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THE EFFECT OF SOME PHYSICO-CHEMICAL PARAMETERS ON THE SURVIVAL AND TOXIGENICITY OF *VIBRIO CHOLERAE*

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A Thesis submitted to the Faculty of Medicine, University of the Witwatersrand, Johannesburg, for the degree of Doctor of Philosophy (Med).

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ABSTRACT

BACKGROUND

Chemical pollution of the environment is a major problem attracting justified concern world wide. To date, most of the focus has been on the impact of pollution on humans, animals and plants. It is however, necessary to look at its effects on pathogenic microorganisms many of which have very delicately balanced ecological relationships within their habitats. This study investigates the effect of one major industrial pollutant, iron, and pH on *V. cholerae* in both natural and man-made aquatic habitats.

METHODS

Three separate studies measured the survival of cholera bacilli in the presence of increasing concentrations of iron and varying pH values in clean as well as industrially polluted water and, by means of ELISA and mouse adrenal cell line assays, evaluated the effect of iron on cholera toxin production. Several strains of *V. cholerae* were used, namely *V. cholerae* non O1 (including O139), O1 El Tor and Classical.

RESULTS

The well known enhancement of *V. cholerae* survival in the pH range of 7-11 was demonstrated in clean water in this study but, at any pH value, survival was enhanced with increasing

iron (ferric oxide) levels up to an optimum of 0.01-1g/l after which it declined. In industrially polluted water this boosting effect also occurred, but tended to be seen at pH values above 8. Generally, the length of survival and *V. cholerae* counts were greater in polluted than in clean water, which may be a function of additional nutrient availability in the former. Cholera toxin production was significantly enhanced in iron concentrations above 1 g/l, i.e. this occurred in a range different from that affecting survival of the organism. There were significant differences between the survival of the 01 and non-01 strains, but in their patterns of behaviour in response to different pH and iron levels were
similar.

CONCLUSIONS

The above findings may have important implications with regard to both the epidemiology and pathogenesis of cholera:

1. It is postulated that increased pollution due to industrialisation may create more favourable conditions for *V. cholerae* survival. This may have played an important role in the post World War II re-emergence of cholera in its severe pandemic form.
2. It is also postulated that increased toxin production may cause clinical cholera following lower infective doses than is the case with organsims of'normal' toxigenicity.

3. Finally, it is suggested that metal based water distribution systems, as they become older and more subject to internal corrosion, become increasingly a risk factor in the survival and distribution of *V. cholerae* once it has gained access to such a system.

A proposed model for the postulated impact of environmental pollution on the ecology and epidemiology of *V. cholerae* has been constructed. The same factors may also be valid for other waterborne pathogenic microorganisms.

DECLARATION

I declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any university.

I declare that this thesis has the approval of the Committee for Research on Animals of the University of the Witwatersrand and that the number of the certificate of approval is: AEC NO 93/15/3.

Patel

31st day of JULY, 1996.

Part of the work described in this thesis has been published under the title: The effect of pH and iron on the survival of *V. cholerae* (Patel *et al.* Trans. Roy. Soc. Trop. Med. Hyg., 1995, 89: 175-177).

DEDICATION

To the Almighty who is the Creator of the universe, source of souls and supreme bliss whose grace makes the dumb eloquent and the cripple cross mountains, who is the embodiment of knowledge and light, who is the remover of all sins and ignorance.

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LIST OF ABBREVIATIONS

A	Angström
APW	Alkaline Peptone Water
C	Centigrade (Celsius)
cfu	Colony forming unit
CT	Cholera Toxin
CAYEG	Casamino Acid Yeast Extract Glucose
DEA	Diethanolamine
DMSO	Dimethyl Sulphoxide
EDTA	Ethylene Diamine Tetra acetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
FCS	Fetal Calf Serum
g	Gram
HBSS	Hanks' Balanced Salt Solution
HCl	Hydrochloric Acid
hrs	Hours
l	Litre
M	Molar
mg	Milligram
ml	Millilitre
MRC	Medical Research Council
NaOH	Sodium hydroxide
ng	Nanogram
OD	Optical density
pg	Picogram

RPMI	Roswell Park Memorial Institute
rpm	Revolutions per minute
RWB	Rand Water (formerly Rand Water Board)
SAIMR	South African Institute for Medical Research
SD	Standard deviation
TCBS	Thiosulphate Citrate Bilesalts Sucrose
µg	Microgram
µl	Microlitre
YEP	Yeast Extract Peptone

TABLE OF CONTENTS

	Page
Abstract	*i*
Declaration	*iv*
Dedication	*v*
Acknowledgements	*vi*
List of abbreviations	*viii*
Table of contents	*x*
Index	*xi*
List of Figures	*xvii*
List of Tables	*xx*

INDEX

Page

CHAPTER 1:

Historical overview of the global spread of cholera.

1.1	Introduction.....	1
1.2	The 7th cholera pandemic.....	3
1.3	Current perspective on cholera pandemics..	11
1.4	Summary.....	13
1.5	References.....	14

CHAPTER 2:

A literature review of research on cholera with special reference to its ecology and epidemiology

2.1	Introduction.....	19
2.2	The etiology of cholera with special reference to the role of cholera toxin ...	21
2.2.1	The cholera bacillus	23
2.2.2	Cholera toxins	24
2.2.2.1	Structure and activity of cholera toxin ..	25
2.2.2.2	In vitro cholera toxin production	25
2.2.2.3	Assays for the detection of cholera toxin.	27
2.2.2.4	Iron in relation to *V. cholerae* and cholera toxin production.....	27

Page

2.3 The human host and basic mechanisms of cholera transmission..... 28

2.3.1 Waterborne versus non-waterborne transmission of cholera 28

2.3.2 The role of carriers in cholera 32

2.3.3 Person-to-person transmission of cholera.. 35

2.3.4 Transmission of cholera by contaminated foods..... 37

2.4 The aquatic reservoir and the role of aquatic environmental factors in the maintenance of cholera outside the human host 41

2.4.1 Zooplankton and phytoplankton 46

2.4.2 Estuarine sediments, salinity, pH, temperature and cations 47

2.4.3 Iron 49

2.5 Summary 49

2.6 References 52

CHAPTER 3:

Study design 74

3.1 References 81

Page

CHAPTER 4:

The interaction of pH and iron in their effect on the survival of *V. cholerae*.

4.1 Introduction 82

4.2 Materials and methods..... 82

4.2.1 Bacterial cultures and inocula..... 83

4.2.2 Solubility of ferric oxide in water..... 85

4.2.3 *V. cholerae* survival studies 85

4.2.4 Statistical analysis 86

4.3 Results 87

4.3.1 Solubility of ferric oxide in water..... 87

4.3.2 *V. cholerae* survival study..... 87

4.4 Discussion 97

4.5 Conclusions..... 104

4.6 References..... 106

4.7 Appendix..... 109

CHAPTER 5:

Survival of *V. cholerae* in water polluted by industrial effluents.

5.1 Introduction 111

5.2 Materials and Methods 112

5.2.1 Water sampling sites 113

Page

5.2.2 Collection and pre-inoculation testing of water samples 113
5.2.3 Bacterial cultures and inocula 117
5.2.4 Inoculation of water samples and determination of *V. cholerae* survival 119
5.2.5 Statistical analyses..... 120
5.3 Results and discussion 120
5.3.1 Microbiological analysis of pre-inoculation water samples 120
5.3.2 Survival of *V. cholerae*..... 122
5.4 Conclusions 148
5.5 References 150

CHAPTER 6:

The effect of iron on the toxigenicity of *V. cholerae*.

6.1 Introduction..... 151
6.2 Materials and methods 152
6.2.1 Cholera toxin titration 152
6.2.2 Bacterial cultures..... 154
6.2.3 Procedure used to compare toxin production in YEP and CAYEG media..... 154
6.2.4 Preparation of bacterial inoculum and culture medium titration 156

		Page
6.2.5	Procedure to determine the effect of iron on toxin production by *V. cholerae*	158
6.2.6	Microtitre ganglioside ELISA assay.....	159
6.2.7	Y1 mouse adrenal cell line assay	162
6.2.7.1	Cell culture, storage, recovery and subculture.....	162
6.2.7.2	Toxin assay.....	163
6.3	Results	164
6.3.1	Titration of known cholera toxin (CT) concentrations.....	164
6.3.2	Cholera toxin production in YEP medium ...	167
6.3.3	Effect of iron on toxin production by *V. cholerae* as demonstrated by the microtitre GM_1-ELISA technique.....	167
6.3.3.1	Results with the microtitre GM_1-ELISA technique.....	169
6.3.3.2	Results with the Y_1 cell line technique...	176
6.4	Discussion.....	178
6.5	Conclusions	189
6.6	References	191
6.7	Appendix	200
6.7.1	Recipes	200
6.7.2	Tables	203

Page

CHAPTER 7:

Summary and correlation of results: a proposed model for the ecology and epidemiology of cholera in the context of environmental pollution 207

7.1 References 217

LIST OF FIGURES

CHAPTER ONE

Page

1.1 Global spread of cholera (1961-1993) Based on (Glass *et al.* 1991 and WHO, 1994) 4

CHAPTER FOUR

4.1 Survival of *V. cholerae* in sterile distilled water at pH 7 and in the presence of increasing iron concentrations 93

4.2 Survival of *V. cholerae* in sterile distilled water at pH 9 and in the presence of increasing iron concentrations 94

4.3 Survival of *V. cholerae* in sterile distilled water at pH 11 and in the presence of increasing iron concentrations 95

4.4 Metal water conduit clearly showing iron corrosion tubercles 102

CHAPTER FIVE

Page

5.1 Locations of water sampling points in the Vaal river catchment area (Rand Water Map 1) 115

5.2 Locations of water sampling points in the Vaal river catchment area (Rand Water Map 2) 116

5.3 Survival of *V. cholerae* at increasing pH values in effluent polluted water (all sampling points). Survival in six pH-adjusted samples from point N7 are indicated in red..... 139

5.4 Survival of *V. cholerae* by increasing iron concentration in effluent polluted water 141

5.5 Survival of *V. cholerae* by increasing iron concentration in effluent polluted water - separating the results at iron concentrations below 1 mg/l..... 142

CHAPTER SIX

6.1 GM_1 ganglioside microtitre ELISA plate..... 161

6.2 Titration slope using linear regression for cholera toxin values obtained by GM_1 ganglioside micro-ELISA 166

6.3 Cholera toxin production by *V. cholerae* O1 classical in response to increasing iron concentrations..... 173

Page

6.4 Cholera toxin production by *V. cholerae* 01 El Tor in response to increasing iron concentrations 174

6.5 Cholera toxin production by *V. cholerae* non-01 in response to increasing iron concentrations 175

6.6 Illustrating different views of water hyacinths. Note the extensive coverage of the water surface by this plant..... 187

CHAPTER SEVEN

7.1 The effect of iron on the survival and toxin production of *V. cholerae*..... 208

7.2 Model for potential impact of environmental pollution on the ecology and epidemiology of *V. cholerae* 215

LIST OF TABLES

Page

CHAPTER ONE

1.1 Known cholera pandemics 2

CHAPTER FOUR

4.1 *Vibrio cholerae* cultures used in this study 84

4.2 Solubility of ferric oxide as measured 14 days after addition of the oxide to water 88

4.3 Survival of *V. cholerae* non-O1 (VC1 and VC2) in sterile distilled water at increasing pH values and iron concentrations 89

4.4 Survival of *V. cholerae* O1 El Tor (VC3 and VC4) in in sterile distilled water at increasing pH values and iron concentrations 90

4.5 Survival of *V. cholerae* O1 classical (VC5 and VC6) in sterile distilled water at increasing pH values and iron concentrations 91

4.6 Statistical significance (by 2-way ANOVA) of *V. cholerae* survival differences in sterile distilled water at increasing pH values and iron concentrations..... 96

Page

4.7 Statistical significance (by 1-way ANOVA) of the effect of iron on the survival of *V. cholerae* in sterile distilled water at different pH levels ... 98

CHAPTER FIVE

5.1 Sampling sites for the collection of industrially polluted water samples (April 1989 - January 1990)..... 114
5.2 *V. cholerae* cultures used in this study 118
5.3 Microbiological analysis of industrially polluted water samples..... 121
5.4 Survival of *V. cholerae* in sample N7..... 123
5.5 Survival of *V. cholerae* in sample E7..... 124
5.6 Survival of *V. cholerae* in sample E17..... 125
5.7 Survival of *V. cholerae* in sample V17..... 126
5.8 Survival of *V. cholerae* in sample V18..... 127
5.9 Survival of *V. cholerae* in sample T1..... 128
5.10 Survival of *V. cholerae* in sample R5..... 129
5.11 Survival of *V. cholerae* in sample R4..... 130
5.12 Survival of *V. cholerae* in sample E11..... 131
5.13 Survival of *V. cholerae* in sample WJ..... 132
5.14 Survival of *V. cholerae* in sample WC..... 133
5.15 Survival of *V. cholerae* in sample VAS..... 134
5.16 Pooled *V. cholerae* survival data, sorted by sampling point (n=96)..... 136

Page

5.17 Pooled *V. cholerae* survival data, sorted by pH and separated in groups of n=16 each 138

5.18 Pooled *V. cholerae* survival data, sorted by iron and separated in groups of n=16 each 140

5.19 Analysis of variance (ANOVA) of 96 pooled *V. cholerae* survival data by pH from 3.3 to 9.2 .. 144

5.20 Analysis of variance (ANOVA) of 48 pooled *V. cholerae* survival data by iron concentration from 0.03 mg/l to 33.0 mg/l..... 147

CHAPTER SIX

6.1 Microtitre ganglioside ELISA method used for determination of cholera toxin concentration..... 153

6.2 Description of *V. cholerae* strains used in this study..... 155

6.3 Optical densities (at 405nm) of ten cholera toxin titrations using the GM_1 ganglioside ELISA 165

6.4 Production of cholera toxin by ten strains of *V. cholerae* in YEP and CAYEG medium 168

6.5(a) Summarised results: Effect of iron (Fe_2O_3) on cholera toxin production by *V. cholerae* O1 classical strains. Toxin measured by GM_1 micro-ELISA (A 405)..... 170

Page

6.5(b) Full results: Effect of iron (Fe_2O_3) on cholera toxin production by *V.cholerae* O1 classical strains. Toxin measured by GM_1 micro-ELISA (A 405)..... 204

6.6(a) Summarised results: Effect of iron (Fe_2O_3) on cholera toxin production by *V. cholerae* O1 El Tor strains. Toxin measured by GM_1 micro-ELISA (A 405)..... 171

6.6(b) Full results: Effect of iron (Fe_2O_3) on cholera toxin production by *V.cholerae* O1 El Tor strains. Toxin measured by GM_1 micro-ELISA (A 405) 205

6.7(a) Summarised results: Effect of iron (Fe_2O_3) on cholera toxin production by *V. cholerae* non-O1 strains. Toxin measured by GM_1 micro-ELISA (A 405)..... 172

6.7(b) Full results: Effect of iron (Fe_2O_3) on cholera toxin production by *V. cholerae* non-O1 strains. Toxin measured by GM_1 micro-ELISA (A 405) 206

6.8 Effect of iron (Fe_2O_3) on cholera toxin production by *V. cholerae*. Toxin measured by Y_1 mouse adrenal cell line method..... 177

6.9 Range of published iron concentrations in surface waters and sediments..... 184

CHAPTER 1

HISTORICAL OVERVIEW OF THE GLOBAL SPREAD OF CHOLERA

1.1 INTRODUCTION

True cholera, caused by *Vibrio cholerae*, probably existed in Asia since the times of Hippocrates and the Lord Buddha (Barua, 1992). However, the lack of knowledge about its etiology and confusion caused by many clinically similar diarrhoeal diseases due to other pathogens, has handicapped a complete, accurate chronological and geographic description of cholera in ancient times.

According to Pollitzer (1959), the year 1817 marks the onset of the first of a series of seven pandemics during which cholera, until then mainly of local Indian importance, gained impetus there and extended to other parts of the world, thus causing international concern.

The first pandemic of cholera started in Bengal in 1817 and came to involve most of Asia by 1823. The second pandemic (1826-1827) affected the whole of Asia and parts of Europe and Africa. Similar pandemics and epidemics followed at intervals (table 1.1).

TABLE 1.1 Known Cholera Pandemics

Pandemic (Traditional designation)	Period (year)	Causative bacterial strain	Pandemic (Putative designation)
1	1817-1823	unknown	1 (1817-1923)
2	1829-1851	unknown	
3	1852-1859	unknown	
4	1863-1879	unknown	
5	1881-1896	classical V.cholerae 01	
6	1899-1923	classical V.cholerae 01	
7	1961-	El Tor V.cholerae 01	2 (1961-)
"8"	1992-	'Bengal' V.cholerae 0139	3 (1992-)

The seventh pandemic started in 1961 and involved virtually the whole world, with the exception of Australia. Unlike the 5th and 6th pandemics which were thought to be caused by the so-called classical biotype of *V. cholerae* O1, the strain responsible for the 7th pandemic was the El Tor biotype. The latter which was initially found to differ from the classical biotype by its haemolytic properties, was first isolated by Gotschlich in 1905 from dead Mecca pilgrims at the quarantine camp of El Tor in the Red Sea (Pollitzer, 1959). From the year 1937, localised epidemics of El Tor cholera were recognised on the island of Sulawesi (then Celebes), Indonesia, but signs of pandemic spread was not recognised until the early 1960s.

South Africa became affected extensively by cholera for the first time in its recorded history during the 7th pandemic which in due course stimulated the study described in this thesis. It is therefore relevant and interesting to consider the events that ultimately led to the arrival of cholera in Southern Africa.

