} Zn. For instance at a growth stage with 300 mg l^{-1} dry weight (Fig. 6.4) there is a 4.8-fold increase in algal Zn for a 10-fold increase in environmental Zn.

No investigation was carried out to study the effect of other factors on Zn accumulation by *A. nidulans*, but Katagiri (1975) found that Cd accumulation is pH dependent, more accumulation occurring at neutral pH than at more alkaline values. A similar observation has been made with *Chlorella* (Hart and Scaife 1977). This phenomenon may be related to the fact that solubility of metal is reduced at more alkaline conditions; the Cd may be present in a form which cannot be transported by the cell.

8.5 Tolerance and adaptation shown by field material

It is clear that some blue-green algae are capable of resisting very high levels of Zn. At least in streams these are usually very narrow forms which can often be referred to *Plectonema* (section 1.1).

Members of Chlorococcales may also be present, and, although usually small-celled, larger forms may occur. Laboratory assays (Table 7.4) have shown that most isolates from high Zn sites are adapted forms, being more tolerant of high Zn levels than most isolates from sites lacking Zn-enrichment, thus resembling observations for *Stigeoclonum tenue* (Harding and Whitton 1977) and *Hormidium rivulare* (Say and Whitton 1977). However the results of *Calothrix* D 473 and *Calothrix parietina* D 550 did not fit with the other results. Although *Calothrix* D 473 was isolated from sediments relatively rich in Zn, it was relatively sensitive in the laboratory assay. A possible explanation is that the site is highly calcareous, a factor reducing the toxicity of Zn to most organisms (section 1.4). *C. parietina* D 550 was relatively Zn-resistant although it comes from a site with low Zn levels (Table 2.7). It is difficult to suggest any explanation. The alga was isolated by physical means and subcultured in a medium with 0.04 mg l^{-1} Zn until the time of assay. A study was carried out by Stokes *et al.* (1973) on the heavy metal tolerance of algae from contaminated lakes in the Sudbury mining area of Ontario. Whilst not giving clear evidence for genetic adaptation of species of *Chlorella vulgaris* and *Scenedesmus acuminatus* to the toxicity of Cu and Ni, it did show that strains isolated from contaminated lakes were more tolerant than closely related laboratory strains of species in the same genera. In discussing their results, Stokes *et al.* postulate that the two genera may be inherently more adaptable to the toxic effect of the metals than most other algae.

In spite of observations such as these, Whitton and Say (1975) conclude that it seems probable that not all algae growing in high concentrations of heavy metals in the field have developed special

tolerance to the particular metal(s). As the number of species of algae which can grow in non-polluted streams is so large, several species may exist which may possess the capacity to grow in elevated concentrations of metal 'by accident' and that might be what happened with *Calothrix parietina* D 550.

8.51 Nitrogen fixation

There are two possible explanations for the possible absence of nitrogen fixation in streams with levels of Zn above 10 mg l^{-1}, and its rarity (judged by frequency of heterocystous organisms (Whitton 1980)) at slightly lower levels. It may be due simply to these environments carrying sufficient combined nitrogen that potential nitrogen fixers have no selective advantage. On the other hand it may be due to the particular sensitivity to Zn of nitrogenase or some other feature of the nitrogen fixers. The greater sensitivity of strains when grown in the absence of combined nitrogen rather than its presence (Table 7.1) suggests that the second explanation may be important. Nevertheless it can not be the sole reason because *Calothrix* D 184 is capable of growing in a medium free of combined nitrogen (Fig. 7.1), yet with a Zn level higher than yet recorded in the field for a nitrogen-fixing blue-green alga.

8.6 Concluding remarks

The occurrence of blue-green algae at sites polluted by heavy metals was reviewed at the beginning of this thesis. During the present study it has been shown that *Anacystis nidulans* can adapt to the heavy metals Co, Ni, Cu, Zn and Cd, after repeated subculture in the presence of these respective metals. A highly-inhibitory level of metals was essential for obtaining these adaptations. It thus seems likely that such adaptation can take place quite easily in nature where a wide range of metal levels

are likely to occur within one region such as the vicinity of a mine. It is to be hoped that further work, using a range of laboratory strains and both liquid and solid media, may lead to a more complete understanding of adaptation for heavy metal resistance by blue-green algae.

The indication that factors such as Ca, Mg and PO_4-P play a role in reducing metal toxicity to *A. nidulans* fits the hypothesis that more than one tolerance mechanism may be present and shows how complicated the situation may be in the field. A passive binding of Zn to exchange sites in the region of the cell wall might be subjected to competition from other ions such as Ca and Mg. From the morphological observations on *A. nidulans* strains it was possible only to speculate how the Zn sorbed to the cell walls may interfere in some way, such as with cell division, leading to the production of filamentous or sub-spherical forms. It is clear that more detailed study of the morphology of *A. nidulans* would help clarify the possible role of Zn in producing morphological changes. Other studies which would help understanding of the influence of Zn are ones using ^{65}Zn to follow up the accumulation work reported here.

While most of the experiments reported here were performed with laboratory cultures, some toxicity tests were carried out on algae isolated from high Zn sites. These algae still showed high Zn tolerance in the laboratory after isolation. The fact that non-heterocystous forms of blue-green algae occurred at higher levels of Zn in the field than did heterocystous forms also requires further study to see if Zn inhibits nitrogen fixation directly.

SUMMARY

(i) Relatively low concentrations of heavy metals inhibited the growth of *Anacystis nidulans*. The maximum concentrations of Co, Ni, Cu, Zn and Cd tolerated by wild-type alga, as judged by the levels of metals proving strongly inhibitory, were 0.32, 0.16, 0.15, 1.5 and 0.55 mg l^{-1}, respectively. In concentrations $<$ 0.5 mg l^{-1} Zn, growth was almost the same as in control, both as regards the length of the lag phase and the final population density.

(ii) Wild-type *Anacystis nidulans* was found also to be very sensitive to low concentrations of antibiotics, inhibition of growth occurring at 0.01 μg ml^{-1} of penicillin, polymyxin or streptomycin, as judged by the concentration leading to a 50% reduction in growth rate.

(iii) Mutants of *Anacystis nidulans* tolerant to high levels of Co, Ni, Cu, Zn or Cd were obtained by repeated subculturing at strongly inhibitory levels of metals. For instance the level of Zn at which strong inhibition occurred was raised from 1.45 to 16.5 mg l^{-1} Zn after 75 subcultures. For Co, Ni, Cu or Cd the levels were raised to 2.45, 1.30, 0.55 and 2.5 mg l^{-1}, respectively, after 25 subcultures. The comparative response of various strains to a particular metal was in general quite similar whether judged by growth rate, lag or yield

(iv) Colony formation on agar with different levels of Zn showed that the acquisition of increased resistance was due to the production of mutants.

The mutants correspond to those regarded as spontaneous elsewhere in the blue-green algal literature, but critical experiments were not made to rule out the possibility that Zn itself has a role as a mutagenic agent.

(v) It proved impossible to demonstrate the presence of mutants when making subcultures from medium lacking Zn-enrichment. On the other hand the presence of sub-inhibitory Zn did not lead to any increase in the rate of 'spontaneous' mutation for penicillin or streptomycin tolerance. The use of NTG (N-methyl-N'-nitro-N-nitrosoguanidine) as a mutagenic agent failed to lead to any detectable increase in the rate of mutation for Zn-tolerance. NTG also failed to lead to detectable mutation (1 in 5 x 10^8 units) in cultures lacking Zn-enrichment.

(vi) All the metal tolerant strains still grew well in basal medium. There was no indication that they now required higher levels of these metals for optimum growth; only very slight changes were detectable in the lag, exponential growth rate and yield as compared with the wild-type.

(vii) Two of the mutants with high tolerance for Zn and one for each of the other metals were chosen for comparative studies. Assays of cross-resistance of each of the five types of mutant were made to other four metals. In most cases changes in cross-resistance were only slight, with about equal numbers of examples of increased and decreased resistance. Examples of marked changes were increased Co-resistance of a Cd-tolerant strain, and decreased Cd-resistance of a Ni-tolerant strain.

(viii) The influence was tested of a range of factors on the toxicity of Cu, Zn and Cd to the wild-type, of Zn to two Zn-tolerant strains, and of Cu to a Cu-tolerant strain. K (5 - 500 mg l^{-1}) and Cl (10 - 200 mg l^{-1}) had no detectable effect. HEPES N-2 hydroxyethylpiperazine-N'-2-ethanesulphonic acid (0.3 - 1.2 mg l^{-1}) led to slight reduction in Zn toxicity to wild-type; not surprisingly, EDTA caused a marked reduction in toxicity in all cases.

Increasing levels of Cu, Cd and Hg all led to an increased toxicity, the effects of concentrations of the individual metals apparently being additive rather than synergistic. Increases in Ca, Mn, Fe, Mg and P reduced Zn toxicity to both wild-type and Zn-tolerant strains, but the strains differed in their response to pH. With the wild-type a rise in pH (6.0 - 8.0) brought about major reductions in toxicity, while that rise brought about an increase in the toxicity to Zn-tolerant strains.

(ix) The responses of Zn-t5.0 and Zn-t12.0 strains to penicillin, polymyxin and streptomycin were also tested. In every case there was a slight increase in resistance to about 6x the level tolerated by wild-type.

(x) Morphological changes were noted at the higher levels of metals and in some cases there was considerable diversity within a single flask. At the level of metals sub-inhibitory for each of these strains filaments were produced. The morphological response to Cu was different. At inhibitory levels of Cu, wild-type, Cu-t0.5 and Zn-t5.0 all formed many sub-spherical units. A further marked change took place with Cu-t0.5 during subcultures 22 to 25. Although there was no detectable change in the Cu-tolerance of the population as a whole, the subspherical structure became replaced completely by filaments. Subspherical structures similar to those found at high levels of Cu were also noted with Co-t1.8 grown at high Co levels, but here these units formed less than 1% of the population.

(xi) No big shifts in absorption maxima of pigments between mutants and wild-type were observed, but particularly at intermediate Cd-concentrations, there was a marked increase in the relative phycocyanin

content of wild-type *Anacystis nidulans*. Phycocyanin was not observed at any concentrations of Cd in the growth of Cd-tolerant strains.

(xii) The Zn concentrations in the alga (μg g^{-1} dry weight) increased with increased Zn in the medium and generally also with the age of the culture.

(xiii) A resistant strain of *Anacystis* (Zn-t12.0) took up approximately the same amount of Zn as wild-type from environmental concentrations at which they both grew (0.1 and 1.0 mg l^{-1} Zn).

(xiv) Algae isolated from high concentrations of Zn in the field had in general a much greater resistance to the metal than ones growing in lower concentrations or obtained from algal culture collections. The situation is however not completely clear-cut, because one isolate of *Calothrix* (*C. parietina* D 550) from a low Zn site showed considerable Zn resistance.

(xv) A laboratory comparison was made of the influence of Zn on nitrogen fixation from a strain from a presumed low Zn site (*Anabaena cylindrica*) and one from a high Zn site (*Calothrix* D 184) shows that the former was only slightly more sensitive than *Calothrix* when Zn was first added. The effect was however pronounced 24 h later.

REFERENCES

ABELSON P. H. and ALDOUS E. (1950) Ion antagonisms in microorganisms. Interference of normal magnesium metabolism by nickel, cobalt, cadmium, zinc and manganese. *J. Bact.* 60: 401-413.

ADAMS F. S., COLE H. Jr and LOWRY L. B. (1973) Element constitution of selected aquatic vascular plants from Pennsylvania: submerged and floating leaved species and rooted emergent species. *Environ. Pollut.* 5: 117-147.

ALLEN M. B. and ARNON D. I. (1955) Studies on nitrogen-fixing blue-green algae. 1. Growth and nitrogen fixation by *Anabaena cylindrica* Lemm. *J . Physiol.* 30: 366-372.

ALLEN M. M. (1968) Simple conditions for growth of unicellular blue-green algae on plates. *J. Phycol.* 4: 1-4.

ARNON D. I. (1958) The role of micronutrients in plant nutrition with special reference to photosynthesis and nitrogen assimilation. In: Lamb C. A., Bentley O. G. and Beattie J. M. (eds) pp. 1 - 32. Trace Elements. Academic Press, Inc. New York.

ASHIDA J. (1965) Adaptation of fungi to metal toxicants. *A. Rev. Phytopathol.* 3: 153-174.

BARTLETT L., RABE F. W. and FUNK W. H. (1974) Effects of copper, zinc and cadmium on *Selenastrum capricornum.* *Wat. Res.* 8: 179-185.

BREAK G. S., JENSEN A. and MOTHUS A. (1976) Heavy metal tolerance of marine phytoplankton. III. Combined effects of copper and zinc ions on cultures of four common species. *J. exp. mar. Biol. Ecol.* 25: 37-50.

COLEMAN R. D., COLEMAN R. L. and RICE E. L. (1971) Zinc and cobalt bio-concentration and toxicity in selected algal species. *Bot. Gaz.* 132: 102-109.

CRAIG I. W. and CARR N.G. (1968) C-phycocyanin and allophycocyanin in two species of blue-green algae. *Biochem. J.* 106: 361-366.

CRESPI H. L., MANDEVILLE S. E. and KATZ J. J. (1962) The action of lysozyme on several blue-green algae. *Biochem. Biophys. Res. Commun.* 9: 569-573.

DAVIES P. H., GOETL Jr, J. P., SINLEY J. R. and SMITH N. F. (1976) Acute and chronic toxicity of lead to Rainbow Trout, *Salmo gairdneri*, in hard and soft water. *Wat. Res.* 10: 199-206.

DE FILLIPPIS L. F. and PALLAGHY C. K. (1976a) The effect of sub-lethal concentrations of mercury and zinc on *Chlorella*. II. Photosynthesis and pigment composition. *Z. Pflanzenphysiol.* 78: 314-322.

DE FILLIPPIS L. F. and PALLAGHY C. K. (1976b) The effect of sub-lethal concentrations of mercury and zinc on *Chlorella*. III. Development and possible mechanisms of resistance to metals. *Z. Pflanzenphysiol.* 79: 323-335.

DELMOTTE A. (1980) Influence of cadmium on growth and nitrogen metabolism of *Anabaena cylindrica* Lemm. *J. exp. Bot.* 31: 1107-1118.

DROOP M. R. (1967) A procedure for routine purification of algal cultures with antibiotics. *Br. phycol. Bull.* 3: 295-297.

EILERS H. (1926) Zur Kenntnis der Ernahrungsphysiologie von *Stichococcus bacillaris* Näg. *Rec. trav. botan. neerl.* 23: 362-395.

ELDER J. F. and HORNE A. J. (1978) Copper cycles and $CuSO_4$ algicidal capacity in two California lakes. *Environ. Management* 2: 17-30.

ERNST W. H. O. (1974) Schwermetallvegetation der Erde (= *Geobotanica Selecta* V) 194 pp. Gustav Fischer, Stuttgart, Germany.

FALCHUK K. H., FAWCETT D. W. and VALLEE B. L. (1975) Competitive antagonism of cadmium and zinc in the morphology and cell division of *Euglena gracilis*. *J. submicr. Cytol.* 7: 139-152.

FEENEY R. E., LIGHTBODY H. D. and GARIBALDI J. A. (1947) Zinc as an essential element for growth and subtilin formation by *Bacillus subtilis*. *Arch. Biochem.* 15: 13-17.

FEUILLADE J. et FEUILLADE M. (1977) Relations entre la croissance d'*Oscillatoria rubescens* D.C. et la teneur en cuiver de son mileu de culture (Relation between the growth of *Oscillatoria rubescens* D.C. and the copper content of the culture medium). *Ann. Hydrobiol.* 8: 389-399.

FOGG G. E. (1973) Physiology and ecology of marine blue-green algae. In: Carr N. G. and Whitton B. A. (eds) The Biology of the Blue-Green Algae, 676 pp., pp. 368-378. Blackwell, Oxford.

FOGG G. E. and WESTLAKE D. F. (1955) The importance of extracellular products of algae in freshwater. *Verh. int. Verein. theor. angew. Limnol.* 12: 219-232.

GÄCHTER R. (1976) Untersuchungen über die Beeinflussung der plankischen Photosynthese durch anorganische Metallsalze im eutrophen Alpnachersee und der mesotrophen Horwer Bucht. *Schweiz. Z. Hydrol.* 38: 97-119.

GALE N. L., HARDIE M. G.,JENNETT J. C. and ALETI A. (1973) Transport of trace pollutants in lead mining wastewaters. In: Hemphill D. D. (ed.) Trace Substances in Environmental Health - VI. 399 pp. pp. 95-106 University of Missouri-Columbia.

GOLOMZIK A.I. and IVANOV V. I. (1965) Adaptation of *Thiobacillus ferrooxidans* to increased concentrations of hydrogen and iron ion. *Microbiologiya, Transl.* 34: 465-468.

GOOD N. E., WINGET G. D., WINTER W., CONNOLLY T. N., ISAWA S. and SINGH R. N. (1966) Hydrogen ion buffers for biological research. *Biochemistry, N. Y.* 5: 467-477.

GOPAL T., RANA B. C. and KUMAR H. D. (1975) Autecology of the blue-green alga *Nodularia spumigena* Mertens. *Nova Hedwigia* 26: 225-232.

GREEN J. C., MILLER W. E., SHIROYAMA T. and MERWIN E. (1973) Toxicity of zinc to the green alga *Selenastrum capricornutum* as a function of phosphorus or ionic strength. In: Proceeding: Biostimulation and nutrient assessment workshop held at Corvallis, Oregon. 325 pp., pp. 28-43.

GRIMM P. W. and ALLEN P. (1954) Promotion by zinc of the formation of cytochromes in *Ustilago sphaerogena*. *Pl. Physiol.* 29: 369-377.

GUPTA R. S. and KUMAR H. D. (1970) Action of mutagenic chemicals on *Anacystis nidulans*. V. Diethyl sulphate. *Arch. Mikrobiol.* 70: 313-329.

HAAVIK H. I. (1976) On the role of bacitracin peptides in trace metal transport by *Bacillus licheniformis*. *J. gen. Microbiol.* 96: 393-399.

HALL R. H. (1974) Trace metal-chelator effect on growth of *Microcystis*. 37th Ann. Meeting Amer. Soc. Limnol. and Oceanogr. Inc., Seattle, Wash.

HARDING J. P. C. (1978) Studies on Heavy Metal Toxicity and Accumulation in the Catchment Area of the Derwent Reservoir. 482 pp. Ph.D. Thesis, University of Durham, England.

HARDING J. P. C. and WHITTON B. A. (1976) Resistance to zinc of *Stigeoclonium tenue* in the field and the laboratory. *Br. phycol. J.* 11: 417-426.

HARDING J. P. C. and WHITTON B. A. (1977) Environmental factors reducing the toxicity of zinc to *Stigeoclonium tenue*. *Br. phycol. J.* 12: 17-21.

HARDY R. W. F., BURNS R. C. and HOLSTEN R. D. (1973) Application of the acetylene-ethylene assay for measurement of nitrogen fixation. *Soil Biol. Biochem.* 5: 47-81.

HARGREAVES J. W. and WHITTON B. A. (1976) Effect of pH on tolerance of *Hormidium rivulare* to zinc and copper. *Ecologia, Berl.* 26: 235-243.

HART B. A. and SCAIFF B. D. (1977) Toxicity and bioaccumulation of cadmium in *Chlorella pyrenoidosa*. *Environ. Res.* 14: 401-413.

HENRIKSSON L. E. and DA SILVA E. J. (1978) Effects of some inorganic elements on nitrogen-fixation in blue-green algae and some ecological aspects of pollution. *Z. allg. Mikrobiol.* 18: 487-494.

