

Arsenite Toxicity and Tolerance in Three Microalgae, Synechococcus
leopoliensis, Oscillatoria limnetica and Chlorella pyrenoidosa.

by

Norman Chege Njoroge

A thesis submitted to the Department of Biology in conformity with the
requirements for the degree of Master of Science.

Queen's University
Kingston, Ontario, Canada
December, 1988

Copyright © Norman Chege Njoroge, 1988

UNIVERSITY OF NAIROBI
LIBRARY

Abstract

The cyanobacteria Oscillatoria limnetica and Synechococcus leopoliensis manifested concentration-dependent growth lags in presence of arsenite above 200 μ M. There were no detectable growth lags in the Chlorophyte Chlorella pyrenoidosa in presence of arsenite concentrations above 1mM and arsenite affected the log rate of growth. C. pyrenoidosa was less sensitive to arsenite than the two cyanobacteria.

Arsenite toxicity in S. leopoliensis was affected by pH of the medium. It decreased with an increase in pH. 1mM potassium acetate also alleviated toxicity in cells grown in presence of 200 μ M arsenite. Acetate did not alleviate toxicity of C. pyrenoidosa grown in presence of 3mM arsenite.

Cells of the two cyanobacteria grown in presence of 10 μ M arsenite for up to 12 hours became tolerant to 200 μ M arsenite with growth being similar to that of normal cells. 1.0 μ M arsenite was also adequate to induce arsenite tolerance but not 0.1 μ M arsenite. Pre-exposure of C. pyrenoidosa to 500 μ M arsenite for 12 hours induced some "tolerance" to arsenite concentration of up to 4mM.

The amounts of arsenic in whole air-dried tolerant and sensitive cells of S. leopoliensis were similar after exposure to 200 μ M arsenite for 8 or 9 hours. The cyanobacteria accumulated arsenic to about 18-40 times the medium concentration. The relative amounts of arsenite and arsenate in ice-cold water extract from tolerant cells were the same as in sensitive cells. However in recovered cells (after a 30-hour growth lag) almost all arsenic in the ice-cold water extract was in form of arsenate. The mechanism of tolerance in this culture was apparently due to conversion of arsenite to arsenate. C. pyrenoidosa grown in non-toxic concentration of arsenic (500 μ M) accumulated arsenic to about 80 times the medium concentration after 11 hours.

Acknowledgements

Possible mechanisms of arsenite tolerance in S. leopoliensis were investigated. The data did not support a tolerance mechanism based upon exclusion of arsenite, decreased uptake of arsenite, external or internal conversion of arsenite to the less toxic arsenate form, (except in recovered cells), or increase in any identified sulfhydryl compounds. An alternative pathway for synthesis of acetyl-CoA in which pyruvate dehydrogenase complex is not involved should be considered.

I would also like to express my deepest appreciation to Drs. J.D. MacArthur and S.L. Palmer (Department of Physics, Queen's University), Dr. J.B. Polard (Chemistry Department, Queen's University) for their timely help in detection of arsenic by PIXE. I would also like to thank Dr. P. Beeley (Royal Military College, Kingston) for his help in the use of NAA to determine arsenic in algal samples.

Thanks also go to W.M. Hickman, K. Reid and Harriet Hemmingson for their help in gel electrophoresis and fluorography of algal proteins.

I would also like to thank my colleague in the laboratory, D. Kelly whose company gave me invaluable moral support.

Thanks a lot to J. Surette for her patience in typing the thesis and to D. Kahan for the wonderful graphics.

Lastly, but not the least, I would like to express my deepest thanks and appreciation to my wife A. Wangui, my son P.M. Njoroge and daughter A.C. Njeri for their encouragement and patience during this study.

This work was funded by the Kenya-Canada General Training Fund and by the Natural Sciences and Engineering Research Council of Canada.

Acknowledgements

First and foremost many thanks go to my supervisor Dr. K. Budd, for making me feel at home in Canada and for giving me useful suggestions and advice that made my research and writing of the thesis so successful. 111

Special thanks also go to my co-supervisors, Drs. D.H. Turpin and A. Crowder for their helpful suggestions and encouragement throughout this study.

I would also like to express my deepest appreciation to Drs. J.D. MacArthur and G.R. Palmer (Department of Physics, Queen's University), Dr. J.S. Poland (Chemistry Department, Queen's University) for their timely help in estimation of arsenic by PIXE. I would also like to thank Dr. P. Beeley (Royal Military College, Kingston) for his help in the use of NAA to determine arsenic in algal samples. of arsenic in aquatic plants and animals..... 9

Thanks also go to W.E. Hekman, K. Reid and Harriet Hemmingson for their help in gel electrophoresis and fluorography of algal proteins. 13

I would also like to thank my colleague in the laboratory, D. Kelly whose company gave me invaluable moral support. 27

Thanks a lot to J. Surette for her patience in typing the thesis and to D. Reimer for the wonderful graphics. 37

Lastly, but not the least, I would like to express my deepest thanks and appreciation to my wife A. Wangui, my son P.M. Njoroge and daughter A.C. Njeri for their encouragement and patience during this study. 43

This work was funded by the Kenya-Canada General Training Fund and by the Natural Sciences and Engineering Research Council of Canada. 51

and *C. pyrenoidosa*..... 52

5. Levels of free (water soluble) and bound arsenic in *S.*

inopollensis as determined by NAA (Neutron Activation

Analysis)..... 53

Table of Contents

Abstract.....	i
Acknowledgement.....	iii
List of Tables.....	vi
List of Figures.....	viii
General Introduction.....	1
Literature Review.....	3
1. Arsenic in the environment.....	3
2. Nature and transformation of arsenic in water.....	6
3. Penetration of arsenicals into cells.....	9
4. Bioaccumulation of arsenic in aquatic plants and animals.....	9
5. Toxicity of arsenic to aquatic plants and animals.....	11
6. Effect of arsenic on cellular metabolism.....	13
7. Tolerance mechanisms to toxicants.....	17
Materials and Methods.....	27
Results.....	37
1. Toxicity of arsenite.....	37
2. Alleviation of arsenite toxicity by potassium acetate.....	38
3. Effect of pH on arsenite toxicity and arsenic content in <u>S.leopoliensis</u>	45
4. Arsenite tolerance in the microalgae.....	51
5. Arsenic levels in whole air-dried cells of <u>S. leopoliensis</u> and <u>C. pyrenoidosa</u>	52
6. Levels of free (water soluble) and bound arsenic in <u>S.</u> <u>leopoliensis</u> as determined by NAA (Neutron Activation Analysis).....	53

7.	Analysis of arsenic on paper chromatogram by Protein Induced X-ray Emission (PIXE).....	54
8.	Analysis of arsenic in water extracts from tolerant and sensitive cells of <i>S. leopoliensis</i> and paper chromatogram by NAA.....	54
9.	Uptake of $^{35}\text{SO}_4^{2-}$ by normal and tolerant cells of <i>S. leopoliensis</i>	55
10.	Thin layer chromatography of ethanol extracts from normal and tolerant cultures of <i>S. leopoliensis</i> provided with ^{35}S in the growth medium.....	56
11.	Polyacrylamide Slab Gel electrophoresis of tolerant and sensitive cell extracts of <i>S. leopoliensis</i>	56
	Discussion.....	78
1.	Arsenite sensitivity in the microalgae.....	78
2.	Effect of exogenous acetate on arsenite toxicity.....	79
3.	Effect of pH on arsenite toxicity.....	80
4.	Inducibility of arsenite tolerance.....	81
5.	Tolerance mechanism(s) to arsenite in <i>S. leopoliensis</i>	82
	Suggestions for further research.....	88
	Literature cited.....	89
	Appendices.....	99
	Vita.....	123
10.	Next generation electrical network and reduced utilization of	
11.	<i>S. leopoliensis</i> when transformed to 35S, 35S and 35S.....	
11.	Arsenic levels in whole cell-derived vesicles and tolerance levels of	
	<i>S. leopoliensis</i> as determined by Protein Induction Analysis	
	(NAA)	85

LIST OF TABLES

Table

1. Range in amounts (ppb) and chemical forms (species) of arsenic in aquatic systems.	5
2. Alleviation of arsenite toxicity by acetate in <u>S. leopoliensis</u>	43
3. Growth lags of <u>S. leopoliensis</u> grown under different pH's.	49
4. Amount of arsenic in filtered layers of <u>S. leopoliensis</u> grown in media containing 200 μ M arsenite at different pH's.	50
5. Growth lags of sensitive and tolerant cells of the cyanophytes in arsenite.	58
6. Growth lags of <u>S. leopoliensis</u> in 200 μ M arsenite after induction with 10 μ M and 1.0 μ M arsenite for different time periods.	59
7. Generation times of <u>C. pyrenoidosa</u> in 1-5mM arsenite with or without exposure to non-inhibiting concentration of arsenite (500 μ M).	60
8. Cell numbers of normal (control) cultures and cultures undergoing induction of arsenite tolerance.	61
9. Growth lags of tolerant cells of <u>S. leopoliensis</u> in 200 μ M arsenite after transfer from an arsenite-free medium.	63
10. Mean generation times of tolerant and recovered cultures of <u>S. leopoliensis</u> when transferred to 300, 400 and 500 μ M As.	64
11. Arsenic levels in whole air-dried sensitive and tolerant cells of <u>S. Leopoliensis</u> as determined by Neutron Activation Analysis (NAA)	65

12. Amount of arsenic (ppm, dry wt) in filtered layers of <u>C. pyrenoidosa</u> grown in presence of 0.5 - 3.0mM arsenite	66
13. Levels of free (water soluble) and bound arsenic in <u>S. leopoliensis</u> as determined by NAA.	67
14. Amount of ^{35}S in ethanol and NaOH/HCl extracts from normal and tolerant cells.	73
15. Amount of ^{35}S in ethanol extracts (inclusive of lipid and water soluble compounds) and in NaOH/HCl extracts from tolerant and normal cells of <u>S. leopoliensis</u> allowed to grow up to three generations ($A_{660\text{nm}}$ 0.12 - 1.05).	74
16. Molecular weights of proteins in the bands of polyacrylamide gel and the fluorograph.	75
6. Alleviation of arsenite toxicity by 1mM potassium acetate in <u>S. leopoliensis</u>	44
7. Effect of pH on arsenite toxicity in <u>S. leopoliensis</u>	47
8. The controls for the "Effect of pH on arsenite toxicity in <u>S. leopoliensis</u> ".	48
9. Decay of tolerance with time in <u>S. leopoliensis</u>	62
10a. Distribution of arsenic in ice-cold water extracts of sensitive and tolerant cells on paper chromatogram as determined by Proton Induced X-Ray Emission (PIXE).	58
11. Distribution of arsenic in ice-cold water extracts of tolerant cells on paper chromatogram as determined by Neutron Activation Analysis (NAA).	69
12. Distribution of arsenic in ice-cold water extracts of sensitive cells on paper chromatogram as determined by NAA.	70

LIST OF FIGURES:

Figure

1. Eh-pH diagram for arsenic at 25°C and 1 atmosphere (Ferguson and Gavis 1972) (Note: solid species are enclosed in parentheses in cross-hatched areas).	8
2. Effect of arsenite on growth of <u>S. leopoliensis</u>	39
3. Effect of arsenite on growth of <u>Oscillatoria limnetica</u>	40
4. Effect of different concentrations of sodium arsenite on the growth of <u>Chlorella pyrenoidosa</u>	41
5. Alleviation of arsenite toxicity by 1mM potassium acetate in <u>S. leopoliensis</u>	42
6. Alleviation of arsenite toxicity by 1mM potassium acetate in <u>O. limnetica</u>	44
7. Effect of pH on arsenite toxicity in <u>S. leopoliensis</u>	47
8. The controls for the "Effect of pH on arsenite toxicity in <u>S. leopoliensis</u> ".	48
9. Decay of tolerance with time in <u>S. leopoliensis</u>	62
10a,b Distribution of arsenic in ice-cold water extracts of sensitive and tolerant cells on paper chromatogram as determined by Proton Induced X-Ray Emission (PIXE).	68
11. Distribution of arsenic in ice-cold water extracts of tolerant cells on paper chromatogram as determined by Neutron Activation Analysis (NAA).	69
12. Distribution of arsenic in ice-cold water extracts of sensitive cells on paper chromatogram as determined by NAA.	70

13. Distribution on paper chromatogram of arsenic in ice-cold water extract from sensitive cells that were allowed to recover (after 30 hrs). (Analysis by NAA) 71
14. Amounts of ^{35}S (in ppm) in ethanol extracts from normal and tolerant cultures. 72
15. Polyacrylamide SDS Gel Electrophoresis of proteins in cell extracts from S. leopoliensis that were not provided with radioactive sulphate in the growth medium. 76
16. Fluorograph of proteins in cell extracts from S. leopoliensis that were provided with radioactive sulphate in the growth medium. 77

General Introduction

Arsenic is a member of Group VA elements (N, P, As, Sb, Bi). It is at the borderline between non-metals and metals and as such is sometimes called a metalloid. Its chemical properties are similar to those of phosphorus. It is widely distributed in the environment and in plant and animal tissues. The different forms of arsenic in the environment have different toxicities.

Arsenate generally is less toxic than arsenite and does not inhibit any known enzyme system. However, the former inhibits ATP synthesis by uncoupling oxidative phosphorylation and replacing the stable phosphoryl group. Arsenite, on the other hand, inhibits sulfhydryl group-dependent enzymes and is retained in the body tissue proteins, such as keratin disulfides in hair, nails and skin. (Moore and Ramamoorthy, 1984).

In spite of, and in some cases because of, its known toxicity, arsenic has been used for numerous purposes e.g. as a wood preservative, paint pigment and pesticide; in the production of glass; in the manufacture of transistors, lasers and semiconductor devices; and as a therapeutic agent in numerous animal and human diseases including trypanosomiasis and syphilis.

With respect to human health, Mushak (1985) has expressed concern about the potential impact of acidic precipitation on the environmental mobility of arsenic. Arsenic may be mobilized from household plumbing into tap water by the corrosive action of acidic water. Surface water from catchment areas where acidic deposition occurs may contain elevated levels of soluble arsenic. In addition, acidification of aquatic ecosystems that are sources of drinking water, may result in enhanced release of arsenic from sediments to water. Therefore the change in acidity of both soil and aquatic ecosystems may lead to altered biotransformation processes of arsenic. This would affect the relative amounts of different forms of arsenic varying in their toxicity to humans.

The toxicity of arsenicals to plants and animals in general is well known. However, there are few published data on mechanisms of arsenic toxicity and tolerance in phytoplankton. Budd et al. (1986) reported that arsenite toxicity in Synechococcus leopoliensis was apparently due to inhibition of pyruvate dehydrogenase complex, an enzyme which catalyzes the synthesis of acetyl-CoA from pyruvate. However, when cells of S. leopoliensis were grown in presence of 10 μ M arsenite (a concentration that did not affect growth) for 12 hours, they became tolerant to concentrations of arsenite that inhibited growth of untreated cells. Cells of S. leopoliensis also recovered when incubated in media containing growth-inhibiting levels of arsenite. Arsenite tolerance did not appear to be due to the presence of an arsenite-insensitive form of pyruvate dehydrogenase complex, to differences in arsenic uptake, or to modification of exogenous arsenite.

In this study, the sensitivity to arsenite toxicity of S. leopoliensis and O. limnetica (Cyanobacteria) was compared to that of C. pyrenoidosa (Chlorophyta). The two phyla exhibited a remarkable difference in this respect. The alleviation of arsenite toxicity by acetate in the three microalgae and the effect of pH (in the range 6.2 - 8) on arsenite toxicity in S. leopoliensis in particular, were also investigated. The inducibility of arsenite tolerance in the cyanobacteria was compared to that of C. pyrenoidosa. The effect, on tolerance, of transferring tolerant cells of S. leopoliensis to an arsenite-free medium was also investigated. Lastly further attempts were made to identify the mechanism(s) of tolerance to arsenite in S. leopoliensis. The possible mechanisms investigated were: internal or external conversion of arsenite to the less toxic arsenate form, and synthesis of low or high molecular weight sulfhydryl compounds that could bind arsenite into a non-toxic form.

LITERATURE REVIEW

1. Arsenic in the environment.

Arsenic does not normally occur as a free element in the environment. More frequently it occurs as a major component of sulfide minerals. The abundance in the continental earth's crust is about 1.5 - 2 ppm As (NAS 1977). It is mobilized in the environment through natural processes such as weathering of rocks and minerals, volcanic eruptions (7.8×10^9 g/y) and human activities such as smelting of non-ferrous metals, combustion of coal for generation of electricity and space heating, and use of arsenical pesticides (23.6×10^9 g/y) (Walsh et al 1979). (cited in Adriano 1986).

In the air, the natural level of arsenic is about $0.005 - 1.0 \mu\text{g}/\text{m}^3$ (NRCC, 1978). Its sources are volcanoes, forest fires and microbial reduction of arsenicals in soil and water to volatile arsines which are rapidly oxidized in presence of sunlight to arsenious oxides (Braman 1975, Wood 1974). Human activities such as burning coal for power production, burning vegetation treated with arsenical pesticides and lead arsenate spraying in orchards have raised airborne arsenic temporarily and locally to $19-69 \mu\text{g}/\text{m}^3$, $400 \mu\text{g}/\text{m}^3$ and $20 - 260,000 \mu\text{g}/\text{m}^3$ respectively (NRCC 1978). Arsenic in air is composed primarily of inorganic arsenic oxides and arsenates (Braman 1975; Kamori and Sugawara 1965) and is contained in particles of about 1 μm in diameter.

Uncontaminated soils contain about 10ppm arsenic but agricultural use of arsenicals can increase it to 600 ppm or more (Adriano 1986). In areas near mineral deposits of arsenic, soil levels may average 400 to 900 ppm (NRCC 1978). Inorganic arsenite may be bound to iron and aluminium oxides. Organoarsenicals adsorb to iron hydroxides on the clay surface (Wauchope, 1975) at the same site as phosphate. They are adsorbed more strongly than inorganic arsenicals, with sorption increasing in the order phosphate <

cacodylate < arsenate ~ methylarsonate (Fassbender, 1975).

In uncontaminated surface waters, arsenic content varies greatly but in general it averages less than 10 ppb (Waldbott, 1973). The amounts (in ppb) and chemical forms (species) of arsenic reported by several workers have been summarized by Braman, 1983 (cited in Fowler, 1983) (Table 1). Arsenic in water comes from natural sources such as weathering of rocks and minerals (45×10^3 tons/y on global basis) and land erosion (0.01 mg/L); anthropogenic sources contribute about 3 times (110×10^3 tons/y) that contributed from natural weathering (Ferguson and Gavis, 1972). Some anthropogenic sources and their arsenic contents are fossil fuels (2 - 25 ppm As in coal; 0.05 - 1.1 ppm in petroleum), household detergents - mostly of high-phosphate type (1 - 73 ppm As, Anginos et al 1970), urban sewage effluent (about 1.1 ppm, Carricker et al 1976) and runoff/leachate solutions from agricultural land (about 723 ppm As, Bails and Ball (1966).

In freshwater sediments, high levels of arsenic may be found adsorbed to hydrous iron oxides or various sedimentary colloids, e.g. aluminosilicates or organosilicates (NRCC 1978). Huang and Liao (1978) reported a range of total arsenic content of sediments of ten selected freshwater lakes in Canada of 2.7 to 13.2 mg kg⁻¹. Clement and Faust (1981) reported that the desorption of arsenic from contaminated sediments was highly dependent on reduction of Fe(+3) to Fe(+2). Anaerobic conditions which favour formation of Fe²⁺, produced ten times higher concentrations of As(+3) compared to aerobic conditions.

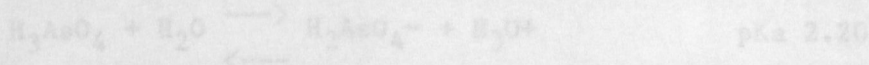
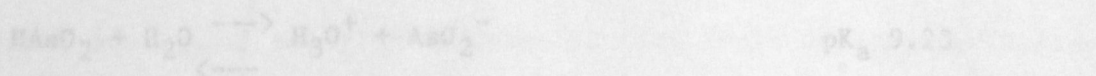
Table 1

Range in amounts (ppb) and chemical forms (species) of arsenic in aquatic systems.

Chemical form	Freshwater	Seawater
Arsenic (III)	<0.02-2.74	0.016-0.62
Arsenic (V)	0.16-64	1.20-1.75
Methyl arsonic acid (MAA)	<0.02-14.4	<0.002-0.08
Dimethyl arsenic acid (DMAA)	<0.02-6.31	0.002-0.29
Trimethyl arsine (TMA)	5.31	

1 ppb = 1 $\mu\text{g L}^{-1}$ = 13.3nM.

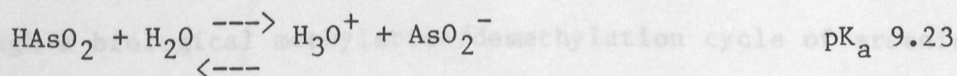
Braman, 1983 (cited in Fowler, 1983).



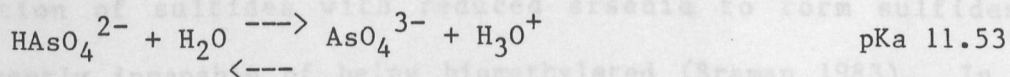
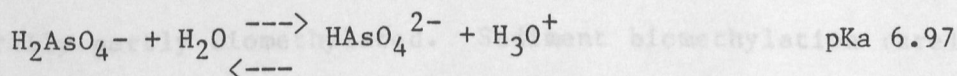
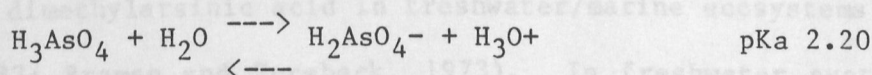
2. Nature and transformation of arsenic in water.

Most of the arsenic in water is in soluble form. Of the inorganic arsenic in water, arsenate is the most stable and predominates under equilibrium conditions. However, certain marine bacteria and corals have the ability to reduce arsenate to arsenite resulting in predominance of arsenite over arsenate (NRCC, 1978). The chemical species of arsenic present in water also depend on pH, Eh (standard oxidation-reduction potential) and dissolved oxygen content of water. Low pH favours formation of lower oxidation states of arsenic (i.e. +3, 0, -3) and pH 7-14 favour increased percentage of ionic species and pentavalent arsenic (Gulledge and O'Connors, 1973). At environmental ranges of pH 4-8, arsenious acid (HAsO_2) is essentially not dissociated while arsenic acid (H_3AsO_4) is mainly in some ionic form as indicated in the following equilibria.

Arsenious acid



Arsenic acid



Ferguson and Gavis (1972) proposed an interaction scheme of pH and Eh in determining the chemical species of arsenic in water. (Figure 1). High Eh (oxygenated waters), favours higher oxidation states and the predominant form is arsenic acid and its ionic forms (H_2AsO_4^- , HAsO_4^- , and AsO_4^{3-}). Under moderate Eh (or mildly reducing conditions) arsenious acid, H_3AsO_3 and its ionic forms (H_2AsO_3^- , HASO_3^{2-}) prevail.

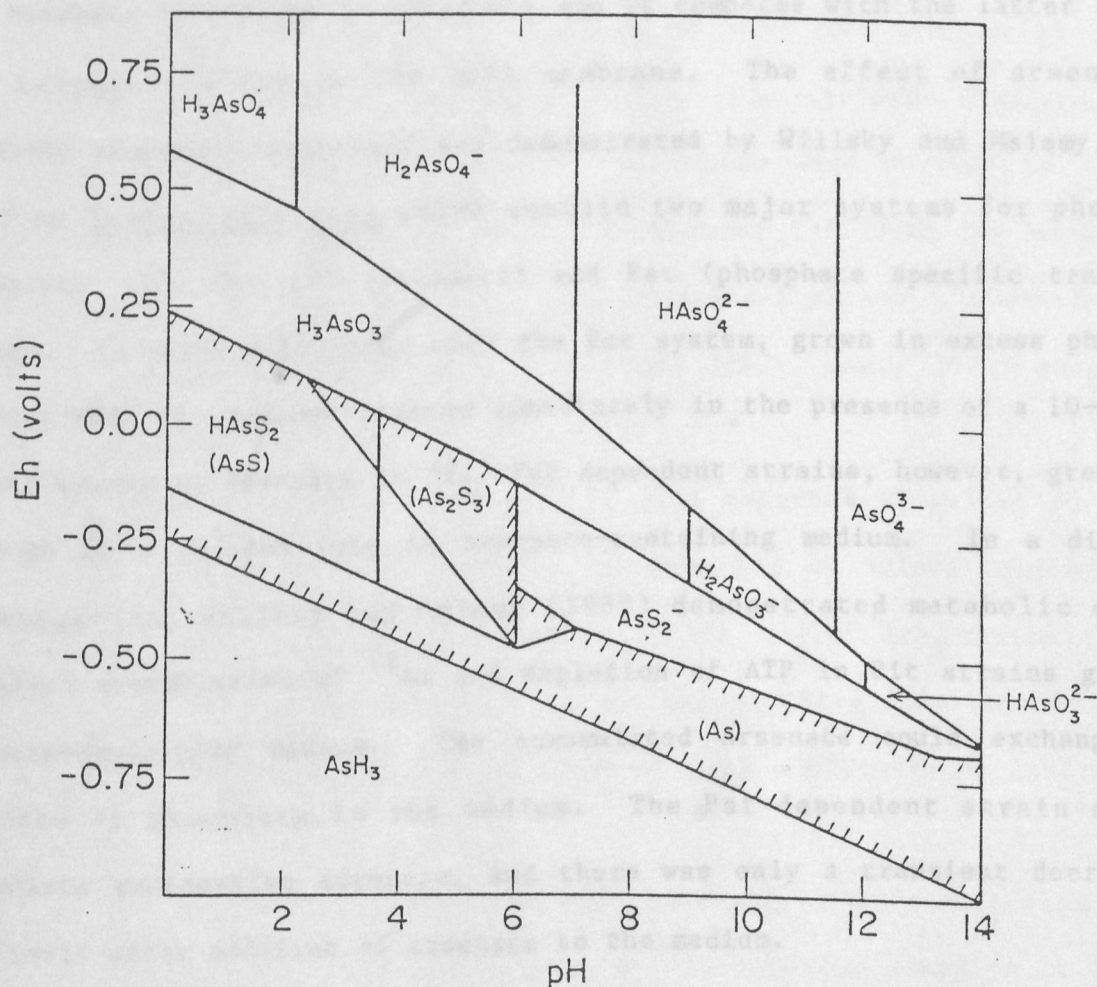
The metallic form ($\text{As}(0)$) and arsine (AsH_3) are the common species at low Eh (reducing conditions) and if sulphur is present, arsenic sulfides (AsS_2^- , HAS_2 , As_2S_3 and AsS) may also be formed. (Ferguson and Gavis 1972). Therefore, arsenate (As(V)) and arsenite (As(III)) prevail under aerobic and anaerobic conditions respectively. In addition, Johnson and Pilson (1975) demonstrated that increased salinity and temperature increased oxidation of arsenic in seawater.

In addition to arsenate, other chemical species of arsenic may be formed through a biological methylation/demethylation cycle of arsenic (Ridley et al, 1977). This process leads to presence of arsenite (As(III)), methylarsonic acid, and dimethylarsinic acid in freshwater/marine ecosystems (Andreae, 1978; Braman 1983; Braman and Foreback, 1973). In freshwater systems, arsenic is generally partly biomethylated. Sediment biomethylation rarely occurs due to reaction of sulfides with reduced arsenic to form sulfides which are apparently incapable of being biomethylated (Braman 1983). In saline water systems, methylated forms of arsenic are found mainly in the photic zone and not much below the thermocline. Phytoplankton and zooplankton activity is probably responsible for production of methylated forms of arsenic in the photic zone (Ridley et al, 1977).

Figure 1.

Eh-pH diagram for arsenic at 25°C and 1 atmosphere (Ferguson and Gavis 1972) (Note: solid species are enclosed in parentheses in cross-hatched areas).

Figure 1.



3. Penetration of arsenicals into cells.

Brooks (1923-1925) studied the entrance of arsenite into Valonia cells and found almost all taken up into the protoplasm, probably because of binding to proteins. The uptake of arsenic at an external pH of about 7 was minimal. At physiological pH, the un-ionized arsenious acid (pKa 9.23) is the dominant form and would be expected to enter into cells easily (Webb, 1966). Almost nothing is known on arsenite ions (AsO_2^-) transport into cells.

Arsenate is similar to phosphate and it competes with the latter for the same transport sites on the cell membrane. The effect of arsenate on inorganic phosphate transport was demonstrated by Willsky and Malamy (1976, 1980) in Escherichia coli which contain two major systems for phosphate transport: the Pit (Pi transport) and Pst (phosphate specific transport) systems. In cells containing only the Pst system, grown in excess phosphate medium (1mM), Pi transport ceased immediately in the presence of a 10-fold or greater excess of arsenate to Pi. Pst dependent strains, however, grew on Pi although at a reduced rate in arsenate-containing medium. In a different investigation, Willsky and Malamy (1980) demonstrated metabolic energy-dependent accumulation of ^{74}As and depletion of ATP in Pit strains grown in arsenate-containing medium. The accumulated arsenate could exchange with arsenate or phosphate in the medium. The Pst-dependent strain did not accumulate radioactive arsenate, and there was only a transient decrease in ATP levels after addition of arsenate to the medium.

Information on the penetration of organoarsenicals was unavailable, but since they are lipid soluble, they would be expected to enter into the cells easily.

4. Bioaccumulation of arsenic in aquatic plants and animals.

Arsenic may be accumulated by aquatic organisms. The bioaccumulation ratio (BR) for arsenic, as used in this study, is defined as the ratio of

arsenic in ppm (w/v) in algal cell water or ppm As(D.W.) in algae divided by the concentration of arsenic in ppm (w/v) in the growth medium.

In New Zealand, it was found that algae growing in lakes fed by hot springs with arsenic contents as high as 0.1 ppm had arsenic concentrations between 20 and 1,450 ppm (BR 200-14,500) on a dry matter basis (Lancaster et al., 1971). Reay (1972) also reported a BR of 20,000 for submerged weeds in the Waikato River (from geothermal origin with high As) in New Zealand. Emerged weeds from the same river had a BR of only 100. Cacodylic acid was accumulated by algae to a much higher degree than arsenate (Isensee et al., 1973) but monosodium methane arsonate BR values were similar to those of arsenate (Woolson et al. 1974) cited in Woolson (1975). Marine plants were found to have much higher BR values than freshwater plants. Seaweeds had BR values of 350 to 71,000 (Wilson and Fieldes, 1941; Lunde, 1973) while algae had BR of between 50 and 47,500.

Among aquatic animals, Pratt et al. (1972) reported BR values of 3 to 30 for various freshwater fish. Marine organisms had BR values ranging from 9 for crustacea and shellfish (Chapman, 1926) to 64,100 for shrimp (dry wt) (Coulson et al. 1935). In general, for various assorted salt water fish, the BR were 10 to 100 times higher than those of fish and Crustacea in freshwater (Woolson 1975).

Ball and Hooper (1965) studied experimentally the relationship between the water concentration and the uptake of ⁷⁴As-labelled arsenite by algae in aquariums containing several species of algae, fish and soil. High concentrations of arsenic were found in all species of algae. Since the arsenic could be washed off with dilute hydrochloric acid, it was considered to be loosely held on the surface rather than absorbed into the tissues. The high degree of adsorption may be attributed to the larger surface:volume ratio

of algae. Freshwater and marine algae may also accumulate arsenic through the synthesis of both fat-soluble and water-soluble organic arsenic compounds (Lunde, 1973).

5. Toxicity of arsenic to aquatic plants and animals

In general, inorganic arsenicals are more toxic than organic arsenicals and arsenites more toxic than arsenates. The toxicity of arsenical compounds decreases as follows: arsine (-III) > organo-arsine derivatives > arsenites (+III) > arsenoxides (+III) > arsenates (+V) > pentavalent organic compounds (+V) > arsonium metals (+I) > metallic arsenic (0) (NRCC, 1978).

Christensen and Zielki (1980) studied the effects of arsenate on growth of the freshwater alga Chlamydomonas over a 25-day period. They observed that As(V) inhibited growth at a concentration of 0.075 mg As L⁻¹. Budd et al (1981) reported that 200uM arsenite temporarily stopped growth of the blue-green alga S. leopoliensis. In another study using the same alga, Budd et al (1980) had also demonstrated that S. leopoliensis was resistant to arsenate at up to 200µM. Arsenate uptake was low and was not affected by light or exogenous phosphate.