1.2 THE 7TH CHOLERA PANDEMIC

This section describes some of the highlights of the 7th pandemic which is currently still in progress (fig 1.1) and

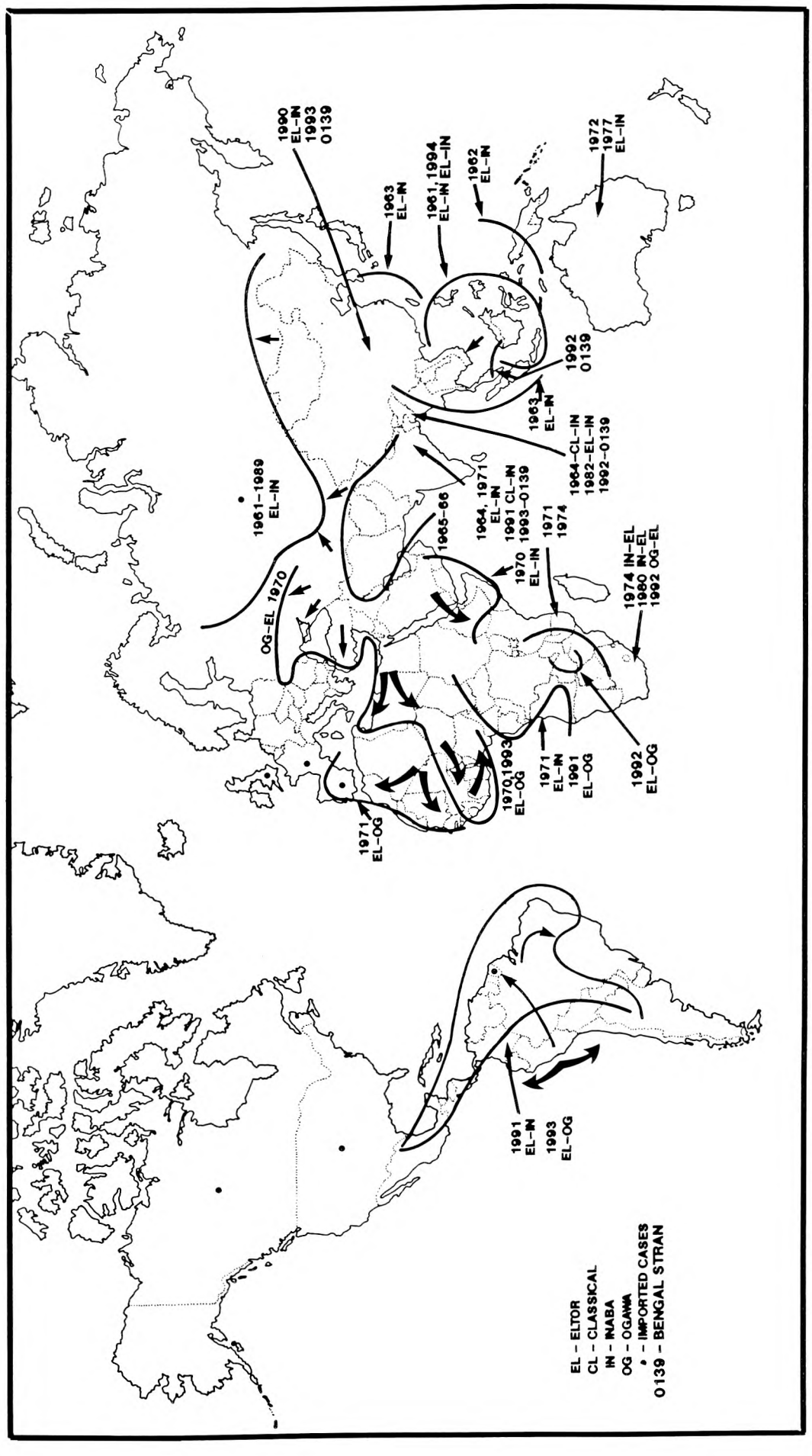

Fig 1.1 Global spread of cholera 1961 – 1994. (Based on Glass et al, 1991, Wkly. Epidemiol. Rec., 1994)

which, according to Kamal (1974), occurred in distinct stages.

Invasion of South East Asia: 1961-1962.

In January 1961 cholera broke out again in Sulawesi, Indonesia. It spread to the other Indonesian islands, the Philippines and Hong Kong. During 1961, 12197 cases were recorded in that area (World Health Organization, 1965).

The disease continued to spread to other parts of the South-East Asian archipelago (including Sabah, Taiwan, Irian Barat), presumably by carriers using seaborne transportation. The total number of cholera cases in these islands during 1962 was 13393 (Kamal, 1974).

There are various explanations for the start and spread of the 1961-1962 outbreak. The population was highly susceptible because cholera occurred after an absence of a decade and a half. The volume of infection was large in that more than one island was infected. Furthermore, due to the political situation there were major troop movements and civil population emigrating in Indonesia. The transportation and travel promoted the spread of cholera (Kamal, 1974).

Cholera spread through mainland Asia: 1963-1969.

By 1963 the mainland of Asia had become affected. At the same time, areas where pandemic spread had occurred during 1961-1962, were reinfected, except for Taiwan. Malaysia, Thailand, Singapore, Cambodia, Burma and Vietnam were affected by the beginning of 1964, and Korea and Bangladesh were affected later that year (Kamal, 1974).

The spread by *V. cholerae* El Tor to India was announced by Barua et al in 1964. Within a year, the whole of India was affected. The introduction of the infection to this country appeared to be predominantly via sea routes. From India it spread to contiguous territories of West Pakistan, Bangladesh, Afghanistan, and the former USSR. However, at the same time classical *V.cholerae* was still the predominant biotype in West Pakistan and Bangladesh.

The Middle East Afro-European phase of the pandemic: 1970-1991.

By 1970 cholera El Tor had spread to 24 new countries: six in Asia, fifteen in Africa and three in Europe. It was the worst year of the pandemic because the geographic distribution of cholera was now widespread. It is important to note that two different serotypes of *Vibrio cholerae*

caused the outbreaks in the Middle East. In Lebanon, Libya, Syria, Tunisia and the Black Sea ports serotype Ogawa predomiated while in Israel, Jordan, Saudi Arabia and Dubai it was Inaba. This may indicate the possibility of two sources of infection or of antigenic conversion between the two serotypes during the course of an epidemic. This difference in the prevailing serotype of the vibrio continued to be reported from time to time during the progression of the pandemic. In 1971, *V.cholerae* El Tor Inaba was prevalent in much of East Africa whilst West Africa appeared to be part of the Ogawa domain.

From 1970 onwards cholera El Tor invaded one West African country after another. The spread of the pandemic appeared to be seaborne at first, affecting particularly coastal towns and fishing villages. Then, as the disease took a foothold within countries, the tribal customs of the populations, especially funeral rites involving ceremonial meals and contact with the dead, their nomadic type of movement and the use of rivers for transportation, trade and travel were important factors in the spread of the disease.

In Europe, cholera due to *V. cholerae* El Tor Inaba appeared in Istanbul, Turkey, where its explosive nature was characteristic of waterborne spread. In Czechoslovakia,

cases of cholera due to the Ogawa serotype were reported in 1970.

In 1971 a particularly large outbreak of El Tor cholera occurred in Indonesia and affected inhabitants of islands where cholera had not occurred for many years. A major epidemic also occurred in India among refugees from Bangladesh. A study on cholera strains isolated from these patients showed that Inaba and Ogawa serotypes of both El Tor and classical biotypes were involved (Kamal, 1974).

The first locally acquired cases of cholera due to El Tor Inaba in Australia occurred in a caravan park in South Eastern Queensland in 1977 (Rogers *et al.*, 1980). Small outbreaks occurred in Italy (1973-1979), Portugal (1974), Japan (1978), Bahrain (1978), Guam, Oceania (1974), Gilbert Islands (1977), Sri Lanka (1973) and Louisiana, USA (1978). In South Africa, a small outbreak of cholera appeared in 1974 among migrant workers in gold mines (Isaäcson *et al.*, 1974). However, the disease occurred in this country in major epidemic form only from 1980 and 25251 laboratory confirmed cases were reported between 1980 and 1986. The outbreak was caused by *V. cholerae* El Tor Inaba (Küstner and Du Plessis, 1991). The number of cholera cases declined thereafter, but in 1992-1993 a small resurgence occurred (Wilkinson, D., 1993), mainly due to the Ogawa

serotype.

During 1982, after an absence of 10 years, the classical biotype of *V. cholerae* reappeared in Bangladesh in epidemic form and rapidly became dominant (Samadi *et al.*, 1983).

Invasion of South and Central America: 1991 - to date.

In 1991, the pandemic of cholera El Tor which had started in 1961 in Sulawesi, Indonesia, finally completed its encirclement of the globe by invading South America. A very large outbreak due to the Inaba serotype started in Peru as a result of consumption of unchlorinated piped water, and in due course affected all but seven of the countries of South and Central America. At the same time, a major resurgence of cholera occurred in Africa, affecting 21 countries. This number of newly affected countries was more than in any other year since the arrival of the pandemic in Africa in 1970. Asian countries reported 49791 cases while Europe notified 316 cases. By 1992 serotype Ogawa of El Tor *V. cholerae* was also introduced into South America. Alternatively, it is possible that serotype conversion from Inaba to Ogawa may have ocurred (Manning *et al.*,1994).

To summarize, the year 1991 saw more cases occurring in more countries than any other year on record (WHO, 1992). This may have been the result of worldwide economic depression, massive social disruption and population movements as well as, in some areas, a decline in living standards. These phenomena occurred in Africa as well as in Latin America in 1991, providing ideal conditions, especially a deterioration in personal hygiene and environmental sanitation for cholera to flourish. In part, improved surveillance may have contributed to the increase in recorded cases.

The eighth pandemic?

Towards the end of 1992 and early in 1993 unusual strains of *V. cholerae* were isolated from typical cholera-like outbreaks in India. They were found to be *V. cholerae* non O1, but they could not be typed by the panel of 138 reference sera developed for *V. cholerae* non O1 at the National Institute of Health in Japan. The strains were shown to produce cholera toxin and have been designated *V. cholerae* 0139 or Bengal strain (Ramamurthy *et al.*, 1993).

The epidemic caused by this strain spread to Bangladesh where it rapidly spread throughout the country. Within 4 months, 107297 cases of diarrhoea with 1473 deaths had been

reported. The disease was identical to cholera in its clinical features and response to treatment, but most cases occurred in adults, suggesting that the population had had no previous immunological experience with the causative agent. Subsequent studies showed a lack of cross protection against diarrhoea due to this strain in rabbits immunized against *V. cholerae* O1 (Albert *et al.*, 1994a). These workers also showed that antibacterial immunity rather than antitoxic immunity may be most important for protection against *V. cholerae* 0139. Conversely, rabbits immunized against *V. cholerae* 0139 were not protected against *V. cholerae* O1 (Albert *et al.*, 1994b). The epidemic potential of this new strain, and the universal lack of immunity to it may herald the beginning of an eighth cholera pandemic. Speedy transportation with increasing international travel and population movements may promote rapid dissemination of this new strain (Swerdlow and Ries, 1993; Albert *et al.*, 1993). Dispersion of *V. cholerae* 0139 beyond India and Bangladesh has rapidly taken place to Thailand, China, Malaysia, Pakistan, Nepal and Sri Lanka (WHO, 1994).

1.3 Current perspective on cholera pandemics

Cholera-like disease has been known since the time of Hippocrates and Lord Buddha (Barua, 1992). The diagnosis

was based only on clinical observations until Robert Koch isolated the causative bacterium. It has been shown that the fifth and sixth pandemics were caused by *V. cholerae* O1 of the classical biotype, but the biotype(s) responsible for the first four pandemics is unknown (Barua, 1992). Although there is no bacteriological evidence that the first four pandemics were caused by the classical biotype of *V. cholerae* O1, there is some justification for considering the first six pandemics as a single pandemic (Blake, 1994). Firstly, the six pandemics cannot be separated clearly and there is no evidence of the intervals having been cholera free. Although the interval between the sixth and the seventh pandemic was also not entirely cholera free, the biotypes of the causative agents, however, were different, i.e. classical and El Tor respectively. Secondly, we now know that *V. cholerae* can survive and multiply silently in some natural environments for an indefinite length of time (Blake *et al.*, 1980; Bourke *et al.*, 1986; Colwell and Spira, 1992). Pandemics one to six may have originated from organisms persisting in the environment in many countries or from small foci of human disease.

There may now be an eigth pandemic caused by *V. cholerae* of a different O group, namely 0139, in the process of evolving. Therefore, in future years it may be thought that

only three cholera pandemics have affected the world, each caused by a distinctive *V. cholerae* strain (Table 1.1) (Blake, 1994).

1.4 SUMMARY

V. cholera is known to have caused seven pandemics since 1817 (table 1.1) but the bacterial strains are unknown for the first four of these pandemics. However, the classical biotype of *V. cholerae* was responsible for the 5th and 6th pandemics. The El Tor biotype, which spread from Sulawesi (Indonesia), caused the start of the 7th pandemic in 1961. This pandemic is still in progress. In 1992, a newly recognised strain of *V. cholerae* non-O1, namely O139, also known as the Bengal strain, emerged in India where it caused epidemic cholera and rapidly spread in epidemic form to Bangladesh, Burma, China, Malaysia, Pakistan and Sri Lanka.

Today, cholera remains one of the world's major health problems.

1.5 REFERENCES

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CHAPTER 2

A LITERATURE REVIEW OF RESEARCH ON CHOLERA WITH SPECIAL REFERENCE TO ITS ECOLOGY AND EPIDEMIOLOGY

2.1 INTRODUCTION

The following review of research on cholera epidemiology should place the current study, which investigates the effects of inorganic aquatic habitat pollution, in its correct perspective.

Two distinct approaches have been taken by previous investigators in regard to the understanding of cholera.

The first approach, which started during the early 19th century and gained momentum with Robert Koch's discovery of *Vibrio cholerae* (the 'comma bacillus') as the causative agent of the disease, concerned itself almost exclusively with humans as the principal reservoir (Pollitzer, 1959). Koch and other workers were unable to infect laboratory animals and the idea of an animal reservoir was never seriously entertained. The concepts of water borne and contact (person-to-person) transmission, and of healthy

carriers, became topics of intense investigation. Food became recognized as an important vehicle of cholera since the end of the 19th century.

Although the second approach also originated with Koch who, as early as 1884, suggested that the comma bacillus was an aquatic organism, the idea of water as an actual reservoir of cholera did not receive serious attention until the 1970s (Koch, 1884). The seminal work of Colwell and other workers drew attention to this neglected aspect and ushered in the aquatic reservoir era of research on cholera epidemiology. Additionally, scientists looked into related questions, such as those of maintenance and bacterial amplification mechanisms within the aquatic habitat. As will be seen later (Chapter 6), researchers outside the medical biological sphere (e.g. water engineering and industrial microbiology) made observations which, interpreted in the light of cholera epidemiology, are proving to be invaluable contributions to the growing body of knowledge necessary to understand why cholera is still rampant during the 1990s.

2.2 THE AETIOLOGY OF CHOLERA WITH SPECIAL REFERENCE TO THE ROLE OF CHOLERA TOXIN

John Snow did the most influential and pioneering work on cholera in the 18th century. His important paper on the mode of communication was published in 1855 (Snow, 1855). He studied the continuous attacks among close contacts in various parts of England and Scotland during 1831-1832 and 1848-1849. He thought that cholera was communicable from the sick to the healthy and that the loss of fluid from the proliferation of "morbid material" or "cholera poison" in the intestines was responsible for the disease. Snow realized that cholera spreads by faecal-oral transmission. He emphasized that the poverty and the habits of the working classes influenced the transmission and he categorized unhygienic and cramped working conditions as high risk factors. He also drew attention to the seasonal variation of cholera incidence and considered the possible role of houseflies in spreading cholera in summer. Snow concluded with a six point strategy for cholera:

- Good drainage
- Good water supply
- Improved housing with less crowding
- Quarantining of sick persons on incoming ships

- Public wash houses
- Education in personal and domestic cleanliness

During that era, a group led by Von Pettenkofer vigorously criticised Snow, Koch and other thinkers who believed in the "germ theory" of disease transmission. He postulated that soil contaminated by human faeces and urine gave rise to a "cholera- miasma" (Howard-Jones, 1973). Later, he suggested that the cholera germ, designated X, needed to contact a substrate in soil, Y, to produce the cholera poison Z.

Using a microscope, Pacini, in 1854, was the first to observe the cholera vibrio, subsequently named *Vibrio cholerae*, in the bowel contents of a patient who had died of cholera (Sakazaki, R., 1992). In 1884, at the Berlin Conference convened for the discussion of cholera, Robert Koch announced the discovery of the vibrio as the causative organism of the disease. He stressed the probable importance of extracellular "poison" produced by the comma-bacillus in asymptomatic or mildly ill cholera excreters. Koch was also the first to discuss the survival and multiplication of the comma bacillus in the environment.

2.2.1 The cholera bacillus

Vibrio cholerae is an aerobic, facultatively anaerobic,non-sporing, gram negative bacillus which is highly motile. On the basis of their somatic or O antigens, a large number of serotypes have been recognised. For the sake of convenience these have been referred to as group O1 and group non-O1 since, until recently, choleragenic *V. cholerae* belonged only in the O1 group. Non-O1 *V. cholerae*, also known as NAG (non-agglutinable with O1 antiserum) was not known to cause cholera, although such strains could produce a milder (usually) diarrhoeal illness. Non-O1, furthermore, did not tend to exhibit the rapidly and widely disseminating characteristics shown by the choleragenic O1 strains. Two biogroups or biovars of *V. cholerae* are recognised to constitute the O1 group; they are known as 'classical' and 'El Tor' *V. cholerae*. The classical variety is the organism thought to be responsible for the 5th and 6th pandemics. The El Tor cholera vibrio was first isolated in 1905 by Gotschlich from dead bodies of Mecca pilgrims at the El Tor quarantine station in the Red Sea (Pollitzer R, 1959). Although the non-O1 group of *V. cholerae* bacilli was thought to be non-choleragenic, this belief had to be revised in view of the finding of a new non-O1 strain which was able to cause rapidly disseminating, severe epidemics of cholera in Asia. This

strain, typed as *V. cholerae* O139, is also known as the Bengal strain after its site of first known appearance. The classical and El Tor varieties are not cross-immunogenic with the Bengal strain. The classical and El Tor varieties have been classified into three serotypes: Inaba, Ogawa and Hikojima. Only the Inaba and Ogawa serotypes have featured significantly as causes of individual epidemics.

2.2.2 Cholera toxins

Whilst *V. cholerae* is clearly the causative agent of cholera, it is entirely non-invasive and can cause clinical disease only by means of one of its toxins. *V. cholerae* yields several extra- cellular products which adversely affect eukaryotic cells. Probably the most important of these is the heat labile cholera toxin (CT), also known as choleragen, which is an enterotoxin capable of causing severe diarrhoea in humans.

