Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

DISINFECTION AND ENVIRONMENTAL STUDIES ON PATHOGENIC FREE-LIVING AMOEBAE

A thesis presented in partial fulfillment of the

requirements for the degree of

Master of Science in Microbiology

at

Massey University, Palmerston North

New Zealand

Mark William Dawson

1982

593.117
Daw
MASSEY UNIVERSITY
LIBRARY
82_10040

ABSTRACT

Over the last fifteen years, there has been an increasing awareness of sporadic cases of Primary Amoebic Meningoencephalitis (PAM), affecting primarily younger age groups and appearing in an acute fulminant form. The earliest positive case known, may have been in England in 1909.

The pathogenic free-living amoebae (PFLA), which comprises the genera Naegleria and Acanthamoeba, are the causative organisms of PAM and Granulomatous Amoebic Encephalitis (GAE) respectively. PAM is a rapidly fatal disease affecting the central nervous system (CNS), and GAE although essentially confined to the CNS, may also take the form of granulomata in the liver, spleen, uterus and kidneys.

A study on the disinfecting potential of Baquacil in axenic conditions, for comparison with the disinfecting potential of the chlorine, chlorine dioxide, ozone and Deciquam 222, showed that the order of effectiveness as amoebicides was Baquacil, chlorine, chlorine dioxide, ozone and Deciquam 222 in hard water. In soft water the order is Deciquam 222, Baquacil, chlorine, chlorine dioxide and ozone.

Further study on the effect of Baquacil, chlorine and chlorine dioxide on amoebae, in conditions involving the use of a known Biochemical Oxygen Demand (BOD), a known bacteria concentration, and a combination of BOD and bacteria, confirmed Baquacil as a more effective amoebicide than chlorine, which in turn was more effective than chlorine dioxide. The concentrations of each disinfectant required were increased by the presence of a BOD, and of bacteria. The bacteria were preferentially destroyed over the amoebae with all three disinfectants.

Baquacil resistant clones of Naegleria fowleri were isolated, although it is not known whether this resistance is due to genetic or physiological variation.

Axenically and Monoxenically cultured amoebae were used, the

latter to increase the resemblance of the amoebae to those found in the environment. Differences in survival rates were observed, the monoxenically cultured amoebae invariably having higher survival rates.

Competition studies were done with *Naegleria* spp. and *T.pyriformis* on three bacteria species, after preliminary studies on the ability of the two protozoa to grow on eight species of bacteria. Of the three bacteria used in the competiton studies, *Escherichia coli* and *Enterobacter cloacae* were shown to support both *Naegleria* spp. and *T.pyriformis*, with the ciliate increasing in numbers by up to 3 fold over the controls, but the amoebae were affected only slightly, with a small decrease in numbers compared to the controls. A synergistic relationship was evident on the third bacteria species, *Pseudomonas fluorescens*, between *Naegleria* spp. and *T.pyriformis*, where as in the controls, this bacterium was not a good growth support bacteria for either protozoan.

ACKNOWLEDGEMENTS

I would like to personally thank the following people:

The Department of Microbiology and Genetics, Massey University, for providing the opportunity and facilities for this investigation.

My supervisor, Dr. Tim Brown, for his patience and guidance through out this study.

Professor D.F. Bacon and the other academic and technical staff of the department.

Mrs. Elizabeth Keys, and Mr. Craig Biddick for their technical assistance and friendship during this study.

Mr. D. Till, of the National Health Institute, Wellington for his cooperation in the disinfection study.

The Massey University Library, for their assistance.

The Central Photgraphic Unit.

The New Zealand Health Department , and the Medical Research Council for financil support during this study.

ICI (NZ) Ltd, for the supply of Baquacil.

Henry Berry Ltd, for the loan of their typewriter.

My parents, for their support and faith in me throughout this project.

An last, but by no means least, my wife, Debbie, for her support, and trust in my ability and for not only putting up with me, but also typing the final copy of this thesis.

TABLE OF CONTENTS

ABSTRACT	ii
ACKNOWLEDGEMENTS	iv
LIST OF TABLES	ix
LIST OF FIGURES	xii
CHAPTER ONE: INTRODUCTION	1
1.1 The History of Free-Living Amoebae as Disease Agents.	1
1.2 Classification	3
1.3 Pathogenicity	5
1.4 Immunity	7
1.5 Diagnosis	8
1.6 Treatment	11
1.7 Occurrence and Distribution	15
1.8 Disinfection and Control Measures	18
CHAPTER TWO: MATERIALS	21
2.1 Amoeba Cultures Used	21
2.2 Ciliate Culture Used	22
2.3 Bacteria Cultures Used	22
2.4 Plate Media	22
2.4.1 Amoeba Saline Agar	22
2.4.2 Amoeba 1% Saline Agar	23
2.4.3 Amoeba Saline Bacto Agar	23
2.4.4 Nutrient Media Agar	24
2.4.5 Plate Count Agar	24
2.4.6 Brain Heart Infusion Agar	24
2.5 Axenic Media for Amoebae	24
2.5.1 Pages Amoeba Saline	24
2.5.2 CYM Medium	25
2.5.3 4% Neff Medium	26
2.6 Axenic Media for Ciliates	26
2.6.1 Tetrahymena Medium	26
2.7 Bacterial Growth Medium	27
2.7.1 Brain Heart Infusion	27
2.7.2 Pages Amoeba Saline Glucose	27
2.8 Miscellaneous Solutions	28
2.8.1 Sodium Phosphate Buffer	28

2.8.2 Phosphate Buffered Saline 28
2.8.3 Baquacil Indicator Solution 28
2.8.4 200ppm Hard Water 28
2.8.5 D.P.D Reagents 29
2.8.6 Reagents for BOD 29
2.8.7 20% Bovine Faecal Matter Solution 29
2.9 Disinfectant Solutions 29
2.9.1 Water 29
2.9.2 Chlorine 29
2.9.3 Chlorine dioxide 29
2.9.4 Baquacil 29

CHAPTER THREE: METHODS 30
3.1 Sterilisation 30
3.2 Axenic Culture Techniques 30
3.2.1 Maintainance of amoeba stock cultures 30
3.2.2 Maintainance of *Tetrahymena* stock cultures 30
3.2.3 Bacteria culture 30
3.3 Monoxenic Amoeba Culture Techniques 31
3.4 Growth Experiments 31
3.4.1 Amoebae 31
3.4.2 *Tetrahymena* 32
3.4.3 Amoeba - *Tetrahymena* competition 32
3.5 Biochemical Oxygen Demand Test 32
3.6 Production of Disinfectants 33
3.6.1 Chlorine 33
3.6.2 Chlorine dioxide 33
3.6.3 Baquacil 33
3.7 Chemical Analysis of Disinfectants 33
3.8 Disinfection Tests 35
3.8.1 Axenic tests 35
3.8.2 Monoxenic tests 36
3.8.3 Non-viable organic demand test 37
3.8.4 Monoxenic plus organic demand test 38

CHAPTER FOUR: RESULTS 39
4.1 The Use of Baquacil Against Pathogenic Free-Living Amoebae 39
4.1.1 Axenically Grown Amoebae in Axenic Conditions 39
4.1.2 Axenically Grown Amoebae plus Bacteria 48

4.1.3 Axenically Grown Amoebae plus a BOD 51
4.1.4 Axenically Grown Amoebae plus a BOD and Bacteria 51
4.1.5 Monoxenically Grown Amoebae plus Bacteria 61
4.1.6 Monoxenically Grown Amoebae plus a BOD 64
4.1.7 Monoxenically Grown Amoebae plus a BOD and Bacteria 64
4.1.8 Axenically Grown Amoebae and Extended Exposure Time 73
4.1.9 Axenically Grown Amoebae plus Bacteria with Increased Baquacil concentration 76
4.1.10 Axenically Grown Amoebae plus Bacteria and Increased Exposure Time and Baquacil Concentration 79
4.1.11 Monoxenically Grown Amoebae plus Bacteria with Increased Exposure Time and Baquacil Concentration 82
4.1.12 Suspected Baquacil Resistant Clones of _Naegleria_ spp. 87
4.2 The Use of Chlorine as a Disinfectant Against Pathogenic Free-Living Amoebae 89
4.2.1 Axenically Grown Amoebae plus Bacteria 89
4.2.2 Axenically Grown Amoebae plus a BOD 92
4.2.3 Axenically Grown Amoebae plus a BOD and Bacteria 97
4.2.4 Monoxenically Grown Amoebae plus Bacteria 100
4.2.5 Monoxenically Grown Amoebae plus a BOD 103
4.2.6 Monoxenically Grown Amoebae plus a BOD and Bacteria 108
4.3 The Use of Chlorine Dioxide as a Disinfectant Against Pathogenic Free-Living Amoebae 111
4.3.1 Axenically Grown Amoebae plus Bacteria 111
4.3.2 Axenically Grown Amoebae plus a BOD 114
4.3.3 Axenically Grown Amoebae plus a BOD and Bacteria 119
4.3.4 Monoxenically Grown Amoebae plus Bacteria 122
4.3.5 Monoxenically Grown Amoebae plus a BOD 125
4.3.6 Monoxenically Grown Amoebae plus a BOD and Bacteria 130
4.4 Growth and Competition Studies using _Naegleria_ and _Tetrahymena_ on Eight Bacteria species at 30°C 133
4.4.1 The Growth of _Naegleria_ on Eight Bacteria Species 133
4.4.2 The Growth of _T.pyriformis_ on Eight Bacteria Species 153
4.4.3 Competition between _T.pyriformis_ and four _Naegleria_ strains 159

CHAPTER FIVE: DISCUSSION 168
5.1 The Disinfection of Pathogenic Free-Living Amoebae 168

5.2 Competition Between *Naegleria* and *Tetrahymena* for Bacteria 177

BIBLIOGRAPHY 186

LIST OF TABLES

I.	Amoeba Cultures Used	21
II.	Bacteria Cultures Used	22
III.	The Effect of Baquacil on Axenically Grown Amoebae, for 30 minutes, at 25°C	43
IV.	The Effect of Baquacil on Axenically Grown Amoebae, for 30 minutes, at 30°C	45
V.	The Effect of Baquacil on Axenically Grown Amoebae, for 30 minutes, at 37°C	47
VI.	The Effect of Baquacil on Axenically Grown Amoebae, plus 1 x $10^5.cm^{-3}$ bacteria, for 30 minutes, at 30°C	50
VII.	The Effect of Baquacil on Axenically Grown Amoebae, plus a BOD of 1.2 $mg.l^{-1}$, for 30 minutes at 30°C	54
VIII.	The Effect of Baquacil on Axenically Grown Amoebae, plus a BOD of 1.2 $mg.l^{-1}$, plus 1 x $10^5.cm^{-3}$ bacteria, for 30 minutes, at 30°C	56
IX.	The Effect of Baquacil on Axenically Grown Amoebae, plus a BOD of 4.7 $mg.l^{-1}$, for 30 minutes, at 30°C	58
X.	The Effect of Baquacil on Axenically Grown Amoebae, plus a BOD of 4.7 $mg.l^{-1}$, plus 1 x $10^5.cm^{-3}$ bacteria, for 30 minutes, at 30°C	60
XI.	The Effect of Baquacil on Monoxenically Grown Amoebae, plus 1 x $10^5.cm^{-3}$ bacteria, for 30 minutes, at 30°C	63
XII.	The Effect of Baquacil on Monoxenically Grown Amoebae, plus a BOD of 1.2 $mg.l^{-1}$, for 30 minutes, at 30°C	67
XIII.	The Effect of Baquacil on Monoxenically Grown Amoebae, plus a BOD of 1.2 $mg.l^{-1}$, plus 1 x $10^5.cm^{-3}$ bacteria, for 30 minutes, at 30°C	69
XIV.	The Effect of Baquacil on Monoxenically Grown Amoebae, plus a BOD of 4.7 $mg.l^{-1}$, for 30 minutes, at 30°C	71
XV.	The Effect of Baquacil on Monoxenically Grown Amoebae, plus a BOD of 4.7 $mg.l^{-1}$, plus 1 x $10^5.cm^{-3}$ bacteria, for 30 minutes, at 30°C	72
XVI.	The Effect of 50$mg.l^{-1}$ Baquacil on Three Axenically Grown Naegleria strains, for 240 minutes, at 30°C	75
XVII.	The Effect of up to 80 $mg.l^{-1}$ Baquacil on Axenically Grown Amoebae, for 30 minutes, at 30°C	78

XVIII.	The Effect of up to 80 $mg.l^{-1}$ Baquacil on Axenically Grown Amoebae, plus 1 x $10^5.cm^{-3}$ bacteria, for 60 minutes, at 30^oC	81
XIX.	The Effect of Baquacil on Monoxenically Grown Amoebae, plus 1 x $10^5.cm^{-3}$ bacteria, for 60 minutes, at 30^oC	84
XX.	The Effect of up to 80 $mg.l^{-1}$ Baquacil on Monoxenically Grown Amoebae, plus 1 x $10^5.cm^{-3}$ bacteria, for 60 minutes, at 30^oC	86
XXI.	The Effect of 50 $mg.l^{-1}$ Baquacil on suspected Baquacil resistant clones of Naegleria strains	88
XXII.	The Effect of Chlorine on Axenically Grown Amoebae, plus 1 x $10^5.cm^{-3}$ bacteria, for 30 minutes, at 30^oC	91
XXIII.	The Effect of Chlorine on Axenically Grown Amoebae, plus a BOD of 1.2 $mg.l^{-1}$, for 30 minutes, at 30^oC	94
XXIV.	The Effect of Chlorine on Axenically Grown Amoebae, plus a BOD of 4.7 $mg.l^{-1}$, for 30 minutes, at 30^oC	96
XXV.	The Effect of Chlorine on Axenically Grown Amoebae, plus a BOD of 1.2 $mg.l^{-1}$, plus 1 x $10^5.cm^{-3}$ bacteria, for 30 minutes, at 30^oC	99
XXVI.	The Effect of Chlorine on Monoxenically Grown Amoebae, plus 1 x $10^5.cm^{-3}$ bacteria, for 30 minutes, at 30^oC	102
XXVII.	The Effect of Chlorine on Monoxenically Grown Amoebae, plus a BOD of 1.2 $mg.l^{-1}$, for 30 minutes, at 30^oC	105
XXVIII.	The Effect of Chlorine on Monoxenically Grown Amoebae, plus a BOD of 4.7 $mg.l^{-1}$, for 30 minutes, at 30^oC	106
XXIX.	The Effect of Chlorine on Monoxenically Grown Amoebae, plus a BOD of 1.2 $mg.l^{-1}$, for 30 minutes, at 30^oC	110
XXX.	The Effect of Chlorine Dioxide on Axenically Grown Amoebae, plus 1 x $10^5.cm^{-3}$ bacteria, for 30 minutes, at 30^oC	113
XXXI.	The Effect of Chlorine Dioxide on Axenically Grown Amoebae, plus a BOD of 1.2 $mg.l^{-1}$, for 30 minutes, at 30^oC	116
XXXII.	The Effect of Chlorine Dioxide on Axenically Grown Amoebae, plus a BOD OF 4.7 $mg.l^{-1}$, for 30 minutes, at 30^oC	118
XXXIII.	The Effect of Chlorine Dioxide on Axenically Grown Amoebae, plus a BOD of 1.2 $mg.l^{-1}$, plus 1 x $10^5.cm^{-3}$ bacteria, for 30 minutes, at 30^oC	121
XXXIV.	The Effect of Chlorine Dioxide on Monoxenically Grown Amoebae, plus 1 x $10^5.cm^{-3}$ bacteria, for 30 minutes, at 30^oC	124

XXXV.	The Effect of Chlorine Dioxide on Monoxenically Grown Amoebae, plus a BOD of 1.2 $mg.l^{-1}$, for 30 minutes, at 30°C	127
XXXVI.	The Effect of Chlorine Dioxide on Monoxenically Grown Amoebae, plus a BOD of 4.7 $mg.l^{-1}$, for 30 minutes, at 30°C	129
XXXVII.	The Effect of Chlorine Dioxide on Monoxenically Grown Amoebae, plus a BOD of 1.2 $mg.l^{-1}$, plus 1 x $10^5.cm^{-3}$ bacteria, for 30 minutes, at 30°C	132
XXXVIII.	Growth of Four Naegleria strains on E.coli at 30°C	138
XXXIX.	Growth of Four Naegleria strains on K.aerogenes at 30°C	140
XL.	Growth of Four Naegleria strains on E.cloacae at 30°C	142
XLI.	Growth of Four Naegleria strains on M.luteus at 30°C	144
XLII.	Growth of Four Naegleria strains on B.subtilis at 30°C	146
XLIII.	Growth of Four Naegleria strains on S.marcescens at 30°C	148
XLIV.	Growth of Four Naegleria strains on Ps.aeruginosa at 30°C	150
XLV.	Growth of Four Naegleria strains on Ps.fluorescens at 30°C	152
XLVI.	Growth of T.pyriformis on the Eight Species of Bacteria at 30°C	158
XLVII.	Competition Between T.pyriformis and each of Four Naegleria strains on E.coli, at 30°C	163
XLVIII.	Competition Between T.pyriformis and each of Four Naegleria strains on E.cloacae, at 30°C	165
XLIX.	Competition Between T.pyriformis and each of Four Naegleria strains on Ps.fluorescens, at 30°C	167

LIST OF FIGURES

1. Method Used for the Production of Chlorine Dioxide. 34

2. The Effect of Baquacil on Axenically Grown Amoebae, for 30 minutes, at 25°C 42

3. The Effect of Baquacil on Axenically Grown Amoebae, for 30 minutes, at 30°C 44

4. The Effect of Baquacil on Axenically Grown Amoebae, for 30 minutes, at 37°C 46

5. The Effect of Baquacil on Axenically Grown Amoebae, plus $1 \times 10^5.cm^{-3}$ bacteria, for 30 minutes, at 30°C 49

6. The Effect of Baquacil on Axenically Grown Amoebae, plus a BOD of $1.2 mg.l^{-1}$, for 30 minutes, at 30°C 53

7. The Effect of Baquacil on Axenically Grown Amoebae, plus a BOD of $1.2 mg.l^{-1}$, plus $1 \times 10^5.cm^{-3}$ bacteria, for 30 minutes, at 30°C 55

8. The Effect of Baquacil on Axenically Grown Amoebae, plus a BOD of $4.7 mg.l^{-1}$, for 30 minutes, at 30°C 57

9. The Effect of Baquacil on Axenically Grown Amoebae, plus a BOD of $4.7 mg.l^{-1}$, plus $1 \times 10^5.cm^{-3}$ bacteria, for 30 minutes, at 30°C 59

10. The Effect of Baquacil on Monoxenically Grown Amoebae, plus $1 \times 10^5.cm^{-3}$ bacteria, for 30 minutes, at 30°C 62

11. The Effect of Baquacil on Monoxenically Grown Amoebae, plus a BOD of $1.2 mg.l^{-1}$, for 30 minutes, at 30°C 66

12. The Effect of Baquacil on Monoxenically Grown Amoebae, plus a BOD of $1.2 mg.l^{-1}$, plus $1 \times 10^5.cm^{-3}$ bacteria, for 30 minutes, at 30°C 68

13. The Effect of Baquacil on Monoxenically Grown Amoebae, plus a BOD of $4.7 mg.l^{-1}$, for 30 minutes, at 30°C 70

14. The Effect of $50 mg.l^{-1}$ Baquacil on Three Axenically Grown Naegleria strains, for 240 minutes, at 30°C 74

15. The Effect of up to $80 mg.l^{-1}$ Baquacil on Axenically Grown Amoebae, for 30 minutes, at 30°C 77

16. The Effect of up to $80 mg.l^{-1}$ Baquacil on Axenically Grown Amoebae, plus $1 \times 10^5.cm^{-3}$, for 60 minutes, at 30°C 80

17. The Effect of Baquacil on Monoxenically Grown Amoebae, plus $1 \times 10^5.cm^{-3}$ bacteria, for 60 minutes, at 30°C 83

18. The Effect of up to 80 $mg.l^{-1}$ Baquacil on Monoxenically Grown Amoebae, plus $1 \times 10^5.cm^{-3}$ bacteria, for 60 minutes, at 30^oC 85

19. The Effect of Chlorine on Axenically Grown Amoebae, plus $1 \times 10^5.cm^{-3}$ bacteria, for 30 minutes, at 30^oC 90

20. The Effect of Chlorine on Axenically Grown Amoebae, plus a BOD of 1.2 $mg.l^{-1}$, for 30 minutes, at 30^oC 93

21. The Effect of Chlorine on Axenically Grown Amoebae, plus a BOD of 4.7 $mg.l^{-1}$, for 30 minutes, at 30^oC 95

22. The Effect of Chlorine on Axenically Grown Amoebae, plus a BOD of 1.2 $mg.l^{-1}$, plus $1 \times 10^5.cm^{-3}$ bacteria, for 30 minutes, at 30^oC 98

23. The Effect of Chlorine on Monoxenically Grown Amoebae, plus $1 \times 10^5.cm^{-3}$ bacteria, for 30 minutes, at 30^oC 101

24. The Effect of Chlorine on Monoxenically Grown Amoebae, plus a BOD of 1.2 $mg.l^{-1}$, for 30 minutes, at 30^oC 104

25. The Effect of Chlorine on Monoxenically Grown Amoebae, plus a BOD of 4.7 $mg.l^{-1}$, for 30 minutes, at 30^oC 106

26. The Effect of Chlorine on Monoxenically Grown Amoebae, plus a BOD of 1.2 $mg.l^{-1}$, for 30 minutes, at 30^oC 109

27. The Effect of Chlorine Dioxide on Axenically Grown Amoebae, plus $1 \times 10^5.cm^{-3}$ bacteria, for 30 minutes, at 30^oC 112

28. The Effect of Chlorine Dioxide on Axenically Grown Amoebae, plus a BOD of 1.2 $mg.l^{-1}$, for 30 minutes, at 30^oC 115

29. The Effect of Chlorine Dioxide on Axenically Grown Amoebae, plus a BOD of 4.7 $mg.l^{-1}$, for 30 minutes, at 30^oC 117

30. The Effect of Chlorine Dioxide on Axenically Grown Amoebae, plus a BOD of 1.2 $mg.l^{-1}$, plus $1 \times 10^5.cm^{-3}$ bacteria, for 30 minutes, at 30^oC 120

31. The Effect of Chlorine Dioxide on Monoxenically Grown Amoebae plus $1 \times 10^5.cm^{-3}$ bacteria, for 30 minutes, at 30^oC 123

32. The Effect of Chlorine Dioxide on Monoxenically Grown Amoebae, plus a BOD of 1.2 $mg.l^{-1}$, for 30 minutes, at 30^oC 126

33. The Effect of Chlorine Dioxide on Monoxenically Grown Amoebae plus a BOD of 4.7 $mg.l^{-1}$, for 30 minutes, at 30^oC 128

34. The Effect of Chlorine Dioxide on Monoxenically Grown Amoebae plus a BOD of 1.2 $mg.l^{-1}$, plus $1 \times 10^5.cm^{-3}$ bacteria, for 30 minutes at 30^oC 131

35. Growth of _Naegleria_ on _E.coli_ at 30^oC 137

36. Growth of Naegleria on K.aerogenes at 30°C 139

37. Growth of Naegleria on E.cloacae at 30°C 141

38. Growth of Naegleria on M.luteus at 30°C 143

39. Growth of Naegleria on B.subtilis at 30°C 145

40. Growth of Naegleria on S.marcescens at 30°C 147

41. Growth of Naegleria on Ps.aeruginosa at 30°C 149

42. Growth of Naegleria on Ps.fluorescens at 30°C 151

43. Growth of T.pyriformis on E.cloacae, K.aerogenes, E.coli, M.luteus at 30°C 155

44. Growth of T.pyriformis on Ps.aeruginosa, Ps.fluorescens, B.subtilis, S.marcescens at 30°C 157

45. Competition between T.pyriformis and N.fowleri, and T.pyriformis and N.gruberi, on E.coli, at 30°C 162

46. Competition between T.pyriformis and N.fowleri, and T.pyriformis and N.gruberi, on E.cloacae, at 30°C 164

47. Competition between T.pyriformis and N.fowleri, and T.pyriformis and N.gruberi, on Ps.fluorescens, at 30°C 166

CHAPTER ONE: INTRODUCTION

1.1. The History of Free-Living Amoebae as Disease Agents.

The History of Pathogenic Free-Living Amoebae (PFLA) of the genera Acanthamoeba and Naegleria has been extensively reviewed by Carter (1972), Cursons (1974), Culbertson (1971), and Cursons and Brown (1976).

The commonest disease caused by PFLA is Primary Amoebic Meningo-encephalitis (PAM). These PFLA are quite different to those traditionally regarded as parasitic in man and are not parasitic in the strict sense. They are ubiquitous in the environment, and are free-living in water, sewage, soil, and other decaying organic matter (Carter, 1972; Duma, 1981) and opportunist pathogens in Man.

Willaert in 1974, tabulated 84 cases from all the continents with the exception of Antarctica , since then at least 36 additional cases have been reported.

Acanthamoeba spp. were the first agents implicated in this disease although PAM is now known to be caused by a free-living amoeba of the genus Naegleria. This was due mainly to the first case prototype of this illness described by Fowler and Carter in 1965. In 1968, Culbertson et al., showed that the incriminating species of most reported cases belonged to the genus Naegleria. In 1970, on the basis of morphological, cultural, and pathogenicity differences, Carter named the pathogenic species Naegleria fowleri to distinguish it from the non-pathogenic species Naegleria gruberi .

Prior to 1968, all cases of PAM were attributed to Acanthamoeba spp. probably because of the pioneering work of Culbertson et al., (1958, 1959, 1965) who, whilst working on the production of polio vaccine, found an amoeba contaminant of the

cultures of monkey kidney cells. These cultures, when inoculated intracerebrally into mice, produced a necrotizing haemorrhagic meningo-encephalitis that killed mice in 4 - 7 days. The amoeba responsible were identified as an Acanthamoeba, and they predicted that on the basis of this finding, this amoeba could be capable of producing disease in humans. This amoeba was previously considered to be a harmless free-living organism.

The diseases caused by PFLA can be divided into two entities (Chang, 1974).

a) a swimming - associated acute meningo-encephalitis known as Primary Amoebic Meningo-encephalitis (PAM) (Martinez et al., 1977). This is the more important of the two, and is caused by the pathogenic Naegleria fowleri.

   Infection is thought to occur in two ways:

   (i) Naegleria contaminated water is introduced into the upper nasal passages.

   (ii) the washing of trophoziotes residing in the lower nasal passages of a carrier into the upper nasal area. A pathogenic strain of Naegleria has been isolated from a healthy carrier (Visvesvara, et al., 1974)

b) a non-swimming-associated chronic meningo-encephalitis, now known as Granulomatous Amoebic Encephalitis. (GAE) (Martinez et al.,1980; Martinez, 1980), cuased by amoeba of the Acanthamoeba/Hartmanella group.

Considered opinion is that A.rhysodes along with A.culbertsoni are the most likely species to cause GAE. The involvement of the Central

Nervous System (CNS) appears to be a secondary phenomenion in GAE, representing metatastic spread from a primary focus in the skin, genitourinary tract, or respiratory tract (Martinez et al., 1977, 1980; Martinez, 1980). Cutaneous ulceration is a possible point of entry, with haemotogenous spread to the CNS and lower respiratory tract. Infections in experimental animals have been reported as being due to this spread (Martinez et al., 1977).

Subsequently, Acanthamoeba spp. have been implicated in a number of chronic illnesses, such as respiratory infections (Martinez et al., 1975, 1980; Martinez, 1980), corneal ulceration of the eye leading to blindness (Nagington et al.,1974; Visversvara et al., 1974), and together with Naegleria spp. in humidifier disease (MRC symposium, 1977; Rowbottom, 1980; Edwards et al., 1978), and chronic keratitis (Ma et al., 1981; Bos et al., 1981). The reidentification of the etiological agents of the 1968 cases of PAM in New Zealand as N.fowleri (Cursons & Brown, 1975, 1976) has dismissed the notion of Mandal et al., (1970), that slime moulds are involved in the etiology of PAM.

1.2 Classification

Study of the basic classification of the small free-living amoebae has been stimulated by the discovery of their role in human disease. Schuster (1979) has made clear the fact that slime moulds, both cellular and true, are excluded from the small amoebae and amoeboflagellate group. Carter (1970) accepted the decision of Page to retain the family designation of Vahlkampfidae (for Naegleria spp.) in preference to the revision of Singh and Das (1970). Studies by Fulton (1970) and Schuster (1975) on the mitosis of Naegleria spp. and related amoebae displaying promitosis have confirmed the lack of validity in using details such as "interzonal body" and

"polar caps" to establish a higher taxa, such as Schizopyrenidae. Schuster (1975) reviews these and other points in some detail.

Differentiation into species is quite difficult. Lastovica (1974) has shown in electron micrographs, presence of preformed exit pores in N.gruberi cysts, but not in N.fowleri. Bakers yeast has also been used, with N.fowleri showing no phagocytosis where N.gruberi shows marked ingestion of the yeasts (Thong and Ferrante, 1978), and starch gel electrophoresis has been used to separate pathogenic and non-pathogenic Naegleria spp. (Nerad & Daggett, 1979). A new species of Naegleria , N.lovaniensis has been isolated. It is found in association with N.fowleri, and causes Cytopathic Effects (CPE) in vero cell culture, although it is non-pathogenic to experimental animals and is thermophilic (Stevens et al., 1980).

Acanthamoeba classification has been changing with regularity. Acanthamoeba used to be called soil amoebae, but are now considered part of the PFLA group. A number of previously distinct species are now regarded as strains of Acanthamoeba castellanii. Attempts have been made to distinguish species by serological analysis techniques (Schuster, 1979).

In a review by Cursons and Brown (1976), the controversy regarding the classification of the PFLA appeared to be settled with the majority of workers preferring Chang's 1971 classification scheme. Accurate identification of isolates requires the use of a range of specific cytological, morphological, physiological, immunological, growth and pathogenicity characteristics in an ordered sequence, readily usable by hospital and public health laboratory staff (Cursons & Brown, 1976).

1.3 Pathogenicity

The invasion of organs and tissues by PFLA is now well documented (Culbertson et al., 1959, 1968, 1972; Carter, 1968, 1970, 1972; Callicot et al., 1968; Chang, 1971, 1974, 1976; Culbertson, 1971; Martinez et al., 1973, 1975, 1977, 1980; Martinez, 1980; Visvesvara & Balamuth, 1975; Wong et al., 1975a&b). The invasion of the CNS by Naegleria spp. occurs primarily via the nasal mucosal epithelium, mainly due to the pathological condition of the cribriform plate and subjacent nasal passages (Culbertson, 1971; Carter, 1972), which has been experimentally verified by Martinez et al., (1973). Using mice, they showed that amoebic invasion occurs through the disruption of the olfactory mucosa, penetration into the submucosal plexus, probably by phagacytosis by the amoebae, of the sustantacular cells of the olfactory neuroepithelium, and finally through the cribriform plate into the CNS. Virus-like particles have been seen inside these amoebae which produce a CPE in chick cells, and are possibly related to human pathogencity (Schuster & Dunnebacke, 1974), though Cursons & Brown (1976) believe this factor resides in the secreted phospholipases . Some properties of these particles e.g. Ultra Violet (U.V.) light resistance, are similar to the scrapie agent (Schuster & Dunnebacke, 1974; Dunnebacke & Schuster, 1971).

In cases of GAE , the involvement of the CNS appears to be a secondary phenomena representing metatastic spread from a primary focus in the skin, genitourinary, or respiratory tracts. (Martinez et al., 1975, 1977, 1980; Martinez, 1980; Culbertson, 1971; Schuster, 1979). Martinez et al., (1975) reported lower respiratory tract infections in experimental animals.

GAE appears to be an opportunistic infection of the CNS (Martinez, 1980; Martinez et al., 1980; Martinez & De Jonckheere, 1981) and occurs in patients who are chronically ill, debilitated, or in those whose cell mediated immune responses have been impaired as a result of either underlying systemic disease or its treatment by immunosuppressive methods (Robert & Rorke, 1973; Martinez, 1980).

Acanthamoeba infections of sites with reduced accessibility to the immune system e.g. the eye also demonstrates the opportunistic nature of these infections. Isolations of Acanthamoeba spp. from the cornea of the eye (Nagington et al., 1974) were shown to be of low virulence, and infection only resulted after damage to the cornea (Visvesvara et al., 1975).

Once CNS invasion has occurred, destruction of the surrounding tissue is thought to be brought about by phagocytosis of host tissue by N.fowleri, and pinocytosis in combination with phospholipase secretion by A.culbertsoni (Visvesvara & Callaway, 1974; Cursons & Brown, 1976). There have been many reports of the possession of lysosomal and hydrolytic enzymes (Martinez et al., 1975; Chang, 1976; Cursons & Brown, 1976), and there has been speculation that the levels of the cytopathic enzymes produced, may explain the degrees of virulence among the Acanthamoeba and N.fowleri isolates (Cursons, 1978; Culbertson, 1971; De Jonckheere & Van de Voorde, 1977).

An N.fowleri isolate from a child who died of PAM in Florida, was intranasally inoculated into sheep, to determine whether livestock are susceptible to PFLA infection. The sheep died seven days later from amoebic invasion of the CNS (Young et al., 1980).

Byers (1979), has also speculated on the possiblity of

Acanthamoeba spp. being vectors for Mycobacterium leprae (leprosy bacillus).

Wyburn-Mason (1979) has speculated on PFLA involvement in rheumatoid and autoimmune diseases, but his views are not supported by other investigators.

1.4 Immunity

Many authors have pondered over the low incidence of PAM and GAE cases with regards the ease and frequency of isolation of PFLA form the environment (Anderson & Jamieson, 1971; Cursons et al., 1977, 1977b; John et al., 1977; Wellings et al., 1977; Wellings, 1979; Haggerty & John, 1978; Duma 1978, 1980). This has led many to speculate on the existance of probable host related susceptibility factors, and the demonstration of specific antibodies to free-living amoebae in human sera has been reported (Edwards et al., 1976; Cursons et al., 1977, 1980a&b; MRC Symposium, 1977; Cursons, 1978).

Adams et al. (1976), reported that mice surviving a primary intravenous injection of N.fowleri were subsequently resistant to further challenge by the same route with a dose that produced a uniformly fatal disease in untreated control mice. It was further shown by this group, that mice immunized with live or formalised N.fowleri or N.gruberi either subcutaneously, intraperitoneally, intravenously, or intramuscularly, were significantly protected from a subsequent challenge with N.fowleri (John et al., 1977), and Thong et al. (1980) showed that amoebic lysate and cell culture supernatent also immunized mice against N.fowleri meningo-encephalitis. Cursons et al. (1980a), have shown that the humoral antibody response consists of very high IgM levels, and since the immune response is a progressive change from IgM to IgG, this suggested

persistant exposure to PFLA. The same group found a factor, present only in fresh adult sera capable of inhibiting a CPE in vero cell cultures from Acanthamoeba culbertsoni (A-1), which suggests normal human sera has "natural antibody" to Acanthamoeba spp.

The role of cell mediated immunity (CMI) in resistance to infection by N.fowleri has been reported. Guinea pigs surviving a normally fatal challenge with N.fowleri, exhibited a delayed hypersensitivity when tested intradermally with a soluble fraction derived from N.fowleri (Diffley et al., 1976: Cursons, et al., 1977, 1980b). Thong (1978) stated that protective immunity to PAM could be transferred to syngeneic mice by immune sera but not by immune spleen cells. Whether agglutination antibodies demonstrated in immune sera, or antitoxic antibodies in immune sera are the active principle is not certain. All this evidence lends support to the hypothesis that unwitting exposure to the more ubiquitous non-pathogenic N.gruberi may immunize against N.fowleri, and the same may occur with Acanthamoeba spp. (Martinez, 1980). The fact that some underlying immunity exists, was demonstrated by Wong et al., (1975), who showed that primates were apparantly immune to intranasal or intravenous inoculations of N.fowleri or A.culbertsoni unless on immunosuppressive drugs. However intrathecal inoculations were shown to cause amoebic meningo-encephalitis. Culbertson (1971) has shown that mice immunized with Acanthamoeba spp. are resistant to intranasal challenge with A.culbertsoni, but the same was not able to be shown with Naegleria spp.

1.5. Diagnosis

Early diagnosis and treatment, along with careful intensive care therapy is extremely important in the treatment of infections due to PFLA; more so in those caused by Naegleria spp. The survival of a nine year old female in Torrance, California (Seidel et al., pers.comm.),

and that of a fourteen year old male in Australia (Anderson & Jamieson, 1972) could be attributed to this.

Infections due to _Naegleria_ spp. are usually characterized by a previous history of swimming in freshwater some 7 - 14 days before expressing typical meningitis symptoms (Cursons _et al._, 1977; Carter, 1972; Chang, 1974). The symptoms include severe headache (usually frontal), sore throat, nausea, vomiting, fever (39 - 41°C) accompanied by a stiff neck. Clinical isolation of amoebae can routinely be done by cultivation of Cerebral Spinal Fluid (CSF), brain tissue or nasal discharge on Pages's Amoeba Saline agar (PAS agar) spread with live _Escherichia coli_ or _Enterobacter cloacae_; by axenic CYM culture; or passaging suspected material through cell culture, at 37 - 45°C (Cursons _et al._, 1978). The examination of CSF is probably the most routine method of diagnosing general meningitis. The differences between amoebic and bacterial meningitis are slight, and although in positive amoebic cases there tends to be a predominance of neutrophils in the CSF, a high protein and low sugar levels, complete diagnosis relies on finding amoebae in the fluid and further cultivation of these for complete diagnosis. Species identification can then be achieved by a method outlined by Cursons & Brown (1976), an alternative but similar method was outlined by Cerva (1980).

In post-mortem diagnosis, a degree of encephalitis is invariably present. Severe brain swelling and redness, combined with purulent and haemorrhagic exudate containing numerous amoebae is more extensive on the ventral surface of the cerebrum or cerebellum and over the brain stem. Amoebae are also numerous in the olfactory nerve bundles which are virtually destroyed by purulent inflammation (Carter, 1969, 1972; Duma, 1978). The grey matter of the cerebral hemispheres and cerebellum shows variable sized lesions which tend to

be haemorrhagic and quite soft when large (Culbertson, 1971). Purulent meningitis is usually inconspicuous and confined to the antero-basal aspects of the brain, and it is only rarely that one can find inflammation or amoebic invasion in the posterior cerebral hemispheres, brain stem or cerebellum, and never in the spinal cord (Carter, 1969, 1972; Duma, 1978).

Indirect Fluorescent Antibody (IFAB) technique applied to either Naegleria or Acanthamoeba spp. is a valuable tool in the identification of species (Cerva & Kramar, 1973; Cursons et al., 1976a ; De Jonckheere et al., 1974). It can also be applied to identify amoebae in brain sections of victims. IFAB methods can also be used to provide rapid screening methods for detection of PFLA in swimming pools, tap and other domestic and recreational water supplies.

Immunoperoxidase methods have been used to demonstrate both Naegleria and Acanthamoeba spp. in brain sections of patients who have died from PAM and GAE respectively, by Culbertson (1975) and Cursons et al. (1976). This is a method that may be shown to be more valuable in the future than immunofluorescence techniques. It has certain advantages over IFAB in that permanent preparations can be made, no specialised equipment is necessary and clear definitive staining of tissue elements results (Culbertson, 1975).

Isoenzyme electrophoresis is rapid, reproducible, simple, and inexpensive, and does not require aminal handling facilities, nor is it reliant on morphological cyst structures which vary with culture conditions. It can be used for diagnosis as well as environmental monitoring (Nerad, 1979).

GAE infections are difficult to diagnose even in advanced cases, due to the lack of specific symptoms and the apparent lack of

amoebae in the CSF (Chang, 1974), although Martinez (1980) reported a method of staining CSF and distinguishing Acanthamoeba in the CSF. There is usually a history of poor health and immunological incompetence with few patients giving a past history of swimming (Martinez, 1980). The onset is slow (longer than 10 days) and insidious, with the lung, brain, and kidneys being infected (Martinez et al., 1977; Martinez 1979). Acanthamoeba infections may initially produce a severe bronchopneumonia, the organisms then disseminating and reaching the CNS via the bloodstream (Marino, 1975; Martinez et al., 1980).

Post mortem diagnosis relies on the presence of superficial lesions in the grey matter with granulomatous inflammation, and the presence of trophozoites and double walled wrinkled cysts in apparently normal tissue bordering the lesion (Chang, 1974; Carter, 1972; Culbertson, 1971; Hoffman et al., 1978; Martinez et al., 1980; Martinez, 1980), and these authors regard this as diagnostic of Acanthamoeba infections.

In the case of eye infections reported by Nagington et al. (1974) and Jones et al. (1975), positive diagnosis was possible by taking corneal scrapings, with subsequent isolation and identification of Acanthamoeba spp.

1.6 Treatment

The drug treatment of PAM has been very discouraging, with only 2 documented cases of survivors of the disease with 9 other possible survivors (Elmsly, 1980). In the earlier cases where the amoebic nature of the disease had not been suspected, treatment consisted only of antibacterial agents such as supha-drugs, penicillin, streptomycin, tetracyclines and chloramphenicol (Fowler & Carter, 1965; Butt et al., 1968; Cerva & Novak, 1968; Dos Santos, 1970; Van den Driessche et al.,

1973). However, even in later cases where the antiprotozoal drugs emetine, chloroquine, and metronidazole, were often used, the course of the disease was not affected in the slightest (Carter, 1968, 1970, 1972; Duma *et al.*, 1971), except for one unproven case (Grundy & Blowers, 1970) in which survival was attributed to chloroquine. *Naegleria* were supposedly isolated from the CSF but failed to survive for any length of time in culture and were consequently not positively identified. Also, the patient presented atypical symptoms.

The *in vitro* activity of antibacteria agents against pathogenic *Naegleria* has been extensively reviewed (Carter, 1969; Mandal *et al.*, 1970; Thong *et al.*, 1977; Lee *et al.*, 1979; Donald *et al.*, 1979). Of the antiprotozoal drugs, emetine hydrochloride was shown to be effective *in vitro* against *N.fowleri* (Carter, 1969; Prasad, 1972; Das, 1975) although it does not protect animals from the disease (Culbertson *et al.*, 1968), probably due to its inability to pass the blood-brain barrier (Parmer & Cottrill, 1949). Chloroquine and metronidazole have also been shown to be ineffective, both in *in vitro* and *in vivo* studies (Carter, 1969; Mandal *et al.*, 1970; Duma *et al.*, 1971). Amphotericin B, is an antifungal polyene antibiotic, which has been shown to be very effective in *in vitro* studies against pathogenic *Naegleria* spp. (Carter, 1969; Mandal *et al.*, 1970; Duma *et al.*, 1971; Schuster & Rechthand, 1975; Visvesvara & Balamuth, 1975; Duma & Finley 1976; De Jockheere & Van de Voorde, 1977; Donald *et al.*, 1979) and to show *in vivo* promise (Culbertson *et al.*, 1968; Carter, 1969; Das, 1971; Thong, 1978, 1979, 1980; Elmsly, 1980; Seidel *et al.*, pers. comm.). It is however a very toxic drug, probably binding with sterols in the plasma membrane, causing "holes" in it and consequent leakage of cellular contents, not only of *Naegleria* spp. cells, but all cells in contact with this drug.

Amphotericin B was used in the treatment of all survivors except the unproven case of Grundy and Blowers (1970) and Callicott et al. (1968). In vitro and in vivo tests have shown a synergistic activity between amphotericin B and tetracycline (Donald, 1979; Thong et al., 1979; Elmsly, 1980) and amphotericin B and oxytetracycline (Elmsly, 1980).

The most recent successful treatment of an N.fowleri PAM case in 1978, is that of a nine year old female caucasian from Torrance, California, who showed the typical symptoms of meningo-encephalitis three days prior to admission to hospital (Seidel et al., pers. comm.). Routine CSF cell count procedures revealed organisms with amoeboid movements. The following treatment was given:

Amphotericin B	-	1.5 mg Intrathecal (IT)
Amphotericin B	-	40 mg Intravenous (IV)
Sulfadiazine	-	2 g Intravenous (IV) (50 $mg.kg^{-1}$)
Chloramphenicol	-	1 g Intravenous (IV) (25 $mg.kg^{-1}$)
Penicillin	-	3.4×10^6 units Intravenous (IV)

The patient was then transferred to Harbour General Hospital, where on admission, she was in a coma, but responsive to tactile stimulation and pain. The following treatment was administered:

(i) Amphotericin B was given IV at a dose of 1.5 $mg.kg^{-1}.day^{-1}$ given in two doses daily for three days, after which it was decreased to 1 $mg.kg^{-1}.day^{-1}$ given in a single daily dose for six days.

(ii) Amphotericin B was also given IT at 1.5 $mg.day^{-1}$ for two days, after which it was decreased to 1 mg every other day for eight days, administered through a lumbar intrathecal catheter.

(iii) Miconazole was given IV at 350 $mg.m^{-2}.day^{-1}$ three times a day for nine days.

(iv) Miconazole was given IT at a dose of 10 $mg.day^{-1}$ for two days then 10 mg every other day for eight days.

(v) Rifampin was given orally at a dose of 10 $mg.kg^{-1}.day^{-1}$ three times a day for nine days.

Sulphadiazine was given at a dose of 4 $g.day^{-1}$ for three days until after the diagnosis of N.fowleri meningo-encephalitis was confirmed. Penicillin and chloramphenicol were continued for three days until bacterial CSF cultures were found to be negative. Dexamethasone and diphenylhydantoine were given for increased intracranial pressure and seizure activity respectively. The patient stabilized clinically over the first forty eight hours. Gradually over the subsequent month of hospitalisation, her mental status improved, and she had no significant neurological deficits noted at discharge (Seidel et al., pers. comm.).

The treatment of Acanthamoeba infections has been far from successful (Kerrohan et al., 1960; Jager & Stamm, 1972; Robert & Rourke, 1973; Nagington et al., 1974; Bhagwandeen et al., 1975; Martinez et al., 1977, 1980; Ma et al., 1981, Seilhamer & Byers, 1978). Except in the case of eye infections (Nagington et al., 1974, 1976) the main problem in Acanthamoeba infections is the difficulty of diagnosis, and all recorded cases of GAE have been diagnosed post mortem. Even in advanced cases, there is often a lack of specific signs and symptoms indicative of the disease, and no amoebae are seen in the CSF (Chang, 1974).

Carter (1972) suggested that sulphadiazine be used in addition to amphotericin B, in the treatment of PAM in case the causative agent should prove to be an Acanthamoeba spp. To date, the only clincical experience with sulphadiazine in the treatment of humans, is that reported by Nagington et al. (1974), where it was used to treat an eye infection. Amphotericin B has been shown to be ineffective against Acanthamoeba spp.

aganist Acanthamoeba spp. at all levels attainable in humans (Duma & Findley, 1976; Visvevara & Balamuth, 1975; Casemore, 1970). However, Donald (1979) found that A.castellanii (1501) was not susceptible but 100 $ug.cm^{-3}$ was amoebicidal for A.culbertsoni (A-1). Experiments with 5-fluorocytosine (Stevens & O'Dell, 1974; Donald, 1979) have shown it to be of limited use in human Acanthamoeba infections, especially if treatment was not initiated until some time after infection.

1.7 Occurrence and Distribution

The ability of PFLA to form resistant cysts undoubtedly enables them to withstand unfavourable conditions, e.g. Acanthamoeba spp. have been isolated from Antarctic soils (Brown et al. pers. comm.) and from water at $2^{o}C$ (Brown & Cursons, 1977), but also to take advantage of intermittent favourable conditions. The PFLA appear to be truly ubiquitous organisms, as isolations have been recorded from a variety of environmental sources such as air (Kingston & Warhurst, 1968; Lawande et al., 1979) humidifier systems (MRC Symposium, 1977; Rowbottom, 1980; Edwards et al., 1978) freshwater, marine and brackish waters (De Jonckheere et al., 1975; Brown & Cursons, 1977; Mascaro et al., 1981; Stevens et al., 1977; Wellings et al., 1977, 1979; Duma, 1981) factory effluent (De Jonckheere 1978, 1981; De Jonckheere et al., 1977; Dive et al., 1981; Duma, 1981) chlorinated swimming and domestic waters (Cerva, 1971; Anderson & Jamieson, 1977, Cerva & Huldt, 1974) from bottled drinking water (Desmet-Paix, 1974) from a home dialysis unit (Casemore, 1977) from soil (Anderson & Jamieson, 1972, 1974; Cursons et al., 1978b; Cursons et al., 1979a) from sewage (Singh & Das, 1972; Chang, 1974) they have also been isolated from a municipal drinking water supply (Chang, 1972) in chlorinated swimming pools (De Jonckheere, 1979; Kadlec et al., 1978, 1980; Cerva & Huldt, 1974). Isolations have slso been recorded from cell cultures (Jahnes et al., 1957; Stevens &

O'Dell, 1973; Willaert et al., 1978) throat and nasal cavities (Elridge & Tobin, 1967; Cerva et al., 1973; Chang et al., 1975) eye infections (Nagington et al., 1974; Visvesvara et al., 1975) gastriontestinal washings (Hoeffler & Rubel, 1974), cold-blooded vertebrates (Frank, 1974), snails (Kingston & Taylor, 1976) fish tanks, and fish (Taylor, 1977; De Jonckheere et al., 1979b), dogs (Glastonbury & Frauenfelder, 1981) and sheep (Young et al., 1980).

Temperature and pH are equally tolerated over a wide range (Dawson et al., 1982) with *in vitro* growth reported up to 45°C (Griffin, 1972) and as low as 4°C for cyst survival (Warhurst et al., 1980), and a pH range of 4.6 to 9.5 (Carter, 1970).

The distribution of the pathogenic species in relation to non-pathogenic ones is not yet know (Cursons, 1978). In general, non-pathogenic species are more prevalent at ambient temperatures in temperate zones than the pathogenic species which occur at supra-normal temperatures. The repeated isolations of PFLA from waters above ambient temperature, i.e. greater or equal to 30°C (De Jonckheere et al., 1975, 1977; Stevens et al., 1977; Wellings et al., 1977, 1977a; Wellings, 1979; Cursons et al., 1978b; De Jonckheere, 1978, 1980) combined with their higher optimu- temperature of growth (Griffin, 1972) suggests that the pathogenic species are environmentally selected over non-pathogenic species in waters above ambient temperature. Wellings (1979) has shown that fluctuating temperatures are detrimental to pathogenic *Naegleria* cysts, and suggest that this may cause the low level of pathogenic *Naegleria* populations in thermally enriched lakes or rivers, where the thermal enrichment is periodic rather than constant. However, where thermal water discharge was constant De Jonckheere et al., (1975, 1977) and Duma. (1981), found that a selection for pathogenic *Naegleria* spp. existed. In a study of one

particular lake, the water of which was used to cool a power generating plant, Duma (1981) found that prior to the plant opening, and after it was closed, the number of pathogenic Naegleria spp. isolated was no greater than other lakes tested in the same study, but during thermal enrichment , pathogenic Naegleria spp. were isolated in significantly greater numbers. The period of this study was 3 years, 1 year prior to thermal enrichment, 1 year during, and 1 year after enrichment ceased.

PFLA are significant members of the soil community, and are possibly the chief contributing factor to the decline of certain bacterial populations in the soil (Anderson & Jamieson, 1974). Cursons et al., (1978b) and Wellings et al., (1977), and Wellings, (1979) have isolate PFLA from the soil also , which according to Singh (1978) is the preferred habitat of PFLA, implicates the soil as being the resevoir of pathogens, and that contamination occurs via run off after rain (Cursons, 1978). However, De Jonckheere (1979) suggests that because of the relatively high numbers of amoebae in nasal and pharyngeal swabs that humans could contaminate pools, rather than, or more likely, as well as the rain-soil-pool-human route. Wellings (1979) found that Naegleria spp. were able to overwinter in the sediment of some Florida lake beds, with the discovery that both pathogenic and non-pathogenic Naegleria cysts can survive under anaerobic conditions.

Acanthamoeba spp. are isolated from the environment more often than Naegleria spp. In a lake in the U.S.A. Naegleria spp. were isolated monthly over a one year period, the numbers being low but constant. Acanthamoeba spp. were found more often particularly on the bottom of the lake, the numbers fluctuated over the same period, but were not correlated to any known factor e.g. pH, temperature, bacterial count, or nitrate and phosphate levels (O'Dell, 1979).

In a surey, De Jonckheere (1979c), found the swimming pools,

or filters were contaminated in about 65% of the European pools tested, and in up to 90% of the pools tested in U.S.A. although the numbers per pool in the U.S.A. were lower, and no virulence tests were carried out. Occurrence of amoebae in chlorinated swimming pools is probably due to the introduction of amoebae by persons swimming in thermal waters, walking in wet soil (De Jonckheere et al., 1977; Cursons, 1978; Cursons et al., 1980).

In a study of lakes in Virginia, U.S.A., Duma (1981) attempted to correlate various factors i.e. pH, temperature, bacterial flora, limnological content, etc., of the water, with the isolation of pathogenic Naegleria spp. Water temperature was found to correlate best with the presence of these amoebae, with temperatures of above 30°C indicating that the presence of these pathogenic amoebae is more likely. However other factors, which may also be important, involved the presence or absence of two species of bacteria, and the ion content of the water. Two bacteria species frequently isolated from lake waters, Pseudomonas fluorescens and Serratia marsescens, were found in laboratory test to be inhibitory to pathogenic Naegleria spp., and further, Duma found that every lake from which Ps.fluorescens was consistantly isolated no pathogenic Naegleria were isolated.

1.8 Control Measures

Free-living amoebae are widely dispersed in the environment and the fact that they can be isolated from chlorinated domestic and swimming waters (Cerva, 1971, 1977, 1980; Cerva & Huldt, 1974; Kadlec et al., 1978, 1980; De Jonckheere et al., 1976; De Jonckheere & Van de Voorde, 1976; De Jonckheere, 1979a; Cursons, 1978; Cursons et al., 1980), as well as untreated recreational waters has led to an expression of concern by public health authorities over the possible contraction of PAM or GAE via these sources.

Cerva (1971), after reviewing 16 fatal cases of PAM from an indoor chlorinated swimming pool, stated that there will always be the constant presence of limax amoebae, even under the strictest obervations of all routine safety measures applied to swimming pools and water systems. This was supported by a reported case of PAM in South Australia by Anderson and Jamieson (1972), in which the victim contracted the disease from domestic bath water, and that superchlorination to 10 $mg.l^{-1}$ failed to erradicate Naegleria from the contaminated pool. However, Lyons and Kapur (1977), in a survey of 30 halogenated public swimming pools concluded that the low amoebic densities (less than one per litre), in the majority of pools demonstrated that these amoebae could be adequately controlled by proper pool maintainance. However, the possession of resistant cysts complicates the disinfection process.

Derreumaux et al. (1974) demonstrated that 0.5 $mg.l^{-1}$ of HOCl, the active disinfecting component of chlorine disinfection, was able to erradicate both Naegleria and Acanthamoeba spp., although Cursons et al. (1980) agreed for Naegleria, they found that a higher concentration of 1.25 $mg.l^{-1}$ was required to erradicate Acanthamoeba spp. De Jonckheere and Van de Voorde (1976) showed that chlorination of pools was practicable, in that an initial concentration of chlorine between 0.5 - 1.0 $mg.l^{-1}$ was cystidal for Naegleria spp. but that A.culbertsoni cysts were not inactivated by levels up to 40 $mg.l^{-1}$.