The relative toxicities of arsenite As(III) and arsenate As(V) in phytoplankton are difficult to generalize. Bottino et al (1978) investigated the effects of As(V) and As(III) on the growth of the marine phytoplankton Tetraselmis chui and Hymenomas carterae. At 10 ppm As and above, both chemical forms affected growth of the former but not that of the latter. As(III) did not affect growth in either species. Sanders (1979) also examined the effect of As(V) and As(III) on the growth of Skeletonoma costatum and the influence of exogenous phosphate on these effects. Arsenate As(V) inhibited primary production of S. costatum at concentrations as low as 67nM in presence of low phosphate concentrations but 3uM phosphate abolished this effect. Arsenite, As(III) was toxic in the same range of concentrations as arsenate

As(V).

Several workers have also investigated the effect of arsenic in invertebrates. Spehar et al (1980) reported that exposure of the amphipod Gammarus pseudolimnaeus to 0.96 mg As(III) L⁻¹ resulted in 100% mortality within 14 days. Additional exposure of the amphipods to 0.97 mg As(V) L⁻¹ did not affect them. Marine invertebrates have been found to exhibit a wide range of susceptibility to As(III). Nelson et al (1976) demonstrated in the juvenile bay scallop, Argopecten irradians, a 96-hour LC₅₀ for As(III) of 3.49 mg L⁻¹ in sea water at 20°C and 25‰. Curtis et al (1979) using the grass shrimp Palemonetes pugio, found a mean LC₅₀ for this species of 40.6 mg AsL⁻¹ with arsenic trisulfide As(III).

Among the fish, Sorenson (1976) studied the toxicity of As(V) to green sunfish (Lepomis cyanellus) at concentrations of up to 1,000 mg L⁻¹ As over a 15-day period. The mean LC₅₀ for 46, 17 and 12 hours were 100, 500 and 1,000 ppm As(V) respectively. Pandey and Shukla (1979) reported mean LC₅₀ for 24 and 48 hours of 24 ppm and 18 ppm arsenic trioxide As(III) respectively for the blue tropical fish, Puntius sophore.

Arsenite toxicity may be influenced by pH, phosphate and temperature. Toxicity to aquatic plants and invertebrates generally decrease with an increase in the pH of the medium. High pH favours formation of higher oxidation states of arsenic which are less toxic (Moore and Ramamoorthy, 1984). In presence of low exogenous phosphate, 67nM arsenate was found to inhibit growth of Skeletonoma costatum and the addition of 3μM phosphate abolished this effect (Sanders 1979). Keeley and Thullen (1971a, b) reported that disodium methane arsonate controlled purple nutsedge better at 20° and 29° than at 13°C.

Depending on the period of exposure of an organism to arsenic, toxicity

may be described as acute or chronic. Acute toxicity refers to harmful effects or mortality of an organism after a brief exposure to arsenic. The period of exposure is usually 4 days or less and the exposure may be single or multiple. The most common test for acute toxicity is the LC_{50} (median lethal concentration). LC_{50} is the concentration of arsenic that is estimated to be fatal to 50% of the test organisms under stated conditions of the test. Sometimes a similar term LD_{50} (median lethal dose) is used. In cases where mortality is difficult to determine e.g. in invertebrates, an EC_{50} (median effective concentration) is estimated rather than LC_{50} . The acute effect in such a case is considered as immobilization or inability to maintain posture. In phytotoxicity, absorbance of chlorophyll a, cell counts or dry weight may be determined to assess acute effects. Chronic toxicity, on the other hand, refers to adverse effects that appear after a long period of uptake of small quantities of arsenic. The concentration or amount is small enough so that no acute effects appear. The period of exposure is frequently a significant part of the normal life of an organism. Manifestations of chronic toxicity may be reproductive or behavioural effects.

6. Effect of arsenic on cellular metabolism

(a) Transport across the cell membrane

Scarborough (1975) suggested that arsenite could react with membrane-bound sulfhydryl enzymes resulting in inhibition of glucose active transport. Cell respiration and therefore energy necessary to maintain cell membrane integrity decreases. This eventually leads to increased permeability and destruction of the cell (Ochme, 1972).

Arsenate, a competitive inhibitor of phosphate transport, altered phosphate transport system in Escherichia coli (Willsky and Malamy, 1980), an organism which has two major phosphate transport systems (phosphate transport,

Pit and phosphate specific transport, Pst). The capacity (V_{max}) of the Pst system in cells grown with a 10-fold excess of arsenate to phosphate (1mM) increased by a factor of almost two but the affinity (K_m) for phosphate remained unchanged. The Pit dependent strain stopped growth in arsenate-containing media. Willsky and Malamy (1980) proposed that the increased capacity for phosphate transport of the Pst system after exposure to arsenate in the medium was due to either (I) competitive inhibition of phosphate transport by arsenate through the Pst system and derepression of the transport system due to apparent phosphate starvation or (II) involvement of arsenate as a substrate of the Pst system, which also functions as a rapid arsenate exit system.

(b) Glycolysis

Yamazaki and Haynes (1975) noted that 2×10^{-4} M arsenite completely inhibited pyruvate dehydrogenase, an enzyme which catalyzes the oxidative decarboxylation of pyruvate resulting in formation of CO_2 and acetyl-CoA. The latter provides acetyl groups to the citric acid cycle. Budd et al (1986) also suggested that toxicity of arsenite in the blue-green algae S. leopoliensis was due to inhibition of pyruvate dehydrogenase.

Arsenate can substitute for phosphate in some glycolytic reactions to form unstable arsenylated compounds which undergo spontaneous hydrolysis (arsenolysis). This leads to increased rate of oxidation but without production of a high energy phosphate bond as in ATP (Frost 1967a).

(c) Citric Acid Cycle

At low levels, arsenate may stimulate respiration for succinate but not NAD-linked substrates, while high levels uncouple phosphorylation reactions (Wickes and Wiskich, 1976). Arsenate also activates fumarase more than arsenite does (Dixon and Webb, 1958). Fumarase is responsible for conversion of fumarate to L-malate, an important step in the cycle (Woolson, 1983).

Isocitrate dehydrogenase, an important enzyme at the point of control in both lipid and carbohydrate metabolism was only 30% inhibited by 10^{-3} M arsenate but 100% inhibited by a similar concentration of 2-amino-4-arsenosphenol (an organic trivalent arsenical) (Bhuvaneswaran et al, 1972; Johnstone 1963).

(d) Oxidative Phosphorylation

Sandoval et al (1970) have shown that arsenate induces ATPase activity but Barron and Singer (1943) reported inhibition of ATPase by organic arsenicals. Melandri et al (1975) proposed that arsenate stimulates ATPase activity leading to reduced ATP production. In bacterial chromatophores and higher plant chloroplasts, arsenate stimulates ATP-driven electron transport but inhibits ATP production driven by light energy (photophosphorylation) (NRCC 1978).

Fong et al (1980) have shown directly that cellular ATP levels are significantly decreased in Escherichia coli exposed to arsenite. Recently, Squibb and Fowler (1983) suggested that an inhibition of mitochondrial oxidative phosphorylation is a primary effect of arsenic on cells.

(e) Lipid metabolism

Kannangara and Stumpf (1972) reported that arsenite caused accumulation of C_{16} acids in the de novo fatty acid synthesis. They suggested that arsenite acts on the enzyme system involved exclusively in the $C_{16} \rightarrow C_{18}$ step and not in earlier stages. This effect has been demonstrated for germinating seeds, the chloroplast system and the potato synthetase system (Huang and Stumpf, 1971).

Arsenic also inhibits lipid oxidation. β -hydroxybutyrate oxidation was completely inhibited by 5×10^{-4} M (38 ppm) p-arsenoso-benzoic acid (Johnstone 1963). A concentration of 10^{-4} M trivalent organic arsenical inhibited lipase

activity by 62% (Barron and Singer 1943) and stearate oxidase by 72-100%.

(f) Protein and enzyme synthesis

The rate of incorporation of labelled amino acids in vitro was lowered by 41% at a sodium arsenate concentration of $100\mu\text{g ml}^{-1}$ of medium (Baron et al, 1975) but not at $10\mu\text{g arsenate ml}^{-1}$. Budd et al (1986) reported that $200\mu\text{M}$ arsenite decreased the incorporation of carbon in the light into α -amino acids, especially glutamate, a precursor of proline and arginine, and an amino-group donor for synthesis of other amino acids. An inhibition of amino acid synthesis would lead to decreased protein and enzyme synthesis.

(g) Nucleic Acid Metabolism

Baron et al (1975) demonstrated that in vitro incorporation of triphosphates into DNA and RNA was reduced by 3-4% at sodium arsenate concentrations exceeding $1\mu\text{g ml}^{-1}$ and was reduced 10-35% at arsenate levels above $10\mu\text{g ml}^{-1}$. Petres et al (1977) reported that arsenate added in vitro to human lymphocytes, inhibits synthesis of DNA and RNA in a dose-dependent manner. $10\mu\text{M}$ sodium arsenite was found to inhibit DNA synthesis and histone phosphorylation (Guiley et al, 1980).

These findings suggest that after arsenic enters the nucleus it inhibits enzymes involved in nucleic acid synthesis, most likely those containing sulfhydryl groups.

The foregoing examples illustrate the many ways in which arsenic may upset cell metabolism and interfere with normal growth. Arsenic interferes with cellular metabolism by combining chemically with sulfhydryl groups of enzymes and their co-factors e.g. thiamine pyrophosphate or lipoic acid which are essential co-factors in the α -keto acid (R-CO-COOH) oxidation (Luh et al 1973). By reacting with the sulfhydryl groups arsenic destroys the tertiary structure which is essential for enzyme activity. The ability of arsenate to enter into reactions in place of phosphate is another major mechanism of

interference of arsenic with cellular metabolism. Therefore, when one considers the number of reactions in organisms that involve sulfhydryl groups and phosphorus, it is easy to understand the versatile nature of arsenic as a toxicant.

7. Tolerance mechanisms to toxicants

Introduction

Tolerance is a form of adaptation and is controlled by the genetic constitution of an individual organism. It is an adaptation of the individual rather than the population and therefore the stressor or toxicant does not usually operate at a physiological concentration great enough to destroy the organism. Although individual organisms may tolerate a toxicant during a single lifetime, the level of such adaptations are usually low, on the order of tenfold or less (Hodgson and Gulthrie, 1980).

The induction of tolerance to a toxicant may require appreciable periods of time when complicated mechanisms of adaptation are required, such as increased phagocytosis or morphological changes. However, tolerance due to increased levels of enzymes or other protein-related processes is often acquired quickly. For example, increased amounts of metallothionein, a protein that binds cadmium and stores it away from the site of action, are detectable within a few hours after stress by an appropriate toxic metal.

When the stressing chemical is removed, tolerance usually does not last for more than a few weeks although an occasional tolerance may last for a longer period (Hodgson and Guthrie, 1980).

Although the terms resistance and tolerance are sometimes used interchangeably to describe adaptation to toxicants, they are quite different in meaning. In acquired resistance, unlike tolerance, a substantial part of the population must be destroyed for adaptation to occur. It may occur within

increased uptake of a non-metal has also been reported. Nichol et al. (1978) reported that resistance to fluoride in *S. leucon* was associated with a decrease in fluoride uptake. Resistance to fluoride was associated only in the life of a single individual but also in generations that follow. When a stressing chemical is removed, resistance may persist for several weeks, months or years in some cases (Hodgson and Guthrie, 1980).

Cases of resistance and tolerance mechanisms to stressing chemicals or toxicants have been reported by several workers.

(a) Changes in cell membrane permeability and decreased uptake of toxicants.

Changes in cell membranes such as decreased permeability allow adaptation both by decreasing the amounts of toxicant and affording greater opportunity for detoxication during any given period. Wainwright and Woolhouse (1975) demonstrated changes in cell membrane permeability in Agrostis tenuis. They showed that increasing the concentration of copper caused greater leakage of K^+ from roots of non-tolerant than from the tolerant plants. This was interpreted as damage of the plasmalemma by copper in the non-tolerant plants. It was therefore suggested that plasmalemma modifications were involved in copper-tolerance.

Beppu and Arima, (1964) reported that reduced penetration of arsenite was the adaptive mechanism in Pseudomonas pseudomallei. More recently, Rosen and Borbolla (1984) demonstrated that arsenite-resistant cells of Escherichia coli showed reduced accumulation of $^{74}AsO_2^-$ compared to arsenite-sensitive cells. The former exhibited energy-dependent extrusion of AsO_2^- but not the latter. Silver and Keach (1982) reported decreased net uptake resulting from accelerated efflux of arsenate by induced resistant cells of Staphylococcus aureus. The rate of efflux was relatively independent of phosphate level or external pH. Weiss et al (1978) demonstrated lower accumulation of Cd^{2+} ions in plasmid-bearing resistant cells of S. aureus than in the sensitive cells.

Decreased uptake of a non-metal has also been reported. Nichol et al (1987) suggested that fluoride resistance in S. leopoliensis was associated with a lower accumulation of fluoride in fluoride-resistant cells than in the sensitive cells. They proposed that passive F-anion movement across the membranes of the former could occur leading to less accumulation of fluoride than in the latter.

(b) Exclusion of toxicant

Lead is normally accumulated in roots with little transported to the leaves. Barry and Clark (1978) reported that there was little increase in foliar lead in Agrostis tenuis, Festuca ovina and Minuartia verna over the range of 500-20,000 $\mu\text{g lead g}^{-1}$ in the soil around abandoned lead mines. The mechanism of metal exclusion and restricted transport has been well established (Peterson, 1983).

Aluminium-tolerant species of wheat are able to raise pH and precipitate aluminium at the surface of the roots. The rise in pH is a consequence of preferential uptake of NO_3^- resulting in release of OH^- . Aluminium-sensitive varieties of wheat are not able to do the same (Dodge and Hiatt, 1972).

It has also been reported that some algae exclude metals by releasing organic materials which chelate free metal ions in solution (Davey et al 1973; Swallow et al 1978; Jackson and Morgan 1978).

(c) Sequestration of toxicant.

The differential solvent extraction of root tissue homogenates has revealed that the washed cell wall fraction is the major site of zinc or copper localization in tolerant ecotypes of A. tenuis and A. stolonifera (Peterson, 1969; Turner, 1970; Turner and Marshall 1971, 1972) (cited in Robb and Pierpoint 1983). In addition the extracted residues from tolerant plant roots contained a higher percentage of zinc than those of the non-tolerant plants (Peterson, 1969).

The mature leaves of tea, the best-known accumulator of aluminium, may contain about 20000 ppm aluminium (Sivasubramaniam and Talibudeen 1972). The aluminium is concentrated in thickened epidermal cells (Matsumoto et al 1976). Vaughan and Linehan (1976) have suggested that aluminium accumulator species such as tea may store aluminium in vacuoles in form of complexes.

In the algae, Silverberg (1975) (cited in Rand and Petrocelli 1985) found that metal tolerance exhibited by the green alga Stigeoclonium tenue resulted from partial exclusion of metal ions by the cell wall alginates.

(d) Biological oxidation, reduction or hydrolysis

It has been suggested that flooding tolerance to Mn^{2+} toxicity in rice and many other species involves the capacity to sustain diffusion of oxygen through the intercellular air space system to the root surface for oxidation of Mn^{2+} to the nontoxic form Mn^{4+} (Woolhouse 1983).

Iron tolerance in rice may also involve oxidation of the toxic Fe^{2+} to the nontoxic Fe^{3+} . Lowland varieties of rice are better adapted to withstand both waterlogging and Fe toxicity than upland varieties because they have larger airtspaces and their roots are able to secrete O_2 for oxidation of Fe^{2+} to Fe^{3+} (Woolhouse, 1983) which forms iron plaques (reddish-brown deposits) at the root surfaces. Taylor (1983) hypothesized that the iron plaques play a role in the exclusion of Fe^{2+} from the plants. The potential iron toxicity within the plant is reduced through storage of a non-toxic form of iron in plastids (Smith 1984). About 80% of a plant's total iron was found in chloroplasts in form of phytoferretin, cytochromes, iron-sulfur proteins and heme proteins (Pushnick et al 1984, Harrison and Hoare 1980). A link between tolerance of iron toxicity with control of the rhizosphere microflora by certain nutrients has been suggested by some workers (Trolldenier, 1973, 1977; Benckiser et al 1984a; 1984b). For example, N-, P- or K- deficient culture

solutions exhibited the greatest increase in iron-reducing bacteria and hence Fe^{2+} toxicity.

A trimeric mercuric reductase enzyme which was characterized as a flavoprotein with a molecular weight of about 180,000 (Scottell 1978) has been purified from two plasmid-containing E. coli strains. The cofactor for the enzyme is NADPH and NADH works poorly or not at all (Komura et al 1971; Furukawa and Tonomura, 1972; Summers and Sugarman, 1974). The enzyme also required a reduced thiol reagent nearly 100-fold in excess of Hg^{2+} . Under these conditions virtually all mercury was in form of RSHg^+ and possibly RSHgSR (Furukawa and Tonomura, 1972; Izaki, 1977; Izaki et al, 1974; Schottel, 1978).

The capacity of some algae to reduce mercury to metallic mercury which may then volatilize has also been reported. DeFillipis and Pallaghy (1976) suggested that resistant cells of Chlorella had a greater releasing power of elemental mercury than sensitive ones.

In the accumulator species of Sebertia acuminata hydrolysis of Ni^{2+} has been reported (Lee et al, 1978). High concentrations of the hydrated cation $\text{Ni}(\text{H}_2\text{O})_6^{2+}$ were found.

(e) Incorporation of toxicants into harmless low molecular weight compounds

In many selenium accumulators including several dozen species of the legume genus Astragalus, e.g. A. pectinatus and A. bisulcatus, selenium is in form of seleno-amino acids, the principal ones being selenocystathione and selenomethylselenocysteine (Nigam and McConnell, 1976). Non-accumulators and selenium-sensitive plants synthesize mainly selenomethionine and selenocysteine, both amino acids being incorporated into proteins through the usual aminoacyl-tRNA synthetase enzymes. Seleno-amino acids of accumulators are not incorporated into proteins and therefore selenium tolerance involves exclusion of selenium from proteins or enzymes.

In the nickel tolerant plants such as Alyssum bertolonii, a nickel accumulator (Pelosi et al, 1976), Alyssum serpyllifolium and Pearsonia metallifera (Lee et al, 1978) nickel is complexed with malic and malonic acids. In other nickel accumulators (Hybanthus austro-caledonicus, H. caledonicus, Sebertia acuminata) the citro-nickel (II) complex predominates (Lee et al 1978). Conditions of nickel toxicity may therefore arise when levels of the complexing agents are inadequate giving rise to free Ni^{2+} ions which are toxic. The complexes may then be stored in vacuoles of the plant cells.

Tolerance of chromium ions Cr^{3+} in Leptospermum scoparium (Lyon et al, 1969) and of zinc in Silene cucubalus, Thlaspi alpestre, Rumex acetosa and Agrostis tenuis (Mathys, 1977) has been associated with formation of trioxalatochromate (III) ion, $[\text{Cr}(\text{C}_2\text{O}_4)_3]^{3-}$ and zinc malate/oxalate complexes respectively.

Heavy-metal complexing peptides (or phytochelatins) whose structure was established as n-glycine ($n = 3$ to 7) have been isolated from plant cell suspension cultures of Rauwolfia serpentina (Grill et al, 1985). The synthesis of phytochelatins was induced by $200\mu\text{M}$ CdSO_4 in the medium. Other heavy metal ions such as Cu^{2+} , Hg^{2+} , Pb^{2+} and Zn^{2+} were also able to induce synthesis of phytochelatins. The induction of phytochelatin synthesis was also observed in cell cultures of nine other plants. In all these cases, more than 90% of Cd^{2+} taken up by cells was complexed to the phytochelatin peptides. Cells not exposed to metals did not synthesize any phytochelatins.

Stokes et al (1977) suggested that copper tolerance in the alga Scenedesmus acutiformis was associated with synthesis of a low molecular weight copper binding protein (in the range of 10,000 mol. wt). In cells treated with 0.2 ppm Cu, the ratio of Cu to protein was 2.5 atom/mole compared

to 0.2 g atom/mole for untreated cells. The copper binding protein increased in response to increased Cu.

(f) Incorporation of metal ions into harmless high molecular weight compounds

Curvetto and Rauser (1979) and Rauser and Curvetto (1980) reported presence of metallo proteins in accumulator plants. They isolated a copper-thionein from roots of copper-tolerant Agrostis gigantea.

In the blue-green alga Anacystis nidulans cadmium-binding proteins have been isolated (Maclean et al, 1972). Olafson et al (1979) also demonstrated the synthesis of metallothioneins or proteins similar to metallothioneins in a marine blue-green alga, Synechococcus sp. Cells exposed to $2.5 \times 10^{-5} \text{M CdCl}_2$ for one month, produced about 50ng metallothionein/mg wet cells. Non-exposed cells produced no measurable metallothionein.

Higham et al (1984) reported synthesis of three cysteine-rich proteins of molecular weight 4000 to 7000 in cadmium-resistant Pseudomonas putida grown in 3mM cadmium.

Synthesis of metallothioneins may be induced by several metals, including Zn, Cu, Cd, Ag and Hg. Induction by one metal may give resistance to another because of the non-specific nature of the metallothioneins (Weis and Weis, 1983).

(g) Formation of resistant enzymes to toxicants

An acid phosphatase covalently linked to the cell walls of Agrostis tenuis was found to have a greater Cu^{2+} inhibitor constant in preparations from a Cu-tolerant clone ($K_i(\text{Cu}) \text{M } 1.23 \times 10^{-4}$) compared to the enzyme from normal pasture clone ($K_i(\text{Cu}) \text{M } 4.33 \times 10^{-5}$) (Wainwright 1975; Wainwright and Woolhouse 1975). Values of K_i were calculated from the double reciprocal plots using the modified rate equation by Westley (1969) (cited in Woolhouse, 1983).

$$K_i = \frac{[I]}{cV_{\max} - 1}$$

Where I = inhibitor concentration

c = the intercept on the ordinate

when $1/v$ is plotted against $1/s$.

Cox et al (1976), Ernst (1976) and Mathys (1975) were not able to find enzymes with altered properties internal to the cell membrane in metal-tolerant clones. So it is likely that such enzymes are protected by exclusion and compartmentation of copper.

An enzyme resistant to the herbicide glyphosate (N-phosphonomethylglycine), and inhibitor of 5-enolpyruvyl-3-phosphoshikimate synthesis, has been isolated from mutants of Salmonella typhimurium. Comai et al (1983) isolated 5-enolpyruvyl-3-phosphoshikimate synthetase that was resistant to inhibition by glyphosate from the mutants.

(h) Increased amount of target molecule

If the amount of the target molecule is increased to compensate for occupation of the target site, and thus maintain normal physiological processes, harmful effects of a toxicant can be overcome.

It has been suggested that methotrexate resistance in cultured mammalian cells is due to overproduction of the target molecule dihydrofolate. The overproduction of dihydrofolate may be the result of a dose-related amplification of the dihydrofolate gene (Alt et al, 1983; Horns et al, 1984, cited in Schimke 1984).

Arsenic tolerance may also be associated with increased amounts of free sulfhydryl groups. Harrington (1959) found that strains of the blue tick (Boophilus decoloratus) that were resistant to arsenious oxide, contained more total free sulfhydryl groups than sensitive strains. The affinity of arsenite for sulfhydryl groups has been suggested as being responsible for the toxic action and hence increased synthesis of sulfhydryl compounds would be

beneficial. However, arsenite resistance is not well understood (Foster, 1983) and there is no adequate evidence to support the idea of increased synthesis of the target sulfhydryl compounds.

(i) Bypass of toxicant receptor

Cases of resistance have been cited in which organisms have evolved mechanisms that bypass the affected site of action. Some scale insects have acquired resistance to hydrogen cyanide (HCN) by use of a cytochrome chain sequence that avoids the terminal cytochrome, the site of inhibition by HCN (Hodgson and Guthrie, 1980).

The mitochondria isolated from skunk cabbage and mung bean hypocotyls exhibit cyanide-resistant respiratory activity (Bendall and Bonner, 1971). The electron flux through cytochrome c has been shown to be small compared with the flux through an unidentified alternative oxidase which is known to have a high affinity for oxygen. Cytochrome b undergoes oxidation and reduction in presence of cyanide but does not play any role in the cyanide-resistant pathway.

The use of an alternative enzyme system has been associated with resistance to sulfonamide drugs in bacterial cells. Sulfonamides inhibit growth in bacterial cells by interfering with the synthesis of folic acid (Brown, 1962; cited in Foster, 1983). The point of interference is the condensation of p-aminobenzoic acid with dihydropteridine to form dihydropteroic acid. The drug competitively inhibits dihydropteroate synthase (DHPS) and drains off the supply of pteridine precursor into a sulfonamide analog of dihydropteroate which is excreted into the medium (Foster, 1983). In E. coli, there are two types of DHPS: chromosomal and plasmid-encoded enzymes (Svedberg and Skold, 1980 cited in Foster 1983). The former binds sulfonamide slightly more efficiently than the natural substrate p-aminobenzoic acid but the latter binds sulfonamide 10,000-fold less

MATERIALS AND METHODS

efficiently. Resistant E. coli are therefore able to use the plasmid-encoded DHPS to synthesize dihydropteroic acid and grow normally.

The following cyanophytes and chlorophyte were used:

1. Cocconeis lineatica Lemmermann (Cyanophyta), isolated by Karen E. Jones from Lake Opinicon, Ontario, Canada in 1976.
2. Microcystis leopoldiana (Racib) Komarek (Cyanophyta), strain UTEX No. 613 from the University of Texas culture collection. (Old name: Anacystis leopoldiana (Racib) Brown and Gailly).
3. Chlorella pyrenoidosa Chick (Chlorophyta), strain UTEX No. 343 from the University of Texas culture collection.
4. Chlorella glaucochloris

Chemicals used were of analytical or certified grade from J.J. Baker Co., Fisher Scientific Co., Sigma Chemical Co., B.D.H. Co., and Mallinckrodt, Inc.

Demineralized water was obtained by passing distilled water from a Barnstead waterstill through ultrapure resin beds (Barnstead). The resistance of the resulting water was greater than $10 \text{ megohm cm}^{-1}$.

Glassware was cleaned with Triton X-100 detergent and sometimes by chromic acid to remove stubborn stains. It was then rinsed several times with deionized water and once or twice with distilled water.

3. Growth media

The growth medium was Daley and Brown's (1973) modification of No. 11 medium (Hughes et al 1958). The composition and level of nutrients of a half strength medium are given in Appendix I. The stock cultures were maintained in full strength medium while algae used in experiments were grown in half strength medium. Growth media and equipment were sterilized in an autoclave (AMSCO, Canada) for 20 minutes at 221.5°F before use.

Growth media containing acetate at 1mM were obtained by dissolving 0.8916g. of potassium acetate in 200 ml of medium.

MATERIALS AND METHODS

1. Microalgae

The following cyanophytes and chlorophyte were used:

- a. Oscillatoria limnetica Lemmermann (Cyanophyta), isolated by Karen E. Jones from Lake Opinicon, Ontario, Canada in 1976.
- b. Synechococcus leopoliensis (Racib) Komarek (Cyanophyta), strain UTEX No. 625 from the University of Texas culture collection. (Old name: Anacystis nidulans (Lyngb.) Drouet and Daily).
- c. Chlorella pyrenoidosa Chick (Chlorophyta), strain UTEX No. 343 from the University of Texas culture collection.

2. Reagents, glassware

Chemicals used were of analytical or certified grade from J.J. Baker Co., Fisher Scientific Co., Sigma Chemical Co., B.D.H. Co., and Mallinckrodt, Inc.

Demineralized water was obtained by passing distilled water from a Barnstead waterstill through ultrapure resin beds (Barnstead). The resistance of the resulting water was greater than $16 \text{ megohm cm}^{-3}$.

Glassware was cleaned with Triton X-100 detergent and sometimes by chromic acid to remove stubborn stains. It was then rinsed several times with hot and cold tap water and once or twice with distilled water.

3. Growth media

The growth medium was Daley and Brown's (1973) modification of No. 11 medium (Hughes et al 1958). The composition and level of nutrients of a half strength medium are given in Appendix 1. The stock cultures were maintained in full strength medium while algae used in experiments were grown in half strength medium. Growth media and equipment were sterilized in an autoclave (AMSCO, Canada) for 20 minutes at 221.5° F before use.

Growth media containing acetate at 1mM were obtained by dissolving 0.0916g. of potassium acetate in 200 ml of medium.

4. Stock cultures

The stock cultures were maintained in 2.8-litre Fernbach flasks kept in a culture room at a temperature of $27 \pm 1^{\circ} \text{C}$ and light intensity of about $10 \mu\text{E. m}^{-2} \text{s}^{-1}$ (latter measured with a photometer, Li-Cor Inc. Model Ll-185B with an Ll-190 5B Quantum Sensor).

New stock cultures were started after about 2 or 3 months and transfers were done in a Laminar Flow Hood (Environmental R & D Corp., Conn.). A new stock culture was not used as a source of inocula until some of it had been transferred to fresh full strength medium.

5. Preparation of Inoculum for growth experiments.

Inoculum was prepared by transferring algae from the stock culture into sterilized half strength growth medium. Transfer was done in the Laminar Flow Hood, previously cleaned with 95% ethanol spray. The inoculum from the stock culture was usually started at an absorbance (A_{660}) of 0.1.

6. Growth Experiments.

The growth experiments were carried out using batch culture technique. The bottles used (400ml) were fitted with a glass bubbler-tube mounted through a foam stopper. Sometimes the algae were grown in the 2.8 - litre flasks when large amounts of algal material were needed.

The cultures were bubbled with air (Departmental air line) enriched with 0.5% (v/v) carbon dioxide (Cdn. Welding Gases Ltd. Kingston). The flow rates of air and carbon dioxide were measured by meters (Matheson of Canada Ltd. Whitby, Ontario). The rate of bubbling was sufficient to keep the algae in suspension. Light was supplied by four fluorescent tubes (two cool white and two "Grow-Lux" tubes, Sylvania Corp.) which gave a light intensity of $80\text{--}90 \mu\text{E m}^{-2} \text{sec}^{-1}$ at the face of the culture bottles. The culture room was air-conditioned with a temperature of $27 \pm 1^{\circ} \text{C}$.

The growth of algae was measured by decanting samples from the bottles into spectrophotometer tubes in an alcohol-sterilized Laminar Flow Hood. The A_{660} was measured with a spectrophotometer (Bausch and Lomb "Spectronic 20").

Growth was plotted on semilogarithmic graph papers. Growth lags of arsenite-treated cultures were estimated by extrapolating the exponential portions of growth curves back to the absorbance value when arsenite was added. In cases where initial growth lags were absent, the generation time (A_{660} or cell numbers) was used for comparison of growth between cultures of algae.

7. Preparation of Tolerant cells.

Cyanophytes at logarithmic phase of growth were spun down in a centrifuge (Beckman, Model J2-21M) at 4000rpm., 25° C for 10 minutes. The cells were then resuspended in sterile half strength medium with an arsenite concentration of 10uM and allowed to grow for 10-12 hours. These cells were then spun down as above and used as secondary inoculum.

Induction of arsenite-tolerance was confirmed in all cases by transferring the cells to half strength medium with an arsenite concentration of 200uM. At this concentration, arsenite did not affect growth of tolerant cells of the cyanophytes.

8. Cell Counts.

Cells of S. leopoliensis were counted at certain time intervals during induction of arsenite tolerance by use of the Petroff-Hauser counting chamber (Hauser Scientific, Blue Bell,, Pa. 19422) under a microscope at 400X (Carl Zeiss Winkel). A_{660} was also measured before each count.

1 ml. of the tolerant culture was mixed with 100ul of Lugol's solution to make cells settle on the slide during counting.

A control (non-induced) culture was counted in a similar manner.

9. Paper Chromatography.

a. Washing of chromatography paper

A Whatman Chromatography Paper No. 1. (W & R Balston, Ltd. England) was cleaned by allowing HNO_3 to run through the paper in a chromatography tank for about 72 hours. Cleaning of the paper was necessary in order to remove salts of calcium and magnesium that could interfere with the migration of arsenic. The paper was then rinsed with demineralized water until all the acid was removed.

b. Chromatography.