HORIKOSHI T., NAKAJIMA A. and SAKAGUCHI T. (1979) Uptake of uranium by *Chlorella regularis*. *Agric. Biol. Chem.* 43: 617-623.

HORNE A. J. and GOLDMAN C. R. (1974) Suppression of nitrogen-fixation by blue-green algae in a eutrophic lake with trace additions of copper. *Science, N. Y.* 183: 409-411.

HOSHAW R. W. and ROSOWSKI J. R. (1973) Methods for microscopic algae. In: Stein J. R. (ed.) Handbook of Phycological Methods 448 pp., pp. 53-67. Cambridge University Press, Cambridge.

HUTCHINSON T. C. (1973) Comparative studies of the toxicity of heavy metals to phytoplankton and their synergistic interactions. *Wat. Pollut. Res. Can.* 8: 68-89.

HUTCHINSON T. C. and CZYRSKA H. (1972) Cadmium and zinc toxicity and synerigism to floating aquatics. *Wat. Pollut. Res. Can.* 7: 59-65.

IWASAKI H. (1967) Nutritional studies of the edible seaweed *Porphyra tenera*. II. Nutrition of conchocelis. *J. Phycol.* 3: 30-34.

JENNETT J. C., HASSETT, J. M. and SMITH J. E. (1980) The use of algae to control heavy metals in the environment. *Minerals and the Environment* 2: 26-31.

JONES G. E., MURRAY L. and CARR N. G. (1978) Trace element composition of five cyanobacteria. In: Krumbein W. E. (ed.) Environmental Biogeochemistry and Geomicrobiology vol. 3: Methods, Metals and Assessment pp. 967-973. Ann Arbor Sciences, Michigan, U.S.A.

JONES J. R. E. (1964) Fish and River pollution. viii + 203 pp. Butterworth, London.

KATAGIRI K. J. (1975) The effect of cadmium on *Anacystis nidulans* 53 pp. M. Sc. Thesis, University of Vermont, U.S.A.

KUMAR H. D. (1964) Streptomycin- and penicillin-induced inhibition of growth and pigment production of strains of *Anacystis nidulans* resistant to these antibiotics. *J. exp. Bot.* 15: 232-250.

KUMAR H. D. (1968) Action of mutagenic chemicals on *Anacystis nidulans* III. N-Methyl-N'-Nitro-N-Nitrosoguanidine. *Arch. Mikrobiol.* 63: 95-102.

KUNISAWA R. and COHEN-BAZIRE G. (1970) Mutation of *Anacystis nidulans* that affect celldivision. *Arch. Mikrobiol.* 71: 49-59.

KRATZ W. A. and MYERS J. (1955) Nutrition and growth of several blue-green algae. *Am. J. Bot.* 42: 282-287.

LADHA J. K. and KUMAR H. D. (1978) Genetics of blue-green algae. *Biol. Revs* 53: 355-386.

LANGE W. (1971) Limiting nutrient elements in filtered Lake Erie water. *Wat. Res.* 5: 1031-1048.

MALANCHUK J. L. and GRUENDLING G. K. (1973) Toxicity of lead nitrate to algae. *Water Air Soil Pollut.* 2: 181-190.

MARKER A. F. H. (1972) The use of acetone and methanol in the estimation of chlorophyll in the presence of phaeophytin. *Freshwat. Biol.* 2: 361-385.

MATHYS W. (1980) Zinc tolerance by plants. In: Nriagu J. O. (ed.) Zinc in the Environment (Part 2: Health Effects) 480 pp. pp. 415-437. Wiley-Interscience, New York.

MAZÉ P. (1914) Influence respective des éléments de la solution minérale sur le développement du maus. *Ann. Inst. Pasteur* 28: 1-5.

MITRA R. S., GRAY R. H., CHIN B. and BERNSTEIN I. A. (1975) Molecular mechanisms of accommodation in *Escherichia coli* to toxic levels of Cd^{2+}. *J. Bact.* 121: 1180-1188.

MOUNT D. I. (1966) The effect of total hardness and pH on acute toxicity of zinc to fish. *Air Wat. Pollut. Int. J.* 10: 49-56.

MYERS J. and KRATZ W. A. (1955) Relations between pigment content and photosynthetic characteristics in a blue-green alga. *J. Gen. physiol.* 39: 11-22.

McKNIGHT, D. M. and MOREL F. M. M. (1979) Release of weak and strong copper-complexing agents by algae. *Limnol. Oceanogr.* 24: 823-827.

NAKANO Y., OKAMOTO K., TODA S. and FUWA K. (1978) Toxic effects of cadmium on *Euglena gracilis* grown in zinc deficient and zinc sufficient media. *Agric. Biol. Chem.* 42: 901-907.

NASON A., KAPLAN N. O. and COLOWICK S. P. (1951) Changes in enzymatic constitution in zinc-deficient *Neurospora*. *J. biol. Chem.* 188: 397-406.

NICHOLAS D. J. D. and FIELDING A. H. (1951) The use of *Aspergillus niger* (M.) for the determination of magnesium, zinc, copper and molybdenum available in soils to crop plant. *J. hort. Sci.* 26: 125-147.

OKAMOTO K., SUZUKI M., FUKAMI M., TODA S. and FUWA K. (1977) Uptake of heavy metals by a copper-tolerant fungus, *Penicillum ochro-chloron*. *Agric. Biol. Chem.* 41: 17-22.

OVERNELL J (1975) The effect of some heavy metal ions on photosynthesis in a freshwater alga. *Pesticide Biochemistry and Physiology* 5: 19-26.

OVERNELL J. (1976) Inhibition of marine algal photosynthesis by heavy metals. *Mar. Biol.* 38: 335-342.

PAKALNE D., NOLLENDORFA A. and UPITIS V. (1970) Little investigated trace elements in *Chlorella* culture; cadmium. *Latv. PSR. zinat. Akad. vestis.* 11: 16-24.

PALMER C. M. (1969) A composite rating of algae tolerating organic pollution. *J. Phycol.* 5: 78-82.

PATON W. H. N. and BUDD K. (1972) Zinc uptake in *Neocosmospora vasinfecta*. *J. gen. Microbiol.* 72: 173-184.

PICKERING D. C. and PUIA I. L. (1969) Mechanism for the uptake of zinc by *Fontinalis antipyretica*. *Physiologia Pl.* 22: 653-661.

PICKETT A. W. and DEAN A. C. R. (1976) Cadmium and zinc sensitivity and tolerance in *Klebsiella* (*Aerobacter*) *aerogenes*. *Microbios* 15: 79-91.

PICKETT A. W. and DEAN A. C. R. (1979) Cadmium and zinc sensitivity and tolerance in *Bacillus subtilis* subsp. *niger* and in a *Pseudomonas* sp. *Microbios* 24: 51-64.

PRASK J. A. and PLOCKE D. J. (1971) A role of zinc in the structural integrity of the cyto-plasmic ribosomes of *Euglena gracilis*. *Pl. Physiol.* 48: 150-155.

RANA B. C., GOPAL, T. and KUMAR, H. D. (1971) Studies on the biological effects of industrial wastes on the growth of algae. *Environ. Health* 13: 138-143.

RANA B. C. and KUMAR H. D. (1974a) The toxicity of zinc to *Chlorella vulgaris* and *Plectonema boryanum* and its protection by phosphate. *Phykos* 13: 60-66.

RANA B. C. and KUMAR H. D. (1974b) Effects of toxic waste and waste water components on algae. *Phykos* 13: 67-83.

RAULIN J. (1869) Études cliniques sur la végétation. *Ann. Sci. Nat. Botan. Biol. végétale* 11: 93-299.

RIGBY C. H., CRAIG S. R. and BUDD K. (1980) Phosphate uptake by *Synechococcus leopoliensis* (Cyanophyceae): enhancement by calcium ion. *J. Phycol.* 16: 389-393.

RIPPKA R., DERULLES, J., WATERBURY J. B., HERDMAN M. and STANIER R. Y. (1979) Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J. gen. Microbiol.* 111: 1-61.

ROSKO J. J. and RACHLIN J. W. (1977) The effect of cadmium, copper, mercury, zinc and lead on cell division, growth, and chlorophyll *a* content of the chlorophyte *Chlorella vulgaris*. *Bull. Torrey bot. Club* 104: 226-233.

SADLER W. R. and TRUDINGER P. A. (1976) The inhibition of microorganisms by heavy metals. *Mineral. Dep.* 2: 158-168.

SARMA T. A. (1979) An attempt to find copper resistance in *Anacystis nidulans*. In: Singh S. P., Tiwari D. N., Kashyap A. K. and Yadawa P. K. (eds) *Advances in Cyanophyta Research*. 139 pp., pp. 73-76. Department of Botany, Banaras Hindu University, India.

SAY P. J. (1977) Microbial Ecology of High Zinc Level Streams. 295 pp. Ph. D. Thesis, University of Durham, England.

SAY P. J. and WHITTON B. A. (1977) Influence of zinc on lotic plants. II. Environmental effects on toxicity of zinc to *Hormidium rivulare*. *Freshwat. Biol.* 7: 377-384.

SAY P. J. and WHITTON B. A. (1978) Chemistry and benthic algae of a zinc-polluted stream in the Northern Pennines. *Br. phycol. J.* 13: 206.

SAY P. J., DIAZ B. M. and WHITTON B. A. (1977) Influence of zinc on lotic plants. 1. Tolerance of *Hormidium* species to zinc. *Freshwat. Biol.* 7: 357-376.

SINCLAIR C. and WHITTON B. A. (1977) Influence of nutrient deficiency on hair formation in the Rivulariaceae. *Br. phycol. J.* 12: 297-313.

SINGH R. N. and SINHA R. (1965) Genetic recombination in a blue-green alga *Cylindrospermum majus* Kütz. *Nature, Lond.* 207: 782-783.

SINGH H. N., VAISHAMPAYAN A. and SINGH R. K. (1978) Evidence for the involvement of a genetic determinant controlling functional specificity of group VI B elements in the metabolism of N_2 and NO_3^- in the blue-green alga. *Biochem. Biophys. Res. Commun.* 81: 67-74.

SMITH R. V. and FOY R. H. (1974) Improved hydrogen ion buffering of media for the culture of freshwater algae. *Br. phycol. J.* 9: 239-245.

SPARLING A. B. (1968) Interaction between blue-green algae and heavy metals. 106 pp. Ph. D. Thesis, University of Washington, U.S.A.

STEEMANN NIELSEN E. and KAMP-NIELSEN L. (1970) Influence of deleterious concentrations of copper on the growth of *Chlorella pyrenoidosa*. *Physiol. Plant.* 23: 828-840.

STEINBERG R. A. (1939) Growth of fungi in synthetic nutrient solutions. *Bot. Rev.* 5: 327-350.

STEWART W. D. P. and LEX M. (1970) Nitrogenase activity in the blue-green alga *Plectonema boryanum* strain 594. *Arch. Mikrobiol.* 73: 250-260.

STOKES P. and HUTCHINSON T. C. (1976) Copper toxicity to phytoplankton, as affected by organic ligands, other cations and inherent tolerance of algae to copper. In: Andrews R. W., Hodson P. V. and Konasewich D. E. (eds) Toxicity to BIOTA of Metal Forms in Natural Water. Windsor, Ontario International Joint Commission pp. 159-185.

STOKES P. M., HUTCHINSON T. C. and KRAUTER K. (1973) Heavy metal tolerance in algae isolated from contaminated lakes near Sudbury, Ontario. *Can J. Bot.* 51: 2155-2168.

STRATTON G. W. and CORKE C. T. (1979) The effect of mercuric, cadmium, and nickel ion combinations on a blue-green alga. *Chemosphere* 8: 731-740.

TEVLIN M. P. (1978) An improved experimental medium for freshwater toxicity studies using *Daphnia magna*. *Wat. Res.* 12: 1027-1034.

THOMAS W. H., HOLLIBAUGH J. T. and SIEBERT D. L. R. (1980) Effects of heavy metals on the morphology of some marine phytoplankton. *Phycologia* 19: 202-209.

TROLLOPE D. R. and EVANS B. (1976) Concentrations of copper, iron, lead, nickel and zinc in freshwater algal blooms. *Environ. Pollut.* 11: 109-116.

TSUI C. (1948) The role of zinc in auxin synthesis in the tomato plant. *Am. J. Bot.* 35: 172-179.

UPITIS V. V., PAKALNE D. S. and NOLLENDORF A. F. (1973) The dosage of trace elements in the nutrient medium as a factor in increasing the resistance of *Chlorella* to unfavourable conditions of culturing. *Mikrobiologiya, Transl.* 42: 854-858.

VALLEE B. L. (1962) Zinc. In: Comar C. L. and Bronner F. (eds) Mineral Metabolism; an Advance Treatise pp. 443-482. Academic Press, New York.

VAN-BAALEN C. (1965) Quantitative surface plating of coccoid blue-green algae. *J. Phycol.* 1: 19-22.

VELICHKO I. M. (1968) O fizologicheskei aktivnosti nekotorykh ionov po otnosheniyu K *Microcystis* (Kütz.) Elenk. In: Topachevsky A. V. (ed.) '*Tsvetenie*' *Vody*, 387 pp., pp. 217-222. Naukova Dumka, Kiev, U.S.S.R. Russian. (Physiological activity of some ions in relation to *Microcystis* (Kütz.) Elenk. : translation of this chapter held at National Lending Library, Boston Spa, England.

WACKER W. E. C. and VALLEE B. L. (1959) Nucleic acids and metals. 1. Chromium, manganese, nickel iron and other metals in ribonucleic acid from diverse biological sources. *J. biol. Chem.* 234: 3257-3262.

WHITTON B. A. (1970a) Toxicity of zinc, copper and lead to *Chlorophyta* from flowing water. *Arch. Mikrobiol.* 72: 353-360.

WHITTON B. A. (1970b) Toxicity of heavy metals to freshwater algae: A review. *Phykos* 9: 116-125.

WHITTON B. A. (1980) Zinc and plants in rivers and streams. In: Nriagu J. O. (ed.) Zinc in the Environment Part 2: Health Effects 480 pp., pp. 363-400. Wiley-Interscience, New York.

WHITTON B. A. and SAY P. J. (1975) Heavy metals. In: Whitton B. A. (ed.) River Ecology 724 pp., pp. 286-311. Blackwell, Oxford.

WILD H. (1968) Geobotanical anomalies in Rhodesia 1: The vegetation of copper-bearing soils. *Kirkia* 7: 1-71.

WILSON D. O. and REISENAUER H. M. (1970) Effect of manganese and zinc ions on the growth of *Rhizobium*. *J. Bact.* 102: 729-732.

WIXSON B. G. and GALE N. L. (1975) Some limnological effects of lead and associated heavy metals from mineral production in southeast Missouri, U.S.A. 10 pp. In: International Symposium, Interaction between water and living matter, Odessa, USSR.

WOLD W. S. M. and SUZUKI I (1976) The citric acid fermentation by *Aspergillus niger* regulation by zinc of growth and acidogenesis. *Can. J. Microbiol.* 22: 1083-1092.

WYN JONES R. G. and LUNT O. R. (1967) The function of calcium in plants. *Bot. Rev.* 33: 407-426.

APPENDIX

Content list A3

		page
Table A3.1	Influence of Co on the growth of Co-t1.8.	244
A3.2	Influence of Ni on the growth of Ni-t1.0.	244
A3.3	Influence of Cu on the growth of Cu-t0.5.	245
A3.4	Influence of Zn on the growth of Zn-t5.0.	245
A3.5	Influence of Zn on the growth of Zn-t12.0..........	246
A3.6	Influence of Cd on the growth of Cd-t2.0.	246
A3.7	Influence of Zn and time of harvesting in .... batch culture on length of wild-type *Anacystis*.	247
A3.8	Influence of Zn and time of harvesting in .... batch culture on length of Zn-t5.0; inoculum from basal medium.	248
A.3.9	Influence of Zn and time of harvesting in .... batch culture on length of Zn-t5.0; inoculum from strongly inhibitory level of Zn.	249

Content list A4.0

Table A4.1 Influence of Co on the growth of wild-type 250

A4.2 Influence of Co on the growth of Ni-t1.0. 250

A4.3 Influence of Co on the growth of Cu-t0.5. 251

A4.4 Influence of Co on the growth of Zn-t5.0. 251

A4.5 Influence of Co on the growth of Zn-t12.0. 252

A4.6 Influence of Co on the growth of Cd-t2.0. 252

A4.7 Influence of Ni on the growth of wild-type....... 253

A4.8 Influence of Ni on the growth of Co-t1.8. 253

A4.9 Influence of Ni on the growth of Cu-t0.5. 254

A4.10 Influence of Ni on the growth of Zn-t5.0. 254

A4.11 Influence of Ni on the growth of Zn-t12.0. 255

A4.12 Influence of Ni on the growth of Cd-t2.0. 255

A4.13 Influence of Cu on the growth of wild-type....... 256

A4.14 Influence of Cu on the growth of Co-t1.8. 256

A4.15 Influence of Cu on the growth of Ni-t1.0. 257

A4.16 Influence of Cu on the growth of Zn-t5.0. 257

A4.17 Influence of Cu on the growth of Zn-t12.0. 258

A4.18 Influence of Cu on the growth of Cd-t2.0. 258

A4.19 Influence of Zn on the growth of wild-type....... 259

A4.20 Influence of Zn on the growth of Co-t1.8. 259

A4.21 Influence of Zn on the growth of Ni-t1.0 260

A4.22 Influence of Zn on the growth of Cu-t0.5. 260

A4.23 Influence of Zn on the growth of Cd-t2.0. 261

A4.24 Influence of Cd on the growth of wild-type....... 261

A4.25 Influence of Cd on the growth of Co-t1.8. 262

A4.26 Influence of Cd on the growth of Ni-t1.0. 262

A4.27 Influence of Cd on the growth of Cu-t0.5. 263

A4.28 Influence of Cd on the growth of Zn-t5.0. 263

A4.29 Influence of Cd on the growth of Zn-t12.0 264

A4.30 Influence of Hg on the growth of wild-type....... 264

A4.31 Influence of Pb on the growth of wild-type. 265

A4.32 Pigment changes during growth of wild-type 266
Anacystis at different Cd concentrations.