Chang (1978) examined the response to some physical and chemical agents, e.g. drying, heat, lyophilisation, chlorination. He found that the pathogenic N.fowleri was only better able to survive at higher temperatures, in all other conditions tested, the non-pathogenic N.gruberi was more tolerant and remained viable for longer periods. He also said that measurements of chlorine levels in water should

measure the residual free chlorine, not the amount of chlorine added, and that this is a possible reason for the PAM cases in South Australia.

CHAPTER TWO: MATERIALS

2.1 Amoeba Cultures Used

Table I: Amoeba Cultures Used

Species	Strain	Pathogenic	Source
Naegleria fowleri	MsM	+	MU
Naegleria fowleri	$MsMrb_1$	+	MU
Naegleria fowleri	$MsMrb_2$	+	MU
Naegleria fowleri	$MsMrb_3$	+	MU
Naegleria fowleri	$MsMrb_4$	+	MU
Naegleria fowleri	MsT	+	MU
Naegleria fowleri	$MsTrb_1$	+	MU
Naegleria fowleri	Northcott	+	ACH
Naegleria gruberi	P1200f	-	NHI
Naegleria gruberi	$P1200frb_1$	-	MU
Naeglerai gruberi	$P1200frb_2$	-	MU
Acanthamoeba culbertsoni	A-1	+	CCAP
Acanthamoeba castellanii	1501	-	IMTPL

+ = Positive

- = Negative

NHI = National Health Institute, Wellington, N.Z.

CCAP = Culture Centre for Algae and Protozoa, England.

IMTPL = Institute de Medicine Tropical Prins Leopold, Belgium.

MU = Massey University, Palmerston North, N.Z.

ACH = Adelaide Childrens Hospital, Australia.

2.2 Ciliate Culture Used

Tetrahymena pyriformis L1630/1W obtained from CCAP.

2.3 Bacteria Cultures Used

Table II: Bacteria Cultures Used.

Species	Gram Reaction	Motility	Pigmentation
Enterobacter cloacae	-	+	-
Klebsiella aerogenes	-	+	-
Pseudomonas aeruginosa	-	+	+
Pseudomonas fluorescens	-	+	+
Escherichia coli	-	+	-
Micrococcus luteus	+	+	-
Serratia marcescens	-	+	+
Bacillus subtilis	+	-	+

- = negative

+ = positive

2.4 Plate media

2.4.1 Amoeba Saline Agar (Page, 1967; Cursons et al., 1978a) for the isolation of Naegleria spp.

NaCl	=	0.12g
$MgSO_4.7H_2O$	=	0.004g
$CaCl_2.2H_2O$	=	0.004g
Na_2HPO_4	=	0.142g
KH_2PO_4	=	0.136g
Agar	=	15.0g
Distilled water	=	1.0 litre

pH 6.8

Autoclave at 103.4 KPa, 121°C, for 15 minutes

2.4.2 Amoeba 1% Saline Agar (Cursons, 1978, modified from Page, 1967) for the isolation of Acanthamoeba spp

NaCl	=	10.0g
$MgSO_4.7H_2O$	=	0.004g
$CaCl_2.2H_2O$	=	0.004g
Na_2HPO_4	=	0.142g
KH_2PO_4	=	0.136g
Agar	=	15.0g
Distilled water	=	1.0 litre

pH 6.8

Autoclave at 103.4 KPa, 121°C, for 15 minutes

2.4.3 Amoeba Saline Bacto Agar (PASB) (Cursons, 1978, modified from Fulton, 1970) for plaque counting of Naegleria spp. and Acanthamoeba spp.

NaCl	=	0.12g
$MgSO_4.7H_2O$	=	0.004g
$CaCl_2.2H_2O$	=	0.004g
Na_2HPO_4	=	0.142g
KH_2PO_4	=	0.136g
Difco Bacto-peptone	=	2.0g
Agar	=	15.0g
Distilled water	=	1.0 litre

pH 6.8

Autoclave at 103.4 KPa, 121°C, for 15 minutes

2.4.4 Nutrient Media Agar (Fulton, 1970) for plaque counting of *Naegleria* spp. and *Acanthamoeba* spp.

Difco Bacto-peptone	=	2.0g
KH_2PO_4	=	1.0g
K_2HPO_4	=	1.5g
Glucose	=	2.0g
Agar	=	20.0g
Distilled Water	=	1.0 litre

pH 6.8

Autoclave at 103.4 KPa, 121°C, for 15 minutes.

2.4.5 Plate Count Agar for dilution plate counting of bacteria

Difco Bacto Plate Count Agar	=	23.0g
Distilled Water	=	1.0 litre

pH 6.8

Autoclave at 103.4 KPa, 121°C, for 15 minutes.

2.4.6 Brain Heart Infusion Agar for the culture of bacteria

Difco Bacto Brain Heart Infusion	=	37.0g
Agar	=	20.0g
Distilled Water	=	1.0 litre

pH 6.8

Autoclave at 103.4 KPa, 121°C, for 15 minutes.

2.5 Axenic Media for Amoebae

2.5.1 Pages Amoeba Saline (PAS) (Page, 1967) for diluting out either *Naegleria* spp. or *Acanthamoeba* spp. and as a base for CYM Medium.

NaCl	=	0.12g
$CaCl_2.2H_2O$	=	0.004g
$MgSO_4.7H_2O$	=	0.004g

Na_2HPO_4	=	0.142g
KH_2PO_4	=	0.136g
Thiamine HCl	=	0.001g
d - Biotin	=	0.002g
Vitamin B12	=	0.000001g
L - Methionine	=	0.0895g
Distilled Water	=	1.0 litre

pH 6.8

Autoclave at 103.4 KPa, 121°C, for 15 minutes.

2.5.2 CYM Medium (Cursons et al., 1978; modified from O'Dell and Stevens, 1973a) for the axenic cultivation of Naegleria spp.

Gluxose	=	10.0g
Difco Yeast Extract	=	5.0g
Difco Casitone	=	10.0g
Pages Amoeba Saline	=	1.0 litre

pH 6.8

Autoclave at 103.4 KPa, 121°C, for 15 minutes.

To 4.5 cm^3 CYM add aseptically 0.5 cm^3 of the following cocktail:

Sterile Serum	=	50.0 cm^3
Sterile Hemin	=	10.0 cm^3
Sterile Distilled Water	=	40.0 cm^3
Penicillin/Streptomycin	=	200,000 units. cm^{-3}

2.5.3 4.0% Neff Medium (Stevens & O'Dell, 1973) for the axenic cultivation of *Acanthamoeba* spp.

Difco Proteose Peptone	=	40.0g
Glucose	=	15.0g
Difco Yeast extract	=	7.5g
$MgSO_4.7H_2O$	=	0.2465g
$CaCl_2.2H_2O$	=	0.0109g
KH_2PO_4	=	0.27218g
Ferric Citrate	=	0.0335g
d - Biotin	=	0.002g
Thiamine HCl	=	0.001g
Vitamin B12	=	0.000001g
Distilled Water	=	1.0 litre

pH 6.8

Autoclave at 103.4 KPa, 121°C, for 10 minutes.

To 9.0 cm^3 of 4.0% Neff medium, is added 1.0 cm^3 of Penicillin/Streptomycin solution (200,000 units.cm^{-3}).

2.6 Axenic Media for Ciliates

2.6.1 Tetrahymena medium (Dobra *et al.*, 1980, modified by Dawson) for the axenic cultivation of *Tetrahymena pyriformis*

Glucose	=	14.0g
Bacto Yeast Extract	=	14.0g
Casamino Acids	=	32.0g
$MgCl_2$	=	0.5g
KH_2PO_4	=	3.5g
K_2HPO_4	=	3.5g
Panmycin	=	0.0175g

Distilled Water = 3.650 litre

pH 6.8

Autoclave at 103.4 KPa, 121°C, for 15 minutes.

2.7 Bacterial Growth Medium

2.7.1 Brain Heart Infusion (BHI)

Difco Bacto Brain Heart Infusion = 37.0g

Distilled water = 1.0 litre

pH 6.8

Autoclave at 103.4 PKa, 121°C, for 15 minutes.

2.7.2 Pages Amoeba Saline Glucose (Brown pers. comm. 1981 modified from Page, 1967.) for slow growth of bacteria in amoeba growth support experiments.

NaCl	=	0.12g
$MgSO_4.7H_2O$	=	0.004g
$CaCl_2.2H_2O$	=	0.004g
Na_2HPO_4	=	0.142g
KH_2PO_4	=	0.136g
Glucose	=	0.0005g
Thiamine HCl	=	0.001g
d - Biotin	=	0.002g
Vitamin B12	=	0.000001g
L - Methionine	=	0.0895g
Distilled water	=	1.0 litre

pH 6.8

Autoclave at 103.4 KPa, 121°C, for 15 minutes.

2.8 Miscellaneous Solutions

2.8.1 Sodium Phosphate Buffer 0.01M

Na_2HPO_4	=	14.2g
$NaH_2PO_4.2H_2O$	=	18.0g
Distilled Water	=	1.0 litre

pH 7.0

Autoclave at 103.4 KPa, 121°C, for 15 minutes.

2.8.2 Phosphate Fuffered Saline (PBS)

NaCl	=	8.5g
Na_2HPO_4	=	1.28g
NaH_2PO_4	=	0.156g
Distilled Water	=	1.0 litre

pH 7.6

Autoclave at 103.4 KPa, 121°C, for 15 minutes.

2.8.3 Baquacil Indicator Solution (Alford, pers. comm. 1980)

for the estimation of Baquacil in aqueous solution.

Gelatin	=	2.5g
Eosine Y	=	0.06g
Distilled Water	=	0.260 litre

Dissolve gelatin in a little warm distilled water, make up to 250.0 cm^3 with distilled water. Add 10.0 cm^3 of 0.6 % Eosine Y solution, 0.06g Eosine Y dissolved in 10.0 cm^3 distilled water.

2.8.4 200 ppm Hard Water

$CaCl_2.2H_2O$	=	0.00043g
$MgCl_2.2H_2O$	=	0.00028g
$NaHCO_3$	=	0.00045g
Distilled Water	=	1.0 litre

2.8.5 DPD Reagents (Palin, 1974) for the chemical analysis of chlorine and chlorine dioxide.

2.8.6 Reagents for BOD (World Health Organisation, 1963) for the determination of the BOD of a body of water.

2.8.7 20 % Bovine Faecal Matter Solution (BFM)

Dried and Powdered Bovine Faecal Matter = 20.0g

Distilled Water = 1.0 litre

Autoclave at 103.4 KPa, 121°C, for 15 minutes.

Prepared BFM in the laboratory by obtaining a large cowpat, drying at 120°C, for 4 hours , then ground in a mortar and pestle.

2.9 Disinfectant Solutions Used

2.9.1 Water

All Water was distilled and deionized.

2.9.2 Chlorine

A colution of sodium hypochlorite (NaOCl) was obtained from BDH and diluted as required with sterile, chlorine free, distilled, deionized water.

2.9.3 Chlorine Dioxide

Chlorine dioxide was generated in the laboratory (See Method 3.5.7.).

2.9.4 Baquacil

Baquacil (Poly hexamethylene biguanide hydrochloride) solution was obtained from ICI N.Z. Ltd.

CHAPTER THREE: METHODS

3.1 Sterilisation

All Glassware, except that used for analytical work was sterilised at 103.4KPa, 121°C, for 15 minutes, Analytical Glassware was sterilised by dry heat at a minimum of 160°C for two hours.

3.2 Axenic Culture Techniques

3.2.1. Maintaninance of amoeba stock cultures.

Axenic cultures of all N.fowleri and N.gruberi strains were maintained in CYM Medium, A.culbertsoni (A-1) and A.castellanii (1501) were maintained in 4.0% Neff medium. Pathogenic species were incubated at 37°C and subcultured every 24 hours, and non-pathogenic species were incubated at 30°C and subcultured every 48 hours. All stock cultures were maintained in universal bottles and incubated on rotary gyroshakers (120 r.p.m.)

3.2.2. Maintainance of Tetrahymena stock cultures

T.pyriformis were subcultured every 4 days, 0.4cm^3 of culture inoculated into 10 cm^3 of sterile Tetrahymena medium. The cultures were incubated at room temperature.

3.2.3. Bacteria culture

Bacteria of the required species were inoculated into 10 cm^3 sterile BHI broth, from a spread plate on BHI agar, by sterile loop, and incubated overnight at the relevant temperature. Then a sterile loop inoculum of the overnight culture was subcultured into a further

10 cm^3 sterile BHI broth, and incubated overnight at the relevant temperature. These second broth cultures were used for the tests.

3.3 Monoxenic Amoeba Culture Techniques.

Axenic cultures of amoebae, 18 - 24 hours old, were centrifuged at 425 x g for 10 minutes, washed with sterile PAS, and centrifuged again, this was repeated three times. After resuspending in 5 cm^3 PAS, 0.5 cm^3 of this amoeba suspension was transferred aseptically to a sterile PAS agar plate. Then 0.1 cm^3 of an overnight culture of Enterobacter cloacae grown in BHI broth was added. To this was added 5 cm^3 PAS solution. All strains of amoeba plus bacteria suspension on plates, were incubated at the appropriate temperature i.e. Pathogens at 37°C and non-pathogens at 30°C - and subcultured every 48 hours. This was by 2 cm^3 of amoeba plus bacteria suspension transferred to a fresh sterile PAS agar plate, and 5 cm^3 PAS solution added.

3.4 Growth Experiments

3.4.1 Amoebae

To determine whether a particular species of bacteria would support the growth of amoebae, standard batch culture techniques were used. Into a 250 cm^3 erlenmeyer flask, was dispersed 48 cm^3 of sterile PAS Glucose solution. Amoebae were centrifuged from the culture media at 425 x g for 10 minutes, then resuspended in sterile PAS Glucose solution and washed. This was repeated three times. The amoebae were then counted

in a modified Fuchs-Rosenthal haemocytometer, and diluted to a final concentration of 5×10^5 cells.cm^{-3}, 1.0 cm^3 of which was then added to the flask. The overnight culture of bacteria was diluted to 5×10^7 cells.cm^{-3} with reference to an optical density standard curve, and 1.0 cm^3 of this suspension was added to the flask to make a final volume of 50 cm^3 test culture. These cultures were incubated at 30^oC on a gyroshaker at 120 r.p.m. Daily samples were taken for up to 7 days, the amoebae were counted in a modified Fushs-Rosenthal haemocytometer, and bacteria by the dilution plate method, in plate count agar.

3.4.2 Tetrahymena

The same bacteria species were used as in Methods 3.4.1., the method was the same, and the initial inoculum of T.pyriformis was 1×10^4 cells.cm^{-3} also (1.0 cm^3 of a 5×10^5.cm^{-3} suspension).

3.4.3. Amoeba - Tetrahymena Competition

The species of bacteria tested in Methods 3.4.1. and 3.4.2. that supported the growth of either or both of the protozoans tested, were used as the growth support organism to determine the extent of competition for the bacteria between amoebae and ciliates. The method was as in Methods 3.4.1. and 3.4.2.

3.5 Biochemical Oxygen Demand Test (BOD_5)

Standard methods for BOD_5 determination were used (World Health Organisation, 1963.), except that in the preparation of BFM, both powder and solution, it was sterilised, and sterile deionised water was used as the

diluent for the powder, and to make the solution. For these reasons, bacteria had to be introduced to the system. This was done using 1.0 cm^3 of a 2.5 x 10^6 cells.cm^{-3} suspension, diluted with reference to an optical density standard curve, of an overnight culture of E.cloacae.

3.6 Production of Disinfectants

3.6.1 Chlorine

A stock solution of sodium hypochlorite (BDH) was diluted to the appropriate concentration using sterile, chlorine free, deionized distilled water.

3.6.2 Chlorine dioxide

This was produced as recommended by the American Public Health Association (1971) see fig. 1. 5.0 g of sodium chlorite was dissolved in 375.0 cm^3 of deionized water, mixed and transferred to flask E. A smooth current of air was passed through the system and 5.0 cm^3 increments of H_2SO_4 introduced from E into B at 5 minute intervals. The airflow was continued for a further 30 minutes, and the solution in bottle D stored in a brown bottle at 4 ^oC.

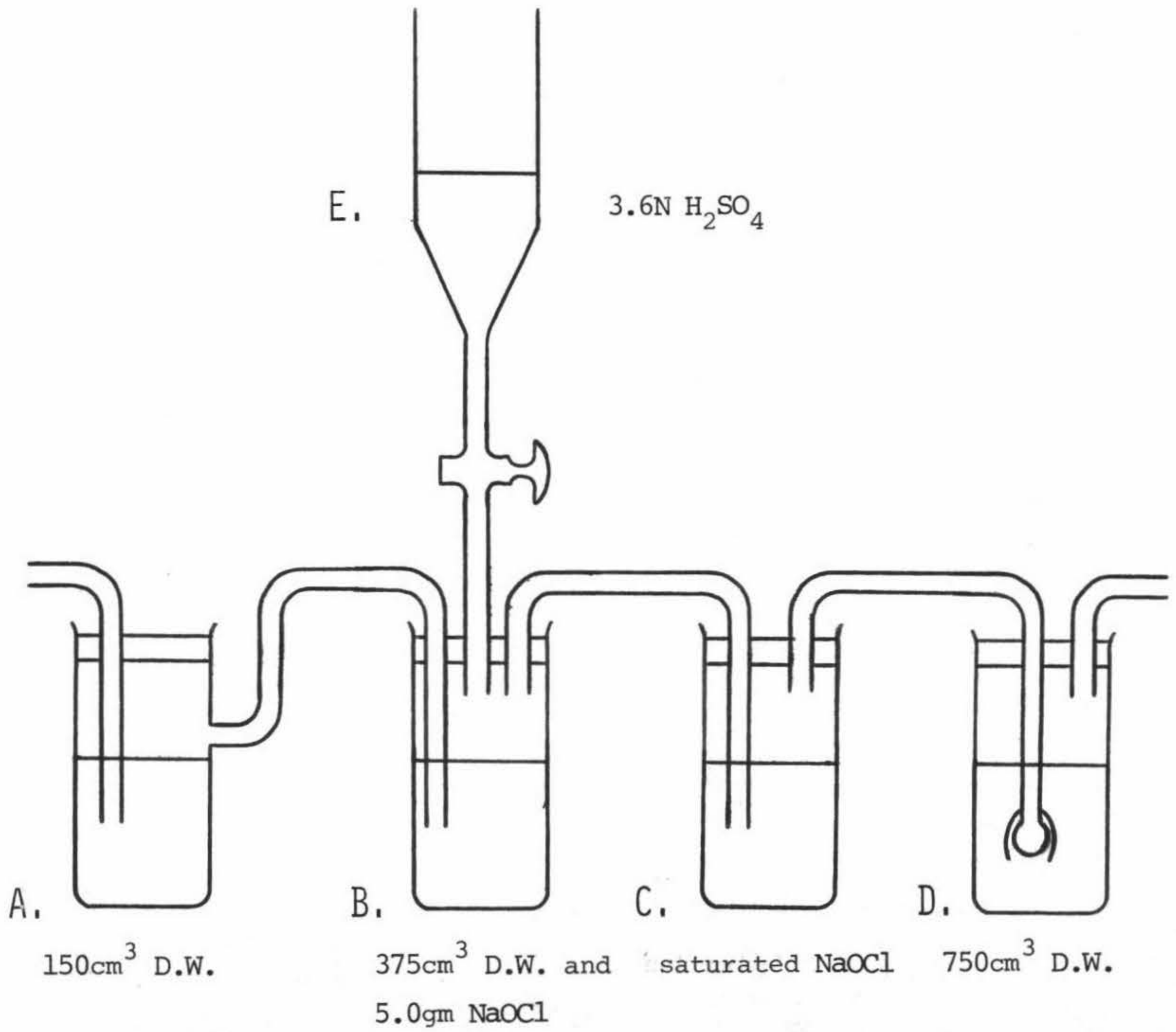
3.63 Baquacil

A stock solution of baquacil was obtained from ICI (NZ) Ltd and diluted to the appropriate concentration, using sterile 200ppm hard water.

3.7 Chemical Analysis of Disinfectants

Chlorine and chlorine dioxide were analysed by the DPD method (Palin, 1974). Baquacil was analysed by the method.

Figure 1 Method used for the production of chlorine dioxide.
(Cursons, 1978).



recommended by the manufacturers. The indicator solution was made as in Materials 2.8.3. Of this indicator solution, 1.0 cm^3 was added to 10 cm^3 of sample, or standard solution. A standard curve, of absorbance at 540 nm vs Baquacil concentration was constructed and used to determine the residual baquacil concentration. The colorimeter used was a Unicam SP1800.

3.8 Disinfection Tests.

3.8.1 Axenic Tests (Cursons et al., 1980, modified)

Amoeba cultures of 18 - 24 hours were centrifuged from the culture media (CYM for Naegleria spp., 4 % Neff for Acanthamoeba spp.) at 425 x g for 10 minutes and resuspended in sterile 0.01 M phosphate buffer. They were washed and centrifuged three times in the same buffer, counted in a modified Fuchs-Rosenthal haemocytometer and resuspended to a final concentration of 2.5×10^5 $cells.cm^{-3}$. A viable count was done on them using the plaque formation method on PASB agar (Cursons, 1978, modified from Fulton, 1970). For the test, a total of 24.0 cm^3 of 0.01 M phosphate buffer, pH 7.0, containing the desired concentration of disinfectant was dispersed in 250 cm^3 erlenmeyer flasks. A total of 1.0 cm^3 washed amoebae was added to each flask, which were then incubated at the desired temperature for the required period on a gyroshaker at 120 r.p.m. Disinfectant levels were assayed at the commencement of incubation, and at the end of the incubation period on 10 cm^3 of the test culture. The remaining 15 cm^3

was filtered through a 5um cellulose acetate filter (no neutralising agent for baquacil was known). The filter was then washed with 1.0 cm^3 of sterile PAS, and 0.1 cm^3 of this suspension was plated on to a lawn of E.cloacae on PASB agar. The resulting plaques formed by the amoebae feeding on the bacteria and leaving a clear area, were counted as representing the number of viable amoebae present in the original sample.

3.8.2 Monoxenic Tests

Monoxenically cultured amoebae, subcultured a minium of 10 times, of between 24 - 30 hours incubation were centrifuged at 425 x g, resuspended in 0.01 M phosphate buffer, pH 7.0. The amoebae were washed and counted as in Methods 3.7.1. Then 21.0 cm^3 of the same buffer, was dispersed in 250 cm^3 erlenmeyer flasks, 0.5 cm^3 of a 1.25×10^7 $cells.cm^{-3}$ bacteria suspension, as measured by an optical density standard curve and confirmed by a dilution plate count, of an overnight culture of E.cloacae, and 1.0 cm^3 of amoeba suspension was then added to the flask. Finally 2.5 cm^3 of buffer containing the required disinfectant concentration was added. The flasks were incubated at the required temperature for the required period on a gyroshaker at 120 r.p.m. At the commencement of incubation and after the end of the incubation period, 10 cm^3 of test culture was used for chemical analysis of disinfectant levels. At this point, remaining chlorine and chlorine dioxide were neutralised with a small crystal of sodium thiosulphate, baquacil was not neutralised. This

remaining 15 cm^3 was titrated as in Methods 3.7.1. except for 0.1 cm^3 which was plated in plate count agar, for a total bacteria count.

Axenically grown amoebae were also used in monoxenic tests as a comparison, to determine the effect of growth on bacteria versus axenic growth of amoebae. These axenically grown amoebae were prepared as in Methods 3.7.1. the test carried out as with the monoxenically grown amoebae.

3.8.3. Non - Viable Organic Demand Tests

A Biochemical Oxygen Demand (BOD_5) test was done on a range of concentrations of dried Bovine Faecal Matter (BFM) powder in water, and BFM solution. The concentrations of powder causing a BOD_5 of 4.7 $mg.l^{-1}$ dissolved oxygen (D.O.) and solution causing that of 1.3 and 4.7 $mg.l^{-1}$ D.O. were used in the disinfection tests to create an organic demand. The concentrations used were 0.1 % and 0.4 % (w/v) respectively. For the tests, the amoebae were treated as in Methods 3.7.2. A total of 20 cm^3 of 0.01M phosphate buffer was dispersed in 250 cm^3 erlenmeyer flasks containing the desired amount of BFM powder, or concentration of BFM solution.

Then 1.0 cm^3 of the amoeba suspension was added, and then 4.0 cm^3 of the same buffer, containing the desired concentration of disinfectant was added to each flask.

The flasks were then incubated at the required temperature for the required period on a gyroshaker at 120 r.p.m.

Chemical analysis of disinfectants, and plaque counts tests were carried out as in Methods 3.7.2. Axenically grown amoeba were used as well as monoxenically grown amoebae,

for the reasons outlined in Methods 3.7.2.

3.8.4. Monoxenic plus Organic Demand Tests

The amoebae were prepared as in Methods 3.7.2., the non-viable organic demand created as in Methods 3.7.3. the bacteria and disinfectant concentrations as Methods 3.7.2. Incubation of test cultures, neutralisation of disinfectants, chemical analysis of disinfectants, and plaque count tests for amoebae, and plate count tests for bacteria, were all carried out as in Methods 3.7.2. For the reasons outlined in Methods 3.7.2. axenically grown and monoxenically grown amoebae were both tested.

CHAPTER FOUR: RESULTS

4.1 The Use of Baquacil Against Pathogenic Free-Living Amoebae

4.1.1 Axenically Grown Amoebae in Axenic Conditions

The amoebicidal capacity of Baquacil in axenic conditions is shown in Figs. 2,3,4, and Tables III,IV,V. The control culture survival rates at the three incubation temperatures of 25,30, 37°C, supported the idea of temperature optima for pathogenic and non-pathogenic amoebae. At 25°C (Fig.2) the pathogen survival rates were 16-21%, where as the non-pathogen survival rates were 61 and 67%, for Naegleria and Acanthamoeba spp. respectively. At 37°C (Fig.4) the pathogen survival rates were 91-98%, while the non-pathogen survival rates were 21 and 30% for Naegleria and Acanthamoeba spp. respectively. At 30°C (Fig.3), the survival rates were greater than 88% for all strains.

Figure 2 and Table III show that at 25°C, of the strains tested, only N.fowleri (MsT) and A.castellanii (1501) could survive 50 mg.l^{-1} of Baquacil, which is the recommended swimming pool maintainance concentration, although all of the other strains tested survived 40 mg.l^{-1}. The Baquacil demand created by the organic content of the inoculum, i.e. the amoebae, reacting with the active component of Baquacil, varied between 4.5 and 15.0 mg.l^{-1} (mean 9.0 mg.l^{-1}). Figure 3, and Table IV show that at 30°C, of the strains tested, N.fowleri (Northcott) was sterilised, N.fowleri (MsM) had a survival rate of 7.5%, and the other four strains had survival rates of $< 3.0\%$ at 50 mg.l^{-1} Baquacil. The Baquacil demand varied between 7.5 and 19.0 mg.l^{-1} (mean 10.0 mg.l^{-1}). Figure 4, and Table V show that N.fowleri (Northcott) and N.gruberi (P1200f) were sterilised by 50 mg.l^{-1} Baquacil, although all of the other strains tested of both Naegleria and Acanthamoeba spp. had a survival rate of $< 3.0\%$ at 50 mg.l^{-1}.

The Baquacil demand varied between 8.0 and 21.0 $mg.l^{-1}$. The mean demand was 9.0 $mg.l^{-1}$ for Naegleria and 17.0 $mg.l^{-1}$ for Acanthamoeba spp.

At all three incubation temperatures, there was no marked difference in sensitivity to Baquacil between Naegleria and Acanthamoeba apparent. There was however, at 37°C a greater Baquacil demand by the two Acanthamoeba strains over the Naegleria strains, although at 25 and 30°C there were no such marked differences in Baquacil demand. The only strain to be eliminated at all three incubation temperatures was N.fowleri (Northcott), demonstrating a greater sensitivity of Baquacil under these conditions than the other five strains tested.

In Figures 2 - 34 the following legend applies:

○ = _N.fowleri_ (MsM)

● = _N.fowleri_ (MsT)

△ = _N.fowleri_ (Northcott)

■ = _A.culbertsoni_ (A-1)

□ = _N.gruberi_ (P1200f)

▲ = _A.castellanii_ (1501)

X = _E.cloacae_

Figure 2 (Table III opposite) The Effect of Baquacil on Axenically Grown Amoebae, for 30 minutes, at 25°C.

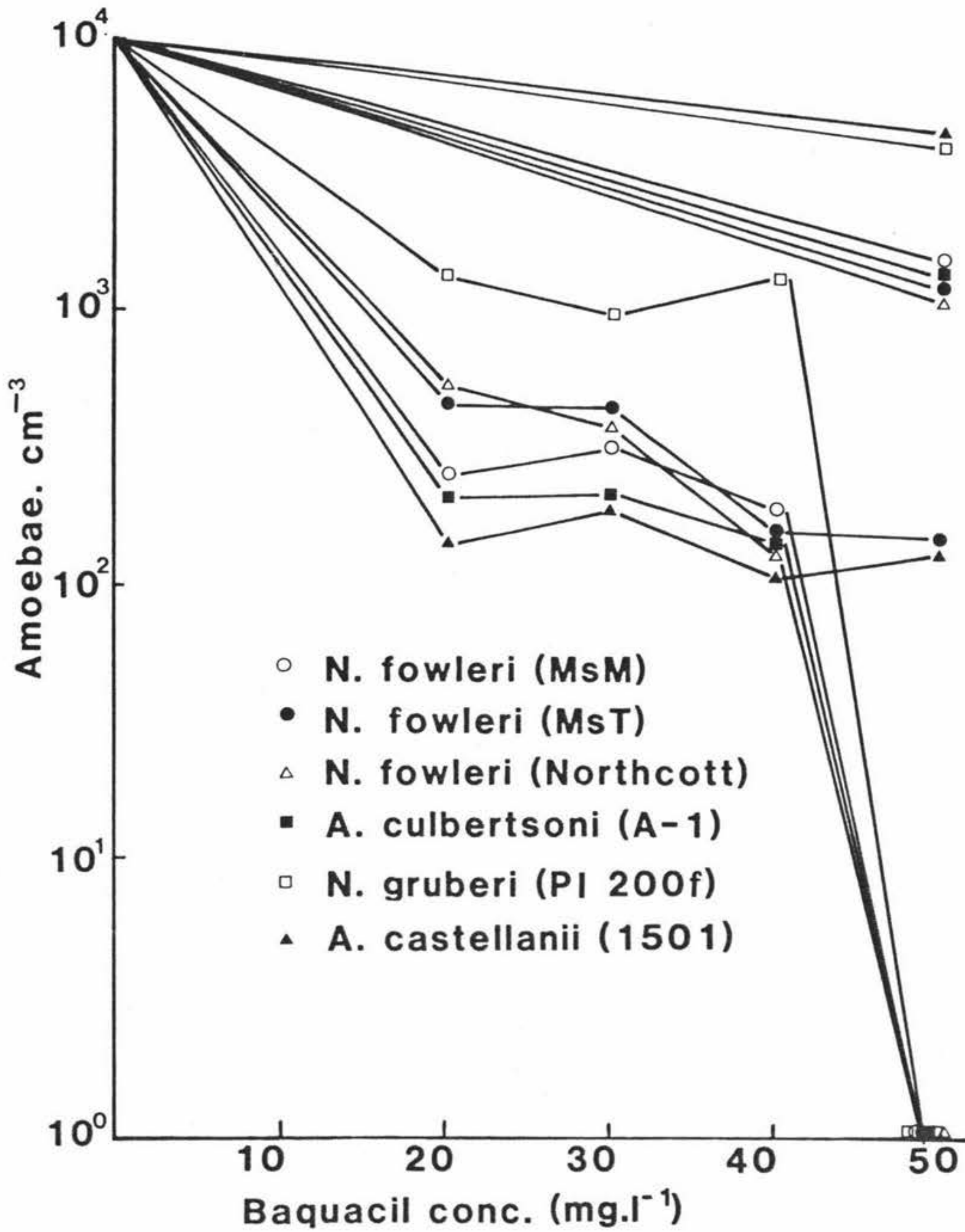


Table III

Amoeba	Baquacil ($mg.l^{-1}$)			Survivors	
	Initial	Final	Demand	Cells.cm^{-3}	%
N.fowleri (MsM)	0	0	0	2000	20.0
	20	8	12.0	375	3.75
	30	25.5	4.5	475	4.75
	40	28.0	12.0	225	2.25
	50	41.2	8.8	0	0
N.fowleri (MsT)	0	0	0	1500	15.0
	20	6.5	13.5	675	6.75
	30	18.5	11.5	675	6.75
	40	32.2	7.5	225	2.25
	50	42.0	8.0	225	2.25
N.fowleri (Northcott)	0	0	0	1800	18.0
	20	9.0	11.0	600	6.0
	30	23.0	7.0	600	6.0
	40	31.5	8.5	75	0.75
	50	42.5	7.5	0	0
N.gruberi (PL200f)	0	0	0	6100	61.0
	20	12.0	8.0	1825	18.25
	30	16.0	14.0	1050	10.5
	40	26.0	14.0	1275	12.75
	50	42.0	8.0	0	0
A.culbertsoni (A-1)	0	0	0	1600	16.0
	20	3.0	17.0	375	3.75
	30	19.0	11.0	375	3.75
	40	33.0	7.0	225	2.25
	50	46.0	4.0	0	0
A.castellanii (1501)	0	0	0	6700	67.0
	20	9.0	11.0	150	1.5
	30	16.0	14.0	300	3.0
	40	33.0	7.0	75	0.75
	50	43.0	7.0	150	1.5

Figure 3 (Table IV opposite) The Effect of Baquacil on Axenically Grown Amoebae, for 30 minutes, at 30°C.

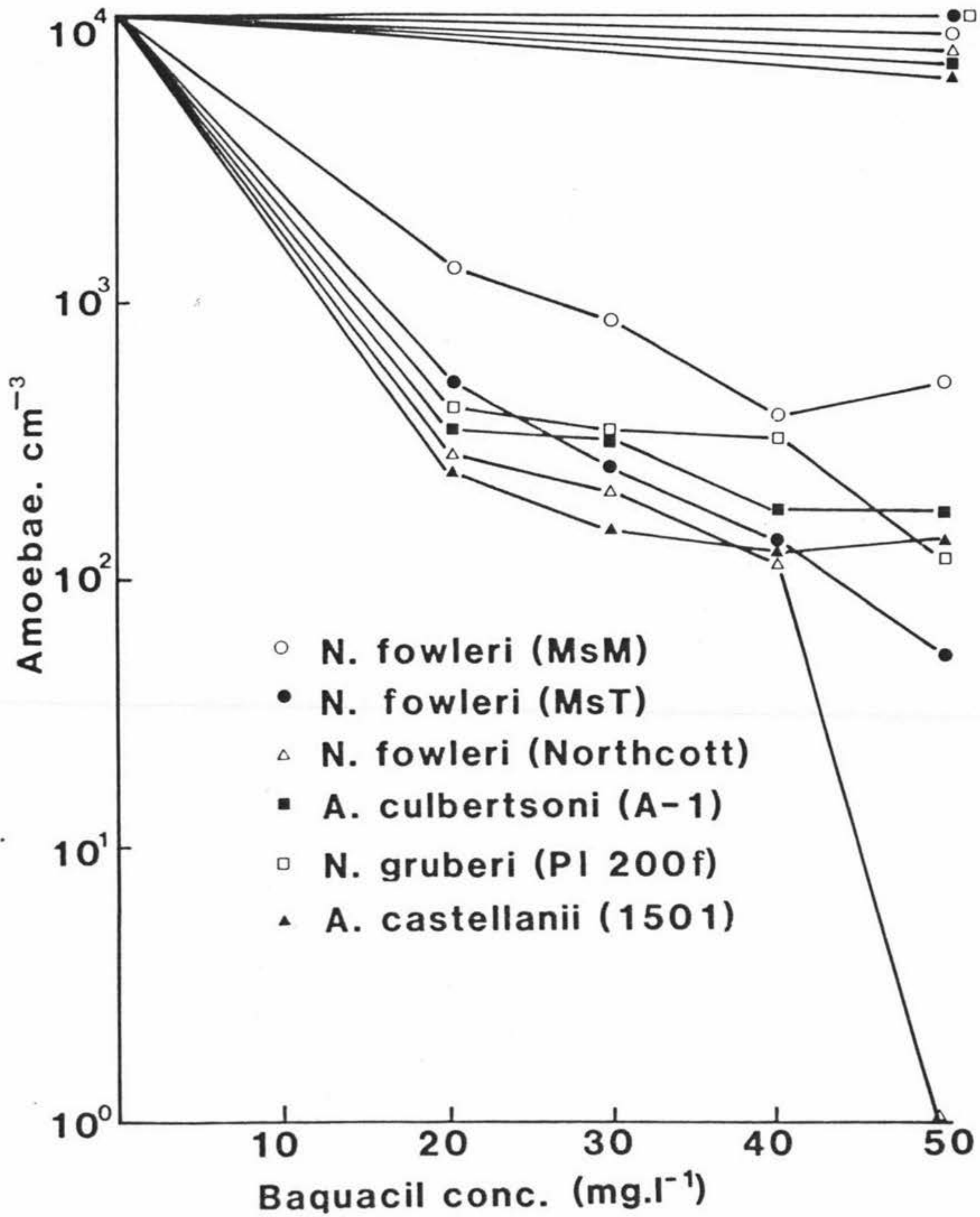


Table IV

Amoeba	Baquacil(mg.l^{-1})			Survivors	
	Initial	Final	Demand	Cells.cm^{-3}	%
N.fowleri (MsM)	0	0	0	9700	97.0
	20	2.0	18.0	1250	12.5
	30	3.0	27.0	975	9.75
	40	28.0	12.0	600	6.0
	50	38.0	12.0	750	7.5
N.fowleri (MsT)	0	0	0	9900	100.0
	20	10.0	10.0	780	7.8
	30	17.0	13.0	375	3.75
	40	26.5	13.5	300	3.0
	50	40.5	9.5	300	3.0
N.fowleri (Northcott)	0	0	0	9200	92.0
	20	13.0	7.0	600	6.0
	30	19.0	11.0	400	4.0
	40	34.0	6.0	150	1.5
	50	39.0	11.0	0	0
N.gruberi (PL200f)	0	0	0	9900	100.0
	20	1.0	19.0	525	5.25
	30	18.0	12.0	450	4.5
	40	27.3	12.7	500	5.0
	50	34.0	16.0	300	3.0
A.culbertsoni (A-1)	0	0	0	8800	88.0
	20	8.0	12.0	450	4.5
	30	22.0	8.0	450	4.5
	40	32.5	7.5	275	2.75
	50	42.0	8.0	300	3.0
A.castellanii (1501)	0	0	0	9000	90.0
	20	10.0	10.0	375	3.75
	30	20.0	10.0	275	2.75
	40	26.3	13.7	150	1.5
	50	34.0	16.0	300	3.0

Figure 4 (Table V opposite) The Effect of Baquacil on Axenically Grown Amoebae, for 30 minutes, at 37°C.

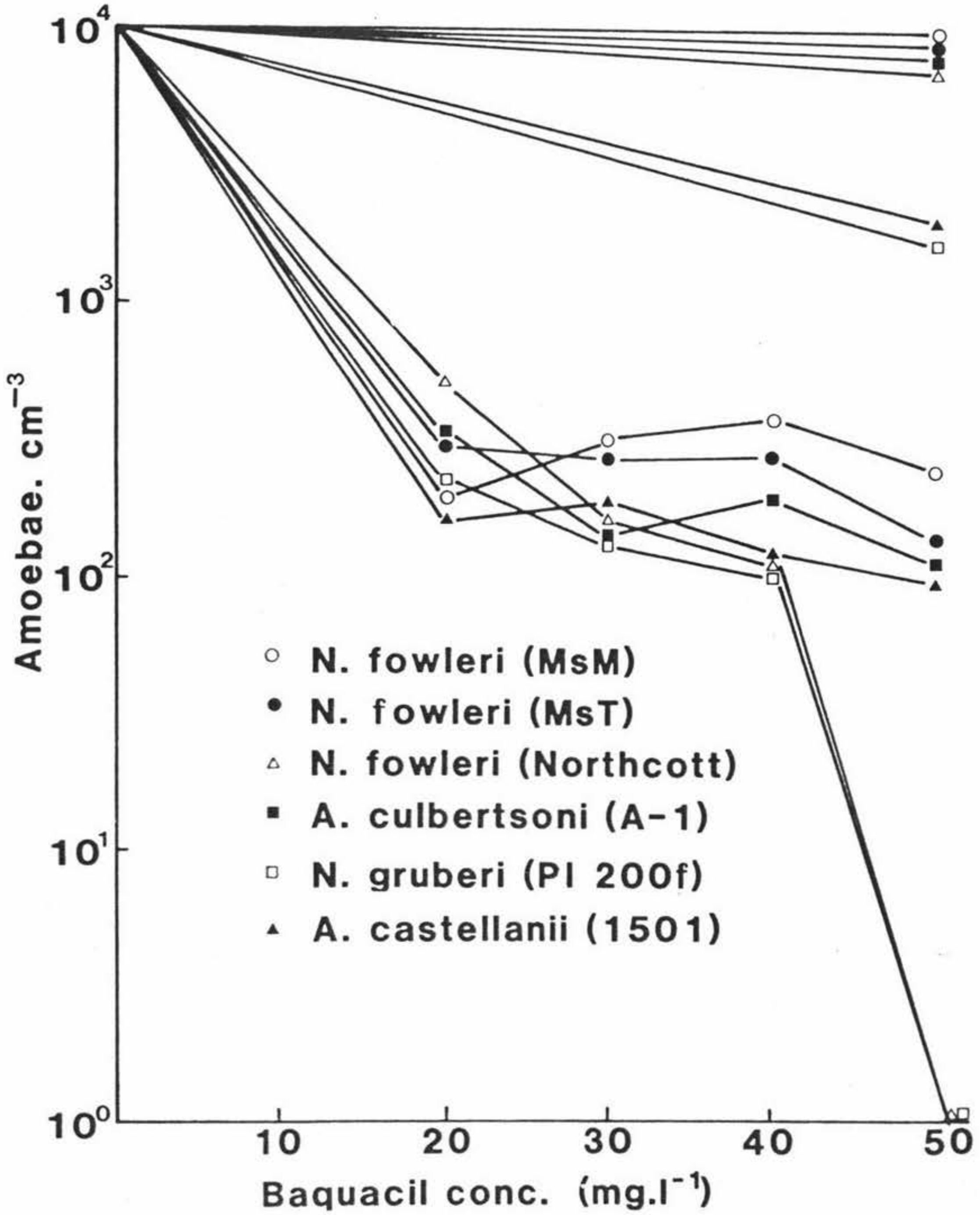


Table V

Amoeba	Baquacil $(mg.l^{-1})$			Survivors	
	Initial	Final	Demand	$Cells.cm^{-3}$	%
N.fowleri (MsM)	0	0	0	9800	98.0
	20	12.0	8.0	300	3.0
	30	18.5	11.5	525	5.25
	40	31.0	9.0	525	5.25
	50	40.0	10.0	225	2.25
N.fowleri (MsT)	0	0	0	9600	96.0
	20	11.0	9.0	525	5.25
	30	16.3	13.7	450	4.5
	40	32.0	8.0	525	5.25
	50	41.0	9.0	150	1.5
N.fowleri (Northcott)	0	0	0	9400	94.0
	20	11.0	9.0	600	6.0
	30	19.0	11.0	225	2.25
	40	31.0	9.0	150	1.5
	50	42.0	8.0	0	0
N.gruberi (PL200f)	0	0	0	2100	21.0
	20	12.0	8.0	300	3.0
	30	17.5	12.5	150	1.5
	40	29.0	11.0	75	0.75
	50	39.6	11.4	0	0
A.culbertsoni (A-1)	0	0	0	9100	91.0
	20	2.0	18.0	525	5.25
	30	12.0	8.0	150	1.5
	40	17.5	12.5	300	3.0
	50	29.0	21.0	150	1.5
A.castellanii (1501)	0	0	0	3000	30.0
	20	2.0	18.0	275	2.75
	30	8.0	12.0	300	3.0
	40	18.0	12.0	150	1.5
	50	36.0	14.0	75	0.75

4.1.2 Axenically Grown Amoebae plus Bacteria

The results shown in Fig. 5 and Table VI show that when $1 \times 10^{5}.cm^{-3}$ E.cloacae are added, the survival rate of all strains increased by between 0.5 and 4.0%. The Baquacil demand increased by between 0.5 and 2.0 $mg.l^{-1}$, to a mean demand of 10.5 $mg.l^{-1}$ for Naegleria, although the demand of Acanthamoeba spp. remained at 9.0 $mg.l^{-1}$. None of the six strains tested was sterilised by 50 $mg.l^{-1}$ Baquacil, although the survival rate at this concentration was $<$ 4.0% for all four pathogens, and $<$ 6.5% for both non-pathogenic strians. The bacteria were killed by 30 $mg.l^{-1}$ Baquacil, although $<$ 1.0% survived 20 $mg.l^{-1}$.

Figure 5 (Table VI opposite) The Effect of Baquacil on Axenically Grown Amoebae, plus $1 \times 10^5.cm^{-3}$ bacteria, for 30 minutes, at $30^{o}C$.

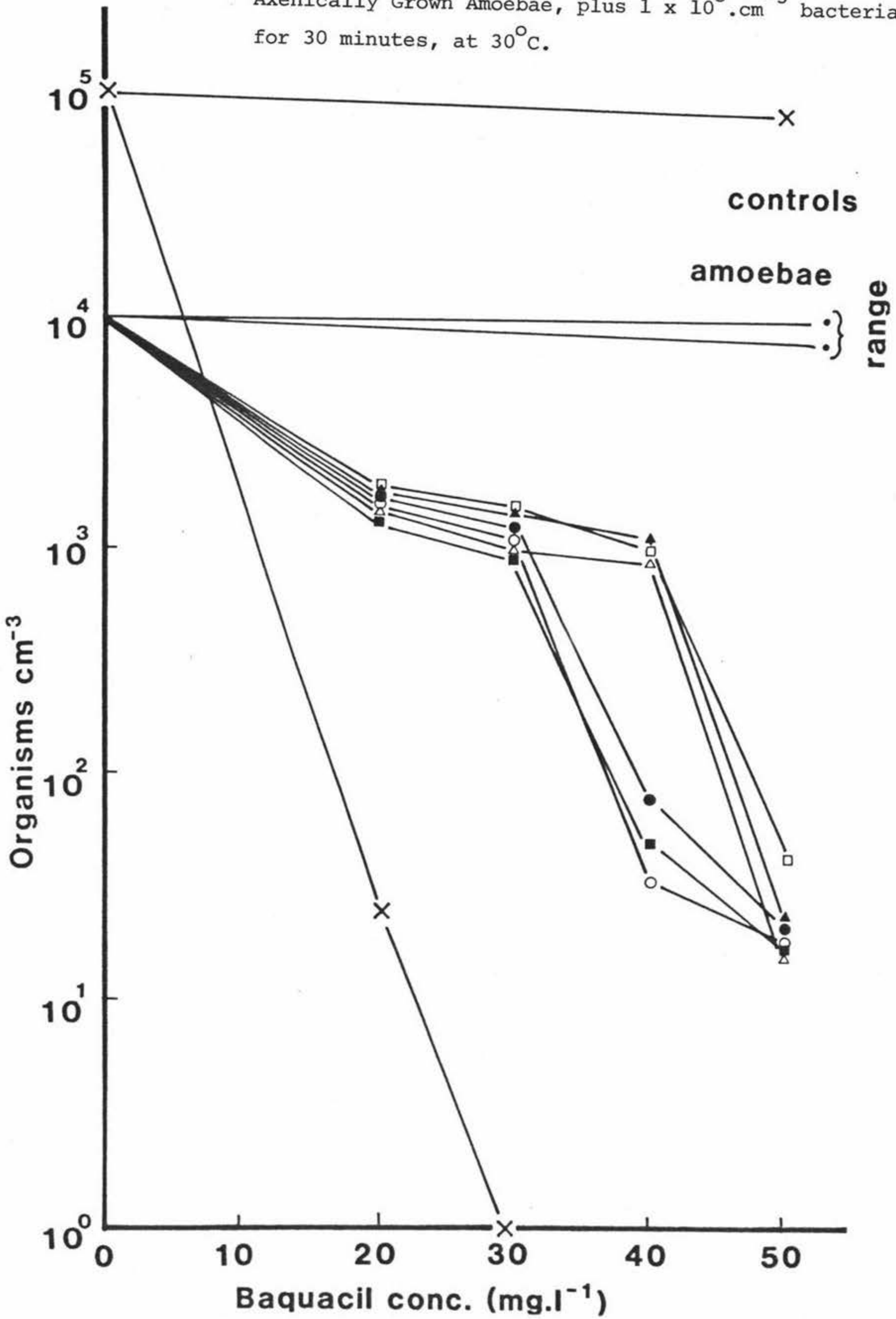


Table VI

Amoeba	Baquacil ($mg.l^{-1}$)			Survivors		
	Initial	Final	Demand	$Amoebae.cm^{-3}$	%	$Bacteria.cm^{-3}$
N.fowleri (MsM)	0	0	0	9300	93.0	81000
	20	9.0	11.0	1575	15.75	40
	30	19.5	10.5	1200	12.0	0
	40	30.5	9.5	525	5.25	0
	50	38.0	12.0	375	3.75	0
N.fowleri (MsT)	0	0	0	9600	96.0	83000
	20	9.0	11.0	1650	16.5	40
	30	20.0	10.0	1200	12.0	0
	40	29.5	10.5	825	8.25	0
	50	41.0	9.0	375	3.75	0
N.fowleri (Northcott)	0	0	0	9000	90.0	80000
	20	10.0	10.0	1850	18.5	60
	30	21.5	8.5	1600	16.0	0
	40	33.0	7.0	950	9.5	0
	50	42.0	8.0	375	3.75	0
N.gruberi (P1200f)	0	0	0	9750	97.5	80000
	20	9.0	11.0	2300	23.0	100
	30	20.0	10.0	1700	17.0	0
	40	30.0	10.0	1050	10.5	0
	50	41.0	9.0	600	6.0	0
A.culbertsoni (A-1)	0	0	0	9000	90.0	79000
	20	8.5	11.5	1700	17.0	20
	30	23.0	7.0	1500	15.0	0
	40	31.0	9.0	750	7.5	0
	50	42.0	8.0	395	3.95	0
A.castellanii (1501)	0	0	0	9000	90.0	83000
	20	11.0	9.0	1800	18.0	85
	30	21.0	9.0	1600	16.0	0
	40	32.0	8.0	1350	13.5	0
	50	41.5	8.5	525	5.25	0

4.1.3 Axenically Grown Amoebae plus a BOD

Figures 6 and 8, and Tables VII and IX show the effect of an increased organic demand in the form of BFM solution, a natural pollutant commonly found in natural water. 0.1% and 0.4% BFM were used to create BODs of 1.2 and 4.7 $mg.l^{-1}$, in an attempt to recreate the conditions of slightly polluted, and heavily polluted water respectively. Figure 6 and Table VII show that at a BOD of 1.2 $mg.l^{-1}$, the survival rate was not markedly different to those in axenic conditions at the 30°C incubation temperature (section 4.1.1, Fig.3, Table IV), except that the N.fowleri (Northcott) survival rate was 0.75%, and A.castellanii (1501) was sterilised at 50 $mg.l^{-1}$ Baquacil. The other four strains' survival rates were between 0.75 and 3.75%. The Baquacil demand was more than doubled, to between 21.0 and 27.0 $mg.l^{-1}$ (mean 22.0 $mg.l^{-1}$). There was no marked difference between the Naegleria and Acanthamoeba strains, both in sensitivity to Baquacil, and Baquacil demand created.

Figure 8 and Table IX show at a BOD of 4.7 $mg.l^{-1}$, a survival rate of between 38.0 and 49.5%, at a Baquacil concentration of 50 $mg.l^{-1}$, for all strains.

4.1.4 Axenically Grown Amoebae plus a BOD and Bacteria

Figure 7 and Table VIII show that the lower BOD of 1.2 $mg.l^{-1}$, in combination with 1 x $10^{5}.cm^{-3}$ E.cloacae causes a greater Baquacil demand. However the survival rates were $<$3.0% for all pathogenic strains, and for the non-pathogenic strains, 6.75% for N.gruberi (P1200f), and A.castellanii (1501) was sterilised, by 50 $mg.l^{-1}$ Baquacil. The number of bacteria surviving 20 $mg.l^{-1}$ Baquacil was up to 10 times the number surviving in the absence of a BOD (section 4.1.2, Fig.5, Table VI), although the bacteria were killed by 30 $mg.l^{-1}$. The Baquacil demand was between 25.0 and 27.0 $mg.l^{-1}$.

Neither the Baquacil demand, nor the sensitivity to Baquacil, differed significantly between the two genera.

Figure 9 and Table X show that when the combination of a high BOD of 4.7 $mg.l^{-1}$ and $1.x\ 10^{5}.cm^{-3}$ E.cloacae was added, the survival rate at 50 $mg.l^{-1}$ Baquacil was greater than 66.0% for all strains, and in all test flasks, there were between 10 and $50.cm^{-3}$ E.cloacae survivors. As in section 4.1.3 (Fig.8 and Table IX), the demand was equal to the initial Baquacil concentrations used.

Figure 6 (Table VII opposite) The Effect of Baquacil on Axenically Grown Amoebae, plus a BOD of 1.2 mg.l^{-1}, for 30 minutes, at 30°C.