When genes coding for CT are deleted from *V. cholerae* strains, their ability to produce diarrhoea of reduced severity is frequently still present. This observation resulted in the search for, and the finding of other toxins. Several other toxins produced by *V. cholerae*, including haemolysin, cytolysin, zonula occludens toxin,

heat stable enterotoxin, shiga-like toxin, accessory cholera enterotoxin, 'new' cholera toxin and others have now been described (Kaper *et al.*, 1994). They were, however, not included in this study.

2.2.2.1 Structure and activity of cholera toxin

CT has 6 subunits, 1 of type A and 5 of type B (Gill, D.M., 1976). The B subunits serve to bind the holotoxin to intestinal epithelial cell membrane receptors consisting of a ganglioside known as GM_1. This binding enables the A subunit to effect its specific intracellular enzymatic function which ultimately results in a dramatic loss of fluids and electrolytes from the intestinal tract. Antibodies against the immunodominant B subunit are much more efficient at neutralising CT activity than those directed at the A subunit (Petersen *et al.*, 1979).

2.2.2.2 In vitro cholera toxin production

Several methods and media exist for enhancing in vitro CT production.

Media for this purpose include the syncase medium (Finkelstein *et al.*, 1966), casamino acid yeast extract (CAYE) medium (Kusama and Craig, 1970) and yeast extract peptone water medium (Iwanaga and Kuyyakanond, 1987).

Culture conditions are also important to optimise CT production. For example, switching from a stationary position to shaking the culture when the vibrios are at the end of their exponential growth phase, stimulates their CT production. Lincomycin, a protein synthesis inhibitor, was found to increase the rate of CT synthesis (Levener *et al.*, 1977). Cholera cultures grown in the presence of 5-10% CO_2 also produce more toxin despite the observation that cell growth is actually slightly decreased in this system (Shimamura *et al.*, 1985).

Tamplin and Colwell (1986) studied the effects of salt and organic nutrient (Casamino Acids) concentrations in defined seawater media on the production of CT. They showed that, in the presence of 10 g organic nutrient per litre, CT production increased markedly with increasing salinity in the range of 0-3%. This effect was reduced with 1 g organic nutrient per litre, whereas CT was undetectable at organic nutrient concentrations below 0.1 g/l.

Islam (1990) found an increase in toxin production by *V. cholerae* when the latter was in association with green algae (*Rhizoclonium fontanum*).

2.2.2.3 Assays for the detection of cholera toxin

Various biological and immunological techniques are available for the detection of CT. They include the ligated rabbit ileal loop assay, tissue culture, passive haemagglutination, reverse passive latex agglutination (RPLA), enzyme linked immunosorbent assay (ELISA), GM_1 erythroassay, passive immune haemolysis and solid phase radioimmunoassay .

2.2.2.4 Iron in relation to *V. cholerae* and cholera toxin production

The role of iron in relation to cholera virulence has been studied by several workers. *V. cholerae* produces iron chelating siderophores known as vibriobactin. This high-affinity iron-binding compound enables the organism to obtain iron from highly insoluble environmental iron sources or from protein-bound host iron. However, by studying *V. cholerae* mutants which were defective in the iron-vibriobactin transport system, Sigel et al. (1985) established that a functional system is not essential for the pathogenesis of cholera, although it may enhance multiplication of the organism in the gut lumen. In addition, low-iron conditions stimulate the expression of new outer membrane proteins which channel chelated iron

into the cells. At the same time, the expression of some existing proteins may decrease (Sigel and Payne, 1982). Goldberg *et al.* (1990) showed that iron regulated genes are important in the virulence of *V. cholerae*.

2.3 THE HUMAN HOST AND BASIC MECHANISMS OF CHOLERA TRANSMISSION

Humans are the only natural hosts of the cholera vibrios and usually aquire the infection by ingestion of contaminated water or food.

2.3.1 Waterborne versus non-waterborne transmission of cholera

Cholera characteristically occurs in crowded communities of a low socio-economic level. It tends to persist in such communities which share natural water supplies such as "tanks", ponds, canals or rivers for bathing, laundering of linen and other domestic uses. Cholera outbreaks in poor communities are almost invariably associated with a finding of heavy contamination of water sources with coliforms, *Escherichia coli* type I (faecal coli) and usually with *V. cholerae*. Some authors have therefore questioned the validity of others concluding a causal relationship between contaminated water sources and a cholera outbreak when

there is often no hard evidence of such a relationship. In such cases, the presence of *V. cholerae* in the water may be the effect, rather than the source of the outbreak, the true source of which may remain unidentified. Some of the outbreaks where waterborne transmission was involved are described here.

Feachem et al (1981) reviewed work by Godbole and Wagle (1970). These authors studied a cholera outbreak in 1967 in Poona, India, where a community with a cluster of cases was found to have access to both treated tap water and faecally contaminated open well water. *V. cholerae* isolated from the well water was the same as that from the patients. After chlorination of the well, the outbreak subsided. The authors concluded that the well water was responsible for the outbreak. Feachem et al (1981) felt this conclusion to be unjustified. For one thing, when an outbreak of cholera occurs, open wells in the neighbourhood are likely to become contaminated by *V. cholerae*. For another, outbreaks are likely to subside regardless of any intervention. Although it is possible that well water did play a role in this outbreak, there is nothing specific in the data provided by Godbole and Wagle to incriminate this type of transmission in preference to other possible types.

A large cholera outbreak in Russia during 1970-1971 was found to be due to waterborne and foodborne transmission. Water for recreational purposes, drinking water and surface waters were all found to be contaminated with sewage but consumption of seafood was another mode of transmission implicated in this outbreak (Narkevich *et al.*, 1993).

Velimirovic and colleagues (1975) studied 711 hospitalized cholera patients in Manila during 1971-1972. They noted that 93% of cholera cases came from poor families using chlorinated municipal water. Therefore they concluded that water was not primarily responsible for the spread of infection and any role that water might have played could only have been through secondary contamination of stored water in the home.

Patel and Isaäcson (1989) showed that under laboratory conditions, *V. cholerae* O1 as well as non-O1 could survive in potable water stored in containers for up to 27 days depending on the type of container and the chlorine level of the stored water.

In 1980, epidemic cholera in South Africa was traced to consumption of water from an irrigation canal which was contaminated with *V. cholerae* (Küstner *et al.*, 1981). It was suggested that the water had been contaminated by an

asymptomatic carrier originating from an endemic area elsewhere in Africa.

In 1991 epidemic cholera appeared for the first time in Latin America. The transmission of *V. cholerae* may have occurred through many different routes. Urban infrastructures appeared to be of major importance. Large, often poorly maintained municipal water supply systems had intermittent flows making sewage cross contamination likely, while chlorination was often intermittent or inadequate. Municipal sewers discharged into rivers and harbours adjacent to shellfish beds and fishing docks. Raw sewage was used to irrigate and fertilize fruit and vegetables that were often eaten raw, as were fish and shellfish. Within Peru, documented routes of transmission included consumption of unboiled municipal water, foods and beverages from street vendors, and of rice left at ambient temperature for many hours after cooking (Tauxe and Blake, 1992; Ries *et al.*, 1992).

The above review shows that although water plays an important role in cholera transmission, in most outbreaks a combination of several modes of spread can usually be demonstrated.

2.3.2 The role of carriers in cholera

Cholera has spread along the line of human communication by patients and healthy carriers. The latter can be incubatory, convalescent or healthy contact carriers. Carriers transmit *V. cholerae* infection to other family members by contaminating stored water and cooked food with contaminated fingers (Deb *et al.*, 1982). According to Feachem (1982), the median carriage time for asymptomatic carriers is between 1 and 8 days. However, researchers have reported intermittent excretion of *V. cholerae* by a few individuals over much longer periods, for instance up to 99 days in Calcutta (Joint ICMR-GNB-WHO Cholera study group, 1970) and in a single, exceptional case in the Philippines (Cholera Dolores) 42 months (Azurin *et al.*, 1967). Dolores was suffering from hookworm disease with anaemia and it was suggested that this may have lowered her general resistance. She also had a chronic cholecystitis which may have further contributed to her long term carrier state.

Sinha *et al.*(1967) investigated cholera among slum dwellers in eastern Calcutta and showed the importance of carriers and person-to-person transmission. The population studied included 12 800 persons in 2 286 families living in 361 houses. Tube wells and a reticulated municipal supply

served as drinking water sources. There were 433 latrines of which 93% were of the bucket type. During a 10 months sampling period, 46% of latrines yielded *V. cholerae* on at least one occasion. Among users of positive latrines, 4.7% were found to be *V. cholerae* excretors, whereas among users of negative latrines, none were *V. cholerae* excretors. Isolation of *V. cholerae* from carriers as well as from latrines occurred throughout the year. The prevalence of *V. cholerae* excretion among healthy members of the study population was 1.3%, but in healthy contacts of hospitalised cholera cases it was 6%. The mean duration of confirmed cholera carriage was 19 days, with a maximum of 43 days. This study showed that carriers maintained cholera transmission in the community. Carriers contaminated the latrines which, being mostly of the bucket type, were handled by people, thus promoting person-to-person transmission.

Similarly, the percentage of asymptomatic contacts was found to be high when McCormack *et al.* (1969) reviewed the pattern of endemic cholera in the Matlab area of Bangladesh during 1963-1966. Another episode of infection was studied by the Joint ICMR-GWB-WHO cholera study group (1970) in a small neighbourhood in Calcutta. This community of 59 persons in 14 families lived in very crowded and unhygienic conditions. Water was collected and stored in metal or

earthenware pots. Two bacteriologically confirmed cholera cases ocurred simultaneously in one family and comprised the index cases for an outbreak. During the next 100 days of detailed surveillance, 78% of family contacts of the index cases and 63% of persons in other families became asymptomatically infected. Of the 14 families, 13 had at least one infection. Bencic and Sinha (1972) also reviewed work on cholera carriers in the Calcutta area. Although *V. cholerae* was rarely isolated from persons hospitalised with diarrhoea during January and February of each study year, it was regularly isolated from latrines and healthy carriers during these months. Clearly, healthy carriers could therefore silently continue transmission of cholera.

These observations show that the incidence of infection can be very high and that infection in a community can last for a long time even in the absence of overt disease. However, continuous transmission among humans alone is not likely to continue indefinitely for various reasons such as the high dose necessary for infection (Hornick *et al.*, 1971), the frequently short survival time of *V. cholerae* in clean water at higher ambient temperatures($20^{o}C$-$30^{o}C$) (Feachem *et al.*, 1981) and the isolation of different phage types along a chain of apparently continuous transmission (Glass *et al.*, 1983).

2.3.3 Person-to-person transmission of cholera

From the time of John Snow to the present, many reports on cholera have implicated person-to-person transmission. The main features of such transmission are poverty and lack of education. When cholera broke out in Taiwan in 1962 after 16 years absence, it lasted only three months during which there were 303 confirmed cases and 24 deaths. Yen (1964) studied this short outbreak and suggested that person-to-person transmission within households was important as shown by a 9.5% household contact infection rate and that asymptomatic carriers were probably responsible for spreading the infection from area to area.

Two outbreaks of cholera in Negros Occidental in the Philippines in 1961-1962 were thought to have been spread from person-to-person since cholera infections invariably were highly localised among close contacts, even in densely populated areas with poor sanitation (Dizon *et al.*, 1965; Joseph *et al.*, 1965; Tamayo *et al.*, 1965; Mosley *et al.*, 1965). Similar results were obtained by the Philippines Cholera Committee (1970) in studies on cholera transmission during 1967 in the fishing and farming village of Can Itom in Negros Occidental.

Certain attributes of the index case are important in person-to-person transmission. For example, infection among family contacts is high when the index case is female or a child (Oseasohn *et al.*, 1966; Khan and Shahidullah, 1979; Khan and Shahidullah, 1980). Females are more likely to contaminate stored water and food, whereas children tend to have a lower standard of personal hygiene than adults. Also, the infection rate in contacts of clinical cholera patients is high compared to that in contacts of asymptomatic carriers (Mosley *et al.*, 1968). This is probably due to the handling of patients with diarrhoea and high excretion rates of *V. cholerae*.

There are more examples indicating the occurrence of person-to-person transmission. Forbes *et al.* (1968) reviewed cholera in Hong Kong during 1961-1966 and concluded that "the main means of spread was by direct contact with a case or carrier or the consumption of food contaminated by a case or carrier". This conclusion is justified in view of the fact that the majority of people in the urban area of Hong Kong had ready access to chlorinated piped water. Also, the incidence of cholera was high among bucket latrine users compared to people who used flush toilets. Handling of such buckets is clearly more likely to result in person-to-person cholera transmission.

Person-to-person transmission was reported to be predominant in the 1970 cholera epidemic in West Africa, especially during festivals, assemblies and funerals (Merson *et al.*,1980). Survival of *V. cholerae* in human sweat can enhance person-to-person transmission (Dodin and Felix, 1972; Isaäcson and Smit, 1979).

2.3.4 Transmission of cholera by contaminated foods.

Cholera may be acquired by ingestion of food which has been contaminated by a person attending a patient, or by flies feeding on stools and vomitus of patients, or by the marine environment. The hands of cholera patients have frequently been found to harbour *V. cholerae* O1 (Neogy *et al.*, 1979) and the organism multiplies within hours in cooked foods, such as rice, to levels of 10^8/g (Kolvin and Roberts, 1982). Thus, inoculation of food with only a few organisms may rapidly result in an infectious dose sufficient to cause disease after ingestion.

Cholera attack rates in a village in Truk (Micronesia) were shown to be significantly higher in households in which the index case was a food handler or in which food had been eaten prepared by a food handler who had recently been ill, than when these variables did not apply (Holmberg *et al.*, 1984). This study also suggested that foodborne

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transmission of *V. cholerae* was more important than person-to-person spread in this particular outbreak (Holmberg *et al.*, 1984).

In Jerusalem, vegetables fertilised with contaminated night soil were thought to have caused a cholera outbreak in 1970 (Cohen *et al.*, 1971). Isaäcson *et al* (1986) isolated *V. cholerae* from carrots grown in soil accidentally contaminated with sewage. In that incident *V. cholerae* was shown to survive in the soil for up to 10 days after contamination occurred. No cholera cases resulted as the vegetables were destroyed before consumption.

Seafood is a good vehicle of *V. cholerae*. A single case of cholera occurred in Texas in 1973 and, in the summer of 1978, eight symptomatic and three asymptomatic infections in four clusters were detected in Louisiana. They were associated with the ingestion of boiled or steamed crabs. Laboratory studies showed that *V. cholerae* could be isolated from artificially infected crabs even after boiling for 8 minutes or steaming for 25 minutes. The *V. cholerae* strains isolated from seafood in Louisiana were very similar to the strain isolated from the 1973 case in Texas. This suggests that the organisms persisted in the environment or that there was a human reservoir of infection along the Gulf coast over at least 5 years (Blake

et al., 1980a; 1980b). This mechanism can also explain an outbreak in Kelantan, Malaysia, in 1969 which was described by Dutt *et al* (1971).

In 1974, Portugal reported a cholera epidemic with 2 467 confirmed cases, and 48 deaths. Most of the country was affected, but the incidence was highest in the extreme south. *V. cholerae* was isolated from sea water and shell fish, including oysters, clams, cockles and mussels from the Tavira-Faro area. Tavira and Faro are separated from the open sea by the Ria de Faro, a complex of islands and mud flats that extends approximately 50km along the southern coast. Sewage from the coastal towns emptied into the Ria. Most shellfish consumed throughout Portugal are caught in this area. Blake *et al.* (1977a) concluded that, although the reason for the rapid spread of cholera throughout Portugal was not known, contaminated shellfish shipped from the Ria de Faro caused a few cases in many different areas and the excreta of these persons contaminated the environment with vibrios whose subsequent transmission involved many different vehicles. One of these was a non-carbonated spring water bottled at a spa near Lisbon. The spring where the water was collected was found to be contaminated (Blake *et al.*, 1977b).

A similar outbreak occurred in Sardinia, Italy, between August and October 1973. Seafood was identified as the probable major source of infection in these outbreaks. There was no evidence that drinking water or raw vegetables played a role (Baine *et al.*, 1974). In 1979 (Salmaso *et al.*, 1980), the same strain of *V. cholerae* reappeared in Sardinia, resulting in 10 clinical cases and two asymptomatic infections. Again the source was seafood which was eaten raw. It had been harvested from polluted marine waters, including a salt water lagoon which received raw sewage. *V. cholerae* was isolated from the lagoon and from the seafood in the lagoon. A marine reservoir of this strain of vibrio might have been maintained from 1973-1979. Today there is enough evidence to justify such a conclusion. For example, *V. cholerae* has been isolated from sea water and from seafood in endemic as well as non-endemic areas (Colwell *et al.*,1981; Kaper *et al.*, 1979; Lee *et al.*, 1982).

Even preserved seafood can serve as a vehicle of cholera transmission. Salted fish, home-preserved and eaten whole, were thus thought to be the vehicle of transmission of cholera in Guam for six confirmed cases in 1974 (Merson *et al.*, 1977).

Seafood may also be involved in the transmission of non-O1 *V. cholerae*. Wilson (1980) reported five cases of acute diarrhoea associated with non-O1 *V. cholerae* in Florida. There was a strong association between these cases and a recent history of raw oyster consumption. *V. cholerae* non O1 was isolated from water and oysters at two places where some of the incriminated oysters had originated. This may prove to be important in relation to the epidemiology of the epidemic cholera-like illness caused by the recently identified non O1 *V. cholerae* 0139 (Ramamurthy *et al.*, 1993).

2.4 THE AQUATIC RESERVOIR AND THE ROLE OF AQUATIC ENVIRONMENTAL FACTORS IN THE MAINTENANCE OF CHOLERA OUTSIDE THE HUMAN HOST

As early as 1884 Robert Koch hypothesized that the comma-bacillus was an aquatic organism established only at the uninhabited southern edges of the Ganges and Brahmaputra deltas in India. The relationship between *V. cholerae* and water is crucial to an understanding of the epidemiology of cholera. Koch's aquatic reservoir theory was largely ignored until the 1970's when new information became available regarding the presence of atypical *V. cholerae* O1 and non-O1 in aquatic environments.