Content list A5.0

page

Table A5.1 Influence of Na on Cd toxicity to wild-type *Anacystis*.... 267

A5.2 Influence of K on Zn toxicity to wild-type *Anacystis*..... 267

A5.3 Influence of K on Cd toxicity to wild-type *Anacystis*..... 268

A5.4 Influence of Ca on Zn toxicity to wild-type *Anacystis*.... 268

A5.5 Influence of Ca on Zn toxicity to Zn-t5.0 *Anacystis*. 269

A5.6 Influence of Ca on Zn toxicity to Zn-t12.0 *Anacystis*..... 269

A5.7 Influence of Ca on Cd toxicity to wild-type *Anacystis*.... 270

A5.8 Influence of Mn on Zn toxicity to Zn-t5.0 *Anacystis*. 270

A5.9 Influence of Mn on Zn toxicity to Zn-12.0 *Anacystis*. 271

A5.10 Influence of Fe on Zn toxicity to Zn-t5.0 *Anacystis*. 271

A5.11 Influence of Fe on Zn toxicity to Zn-t12.0 *Anacystis*..... 272

A5.12 Influence of Fe on Cu toxicity to wild-type *Anacystis*.... 272

A5.13 Influence of Fe on Cu toxicity to Cu-t0.5 *Anacystis*. 273

A5.14 Influence of PO_4-P on Zn toxitity to wild-type *Anacystis*. 273

A5.15 Influence of PO_4-P on Zn toxicity to Zn-t5.0 *Anacystis*... 274

A5.16 Influence of PO_4-P on Zn toxicity to Zn-t12.0 *Anacystis*.. 274

A5.17 Influence of PO_4-P on Cd toxicity to wild-type *Anacystis*. 275

A5.18 Influence of NO_3-N on Zn toxicity to wild-type *Anacystis*. 275

A5.19 Influence of EDTA on Zn toxicity to wild-type *Anacystis*.. 276

A5.20 Influence of EDTA on Zn toxicity to Zn-t5.0 *Anacystis*.... 276

A5.21 Influence of EDTA on Zn toxicity to Zn-t12.0 *Anacystis*... 277

A5.22 Influence of EDTA on Cd toxicity to wild-type *Anacystis*.. 277

A5.23 Influence of Zn on Cd toxicity to wild-type *Anacystis*.... 278

units ml^{-1} was used as a growth criterion.

page

Table A5.24 Influence of Zn on Cd toxicity to wild-type *Anacystis*; chl *a* was used as a growth criterion. ... 278

A5.25 Influence of low Zn levels on Cd toxicity to Zn-t5.0 *Anacystis*. 279

A5.26 Influence of high Zn levels on Cd toxicity to Zn-t5.0 *Anacystis*. 279

A5.27 Influence of pH on Zn toxicity to wild-type *Anacystis*..... 280

A5.28 Influence of pH on Zn toxicity to Zn-t5.0 *Anacystis*. 280

A5.29 Influence of pH on Zn toxicity to Zn-t12.0 *Anacystis*...... 281

A5.30 Influence of pH on Cd toxicity to wild-type *Anacystis*...... 281

A5.31 Influence of inoculum size on Zn toxicity to wild-type *Anacystis*; units ml^{-1} was used as a growth criterion. 282

A5.32 Influence of inoculum size on Zn toxicity to wild-type *Anacystis*; chl *a* was used as a growth criterion. 282

Content list A6.0

6.1 Accumulation of Zn during batch culture growth of wild-type *Anacystis*. Results show total values for alga + filter. 283

6.2 Accumulation of Zn during batch culture growth of Zn-t12.0 *Anacystis*. Results show total values for alga + filter. 284

Table A3.1 Influence of Co on the growth of Co-tl.8

time (h)	Source of inoculum				
	from strongly inhibitory level of metal				
	Co (mg l^{-1})				
	0	0.3	0.6	1.2	1.8
0	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$
24	$7.5x10^5$	$6.3x10^5$	$4x10^5$	$3.2x10^5$	$2.5x10^5$
48	$3.2x10^6$	$2.8x10^6$	$1.8x10^6$	$8x10^5$	$4x10^5$
72	$2.5x10^7$	$2.2x10^7$	$1.3x10^7$	$2.5x10^6$	$1.3x10^6$
96	$1x10^8$	$8x10^7$	$5x10^7$	$8x10^6$	$3.2x10^6$
120	$1.3x10^8$	$1x10^8$	$8x10^7$	$1.8x10^7$	$5.6x10^6$
144	$1.6x10^8$	$1.4x10^8$	$1x10^8$	$3.2x10^7$	$1.4x10^7$
168	$1.8x10^8$	$1.6x10^8$	$1.1x10^8$	$5x10^7$	$2x10^7$

Table A3.2 Influence of Ni on the growth of Ni-tl.0

time (h)	Source of inoculum				
	from strongly inhibitory level of metal				
	Ni (mg l^{-1})				
	0	0.2	0.4	0.8	1.0
0	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$
24	$8x10^5$	$6.3x10^5$	$5x10^5$	$3.6x10^5$	$2.8x10^5$
48	$5.6x10^6$	$4.5x10^6$	$2.5x10^6$	$1.4x10^6$	$6.3x10^5$
72	$3.2x10^7$	$2.2x10^7$	$1x10^7$	$5.6x10^6$	$2.5x10^6$
96	$1x10^8$	$5.6x10^7$	$3.2x10^7$	$1.8x10^7$	$8x10^6$
120	$1.4x10^8$	$1x10^8$	$5x10^7$	$3.2x10^7$	$1.8x10^7$
144	$1.6x10^8$	$1.1x10^8$	$6.3x10^7$	$4.5x10^7$	$2.8x10^7$
168	$1.8x10^8$	$1.3x10^8$	$8x10^7$	$5.6x10^7$	$4x10^7$

Table A3.3 Influence of Cu on growth of Cu-t0.5

time (h)	Source of inoculum: from strongly inhibitory level of metal, Cu (mg l^{-1})				
	0	0.1	0.2	0.4	0.5
0	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$
24	$8x10^5$	$7.1x10^5$	$5x10^5$	$3.2x10^5$	$2.5x10^5$
48	$6.3x10^6$	$5.6x10^6$	$3.2x10^6$	$1.4x10^6$	$1x10^6$
72	$3.2x10^7$	$2.5x10^7$	$1.6x10^7$	$6.3x10^6$	$2.8x10^6$
96	$1x10^8$	$8x10^7$	$5.6x10^7$	$2x10^7$	$8x10^6$
120	$1.6x10^8$	$1.3x10^8$	$8x10^7$	$3.2x10^7$	$1.6x10^7$
144	$1.6x10^8$	$1.3x10^8$	$1x10^8$	$5x10^7$	$2.5x10^7$
168	$1.8x10^8$	$1.4x10^8$	$1x10^8$	$5.6x10^7$	$3.2x10^7$

Table A3.4 Influence of Zn on the growth of Zn-t5.0

time (h)	Source of inoculum: basal medium, Zn (mg l^{-1})				Source of inoculum: strongly inhibitory level of metal at which adapted, Zn (mg l^{-1})				
	0.04	2.0	4.0	5.0	0.04	2.0	4.0	5.0	6.0
0	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$
24	$2x10^6$	$8x10^5$	$2.5x10^5$	$1.1x10^5$	$2x10^6$	$1.8x10^6$	$3.2x10^5$	$2x10^5$	$2x10^5$
48	$1.1x10^7$	$6x10^6$	$4x10^5$	$1.4x10^5$	$1.8x10^7$	$1.6x10^7$	$5.6x10^6$	$1x10^6$	$2x10^5$
72	$1x10^8$	$4.2x10^7$	$3.2x10^6$	$1.1x10^6$	$8x10^7$	$6.3x10^7$	$3.6x10^7$	$8x10^6$	$2.5x10^5$
96	$4x10^8$	$1.1x10^8$	$2.2x10^7$	$5x10^6$	$2.2x10^8$	$2x10^8$	$1x10^8$	$3.2x10^7$	$3.6x10^5$
120	$5x10^8$	$3x10^8$	$7.1x10^7$	$1x10^7$	$3.6x10^8$	$3.2x10^8$	$1.6x10^8$	$5.6x10^7$	$5.6x10^5$
144	$5.3x10^8$	$4x10^8$	$1.6x10^8$	$4x10^7$	$5.1x10^8$	$4x10^8$	$1.8x10^8$	$6.3x10^7$	$8x10^5$
168	$5.6x10^8$	$4.5x10^8$	$1.5x10^8$	$6x10^7$	$5.6x10^8$	$4.5x10^8$	$2x10^8$	$8x10^7$	$1.1x10^6$

Table A3.5 Influence of Zn on the growth of Zn-t12.0

time (h)	Source of inoculum											
	basal medium						strongly inhibitory level of metal at which adapted					
	Zn (mg l^{-1})						Zn (mg l^{-1})					
	0.04	4	8	10	11	12	0.04	4	8	10	11	12
0	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}
24	1×10^{6}	5.6×10^{5}	3.2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	1.4×10^{6}	4.2×10^{5}	1.8×10^{5}	1.4×10^{5}	1.4×10^{5}	1.1×10^{5}
48	7.1×10^{6}	3.2×10^{6}	1.6×10^{6}	5.6×10^{5}	3.6×10^{5}	2.5×10^{5}	5.1×10^{6}	2×10^{6}	4.2×10^{5}	3.2×10^{5}	1.4×10^{5}	1.1×10^{5}
72	1.8×10^{7}	1×10^{7}	4×10^{6}	1.4×10^{6}	1.4×10^{6}	4×10^{5}	2.2×10^{7}	2×10^{7}	5.1×10^{6}	1.6×10^{6}	1.4×10^{6}	5.1×10^{5}
96	2.8×10^{7}	1.8×10^{7}	8×10^{6}	3.2×10^{6}	1.8×10^{6}	7.1×10^{5}	5.1×10^{7}	4×10^{7}	2.5×10^{7}	1.6×10^{7}	5.6×10^{6}	1.3×10^{6}
120	6.3×10^{7}	4×10^{7}	1.8×10^{7}	5.6×10^{6}	3.6×10^{6}	1.8×10^{6}	8×10^{7}	8×10^{7}	5.6×10^{7}	4.2×10^{7}	1.6×10^{7}	2×10^{6}
144	8×10^{7}	6.3×10^{7}	3.2×10^{7}	1.4×10^{7}	8×10^{6}	4×10^{6}	1.6×10^{8}	1.4×10^{8}	1×10^{8}	7.1×10^{7}	3.2×10^{7}	6.3×10^{6}
168	1.2×10^{8}	1.1×10^{8}	8.8×10^{7}	5.2×10^{7}	3.2×10^{7}	1.0×10^{7}	1.6×10^{8}	1.5×10^{8}	1.1×10^{8}	1×10^{8}	7.1×10^{7}	2.3×10^{7}

Table A3.6 Influence of Cd on growth of Cd-t2.0

time (h)	Source of inoculum				
	from strongly inhibitory level of metal				
	Cd (mg l^{-1})				
	0.0	0.5	1.0	1.5	2.5
0	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}
24	1×10^{6}	7.1×10^{5}	5×10^{5}	3.6×10^{5}	2.8×10^{5}
48	1.3×10^{7}	8×10^{6}	5.6×10^{6}	2.5×10^{6}	8×10^{5}
72	3.6×10^{7}	2.5×10^{7}	1.4×10^{7}	5.6×10^{6}	1.8×10^{6}
96	1×10^{8}	7.1×10^{7}	3.6×10^{7}	1.3×10^{7}	3.2×10^{6}
120	1.3×10^{8}	8×10^{7}	5×10^{7}	2.2×10^{7}	8×10^{6}
144	1.4×10^{8}	1×10^{8}	6.3×10^{7}	3.2×10^{7}	1.4×10^{7}
168	1.8×10^{8}	1.1×10^{8}	7.1×10^{7}	4×10^{7}	2.5×10^{7}

Table A3.7 Influence of Zn and time of harvesting in batch culture on length of wild-type *Anacystis*.

time (h)	Zn (mg l^{-1})	length classes of cells			
		$3 \leq 6.0$	$6.0 \leq 9.0$	$9.0 \leq 12.0$	$12 \leq 18.0$
24	0.04	90 ± 8.2	10 ± 2.6		
	0.5	88 ± 8.5	12 ± 3.0		
	0.75	61 ± 6.6	38.4 ± 6.1		
	1.0	45.4 ± 8.1	53.6 ± 11		
48	0.04	85 ± 5.7	15 ± 3.2		
	0.5	76 ± 10.1	24 ± 4.5		
	0.75	52.3 ± 8.1	47.7 ± 7.5		
	1.0	39.5 ± 7.5	60.5 ± 13.4		
	1.25	27.4 ± 1.3	72.6 ± 9.8		
96	0.04	62 ± 6	38 ± 9.4		
	0.5	50 ± 8.5	50 ± 8.9		
	0.75	24 ± 3.6	76 ± 10.7		
	1.0	16 ± 1.5	84 ± 9.1		
	1.25	0	92.8 ± 7.9	7.2 ± 0.8	
192	0.04	47.4 ± 5	42.6 ± 7.0	0	
	0.5	29.7 ± 5.4	70.3 ± 7.2	0	
	0.75	9.6 ± 2.2	90.4 ± 6.3	0	
	1.0	0	69.2 ± 7.5	30.8 ± 5.6	
	1.25	0	29.4 ± 4.8	70.6 ± 11.5	

Table A3.8 Influence of Zn a-d time of harvesting in batch culture on length of Zn-t5.0; inoculum was taken from basal medium.

time (h)	Zn (mg l^{-1})	length classes of cells			
		9 ≤ 12.0	12 ≤ 24	24 ≤ 36	36 ≤ 48
24	0.04	84.6 ± 10	15.4 ± 3.2		
	1.0	83.6 ± 6.6	16.4 ± 3.3		
	2.0	78 ± 9.2	22 ± 4.8		
	3.0	66.7 ± 14	33.3 ± 7.8		
	4.0	27.4 ± 6	72.6 ± 16.9		
	5.0	16.2 ± 4.5	83.8 ± 13.6		
48	0.04	79.3 ± 8.5	20.7 ± 4.3		
	1.0	71.2 ± 10.4	28.8 ± 5.4		
	2.0	64 ± 13.1	36 ± 8.0		
	3.0	52.7 ± 8.2	47.3 ± 12.8		
	4.0	32.3 ± 9.3	67.7 ± 15.1		
	5.0	25.6 ± 6.4	74.4 ± 12.2		
96	0.04	65.4 ± 10	34.6 ± 10	0	
	1.0	42.7 ± 9	57.3 ± 9.8	0	
	2.0	25.6 ± 6.2	74.4 ± 12.4	0	
	3.0	24.7 ± 6.8	75.3 ± 14.8	0	
	4.0	21.7 ± 5.5	59.7 ± 12.5	18.6 ± 3.2	
	5.0	11.7 ± 3.8	63.1 ± 10.8	25.2 ± 3.5	
192	0.04	61.6 ± 7.2	38.4 ± 8.4	0	
	1.0	33.6 ± 6.5	46 ± 12.5	20.4 ± 3.2	
	2.0	17.4 ± 3.6	52 ± 17.2	30.6 ± 6.6	
	3.0	5.7 ± 1.4	48 ± 11.8	46.3 ± 1.5	
	4.0	0	45 ± 14.1	42.5 ± 3.4	13.5 ± 4.2
	5.0	0	40 ± 13.7	47.6 ± 5.4	12.4 ± 3.7

Table A3.9 Influence of Zn and time of harvesting in batch culture on length of Zn-t5.0; inoculum was taken from strongly inhibitory level.

time (h)	Zn (mg l^{-1})	length classes of cells			
		6≤9.0	9≤12	12≤24	24≤36
24	0.04	21.9 ± 3	78.1 ± 6.7	0	
	1.0	20.30	64.5 ± 2.6	15.2	
	2.0	16.6 ± 4.3	65.1 ± 6.8	18.4 ± 2.5	
	3.0	13.5 ± 5.4	43.2 ± 5.8	29.7 ± 4.2	
	4.0	10 ± 3.6	45.2 ± 7.5	44.8 ± 8.5	
	5.0	8.9 ± 2.5	29.8 ± 8.3	61.3 ± 3.3	
48	0.04	16.8	83.8 ± 9.5	0	
	1.0	0	80.9 ± 10	19.1 ± 6.8	
	2.0	0	70.4 ± 7.8	29.6 ± 3.9	
	3.0	0	61.2 ± 11.2	38.8 ± 7.2	
	4.0	0	42.9 ± 10.6	57.1 ± 4.3	
	5.0		31.8 ± 6.6	68.2 ± 6.5	
96	0.04		86.2 ± 6.8	13.8 ± 1.8	0
	1.0		71.4 ± 7.5	28.6 ± 6.9	0
	2.0		58.3 ± 11	41.7 ± 4.1	0
	3.0		28.4 ± 3.7	38.9 ± 7.2	32.7 ± 6.3
	4.0		13 ± 3.8	42 ± 5.6	45 ± 8.5
	5.0		-	47.2 ± 5.4	52.8 ± 5.3
192	0.04		67.3 ± 7.1	32.7 ± 4.8	0
	1.0		48.4 ± 5.6	51.6 ± 8.2	0
	2.0		21.6 ± 3.8	78.4 ± 7.5	0
	3.0		16.6 ± 4.0	52.4 ± 5.7	31 ± 5.7
	4.0		13.4 ± 3.8	45.6 ± 6.6	41 ± 4.8
	5.0		0	36.2 ± 5.4	63.8 ± 8.1

Table A4.1 Influence of Co on the growth of wild-type

time (h)	Source of inoculum															
	basal medium								strongly inhibitory level of metal							
	Co (mg l^{-1})								Co (mg l^{-1})							
	0	0.02	0.04	0.08	0.1	0.16	0.2	0.32	0	0.02	0.04	0.08	0.10	0.16	0.2	0.4
0	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}
24	1.3×10^{6}	7.1×10^{5}	6.3×10^{5}	4.5×10^{5}	3.6×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	1×10^{6}	7.1×10^{5}	5.6×10^{5}	4×10^{5}	3.2×10^{5}	3.2×10^{5}	3.2×10^{5}	3.2×10^{5}
48	8×10^{6}	5.1×10^{6}	3.6×10^{6}	1.8×10^{6}	1.1×10^{6}	5.6×10^{5}	3.6×10^{5}	2.5×10^{5}	7.1×10^{6}	5×10^{6}	2.5×10^{6}	1.6×10^{6}	8×10^{5}	5.1×10^{5}	4×10^{5}	3.2×10^{5}
72	3.2×10^{7}	2×10^{7}	8×10^{6}	6.3×10^{6}	4×10^{6}	1×10^{6}	6.3×10^{5}	4×10^{5}	2.5×10^{7}	1.8×10^{6}	1×10^{7}	5.6×10^{6}	2.8×10^{6}	1×10^{6}	7.1×10^{5}	5.1×10^{5}
96	9×10^{7}	6.3×10^{7}	2.5×10^{7}	2×10^{7}	1.1×10^{7}	2.5×10^{6}	1×10^{6}	5.1×10^{5}	7.1×10^{7}	5.1×10^{7}	3.2×10^{7}	1.8×10^{7}	8×10^{6}	3.2×10^{6}	1.6×10^{6}	7.1×10^{5}
120	1.3×10^{8}	8×10^{7}	6.3×10^{7}	3.6×10^{7}	2×10^{7}	4×10^{6}	3.6×10^{6}	6.3×10^{5}	1.6×10^{6}	1×10^{8}	5.1×10^{7}	3.2×10^{7}	1.6×10^{7}	7.1×10^{6}	4×10^{6}	1.8×10^{6}
144	1.6×10^{8}	1.3×10^{8}	8×10^{7}	5.6×10^{7}	3.2×10^{7}	6.3×10^{6}	1.8×10^{6}	8×10^{6}	2×10^{8}	1.3×10^{8}	8×10^{7}	5.1×10^{7}	2.5×10^{7}	1×10^{7}	5.6×10^{6}	2.5×10^{6}
168	2.5×10^{8}	1.6×10^{8}	1×10^{8}	6.3×10^{7}	4×10^{7}	1×10^{7}	3.2×10^{6}	1.3×10^{6}	2.5×10^{8}	1.8×10^{8}	1.3×10^{8}	8×10^{7}	4.5×10^{7}	1.8×10^{7}	9×10^{6}	3.6×10^{6}