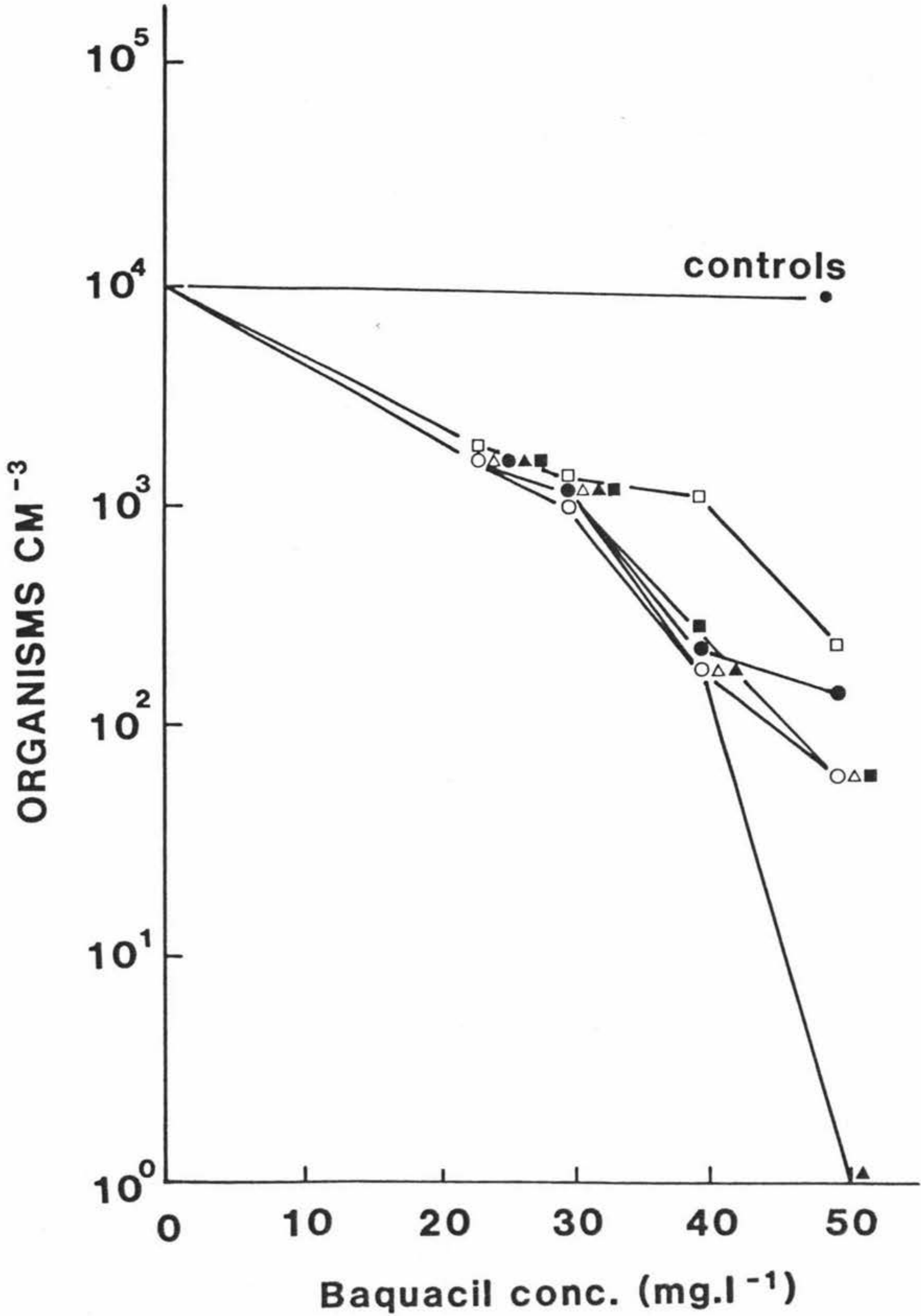


Table VII

Amoeba	Baquacil($mg.l^{-1}$)			Survivors	
	Initial	Final	Demand	$Cells.cm^{-3}$	%
N.fowleri (MsM)	0	0	0	9300	93.0
	20	0	20.0	1850	18.5
	30	6.0	24.0	1000	10.0
	40	19.0	21.0	250	2.5
	50	28.0	22.0	75	0.75
N.fowleri (MsT)	0	0	0	9450	94.5
	20	0	20.0	1700	17.0
	30	5.0	25.0	1200	12.0
	40	18.0	22.0	300	3.0
	50	28.0	22.0	150	1.5
N.fowleri (Northcott)	0	0	0	9150	91.5
	20	0	20.0	1800	18.0
	30	8.0	22.0	1200	12.0
	40	20.0	20.0	250	2.5
	50	29.0	21.0	75	0.75
N.gruberi (P1200f)	0	0	0	9600	96.0
	20	0	20.0	2200	22.0
	30	4.0	26.0	1600	16.0
	40	18.0	22.0	1000	10.0
	50	29.0	21.0	375	3.75
A.culbertsoni (A -1)	0	0	0	9300	93.0
	20	0	20.0	1800	18.0
	30	4.0	26.0	1200	12.0
	40	18.0	22.0	450	4.5
	50	29.0	21.0	75	0.75
A.castellanii (1501)	0	0	0	9300	93.0
	20	0	20.0	1800	18.0
	30	4.0	26.0	1200	12.0
	40	16.0	24.0	250	2.5
	50	29.0	21.0	0	0

Figure 7 (Table VIII opposite) The Effect of Baquacil on Axenically Grown Amoebae, plus a BOD of 1.2 $mg.l^{-1}$, plus $1 \times 10^5.cm^{-3}$ bacteria, for 30 minutes, at 30^oC.

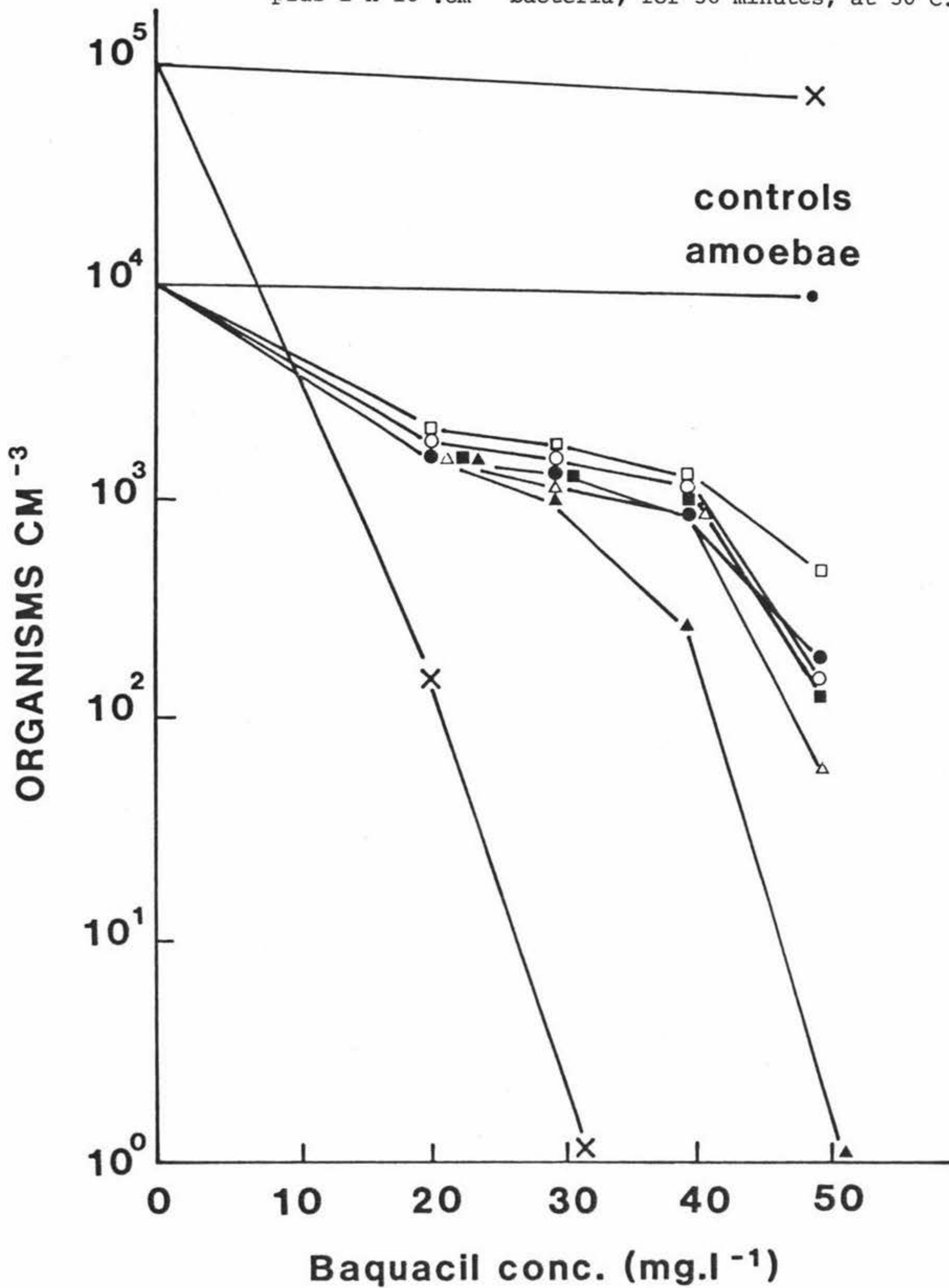


Table VIII

Amoeba	Baquacil(mg.l^{-1})			Survivors		
	Initial	Final	Demand	Cells.cm^{-3}	%	Bacteria .cm^{-3}
N.fowleri (MsM)	0	0	0	9300	93.0	80000
	20	0	20.0	2400	24.0	100
	30	5.0	25.0	1800	18.0	0
	40	14.0	26.0	1150	11.5	0
	50	24.0	26.0	175	1.75	0
N.fowleri (MsT)	0	0	0	9300	93.0	81000
	20	0	20.0	2100	21.0	121
	30	7.0	23.0	1650	16.5	0
	40	14.0	26.0	950	9.5	0
	50	25.0	25.0	250	2.5	0
N.fowleri (Northcott)	0	0	0	9300	93.0	90000
	20	0	20.0	2100	21.0	108
	30	6.0	24.0	1400	14.0	0
	40	15.0	25.0	950	9.5	0
	50	25.0	25.0	75	0.75	0
N.gruberi (P1200f)	0	0	0	9600	96.0	82000
	20	0	20.0	2550	25.5	108
	30	5.0	25.0	2100	21.0	0
	40	13.0	27.0	1200	12.0	0
	50	23.0	27.0	675	6.75	0
A.culbertsoni (A-1)	0	0	0	9250	92.5	79000
	20	0	20.0	2100	21.0	120
	30	3.5	26.5	1400	14.0	0
	40	13.5	26.5	1000	10.0	0
	50	23.0	27.0	100	1.0	0
A.castellanii (1501)	0	0	0	9300	93.0	88000
	20	0	20.0	2100	21.0	110
	30	5.0	25.0	1200	12.0	0
	40	14.0	26.0	400	4.0	0
	50	24.0	26.0	0	0	0

Figure 8 (Table IX opposite) The Effect of Baquacil on Axenically Grown Amoebae, plus a BOD of 4.7 mg.l^{-1}, for 30 minutes, at $30^{\circ}C$.

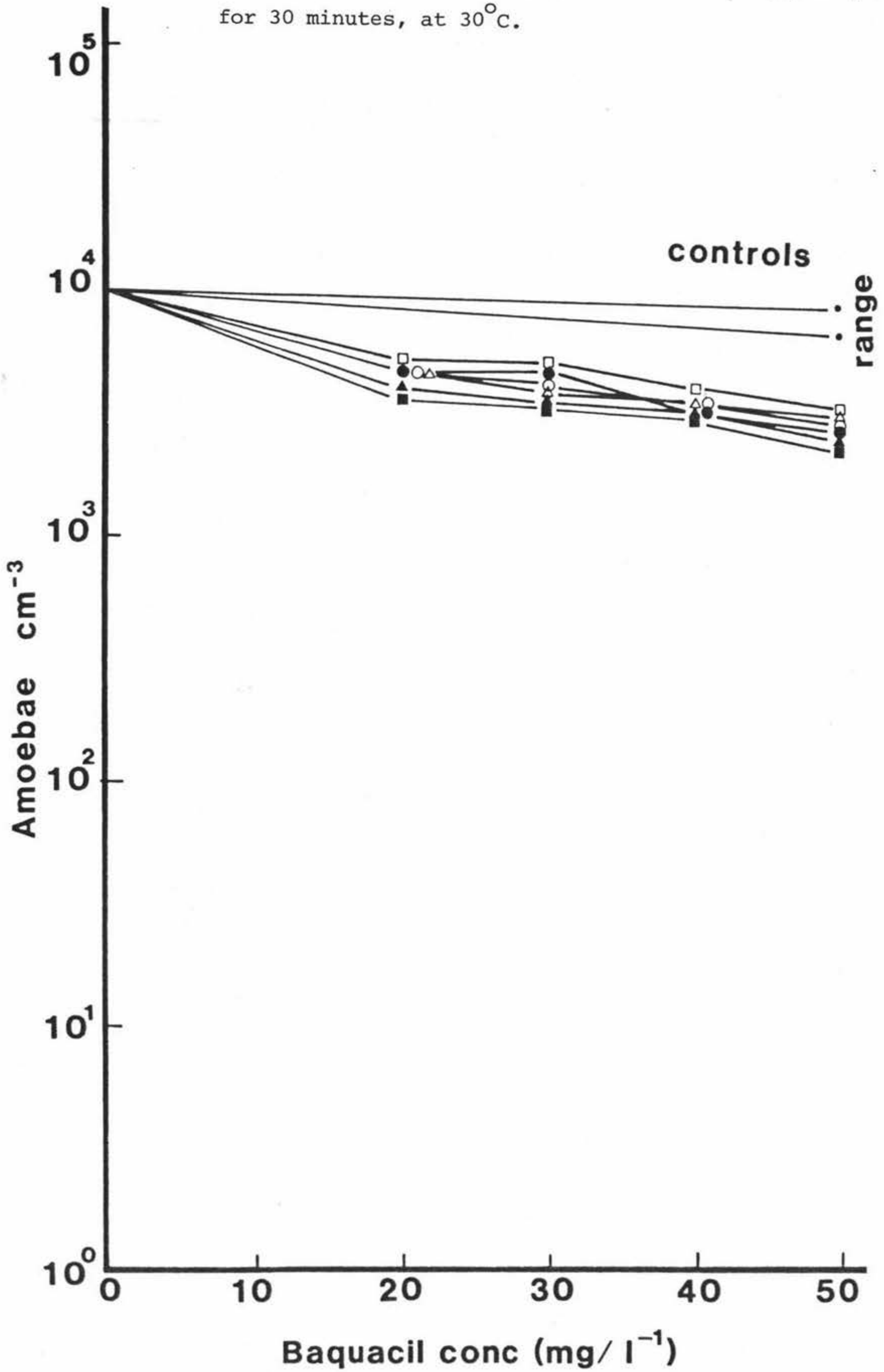


Table IX

Amoeba	Baquacil ($mg.l^{-1}$)			Survivors	
	Initial	Final	Demand	Cells.cm^{-3}	%
N.fowleri (MsM)	0	0	0	8800	88.0
	20	0	20	6600	66.0
	30	0	30	6750	67.5
	40	0	40	5050	50.5
	50	0	50	4100	41.5
N.fowleri (MsT)	0	0	0	8800	88.0
	20	0	20	6600	66.0
	30	0	30	6550	65.5
	40	0	40	5100	51.0
	50	0	50	4250	42.5
N.fowleri (Northcott)	0	0	0	8900	89.0
	20	0	20	6750	67.5
	30	0	30	6150	61.5
	40	0	40	5250	52.5
	50	0	50	4400	44.0
N.gruberi (PL200f)	0	0	0	9200	92.0
	20	0	20	7050	70.5
	30	0	30	6450	64.5
	40	0	40	5850	58.5
	50	0	50	4950	49.5
A.culbertsoni (A-1)	0	0	0	8500	85.0
	20	0	20	6050	60.5
	30	0	30	6100	61.0
	40	0	40	5150	51.5
	50	0	50	4400	44.0
A.castellanii (1501)	0	0	0	8200	82.0
	20	0	20	5700	57.0
	30	0	30	5775	57.75
	40	0	40	4700	47.0
	50	0	50	3800	38.0

Figure 9 (Table X opposite) The Effect of Baquacil on Axenically Grown Amoebae, plus a BOD of 4.7 $mg.l^{-1}$, plus 1 x $10^5.cm^{-3}$ bacteria, for 30 minutes, at 30°C.

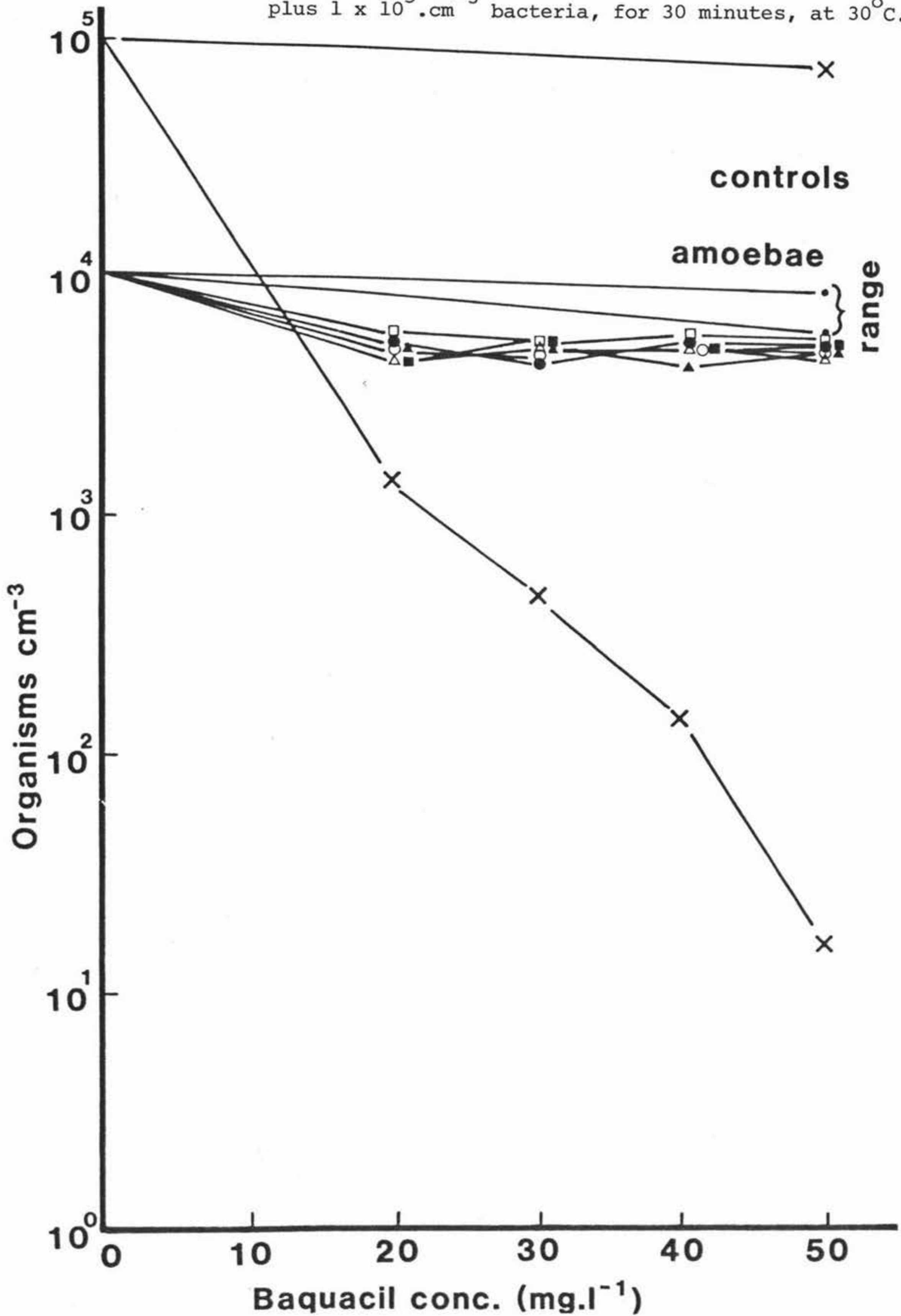


Table X

Amoebae	Baquacil ($mg.l^{-1}$)			Survivors		
	Initial	Final	Demand	Amoebae.cm^{-3}	%	Bacteria.cm^{-3}
N.fowleri (MsM)	0	0	0	8650	86.5	85000
	20	0	20	6700	67.0	1250
	30	0	30	6700	67.0	650
	40	0	40	6600	66.0	150
	50	0	50	6700	67.0	20
N.fowleri (MsT)	0	0	0	8800	88.0	87000
	20	0	20	6950	69.5	1200
	30	0	30	6675	66.75	750
	40	0	40	6800	68.0	100
	50	0	50	6975	66.75	20
N.fowleri (Northcott)	0	0	0	8500	85.0	85500
	20	0	20	6675	66.75	1150
	30	0	30	6750	67.5	670
	40	0	40	6600	66.0	120
	50	0	50	6675	66.75	20
N.gruberi (P1200f)	0	0	0	9150	91.5	86000
	20	0	20	7250	72.5	1300
	30	0	30	7100	71.0	500
	40	0	40	7400	74.0	90
	50	0	50	7250	72.5	10
A.culbertsoni (A-1)	0	0	0	8200	82.0	85000
	20	0	20	6675	66.75	1300
	30	0	30	6900	69.0	600
	40	0	40	6600	66.0	140
	50	0	50	6975	69.75	40
A.castellanii (1501)	0	0	0	7750	77.5	85000
	20	0	20	6750	67.5	1200
	30	0	30	6750	67.5	700
	40	0	40	6375	63.75	120
	50	0	50	6800	68.0	50

4.1.5 Monoxenically Grown Amoebae plus Bacteria

The results in Fig. 10 and Table XI show that at 50 $mg.l^{-1}$ Baquacil, none of the six strains were sterilised, but all had a survival rate of between 6.0 and 8.0%. At 20 $mg.l^{-1}$ Baquacil, there were $< 1.0\%$ bacteria surviving, and at 30 $mg.l^{-1}$ these were killed. There was no significant difference in sensitivity to Baquacil between *Acanthamoeba* and *Naegleria* in these conditions. The Baquacil demand varied from 6.0 to 14.0 $mg.l^{-1}$, with 12 $mg.l^{-1}$ as the mean for *Naegleria*, and 5.5 to 13.0 $mg.l^{-1}$ with 10.0 $mg.l^{-1}$ as the mean for *Acanthamoeba*.

Figure 10 (Table XI opposite) The Effect of Baquacil on Monoxenically Grown Amoebae, plus 1 x 10^5.cm^{-3} bacteria, for 30 minutes, at $30^{o}C$.

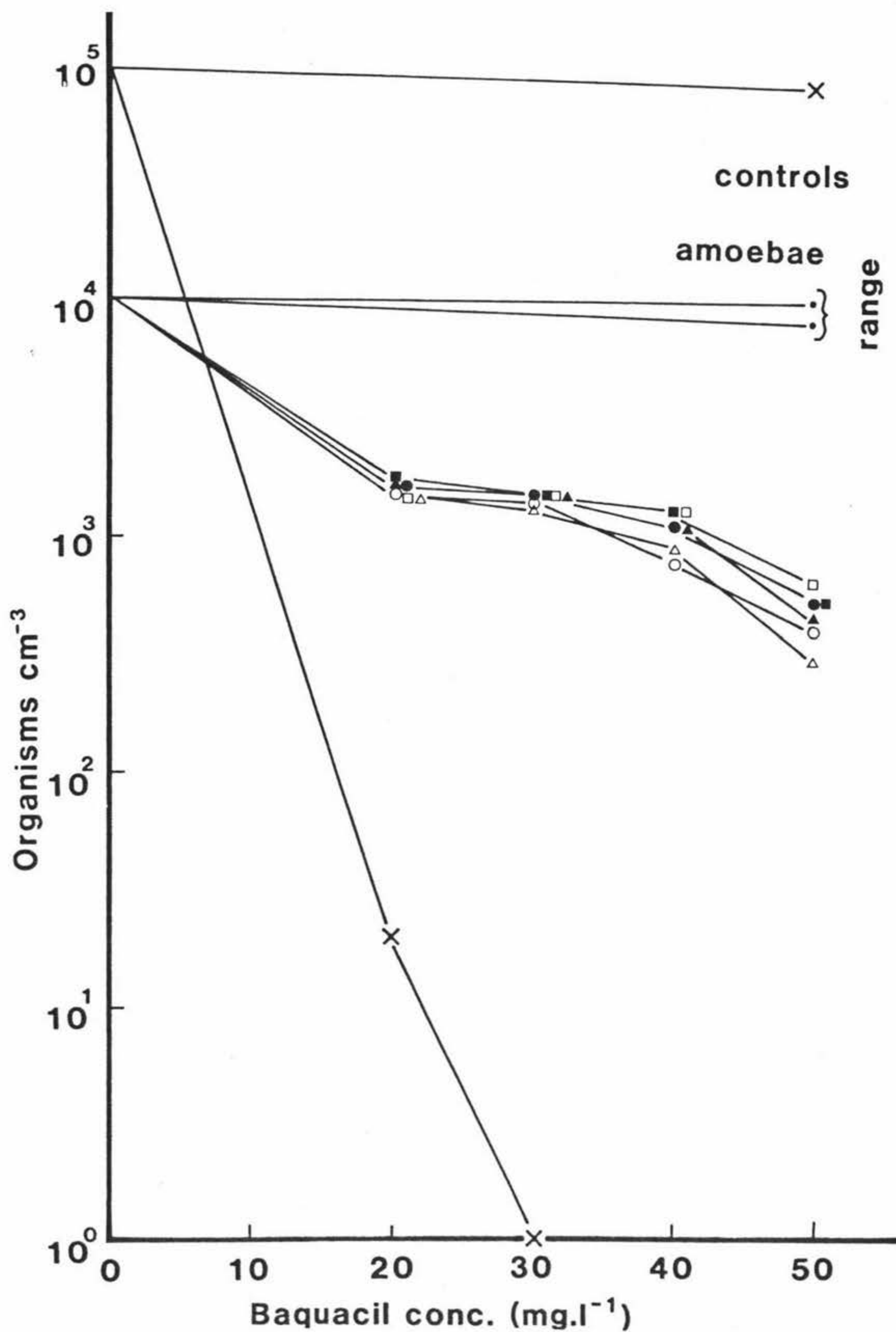


Table XI

Amoeba	Baquacil ($mg.l^{-1}$)			Survivors		
	Initial	Final	Demand	$Amoebae.cm^{-3}$	%	$Bacteria.cm^{-3}$
N.fowleri (MsM)	0	0	0	9500	95.0	90000
	20	8.0	12.0	1800	18.0	50
	30	19.0	11.0	1575	15.75	0
	40	30.0	10.0	900	9.0	0
	50	40.5	9.5	600	6.0	0
N.fowleri (MsT)	0	0	0	9600	96.0	90500
	20	8.0	12.0	1950	19.5	40
	30	18.0	12.0	1600	16.0	0
	40	29.0	11.0	1050	10.5	0
	50	39.5	10.5	750	7.5	0
N.fowleri (Northcott)	0	0	0	9300	93.0	90000
	20	14.0	6.0	1850	18.5	30
	30	22.0	8.0	1450	14.5	0
	40	33.0	7.0	950	9.5	0
	50	41.5	8.5	450	4.5	0
N.gruberi (P1200f)	0	0	0	9800	98.0	89500
	20	14.0	6.0	2450	24.5	30
	30	21.0	9.0	1700	17.0	0
	40	26.0	14.0	1150	11.5	0
	50	42.0	8.0	800	8.0	0
A.culbertsoni (A-1)	0	0	0	9100	91.0	91000
	20	13.0	7.0	1900	19.0	25
	30	21.0	9.0	1750	17.5	0
	40	31.5	8.5	1200	12.0	0
	50	37.0	13.0	750	7.5	0
A.castellanii (1501)	0	0	0	9250	92.5	90000
	20	14.5	5.5	2000	20.0	38
	30	21.0	9.0	1650	16.5	0
	40	33.0	7.0	1050	10.5	0
	50	41.0	9.0	675	6.75	0

4.1.6 Monoxenically Grown Amoebae plus a BOD

The lower BOD of 1.2 $mg.l^{-1}$ (Fig.11 and Table XII), allowed survival rates, at all concentrations of baquacil tested, of between 1.0 and 6.0% higher than the corresponding experiment with axenically grown amoebae (Fig. 6 and Table VII). The survival rates at 50 $mg.l^{-1}$ Baquacil were $< 3.0\%$ for the four pathogenic strains, and 5.25% for N.gurberi (P1200f). A.castellanii (1501) was sterilised at this concentration. The Baquacil demand was between 20.0 and 28.0 $mg.l^{-1}$, with the mean demand of 23.0 $mg.l^{-1}$. There was no marked difference between the two genera in either Baquacil demand or sensitivity to Baquacil.

Figure 12 and Table XIV show that with the addition of a higher BOD of 4.7 $mg.l^{-1}$ there is very little difference in the survival rate of all six strains, between control and test cultures, the largest difference being 9.5% with N.fowleri (Northcott). The Baquacil demand was equal to the initial Baquacil concentrations used. There was no significant difference in sensitivity between Naegleria and Acanthamoeba spp. to Baquacil in these conditions.

4.1.7 Monoxenically Grown Amoebae plus a BOD and Bacteria

Figure 13 and Table XIII show that the survival rates of all six strains of amoebae are increased by between 1.0 and 4.0% over those in the corresponding experiment with axenically grown amoebae (section 4.1.4, Fig.7 and Table VIII). The survival rates were $< 5.0\%$ for all strains tested, except N.gruberi (P1200f), which had a survival rate of 8.75%. The number of bacteria surviving was not significantly different from those in the corresponding axenically grown amoebae experiment. This was between 80 and 170.cm^{-3} E.cloacae at 20 $mg.l^{-1}$, and killed at 30 $mg.l^{-1}$ Baquacil. There was no significant difference between Naegleria and Acanthamoeba in sensitivity to Baquacil, and the Baquacil demand was between 26.0 and 29.5 $mg.l^{-1}$ (mean 27.5 $mg.l^{-1}$).

Table XV shows that there was no significant difference between the survival rates of test cultures and the control cultures in all six strains. There were between 18 and 50.cm^{-3} bacteria surviving at the 50 $mg.l^{-1}$ Baquacil concentration. The Baquacil demand was equal to the initial concentrations of Baquacil used.

Figure 11 (Table XII opposite) The Effect of Baquacil on Monoxenically Grown Amoebae, plus a BOD of 1.2 mg.l^{-1}, for 30 minutes, at 30°C.

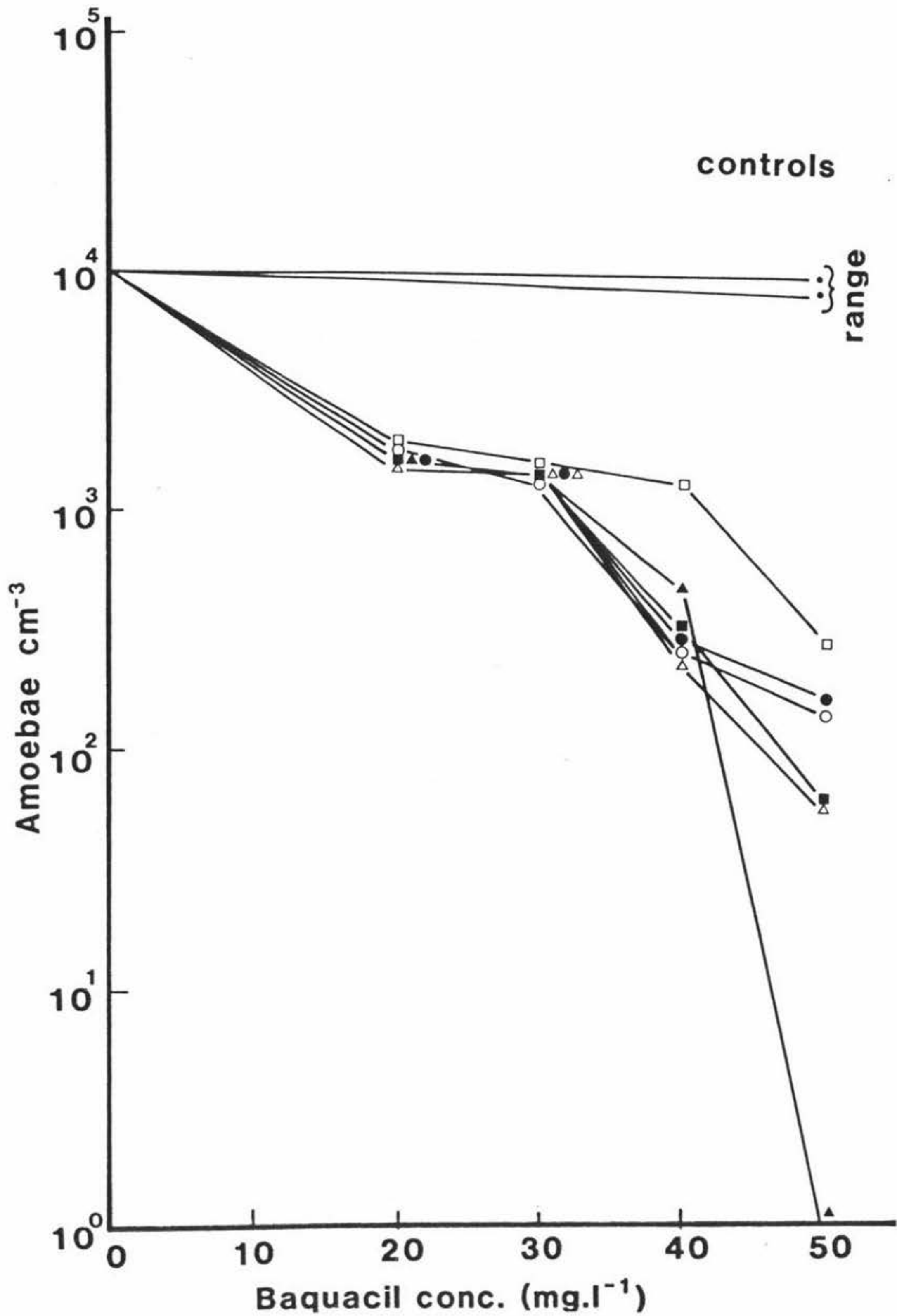


Table XII

Amoeba	Baquacil ($mg.l^{-1}$)			Survivors	
	Initial	Final	Demand	Cells.cm^{-3}	%
N.fowleri (MsM)	0	0	0	9300	93.0
	20	0	20.0	2250	22.5
	30	4.0	26.0	1175	11.75
	40	18.0	22.0	375	3.75
	50	29.0	21.0	150	1.5
N.fowleri (MsT)	0	0	0	9450	94.5
	20	0	20.0	1900	19.0
	30	3.0	27.0	1350	13.5
	40	17.5	22.5	450	4.5
	50	29.0	21.0	275	2.75
N.fowleri (Northcott)	0	0	0	9150	91.5
	20	0	20.0	2025	20.25
	30	2.0	28.0	1350	13.5
	40	18.0	22.0	375	3.75
	50	28.5	21.5	75	0.75
N.gruberi (P1200f)	0	0	0	9600	96.0
	20	0	20.0	2600	26.0
	30	3.0	27.0	1900	19.0
	40	18.5	21.5	1500	15.0
	50	29.5	20.5	525	5.25
A.culbertsoni (A-1)	0	0	0	9300	93.0
	20	0	20.0	2100	21.0
	30	4.0	26.0	1350	13.5
	40	17.0	23.0	525	5.25
	50	28.0	22.0	75	0.75
A.castellanii (1501)	0	0	0	9300	93.0
	20	0	20.0	2025	20.25
	30	4.0	26.0	1350	13.5
	40	18.0	22.0	675	6.75
	50	27.5	22.5	0	0

Figure 12 (Table XIII opposite) The Effect of Baquacil on Monoxenically Grown Amoebae, plus a BOD of 1.2 mg.l^{-1}, plus 1 x 10^{5}.cm^{-3} bacteria, for 30 minutes, at 30°C.

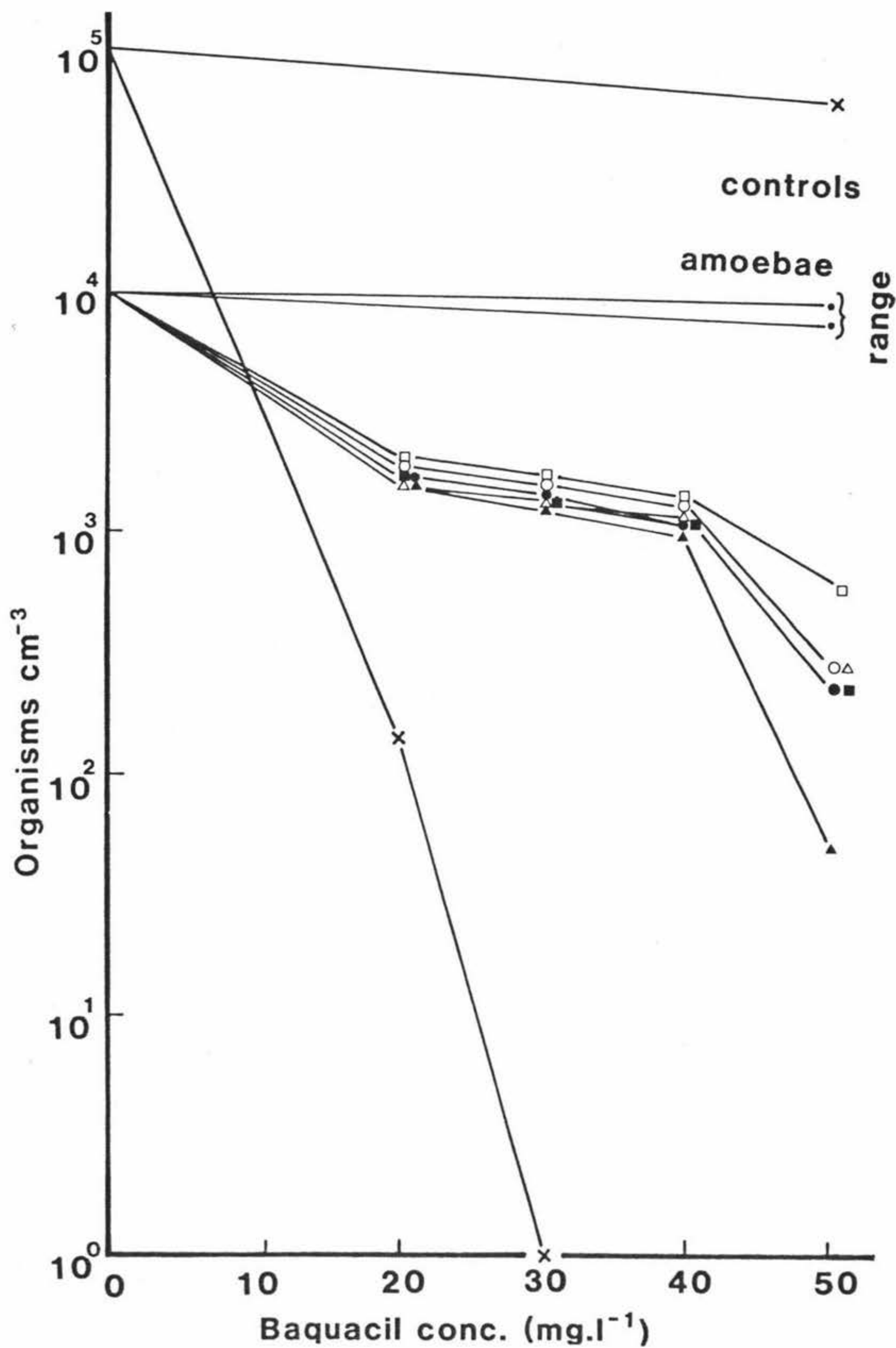


Table XIII

Amoeba	Baquacil ($mg.l^{-1}$)			Survivors		
	Initial	Final	Demand	$Amoebae.cm^{-3}$	%	$Bacteria.cm^{-3}$
N.fowleri (MsM)	0	0	0	9300	93.0	80000
	20	0	20.0	2650	26.5	95
	30	3.0	27.0	2050	20.5	0
	40	11.0	29.0	1350	13.5	0
	50	22.0	28.0	450	4.5	0
N.fowleri (MsT)	0	0	0	9300	93.0	81000
	20	0	20.0	2300	23.0	165
	30	4.0	26.0	1875	18.75	0
	40	11.5	28.5	1050	10.5	0
	50	21.5	28.5	375	3.75	0
N.fowleri (Northcott)	0	0	0	9450	94.5	90000
	20	0	20.0	2450	24.5	85
	30	3.5	26.5	1650	16.5	0
	40	11.0	29.0	1175	11.75	0
	50	22.0	28.5	450	4.5	0
N.gruberi (P1200f)	0	0	0	9600	96.0	82000
	20	0	20.0	2850	28.5	125
	30	3.0	27.0	2175	21.75	0
	40	12.0	28.0	1450	14.5	0
	50	23.0	27.0	875	8.75	0
A.culbertsoni (A-1)	0	0	0	9000	90.0	75000
	20	0	20.0	2300	23.0	150
	30	3.5	26.5	1650	16.5	0
	40	10.5	29.5	1200	12.0	0
	50	20.5	29.5	375	3.75	0
A.castellanii (1501)	0	0	0	9150	91.5	90000
	20	0	20.0	2400	24.0	95
	30	3.0	27.0	1350	13.5	0
	40	11.5	28.5	975	9.75	0
	50	21.0	29.0	75	0.75	0

Figure 13 (Table XIV opposite) The Effect of Baquacil on Monoxenically Grown Amoebae, plus a BOD of 4.7 $mg.l^{-1}$, for 30 minutes, at 30°C.

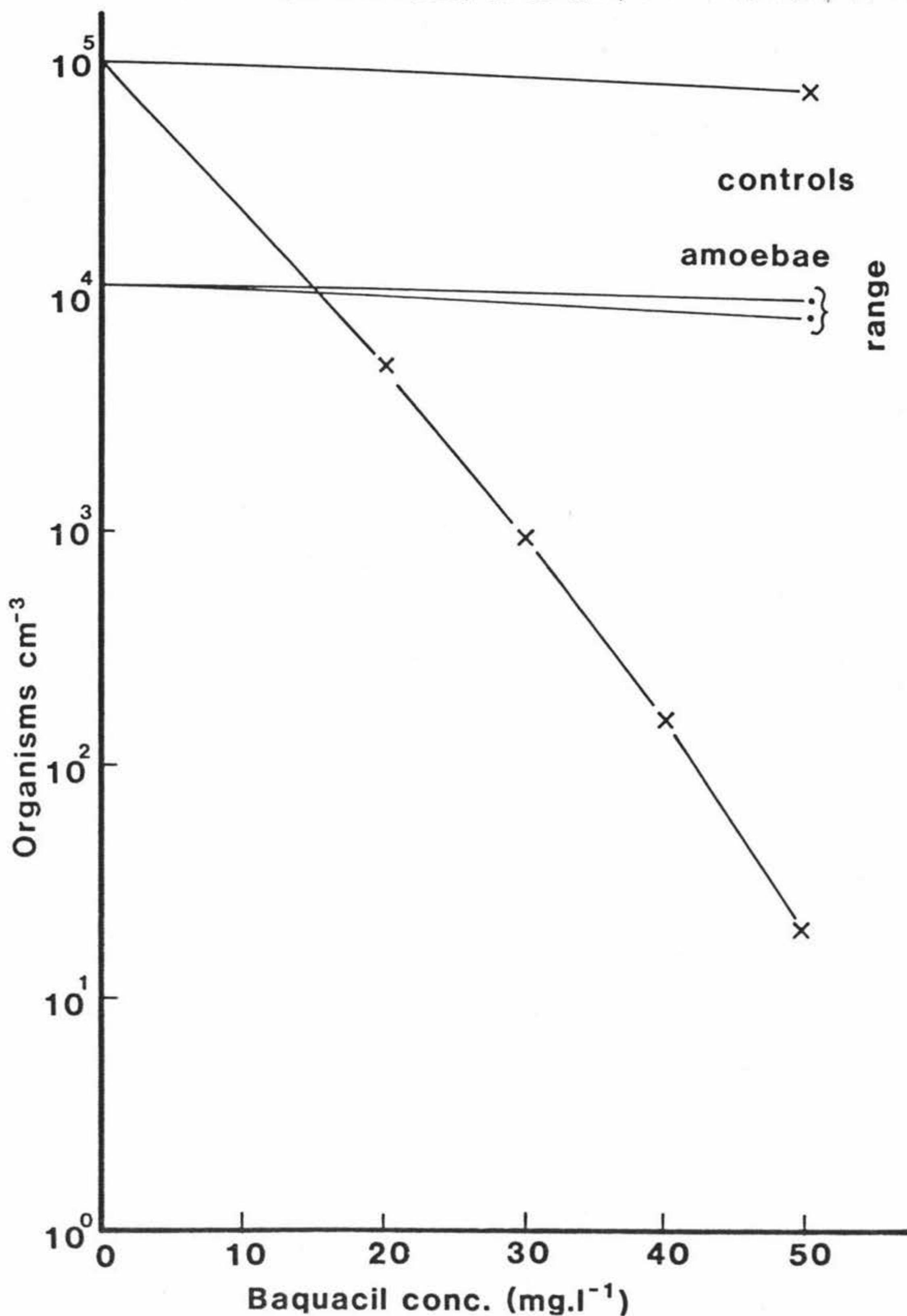


Table XIV

Amoeba	Baquacil ($mg.l^{-1}$)			Survivors	
	Initial	Final	Demand	$Cells.cm^{-3}$	%
N.fowleri (MsM)	0	0	0	8500	85.0
	20	0	20	8400	84.0
	30	0	30	8400	84.0
	40	0	40	8400	84.0
	50	0	50	8400	84.0
N.fowleri (MsT)	0	0	0	8650	86.5
	20	0	20	8600	86.0
	30	0	30	8600	86.0
	40	0	40	8700	87.0
	50	0	50	8650	86.5
N.fowleri (Northcott)	0	0	0	8650	86.5
	20	0	20	8500	85.0
	30	0	30	8000	80.0
	40	0	40	7750	77.5
	50	0	50	7600	76.0
N.gruberi (P1200f)	0	0	0	9450	94.5
	20	0	20	9100	91.0
	30	0	30	9200	92.0
	40	0	40	8700	87.0
	50	0	50	8000	80.0
A.culbertsoni (A-1)	0	0	0	8450	84.5
	20	0	20	8500	85.0
	30	0	30	8200	82.0
	40	0	40	8100	81.0
	50	0	50	8250	82.5
A.castellanii (1501)	0	0	0	8750	87.5
	20	0	20	8250	82.5
	30	0	30	8250	82.5
	40	0	40	8200	82.0
	50	0	50	8000	80.0

Table XV The Effect of Baquacil on Monoxenically Grown Amoebae, plus a BOD of 4.7 $mg.l^{-1}$, plus $1 \times 10^{5}.cm^{-3}$ bacteria, for 30 minutes, at $30^{o}C$

Amoeba	Baquacil ($mg.l^{-1}$)			Survivors		
	Initial	Final	Demand	$Amoebae.cm^{-3}$	%	$Bacteria.cm^{-3}$
N.fowleri (MsM)	0	0	0	9000	90.0	90000
	20	0	20	9000	90.0	7000
	30	0	30	9000	90.0	950
	40	0	40	8900	89.0	200
	50	0	50	9000	90.0	20
N.fowleri (MsT)	0	0	0	9000	90.0	89000
	20	0	20	9100	91.0	6500
	30	0	30	9150	91.5	1000
	40	0	40	9100	91.0	225
	50	0	50	9050	90.5	15
N.fowleri (Northcott)	0	0	0	8600	86.0	91000
	20	0	20	8700	87.0	9000
	30	0	30	8850	88.5	1000
	40	0	40	8950	89.5	250
	50	0	50	8800	88.0	18
N.gruberi (P1200f)	0	0	0	9200	92.0	10500
	20	0	20	9150	91.5	8500
	30	0	30	9100	91.0	980
	40	0	40	9075	90.75	190
	50	0	50	8900	89.0	12
A.culbertsoni (A-1)	0	0	0	8200	82.0	90000
	20	0	20	8300	83.0	9700
	30	0	30	8450	84.5	1000
	40	0	40	8100	81.0	280
	50	0	50	8250	82.5	50
A.castellanii (1501)	0	0	0	8300	83.0	91000
	20	0	20	8400	84.0	8800
	30	0	30	8450	84.5	890
	40	0	40	8150	81.5	190
	50	0	50	8400	84.0	22

4.1.8 Axenically Grown Amoebae and Extended Exposure Time

The time of exposure of the amoebae of 50 $mg.l^{-1}$ Baquacil was increased to 240 minutes. N.fowleri (MsM) and (MsT), and N.gruberi (Pl200f) were used as these three strains had the highest survival rates at 30°C (section 4.1.1, Fig.4, and Table V), when the exposure time was only 30 minutes. Figure 14 and Table XVI show the survival rates at 60 minutes, which were N.gruberi (Pl200f) 0.75%, N.fowleri (MsT) was sterilised, and N.fowleri (MsM) 1.5%. At 105 minutes N.gruberi (Pl200f) was sterilised, but N.fowleri (MsM) was not sterilised until 150 minutes. However at 60 minutes, the survival rates for all three were $<$ 2.0%. The Baquacil demand was 10.5 $mg.l^{-1}$ for N.gruberi (Pl200f), a mean demand of 11.0 $mg.l^{-1}$ for N.fowleri (MsT), and 13.0 $mg.l^{-1}$ for N.fowleri (MsM). Over the same exposure period, the survival rates of control cultures, in buffer, dropped by $<$ 19%.

Figure 14 (Table XVI opposite) The Effect of 50 $mg.l^{-1}$ Baquacil on Three Axenically Grown *Naegleria* strains, for 240 minutes, at 30°C.

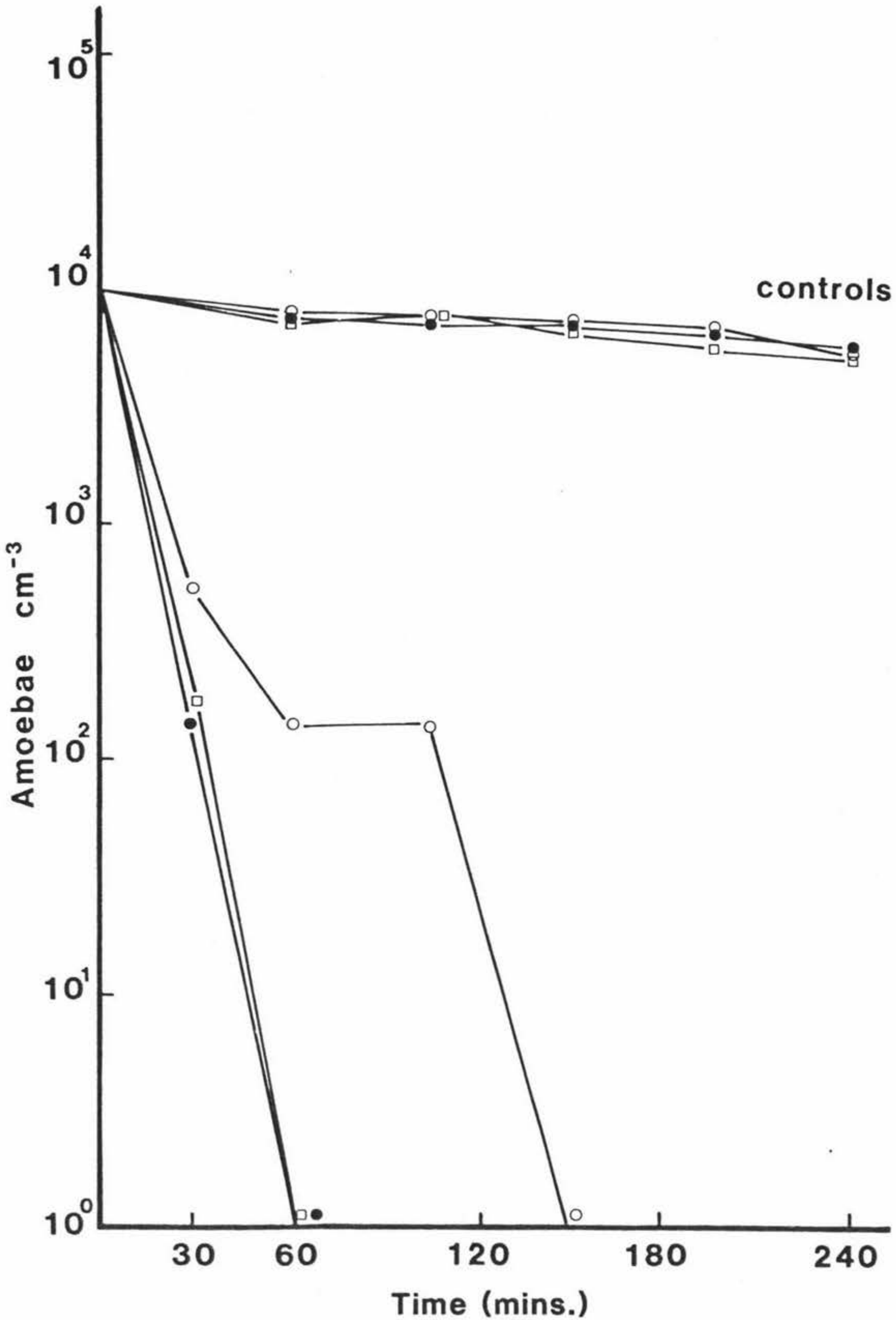


Table XVI

Amoeba	Time (mins)	Baquacil ($mg.l^{-1}$)			Survivors	
		Initial	Final	Demand	$Cells.cm^{-3}$	%
N.fowleri (MsM)	60	0	0	0	8700	87.0
	105	0	0	0	8700	87.0
	150	0	0	0	8400	84.0
	195	0	0	0	8100	81.0
	240	0	0	0	7950	79.5
	60	50	40.5	9.5	150	1.5
	105	50	37.0	13.0	75	0.75
	150	50	37.0	13.0	0	0
	195	50	36.5	13.5	0	0
	240	50	35.0	15.0	0	0
N.fowleri (MsT)	60	0	0	0	9000	90.0
	105	0	0	0	8550	85.5
	150	0	0	0	8250	82.5
	195	0	0	0	7950	79.5
	240	0	0	0	7500	75.0
	60	50	37.0	13.0	0	0
	105	50	39.5	10.5	0	0
	150	50	37.0	13.0	0	0
	195	50	39.5	10.5	0	0
	240	50	39.5	10.5	0	0
N.gruberi (P1200f)	60	0	0	0	8850	88.5
	105	0	0	0	8550	85.5
	150	0	0	0	8550	85.5
	195	0	0	0	8100	81.0
	240	0	0	0	7800	78.0
	60	50	39.5	10.5	75	0.75
	105	50	39.5	10.5	0	0
	150	50	39.5	10.5	0	0
	195	50	39.5	10.5	0	0
	240	50	39.5	10.5	0	0

4.1.9 Axenically Grown Amoebae plus Bacteria with Increased Baquacil Concentration.

Due to the inability to sterilise the test cultures up to this stage, an increase in initial Baquacil concentration was made (Fig.15 and Table XVII).

Figure 15 and Table XVII show that at an exposure time of 30 minutes, N.fowleri (Northcott) was sterilised by 60 $mg.l^{-1}$, both Acanthamoeba spp. and N.fowleri (MsM) were sterilised by 70 $mg.l^{-1}$, and N.fowleri (MsT) was sterilised by 80 $mg.l^{-1}$ Baquacil. N.gruberi (P1200f) was not sterilised by any concentration tested, although at all concentrations, the survival rates were $< 8.0\%$. The Baquacil demand varied between 8.0 and 28.0 $mg.l^{-1}$ (mean 11.0 $mg.l^{-1}$). There was no significant difference between the two genera in either Baquacil demand or sensitivity to Baquacil.

Figure 15 (Table XVII opposite) The Effect of up to 80 mg.l^{-1} Baquacil on Axenically Grown Amoebae, for 30 minutes, at 30°C.