The method of separation of arsenate and arsenite was developed by Miletukova et al (1968). The solvent was a methanol-ammonium hydroxide-water mixture (83:4:13 by vol.). Prior to chromatography, the tank was saturated with ammonia vapour. Development was for 2.5 hours at laboratory temperature. The two forms of arsenic, arsenite and arsenate, were detected by use of ammoniacal silver nitrate spray (5 ml of 28% NH_4OH , 45 ml demineralized water and 4 g. of AgNO_3).

Arsenic was extracted from algal pellets with ice-cold water (see MacArthur et al, 1985). 100 μl of the ice-cold water extract was chromatographed as above simultaneously with arsenate and arsenite standards in outside lanes on the paper.

10. Estimation of arsenic in whole cells and algal residues and on paper chromatograms.

a. Proton Induced X-Ray Emission (PIXE)

This technique is used to determine the concentration of various elements in a cell (MacArthur et al 1983, 1985). A simplified diagram of the algal sample and detectors is shown in Appendix 2.

Algal samples were prepared by filtering cells onto membranes (Millipore

Corporation, Bedford, Mass., U.S.A.) of 0.45 μ m pore size and 24 mm in diameter. Filtration was done under reduced pressure and the volume of culture filtered was chosen to give approximately 10^9 cells (Synechococcus leopoliensis) or 9.0×10^7 cells (Chlorella pyrenoidosa). Appendix 10 and 11 show curves that were used to determine the volume of culture necessary to give the required number of cells over a range of cell densities. The cells were air-dried before analysis.

The amount of arsenic in algal residues of S. leopoliensis after ice-cold water extraction of cells was determined by resuspending the residue in fresh medium equal in volume to the growth medium. A sample of resuspended algal residue equal in volume to that of the original culture of whole cells was then filtered onto the membrane and air-dried.

Paper chromatograms of water extracts from tolerant and sensitive cells of S. leopoliensis were scanned by the proton beam along the path of migration of arsenic. Measurements were done at intervals of 1 cm. and beam spots formed on the paper were about 3 mm in diameter. Arsenic was detected by its characteristic X-Ray energy of $K_{\alpha} = 10.54$ keV (Kiloelectronvolts). (Palmer, 1988 personal communication).

b. Neutron Activation Analysis (NAA).

Neutron Activation followed by gamma ray spectrometry was used for the determination of arsenic in algal samples. The technique works as follows. In the radioactivation step, ^{75}As accepts a neutron to produce radioactive ^{76}As . The ^{76}As produced by irradiation emits gamma rays with greatest intensity at 0.559 MeV.

NAA is a highly sensitive technique with a very low detection limit. Under minimal spectral interferences, a reactor neutron flux of 10^{13} neutron/cm²/sec and a 50-hour irradiation results in a sensitivity of 3×10^{-11} g arsenic with a 3-10% precision, (3×10^{-11} g of ^{75}As produce enough ^{76}As

to yield 10^3 counts/min. measured on germanium-lithium detector). (Jervis et al 1970). The concentration of arsenic in a sample is calculated by comparing its peak area with that of a standard irradiated and counted under similar conditions.

Algal samples for NAA were prepared by filtering cells onto membranes (Nuclepore Corp., Pleasanton, California) of 0.4 μ m pore size and 47 mm in diameter. The membrane with the mounted algal material was put in an open plastic vial and dried in a desiccator containing anhydrous calcium sulphate. The dry algal samples were weighed and the vials sealed before irradiation. The samples were irradiated with neutrons in SLOWPOKE-2 (Appendix 3) at a neutron flux of 5.1×10^{11} neutrons/cm²/sec. for about 1 hour. The gamma ray energies of arsenic were detected by lithium-drifted germanium crystals and the peak areas integrated by a multi-channel analyzer (MCA). For liquid samples, a known volume (1ml) was put into a vial which was then sealed and analysis done as for the solid algal material. The activity of the samples was counted for 33 minutes. (Appendix 3).

For paper chromatograms, thin strips of paper (1 x 2 cm) were cut off down the path of migration of arsenic. The width of the paper strips was made slightly larger than that observed for arsenite and arsenate spots after spraying with ammoniacal silver nitrate. The paper strips were put into vials which were sealed and each irradiated for 2 hours. The activity of arsenic in the paper strips was counted for 66 minutes.

11. Estimation of $^{35}\text{SO}_4^{2-}$ uptake by cells of *S. leopoliensis* during induction of arsenite tolerance.

Two cultures of cells during active growth were each treated with 0.5 ml of a mixture of 1mM MgSO_4 and $^{35}\text{SO}_4^{2-}$ in aqueous solution (latter from Amersham International, U.K.). The specific activity of ^{35}S differed in the

different experiments but was in the range of 2.6×10^6 to 5.4×10^6 cpm/ $\mu\text{mol SO}_4^{2-}$. One of the cultures was further treated with 10 μM arsenite. After 2 and 4 hours, samples of the cultures were taken and extracted with 5ml of 80% aqueous ethanol. The ethanol-insoluble fraction was digested in 2ml 1N NaOH and neutralized with 2ml 1N HCl. Neutralization was necessary in order to eliminate chemiluminescence during counting in the scintillation counter.

5ml of a scintillation liquid (mixture of 0.4% PPO/0.01% POPOP in toluene: Triton 2:1, v/v) was put into vials and the cell extracts injected into them with micropipettes (Pipetman, Gilson Medical Electronics (France) S.A.). Radioactivity of ^{35}S was counted for 1 minute at 10K by liquid scintillation spectrometry using a Searle Delta-300 counter (^{35}S on thin layer chromatogram) or by RackBeta Liquid Scintillation counter for 10 minutes. (Experiments 1 and 2 in Table 14 and 15).

The ethanol extract was further analysed for non-polar and polar compounds. It was put in 12ml-graduated centrifuge tubes, immersed in warm water and nitrogen blown over it to speed up evaporation of ethanol. The residue was extracted successively with petroleum ether, methanol/chloroform mixture (2:1 v/v) and demineralized water to obtain lipid-soluble polar and non-polar substances and water soluble compounds respectively. ^{35}S was determined in the different fractions with the RackBeta Scintillation counter.

The amounts of ^{35}S were calculated in $\mu\text{mol } ^{35}\text{S mg}^{-1} \text{ Chla}$. In terms of ppm of ^{35}S (dry weight) the following equation was used:

$$\text{ppm } ^{35}\text{S, dry wt} = \frac{\mu\text{mol } ^{35}\text{S}}{160 \text{ mg dry wt.}} \frac{\text{mg}^{-1} \text{ Chla}}{\text{mg}^{-1} \text{ Chla}} \times \frac{32 \mu\text{g S}}{\mu\text{mol S}} \times 10^3$$

$$= \mu\text{mol S mg}^{-1} \text{ Chla} \times 2 \times 10^2$$

1 mg Chla = 160 mg dry wt (Budd, 1988 personal communications, Appendix 16).

12. Thin-layer chromatography for the separation and estimation of ^{35}S -labelled sulfhydryl compounds.

A cellulose slurry (22.5g cellulose and 150 ml demineralized water homogenized in blender) was applied on glass plates at a thickness of about 0.2 mm with a slurry spreader (Desaga, Heidelberg, W. Germany). The plates were air-dried overnight before use.

Standard solutions of cysteine, glutathione and reduced lipoic acid were prepared by dissolving 2mg of the first two and 5ul of lipoic acid in 50ml of acetone-water solution (50:50 v/v). 10ul of each standard solution was applied on a spot 2 cm from the bottom edge. 10ul of $\text{Mg}^{35}\text{SO}_4$ was also applied on a spot as another standard.

Ethanol extracts from normal and tolerant cells were applied on horizontal streaks at the bottom of the plate. The plate was then put in a chromatography tank containing 50ml of a mixture of n-butanol, acetic acid and water (60:15:25 by volume). The chromatogram was allowed to develop for about 2 hours.

Sulfhydryl compounds were located on the chromatogram by use of a spray reagent (0.003% 2,2¹-Dithiobis-(5-Nitropyridine) in acetone). Appearance of a yellow colour revealed the presence of a sulfhydryl compound. The radioactive zones were then scraped off and transferred to scintillation vials for estimation of ^{35}S .

13. Polyacrylamide Slab Gel Electrophoresis of tolerant and normal cell extracts.

a. Preparation of algal samples.

Three types of cultures were harvested and freeze-dried. One was a normal culture, the second was a tolerant culture just induced with 10uM arsenite and the third type was a tolerant culture that had been transferred to medium containing 200uM arsenite for 4 hours. The $A_{660\text{nm}}$ at harvest time

were 0.45, 0.43 and 0.43 respectively. The freeze-dried algae were resuspended in 10ml of EDTA-Tris buffer, pH 8.2 and cells ruptured once in a French Pressure Cell (SLM-Aminco, Urbana, IL. U.S.A.) at 18,000 psi. Some of the crude extract was spun down in a centrifuge at 3000 rpm, 5° C for 10 minutes and the supernatant and pellet preserved in different test tubes.

b. Preparation of the gel.

A pair of clean plates was set up in the Bio-Rad Protein 11 Slab Cell (E. 1. DuPont de Nemours and Co.) and spaced for a 1.5 mm-thick gel. The bottom of the plates was sealed by injecting very hot agarose (0.5% agarose w/v in demineralized water) with a pasteur pipet between the plates at the top corners of the glass plates. The agarose seal was about 1 cm deep.

A 12% running gel (Appendix 12b) was immediately poured between the plates after addition of TEMED (N,N,N^1, N^1 -tetramethyl ethylenediamine to the other components of the running gel mixture. The running gel filled about 3/4 of the height of the plates. Butanol was poured on top to protect the gel from drying. The gel was then allowed to polymerize for about 20-30 minutes. The butanol was then removed from the top with a pasteur pipet and a 2.5% stacking gel (Appendix 12a) was also immediately added on top of the running gel. A "comb" to allow the formation of wells was inserted into the stacking gel which took about 30 minutes to set.

The cell extracts were put in 1.5 ml Eppendorf micro test tubes (Brinkman Instruments Co. U.S.A) to which were added SDS/glycerol Tris buffer pH 6.8 and 75% glycerol containing bromphenol blue (See Appendix 14 for volumes of different components in the micro test tubes). The tubes were heated over boiling water for 3 minutes and then spun in the Eppendorf Microcentrifuge. A standard mixture of low molecular weight proteins (mol. wt. between 14000 and 92000 daltons) was similarly treated. The cell extracts and standard protein

mixture were injected into the wells with a microliter pipette (Hamilton Co., Reno, Nevada, U.S.A.)

The gel was run for about 12 hours on constant voltage of 100 volts and a current of 30 milliamps. It was then stained in Coomassie blue (Appendix 12c) for 1 hour and finally destained for about 2 hours in a methanol-acetic acid mixture.

14. Fluorography of polyacrylamide gel for the detection of ^{35}S -labelled proteins in normal and tolerant cells of *S. leopoliensis*.

Normal and tolerant cultures grown in presence of $^{35}\text{SO}_4^{2-}$ were freeze-dried and extracted as described above. A 12% polyacrylamide gel was run as above using 20 μl of the extracts. (Appendix 13). After staining as above, the polyacrylamide gel was further destained overnight in 7% acetic acid (v/v) in water and then immersed in 5% glycerol dissolved in 7% acetic acid. The gel was then soaked in 20ml of 22% 2,5-diphenyloxazole (PPO) in acetic acid. PPO was incorporated into the gel to increase the efficiency of radioactive detection. The PPO solution was changed 4 times during the soaking period. The gel was then washed in demineralized water for 10 minutes and in 50% ethanol for 5 minutes.

The gel was dried at 80 $^{\circ}\text{C}$ in a Bio-Rad gel dryer and exposed to Cromex Medical X-Ray film (E.I. Dupont De Nemours & Co. Inc. U.S.A.) for 18 days followed by another exposure for 6 months.

RESULTS

1. Toxicity of arsenite

a. Synechococcus leopoliensis

Arsenite caused transient growth lags in S. leopoliensis. The duration of the growth lags depended on the concentration of arsenite in the growth medium (Figure 2). In presence of 200, 300 and 400 μ M arsenite, the growth lags were 74, 100 and 178 hours respectively. After recovery, the rates of growth were in general close to that of the control. In presence of 200 μ M arsenite, the doubling (or generation) time could not be determined because the growth of the culture was stopped before logarithmic phase proper. However, in other experiments done in this study (data not presented) the doubling time in presence of 200 μ M arsenite after recovery was 11.66 ± 0.88 h ($\pm$ SE, $n = 3$). In presence of 300 and 400 μ M arsenite the doubling times were 12 and 14 hours respectively compared to 10 hours for the control.

Thin layer chromatography of the spent medium from recovered cells showed that arsenic was almost entirely present as arsenite after 70 hours.

b. Oscillatoria limnetica

Arsenite also caused transient growth lags in O. limnetica. In presence of 200, 300 and 400 μ M arsenite, the growth lags were 27, 34 and 75 hours respectively, compared to 1 h for the control. After recovery of growth, the doubling times were 16, 21 and 17 hours respectively compared to 8 hours for the control (Figure 3). The concentration dependent growth lags were significantly different from the control. (Appendix 4 calculated $F = 86.8$, tabulated $F = 15.8$, $n = 3$, $n_2 = 8$, $p = 0.001$). The experiment was stopped after 108 hours after all cultures entered into logarithmic phase of growth.

The duration of growth lags and doubling times showed some variation between experiments. In another investigation conducted in this study, the

growth lags in presence of 100, 200, 300, 400 and 500 μ M arsenite were 11, 32, 34, 49 and 57 hours respectively. The doubling times were 7, 14, 15, 13 and 16 hours respectively, compared to 13 hours for the control.

c. Chlorella pyrenoidosa

In C. pyrenoidosa, unlike the cyanophytes, arsenite did not cause detectable growth lags (Figure 4). The effect of arsenite was mainly on the log rate of growth. The generation times in presence of 1 and 2mM arsenite were 20 and 52 hours respectively, compared to 12 hours for the control. Growth in 3mM arsenite was completely inhibited (Figure 4). The concentration dependent generation times were significantly different from the control. (ANOVA calculated $F = 64$, tabulated $F = 27$, $n_1 = 2$, $n_2 = 6$ and $p = 0.001$).

The generation times were found to vary between experiments. In another experiment done in this study (data not presented) the generation times in presence of 1 and 2mM arsenite were 11 and 18 hours respectively, compared to 10 hours for the control. Growth in 3, 4 and 5mM was completely inhibited.

2. Alleviation of arsenite toxicity by potassium acetate.

a. S. leopoliensis

1mM of potassium acetate in the growth medium containing 200 μ M arsenite significantly alleviated arsenite toxicity in S. leopoliensis. This was evident in the decrease of growth lags. (Figure 5 and Table 2)

b. O. limnetica

1mM acetate eliminated arsenite toxicity in O. limnetica. The growth lags of the culture grown in presence of acetate and 200 μ M arsenite was 0 hours and that grown in 200 μ M arsenite without acetate (the KCl control) was 42 hours. (Figure 6)

c. C. pyrenoidosa.

Acetate did not alleviate toxicity of 3mM arsenite in C. pyrenoidosa (data not presented).

Figure 2

Effect of arsenite on growth of S. leopoliensis. Numbers next to curves represent the concentration of arsenic (μM) in the growth medium.

Figure 2.

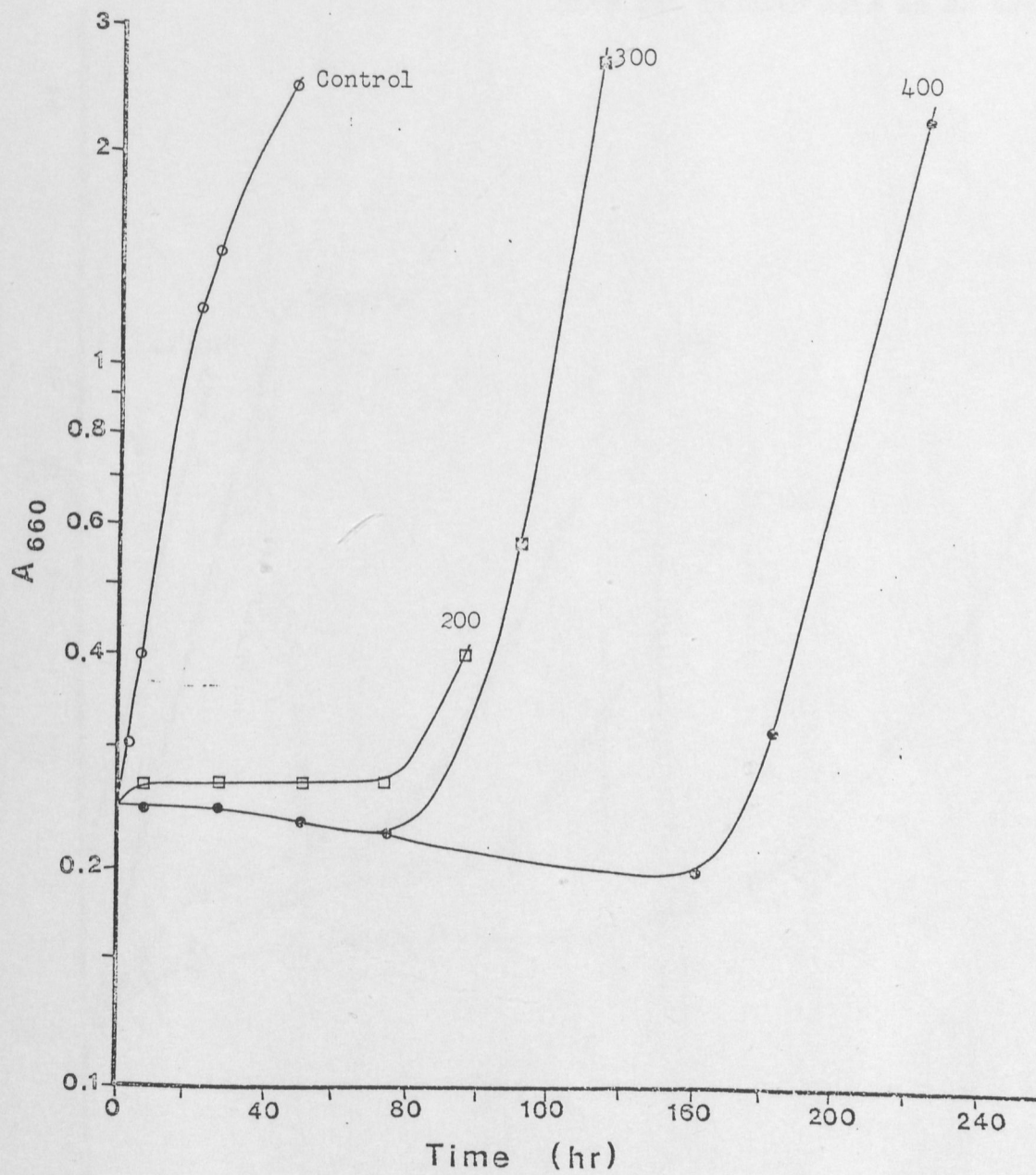


Figure 3.

Effect of arsenite on growth of Oscillatoria limnetica. Numbers next to the curves represent the concentration of arsenite (μM) in the growth medium. The points on the curves are means of triplicate samples ($\text{Mean} \pm \text{SE}$, $n = 3$). Error bars are one SE on each side of the mean.

Figure 3.

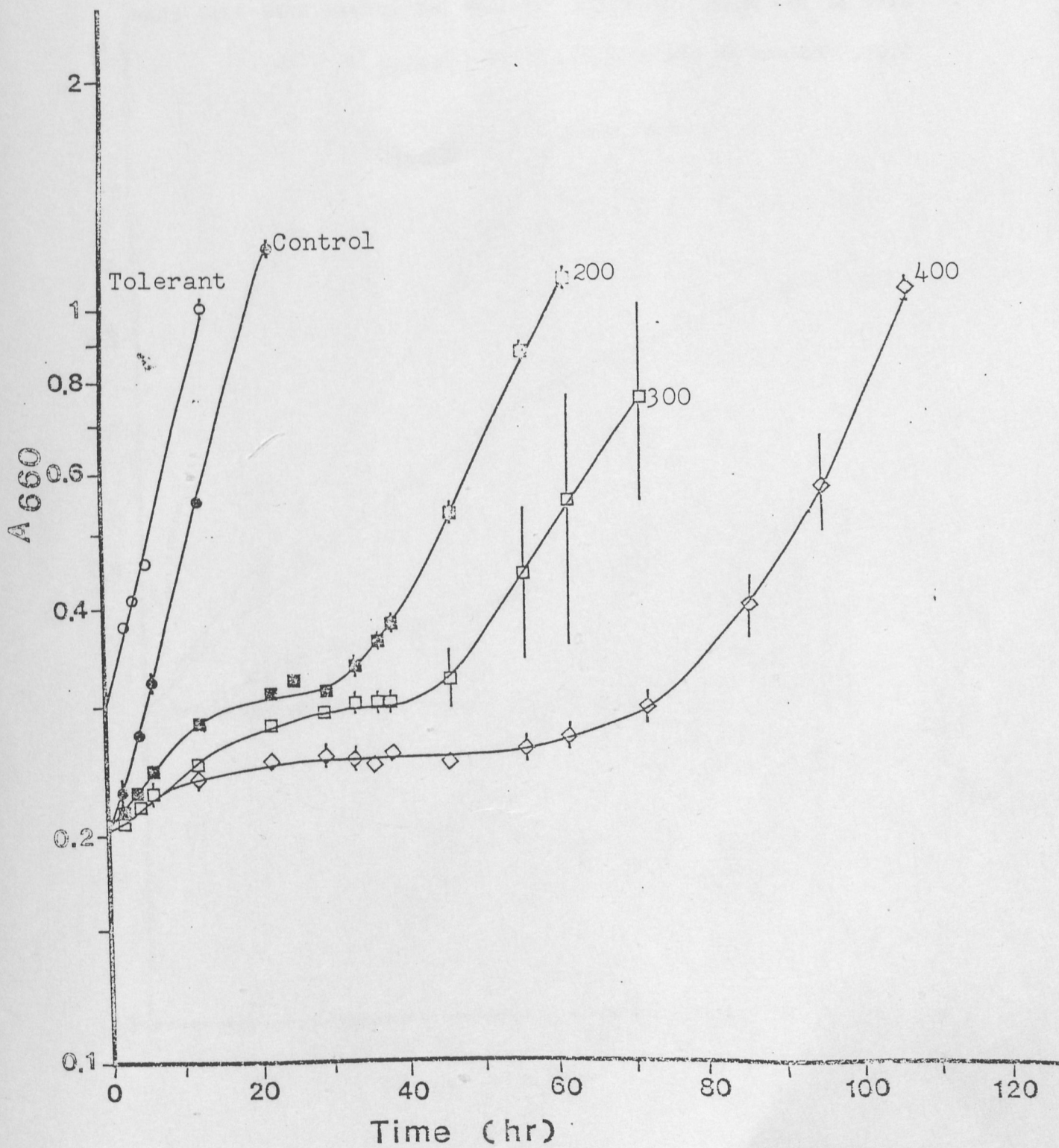


Figure 4

Effect of different concentrations of sodium arsenite on the growth of Chlorella pyrenoidosa.

Numbers next to curves represent concentrations (mM) of arsenite in the culture medium. Error bars are one SE on each side of the mean. SE's for 2mM and 3mM curves were less than 0.01. Points on the curves are mean A_{660nm} ($n = 3$).

Figure 4.

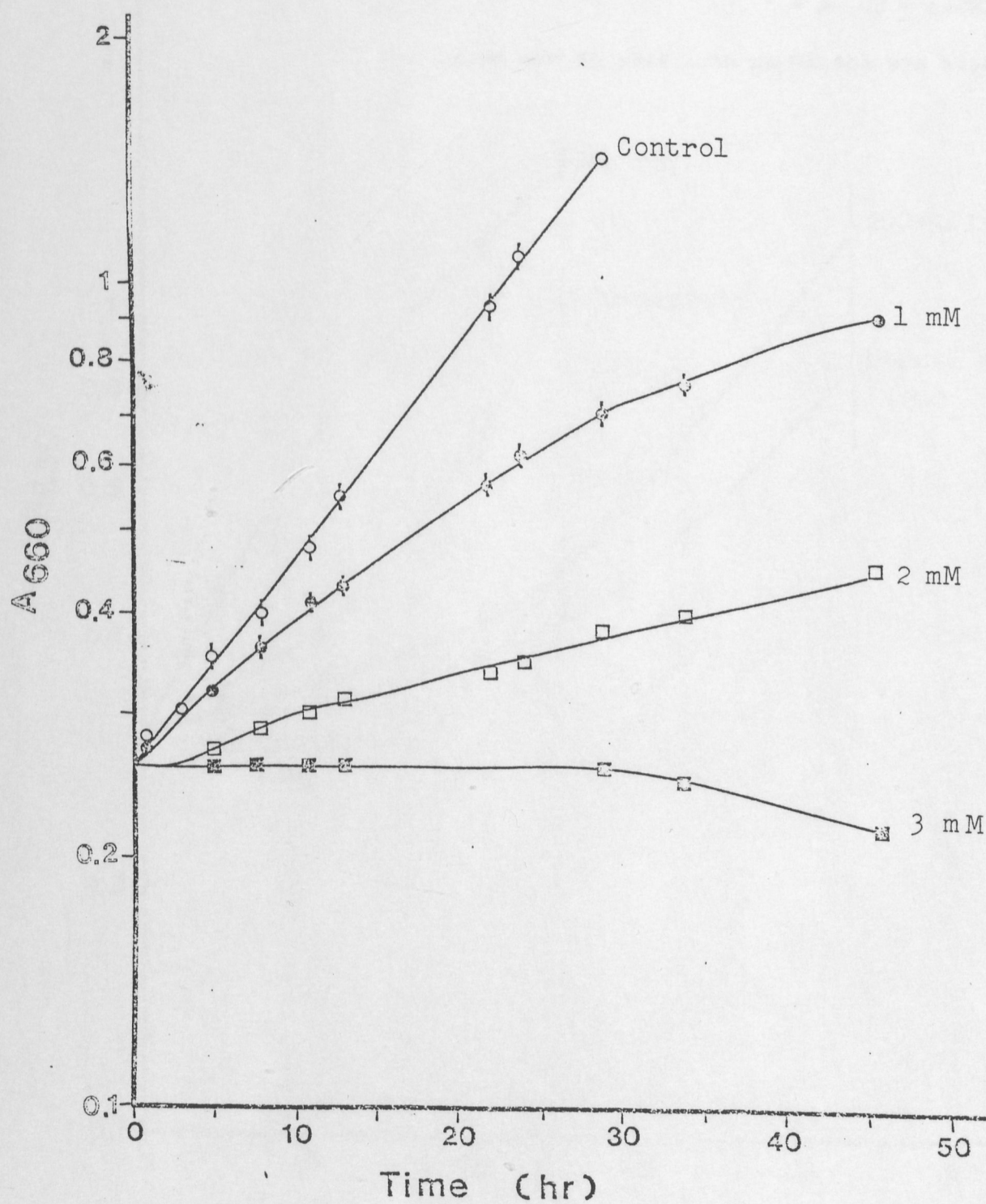


Figure 5

Alleviation of arsenite toxicity by 1mM potassium acetate in S. leopoliensis. Numbers next to the curves represent concentration of arsenite (μM) in the growth medium.

The points on the curves are means of triplicate samples (Mean $\pm$ SD, n = 3).

Bars are one SD on each side of the mean

Figure 5.

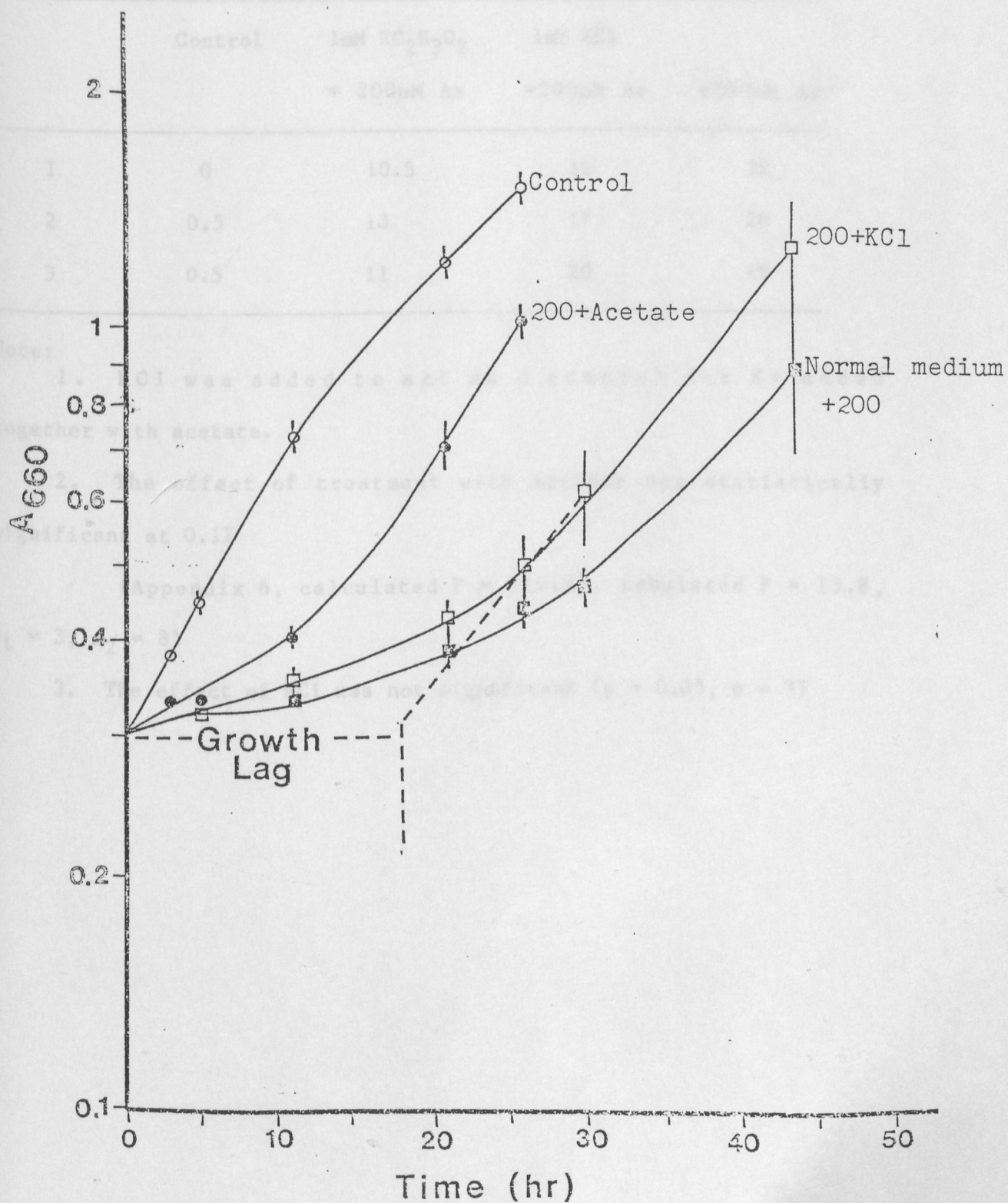


Table 2

Alleviation of arsenite toxicity by acetate in S. leopoliensis

Replicate No.	Growth lags hours in normal medium with addition of			
	Control	1mM $\text{KC}_2\text{H}_3\text{O}_2$ + 200 μM As	1mM KCl +200 μM As	+200 μM As
1	0	10.5	16	22
2	0.5	13	17	20
3	0.5	11	20	19

Note:

1. KCl was added to act as a control for K⁺ added together with acetate.

2. The effect of treatment with acetate was statistically significant at 0.1%

(Appendix 6, calculated F = 111.82, tabulated F = 15.8,

$n_1 = 3$, $n_2 = 8$)

3. The effect of KCl was not significant ($p = 0.05$, $n = 3$)

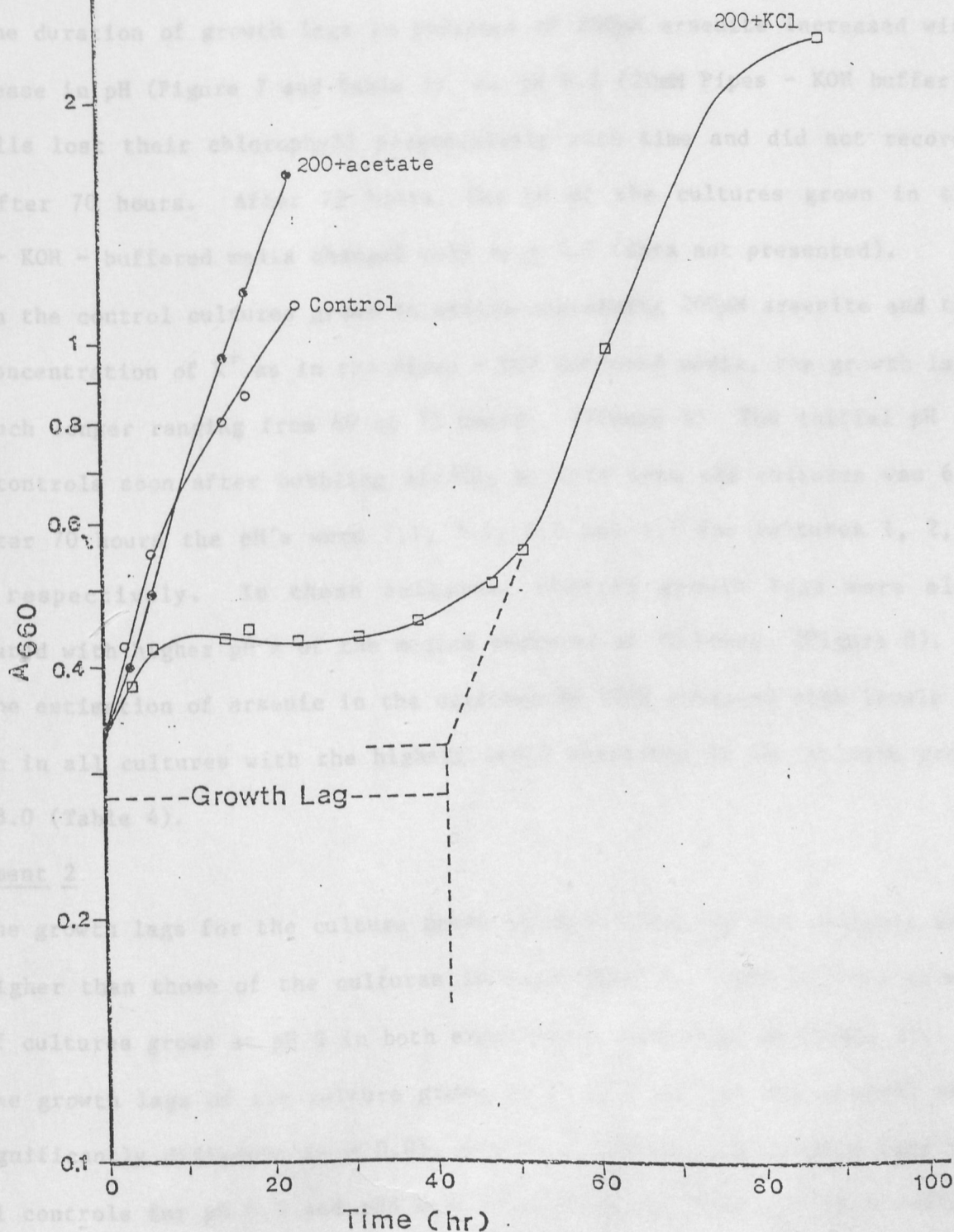
Figure 6

Alleviation of arsenite toxicity by 1mM potassium acetate in 0.
limnetica. Numbers next to curves represent concentration of arsenite (μM) in
the growth medium.