Table A4.2 Influence of Co on the growth of Ni-t1.0

time (h)	Source of inoculum												
	basal medium							strongly inhibitory level of metal at which adapted					
	Co (mg l^{-1})							Co (mg l^{-1})					
	0	0.05	0.1	0.2	0.4	0.6	0.8	0	0.05	0.1	0.2	0.4	0.6
0	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}
24	1.3×10^{6}	8×10^{5}	6.3×10^{5}	5.6×10^{5}	4×10^{5}	2×10^{5}	1.8×10^{5}	1.1×10^{6}	1×10^{6}	7.1×10^{5}	4×10^{5}	2.5×10^{5}	1.8×10^{5}
48	1.1×10^{7}	8×10^{6}	4×10^{6}	3.2×10^{6}	1.3×10^{6}	3.2×10^{5}	1.6×10^{5}	1.1×10^{7}	8×10^{6}	3.6×10^{6}	1×10^{6}	4.5×10^{5}	1.3×10^{5}
72	5×10^{7}	4.5×10^{7}	1.6×10^{7}	1×10^{7}	2.5×10^{6}	3.6×10^{5}	1×10^{5}	3.8×10^{7}	2.5×10^{7}	1×10^{7}	3.2×10^{6}	8×10^{5}	2×10^{5}
96	8×10^{7}	7.1×10^{7}	4×10^{7}	2.5×10^{7}	4×10^{6}	6.3×10^{5}	1.3×10^{5}	1×10^{8}	6.3×10^{7}	2.5×10^{7}	6.3×10^{6}	1.6×10^{6}	3.6×10^{5}
120	1.6×10^{8}	1.3×10^{8}	8×10^{7}	6.3×10^{7}	8×10^{6}	1×10^{6}	1.8×10^{5}	1.6×10^{8}	8×10^{7}	3.6×10^{7}	1.8×10^{7}	2.5×10^{6}	5×10^{5}
144	2.5×10^{8}	1.6×10^{8}	1.1×10^{8}	8×10^{7}	1.8×10^{7}	2.5×10^{6}	2×10^{5}	2.5×10^{8}	1.3×10^{8}	6.3×10^{7}	3.6×10^{7}	5.6×10^{6}	7.1×10^{5}
168	3.2×10^{8}	2.5×10^{8}	1.4×10^{8}	1.3×10^{8}	2.8×10^{7}	5.6×10^{6}	4×10^{5}	2.8×10^{5}	1.8×10^{8}	8×10^{7}	4.5×10^{7}	1.3×10^{7}	1×10^{6}

Table A4.3 Influence of Co on the growth of Cu-t0.5

time (h)	Source of inoculum											
	basal medium						strongly inhibitory level of metal to which adapted					
	Co (mg l^{-1})						Co (mg l^{-1})					
	0	0.05	0.1	0.2	0.4	0.6	0	0.05	0.1	0.2	0.4	0.6
0	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}
24	1×10^{6}	7.1×10^{5}	4.5×10^{5}	3.2×10^{5}	2.5×10^{5}	2.3×10^{5}	1×10^{6}	8×10^{5}	4×10^{5}	2.5×10^{5}	2×10^{5}	1.4×10^{5}
48	6.3×10^{6}	3.2×10^{6}	1×10^{6}	5.6×10^{5}	4×10^{5}	2.5×10^{5}	6.3×10^{6}	3.2×10^{6}	1×10^{6}	4×10^{5}	2.5×10^{5}	1.4×10^{5}
72	3.2×10^{7}	1.5×10^{7}	4×10^{6}	2×10^{6}	8×10^{5}	3.6×10^{5}	3.2×10^{7}	1.8×10^{7}	3.2×10^{6}	8×10^{5}	3.2×10^{5}	1.4×10^{5}
96	1.8×10^{8}	1.3×10^{8}	1.3×10^{7}	4.5×10^{6}	1.6×10^{6}	5.1×10^{5}	1×10^{8}	5.1×10^{7}	6.3×10^{6}	1.4×10^{6}	5×10^{5}	d
120	2.5×10^{8}	2×10^{8}	2.5×10^{7}	1.3×10^{7}	2.5×10^{6}	7.1×10^{5}	1.6×10^{8}	1×10^{8}	1.8×10^{7}	2.5×10^{6}	8×10^{5}	d
144	3.2×10^{8}	2.5×10^{8}	5×10^{7}	2×10^{7}	6.3×10^{6}	1.3×10^{6}	1.8×10^{8}	1.4×10^{8}	3.2×10^{7}	5×10^{6}	1.4×10^{6}	d
168	3.6×10^{8}	2.5×10^{8}	8×10^{7}	4×10^{7}	1×10^{7}	2×10^{6}	1.8×10^{8}	1.6×10^{8}	3.6×10^{7}	8×10^{6}	2.5×10^{6}	d

Table A4.4 Influence of Co on the growth of Zn-t5.0

time (h)	Source of inoculum											
	basal medium						strongly inhibitory level of metal at which adapted					
	Co (mg l^{-1})						Co (mg l^{-1})					
	0	0.05	0.1	0.2	0.3	0.4	0	0.05	0.1	0.2	0.3	0.4
0	1.8×10^{5}	1.8×10^{5}	1.8×10^{5}	1.8×10^{5}	1.8×10^{5}	1.8×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}
24	1×10^{6}	5×10^{5}	4×10^{5}	3.6×10^{5}	2×10^{5}	1.8×10^{5}	1×10^{6}	5.6×10^{5}	4×10^{5}	3.2×10^{5}	2.5×10^{5}	2×10^{5}
48	8×10^{6}	2.8×10^{6}	1.3×10^{6}	8×10^{5}	2.5×10^{5}	1.8×10^{5}	8×10^{6}	2.5×10^{6}	1.3×10^{6}	8×10^{5}	3.6×10^{5}	2.5×10^{5}
72	3.3×10^{7}	1.3×10^{7}	6.3×10^{6}	3.2×10^{6}	1×10^{6}	2×10^{5}	2.8×10^{7}	1.2×10^{7}	6.3×10^{6}	2.8×10^{6}	1×10^{6}	5×10^{5}
96	1.1×10^{8}	4×10^{7}	1.8×10^{7}	1×10^{7}	2.5×10^{6}	4×10^{5}	1×10^{8}	6.3×10^{7}	2.5×10^{7}	1.1×10^{7}	2.5×10^{6}	1.3×10^{6}
120	1.8×10^{8}	8×10^{7}	4.0×10^{7}	2.5×10^{7}	6.3×10^{6}	1×10^{6}	1.6×10^{8}	1×10^{8}	4.5×10^{7}	3.1×10^{7}	8×10^{6}	2.8×10^{6}
144	2×10^{8}	1.3×10^{8}	8×10^{7}	4×10^{7}	1.1×10^{7}	1.6×10^{6}	2×10^{8}	1.6×10^{8}	8×10^{7}	4.5×10^{7}	1.8×10^{7}	4.5×10^{6}
168	3.1×10^{8}	1.6×10^{8}	1×10^{8}	9×10^{7}	1.6×10^{7}	2.5×10^{6}	2.8×10^{8}	1.8×10^{8}	1×10^{8}	6.3×10^{7}	2.5×10^{7}	7.1×10^{6}

Table A4.5 Influence of Co on the growth of Zn-t12.0

time (h)	Source of inoculum											
	basal medium						strongly inhibitory level of metal at which adapted					
	Co (mg l^{-1})						Co (mg l^{-1})					
	0	0.05	0.10	0.20	0.30	0.40	0	0.05	0.10	0.20	0.30	0.40
0	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}
24	1.0×10^{6}	8×10^{5}	4.5×10^{5}	4×10^{5}	2.5×10^{5}	2×10^{5}	1.0×10^{6}	5.6×10^{5}	3.2×10^{5}	2.5×10^{5}	2×10^{5}	1.8×10^{5}
48	1.3×10^{7}	2.5×10^{6}	1.8×10^{6}	9×10^{5}	2.5×10^{5}	2×10^{5}	1.1×10^{7}	1.3×10^{6}	1×10^{6}	4.5×10^{5}	2.8×10^{5}	1.8×10^{5}
72	4×10^{7}	1.3×10^{7}	6.3×10^{6}	2×10^{6}	4×10^{5}	2×10^{5}	4×10^{7}	9×10^{6}	2.5×10^{6}	6.3×10^{5}	4.1×10^{5}	2.5×10^{5}
96	1×10^{8}	4×10^{7}	2.5×10^{7}	4×10^{6}	6.3×10^{5}	2.8×10^{5}	1×10^{8}	3.2×10^{7}	6.3×10^{6}	1.8×10^{6}	1×10^{6}	2.8×10^{5}
120	1.8×10^{8}	6.3×10^{7}	4×10^{7}	8×10^{6}	1×10^{6}	3.6×10^{5}	1.8×10^{8}	5×10^{7}	1.1×10^{7}	2.8×10^{6}	1.4×10^{6}	2.8×10^{5}
144	2.5×10^{8}	9×10^{7}	6.3×10^{7}	1.3×10^{7}	2×10^{6}	4×10^{5}	2.6×10^{8}	6.3×10^{7}	2×10^{7}	8×10^{6}	2.8×10^{6}	6.3×10^{5}
168	2.8×10^{8}	1×10^{8}	8×10^{7}	2×10^{7}	2.6×10^{6}	6.3×10^{5}	3.2×10^{8}	1×10^{8}	4×10^{7}	1.3×10^{7}	4×10^{6}	1.4×10^{5}

Table A4.6 Influence of Co on the growth of Cd-t2.0

time (h)	Source of inoculum											
	basal medium					strongly inhibitory level of metal at which adapted						
	Co (mg l^{-1})					Co (mg l^{-1})						
	0	0.2	0.4	0.6	0.8	0	0.2	0.4	0.6	0.8	1.0	1.5
0	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}
24	1.3×10^{6}	9×10^{5}	5.6×10^{5}	3.6×10^{5}	1.8×10^{5}	1×10^{6}	1×10^{6}	8×10^{5}	6.3×10^{5}	4×10^{5}	2.5×10^{5}	1×10^{5}
48	1.3×10^{7}	8.8×10^{6}	2.5×10^{6}	8×10^{5}	2.5×10^{5}	1.1×10^{7}	1×10^{7}	4.5×10^{6}	3.2×10^{6}	9×10^{5}	2.5×10^{5}	d
72	3.2×10^{7}	2.5×10^{7}	8×10^{6}	1.3×10^{6}	3.2×10^{5}	3.2×10^{7}	4×10^{7}	1.8×10^{7}	8×10^{6}	1.6×10^{6}	4×10^{5}	d
96	8×10^{7}	5×10^{7}	2.5×10^{7}	2.8×10^{6}	5.6×10^{5}	9×10^{7}	5.6×10^{7}	4×10^{7}	2.5×10^{7}	3.2×10^{6}	7.1×10^{5}	d
120	1.3×10^{8}	7.1×10^{7}	4×10^{7}	6.3×10^{6}	1.3×10^{6}	1.6×10^{8}	8×10^{7}	5.6×10^{7}	4×10^{7}	5×10^{6}	1.3×10^{6}	d
144	1.8×10^{8}	1.1×10^{8}	6.3×10^{7}	1.3×10^{7}	1.6×10^{6}	1.8×10^{8}	1×10^{8}	8×10^{7}	5.6×10^{7}	1.3×10^{7}	1.8×10^{6}	d
168	2.8×10^{8}	1.3×10^{8}	8×10^{7}	1.8×10^{7}	2×10^{6}	2.5×10^{8}	1.6×10^{8}	8×10^{7}	6.3×10^{7}	2.5×10^{7}	2.5×10^{6}	d

Table A4.7 Influence of Ni on the growth of Wild-type

time (h)	Source of inoculum															
	basal medium								strongly inhibitory level of metal							
	Ni (mg l^{-1})								Ni (mg l^{-1})							
	0	0.02	0.04	0.08	0.1	0.16	0.2	0.24	0	0.02	0.04	0.08	0.1	0.16	0.2	0.24
0	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}
24	1×10^{6}	6.3×10^{5}	5.1×10^{5}	2.5×10^{5}	2.2×10^{5}	2×10^{5}	1.3×10^{5}	d	1.3×10^{6}	7.1×10^{5}	5×10^{5}	4×10^{5}	3.2×10^{5}	2.8×10^{5}	2.2×10^{5}	d
48	8×10^{6}	4.5×10^{6}	2.8×10^{6}	6.3×10^{5}	5.1×10^{5}	2.5×10^{5}	1.3×10^{5}	d	7.2×10^{6}	2.5×10^{6}	1.6×10^{6}	8×10^{5}	5.6×10^{5}	5×10^{5}	3.6×10^{5}	d
72	3.2×10^{7}	1.8×10^{7}	8×10^{6}	2.5×10^{6}	1.3×10^{6}	2.5×10^{5}	1.8×10^{5}	d	2.2×10^{7}	1×10^{7}	5.6×10^{6}	1.8×10^{6}	1.6×10^{6}	8×10^{5}	6.3×10^{5}	d
96	8×10^{7}	4.5×10^{7}	2.5×10^{7}	1×10^{7}	4×10^{6}	6.3×10^{5}	2.5×10^{5}	d	8×10^{7}	3.2×10^{7}	1.6×10^{7}	4×10^{6}	3.6×10^{6}	1.8×10^{6}	1.3×10^{6}	d
120	1×10^{8}	6.3×10^{7}	3.6×10^{7}	1.8×10^{7}	8×10^{6}	1.6×10^{6}	4×10^{5}	d	1.3×10^{8}	5.6×10^{7}	3.2×10^{7}	1.6×10^{7}	8×10^{6}	3.2×10^{6}	2.2×10^{6}	d
144	1.1×10^{8}	8×10^{7}	5×10^{7}	2.5×10^{7}	1.1×10^{7}	2.5×10^{6}	4×10^{5}	d	2×10^{8}	1×10^{8}	6.3×10^{7}	2.8×10^{7}	1.6×10^{7}	5.6×10^{6}	3.6×10^{6}	d
168	1.6×10^{8}	8.2×10^{7}	5.6×10^{7}	3.6×10^{7}	1.8×10^{7}	5×10^{6}	8×10^{5}	d	2.8×10^{8}	1.6×10^{8}	1×10^{8}	5×10^{7}	2.5×10^{7}	1×10^{7}	5.6×10^{6}	d

Table A4.8 Influence of Ni on the growth of Co-t1.8

time (h)	Source of inoculum									
	basal medium					strongly inhibitory level of metal at which adapted				
	Ni (mg l^{-1})					Ni (mg l^{-1})				
	0	0.05	0.1	0.15	0.2	0	0.05	0.1	0.15	0.2
0	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}
24	1.2×10^{6}	1×10^{6}	8×10^{5}	4×10^{5}	2.5×10^{5}	1.3×10^{6}	8×10^{5}	6.2×10^{5}	3.6×10^{5}	2.5×10^{5}
48	1.3×10^{7}	6.3×10^{6}	4×10^{6}	1×10^{6}	4×10^{5}	1×10^{7}	4×10^{6}	1.3×10^{6}	9×10^{5}	4×10^{5}
72	4.5×10^{7}	2.5×10^{7}	1×10^{7}	2.5×10^{6}	5×10^{5}	4.6×10^{7}	1.1×10^{7}	8×10^{6}	3.2×10^{6}	4.5×10^{5}
96	1.1×10^{8}	6.3×10^{7}	2.8×10^{7}	6.3×10^{6}	5.6×10^{5}	8×10^{7}	3.2×10^{7}	2×10^{7}	8×10^{6}	8×10^{5}
120	1.8×10^{8}	1.3×10^{8}	5×10^{7}	2×10^{7}	8×10^{5}	1.6×10^{8}	6.3×10^{7}	4×10^{7}	1.6×10^{7}	1.3×10^{6}
144	3.2×10^{8}	1.6×10^{8}	9×10^{7}	2.8×10^{7}	1.3×10^{6}	2.8×10^{8}	9×10^{7}	7.1×10^{7}	2.8×10^{7}	2.5×10^{6}
168	3.2×10^{8}	2×10^{8}	1.1×10^{8}	4×10^{7}	2.5×10^{6}	3.6×10^{8}	1.4×10^{8}	1×10^{8}	3.6×10^{7}	4×10^{6}

Table A4.9 Influence of Ni on the growth of Cu-t0.5

time (h)	Source of inoculum									
	basal medium					strongly inhibitory level of metal at which adapted				
	Ni (mg l^{-1})					Ni (mg l^{-1})				
	0	0.05	0.1	0.15	0.2	0	0.05	0.1	0.15	0.2
0	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}
24	1.1×10^{6}	6.3×10^{5}	4×10^{5}	3.2×10^{5}	2×10^{5}	1×10^{6}	7.1×10^{5}	4×10^{5}	2.5×10^{5}	2×10^{5}
48	1×10^{7}	4×10^{6}	1×10^{6}	7.1×10^{5}	2.5×10^{5}	1×10^{7}	5.1×10^{6}	2×10^{6}	8×10^{5}	2.5×10^{5}
72	3.6×10^{7}	1×10^{7}	3.2×10^{6}	1.6×10^{6}	4×10^{5}	3.4×10^{7}	2.5×10^{7}	6.3×10^{6}	2.5×10^{6}	3.2×10^{5}
96	1×10^{8}	3.2×10^{7}	5.6×10^{6}	3.2×10^{6}	7.1×10^{5}	1×10^{8}	5×10^{7}	1.4×10^{7}	5×10^{6}	4×10^{5}
120	1.8×10^{8}	6.3×10^{7}	1.4×10^{7}	6.3×10^{6}	1×10^{6}	1.8×10^{8}	8×10^{7}	2.5×10^{7}	1×10^{7}	4.2×10^{5}
144	2.5×10^{8}	8×10^{7}	2.5×10^{7}	1×10^{7}	1.6×10^{6}	3.2×10^{8}	1.6×10^{8}	4×10^{7}	1.1×10^{7}	6.3×10^{5}
168	3.2×10^{8}	1×10^{8}	3.6×10^{7}	1.8×10^{7}	2.5×10^{6}	3.2×10^{8}	1.8×10^{8}	6.3×10^{7}	2.5×10^{7}	1.1×10^{6}

Table A4.10 Influence of Ni on the growth of Zn-t5.0

time (h)	Source of inoculum											
	basal medium						strongly inhibitory level of metal at which adapted					
	Ni (mg l^{-1})						Ni (mg l^{-1})					
	0	0.05	0.1	0.2	0.22	0.25	0	0.05	0.1	0.2	0.22	0.25
0	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}
24	1×10^{6}	5.6×10^{5}	4×10^{5}	2.5×10^{5}	1.8×10^{5}	1.8×10^{5}	1×10^{6}	8×10^{5}	4×10^{5}	2.5×10^{5}	1.8×10^{5}	1.8×10^{5}
48	8×10^{6}	2.8×10^{6}	7.1×10^{5}	3.2×10^{5}	2.5×10^{5}	1.8×10^{5}	1×10^{7}	3.6×10^{6}	8×10^{5}	3.6×10^{5}	1.8×10^{5}	d
72	3.6×10^{7}	1.4×10^{7}	2.8×10^{6}	6.3×10^{5}	4.5×10^{5}	2×10^{5}	4×10^{7}	1.3×10^{7}	2×10^{6}	5.8×10^{5}	2.5×10^{5}	d
96	1×10^{8}	2.8×10^{7}	5×10^{6}	1.3×10^{6}	8×10^{5}	2.5×10^{5}	1×10^{8}	2×10^{7}	3.1×10^{6}	8×10^{5}	3.2×10^{5}	d
120	1.8×10^{8}	5×10^{7}	1.3×10^{7}	2.5×10^{6}	1.3×10^{6}	3.6×10^{5}	1.8×10^{8}	4.5×10^{7}	7.2×10^{6}	1.6×10^{6}	4×10^{5}	d
144	2×10^{8}	8×10^{7}	1.8×10^{7}	3.6×10^{6}	1.8×10^{6}	4.5×10^{5}	2.6×10^{8}	6.3×10^{7}	1.8×10^{7}	2.5×10^{6}	7.1×10^{5}	d
168	3.2×10^{8}	1.3×10^{8}	3.6×10^{7}	6.3×10^{6}	2.5×10^{6}	7.1×10^{5}	3.1×10^{8}	1×10^{8}	3.2×10^{7}	5.1×10^{6}	8×10^{5}	d