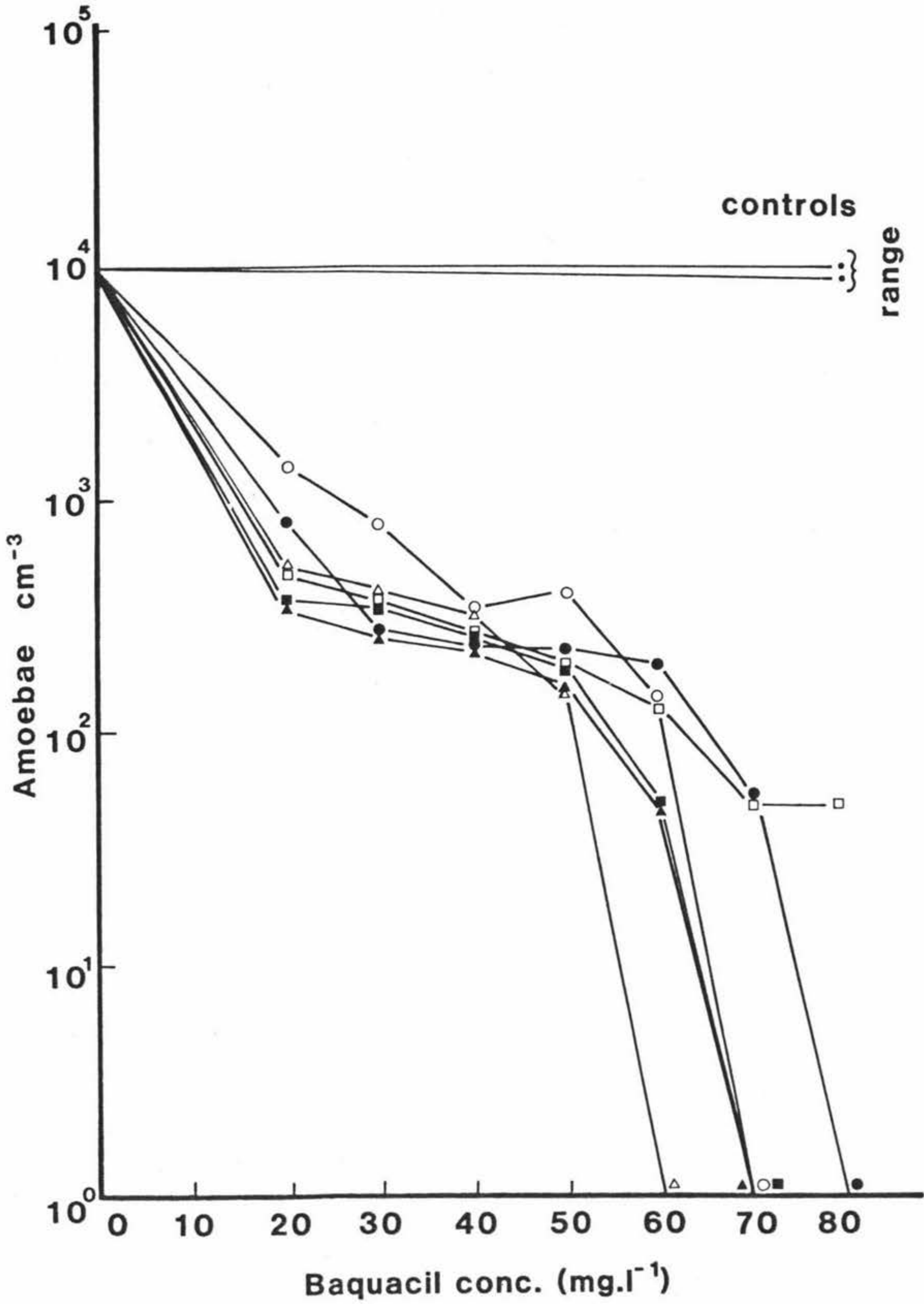


Table XVII

Amoeba	Baquacil (mg.l^{-1})			Survivors	
	Initial	Final	Demand	Cells.cm^{-3}	%
N.fowleri (MsM)	0	0	0	9600	96.0
	20	2.0	18.0	1250	12.5
	30	4.0	26.0	900	9.0
	40	26.0	14.0	525	5.25
	50	39.0	11.0	600	6.0
	60	48.0	12.0	150	1.5
	70	69.0	11.0	0	0
	80	70.5	9.5	0	0
N.fowleri (MsT)	0	0	0	9800	98.0
	20	10.0	10.0	900	9.0
	30	16.0	14.0	450	4.5
	40	25.0	15.0	375	3.75
	50	39.0	11.0	375	3.75
	60	50.0	10.0	300	3.0
	70	59.5	10.5	75	0.75
	80	71.0	9.0	0	0
N.fowleri (Northcott)	0	0	0	9300	93.0
	20	12.0	8.0	750	7.5
	30	20.0	10.0	600	6.0
	40	30.0	10.0	525	5.25
	50	39.5	11.5	150	1.5
	60	48.8	11.2	0	0
	70	59.5	10.5	0	0
	80	70.0	10.0	0	0
N.gruberi (P1200f)	0	0	0	10000	100.0
	20	8.0	12.0	750	7.5
	30	21.0	9.0	600	6.0
	40	30.5	9.5	450	4.5
	50	39.0	9.0	300	3.0
	60	48.5	11.5	150	1.5
	70	59.0	11.0	75	0.75
	80	71.0	9.0	75	0.75
A.culbertsoni (A-1)	0	0	0	8800	88.0
	20	8.0	12.0	525	5.25
	30	20.0	10.0	525	5.25
	40	28.0	12.0	450	4.5
	50	41.0	9.0	275	2.75
	60	52.0	8.0	75	0.75
	70	60.5	9.5	0	0
	80	69.0	11.0	0	0
A.castellanii (1501)	0	0	0	9100	91.0
	20	9.0	11.0	525	5.25
	30	18.0	12.0	450	4.5
	40	29.5	10.5	375	3.75
	50	41.0	9.0	150	1.5
	60	49.5	10.5	75	0.75
	70	58.0	12.0	0	0
	80	70.0	10.0	0	0

4.1.10 Axenically Grown Amoebae plus Bacteria and Increased Exposure Time and Baquacil Concentration.

As is shown by Fig. 16 and Table XVIII, 80 $mg.l^{-1}$ Baquacil sterilised all six strains. All except *N.gruberi* (P1200f) were sterilised by 70 $mg.l^{-1}$, and *N.fowleri* (Northcott) by 60 $mg.l^{-1}$, at an exposure time of 60 minutes. The bacteria were killed in all flasks by 20 $mg.l^{-1}$ Baquacil. The Baquacil demand varied from 7.0 to 12.0 $mg.l^{-1}$ (mean 10.0 $mg.l^{-1}$). No marked difference in sensitivity to Baquacil or Baquacil demand was apparent between *Naegleria* and *Acanthamoeba* spp.

Figure 16 (Table XVIII opposite) The Effect of up to 80 $mg.l^{-1}$ Baquacil on Axenically Grown Amoebae, plus $1 \times 10^5.cm^{-3}$ bacteria, for 60 minutes, at 30°C.

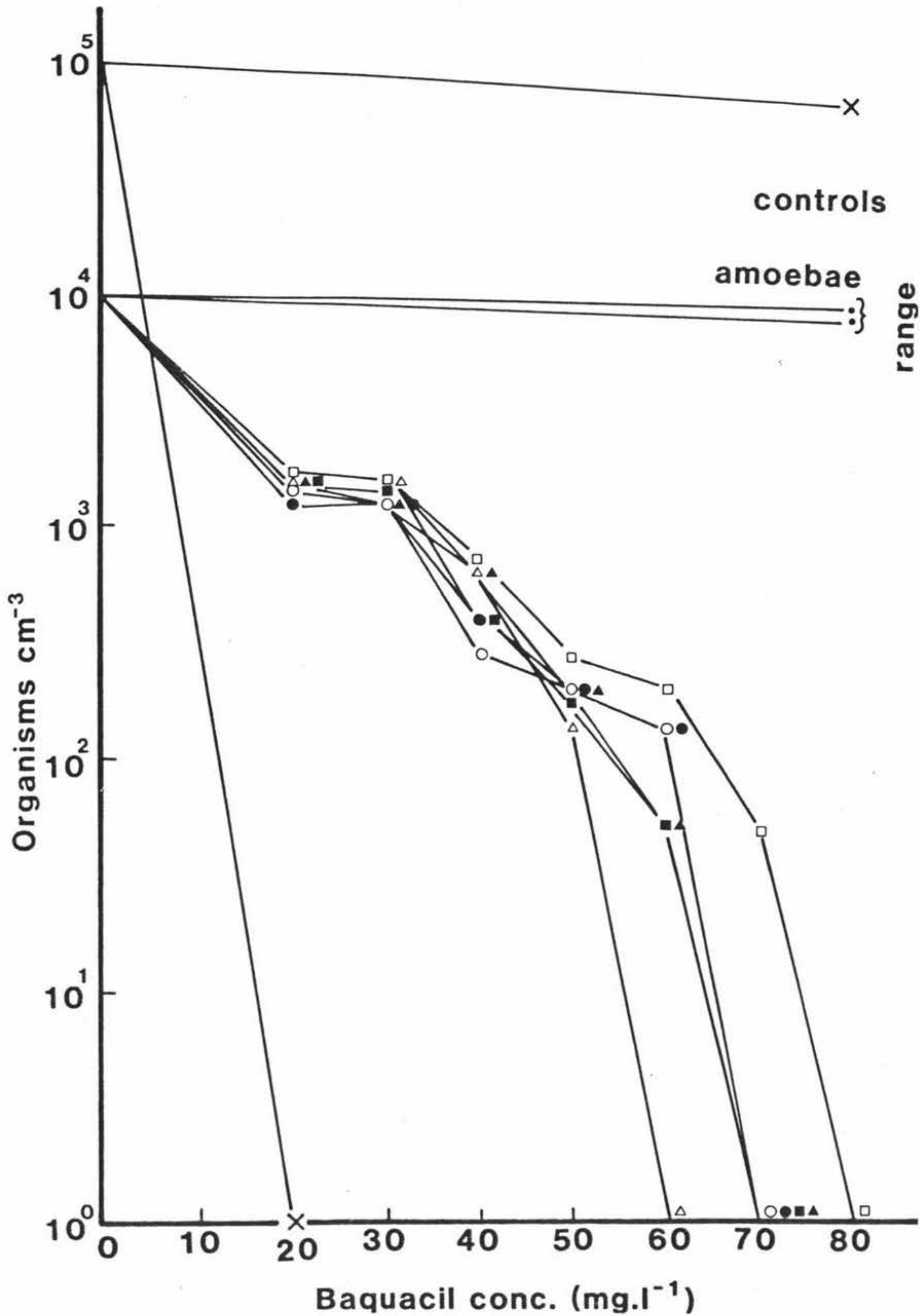


Table XVIII

Amoeba	Baquacil ($mg.l^{-1}$)			Survivors		
	Initial	Final	Demand	Amoebae.cm^{-3}	%	Bacteria.cm^{-3}
N.fowleri (MsM)	0	0	0	9300	93.0	81000
	20	9.0	11.0	1575	15.75	0
	30	19.0	11.0	1050	10.5	0
	40	29.5	10.5	450	4.5	0
	50	39.0	11.0	300	3.0	0
	60	48.5	11.5	150	1.5	0
	70	60.0	10.0	0	0	0
	80	69.0	11.0	0	0	0
N.fowleri (MsT)	0	0	0	9450	94.5	81500
	20	9.0	11.0	1500	15.0	0
	30	21.0	9.0	1050	10.5	0
	40	28.5	11.5	600	6.0	0
	50	40.0	10.0	300	3.0	0
	60	49.5	10.5	150	1.5	0
	70	62.0	8.0	0	0	0
	80	70.0	10.0	0	0	0
N.fowleri (Northcott)	0	0	0	9000	90.0	79000
	20	10.0	10.0	1600	16.0	0
	30	20.0	10.0	1450	14.5	0
	40	33.0	7.0	800	8.0	0
	50	43.0	7.0	150	1.5	0
	60	52.5	7.5	0	0	0
	70	60.0	10.0	0	0	0
	80	71.0	9.0	0	0	0
N.gruberi (P1200f)	0	0	0	9200	92.0	77000
	20	8.5	11.5	2000	20.0	0
	30	19.5	10.5	1500	15.0	0
	40	28.0	12.0	850	8.5	0
	50	40.5	9.5	450	4.5	0
	60	51.0	9.0	300	3.0	0
	70	59.0	11.0	75	0.75	0
	80	60.5	9.5	0	0	0
A.culbertsoni (A-1)	0	0	0	8850	88.5	80000
	20	8.0	12.0	1600	16.0	0
	30	21.0	9.0	1200	12.0	0
	40	29.5	10.5	600	6.0	0
	50	39.0	11.0	275	2.75	0
	60	48.0	12.0	75	0.75	0
	70	60.0	10.0	0	0	0
	80	69.5	10.5	0	0	0
A.castellanii (1501)	0	0	0	8900	89.0	81000
	20	11.2	8.8	1600	16.0	0
	30	21.6	8.4	1050	10.5	0
	40	31.5	8.5	800	8.0	0
	50	41.0	9.0	300	3.0	0
	60	50.0	10.0	75	0.75	0
	70	59.8	10.2	0	0	0
	80	70.0	10.0	0	0	0

4.1.11 Monoxenically Grown Amoebae plus Bacteria with Increased Exposure Time and Increased Baquacil Concentration

Figure 17 and Table XIX show that increased exposure time significantly decreased the survival rate of all six strains of amoebae, by between 2.0 and 4.5%, to those shown in Fig.10 and Table XI (section 4.1.5). The survival rates were $<$ 5.0% at 50 mg.l^{-1} Baquacil, and N.fowleri (Northcott) was sterilised at this concentration. The bacteria were killed by 20 mg.l^{-1} Baquacil.

When both exposure time and initial Baquacil concentration were increased, Fig.18 and Table XX show that at 60 mg.l^{-1} N.fowleri (Northcott) is sterilised, at 70 mg.l^{-1}, at 70 mg.l^{-1} all strains except N.gruberi (P1200f) were sterilised. N.gruberi (P1200f) was then sterilised by 80 mg.l^{-1} Baquacil. These were the same as the axenically grown amoebae in the same test (section 4.1.10, Fig.16 and Table XVIII). The bacteria were killed by 20 mg.l^{-1} Baquacil. The Baquacil demand was between 10.0 and 12.0 mg.l^{-1}. There was no apparent difference in sensitivity between the two genera, and nor was there any difference in Baquacil demand.

Figure 17 (Table XIX opposite) The Effect of Baquacil on Monoxenically Grown Amoebae, plus $1 \times 10^5.cm^{-3}$ bacteria, for 60 minutes, at 30^oC.

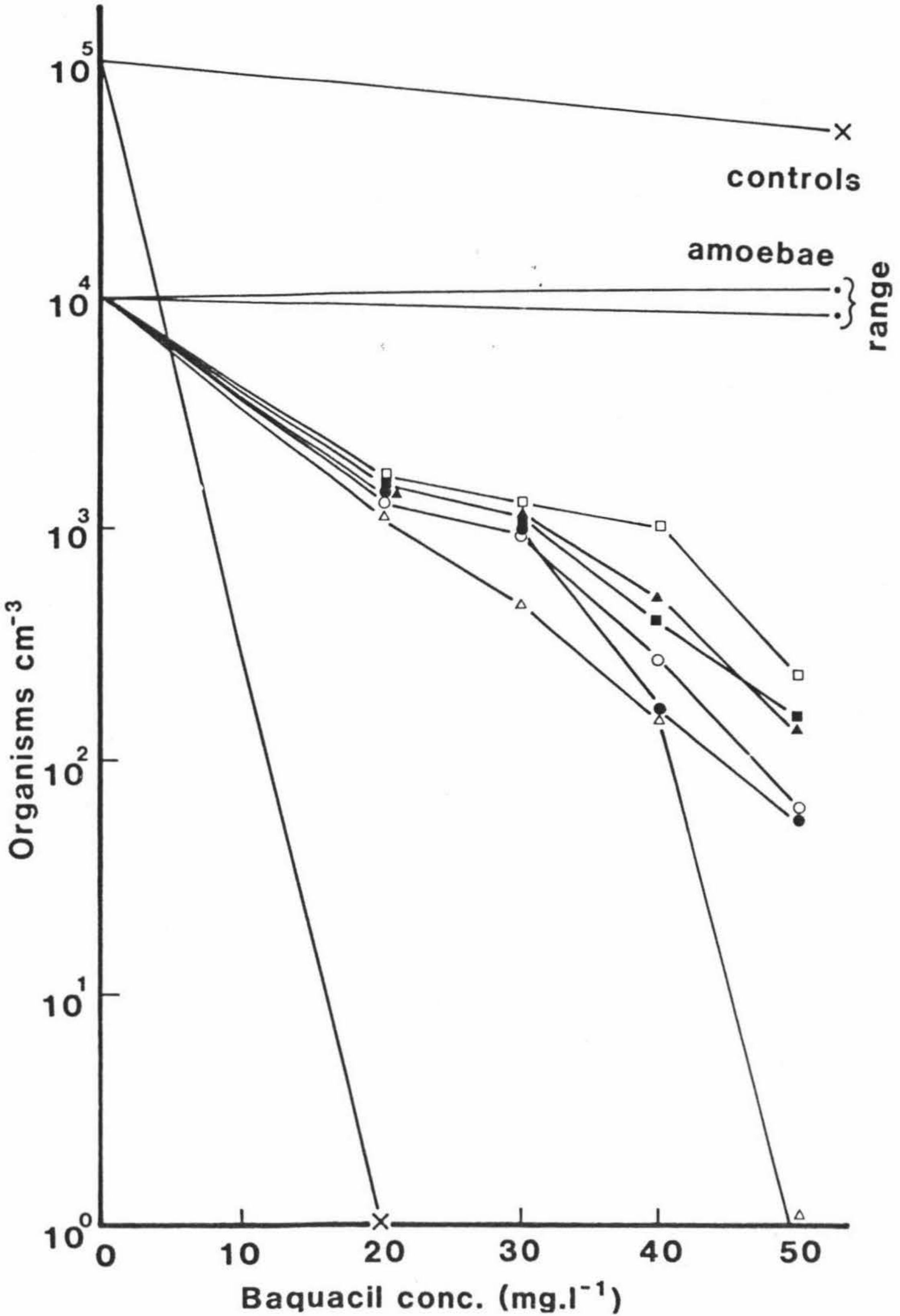


Table XIX

Amoeba	Baquacil (mg.l^{-1})			Survivors		
	Initial	Final	Demand	Amoebae.cm^{-3}	%	Bacteria.cm^{-3}
N.fowleri (MsM)	0	0	0	9600	96.0	74000
	20	7.0	13.0	1400	14.0	0
	30	18.0	12.0	975	9.75	0
	40	28.0	12.0	550	5.5	0
	50	40.0	10.0	375	3.75	0
N.fowleri (MsT)	0	0	0	9800	98.0	78000
	20	7	13.0	1450	14.5	0
	30	17.5	12.5	900	9.0	0
	40	26.0	14.0	425	4.25	0
	50	39.0	11.0	275	2.75	0
N.fowleri (Northcott)	0	0	0	9400	94.0	76000
	20	14.0	6.0	1200	12.0	0
	30	21.0	9.0	675	6.75	0
	40	32.0	8.0	300	3.0	0
	50	39.0	11.0	0	0	0
N.gruberi (P1200f)	0	0	0	10350	103.5	72000
	20	13.0	7.0	1950	19.5	0
	30	20.0	10.0	1500	15.0	0
	40	31.5	8.5	975	9.75	0
	50	42.0	8.0	475	4.75	0
A.culbertsoni (A-1)	0	0	0	9400	94.0	76500
	20	13.0	7.0	1700	17.0	0
	30	22.0	8.0	1150	11.5	0
	40	30.0	10.0	600	6.0	0
	50	36.0	14.0	375	3.75	0
A.castellanii (1501)	0	0	0	9600	96.0	76000
	20	13.0	7.0	1650	16.5	0
	30	22.0	8.0	1400	14.0	0
	40	30.0	10.0	750	7.5	0
	50	40.0	10.0	275	2.75	0

Figure 18 (Table XX opposite) The Effect of up to 80 $mg.l^{-1}$ Baquacil on Monoxenically Grown Amoebae, plus $1 \times 10^5.cm^{-3}$ bacteria, for 60 minutes, at 30^oC.

controls

amoebae range

ORGANISMS CM^{-3}

10^5

10^4

10^3

10^2

10^1

10^0

0 10 20 30 40 50 60 70 80

Baquacil conc. ($mg.l^{-1}$)

Table XX

Amoeba	Baquacil ($mg.l^{-1}$)			Survivors		
	Initial	Final	Demand	$Amoebae.cm^{-3}$	%	$Bacteria.cm^{-3}$
N.fowleri (MsM)	0	0	0	9400	94.0	82000
	20	8.0	12.0	1650	16.5	0
	30	19.0	11.0	1200	12.0	0
	40	28.0	12.0	550	5.5	0
	50	38.5	11.5	150	1.5	0
	60	49.0	11.0	75	0.75	0
	70	59.0	11.0	0	0	0
	80	68.5	11.5	0	0	0
N.fowleri (MsT)	0	0	0	9500	95.0	83000
	20	9.0	11.0	1600	16.0	0
	30	18.5	11.5	1300	13.0	0
	40	29.2	10.8	575	5.75	0
	50	39.0	11.0	200	2.0	0
	60	48.5	11.5	75	0.75	0
	70	58.0	12.0	0	0	0
	80	69.0	11.0	0	0	0
N.fowleri (Northcott)	0	0	0	9300	93.0	81000
	20	9.0	11.0	1600	16.0	0
	30	20.0	10.0	1250	12.5	0
	40	29.0	11.0	350	3.5	0
	50	39.5	10.5	150	1.5	0
	60	49.2	10.8	0	0	0
	70	59.5	10.5	0	0	0
	80	69.0	11.0	0	0	0
N.gruberi (P1200f)	0	0	0	9400	94.0	83000
	20	9.0	11.0	2100	21.0	0
	30	18.8	11.2	1800	18.0	0
	40	29.0	11.0	1000	10.0	0
	50	38.0	12.0	550	5.5	0
	60	48.5	11.5	375	3.75	0
	70	58.0	12.0	75	0.75	0
	80	68.4	11.6	0	0	0
A.culbertsoni (A-1)	0	0	0	9100	91.0	84000
	20	10.0	10.0	1700	17.0	0
	30	19.0	11.0	1300	13.0	0
	40	29.5	10.5	800	8.0	0
	50	39.0	11.0	350	3.5	0
	60	49.2	10.8	150	1.5	0
	70	58.5	11.5	0	0	0
	80	68.8	11.2	0	0	0
A.castellanii (1501)	0	0	0	9000	90.0	82000
	20	9.5	10.5	1800	18.0	0
	30	18.5	11.5	1350	13.5	0
	40	29.0	11.0	850	8.5	0
	50	39.5	10.5	325	3.25	0
	60	49.0	11.0	150	1.5	0
	70	59.0	11.0	0	0	0
	80	69.0	10.5	0	0	0

4.1.12 Suspected Baquacil Resistant Clones of Naegleria spp.

From the axenically grown and tested amoebae, in section 4.1.1 Tables III,IV and V, as many plaques as possible were cultured as clones in CYM medium and subcultured every 48 hours for three months prior to this experiment. Table XXI shows that of the clones that survived the three months, four strains had much higher survival rates than the corresponding "parent" strain. Both N.gruberi (P1200f) suspected resistant strains' survival rates were not significantly different to N.gruberi (P1200f). The same applied to N.fowleri ($MsMrb_1$) and N.fowleri (MsM). N.fowleri ($MsMrb_2$) and ($MsMrb_4$) survival rates indicate some resistance but not total resistance to Baquacil. However the N.fowleri ($MsMrb_3$) and ($MsTrb_1$) survival rates indicate almost total resistance to Baquacil. The Baquacil demand for the confirmed non-resistant strains was a mean of 12.0 $mg.l^{-1}$, for the partially resistant a mean of 7.0 $mg.l^{-1}$, and for the resistant strains, the demand was 4.0 $mg.l^{-1}$.

Table XXI. The Effect of 50 mg.l^{-1} Baquacil on Suspected Resistant Clones of *Naegleria* strains.

Amoeba	Baquacil (mg.l^{-1})			Survivors	
	Initial	Final	Demand	Cells.cm^{-3}	%
N.fowleri (MsM)	0 50	0 36.0	0 14.0	8250 300	82.5 3.0
N.fowleri ($MsMrb_1$)	0 50	0 36.0	0 14.0	8100 750	81.0 7.5
N.fowleri ($MsMrb_2$)	0 50	0 42.0	0 8.0	9150 3000	91.5 30.0
N.fowleri ($MsMrb_3$)	0 50	0 46.0	0 4.0	9000 9600	90.0 96.0
N.fowleri ($MsMrb_4$)	0 50	0 43.5	0 6.5	8700 5700	87.0 57.0
N.fowleri (MsT)	0 50	0 39.0	0 11.0	8900 300	89.0 3.0
N.fowleri ($MsTrb_1$)	0 50	0 46.0	0 4.0	9450 9300	94.5 93.0
N.gruberi (P1200f)	0 50	0 38.0	0 12.0	9400 575	94.0 5.75
N.gruberi ($P1200frb_1$)	0 50	0 38.0	0 12.0	9450 525	94.5 5.25
N.gruberi ($P1200frb_2$)	0 50	0 39.0	0 11.0	9300 550	93.0 5.5

4.2 The Use of Chlorine as a Disinfectant Against Pathogenic Free-Living Amoebae

Because the deionized water used exerted a negligible chlorine demand, the Total Available Chlorine (TAC) at zero time was equal to the Free Available Chlorine (FAC) at that time (Tables XXII to XXIX). The difference (TAC - FAC), known as Combined Available Chlorine (CAC), represents the chlorine demand due to the organic content of the inoculum reacting with the chlorine during the 30 minute exposure time.

4.2.1 Axenically Grown Amoebae plus Bacteria

Figure 19 and Table XXII show that, although not amoebicidal, 0.75 $mg.l^{-1}$ chlorine is bactericidal. The lowest amoebicidal concentration was 0.96 $mg.l^{-1}$ for *Naegleria* spp. and 2.05 $mg.l^{-1}$ for *Acanthamoeba* spp. *Naegleria* were more sensitive than *Acanthamoeba* spp., and the bacteria were preferentially killed before the amoebae.

Figure 19 (Table XXII opposite) The Effect of Chlorine on Axenically Grown Amoebae, plus 1 x 10^5.cm^{-3} bacteria, for 30 minutes, at 30^oC.

controls

amoebae

10^5
10^4
10^3
10^2
10^1
10^0

Organisms cm^{-3}

0 1.5 3.0 4.5

Total Available Chlorines (TAC) ($mg.l^{-1}$)

Table XXII

Amoeba	Chlorine ($mg.l^{-1}$)			Survivors		
	TAC	FAC	CAC	$Amoebae.cm^{-3}$	%	$Bacteria.cm^{-3}$
N.fowleri (MsM)	0	0	0	8950	89.5	91000
	0.75	0	0.75	4550	45.5	0
	1.5	0.45	1.05	0	0	0
	3.0	2.0	1.0	0	0	0
	4.5	3.47	1.03	0	0	0
N.fowleri (MsT)	0	0	0	8900	89.0	91000
	0.75	0	0.75	4800	48.0	0
	1.5	0.52	0.98	0	0	0
	3.0	2.0	1.0	0	0	0
	4.5	3.47	1.03	0	0	0
N.fowleri (Northcott)	0	0	0	8800	88.0	92000
	0.75	0	0.75	4300	43.0	0
	1.5	0.54	0.96	0	0	0
	3.0	2.0	1.0	0	0	0
	4.5	3.45	1.05	0	0	0
N.gruberi (P1200f)	0	0	0	9000	90.0	91000
	0.75	0	0.75	4400	44.0	0
	1.5	0.52	0.48	0	0	0
	3.0	2.0	1.0	0	0	0
	4.5	3.5	1.0	0	0	0
A.culbertsoni (A-1)	0	0	0	8800	88.0	93000
	0.75	0	0.75	7050	70.5	0
	1.5	0	1.5	2550	25.5	0
	3.0	0.95	2.05	0	0	0
	4.5	2.4	2.1	0	0	0
A.castellanii (1501)	0	0	0	9000	90.0	90000
	0.75	0	0.75	6850	68.5	0
	1.5	0	1.5	2250	22.5	0
	3.0	0.9	2.1	0	0	0
	4.5	2.42	2.08	0	0	0

4.2.2 Axenically Grown Amoebae plus a BOD

To enable a comparison with Baquacil and chlorine dioxide, both BOD levels, i.e. 1.2 and 4.7 $mg.l^{-1}$ were tested. The lower BOD of 1.2 $mg.l^{-1}$, together with the inoculum of amoebae, caused an organic chlorine demand of 2.6 $mg.l^{-1}$ for *Naegleria*, and 4.9 $mg.l^{-1}$ for *Acanthamoeba* spp. (Fig. 20 and Table XXIII). In the case of *Acanthamoeba* spp. this was not amoebicidal. This again highlights the greater sensitivity of *Naegleria* spp. to chlorine than *Acanthamoeba* spp., as do the results shown in Fig. 21 and Table XXIV. These show that 6.0 $mg.l^{-1}$ chlorine (the highest concentration used) was not amoebicidal for either genera of amoebae in the presence of a high BOD of 4.7 $mg.l^{-1}$. The survival rates were between 15.5 and 18.5% for *Naegleria* spp., and 56.75 and 58.5% for *Acanthamoeba* spp. at this chlorine concentration.

Figure 20 (Table XXIII opposite) The Effect of Chlorine on Axenically Grown Amoebae, plus a BOD of 1.2 $mg.l^{-1}$, for 30 minutes, at $30^{o}C$.

controls range

Amoebae.cm^{-3}

10^4 10^3 10^2 10^1 10^0

0 1.0 2.0 3.0 4.0 5.0 6.0

TAC ($mg.l^{-1}$)

Table XXIII

Amoeba	Chlorine ($mg.l^{-1}$)			Survivors	
	TAC	FAC	CAC	$cells.cm^{-3}$	%
N.fowleri (MsM)	0	0	0	8900	89.0
	1.5	0	1.5	5650	56.5
	3.0	0.42	2.58	150	1.5
	4.5	1.9	2.6	0	0
	6.0	3.39	2.61	0	0
N.fowleri (MsT)	0	0	0	8600	86.0
	1.5	0	1.5	5500	55.0
	3.03	0.43	2.6	75	0.75
	4.5	1.49	2.61	0	0
	6.0	3.37	2.63	0	0
N.fowleri (Northcott)	0	0	0	8550	85.5
	1.5	0	1.5	5250	52.5
	3.0	0.43	2.57	0	0
	4.5	1.9	2.6	0	0
	5.95	3.31	2.62	0	0
N.gruberi (P1200f)	0	0	0	8800	88.0
	1.5	0	1.5	5300	53.0
	2.97	0.37	2.6	75	0.75
	4.48	1.47	2.61	0	0
	6.0	3.39	2.61	0	0
A.culbertsoni (A-1)	0	0	0	8800	88.0
	1.5	0	1.5	8400	84.0
	3.0	0	3.0	7500	75.0
	4.52	0	4.52	6000	60.0
	6.0	1.04	4.96	3000	30.0
A.castellanii (1501)	0	0	0	8700	87.0
	1.5	0	1.5	8700	87.0
	3.0	0	3.0	7600	76.0
	4.5	0	4.5	6000	60.0
	6.03	1.13	4.9	2850	28.5

Figure 21 (Table XXIV opposite) The Effect of Chlorine on Axenically Grown Amoebae, plus a BOD of 4.7 $mg.l^{-1}$, for 30 minutes, at $30^{o}C$.

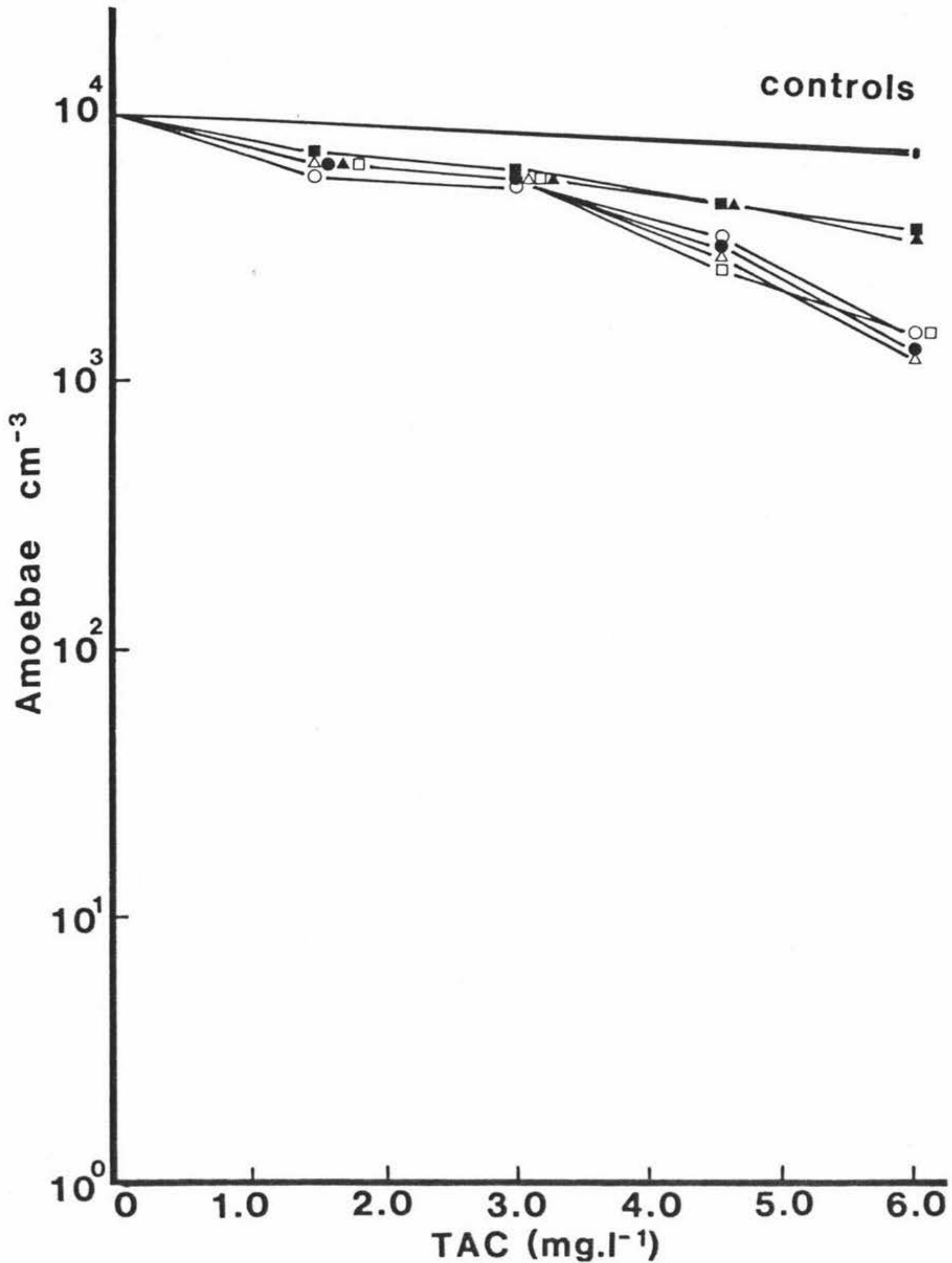


Table XXIV

Amoeba	Chlorine ($mg.l^{-1}$)			Survivors	
	TAC	FAC	CAC	$cells.cm^{-3}$	%
N.fowleri (MsM)	0	0	0	8700	87.0
	1.5	0	1.5	7950	79.5
	3.0	0	3.0	7400	74.0
	4.5	0	4.5	5500	55.0
	6.0	0	6.0	1800	18.0
N.fowleri (MsT)	0	0	0	8700	87.0
	1.5	0	1.5	8250	82.5
	3.0	0	3.0	7700	77.0
	4.51	0	4.51	5100	51.0
	6.0	0	6.0	1650	16.5
N.fowleri (Northcott)	0	0	0	8600	86.0
	1.51	0	1.51	8250	82.5
	3.0	0	3.0	7350	73.5
	4.52	0	4.52	4950	49.5
	6.0	0	6.0	1550	15.5
N.gruberi (P1200f)	0	0	0	8850	88.5
	1.48	0	1.48	8300	83.0
	3.0	0	3.0	7450	74.5
	4.5	0	4.5	4500	45.0
	6.0	0	6.0	1850	18.5
A.culbertsoni (A-1)	0	0	0	8850	88.5
	1.5	0	1.5	8550	85.5
	2.97	0	2.97	7600	76.0
	4.5	0	4.5	6850	68.5
	6.0	0	6.0	5850	58.5
A.castellanii (1501)	0	0	0	8700	87.0
	1.5	0	1.5	8250	82.5
	3.0	0	3.0	7550	75.5
	4.5	0	4.5	6800	68.0
	5.97	0	5.97	5675	56.75

4.2.3 Axenically Grown Amoebae plus a BOD and Bacteria

Figure 22 and Table XXV show that the addition of $1 \times 10^{5}.cm^{-3}$ E.cloacae increases the organic chlorine demand, as does a BOD of $1.2\ mg.l^{-1}$. Table XXV shows that Naegleria were more sensitive to chlorine than Acanthamoeba, with amoebicidal concentrations of $3.9\ mg.l^{-1}$ and $5.3\ mg.l^{-1}$ respectively. Bacteria were killed by $1.5\ mg.l^{-1}$ chlorine.

The higher BOD of $4.7\ mg.l^{-1}$ was not tested in combination with bacteria due to the "protective" effect for the amoebae of the high BOD load.

Figure 22 (Table XXV opposite) The Effect of Chlorine on Axenically Grown Amoebae, plus a BOD of 1.2 $mg.l^{-1}$, plus $1 \times 10^5.cm^{-3}$ bacteria, for 30 minutes, at 30^oC.

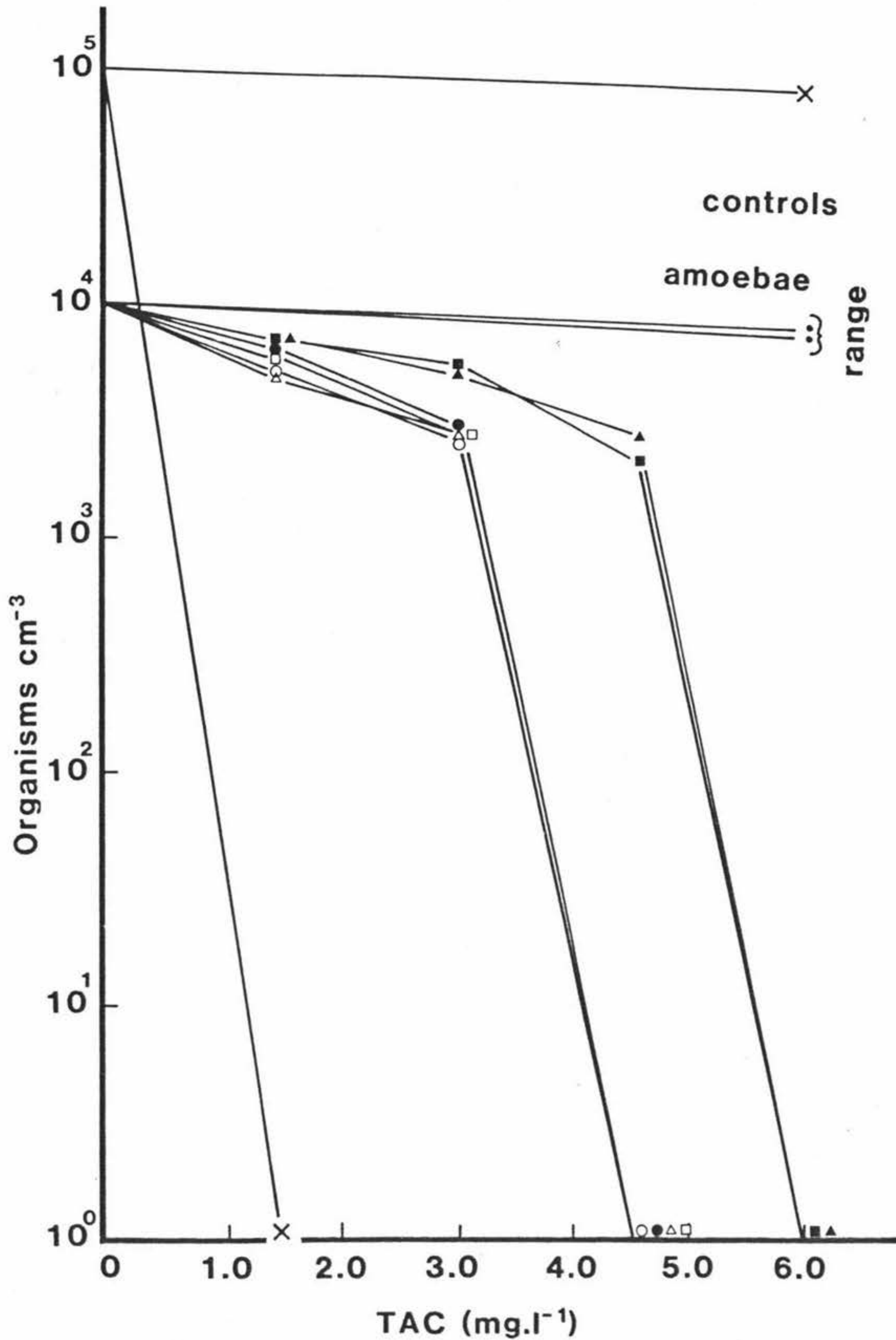


Table XXV

Amoeba	Chlorine (mg.l^{-1})			Survivors		
	TAC	FAC	CAC	Amoebae.cm^{-3}	%	Bacteria.cm^{-3}
N.fowleri (MsM)	0	0	0	8900	89.0	91000
	1.5	0	1.5	7100	71.0	0
	3.0	0	3.0	4100	41.0	0
	4.5	0.6	3.9	0	0	0
	6.0	2.12	3.88	0	0	0
N.fowleri (MsT)	0	0	0	8850	88.5	91000
	1.5	0	1.5	8000	80.0	0
	3.0	0	3.0	4600	46.0	0
	4.47	0.61	3.86	0	0	0
	6.0	2.11	3.89	0	0	0
N.fowleri (Northcott)	0	0	0	9000	90.0	90000
	1.48	0	1.48	7000	70.0	0
	3.0	0	3.0	4300	43.0	0
	4.5	0.59	3.91	0	0	0
	6.0	2.12	3.88	0	0	0
N.gruberi (P1200f)	0	0	0	8900	89.0	92000
	1.5	0	1.5	7500	75.0	0
	2.98	0	2.98	4300	43.0	0
	4.5	0.6	3.9	0	0	0
	5.96	2.06	3.9	0	0	0
A.culbertsoni (A-1)	0	0	0	9000	90.0	91000
	1.48	0	1.48	8200	82.0	0
	2.96	0	2.96	7600	76.0	0
	4.5	0	4.5	3350	33.5	0
	6.0	0.7	5.3	0	0	0
A.castellanii (1501)	0	0	0	9000	90.0	93000
	1.5	0	1.5	8300	83.0	0
	3,0	0	3.0	7500	75.0	0
	4.5	0	4.5	4500	45.0	0
	6.02	0.72	5.3	0	0	0

MASSEY UNIVERSITY
LIBRARY

4.2.4 Monoxenically Grown Amoebae plus Bacteria

Figure 23 and Table XXVI show that when the organic chlorine demand is increased by the addition of 1 x $10^5.cm^{-3}$ *E.cloacae*, there is still a difference in sensitivity to chlorine between *Naegleria* and *Acanthamoeba* spp., the former being more sensitive than the latter. The chlorine demand was increased to 1.1 $mg.l^{-1}$ for *Naegleria* and 2.2 $mg.l^{-1}$ for *Acanthamoeba* spp., both these concentrations were amoebicidal. This demand was an increase of 0.1 $mg.l^{-1}$ chlorine over the corresponding axenically grown amoebae experiment (section 4.2.1, Table XXII). The bacteria were killed by 0.75 $mg.l^{-1}$ chlorine.

Figure 23 (Table XXVI opposite) The Effect of Chlorine on Monoxenically Grown Amoebae, plus $1 \times 10^5.cm^{-3}$ bacteria, for 30 minutes, at 30^oC.

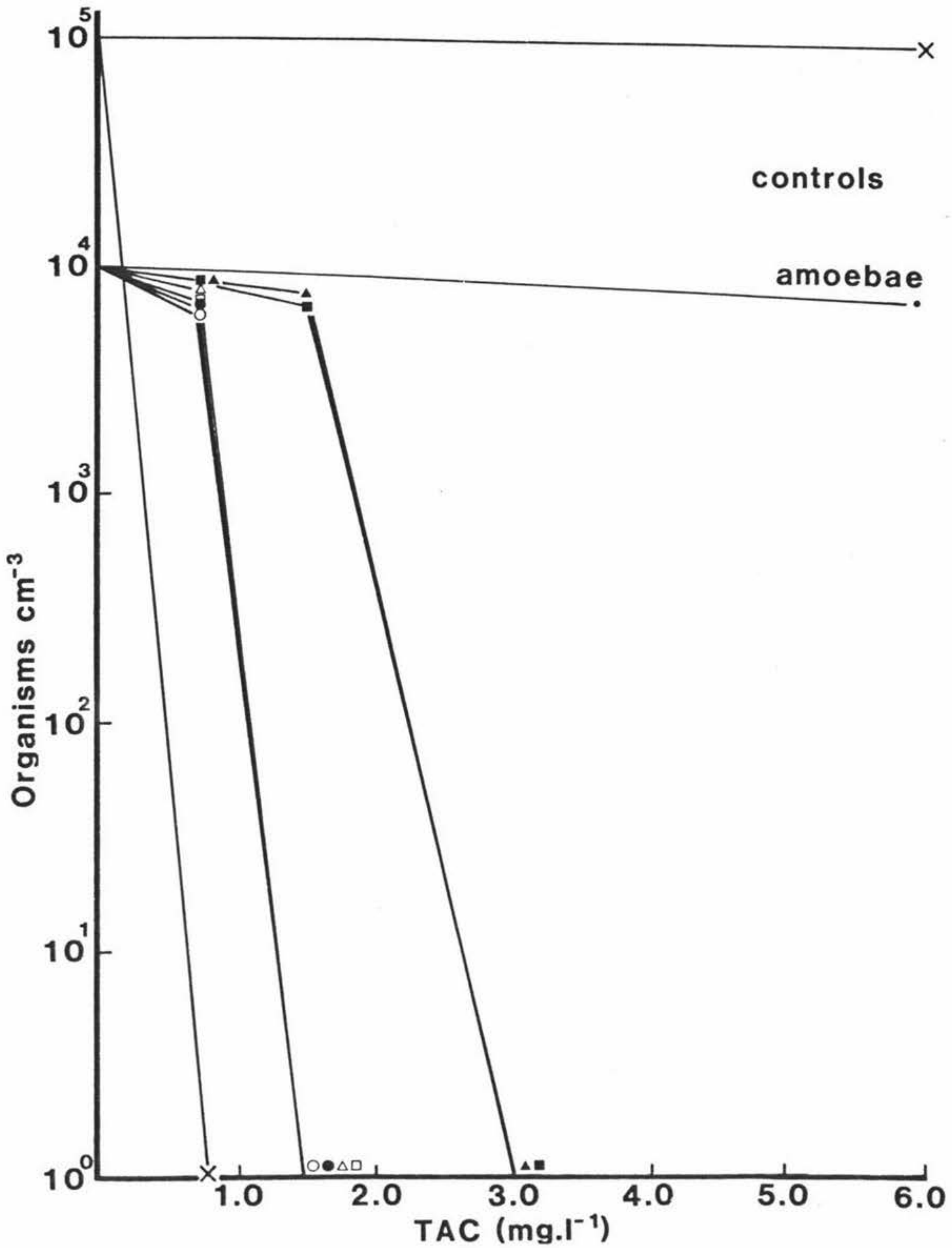


Table XXVI

Amoeba	Chlorine (mg.l^{-1})			Survivors		
	TAC	FAC	CAC	Amoebae.cm^{-3}	%	Bacteria.cm^{-3}
N.fowleri (MsM)	0	0	0	8550	85.5	90000
	0.75	0	0.75	8400	84.0	0
	1.5	0.4	1.1	0	0	0
	3.0	1.87	1.13	0	0	0
	4.5	3.38	1.12	0	0	0
N.fowleri (MsT)	0	0	0	8850	88.5	92000
	0.75	0	0.75	8600	86.0	0
	1.48	0.35	1.13	0	0	0
	3.0	1.85	1.15	0	0	0
	4.5	3.4	1.1	0	0	0
N.fowleri (Northcott)	0	0	0	8550	85.5	98000
	0.75	0	0.75	8500	85.0	0
	1.5	0.36	1.14	0	0	0
	2.95	1.84	1.11	0	0	0
	4.5	3.35	1.15	0	0	0
N.gruberi (P1200f)	0	0	0	8850	88.5	92000
	0.75	0	0.75	8700	87.0	0
	1.5	0.4	1.1	0	0	0
	3.0	1.87	1.13	0	0	0
	4.5	3.38	1.12	0	0	0
A.culbertsoni (A-1)	0	0	0	8850	88.5	93000
	0.75	0	0.75	8775	87.75	0
	1.5	0	1.5	8000	80.0	0
	3.0	0.8	2.2	0	0	0
	4.51	2.3	2.21	0	0	0
A.castellanii (1501)	0	0	0	8700	87.0	91000
	0.75	0	0.75	8530	85.5	0
	1.5	0	1.5	8150	81.5	0
	3.0	0.79	2.21	0	0	0
	4.5	2.29	2.21	0	0	0

4.2.5 Monoxenically Grown Amoebae plus a BOD

Figure 24 and Table XXVII show that the low BOD of 1.2 $mg.l^{-1}$, caused when in combination with *Naegleria*, a chlorine demand of 2.7 $mg.l^{-1}$, and a chlorine demand of 5.0 $mg.l^{-1}$ for *Acanthamoeba*, though the latter concentration was not amoebicidal.

The effect of the high BOD of 4.7 $mg.l^{-1}$ on the amoebicidal capacity of chlorine is shown in Fig. 25 and Table XXVIII. *Naegleria* were shown to be more sensitive to chlorine than *Acanthamoeba* spp. Although 6.0 $mg.l^{-1}$, the highest concentration used, was not amoebicidal, the survival rates of all six strains of amoebae were increased by between 1.0 and 4.0%, over those of the corresponding experiment with axenically grown amoebae (section 4.2.3, Fig.21 and Table XXIV).

Figure 24 (Table XXVII opposite) The Effect of Chlorine on Monoxenically Grown Amoebae, plus a BOD of 1.2 $mg.l^{-1}$, for 30 minutes, at $30^{\circ}C$.

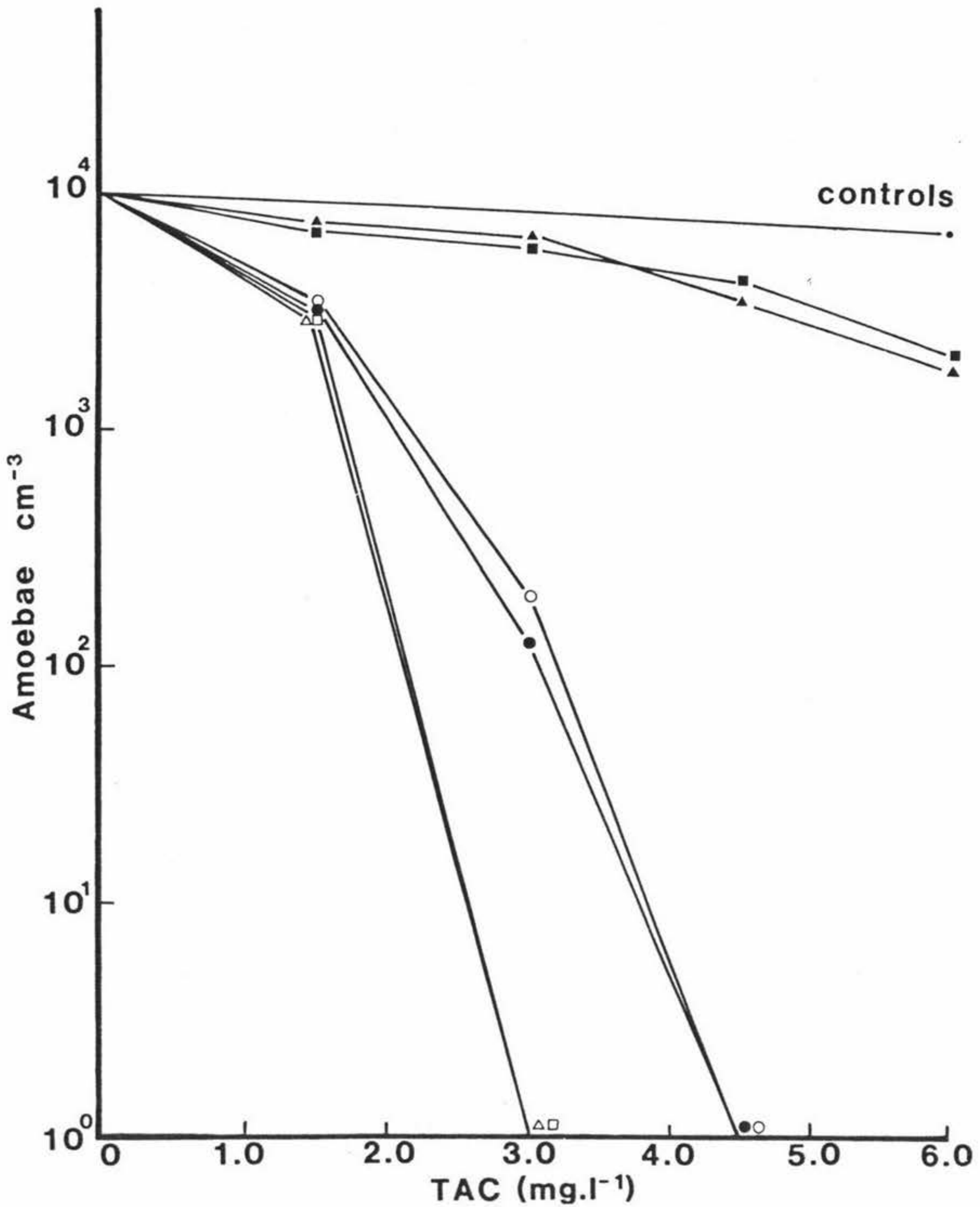


Table XXVII

Amoeba	Chlorine (mg.l^{-1})			Survivors	
	TAC	FAC	CAC	Cells.cm^{-3}	%
N.fowleri (MsM)	0	0	0	8850	85.5
	1.5	0	1.5	5625	56.25
	2.9	0.3	2.6	325	3.25
	4.5	1.76	2.74	0	0
	5.9	3.23	2.67	0	0
N.fowleri (MsT)	0	0	0	8400	84.0
	1.5	0	1.5	5300	53.0
	3.0	0.3	2.7	175	1.75
	4.5	1.78	2.72	0	0
	6.0	3.3	2.7	0	0
N.fowleri (Northcott)	0	0	0	8550	85.5
	1.5	0	1.5	5250	52.5
	3.0	0.25	2.75	0	0
	4.5	1.75	2.75	0	0
	6.0	3.28	2.72	0	0
N.gruberi (P1200f)	0	0	0	8700	87.0
	1.45	0	1.45	5375	53.75
	3.0	0.25	2.75	0	0
	4.45	1.8	2.65	0	0
	6.0	3.3	2.7	0	0
A.culbertsoni (A-1)	0	0	0	8700	87.0
	1.5	0	1.5	8450	84.5
	3.0	0	3.0	7800	78.0
	4.5	0	4.5	6100	61.0
	6.0	0.95	5.05	3150	31.5
A.castellanii (1501)	0	0	0	8700	87.0
	1.5	0	1.5	8600	86.0
	3.0	0	3.0	7950	79.5
	4.5	0	4.5	5500	55.0
	6.0	1.0	5.0	2700	27.0

Figure 25 (Table XXVIII opposite) The Effect of Chlorine on Monoxenically Grown Amoebae, plus a BOD of 4.7 $mg.l^{-1}$, for 30 minutes, at $30^{\circ}C$.