3. Effect of pH on arsenite sensitivity and arsenite content in *S. leopoliensis*.

Experiment 1

Figure 6



Experiment 2

The growth lag for the culture grown at pH 8.0 was significantly higher than those of the cultures grown at pH 6.5 and pH 5.0.

The growth lag of the culture grown at pH 8.0 was also significantly higher than those of the cultures grown at pH 6.5 and pH 5.0.

The growth lag of the culture grown at pH 8.0 was also significantly higher than those of the cultures grown at pH 6.5 and pH 5.0.

The growth lag of the culture grown at pH 8.0 was also significantly higher than those of the cultures grown at pH 6.5 and pH 5.0.

The growth lag of the culture grown at pH 8.0 was also significantly higher than those of the cultures grown at pH 6.5 and pH 5.0.

The growth lag of the culture grown at pH 8.0 was also significantly higher than those of the cultures grown at pH 6.5 and pH 5.0.

The growth lag of the culture grown at pH 8.0 was also significantly higher than those of the cultures grown at pH 6.5 and pH 5.0.

The growth lag of the culture grown at pH 8.0 was also significantly higher than those of the cultures grown at pH 6.5 and pH 5.0.

3. Effect of pH on arsenite toxicity and arsenic content in *S. leopoliensis*.

Experiment 1

The duration of growth lags in presence of 200 μ M arsenite increased with a decrease in pH (Figure 7 and Table 3) At pH 6.2 (20mM Pipes - KOH buffer), the cells lost their chlorophyll progressively with time and did not recover even after 70 hours. After 70 hours, the pH of the cultures grown in the pipes - KOH - buffered media changed only by ± 0.2 (data not presented).

In the control cultures grown in medium containing 200 μ M arsenite and the same concentration of K⁺ as in the pipes - KOH buffered media, the growth lags were much longer ranging from 60 to 73 hours. (Figure 8) The initial pH of these controls soon after bubbling air/CO₂ mixture into the cultures was 6.5 and after 70 hours the pH's were 7.1, 7.1, 6.8 and 6.7 for cultures 1, 2, 3 and 4 respectively. In these cultures, shorter growth lags were also associated with higher pH's of the medium measured at 70 hours, (Figure 8).

The estimation of arsenic in the cultures by PIXE revealed high levels of arsenic in all cultures with the highest level occurring in the culture grown at pH 8.0 (Table 4).

Experiment 2

The growth lags for the culture grown at pH 6.5 and the KCl controls were much higher than those of the cultures in experiment 1. However, the growth lags of cultures grown at pH 8 in both experiments were similar (Table 3).

The growth lags of the culture grown at pH 6.5 and its KCl control were not significantly different ($p = 0.05$, $n = 3$). However, the growth lags for the KCl controls for pH 6.5 and pH8 were significantly different ($p = 0.05$, $n = 3$).

Thin layer chromatography of the spent medium from the culture at pH 8.0 showed that arsenic was almost entirely present as arsenite after 23 hours of

growth (cf. Figure 1).

Figure 7

Effects of pH on arsenite toxicity in S. leopoliensis.
The growth medium in all cases contained 200 μ M arsenite.
Numbers next to curves represent the pH's.

Figure 7.

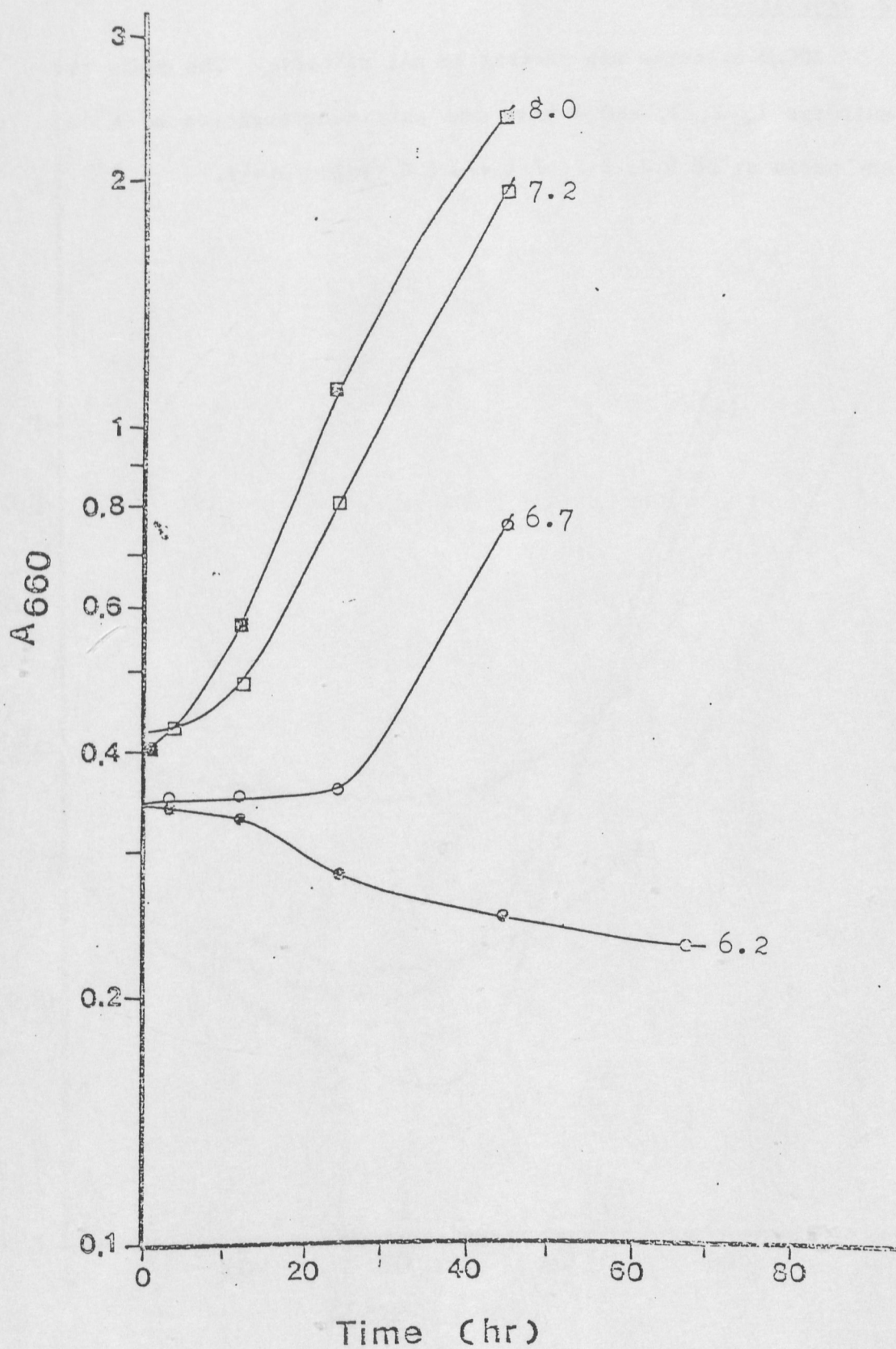


Figure 8

The controls for the "Effect of pH on arsenite toxicity in S. leopoliensis".

200 μ M arsenite was present in all cultures. The media for cultures 1, 2, 3, and 4 have the same concentration of K^+ as the media at pH 6.2, 6.7, 7.2 and 8.0 respectively.

Figure 8.

Table 3

Growth lags of *S. leopoliensis* grown under different pH's.

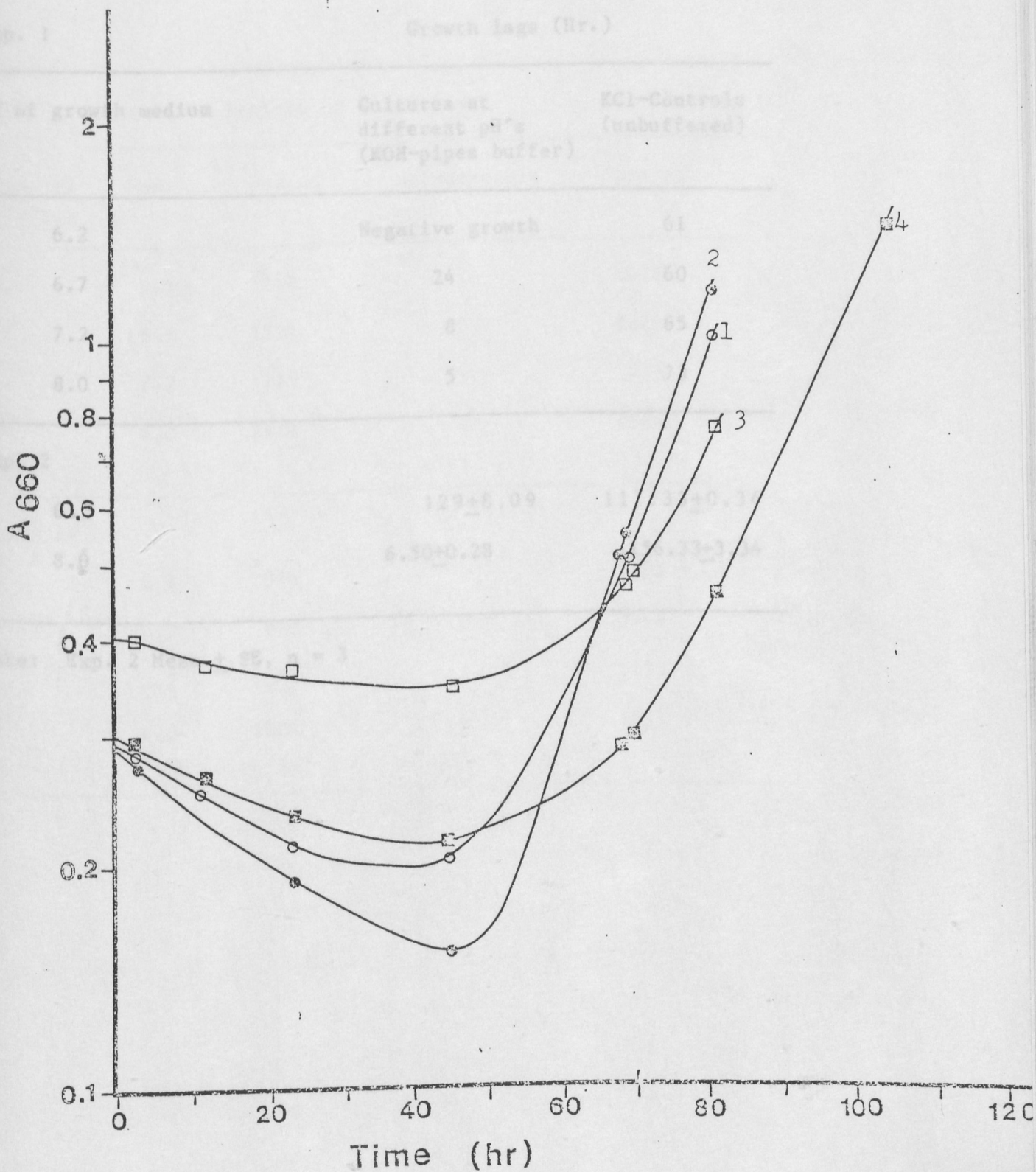


Table 3

Growth lags of S. leopoliensis grown under different pH's.

Growth lags (Hr.)			
Exp. 1	pH of growth medium	Arsenic in media containing 200ppm arsenite at different pH's.	Cultures at different pH's (KOH-pipes buffer)
			KCl-Cóntrols (unbuffered)
	6.2		Negative growth
	6.7	1768	24
	7.2	1294	8
	8.0	1270	5
Exp. 2	6.5		129 $\pm$ 8.09
	8.0		6.50 $\pm$ 0.28
			117.33 $\pm$ 0.34
			156.33 $\pm$ 3.34

Note: Exp. 2 Mean $\pm$ SE, n = 3

Table 4

Amount of arsenic in filtered layers of S. leopoliensis grown in media containing 200 μ M arsenite at different pH's.

Amount of Arsenic in the cells				
After 12 hours	pH	ppm As	KCl-Controls	ppm As
	6.2	1768	1	1478
	6.7	1294	2	2626
	7.2	1270	3	971
	8.0	2502	4	1644
After 24 hours	6.2	976	1	1798
	6.7	970	2	3515
	7.2	1309	3	1131
	8.0	1904	4	1987

4. Arsenite tolerance in the microalgae.

a. Inducibility of arsenite tolerance.

(i) Oscillatoria limnetica and Synechococcus leopoliensis

When cultures of these cyanophytes were grown in presence of 10 μ M arsenite for 10 hours and then transferred to the higher concentrations of arsenite, shorter growth lags than those for non-exposed cells (sensitive cells) were observed (Table 5). Therefore, 10 μ M arsenite induced some arsenite tolerance in the two cyanophytes. Tolerance was most noticeable for the lower arsenite concentrations tested.

1.0 μ M arsenite was also capable of inducing arsenite tolerance in S. leopoliensis. For the same duration of exposure, the level of arsenite tolerance appeared to be greater for the cultures exposed to 10 μ M arsenite. This was evident in the growth lags which were shorter for the latter than the former when cultures were transferred to 200 μ M arsenite (Table 6).

0.1 μ M arsenite did not induce arsenite tolerance in S. leopoliensis (data not presented).

(ii) Chlorella pyrenoidosa.

When cultures of C. pyrenoidosa were pre-exposed to an arsenite concentration that did not inhibit growth (0.5mM) for about 14 hours, they developed some tolerance to 3mM and 4mM arsenite, concentrations that normally inhibited growth completely. The pre-exposed cultures had measurable generation times unlike the non-exposed. (Table 7)

b. Cell numbers of S. leopoliensis during induction of arsenite tolerance.

In general, the mean cell numbers (per small square) of cultures during exposure to 10 μ M As and of the control cultures were not significantly different ($p = 0.5$, $n = 80$). (Table 8). It was only cultures counted after 7 hours that had significantly different means at $p = 0.05$ but not at $p = 0.02$. The growth rates, measured as absorbance of chlorophyll at 660nm, of the two

cultures were also similar.

c. Degree of tolerance in tolerant cells of *S. leopoliensis* grown in arsenite-free medium.

Arsenite tolerance was lost or decayed when tolerant cells were allowed to grow in an arsenite-free medium. When such cells were transferred to a medium containing 200 μ M arsenite, varying growth lags were observed. Their duration depended on the period of growth in the arsenite-free medium. (Figure 9 and Table 9).

d. Sensitivity of tolerant and recovered cultures of *S. leopoliensis* to arsenite concentrations above 200 μ M.

Both the tolerant and recovered cells (after a 70-hour growth lag in 200 μ M arsenite) did not show any detectable growth lags when transferred to media containing 300, 400 and 500 μ M arsenite. The effect of arsenite was on log rate of growth. The generation times of tolerant cells were significantly shorter than those of recovered cells in presence of 300 and 400 μ M arsenite (Table 10). However, in presence of 500 μ M arsenite, the recovered cells had a significantly shorter growth lag.

The level of arsenic in cells grown in a concentration of arsenic that stops growth completely i.e. 3mM arsenite was higher than in cells grown at 0.5 and 1mM. However, the bioconcentration factors in the former were in general lower than in the latter.

5. Arsenic levels in whole air-dried cells of *S. leopoliensis* and *C. pyrenoidosa*.

S. leopoliensis.

The estimation of arsenic in *S. leopoliensis* by Neutron Activation Analysis (NAA) showed that tolerant and sensitive cultures had similar levels of arsenic. In both cultures, the concentration of arsenic in the cells was

higher than in the growth medium. Tolerant and sensitive cultures concentrated arsenic to about 30 fold (on average) the medium concentration respectively. One sensitive culture that was allowed to recover growth (after 30 hours) concentrated arsenic to 63 times the medium concentration. (Table 11).

C. pyrenoidosa

Analysis by PIXE revealed high levels of arsenic compared to S. leopoliensis, in C. pyrenoidosa grown in presence of arsenic concentration that do not have a significant effect on growth i.e. 0.5 mM. (Table 12). The amounts of arsenic determined by NAA in two of the cultures grown in presence of 0.5mM (see table 12, cultures marked with asterisks) were close to those determined by PIXE. By the former determination the amounts of arsenic in cultures harvested after 4 and 11 hours were 2257 and 3078 ppm arsenic respectively and by PIXE the amounts were 1692 and 3142 respectively.

6. Levels of free (water soluble) and bound arsenic in S. leopoliensis as determined by NAA.

The determination of arsenic in S.leopoliensis was aimed at finding out whether tolerant and sensitive cultures differ with respect to free water-soluble and bound arsenic.

The recovery of arsenic from the cells was incomplete, ranging from 12.4 to 60.2% of whole-cell arsenic (Table 13). In experiment 1 the generally lower recoveries as compared to experiment 2 may be due to not measuring arsenic in the filtrates. However, the reasons for arsenic loss are not known.

Arsenic in the cells occurs in both free and bound forms and in roughly similar amounts. However the incomplete recovery of arsenic makes this conclusion weak.

When sensitive cells are compared to tolerant cells as regards percentage

of arsenic in free form (or in bound form), no pattern emerges. The tolerant cells do not have consistently more bound or free arsenic than do sensitive ones, either on an absolute or percentage basis. But again, low recovery of arsenic weakens this conclusion. Recovered cells differed from the other two types of cultures.

The total amount of arsenic in the ice-cold water extract and pellet from tolerant and sensitive cells was much lower than that estimated in whole cells (Table 11). The highest recovery of arsenic in tolerant and sensitive cells was 60 and 35% of whole-cell arsenic respectively (Table 13, experiment 2).

7. Analysis of arsenic on paper chromatogram by Proton Induced X-Ray Emission (PIXE)

There were peaks of arsenic at positions with R_f values that would be expected of arsenate (R_f 0.34) and arsenite (R_f 0.58-0.63).

A comparison of the peak areas with a standard arsenite/arsenate chromatogram (Appendix 9) revealed that the total amount of arsenic in arsenite plus arsenate were 0.0037 and 0.0017 g for tolerant and sensitive cells respectively. Of these totals, the percentage of arsenate in tolerant and sensitive cells was about 14 and 18%; so arsenite was 86 and 82% respectively. (Figures 10a and 10b).

8. Analysis of arsenic in water extracts from tolerant and sensitive cells of S. leopoliensis on paper chromatogram by Neutron Activation Analysis (NAA)

There were peaks of arsenic at positions with R_f values of 0.22 and 0.56. The values were similar to those of standard arsenate (R_f 0.28) and arsenite (R_f 0.56).

In the water extracts from tolerant and sensitive cultures harvested after 9 hours, the percentage of arsenic as arsenite in tolerant cells (45%) was the same as that in sensitive (47%) cells within error. Similarly, the

percentage of arsenic as arsenate was the same in both types of cells (23% and 26% respectively). (Figures 11 & 12). In the water extract from sensitive cells that recovered after a long growth lag (about 30 hours), most of the arsenic was in the form of arsenate (86%) and arsenite was hardly detectable (0.75%). (Figure 13).

9. Uptake of $^{35}\text{SO}_4^{2-}$ by normal and tolerant cells of *S. leopoliensis*.

a. During induction of arsenite tolerance.

Two cultures were grown in presence of ^{35}S - labelled MgSO_4 . One of them was also treated with 10uM arsenite. Radioactivity of ^{35}S was counted in ethanol extracts and in NaOH/HCl extracts (dissolved cell-residue from ethanol extraction). The results are in Table 14.

The amounts of ^{35}S in the NaOH/HCl extracts of normal and tolerant cultures were not significantly different ($p = 0.05$, $n = 3$). It was only in experiment 1 after 2 hours that the amounts of ^{35}S in the ethanol extracts from normal and tolerant cultures were significantly different with more ^{35}S being found in the normal cultures. (Table 14).

b. During growth within three generations of cells.

The study was aimed at finding out whether there was a difference in the partitioning of ^{35}S in normal and tolerant cells. Cultures were allowed to grow for three generations in presence of ^{35}S - labelled MgSO_4 . Results are in Table 15.

As in the uptake of ^{35}S within one generation (Table 13) the amounts of ^{35}S in NaOH/HCl extracts of tolerant cultures and normal cultures were not significantly different ($p = 0.05$, $n = 3$) (Table 14). Radioactivity of ^{35}S in the small molecules (ethanol, petroleum ether and $\text{CH}_4\text{OH}/\text{CHCl}_3$ extracts) from tolerant and normal cultures was in general not significantly different. It was only in two cases, (Table 15, experiment 2, values marked with asterisks) where the ethanol and petroleum ether extracts from tolerant and

control without arsenite in the growth medium. (Figure 10). The running of a polyacrylamide gel of ^{35}S -labelled proteins in cell extracts followed by fluorography revealed ^{35}S -labelled proteins of molecular weight between 15 and 72 kD. (see Appendix 15 and Table 16). In this range normal cultures had significantly different amounts of ^{35}S , with the higher amounts occurring in the normal cultures.

10. Thin layer chromatography of ethanol extracts from normal and tolerant cultures of *S. leopoliensis* provided with ^{35}S in the growth medium.

This analysis of ethanol extracts was aimed at identifying and estimating amounts of ^{35}S -labelled compounds in tolerant and normal cells.

Two areas on the chromatogram had the highest counts, one between 1.5 and 6 cm and the other between 10.5 and 15 cm from the origin. The peaks at the latter position corresponded to the position of lipoic acid according to R_f value (approximately 0.93). The amounts of ^{35}S at this position for both the normal and tolerant cultures were similar (Figure 14).

The amount of ^{35}S at position 1.5-6cm did not correspond with any of the other markers used, namely glutathione (R_f 0.40), cysteine (R_f 0.45) or methionine (R_f 0.63). In addition, the amount of ^{35}S at this position in normal cultures was higher than in tolerant cultures.

The peak at the position of lipoic acid on the thin layer chromatogram could not be identified with certainty because of yellow carotenoids which had the same mobility as lipoic acid. The colour of lipoic acid and the detection reagent (2, 2¹-Dithiobis-(5-Nitropyridine) was also yellow.

11. Polyacrylamide Slab Gel Electrophoresis of tolerant and sensitive cell extracts of *S. leopoliensis*.

The purpose of the analysis was to find out whether induction of arsenite tolerance was associated with synthesis of special protein(s).

The gel electrophoresis of proteins in cell extracts from cells that were not provided with SO_4^{2-} revealed proteins of molecular weight between 15.5 kD and 72 kD. (see Appendix 15 and Table 16). In these proteins, no special protein bands were detectable to the eye in tolerant cells compared to the

control without arsenite in the growth medium. (Figure 15).

The running of a polyacrylamide gel of ^{35}S -labelled proteins in cell extracts followed by fluorography revealed ^{35}S -labelled proteins of molecular weight between 28 and 54 kD. (Appendix 15 and Table 16). Within this range of molecular weights, the patterns of protein bands in tolerant cells and normal cells without arsenite in the medium were similar. The results also showed lack of protein bands in lanes containing cell extracts from sensitive cells treated with 200 μM arsenite. (Figure 16 lanes 6 and 6'). These cells apparently were not capable of incorporating radioactive sulfate into proteins. In contrast to this, cells that were induced with 10 μM arsenite for arsenite tolerance and later transferred to 200 μM arsenite showed protein bands similar to those of the normal cells grown in arsenite-free medium. (Figure 16 lanes 7 and 7'). Very little incorporation of $^{35}\text{SO}_4^{2-}$ was noted for proteins with a molecular weight less than 28 kD in all cases (Table 16 and Figure 16).

Table 5

Growth lags of sensitive and tolerant cells of the cyanophytes in arsenite.

Concentration of arsenite (μM)	Growth lags (hr.)			
	<u>O. limnetica</u>		<u>S. leopoliensis</u>	
	Sensitive cells	Tolerant cells	Sensitive cells	Tolerant cells
0	2	2	0	0
200	32	2	80	0
300	34	3	100	0
400	49	30	180	12

Table 6

Growth lags of S. leopoliensis in 200 μ M arsenite after induction with 10 μ M and 1.0 μ M arsenite for different time periods.

Period of incubation (hr) in 10 or 1.0 μ M arsenite	Growth lags (hr) in 200 μ M arsenite when induction is by:	
	10 μ M arsenite	1.0 μ M arsenite
0.5	4	8.5
1.0	4	8.0
1.5	3	7.0
2.0	0	5.5

Table 7

Generation times of C. pyrenoidosa in 1-5mM arsenite with or without exposure to non-inhibitory concentration of arsenite (500 μ M).

Concentration of arsenite (mM)	Generation Times (Hr)	
	Non-exposed	Pre-exposed
0	10	10
1	11.5	10
2	17.5	12
3	No growth	15
4	No growth	28
5	No growth	No growth

Table 8

Cell numbers of normal (control) cultures and cultures undergoing induction of arsenite tolerance.

Counting done after (hr)	Absorbance, 660nm		Number per ml. x 10 ⁶	
	Control culture	Tolerant culture	Control culture	Tolerant culture
0	0.26	0.26	-	-
1	0.30	0.30	65.3	62.8
3	0.35	0.34	87.3	77.8
5	0.39	0.39	88.0	75.5
7	0.41	0.40	90.0	84.3

Note:

No. of cells per ml = No. in 80 small squares x 250,000.

Figure 9

Decay of tolerance with time in S. leopoliensis.

Numbers next to curves indicate the period of growth (hrs) of tolerant cells in arsenite-free medium before transfer to a medium containing 200 μ M arsenite.

c = control.

Table 9

Growth lags of tolerant cells of *S. leopoliensis* after transfer from an arsenite-free medium to 200µM arsenite.

Figure 9.

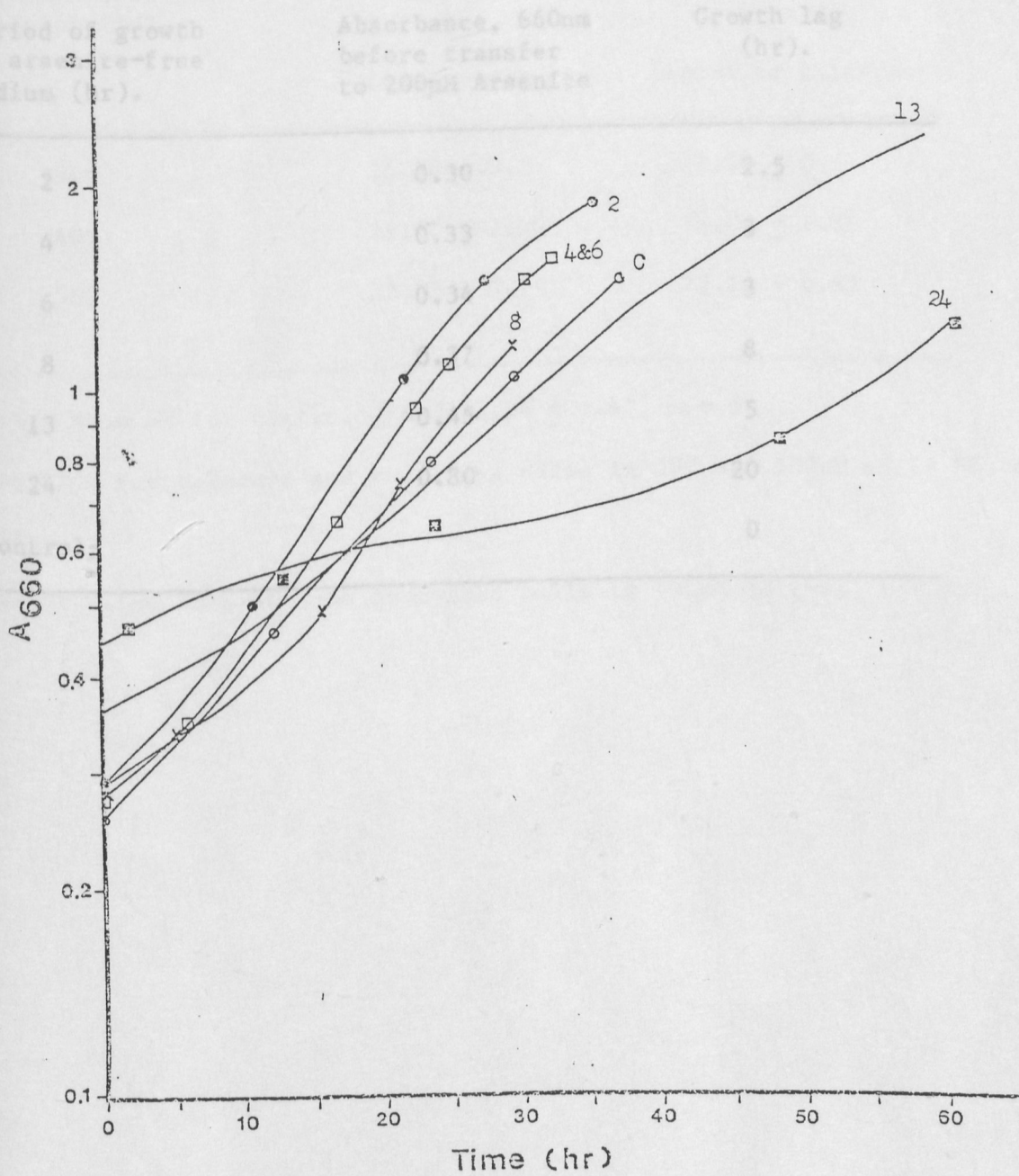


Table 9

Growth lags of tolerant cells of S. leopoliensis after transfer from an arsenite-free medium to 200 μ M arsenite.