Table A4.11 Influence of Ni on the growth of Zn-t12.0

time (h)	Source of inoculum									
	basal medium					strongly inhibitory level of metal at which adapted				
	Ni (mg l^{-1})					Ni (mg l^{-1})				
	0	0.05	0.10	0.15	0.2	0	0.05	0.10	0.15	0.2
0	2x10^{5}	2x10^{5}	2x10^{5}	2x10^{5}	2x10^{5}	2x10^{5}	2x10^{5}	2x10^{5}	2x10^{5}	2x10^{5}
24	1.8x10^{6}	6.3x10^{5}	3.6x10^{5}	2.5x10^{5}	1.8x10^{5}	1.8x10^{6}	5.5x10^{5}	2.5x10^{5}	1.8x10^{5}	1.1x10^{5}
48	1.3x10^{7}	4.5x10^{6}	8x10^{5}	4x10^{5}	1.8x10^{5}	1.1x10^{7}	4x10^{6}	5.6x10^{5}	2.5x10^{5}	1.0x10^{5}
72	4x10^{7}	8x10^{6}	2.5x10^{6}	8x10^{5}	2x10^{5}	4x10^{7}	7.1x10^{6}	1.3x10^{6}	3.2x10^{5}	d
96	1x10^{8}	2.5x10^{7}	4.5x10^{6}	1.8x10^{6}	2x10^{5}	1x10^{8}	1.8x10^{7}	2x10^{6}	4x10^{5}	d
120	1.8x10^{8}	4x10^{7}	1.3x10^{7}	2.8x10^{6}	3.6x10^{5}	1.6x10^{8}	3.6x10^{7}	4x10^{6}	5.6x10^{5}	d
144	2.8x10^{8}	6.3x10^{7}	1.8x10^{7}	4.1x10^{6}	5.6x10^{5}	2.8x10^{8}	8x10^{7}	5.6x10^{6}	1x10^{6}	d
168	3.2x10^{8}	1x10^{8}	3.2x10^{7}	6.3x10^{6}	1x10^{6}	2.8x10^{8}	1.1x10^{8}	1.3x10^{7}	1.6x10^{6}	d

Table A4.12 Influence of Ni on the growth of Cd-t2.0

time (h)	Source of inoculum											
	basal medium						strongly inhibitory level of metal at which adapted					
	Ni (mg l^{-1})						Ni (mg l^{-1})					
	0	0.05	0.10	0.15	0.2	0.25	0	0.05	0.1	0.2	0.25	0.3
0	2x10^{5}	2x10^{5}	2x10^{5}	2x10^{5}	2x10^{5}	2x10^{5}	2x10^{5}	2x10^{5}	2x10^{5}	2x10^{5}	2x10^{5}	2x10^{5}
24	1.3x10^{6}	9x10^{5}	6.3x10^{5}	4x10^{5}	2.5x10^{5}	1x10^{5}	1.3x10^{6}	8x10^{5}	5.6x10^{5}	5.6x10^{5}	2.5x10^{5}	1.8x10^{5}
48	1x10^{7}	5.6x10^{6}	4x10^{6}	1.3x10^{6}	2.5x10^{5}	1.2x10^{5}	1.8x10^{7}	6.3x10^{6}	4x10^{6}	2x10^{6}	5x10^{5}	1.8x10^{5}
72	3.2x10^{7}	1.6x10^{7}	8.8x10^{6}	2.5x10^{6}	3.2x10^{5}	d	3.6x10^{7}	2.8x10^{7}	1.8x10^{7}	1x10^{7}	9x10^{5}	2.5x10^{5}
96	1x10^{8}	4x10^{7}	1.6x10^{7}	7.1x10^{6}	7.1x10^{5}	d	1x10^{8}	5.6x10^{7}	4x10^{7}	1.6x10^{7}	1.6x10^{6}	4x10^{5}
120	1.8x10^{8}	8x10^{7}	4x10^{7}	1.6x10^{7}	1.3x10^{6}	d	1.8x10^{8}	1.1x10^{8}	8x10^{7}	2.8x10^{7}	2.5x10^{6}	5x10^{5}
144	2.5x10^{8}	1.6x10^{8}	8x10^{7}	3.6x10^{7}	2.5x10^{6}	d	2.5x10^{8}	1.4x10^{8}	1x10^{8}	4x10^{7}	4x10^{6}	7.1x10^{5}
168	3.2x10^{8}	1.8x10^{8}	1x10^{8}	5x10^{7}	3.6x10^{6}	d	3.2x10^{8}	1.3x10^{8}	1.1x10^{8}	5.6x10^{7}	6.3x10^{6}	1x10^{6}

Table A4.13 Influence of Cu on the growth of wild-type

time (h)	Source of inoculum															
	basal medium								strongly inhibitory level of metal							
	Cu (mg l^{-1})								Cu (mg l^{-1})							
	0	0.01	0.02	0.04	0.08	0.1	0.12	0.16	0	0.01	0.02	0.04	0.08	0.1	0.12	0.16
0	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}
24	1×10^{6}	6.3×10^{5}	4×10^{5}	3.2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	1×10^{6}	7.1×10^{5}	4×10^{5}	3.2×10^{5}	2.5×10^{5}	2.5×10^{5}	2.5×10^{5}	1.8×10^{5}
48	6.3×10^{6}	2.5×10^{6}	1.3×10^{6}	5.6×10^{5}	4×10^{5}	2.5×10^{5}	2.5×10^{5}	1.3×10^{5}	5.6×10^{6}	4×10^{6}	8×10^{5}	4.5×10^{5}	4.3×10^{5}	2.5×10^{5}	2.2×10^{5}	1.8×10^{5}
72	2.8×10^{7}	1.6×10^{7}	6.3×10^{6}	4×10^{6}	1.6×10^{6}	6.3×10^{5}	3.2×10^{5}	1.6×10^{5}	2.4×10^{7}	1.6×10^{7}	4×10^{6}	2.2×10^{6}	1.4×10^{6}	1×10^{6}	4×10^{5}	2.5×10^{5}
96	1×10^{8}	6.3×10^{7}	3.6×10^{7}	2×10^{7}	1×10^{7}	2×10^{6}	3.6×10^{5}	2×10^{5}	7.1×10^{7}	5×10^{7}	2.8×10^{7}	1.4×10^{7}	5.6×10^{6}	2.8×10^{6}	1.3×10^{6}	4.5×10^{5}
120	1.3×10^{8}	1×10^{8}	5.6×10^{7}	4×10^{7}	1.8×10^{7}	4×10^{6}	6.3×10^{5}	2.5×10^{5}	1.3×10^{8}	7.1×10^{7}	5×10^{7}	2.8×10^{7}	1.4×10^{7}	5.6×10^{6}	2.8×10^{6}	1.1×10^{6}
144	1.8×10^{8}	1.1×10^{8}	6.3×10^{7}	5×10^{7}	2.3×10^{7}	7.1×10^{6}	1.3×10^{6}	3.6×10^{5}	1.6×10^{8}	1.3×10^{8}	8×10^{7}	5×10^{7}	2.2×10^{7}	1.1×10^{7}	6.3×10^{6}	2.5×10^{6}
168	2×10^{8}	1.2×10^{8}	7.1×10^{7}	5.1×10^{7}	2.8×10^{7}	1×10^{7}	2×10^{6}	5.6×10^{5}	2×10^{8}	1.4×10^{8}	9×10^{7}	5.1×10^{7}	3.8×10^{7}	2×10^{7}	1×10^{7}	4×10^{6}

Table A4.14 Influence of Cu on the growth of Co-tl.8

time (h)	Source of inoculum									
	basal medium					strongly inhibitory level of metal at which adapted				
	Cu (mg l^{-1})					Cu (mg l^{-1})				
	0	0.05	0.1	0.15	0.2	0	0.05	0.1	0.15	0.2
0	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}
24	1.6×10^{6}	9×10^{5}	5×10^{5}	2.5×10^{5}	d	1.2×10^{6}	6.3×10^{5}	4×10^{5}	2.5×10^{5}	d
48	1.1×10^{7}	3.6×10^{6}	1.3×10^{6}	5×10^{5}	d	1×10^{7}	1.4×10^{6}	8×10^{5}	3.6×10^{5}	d
72	5×10^{7}	8×10^{6}	3.2×10^{6}	7.1×10^{5}	d	4.5×10^{7}	3.6×10^{6}	2×10^{6}	6.3×10^{5}	d
96	1×10^{8}	2×10^{7}	8×10^{6}	1.3×10^{6}	d	1×10^{8}	8×10^{6}	4.5×10^{6}	1.3×10^{6}	d
120	1.6×10^{8}	5×10^{7}	1.8×10^{7}	2×10^{6}	d	1.8×10^{8}	2×10^{7}	8×10^{6}	2.5×10^{6}	d
144	3.2×10^{8}	7.1×10^{7}	3.6×10^{7}	3.6×10^{6}	d	3.2×10^{8}	3.6×10^{7}	1.8×10^{7}	3.6×10^{6}	d
168	3.6×10^{8}	1.1×10^{8}	6.3×10^{7}	5.6×10^{6}	d	3.2×10^{8}	6.3×10^{7}	2.5×10^{7}	4×10^{6}	d

Table A 4.15 Influence of Cu on the growth of Ni-t1.0

time (h)	Source of inoculum									
	basal medium					strongly inhibitory level of metal at which adapted				
	Cu (mg l^{-1})					Cu (mg l^{-1})				
	0	0.05	0.10	0.15	0.20	0	0.05	0.10	0.15	0.20
0	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}
24	1.1×10^{6}	9×10^{5}	5×10^{5}	2.2×10^{5}	2.2×10^{5}	1.4×10^{6}	6.3×10^{5}	3.6×10^{5}	2.5×10^{5}	d
48	1×10^{7}	3.6×10^{6}	1×10^{6}	2.5×10^{5}	1.8×10^{5}	1.1×10^{7}	2.8×10^{6}	4.5×10^{5}	1.8×10^{5}	d
72	4×10^{7}	1.3×10^{7}	2.5×10^{6}	4×10^{5}	1.8×10^{5}	3.6×10^{7}	6.3×10^{6}	8×10^{5}	1.3×10^{5}	d
96	1×10^{8}	2.5×10^{7}	5.6×10^{6}	3.2×10^{5}	1.6×10^{5}	8×10^{7}	1×10^{7}	1.0×10^{6}	1.3×10^{5}	d
120	1.8×10^{8}	5×10^{7}	1.1×10^{7}	8×10^{5}	3.2×10^{5}	1.3×10^{8}	1.4×10^{7}	1.6×10^{6}	1.8×10^{5}	d
144	2×10^{8}	8×10^{7}	2.5×10^{7}	1.6×10^{6}	4×10^{5}	2.8×10^{8}	3.6×10^{7}	2.5×10^{6}	2.5×10^{5}	d
168	3.6×10^{8}	1.1×10^{8}	3.6×10^{7}	3.2×10^{6}	6.3×10^{5}	3.2×10^{8}	6.3×10^{7}	4×10^{6}	2.5×10^{5}	d

Table A4.16 Influence of Cu on the growth of Zn-t5.0

time (h)	Source of inoculum											
	basal medium						strongly inhibitory level of metal at which adapted					
	Cu (mg l^{-1})						Cu (mg l^{-1})					
	0	0.05	0.1	0.2	0.25	0.3	0	0.05	0.1	0.2	0.25	0.3
0	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}
24	1.3×10^{6}	7×10^{5}	5.6×10^{5}	2.5×10^{5}	1.7×10^{5}	1.7×10^{5}	1×10^{6}	6.5×10^{5}	2.5×10^{5}	2×10^{5}	2×10^{5}	1.5×10^{5}
48	1.0×10^{7}	7.9×10^{6}	2×10^{6}	3.2×10^{5}	1.7×10^{5}	1.0×10^{5}	8×10^{6}	3×10^{6}	4.5×10^{5}	3.1×10^{5}	2×10^{5}	d
72	4×10^{7}	2×10^{7}	6×10^{6}	4.6×10^{5}	2.8×10^{5}	d	2.8×10^{7}	1.6×10^{7}	1.3×10^{6}	5×10^{5}	2.2×10^{5}	d
96	2×10^{8}	1×10^{8}	2×10^{7}	1×10^{6}	5.6×10^{5}	d	1×10^{8}	4×10^{7}	4.5×10^{6}	1×10^{6}	2.8×10^{5}	d
120	2.5×10^{8}	2×10^{8}	7×10^{7}	2.8×10^{6}	1.6×10^{6}	d	1.6×10^{8}	8×10^{7}	1×10^{7}	1.7×10^{6}	6.3×10^{5}	d
144	3.2×10^{8}	2.5×10^{8}	1.2×10^{8}	8×10^{6}	3×10^{6}	d	2×10^{8}	1×10^{8}	2.5×10^{7}	4×10^{6}	8×10^{5}	d
168	3.2×10^{8}	3.2×10^{8}	1.6×10^{8}	2×10^{7}	5.6×10^{6}	d	2.8×10^{8}	1.6×10^{8}	4.5×10^{7}	7.1×10^{6}	1.1×10^{6}	d

Table A4.17 Influence of Cu on the growth of Zn-t12 o

time (h)	Source of inoculum									
	basal medium					strongly inhibitory level of metal at which adapted				
	Cu (mg l^{-1})					Cu (mg l^{-1})				
	0	0.05	0.10	0.20	0.25	0	0.05	0.10	0.20	0.25
0	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}
24	1.0×10^{6}	8×10^{5}	4×10^{5}	2.5×10^{5}	1.8×10^{5}	8×10^{5}	5×10^{5}	2.5×10^{5}	1×10^{5}	1×10^{5}
48	1×10^{7}	5.6×10^{6}	1.3×10^{6}	2.8×10^{5}	2×10^{5}	1×10^{7}	3.2×10^{6}	6.3×10^{5}	1×10^{5}	d
72	2.5×10^{7}	9×10^{6}	3.2×10^{6}	4×10^{5}	2.5×10^{5}	2×10^{7}	8×10^{6}	1.3×10^{6}	1.3×10^{5}	d
96	1×10^{8}	2×10^{7}	8×10^{6}	8×10^{5}	4×10^{5}	5×10^{7}	1.4×10^{7}	3.6×10^{6}	2.5×10^{5}	d
120	1.6×10^{8}	3.6×10^{7}	1.3×10^{7}	2.8×10^{6}	8×10^{5}	1.10^{8}	3.6×10^{7}	8×10^{6}	4×10^{5}	d
144	2.[illegible]$\times10^{8}$	6.3×10^{7}	2.5×10^{7}	8×10^{6}	1.4×10^{6}	1.8×10^{8}	6.3×10^{7}	1.4×10^{7}	8×10^{5}	d
168	2.8×10^{8}	9×10^{7}	4.5×10^{7}	1.4×10^{7}	5×10^{6}	2×10^{8}	8×10^{7}	2×10^{7}	1.3×10^{6}	d

Table A4.18 Influence of Cu on the growth of Cd-t20

time (h)	Source of inoculum											
	basal medium						strongly inhibitory level of metal at which adapted					
	Cu (mg l^{-1})						Cu (mg l^{-1})					
	0	0.05	0.1	0.15	0.2	0.25	0.05	0.1	0.15	0.2	0.25	0.3
0	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}
24	9×10^{5}	5×10^{5}	3.2×10^{5}	2.5×10^{5}	1.8×10^{5}	1.2×10^{5}	9×10^{5}	5.6×10^{5}	4×10^{5}	2.8×10^{5}	2×10^{5}	2×10^{5}
48	1×10^{7}	6.3×10^{6}	1.3×10^{6}	4×10^{5}	1.3×10^{5}	d	1×10^{7}	4×10^{6}	2×10^{6}	9×10^{5}	4.5×10^{5}	2.5×10^{5}
72	3.2×10^{7}	2×10^{7}	4×10^{6}	8×10^{5}	2.5×10^{5}	d	3.2×10^{7}	2×10^{7}	6.3×10^{6}	2.5×10^{6}	1.3×10^{6}	1.8×10^{5}
96	8.8×10^{7}	6.3×10^{7}	9×10^{6}	2.2×10^{6}	4×10^{5}	d	1×10^{8}	5×10^{7}	2×10^{7}	5.1×10^{6}	3.2×10^{6}	3.2×10^{5}
120	1.8×10^{8}	1.3×10^{8}	2.8×10^{7}	4×10^{6}	8×10^{5}	d	1.8×10^{8}	9×10^{7}	4×10^{7}	9×10^{6}	4.5×10^{6}	3.6×10^{5}
144	3.2×10^{8}	2×10^{8}	4×10^{7}	8×10^{6}	1.1×10^{6}	d	2.8×10^{8}	1.6×10^{8}	6.3×10^{7}	1.6×10^{7}	8.8×10^{6}	6.3×10^{5}
168	3.2×10^{8}	2.5×10^{8}	9×10^{7}	2×10^{7}	1.4×10^{6}	d	3.2×10^{8}	2×10^{8}	1×10^{8}	2.8×10^{7}	1.3×10^{7}	1.3×10^{6}

Table A4.19 Influence of Zn on the growth of wild-type

time (h)	Source of inoculum											
	basal medium						strongly inhibitory level of metal					
	Zn (mg l^{-1})						Zn (mg l^{-1})					
	0.04	0.5	1.0	1.25	1.5	1.75	0.04	0.5	1.0	1.25	1.5	1.75
0	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}
24	8×10^{6}	7.5×10^{6}	4×10^{6}	5×10^{5}	1.5×10^{5}	1.5×10^{5}	1.6×10^{6}	9×10^{5}	6.3×10^{5}	5.1×10^{5}	3.2×10^{5}	1.8×10^{5}
48	8×10^{7}	6×10^{7}	4×10^{7}	7.1×10^{6}	3.2×10^{5}	d	1×10^{7}	6.3×10^{6}	3.2×10^{6}	1.8×10^{6}	6×10^{5}	2.2×10^{5}
72	4×10^{8}	1.2×10^{8}	1×10^{8}	1.8×10^{7}	7.1×10^{5}	d	5.1×10^{7}	2.2×10^{7}	1.6×10^{7}	8×10^{6}	1.8×10^{6}	5.1×10^{5}
96	5×10^{8}	2×10^{8}	1.1×10^{8}	2.8×10^{7}	2.2×10^{6}	d	2×10^{8}	8×10^{7}	5.6×10^{7}	3.2×10^{7}	4×10^{6}	8×10^{5}
120	5.6×10^{8}	2.5×10^{8}	1.3×10^{8}	4.5×10^{7}	5.6×10^{6}	d	4×10^{8}	1.8×10^{8}	1.3×10^{8}	8×10^{7}	8×10^{6}	1.6×10^{6}
144	6.3×10^{8}	2.8×10^{8}	1.6×10^{8}	6.3×10^{7}	1×10^{7}	d	5.1×10^{8}	2.5×10^{8}	1.6×10^{8}	1×10^{8}	1.6×10^{7}	2.1×10^{6}
168	7.9×10^{8}	3.2×10^{8}	1.8×10^{8}	8×10^{7}	1.8×10^{7}	d	7.1×10^{8}	3.2×10^{8}	1.8×10^{8}	1.1×10^{8}	2.5×10^{7}	4×10^{6}