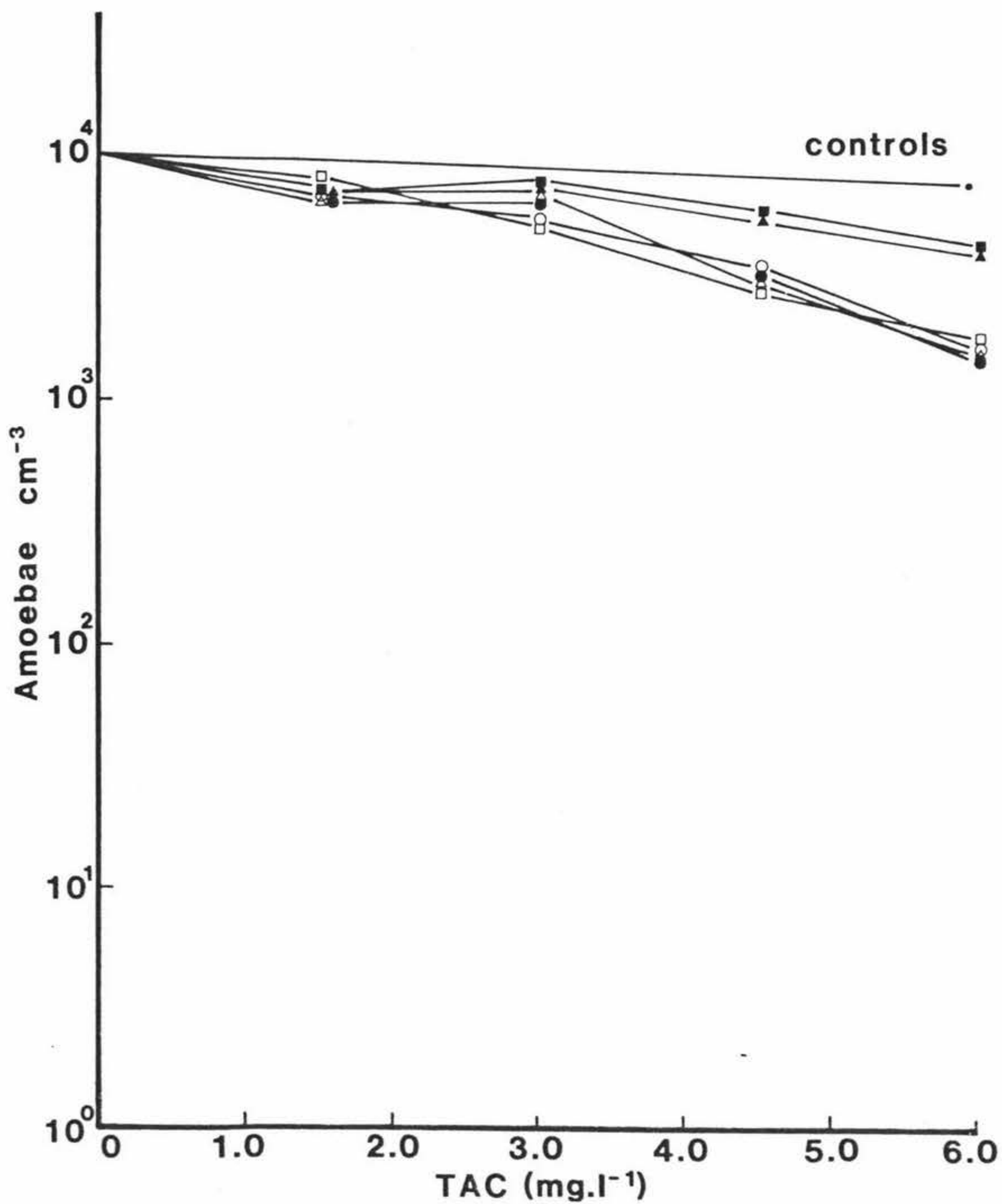


Table XXVIII

Amoeba	Chlorine (mg.l^{-1})			Survivors	
	TAC	FAC	CAC	Cells.cm^{-3}	%
N.fowleri (MsM)	0	0	0	8550	85.5
	1.5	0	1.5	8250	82.5
	3.0	0	3.0	7500	75.0
	4.5	0	4.5	5600	56.0
	6.0	0	6.0	1950	19.5
N.fowleri (MsT)	0	0	0	8700	87.0
	1.5	0	1.5	8350	83.5
	3.0	0	3.0	7875	78.75
	4.5	0	4.5	5350	53.5
	6.0	0	6.0	1725	17.25
N.fowleri (Northcott)	0	0	0	8550	85.5
	1.5	0	1.5	8300	83.0
	3.0	0	3.0	7750	77.5
	4.5	0	4.5	5025	50.25
	6.0	0	6.0	1800	18.0
N.gruberi (P1200f)	0	0	0	8850	88.5
	1.5	0	1.5	8650	86.5
	3.0	0	3.0	7750	77.5
	4.5	0	4.5	4650	46.5
	6.0	0	6.0	2350	23.5
A.culbertsoni (A-1)	0	0	0	8850	88.5
	1.5	0	1.5	8600	86.0
	3.0	0	3.0	8100	81.0
	4.5	0	4.5	6950	69.5
	6.0	0	6.0	5950	59.5
A.castellanii (1501)	0	0	0	8700	87.0
	1.5	0	1.5	8325	83.25
	3.0	0	3.0	7750	77.5
	4.5	0	4.5	6800	68.0
	6.0	0	6.0	6050	60.5

4.2.6 Monoxenically Grown Amoebae plus a BOD and Bacteria

As in section 4.2.3, where the BOD was sufficiently high to offer "protection" to the amoebae i.e. 4.7 $mg.l^{-1}$, the bacteria/BOD combination was not tested. The combination of $1.x\ 10^{4}.cm^{-3}$ amoebae, $1 \times 10^{5}.cm^{-3}$ E.cloacae, and a BOD of 1.2 $mg.l^{-1}$ (Fig. 26 and Table XXIX) increased the chlorine demand to 3.2 $mg.l^{-1}$ for Naegleria and 5.5 $mg.l^{-1}$ for Acanthamoeba spp. Both of these concentrations were amoebicidal.

Naegleria again shows greater snesitivity to chlorine over Acanthamoeba spp. Preferential removal of the bacteria, before the amoebae is also shown.

Figure 26 (Table XXIX opposite) The Effect of Chlorine on Monoxenically Grown Amoebae, plus a BOD of 1.2 $mg.l^{-1}$, plus 1 x $10^5.cm^{-3}$ bacteria, for 30 minutes, at 30^oC.

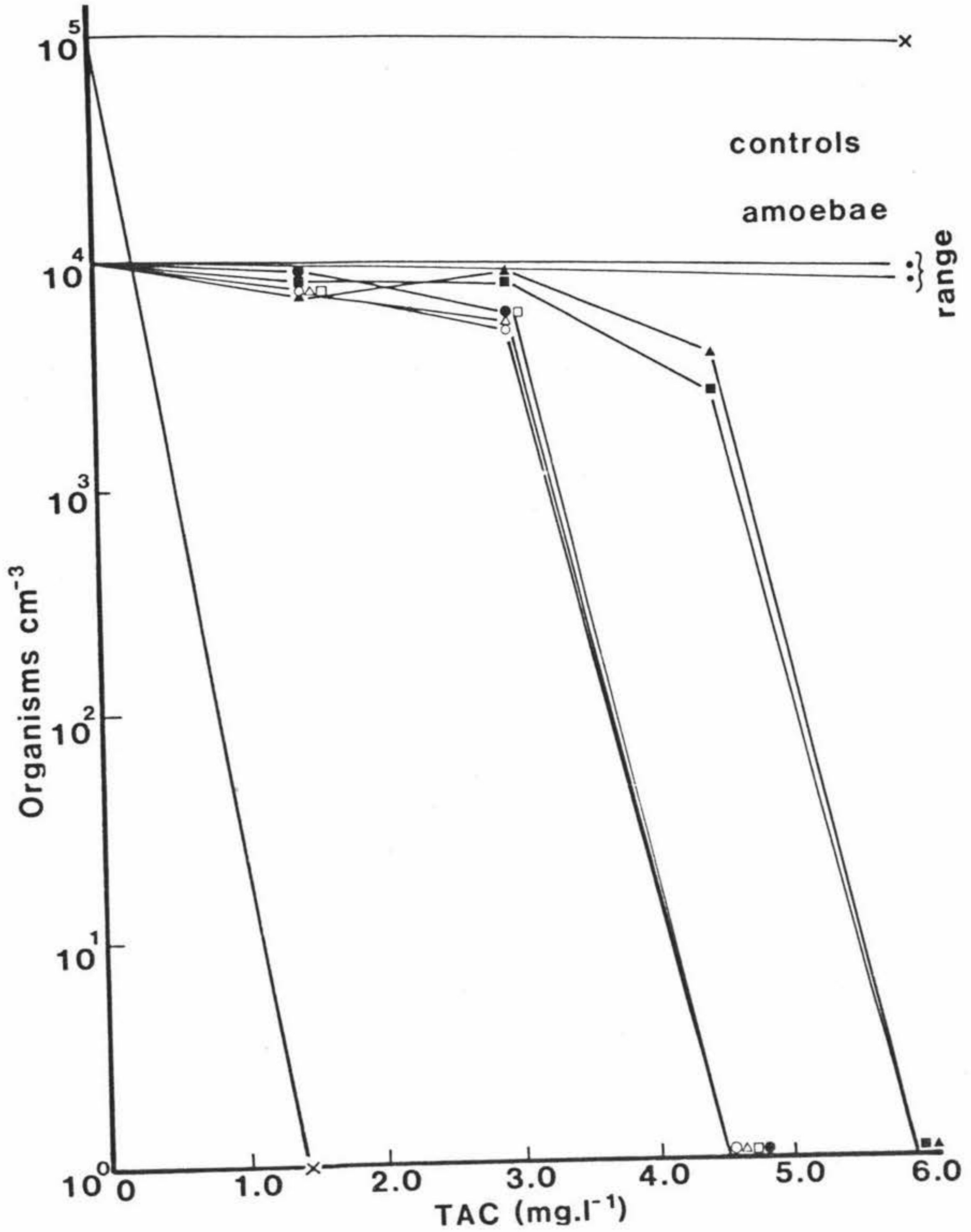


Table XXIX

Amoeba	Chlorine ($mg.l^{-1}$)			Survivors		
	TAC	FAC	CAC	$Amoebae.cm^{-3}$	%	$Bacteria.cm^{-3}$
N.fowleri (MsM)	0	0	0	8850	88.5	90000
	1.5	0	1.5	8775	87.75	0
	3.0	0	3.0	6850	68.5	0
	4.5	1.29	3.21	0	0	0
	6.0	2.81	3.19	0	0	0
N.fowleri (MsT)	0	0	0	9150	91.5	88000
	1.5	0	1.5	8900	89.0	0
	2.98	0	2.98	7200	72.0	0
	4.46	1.39	3.07	0	0	0
	6.0	2.77	3.23	0	0	0
N.fowleri (Northcott)	0	0	0	8850	88.5	92000
	1.51	0	1.51	8700	87.0	0
	3.0	0	3.0	6950	69.5	0
	4.5	1.31	3.19	0	0	0
	6.05	2.87	3.18	0	0	0
N.gruberi (P1200f)	0	0	0	9000	90.0	89000
	1.5	0	1.5	8775	87.75	0
	2.97	0	2.97	7100	71.0	0
	4.5	1.41	3.09	0	0	0
	6.0	2.87	3.18	0	0	0
A.culbertsoni (A-1)	0	0	0	9000	90.0	90000
	1.5	0	1.5	8825	88.25	0
	3.0	0	3.0	7850	78.5	0
	4.5	0	4.5	3850	38.5	0
	6.0	0.52	5.48	0	0	0
A.castellanii (1501)	0	0	0	8850	88.5	93000
	1.5	0	1.5	8650	86.5	0
	3.0	0	3.0	8175	81.75	0
	4.5	0	4.5	4800	48.0	0
	6.0	0.5	5.5	0	0	0

4.3 The Use of Chlorine Dioxide as a Disinfectant Against Pathogenic Free-Living Amoebae

4.3.1 Axenically Grown Amoebae plus Bacteria

Figure 27 and Table XXX show that although none of the concentrations tested were amoebicidal, it was evident that *Naegleria* was more sensitive than *Acanthamoeba* to chlorine dioxide. The respective chlorine dioxide demands were between 2.85 and 3.0 $mg.l^{-1}$ for *Naegleria* and 3.0 $mg.l^{-1}$ for *Acanthamoeba* spp. When the chlorine dioxide demand was 0.85 $mg.l^{-1}$ there were bacterial survivors, though the survival rate was $< 1.0\%$, and at 1.65 $mg.l^{-1}$, the bacteria were all killed.

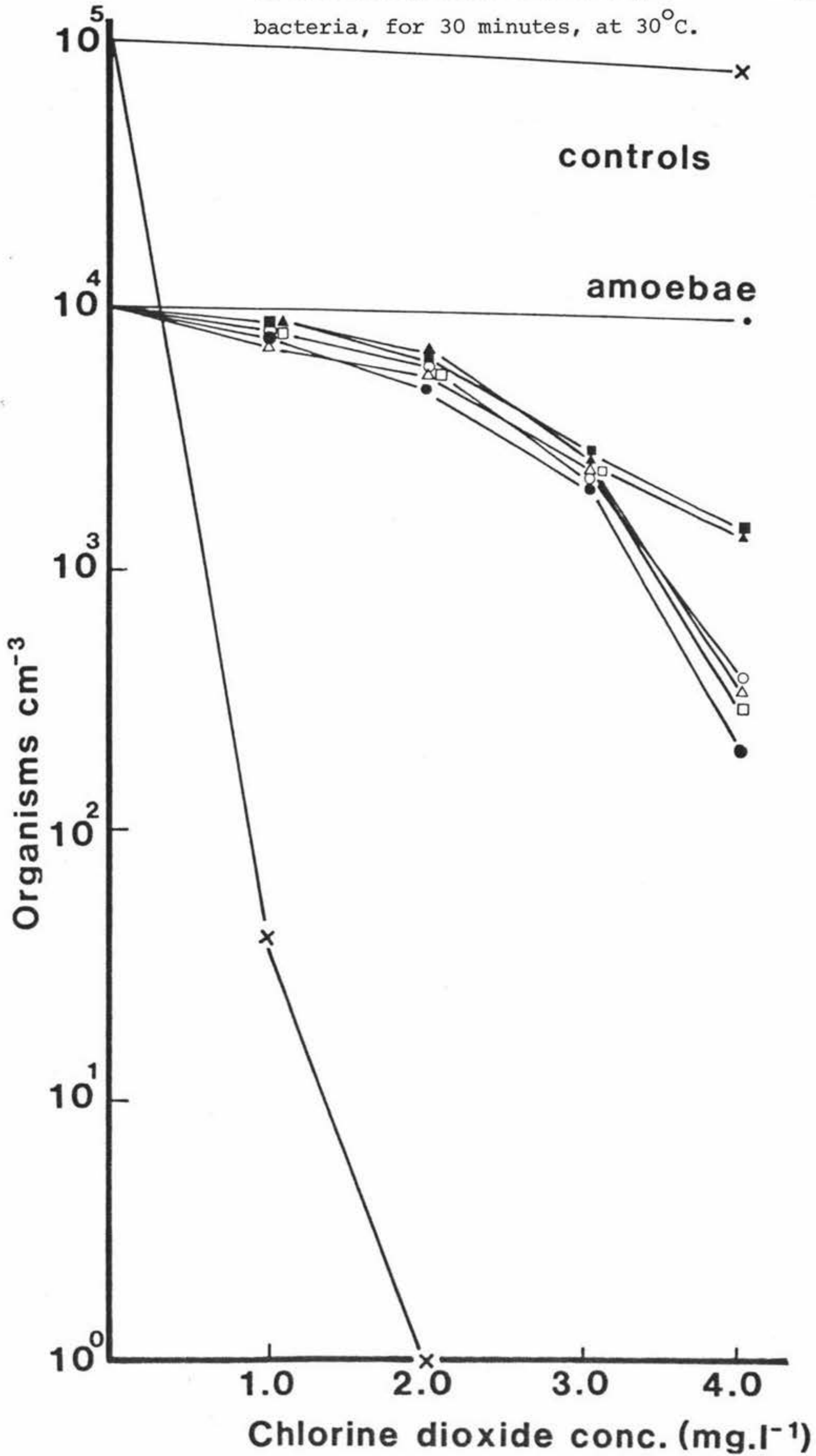


Figure 27 (Table XXX opposite) The Effect of Chlorine Dioxide on Axenically Grown Amoebae, plus $1 \times 10^5.cm^{-3}$ bacteria, for 30 minutes, at 30°C.

Table XXX

Amoeba	Chlorine dioxide (mg.l^{-1})			Survivors		
	Initial	Final	Demand	Amoebae.cm^{-3}	%	Bacteria.cm^{-3}
N.fowleri (MsM)	0	0	0	9300	93.0	91000
	1.0	0.17	0.83	9000	90.0	60
	2.0	0.33	1.67	7600	76.0	0
	3.0	0.85	2.15	3500	35.0	0
	4.0	1.15	2.85	600	6.0	0
N.fowleri (MsT)	0	0	0	9300	93.0	92000
	1.0	0.19	0.81	8800	88.0	50
	2.0	0.38	1.62	6800	68.0	0
	3.0	0.81	2.19	3100	31.0	0
	4.0	1.05	2.95	300	3.0	0
N.fowleri (Northcott)	0	0	0	9200	92.0	93000
	1.0	0.12	0.88	8700	87.0	60
	2.0	0.31	1.69	7200	72.0	0
	3.0	0.83	2.17	3600	36.0	0
	4.0	1.0	3.0	575	5.75	0
N.gruberi (P1200f)	0	0	0	9600	96.0	89000
	1.0	0.13	0.87	9100	91.0	30
	2.0	0.3	1.7	7300	73.0	0
	3.0	0.86	2.14	3400	34.0	0
	4.0	1.03	2.97	500	5.0	0
A.culberstoni (A-1)	0	0	0	9200	92.0	90000
	1.0	0.18	0.82	8850	88.5	80
	2.0	0.3	1.7	8000	80.0	0
	3.0	0.6	2.4	4500	45.0	0
	4.0	1.0	3.0	1600	16.0	0
A.castellanii (1501)	0	0	0	9150	91.5	90000
	1.0	0.15	0.85	9000	90.0	70
	2.0	0.38	1.62	8200	82.0	0
	3.0	0.6	2.4	4050	40.5	0
	4.0	1.0	3.0	1500	15.0	0

4.3.2 Axenically Grown Amoebae plus a BOD

Figure 28 and Table XXXI show that when the low BOD of 1.2 $mg.l^{-1}$ was applied, 7.5 $mg.l^{-1}$ initial chlorine dioxide concentration was not amoebicidal, although for Naegleria the survival rate was $\lessdot$ 3.5%, and for Acanthamoeba $\lessdot$ 11.0%. The Naegleria spp. were shown to be more sensitive to chlorine dioxide than the Acanthamoeba spp. The respective chlorine dioxide demands were 5.6 and 6.7 $mg.l^{-1}$.

At the high BOD of 4.7 $mg.l^{-1}$, Fig.29 and Table XXXII show that although not amoebicidal to either Naegleria or Acanthamoeba spp., the former was more sensitive to chlorine dioxide than the latter, at the concentrations of chlorine dioxide used. The survival rates of Naegleria spp. were between 24.0 and 26.5%, and 60.0% for Acanthamoeba spp. The chlorine dioxide demand was 7.2 $mg.l^{-1}$ for both genera.

Figure 28 (Table XXXI opposite) The Effect of Chlorine Dioxide on Axenically Grown Amoebae, plus a BOD of 1.2 $mg.l^{-1}$, for 30 minutes, at $30^{o}C$.

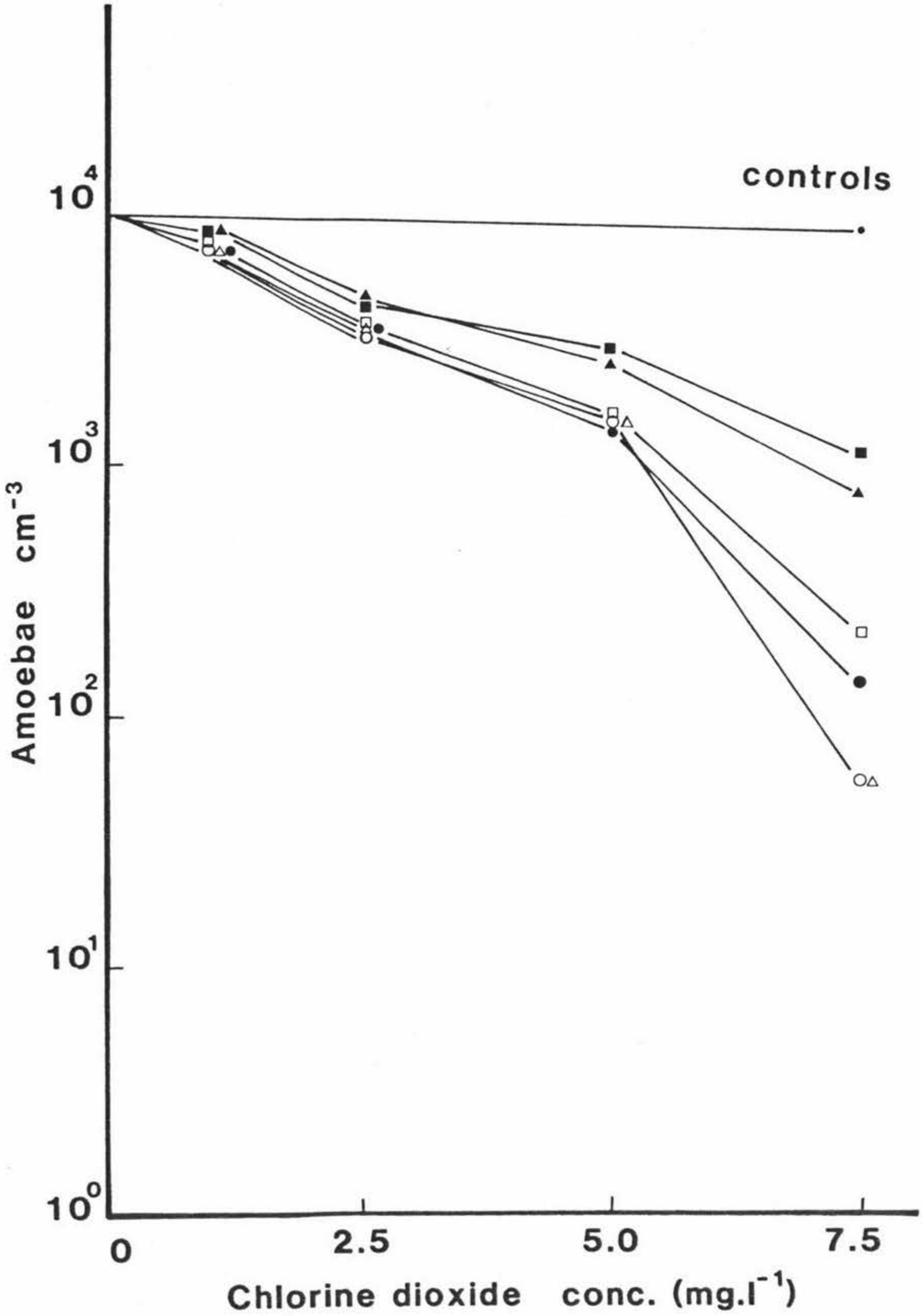


Table XXXI

Amoeba	Chlorine dioxide $(mg.l^{-1})$			Survivors	
	Initial	Final	Demand	$Cells.cm^{-3}$	%
N.fowleri (MsM)	0	0	0	9300	93.0
	1.0	0.13	0.87	8900	89.0
	2.5	0.18	2.32	5050	50.5
	5.0	0.48	4.52	1750	17.5
	7.5	1.9	5.6	75	0.75
N.fowleri (MsT)	0	0	0	9200	92.0
	1.0	0.15	0.85	8950	89.5
	2.5	0.2	2.3	5200	52.0
	5.0	0.45	4.55	1500	15.0
	7.5	1.7	5.8	125	1.25
N.fowleri (Northcott)	0	0	0	9300	93.0
	1.0	0.15	0.85	8850	88.5
	2.5	0.2	2.3	5300	53.0
	5.0	0.46	4.54	1600	16.0
	7.5	1.9	5.6	75	0.75
N.gruberi (P1200f)	0	0	0	9600	96.0
	1.0	0.1	0.9	9000	90.0
	2.5	0.2	2.3	5450	54.5
	5.0	0.4	4.6	1800	18.0
	7.5	1.9	5.6	300	3.0
A.culbertsoni (A-1)	0	0	0	9300	93.0
	1.0	0.1	0.9	9100	91.0
	2.5	0.2	2.3	6350	63.5
	5.0	0.8	4.2	4500	45.0
	7.5	0.9	6.6	1050	10.5
A.castellanii (1501)	0	0	0	9300	93.0
	1.0	0.1	0.9	9000	40.0
	2.5	0.2	2.3	6500	65.0
	5.0	0.5	4.5	4000	40.0
	7.5	0.8	6.7	900	9.0

Figure 29 (Table XXXII opposite) The Effect of Chlorine Dioxide on Axenically Grown Amoebae, plus a BOD of 4.7 $mg.l^{-1}$, for 30 minutes, at $30^{\circ}C$.

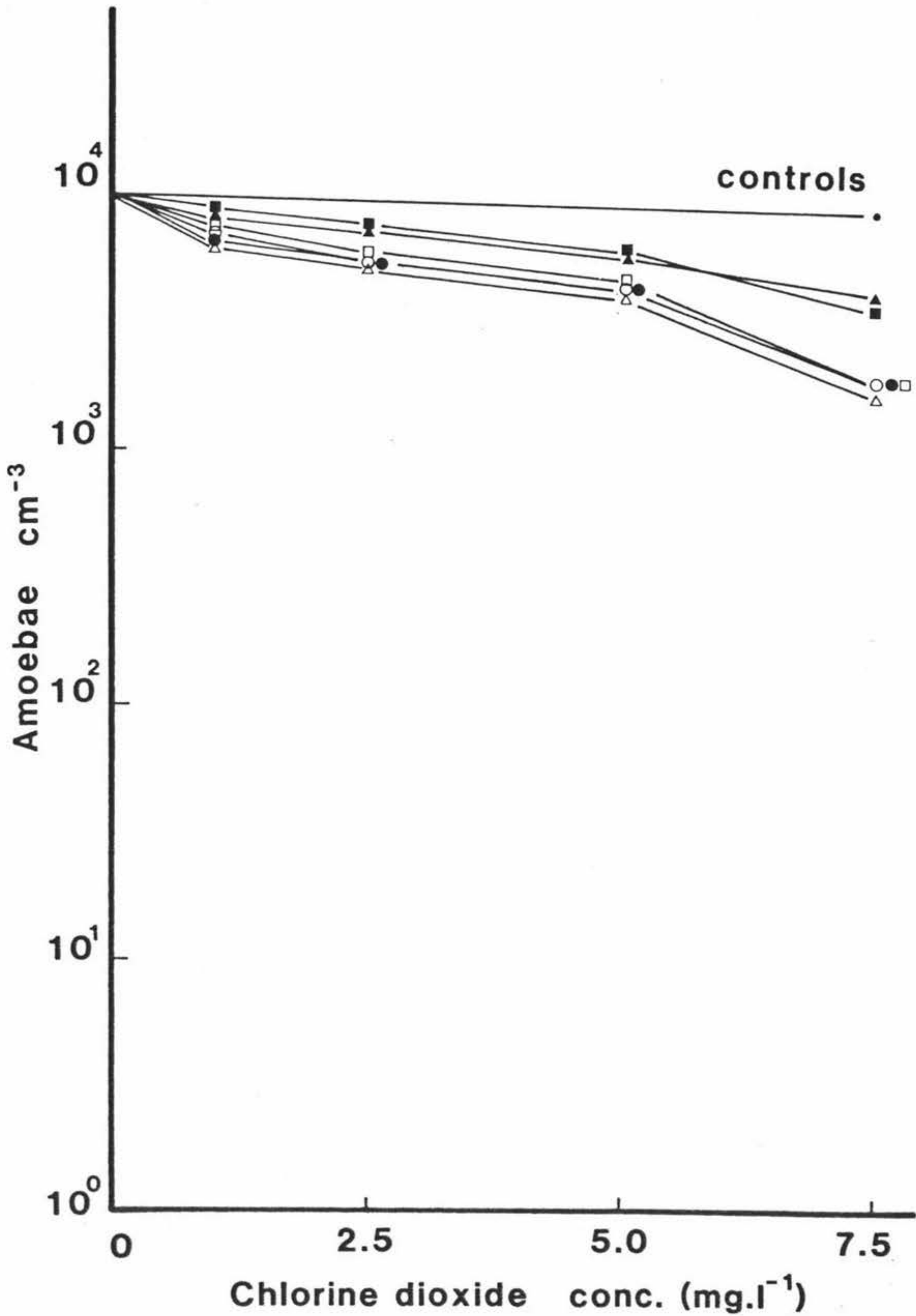


Table XXXII

Amoeba	Chlorine dioxide ($mg.l^{-1}$)			Survivors	
	Initial	Final	Demand	$Cells.cm^{-3}$	%
N.fowleri	0	0	0	9400	94.0
(MsM)	1.0	0.1	0.9	8600	86.0
	2.5	0.2	2.3	7400	74.0
	5.0	0.25	4.75	6250	62.5
	7.5	0.2	7.3	2650	26.5
N.fowleri	0	0	0	9300	93.0
(MsT)	1.0	0.07	0.93	8450	84.5
	2.5	0.15	2.35	7300	73.0
	5.0	0.21	4.79	6150	61.5
	7.5	0.19	7.31	2500	25.0
N.fowleri	0	0	0	9300	93.0
(Northcott)	1.0	0.08	0.92	8300	83.0
	2.5	0.16	2.34	7150	71.5
	5.0	0.21	4.79	6000	60.0
	7.5	0.24	7.26	2400	24.0
N.gruberi	0	0	0	9450	94.5
(P1200f)	1.0	0.05	0.95	8800	88.0
	2.5	0.12	2.38	7500	75.0
	5.0	0.14	4.86	6300	63.0
	7.5	0.2	7.3	2650	26.5
A.culbertsoni	0	0	0	9300	93.0
(A-1)	1.0	0	1.0	9300	93.0
	2.5	0.1	2.4	8900	89.0
	5.0	0.11	4.89	7800	78.0
	7.5	0.12	7.38	5950	59.5
A.castellanii	0	0	0	9200	92.0
(1501)	1.0	0	1.0	9000	90.0
	2.5	0.09	2.41	8800	88.0
	5.0	0.1	4.9	7600	76.0
	7.5	0.1	7.4	6000	60.0

4.3.3. Axenically Grown Amoebae plus a BOD and Bacteria

Figure 30 and Table XXXIII show the effect of the addition of $1 \times 10^5.cm^{-3}$ E.cloacae, along with a BOD of $1.2\ mg.l^{-1}$. This increased the chlorine dioxide demand, and allowed survival rates of between 3.75 and 7.5% for Naegleria, and between 16.0 and 17.0% for Acanthamoeba spp. Also shown is that $2.5\ mg.l^{-1}$ chlorine dioxide was not bactericidal under these conditions, although the bacterial survival rate was $\lessdot$ 1.0%. Naegleria were shown to be more sensitive to chlorine dioxide than Acanthamoeba. The respective chlorine dioxide demands were not significantly different.

As in section 4.2.3, the combination of high BOD ($4.7\ mg.l^{-1}$) and bacteria was not tested.

Figure 30 (Table XXXIII opposite) The Effect of Chlorine Dioxide on Axenically Grown Amoebae, plus a BOD of 1.2 $mg.l^{-1}$, plus 1 x $10^5.cm^{-3}$ bacteria, for 30 minutes, at 30^oC.

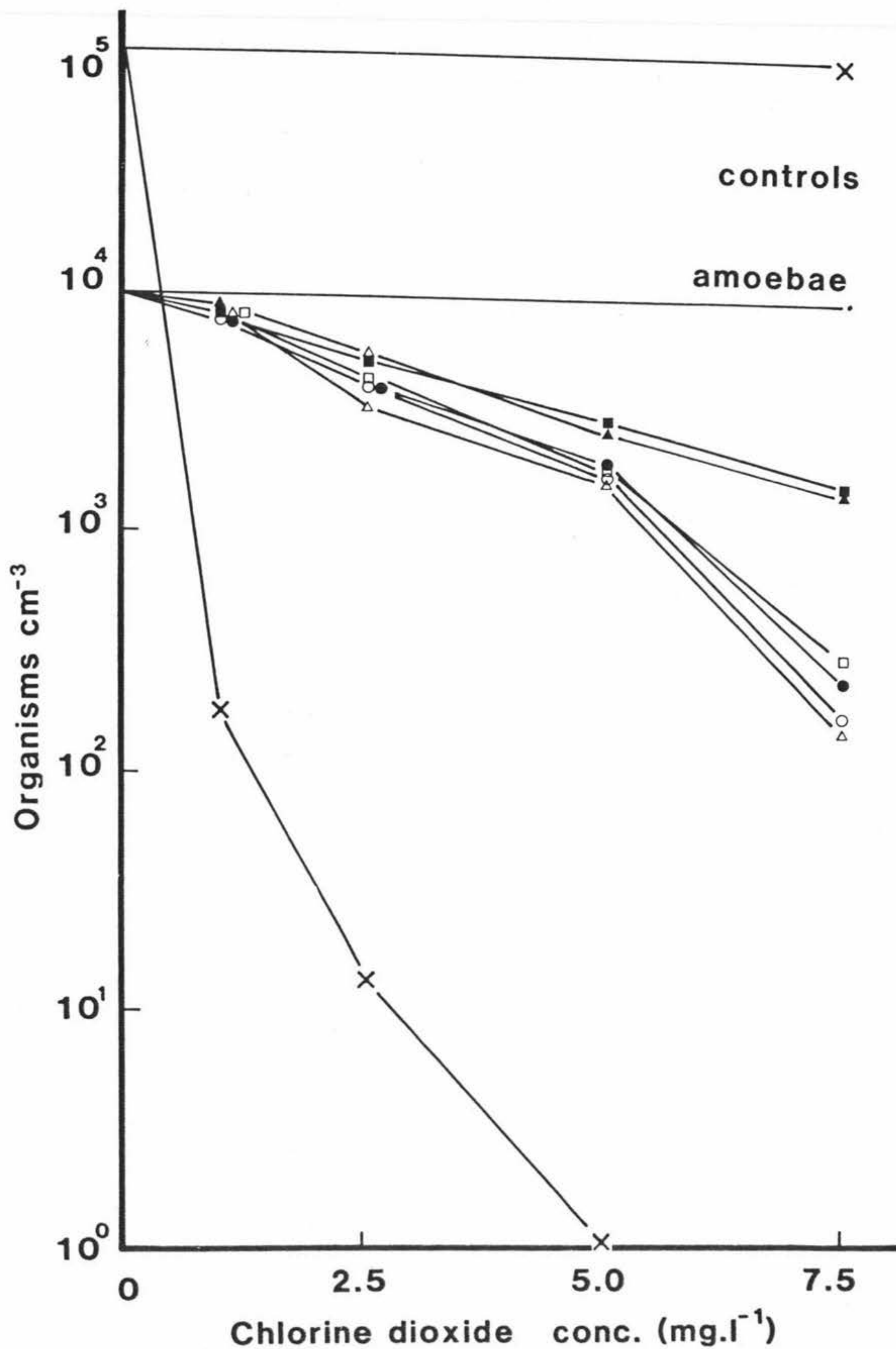


Table XXXIII

Amoeba	Chlorine dioxide ($mg.l^{-1}$)			Survivors		
	Initial	Final	Demand	$Amoebae.cm^{-3}$	%	$Bacteria.cm^{-3}$
N.fowleri (MsM)	0	0	0	9300	93.0	92000
	1.0	0.1	0.9	8900	89.0	250
	2.5	0.2	2.3	6000	60.0	10
	5.0	0.4	4.6	2550	25.5	0
	7.5	0.5	7.0	700	7.0	0
N.fowleri (MsT)	0	0	0	9200	92.0	91000
	1.0	0.1	0.9	8950	89.5	200
	2.5	0.2	2.3	5900	59.0	20
	5.0	0.3	4.7	2700	27.0	0
	7.5	0.6	6.9	375	3.75	0
N.fowleri (Northcott)	0	0	0	9300	93.0	90000
	1.0	0.15	0.85	9000	90.0	180
	2.5	0.2	2.3	5050	50.5	16
	5.0	0.3	4.7	2000	20.0	0
	7.5	0.55	6.95	750	7.5	0
N.gruberi (P1200f)	0	0	0	9200	92.0	93000
	1.0	0.15	0.85	9000	90.0	280
	2.5	0.3	2.2	6300	63.0	21
	5.0	0.4	4.6	2300	23.0	0
	7.5	0.5	7.0	450	4.5	0
A.culbertsoni (A-1)	0	0	0	9150	91.5	91000
	1.0	0.1	0.9	9000	90.0	180
	2.5	0.2	2.3	7000	70.0	10
	5.0	0.5	4.5	4200	42.0	0
	7.5	0.7	6.8	1600	16.0	0
A.castellanii (1501)	0	0	0	9200	92.0	90000
	1.0	0.1	0.9	9100	91.0	120
	2.5	0.2	2.3	7050	70.5	6
	5.0	0.4	4.6	4100	41.0	0
	7.5	0.8	6.7	1700	17.0	0

4.3.4 Monoxenically Grown Amoeba plus Bacteria

Figure 31 and Table XXXIV show that the addition of 1 x $10^5.cm^{-3}$ E.cloacae to monoxenically cultured amoebae caused an increased survival rate of between 1.0 and 2.25% for Naegleria and 3.5 to 4.0% for Acanthamoeba spp. over the corresponding experiment with axenically cultured amoebae (section 4.3.1, Fig.27 and Table XXX). The chlorine dioxide demand did not differ significantly between the two genera, nor between axenically cultured and monoxenically cultured amoebae. The highest concentation of chlorine dioxide used was not amoebicidal. The bactericidal concentrations of chlorine dioxide did not significantly differ between this and those in the corresponding axenically grown amoebae experiment (Table XXX). Naegleria was shown to be more sensitive than Acanthamoeba to chlorine dioxide, with survival rates of between 4.25 and 8.25%, and 18.5 and 20% respectively.

Figure 31 (Table XXXIV opposite) The Effect of Chlorine Dioxide on Monoxenically Grown Amoebae, plus 1 x $10^5.cm^{-3}$ bacteria, for 30 minutes, at 30^oC.

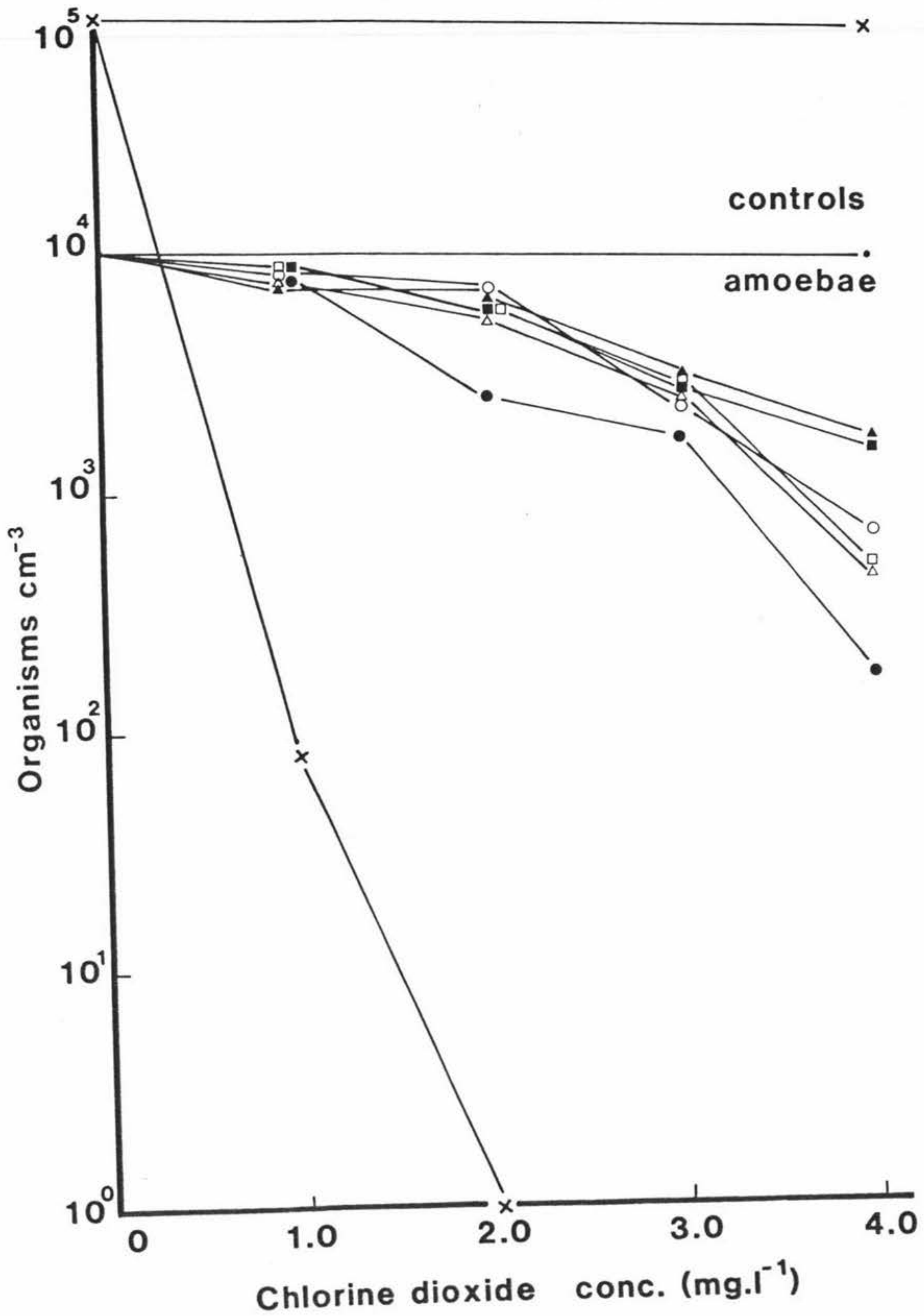


Table XXXIV

Amoeba	Chlorine dioxide ($mg.l^{-1}$)			Survivors		
	Initial	Final	Demand	$Amoebae.cm^{-3}$	%	$Bacteria.cm^{-3}$
N.fowleri (MsM)	0	0	0	9300	93.0	93000
	1.0	0.17	0.83	9100	91.0	92
	2.0	0.33	1.67	8100	81.0	0
	3.0	0.9	2.1	3800	38.0	0
	4.0	1.15	2.85	825	8.25	0
N.fowleri (MsT)	0	0	0	9000	90.0	91000
	1.0	0.19	0.81	8850	88.5	90
	2.0	0.35	1.65	3900	39.0	0
	3.0	0.8	2.2	2300	23.0	0
	4.0	1.1.	2.9	425	4.25	0
N.fowleri (Northcott)	0	0	0	9000	90.0	90000
	1.0	0.12	0,88	8900	89.0	150
	2.0	0.31	1.69	7200	72.0	0
	3.0	0.85	2.15	3750	37.5	0
	4.0	1.1	2.9	675	6.75	0
N.gruberi (P1200f)	0	0	0.	9600	96.0	91000
	1.0	0.12	0.88	9300	93.0	95
	2.0	0.3	1.7	6950	69.5	0
	3.0	0.85	2.15	3900	39.0	0
	4.0	1.1.	2.9	700	7.0	0
A.culbertsoni (A-1)	0	0	0	9000	90.0	90000
	1.0	0.13	0.87	8850	88.5	180
	2.0	0.31	1.69	8100	81.0	0
	3.0	0.65	2.35	4600	46.0	0
	4.0	1.2	2.8	2000	20.0	0
A.castellanii (1501)	0	0	0	9000	90.0	94000
	1.0	0.15	0.85	9000	90.0	95
	2.0	0.39	1.61	7900	79.0	0
	3.0	0.7	2.3	4300	43.0	0
	4.0	1.1	2.9	1850	18.5	0

4.3.5 Monoxenically Grown Amoebae plus a BOD

The effect of both the low BOD (1.2 $mg.l^{-1}$) and high BOD (4.7 $mg.l^{-1}$) on the amoebicidal capacity of chlorine dioxide are shown in Figs. 32 and 33, and Tables XXXV and XXXVI respectively. The results shown in Fig. 32 and Table XXXV show that the survival rates for the four pathogenic strains were not significantly different to the corresponding experiment with axenically grown amoebae (section 4.3.2, Fig.28 and Table XXXI). However, the other two strains had higher survival rates, increased by 2.25 and 3.0% for N.gruberi (P1200f) and A.castellanii (1501) respectively. The chlorine dioxide demand was 5.9 $mg.l^{-1}$ for Naegleria and 6.7 $mg.l^{-1}$ for Acanthamoeba, an increase 0.3 $mg.l^{-1}$ for Naegleria over that shown in Table XXXI (the corresponding axenically cultured amoebae experiment). There was no difference in the chlorine dioxide demand for Acanthamoeba spp. Naegleria was again shown to be more sensitive to chlorine dioxide than was Acanthamoeba

Figure 33 and Table XXXVI indicate that the survival rate of monoxenically cultured amoebae is up to 11.0% higher than the survival rates with the axenically cultured amoebae experiment (section 4.3.2, Fig. 29 and Table XXXII). The chlorine dioxide demand was not significantly different between the two genera, although Naegleria were more sensitive than Acanthamoeba spp.

Figure 32 (Table XXXV opposite) The Effect of Chlorine Dioxide on Monoxenically Grown Amoebae, plus a BOD of 1.2 $mg.l^{-1}$, for 30 minutes, at $30^{o}C$.

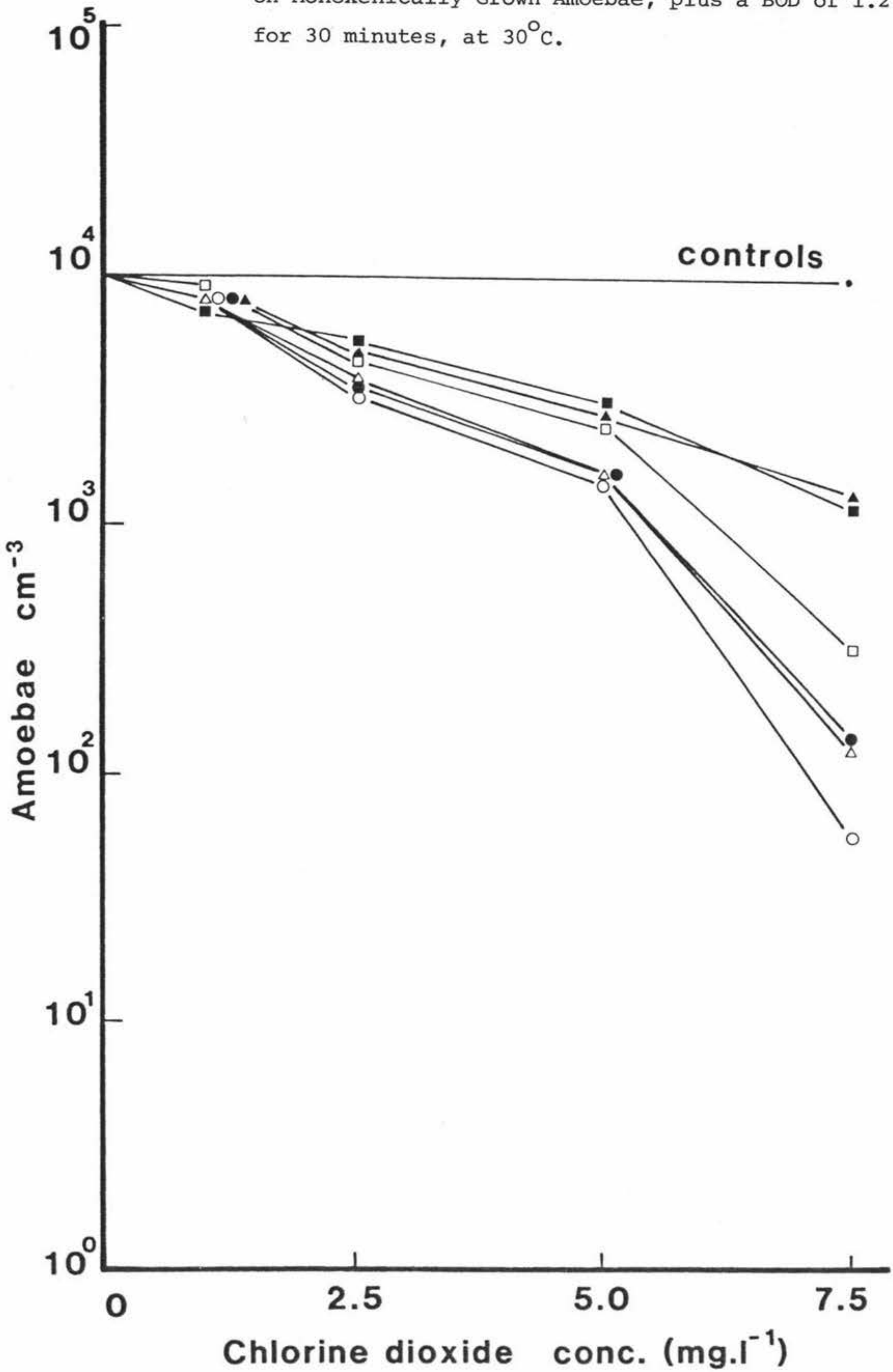


Table XXXV

Amoeba	Chlorine dioxide ($mg.l^{-1}$)			Survivors	
	Initial	Final	Demand	Cells.cm^{-3}	%
N.fowleri (MsM)	0	0	0	9300	93.0
	1.0	0.13	0.87	8900	89.0
	2.5	0.18	2.32	5150	51.5
	5.0	0.45	4.55	1850	18.5
	7.5	1.85	5.64	75	0.75
N.fowleri (MsT)	0	0	0	9000	90.0
	1.0	0.13	0.87	9000	90.0
	2.5	0.19	2.31	5400	54.0
	5.0	0.43	4.57	1800	18.0
	7.5	1.65	5.85	150	1.5
N.fowleri (Northcott)	0	0	0	9000	90.0
	1.0	0.1	0.9	9000	90.0
	2.5	0.18	2.32	5300	53.0
	5.0	0.39	4.61	1900	19.0
	7.5	1.6	5.9	150	1.5
N.gruberi (P1200f)	0	0	0	9600	96.0
	1.0	0.1	0.9	9500	95.0
	2.5	0.2	2.3	6300	63.0
	5.0	0.35	4.65	2550	25.5
	7.5	1.7	5.8	525	5.25
A.culbertsoni (A-1)	0	0	0	9000	90.0
	1.0	0.1	0.9	8850	88.5
	2.5	0.19	2.31	6900	69.0
	5.0	0.5	4.5	4600	46.0
	7.5	0.85	6.65	1100	11.0
A.castellanii (1501)	0	0	0	9150	91.5
	1.0	0.1	0.9	9000	90.0
	2.5	0.2	2.3	6800	68.0
	5.0	0.4	4.1	4500	45.0
	7.5	0.78	6.72	1200	12.0

Figure 33 (Table XXXVI opposite) The Effect of Chlorine Dioxide on Monoxenically Grown Amoebae, plus a BOD of 4.7 $mg.l^{-1}$, for 30 minutes, at $30^{o}C$.

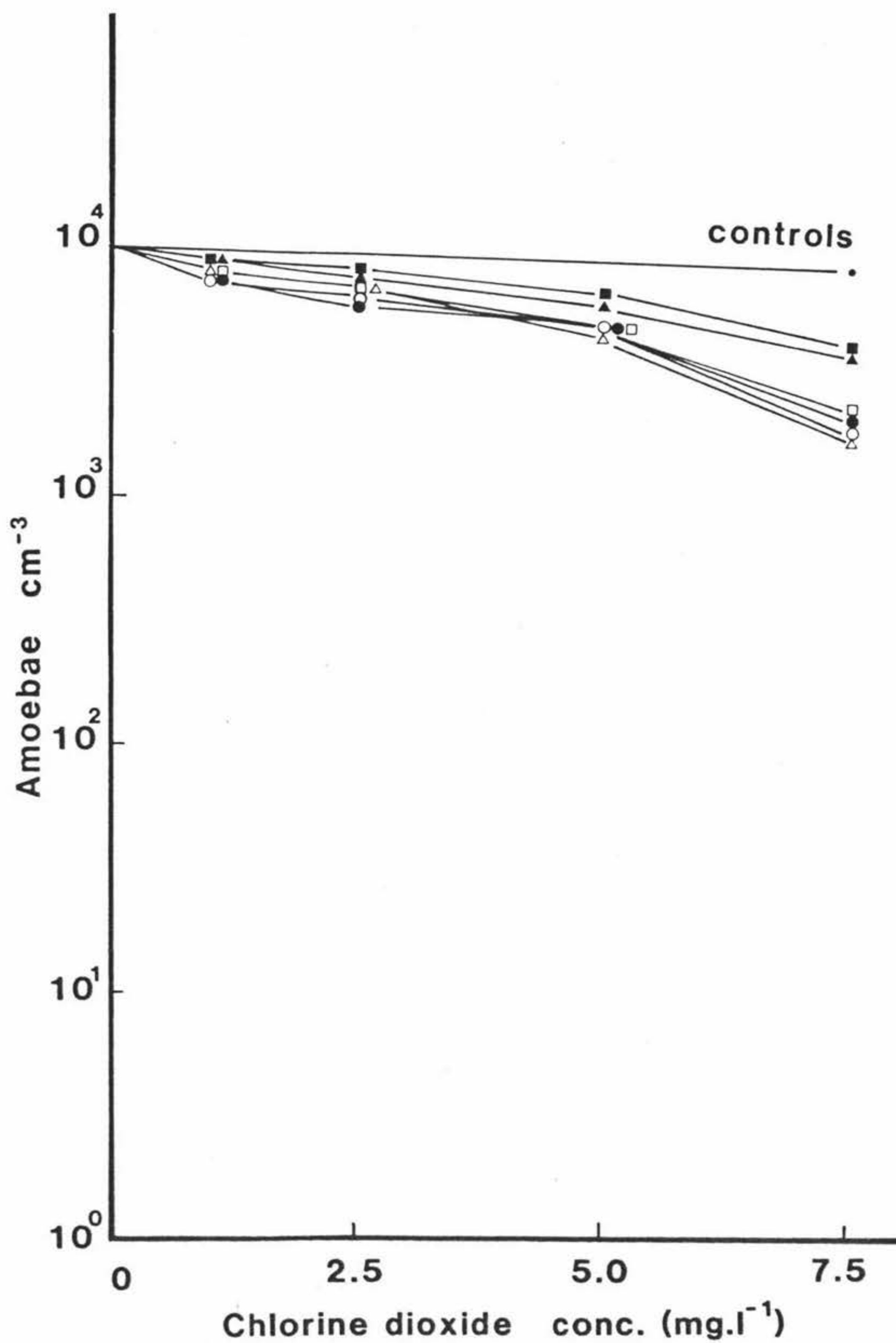


Table XXXVI

Amoeba	Chlorine dioxide ($mg.l^{-1}$)			Survivors	
	Initial	Final	Demand	$Cells.cm^{-3}$	%
N.fowleri (MsM)	0	0	0	9400	94.0
	1.0	0.05	0.95	8900	89.0
	2.5	0.09	2.41	7800	78.0
	5.0	0.13	4.87	6950	69.5
	7.5	0.2	7.3	3000	30.0
N.fowleri (MsT)	0	0	0	9300	93.0
	1.0	0.04	0.96	8850	88.5
	2.5	0.08	2.42	7600	76.0
	5.0	0.15	4.85	6800	68.0
	7.5	0.22	7.28	3300	33.0
N.fowleri (Northcott)	0	0	0	9350	93.5
	1.0	0.06	0.94	9000	90.0
	2.5	0.09	2.41	8100	81.0
	5.0	0.14	4.86	6700	67.0
	7.5	0.2	7.3	2850	28.5
N.gruberi (P1200f)	0	0	0	9400	94.0
	1.0	0.05	0.95	9100	91.0
	2.5	0.08	2.42	8300	83.0
	5.0	0.13	4.87	7000	70.0
	7.5	0.21	7.29	3500	35.0
A.culbertsoni (A-1)	0	0	0	9300	93.0
	1.0	0	1.0	9300	93.0
	2.5	0.05	2.45	9100	91.0
	5.0	0.09	4.91	8300	83.0
	7.5	0.15	7.35	6200	62.0
A.castellanii (1501)	0	0	0	9350	93.5
	1.0	0	1.0	9200	92.0
	2.5	0.09	2.41	8900	89.0
	5.0	0.08	4.92	7800	78.0
	7.5	0.1	7.4	6000	60.0

4.3.6 Monoxenically Grown Amoebae plus a BOD and Bacteria

As with chlorine, only the low BOD of 1.2 $mg.l^{-1}$ was tested. Figure 34 and Table XXXVII show that with the exception of N.fowleri (MsT) the survival rate of which at 7.5 $mg.l^{-1}$ chlorine dioxide was 4.5%, all Naegleria strains tested had survival rates of between 7.25 and 8.5% at that chlorine dioxide concentration. The Acanthamoeba survival rates were 20.0 and 21.0% for pathogen and non-pathogen respectively. This is an increase of between 1.0 and 4.0% for Naegleria, and 4.0% for Acanthamoeba, over the corresponding experiment using axenically grown amoebae (section 4.3.4, Fig. 30 and Table XXXIII). The chlorine dioxide demands of the two genera were not significantly different. The chlorine dioxide concentration of 2.5 $mg.l^{-1}$ was not bactericidal, although there was $\triangleleft$ 1.0% survival rate, under these conditions. This is not significantly different to the corresponding experiment using axenically grown amoeba.