Period of growth in arsenite-free medium (hr).	Absorbance, 660nm before transfer to 200 μ M Arsenite	Growth lag (hr).
2	0.30	2.5
4	0.33	3 $\pm$ 1.00
6	0.34	13 $\pm$ 0.93
8	0.37	8
13	0.45	5
24	0.80	20
Control	-	0

Table 10

Mean generation times of tolerant and recovered cultures of S. leopoliensis when transferred to 300, 400 and 500 μ M As.

Concentration of arsenite (μ M)	Mean generations times (hr) (GT)	
	Tolerant cultures	Recovered cultures
300	15.33 $\pm$ 0.34	18.00 $\pm$ 0
400	26.00 $\pm$ 1.00	32.00 $\pm$ 1.00
500	32.33 $\pm$ 0.72	25.16 $\pm$ 0.93

Note: Mean GT for control (-As) 11.00 $\pm$ 3.67, n = 5

Mean GT's for tolerant and recovered cells in 300 and 500 μ M As ($\pm$ SE, n = 3)

Mean GT's for tolerant and recovered cells in 400 μ M As ($\pm$ SE, n = 2).

Table 11

Arsenic levels in whole air-dried sensitive and tolerant cells of S. leopoliensis as determined by Neutron Activation Analysis (NAA)

A_{660nm} of cultures at harvest ranged from 0.40 to 0.75 and volume of cultures were 1800-2000ml. The indicated errors are not statistical standard errors. They are counting errors inherent in the NAA counter.

Table 11

Type of culture	Weight of dry algae (g)	$\mu\text{g As mg}^{-1}$ Chla	ppm As	BR
<u>Experiment 1</u>				
<u>Tolerant</u>				
10 μM As induction, 9 hours in 200 μM As	0.6368	98.96 $\pm$ 3.48	508.12 $\pm$ 17.88	40
<u>Sensitive</u>				
9 hrs in 200 μM As	0.4814	89.33 $\pm$ 3.32	575.23 $\pm$ 21.38	36
<u>Recovered</u>				
In 200 μM As until recovery, (30 hrs)	1.1008	156.40 $\pm$ 4.58	650.78 $\pm$ 19.05	63
<u>Experiment 2</u>				
<u>Tolerant</u>				
10 μM As induction, 8 hr in 200 μM As	0.2286	44.33 $\pm$ 0.51	486.92 $\pm$ 5.60	18
<u>Sensitive</u>				
8 hr in 200 μM As	0.1998	51.34 $\pm$ 0.60	573.56 $\pm$ 6.71	21

Key:

BR = Bioaccumulation Ratio
 $= \frac{\text{ppm (w/v) arsenic in algal cell water}}{\text{ppm (w/v) arsenic in growth medium}}$

200 μM arsenite = 15 ppm (w/v) arsenite

As = arsenic

Note: Arsenic was estimated by PHS.

As = ppm (D.W.) As in algae

ppm (w/v) As in growth medium

* Values obtained by HPLC were 75% and 70% respectively.

Table 12

Amount of arsenic (ppm, dry wt) in filtered layers of C. pyrenoidosa grown in presence of 0.5 - 3.0mM arsenite.

Concentration of arsenite (mM)	Hours of growth	As, ppm dry	Bioaccumu- lation ratio (BR)
0.5	0	2615	66
	2	1846	46
	4	*1692	42
	11	*3142	80
	22	4000	102
	46	2166	54
1.0	0	5250	66
	3.5	6000	76
	7.5	6333	80
	12.5	6000	76
	24.5	4166	52
3.0	0	5375	22
	3.5	7666	32
	7.5	21000	88
	12.5	22000	93
	24.5	8364	35

Note: Arsenic was estimated by PIXE.

BR = $\frac{\text{ppm (D.W.) As in algae}}{\text{ppm (w/v) As in growth medium.}}$

* Values obtained by NAA were 2257 and 3078 ppm after 4 and 11 hours respectively.

Table 13

Levels of free (water soluble) and bound arsenic in S. leopoliensis as determined by NAA.

The cultures in Table 10 were extracted in 20ml ice-cold water and flash evaporated (in vacuo at 40° C) to 4ml (Experiment 1) and 5ml of ice-cold water (Experiment 2).

Table 13

Culture	$\mu\text{g arsenic mg}^{-1} \text{ Chla}$				
	Ice-cold water extract	Filtrate from resuspended pellet	Pellet left after ice- cold water extraction	Total	% of whole- cell arsenic
<u>Exp. 1</u>					
Tolerant	5.03 ± 0.08	-	7.28 ± 2.20	12.31 ± 2.28	12.44
Sensitive	7.92 ± 0.10	-	8.73 ± 2.22	16.65 ± 2.32	18.64
Recovered	15.79 ± 0.12	-	34.74 ± 2.88	50.53 ± 3.00	32.31
<u>Exp. 2</u>					
Tolerant	12.32 ± 0.23	3.30 ± 0.14	11.06 ± 0.06	26.68 ± 0.43	60.18
Sensitive	10.85 ± 0.20	2.85 ± 0.14	4.17 ± 0.06	17.86 ± 0.41	34.79

Note:

1. These experiments are the same as experiments 1 and 2 in Table 11.
2. The indicated errors are not statistical standard errors. They are counting errors inherent in the NAA counter.

Figure 10 a, b.

Distribution of arsenic in ice-cold water extracts of sensitive and tolerant cells on paper chromatogram as determined by PIXE.

Figure 10.

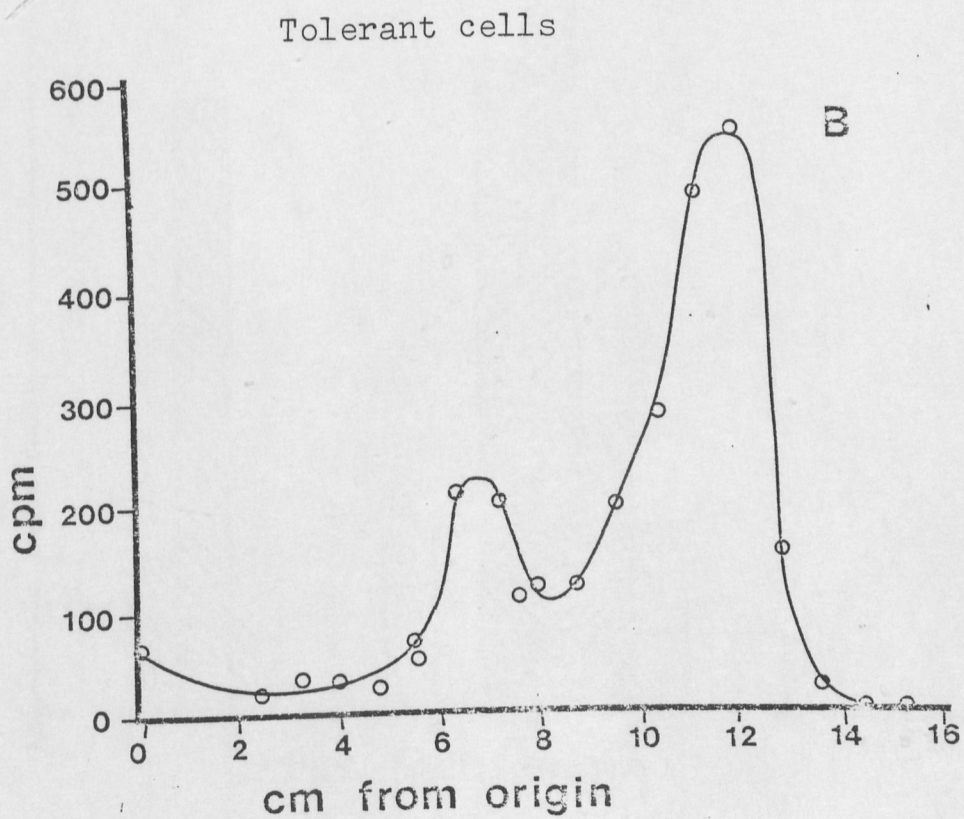
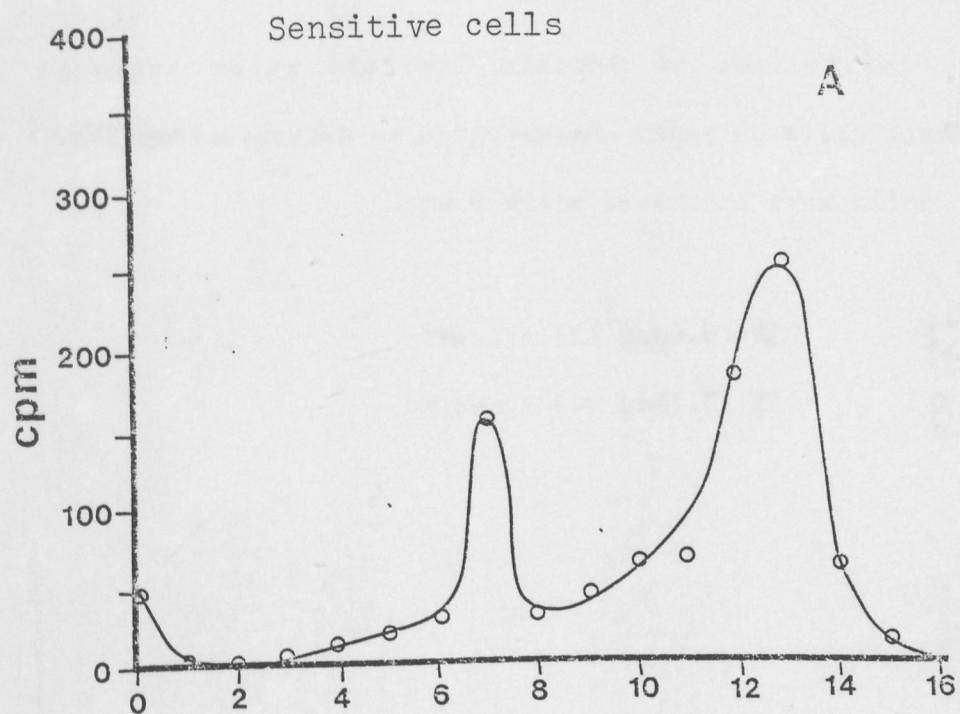


Figure 11

Distribution of arsenic ice-cold water extracts of tolerant cells on paper chromatogram as determined by NAA.

Cells were harvested after 9 hrs.

KEY:



23% (0.08 μ g As) arsenate



45% (0.14 μ g As) arsenite

Figure 11.

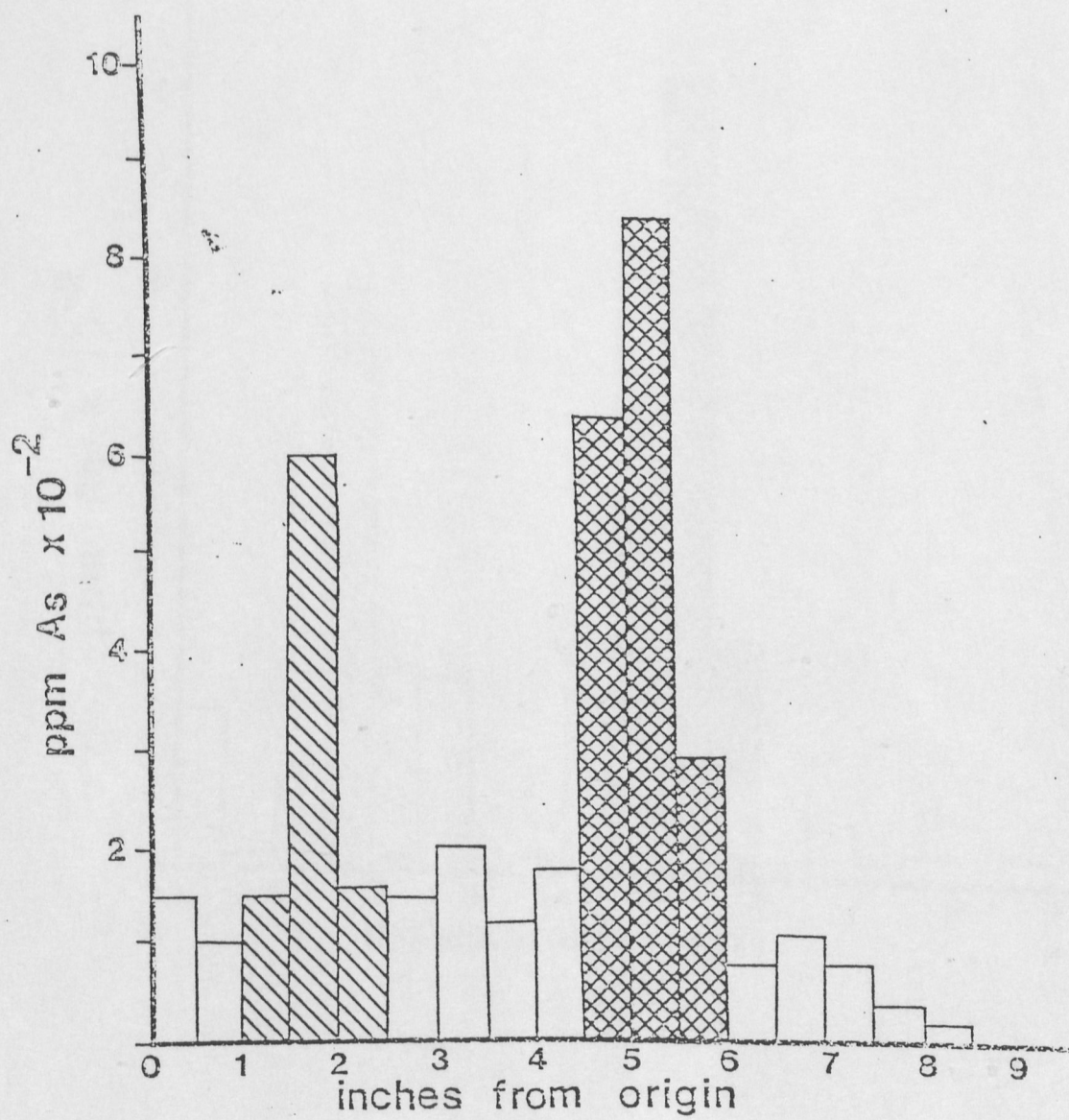


Figure 12

Distribution of arsenic in ice-cold water extracts of sensitive cells on paper chromatogram.

Cells were harvested after 9 hrs.

KEY:



26% (0.13 μ g As) arsenate



47% (0.23 μ g As) arsenite

Figure 12.

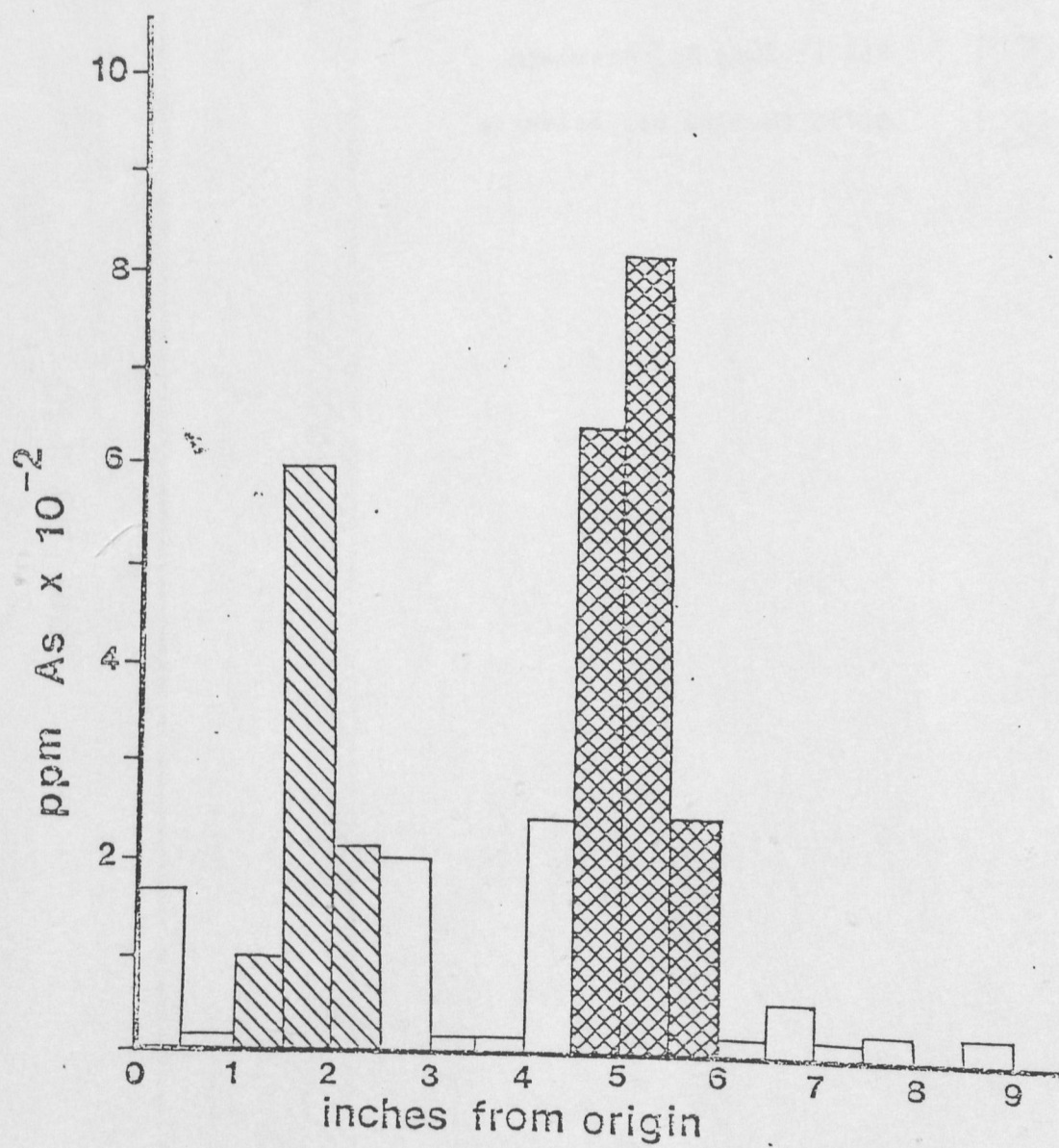


Figure 13

Distribution on paper chromatogram of arsenic in ice-cold water extract from sensitive cells that were allowed to recover. (Analysis by NAA)

Cells were harvested after 30 hrs.

KEY:



86% (1.24 μ g AS) arsenate



0.75% (0.02 μ g As) arsenite

Figure 13.

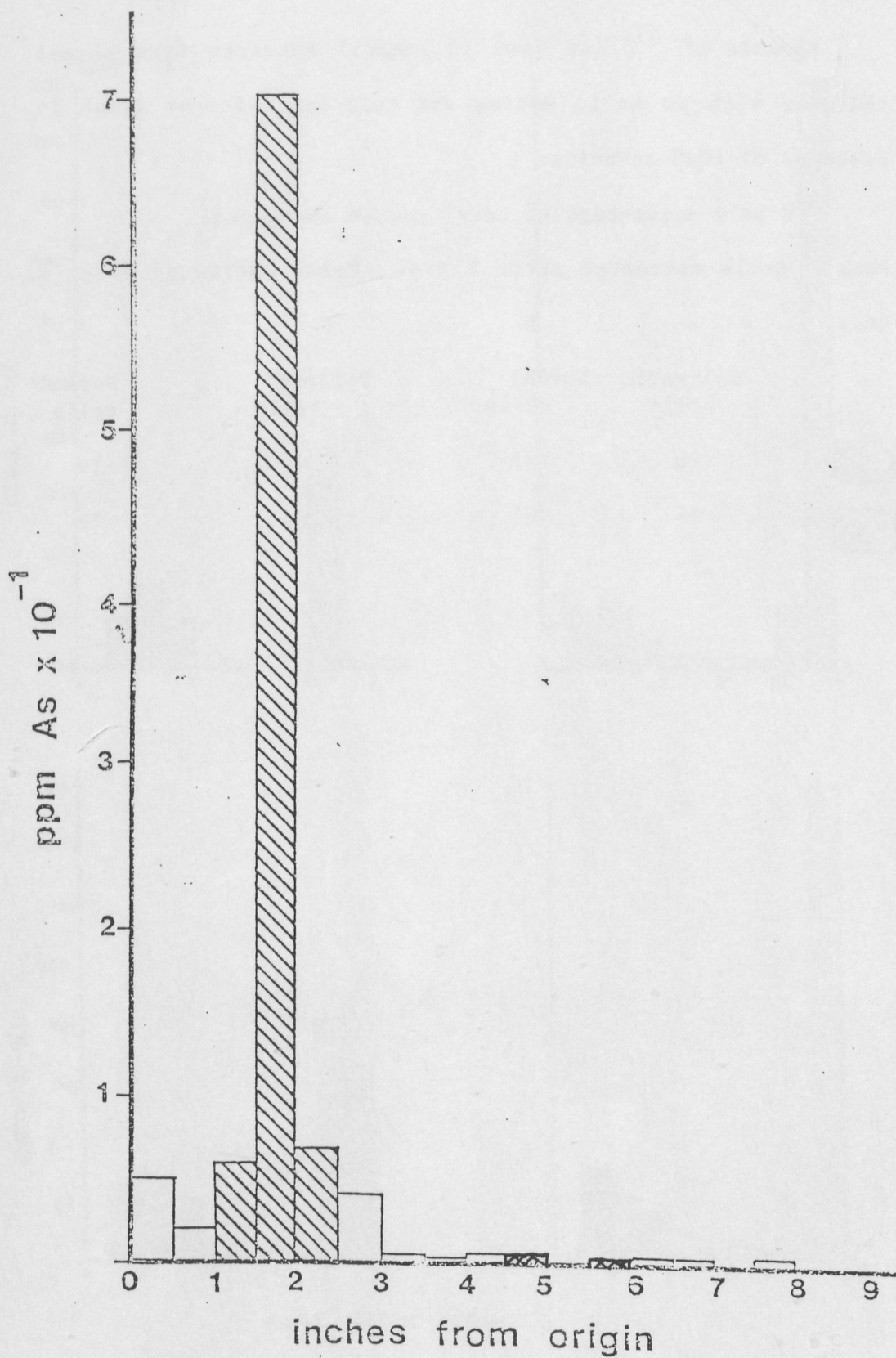


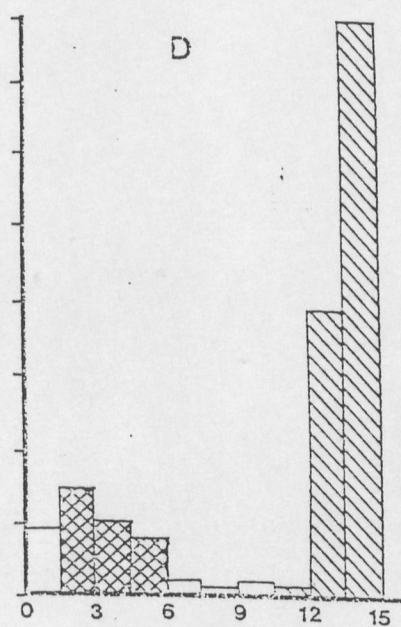
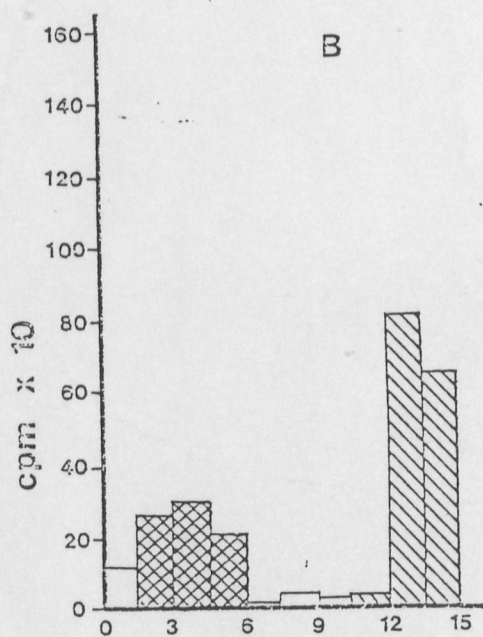
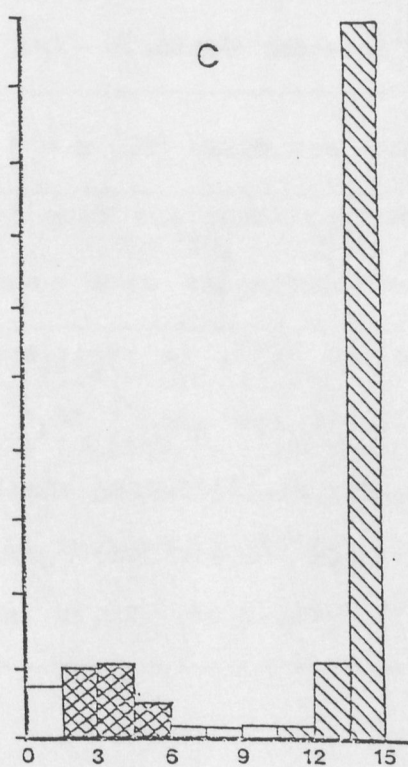
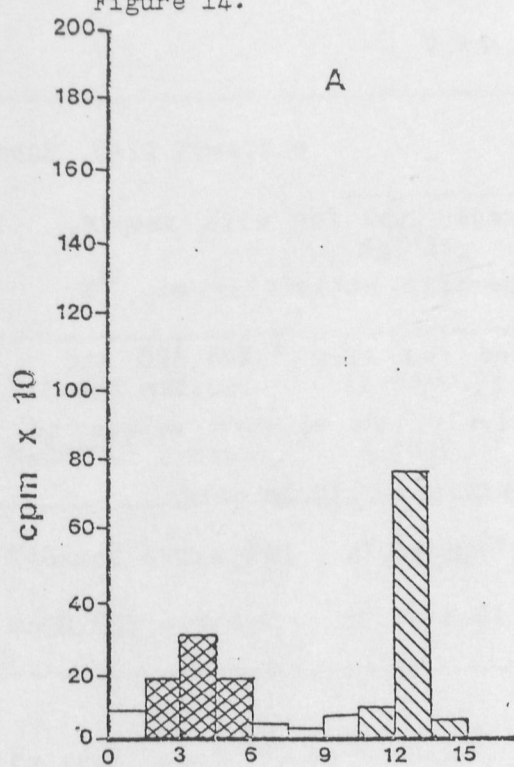
Figure 14

Amounts of ^{35}S (in cpm) in ethanol extracts from normal cultures with no As in medium and tolerant cultures grown in presence of $10\mu\text{M}$ arsenite.

^{35}S as a percentage of total cpm at the peaks.

Peak	Cells extracted after 2 hrs.		Cells extracted after 6 hrs.	
hrs.	a	b	c	d
	Tolerant cells	Normal cells	Tolerant cells	Normal cells
	26	32	16	19
	64	61	76	72

Figure 14.



cms from origin

Table 14

Amount of ^{35}S in ethanol and NaOH/HCl extracts from normal and tolerant cells.

Values are means $\pm$ SE, n = 3

Each experiment was done in triplicates and for each sample, three subsamples were counted. Specific activities of ^{35}S -labelled MgSO_4 in experiment one and two were 5,244,320 and 2,623,846 cpm $\mu\text{mol}^{-1} \text{SO}_4^{2-}$ respectively. To convert values to ppm, dry wt. following simplified equation could be used:

$$\text{ppm } ^{35}\text{S (dry wt)} = \mu\text{mol } ^{35}\text{S mg}^{-1}\text{Chla} \times 2 \times 10^2.$$

Table 14

		Treatment and Hours of Incubation			
Experiment	Cell Fraction	2 Hours		4 Hours	
		Mg ³⁵ SO ₄	10μM As +Mg ³⁵ SO ₄	Mg ³⁵ SO ₄	10μM As +Mg ³⁵ SO ₄
1	Ethanol extract	*	*	*	*
		12.05±0.71	6.94±0.01	15.99±0.01	11.79±1.36
	NaOH/HCl extract	6.26±0.01	6.68±0.001	10.56±0.01	11.34±0.01
2	Ethanol extract	18.36±1.47	19.12±1.91	34.23±0.93	33.53±3.55
	NaOH/HCl extract	38.98±3.81	36.46±1.47	69.32±2.88	61.29±5.71

Note:

1. Units are: μmol ³⁵S mg⁻¹ Chla.
2. All values to be multiplied by 10⁻².
3. * Their differences are statistically significant (p = 0.05 n = 3)

Table 15

Amount of ^{35}S in ethanol extracts (inclusive of lipid and water soluble compounds) and in NaOH/HCl extracts from tolerant and normal cells of S. leopoliensis allowed to grow up to three generations ($A_{660\text{nm}}$ 0.12 - 1.05).

Specific activities of S- labelled MgSO_4 in experiment one and two were 5,448,688 and 4,577,843 cpm per $\mu\text{mol}^{-1} \text{SO}_4^{2-}$ respectively.

Each experiment was done in triplicates and for each sample, three subsamples were counted.

To convert values to ppm dry wt. following simplified equation could be used.

$$\text{ppm } ^{35}\text{S}(\text{dry wt.}) = \mu\text{mol } ^{35}\text{S mg}^{-1}\text{CH1a} \times 2 \times 10^2.$$

Table 15

Cell fraction	Normal cells ($^{35}\text{SO}_4^{2-}$)	Tolerant cells ($^{35}\text{SO}_4^{2-}$ + 10 μM As)	Tolerant cells ($^{35}\text{SO}_4^{2-}$ + 10 μM As) induction + 200 μM As)
<u>Experiment 1</u>			
1. Ethanol extract	1.13 $\pm$ 0.08	1.15 $\pm$ 0.11	-
a. Petroleum ether	0.51 $\pm$ 0.02	0.54 $\pm$ 0.01	-
b. $\text{CH}_3\text{OH}/\text{CHCl}_3$	0.26 $\pm$ 0.02	0.30 $\pm$ 0.03	-
c. water	0.05 $\pm$ 0.01	0.05 $\pm$ 0.02	-
2. NaOH/HCl extract	5.56 $\pm$ 0.09	5.67 $\pm$ 0.21	-
<u>Experiment 2</u>			
1. Ethanol extract	*1.35 $\pm$ 0.03	*1.02 $\pm$ 0.06	0.99 $\pm$ 0.06
a. Petroleum ether	*0.65 $\pm$ 0.01	0.58 $\pm$ 0.02	*0.53 $\pm$ 0.03
b. $\text{CH}_3\text{OH}/\text{CHCl}_3$	0.41 $\pm$ 0.03	0.31 $\pm$ 0.02	0.34 $\pm$ 0.01
c. water	0.15 $\pm$ 0.03	0.06 $\pm$ 0.01	0.05 $\pm$ 0.01
2. NaOH/HCl extract	5.65 $\pm$ 0.13	5.24 $\pm$ 0.20	5.09 $\pm$ 0.34

Note:

- (1) Units are: $\mu\text{mol } ^{35}\text{S mg}_{-1} \text{ Chl a.}$
- (2) Values are means $\pm$ SE, $n = 3$.
- (3) * Their differences were statistically significant.
($p = 0.05$, $n = 3$).

Table 16

Molecular weights of proteins in the bands of polyacrylamide gel and the fluorograph.

Proteins not labelled with ^{35}S		Proteins labelled with ^{35}S	
Distance from origin (cm)	Molecular weight kD	Distance from origin (cm)	Molecular weight kD
1.2	72	4.0	54
1.4	68	4.2	52
1.6	64	4.5	49
2.0	57	5.5	41
2.2	54	5.8	38.5
2.4	51	6.2	36
3.7	35	6.5	34
4.1	31	6.9	31.5
4.6	27	7.2	30
5.0	24	7.5	28
5.5	21	-	-
6.5	15.5	-	-

Appendix 15 shows how the molecular weights were determined from the semilogarithmic plot of molecular weights of standard proteins against the distance they travelled in the gel.

Figure 15

Polyacrylamide SDS Gel Electrophoresis of proteins in cell extracts from S. leopoliensis that were not provided with radioactive sulphate in the medium.

KEY

Crude extracts. (12 μ l of sample)

1. Control (in arsenite-free medium)
2. Tolerant cells (just induced at 10 μ M arsenite)
3. Tolerant cells (induced at 10 μ M As and transferred to 200 μ M As.