Table A4.20 Influence of Zn on the growth of Co-tl.r

time (h)	Source of inoculum									
	basal medium					strongly inhibitory level of metal at which adapted				
	Zn (mg l^{-1})					Zn (mg l^{-1})				
	0.04	0.5	1.0	1.5	2.0	0.04	0.5	1.0	1.25	1.5
0	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}
24	1.1×10^{6}	1.1×10^{6}	5×10^{5}	2.5×10^{5}	1×10^{5}	1.0×10^{6}	1×10^{6}	4×10^{5}	2.5×10^{5}	1×10^{5}
48	1.3×10^{7}	5×10^{6}	1×10^{6}	3.2×10^{5}	8×10^{4}	1.1×10^{7}	4.5×10^{6}	8×10^{5}	2.5×10^{5}	1×10^{5}
72	4.5×10^{7}	2×10^{7}	3.6×10^{6}	5×10^{5}	d	3.6×10^{7}	1.6×10^{7}	1.1×10^{6}	3.2×10^{5}	d
96	1.1×10^{8}	5.1×10^{7}	7.1×10^{6}	8×10^{5}	d	1×10^{8}	3.6×10^{7}	2.5×10^{6}	4×10^{5}	d
120	1.8×10^{8}	8×10^{7}	1.8×10^{7}	1.6×10^{6}	d	1.8×10^{8}	8×10^{7}	3.6×10^{6}	8×10^{5}	d
144	3.2×10^{8}	1.3×10^{8}	3.6×10^{7}	2.5×10^{6}	d	3.2×10^{8}	1.6×10^{8}	6.3×10^{6}	1×10^{6}	d
168	3.4×10^{8}	1.6×10^{8}	4×10^{7}	3.2×10^{6}	d	3.6×10^{8}	2.8×10^{8}	1×10^{7}	[illegible]	d

Table A4.21 Influence of Zn on the growth of Ni-t1.0

time (h)	Source of inoculum							
	basal medium				strongly inhibitory level of metal at which adapted			
	Zn (mg l^{-1})				Zn (mg l^{-1})			
	0.04	0.5	1.0	1.5	0.04	0.5	1.0	1.5
0	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}
24	1.2×10^{6}	8×10^{5}	4×10^{5}	2.5×10^{5}	1.3×10^{6}	5.6×10^{5}	4×10^{5}	2.5×10^{5}
48	1×10^{7}	5×10^{6}	1.3×10^{6}	4×10^{5}	1.1×10^{7}	3.2×10^{6}	9×10^{5}	2×10^{5}
72	4.6×10^{7}	2×10^{7}	2.5×10^{6}	6.3×10^{5}	4.6×10^{7}	9×10^{6}	2×10^{6}	1.8×10^{5}
96	1×10^{8}	5×10^{7}	5.6×10^{6}	8×10^{5}	1×10^{8}	1.6×10^{7}	3.6×10^{6}	2.5×10^{5}
120	1.8×10^{8}	9×10^{7}	1.1×10^{7}	1.3×10^{6}	1.8×10^{8}	2.8×10^{7}	7.1×10^{6}	4×10^{5}
144	2.5×10^{8}	1.3×10^{8}	2×10^{7}	1.8×10^{6}	2.5×10^{8}	4×10^{7}	2.5×10^{7}	8×10^{5}
168	3.2×10^{8}	2×10^{8}	3.6×10^{7}	2.5×10^{6}	3.6×10^{8}	8×10^{7}	4.5×10^{7}	1.1×10^{6}

Table A4.22 Influence of Zn on the growth of Cu-t0.5

time (h)	Source of inoculum									
	basal medium					strongly inhibitory level of metal to which adapted				
	Zn (mg l^{-1})					Zn (mg l^{-1})				
	0.04	0.5	1.0	1.5	2.0	0.04	0.5	1.0	1.2	1.5
0	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}
24	1×10^{6}	7.1×10^{5}	4.5×10^{5}	2.5×10^{5}	1.6×10^{5}	1×10^{6}	5.6×10^{5}	2.5×10^{5}	1.8×10^{5}	1.6×10^{5}
48	6.3×10^{6}	5.1×10^{6}	1×10^{6}	3.6×10^{5}	1.8×10^{5}	6.3×10^{6}	3.2×10^{6}	5.1×10^{5}	2.5×10^{5}	1.8×10^{5}
72	3.2×10^{7}	2×10^{7}	4.5×10^{6}	6.3×10^{5}	2.5×10^{5}	3.2×10^{7}	1.3×10^{7}	1×10^{6}	3.6×10^{5}	2×10^{5}
96	1×10^{8}	5.6×10^{7}	1.3×10^{7}	1×10^{6}	3.6×10^{5}	1×10^{8}	3.6×10^{7}	2×10^{6}	5.6×10^{5}	2.5×10^{5}
120	1.3×10^{8}	8×10^{7}	8×10^{7}	2×10^{6}	6.3×10^{5}	1.6×10^{8}	6.3×10^{7}	3.6×10^{6}	7.1×10^{5}	3.2×10^{5}
144	1.6×10^{8}	1×10^{8}	1×10^{8}	3.6×10^{6}	8×10^{5}	1.8×10^{8}	7.1×10^{7}	6.3×10^{6}	1×10^{6}	3.6×10^{5}
168	1.8×10^{8}	1.3×10^{8}	1.1×10^{8}	6.3×10^{6}	1.6×10^{6}	1.8×10^{8}	1×10^{8}	1×10^{7}	1.6×10^{6}	5.1×10^{5}

Table A4.23 Influence of Zn on the growth of Cd-t2.0

time (h)	Source of inoculum											
	basal medium					strongly inhibitory level of metal at which adapted						
	Zn (mg l^{-1})					Zn (mg l^{-1})						
	0.04	0.5	1.0	1.5	2.0	0.04	1.0	1.5	2.0	2.5	3.0	3.5
0	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$
24	$1x10^6$	$6.3x10^5$	$3.6x10^5$	$2.5x10^5$	$1.8x10^5$	$8x10^5$	$5.6x10^5$	$4x10^5$	$4x10^5$	$2.8x10^5$	$2.5x10^5$	$1.8x10^5$
48	$1.3x10^7$	$5x10^6$	$5x10^5$	$2.5x10^5$	$1.8x10^5$	$1x10^7$	$6.3x10^6$	$3.2x10^6$	$2.5x10^6$	$8x10^5$	$4x10^5$	$3.2x10^5$
72	$3.2x10^7$	$2x10^7$	$1x10^6$	$4.5x10^5$	$1.8x10^5$	$2.8x10^7$	$2x10^7$	$8x10^6$	$4x10^6$	$1.4x10^6$	$8x10^5$	$3.2x10^5$
96	$8x10^7$	$5x10^7$	$3.2x10^6$	$6.3x10^5$	d	$8x10^7$	$4.5x10^7$	$2.5x10^7$	$1.3x10^7$	$3.6x10^6$	$1.8x10^6$	$4.5x10^5$
120	$1.3x10^8$	$8x10^7$	$5.6x10^6$	$1.3x10^6$	d	$1.4x10^8$	$1x10^8$	$4.5x10^7$	$2.8x10^7$	$1x10^7$	$4x10^6$	$8x10^5$
144	$1.6x10^8$	$1.3x10^8$	$1x10^7$	$2.5x10^6$	d	$2x10^8$	$1.4x10^8$	$8x10^7$	$5.6x10^7$	$1.8x10^7$	$8.8x10^6$	$1.3x10^6$
168	$2.8x10^8$	$1.5x10^8$	$1.8x10^7$	$4x10^6$	d	$2.8x10^8$	$1.4x10^8$	$1x10^8$	$7.1x10^7$	$2.6x10^7$	$1.3x10^7$	$1.8x10^6$

Table A4.24 Influence of Cd on the growth of wild-type

time (h)	Source of inoculum													
	basal medium							strongly inhibitory level of metal						
	Cd (mg l^{-1})							Cd (mg l^{-1})						
	0	0.15	0.3	0.35	0.4	0.5	0.6	0	0.15	0.3	0.35	0.4	0.5	0.6
0	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$
24	$2x10^6$	$1.6x10^6$	$6.3x10^5$	$2x10^5$	$1.8x10^5$	$1.6x10^5$	$1.6x10^5$	$1.8x10^6$	$1.3x10^6$	$8x10^5$	d	$3.2x10^5$	$2.5x10^5$	$2.5x10^5$
48	$3.6x10^7$	$2.1x10^7$	$1x10^6$	$2.5x10^5$	$2.1x10^5$	$2x10^5$	$1.8x10^5$	$1.8x10^7$	$1.3x10^7$	$1.8x10^6$	d	$8x10^5$	$5.1x10^5$	$3.2x10^5$
72	$1.6x10^8$	$1.4x10^8$	$2.8x10^6$	$5.6x10^5$	$4x10^5$	$2.8x10^5$	$2.5x10^5$	$7.1x10^7$	$4.5x10^7$	$5.1x10^6$	d	$1.8x10^6$	$1x10^6$	$5.6x10^5$
96	$2.8x10^8$	$2.2x10^8$	$1.4x10^7$	$4.5x10^6$	$3.2x10^6$	$1.8x10^6$	$1x10^6$	$1.6x10^8$	$1x10^8$	$1.4x10^7$	d	$3.6x10^6$	$2x10^6$	$9x10^5$
120	$3.6x10^8$	$2.5x10^8$	$2.5x10^7$	$1.1x10^7$	$6.3x10^6$	$4x10^6$	$2x10^6$	$2.2x10^8$	$1.6x10^8$	$3.6x10^7$	d	$5.6x10^6$	$3.2x10^6$	$1.6x10^6$
144	$4x10^8$	$2.8x10^8$	$4.5x10^7$	$1.6x10^7$	$8x10^6$	$5.6x10^6$	$3.2x10^6$	$2.8x10^8$	$2x10^8$	$7.1x10^7$	d	$1.1x10^7$	$6.3x10^6$	$2.5x10^6$
168	$5.1x10^8$	$2.8x10^8$	$7.1x10^7$	$2.8x10^7$	$1.4x10^7$	$8x10^6$	$4x10^6$	$4.2x10^8$	$2.5x10^8$	$8.2x10^7$	d	$2.2x10^7$	$1.3x10^7$	$5.6x10^6$

Table A4.25 Influence of Cd on the growth of Co-tl.8

time (h)	Source of inoculum											
	basal medium						strongly inhibitory level of metal at which adapted					
	Cd (mg l^{-1})						Cd (mg l^{-1})					
	0	0.05	0.10	0.2	0.3	0.4	0	0.05	0.1	0.15	0.2	0.3
0	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}
24	1.1×10^{6}	7.1×10^{5}	4.5×10^{5}	3.2×10^{5}	2.5×10^{5}	2.5×10^{5}	8×10^{5}	4×10^{5}	2.5×10^{5}	2.2×10^{5}	2.2×10^{5}	d
48	8×10^{6}	5×10^{6}	1.8×10^{6}	1×10^{6}	5.1×10^{5}	2.5×10^{5}	8×10^{6}	2×10^{6}	6.3×10^{5}	2.5×10^{5}	2.5×10^{5}	d
72	4.2×10^{7}	1.3×10^{7}	6.3×10^{6}	2.5×10^{6}	8×10^{5}	2.5×10^{5}	2.8×10^{7}	7.1×10^{6}	1.8×10^{6}	6.3×10^{5}	2.5×10^{5}	d
96	1×10^{8}	2.5×10^{7}	1.3×10^{7}	5.6×10^{6}	2×10^{6}	4×10^{5}	1×10^{8}	2×10^{7}	3.6×10^{6}	1.8×10^{6}	4×10^{5}	d
120	1.6×10^{8}	6.3×10^{7}	2.5×10^{7}	1.3×10^{7}	4×10^{6}	6.3×10^{5}	1.4×10^{8}	3.6×10^{7}	6.3×10^{6}	2.5×10^{6}	8×10^{5}	d
144	2×10^{8}	1×10^{8}	4.5×10^{7}	1.8×10^{7}	6.3×10^{6}	1×10^{6}	1.8×10^{8}	5.1×10^{7}	1.3×10^{7}	5.1×10^{6}	1.4×10^{6}	d
168	2.1×10^{8}	1.3×10^{8}	6.3×10^{7}	2.8×10^{7}	1.4×10^{7}	1.4×10^{6}	2×10^{8}	6.3×10^{7}	2×10^{7}	7.1×10^{6}	3.2×10^{6}	d

Table A4.26 Influence of Cd on the growth of Ni-tl.0

time (h)	Source of inoculum											
	basal medium						strongly inhibitory level of metal at which adapted					
	Cd (mg l^{-1})						Cd (mg l^{-1})					
	0	0.05	0.10	0.15	0.20	0.25	0	0.05	0.10	0.15	0.20	0.25
0	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}
24	1×10^{6}	5.1×10^{5}	3.2×10^{5}	3.2×10^{5}	3.2×10^{5}	d	8×10^{5}	6.3×10^{5}	3.2×10^{5}	2.5×10^{5}	2×10^{5}	d
48	6.3×10^{6}	2.8×10^{6}	1×10^{6}	4×10^{5}	3.6×10^{5}	d	6.3×10^{6}	1.6×10^{6}	8×10^{5}	6.3×10^{5}	2.8×10^{5}	d
72	3.6×10^{7}	1×10^{7}	3.5×10^{6}	8×10^{5}	4×10^{5}	d	3.2×10^{7}	6.3×10^{6}	2.5×10^{6}	8×10^{5}	4.5×10^{5}	d
96	8×10^{7}	3.2×10^{7}	1×10^{7}	1.8×10^{6}	6.3×10^{5}	d	1×10^{8}	1.6×10^{7}	6.3×10^{6}	1.3×10^{6}	8×10^{5}	d
120	1.3×10^{8}	6.3×10^{7}	2×10^{7}	2.8×10^{6}	1×10^{6}	d	1.4×10^{8}	4×10^{7}	1.4×10^{7}	2.5×10^{6}	1.6×10^{6}	d
144	1.6×10^{8}	8×10^{7}	3.2×10^{7}	5.6×10^{6}	1.8×10^{6}	d	2×10^{8}	5.6×10^{7}	2.5×10^{7}	5.6×10^{5}	2.5×10^{6}	d
168	2×10^{8}	1×10^{8}	4×10^{7}	1×10^{7}	3.2×10^{6}	d	2.5×10^{8}	7.1×10^{7}	3.6×10^{7}	1.4×10^{7}	3.2×10^{6}	d

Table A4.27 Influence of Cd on the growth of Cu-t0.5

time (h)	Source of inoculum										
	basal medium						strongly inhibitory level of metal to which adapted				
	Cd (mg l^{-1})						Cd (mg l^{-1})				
	0	0.05	0.10	0.20	0.40	0.60	0	0.05	0.10	0.15	0.20
0	2×10^5	2×10^5	2×10^5	2×10^5	2×10^5	2×10^5	2×10^5	2×10^5	2×10^5	2×10^5	2×10^5
24	1×10^6	6.3×10^5	5.1×10^5	3.6×10^5	2.5×10^5	2.5×10^5	1×10^6	4×10^5	2.5×10^5	2×10^5	1.6×10^5
48	6.3×10^6	3.6×10^6	2×10^6	1×10^6	5.6×10^5	2.8×10^5	6.3×10^6	2×10^6	7.1×10^5	3.6×10^5	2.0×10^5
96	1×10^8	2×10^7	8×10^6	5.1×10^6	1.6×10^6	6.3×10^5	1×10^8	2×10^7	4×10^6	1×10^6	3.2×10^5
120	1.3×10^8	4×10^7	1.3×10^7	1×10^7	4.5×10^6	1×10^6	1.6×10^8	4×10^7	6.3×10^6	2.5×10^6	4×10^5
144	1.6×10^8	8×10^7	2.5×10^7	1.6×10^7	8×10^6	1.6×10^6	1.8×10^8	6.3×10^7	8×10^6	3.6×10^6	7.1×10^5
168	1.8×10^8	1×10^8	3.6×10^7	2×10^7	4.3×10^6	2×10^6	1.8×10^8	7.1×10^7	2×10^7	6.3×10^6	1×10^6

Table A4.28 Influence of Cd on the growth of Zn-t5.0

time (h)	Source of inoculum												
	basal medium					strongly inhibitory level of metal at which adapted							
	Cd (mg l^{-1})					Cd (mg l^{-1})							
	0	0.2	0.3	0.4	0.5	0	0.2	0.3	0.4	0.5	0.6	0.8	1.0
0	2×10^5	2×10^5	2×10^5	2×10^5	2×10^5	2×10^5	2×10^5	2×10^5	2×10^5	2×10^5	2×10^5	2×10^5	2×10^5
24	1×10^6	4×10^5	1.8×10^5	1.8×10^5	1.8×10^5	9×10^5	7.1×10^5	4×10^5	3.2×10^5	2.8×10^5	2.5×10^5	2×10^5	1.8×10^5
48	8×10^6	1.3×10^6	2.5×10^5	1.8×10^5	1.8×10^5	5.1×10^6	2.8×10^6	1×10^6	5×10^5	4×10^5	4×10^5	3.2×10^5	1.8×10^5
72	4.5×10^7	3.6×10^6	3.2×10^5	1.8×10^5	1.8×10^5	3.6×10^7	1.6×10^7	4×10^6	1.3×10^6	8×10^5	5.6×10^5	4×10^5	2×10^5
96	1×10^8	8×10^6	8×10^5	2.5×10^5	1.8×10^5	1×10^8	5.6×10^7	1.6×10^7	3.6×10^6	2.5×10^6	1×10^6	5×10^5	3.6×10^5
120	1.6×10^8	1.8×10^7	1.3×10^6	4×10^5	2.4×10^5	1.8×10^8	1×10^8	4×10^7	8×10^6	5.6×10^6	3.2×10^6	1.3×10^6	5×10^5
144	2×10^8	4×10^7	1.6×10^6	6.3×10^5	2.8×10^5	2.0×10^8	1.6×10^8	8×10^7	2.5×10^7	1.4×10^7	5.6×10^6	2.8×10^6	8×10^5
168	3.2×10^8	6.3×10^7	3.6×10^6	1.1×10^6	3.6×10^5	3.2×10^8	1.6×10^8	1×10^8	3.6×10^7	2.5×10^7	1.8×10^7	5.6×10^6	1.4×10^6

Table A4.29 Influence of Cd on the growth of Zn-t12.0

time (h)	Source of inoculum												
	basal medium					strongly inhibitory level of metal at which adapted							
	Cd (mg l^{-1})					Cd (mg l^{-1})							
	0	0.2	0.3	0.4	0.5	0	0.2	0.3	0.4	0.5	0.6	0.8	1.0
0	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}
24	1.0×10^{6}	6.3×10^{5}	3.2×10^{5}	1.8×10^{5}	1.8×10^{5}	1.0×10^{6}	6.3×10^{5}	4×10^{5}	3.6×10^{5}	3.2×10^{5}	2.5×10^{5}	2×10^{5}	1.8×10^{5}
48	1.3×10^{7}	1.8×10^{6}	4×10^{5}	1.8×10^{5}	1.8×10^{5}	1.1×10^{7}	2.5×10^{6}	1.3×10^{6}	8×10^{5}	4.5×10^{5}	4×10^{5}	2.8×10^{5}	1.8×10^{5}
72	4×10^{7}	6.3×10^{6}	5.6×10^{5}	1.8×10^{5}	1.8×10^{5}	4×10^{7}	8×10^{6}	3.6×10^{6}	2.5×10^{6}	8×10^{5}	5.6×10^{5}	4×10^{5}	2.5×10^{5}
96	1×10^{8}	1.3×10^{7}	1.3×10^{6}	3.6×10^{5}	1.8×10^{5}	1×10^{8}	2.8×10^{7}	1×10^{7}	6.3×10^{6}	2.5×10^{6}	1.4×10^{6}	8×10^{5}	3.2×10^{5}
120	1.8×10^{8}	3.2×10^{7}	2.5×10^{6}	1×10^{6}	2.5×10^{5}	1.8×10^{8}	8×10^{7}	2.8×10^{7}	1.3×10^{7}	6.3×10^{6}	3.2×10^{6}	1.8×10^{6}	4×10^{5}
144	2.8×10^{8}	7.1×10^{7}	3.2×10^{6}	1.6×10^{6}	2.8×10^{5}	3.2×10^{8}	1.4×10^{8}	5×10^{7}	2.8×10^{7}	1.3×10^{7}	5.6×10^{6}	2.8×10^{6}	6.3×10^{5}
168	3.2×10^{8}	1×10^{8}	8×10^{6}	2.8×10^{6}	4.5×10^{5}	3.2×10^{8}	2×10^{8}	8×10^{7}	5×10^{7}	2.8×10^{7}	8×10^{6}	4×10^{6}	1×10^{6}