In Australia, the first locally acquired case of cholera was identified in a caravan park in 1977 (Rogers *et al.*, 1980). Only one other case occurred, 10 days after the onset of illness in the index patient. It was not clear whether the second patient was a co-primary case or whether he was infected as a result of contact with soiled garments of the first. The water supply to the caravan park, and the river system which served as the water source, yielded *V. cholerae* but no source of contamination could be found. For almost 2 years, cholera bacilli continued to be isolated intermittently from the 2 rivers which traverse sparsely populated areas. According to these workers their observations were "the first to suggest strongly that certain types of surface, and possibly subsurface, water having the right hydrogen ion concentration and containing the necessary nutrients may serve as silent sources of *V. cholerae* for long periods after contamination. Such foci might play a significant role in both the world wide dissemination of cholera and the maintenance of endemic infection in developing countries." This seminal work was carried out almost concurrently with Colwell's equally important studies on the survival of *V. cholerae* in Chesapeake Bay (Colwell *et al.*, 1977).

Two different types of data exist on the occurrence of *V. cholerae* in water. Firstly, there are many records of

V. cholerae isolations from tanks, wells, ponds, household water jars and rivers in or near communities where cholera cases or infections have been reported. The second type concern the recent isolations of *V. cholerae* O1, non-O1 and atypical O1 in water and waste water at places distant from any known human *V. cholerae* infection, such as those in the United States (Colwell *et al.*, 1981; Kaper *et al.*, 1979), England (Bashford *et al.*, 1979; Lee *et al.*, 1982), Germany (Bockemühl *et al.*, 1986), Japan (Osaka City Institute of Public Health and Environmental Science, 1983), Australia (Rogers *et al.*, 1980). This second group of findings could be explained on the basis of contamination by bird excreta. There is ample evidence of aquatic birds harbouring *V. cholerae*. Cholera bacilli were recovered from bird faeces collected in Louisiana (Roberts *et al.*, 1984), domestic geese in Florida (Schlater *et al.*, 1981), intestinal contents of mallards in Hungary (Szeness *et al.*, 1979), gulls in England (Lee *et al.*, 1982) and from aquatic birds in Colorado and Utah (Ogg *et al.*, 1989). In view of these findings and assuming that *V. cholerae* can survive in the gut of some migrating birds for a few days or longer, birds could potentially transport the organism over long distances but are not considered to be important as reservoir hosts.

A major contribution to the development of the aquatic reservoir concept was the finding that *V. cholerae* can survive in a dormant, non-culturable form in water (Xu, H-S *et al.*, 1982, Colwell and Huq, 1994). Singleton *et al.* (1982), compared quantitative methods for both culturable and non-culturable *V. cholerae*. They found that, although the method for non-culturable organisms (which demonstrates both culturable and non-culturable organisms) yielded higher bacterial counts, the latter's rate of decrease over time was similar for both methods.

Applying the concept of an aquatic reservoir to endemic cholera in Bangladesh, it was proposed by Feachem (1982) that the reservoir had gone undetected in the non-cholera season when cholera infections were not occurring locally. He suggested that the reasons for the non-detection of vibrios include their association with selected microhabitats which may have been inadequately sampled (such as plant surfaces, sediments or zooplankton), or that they exhibit biochemical or antigenic properties making them difficult to recognise by standard water sample collection and laboratory procedures.

Current evidence indicates the latter is the most likely reason. It is known that during starvation *V. cholerae* O1 and other vibrio species undergo physiological and

morphological changes. These include novel bacterial protein production and changes in fatty acids (Baker *et al.*, 1983; Guckert *et al.*, 1986; Hood *et al.*, 1986; Oliver and Stringer, 1984). It has been shown in other vibrio species that adherence properties can also be enhanced (Dawson *et al.*, 1981). However, most important may be the ability of *V. cholerae* to remain in a viable but non-culturable condition which is induced by nutrient deficient environments, as suggested by Colwell and Huq (1994).

There is ample evidence that *V. cholerae* O1 is well adapted to life in saline waters and probably forms part of the autochthonous flora of some estuarine environments (Baker *et al.*, 1983; Colwell *et al.*, 1981; Kaysner *et al.*, 1987; Tison *et al.*, 1986; Miller *et al.*, 1984; Hood *et al.*, 1981; Kaper *et al.*, 1979). Other workers found *V. cholerae* to be indigenous also in fresh water bodies in England (Lee et al., 1982), Japan (Osaka, 1983) and Germany (Bockemühl et al, 1986). Many factors affect the survival and growth of *V. cholerae* in such environments, such as zooplankton (Kaysner *et al.*, 1990; Nalin *et al.*, 1979; Tamplin *et al.*, 1990), salinity and organic nutrients (Singleton *et al.*,1982), pH, temperature, cation composition (Miller *et al.*,1984) and iron levels (Patel and Isaäcson, 1994).

2.4.1 Zooplankton and phytoplankton

Dawson *et al.* (1981) found that bacteria could survive in adverse aquatic conditions, such as a suboptimal supply of nutrients, when they were closely associated with various surfaces. Such surfaces are commonly presented to aquatic bacteria by plankton and other microscopic plant and animal life that dominate the microflora of aquatic ecosystems. An example is the finding that *V. cholerae* could survive on water hyacinth (Spira *et al.*, 1981) and on common duckweed. Such findings may be explained on the basis of dissolved organic carbon excreted by these plants and utilised by bacteria (Islam *et al.*, 1990).

Tamplin *et al.* (1990) showed that *V. cholerae* O1 concentrated on chitinous surfaces of plankton, thus increasing the number of these organisms in a given unit of water. A direct relationship between human disease and attachment of *V. cholerae* to chitinous surfaces was proposed by Nalin *et al.* (1979). These workers found that adsorption to chitin actually protected cholera bacilli by showing that they could multiply in acidic chitin-saline suspensions. It was suggested that such a protective association with chitin may operate and enable *V. cholerae* to bypass the gastric acid barrier when the organisms are ingested together with chitinous seafood.

2.4.2 Estuarine sediments, salinity, pH, temperature and cations

Research by Hood and Ness (1982) on estuarine water and sediments showed that prolonged survival of *V. cholerae* occurred in raw water, but not in raw sediment. Improved survival of *V. cholerae*, obtained in sterilized sediment, which had an abundance of nutrients, was thought to be due to elimination of competing microorganisms by the sterilization process. Prolonged survival and growth of *V. cholerae* in raw water suggested that this organism is well adapted to life in an estuarine environment.

Salinity appears to be very important for prolonged aquatic maintenance of a *V. cholerae* population. As early as 1959, Chatterjee and Gupta reported a striking correlation between the incidence of cholera in Calcutta, India and the salinity of the Hoogly River. Epidemic cholera occurred when salinity ranged from 0.01-0.1% and the highest incidence rates were associated with the highest degrees of salinity. Although *V. cholerae* required a salinity of at least 0.01% to survive beyond 24 hours, Miller *et al.* (1982) showed that higher salt concentrations are probably required for its prolonged aquatic maintenance. In 1984 these same workers reported their results of extensive studies on the survival and growth of

toxigenic *V. cholerae* O1 in water under various conditions. They found that although the optimal salt concentration for survival was 2%, good support for the organism was obtained at all concentrations from 0.25-3.0%. They also studied the interaction between temperature and salinity in determining the length of survival of the cholera bacillus. Survival was found to be longer at 25°C than at 4°C at all the concentrations of salt in the range studied (0.01%-1.0%). There was no survival at 4°C even in the presence of nutrients. An alkaline pH and cations such as Na^+ were shown to favour growth and survival of *V. cholerae*. With the provision of an estuarine level of salt concentration, a pH of 7.0-8.5 and a warm temperature, the organism survived for long periods of time without nutrients. Its mortality rate whilst in this state was very low.

It has been reported that cholera toxin may be able to alter sodium output by other aquatic organisms (Lacey, 1995). This, in turn, may enhance *V. cholerae's* own survival by stimulating the prevailing levels of salinity.

2.4.3 Iron

Enhanced survival of *V. cholerae* (Patel and Isaäcson, 1989 and 1994) and *Salmonella typhi* (Wegerhoff, 1989) in corroding metal drums used as household water storage containers has been shown to be due to iron released as the result of oxidation and which was then utilized by the organisms. The concentration of iron in well aerated water bodies is usually low. In surface waters it is generally present in the ferric (Fe^{+++}) state but, in the presence of low levels of oxygen, the ferrous (Fe^{++}) form can persist. Although iron is naturally released into environmental waters from e.g. rock, much of its presence is a result of human activities such as acid mine drainage, burning of coke and coal, human waste disposal, leaching from land fills, mineral processes and iron related industries, and the corrosion of iron and steel (WHO, 1984).

2.5 SUMMARY

V. cholerae is the causative agent of cholera and is classified serologically into groups O1 and non-O1. Group O1 comprises classical and El Tor biotypes each of which is further classified into the three serotypes Inaba, Ogawa and Hikojima.

Clinical disease is caused by the production of cholera toxin (CT) in the intestinal tract. In vitro, CT production is affected by numerous factors including the composition of the culture medium, the presence and quantity of carbon dioxide, salinity, protein synthesis inhibitors, organic nutrients and association of the organism with algae.

Water plays an important role in cholera transmission although, in most outbreaks, a combination with additional modes of spread, such as carriers, person-to-person transmission and/or contaminated food, can be demonstrated.

The possibility of an aquatic reservoir was suggested as early as 1884 by Robert Koch, but this important concept did not receive serious attention until the 1970's. Today there is ample evidence that *V. cholerae* survives well in an aquatic environment. It can, furthermore, transform into a non-culturable form which is able to withstand adverse conditions.

Various physico-chemical factors affect the survival of *V. cholerae* in the aquatic environment, including pH, salinity, organic nutrients, sediments, zooplankton, phytoplankton and other components.

Much research has been carried out on *V. cholerae* in relation to brackish water but fewer studies have been done on the organism's survival in fresh water, especially in the presence of inorganic pollutants. This literature review yielded no information on the effect of inorganic water pollutants on the toxigenicity of *V. cholerae*. These aspects are subject to the investigations described in this thesis.

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CHAPTER 3

STUDY DESIGN

Epidemic cholera which, by the end of the nineteen fifties, had been driven back mainly to the Indian subcontinent, resumed its world wide spread in the early nineteen sixties. After the end of World War II, economic rebuiling and decolonisation resulted in a vast increase of industrialisation. The potential dangers of industrial pollution, especially of water, were at first not generally appreciated. Air pollution, so much more visible, was one of the earliest concerns about pollution. Public concern about water pollution tended to lag behind.

The infectious dose required for clinical cholera to manifest itself is several logs (base 10) higher than the concentration of free living cholera bacilli per unit volume found in natural marine or fresh water bodies. Sufficient bacterial multiplication must therefore occur prior to ingestion of such water by a susceptible host. It is likely that several physico-chemical factors in the aquatic environment may promote the survival and multiplication of *V. cholerae*, resulting in overt disease in the community concerned.

A previous study (Patel and Isaäcson 1989) on the survival time of *V. cholerae* in African rural domestic water storage containers showed that this organism survived much longer in corroding metal water storage drums than in plastic, clay or wooden containers. The improved survival was shown to be due to the presence of insoluble iron. These observations stimulated questions concerning the possible role of iron as a bacterial growth stimulating factor in natural surface water bodies when these become contaminated with *V. cholerae*. Such questions related more specifically to two different settings.

The first setting relates to rivers and streams which are sometimes polluted by mining and other industrial effluents containing high levels of iron. The Gauteng province of South Africa comprises the Pretoria/Johannesburg/ Vereeniging axis with its satellite towns and surrounds. It is the country's commercial centre and its most heavily industrialised province.

The Department of Water Affairs is responsible for the management of all South African water resources and in Gauteng the Department supplies raw water to Rand Water, a major water supply company formerly known as the Rand Water Board. Rand water purifies the water for bulk supply to local authorities, mines and other large industrial

concerns. Various local authorities are responsible for redistribution of the purified water to domestic and other consumers.

In Gauteng, routine periodic monitoring by Rand Water of industrially polluted water bodies has shown the pH to vary from 3 to 9 (Rand Water Board, 1987). There usually was a direct relationship between the degree of pollution and the pH level with the more extreme pH values being more frequent in heavily polluted waters. Iron concentrations ranged from <0.05-1000 mg/l water. The possible effects of different pH and iron levels on cholera survival in this polluted water have never been investigated. During recent years, vast numbers of people, estimated at more than a million, have moved into Gauteng where many have settled in squatter camps on the banks of rivers and streams which often serve as a source of domestic water.

The second setting involves domestic water distribution systems, many of which still consist largely of metal piping instead of the synthetic materials commonly used in modern construction. Problems arising from the presence of iron in drinking water may affect its appearance and taste. Iron does, however, also create more serious problems. Ferrous salts are unstable at the pH (neutral) usually *pertaining to potable water supplies and they precipitate*

as insoluble ferric hydroxide which settles out as a rust coloured silt. With time, iron settling out in distribution systems tends to reduce the water flow rate. It also promotes proliferation of 'iron bacteria' such as *Sphaerotilus species* which oxidize iron from the ferrous to the ferric state. This manifests as a slimy coating on the interior of the piping (Dondero, 1975). These problems generally arise when the iron concentration in the water distribution system approaches 0.3 mg/l (WHO, 1984).

Oxidation of such piping may also lead to cracks in the system through which various bacteria, including intestinal pathogens such as *V. cholerae*, may gain access from the surrounding soil, especially where sewerage systems are absent, or also old, cracked and in poor repair. Repairs on piping in themselves can create opportunities for bacterial invasion. Lack of proper chlorination, or the absence of any water treatment can, under these circumstances, result in outbreaks of water borne diseases.

Several questions arose that needed answering:

- Is internal corrosion of water distribution systems likely to enhance growth and survival of *V. cholerae*?

Briefly, this question was studied in chapter 4 by culturing six strains of *V. cholerae* (non-O1, O1 classical and O1 El Tor) at ambient temperature in sterile distilled water. Iron (ferric oxide, an insoluble form) was added to obtain a series of concentrations ranging from 0-2 g/l and pH was adjusted in a range of values from 5 to 11 which is a range outside which *V. cholerae* fails to grow or grows poorly (Pollitzer R, 1959).

The results obtained with the various pH/iron combinations were subjected to 1-way and 2-way statistical analyses of variance (ANOVA) to determine the effect, if any, of iron and pH, separately and in combination, on the survival time of *V. cholerae*.

- Is the growth and length of survival of *V. cholerae* affected by the pH and iron levels in industrially polluted waters? If yes, do pH and iron levels interact in such an effect?

To address this question, water samples from industrially polluted surface waters (rivers and streams) in Gauteng were collected monthly for one year. Rand Water was approached to assist in identifying 12 suitable water bodies, collection of the samples and provision of their initial pH and iron values. *V. cholerae* (1 strain each of

non-O1, O1 classical and O1 El Tor) were inoculated into the samples to determine their surival time under ambient temperature and prevailing pH and iron concentration. The samples were first filtered or autoclaved to prevent overgrowth of *V. cholerae* by other bacteria present in water. The presence of *V. cholerae*, quantitatively and in terms of length of survival was determined by means of common bacteriological methods.

The results were statistically analysed by means of a 1-way ANOVA. The study will be covered in full in chapter 5.

- Finally, does iron concentration in water affect cholera toxin production by *V. cholerae*?

Virulence factors of some systemically invasive bacteria (e.g. *Yersinia pestis* and *Salmonella typhi* are stimulated in vivo by increased iron levels. In contrast, *V. cholerae* is a non- invasive bacillus which exerts its pathogenic effect only by virtue of its enterotoxin which is produced in the lumen of the bowel. If the growth of *V. cholerae* is stimulated by increased iron in its aquatic environment, the question arises if iron may at the same time enhance the toxigenicity of this organism. An answer to this important question has not been found in the literature.

For this study, known concentrations of purified CT were assayed to produce a titration slope for calculation of the amounts of toxin produced by the cholera strains under study. Eight toxigenic strains of *V. cholerae* including 0139 (Bengal), and two non-toxigenic strains of *V. cholerae* were cultured in the presence of iron concentrations ranging from 0-10.0 g/l. Their cholera toxin (CT) producing ability were then investigated by means of a microtitre ganglioside ELISA and a Y_1 mouse adrenal cell line assay. Significance of the results was calculated by means of multiple regression analysis. This study and its results are detailed in chapter 6.

The findings of the three studies are summarised and an attempt is made to link and reconcile them by constructing a model illustrating the importance of iron in 'pure' reticulated water and of industrial water pollution in relation to the ecology and epidemiology of cholera today (chapter 7).

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CHAPTER 4

THE INTERACTION OF pH AND IRON IN THEIR EFFECT ON THE SURVIVAL OF *V. CHOLERAE.*

4.1 INTRODUCTION

The effect of iron, one of the major industrial pollutants, and of pH on the survival of *V. cholerae* is investigated in this chapter which attempts to answer the question 'Is the internal corrosion of water distribution systems likely to enhance the growth and survival of *V. cholerae*?.

The effect of individual environmental variables on a population of microorganisms is difficult to assess in situ because of constantly changing environmental conditions. The study described in this chapter was therefore done at ambient temperature in otherwise controlled laboratory conditions.

4.2 MATERIALS AND METHODS.

Details of the media and solutions used in this study may be found at the end of this chapter.

4.2.1 Bacterial cultures and inocula.

Six strains of *V. cholerae* were used in this study. Their characteristics and origin are presented in table 4.1.

All the *V. cholerae* cultures had been maintained in semi-solid agar at room temperature. For this study they were subcultured on blood agar plates and incubated at 37°C for 24 hours. A loopful (2 mm diameter) of growth was picked off each plate and stabbed into 10 ml aliquots of alkaline peptone water (APW) which were then incubated overnight. Counts of *V. cholerae* in the inoculum were based on a microdilution technique (Kramer, J., 1977) (with minor modifications) and consistently found to be in the order of 10^9 CFU (colony forming units) per ml.

Minor modifications to Kramer's method included:

- the use of blood agar instead of plate count agar as this facilitated visual differentiation between *V. cholerae* and other organisms.
- Four droplets (20 μl), instead of remaining in situ, were allowed to run in parallel lines across one blood agar plate per dilution. This prevented confluent bacterial growth, enabling more accurate colony counts.

TABLE 4.1 Vibrio cholerae cultures used in this study.

Ref no.	Origin	Biotype	Serotype
VC1	Unknown	-	non 01
VC2	Environmental (Umgeni river)	-	non 01
VC3	Clinical strain (S.A patient)	El Tor	01 Inaba
VC4	Clinical strain (S.A patient)	El Tor	01 Inaba
VC5	Clinical strain (Bangladeshi patient)	Classical	01 Inaba
VC6	CDC vaccine strain	Classical	01 Ogawa

After incubation and performance of the *V. cholerae* count, each 10 ml aliquot of APW was centrifuged at 4000 rpm. The bacterial cells were washed twice with quarter strength Ringers solution and about 1 ml fluid, containing the final sediment, was resuspended in 9 ml of quarter strength Ringers solution. This reconstituted the original concentration of cells to approximately 10^9 CFU/ml.