Supernatants. (12 μ l sample)

- 1'. Control (in arsenite-free medium)
- 2'. Tolerant cells (just induced at 10 μ M arsenite)
- 3'. Tolerant cells (induced at 10 μ M As and transferred to 200 μ M As.
4. Pellet from crude extract of tolerant cells (10 μ M As) digested in 2ml IN NaOH/2ml IN HCl.
5. Pellet of crude extract of tolerant cells (10 μ M As $\rightarrow$ 200 μ M As) similarly treated.

S standard proteins

Unlabelled lanes contained half the amount of cell extracts used in labelled lanes.

Figure 15

1 2 3 1' 2' 3' 4 5 S
↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓

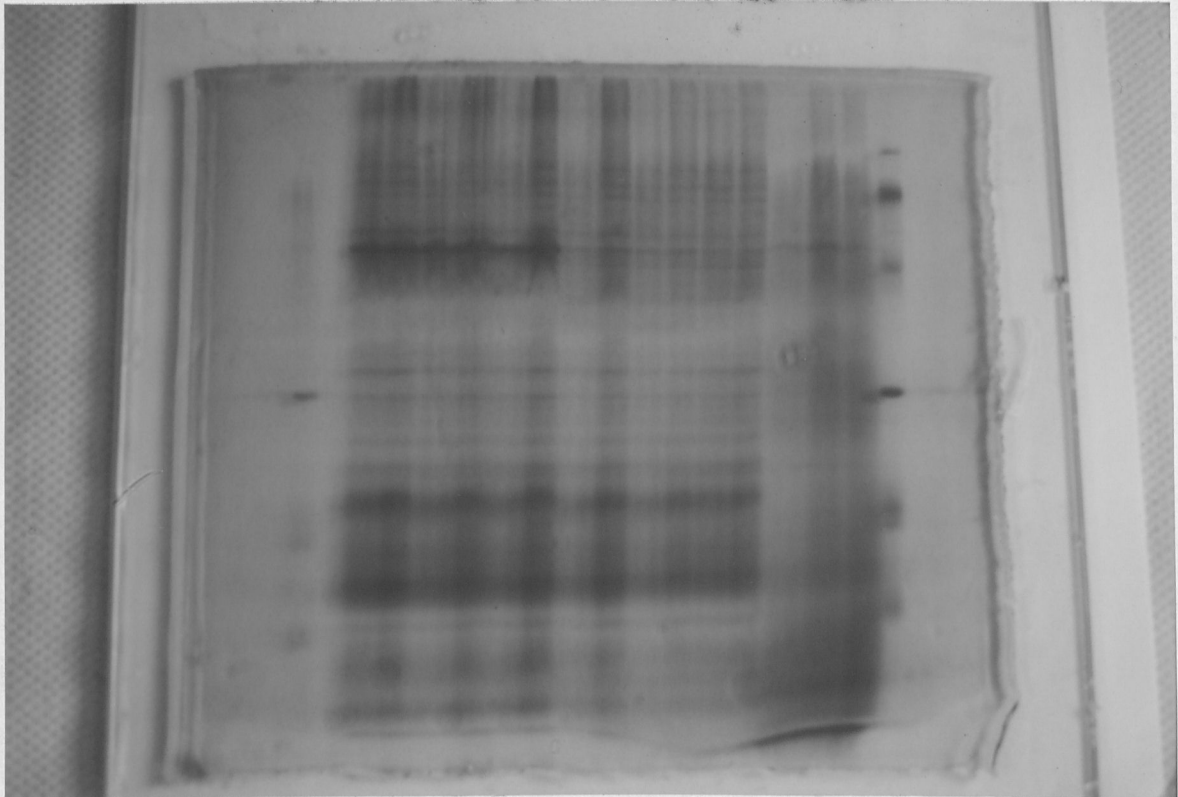


Figure 16

Fluorograph of proteins in cell extracts from S. leopoliensis that were provided with radioactive sulphate in the growth medium.

Exposure of the dry gel to an X-Ray film for six months did not produce protein bands of greater intensity than exposure for 18 days.

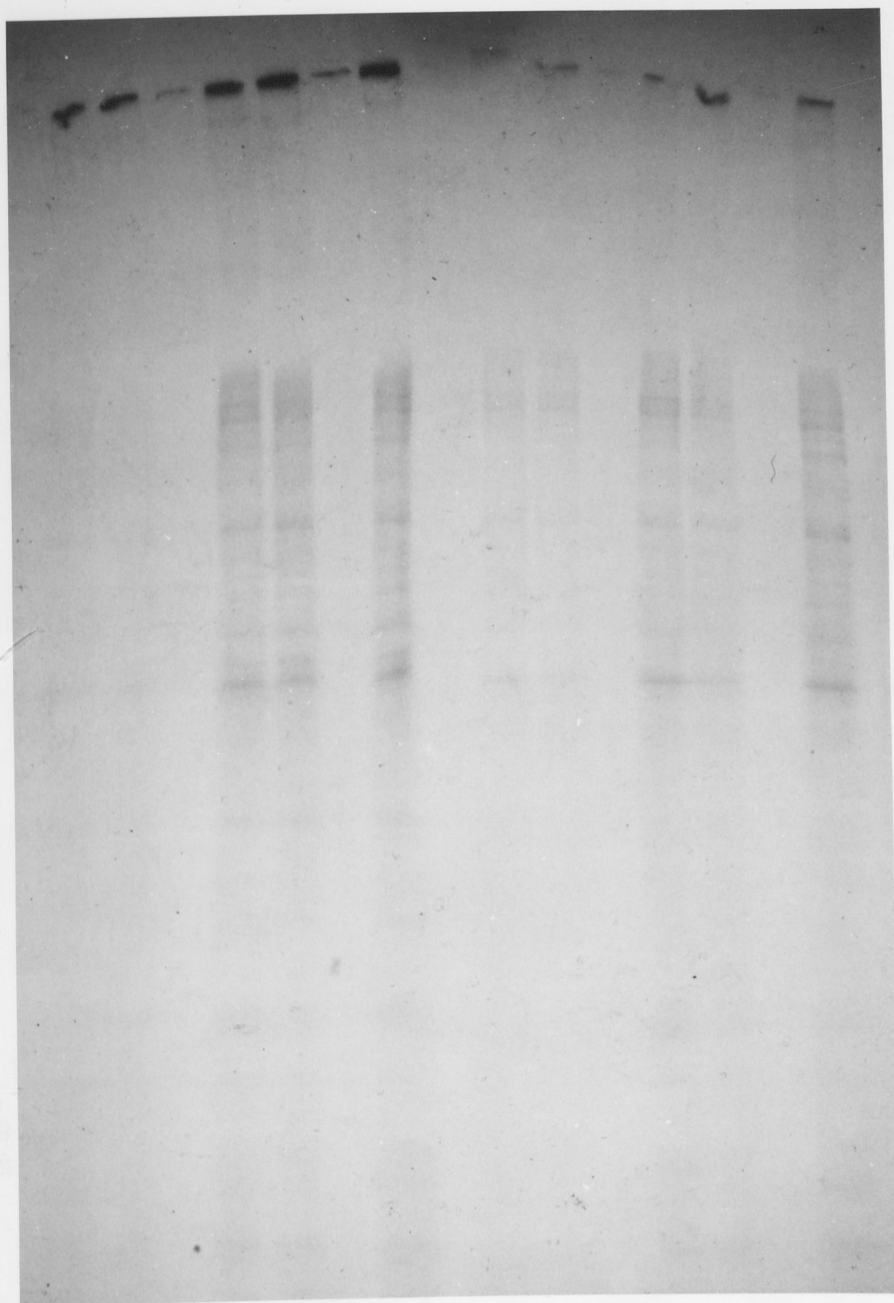
KEY:

Lane

1. Normal cells (in As-free medium containing $^{35}\text{SO}_4^{2-}$)
 2. Tolerant cells (10 μM As and $^{35}\text{SO}_4^{2-}$ in medium)
 3. Sensitive cells (200 μM As and $^{35}\text{SO}_4^{2-}$ in medium)
(1-3 cultures harvested after 2 hours).
 4. Normal cells (in As-free medium containing $^{35}\text{SO}_4^{2-}$)
 5. Tolerant cells (10 μM As and $^{35}\text{SO}_4^{2-}$ in medium)
 6. Sensitive cells (200 μM As and $^{35}\text{SO}_4^{2-}$ in medium)
(4-6 cultures harvested after 4 hours).
 7. Tolerant cells ($^{35}\text{SO}_4^{2-}$ and 10 μM As (induction)
(and later transferred to 200 μM As)
- 1' - 7' Supernatant obtained from spinning down crude extracts
(1 - 7) in a centrifuge.

Figure 16

1 2 3 4 5 6 7 1' 2' 3' 4' 5' 6' 7'



Discussion

In this discussion, I would like to examine the results of this study with respect to arsenite toxicity and tolerance in the microalgae that have been investigated. The specific issues I want to address are the following:

1. Arsenite sensitivity in the microalgae
2. Effect of exogenous acetate on arsenite toxicity.
3. Effect of pH on arsenite toxicity in S. leopoliensis.
4. Inducibility of arsenite tolerance
5. Tolerance mechanism(s) to arsenite in S. leopoliensis.

1. Arsenite sensitivity in the microalgae.

Both cyanophytes were sensitive to arsenite in the micromolar range. O. limnetica however, appeared to be slightly less sensitive to arsenite than S. leopoliensis. The growth lags of the former in presence of the different concentrations of arsenite were shorter than for the latter. Budd et al (1986) obtained similar growth lags for S. leopoliensis.

The sensitivity of S. leopoliensis to 200 μ M arsenite was found to vary between experiments. In a number of experiments performed in this study, the average growth lag was 34.2 ± 9.8 h ($\pm$ SD, n = 7). Budd et al (1986) noted similar variation and reported an average growth lag of 44.6 ± 19.2 h ($\pm$ SD, n = 13). The variation was probably due to different pH's of the growth media at the time of arsenite addition. The pH of the medium has been shown to have an effect on arsenite toxicity in S. leopoliensis (Figure 7). Increase in pH reduced arsenite toxicity.

After recovery of growth in the different concentrations of arsenite, the generation times of S. leopoliensis were close to that of the control grown in arsenite-free medium. However, in O. limnetica the generation times were variable. This was probably due to aggregation of algal filaments and settling out of alga to the bottom of culture bottles.

Unlike the cyanophytes, C. pyrenoidosa was remarkably different in its sensitivity to arsenite. It was not sensitive to arsenite concentrations up to 800 μ M (data not presented). The sensitivity range was between 1mM and 3mM arsenite and at the latter concentration growth was completely inhibited. The lack of growth lags in C. pyrenoidosa suggests a different mechanism of arsenite toxicity in this chlorophyte from that of S. leopoliensis. Budd et al (1986) suggested that inhibition of pyruvate dehydrogenase was the cause of growth lags in S. leopoliensis.

The high levels of arsenic in the cells of C. pyrenoidosa (Table 12), suggest that the inherent resistance to arsenite toxicity is not through an exclusion mechanism of arsenite. The cells even accumulated arsenic well above that of the medium.

The "tolerance" to the high concentrations of arsenite in C. pyrenoidosa may be better described as natural resistance. In this case, the population of cells had the genes for resistance to arsenite at high frequency and did not require prior exposure to the toxicant for selection of the adaptive mechanism. The physiological and/or biochemical mechanism of arsenite toxicity and resistance in C. pyrenoidosa needs to be investigated.

2. Effect of exogenous acetate on arsenite toxicity.

The alleviation of arsenite toxicity by acetate in the cyanophytes implies a similar mechanism of toxicity. The reduction of growth lag in S. leopoliensis and elimination in O. limnetica suggests a faster uptake and/or utilization of acetate in the latter than in the former (Figure 5 and 6). The uptake and utilization of acetate by S. leopoliensis has been reported by Hoare et al (1967) and Ihlenfeldt and Gibson (1977). By use of labelled acetate, they demonstrated accumulation of radioactivity in lipids and amino acids, particularly in glutamate, proline, arginine and leucine. When cells

are therefore provided with acetate in presence of arsenite in the medium, pyruvate dehydrogenase complex (PDC) is inhibited (Budd et al, 1986) but acetyl-CoA is apparently synthesized via a pathway not involving PDC. Acetyl-CoA is then used as a precursor in biosynthesis of lipids and amino acids necessary for growth. The acetate metabolism of O. limnetica in presence of arsenite may be similar to that of S. leopoliensis.

The lack of alleviation of arsenite toxicity in C. pyrenoidosa by acetate suggests a different mechanism of toxicity in this chlorophyte, as already concluded on other grounds (see above).

3. Effect of pH on arsenite toxicity

The pH drift in unbuffered cultures was in the range of 0.2 to 0.6 above the initial pH of 6.5 that prevails in the growth medium soon after bubbling air/CO₂ mixture. On the other hand, the pH drift in cultures grown in PIPES-KOH buffer was small, about ± 0.2 . The response of the cells to arsenite in these cultures was therefore closely associated with the specific pH levels of the medium. The results strongly indicated that at acidic pH, arsenite was more toxic than at neutral or alkaline pH. The lower toxicity of arsenite at alkaline pH values was not associated with restricted uptake of arsenite by cells of S. leopoliensis (Table 4). This is in contrast to the report by Ricks and Hoskins (1948) that the penetration of arsenite into flesh fly larvae decreased with rise in pH.

The KCl controls with the higher pH's (7.1) had shorter growth lags. As in the PIPES-KOH buffered cultures at alkaline pH, the lowered toxicity, as indicated by shorter growth lags in these KCl-controls, was not associated with decreased uptake of arsenite (Table 4). The high levels of arsenic in the cultures grown in medium at pH 6.2-8.0 is consistent with the suggestion by Braman (1983) that in the pH range 4-8 arsenious acid is essentially in un-ionized form and would therefore enter cells easily. The thin layer

chromatography (TLC) of medium from a culture grown at pH 8.0 also revealed that most arsenic was in the form of arsenite. The TLC of medium from a culture grown at pH 6.5 showed similar results.

According to the Henderson - Hasselbalch Equation, i.e.

$$\text{pH} = \text{pKa} + \log \frac{[\text{conjugate base}]}{[\text{acid}]}$$

the percentage of the un-ionized arsenious acid (HAsO_2) at pH 6.5 and pH 8.0 would be about 99 and 94 percent respectively. So cultures grown at pH 6.5 should have more internal arsenic than at pH 8. However, this was not the case (Table 4) and cultures grown at pH 6.2 and 6.7 had lower contents of arsenic than the one grown at pH 8.0.

It may be concluded that the decrease in arsenite toxicity with increase in pH of the growth medium is not associated with decreased uptake of arsenite by S. leopoliensis. A possible explanation for lower toxicity at high pH is that once arsenious acid enters into cells, it is converted to a less toxic form e.g. arsenate. Alternatively cells may change the conditions in the medium in a way that reduces arsenite toxicity. The lower arsenite toxicity at high pH is an area that certainly requires further investigation.

4. Inducibility of arsenite tolerance.

The two cyanophytes were more easily induced to show arsenite tolerance than was C. pyrenoidosa. Tolerant cultures of the former two grew like normal cells in presence of $200\mu\text{M}$ arsenite (Table 5), a concentration that caused a 20-70 hours growth lag in cultures that had not been pre-exposed to arsenite. "Tolerant" cultures of C. pyrenoidosa did not grow like normal cells in presence of the growth-inhibitory levels of arsenite, 2mM or 3mM (Table 7). The difference in inducibility of arsenite tolerance between the two cyanophytes and the chlorophyte suggests that the adaptive mechanisms to arsenite may not be the same in these two phyla of algae.

The induction of arsenite tolerance in S. leopoliensis seemed to depend on the concentration of arsenite in the medium. 10 μ M arsenite induced a higher degree of tolerance than 10 μ M arsenite within a given period of time. A culture induced with 10 μ M arsenite for 2 hours did not show any growth lag in 200 μ M arsenite while that induced with 1 μ M arsenite for the same period of time showed a growth lag of 5.5 hours (Table 6). A similar observation was made by Budd et al (1986) who reported that 2 hours was required for maximal induction of arsenite tolerance by 10 μ M arsenite and that 14 hours were required for 1.0 μ M arsenite to be as effective as 10 μ M arsenite in inducing tolerance.

When tolerant cells of S. leopoliensis were allowed to grow in an arsenite-free medium, arsenite tolerance was found to decrease with time (Figure 9 and Table 9). This observation also supports the suggestion that acquisition of arsenite tolerance is by induction rather than selection of arsenite-tolerant genotypes from the population.

5. Tolerance mechanism(s) to arsenite in S. leopoliensis.

The possible hypotheses of tolerance mechanism to arsenite in S. leopoliensis may be one or more of the following under four main areas:

a. Decreased uptake of arsenite

i) Release of organic material which binds arsenite in solution making it unavailable for uptake.

ii) Change in cell membrane permeability to restrict entry of arsenite into the cells.

iii) Secretion of slime or other substances by cells which reduce diffusion rates of arsenite to the cell surfaces.

iv) Exclusion of arsenite by sequestration in the cell wall or membrane.

b. Detoxification of arsenite outside the cell. Transformation of

arsenite into less toxic form e.g. arsenate.

c. Internal detoxification.

i) Transformation of arsenite inside the cell into less toxic form or more easily excreted form.

ii) Formation of low molecular weight-sulfhydryl compounds that bind arsenite, thus making it unavailable as a toxicant.

iii) Formation of high molecular weight sulfhydryl compounds that also bind arsenite into inactive form.

d. Enzyme adaptations

i) Synthesis of excess or resistant pyruvate dehydrogenase.

ii) Synthesis of alternative enzyme system for production of acetyl-CoA.

a. Decreased uptake of arsenite.

Available data do not support this mechanism of arsenite tolerance in S. leopoliensis. The levels of arsenic in tolerant and sensitive cells, as determined by NAA, were similar (Table 11). A similar observation was made by MacArthur et al (1985). By use of Proton Induced X-Ray Emission (PIXE) they showed that tolerant and sensitive cultures of S. leopoliensis had the same level of arsenic, about 35 ppm, dry wt. The levels of arsenic obtained in this study in tolerant and sensitive cells were on average 498 ± 11 ppm ($\pm$ SE, $n = 2$) and 574 ± 1 ($\pm$ SE, $n = 2$) ppm respectively. MacArthur et al (1985) washed the cells before analysis and considerable amounts of arsenic might have been leached out during washing. Hekman (1983) has reported that fluoride is removed from the cell surface of S. leopoliensis by washing with demineralized water. In this study, however, the cells were not washed and that could be a possible explanation of the discrepancy between the two estimations of arsenic.

Cells of S. leopoliensis accumulated arsenic (Table 11). Other cases of bioaccumulation of arsenic by freshwater algae have been reported by Woolson

recovery of growth of sensitive cells after a 30-hour growth lag (Figure 13).
et al (1974).

Decreased uptake of arsenite therefore does not appear to be the mechanism of arsenite tolerance in S. leopoliensis. However, binding of arsenite to cell walls (Ref. hypothesis a (iv)) may be a possible mechanism.

b. Detoxification of arsenite outside the cell.

There is no evidence to support the idea (see fig. 1) that arsenite was transformed into the less toxic form (arsenate) outside the cells of S. leopoliensis. Budd and Craig (1981) have already demonstrated that the growth of S. leopoliensis is not affected by external arsenate at up to 200 μ M. By use of radioactive arsenite, Budd et al (1985) showed that there was no significant conversion of arsenite to arsenate or other forms in the medium of sensitive and tolerant cultures. Thin layer chromatography of a medium obtained from a recovered culture (after a growth lag of about 70 hours in 200 μ M arsenite) has also revealed that most of the arsenic is in the form of arsenite (Experiment of Table 10).

c. Internal detoxication.

(i) Transformation of arsenite into less toxic form.

The analysis of arsenic in ice-cold water extracts from tolerant and sensitive cells by PIXE and NAA revealed the presence of arsenate and arsenite. In both cultures the percentage of arsenite was about twice that of arsenate (Figures 11 and 12). Furthermore, the percentage of arsenic as arsenite in tolerant cells (45%) was the same (within error) as that in sensitive cells (47%). Similarly the percentage of arsenate was the same in both cultures, 23% and 26% respectively. These results suggest that tolerance mechanism in S. leopoliensis is not due to oxidation of arsenite to less toxic forms such as arsenate.

However, oxidation of arsenite to arsenate was associated with the

recovery of growth of sensitive cells after a 30-hour growth lag (Figure 13). The significantly longer generation time of recovered cultures in 300 and 400 μ M arsenite compared to tolerant cultures (Table 10) suggest conversion of arsenite to arsenate as the only mechanism of tolerance in the former. The data also suggests that the latter have a more powerful arsenite tolerance mechanism than the former. However, that conclusion is not supported by the growth lags obtained when tolerant and recovered cells were transferred to 500 μ M arsenite. At this concentration, the recovered cells had a significantly shorter growth lag than the tolerant cells (Table 10). This observation suggests a second mechanism of tolerance in recovered cells in addition to the proposed conversion of arsenite to arsenate.

(ii) Formation of low molecular weight sulfhydryl compounds that bind arsenite into an inactive form.

The total amounts of ^{35}S in the tolerant and normal cultures of S. leopoliensis harvested after 2 and 4 hours were similar (Table 14). The amounts of ^{35}S in the small molecules with a molecular weight of less than 500 daltons (e.g. lipoic acid, amino acids in ethanol extract) in both cultures were in general not significantly different. A similar observation was made in cultures allowed to multiply up to three generations. In a few cases where the difference was significant, the larger amounts of ^{35}S occurred in normal cultures grown in arsenite-free medium (Tables 14 and 15).

The lipid-soluble and water-soluble compounds in ethanol extracts had similar amounts of ^{35}S . Most of the ^{35}S was in the lipid-soluble fraction. Again, in cases where the difference in these two fractions were significant, the larger amounts of ^{35}S were found in normal cells (Table 15).

The estimation of ^{35}S in the ethanol-soluble fractions on the thin-layer chromatogram showed that the distribution of radioactive sulfur in normal and tolerant cells was similar. However, the chromatographic separations achieved

were not optimal (Figure 14).

These findings do not support the synthesis of a low molecular weight sulfhydryl compound by tolerant cells that was not present in the normal cells. Therefore the above hypothesis is not supported by available evidence.

(iii) Formation of high molecular weight sulfhydryl compounds that could also bind arsenite into inactive form.

The amounts of ^{35}S in large molecules e.g. proteins (NaOH/HCl extract) in tolerant and normal cells were in all cases not significantly different. (Tables 14 and 15). The amount of arsenic that would be associated with these molecules (in the pellets, Table 13) in tolerant cells (7-25%) and in sensitive cells (10-8%) were rather low compared to arsenic in the whole air-dried cells (Table 11). However, recovery of total arsenic in these experiments was low.

The separation of proteins by polyacrylamide gel electrophoresis and the fluorographic analysis did not show protein bands in tolerant cells that were not present in normal cells grown in arsenite-free medium.

The findings do not support a tolerance mechanism in which high molecular weight sulfhydryl compounds are synthesized to scavenge arsenite making it ineffective as a toxicant.

d. Enzyme adaptations

(i) Synthesis of excess or resistant pyruvate dehydrogenase.

There is no evidence to support synthesis of excess or resistant pyruvate dehydrogenase as a mechanism of arsenite tolerance in S. leopoliensis. The report by Budd et al. (1985) indicate that pyruvate dehydrogenase from arsenite-tolerant cells had the same activity as that from sensitive cells.

(ii) Alternative pathway for synthesis of Acetyl-CoA

The alleviation of arsenite toxicity in S. leopoliensis by acetate

(Figure 4) suggests a mechanism for the synthesis of acetyl-CoA via a pathway not involving pyruvate dehydrogenase complex. Hoare et al. (1967) reported the presence of acetyl-CoA synthetase (E.C. 6.2.1.1) that was involved in the assimilation of exogenous acetate into amino acids (glutamate, proline, arginine, leucine) and protein in Anacystis nidulans. Therefore with this enzyme, tolerant cells need to synthesize only acetate in order to bypass synthesis of acetyl-CoA via pyruvate dehydrogenase pathway. Alternatively, they could use pyruvate to make acetyl-CoA via an enzyme other than pyruvate dehydrogenase.

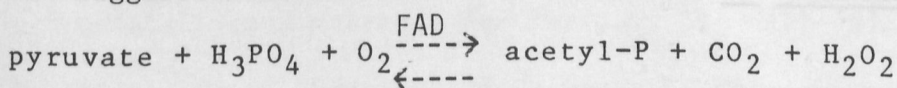
Takeuchi et al. (1975) reported the presence of a new type of pyruvate oxidase (CoA acetylating) in Entamoeba histolytica which catalyzed acetylation of CoA in presence of pyruvate, oxygen and FAD. The suggested reaction was

$$\text{pyruvate} + \text{CoA} + \text{O}_2 \longrightarrow \text{acetyl-CoA} + \text{CO}_2 + \text{H}_2\text{O}_2$$

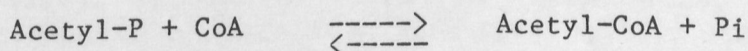
The enzyme was insensitive to 5mM arsenite.

In the bacterial system, Hager et al. (1954) reported a mercury-resistant enzyme in Lactobacillus delbrueckii which catalyzed oxidation of pyruvate to acetyl phosphate and CO₂ without involvement of either CoA or lipoic acid.

The suggested reaction was:



This enzyme was also insensitive to arsenite. Acetyl-phosphate can be converted to acetyl-CoA via, phosphotransacetylase (E.C. 2.3.1.8) thus:



These and alternative pathways of acetyl-CoA synthesis are currently under investigation as possible arsenite tolerance mechanism(s) in S. leopoliensis.

Suggestions for further research

A number of questions have arisen from this study and further investigations should be conducted to find out:

1. Whether resistance to arsenite toxicity in C. pyrenoidosa is associated with sequestration of arsenic in the cell wall or external/internal conversion of arsenite to the less toxic arsenate form.
2. Whether pH has any effect on arsenite toxicity in C. pyrenoidosa and O. limnetica.
3. The relative concentrations or amounts of arsenite and arsenate in ice-cold water extracts from cells of S. leopoliensis grown in presence of 200uM arsenite at different pH's.
4. The mechanism of alleviation of arsenite toxicity by alkaline pH in S. leopoliensis.
5. Whether alleviation of arsenite toxicity by acetate is associated with decreased uptake of arsenite.
6. Whether the inhibition of growth by arsenite in O. limnetica is also due to inhibition of pyruvate dehydrogenase complex.
7. The mechanism of arsenite tolerance in O. limnetica.

LITERATURE CITED

- Adriano, D.C. (1986). Trace elements in the terrestrial environment. Springer-Verlag, N.Y.
- Alt, F.W., R.E. Kellems and R.T. Schimke. (1976). Synthesis and degradation of folate reductase in sensitive and methotrexate-resistant lines of S180 cells. J. Biol. Chem. 251: 3063-3074.
- Andrea, M.O. (1978). Distribution and speciation of arsenic in natural waters and some marine algae. Deep-Sea Res. 25: 391-402.
- Angino, E.E., L.M. Magnuson, T.C. Waugh, O.K. Galle and J. Bredfeldt. (1970). Arsenic in detergents: possible danger and pollution hazard. Science 168: 389-390.
- Bails, J.D. and R.C. Ball. (1966). Response of pond metabolism to sodium arsenite. Mich. Acad. Sci. Arts Letters, Pap. 11: 193-208.
- Ball, R.C. and F.H. Hooper. (1965). The use of ⁷⁴-tagged sodium arsenite in a study of effects of a herbicide on pond ecology. In: Isotopes in Weed Research Proceedings of a Symposium, Vienna: International Atomic Energy Agency, 1966.
- Baron, D., I. Kumick, I. Frischmuth and J. Petres. (1975). Further "in vitro" studies on the biochemistry of the inhibition of nucleic acid and protein synthesis induced by arsenic. Arch. Dermatol. Res. 253(1): 15-22.
- Barron, E.S. and T.P. Singer. (1943). Enzyme systems containing active sulfhydryl groups. The role of glutathione. Science 97(2520): 356-358.
- Barry, S.A.S. and S.C. Clark. (1978). Problems of interpreting the relationship between the amounts of lead and zinc in plants and soil on metalliferous wastes. New Phytol. 81: 773-783.
- Benckiser, G., S. Santiago, H.U. Neue, I. Watanabe and J.C.G. Ottow. (1984a). Effects of fertilization on exudation, dehydrogenase activity, iron-reducing populations and Fe²⁺ formation in the rhizosphere of rice (Oryza sativa L.) in relation to iron toxicity. Plant Soil, 79: 306-316.
- Benckiser, G., J.C.G. Ottow, I. Watanabe and S. Santiago. (1984b). The mechanism of excessive iron-uptake (iron toxicity) of wetland rice. J. Plant Nut., 7: 177-185.
- Bendall, D.S. and W.D. Bonner, Jr. (1971). Cyanide-insensitive respiration in plant mitochondria. Plant Physiol. 47: 236-245.
- Bhuvaneswaran, C., C-H. Ho and C.L. Wadkins. (1972). Inactivation of coupled respiration of mitochondria by inorganic arsenate and partial restoration by ATP. Biochem. Biophys. Res. Commun. 49(3): 690-697.
- Bishop, O.N. (1981). Statistics for biology: A practical guide for the experimental biologist. Longman Group Ltd.

- Bostock and Tyler-Smith. (1981). In" R.T. Schimke. Gene amplification in cultured animal cells. Cell, Vol. 37, 705-713. 1984.
- Bottino, N.R., R.S. Newman, E.R. Cox, R. Stockton, M. Hoban, R.A. Zongaro and K.J. Irgolic. (1978). In: B.A. Fowler (ed.). Biological and environmental effects of arsenic. Elsevier Science Publishers (1983).
- Braman, R.S. (1975). Arsenic in the environment. In: E.A. Woolson. Arsenical pesticides. ACS Symp. Ser. 7, Am. Chem. Soc. pp. 108-123.
- Braman, R.S. (1983). Environmental reaction and analysis method. In: B.A. Fowler (ed.). Biological and environmental effects of arsenic. Vol. 6. Elsevier Science Publishers. pp. 141-154.
- Braman, R.S. and C.C. Foreback. (1973). Methylated forms of arsenic in the environment. Science 182(4118): 1247-1249.
- Brooks. 1923, 1925 cited in J.L. Webb. Enzyme and Metabolic Inhibitors. Vol. II. p. 698-699. Academic Press. (1966).
- Brown, G.M. (1962). The biosynthesis of folic acid. 11. Inhibition of sulfonamides. In: T.J. Foster. Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. Microbiological reviews, 1983. p. 361-409.
- Budd, K. and S.R. Craig. (1981). Resistance to arsenate toxicity in the blue-green alga Synechococcus leopoliensis. Can. J. Bot. 59: 1518-1521.
- Budd, K., J.R. Casey and J.D. MacArthur. (1986). Arsenite toxicity and arsenite tolerance in the cyanobacterium Synechococcus leopoliensis. Can. J. Bot. 64: 2433-2440.
- Carriker, N.E., W.T. Gillespie and P.L. Brezonik. (1976). Boron and arsenic studies in Florida waters. Water Resour. Research Centre, Univ. Florida Pub. No. 34. 100 pp.
- Carman et al. (1984). In" R.T. Schimke. Gene Amplification in cultured animal cells. Cell, Vol. 37, 705-713.
- Chapman, A.C. On the presence of compounds of arsenic in marine crustaceans and shell fish. In: National Research Council. Committee on Medical and Biologic Effects of Environmental Pollutants. 1977.
- Clement, W.H. and S.D. Faust. (1981). The release of arsenic from contaminated sediments and muds. Journal of Environmental Science and Health. Part A 16: 87-122.
- Davey, E.W., M.J. Morgan and S.J. Erickson. (1973). A biological measurement of the copper complexation capacity of seawater. Limnol. Oceanogr. 18: 993-997.
- Fillipis de, L.F. and C.K. Pallaghy. (1976). The effect of sublethal concentrations of mercury and zinc on Chlorella Z. Pflanz. 78(5): 197-207.