Table A4.30 Influence of Hg on the growth of wild-type

time (h)	Source of inoculum						
	basal medium						
	Hg (mg l^{-1})						
	0	0.01	0.02	0.03	0.04	0.05	0.06
0	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}	2×10^{5}
24	8×10^{5}	6.3×10^{5}	4.5×10^{5}	3.2×10^{5}	2×10^{5}	1.6×10^{5}	d
48	6.3×10^{6}	3.6×10^{6}	1.8×10^{6}	3.6×10^{5}	2.5×10^{5}	1.3×10^{5}	d
72	3.2×10^{7}	2×10^{7}	4×10^{6}	5.1×10^{5}	2.6×10^{5}	1×10^{5}	d
96	1×10^{8}	5.1×10^{7}	1×10^{7}	1.3×10^{6}	3.2×10^{5}	1.3×10^{5}	d
120	1.4×10^{8}	7.2×10^{7}	1.4×10^{7}	2.5×10^{6}	6.3×10^{5}	3.6×10^{5}	d
144	1.6×10^{8}	9×10^{7}	2×10^{7}	4.5×10^{6}	1×10^{6}	6.3×10^{5}	d
168	2×10^{8}	1×10^{8}	2.5×10^{7}	7.1×10^{6}	2×10^{6}	1.3×10^{6}	d

Table A4.31 Influence of Pb on the growth of wild-type

time (h)	Source of inoculum						
	basal medium						
	Pb (mg l^{-1})						
	0	10	15	20	30	35	40
0	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$	$2x10^5$
24	$1x10^6$	$8x10^5$	$5x10^5$	$3.2x10^5$	$2.3x10^5$	$1.6x10^5$	d
48	$9x10^6$	$3.6x10^6$	$1.8x10^6$	$7.1x10^5$	$2.5x10^5$	$2x10^5$	d
72	$3.2x10^7$	$1.3x10^7$	$4x10^6$	$1.3x10^6$	$5.1x10^5$	$2x10^5$	d
96	$1x10^8$	$3.2x10^7$	$1.3x10^7$	$2.5x10^6$	$7.1x10^5$	$2.5x10^5$	d
120	$1.6x10^8$	$5.6x10^7$	$2.5x10^7$	$4.5x10^6$	$1.1x10^6$	$4.5x10^5$	d
144	$1.8x10^8$	$6.3x10^7$	$3.6x10^7$	$8x10^6$	$1.8x10^6$	$5.1x10^5$	d
168	$2x10^8$	$8x10^7$	$5x10^7$	$1.1x10^7$	$2.5x10^6$	$8x10^5$	d

Table A4.32 Pigment changes during growth of wild-type *Anacystis* at different Cd concentrations.

time (days)	Cd mg l^{-1}	dry wt (mg l^{-1})	chl *a* (mg l^{-1})	phyco. (mg l^{-1})	% chl *a* dry wt	% phyco. dry wt	phyco: chl *a* (by dry wt.)
0	0.0	20	0.20	ND	1.0	ND	ND
	0.05	20	0.20	ND	1.0	ND	ND
	0.15	20	0.20	ND	1.0	ND	ND
	0.25	20	0.20	ND	1.0	ND	ND
2	0	80	0.83	0.33	1.04	0.42	0.40
	0.05	80	0.70	0.67	0.88	0.84	0.96
	0.15	25	0.24	ND	0.96	ND	ND
	0.25	20	0.18	ND	0.96	ND	ND
4	0.0	130	1.26	0.91	0.97	0.70	0.72
	0.05	116	1.39	2.25	1.20	1.93	1.60
	0.15	60	0.28	0.17	0.47	0.28	0.61
	0.25	55	0.28	0.15	0.51	0.27	0.53
6	0.0	180	1.81	1.25	1.0	0.70	0.70
	0.05	182	0.83	4.1	0.46	2.23	5.0
	0.15	120	0.83	1.0	0.70	0.84	1.2
	0.25	130	0.70	0.91	0.54		1.15
8	0.0	216	2.22	1.75	1.03	0.81	0.78
	0.05	222	0.56	4.3	0.25	1.95	7.68
	0.15	142	1.39	2.57	0.98	1.80	1.85
	0.25	136	1.11	2.45	0.82	1.80	2.21
10	0.0	224	2.36	2.5	1.05	1.11	1.06
	0.05	200	1.53	2.22	0.77	1.11	1.45
	0.15	180	1.67	4.10	0.93	2.23	2.46
	0.25	180	1.53	3.76	0.85	2.10	2.47
12	0.0	240	2.10	2.67	0.88	1.11	1.27
	0.05	228	1.95	2.80	0.85	1.23	1.43
	0.15	200	1.95	5.57	0.98	2.78	2.96
	0.25	196	1.67	5.45	0.85	2.78	3.26
14	0.0	256	2.26	2.60	0.88	1.0	1.15
	0.05	248	1.98	2.73	0.80	1.1	1.38
	0.15	225	1.82	6.0	0.81	2.67	3.36
	0.25	225	1.76	6.10	0.78	2.70	3.47

Table A5.1 Influence of Na on Cd toxicity to wild-type *Anacystis*.

Cd ($mg\ l^{-1}$)	Na ($mg\ l^{-1}$)								
	2.5	5	10	20	40	50	100	200	300
0	8.0	8.6	8.6	8.6	8.6	8.8	8.2	8.5	8.6
0.15	6.8	6.8	6.4	6.4	6.6	6.4	6.2	6.6	6.5
0.30	5.4	5.4	5.5	5.2	5.6	5.1	5.4	5.5	5.4
0.45	4.3	4.2	4.4	4.3	4.6	4.3	4.6	4.6	4.4
0.6	1.3	1.5	1.3	1.4	2.8	1.6	1.6	1.5	1.6
0.9	d	d	d	d	d	d	d	d	d

Table A5.2 Influence of K on Zn toxicity to wild-type *Anacystis*.

Zn ($mg\ l^{-1}$)	K ($mg\ l^{-1}$)									
	5	10	20	40	80	100	200	300	400	500
0.04	8.8	8.6	8.5	8.4	8.5	8.2	8.1	8.2	8.2	8.1
0.10	8.8	8.7	8.4	8.4	8.6	8.2	8.0	8.2	8.1	8.0
0.20	8.8	8.7	8.4	8.5	8.4	8.0	8.0	8.2	8.0	8.1
0.40	8.6	8.7	8.6	8.2	8.4	8.4	8.2	7.8	7.4	7.5
0.60	8.5	8.6	8.3	8.0	8.5	8.0	8.1	7.6	7.6	7.1
0.70	8.4	8.2	7.8	7.7	7.8	7.5	7.4	7.4	7.6	7.2
0.80	8.4	7.6	7.6	7.5	7.5	7.2	7.4	7.6	7.0	7.2
1.0	8.2	7.4	7.2	7.6	7.5	7.2	7.2	7.5	7.2	7.0
1.25	7.8	7.1	7.0	7.5	7.5	6.8	6.8	6.8	7.0	6.9
1.50	0.3	0.3	0.24	0.06	0.07	0.05	0.06	0.03	0.06	0.04
1.75	d	d	d	d	d	d	d	d	d	d

Table A5.3 Influence of K on Cd toxicity to wild-type *Anacystis*.

Cd (mg l^{-1})	K (mg l^{-1})									
	5	10	20	40	80	100	200	300	400	500
0.04	9.2	8.8	8.8	8.7	8.6	8.8	8.8	8.8	8.8	8.8
0.15	8.4	8.2	8.2	8.0	7.8	7.8	7.6	7.4	7.5	7.5
0.30	5.4	5.2	5.5	5.6	5.4	5.4	5.2	5.0	5.1	5.2
0.45	4.4	4.5	4.8	4.2	4.2	3.8	3.6	3.2	3.2	3.2
0.60	1.5	1.8	1.8	1.4	1.6	1.4	1.5	1.4	1.4	1.4
0.90	d	d	d	d	d	d	d	d	d	d

Table A5.4 Influence of Ca on Zn toxicity to wild-type *Anacystis*.

Zn (mg l^{-1})	Ca (mg l^{-1})							
	2.5	5	10	20	40	80	100	200
0.04	7.5	7.9	8.5	8.8	9.4	10.6	10.7	11.6
0.25	7.8	7.9	8.4	8.6	9.2	10.6	10.8	11.2
0.50	7.8	7.8	8.4	8.5	8.9	10.2	10.4	10.6
0.75	7.5	7.6	7.8	8.2	8.9	9.3	10.2	10.4
1.0	7.4	7.5	7.7	8.0	8.5	9.3	9.6	10.4
1.25	6.0	7.0	7.4	7.5	8.3	9.4	9.6	9.8
1.50	0.05	0.4	0.66	4.3	6.2	7.7	7.8	8.2
2.0	d	d	0.05	3.7	5.6	6.7	6.8	7.1

Table A5.5 Influence of Ca on Zn toxicity to Zn-t5.0 *Anacystis*.

Zn (mg l^{-1})	Ca (mg l^{-1})							
	2.5	5	10	20	40	80	100	200
0.04	8.2	8.3	8.4	8.6	8.8	9.0	9.2	9.4
2	7.8	8.0	8.1	8.2	8.5	8.4	8.6	8.7
4	4.6	5.8	7.5	7.8	8.2	8.0	8.5	8.5
5	2.8	3.5	4.0	6.8	7.5	8.2	8.2	8.4
6	d	d	d	4.8	5.2	8.0	8.0	8.3
8	d	d	d	0.05	2.3	7.5	7.5	8.0
10	d	d	d	d	d	6.8	6.8	8.0
12	d	d	d	d	d	5.6	5.6	7.8

Table A5.6 Influence of Ca on Zn toxicity to Zn-t12.0 *Anacystis*.

Zn (mg l^{-1})	Ca (mg l^{-1})							
	2.5	5	10	20	40	80	160	320
0.04	8.2	8.3	8.4	8.6	8.8	9.0	9.2	9.4
4	7.8	8.0	8.0	8.2	8.5	8.4	8.6	8.8
8	4.6	5.8	7.5	7.8	8.2	8.0	8.5	8.5
10	3.8	4.8	5.0	6.8	7.5	8.2	8.2	8.4
12	3.2	3.6	4.2	4.8	5.4	8.0	8.0	8.3
16	d	d	d	0.05	2.3	7.5	7.5	8.0
20	d	d	d	d	d	6.8	6.8	8.0
24	d	d	d	d	d	5.6	5.6	7.8

Table A5.7 Influence of Ca on Cd toxicity to wild-type *Anacystis*.

Cd (mg l^{-1})	Ca (mg l^{-1})							
	2.5	5	10	20	40	80	100	200
0	8.0	8.8	8.8	8.8	8.4	8.8	8.8	9.4
0.3	4.8	5.4	6.8	8.4	8.0	8.8	9.0	9.6
0.45	4.0	4.2	4.0	5.4	5.4	5.8	6.0	6.4
0.6	1.8	3.0	4.6	4.6	4.8	4.8	4.8	5.4
0.9	d	2.0	3.0	3.2	3.6	4.2	4.4	4.8
1.2	d	d	d	1.8	2.1	4.0	4.2	4.5

Table A5.8 Influence of Mn on Zn toxicity to Zn-t5.0 *Anacystis*.

Zn (mg l^{-1})	Mn (mg l^{-1})							
	0.05	0.10	0.50	1.0	2.5	5	10	20
0.04	8.3	8.3	8.4	8.5	8.6	8.5	8.0	7.2
2	8.4	8.3	8.4	8.5	8.6	7.0	6.2	3.0
4	5.8	5.8	5.7	7.0	8.0	6.8	4.3	d
5	3.4	3.5	3.4	4.8	7.2	6.2	d	d
6	d	d	d	3.4	6.8	5.6	d	d
8	d	d	d	3.4	6.0	3.8	d	d
10	d	d	d	3.2	4.2	3.7	d	d
12	d	d	d	1.8	2.1	1.6	d	d

Table A5.9 Influence of Mn on Zn toxicity to Zn-t12.0 *Anacystis*.

Zn (mg l^{-1})	Mn (mg l^{-1})							
	0.05	0.1	0.5	1.0	2.5	5	10	20
0.04	8.3	8.3	8.4	8.5	8.6	8.5	7.8	7.0
4	8.4	8.3	8.4	8.5	8.6	7.0	6.2	3.0
8	5.8	5.8	5.7	7.0	8.0	6.8	4.5	d
10	3.4	3.5	3.4	4.8	7.2	6.4	d	d
12	d	2.4	3.2	3.4	6.4	5.6	d	d
16	d	d	d	3.4	6.0	3.6	d	d
20	d	d	d	3.2	4.2	3.7	d	d
24	d	d	d	1.8	2.1	1.6	d	d

Table A5.10 Influence of Fe on Zn toxicity to Zn-t5.0 *Anacystis* results based on units $ml^{-1} \times 10^{7}$; age of the alga 5 days- d = died.

Zn (mg l^{-1})	Fe (mg l^{-1})							
	0.05	0.1	0.5	1.0	2.5	5	10	20
0.04	5.4	7.5	7.6	7.6	7.8	7.0	6.5	5.0
2	5.8	7.4	7.3	7.2	7.1	7.0	6.2	4.8
4	5.6	7.2	7.0	7.2	7.0	6.8	6.0	4.5
5	5.2	6.4	7.3	7.2	6.8	6.2	5.1	4.0
6	d	d	d	d	3.1	4.1	3.8	3.6
8	d	d	d	d	d	2.6	3.4	3.2
10	d	d	d	d	d	d	2.2	1.8
12	d	d	d	d	d	d	d	d

Table A5.11 Influence of Fe on Zn toxicity to Zn-t12.0 *Anacystis*. results based on units $ml^{-1} \times 10^7$; age of the alga 5 days; d = died.

Zn ($mg\ l^{-1}$)	Fe ($mg\ l^{-1}$)							
	0.05	0.1	0.5	1.0	2.5	5	10	20
0.04	5.4	7.5	7.6	7.6	7.8	7.0	6.2	5.1
4	5.8	7.4	7.5	7.5	7.3	6.8	5.8	4.6
8	5.6	7.3	7.3	7.4	7.0	6.4	5.4	4.5
10	5.2	7.2	7.2	7.2	6.8	4.1	3.8	3.2
12	5.0	6.8	7.0	7.0	3.2	2.6	3.4	3.2
16	d	d	d	d	d	d	2.2	1.8
20	d	d	d	d	d	d	1.2	d
24	d	d	d	d	d	d	d	d

Table A5.12 Influence of Fe on Cu toxicity to wild-type *Anacystis* the results based on units $ml^{-1} \times 10^7$; age of the alga 5 days; d = died.

Cu ($mg\ l^{-1}$)	Fe ($mg\ l^{-1}$)							
	0.05	0.10	0.50	1.0	2.5	5	10	20
0.0	5.5	7.6	7.8	7.8	7.8	6.9	6.5	4.8
0.02	5.8	7.6	8.0	7.8	7.8	6.8	6.3	5.2
0.04	4.8	5.0	5.2	5.6	5.8	5.2	5.0	5.0
0.08	2.5	2.5	2.8	3.8	4.0	5.0	5.1	4.8
0.10	0.85	0.85	1.3	1.8	3.6	4.0	4.5	4.2
0.16	0.06	0.06	1.0	1.2	1.2	1.4	2.3	3.8
0.20	d	d	d	d	d	d	d	d
0.24	d	d	d	d	d	d	d	d

Table A5.13 Influence of Fe on Cu toxicity to Cu-t0.5 *Anacystis*. results based on units $ml^{-1} \times 10^7$; age of the alga 5 days; d = died

Cu (mg l^{-1})	Fe (mg l^{-1})							
	0.05	0.10	0.50	1.0	2.5	5	10	20
0.0	5.6	7.8	7.8	7.6	7.8	6.9	6.5	5.0
0.2	6.0	5.6	6.2	6.5	6.2	6.0	6.3	5.0
0.4	0.8	1.0	3.6	3.2	4.2	4.6	4.0	3.8
0.5	0.07	0.85	2.1	3.4	3.6	3.0	3.2	3.4
0.6	d	d	0.05	0.38	1.8	1.2	0.45	0.5
0.8	d	d	d	0.02	0.66	0.8	d	d
1.0	d	d	d	d	0.52	0.6	d	d
1.5	d	d	d	d	0.45	d	d	d

Table A5.14 Influence of PO_4-P on Zn toxicity to wild-type *Anacystis*.

Zn (mg l^{-1})	PO_4-P (mg l^{-1})							
	0.88	1.75	3.5	7	14	28	56	112
0.04	6.5	7.8	8.5	8.6	9.4	9.5	10.0	10.2
0.5	5.9	7.6	8.6	8.6	9.6	9.6	10.0	10.0
1.0	5.7	7.4	8.4	8.5	9.3	9.2	9.6	9.8
1.5	3.6	7.4	7.2	7.8	8.2	8.6	9.2	9.4
2.0	0.4	7.4	7.6	7.8	7.9	8.2	8.8	9.1
2.5	d	6.8	7.2	7.5	7.8	7.8	8.5	8.6
3.0	d	6.8	7.2	7.2	7.5	7.4	8.4	8.4
3.5	d	d	4.5	5.8	7.2	7.2	7.8	7.8
4.0	d	d	3.8	4.5	6.8	7.0	7.6	7.6

Table A5.15 Influence of PO_4-P on Zn toxicity to Zn-t5.0 *Anacystis*.

Zn (mg l^{-1})	PO_4-P (mg l^{-1})						
	1.75	3.5	7	14	28	56	112
0.04	8.8	8.8	9.6	9.5	9.8	10.0	10.2
2	8.8	8.8	9.6	9.5	9.8	10.0	10.2
4	5.8	6.2	8.6	9.0	9.4	9.5	9.2
5	4.2	4.8	6.4	8.1	9.0	9.4	9.4
6	d	3.2	5.5	8.0	8.8	9.4	9.5
8	d	d	4.5	8.0	8.6	9.2	9.2
10	d	d	2.3	7.8	8.0	8.4	8.6
12	d	d	d	4.3	7.8	8.0	8.4

Table A5.16 Influence of P^{o}_{4} - P on Zn toxicity to Zn-t12.0 *Anacystis*.