4.2.2 Solubility of ferric oxide in water

To ensure that ferric oxide would remain in water as an insoluble compound, thus giving consistant results for the *V. cholerae* survival study, the following determinations were done.

Eight sterile glass beakers were each prepared with 1 litre sterile distilled water and ferric oxide (Fe_2O_3) concentrations ranging from 0-2.0 g/l. The beakers were allowed to stand at room temperature for two weeks. The water samples were then analysed (by the inductive couples plasma method) for the level of soluble iron by Rand Water, Vereeniging, South Africa.

4.2.3 *V. cholerae* survival studies

Further series of 32 beakers with one litre distilled water

were prepared as described above (section 4.2.2). For each iron concentration, the experiment was run at pH 5.0, 7.0, 9.0 and 11.0. The beakers were covered with glass petri dishes and autoclaved. After autoclaving, the pH was measured again but readjustment was never found necessary. Each beaker was then inoculated with 0.25 ml of bacterial suspension to obtain a final count of approximately 10^5 to 10^6 CFU/ml. After inoculation the beakers were kept at ambient temperature.

The beakers were sampled daily by slipping a sterile pasteur pipette aseptically under the covering petri dish, stirring the contents and removing aliquots of approximately 3 ml. The aliquots were subcultured in 10 ml APW which was incubated at 37°C for 24 hours. Occasional scant contamination by gram positive bacteria occurred. This did not interfere with the recognition and selection of suspected *V. cholerae* colonies which, from time to time, were tested biochemically and/or serologically to confirm their identity. This procedure was continued daily until three consecutive negative results were obtained. This study was repeated once.

4.2.4 Statistical analysis

Where indicated, analysis of variance (ANOVA) was performed

to assess the significance of the effect of pH and iron, separately and combined, on the bacterial survival data.

The Epi-Stat computer application, version 3, developed by Tracy L Gustafson, 1984, which includes, amongst other statistical analyses, the ANOVA methods (1-way and 2-way) was used for this purpose.

4.3 RESULTS

4.3.1 Solubility of ferric oxide in water

Analysis for the presence of total soluble iron showed similar, minute quantities ($1.7x10^{-5}$ to $6.4x10^{-5}$ g/l) in all water samples containing the insoluble Fe_2O_3 (0.005-2 g/l) as well as in the control sample without added Fe_2O_3 (table 4.2).

4.3.2 *V. cholerae* survival study

The results are presented in tables 4.3-4.5 each of which compares the survival of *V. cholerae* at three different pH values of pH 7, 9 and 11 and in the presence of increasing amounts of Fe_2O_3 (0-2 g/l).

TABLE 4.2 Solubility of ferric oxide as measured 14 days after addition of the oxide to water.

Ferric oxide g/l	Soluble iron g/l
0	0.000021
0.005	0.000029
0.01	0.000032
0.05	0.000041
0.1	0.000017
0.5	0.000036
1.0	0.000042
2.0	0.000064

TABLE 4.3 Survival of V.cholerae non-O1 (VC1 and VC2) in sterile distilled water at increasing pH values and iron concentrations.

Fe_2O_3 g/l	Survival of V.cholerae non O1 (No. of days*)					
	VC1			VC2		
	pH 7	pH 9	pH 11	pH 7	pH 9	pH 11
0	0.5±0	8.0±1.41	13.5±2.12	1.0±0	15.5±2.12	42.5±2.12
0.005	0.5±0	10.5±0.71	17.0±0	2.5±0.71	17.5±0.71	38.5±3.54
0.01	0.5±0	10.0±1.41	17.0±0	3.5±0.71	29.0±1.41	57.5±3.54
0.05	2.0±0	10.0±1.41	19.5±2.12	5.0±0	24.5±0.71	77.5±6.36
0.1	4.5±2.12	10.5±0.71	18.0±4.24	6.5±0.71	20.5±0.71	42.0±8.49
0.5	11.0±2.83	8.5±0.71	18.0±4.24	9.5±0.71	19.0±1.41	63.5±14.85
1.0	10.0±2.83	5.0±2.83	17.5±4.95	10.0±1.41	18.5±0.71	41.5±0.71
2.0	6.0±2.83	5.0±2.83	7.0±1.41	1.5±0.71	17.5±0.71	39.0±1.41

* mean ± SD

TABLE 4.4 Survival of V.cholerae O1 El Tor (VC3 and VC4) in sterile distilled water at increasing pH values and iron concentrations.

Fe_2O_3 g/l	Survival of V.cholerae O1 El Tor (No. of days*)					
	VC3			VC4		
	pH 7	pH 9	pH 11	pH 7	pH 9	pH 11
0	0.5±0	7.0±1.4	15.0±9.8	1.5±0.7	4.5±0.7	22.5±6.4
0.005	0.5±0	12.0±0	34.0±1.4	4.5±0.7	10.5±0.7	55.5±9.2
0.01	0.5±0	10.5±3.5	43.5±14.8	5.0±0	25.5±2.1	49.0±4.2
0.05	1.0±0	18.0±4.2	37.5±12.0	5.5±0.7	22.5±2.1	52.5±5.0
0.1	3.0±1.4	16.5±3.5	28.5±0.7	9.0±2.8	16.0±4.2	57.0±5.7
0.5	2.0±0	6.0±1.4	9.5±2.1	11.5±0.7	16.5±3.5	62.0±8.5
1.0	2.0±0	1.5±0.7	8.5±0.7	11.0±0	18.0±5.7	35.0±14.1
2.0	1.0±0	1.5±0.7	4.0±4.2	4.0±4.2	13.5±5.0	31.5±14.9

* mean ± SD

TABLE 4.5 Survival of V.cholerae O1 classical (VC5 and VC6) in sterile distilled water at increasing pH values and iron concentrations.

Fe_2O_3 g/l	Survival of V.cholerae classical (No. of days*)					
	VC5			VC6		
	pH 7	pH 9	pH 11	pH 7	pH 9	pH 11
0	0.5±0	10.5±2.1	20.5±0.7	1.5±0.7	10.0±1.4	23.5±0.7
0.005	0.5±0	14.0±2.8	36.5±2.1	2.0±0	15.5±0.7	23.0±0
0.01	0.5±0	13.5±2.1	50.5±17.6	3.0±0	19.5±0.7	36.0±2.8
0.05	2.0±0	16.0±0	51.5±4.9	3.5±0.7	20.0±0	51.5±6.4
0.1	4.5±0.7	19.0±4.2	45.0±1.4	6.0±0	14.0±1.4	50.0±8.5
0.5	6.0±5.6	11.0±2.81	30.0±2.8	9.5±0.7	12.0±0	32.5±0.7
1.0	2.0±0	9.0±0	26.0±2.8	2.0±0	8.5±0.7	26.0±1.4
2.0	1.5±0.7	3.0±2.8	20.0±0.7	1.5±0.7	5.5±0.7	12.5±0.7

* mean ± SD

At pH 5, survival of *V. cholerae* was less than 1 day irrespective of the iron concentration and these data were omitted. The longest survival was obtained at pH 11 where it was 77.5 days for *V. cholerae* non-O1 (VC2, table 4.3), 62 days for El Tor (VC4, table 4.4) and 51.5 days for the classical strains (VC5 and VC6, table 4.5). The patterns of survival response to increasing pH values and iron concentrations did not differ markedly from strain to strain.

With all six strains combined it was found that, at pH 7, the peak effect of iron on survival times occurred at a concentration of around 0.1-1.0 g/l, at pH 9 it occurred at 0.01-0.1 g/l and at pH 11 enhanced survival occurred over a broader range of iron concentrations with peaks at 0.01-0.1 g/l (figures 4.1-4.3).

The significance of these apparent responses to pH and iron was assessed by performing analyses of variance (ANOVA) on the survival data. A 2-way ANOVA was done on each strain separately to assess the individual effect as well as the interaction of pH and iron concentration on bacterial survival. By this means it was shown (table 4.6) that the survival increased with increasing pH from 7-11 and that the effect of pH was predominant for each strain ($p < 10^{-6}$).

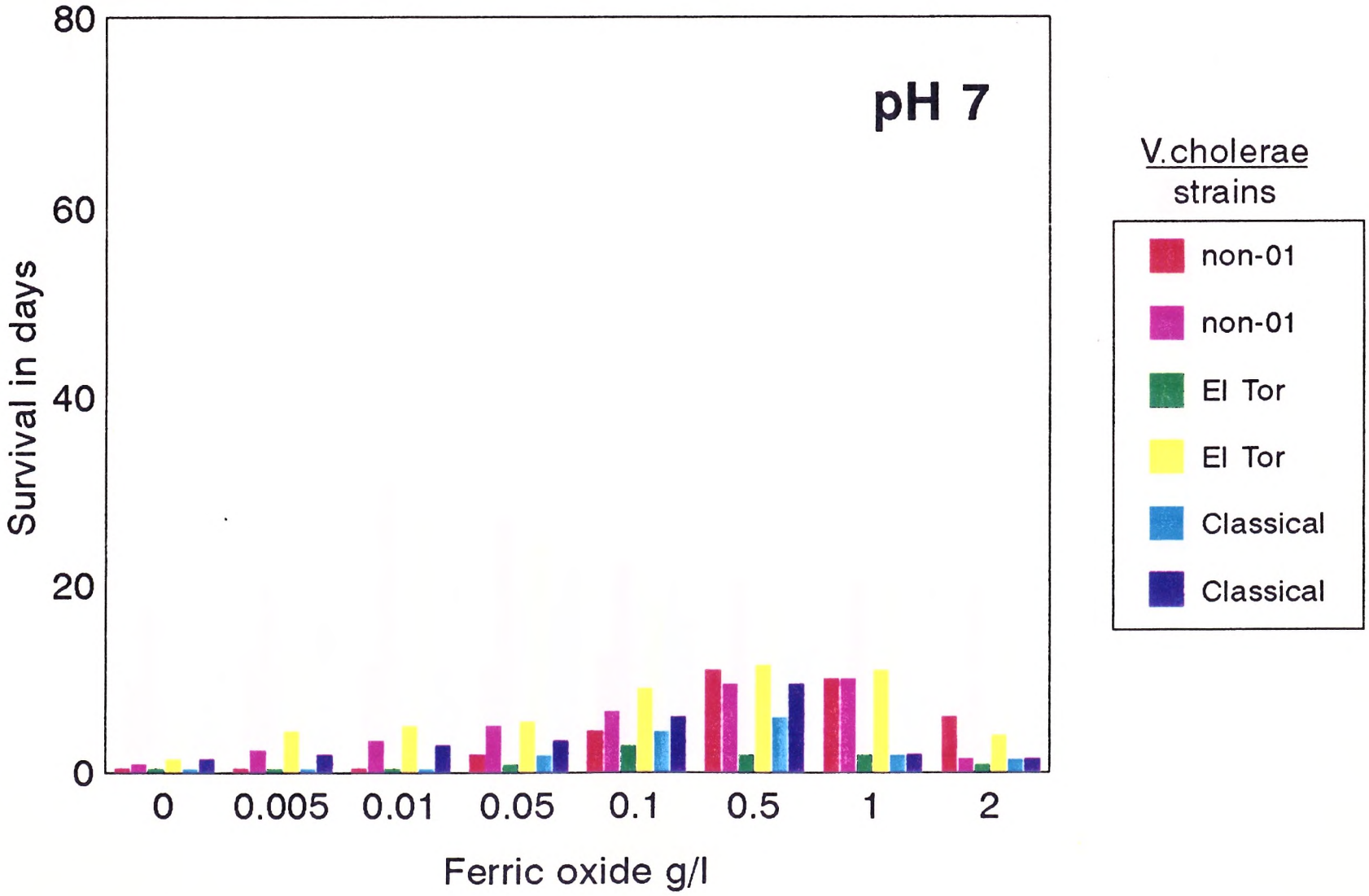

Fig 4.1 Survival of V.cholerae in sterile distilled water at pH 7 and in the presence of increasing iron concentrations.

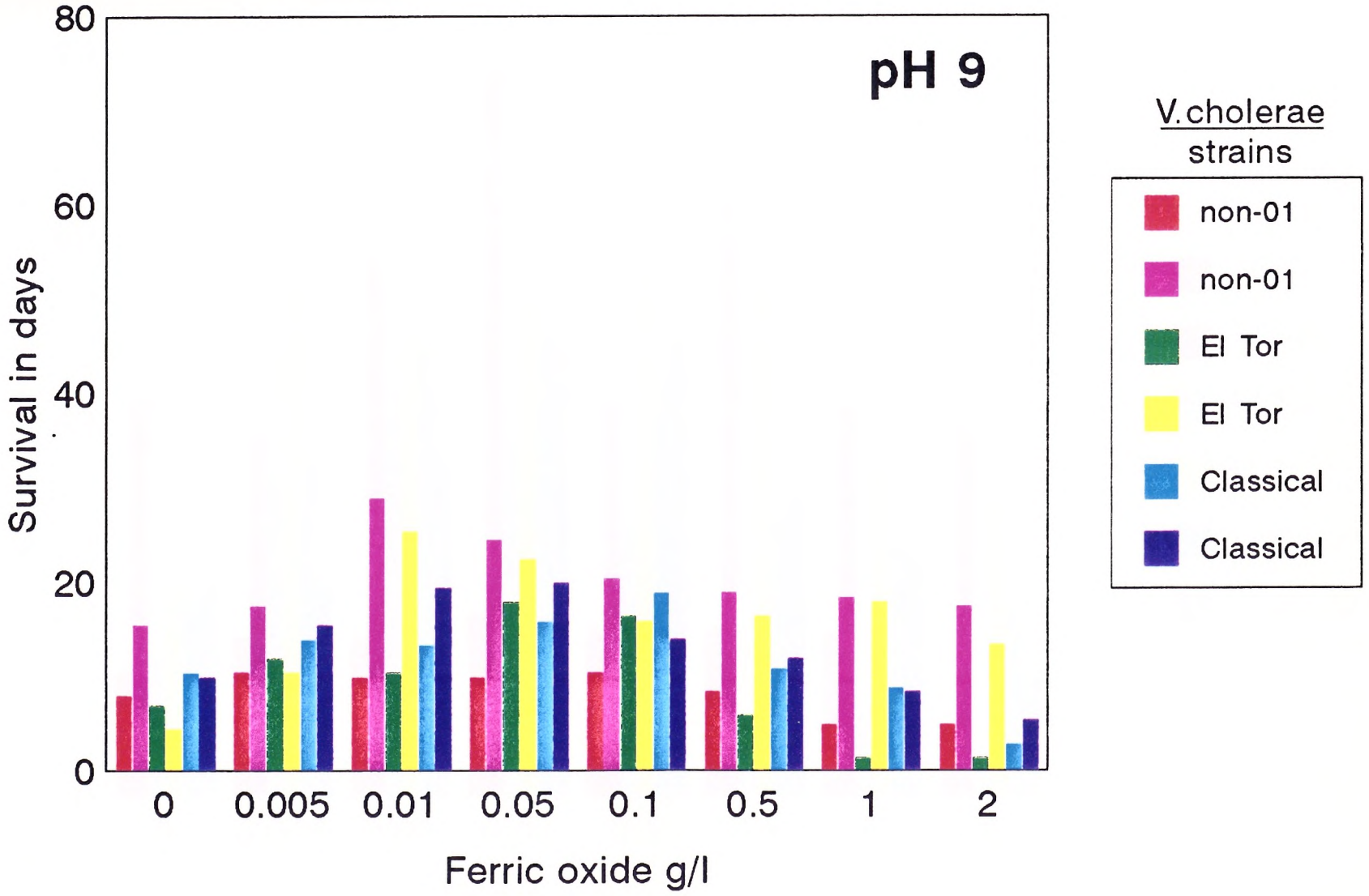

Fig 4.2 Survival of V.cholerae in sterile distilled water at pH 9 and in the presence of increasing iron concentrations.

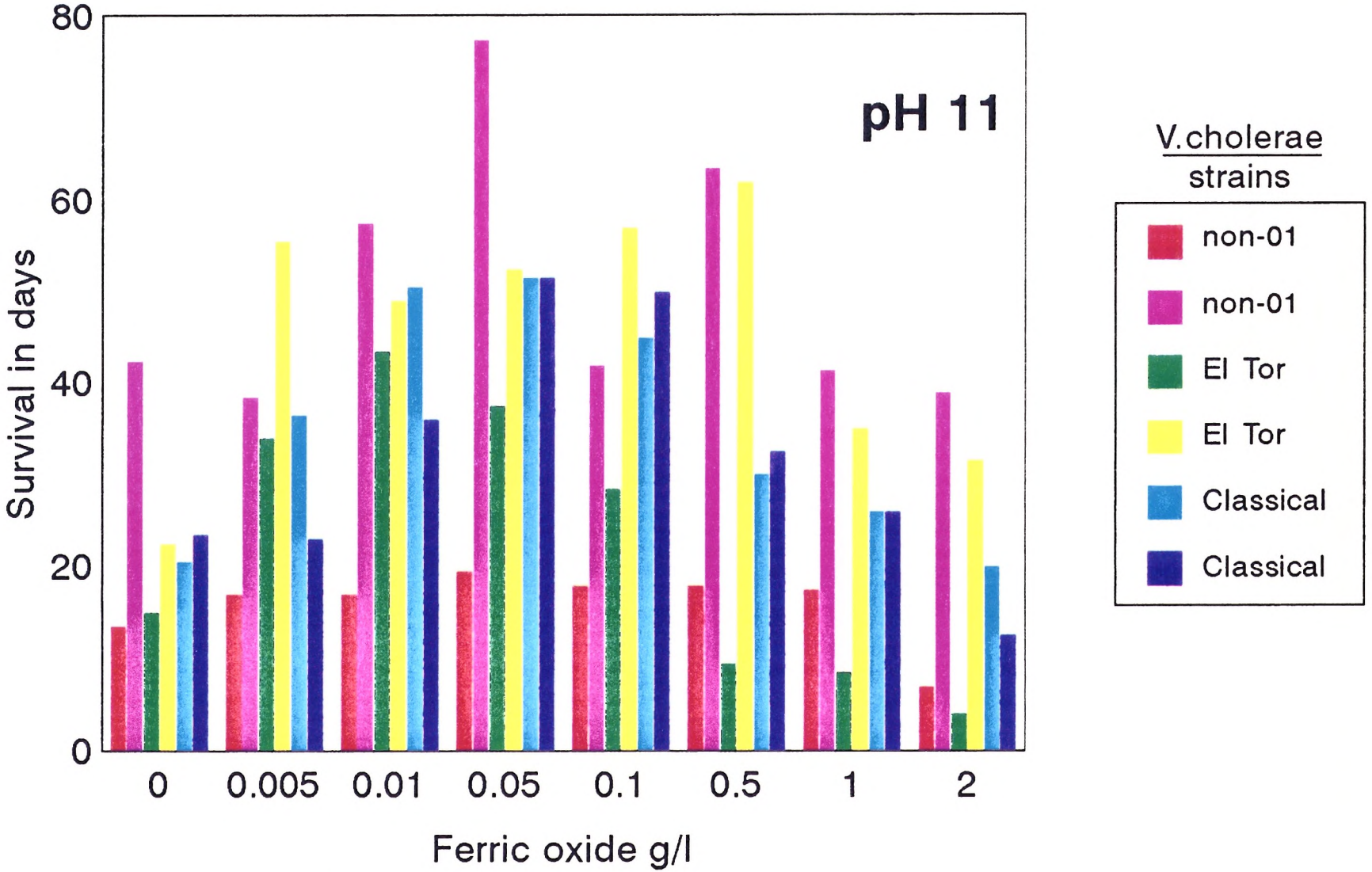

Fig 4.3 Survival of V.cholerae in sterile distilled water at pH 11 and in the presence of increasing iron concentrations.