Figure 34 (Table XXXVII opposite) The Effect of Chlorine Dioxide on Monoxenically Grown Amoebae, plus a BOD of 1.2 $mg.l^{-1}$, plus $1 \times 10^{5}.cm^{-3}$ bacteria, for 30 minutes, at $30^{o}C$.

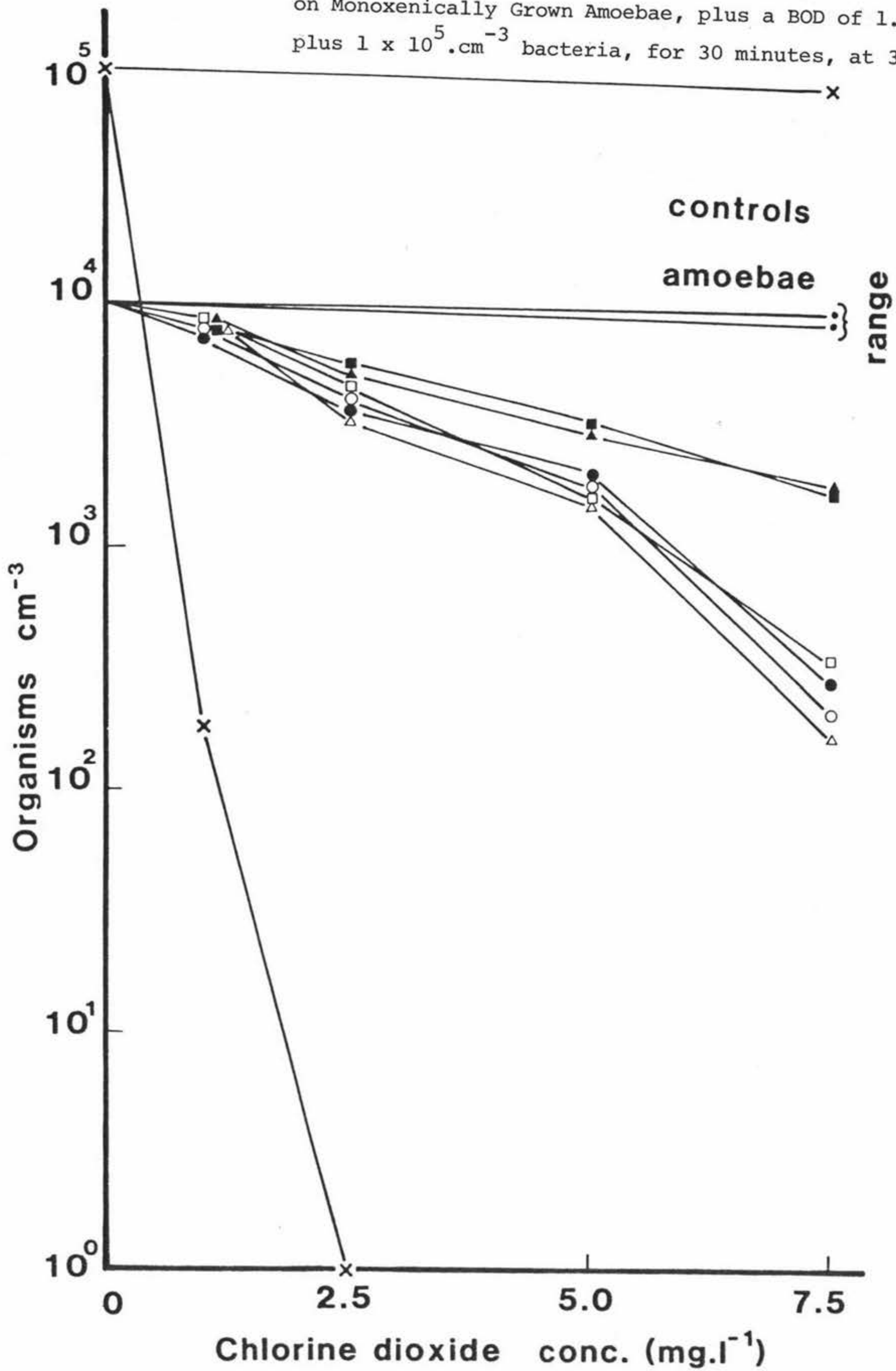


Table XXXVII

Amoebae	Chlorine dioxide ($mg.l^{-1}$)			Survivors		
	Initial	Final	Demand	$Amoebae.cm^{-3}$	%	$Bacteria.cm^{-3}$
N.fowleri (MsM)	0	0	0	9300	93.0	89000
	1.0	0.1	0.9	8900	89.0	300
	2.5	0.2	2.3	6050	60.5	10
	5.0	0.35	4.65	2600	26.0	0
	7.5	0.45	7.05	800	8.0	0
N.fowleri (MsT)	0	0	0	9000	90.0	89000
	1.0	0.1	0.9	8850	88.5	180
	2.5	0.18	2.32	5900	59.0	3
	5.0	0.3	4.7	2775	27.75	0
	7.5	0.55	6.95	450	4.5	0
N.fowleri (Northcott)	0	0	0	8850	88.5	92000
	1.0	0.1	0.9	8600	86.0	200
	2.5	0.19	2.31	5200	52.0	0
	5.0	0.27	4.73	2100	21.0	0
	7.5	0.51	6.99	725	7.25	0
N.gruberi (P1200f)	0	0	0	9600	96.0	92000
	1.0	0.13	0.87	9300	93.0	250
	2.5	0.2	2.3	5600	56.0	16
	5.0	0.35	4.65	2450	24.5	0
	7.5	0.45	7.05	850	8.5	0
A.culbertsoni (A-1)	0	0	0	9150	91.5	93000
	1.0	0.1	0.9	9000	90.5	280
	2.5	0.2	2.3	7400	74.0	15
	5.0	0.45	4.55	4800	48.0	0
	7.5	0.65	6.85	2000	20.0	0
A.castellanii (1501)	0	0	0	9300	93.0	91000
	1.0	0.1	0.9	9150	91.5	240
	2.5	0.23	2.27	7300	73.0	16
	5.0	0.4	4.6	4650	46.5	0
	7.5	0.7	6.8	2100	21.0	0

4.4 Growth and Competition Studies Using Naegleria and Tetrahymena on Eight Bacteria species at 30°C

4.4.1 The Growth of Naegleria on Eight Bacteria species

The results shown in Figs.35 to 42, and Tables XXXVIII to XLV, indicate the relative ability of the bacteria species tested to support the growth of N.fowleri (MsM),(MsT), and (Northcott), and N.gruberi (P1200f). Because of the lack of significant difference between the three N.fowleri strains, Figs.35 to 42, show the difference between N.guberi (P1200f), and the mean of the N.fowleri results in the tables. Of all the bacteria tested, only E.coli (Fig.35, Table XXXVIII) supported the growth of amoebae sufficiently to allow the survival of trophozoites throughout the 7 day experiment. Cysts were too low in numbers to be counted until the second day. It was not unitl the sixth day that the number of surviving trophozoites fell to below the initial inoculum concentration of amoebae, although at no stage did the total number of surviving amoebae (trophozoites plus cysts) fall below the initial inoculum concentration. The peak levels of a 15 fold increase for N.fowleri and 25 times for N.gruberi (P1200f) were both reached on day three (72 hours). The bacteria concentration increased daily and peaked on day 4 (96 hours) at $1 \times 10^{8}.cm^{-3}$, and then decreased to be at the same concentration as the initial inoculum ($1 \times 10^{6}.cm^{-3}$) by day 7 (168 hours). The peak was an increase of 100 times, from 1×10^{6} to $1 \times 10^{8}.cm^{-3}$.

The next best in ability to support amoebic growth were K.aerogenes (Fig.36, Table XXXIX) and M.luteus (Fig.38, Table XLI), both of which supported trophozoite survival until day 6 (144 hours). K.aerogenes caused encystment by day 2 (48 hours), while M.luteus caused encystment by day 1 (24 hours). The greatest levels reached were, for N.fowleri by day 2 (48 hours) 8 times, and for N.gruberi (P1200f)

34 times by day 3 (72 hours) on K.aerogenes ; and 10 times for N.fowleri and 11 times for N.gruberi (P1200f), both by day 3 (72 hours) on M.luteus. At no stage, with either bacterial species, did the total number of surviving amoebae fall below the initial inoculum. The concentrations of both bacterial species increased daily to peak at $3 \times 10^{8}.cm^{-3}$ by day 4 (96 hours), although they decreased to be by day 7 (168 hours), slightly below the initial inoculum for K.aerogenes, and slightly above for M.luteus.

E.cloacae (Fig.37 and Table XL) is also a reasonable growth support organism for Naegleria spp. Encystment was not evident until the second day (48 hours), and although for N.fowleri, the concentration of trophozoites was lower on day 6 (144 hours) than the original inoculum, the total amoeba concentration was always above that level. This low number of trophozoites occurred 24 hours later with N.gruberi (P1200f). The population increase was 12 times for N.fowleri and 40 times for N.gruberi (P1200f), both by day 4 (96 hours). The bacteria concentration increased daily to a peak of $2 \times 10^{8}.cm^{-3}$ by day 4 (96 hours), and then slowly decreased, to be twice the initial inoculum concentraion of $1.x\ 10^{6}.cm^{-3}$ on day 7 (168 hours).

The next best bacteria , as a growth support organism for Naegleria was Ps.aeruginosa (Fig.41 and Table XLIV). The pathogenic strains did not grow as well as the non-pathogen. The pathogens' peak was $7 \times 10^{4}.cm^{-3}$ by day 3 (72 hours), while the non-pathogen peaked at $2.1 \times 10^{5}.cm^{-3}$ by day 4 (96 hours). This coincided with the bacteria peak of $2 \times 10^{8}.cm^{-3}$. No cysts were seen until day 2 (48 hours). No pathogenic trophozoites were seen on day 5 (120 hours), and although non-pathogenic trophozoites were seen until day 5 (120 hours), they were not seen on day 6 (144 hours). No cysts were observed on day 6 (144 hours) of either pathogenic or non-pathogenic strains.

B.subtilis did not support the growth of trophozoites of pathogenic or non-pathogenic Naegleria strains. There were flagellates present on day 1 (24 hours) in all pathogenic strains, and there were cysts present for the whole 7 day (168 hour) period of incubation (they appeared on day 1) with all strains. At no time did the number of trophozoites increase at all, and they had been reduced to uncountable levels by day 3 (72 hours). This was also the peak of bacterial growth at $3 \times 10^{8}.cm^{-3}$, as well as cyst numbers at $1 \times 10^{5}.cm^{-3}$ non-pathogenic and $6 \times 10^{4}.cm^{-3}$ pathogenic cysts. The bacterial population decreased in numbers after the peak, to $6 \times 10^{6}.cm^{-3}$ by day 7 (168 hours). This bacteria species was the only one on which flagellates were seen.

The two least effective growth support bacteria for Naegleria, were S.marcescens (Fig.40 and Table XLIII) and Ps.fluorescens (Fig.42 and Table XLV). As can be seen from the tables, day 3 (72 hours) was the last time countable numbers of trophozoites were seen, except with N.gruberi (P1200f) on S.marcescens when countable numbers of amoebae were observed on day 4 (96 hours). By day 5 (120 hours) the number of cysts was too low to count for all amoebae strains tested on both bacteria. There were increases in trophozoites on both bacteria species, to $2 \times 10^{4}.cm^{-3}$ for N.fowleri and $1 \times 10^{5}.cm^{-3}$ for N.gruberi (P1200f) on S.marcescens, and $6 \times 10^{4}.cm^{-3}$ N.fowleri and $4.5 \times 10^{4}.cm^{-3}$ N.gruberi (P1200f) on Ps.fluorescens, all on day 2 (48 hours). The bacterial growth peaks were by day 3 (72 hours) for S.marcescens and day 5 (120 hours) for Ps.fluorescens. Cysts first appeared on day 2 (48 hours) and had been reduced to uncountable levels by day 5 (120 hours).

In Figures 35 - 42 the following legend applies,

- ■ = *N.fowleri* flagellates
- △ = *N.fowleri* trophozoites
- □ = *N.fowleri* cysts
- ○ = *N.gruberi* trophozoites
- ▲ = *N.gruberi* cysts
- ● = The growth support bacteria used in that experiment.

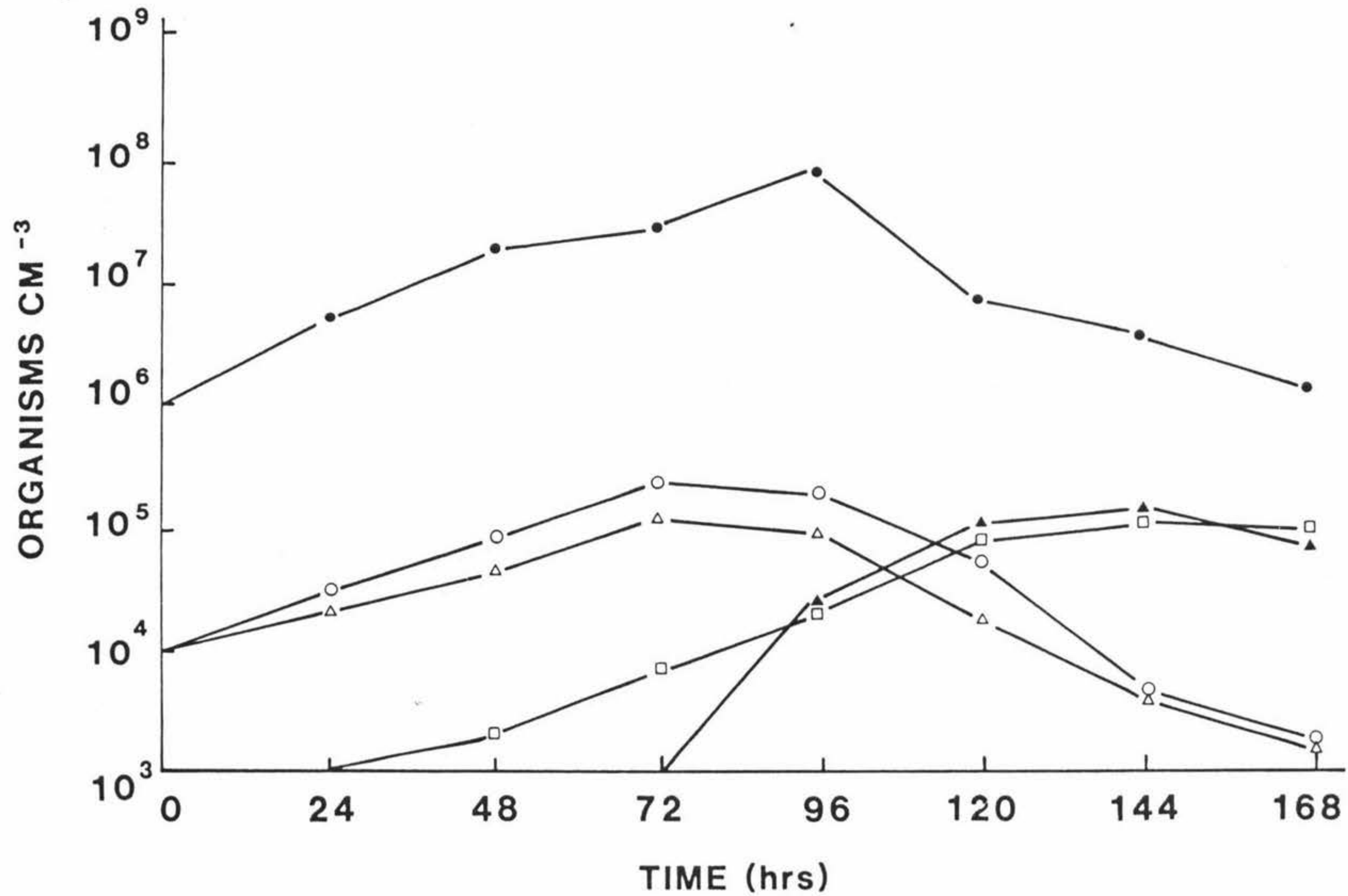


Figure 35 (Table XXXVIII opposite) Growth of <u>Naegleria</u> on <u>E.coli</u> at 30°C.

Table XXXVIII

Organisms	Time (hrs)							
	0	24	48	72	96	120	144	168
N.fowleri (MsM)	1×10^4	4×10^4	6.5×10^4	1×50^5	9×10^4	2×10^4	5×10^3	1×10^3
			3×10^3*	7×10^3*	3×10^4*	9×10^4*	1×10^5*	8.5×10^4*
E.coli	1×10^6	8×10^6	2×10^7	3.8×10^7	8×10^7	8×10^6	4×10^6	1×10^6
N.fowleri (MsT)	1×10^4	4.5×10^4	8×10^4	2×10^5	1×10^5	2×10^4	6×10^3	1×10^3
			1×10^3*	6×10^3*	3×10^4*	8×10^4*	9×10^4*	6×10^4*
E.coli	1×10^6	7×10^6	1×10^7	6×10^7	9×10^7	1×10^7	3×10^6	1×10^6
N.fowleri (Northcott)	1×10^4	3×10^4	7×10^4	1×10^5	1×10^5	1×10^4	5×10^3	1×10^3
			2×10^3*	9×10^3*	4×10^4*	9×10^4*	9×10^4*	3×10^4*
E.coli	1×10^6	8×10^6	2×10^7	4×10^7	2×10^8	8×10^6	4×10^6	1×10^6
N.gruberi (P1200f)	1×10^4	5.5×10^4	1×10^5	2.5×10^5	2×10^5	7×10^4	6×10^3	2×10^3
					4×10^4*	1×10^5*	2×10^5*	7.5×10^4*
E.coli	1×10^6	8×10^6	2×10^7	4×10^7	9×10^7	8×10^6	2×10^6	9×10^5

* = cysts

Figure 36 (Table XXIX opposite) Growth of <u>Naegleria</u> on <u>K.aerogenes</u> at 30°C

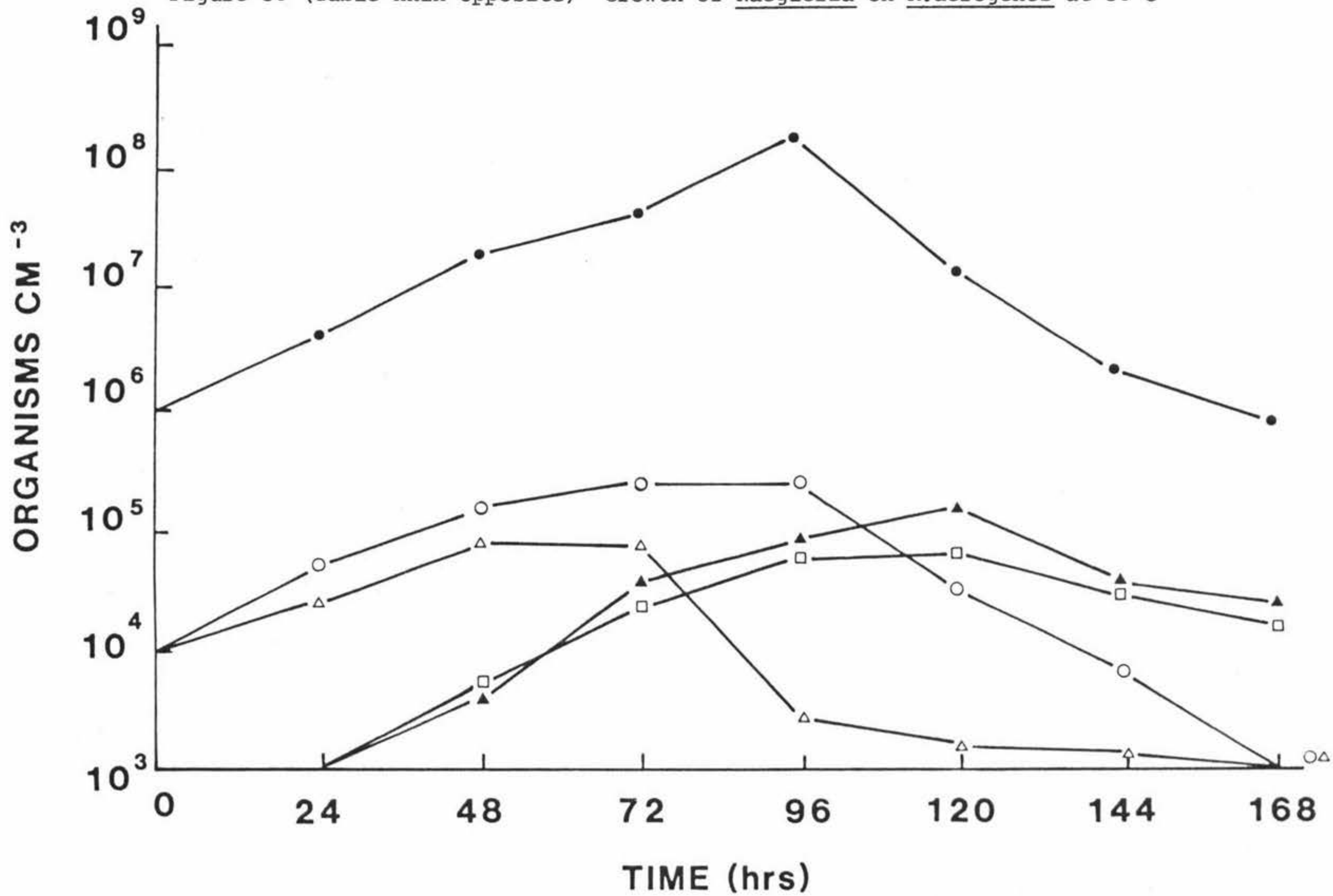


Table XXXIX

Organisms	Time (hrs)							
	0	24	48	72	96	120	144	168
N.fowleri (MsM)	1×10^4	3×10^4	8×10^4	7.7×10^4	4×10^3	1×10^3	1×10^3	$\leq 10^3$
			6×10^3*	2×10^4*	7×10^4*	6.5×10^4*	4×10^4*	1×10^4*
K.aerogenes	1×10^6	6×10^6	3×10^7	7×10^7	2×10^8	1×10^7	2×10^6	9×10^5
N.fowleri (MsT)	1×10^4	4×10^4	1×10^5	7×10^4	7×10^3	2×10^3	1×10^3	$< 10^3$
			7×10^3*	2×10^4*	6×10^4*	7×10^4*	5×10^4*	2×10^4*
K.aerogenes	1×10^6	5×10^6	2×10^7	6×10^7	2×10^8	9×10^6	2×10^6	1×10^6
N.fowleri (Northcott)	1×10^4	3×10^4	7×10^4	7.5×10^4	6×10^3	2×10^3	1×10^3	$\leq 10^3$
			3×10^3*	4×10^4*	5×10^4*	6×10^4*	3×10^4*	2×10^4*
K.aerogenes	1×10^6	6×10^6	2×10^7	7×10^7	4×10^8	9×10^6	2×10^6	9×10^5
N.gruberi (P1200f)	1×10^4	7×10^4	2×10^5	3×10^5	3×10^5	4×10^4	7×10^3	$\leq 10^3$
			5×10^3*	4×10^4*	8×10^4*	2×10^5*	5×10^4*	3×10^4*
K.aerogenes	1×10^6	6×10^6	2×10^7	6×10^7	2×10^8	1×10^7	3×10^6	9×10^5

* = cysts

Figure 37 (Table XL opposite) Growth of <u>Naegleria</u> on <u>E.cloacae</u> at 30°C.

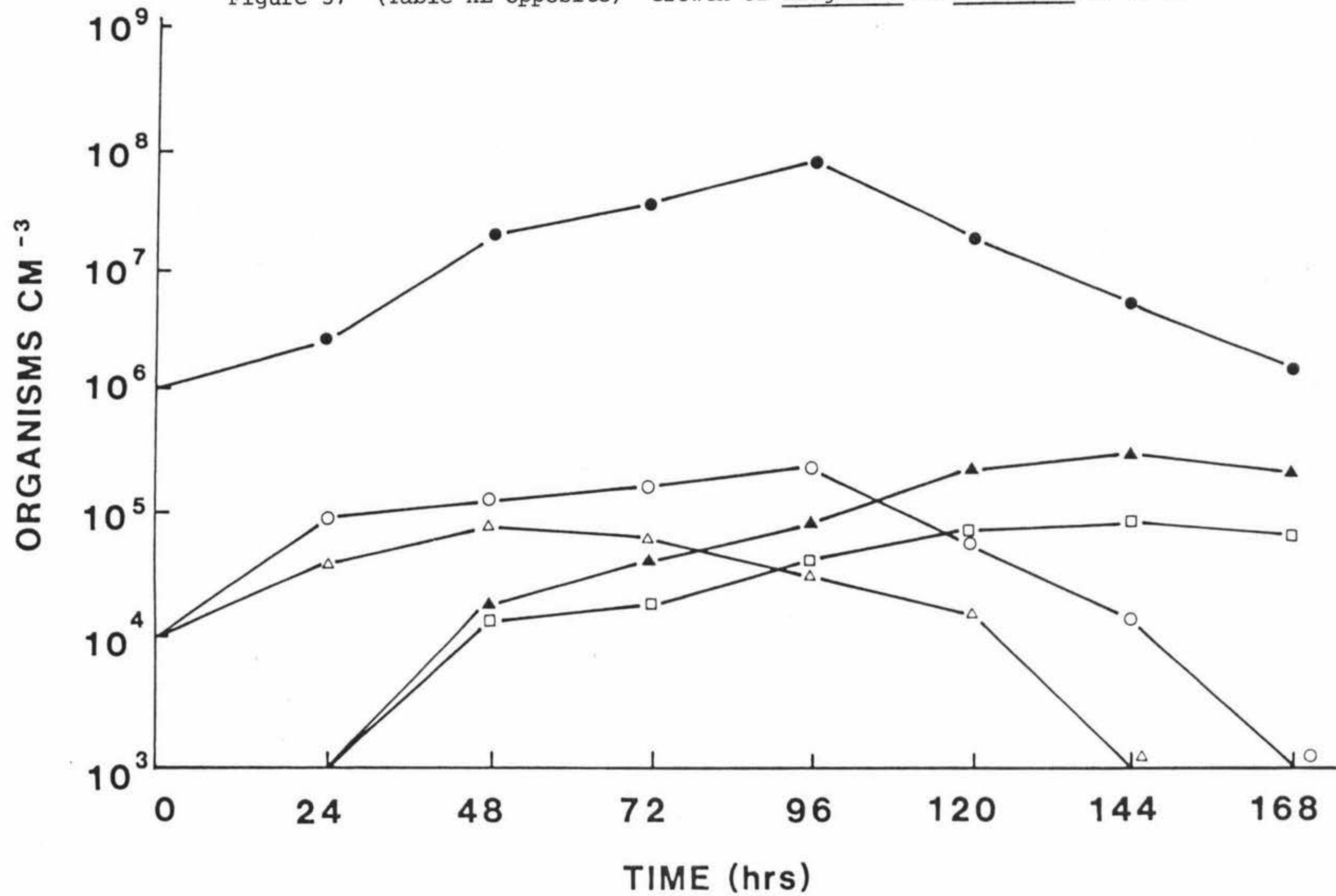


Table XL

Organisms	Time (hrs)							
	0	24	48	72	96	120	144	168
N.fowleri (MsM)	1×10^{4}	6×10^{4}	8×10^{4}	7×10^{4}	5×10^{4}	3×10^{4}	$< 10^{3}$	$< 10^{3}$
			1×10^{4}*	4×10^{4}*	6×10^{4}*	8×10^{4}*	9.5×10^{4}*	9×10^{4}*
E.cloacae	1×10^{6}	4×10^{6}	3×10^{7}	6×10^{7}	1×10^{8}	4×10^{7}	7×10^{6}	2×10^{6}
N.fowleri (MsT)	1×10^{4}	5×10^{4}	8×10^{4}	8×10^{4}	6×10^{4}	3×10^{4}	$< 10^{3}$	$< 10^{3}$
			3×10^{4}*	5×10^{4}*	7×10^{4}*	8×10^{4}*	9×10^{4}*	8×10^{4}*
E.cloacae	1×10^{6}	3×10^{6}	2.5×10^{7}	6×10^{7}	2×10^{8}	2×10^{7}	9×10^{6}	3×10^{6}
N.fowleri (Northcott)	1×10^{4}	7×10^{4}	1×10^{5}	8×10^{4}	5×10^{4}	6×10^{4}	2×10^{3}	$< 10^{3}$
			1×10^{4}*	3×10^{4}*	8×10^{4}*	9×10^{4}*	1×10^{5}*	9×10^{4}*
E.cloacae	1×10^{6}	3×10^{6}	2×10^{7}	5×10^{7}	2×10^{8}	3×10^{7}	8×10^{6}	2×10^{6}
N.gruberi (P1200f)	1×10^{4}	9×10^{4}	1×10^{5}	2.5×10^{5}	4×10^{5}	7.5×10^{4}	1×10^{4}	$< 10^{3}$
			2×10^{4}*	6×10^{4}*	9×10^{4}*	4×10^{5}*	5×10^{5}*	3×10^{5}*
E.cloacae	1×10^{6}	4×10^{6}	3×10^{7}	6×10^{7}	2×10^{8}	3×10^{7}	8×10^{6}	2×10^{6}

* = cysts

Figure 38 (Table XLI opposite) Growth of *Naegleria* on *M.luteus* at 30°C

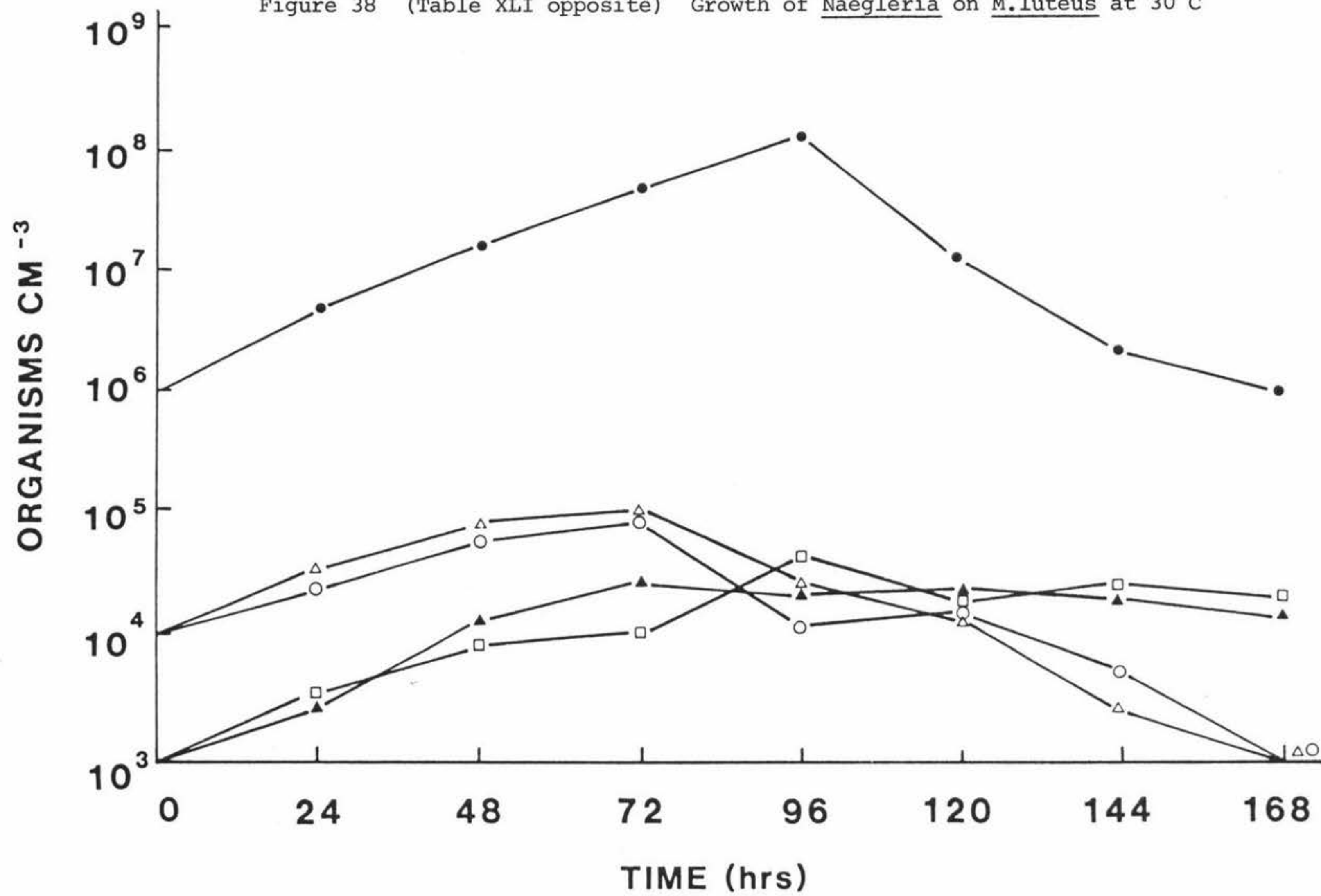


Table XLI

Organisms	Time (hrs)							
	0	24	48	72	96	120	144	168
N.fowleri (MsM)	1×10^4	4×10^4	8×10^4	9×10^4	3×10^4	1×10^4	3×10^3	$< 10^3$
		4.5×10^3*	8×10^3*	9×10^3*	6×10^4*	2×10^4*	3×10^4*	2.5×10^4*
M.luteus	1×10^6	7×10^6	2×10^7	8×10^7	3×10^8	1×10^7	4×10^6	1×10^6
N.fowleri (MsT)	1×10^4	7×10^4	9×10^4	8×10^4	3×10^4	1.4×10^4	2×10^3	$< 10^3$
		3×10^3*	9×10^3*	1.5×10^4*	3×10^4*	9×10^3*	4×10^4*	2×10^4*
M.luteus	1×10^6	6×10^6	3×10^7	8×10^7	2×10^8	1.4×10^7	3×10^6	1.3×10^6
N.fowleri (Northcott)	1×10^4	5×10^4	1.3×10^5	7×10^4	3×10^4	2×10^4	3×10^3	$< 10^3$
		5×10^3*	2×10^4*	1.5×10^4*	7×10^4*	1×10^4*	3×10^4*	1.5×10^4*
M.luteus	1×10^6	6×10^6	4×10^7	8×10^7	3×10^8	7×10^6	5×10^6	1×10^6
N.gruberi (P1200f)	1×10^4	3×10^4	7×10^4	8.5×10^4	9×10^3	1.5×10^4	7×10^3	$< 10^3$
		4×10^3*	1×10^4*	3×10^4*	2.8×10^4*	2.5×10^4*	2×10^4*	1×10^4*
M.luteus	1×10^6	4×10^6	4×10^7	7×10^7	3×10^8	8×10^6	3×10^6	1.4×10^6

* = cysts

Figure 39 (Table XLII opposite) Growth of Naegleria on B.subtilis at 30°C.

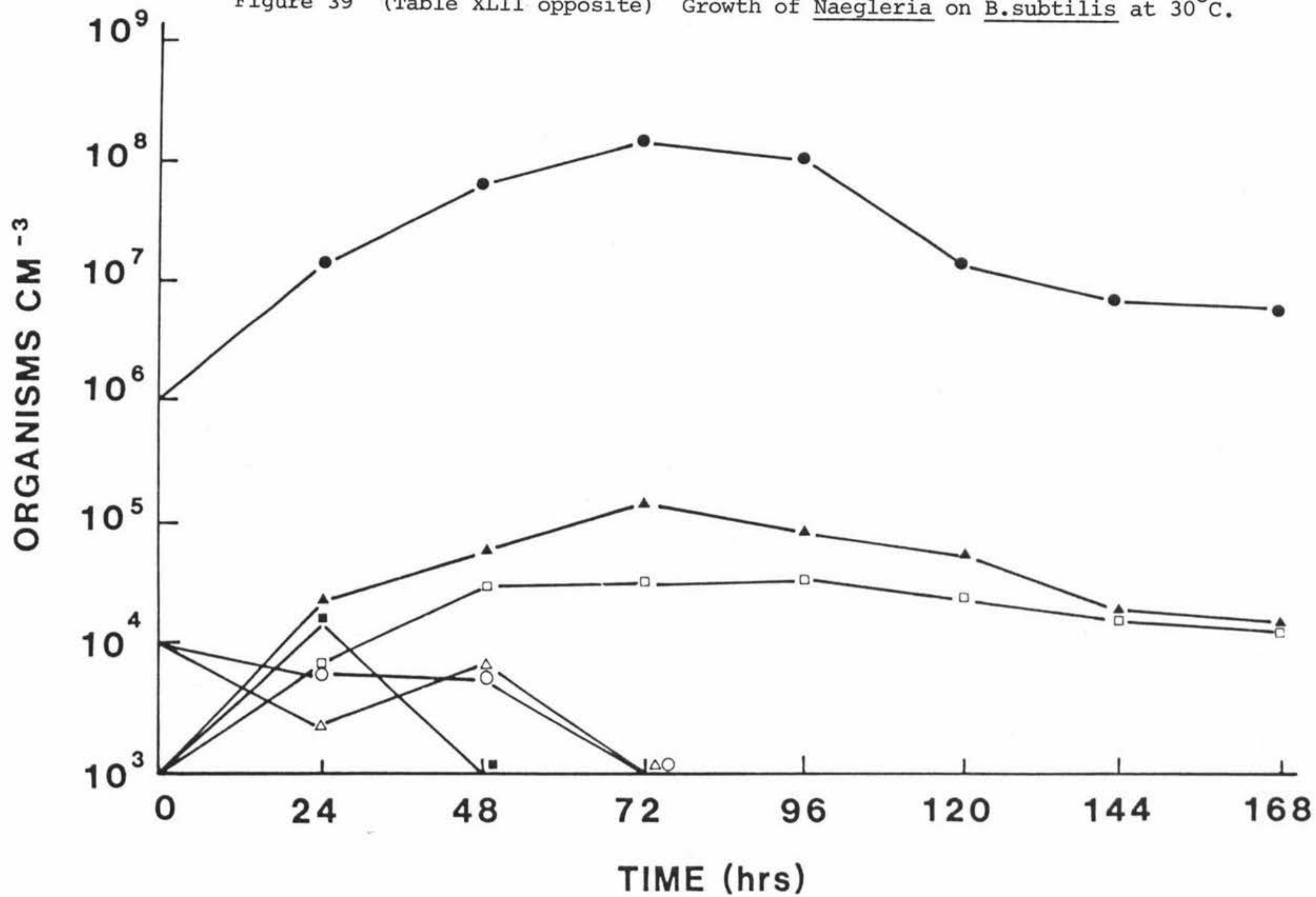


Table XLII

Organisms	Time (hrs)							
	0	24	48	72	96	120	144	168
N.fowleri (MsM)	1×10^4	3×10^3	8×10^3	$<10^3$				
		3×10^4+						
		8×10^3*	4×10^4*	4×10^4*	4×10^4*	3×10^4*	1×10^4*	9×10^3*
B.subtilis	1×10^6	2×10^7	8×10^7	3×10^8	1×10^8	2×10^7	8×10^6	7×10^6
N.fowleri (MsT)	1×10^4	6×10^3	8×10^3	$<10^3$				
		9×10^3+						
		5×10^4*	8×10^4*	8×10^4*	8×10^4*	6×10^4*	2×10^4*	2×10^4*
B.subtilis	1×10^6	3×10^7	8×10^7	2×10^8	9×10^7	2×10^7	8×10^6	5×10^6
N.fowleri (Northcott)	1×10^4	2×10^3	6×10^3	$<10^3$				
		5×10^3+						
		2×10^4*	1×10^5*	7×10^4*	6×10^4*	8×10^4*	2×10^4*	1×10^4*
B.subtilis	1×10^6	4×10^7	9×10^7	4×10^8	8×10^7	1×10^7	7×10^6	6×10^6
N.gruberi (P1200f)	1×10^4	7.5×10^3	7.5×10^3	$<10^3$				
		4×10^4*	7×10^4*	1×10^5*	8×10^4*	6×10^4*	1.5×10^4*	1×10^4*
B.subtilis	1×10^6	3×10^7	9×10^7	2×10^8	9×10^7	2×10^7	6×10^6	6×10^6

+ = flagellates * = cysts

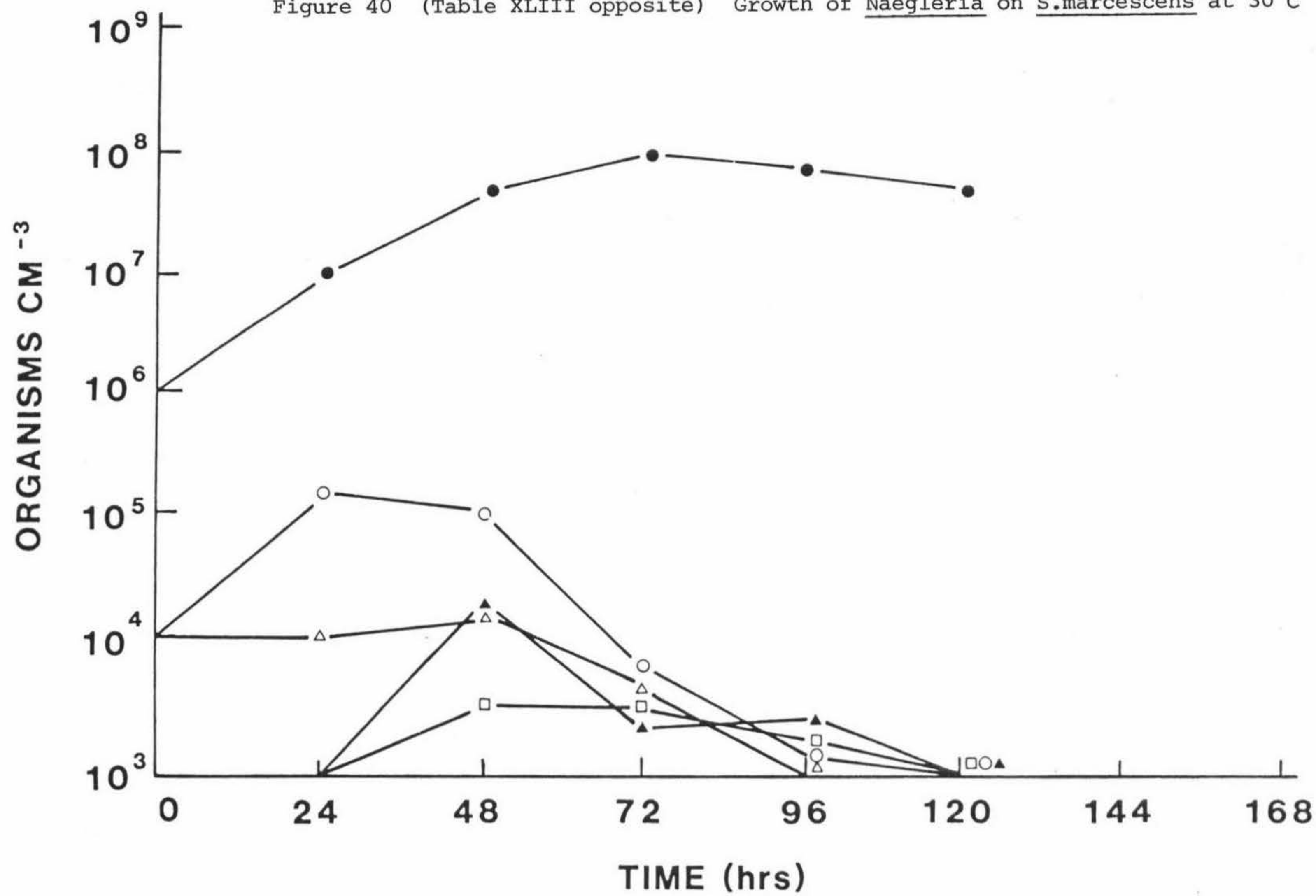


Figure 40 (Table XLIII opposite) Growth of <u>Naegleria</u> on <u>S.marcescens</u> at 30°C

Table XLIII

Organisms	Table (hrs)					
	0	24	48	72	96	120
N.fowleri (MsM)	1×10^4	1×10^4	2×10^4	6×10^3	$< 10^3$	
			5×10^3*	5×10^3*	2×10^3*	$< 10^3$*
S.marcescens	1×10^6	2×10^7	7×10^7	2×10^8	9.5×10^7	8×10^7
N.fowleri (MsT)	1×10^4	2×10^4	2×10^4	4×10^3	$< 10^3$	
			3×10^3*	5×10^3*	2×10^3*	$< 10^3$*
S.marcescens	1×10^6	2.6×10^7	5×10^7	3×10^8	1×10^8	9×10^7
N.fowleri (Northcott)	1×10^4	2×10^4	2.5×10^4	4×10^3	$< 10^3$	
			7×10^3*	6×10^3*	1×10^3*	$< 10^3$*
S.marcescens	1×10^6	4×10^7	6×10^7	1×10^8	2×10^8	8×10^7
N.gruberi (P1200f)	1×10^4	2×10^5	1×10^5	8×10^3	1×10^3	$< 10^3$
			2.8×10^4*	3×10^3*	3×10^3*	$< 10^3$
S.marcescens	1×10^6	1×10^7	6×10^7	1×10^8	9×10^7	8×10^7

* = cysts

Figure 41 (Table XLIV opposite) Growth of <u>Naegleria</u> on <u>Ps.aeruginosa</u> at 30°C.

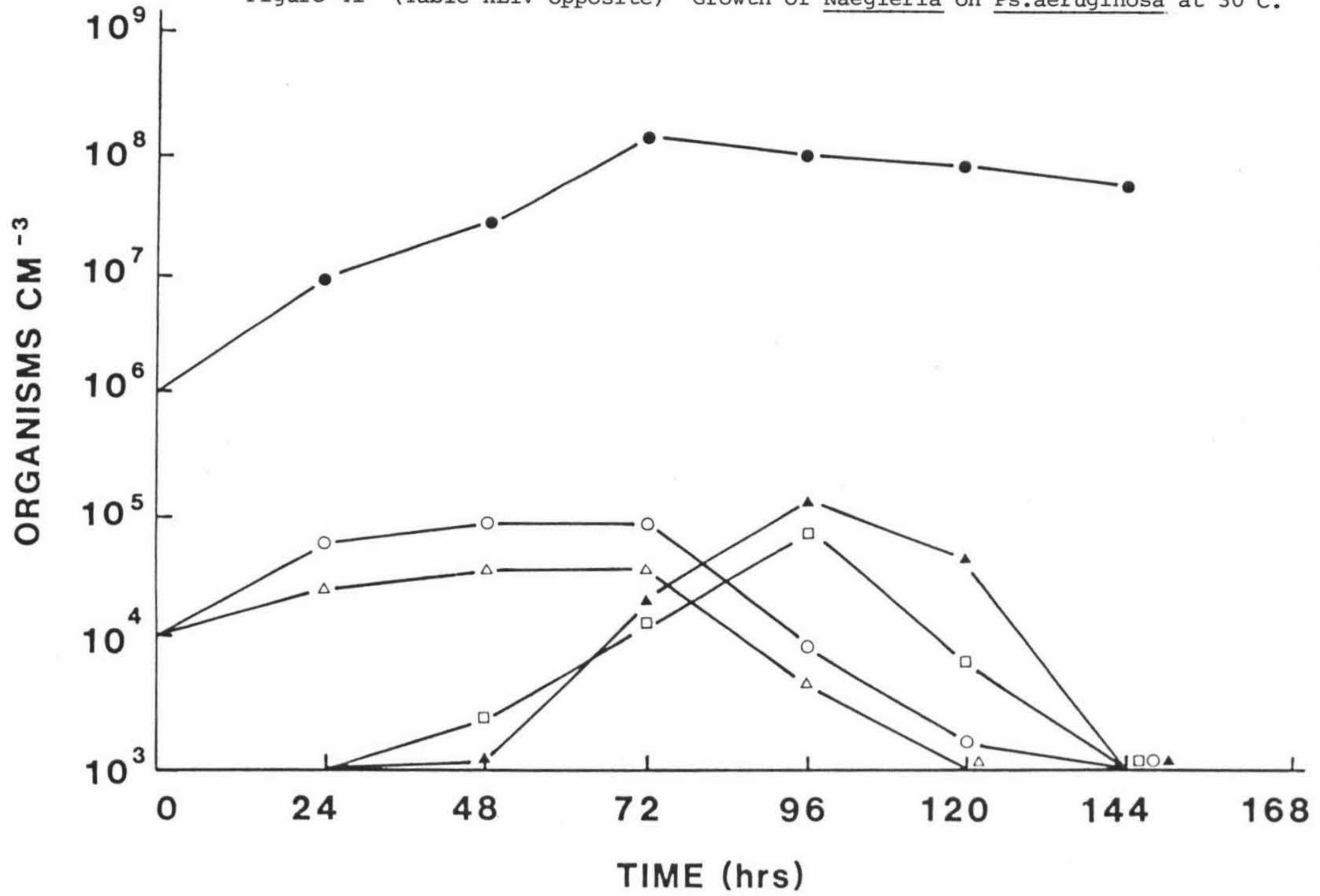


Table XLIV

Organisms	Time (hrs)						
	0	24	48	72	96	120	144
N.fowleri (MsM)	1×10^{4}	3×10^{4}	5×10^{4}	5×10^{4}	7×10^{3}	$< 10^{3}$	
			4×10^{3}*	2×10^{4}*	7×10^{4}*	8×10^{3}*	$< 10^{3}$
Ps.aeruginosa	1×10^{6}	1×10^{7}	4×10^{7}	2×10^{8}	9×10^{7}	8×10^{7}	7×10^{7}
N.fowleri (MsT)	1×10^{4}	5×10^{4}	5×10^{4}	4×10^{4}	6×10^{3}	$< 10^{3}$	
			4×10^{3}*	3×10^{4}*	9×10^{4}*	9×10^{3}*	$< 10^{3}$
Ps.aeruginosa	1×10^{6}	9×10^{6}	6×10^{7}	2×10^{8}	1×10^{8}	9×10^{7}	7×10^{7}
N.fowleri (Northcott)	1×10^{4}	4×10^{4}	6×10^{4}	4×10^{4}	2×10^{3}	$< 10^{3}$	
			6×10^{3}*	3×10^{4}*	4×10^{4}*	1×10^{3}*	$< 10^{3}$
Ps.aeruginosa	1×10^{6}	9×10^{6}	5×10^{7}	2×10^{8}	8×10^{7}	7×10^{7}	5×10^{7}
N.gruberi (Pl200f)	1×10^{4}	7×10^{4}	9×10^{4}	9×10^{4}	1×10^{4}	2×10^{3}	$< 10^{3}$
			1×10^{3}*	3×10^{4}*	2×10^{5}*	7×10^{4}*	$< 10^{3}$
Ps.aeruginosa	1×10^{6}	1×10^{7}	4×10^{7}	1×10^{8}	1×10^{8}	9×10^{7}	6×10^{7}

* = cysts

Figure 42 (Table XLV opposite) Growth of <u>Naegleria</u> on <u>Ps.fluorescens</u> at 30°C.