Zn (mg l^{-1})	P^{o}_{4} - P (mg l^{-1})						
	1.75	3.5	7.0	14	28	56	112
0.04	8.8	8.8	9.6	9.8	9.8	10.0	10.0
4	8.8	8.8	9.6	9.5	9.8	10.0	10.2
8	5.8	5.2	8.6	9.0	9.0	9.5	9.6
10	4.2	4.8	6.4	8.0	9.0	9.4	9.6
12	3.5	4.2	5.5	8.0	8.5	9.4	9.5
16	d	d	4.5	8.0	8.6	9.2	9.2
20	d	d	2.6	7.5	8.0	8.4	8.6
24	d	d	d	4.0	6.8	7.8	8.0

Table A5.17 Influence of PO_4-P on Cd toxicity to wild-type *Anacystis*.

Cd (mg l^{-1})	PO_4-P (mg l^{-1})						
	1.75	3.5	7	14	28	56	112
0	8.8	8.9	8.8	8.9	9.4	9.8	9.4
0.03	8.8	8.8	8.6	8.8	8.9	9.2	9.6
0.06	8.6	8.6	8.8	8.9	9.1	9.0	9.4
0.12	8.8	8.8	8.8	9.0	8.8	8.8	9.0
0.24	7.6	7.8	8.8	8.8	8.8	8.9	8.8
0.36	5.5	5.4	6.8	8.4	8.5	8.6	8.8
0.48	4.4	4.2	4.5	5.4	6.4	7.0	7.8
0.60	3.1	3.0	4.6	4.6	5.8	6.8	7.4
0.90	d	d	3.2	3.8	4.6	5.4	7.0
1.20	d	d	d	2.2	3.1*	5.2	6.8

Table A5.18 Influence of NO_3-N on Zn toxicity to wild-type *Anacystis*.

Zn (mg l^{-1})	NO_3-N (mg l^{-1})									
	1	2.5	5	10	20	40	50	100	200	400
0.04	2.5	2.8	6.8	7.5	7.6	8.2	8.13	8.9	8.8	8.65
0.25	d	d	6.6	7.5	7.5	8.3	8.2	8.3	8.4	8.6
0.50	d	d	5.4	7.4	7.4	7.8	8.4	8.5	8.3	8.5
0.75	d	d	4.8	7.0	7.2	7.7	8.0	8.5	8.6	7.8
1.0	d	d	3.6	6.2	6.8	7.1	7.2	7.8	7.8	7.4
1.25	d	d	d	5.4	5.6	6.5	6.8	7.7	7.4	7.1
1.50	d	d	0.04	0.05	0.06	0.04	0.06	0.04	0.05	0.05
2.0	d	d	d	d	d	d	d	d	d	d

Table A5.19 Influence of EDTA on Zn toxicity to wild-type *Anacystis*.

Zn (mg l^{-1})	EDTA (mg l^{-1})						
	0.5	2	4	8	16	32	64
0.04	8.4	8.8	9.0	9.2	9.6	9.9	10.2
0.5	8.2	8.6	8.8	8.8	8.8	8.6	9.0
1.0	6.4	7.1	8.2	8.2	8.4	8.7	9.0
1.25	4.5	4.8	5.6	8.2	8.3	8.7	9.0
1.5	0.50	0.86	3.8	8.3	8.2	8.2	8.8
2.0	d	d	d	8.0	8.2	8.0	8.4
3.0	d	d	d	3.6	6.5	7.8	8.0
4.0	d	d	d	d	4.5	7.5	8.2
6.0	d	d	d	d	d	5.4	6.2
8.0	d	d	d	d	d	3.6	5.6

Table A5.20 Influence of EDTA on Zn toxicity to Zn-t5.0 *Anacystis*.

Zn (mg l^{-1})	EDTA (mg l^{-1})						
	0.5	1.0	2	4	8	16	32
0.04	8.0	8.0	8.2	8.4	8.8	8.8	8.8
2	7.8	7.6	7.8	7.8	8.8	8.8	8.8
4	5.2	6.4	7.4	7.4	8.0	8.1	8.1
5	4.6	5.4	6.2	6.3	8.0	8.1	8.2
6	d	0.05	0.04	4.4	6.5	7.2	7.4
8	d	d	d	3.2	4.8	7.3	7.5
10	d	d	d	0.08	4.5	7.2	7.3
12	d	d	d	d	3.6	6.4	7.4
14	d	d	d	d	2.5	3.8	6.4
16	d	d	d	d	d	1.6	4.0

Table A5.21 Influence of EDTA on Zn toxicity to Zn-t12.0 *Anacystis*.

Zn (mg l^{-1})	EDTA (mg l^{-1})						
	0.5	2.0	4	8	16	32	64
0.04	9.8	10.4	9.6	9.6	9.6	10.4	11.2
6.0	9.2	9.2	9.3	9.5	10.0	9.8	9.6
12.0	5.6	6.6	7.1	7.8	8.2	8.5	8.8
15.0	d	d	d	3.5	6.5	8.8	8.5
18.0	d	d	d	d	5.8	8.8	8.4
21.0	d	d	d	d	2.5	4.5	6.2
24.0	d	d	d	d	d	1.3	4.8

Table A5.22 Influence of EDTA on Cd toxicity to wild-type *Anacystis*.

Cd (mg l^{-1})	EDTA (mg l^{-1})					
	0.5	2.0	4.0	8.0	16.0	32.0
C	9	8.8	9	9.2	9.0	9.0
0.15	6.4	8.0	8.6	8.2	8.8	9.4
0.30	4.5	6.2	8.6	8.8	8.6	8.6
0.45	4.2	4.5	8.0	8.2	8.8	8.8
0.60	3.0	3.4	4.4	8.0	8.6	8.2
0.90	d	d	3.6	8.0	8.0	8.2
1.2	d	d	2.8	6.4	7.4	8.0
1.50	d	d	1.2	6.5	7.5	8.0

Table A5.23 Influence of Zn on Cd toxicity to wild-type *Anacystis*; units ml^{-1} was used as a growth criterion.

Cd (mg l^{-1})	Zn (mg l^{-1})					
	0.04	0.2	0.4	0.6	0.8	1.0
0	8.6	8.7	8.9	9.0	8.3	8.7
0.15	3.7	8.2	8.8	8.6	8.3	4.8
0.24	4.1	8.9	8.5	8.7	8.7	a
0.30	3.8	8.2	8.5	8.1	8.5	a
0.36	3.3	8.1	8.0	8.0	6.2	a
0.42	a	8.6	8.3	8.2	a	a

Table A5.24 Influence of Zn on Cd toxicity to wild-type *Anacystis*; chl *a* was used as a growth criterion.

Cd (mg l^{-1})	Zn (mg l^{-1})					
	0.04	0.2	0.4	0.6	0.8	1.0
0	0.68	0.74	0.57	0.68	0.78	0.68
0.15	0.34	0.68	0.78	0.78	0.78	0.68
0.24	0.24	0.85	0.57	0.78	0.57	0.40
0.30	0.18	0.78	0.68	0.92	0.57	ND*
0.36	0.23	0.78	0.78	0.57	0.46	ND*
0.42	ND*	0.68	0.57	0.46	0.57	ND*

Table A5.25 Influence of low Zn levels on Cd toxicity to Zn-t5.0 *Anacystis*; chl *a* was used as a growth criterion.

Cd (mg l^{-1})	Zn (mg l^{-1})			
	0.04	0.25	0.5	1.0
0	2.14	2.21	1.92	2.21
0.15	2.14	1.91	1.71	1.71
0.30	2.14	1.88	1.71	1.85
0.45	2.14	1.71	1.71	1.71
0.60	1.85	1.71	1.71	1.71
0.75	1.14	1.57	1.77	1.71
0.90	1.14	1.43	1.78	1.87
1.05	1.07	1.14	1.71	1.07
1.20	ND	1.14	1.86	1.43
1.35	ND	ND	ND	1.43

Table A5.26 Influence of high Zn levels on Cd toxicity to Zn-t5.0 *Anacystis*; chl *a* was used as a growth criterion.

Cd (mg l^{-1})	Zn (mg l^{-1})					
	0.04	2.5	5.0	7.5	10	12.5
0	1.21	1.14	0.92	0.14	d	d
0.10	1.20	0.71	0.71	0.14	d	d
0.20	1.21	0.85	0.71	0.14	d	d
0.30	1.14	0.85	0.76	d o	d	d
0.40	1.20	0.85	0.71	d	d	d
0.50	1.21	0.94	0.87	d	d	d
0.60	1.12	0.71	0.71	d	d	d
0.70	1.14	0.93	0.71	d	d	d
0.80	1.14	0.93	0.78	d	d	d
1.0	0.57	0.93	0.88	d	d	d
1.2	ND	0.87	0.86	d	d	d
1.4	ND	0.57	0.57	d	d	d
1.6	ND	0.28	0.57	d	d	d

Table A5.27 Influence of pH on Zn toxicity to wild-type *Anacystis*.

Zn (mg l^{-1})	pH				
	6.0	6.5	7.0	7.5	8.0
0.04	3.2	5.6	8.6	8.8	9.0
0.25	2.4	4.8	7.8	8.5	8.8
0.50	1.5	4.6	7.5	8.5	8.6
0.75	0.6	4.5	7.4	8.5	8.5
1.0	d	3.8	7.2	8.2	8.1
1.25	d	3.5	6.8	7.2	7.5
1.5	d	d	0.60	6.8	7.2
2.0	d	d	d	6.5	6.8
2.5	d	d	d	2.7	4.3

Table A5.28 Influence of pH on Zn toxicity to Zn-t5.0 *Anacystis*.

Zn (mg l^{-1})	pH				
	6.0	6.5	7.0	7.5	8.0
0.04	6.4	7.6	8.0	8.0	8.0
2	6.2	7.4	7.7	7.6	7.4
4	4.8	5.2	5.3	4.8	4.8
5	4.5	4.6	3.4	2.2	d
6	4.2	4.4	d	d	d
8	1.2	3.6	d	d	d
10	d	d	d	d	d

Table A5.29 Influence of pH on Zn toxicity to Zn-t12.0 *Anacystis*

Zn (mg l^{-1})	pH				
	6.0	6.5	7.0	7.5	8.0
0.04	5.5	7.0	7.4	7.2	7.2
4	4.2	6.3	6.7	4.7	4.7
6	2.6	5.8	6.4	3.2	3.2
8	1.0	5.4	6.0	1.8	1.8
12	d	5.0	2.0	0.05	0.05
16	d	4.3	d	d	d
20	d	2.0	d	d	d
24	d	d	d	d	d

Table A5.30 Influence of pH on Cd toxicity to wild-type *Anacystis*.

Cd (mg l^{-1})	pH				
	6.0	6.5	7.0	7.5	8.0
0.04	3.5	5.8	8.8	9.5	10.2
0.15	d	0.8	6.4	8.8	9.4
0.30	d	0.03	4.2	8.4	8.5
0.45	d	d	3.8	8.6	8.6
0.6	d	d	3.2	6.2	7.2
0.9	d	d	d	5.8	6.2
1.2	d	d	d	4.5	5.8

Table A5.31 Influence of inoculum size on Zn toxicity to wild-type *Anacystis*

Zn (mg l^{-1})	Inoculum size (units ml^{-1})			
	$2x10^4$	$2x10^5$	$2x10^6$	$2x10^7$
0.04	$6.8x10^7$	$8.4x10^7$	$12.2x10^7$	$12.5x10^8$
0.5	$5.4x10^7$	$8.5x10^7$	$9.2x10^7$	$10.4x10^8$
1.0	$3.6x10^7$	$6.4x10^7$	$8.8x10^7$	$9.8x10^8$
1.25	$1.8x10^7$	$1.8x10^7$	$6.5x10^7$	$7.8x10^8$
1.5	d	d	d	$4.0x10^8$
2.0	d	d	d	d

Table A5.32 Influence of inoculum size on Zn toxicity to wild-type *Anacystis*

Zn (mg l^{-1})	Inoculum size (chl a mg l^{-1})			
	0.001	0.01	0.10	1.0
0.04	1.3 ± 0.0	1.38 ± 0.07	1.67 ± 0.07	2.30 ± 0.18
0.5	1.14 ± 0.0	1.21 ± 0.12	1.44 ± 0.17	1.83 ± 0.14
1.0	0.75 ± 0.15	0.75 ± 0.12	1.36 ± 0.07	1.40 ± 0.06
1.25	0.25 ± 0.03	0.28 ± 0.04	0.88 ± 0.08	1.35 ± 0.08
1.5	d	d	0.35 ± 0.005	0.85 ± 0.12
2.0	d	d	d	d
3.0	d	d	d	d

Table A6.1 Accumulation of Zn during batch culture growth of wild-type *Anacystis*. Results show total values for alga + filter (i.e. no allowance made for filter); compare with Tables 6.2, 6.3)

initial Zn (mg l^{-1})	time (h)	dry weight (mg l^{-1})	Zn left in medium (mg l^{-1})	washed + filter at 0.1: Zn in alga	EDTA first	EDTA second	EDTA third	µg Zn g^{-1} dry weight	Zn in alga after washes / Zn left in medium	total Zn recovery (mg l^{-1})	unwashed + filter: Zn in alga	µg Zn g^{-1} dry weight	total Zn / Zn left in medium	total Zn recovery (mg l^{-1})
0.1	0.166	50	0.076	0.010	0.008	0.004	0.002	200	2631.6	0.105	0.024	480	6315.8	0.10
	24	196	0.067	0.012	0.013	0.005	0.003	130.4	1946.3	0.104	0.033	358.7	5353.7	0.10
	48	220	0.062	0.015	0.015	0.006	0.002	136.4	2200	0.10	0.036	327.3	5279	0.099
	96	248	0.054	0.025	0.012	0.007	0.003	201.6	2733.3	0.10	0.046	371	6870.4	0.10
	120	260	0.050	0.031	0.014	0.005	0.002	238.5	4770	0.105	0.050	384.6	7692	0.10
	144	272	0.044	0.038	0.014	0.006	0.002	279.4	6350	0.10	0.055	404.4	9191	0.099
	168	288	0.035	0.042	0.016	0.004	0.002	291.7	8334.3	0.099	0.060	416.6	11902	0.095
	192	310	0.026	0.045	0.016	0.004	0.002	290.3	11165	0.095	0.068	438.7	16873	0.094
										$\bar{x}$ = 0.102 ± 0.003				$\bar{x}$ = 0.0985 ± 0.002
1.0	0.166	50	0.96	0.010	0.022	0.006	0.002	200	208.3	1.0	0.040	800	833.3	1.0
	24	195	0.95	0.012	0.028	0.004	0.003	125	131.6	1.0	0.048	505.3	531.8	0.998
	48	200	0.90	0.035	0.052	0.008	0.004	350	388.8	1.01	0.095	950	1055.5	0.995
	96	212	0.85	0.060	0.070	0.013	0.006	566	665.8	1.0	0.148	1396.3	1642.6	0.998
	120	232	0.76	0.105	0.112	0.013	0.006	905	1190.8	0.994	0.232	2000	2631.6	0.992
	144	256	0.70	0.132	0.132	0.18	0.005	1031.3	1473.3	0.984	0.284	2218.8	3619.7	0.984
	168	272	0.68	0.152	0.135	0.012	0.006	1117.6	1643.5	0.985	0.302	2220.6	3265.6	0.982
	192	304	0.58	0.252	0.146	0.026	0.003	1657	2959	0.989	0.420	2763.2	4934.3	0.980
										$\bar{x}$ = 0.994 ± 0.006				$\bar{x}$ = 0.99 ± 0.007

Table A6.2 Accumulation of Zn during batch culture growth of Zn-12.0 *Anacystis*. Results show total values for alga + filter (i.e. no allowance made for filter); compare with Tables 6.4, 6.5, 6.6.

			washed + filter									unwashed + filter			
initial Zn ($mg\ l^{-1}$)	time (h)	dry weight ($mg\ l^{-1}$)	Zn left in medium ($mg\ l^{-1}$)	Zn in alga	EDTA first	EDTA second	EDTA third	EDTA fourth	μg Zn g^{-1} dry weight	Zn in alga after washes / Zn left in medium	total Zn recovery ($mg\ l^{-1}$)	Zn in alga	μg Zn g^{-1} dry weight	total Zn / Zn left in medium	total Zn recovery ($mg\ l^{-1}$)
0.1	0.166	50	0.071	0.010	0.014	0.003	0.002		200	2817	0.10	0.028	560	788.3	0.099
	24	200	0.065	0.016	0.012	0.005	0.002		160	2461.5	0.10	0.033	330	5077	0.098
	48	216	0.060	0.028	0.010	0.004	0.002		259.3	4321.6	0.102	0.038	352	5863.3	0.098
	96	240	0.052	0.032	0.011	0.004	0.002		266.6	5127	0.10	0.046	383.3	7371.2	0.098
	120	280	0.046	0.040	0.008	0.003	0.002		285.7	6210.8	0.099	0.051	364.3	7920	0.097
	144	292	0.040	0.042	0.012	0.005	0.002		287.7	7192.5	0.099	0.058	397.2	9930	0.096
	168	300	0.032	0.045	0.013	0.005	0.004		300	9375	0.098	0.065	435.3	13541	0.097
	192	320	0.022	0.056	0.012	0.004	0.002		350	15909	0.096	0.074	462.5	21023	0.096
											$\bar{x}$ = 0.099				$\bar{x}$ = 0.0975
											± 0.002				± 0.001
1.0	0.166	50	0.96	0.011	0.030	0.004	0.002		220	229.2	1.01	0.040	800	833	1.0
	24	202	0.92	0.023	0.058	0.006	0.003		230	250	1.01	0.088	880	956.5	1.0
	48	216	0.90	0.045	0.060	0.005	0.003		416.6	403	1.00	0.098	907.4	1008.2	0.998
	96	248	0.80	0.060	0.115	0.007	0.005		483.8	604.8	1.0	0.188	1516.7	1895.8	0.990
	120	280	0.71	0.126	0.140	0.008	0.006		900	1267.6	1.0	0.278	1985.8	2758.1	0.998
	144	296	0.60	0.186	0.20	0.008	0.002		1256.8	2094.6	0.996	0.395	2669	4448.3	0.995
	168	310	0.52	0.215	0.250	0.008	0.003		1387.1	2667.5	0.996	0.470	3032.5	5831.3	0.996
	192	328	0.44	0.302	0.222	0.016	0.002		1841.5	4185.2	0.982	0.542	3305	7511.4	0.982
											$\bar{x}$ = 0.999				$\bar{x}$ = 0995
											± 0.008				± 0.006
10.0	0.166	50	8.55	0.036	0.958	0.35	0.066	0.040	720	84.2	10.0	1.45	29000	3392	10.0
	24	168	8.08	0.045	0.971	0.69	0.148	0.066	535.5	66.3	10.0	1.92	22857	2828.8	10.0
	48	202	7.20	0.240	1.25	0.85	0.380	0.065	2400	333.3	9.98	2.78	25272	3510.1	9.98
	96	248	7.0	0.318	1.26	0.86	0.50	0.04	2564.5	366.4	9.98	2.98	24032	3433.2	9.98
	120	264	6.77	0.407	1.34	0.88	0.52	0.06	3083.3	455.4	9.97	3.208	24303	3589.8	9.98
	144	272	6.77	0.515	1.54	0.75	0.35	0.04	3786.7	560	9.96	3.20	23529	3475.5	9.97
	168	288	6.45	0.645	1.75	0.78	0.32	0.03	4479	694.4	9.97	3.52	24444	3789.8	9.95
	192	300	6.0	1.05	2.15	0.46	0.25	0.04	7000	1166.6	9.95	3.95	26333	4388.8	9.95
											$\bar{x}$ = 9.96				$\bar{x}$ = 9.976
											± 0.016				± 0.018