TABLE 4.6 Statistical significance (by 2-way ANOVA) of V.cholerae survival differences in sterile distilled water at increasing pH values and iron concentrations.

Cholera strain	Significance of effect by		
	pH p=	Iron p=	Interaction of pH and iron p=
Non-O1			
VC1	$<10^{-6}$	$< 5x10^{-3}$	$< 5x10^{-4}$
VC2	$<10^{-6}$	$<10^{-6}$	$<10^{-4}$
El Tor			
VC3	$<10^{-6}$	10^{-6}	10^{-4}
VC4	$<10^{-6}$	$<10^{-4}$	$< 5x10^{-3}$
Classical			
VC5	$<10^{-6}$	$<10^{-5}$	$< 5x10^{-4}$
VC6	$<10^{-6}$	$<10^{-6}$	$<10^{-6}$

The effect of iron was also quite significant (p ranging from $<10^{-6}$ to $<5x10^{-3}$) and so was the interaction of the two factors (p ranging from $<10^{-6}$ to $<5x10^{-3}$).

A 1-way ANOVA was performed in each pH category on all strains combined and on each strain individually to assess the effect of iron, in the presence of a constant pH value, on *V. cholerae* survival. With the data of all the strains combined, iron was shown to significantly prolong survival ($p < 10^{-6}$). Thus, with increasing iron concentrations, *V. cholerae* survival increased from 0.5-11 days at pH 7, from 4.5-29 days at pH 9 and from 13.5-77.5 days at pH 11.

The ANOVA on the individual strains yielded mostly significant and some non-significant results (table 4.7). The latter are thought to be due to the use of individual strains resulting in too few data (4) in each category.

4.4 DISCUSSION

Although much is known about *V. cholerae* survival in salt water, there is less knowledge about the effects on *V. cholerae* survival of different factors in 'clean' or chemically polluted fresh water. Rivers are often shown to be contaminated with cholera bacilli during epidemics and in cholera endemic areas (Lee *et al*, 1982; Venkateswaran *et*

TABLE 4.7 Statistical significance (by 1-way ANOVA) of the effect of iron on the survival of V.cholerae in sterile distilled water at different pH levels.

Cholera strains	pH7 p=	pH9 p=	pH11 p=
Non-O1 (VC1+VC2)	$<10^{-6}$	0.7	0.7
El Tor (VC3+VC4)	0.2	< 0.05	0.07
Classical (VC5+VC6)	$<10^{-3}$	$<10^{-5}$	$<10^{-6}$
All strains combined	$<10^{-6}$	$<10^{-5}$	$<10^{-4}$

al, 1989; Bockemühl *et al*, 1986). In non-endemic areas, fresh water has been known to be contaminated with *V. cholerae* by migratory water birds. In fresh water settings, often far removed from coastal regions but subject to major sources of industrial pollution, it is unknown what the potential would be for survival of this organism which is so harmful to the physical and economical well-being of significant sectors of the world's populations.

An important objective of this study was to evaluate the growth and survival of *V. cholerae* in water under controlled conditions of pH and iron content as a preliminary to a study on its survival in industrially polluted water (chapter 5).

V. cholerae grows better at alkaline than at neutral pH and is highly sensitive to acidic conditions (Sakazaki, R., 1992). The findings with respect to the organism's failure to survive at pH 5 and their improved survival at pH 9 and 11 were therefore not new and not unexpected. In contrast, the findings with regard to the effect of iron, and the interaction of iron and pH on survival of *V. cholerae*, have not previously been reported by other workers.

As inter-strain variation in the response of *V. cholerae* to various factors is known to occur, six different strains were used in this study. Iron significantly boosted survival times, especially in the case of the classical biotype of *V. cholerae* O1 where, despite relatively few data, a significant lengthening of survival was found at all three pH values (7, 9 and 11). The non-O1 strains responded in this manner only at pH 7 whereas El Tor survival was enhanced more markedly by iron increases at pH 9 and pH 11. From a practical point of view the findings obtained in water at pH 11, although similar to those at pH 9, are less important since very high pH values were uncommon, even in polluted water. Although *V. cholerae* non-O1 is thought to survive better in the environment, it is still a matter of debate as to which of the two O-group vibrios, classical or El Tor, is environmentally better adapted.

The results of the 2-way ANOVA indicates that there seems to be a marked interaction between pH and iron levels as far as their prolongation of *V. cholerae* survival time is concerned.

It was mentioned earlier (section 2.2.2.4) that, like many other bacteria, *V. cholerae* produces an iron chelator or siderophore, known as vibriobactin (Griffiths *et al*, 1984)

which serves to solubilise and transport iron into the cell under conditions of iron limitation due to either an inadequate amount of iron or to its presence in an insoluble form. In this way bacteria can utilise the insoluble ferric ions as terminal electron acceptors for anaerobic respiration, thus producing the soluble ferrous ion. The strains of cholera bacillus used in this study may have produced vibriobactin which promoted their survival for prolonged periods of time in the presence of insoluble iron in water without any other nutrients.

These findings may also have implications in quite a different man-made setting, namely that of piped, potable water distribution systems in older, urban settlements. Many urban settlements that were established in the pre-plastics era, have water reticulation systems with metal piping which is subject to internal corrosion (figure 4.4). This is due to the action of numerous factors including water itself and chlorine, and results in the formation of pitting and crevices as well as residual deposits of corroded particles (Clark *et al*, 1994). Such systems, or parts thereof, may periodically be shut down for repairs when their internal integrity is breached and potentially at risk of contamination with bacterial pathogens such as, for example, *V. cholerae* introduced from the external environment. Another source of such contamination may be

Fig 4.4 Metal water conduit clearly showing iron corrosion tubercles.
Courtesy of Houseman (Burnham) Limited, England.



the water supply itself if adequate chlorination is not practiced. Whichever the source, once bacteria gain access to such a reticulation system, it is likely that they will survive and multiply. This was demonstrated with, for example, *Legionella pneumophila* by Fisher-Hoch *et al* (1982).

Extensive research has shown increased microbial resistance due to clumping, aggregation, particle association and modification of growth conditions. During a recent cholera outbreak in Peru, a very rough form of *V cholerae* was isolated and found to be highly resistant to chlorination, much more so than the common smooth strains. This resistance was thought to be promoted by the large aggregates formed by this rough strain. A further factor contributing to the chlorine resistance was a mucoid coating of the organism. According to Clark *et al* (1994) such large bacterial aggregates are easily removed by filtration of water prior to its entry into a distribution system. However, if a rough variant were to enter the system through a breach, it would be difficult to control its subsequent multiplication by chlorination procedures.

Other workers have shown a relationship between corrosion of iron pipes and bacterial resistance to disinfection. LeChevallier *et al* (1993) showed that even low levels of

corrosion can interfere with disinfection by free chlorine. It is thought that the corrosion product reacts with chlorine and prevents it from inactivating bacteria. Chlorine itself is a strong oxidant which increases the rate of corrosion of certain metal objects such as iron pipes. These workers also showed that microorganisms can localise in crevices between corrosion nodules where physical protection than affords increased resistance to disinfectants. These and other studies (Opheim, D. *et al*, 1988; Emde, K.M.E. *et al*, 1992; LeChevallier *et al*, 1987) have shown that various species of coliform bacteria grew in iron tubercles within water distribution pipelines.

4.5 CONCLUSIONS

This study investigated the survival of *V. cholerae* in sterile distilled water which pH and iron levels were carefully measured and controlled. No other nutrients were added. The following were found and have not been reported by other workers:

- *V. cholerae* survival time significantly increased with increasing iron levels up to optimum values of 0.1-1.0 g/l (at pH 7) and 0.01-0.1 g/l (at pH 9 and 11) above which survival decreased. At pH 5, *V. cholerae* survival time was less than 1 day. Although survival was longest at pH 11,

this finding was recongnised to be of little practical value when a subsequent study on industrially polluted surface waters (chapter 5) found pH values to range commonly between 8 and 9 but not to exceed 9.2. Treated water commonly has a neutral pH.

- A 1-way analysis of variance (ANOVA) indicated that, at constant pH levels, iron significantly increased the survival time of all strains of *V. cholerae* studied. A 2-way ANOVA showed that pH and iron significantly interacted to enhance the survival of these organisms.

Other workers have observed that iron corrosion tubercles found in metal water piping and pumping systems can neutralise chlorine and provide shelter to bacteria. It is hereby suggested that such tubercles may, in addition, serve as a source of iron to *V. cholerae* (and other bacteria) that may have gained access to such systems.

Therefore, the finding that *V. cholerae* survival in dechlorinated tap water at neutral and alkaline pH was stimulated significantly by added iron (ferric oxide), has important implications in terms of a possible causal relationship between corroding water reticulation systems and the occurrence of epidemic cholera in urban and rural settings.

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4.7 APPENDIX

1. Blood Agar

Oxoid Columbia agar base.....100 g

Demineralised water.....2.5 l

These are well mixed, autoclaved at 121°C for 30 minutes and then cooled to 50°C. With aseptic precautions sterile, citrated horse blood (at room temperature) 100ml is added; pH is adjusted to 7.5.

2. Semisolid agar

a Bacto agar.....90 g

Demineralised water.....8 l

These are mixed and autoclaved at 121°C for 15 minutes and kept in a hot steamer.

b Nutrient broth No 2.....250 g

Demineralised water.....2 l

These are heated in a steamer to dissolve.

The two mixtures (a) and (b) are added to each other while hot, and, if necessary, the pH is adjusted to 7.2 - 7.4. The medium is distributed in 15ml bottles and autoclaved at 116°C for 10 minutes.

3. 1/4 Strength Ringer Solution

Sodium chloride.....2.25 g
Potassium chloride.....0.105 g
Calcium chloride.....0.12 g
Sodium bicarbonate.....0.05 g
Distilled water.....1.0 l

These ingredients should be dissolved and autoclaved at 121°C for 15 minutes, pH is adjusted to 7.0.

4. Alkaline peptone water

Tryptone.....40 g
Sodium chloride.....20 g
Demineralised water.....4.0 l

These should be well mixed to dissolve and the pH adjusted to 8.4. The medium is distributed and autoclaved at 116°C for 5 minutes.

CHAPTER 5

SURVIVAL OF *V. CHOLERAE* IN WATER POLLUTED BY INDUSTRIAL EFFLUENTS.

5.1 INTRODUCTION

High levels of iron may be found in surface waters as a result of the discharge of certain industrial effluents. This iron, often enters into streams in a soluble form but rapidly becomes insoluble. Iron commonly occurs in two oxidation states, the ferrous state (Fe^{++}) and the ferric state (Fe^{+++}), in surface waters. The redox potential and pH of the aquatic environment play major roles in determining the behaviour of iron in water. In the presence of oxygen, ferrous iron is oxidised to the ferric state which precipitates. Iron in surface waters is therefore usually found as a colloid suspension of ferric hydroxide, which may settle out and harden to a cement-like material. In reducing conditions, ferrous iron may persist and occur in high concentrations, provided that sulphides and carbonates are absent. In the presence of oxygen and pH values less than 3.0, the soluble ferric state of iron is dominant, while under neutral and alkaline conditions, ferric hydroxide and ferric oxide complexes are formed.

Pollution of surface and ground water poses a threat, especially in heavily industrialised areas where the quality of raw water tends to deteriorate. Gauteng, which is the most heavily industrialised area in the country, seemed an appropriate study site to test some of the questions in this thesis, namely 'Is the growth and survival of *V. cholerae* affected by the pH and iron levels in industrially polluted waters? If so, do iron and pH interact in such an effect?'

To monitor pollution, Rand Water routinely inspects the catchment areas from which it draws its raw water. Samples of river water are taken regularly for chemical, bacteriological and biological analyses. The samples used in this study were collected from these same catchment areas.

5.2 MATERIALS AND METHODS

Details of the media and solutions used in this study may be found in the appendix at the end of the previous chapter (section 4.7).

5.2.1 Water sampling sites

Twelve water sampling points where industrial effluents are discharged into rivers or streams were selected to obtain a broad range of iron concentrations (figures 5.1, 5.2 and table 5.1). These points had shown fairly consistent iron levels during the previous 10 years (Rand Water Board, 1987).

5.2.2 Collection and pre-inoculation testing of water samples.

Samples of approximately 5 litres water each were collected from each sampling point once a month in chemically clean plastic bottles by Rand Water who also measured their pH and total iron levels as part of its routine monitoring programme. Chemical analyses were done by means of an Inductive Couples Plasma method. Samples were refrigerated after collection and transported in cool boxes. On arrival in the laboratory total bacterial counts were performed on the water samples by using a pour plate technique. Coliform and *E. coli* type I counts were done once only, on the first monthly series of samples, using the Most Probable Number method (Hoskins J.K., 1934). Before inoculation with *V. cholerae*, water samples were also cultured in nutrient broth to determine the presence of

TABLE 5.1 Sampling sites for the collection of industrially polluted water samples (April 1989 - January 1990).

Site Codes	Sampling Sites
N7	Natalspruit at Alrode
E7	Elsburgspruit at Elsburg Town
E17	Elsburgspruit at Heidelberg Road
V17	Barrage outlet - Vaal river downstream of Vaal Dam
V18	Lindequesdrift Weir - Vaal River downstream of Vaal Dam
T1	Taaibosspruit Weir
R5	Rietspruit at Hardman's Farm
R4	Rietspruit at Alberton, Heidelberg
E11	Stream from Wadeville at South Road Elsburg Spruit system
WJ	Jordaanspruit downstream of Bethlehem Vaal Dam catchment area
WC	Cornelius River - downstream of Warden Vaal Dam catchment area
VAS	Vaal above Standerton - Vaal Dam catchment area

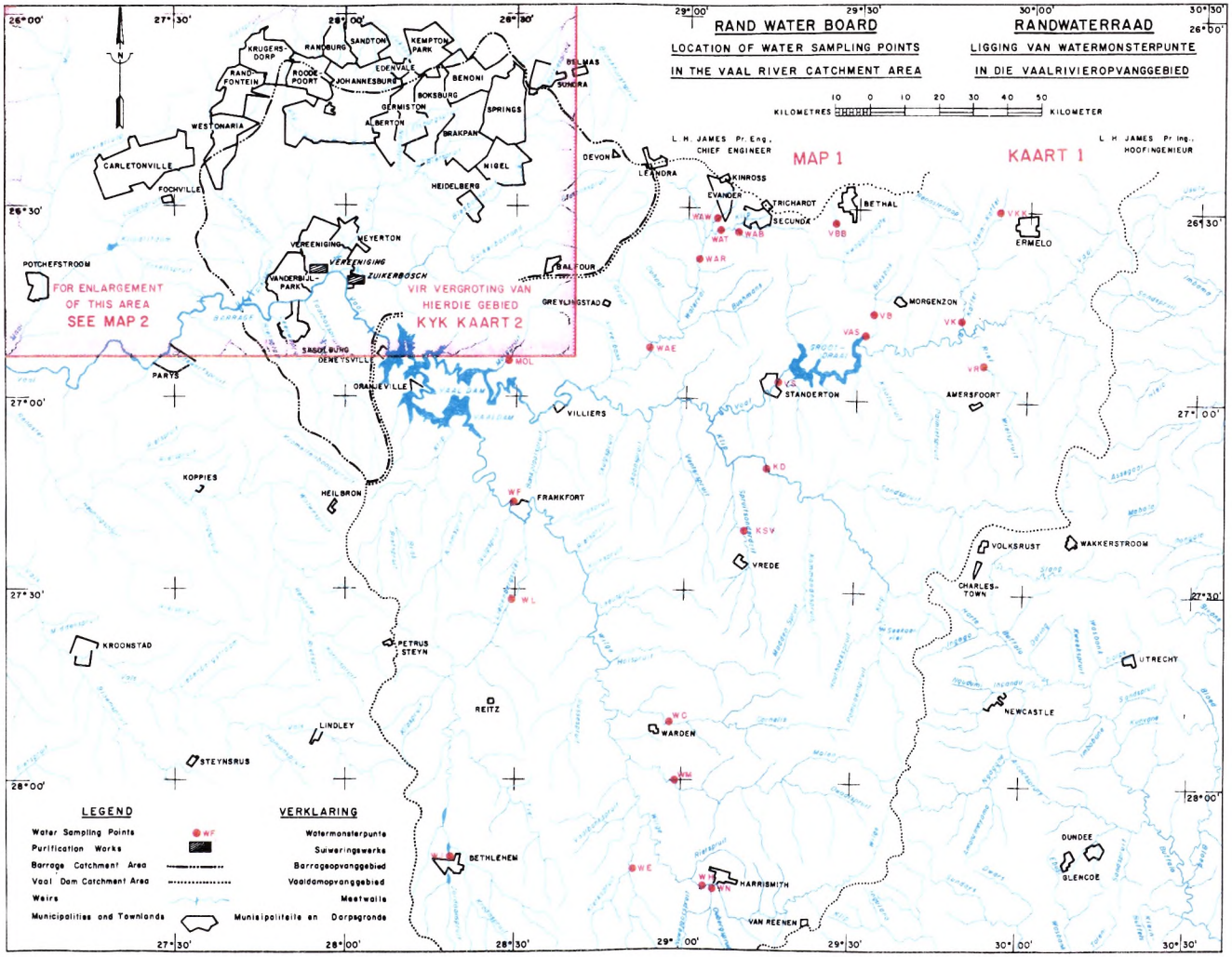

Fig 5.1 Location of water sampling points in the Vaal River catchment area (Rand Water Map 1) (RWB, 1987).