- Dixon, M. and E.C. Webb. (1958). Enzymes. In: B.A. Fowler. Biological and environmental effects of arsenic. Elsevier Science Publishers 1983.
- Dodge, C.D. and A.J. Hiatt. (1972). Relationship of ion uptake imbalance by variations of wheat (Triticale vulgare, L.). In: H.W. Woolhouse. Toxicity and Tolerance in the Responses of Plants to Metals. Ency. of Plant Physiol. New Series Vol. 12C (1983).
- Ernst, W. (1976). Physiological and biochemical aspects of metal tolerance. In: Mansfield TA (ed.). Effects of air pollutants on plants. Soc. Exp. Biol. Semin. Ser. Vol. 1. Cambridge Univ. Press, Cambridge.
- Fassbender, H.W. (1975). Solubility and fractionation criteria for evaluating arsenic-phosphorus relationships in soils. In: B.A. Fowler (ed.). Biological and environmental effects of arsenic. Elsevier Science Publishers. 1983.
- Ferguson, J.F. and J. Gavis. (1972). A review of the arsenic cycle in natural waters. Water Research 6(NII) 1259-1274.
- Fong, K., F. Lee and R. Bockrath. (1980). Effects of sodium arsenite on single-strand DNA break formation and post-replication repair in E. coli following UV irradiation. Mutation Research. 70: 151-156.
- Foster, T.J. (1983). Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in Bacteria Microbiological Reviews, Vol. 47, No. 3 p. 361-409.
- Fowler, B.A. (ed.) (1983). Biological and environmental effects of arsenic. Elsevier Science Publishers.
- Frost, D.V. (1967a). Arsenicals in biology - retrospect and prospect. Fed. Proc. Trace elements in Nutrition 26(1): 194-208.
- Furukawa, K. and K. Tonomura. (1972). In: A.O. Summers and S. Silver. Microbial transformations of metals. Ann. Rev. Microbiol. 1978. 32: 637-72.
- Grill, E., E. Winnacker, M.H. Zenk. (1985). Phytochelatins: The Principal Heavy-Metal Complexing Peptides of Higher Plants. Science. 230: 674-6.
- Guiley, L.R., R.A. Walters, J.H. Jelt and R.A. Tobey. (1980). J. Toxicol. Environ. Health. 6: 87-105.
- Gulledge, J.H. and J.T. O'Connors. (1973). Removal of arsenic (v) from H₂O by adsorption on aluminium and ferric hydroxide. Am. Water Works Assoc. J. 65(8): 548-552.
- Hager, L.P., D.M. Geller and F. Lipmann. (1954). Flavoprotein-catalyzed pyruvate oxidation in Lactobacillus delbrueckii.
- Harrington, J.S. (1959). Contents of cystine-cysteine, glutathione, and total free sulfhydryl in arsenic-resistant and sensitive strains of the blue tick, Bosophilus decoloratus. Nature. 184: 1739-1740.
- Harrison, P.M. and R.J. Hoare. (1980). Metals in biochemistry. Chapman and

Hall, New York.

- Hekman, W.E. (1983). The response of selected planktonic freshwater algae to fluoride (M.Sc. Thesis). Queen's University.
- Higham, D.P., P.J. Sadler and M.D. Scawen. (1984). Cadmium-resistant Pseudomonas putida synthesizes Novel Cadmium Proteins. Science. Vol. 225. 1043-1045.
- Hoare, D.S., S.L. Hoare and R.B. Moore. (1967). The photoassimilation of organic compounds by autotrophic blue-green algae. J. Gen. Microbiol. 49: 351-370.
- Hodgson, E. and F.E. Guthrie. (eds.). (1980). Introduction to Biochemical Toxicology. Elsevier North Holland, Inc.
- Horns, R.C., W.J. Dower and R.T. Schimke. (1984). Gene amplification in a leukemic patient treated with methotrexate. J. Clin. Oncol. 2: 2-7.
- Huang, P.M., and W.K. Liaw. (1978). Distribution and fractionation of arsenic in selected fresh water lake sediments. Internationale Revue der Gesamten Hydrobiologie. 63: 533-543.
- Huang, P.M., and P.K. Stumpf. (1971). Fat metabolism in higher plants. XLIV. Fatty acid synthesis by a soluble fatty acid synthetase from Solanum Tuberosum. Arch. Biochem. Biophys. 143: 412-427.
- Hughes, E.O., P.R. Gorham and A. Zehnder. (1958). Toxicity of an unialgal culture of Microcystis aeruginosa. Can. J. Microbiol. 4, 225-236.
- Ihlenfeldt, M.J.A. and J. Gibson. (1977). Acetate uptake by the unicellular Cyanobacteria Synechococcus and Aphanocapsa. Arch. Microbiol. 113: 231-241.
- Isensee, A.R., P.C. Kearney, E.A. Woolson, G.E. Jones, and V.P. Williams. (1973). Distribution of alkyl arsenicals in model ecosystems. Environmental Science and Technology. 7: 841-845.
- Izaki, K., Y. Tashiro and T. Funaba. (1974). In: A.O. Summers and S. Silver. Microbial transformations of metals. Ann. Rev. Microbiol. 1978. 32: 637-72.
- Izaki, K. (1977). In: A.O. Summers and S. Silver. Microbial transformations of metals. Ann. Rev. Microbiol. 1978. 32: 637-72.
- Jackson, G.A. and J.J. Morgan. (1978). Trace metal-chelator interactions and phytoplankton growth in seawater media; theoretical analysis and comparison with other reported observations. Limnol. Oceanogr. 23: 268-282.
- Jervis, R.E., B. Tiefenbach, W. LePage and J.L. Debrun. (1970). Nuclear activation technique for heavy metal pollutants. Proc. 5th Canadian Symp. Water Pollut. Res. Rep. No. TD 420C21/5th.
- Johnson, D.L. and M.E.Q. Pilson. (1972). Arsenate in the Western North

- Atlantic and adjacent regions. J. Mar. Res. 38(1): 140-149.
- Johnstone, R.M. (1963). Sulfhydryl agents: arsenicals In: R.M. Hochsten and J.H. Quastel. pp. 99-118.
- Kamori, S. and K. Sugawara. (1965). Geochemical study of arsenic in natural waters. I. Arsenic in rain and snow. II. Arsenic in river water. J. Earth Sci. 13(1): 23-45.
- Kannangara, C.G. and P.K. Stumpf. (1972). Fat metabolism in higher plants. XLVII. The effect of nitrite and other anions on the formation of unsaturated fatty acids by isolated chloroplasts. Plant Physiology. 49: 497-501.
- Keeley, P.E. and R.J. Thullen. (1971a). Cotton response to temperature and organic arsenicals. Weed Sci. 19: 297-300.
- Keeley, P.E. and R.J. Thullen. (1971b). Control of nutsedge with organic arsenical herbicides. Weed Sci. 19: 601-606.
- Komura, I., T. Funaba and K. Izaki. (1971). In: A.O. Summers and S. Silver. Microbial Transformations of metals.
- Lancaster, R.J., M.R. Coup and J.W. Hughes. (1971). Toxicity of arsenic present in lake weed. N.Z. Vet. J. 19: 141-145.
- Lee, J., R.D. Reeves, R.R. Brooks and T. Jaffre. (1978). The relation between nickel and citric acid in some nickel-accumulating plants. Phytochemistry. 17: 1033-1035.
- Luh, M.D., R.A. Baker, and D.E. Henley. (1973). Arsenic analysis and toxicity - a review. The Science of the Total Environment. 2: 1-12.
- Lunde, G. (1973). The synthesis of fat and water soluble arsenoorganic compounds in marine and limnetic algae. Acta Chem. Scand. 27: 1586-1594.
- Lyon, G.L., P.J. Peterson and R.R. Brooks. (1969). Chromium-51 distribution in tissues and extracts of Leptospermum scoparium. Planta. 88: 282-287.
- MacArthur, J.D., G.R. Palmer, K. Budd, W.E. Kekman, B.E. Nichol and J.R. Casey. (1985). Element analysis of algae treated with F⁻ or arsenite. Nucl. Instrum. Methods. B 10/11: 653-659.
- Macfie, S.M. (1986). Wetland influence on ferric hydroxide plaque formation. M.Sc. thesis, Queen's University.
- Maclean, F.I., O.J. Lucis, Z.A. Shaikh and E.R. Jansz. (1972). The uptake and subcellular distribution of Cd and Zn in microorganisms. Fed. Proc. Fed. Am. Soc. Exp. Biol. 31: 699.
- Mathys, W. (1975). Enzymes of heavy-metal resistant and non-resistant populations of Silene cucubalus and their interaction with some heavy metals in vitro and in vivo. Physiol Plant. 33: 161-165.

- Mathys, W. (1977). The role of malate, oxalate and mustard oil glucosides in the evolution of zinc-resistance in herbage plants. *Physiol. Plant.* 40: 130-136.
- Matsumoto, H., F. Hirasawa, S. Morimura and E. Takahashi. (1976). Localization of aluminium in tea leaves. *Plant Cell Physiol.* 17: 890-895.
- Melandri, A.B., E. Fabbri and B.A. Melandri. (1975). Energy transduction in photosynthetic bacteria. VII. Activation of the energy-transducing ATPase by inorganic phosphate. *Biochem. Biophys. Acta.* 376: 82-88.
- Melera, P.W., J.A. Lewis, J.L. Biedler and C. Hession. (1980). Antifolate-resistant Chinese hamster cells. Evidence for dihydrofolate reductase gene amplification among independently derived sublines overproducing different dihydrofolate reductases. *J. Biol. Chem.* 255: 7024-7028.
- Miketukova, V., J. Kohlicek and K. Kacil. (1968). Separation of arsenite and arsenate ions by paper chromatography. Study of a methanol-ammonia-water solvent system.
- Mobley, H.L.T. and B.P. Rosen. (1982). Energetics of plasmid-mediated arsenate resistance in Escherichia coli. *Proc. Natl. Acad. Sci. U.S.A.* 79: 6119-6122.
- Moore, J.W. and S. Ramamoorthy. (1984). Heavy metals in Natural Waters. Applied Monitoring and Impact Assessment. Springer-Verlag. N.Y. Inc.
- Mushak, P. (1985). Potential impact of acid precipitation on Arsenic and Selenium. *Environmental Health Perspectives*, Vol. 63 pp. 105-113.
- National Academy of Sciences. (1977). Arsenic. Committee on medical and biological effects of environmental pollutants. National Academy of Sciences, Washington, D.C. 261 pp.
- National Research Council of Canada. (1978). Effects of arsenic in the Canadian environment. Publication No. NRCC 15391 of the Environmental Secretariat Ottawa, Canada. 349 pp.
- Nelson, D.A., A. Calabrese, B.A. Nelson, J.R. MacInnes and D.R. Wenzloff. (1976). In: B.A. Fowler (ed.). Biological and environmental effects of arsenic. Elsevier Science Publishers. 1983.
- Nichol, B.E., K. Budd, G.R. Palmer and J.D. MacArthur. (1987). The mechanism of fluoride toxicity and fluoride resistance in Synechococcus leopoliensis (Cyanophyceae).
- Nigam, S.N. and W.B. McConnell. (1976). Isolation and identification of two isomeric glutamyl selenocystathionines from the seeds of Astragalus pectinatus. *Biochem. Biophys. Acta.* 437: 116-121.
- Oehme, F.W. (1972). Mechanism of heavy metal toxicities. *Clin. Toxicol.* 5(2): 151-167.
- Olafson, R.W., K. Abel and R.G. Sim. (1979). Prokaryotic metallothionein:

- preliminary characterization of blue-green alga heavy metal-binding protein. *Biochem. Biophys. Res. Commun.* 89: 36-43.
- Pandey, K. and J.P. Shukla. (1979). National Acad. Sci. Lett. (India) 2: 425-426.
- Pelosi, P., R. Fiorentini, and C. Galoppini. (1976). *Agri. Biol. Chem.* 40: 1641-1649.
- Peterson, P.J. (1969). The Distribution of Zinc-65 in Agrostis tenuis Sibth. and A. stolonifera L. Tissues. *Journal of Experimental Botany*, Vol. 20, No. 65, pp. 863-75.
- Peterson, P.J. (1983). Adaptation to Toxic Metals. In: D.A. Robb and W.S. Pierpoint. *Metals and Micronutrients: Uptake and Utilization by plants.* Academic Press, 1983.
- Petres, J., D. Baron and M. Hagedorn. (1977). Effects of arsenic cell metabolism and cell proliferation: cytogenetic and biochemical studies. *Environmental Health Perspectives.* 19: 223-227.
- Pratt, D.R., J.S. Bradshaw and B. West. (1972). Arsenic and selenium analyses in fish. *Utah Acad. Proc. Part 1.* 49: 23-26.
- Pushnik, J.C., G.W. Miller and J.H. Manwaring. (1984). The role of iron in higher plant chlorophyll biosynthesis, maintenance and chloroplast biogenesis. *J. Pl. Nutr.* 7(15): 733-758.
- Rand, G.M. and S.R. Petrocelli (eds). (1985). *Fundamentals of Aquatic Toxicology.* Hemisphere Publishing Corporation.
- Rausser, W.E. and N.R. Curvetto. (1980). Metallothionein occurs in roots of Agrostis. *Nature (Lond.).* 287: 563-564.
- Reay, P.F. (1972). The accumulation of arsenic from arsenic-rich natural waters by aquatic plants. *J. Appl. Ecol.* 9(2): 557-565.
- Ridley, W.P., L. Dizikes, A. Cheh and J.M. Woods. (1977). *Environ. Health Perspect.* 19: 43-46.
- Rosen, B.P. and M.G. Borbolla. (1984). A plasmid-encoded arsenite pump produces arsenite resistance in Escherichia coli. *Biochem. Biophys. Res. Commun.* 124: 760-765.
- Sanders, J.G. (1979). Effects of arsenic speciation and phosphate concentration on arsenic inhibition of Skeletonema costatum (Bacillariophyceae). *Phycol.* 15: 424-428.
- Sandoval, F., A. Gomez-Puyou, M. Tuena, E. Chavez and A. Pena. (1970). Effect of sodium and potassium ions on mitochondrial oxidative phosphorylation. Studies with arsenate. *Biochem.* 9(3): 684-689.
- Scarborough, G.A. (1975). The mechanism of arsenate inhibition of the glucose active transport system in Neurospora crassa. *Arch. Biochem. Biophys.* 166(1): 245-250.

- Schimke, R.T. (1984). Gene amplification in cultured animal cells. *Cell*. Vol. 37. 705-713.
- Schottel, J.L. (1978). The mercuric and organomercurial detoxifying enzymes from a plasmid-bearing strain of Escherichia coli. *J. Biol. Chem.* 253: 4341-49.
- Silver, S., K. Budd, K.M. Leahy, W.V. Shaw, D. Hammond, R.P. Novick, G.R. Willsky, M.H. Malamy and H. Rosenberg. (1981). Inducible plasmid-determined resistance to arsenate, arsenite, and Antimony (III) in Escherichia coli and Staphylococcus aureus.
- Silverberg, B.A. (1975). Ultrastructural localization of lead in Stigeoclonium tenue (Chlorophyceae, Ulothrichales) as demonstrated by cytochemical and x-ray microanalysis. *Phycologia*. 14: 265-274.
- Smith, B.N. (1984). Iron in higher plants: storage and metabolic role. *J. Pl. Nutr.* 7(1-5): 759-766.
- Silver, S. and D. Keach. (1982). Energy-dependent arsenate efflux: The mechanism of plasmid-mediated resistance. *Proc. Natl. Acad. Sci.* Vol. 79 pp. 6114-6118.
- Sivasubramanian, S., O. Talibudeen. (1972). Effects of aluminium on the growth of tea (Camellia sinensis) and its uptake of potassium and phosphorus. *Tea* 43: 4-13.
- Sorensen, E.M.B. (1976). Toxicity and accumulation of arsenic in green sunfish, Lepomis cyanellus, exposed to arsenate in water. *Bulletin of Environmental Contamination and Toxicology*. 15: 756-761.
- Spehar, R.L., J.T. Fiandt, R.L. Anderson, and D.L. DeFoe. (1980). Comparative toxicity of arsenic compounds and their accumulation in invertebrates and fish. *Archives of Environmental Contamination and Toxicology*. 9: 53-63.
- Squibb, K.S. and B.A. Fowler. (1983). The toxicity of arsenic and its compounds. In: B.A. Fowler (ed.). *Biological and environmental effects of arsenic*. Elsevier Science Publishers.
- Stokes, P.M., T. Maler and J.R. Riordan. (1977). A low molecular weight copper binding protein in a copper tolerant strain of Scenedesmus acutiformis. In: Hemphill, D.D. (ed.). *Trace substances in environmental health XI*. University of Missouri, Columbia.
- Summers, A.O. and S. Silver. (1978). Microbial transformations of metals. *Ann. Rev. Microbiol.* 32: 637-72.
- Summers, A.O. and L.I. Sugarman. (1974). In: A.O. Summers and S. Silver. *Microbial transformations of metals*. *Ann. Rev. Microbiol.* 32: 637-72.
- Svedberg, G. and O. Skold. (1980). Characterization of different plasmid-borne dihydropteroate synthases mediating bacterial resistance to sulfonamides. *J. Bacteriol.* 142: 1-7.

- Swallow, K.C., J.C. Westall, D.M. Knight, N.M.L. Morel and F.M.N. Morel. Potentiometric determination of copper complexation by phytoplankton exudates. *Limno. Oceanogr.* 23: 538-542.
- Takeuchi, T., E. Weinbach and L. Diamond. (1975). Pyruvate oxidase (CoA accelylating) in Entamoeba histolytica. *Biochem. Biophys. Res. Commun.* 65: 591-6.
- Taylor, G.J. (1983). Copper and nickel tolerance in clones of Typha latifolia L. from contaminated wetlands near Sudbury, Ontario. PhD Thesis, Dept. of Biology, Queen's University, Kingston, Ontario.
- Trent, J.M., R.N. Buick, S. Olson, R.C. Horns and R.T. Schimke. (1984). Cytologic evidence for gene amplification in methotrexate resistant cells obtained from a patient with ovarian adenocarcinoma. *J. Clin. Oncol.* 2: 8-15.
- Trolldenier, G. (1973). Secondary effects of potassium and nitrogen nutrition of rice: change in microbial activity and iron reduction in the rhizosphere. *Plant Soil.* 38: 267-279.
- Turner, R.G. (1970). The subcellular distribution of zinc and copper within the roots of metal-tolerant clones of Agrostis tenuis. *New Phytologist.* 69: 729-731.
- Turner, R.G. and C. Marshall. (1971). The accumulation of ^{65}Zn by root homogenates of zinc-tolerant and non-tolerant clones of Agrostis tenuis. *New Phytol.* 70: 539-545.
- Turner, R.G. and C. Marshall. (1972). The accumulation of zinc by subcellular fractions of roots of Agrostis tenuis in relation to zinc tolerance. *New Phytol.* 71: 671-676.
- Vaughan, D., D.J. Linchan. (1976). The growth of wheat in humic acid solutions under axenic conditions. *Plant Soil.* 44: 448-449.
- Wainwright, S.J. (1975). Some aspects of the mechanism of heavy metal tolerance in Agrostis tenuis. PhD thesis, Leeds University.
- Wainwright, S.J. and H.W. Woolhouse. (1975). Physiological mechanisms of heavy metal tolerance in plants. In: Chadwick M.J. and Goodman G.T. (eds.). *The ecology of resource degradation and renewal.* 15th Symp. Br. Ecol. Soc. Blackwell, Oxford.
- Waldbott, G.L. (1973). Health effects of environmental pollutants. The C.V. Mosby Co., Saint Louis. 316 pp.
- Walsh, P.R., R.A. Duce and J.L. Fasching. (1979). In: D.C. Adriano. Trace elements in terrestrial environment. Springer-Verlag, (1983) N.Y.
- Wauchope, R.D. (1975). In: D.C. Adriano Trace elements in the terrestrial environment. Springer-Verlag (1983) N.Y.
- Webb, J.L. (1966). Enzyme and metabolic inhibitors. Vol. III. Academic Press, N.Y./London.

- Weiss, A.A., S. Silver and T.G. Kinscherf. (1978). Cation transport alteration associated with plasmid-determined resistance to cadmium in Staphylococcus aureus. Antimicrob. Agents Chemother. 14: 856-865.
- Westley. (1969). cited in H.W. Woolhouse. Toxicity and tolerance in the responses of plants to metals. Encyclopedia of Plant Physiology, New Series, 12C 1983, pp. 245-300.
- Wickes, W.A. and J.T. Wiskich. (1976). Arsenate uncoupling of oxidative phosphorylation in isolated plant mitochondria. Aust. J. Plant Physio. 3: 153-162.
- Williams, F.R. and L.P. Hager. (1966). Crystalline flavin pyruvate oxidase from Escherichia coli. i. Isolation and properties of the flavoprotein. Arch. Biochem. Biophys. 116: 168-176.
- Willsky, G.R. and M.H. Malamy. (1976). Control of the synthesis of alkaline phosphatase and the phosphate-binding protein in Escherichia coli. J. Bacteriol. 127: 595-609.
- Willsky, G.R. and M.H. Malamy. (1980). Effect of arsenate on inorganic phosphate transport in Escherichia coli. J. Bacteriol. 144: 366-374.
- Wilson, S.H. and M. Fieldes. (1941). Studies in spectrographic analysis. II. Minor elements in a sea-weed (Macrocystis pyrifera). N.Z.J. Sci. Tech. B. Gen. Sect. 23: 47B-48B.
- Wood, J.M. (1974). Biological cycles for toxic elements in the environment. Science. 183: 1049-1052.
- Woolhouse, H.W. (1983). Toxicity and tolerance in the responses of plants to metals. In: Lange, O.L., P.S. Nobel, C.B. Osmond, H. Ziegler (eds.). Encyclopedia of Plant Physiology, New Series, Vol. 12C. Physiological Plant Ecology III. pp. 245-300.
- Woolson, E.A. (1975). Bioaccumulation of arsenicals. In: E.A. Woolson. Arsenical pesticides. ACS Symp. Ser. 7: pp. 97-107.
- Woolson, E.A. (1983). Emissions, cycling and effects of arsenic in soil ecosystems. In: B.A. Fowler (ed.). Biological and Environmental Effects of Arsenic, Vol. 6, Elsevier Science Publishers, pp. 51-139.

PIPER.

Composition of Daley and Brown's modification of Hughes,

Gorham and Zehnder's no. 11 growth medium.

[K⁺] final

(9)

4.5 M KOH

(ml)

Compound	Final amount	Nutrient	Final concentration
	[mg.l ⁻¹]		[μM]
NaNO ₃	248.0	Na ⁺	3172
MgSO ₄ ·7H ₂ O	37.5	Mg ²⁺	152
CaCl ₂ ·2H ₂ O	18.0	Ca ²⁺	122
Na ₂ CO ₃	10.0	K ⁺	224
K ₂ HPO ₄	19.5	Fe ²⁺	41
Na ₂ EDTA	15.5	NO ₃ ⁻	2900
FeSO ₄ ·7H ₂ O	11.5	SO ₄ ²⁻	193
H ₃ BO ₃	0.1240	Cl ⁻	244
MnSO ₄ ·4H ₂ O	0.0892	CO ₃ ⁻	94
ZnSO ₄ ·7H ₂ O	0.0115	PO ₄ ³⁻	112
(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	0.0035	B ³⁺	2
Co(NO ₃) ₂ ·4H ₂ O	0.0058	Mn ²⁺	0.4
Na ₂ WO ₄ ·2H ₂ O	0.0013	Zn ²⁺	0.04
CuSO ₄ ·5H ₂ O	0.0050	Mo ⁶⁺	0.02
KBr	0.0048	Co ²⁺	0.02
KI	0.0033	W ⁶⁺	0.004
Cd(NO ₃) ₂	0.0062	Cu ²⁺	0.02

(from Hekman, 1983)

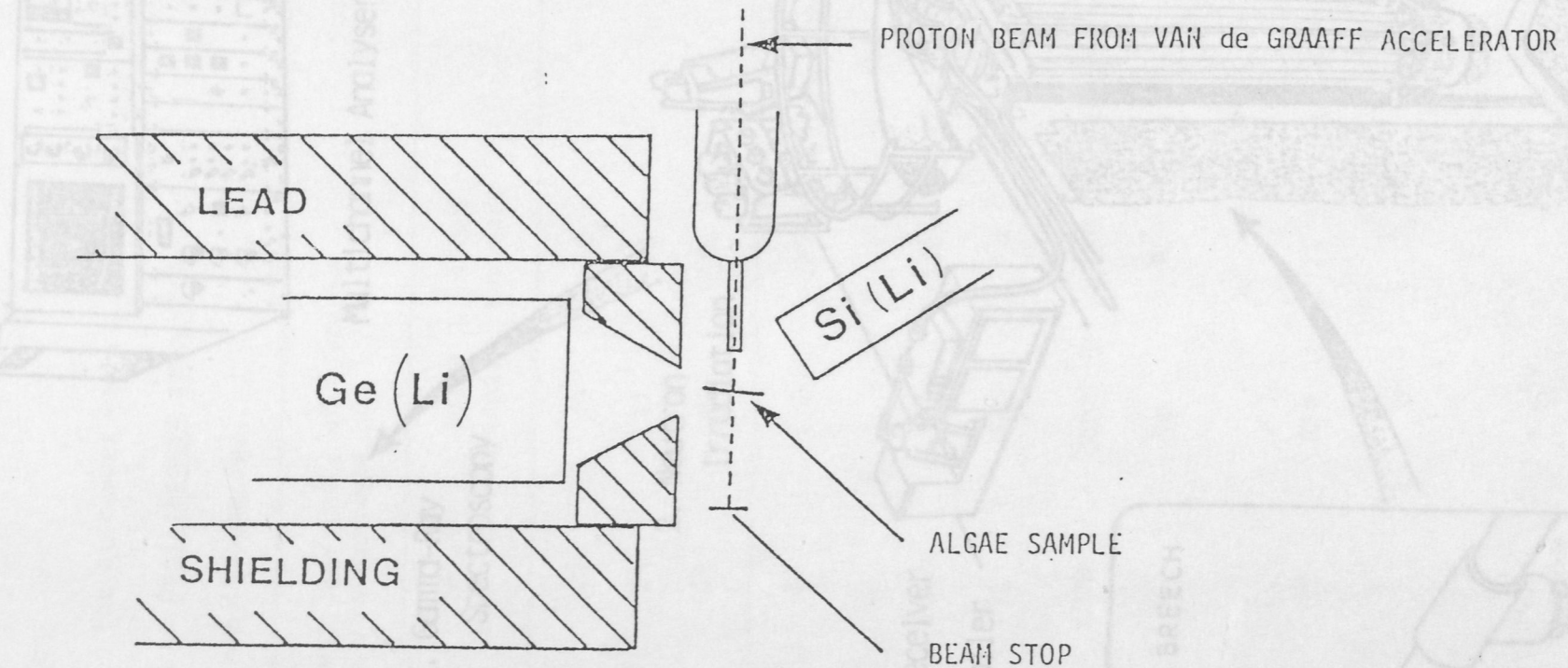
Appendix 1b

Adjustment of pH's of 500ml half strength media containing 20mm PIPES.

PIPES (9)	Volume of 4.5 M KOH (ml)	pH	[K ⁺] final mM
3	3.2	6.2	28.8
3	3.8	6.7	34.2
3	4.5	7.2	40.4
3	5.0	8.0	45.0

Appendix 2

The physical set-up of sample and detectors in
FIGME/PIXE analysis.



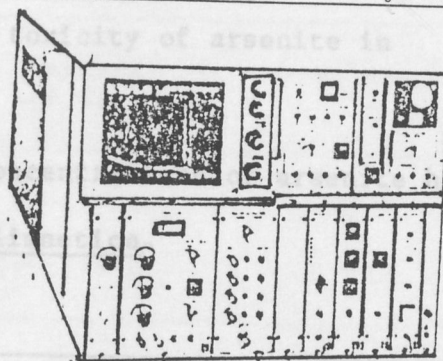
Si (Li) DETECTOR OBSERVES X-RAYS FROM SAMPLE
Ge (Li) DETECTOR OBSERVES GAMMA RAYS FROM SAMPLE

(Hekman, 1983)

Appendix 3 Set up of equipment for Neutron Activation Analysis



Ge Detector



Multichannel Analyser

3. Gamma-Ray Spectroscopy

2. Neutron Irradiation

Sample Receiver
Irradiation Controller

LOADING BREECH

1. Sampling

SLOWPOKE-2

Appendix 4 Analysis of variance

Analysis of variance for data on toxicity of arsenite in Oscillatoria limnetica.

a). Null hypothesis: The different concentrations of arsenite have no effect on the growth of Oscillatoria limnetica.

b). Calculation of sum of squares.

Replicate No.	Growth lags (hrs) of cultures grown in presence of:				Totals
	Control (-As)	200 μ M arsenite	300 μ M arsenite	400 μ M arsenite	
1	1	25	30	70	
2	1	24	47	75	
3	1	29	49	76	
Totals	3	78	126	221	Grand total
Sum of squares of observations	3	2042	5510	16301	23856 = A
Squares of column totals	3	2028	5292	16280	23603 = B
divided by No. of observations	b_1	b_2	b_3	b_4	
Differences (a-b) (or Residual SOS)	0	14	218	21	253 = A - B

Checking

a) $D = 428^2/12 = 15265$

- * Total SOS = $A - D = 23856 - 15265 = 8591$
- Between treatments SOS = $B - D = 23603 - 15265 = 8338$
- Residual SOS = $A - B = 23856 - 23603 = 253$
- * Total SOS = $A - D = 23856 - 15265 = 8591$

c) Analysis of variance

Source of variance	Sum of squares	Degrees of freedom	Mean square
Between treatments (columns)	B-D = 8338	u-1 = 3	2779
Residual	A-B = 253	u(v-1) = 8	32
Total	A-D = 8591	uv-1 = 11	

u = no. of treatments

v = no. of replicates

Mean square = $\frac{\text{sum of squares}}{\text{degrees of freedom}}$

Variance ratios (F)

F = $\frac{\text{between treatment mean square}}{\text{residual mean square}}$

$$= \frac{2779}{32}$$

$$= 86.8$$

From Table 78 (Bishop O.N 1980 p. 196)

When $n_1 = 3$ (degrees of freedom of between - treatments mean square).

$n_2 = 8$ (degrees of freedom of residual) and $p = 0.001$.
F = 15.8.

The calculated F exceeds the tabulated F. Therefore the variance due to effects of treatments is significantly greater ($p = .0001$) than the residual variance due to random error. Therefore reject null hypothesis. Arsenite has an effect on the growth of Oscillatoria limnetica.

Checking

$$a) D = 261^2/9 = 7569$$

$$* \text{ Total SOS} = A-D = 10944.5 - 7569 = 3375.5$$

$$\text{Between treatments SOS} = B - D = 10793.6 - 7569 = 3224.6$$

$$\text{Residual SOS} = A - B = 10944.5 - 10793.6 = 150.9$$

$$* \text{ Total SOS} = A - D = 10944.5 - 7569 = 3375.5$$

Appendix 5

Analysis of variance for data on toxicity of arsenite in *Chlorella pyrenoidosa*.

a) Null hypothesis: The different concentrations of arsenite have no effect on the growth of *C. pyrenoidosa*.

b) Calculation of sum of squares.

Replicate No.	Generation times (hrs) of cultures grown in presence of:			Totals
	Control (-As)	1mM arsenite	2mM arsenite	
1	12.0	19.0	49.0	
2	11.5	19.0	65.0	
3	11.5	22.0	52.0	
Totals	35.0	60.0	166.0	Grand total 261
Sum of squares of observations	408.5	1206	9330	10944.5 = A
Squares of column totals divided by No. of observations	408.3	1200	9185.3	10793.6 = B
Differences (a-b) (or Residual SOS)	0.2	6	144.7	150.9 = A-B

Checking

a) $D = 261^2 / 9 = 7569$

* Total SOS = $A - D = 10944.5 - 7569 = 3375.5$

Between treatments SOS = $B - D = 10793.6 - 7569 = 3224.6$

Residual SOS = $A - B = 10944.5 - 10793.6 = 150.9$

* Total SOS = $A - D = 10944.5 - 7569 = 3375.5$

c) Analysis of variance

Analysis of variance for data from Table

Source of variance	Sum of squares	Degrees of freedom	Mean square
Between treatments (columns)	B-D = 3224.6	u-1 = 2	1612.3
Residual	A-B = 150.9	u(v-1) = 6	25.2
Total	A-D = 3375.5	uv-1 = 8	

u = no. of treatments

v = no. of replicates

Mean square = $\frac{\text{sum of squares}}{\text{degrees of freedom}}$

Variance ratios (F)

F = $\frac{\text{between treatment mean square}}{\text{residual mean squares}}$

= $\frac{1612.3}{25.2}$

= 64.0

From Table 78 (Bishop O.N 1980 p. 196)

When $n_1 = 2$ (degrees of freedom of between - treatments mean square).