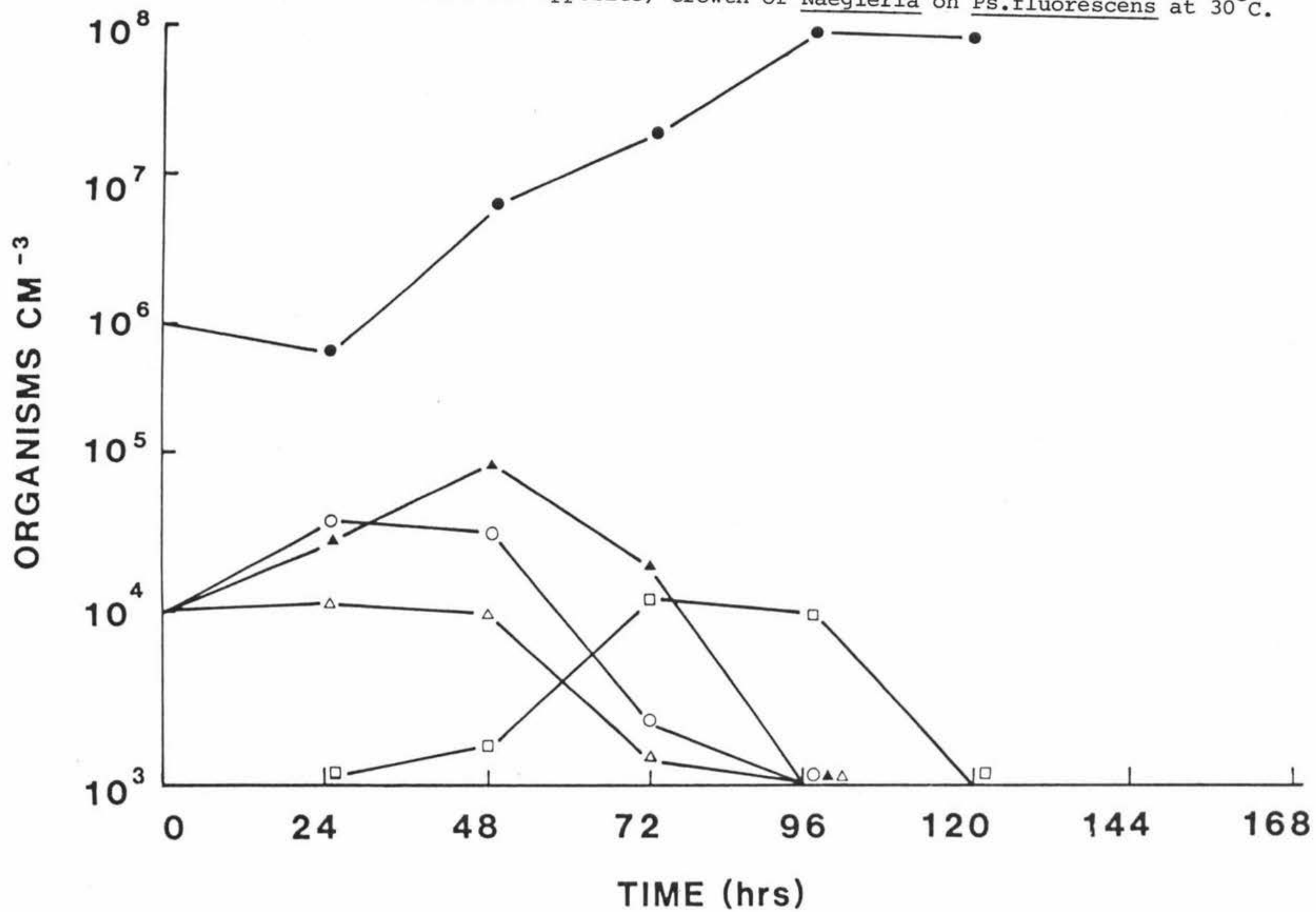


Table XLVI

Organisms	Time (hrs)					
	0	24	48	72	96	120
N.fowleri (MsM)	1×10^4	4×10^4	6×10^4	7×10^3	$< 10^3$	
			4×10^3*	2×10^4*	2×10^4*	$< 10^3$
Ps.fluorescens	1×10^6	4×10^5	8×10^6	3×10^7	9×10^7	1×10^8
N.fowleri (MsT)	1×10^4	7×10^4	5×10^4	3×10^3	$< 10^3$	
			3×10^3*	2×10^4*	3×10^4*	$< 10^3$
Ps.fluorescens	1×10^6	8×10^5	8×10^6	4×10^7	8×10^7	2×10^8
N.fowleri (Northcott)	1×10^4	7×10^3	9×10^3	2×10^3	$< 10^3$	
			1×10^4*	3×10^4*	4×10^4*	$< 10^3$
Ps.fluorescens	1×10^6	1×10^6	6×10^6	6×10^7	9×10^7	2×10^8
N.gruberi (P1200f)	1×10^4	5×10^4	4.5×10^4	3×10^3	$< 10^3$	
		4×10^4*	9×10^4*	4×10^4*	$< 10^3$	
Ps.fluorescens	1×10^6	8×10^5	1×10^7	2×10^7	9×10^7	

* = cysts

4.2.2 The Growth of T.pyriformis on the Eight Bacteria Species

From Figs. 43 and 44, Table XLVI, it can be seen that over a seven day incubation period, of the eight species of bacteria tested for effective growth support, M.luteus, Ps.aeruginosa, K.aerogenes, E.cloacae and E.coli were effective as growth support for T.pyriformis, listed in order of effectiveness, with M.luteus as the most effective. S.marcescens was not effective in this sense, in that by day 7 (168 hours) the number of T.pyriformis was reduced to uncountable levels. This had occurred by day 6 (144 hours) with Ps.fluorescens, and by day 3 (72 hours) with B.subtilis, which was the least effective of the bacteria tested. Most of those bacteria species that were effective as ciliate growth support had actually decreased in number from the initial inoculum by day 2 (48 hours) although most had regained this level by day 5 (120 hours). As can be seen in Figs. 43 and 44, the effective growth support bacteria maintained the ciliates at constant levels for 144 hours of the total incubation period of 168 hours. Those three bacteria species which were not so effective as ciliate growth support, although increased in numbers, only B.subtilis increased by more than ten fold.

Two temperatures were used to determine the ability of T.pyriformis to grow on these bacteria species, i.e. 30 and 37°C. The results shown in Figs. 43 and 44, and Table XLVI are those for 30°C. At 37°C, no T.pyriformis were viable after 24 hours of incubation.

In Figure 43 the following legend applies,

□ = E.cloacae

▲ = K.aerogenes

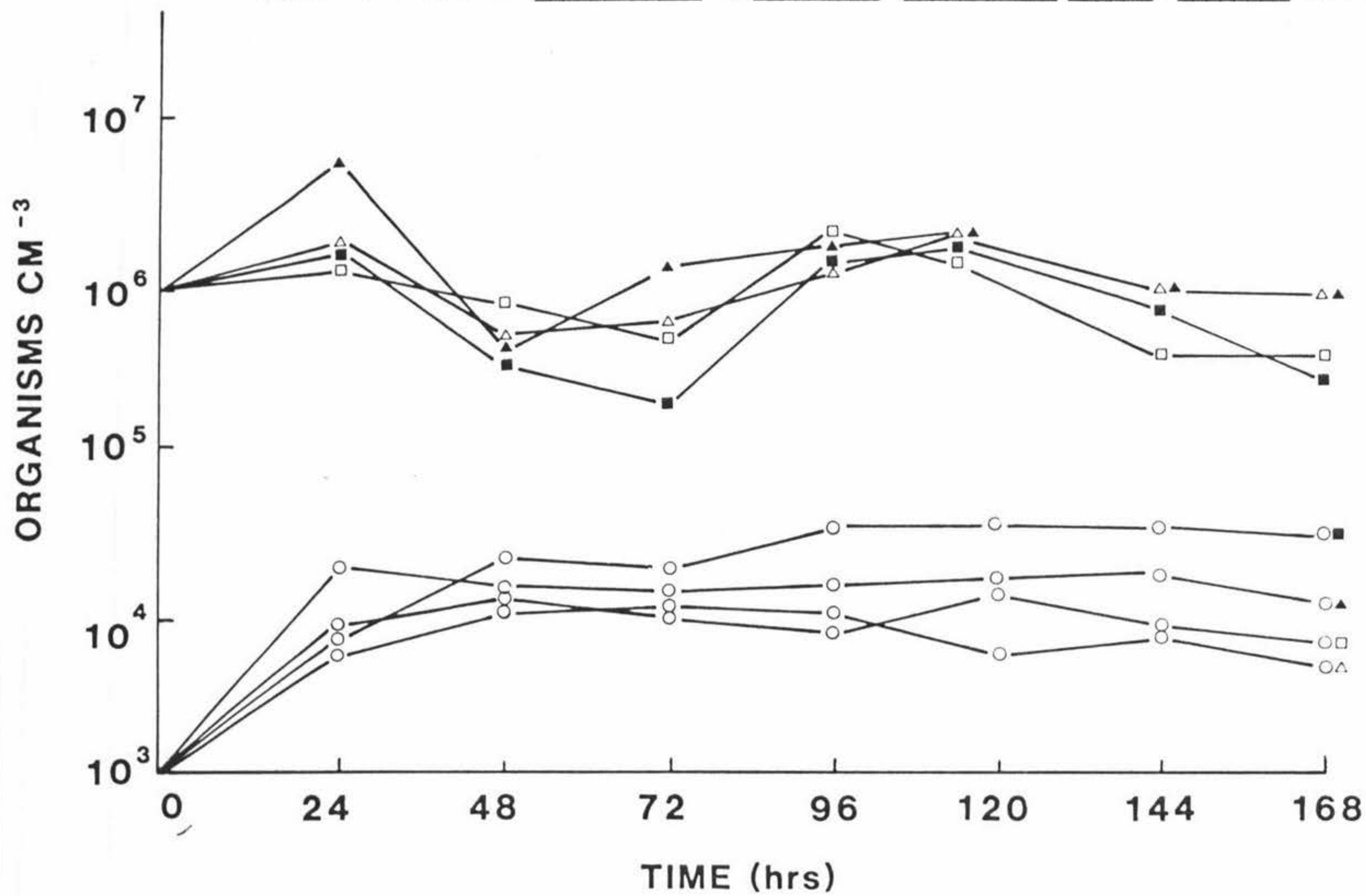
△ = E.coli

■ = M.luteus

○ = T.pyriformis

Note: In Figs. 43 and 44, the symbol for T.pyriformis followed by a bacteria symbol, denotes the growth curve of the ciliate on that bacteria species e.g. ○△ = T.pyriformis growth on E.coli

Figure 43 Growth of _T.pyriformis_ on _E.cloacae_, _K.aerogenes_, _E.coli_, _M.luteus_ at 30°C.



In Figure 44 the following legend applies,

△ = Ps.aeruginosa

▲ = Ps.fluorescens

□ = B.subtilis

■ = S.marcescens

○ = T.pyriformis

Figure 44 Growth of T.pyriformis on Ps.aeruginosa, Ps.fluorescens, B.subtilis, S.marcescens at 30°C.

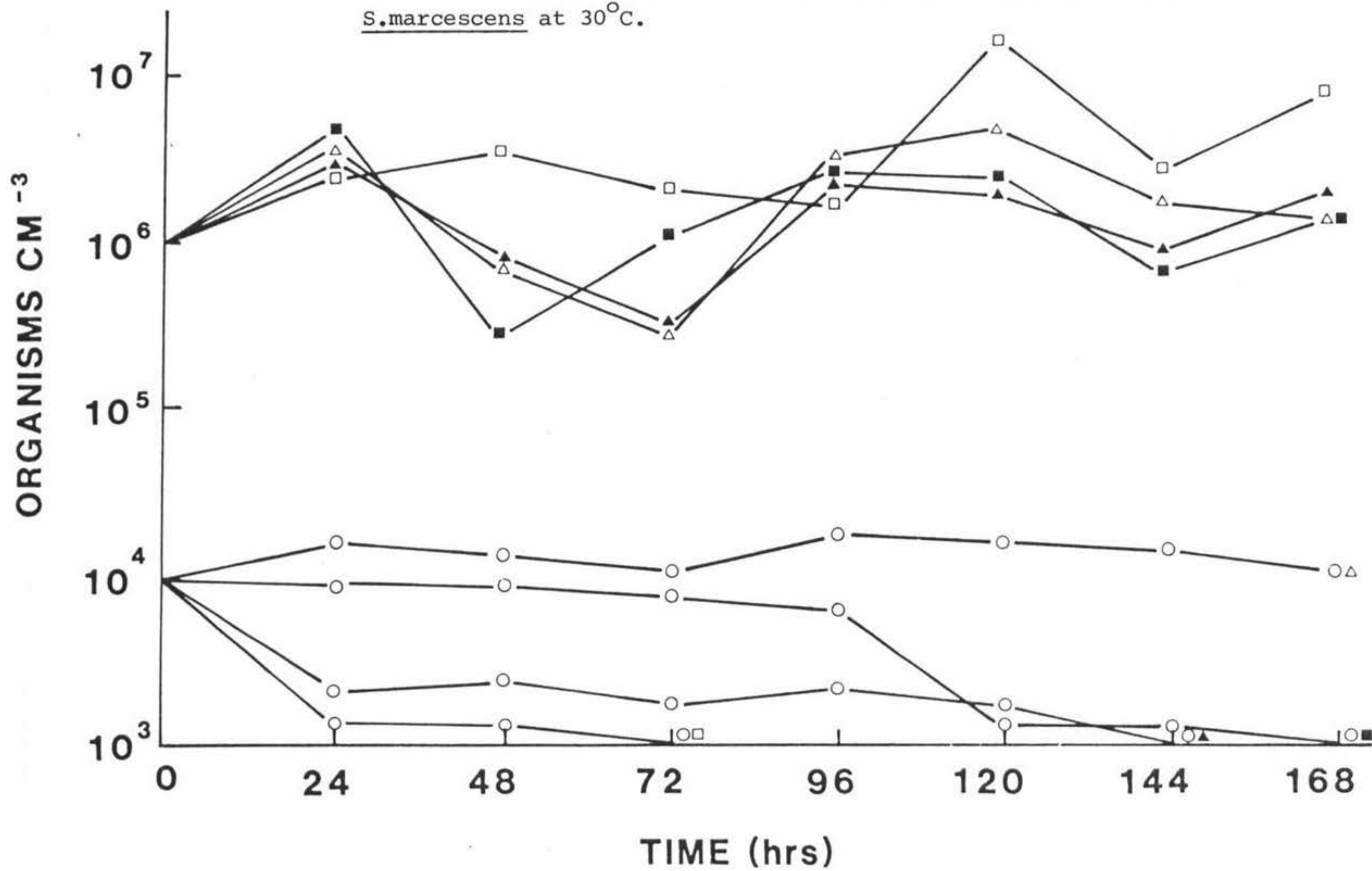


Table XLVI Growth of T.pyriformis on the Eight Species of Bacteria at 30°C.

Organisms	Time (hrs)							
	0	24	48	72	96	120	144	168
T.pyriformis	1×10^4	9×10^3	1.1×10^4	8×10^3	8×10^3	1.3×10^4	8×10^3	6×10^3
E.coli	1×10^6	1×10^6	9×10^5	7×10^5	3×10^6	2.5×10^6	1.4×10^6	1×10^6
T.pyriformis	1×10^4	1.8×10^4	1×10^4	1.8×10^4	1.5×10^4	1.6×10^4	1.7×10^4	1×10^4
K.aerogenes	1×10^6	6×10^6	5×10^5	1.4×10^6	2.2×10^6	3×10^6	8×10^5	8×10^5
T.pyriformis	1×10^4	6×10^3	1.3×10^4	1×10^4	1×10^4	7×10^3	9×10^3	8×10^3
E.cloacae	1×10^6	3×10^6	5×10^5	7.5×10^5	1.5×10^5	3×10^6	8×10^5	8×10^5
T.pyriformis	1×10^4	9×10^3	2.6×10^4	1.9×10^4	5×10^4	5×10^4	4×10^4	2×10^4
M.luteus	1×10^6	2×10^6	4×10^5	2×10^5	2.5×10^6	3×10^6	3×10^5	1×10^5
T.pyriformis	1×10^4	1×10^3	1×10^3	$< 10^3$	$< 10^3$	$< 10^3$	$< 10^3$	$< 10^3$
B.subtilis	1×10^6	3×10^6	5×10^6	3×10^6	2×10^6	2×10^7	4×10^6	9×10^6
T.pyriformis	1×10^4	1.1×10^4	9×10^3	8×10^3	7×10^3	1×10^3	1×10^3	$< 10^3$
S.marcescens	1×10^6	7×10^6	4×10^5	1×10^6	4×10^6	4×10^6	6×10^5	1×10^6
T.pyriformis	1×10^4	2×10^4	1×10^4	1×10^4	2.2×10^4	1.7×10^4	1.5×10^4	9×10^3
Ps.aeruginosa	1×10^6	5×10^6	8×10^5	4×10^5	5×10^6	6×10^6	1.4×10^6	1×10^6
T.pyriformis	1×10^4	3×10^3	4×10^3	2×10^3	3×10^3	2×10^3	$< 10^3$	$< 10^3$
Ps.fluorescens	1×10^6	4×10^6	9×10^5	5×10^5	3×10^6	2.5×10^6	9×10^5	2×10^6

4.4.3 Competition Between T.pyriformis and Four Naegleria Strains

The bacteria species used were E.coli, E.cloacae and Ps.fluorescens. The two former were chosen for their proven effectiveness as growth support for both amoebae and ciliates, Ps.fluorescens was chosen because it was not an effective growth support bacteria for either amoeba or ciliate. As is shown in Figs. 45, 46, and 47, and Tables XLVII, XLVIII and XLIX, all three bacteria species showed effective growth support for both amoebae and ciliates over the 7 day (168 hour) incubation period. For 144 hours of the 168 hour incubation period, the bacteria population was below the initial inoculum level of $1 \times 10^{6}.cm^{-3}$, only returning to this level on day 7. The increase of both Naegleria and T.pyriformis, when grown on each bacteria species was up to eight times the concentration of the initial inoculum of $1 \times 10^{4}.cm^{-3}$. The peak of Naegleria growth was on day 2 (48 hours) on E.cloacae for all strains, day 3 (72 hours) for the pathogens and day 4 (96 hours) for the non-pathogens on E.coli, and day 4 (96 hours) for all strains on Ps.fluorescens. The peak for T.pyriformis, on the on the bacteria in the same order, was day 1 (24 hours), day 4 (96 hours) and day 4 (96 hours) in competition with N.fowleri strains.but day 2 (48 hours) when in competition with N.gruberi (P1200f) on E.cloacae and Ps.fluorescens, and day 3 (72 hours) on E.coli. The lowest concentration of bacteria occurred on day 5 (120 hours) for E.cloacae, day 4 (96 hours) for E.coli, and day 5 (120 hours) for Ps.fluorescens. Although "peaks" of increased concentration of each organism are indicated above, as can be seen in Figs. 45, 46 and 47, the concentrations do not oscillate or vary greatly from day to day, but shows constant population levels. One important factor which must be mentioned is the lack of cysts. No cysts were observed at any stage with any of the Naegleria strains tested,

although the trophozoites were smaller than those seen in CYM medium, and after day 5 (120 hours), the movement of T.pyriformis was observed to be significantly slowed, and dead cells were observed.. This was more obvious with Ps.fluorescens, although present with E.coli and E.cloacae.

The $30^{o}C$ incubation temperature was used, because of the temperature effect on control amoebae cultures, described in section 4.1.1. As described in section 4.4.2, the T.pyriformis strain used did not grow at $37^{o}C$.

In Figures 45, 46 and 47 the following legend applies,

△ = N.fowleri

○ = N.gruberi (P1200f)

▲ = T.pyriformis in competition with N.fowleri

● = T.pyriformis in competition with N.gruberi (P1200f)

□ = The bacteria species used in that experiment

Figure 45 (Table XLVII opposite) Competition between T.pyriformis and N.fowleri and T.pyriformis and N.gruberi, on E.coli at 30°C.

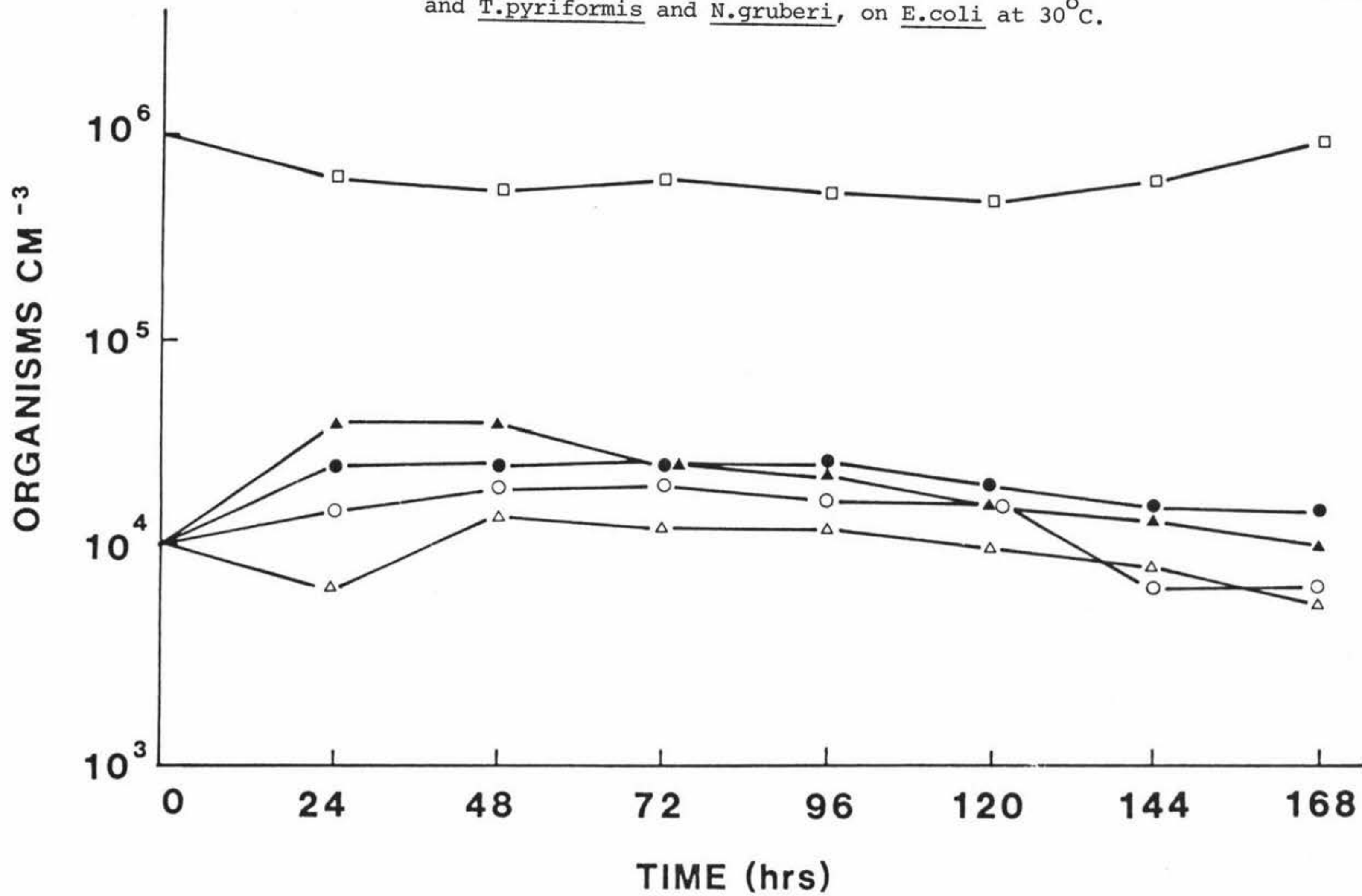


Table XLVII

Organisms	Time (hrs)							
	0	24	48	72	96	120	144	168
N.fowleri (MsM)	1×10^4	7×10^3	2×10^4	2.5×10^4	1.5×10^4	2×10^4	1×10^4	6×10^3
T.pyriformis	1×10^4	5×10^4	6×10^4	7×10^4	6×10^4	5×10^4	3×10^4	2×10^4
E.coli	1×10^6	9×10^5	8×10^5	8×10^5	7×10^5	8×10^5	8×10^5	1×10^6
N.fowleri (MsT)	1×10^4	6×10^3	2×10^4	2.6×10^4	1.8×10^4	1.5×10^4	1.5×10^4	1×10^4
T.pyriformis	1×10^4	5×10^4	7×10^4	7×10^4	8×10^4	5×10^4	3×10^4	2×10^4
E.coli	1×10^6	8×10^5	8×10^5	7×10^5	8×10^5	8×10^5	9×10^5	1.2×10^6
N.fowleri (Northcott)	1×10^4	5×10^3	1×10^4	2×10^4	2×10^4	2×10^4	1×10^4	7×10^3
T.pyriformis	1×10^4	6×10^4	6×10^4	7×10^4	8×10^4	4×10^4	3×10^4	1×10^4
E.coli	1×10^6	8×10^5	9×10^5	7×10^5	6×10^5	8×10^5	8×10^5	1.3×10^6
N.gruberi (P1200f)	1×10^4	3×10^4	3×10^3	3.5×10^4	4×10^4	2×10^4	2×10^4	1×10^4
T.pyriformis	1×10^4	4×10^4	4×10^4	5×10^4	5×10^4	3×10^4	2×10^4	2×10^4
E.coli	1×10^6	9×10^5	8×10^5	8×10^5	7×10^5	8×10^5	9×10^5	1.2×10^6

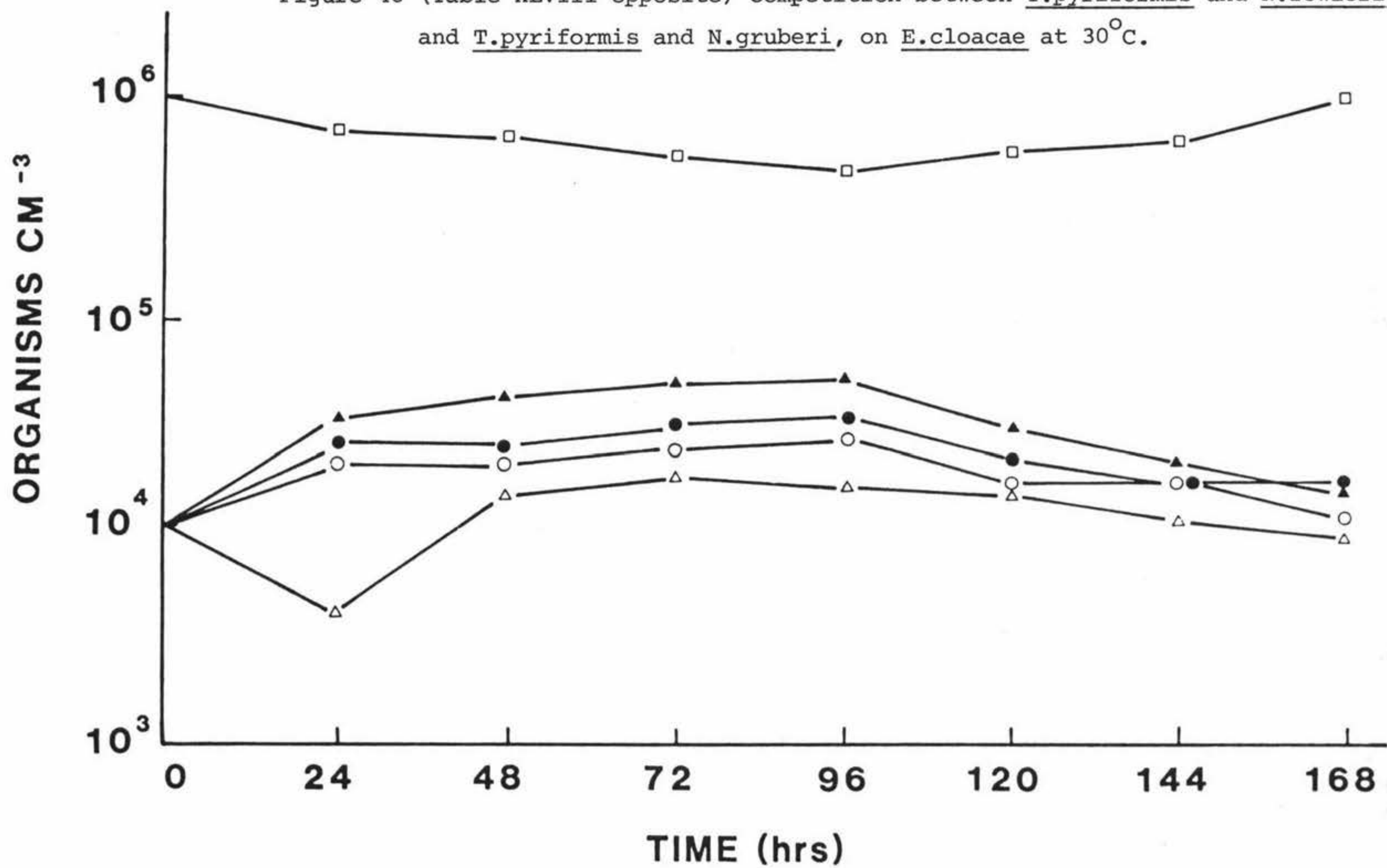


Figure 46 (Table XLVIII opposite) Competition between T.pyriformis and N.fowleri, and T.pyriformis and N.gruberi, on E.cloacae at 30°C.

Table XLVIII

Organisms	Time (hrs)							
	0	24	48	72	96	120	144	168
N.fowleri (MsM)	1×10^4	9×10^3	2×10^4	1.5×10^4	1.3×10^4	1×10^4	8×10^3	5×10^3
T.pyriformis	1×10^4	8×10^4	6×10^4	4×10^4	3.5×10^4	2×10^4	1×10^4	1×10^4
E.cloacae	1×10^6	8×10^5	7×10^5	9×10^5	7×10^5	7×10^5	8×10^5	1×10^6
N.fowleri (MsT)	1×10^4	8×10^3	2×10^4	1.4×10^4	1.1×10^4	1×10^4	1×10^4	8×10^3
T.pyriformis	1×10^4	4×10^4	6×10^4	4×10^4	4×10^4	2×10^4	1.5×10^4	1×10^4
E.cloacae	1×10^6	9×10^5	8×10^5	7×10^5	8×10^5	7×10^5	8×10^5	9×10^5
N.fowleri (Northcott)	1×10^4	7×10^3	1×10^4	1×10^4	1.3×10^4	1×10^4	9×10^3	7×10^3
T.pyriformis	1×10^4	8×10^4	6×10^4	4×10^4	4×10^4	2×10^4	2×10^4	1×10^4
E.cloacae	1×10^6	8×10^5	8×10^5	8×10^5	7×10^5	7×10^5	7×10^5	1×10^6
N.gruberi (P1200f)	1×10^4	2×10^4	3×10^4	3×10^4	2×10^4	2×10^4	8×10^3	8×10^3
T.pyriformis	1×10^4	4×10^4	4×10^4	4×10^4	4×10^4	3×10^4	2×10^4	2×10^4
E.cloacae	1×10^6	8×10^5	7×10^5	8×10^5	8×10^5	7×10^5	9×10^5	1×10^6

Figure 47 (Table XLIX opposite) Competition between T.pyriformis and N.fowleri and T.pyriformis and N.gruberi, on Ps.fluorescens at 30°C.

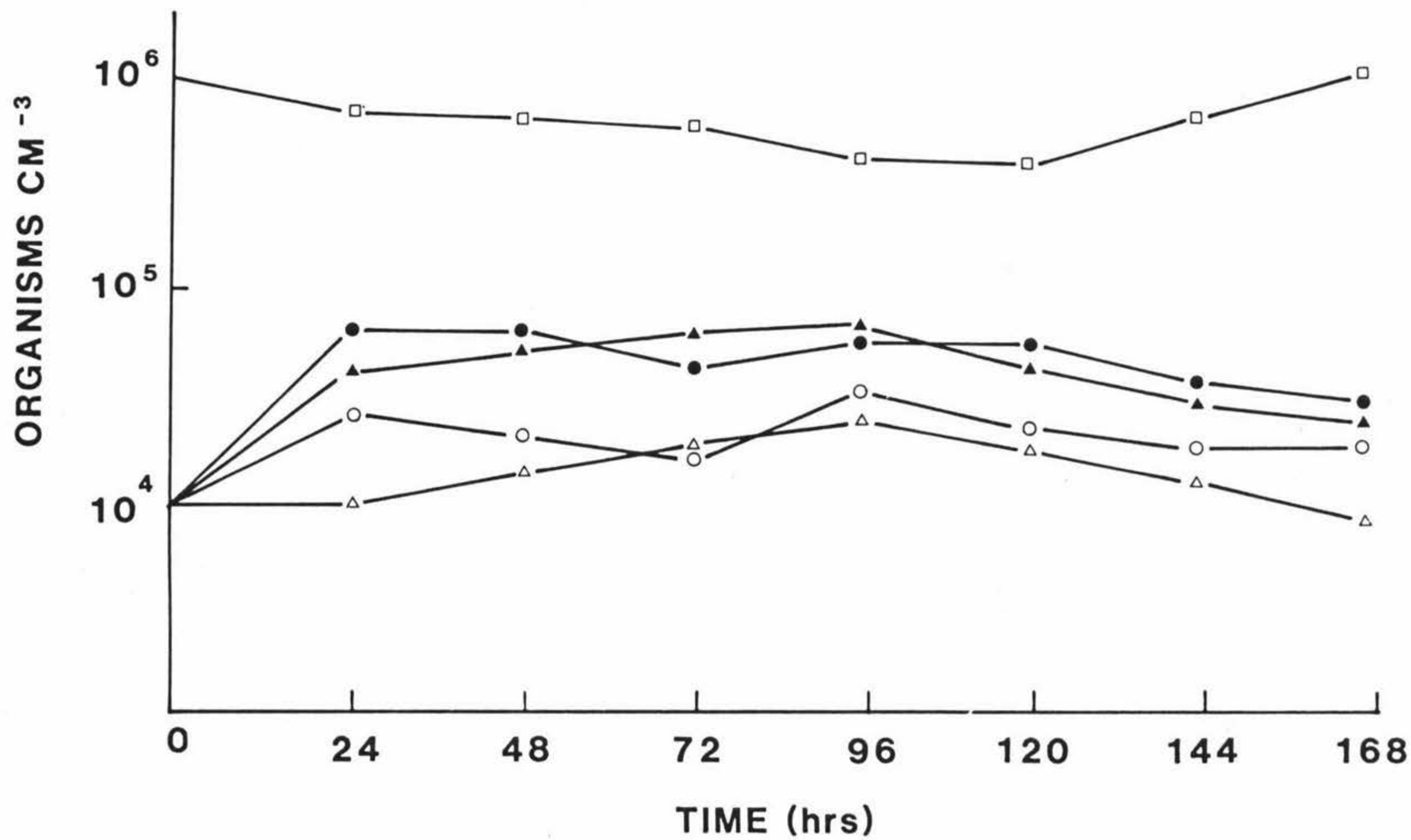


Table XLIX

Organisms	Time (hrs)							
	0	24	48	72	96	120	144	168
N.fowleri (MsM)	1×10^4	1×10^4	2×10^4	3×10^4	3.5×10^4	2×10^4	1×10^4	8×10^3
T.pyriformis	1×10^4	6×10^4	7×10^4	8×10^4	8×10^4	5×10^4	4×10^4	3×10^4
Ps.fluorescens	1×10^6	9×10^5	8×10^5	7×10^5	6×10^5	6×10^5	8×10^5	1.5×10^6
N.fowleri (MsT)	1×10^4	8×10^3	1.5×10^4	2×10^4	3×10^4	2×10^4	1×10^4	7×10^3
T.pyriformis	1×10^4	7×10^4	7×10^4	7×10^4	7×10^4	6×10^4	4×10^4	3.5×10^4
Ps.fluorescens	1×10^6	8×10^5	8×10^5	8×10^5	6×10^5	6×10^5	9×10^5	1×10^5
N.fowleri (Northcott)	1×10^4	9×10^3	1.3×10^4	2.5×10^4	4×10^4	2×10^4	1.3×10^4	1×10^4
T.pyriformis	1×10^4	7×10^4	7×10^4	7×10^4	8×10^4	5×10^4	4×10^4	3×10^4
Ps.fluorescens	1×10^6	9×10^5	8×10^5	8×10^5	7×10^5	6×10^5	9×10^5	1.2×10^6
N.gruberi (P1200f)	1×10^4	4×10^4	3×10^4	2×10^4	5×10^4	3×10^4	2×10^4	2×10^4
T.pyriformis	1×10^4	8×10^4	8×10^4	6×10^4	7×10^4	7×10^4	5×10^4	4×10^4
Ps.fluorescens	1×10^6	8×10^5	8×10^5	7×10^5	6×10^5	5×10^5	6×10^5	6×10^5

CHAPTER FIVE: DISCUSSION

5.1 The Disinfection of Pathogenic Free-Living Amoebae

The use of batch cultures, allows direct comparison of the use of Baquacil, to the results obtained by Cursons et al. (1980), on the amoebicidal capacities of chlorine, chlorine dioxide, ozone, and Deciquam 222, in axenic conditions. It also provides a base line for the follow-up work with bacteria, and Bovine Faecal Matter (BFM) solution, providing a known Biochemical Oxygen Demand (BOD). The results indicated that Baquacil possessed amoebicidal properties and would be able to be used for the disinfection of water contaminated with PFLA. Cursons et al. (1980) also found that chlorine, chlorine dioxide, ozone, and Deciquam 222 had amoebicidal properties. The choice of the disinfectant to be used must be made with regard to the chemical and physical properties of the water to be disinfected, and the relative capabilities of the available disinfectants in those conditions. No matter which disinfectant is ultimately chosen, it is generally accepted that efficiency of that disinfectant is the function of its rate of diffusion through the cell permeability barrier. Once through this barrier, it is assumed that the disinfectant destroys either an integral structural component, or attacks the metabolic machinery of the cell. In amoebae, the initial site of action will be the cell membrane, followed by consequential interruption of metabolism (White, 1972; Venkobacher et al., 1977).

Since its introduction into water treatment almost 80 years ago chlorine has become by far the most predominant method used, for active disinfection of potable water supplies (White, 1972). This is because of its economy, ease of application, relative lack of toxicity and therefore

relative lack of toxicity and therefore side effects, as well as its potency and range of its germicidal capacity, and reasonable persistance in water.

Because it reacts with both organic nitrogenous and ammonia nitrogenous compounds, forming organohalides, and chloramines respectively, when such contaminants are present, sufficient chlorine must be added to satisfy this chlorine demand of the water before any active disinfection can occur. The chlorine demand of the water is the difference between the amount of chlorine applied, or Total Available Chlorine (TAC), and the amount determined analytically as residual chlorine, or Free Available Chlorine (FAC). This technique is known as "break-point chlorination", the break-point being the point after which the addition of any further chlorine shows up as an equivalent increase in FAC (White, 1972).

Cursons *et al.* (1980) showed that 0.74, 0.79, 1.0, and 1.23 $mg.l^{-1}$ Chlorine killed cultures of $2 \times 10^{4}.cm^{-3}$ *N.fowleri*, *N.gruberi*, *A.castellanii* and *A.culbertsoni* respectively, in axenic conditions. The difference in susceptibility to chlorine between the two genera, is thought to be due to the difference in general chemical composition, and more particularly protein composition of the cell membranes, than differences in metabolism (Cursons, 1978). Venkobachar *et al.* (1977), demonstrated that chlorine damages the cell membrane of bacteria, which results in leakage of macromolecules and the complete cessation of oxidative phosphorylation. It is therefore possible for chlorine to exert a double effect on amoebae destroying both cell membrane and metabolism.

However, PFLA have been isolated from chlorinated domestic swimming water (Cerva, 1971; Anderson *et al.*, 1972; Cerva *et al.*, 1974; De Jonckheere *et al.*, 1976; Lyones *et al.*, 1977), which has

led to expressions of concern from public health authorities over the possible contraction of PAM via these waters. After a review of 16 fatal cases of PAM resulting from the use of one indoor chlorinated swimming pool, Cerva (1971) stated that " The constant presence of numerous populations of the limax group of amoebae cannot be prevented even under the strictest observations of all routine safety measures applied to water systems of swimming pools". Chlorine was used by Anderson and Jamieson (1972), when a case of PAM was reported after the victim playfully submerged his head in domestic bath water. They failed to erradicate N.fowleri from a contaminated pool by superchlorination to 10 $mg.l^{-1}$, however the chlorine demand of the water was not tested, and there may have been insufficient FAC available for amoebicidal activity to occur. Subsequently Derreumaux et al. (1974) demonstrated that 0.5 $mg.l^{-1}$ of HOCl, the active disinfecting component of chlorine disinfection, was able to erradicate both Naegleria spp. and Acanthamoeba spp. and that Naegleria were more sensitive to chlorination. De Jonckheere and Van de Voorde (1976) found that an initial concentration of between 0.5 and 1.0 $mg.l^{-1}$ was cysticidal for Naegleria spp. but that A.culbertsoni cysts were not inactivated by 40.0 $mg.l^{-1}$. Cursons et al. (1980) found that Naegleria were more sensitive to chlorination than Acanthamoeba in axenic conditions, and that at pH 7.0, the levels required were 0.79 and 1.25 $mg.l^{-1}$ respectively, which when taking into account the different batch culture methods, and different pH, were similar to those recorded by Derreumaux et al. (1974). Disinfection with chlorine in practice involves break-point chlorination to establish, and subsequently maintain, a reasonable residual concentration for disinfection, which in New Zealand varies from 0.1 - 0.2 $mg.l^{-1}$ for potable water and

0.5 - 0.8 $mg.l^{-1}$ for recreational water (Cursons, 1978). Routine dosing of the water supply is necessary to satisfy the fluctuating chlorine demand of the water, which in a swimming pool situation, because of the increased organic pollution produced by bathers, more FAC will be required for adequate disinfection. The use of chlorine dioxide as a disinfectant is not as well documented as that of chlorine. Its mode of action is thought to be the inhibition of protein synthesis (White, 1972). It is a vigorous oxidising agent, but is 5 times more soluble in water than chlorine. Unlike chlorine, it does not react with water or ammonia, however it does react with water or ammonia, however it does react with proteins, organic nitrogenous compounds, phenols and aromatics, and as such is useful in the removal of tastes and odours from water. It forms a stable residual and is more active at an alkaline pH. Acanthamoeba spp. are more resistant than Naegleria spp. to chlorine dioxide, although it was effective against both genera (Cursons et al., 1980). Because of its properties, chlorine dioxide may be a valid alternative to chlorine in water where there is a high ammonia content and alkaline pH (White, 1972). Baquacil is a 20% solution of polyhexamethylene biguanide hydrochloride, marketed by ICI (NZ)Limited. It has proven bactericidal activity, and has been tested for amoebicidal activity in Australia (Alford pers. comm.).

In comparison with the results of Cursons et al. (1980), using chlorine, chlorine dioxide, onzone, Deciquam 222, Baquacil must be looked upon favourably (Dawson et al., 1982). To assess the capabilities of Baquacil, emphasis must be made on the lack of difference in susceptibility to it between Naegleria and Acanthamoeba spp. This, with its proven amoebicidal capability,

must place it ahead of chlorine in effectiveness as a disinfectant. However, it is not as effective as Deciquam 222, but given the limitations of Deciquam 222, i.e. its inactivation in hard water (Cursons, 1978), Baquacil is more likely to be more generally acceptable. The order of effectiveness as amoebicides in hard water, is Baquacil chlorine, chlorine dioxide, ozone, and Deciquam 222 (Dawson et al., 1982). In soft water, Deciquam 222 would be the most effective, followed by Baquacil, etc.

From the results, it is obvious that with Baquacil, there was a higher survival rate for the monoxenically grown over the axenically grown amoebae. The differences in survival rates with the exceptions of the high BOD experiments, are in the order of 1.0 - 5.0% between the strains of amoeba (Tables VI - XX). However, the actual numbers of amoebae surviving may differ by $\lessdot$ 2 fold between strains. Neither the bacterial survival rates, nor the mean Baquacil demands varied between the use of axenically and monoxenically grown amoebae. Chlorine and chlorine dioxide were tested under the same conditions as Baquacil, but there was little point testing these two disinfectants on the combination of high BOD (of 4.7 $mg.l^{-1}$) plus bacteria, because when the high BOD load was introduced, it created such a chlorine demand that 6.0 $mg.l^{-1}$ chlorine was unable to sterilise either genera, either monoxenically or axenically grown. The monoxenically grown amoebae surivival rates were higher by 2.0% for both Naegleria and Acanthamoeba spp. (Tables XXIV and XXVIII).

In all test conditions, the monoxenically grown amoebae, even after sterilisation, caused a greater organic demand. When there were survivors, the survival rate was higher for the monoxenically grown amoebae. When sterilised, the chlorine demand,

difference was in the order of 0.1 - 0.8 $mg.l^{-1}$. The actual survival rates differed by up to 2.0% which represents a difference in numbers of survivors, of 200 in 1700 $amoeba.cm^{-3}$ in favour of the monoxenically grown amoebae.

Chlorine dioxide was the least effective amoebicide used in this study. The survival rates shown in the results (Figs. 27 - 34, Tables XXX - XXXVII) indicate that the monoxenically grown amoebae survived exposure to chlorine dioxide more readily than the axenically grown amoebae under all conditions tested. As with Baquacil and chlorine, the actual differences were small, in the order of $< 10.5\%$. However, the actual numbers of survivors was higher by up to half as many again, in favour of the monoxenically grown amoebae.

Either the BOD load caused by BFM solution, or the addition of bacteria, caused an increased demand of all three disinfectants, and when used in combination, they caused a cummulative increase in demand. This maybe the reason why superchlorination did not work in the case of Anderson and Jamieson (1972), in that there was insufficient FAC for amoebicidal activity to occur after satisfying the bacterial and organic demand, It is also the probable reason for the amoebae surviving behind the crack in the swimming pool wall, where the chlorine contact with the organic debris would have almost certainly removed the FAC from the water (Cerva, 1971). Cursons _et al._ (1980) speculated that even when only bactericidal doses of disinfectant were used, the amoebae were still affected indirectly through the consequent removal of food. This study has shown that while this may be true (the effect probably being to cause encystment), the presence of bacteria requires increased disinfectant concentrations to produce a rapid

effect on the amoebae in such water. When a BOD is also present, a further increase in initial disinfectant concentration is required for amoebicidal activity.

Comparing the three disinfectant solutions tested, chlorine dioxide must be considered the least effective. It did not sterilise any of the six amoeba strains, in any of the conditions tested, although the kill rate attributed to it, of up to 95.0% at 7.5 $mg.l^{-1}$, was significant. The Naegleria strains were shown to be more susceptible to chlorine dioxide than the Acanthamoeba strains (Figs. 27 - 34, Tables XXX -XXXVII). This is supported by the reults in axenic conditions of Cursons et al. (1980).

The results show that chlorine was a good amoebicidal agent under all test conditions except one (the high BOD load in Sections 4.2.2 and 4.2.5, Figs. 21 and 25, Tables XXIV and XXXIII). However, they also show that up to twice as much chlorine is required to affect Acanthamoeba spp. to the same extent as Naegleria spp. under similar experimental conditions. Thus, with the work of Cursons et al. (1980), and De Jonckheere and Van de Voorde (1976), this study confirms the greater susceptiblity of Naegleria spp. to chlorine than Acanthamoeba spp., in a range of environmental conditions.

Baquacil has the advantage over chlorine and chlorine dioxide, that it is equally effective against both genera. The results show that with the exceptions of when a BOD of 4.7 $mg.l^{-1}$ was present the survival rates were satisfactarily low. Although the results indicate that chlorine is more effective against Naegleria spp. than Baquacil, the chlorine concentrations used were relatively high. Baquacil, however was used at the manufacturers recommended maintainance level, and except for when the higher BOD

was used, the demand never exceeded 60% of that maintainance level. Figure 17 and Table XIX show that by doubling the exposure time of the amoebae to Baquacil, sterilisation of both genera occurs. Therefore Baquacil was the most effective amoebicide of the three examined. With all three disinfectants, there were differences in survival rates between pathogenic and non-pathogenic Naegleria spp. This was most marked with Baquacil although De Jonckheere and Van de Voorde (1976) also observed this with the action of chlorine on Naegleria cysts. Cursons et al. (1980) also observed this effect with both chlorine and chlorine dioxide on Naegleria spp. The reason for this is not clear, but it is possible that their different temperature optima, 30°C for N.gruberi and 37°C for N.fowleri (Cursons et al., 1976; Griffin, 1972) may be involved. This is because all disinfection work was done at incubation temperatures closer to the temperature optima of N.gruberi than N.fowleri, and as shown in Fig. 4 and Table V, when tested at higher temperature Baquacil has a greater effect on the non-pathogenic than pathogenic species.

Monoxenically grown amoebae had higher survival rates than axenically grown amoebae tested under similar conditions, with all three disinfectants. When both axenically and monoxenically grown amoebae were sterilised, the disinfectant demand was higher for the monoxenically grown amoebae. That this difference occurred with all three disinfectants, suggests a common factor is present. This could be due to the expulsion of waste material, which binds to the disinfectant, and therefore protects the amoebae. It could also be due to the attatchment of bacteria to the outside of the amoebae, causing an extra permeability barrier. This possibility is based on the fact that bacteria attatch to particles in liquid media (Henerkellerr & Heller, 1940; Zabell, 1943), and that the amoebae in this case, are

the particles. The monoxenic feeding of the amoebae could cause a minor chemical or physical change in the cell membrane with a consequential slower diffusion rate, or a slightly altered metabolic process related to nutrition, in which one or more enzymes is modified, and less liable to interference by disinfecting agents. The fact that in the increased exposure time experiments with Baquacil, there was no difference in Baquacil demand or survival rates between monoxenically and axenically grown amoebae, suggests that the disinfectant diffusion rate is effected. This could be caused by any one, or a combination of the above. However it must be emphasized, that although the relative differences appear high and therefore significant, in terms of sterilization, when talking of disinfection, the difference between a 95.0% kill and a 97.5% kill is not as significant.

Thus monoxenically grown amoebae are a more exacting test of the amoebicidal capacity of a disinfectant, than axenically grown amoebae.

Figures 2,3,4 and Tables III, IV, V, show the combined effect of temperature and Baquacil on amoebae in axenic conditions. The results show that at 30°C, the amoebicidal effect seems to be due mainly to the Baquacil while at 25°C and 37°C, the temperature effect was added, so the effect was cummulative. This is not surprising, as environmentally, pathogenic Naegleria are usually isolated at temperatures $>26^{\circ}$C (De Jonckheere et al., 1975; Cursons & Brown, 1976; De Jonckheere & Van de Voorde, 1977; Wellings, 1979; Cerva, 1980; Duma, 1981), and the higher optimum growth temperature of the pathogenic species (Griffin, 1972).

The surviving amoebae from section 4.1.1 (Fig.3 and Table IV), were cloned and tested for Baquacil resistance. The results

shown in Table XXI, indicate that of all the clones tested, only two, N.fowleri ($MsMrb_3$) and ($MsTrb_1$), showed almost total resistance. Two other clones, N.fowleri ($MsMrb_4$) and ($MsMrb_2$) showed some resistance, with survival rates at 57.0 and 30.0% respectively. The presence of N.fowleri ($MsMrb_4$) and ($MsMrb_2$) with different resistance characteristics, indicates that there is genetic and physiological variation within the parent clone. This may be related to the involvement of extra-chromosomal DNA, similar to the drug resistance of bacteria residing in plasmids (Davis & Rownd, 1972). Another suggestion is further study on N.fowleri ($MsMrb_3$) and ($MsTrb_1$), to determine among other factors, the possibility of multiple disinfectant resistance, and environmental competitiveness. One point to note is the difference in Baquacil demands, shown in Table XXI for resistant, partially resistant, and non resistant clones. This implies that with the resistant strains, it is irrelevant how concentrated the initial disinfectant level is, and that the cell membrane structure is altered, so as to prevent entry of Baquacil into the cell, rather than altered structure of internal enzymes, which would show a greater Baquacil demand than was evident.

5.2 Competition Between Naegleria and Tetrahymena for Bacteria

In natural conditions, both Naegleria spp. and T.pyriformis use bacteria as a food source (Curds & Cockburn, 1968, 1971; Elliot, 1970; Anderson & Jamieson, 1974). Eight species of bacteria were used in this study, six of which were common water coliforms, i.e. E.cloacae, E.coli, K.aerogenes, Ps.aeruginosa, Ps.fluorescens, and S.marcescens. These were chosen because attempts have been made to correlate mean coliform numbers to Naegleria spp. numbers (Anderson & Jamieson, 1974; Wellings, 1979; Duma, 1981; Brown et al., 1982). The

other two bacteria used were M.luteus and B.subtilis, both gram positive. M.luteus was chosen for tests because it is one of the more common skin bacteria, and with the number of bathers entering a pool, a reasonable level of this bacterium will eventually be present in the water. It is also a relatively slow growing organism when compared to E.cloacae and E.coli. B.subtilis was chosen because it is also a common water bacterium, although gram positive. It is a very fast growing, swarming organism. This factor, as well as its rapid growth capability, make it a good comparison with Ps.aeruginosa, which is also a fast growing bacterium. It did not support Naegleria growth (Anderson & Jamieson, 1974), although these workers found that heat-killed Ps.aeruginosa were utilized by Naegleria spp.

Four of the eight species were unpigmented, i.e. E.cloacae, K.aerogenes, E.coli, and M.luteus, of the other four, S.marcescens was the most obviously pigmented. It can be seen that the four unpigmented bacteria species were better able to support the growth of Naegleria spp. than the pigmented species (section 4.4.1, Figs. 35 - 42, Tables XXXVIII - XLV). The Naegleria spp. population initally increased, peaked, then decreased, but never to below the level of the initial inoculum with the unpigmented bacteria. With the pigmented bacteria, none of the four species supported the growth of Naegleria spp. for more than 5 days (120 hours). B.subtilis, did however, maintain cyst numbers for the 168 hour incubation period. This bacterium was interesting, in that it was the only one in which Naegleria flagellates were seen. This indicates that with B.subtilis, some growth factor is lacking, such that flagellation is stimulated. These results do not agree with those of Duma (1981), who found that S.marcescens completely failed to support the growth of Naegleria spp. due to its production of pigment, and Anderson and Jamieson (1974), who

found that Ps.aeruginosa was totally unable to support growth of Naegleria spp. and presumed this to be due to its ability for rapid growth in the presence of minimal nutrient. They supported this arguement with the positive growth support results using heat-killed bacteria.

The proportions of organisms used were 100:1 bacteria: amoebae initial inoculum, which as can be seen from the results, caused a balance between amoebae and positive growth supporting bacteria. Duma (1981) found in a bacteriological survey of Virginia lakes, that E.cloacae, E.coli, Pseudomonas spp. and S.marcescens were the predominant gram negative bacilli most often isolated. He also found that when Ps.fluorescens was isolated from a lake, no pathogenic Naegleria spp. were isolated from that lake. He then showed that the pigment produced by S.marcescens was inhibitory to Naegleria growth. The results obtained in this study with the unpigmented bacteria, confirmed the results of Anderson and Jamieson (1974), and Duma (1981) that these bacteria were good food sources for Naegleria spp. With Ps. aeruginosa and B.subtilis, both fast growing pigmented bacteria, bacterial overgrowth of the amoebae occured. After showing the red pigment of S.marcescens to be inhibitory to Naegleria spp., Duma (1981) postulated that Pseudomonas spp. pigments may also be inhibitory to Naegleria spp.

The same eight bacteria species were then examined for growth support of T.pyriformis (section 4.4.2, Figs. 43 & 44, Table XLVI). It was known from the continuous culture work of Curds and Cockburn (1971) that K.aerogenes and E.cloacae were good food sources for T.pyriformis, which was confirmed in this study. Because of its ubiquitous distribution and ability to live in a range of water temperatures (Elliot, 1959, 1970), it is not surprising that the same

bacteria which showed positive growth support for T.pyriformis were the same as for Naegleria spp. However, Ps.aeruginosa was also found to be a good food source for T.pyriformis. From this it can be assumed that the pigment of Ps.aeruginosa was not inhibitory to T.pyriformis. T.pyriformis metabolites may otherwise in some way neutralise the effect of pigment being produced by Ps.aeruginosa. Which ever was the case, it was obviously not as successful with S.marcescens or Ps.fluorescens, with neither able to be regarded as good food sources. The poor growth support of B.subtilis may have been due to the speed of growth rather than, or as well as, any toxic effects of its pigment on T.pyriformis. An observation of Curds and Cockburn (1971) regarding the size of T.pyriformis, was also confirmed in this study. This was, just after inoculation, for the first 24 - 48 hours, the ciliate cells were large in size, but after 48 hours, became smaller. This was probably due to there being a plentiful food supply in the initial hours of incubation, but with the voracious feeding of T.pyriformis, and the relatively slow growth rates of the bacteria in PAS glucose, the number of bacteria is reduced. Because the feeding rate of T.pyriformis is governed by the size of the ciliate population, as well as the bacterial concentration (McCashland & Kronschnabel, 1962; Curds & Cockburn, 1968), a balance between feeding, and ciliate cell division at the most economical cell size must arise (Hadjipetrou et al., 1964; Curds & Cockburn, 1968).