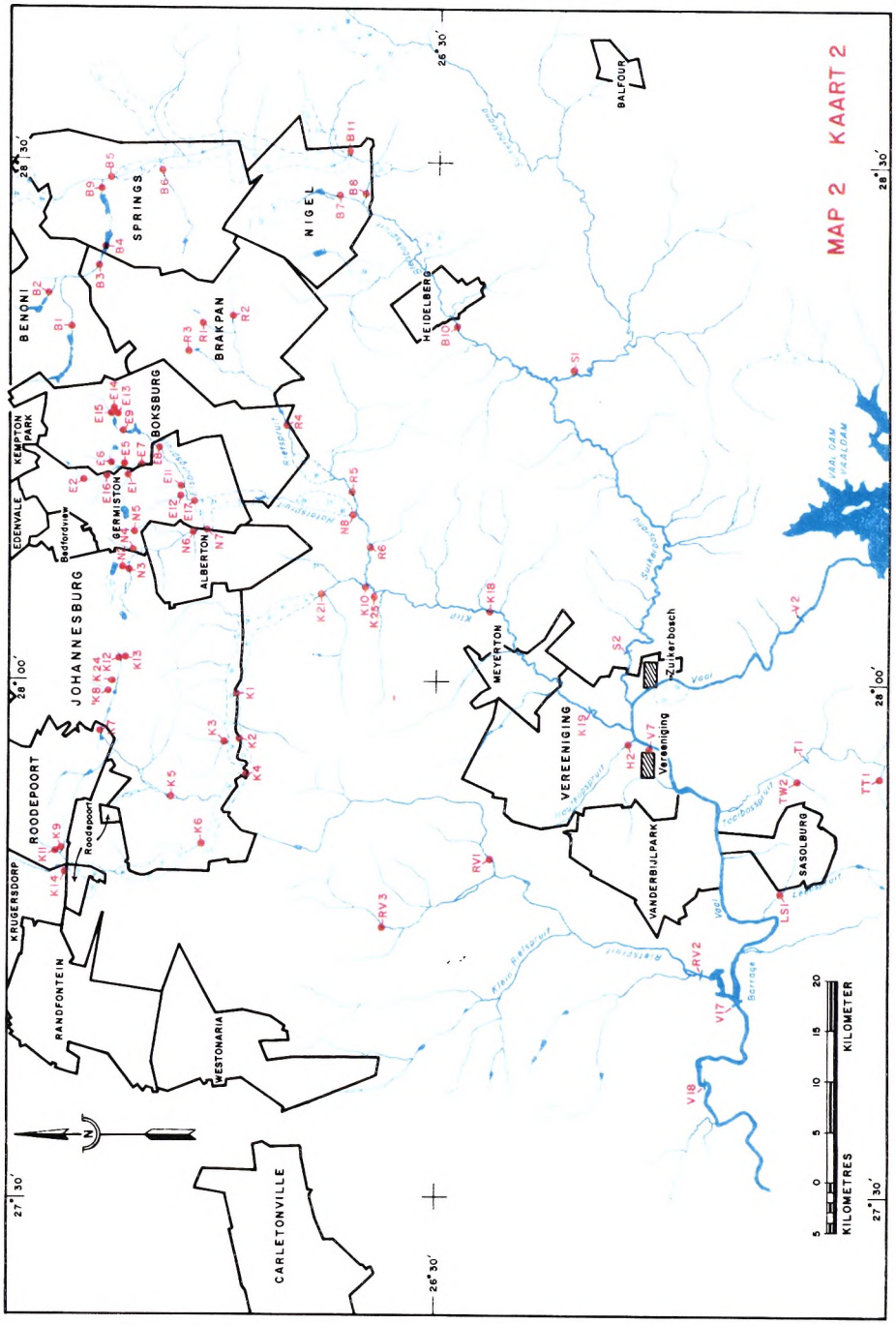

Fig 5.2 Location of water sampling points in the Vaal River catchment area (Rand Water Map 2) (RWB, 1987).

contaminating bacteria. Their identity was established by biochemical and serological tests.

The samples were then either filtered using 0.45 μm pore size, 47 mm diameter filters (April and May, 1989), or autoclaved at 121°C for 20 minutes (June 1989 to January 1990). One litre water from each sample was transferred into each of three sterile one litre glass beakers which were covered with sterile glass petri dishes. Autoclaved samples were allowed to cool to room temperature and their pH was measured again. It was found necessary on only one occasion to adjust the pH by adding 1N NaOH. Iron analysis was done on 200ml of autoclaved water.

5.2.3 Bacterial cultures and inocula.

One strain each of two biotypes of *V. cholerae* O1, namely El Tor and classical, and a strain of *V. cholerae* non-O1 were used in this study (table 5.2). The cultures were maintained in semi-solid agar and on blood agar plates. During the course of the study, appropriate tests were periodically performed to re-confirm the identity of the cultures prior to their inoculation in water samples. Cultures on blood agar were subcultured daily onto fresh blood agar and incubated at 37°C for 24 hours. A loopful (2 mm diameter) of culture was picked off these plates and,

TABLE 5.2 *V. cholerae* cultures used in this study.

Origin	Biotype	Serotype
S.A.I.M.R., JHB., R.S.A.	-	non 01
	El Tor	01 Inaba
Centre for Applied Microbiology & Research, Salisbury, England	Classical	01 Inaba

in a single swift action, dipped to the bottom of McCartney bottles containing 10 ml alkaline peptone water (APW) each. These APW cultures were incubated for 16 hours at 37°C. The inoculum was prepared in this manner for all the experiments. Counts of *V. cholerae* were performed on the inoculum immediately prior to inoculation of water samples, and were found to be in the range of 10^8 to 10^9 organisms/ml.

5.2.4 Inoculation of water samples and determination of *V. cholerae* survival.

For each water sample, three beakers were inoculated with 0.25 ml *V. cholerae* non-O1, O1 El Tor and O1 classical respectively. All inoculated beakers were kept at room temperature and daily counts were done on each sample using a slightly modified microdilution technique (Kramer J, 1977). The modifications were described in chapter 4 (section 4.2.1). In addition, when the bacteria in undiluted water became too few to count (<50 CFU/ml), a small quantity of water was enriched by overnight incubation in APW and then subcultured on blood agar at 37°C for a further 24 hours. The mere presence of *V. cholerae* was then denoted as <50 CFU/ml and absence of growth as a negative result. Samples were discarded after three consecutive negative results.

5.2.5 Statistical analyses

For each *V. cholerae* strain the means and median values of its survival time and the water pH and iron levels were calculated. This was done separately for each sampling point.

Analyses of the duration of survival were performed by means of the 1-way analysis of variance (ANOVA). The objective was to establish whether there were significant *V. cholerae* strain differences in survival, and to establish the significance of the effect of pH and iron, if any, on the duration of survival.

5.3 RESULTS AND DISCUSSION

5.3.1 Microbiological analysis of pre-inoculation water samples.

The organisms isolated from the first series of uninoculated monthly water samples are listed in table 5.3. Most of the isolates were *Bacillus* and *Pseudomonas* species. *V. cholerae* non-O1 was isolated from sample R5. The total bacterial and coliform counts were found to be high in most water samples. Faecal *E. coli* (*E. coli* I) was present in nine of the twelve sampling points. Fungi were not isolated from any water samples.

TABLE 5.3 Microbiological analysis of industrially polluted water samples.

Sample	Total bact. count/ml	Coliform count/100ml	*E. coli* type I count/100ml	Organisms isolated
N7	165	2	0	1. *Pseudomonas aeruginosa* 2. *Bacillus cereus* 3. *Bacillus firmus* fungus - absent
E7	170	0	0	1. *Bacillus species* 2. *Pseudomonas pseudoalcaligenes* 3. *Pseudomonas alcaligenes* fungus - absent
E17	2317	>1800	13	1. *Enterobacter species* 2. *Enterobacter cloacae* 3. *Achromobacter species* 4. *Aeromonas sobria* 5. *Aeromonas hydrophila* 6. *Micrococcus species* 7. *Morganella morgani* fungus - absent
V17	236	>1800	10	1. *Bacillus species* 2. *Pseudomonas pseudoalcaligenes* 3. *Bacillus cereus* 4. *Pseudomonas acidovorans* fungus - absent
V18	204	>1800	12	1. *Pseudomonas putrefaciens* 2. *Aeromonas hydrophila* 3. *Pseudomonas pseudoalcaligenes* 4. *E. coli* fungus - absent
T1	511	550	110	1. *Bacillus firmus* 2. *Bacillus cereus* 3. *Pseudomonas species* 4. *Bacillus species* fungus - absent
R5	489	350	50	1. *Pseudomonas putrefaciens* 2. *Aeromonas hydrophila* 3. *Vibrio cholerae* Non 01 4. *Pseudomonas aeruginosa* fungus - absent

TABLE 5.3 (contd.)

R4	202	900	350	1. Aeromonas hydrophila 2. Aeromonas species 3. Aeromonas sobria 4. Pseudomonas putrefaciens fungus - absent
WJ	260	>1800	6	1. Klebsiella species 2. Aeromonas hydrophila 3. Aeromonas sobria fungus - absent
WC	347	1600	80	1. Aeromonas species 2. Pseudomonas fluorescens 3. Aeromonas species fungus - absent
VAS	154	350	0	1. Aeromonas sobria 2. E. coli fungus - absent

5.3.2 Survival of *V. cholerae*

The water samples were autoclaved or filtered prior to inoculation and aseptic precautions were taken during various procedures, but full sterile conditions were not practical. Rather, samples were monitored for extraneous contaminants and excluded from the analyses if contamination was found. Extraneous contamination of the inoculated water samples was observed from time to time and appeared to adversely affect the survival of the cholera strains. Such survival results were therefore not included in the statistical evaluations.

The basic survival data of the individual *V. cholerae* strains are presented in tables 5.4 - 5.15. Survival is shown in terms of duration in days and of the ranges and medians of the daily bacterial counts. Each table represents a different water sampling point and also indicates the pH values and iron levels of each monthly water sample for that sampling point. In each case, the longest survival was obtained during October, which falls during mid-spring. Although *V. cholerae* non-O1 appeared to have a longer mean survival than the two O1 strains, these differences were not statistically significant ($p \geq 0.05$) between individual sampling points.

Table 5.4 Survival of *V. cholerae* in sample N7.

Sample ID/month	pH	Iron mg/l	*V. cholerae* non-01		*V. cholerae* 01 El Tor		*V. cholerae* 01 Classical	
			Days Survival	Daily median count (range*)	Days Survival	Daily median count (range*)	Days Survival	Daily median count (range*)
N7/4	4.4	17.4	<1	N/A (2.8×10^5-0)	<1	N/A (2.7×10^4-0)	<1	N/A (7.1×10^4-0)
N7/5	3.6	19.0	<1	N/A (1.0×10^4-0)	<1	N/A (2.3×10^3-0)	<1	N/A (2.8×10^3-0)
N7/6	4.4	25.0	<1	N/A (2.5×10^5-0)	<1	N/A (3.6×10^4-0)	<1	N/A (5.4×10^4-0)
N7/7	3.3	20.0	<1	N/A (2.0×10^5-0)	<1	N/A (1.7×10^5-0)	<1	N/A (1.7×10^5-0)
N7/8	7.5	19.0	90	$8.5\text{X}10^2$ (8.0×10^5-0)	31	1.0×10^4 (5.2×10^4-0)	39	1.7×10^5 (6.7×10^5-0)
N7/9	7.5	17.0	7**	<50 (1.5×10^5-0)	55	5.0×10^3 (1.8×10^5-0)	33	<50 (2.5×10^5-0)
N7/10	7.5	25.0	41	$6.5\text{X}10^3$ (2.2×10^6-0)	52	<50 (1.5×10^6-0)	27	1.0×10^3 (1.2×10^6-0)
N7/11	7.5	2.8	13	$1.5\text{X}10^3$ (8.5×10^4-0)	2	<50 (3.6×10^4-0)	2	<50 (8.3×10^4-0)
N7/12	7.5	11.0	40	$5.8\text{X}10^2$ (5.6×10^5-0)	37	9.3×10^4 (5.7×10^5-0)	33	3.1×10^4 (3.9×10^5-0)
N7/1	7.5	11.0	33	$4.1\text{X}10^3$ (5.0×10^5-0)	45	1.2×10^3 (1.9×10^5-0)	21	9.3×10^3 (2.3×10^5-0)
Median -	7.5	14.0	40.0 (n=5,N7/8,N7/10-N7/1)		41 (n=6,N7/8-N7/1)		33 (n=6,N7/8-N7/1)	
Mean -	7.5	14.3	43.4		37		25.8	

* The range includes day 0 counts
** Contaminants present
N/A Not applicable.

The pH of the 6 samples (N7/8 to N7/1) ranged from 2.65 to 6.35. In view of the poor *V. cholerae* samples N7/4 to N7/7, the pH was adjusted to 7.5 (See text in section 5.3.2).

Table 5.5 Survival of *V. cholerae* in sample E7.

Sample ID/month	pH	Iron mg/l	*V. cholerae* non-O1		*V. cholerae* O1 El Tor		*V. cholerae* O1 Classical	
			Days Survival	Daily median count (range*)	Days Survival	Daily median count (range*)	Days Survival	Daily median count (range*)
E7/4	4.6	0.50	<1	N/A (2.8×10^5-0)	1	1.4×10^4 (2.7×10^4-0)	<1	N/A (7.1×10^4-0)
E7/5	6.8	0.25	8	<50 (1.0×10^4-0)	12	8.5×10^2 (1.4×10^4-0)	3	5.0×10^3 (9.0×10^3-0)
E7/6	5.5	0.48	1	1.3×10^5 (2.5×10^5-0)	1	1.8×10^4 (3.6×10^4-0)	1	2.7×10^4 (5.4×10^4-0)
E7/7	4.6	0.22	42	<50 (6.0×10^5-0)	16	8.5×10^2 (1.7×10^5-0)	19	4.7×10^4 (2.9×10^5-0)
E7/8	4.8	0.41	3	4.5×10^2 (1.1×10^5-0)	2	1.0×10^2 (5.1×10^4-0)	2	3.5×10^2 (6.2×10^4-0)
E7/9	6.9	3.20	27	<50 (1.5×10^5-0)	5	<50 (1.2×10^5-0)	7	<50 (1.4×10^4-0)
E7/10	7.2	1.00	26	6.9×10^4 (1.7×10^5-0)	43	<50 (5.0×10^4-0)	34	<50 (4.4×10^4-0)
E7/11	5.6	0.75	2	2.9×10^3 (5.3×10^4-0)	1	1.8×10^4 (3.6×10^4-0)	2	4.5×10^2 (8.3×10^4-0)
E7/12	4.1	0.99	1	1.9×10^4 (3.8×10^4-0)	1	9.0×10^3 (1.8×10^4-0)	1	1.0×10^4 (2.0×10^4-0)
E7/1	4.5	0.99	1	1.9×10^4 (3.8×10^4-0)	1	9.0×10^3 (1.8×10^4-0)	1	1.0×10^4 (2.0×10^4-0)
Median	5.15	0.63	2.5		1.5		2.0	
Mean	5.46	0.88	11.2		8.3		7.1	

* The range includes day 0 counts
N/A Not appplicable.

Table 5.6 Survival of V. *cholerae* in sample E17.

Sample ID/month	pH	Iron mg/l	V. *cholerae* non-O1		V. *cholerae* O1 El Tor		V. *cholerae* O1 Classical	
			Days Survival	Daily median count (range*)	Days Survival	Daily median count (range*)	Days Survival	Daily median count (range*)
E17/4	4.0	0.10	5	$1.5x10^5$ ($3.5x10^6$-0)	5	$1.4x10^6$ ($1.9x10^6$-0)	5	<50 ($2.0x10^5$-0)
E17/5	6.1	<0.05	6	<50 ($1.0x10^4$-0)	3	$6.0x10^3$ ($9.5x10^3$-0)	2	$5.5x10^3$ ($5.5x10^3$-0)
E17/6	3.6	1.37	<1	N/A ($2.5x10^5$-0)	<1	N/A ($3.6x10^4$-0)	<1	N/A ($5.4x10^4$-0)
E17/7	5.8	1.00	3	70 ($2.0x10^5$-0)	3	70 ($1.7x10^5$-0)	<1	N/A ($1.7x10^5$-0)
E17/8	N/D	N/D	N/D	N/D (N/D)	N/D	N/D (N/D)	N/D	N/D (N/D)
E17/9	7.0	0.37	4	50 ($1.5x10^5$-0)	2	50 ($1.2x10^5$-0)	2	$8.3x10^2$ ($1.4x10^4$-0)
E17/10	7.2	0.26	16	<50 ($8.6x10^4$-0)	4	<50 ($5.0x10^4$-0)	6	$1.0x10^3$ ($4.4x10^4$-0)
E17/11	6.5	0.61	6	$4.0x10^3$ ($2.5x10^5$-0)	9	$1.2x10^3$ ($4.4x10^4$-0)	8	$2.8x10^3$ ($3.1x10^4$-0)
E17/12	6.9	0.37	2	$1.0x10^2$ ($3.8x10^4$-0)	1	$9.0x10^3$ ($1.8x10^4$-0)	1	$1.0x10^4$ ($2.0x10^4$-0)
E17/1	7.3	0.41	1	$1.9x10^4$ ($3.8x10^4$-0)	1	$9.0x10^3$ ($1.8x10^4$-0)	2	<50 ($2.0x10^4$-0)
Median	6.5	0.35	4.0		3.0		2.0	
Mean	6.0	0.50	4.8		3.2		3.0	

* The range includes day 0 counts
N/D Not done
N/A Not applicable.

Table 5.7 Survival of *V. cholerae* in sample V17.