$n_2 = 6$ (degrees of freedom of residual) and $p = 0.001$.
F = 27

The calculated F exceeds the tabulated F. Therefore the variance due to effects of treatments is significantly greater ($p = 0.001$) than the residual variance due to random error. Therefore, reject null hypothesis. Arsenite at 1mM and 2mM has a significant effect on growth of C. pyrenoidosa when compared to controls.

Appendix 6

Analysis of variance for data from Table .

a) Calculation of Sum of Squares

Replicate No.	Growth lags in normal medium with addition of: (hrs)				Totals
	Control (-As)	1mM $KC_2H_3O_2$ + 200 μ M As	1mM KCl + 200 μ M As	+200 μ M As	
1	0	10.5	16	22	
2	0.5	13	17	20	
3	0.5	11	20	19	

1	0	10.5	16	22	
2	0.5	13	17	20	
3	0.5	11	20	19	

Totals	1.0	34.5	53	61	Grand total 149.5
Sum of squares of observations	0.5	400.25	945	1245	2590.75 = A
		a_2	a_3	a_4	

Squares of column totals	0.33	396.75	936.33	1240.33	
divided by	b_1	b_2	b_3	b_4	2573.74 = B
No. of observations					

Differences (a-b)	0.17	3.5	8.67	4.67	17.01=A-B (or Residual SOS)
-------------------	------	-----	------	------	-----------------------------

Checking

$$D = 149.5^2/12 = 1862.52$$

$$* \text{ Total SOS} = A - D = 2590.75 - 1862.52 = 728.23$$

$$\text{Between treatments SOS} = B - D = 2573.74 - 1862.52 = 711.22$$

$$\text{Residual SOS} = A - B = 2590.75 - 2573.74 = 17.01$$

$$* \text{ Total SOS} = A - D = 728.23$$

b) Analysis of variance

Source of variance	Sum of squares	Degrees of freedom	Mean square
Between treatments (columns)	B-D = 711.22	u-1 = 3	237.07
Residual	A-B = 17.01	u(v-1) = 8	2.12
Total	A-D = 728.23	uv-1 = 11	

u = no. of treatments

v = no. of replicates

Mean square = $\frac{\text{sum of squares}}{\text{degrees of freedom}}$

c) Variance ratios (F)

$F = \frac{\text{between treatment mean square}}{\text{residual mean squares}}$

$$= \frac{237.07}{2.12}$$

$$= 111.82$$

From Table 78 (Bishop O.N 1980 p. 196)

When $n_1 = 3$ (degrees of freedom of between - treatments mean square).

$n_2 = 8$ (degrees of freedom of residual) and $p = 0.001$.
F = 15.8

The calculated F exceeds the tabulated F. Therefore, the variance due to the effects of treatments is significantly greater ($p = 0.001$) than the residual variance due to random error. Therefore, reject the Null hypothesis. Acetate significantly reduces arsenite toxicity in S. leopoliensis.

Appendix 7a

t-test to find out whether the effect of KCl was significant in the growth of S. Leopoliensis under 200µM arsenite.

- 1) Null hypothesis: There is no effect of extra KCl on the growth of S. leopoliensis under 200µM arsenite compared to that without KCl in presence of the same concentration of arsenite.

1mM KCl	+200µM As
+200uM As	

- 2) Mean growth lags.

$$\bar{x}_1 = 17.67 \pm 1.20 \quad \bar{x}_2 = 20.33 \pm 0.88 \quad (\bar{x} \pm SE, n=3)$$

$$\sigma_2 = 2.08 \quad \sigma = 1.53$$

$$\sigma^2 = 4.33 \quad \sigma^2 = 2.33$$

$$n_1 = 3 \quad n_2 = 3$$

- 3) Difference between means 2.66

$$4) \quad \sigma_d^2 = \frac{4.33}{3} + \frac{2.33}{3} = 1.44 + 0.78$$

$$3 \quad 3 = 2.22$$

$$\sigma_d = 1.49$$

$$5) \quad t = \frac{2.66}{1.49} = 1.78$$

- 6) From table 74 (Bishop, O.N. 1980 p. 192)

For 4 degrees of freedom ($n_1 + n_2 - 2$)

and $p = 0.05$

$$t = 2.78$$

According to null hypothesis, the difference of means should not be greater than $2.78 \times 1.49 = 4.14$. The actual value, 2.66 is less than the expected value of 4.14.

Therefore, the null hypothesis may be accepted. There is no significant difference between the KCl controls and those without KCl.

Therefore, the null hypothesis must be rejected.

ix) - The generation times of tolerant and recovered cells grown in presence of 300µM arsenite are significantly different at 5% level. The recovered cells have a longer generation time than the tolerant cells.

Appendix 7b

t-test to find out whether the generation times of tolerant and recovered cells of S. leopoliensis in 300µM arsenite are significantly different.

i) Null hypothesis:

The generation times of tolerant and recovered cells grown in presence of 300µM arsenite are not different.

ii) Mean generation times
Tolerant cells

$$\bar{x} = 15.33 \pm 0.33 \text{ (+SE)}$$

$$\sigma^2 = 0.33$$

Recovered cells

$$\bar{x} = 18 \pm 0 \text{ (+SE, n= 3)}$$

$$\sigma^2 = 0$$

$$n_1 = 3$$

$$n_2 = 3$$

iii) Difference of means 2.67

iv) Variance for the difference of means (σ_d^2)

$$\sigma_d^2 = \frac{0.33}{3} + \frac{0}{3}$$

$$= 0.11$$

$$\sigma_d = 0.33$$

$$v) \quad t = \frac{2.67}{0.33} = 8.09$$

vi) In Table 74 (Bishop, O.N. 1981, p. 192)

$$\text{When } p = 0.05, (n_1 + n_2 - 1) = 5 \quad t = 2.57$$

vii) According to null hypothesis, the difference of means should not be greater than $2.57 \times 0.33 = 0.84$

viii) The actual value of 2.67 is greater than the expected value of 0.84.

Therefore, the null hypothesis must be rejected.

ix) The generation times of tolerant and recovered cells grown in presence of 300µM arsenite are significantly different at 5% level. The recovered cells have a longer generation time than the tolerant cells.

Appendix 7c

t-test to find out whether the generation times of tolerant and recovered cells of S. leopoliensis in 400uM arsenite are significantly different.

i) Null hypothesis

The generation of tolerant and recovered cells grown in presence of 400uM arsenite are not different

ii) Mean generation times

Tolerant cells

$$\bar{x} = 26 + 1.00 (+SE, n = 2)$$

$$s^2 = 2.00$$

Recovered cells

$$\bar{x} = 32 + 1.00 (+SE, n = 2)$$

$$s^2 = 2.00$$

iii) Difference of means 6.00

iv) Variance of the difference of means (s_d^2)

$$s_d^2 = \frac{2.00}{2} + \frac{2.00}{2}$$

$$= 1.00 + 1.00$$

$$= 2.00$$

$$s_d = 1.41$$

$$v) \quad t = \frac{6.00}{1.41} = 4.26$$

vi) In Table 74 (Bishop, O.N. 1981, p. 192)

When $p = 0.05$, $(n_1 + n_2 - 1) = 3$, $t = 3.18$

vii) According to null hypothesis, the difference of means should not be greater than $3.18 \times 1.41 = 4.48$

viii) The actual value of 6.00 is greater than the expected value of 4.48. Therefore, the null hypothesis must be rejected.

ix) The generation times of tolerant and recovered cells grown in presence of 400uM arsenite are significantly different at the 5% level of confidence. The recovered cells have a longer generation time than the tolerant cells.

Appendix 7d

t-test to find out whether the generation times of tolerant and recovered cells of S. leopoliensis in 500µM arsenite are significantly different.

(Analysis according to Bishop, O.N. 1981, p. 48)

i) Null hypothesis

The generation times of tolerant and recovered cells grown in presence of 500µM arsenite are not different.

ii) Mean generation times

Tolerant cells

Recovered cells

$$\bar{x} = 32.33 \pm 1.26 \text{ (+SD, } n = 3)$$

$$\bar{x} = 25.16 \pm 1.61 \text{ (+SD, } n = 3)$$

$$\sigma^2 = 1.58$$

$$\sigma^2 = 2.59$$

$$n_1 = 3$$

$$n_2 = 3$$

iii) Difference of means 7.17

iv) Variance of the difference of means (σ_d^2)

$$\sigma_d^2 = \frac{1.58}{3} + \frac{2.59}{3} = 0.5266 + 0.8633 = 1.389$$

$$\sigma_d = 1.180$$

$$v) \quad t = \frac{7.17}{1.18} = 6.08$$

vi) In Table 74 (Bishop, O.N. 1981, p. 192)
When $p = 0.05$, $(n_1 + n_2 - 1) = 5$ $t = 2.57$

vii) According to null hypothesis, the difference of means should not be greater than $2.57 \times 1.18 = 3.03$

viii) The actual value of 7.17 is greater than the expected value of 3.03. Therefore, the null hypothesis must be rejected. The GT's of tolerant and recovered cultures in 500µM arsenite are significantly different at 5% level.

ix) Recovered cells have a significantly shorter generation time than tolerant cells when both are transferred to a medium containing 500µM.

Appendix 8

Student t-test to find out whether the difference in $^{35}\text{SO}_4^{2-}$ uptake between normal and tolerant cells is significant

(Analysis according to Bishop, O.N. 1981, p. 48)

a) Null Hypothesis

There is no difference in $^{35}\text{SO}_4^{2-}$ uptake between normal and tolerant cells of *S. leopoliensis*.

b) Mean values (+ SE, n = 3)

Normal cells	$(1.599 \times 10^{-2}) \pm 0.01 \mu\text{mol } ^{35}\text{S mg}^{-1} \text{ Chla}$
Tolerant cells	$(11.79 \times 10^{-2}) \pm 1.36 \mu\text{mol } ^{35}\text{S mg}^{-1} \text{ Chla}$

c) Difference between the means 4.2×10^{-2}

d) Variance of the difference of means (σ_d^2)

$$\sigma_d^2 = \frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}$$

σ_1^2 = variance of mean value of ^{35}S in normal cells

σ_2^2 = variance of mean value of ^{35}S in tolerant cells

$$\sigma_1^2 = \frac{\sigma^2}{n_1} \text{ and } \sigma_2^2 = \frac{\sigma^2}{n_2}$$

$$\therefore \sigma_d^2 = 21.1918 \times 10^{-5}$$

$$\sigma_d = 0.01456$$

σ_d = standard deviation of the difference of means.

e) t = deviation of the difference of means, from zero

standard deviation of the difference of means

$$= \frac{0.042}{0.0146}$$

$$= 2.88$$

f) In Table 74 (Bishop, O.N. 1981, p. 192)

For 5 degree of freedom i.e. $(n_1 + n_2) - 1$ and
 $p = 0.05$, $t = 2.57$

According to null hypothesis, the difference of means should not be greater than

$$2.57 \times 0.0146 \\ = 0.037$$

The actual value, 0.042 is greater than the expected value of 0.0374.

Therefore, the null hypothesis must be rejected. There is a significant difference between the uptake of $^{35}\text{SO}_4^{2-}$ by normal and tolerant cells.

The rest of the observed t values compared to tabulated (expected) values of $t = 2.57$ $p = 0.05$ and 5 degrees of freedom.

Data from Table 14.

Observed t values

Cell fraction	Cultures harvested after (hr)	Experiment 1	Experiment 2
Ethanol extract	2	5.84	0.32
	4	2.88	0.19
NaOH/HCl	2	1.14	0.62
	4	1.15	1.26

Data from Table 15.

Observed t values

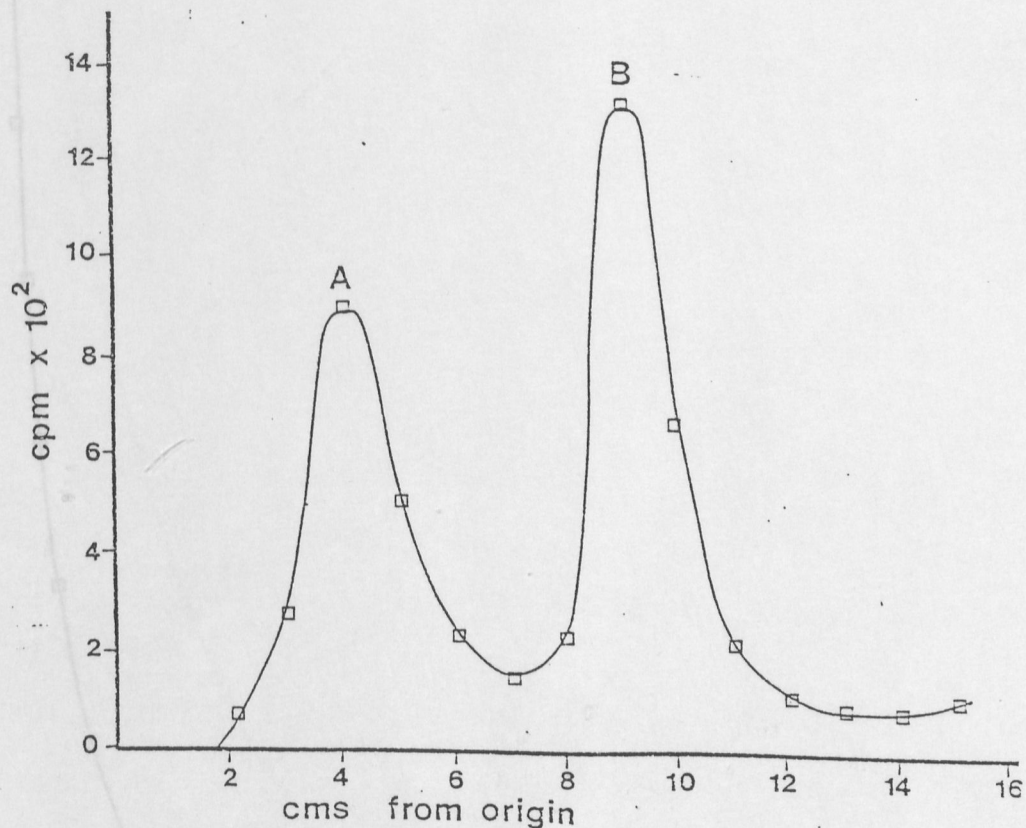
Cell fraction	Experiment 1	Experiment 2
Ethanol extract	0.15	4.76
a. Petroleum ether	1.27	1.15
b. $\text{CH}_3\text{OH}/\text{CHCl}_3$	1.00	2.08
c. Water	0.00	2.47
NaOH/HCl	0.48	1.72

Note: Standard mixture was composed of 10 ug arsenite and 10 ug arsenate.

Peak A arsenate Rf value 0.26

Peak B arsenite Rf value 0.80

Appendix 9 Position of arsenite and arsenate peaks after separation
of of a mixture of both by paper chromatography.

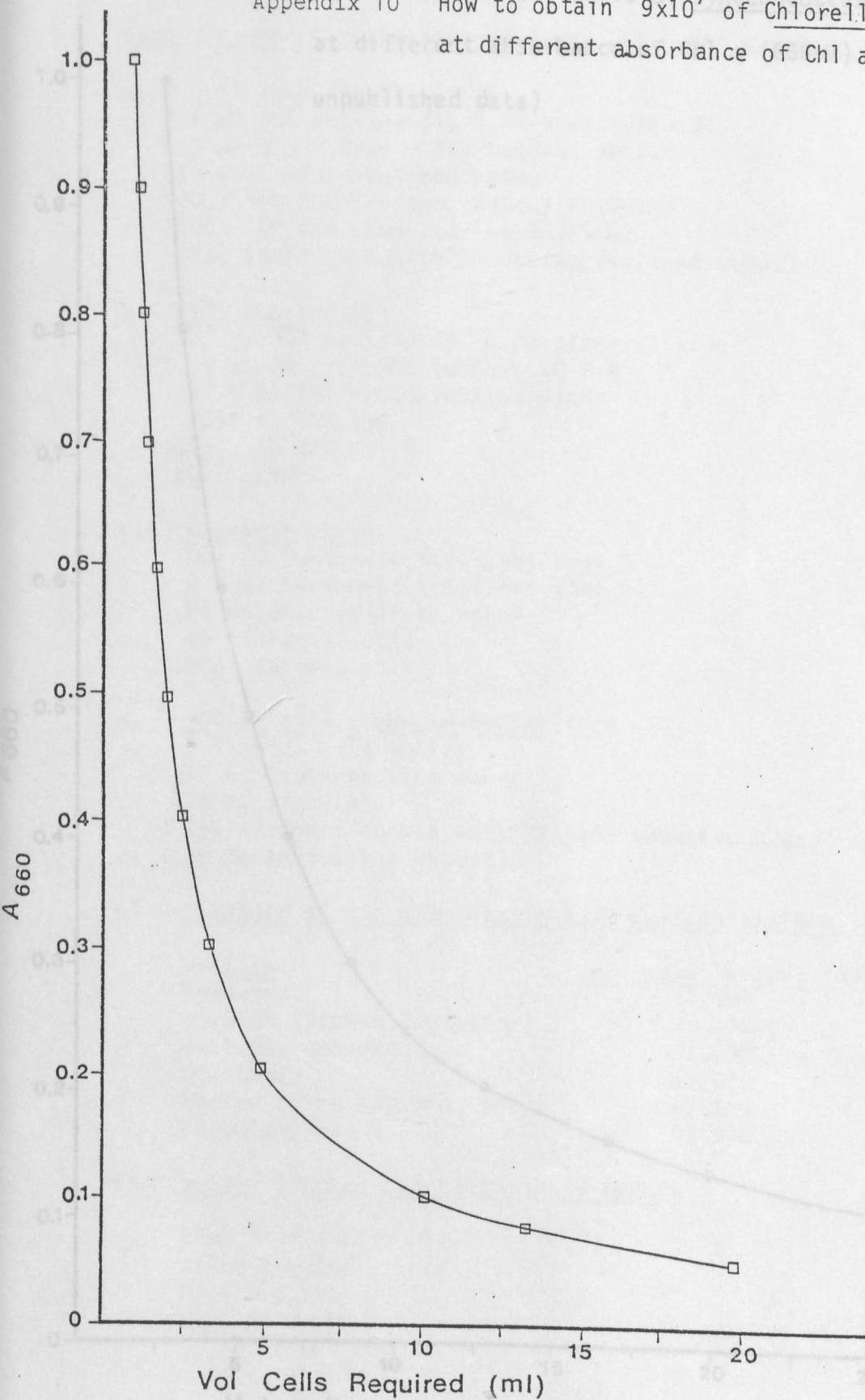


Note: Standard mixture was composed of 10 ug arsenite
and 10 ug arsenate.

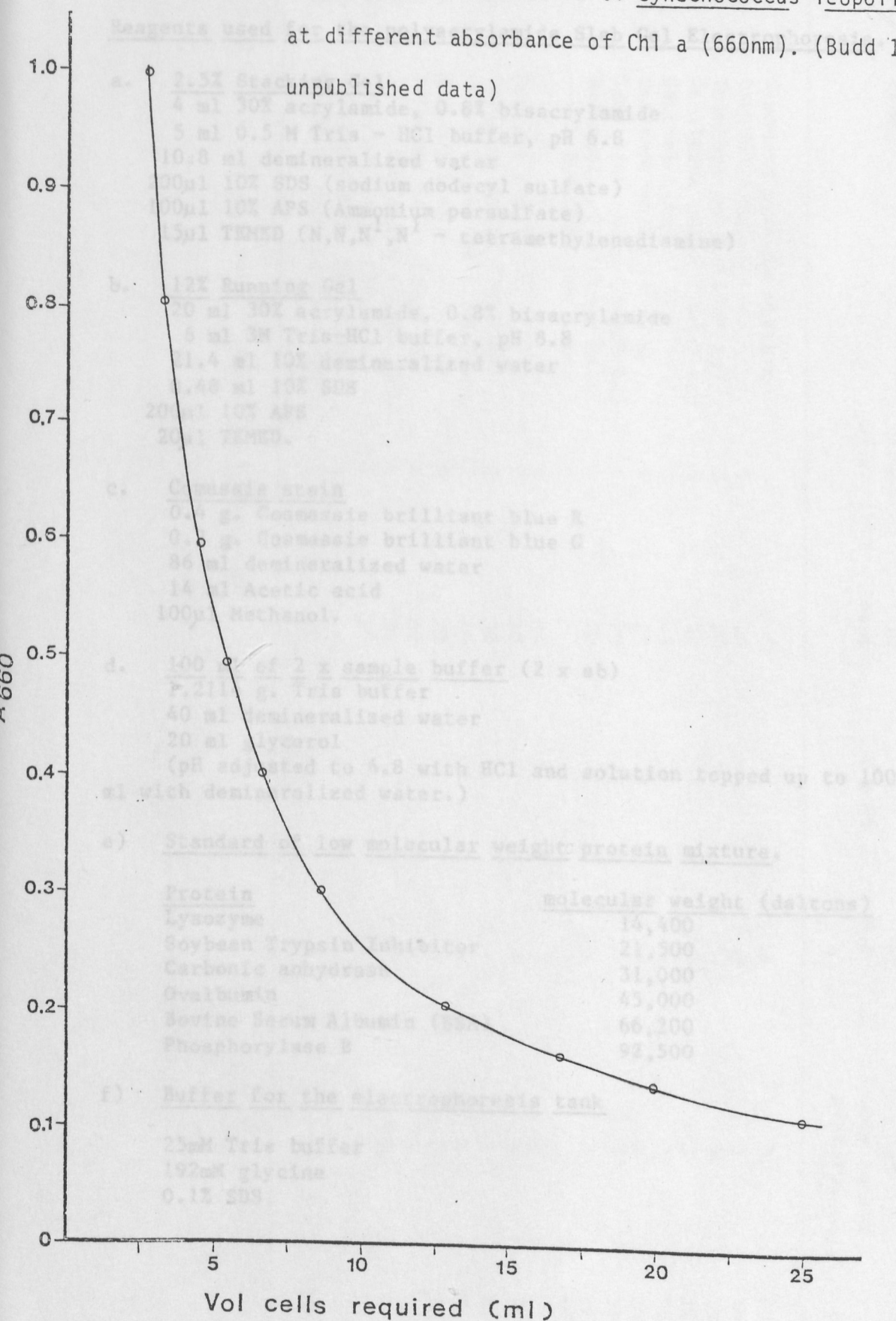
Peak A arsenate Rf value 0.26

Peak B arsenite Rf value 0.60

Appendix 10 How to obtain 9×10^7 of Chlorella pyrenoidosa
at different absorbance of Chl a (660nm).



Appendix 11 How to obtain 10^9 cells of Synechococcus leopoliensis at different absorbance of Chl a (660nm). (Budd 1984, unpublished data)



Appendix 12

Reagents used for the polyacrylamide Slab Gel Electrophoresis.

- a. 2.5% Stacking Gel
 - 4 ml 30% acrylamide, 0.8% bisacrylamide
 - 5 ml 0.5 M Tris - HCl buffer, pH 6.8
 - 10.8 ml demineralized water
 - 200µl 10% SDS (sodium dodecyl sulfate)
 - 100µl 10% APS (Ammonium persulfate)
 - 15µl TEMED (N,N,N',N' - tetramethylenediamine)
- b. 12% Running Gel
 - 20 ml 30% acrylamide, 0.8% bisacrylamide
 - 6 ml 3M Tris-HCl buffer, pH 8.8
 - 21.4 ml 10% demineralized water
 - 0.48 ml 10% SDS
 - 200µl 10% APS
 - 20µl TEMED.
- c. Comassie stain
 - 0.4 g. Cosmassie brilliant blue R
 - 0.2 g. Cosmassie brilliant blue G
 - 86 ml demineralized water
 - 14 ml Acetic acid
 - 100µl Methanol.
- d. 100 ml of 2 x sample buffer (2 x sb)
 - 1.2114 g. Tris buffer
 - 40 ml demineralized water
 - 20 ml glycerol
 - (pH adjusted to 6.8 with HCl and solution topped up to 100 ml with demineralized water.)
- e) Standard of low molecular weight protein mixture.

<u>Protein</u>	<u>molecular weight (daltons)</u>
Lysozyme	14,400
Soybean Trypsin Inhibitor	21,500
Carbonic anhydrase	31,000
Ovalbumin	45,000
Bovine Serum Albumin (BSA)	66,200
Phosphorylase B	92,500
- f) Buffer for the electrophoresis tank
 - 25mM Tris buffer
 - 192mM glycine
 - 0.1% SDS

Volume of cell extracts and other ingredients in various lanes of the polyacrylamide gel.
 (^{35}S - labelled proteins)
 (volume in μl)

Lane No.	Sample in the gel well from:	Cells harvested after (hr)	Volume of sample	Volume of 4 x sb	Volume of H_2O	Volume of blue in 75% glycerol	Total
1	Standard protein mixture	-	1	4	14	3	22
2	Control (-As)	2	20	7	-	3	30
3	Tolerant cells ($10\mu\text{M}$ As)	2	20	7	-	3	30
4	Sensitive cells ($200\mu\text{M}$ As)	2	20	7	-	3	30
5	Control (-As)	4	20	7	-	3	30
6	Tolerant cells ($10\mu\text{M}$ As)	4	20	7	-	3	30
7	Sensitive cells ($200\mu\text{M}$ As)	4	20	7	-	3	30
8	Tolerant cells ($10\mu\text{M}$ As)	4	20	7	-	3	30
9	Control (-As)	2	20	7	-	3	30
10	Tolerant cells ($10\mu\text{M}$ As)	2	20	7	-	3	30
11	Sensitive cells ($200\mu\text{M}$ As)	2	20	7	-	3	30
12	Control (-As)	4	20	7	-	3	30
13	Tolerant cells ($10\mu\text{M}$ As)	4	20	7	-	3	30
14	Sensitive cells ($200\mu\text{M}$ As)	4	20	7	-	3	30
15	Tolerant cells ($10\mu\text{M}$ As)	4	20	7	-	3	30
16	Standard protein mixture	-	1	4	14	3	22

Lane 2 - 8 Crude extracts. Lane 9-15 Supernatant

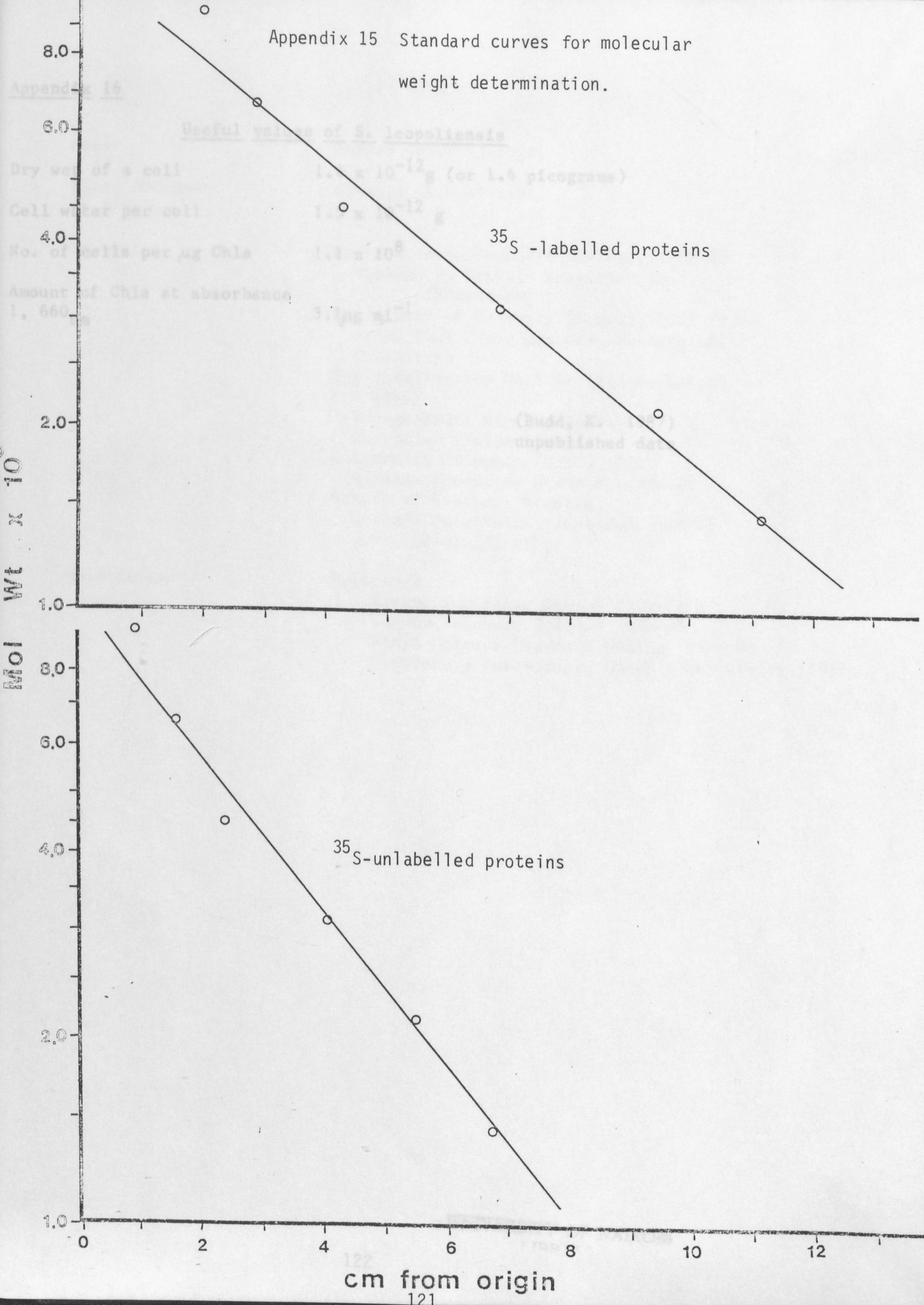
Lane 2 - 7 Crude extracts. Lane 8-13 Supernatant

Volume of cell extracts and other ingredients in various lanes of the polyacrylamide gel.
 (^{35}S - unlabelled proteins)
 (volume in μl)

Lane No.	Sample in the gel from:	Volume of sample	Volume of 2 x sb	Volume of 4 x sb	Volume of H_2O	Volume of Brom-phenol blue in 75% glycerol	Total
1	Standard protein mixture	1	8		7	3	19
2	Control (-As)	6	6		3	3	18
3	"	12	12		3	3	30
4	Tolerant cells ($10\mu\text{M}$ As)	6	6		3	3	18
5	" "	12	12		3	3	30
6	Tolerant cells ($10\mu\text{M}$ As) ----> $200\mu\text{M}$ As for 4hr	6	6		3	3	18
7	" "	12	12		3	3	30
8	Control (-As)	6	6		3	3	18
9	" "	12	12		3	3	30
10	Tolerant cells ($10\mu\text{M}$ As)	6	6		3	3	18
11	" "	12	12		3	3	30
12	Tolerant cells ($10\mu\text{M}$ As) ----> $200\mu\text{M}$ As for 4hr	6	6		3	3	18
13	" "	12	12		3	3	30
14	-	-	-		-	-	-
15	Control pellet in NaOH/HCl	20	-	5	2	3	30
16	Tolerant pellet in NaOH/Hcl ----> $200\mu\text{M}$ As for 2 hr	20	-	5	2	3	30
17	Standard protein mixture	1	8		7	3	19

Lane 2 - 7 Crude extracts. Lane 8-13 Supernatant

Appendix 15 Standard curves for molecular weight determination.



Appendix 16

Useful values of *S. leopoliensis*

Dry wet of a cell 1.4×10^{-12} g (or 1.4 picograms)

Cell water per cell 1.5×10^{-12} g

No. of cells per μ g Chla 1.1×10^8

Amount of Chla at absorbance
1, 660_{nm}

$3.1 \mu\text{g ml}^{-1}$

Experience:

Teaching:

Karima Secondary School 1971-72

Karima Teachers College 1976-78

Kenya Science Teachers College 1978-80

Laboratory Instructor, Queen's University, 1987.

VITA

Name: Norman Chege Njoroge

Place and year of birth: Muranga (Kenya) 1947

Education: Kenya Science Teachers College, 1968-70
Diploma in Science Education (S1)
(Secondary)
University of Nairobi, (Kenya), 1972-75
B.Sc. (2nd Class Honours, Zoology and Chemistry)
Njala University College (Sierra Leone) 1976-77
Postgraduate Diploma in Science Education (Primary)
University of Leeds (U.K.) 1982
Science Education Study Attachment
School of Graduate Studies
Queen's University, (Canada) 1986-88
M.Sc. (Biology) 1988

Experience: Teaching:
Karima Secondary School 1971-72
Kagumo Teachers College 1976-78
Kenya Science Teachers College 1979-86
Laboratory Instructor, Queen's University, 1987.