To examine the effect of competition between Naegleria spp. and T.pyriformis for a bacterial food source, three bacteria species were chosen E.coli, E.cloacae, and Ps.fluorescens. The two former were chosen because of their positive growth support for both amoebae and ciliates, shown in this study and in previous work, (Chang, 1971; Curds & Cockburn, 1971; Anderson & Jamieson, 1974; Cursons et al., 1976;

Cerva, 1980). Ps.fluorescens, was chosen because the results of this study in regard to the relationship between this bacteria and Naegleria spp. did not agree with those of Duma (1981). The results (section 4.4.3, Figs. 45 - 47, and Tables XLVII - XLIX) show that all three bacteria species provided effective growth support, and therefore acted as a food source for both Naegleria spp. and T.pyriformis. Figures 45 - 47 show that the populations of both amoebae and ciliates were constant, with only slight oscillations. The bacteria populations in all three bacteria species decreased, and only started to increase again towards the end of the incubation period. The realationship between populations of T.pyriformis and Naegleria spp. was the same when either E.coli or E.cloacae was the was the food source. This was one of slight inhibition of the amoebae, and a significant increase in ciliate population levels when in combination with each other, as against the controls. The effect on amoebae was expected, as the bacterial inoculum was not changed, i.e. 100:1 bacteria: protozoa, in the controls, but because of the presence of both protozoans, the proportion was 50:1 bacteria: amoebae, and bacteria: ciliate. In effect the proportions were halved.

The increase of the ciliate population was possibly caused by the amoebae excreting a growth factor (Seaman, 1961; McCashland & Kronschnabel, 1962; Duscoff et al., 1964; Stillwell, 1976; Seravin & Orlovskaja, 1977) which facilitated more efficient use of the bacteria by the ciliates. Another possibility is that the amoebae excreted a factor, which when combined with secretions from the ciliate, caused minor flocculation of the bacteria (Curds, 1963). The ciliates can then more easily channel these postulated small flocs into their buccal cavity (Seaman, 1961; Duscoff et al., 1964; Sleigh & Aiello, 1972).

The relationship between T.pyriformis and Naegleria spp.

when grown on Ps.fluorescens, is one of interest, and entirely unexpected, after the results obtained by Duma (1981). This was because both T.pyriformis and Naegleria spp., when grown alone on Ps.fluorescens, were not adequately supported by this bacterium for it to be regarded as a good growth support bacterium, but when the two protozoa were grown together on this bacterium, a synergistic relationship was in evidence. The mechanism of this synergism is not known, but assuming toxic secretions from the bacteria are present (Chang, 1971; Anderson & Jamieson, 1974; Duma, 1981), then when grown alone on Ps.fluorescens, both T.pyriformis and Naegleria spp. are inhibited by one or some of these postulated toxins (Elliot, 1959; Chang, 1971; Menapace et al., 1975; Seravin & Orlovskaja, 1977). However, when grown together in a competition situation each of the two protozoa may excrete a factor which neutralises the bacterial toxin on the other protozoan, so that a T.pyriformis factor neutralises the bacterial toxin for the amoebae, and vice versa. More likely, a factor produced by Naegleria spp., combines with a factor produced by T.pyriformis, which then neutralises sufficient bacterial toxin to enable protozoan growth (Kidder, 1941). Another possibility is that the degree of decrease in the bacterial population, caused by feeding on them in the T.pyriformis and Naegleria spp. controls, is not enough to prevent a threshold level of growth inhibiting factors from being attained. However, when both protozoa are in competition with each for Ps.fluorescens, the decreased population level may be such that the threshold level of growth inhibiting factors is not reached. (Kidder, 1941).

When grown on all bacterial species, with the exception of M.luteus, N.gruberi (P1200f) increased in population size to a greater extent than any of N.fowleri (MsM), (MsT), or (Northcott), as

seen in Figs. 35 - 42, which indicates that at 30°C, the non-pathogenic Naegleria spp. may survive better in natural water bodies. Further study in this area should include mixed populations of pathogenic and non-pathogenic amoebae and later, T.pyriformis also, in continuous culture.

Attempts have been made to correlate various environmental factors with the occurrence in nature, of pathogenic N.fowleri (De Jonckheere et al., 1975; Wellings, 1979; Duma, 1981; Brown et al., 1982). One point made clear in all of these reports, and this present study, is that many environmental factors are involved. Although in all of these reports, elevated water temperature is considered important, with a selection pressure for the pathogenic isolates, temperature alone is not enough to increase the number of pathogenic isolates (De Jonckheere et al., 1975; Duma, 1981). Wellings (1979) reported that increased populations or gram negative bacteria, caused by both direct and indirect sources of organic pollution, appeared to enhance the proliferation of pathogenic Naegleria spp., but offerred no conclusive evidence. Brown et al. (1982) in a New Zealand survey, reported that pools frequently contaminated by coliforms, have a higher rate of PFLA isolation. They also reported the isolation of PFLA from soils with high coliform counts. In the Duma (1981) report, water temperature, salinity, pH, dissolved oxygen (D.O), and conductivity of the water, as well as bacterial flora, limnological content, and climatological data were studied in the field. No definite quantitative relationship between bacterial flora and pathogenic Naegleria spp. was established, apart from showing that S.marcescens and Ps.fluorescens were inhibitory to Naegleria spp. One point brought out in this report is the fact that in the thermally enriched sampling sites, where the greatest number of

pathogenic isolations were made, the D.O content was low, but the water contained high concentrations of iron and manganese, as did the bottom soil, although Brown et al.(1982) found PFLA were absent from some thermal pools with high iron concentrations. The significance of this is obscure, but it is known that pathogenic Naegleria spp. consume erythrocyts, which contain iron in haemoglobin, and that the virulence of these organisms can be revived by the addition of erythrocytes to the culture medium (Culbertson, 1971). It is however, also known that high iron concentrations are lethal to many micro-oganisms (Avakyan, 1974; Cairns, et al., 1974; Weinberg, 1978).

It must be noted that most strains of Ps.fluorescens and of T.pyriformis do not grow at temperatures above $30^{o}C$. In the light of the results in this study, this may help to explain the results of Duma (1981), with respect to the inhibitory action of Ps.fluorescens on Naegleria spp., in that the presence or absence of these ciliates was not reported. Also reported by Duma (1981), was that prolonged periods of temperature $> 30^{o}C$ were required before isolations of pathogenic Naegleria spp. occurred. He cites the example of thermal enrichment of a lake, by a nuclear power plant discharge of coolant water, from which pathogenic isolations did not increase for nearly three months after the first discharge. Elevated temperature alone cannot be responsible for pathogenic isolations, as shown by De Jonckheere et al.(1975) where the workers isolated pathogenic Naegleria spp. from a factory effluent discharge, but none were isolated from the adjacent warm water of an electricity power plant, which was at the same temperature as the factory discharge.

There has been little work done on the effect of D.O. on the distribution of Naegleria spp. Duma (1981) reported that D.O. did not appear to be a critical factor in determining the appearance

or not, of pathogenic Naegleria spp. The finding that N.fowleri requires less oxygen than N.gruberi (Weik & John, 1977 a & b) correlates well with the inversely proportional relationship between D.O. and increased water temperature. McCashland and Kroschnabel (1962) reported that protozoan respiration varied with nutrient availability, and not D.O.

Another point to consider is the demand on the D.O. of any form of organic pollutants. This may cause an increased concentration of gram-negative bacilli (Wellings, 1979), which, as shown in this study, may support the growth of Naegleria spp. The effect of D.O. is the least known and understood area of the environmental factors determining the distribution of pathogenic Naegleria spp. in nature, and with further work in this area, e.g. the use of artificially polluted water, by BFM solution, in continuous culture competition using N.fowleri, N.gruberi, and E.coli, both with and without oxygen enrichment, would be profitable. The effect of organic discharges on D.O. levels and the consequential effects on the bacterial flora of that area must be studied, in order for more efficient control measures to be devised. This is particularly important when considering that N.fowleri uses less oxygen than N.gruberi (Weik & John, 1977 a & b) and the reported discovery by Wellings (1979) that Naegleria cysts can survive in the anaerobic soil of lake bottoms.

BIBLIOGRAPHY

ADAMS, A.C., JOHN, D.T. & BRADLEY, S.D. 1976.
Modification of resistance of mice to Naegleria fowleri infections.
Infect. Immun., 13: 1387 - 1391

AKINS, K. & JAMIESON, A. 1972.
Differentiation promoting factors induced in Acanthamoeba by inhibitors of mitochondrial macromolecule synthesis.
Developmental Biology, 78: 126 - 140

ANDERSON, K. & JAMIESON, A. 1972
Agglutination test for the investigation of the genus Naegleria.
Pathology, 4: 273 - 278

ANDERSON, K., JAMIESON, A., JADIN, F.B., & WILLAERT, E. 1973.
Primary amoebic meningoencephalitis.
Lancet, i: 672

ANDERSON, K. & JAMIESON, A. 1974.
Bacterial suspensions for the growth of Naegleria species.
Pathology, 6: 79 - 84

AVAKYAN, Z.A. 1974.
The toxicity of heavy metals for microorganisms.
P. 3 - 28. In Smirnova S.S. (ed). Microbiology, vol II.
G.K. Hall, Boston. 73p.

BAND, R.N. & BALAMUTH, W. 1974.
Hemin replaces serum as a growth requirement for Naegleria.
Appl. Microbiol., 28: 64 - 65

BARRET, R.A. & ALEXANDER, M. 1977.
Resistance of cysts of amoebae to microbial decomposition.
Appl. Environ. Microbiol., 33: 670 - 674

BOROCHOVIT, E.D., MARTINEZ, A.J. & PATTERSON, G.T. 1981.
Osteomyelitis of a bone graft of the mandible with Acanthamoeba castellanii infection.
Human Pathol., 12: 573 - 576

BOS, H.J., VOLKER-DIEBEN, H.J.M. & KOK-VAN ALPHEN, C.C. 1981.
A case of Acanthamoeba keratitis in the Netherlands.
Trans. Roy. Soc. Trop. Med. Hyg., 75: 86

BROWN, T. 1979.
Observations by immunofluoresence microscopy and electron microscopy on the cytopathogenicity of Naegleria fowleri in mouse embryo-cell cultures.
J. Med. Microbiol., 12: 363 - 371

BROWN, T.J., & CURSONS, R.T.M. 1977.
Pathogenic free-living amoebae (PFLA) from frozen swimming areas in Oslo, Norway.
Scand. J. Infect. Dis., 9: 237 - 240

BROWN, T.J., CURSONS, R.T.M., KEYS, E.A., MARKS, M. & MILES, M. 1982.
The occurrence and distribution of Pathogenic Free-Living Amoebae (PFLA) in thermal areas of the North Island of New Zealand.
Appl. Environ. Microbiol. (In Press)

BUTT, C.G., BARD, C. & KNORR, R.W. 1968.
Naegleria spp. in amoebic encephalitis.
Am. J. Clin. Path., 50: 568 - 574

BYERS, T.J. 1979.
Growth, reproduction & differentiation in Acanthamoeba.
Int. Rev. Cytol., 61: 283 - 337

BYERS, T.J., AKINS, R.A., MAYNARD, B.J., LEFKEN, R.A. & MARTIN, S.C. 1980.
Rapid growth of Acanthamoeba in defined media; induction of encystment by glucose - acetate starvation.
J. Protozool., 27: 216 - 219

CAIN, A.R.R., WIKEY, P.F., BROWNELL, B. & WARHURST, D.C. 1981.
Primary Amoebic Meningoencephalitis.
Arch. Dis. Child., 56: 140 - 144

CAIRNS, J., HEATH, A.G. & PARKER, B.C. 1975.
The effects of temperature on the toxicity of chemicals to aquatic organisms.
Hydrobiologia 47: 135 - 171

CALLICOTT, J.H. 1968.
Amebic meningoencephalitis due to free-living amebas of the Hartmanella (Acanthamoeba) - Naegleria group.
Am. J. Clin. Path., 49: 84 - 91

CALLICOTT, J.H., JONES, M.M., NELSON, E.C.C., DOS SANTOS, J.G., UTZ, J.P., DUMA, R.J. & MORRIS, J.V. 1968.
Meningoencephalitis due to pathogenic free living ameba.
J. Amer. Med. Assoc., 206: 579 - 582

CARTER, R.F. 1968.
Primary amoebic meningoencephalitis: clinical, pathological, and epidemiological features of six fatal cases.
J. Path. Bact., 96: 1 - 27

CARTER, R.F. 1969.
Sensitivity to Amphotericin B of a Naegleria spp. isolated from a case of primary amoebic meningo-encphalitis.
J. Clin. Path., 22: 470 - 474

CARTER, R.F. 1970.
Description of a Naegleria spp. isolated from two cases of primary amoebic meningo-encephalitis and of the experimental pathological changes induced by it.
J. Pathol., 100: 217 - 244

CARTER, R.F. 1972.
Primary amoebic meningoencephalitis. An appraisal of present knowledge.
Trans. R. Soc. Trop. Med. Hyg., 66: 193 - 213

CASEMORE, D.P. 1977.
Free-living amoebae in a home dialysis unit.
Lancet, ii: 1078

CERVA, L. 1970.
Growth of the pathogenic Al strain of Acanthamoeba castellanii in the chick embryo.
Folia. Parasit. (Praha), 17: 315 - 318

CERVA, L. 1971.
Studies of limax amoebae in a swimming pool.
Hydrobiologia, 38: 141 - 161

CERVA, L. 1977.
The influence of temperature on the growth of Naegleria fowleri and N.gruberi in axenic culture.
Folia. Parasit. (Praha), 24: 221 - 228

CERVA, L. 1980.
Laboratory diagnosis of primary amoebic meningoencephalitis and methods for the detection of limax amoebae in the environment.
Folia. Parasit. (Praha), 27: 1 - 9

CERVA, L. & KRAMAR, J. 1973.
Antigenic relationships among several limax amoebae isolates assessed with the indirect fluorescent antibody test (IFAT).
Folia. Parasit. (Praha), 20: 113 - 118

CERVA, L., SERBUS, C. & SKOCIL, V. 1973.
Isolation of limax amoebae from the nasal mucosa of man.
Folia. Parasit. (Praha), 20: 97 - 103

CERVA, L. & HULDT, G. 1974.
Limax amoeba in five swimming pools in Stockholm.
Folia. Parasit. (Praha), 21: 71 - 75

CHAGLA, A.H. & GRIFFITHS, A.J. 1974.
Growth and encystation of Acanthamoeba castellanii.
J. Gen. Microbiol. 84: 139 - 145

CHANG, S.L. 1971.
Small free-living amoebas: cultivation, quantitation, identification, classification, pathogenesis and resistance. p 201 - 254.
In Chang, T.C. ed. Topics in Comparative Pathobiology vol. I.
Academic Press, New York. 314p.

CHANG, S.L. 1972.
Pathogenic free-living amoebae and recreational waters. Presented at the 6th conference of the international water pollution research association, Jerusalem, Israel. June 18 - 24.

CHANG, S.L. 1974.
Etiological, pathological, epidemiological and diagnostical considerations of primary amoebic meningoencephalitis.
Crit. Rev. Microbiol., 3: 135 - 159

CHANG, S.L. 1975.
Resistance of pathogenic Naegleria to some common physical and chemical agents.
Appl. Environ. Microbiol., 35: 365 - 375

CHANG, S.L. 1976.
Pathogenesis of pathogenic Naegleria amoeba.
From Joint Meeting of Am. Soc. Trop. Med. Hyg. and Trans. R. Trop. Med. Hyg., 3 - 5 Nov.

CHANG, S.L., HEALY, S.R., McCABE, L., SHUMAKER, J.B. & SCHULTZ, M.G. 1975.
A strain of pathogenic Naegleria isolated from a human nasal swab.
Health Lab. Sci., 12: 1 - 7

CAIRNS, J. 1979.
Biological indicators of water quality.
James, A. & Evison, L. ed. John Wiley & sons, Chichester.

COTTER, D.A. 1973.
Human meningoencephalitis caused by the free-living amoebae.
Am. J. Med. Tech., 39: 417 - 420

CULBERTSON, C.G., SMITH, J.W. & MINNER, J.R. 1958.
Acanthamoeba: observations on animal pathogenicity.
Science, 127: 1506

CULBERTSON, C.G., SMITH, J.W., COHEN, H.K. & MINNER, J.K. 1959.
Experimental infection of mice and monkeys by Acanthamoeba.
Am. J. Path., 35: 185 - 197

CULBERTSON, C.G. 1961.
Pathogenic Acanthamoeba (Hartmanella).
Am. J. Clin. Path., 35: 195 - 202

CULBERTSON, C.G. 1971.
The pathogenicity of soil amoebas.
Ann. Rev. Microbiol., 25: 231 - 254

CULBERTSON, C.G. 1975.
Soil ameba infection. Specific indirect immunoenzymatic (peroxidase) staining of formalin fixed paraffin sections.
Am. J. Clin. Path., 63: 475 - 482

CULBERTSON, C.G. 1981.
Amoebic meningoencephalitis.
Antibiotics and Chemotherapy, 30: 28 - 53

CULBERTSON, C.G., ENSMINGER, P.W. & OVERTON, W.M. 1965.
The isolation of additional strains of pathogenic Hartmanella spp. (Acanthamoeba spp.): Proposed culture method for application to biological material.
Am. J. Clin. Path. 43: 361 - 364

CULBERTSON, C.G., HOLMES, D.H. & OVERTON, W.M. 1965.
Hartmanella castellanii (Acanthamoeba spp.). Preliminary report on experimental chemotherapy.
Am. J. Clin. Path., 43: 361 - 364

CURDS, C.R. 1963.
The flocculation of suspended matter by Paramecium caudatum.
J. Gen. Microbiol., 33: 357 - 363

CURDS, C.R. & COCKBURN, A. 1968.
Studies on the growth and feeding of Tetrahymena pyriformis in axenic and monoxenic cultures.
J. Gen. Microbiol., 54: 343 - 358

CURDS, C.R. & COCKBURN, A. 1971.
Continuous monoxenic culture of Tetrahymena pyriformis.
J. Gen. Microbiol., 66: 45 - 108

CURSONS, R.T.M. 1974.
Classification and identification of the aetiological agents of primary amoebic meningoencephalitis, together with preliminary investigations of public health measures.
Thesis, MSc., Massey Univ. 107p.

CURSONS, R.T.M. 1978.
Pathogenic free-living amebae - occurrence in New Zealand thermal regions together with investigations into their disinfection, immunity, and virulence.
Thesis PhD. Massey Univ. 207p.

CURSONS, R.T.M. & BROWN, T.J. 1975.
The 1968 cases of primary amoebic meningoencephalitis Myxomycete or Naegleria?
N.Z. Med. J., 82: 123 - 125

CURSONS, R.T.M. & BROWN, T.J. 1976.
Identification and classification of the aetiological agents of primary amebic meningo-encephalitis.
N.Z. J. Mar. Freshwater Res., 10: 245 - 262

CURSONS, R.T.M. & BROWN, T.J. 1978.
Use of cell cultures as an indicator of pathogenicity of free living amoebae.
J. Clin. Path., 31: 1 - 11

CURSONS, R.T.M., BROWN, T.J. & CULBERTSON, C.G. 1976.
Immunoperoxidase staining of trophozoites in primary amebic meningoencephalitis.
Lancet, ii: 479

CURSON, R.T.M., BROWN, T.J. & KEYS, E.A. 1976.
Primary amebic meningo-encephalitis contracted in a thermal tributary of the Waikato River - Taupo: A case report.
N.Z. Med. J., 84: 479 - 481

CURSONS, R.T.M., BROWN, T.J. & KEYS, E.A. 1977.
Immunity to pathogenic free-living amebae.
Lancet, ii: 875

CURSONS, R.T.M., BROWN, T.J. & KEYS, E.A. 1978a.
Diagnosis and identification of the aetiological agents of primary amebic meningoencephalitis (PAM).
N.Z. J. Med. Lab. Tech., 32: 11 - 14

CURSONS, R.T.M., BROWN, T.J. & KEYS, E.A. 1978b.
Primary amebic meningo-encephalitis (PAM) in New Zealand, - aetiological agents, distribution, occurrence and control.
p. 96 - 110. In Proc. 9th N.Z. Biotech. Conf. 1977.

CURSONS, R.T.M., BROWN, T.J. & KEYS, E.A. 1978c.
Virulence of pathogenic free-living amebae.
J. Parasit., 64: 744 - 745

CURSONS, R.T.M., BROWN, T.J. & KEYS, E.A. 1979.
Primary amebic meningo-encephalitis in an indoor heat exchange swimming pool.
N.Z. Med. J., 89: 330 - 331

CURSONS, R.T.M., BROWN, T.J. & KEYS, E.A. 1980.
Effect of disinfectants on pathogenic free-living amoebae: in axenic conditions.
Appl. Environ. Microbiol., 40: 62 - 66

CURSONS, R.T.M., BROWN, T.J., KEYS, E.A., MORIARTY, K.M. & TILL, D. 1980.
Immunity to pathogenic free-living amoebae: role of humoral antibody.
Infect. Immun., 29: 401 - 407

CURSONS, R.T.M., BROWN, T.J., KEYS, E.A., MORIARTY, K.M. & TILL, D. 1980a.
Immunity to pathogenic free-living amoebae: role of cell mediated immunity.
Infect. Immun., 29: 408 - 410

CURSONS, R.T.M., BROWN, T.J., KEYS, E.A., LEARMONTH, J., CAMPBELL, C. & METCALF, P. 1979.
IgA and primary amebic meningoencephalitis.
Lancet i: 223 - 224

CURSONS , R.T.M., DONALD, J.J., BROWN, T.J. & KEYS, E.A. 1979.
Cultivation of pathogenic and non-pathogenic free-living amoebae.
J. Parasit., 65: 189 - 191

CANES, L. & CERVA, L. 1981.
Survival of polioviruses and echoviruses in Acanthamoeba castellanii cultivated in vitro.
J. Hyg. Epidemiol. Microbiol. Imm., 25: 169 - 174

DARBY, C.P., CONRADI, S.E., HOLBROOK, T.W. & CHATELLIER, C. 1979.
Primary amebic meningoencephalitis.
Am. J. Dis. Children, 133: 1025 - 1077

DAVIES, J.F. & ROWND, R. 1972.
Transmissible multiple drug resistance in Enterobacteriaceae.
Science, 176: 758 - 768

DAWSON, M.W., BROWN, T.J. & TILL, D. 1982.
The effect of Baquacil on pathogenic free-living amoebae in axenic conditions.
Appl. Environ. Microbiol. (In Press.)

DE JONCKHEERE, J. 1977.
Use of an axenic medium for differentiation between pathogenic and non-pathogenic Naegleria fowleri isolates.
Appl. Environ. Microbiol., 33: 751 - 757

DE JONCKHEERE, J. 1978.
Quantitative study of Naegleria fowleri in surface water.
Protistologica, XIV: 475 - 481

DE JONCKHEERE, J. 1979.
Pathogenic free living amoebae in swimming pools : survey in Belgium.
Ann. Microbiol. (Inst. Pasteur), 130: 205 - 212

DE JONCKHEERE, J. 1979a.
Occurrence of Naegleria and Acanthamoeba in Aquaria.
Appl. Environ. Microbiol., 38: 590 - 593

DE JONCKHEERE, J. 1979b.
Studies on pathogenic free-living amoebae in swimming pools.
Bulletin De L'Institut Pasteur, 77: 385 - 392

DE JONCKHEERE, J. 1981.
Pathogenic and non-pathogenic Acanthamoeba spp. in thermally polluted discharges and surface waters.
J. Protozool., 28: 56 - 58

DE JONCKHEERE, J. & VAN DE VOORDE, J. 1976.
Differences in destruction of cysts of pathogenic and non-pathogenic Naegleria and Acanthamoeba by chlorine.
Appl. Environ. Microbiol., 31: 294 - 297

DE JONCKHEERE, J. & VAN DE VOORDE, H. 1977.
The distribution of Naegleria fowleri in man - made thermal waters.
Am. J. Trop. Med. Hyg., 26: 10 - 15

DE JONCKHEERE, J. & VAN DE VOORDE, J. 1977a.
Comparative study of six strains of Naegleria with special reference to non-pathogenic variants of Naegleria fowleri.
J. Protozool., 24: 304 - 309

DE JONCKHEERE, J., VAN DIJCK, P. & VAN DE VOORDE, H. 1974.
Evaluation of the indirect fluorescent - antibody technique for identification of Naegleria species.
Appl. Microbiol., 28: 159 - 164

DE JONCKHEERE, J., VAN DIJCK, P. & VAN DE VOORDE, H. 1975.
The effect of thermal pollution on the distribution of Naegleria fowleri
J. Hyg., 75: 7 - 15

DEMPE, S. & JANITSCHKE, K. 1981.
Naegleria fowleri - subacute and chronic meningoencephalitis in mice - intranasal inoculation experiments with a cloned substrain of low virulence.
Tropenmed. Parasitol., 32: 206

DERREUMAUX, A.L., JADIN, J.B., WILLEART, S. & MORET, R. 1974.
Action du chlore sur les amibes de l'eau.
Ann. Soc. Belge. Med. Trop., 54: 415 - 428

DESMET-PAIX, L. 1974.
Rescherche systematique d'amibes dans les eaux de piscines et les eaux minerales.
Ann. Soc. Belge Med. Trop., 54: 409 - 414

DIFFLEY, P., SKEELS, M.R. & SOGANDARES-BERNAL, F. 1976.
Delayed type hypersensitivity in guinea pigs infected subcutaneously with Naegleria fowleri (Carter).
Z. Parasitkde., 49: 133 - 137

DIVE, D.G., LECLERC, H., DE JONCKHEERE, J. & DELATTRE, J.M. 1981.
Isolation of Naegleria fowleri from the cooling pond of an electric power plant in France.
Ann. Microbiol., 132: 97

DOBRA, K.W., McARDLE, E.W. & EHRET, C.W. 1980.
Growth kinetics of three species of Tetrahymena on solid agar.
J. Protozool., 27: 226 - 230

DONALD, J.J. 1979.
The susceptibility of pathogenic free-living amebae to chemotherapeutic agents.
Thesis, MSc., Massey Univ. 149p.

DONALD, J.J., KEYS, E.A., CURSONS, R.T.M. & BROWN, T.J. 1979.
Chemotherapy of primary amebic meningoencephalitis (PAM).
N.Z. J. Med. Lab. Tech., 33: 23 - 26

DOS SANTOS, J.G. 1970.
Fatal primary amebic meningo-encephalitis: a retrospective study in Richmond, Virginia.
Am. J. Clin. Path., 54: 737 - 742

DUMA, R.J. 1978.
Amoebic infections of the nervous system. In Handbook of Clinical Neurology, 35: 25 - 65. Vinken, P.J., & Bruyn, G.W. (ed.)

DUMA, R.J. 1980.
Free-living amebic meningoencephalitis.
Trends in Neurosciences April, 78 - 80

DUMA, R.J. 1981.
Study of pathogenic free-living amebas in freshwater lakes in Virginia. Technical Rep. EPA - 600/S1 - 80 - 037, Health Effects Laboratory, Office of Research and Development, United States Environmental Protection Agency, Cincinati, Ohio. Feb. 1981. 131p.

DUMA, R.J., ROSENBLUM, W.I., MCGEHE, R.F., JONES, M.M. & NELSON, E.C. 1971.
Primary amoebic meningoencephalitis caused by *Naegleria*. Two new cases, response to Amphotericin B and a review.
Ann. Int. Med., *74*: 861 - 869

DUMA, R.J. & FINLEY, H. 1976.
In vitro susceptibility of pathogenic *Naegleria* and *Acanthamoeba* spp. to a variety of chemotherapeutic agents.
Antimicrob. Ag. Chemother., *10*: 370 - 376

DUMA, R.J., HELWIG, W.B. & MARTINEZ, A.J. 1978.
Meningo-encephalitis and brain abscess due to a free living ameba.
Ann. Intern. Med., *88*: 468 - 473

DUSCOFF, H.S., SISTER M. WILLIAMS, SYN, T.B. & BUTLER, B.D. 1964.
Quantitative studies of metabolism in phagotrophic ciliates. I. Growth of *Tetrahymena pyriformis* W on viable and killed bacteria.
J. Protozool. *11*: 309 - 313

EDWARDS, J.H., GRIFFITHS, A.J. & MULLINS, J. 1978.
Protozoa as sources of Antigen in "humidifier fever".
Nature, *264*: 438 - 439

ELLIOTT, A.M. 1959.
Biology of *Tetrahymena*.
Ann. Rev. Microbiol. *13*: 79 - 96

ELLIOTT, A.M. 1970.
The distribution of *Tetrahymena pyriformis*.
J. Protozool., *17*: 162 - 168

ELMSLY, C.J. 1980.
The *in vitro* and *in vivo* testing of chemotherapeutic agents against pathogenic free-living amebae.
Thesis, MSc., Massey Univ. 151p.

ELMSLY, C.J., DONALD, J.J., BROWN, T.J. & KEYS, E.A. 1980.
Chemotherapy of primary amebic meningo-encephalitis (PAM) II. Miconazole and R41,400 (Ketoconazole).
N.Z. J. Med. Lab. Tech., *34*: 37 - 33

ENG, R.J.K. & SELIGMAN, S.J. 1981.
Lumbar puncture induced meningitis.
J. Amer. Med. Assn., *245*: 1456

FOWLER, M. & CARTER, R.F. 1965.
Acute pyogenic meningitis probably due to *Acanthamoeba* spp.: A preliminary report.
Brit. Med. J., *2*: 740 - 742

FRANK, W. 1974.
Limax amoebae from cold - blooded vertebrates.
Ann. Soc. Belge. Med. Trop., 54: 343 - 349

FULTON, C. 1970.
Amebo - flagellates as research partners. In Methods in Cell Biology IV. p 341 - 476. Prescott D.M. (ed.). Academic Press, New York. 514p.

FULTON, C. 1974.
Axenic culture of Naegleria gruberi.
Exp. Cell. Res., 88: 365 - 370

GLASTONBURY, J.R.W. & FRAUENFELDER, A.R. 1981.
Granulomatous meningoencephalomyelitis in a dog.
Aust. Vet. J., 54: 186 - 189

GREEN, D.E. & STUMPF, P.K. 1946.
The mode of action of chlorine.
J. Am. Wat. Wks. Ass., 38: 1301 - 1305

GRIFFIN, J.L. 1972
Temperature tolerance of pathogenic and non-pathogenic free living amebas.
Science, 178: 869 - 870

GRIFFIN, J.L. 1976.
Acanthamoeba meningo-encephalitis in Britain.
Brit. Med. J., 17: 153

GRUNDY, R. & BLOWERS, R. 1970.
A case of primary amoebic meningo-encephalitis treated with chloroquine.
E. African Med. J., 47: 153 - 158

GRUNNET, L., CANNON, G.H. & KUSHNER, J.P. 1981.
Fulminant amebic meningoencephalitis due to Acanthamoeba.
Neurology, 31: 177 - 179

HADJIPETROU, L.P., GERRITS, J.P., TEULINGS, F.A.G. & STOUTHAMER, A.J. 1964.
Relation between energy production and growth of Aerobacter aerogenes.
J. Gen. Microbiol. 36: 139 - 150

HAGGERTY, R.M. & JOHN, D.T. 1978.
Innate resistance of mice to experimental infection with Naegleri fowleri.
Infect. Immun., 20: 73 - 77

HAGGERTY, R.M. & JOHN, D.T. 1980.
Factors affecting the virulence of Naegleria fowleri for mice.
Proc. Helminthol. Soc. Wash., 47: 129 - 134

HARRIS, J.O. 1967.
Non adaptive growth responses of Tetrahymena pyriformis grown on single bacteria species.
J. Protozool. 14: 600 - 602

HECHT, R.J. & COHEN, A.J. 1972.
Primary amebic meningoencephalitis in California.
Calif. Med., 117: 69 - 73

HOFFLER, A.S. & RUBEL, L.R. 1974.
Free living amoebae identified by cytological examination of gastrointestinal washings.
Acta Cytol., 18: 59 - 61

HOFFMAN, E.D., GARCIA, C., LUNSETH, J., McGARRY, P. & COOVER, J. 1978.
A case of primary amoebic meningoencephalitis, light and electron microscopy, and immunohistological studies.
Am. J. Trop. Med. Hyg. 27: 29 - 38

HOLBROOK, T.W. & PARKER, B.W. 1979.
Naegleria fowleri in chick embryos: Effects of embryo age and incubation temperature, and the infectivity of embryo - derived amoebae for mice.
Am. J. Trop. Med. Hyg., 28: 984 - 987

JAGER, B.V. & STAMM, W.P. 1972.
Brain abscesses caused by a free-living amebae probably of the genus Hartmanella in a patient with Hodgkins disease.
Lancet, ii: 1343 - 1345

JAMIESON, A. & ANDERSON, K. 1973.
A method for the isolation of Naegleria species from water samples.
Path., 5: 55 - 58

JEHAN, M., DUTTA, G.P., KAUSHAL, D.C. & SHUKLA, O.P.1975.
Effect of bacterial and fungal extracts and metabolic inhibitors on excystment of soil amoebae.
Ind. J. Exp. Biol., 13: 375 - 377

JOHN, D.T., WEIK, R.R. & ADAMS, A.C. 1977.
Immunisation of mice against Naegleria fowleri infection.
Infect. Immun., 16: 817 - 820

JONES, D.B., VISVESVARA, G.S. & ROBINSON, N.M. 1975.
Acanthamoeba polyphaga keratitis, and Acanthamoeba uveitis associated with amebic meningo-encephalitis.
Trans. Opthal. Soc. U.K., 95: 221 - 231

JOSEPHSON, S.L., WEIK, R.R. & JOHN, D.T. 1977.
Concanavalin A - induced agglutination of Naegleria.
Am. J. Trop. Med. Hyg., 26: 856 - 858

KADLEC, V. 1975.
The effects of some factors on the growth and morphology of Naegleria spp. and three strains of genus Acanthamoeba.
Folia Parasitol. (Praha), 22: 317 - 321

KADLEC, V., CERVA, L, & SKVAROVA, J. 1978.
Virulent Naegleria fowleri in an indoor swimming pool.
Science, 201: 1025

KADLEC, V., SKVAROVA, J., CERVA, L. & NEBAZNIVA, D. 1980.
Virulent Naegleria fowleri in an indoor swimming pool.
Folia Parasitol. (Praha), 27: 11 - 17

KASPRZAK, W. & MAZUR, T. 1972.
Free living amoeba isolated from waters frequented by the people in the vicinity of Poznar, Poland. Experimental studies in mice on the pathogenicity of the isolates.
Z. Tropenmed. Parasit., 23: 391 - 398

KASPRZAK, W., MAZUR, T. & RUCKA, A. 1974.
Studies on some pathogenic strains of free living amoebae isolated from lakes in Poland.
Ann. Soc. Belge. Med. Trop. 54: 351 - 357

KENNY, M. 1971.
The micro - Kolmer complement fixation test in routine screening for soil ameba infection.
Health Lab. Sci., 8: 5 - 10

KERNOHAN, J.W., MAGATH, T.B. & SCHLOSS, G.T. 1960.
Granuloma of the brain probably due to Endolimax williamsi (Iodamoeba butschlii).
Arch. Path., 70: 576 - 580

KIDDER, G.W. 1941.
Growth studies on ciliates. V. The acceleration and inhibition of ciliate growth in biologically conditioned medium.
Physiol. Zool., 14: 209 - 226

KINGSTON, D. & WARHURST, D.C. 1969.
Isolation of amoebae from the air.
J. Med. Microbiol., 2: 27 - 36

KNOX, W.E., STUMPF, P.D., GREEN, D.E. & AVERBACK, V.J. 1948.
The inhibition of sulfhydryl enzymes as a basis of the bactericidal action of chlorine.
J. Bact., 55: 451 - 458

KOCH WEIK, S.M., WEIK, R.R. & JOHN, D.T. 1980.
Inoculum size and pH affecting growth of Naegleria
Proc. Helminthol. Soc. Wash., 47: 270 - 272

LASTOVICA, A.J. 1974.
Scanning electron microscopy of pathogenic and non pathogenic Naegleria cysts.
Int. J. Parasit., 4: 139 - 142

LAWANDE, R.V., ABRAHAM, S.M., JOHN, I.S. & EGLER, L.J. 1979.
Recovery of soil amoebas from nasal passages of children during the dusty Harmattan Period in Zaria.
Am. J. Clin. Path., 71: 201 - 203

LEWIS, E.J. & SAWYER, T.K. 1979.
Acanthamoeba tubiashi n. sp., a new species of fresh water Amoebida (Acanthamoebidae).
Trans. Amer. Microbiol. Soc., 98: 543 - 549

LYONS, T.B. & KAPUR, R. 1977.
Limax amoebae in public swimming pools of Albany, Schenectady, and Rensselaer counties, New York: Their concentration, correlations, and significance.
Appl. Environ. Microbiol., 33: 551 - 555

MA, P., WILLAERT, E., JEUCHTER, K.B. & STEVENS, A.R. 1981.
A case of keratitis due to Acanthamoeba in New York, New York, and features of 10 cases.
J. Infect. Dis., 143: 662 - 667

MANDAL, B.M., GUDEX, D.J., FITCHETT, M.R., PULLON, D.J., WALLAH, J.A. DAVID, C.M. & APTHORP, J. 1970.
Amoebic meningo-encephalitis due to amoebae of the order Myxomycetales (slime mould).
N.Z. Med. J. 71: 16 - 23

MARINO, J.T.M.D. 1975.
Amplification of primary amoebic meningo-encephalitis.
J. Ped., 86: 160 - 161

MARKOWITZ, S.M., SOBIESKI, T., MARTINEZ, A.J. & DUMA, R.J. 1978.
Experimental Acanthamoeba infections in mice pretreated with Methylpredmisolone or tetracycline.
Am. J. Pathol., 92: 733 - 744

MARTINEZ, A.J. 1979.
Free living amoebas in human infections. Symposium on Clinical Parasitology, Philadelphia, Pennsylvania, Nov. 1979.

MARTINEZ, A.J. 1980.
Is Acanthamoeba encephalitis an opportunistic infection?
Neurology, 30: 567 - 574

MARTINEZ, A.J., DUMA, R.J., NELSON, E.C. & MORETTA, F.L. 1973
Experimental Naegleria meningoencephalitis in mice: Penetration of the olfactory mucosal epithelium by Naegleria and the pathologic changes produced: a light and electron microscope study.
Lab. Invest., 25: 121 - 133

MARTINEZ, A.J., DE JONCKHEERE, J.F. 1981.
Infections due to free - living amoebae.
Bulletin de L'Institut Pasteur, 79: 171

MARTINEZ, A.J., MARKOWITZ, S.M. & KUMA, R.J. 1975.
Experimental pneumonitis and encephalitis caused by Acanthamoeba in mice: pathogenesis and ultrastructural features.
J. Infect. Dis., 131: 692 - 699

MARTINEZ, A.J., SOTELO-AVILA, C., GARCIA-TAMAYO, J., MORON, J.T., WILLAERT, E. & STAMM, W.P. 1977.
Meningoencephalitis due to Acanthamoeba spp. pathogenesis and clinico-pathological study.
Acta Neuropath. (Berl.) 37: 183 - 191

MARTINEZ, A.J., SOTELO-AVILA, C., ALCAL, H. & WILLAERT, E. 1980.
Granulomatous encephalitis , intracranial arteritis, and mycotic aneurism due to a free-living ameba.
Acta. Neuropath. (Berl.), 49: 7 - 12

MASCARO, C., FLUVIA, C., OSUNA, A. & GUEVARA, D. 1981.
Virulent Naegleria spp. isolated from a river in Cadiz (Spain).
J. Parasitol., 67: 599

McCASHLAND, B.W. & KRONSCHNABEL, J.M. 1962.
Exogenous factors affecting respiration in Tetrahymena pyriformis.
J. Protozool. 9: 276 - 279

MENAPACE, D., KLEIN, D.A., McCLELLAN, J.F. & MAYEUX, J.V. 1975.
A simplified overlay plaque technique for evaluating responses of small free-living amoebae in grassland soils.
J. Protozool. 22: 405 - 410

M.R.C. SYMPOSIUM. 1977
Humidifier fever.
Thorax, 32: 653 - 663

NAGINGTON, J., WATSON, P.G., PLAYFAIR, T.J., McGILL, J., JONES, B.R. & STEELE, A.D. McG. 1974.
Amoebic infection of the eye.
Lancet ii: 1537 - 1540

NAGINGTON, J. & RICHARDS, J.R. 1976.
Chemotherapeutic compounds and Acanthamoeba from eye infections.
J. Clin. Path. 29: 648 - 651

NAPOLITANO, J.J. 1981.
Opportunistic relationships among Acanthamoeba spp. in a beach sand environment.
Trans. Amer. Microbiol. Soc., 100: 101

NAPOLITANO, J.J., LAVERDE, A.Y. & GAMBLE, H.R. 1977.
Cultivation of Naegleria using alcohol killed bacteria.
Acta Protozool., 16: 207 - 217

NERAD, T.A. & DAGGETT, P.M. 1979.
Starch gel electrophoresis: an effective method for separation of pathogenic and non-pathogenic Naegleria strains.
J. Protozool., 26: 613 - 615

O'DELL, W.D. 1979.
Isolation, enumeration, and identification of amoeba from a Nebraska lake.
J. Protozool., 26: 265 - 269

O'DELL, W.D. & BRENT, M.M. 1974.
Nutritional study of three strains of Naegleria gruberi.
J. Protozool. 21: 129 - 133

O'DELL, W.D. & STEVENS, A.R., 1973.
Quantitative growth of Naegleria in axenic culture.
Appl. Microbiol., 25: 621 - 627

PAGE, F.C. 1967
Taxonomic criteria for limax amebae with descriptions of three new species of Hartmannella and three of Vahlkampfia.
J. Protozool., 14: 499 - 521

PALIN, A.T. 1974.
Analytical control of water disinfection with special reference to differential DPD methods for chlorine, chlorine dioxide, bromine, iodine, ozone.
J. Instn. Wat. Engrs., 28: 139 - 154

PAN, N.R. & GHOSH, T.N. 1971.
Primary amoebic meningo-encephalitis in two Indian children.
J. Indian Med. Ass., 56: 134 - 137

PARMER, L.G. & COTTRILL, C.W. 1949.
Distribution of emetine in tissues.
J. Lab. Clin. Med., 34: 818 - 821

PATRAS, D. & ANDUJAR, J.J. 1966.
Meningo-encephalitis due to Hartmannella (Acanthamoeba).
Am. J. Clin. Path., 46: 226 - 233

PFAFFMAN, M.A. & KLEIN, J. 1966.
Effects of amoebicides on growth of Acanthamoeba spp.
Proc. Soc. Exp. Biol. Med., 121: 539 - 554

PHILLIPS, B.P. 1974.
Naegleria: Another pathogenic amoeba study in germ free guinea pigs.
Am. J. Trop. Med. Hyg., 23: 850 - 855

PIGON, A. 1976.
Cell surface and medium conditioning in Acanthamoeba culture.
Cytobiologie, 13: 107 - 117

RAI, R.D. & MOHAN RAO, V.K. 1978.
Changes in the composition of mouse brain due to experimental amoebic meningoencephalitis.
Ind. J. Exp. Biol., 16: 903 - 905

RINGSTEAD, J., VALJAGER, B., SUK, D. & VISVESVARA, G.S. 1975.
Probable Acanthamoeba meningo-encephalitis in a Korean child.
Am. J. Clin. Path., 66: 723 - 730

ROBERT, V.B. & RORKE, L.B. 1973.
Primary amebic encephalitis, probably from Acanthamoeba.
Ann. Intern. Med., 72: 174 - 179

ROWBOTTOM, T.J. 1980.
Preliminary report on the pathogenicity of Legionella pneumophila for fresh water and soil amoebae.
J. Clin. Path., 33: 1179 - 1183

SAYGI. G., WARHURST, D.C. & ROOME, A.P. 1973.
Primary amebic meningo-encephalitis.
Proc. R. Soc. Med., 66: 277 - 282

SCHUSTER, F.L. 1975.
Ultrastructure of mitosis in the amoeboflagellate Naegleria gruberi.
Tissue Cell, 7: 1

SCHUSTER, F.L. 1979.
Biochemistry and physiology of protozoa.
2nd edit. Academic Press.

SCHUSTER, F.L. & DUNNEBACKE, T.H. 1974.
Growth at 37°C of the EGs strain of the amoeboflagellate Naegleria gruberi containing virus like particles. II Cytoplasmic changes.
J. Invert. Pathol., 23: 182 - 189

SEAMAN. G.R. 1961
Some aspects of phagotrophy in Tetrahymena.
J. Protozool. 8: 204 - 214

SEILHAMER, J.J. & BYERS, T.J. 1978.
Mutants of Acanthamoeba castellanii resistant to erythromycin chloramphenicol, and oligomycin.
J. Protozool., 25: 468 - 489

SERAVIN, L.N. & ORLOVSKAJA, J. 1977.
Feeding behaviour of unicellular animals. I. The main role of chemoreception in the food choice of carnivorous protozoa.
Acta. Protozool., 16: 309 - 332

SINGH, B.N. & DAS, S.R. 1970.
Studies on pathogenic and non-pathogenic free-living amoebae and the bearing of nuclear division on the classification of the order Amoebida.
Phil. Trans. R. Soc. (Lond.)., 259: 435 - 476

SINGH, B.N. & DAS, S.R. 1972.
Occurrence of pathogenic Naegleria aerobia, Hartmanella rhysodes in sewage sludge samples of Lucknow.
Curr. Sci., 41: 277 - 281

SINGH, B.M. & HANUMAIAH, V. 1977.
Temperature tolerance of free-living amoebae and their pathogenicity to mice.
Ind. J. Parasit., 1: 71 - 73

SINGH, B.N. & RAO, V.K.M. 1981.
Studies in experimental meningoencephalitis - changes in amino acid levels and the metabolism in the mouse brain.
Ind. J. Exp. Biol., 19: 186 - 188

SLEIGH, M.A. & AIELLO, E. 1972.
The movement of water by cilia.
Acta Protozool., 11: 265 - 277

STAMM, W.P. 1974.
The staining of free-living amebae by indirect immunofluorescence.
Ann. Soc. Belge. Med. Trop., 54: 321 - 325

STEVENS, A.R., DE JONCKHEERE, J. & WILLAERT, E. 1980.
Naegleria lovaniensis new species: isolation and identification of six thermophilic strains of a new species found in association with Naegleria fowleri.
Int. J. Parasit., 10: 51 - 64

STEVENS, A.R. & O'DELL, W.D. 1973
The influence of growth medium on axenic cultivation of virulent and avirulent Acanthamoeba (37346)
Proc. Soc. Exp. Biol. and Med., 143: 474 - 478

STEVENS, A.R. & O'DELL, W.D. 1974.
In vitro and in vivo activity of 5-fluorocytosine on Acanthamoeba.
Antimicrob. Agents. Chemother., 6: 282 - 289

STEVENS, A.R. & O'DELL, W.D. 1974a.
In vitro growth and virulence of Acanthamoeba.
J. Parasit., 60: 884 - 885

STEVENS, A.R., SHULMAN, S.T., LANSEN, T.A., CICHON, M.J.& WILLAERT, E. 1981
Primary amoebic meningoencephalitis: A report of two cases and antibiotic and immunologic studies.
J. Infect. Dis., 143: 193 - 199

STEVENS, A.R., TYNDALL, R.L., COUTANT, C.C. & WILLAERT, E. 1977.
Isolation of the etiological agent of primary amoebic meningoencephalitis from artificially heated waters.
Appl. Environ. Microbiol., 34: 701 - 705

STEVENS, A.R. & WILLAERT, E. 1980.
Drug sensivity and resistance of four Acanthamoeba species.
Trans. Roy. Soc. Trop. Med. Hyg., 74: 806 - 808

STILLWELL, R.H. 1967.
Colpidium - produced RNA as a growth stimulant for Tetrahymena.
J. Protozool., 14: 19 - 22

SYMMERS, W. St.C. 1969.
Primary amoebic meningo-encephalitis in Britain.
Br. Med. J., ii: 449 - 454

TAYLOR, R.W. 1977.
Isolation and experimantal infection of free-living amoebae in freshwater fishes.
J. Parasit., 63: 232 - 237

THONG, Y.H. 1980.
Primary amoebic meningoencephalitis: fifteen years later.
Med. J. Aust., 1: 352 - 354

THONG, Y.H. & FERRANTE, A. 1978.
Phagocytic behaviour towards bakers yeast distinguishes pathogenic from non-pathogenic Naegleria.
Trans. Roy. Soc. Trop. Med. Hyg. 72: 207 - 209

THONG, Y.H., FERRANTE, A., ROWAN-KELLY, B. & O'KEEFE, D. 1980.
Immunisation with live amoebae, amoebic lysate, and culture supernatent in experimental Naegleria meningoencephalitis.
Trans. Roy. Soc. Trop. Med. Hyg., 74: 570 - 576

THONG, Y.H., ROWAN-KELLY, B. & FERRANTE, A. 1979.
Treatment of experimental Naegleria meningoencephalitis with a combination of amphotericin B and rifamycin.
Scand. J. Infect. Dis., 11: 1 - 2

VAN DEN DRIESSCHE, E., VAN DE PITTE, J., VAN DIJCK, P.J., DE JONCKHEERE, J & VAN DE VOORDE, H. 1973.
Primary amoebic meningoencephalitis after swimming in stream water.
Lancet, ii: 971

VAN DIJCK, P., DE JONCKHEERE, J., REYBROUCK, G. & VAN DE VOORDE, H. 1974.
Rapid identification of Naegleria species by sero-agglutination and fluorescent antibodies - new possibilities for the water supply services.
Zbl. Bakt. Hyg. I. Abt. Orig. B., 158: 541 - 551

VENKOBACHAR, C., IYENGAR, L. & PRABHAKARA RAO, A.V.S. 1977.
Mechanism of disinfection: effect of chlorine on cell membrane functions.
Wat. Res., 11: 727 - 731

VISVESVARA, G.S. & BALAMUTH, W. 1975.
Comparative studies on related free-living and pathogenic amoebae with special reference to Acanthamoeba.
J. Protozool., 22: 245 - 256

VISVESVARA, G.S. & CALLAWAY, G.S. 1974.
Light and electron microscopic observations on the pathogenesis of Naegleria fowleri in mouse brain and tissue culture.
J. Parasit., 21: 239 - 250

VISVESVARA, G.S. & HEALY, G.R. 1974.
Isolation of a strain of pathogenic Naegleria fowleri from a human nasal swab.
J. Protozool., 22: 438

VISVESVARA, G.S. & HEALY, G.R. 1975.
Biologic and serologic characterisation of the 161A strain of Naegleria fowleri.
Health Lab. Sci., 12: 8 - 11

WANG, L.K. & PEK, S.L. 1975.
Cationic surface - active agents as bactericides.
Ind. Eng. Chem. Prod. Res. Rev., 14: 308 - 312

WARHURST, D.C., CARMAN, J.A. & MANN, P.G. 1980.
Survival of Naegleria fowleri cysts at 4°C for eight months with retention of virulence.
Trans. Roy. Soc. Trop. Med. Hyg., 74: 832

WARHURST, D.C., ROOME, A.P.C.H. & SAYGI, G. 1970.
Naegleria sp. from human cerebrospinal fluid.
Trans. Roy. Soc. Trop. Med. Hyg., 64: 19 - 20

WARHURST, D.C. & THOMAS, S.C. 1975.
Acanthamoeba sp. from corneal ulcers.
J. Protozool., 22: 57A

WEIK, R.R. & JOHN, D.T. 1977.
Quantitation and cell size of Naegleria fowleri by electronic particle counting.
J. Parasit., 63: 151 - 152

WEIK, R.R. & JOHN, D.T. 1977a.
Cell size, macromolecular composition, and O_2 consumption during agitated cultivation of Naegleria gruberi.
J. Protozool., 24: 196 - 200

WEIK, R.R. & JOHN, D.T. 1977b.
Agitated mass cultivation of Naegleria fowleri.
J. Parasit., 63: 868 - 871

WEIK, R.R. & JOHN, D.T. 1978.
Macromolecular composition and nuclear number during growth of Naegleria fowleri.
J. Parasit., 64: 746 - 747

WEINBERG, W.D. 1978.
Iron and Infection.
Microbiol. Rev. 42: 45 - 66

WELLINGS, F.M. 1979.
Pathogenic Naegleria: distribution in nature. Technical Rep. EPA - 600 / 1 - 79 - 081, Health Effects Laboratory Office of Research and Development, United States Environmental Protection Agency, Cincinati, Ohio. May, 1979, 1 - 79

WELLINGS, F.M., AMUSO, P.T., CHANG, S.L. & LEWIS, A.L. 1977.
Isolation and identification of pathogenic Naegleria from Florida lakes.
Appl. Environ. Microbiol., 34: 661 - 667

WELLINGS, F.M., LEWIS, A.J., AMUSO, P.T. & CHANG, S.L. 1977a.
Naegleria and water sports.
Lancet, ii: 199 - 200

WHITE, G.C. 1972
Handbook of Chlorination. Van Nostrand Reinhold, New York. 744p.

WILLAERT, E. 1974.
Primary amoebic meningo-encephalitis: a selected bibliography and tabular survey of cases.
Ann. Soc. Belge. Med. Trop., 54: 429 - 440

WILLAERT, E. & STEVENS, A.R. 1980.
Experimental pneumonitis induced by Naegleria fowleri in mice.
Trans. Roy. Soc. Trop. Med. Hyg., 74: 779 - 783

WILLAERT, E., STEVENS, A.R. & TYNDALL, R.L. 1978.
Acanthamoeba royreba sp. N. from a human tumor cell culture.
J. Protozool., 25: 1 - 14

WONG, M.M., KARR, S.L. & BALAMUTH, W.B. 1975.
Experimental infections with pathogenic free-living amoebae in laboratory primate hosts: I(A). A study on susceptibility to Naegleria fowleri.
J. Parasit., 61: 199 - 208

WONG, M.M., KARR, S.L. & BALAMUTH, W.B. 1975a.
Experimental infections with pathogenic free-living amoebae in laboratory primate hosts: I(B). A study on susceptibility to Acanthamoeba culbertsoni.
J. Parasit., 61: 682 - 690

WONG, M.M., KARR, S.L. & CHOW, C.K. 1977.
Changes in the virulence of Naegleria fowleri maintained in vitro.
J. Parasit., 63: 872 - 878

WORLD HEALTH ORGANISATION. 1963.
International standards for drinking water. 2nd ed.
World Health Organisation, Geneva. 207p.

WYBURN-MASON, R. 1979.
The free-living amoebic causation of rheumatoid and autoimmune diseases.
International Medicine, 1: 20 - 24

YOUNG, M.D., WILLAERT, E., NEAL, F.C., SIMPSON, C.F. & STEVENS, A.R. 1980.
Experimental infection of sheep with Naegleria fowleri of human origin.
Am. J. Trop. Med. Hyg., 29: 476 - 477