Sample ID/month	pH	Iron mg/l	*V. cholerae* non-O1		*V. cholerae* O1 El Tor		*V. cholerae* O1 Classical	
			Days Survival	Daily median count (range*)	Days Survival	Daily median count (range*)	Days Survival	Daily median count (range*)
V17/4	7.8	0.05	12	2.5×10^4 (1.8×10^6 -0)	8**	4.0×10^5 (6.5×10^5 -0)	8**	4.5×10^5 (5.1×10^5 -0)
V17/5	7.7	<0.05	7	3.8×10^5 (6.0×10^6 -0)	4	1.7×10^5 (1.7×10^5 -0)	5	4.8×10^6 (5.0×10^6 -0)
V17/6	8.1	0.22	21	7.4×10^4 (8.0×10^5 -0)	27	<50 (5.6×10^4 -0)	27	<50 (5.1×10^4 -0)
V17/7	6.3	0.35	L/A	L/A	L/A	L/A	L/A	L/A
V17/8	7.1	0.47	38	3.6×10^3 (9.5×10^5 -0)	2	<50 (1.4×10^5 -0)	24	<50 (4.4×10^4 -0)
V17/9	8.0	0.06	27	2.8×10^4 (4.2×10^6 -0)	22	2.5×10^2 (2.2×10^5 -0)	35	<50 (4.7×10^6 -0)
V17/10	7.2	0.08	35	1.4×10^2 (1.0×10^6 -0)	26	2.2×10^3 (1.8×10^6 -0)	26	<50 (8.5×10^5 -0)
V17/11	8.1	0.27	27	<50 (5.3×10^4 -0)	10	<50 (3.6×10^4 -0)	23	<50 (1.1×10^5 -0)
V17/12	8.0	0.39	43	<50 (1.6×10^6 -0)	29	<50 (3.0×10^4 -0)	15	<50 (1.1×10^5 -0)
V17/1	7.6	1.20	29	90 (2.1×10^6 -0)	17	<50 (3.0×10^4 -0)	16	<50 (2.0×10^5 -0)
Median	7.75	0.25	27.0		17		23.0	
Mean	7.60	0.31	26.6		16.1		19.9	

* The range includes day 0 counts
** Contaminants present
L/A Laboratory accident.

Table 5.8 Survival of *V. cholerae* in sample V18.

Sample ID/month	pH	Iron mg/l	*V. cholerae* non-01		*V. cholerae* 01 El Tor		*V. cholerae* 01 Classical	
			Days Survival	Daily median count (range*)	Days Survival	Daily median count (range*)	Days Survival	Daily median count (range*)
V18/5	7.7	0.04	21	4.3×10^{2} (3.9×10^{6}-0)	8	5.8×10^{6} (7.9×10^{6}-0)	15	2.5×10^{4} (1.1×10^{7}-0)
V18/5	7.7	<0.05	7	5.6×10^{4} (5.5×10^{6}-0)	7	1.0×10^{5} (1.1×10^{5}-0)	7	2.7×10^{4} (4.8×10^{6}-0)
V18/6	7.9	0.23	28	4.0×10^{4} (4.7×10^{5}-0)	18	1.0×10^{2} (5.6×10^{4}-0)	23	3.1×10^{4} (8.5×10^{4}-0)
V18/7	6.8	0.36	L/A	L/A	L/A	L/A	L/A	L/A
V18/8	7.7	0.36	34	1.4×10^{4} (1.5×10^{6}-0)	1	7.0×10^{4} (1.4×10^{5}-0)	24	<50 (4.4×10^{4}-0)
V18/9	8.1	0.04	24	1.0×10^{2} (7.3×10^{5}-0)	37	<50 (6.4×10^{5}-0)	36	3.5×10^{3} (7.6×10^{5}-0)
V18/10	6.9	0.74	29	3.5×10^{3} (8.6×10^{5}-0)	33	<50 (7.4×10^{5}-0)	16	1.6×10^{5} (7.5×10^{5}-0)
V18/11	8.2	0.27	17	<50 (7.5×10^{4}-0)	20	<50 (3.6×10^{4}-0)	22	<50 (6.2×10^{5}-0)
V18/12	8.1	0.96	23	6.7×10^{3} (5.3×10^{5}-0)	16	<50 (3.0×10^{4}-0)	16	1.0×10^{3} (2.5×10^{5}-0)
V18/1	8.1	1.00	27	7.3×10^{2} (1.8×10^{6}-0)	16	<50 (3.4×10^{5}-0)	15	70 (1.3×10^{5}-0)
Median	7.8	0.32	24.0		17.0		16.0	
Mean	7.7	0.40	23.3		19.4		19.3	

* The range includes day 0 counts
L/A Laboratory accident.

Table 5.9 Survival of *V. cholerae* in sample T1.

Sample ID/month	pH	Iron mg/l	*V. cholerae* non-O1		*V. cholerae* O1 El Tor		*V. cholerae* O1 Classical	
			Days Survival	Daily median count (range*)	Days Survival	Daily median count (range*)	Days Survival	Daily median count (range*)
T1/4	8.1	0.20	5	3.8×10^4 (9.0×10^5-0)	5**	1.6×10^4 (2.4×10^6-0)	8**	5.0×10^3 (4.4×10^6-0)
T1/5	8.1	0.15	10	3.5×10^5 (3.8×10^6-0)	7	5.0×10^3 (1.8×10^5-0)	7	3.9×10^4 (6.0×10^6-0)
T1/6	8.2	0.95	54	7.4×10^3 (1.2×10^6-0)	53	7.9×10^3 (1.5×10^5-0)	41	2.4×10^4 (2.6×10^6-0)
T1/7	7.7	0.61	1	3.0×10^5 (6.0×10^5-0)	1	3.0×10^5 (6.0×10^5-0)	2	8.5×10^4 (1.7×10^5-0)
T1/8	8.3	1.20	42	4.5×10^3 (3.2×10^6-0)	24	8.5×10^4 (2.6×10^5-0)	39	2.4×10^4 (3.0×10^6-0)
T1/9	8.3	2.70	48	1.3×10^4 (1.9×10^6-0)	28	1.5×10^4 (5.0×10^5-0)	29	1.5×10^2 (2.3×10^6-0)
T1/10	N/D	N/D	N/D	N/D (N/D)	N/D	N/D (N/D)	N/D	N/D (N/D)
T1/11	8.7	5.10	45	4.0×10^2 (2.9×10^6-0)	38	<50 (1.8×10^6-0)	28	<50 (2.2×10^6-0)
T1/12	8.2	33.00	39	<50 (2.3×10^6-0)	21	1.0×10^5 (2.6×10^6-0)	28	<50 (2.6×10^6-0)
T1/1	8.2	26.00	29	1.6×10^4 (2.9×10^6-0)	49	<50 (1.9×10^6-0)	34	<50 (1.9×10^6-0)
Median	8.2	1.95	40.5		26.0		28.5	
Mean	8.2	8.66	34.0		28.1		26.8	

* The range includes day 0 counts
** Contaminants present
N/D Not done.

Table 5.10 Survival of *V. cholerae* in sample R5.

Sample ID/month	pH	Iron mg/l	*V. cholerae* non-O1		*V. cholerae* O1 El Tor		*V. cholerae* O1 Classical	
			Days Survival	Daily median count (range*)	Days Survival	Daily median count (range*)	Days Survival	Daily median count (range*)
R5/4	8.0	0.05	15	1.3×10^5 (8.4×10^5-0)	27	2.1×10^5 (1.8×10^6-0)	10	4.6×10^4 (1.5×10^5-0)
R5/5	7.9	0.06	9	4.5×10^2 (1.1×10^5-0)	5	45 (1.0×10^4-0)	4	2.3×10^4 (2.8×10^4-0)
R5/6	7.8	<0.05	12	2.3×10^3 (1.5×10^5-0)	6**	3.9×10^3 (5.6×10^4-0)	8**	1.2×10^4 (5.1×10^4-0)
R5/7	7.5	0.07	1	3.0×10^5 (6.0×10^5-0)	1	3.0×10^5 (6.0×10^5-0)	1	8.5×10^5 (1.7×10^5-0)
R5/8	7.5	0.09	34	1.9×10^3 (1.4×10^5-0)	23	5.3×10^2 (1.4×10^5-0)	15	70 (2.3×10^4-0)
R5/9	7.5	0.08	37	4.3×10^2 (7.5×10^5-0)	25	1.5×10^4 (3.2×10^5-0)	30	<50 (6.3×10^5-0)
R5/10	7.0	0.05	59	6.5×10^2 (3.6×10^5-0)	52	1.3×10^4 (8.0×10^5-0)	27	<50 (4.0×10^5-0)
R5/11	7.4	0.45	37	<50 (5.5×10^5-0)	37	<50 (8.3×10^5-0)	14	1.5×10^2 (3.5×10^5-0)
R5/12	7.4	<0.05	52	<50 (3.4×10^5-0)	19	<50 (3.4×10^5-0)	12	2.0×10^3 (6.1×10^4-0)
R5/1	7.5	<0.05	33	4.0×10^3 (5.3×10^5-0)	14	3.0×10^3 (7.0×10^5-0)	12	2.0×10^4 (3.3×10^5-0)
Median	7.5	0.05	34		23.0		12.0	
Mean	7.6	0.09	32		23.1		14.7	

* The range includes day 0 counts
** Contaminants present.

Table 5.11 Survival of *V. cholerae* in sample R4.

Sample ID/month	pH	Iron mg/l	*V. cholerae* non-O1		*V. cholerae* O1 El Tor		*V. cholerae* O1 Classical	
			Days Survival	Daily median count (range*)	Days Survival	Daily median count (range*)	Days Survival	Daily median count (range*)
R4/4	7.9	<0.05	13	$1.3x10^3$ ($2.3x10^6$ -0)	13	$6.7x10^4$ ($2.3x10^6$ -0)	8	$5.5x10^4$ ($7.3x10^5$ -0)
R4/5	8.0	<0.05	6	$6.0x10^4$ ($2.5x10^5$ -0)	1	$1.1x10^4$ ($2.2x10^4$ -0)	3	$7.3x10^3$ ($2.2x10^4$ -0)
R4/6	7.8	<0.05	14	$1.5x10^3$ ($1.5x10^5$ -0)	8	$2.0x10^3$ ($5.6x10^4$ -0)	11	$3.1x10^3$ ($5.1x10^4$ -0)
R4/7	7.5	<0.05	23	<50 ($9.5x10^4$ -0)	9	$5.6x10^3$ ($4.8x10^4$ -0)	13	$1.6x10^4$ ($2.4x10^5$ -0)
R4/8	7.9	0.07	14	$5.0x10^2$ ($1.1x10^5$ -0)	9	$7.8x10^2$ ($1.4x10^5$ -0)	7	$1.2x10^3$ ($2.3x10^4$ -0)
R4/9	8.0	0.69	36	<50 ($1.9x10^6$ -0)	13	$2.8x10^4$ ($1.2x10^5$ -0)	27	$1.3x10^4$ ($2.1x10^6$ -0)
R4/10	7.2	0.05	51	<50 ($1.1x10^6$ -0)	44	$8.3x10^3$ ($1.2x10^6$ -0)	15	$4.0x10^4$ ($6.0x10^5$ -0)
R4/11	7.6	0.05	20	$1.4x10^3$ ($5.1x10^5$ -0)	27	<50 ($3.3x10^5$ -0)	9	$1.8x10^4$ ($2.5x10^5$ -0)
R4/12	7.6	<0.05	19	$6.6x10^2$ ($1.0x10^5$ -0)	14	<50 ($2.9x10^4$ -0)	7	$3.6x10^3$ ($2.8x10^4$ -0)
R4/1	7.7	<0.05	33	$4.5x10^3$ ($1.5x10^5$ -0)	14	$1.0x10^2$ ($1.6x10^5$ -0)	7	$1.8x10^4$ ($8.8x10^4$ -0)
Median	7.75	0.03	19.5		13.0		8.5	
Mean	7.72	0.10	22.9		16.8		10.7	

* The range includes day 0 counts.

Table 5.12 Survival of *V. cholerae* in sample E11.

Sample ID/month	pH	Iron mg/l	*V. cholerae* non-O1		*V. cholerae* O1 El Tor		*V. cholerae* O1 Classical	
			Days Survival	Daily median count (range*)	Days Survival	Daily median count (range*)	Days Survival	Daily median count (range*)
E11/4	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
E11/5	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
E11/6	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
E11/7	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D
E11/8	6.8	0.36	30	$7.5x10^4$ ($2.6x10^6$-0)	<1	N/A	38	<50 ($2.5x10^6$-0)
E11/9	6.0	0.44	48	<50 ($4.0x10^6$-0)	1	$6.0x10^4$ ($1.2x10^5$-0)	55	<50 ($4.1x10^5$-0)
E11/10	8.1	1.00	37	<50 ($2.9x10^6$-0)	54	<50 ($3.3x10^6$-0)	44	<50 ($2.4x10^6$-0)
E11/11	7.0	3.95	6	$1.2x10^2$ ($2.5x10^5$-0)	5	<50 ($4.4x10^4$-0)	6	<50 ($3.1x10^4$-0)
E11/12	5.6	3.10	<1	N/A	1	$9.0x10^3$ ($1.8x10^4$-0)	2	<50 ($2.0x10^4$-0)
E11/1	4.6	3.20	<1	N/A	1	$9.0x10^3$ ($1.8x10^4$-0)	1	$1.0x10^4$ ($2.0x10^4$-0)
Median	6.4	2.05	18.0		1.0		22.0	
Mean	6.4	2.01	20.3		10.4		24.3	

* The range includes day 0 counts
N/D Not done
N/A Not applicable.

Table 5.13 Survival of *V. cholerae* in sample WJ.

Sample ID/month	pH	Iron mg/l	*V. cholerae* non-O1		*V. cholerae* O1 El Tor		*V. cholerae* O1 Classical	
			Days Survival	Daily median count (range*)	Days Survival	Daily median count (range*)	Days Survival	Daily median count (range*)
WJ/4	7.9	1.40	6**	<50 (1.6×10^6-0)	5**	2.1×10^4 (1.7×10^6-0)	4**	4.4×10^5 (9.0×10^5-0)
WJ/5	7.7	0.12	7	2.5×10^5 (1.4×10^6-0)	3	2.5×10^3 (3.9×10^4-0)	7	6.2×10^4 (1.8×10^5-0)
WJ/6	8.9	1.50	54	2.8×10^3 (6.3×10^6-0)	2	<50 (9.3×10^4-0)	<1	N/A
WJ/7	7.0	1.60	64	<50 (1.9×10^6-0)	12	<50 (6.6×10^4-0)	22	3.5×10^5 (1.8×10^6-0)
WJ/8	8.1	0.39	40	<50 (1.7×10^6-0)	51	1.5×10^3 (2.0×10^6-0)	44	<50 (1.4×10^6-0)
WJ/9	8.5	2.70	34	8.5×10^3 (1.8×10^6-0)	24	6.5×10^4 (4.0×10^5-0)	26	3.6×10^4 (1.6×10^6-0)
WJ/10	8.1	0.96	76	<50 (1.5×10^6-0)	49	1.5×10^2 (1.9×10^6-0)	47	1.8×10^3 (1.5×10^6-0)
WJ/11	8.2	0.80	40	7.0×10^3 (8.3×10^5-0)	57	<50 (1.1×10^6-0)	47	<50 (1.8×10^6-0)
WJ/12	8.2	1.30	21	7.5×10^2 (1.1×10^6-0)	26	<50 (2.0×10^6-0)	14	2.8×10^4 (2.0×10^6-0)
WJ/1	8.6	4.90	19	1.7×10^3 (3.0×10^6-0)	14	2.9×10^4 (1.6×10^6-0)	19	3.5×10^2 (1.9×10^6-0)
Median	8.15	1.35	37.0		19.0		20.5	
Mean	8.10	1.57	36.1		24.3		23.0	

* The range includes day 0 counts
** Contaminants present
N/A Not applicable.

Table 5.14 Survival of *V. cholerae* in sample WC.

Sample ID/month	pH	Iron mg/l	*V. cholerae* non-O1		*V. cholerae* O1 El Tor		*V. cholerae* O1 Classical	
			Days Survival	Daily median count (range*)	Days Survival	Daily median count (range*)	Days Survival	Daily median count (range*)
WC/4	7.9	<0.05	7**	6.2×10^4 (2.6×10^6 -0)	8**	4.1×10^4 (3.0×10^6 -0)	5**	2.2×10^4 (9.5×10^4 -0)
WC/5	8.3	<0.05	11	2.0×10^4 (1.3×10^6 -0)	5	3.8×10^3 (3.9×10^4 -0)	7	<50 (3.4×10^4 -0)
WC/6	9.2	3.00	35	1.8×10^5 (1.5×10^6 -0)	<1	N/A	22	4.1×10^4 (1.7×10^6 -0)
WC/7	7.6	2.00	39	1.5×10^4 (1.1×10^6 -0)	4	1.5×10^2 (6.6×10^4 -0)	42	8.5×10^3 (1.2×10^6 -0)
WC/8	7.7	0.41	34	1.4×10^4 (2.3×10^6 -0)	44	1.5×10^2 (1.8×10^6 -0)	34	<50 (1.4×10^6 -0)
WC/9	8.3	2.10	49	<50 (1.9×10^6 -0)	22	<50 (1.1×10^4 -0)	29	<50 (1.9×10^6 -0)
WC/10	7.9	0.97	36	3.0×10^4 (3.4×10^6 -0)	23	<50 (2.7×10^6 -0)	23	5.0×10^4 (2.2×10^6 -0)
WC/11	8.2	21.00	32	<50 (7.7×10^5 -0)	36	<50 (7.9×10^5 -0)	27	<50 (1.2×10^6 -0)
WC/12	8.6	6.40	18	8.0×10^4 (5.5×10^5 -0)	26	<50 (1.6×10^6 -0)	18	<50 (1.5×10^6 -0)
WC/1	8.6	3.90	38	2.5×10^2 (1.2×10^6 -0)	56	<50 (2.2×10^6 -0)	20	1.0×10^2 (8.0×10^5 -0)
Median	8.25	2.05	34.5		22.5		22.5	
Mean	8.2	3.98	29.9		22.4		22.7	

* The range includes day 0 counts
** Contaminants present
N/A Not applicable.

Table 5.15 Survival of *V. cholerae* in sample VAS

Sample ID/month	pH	Iron mg/l	*V. cholerae* non-O1		*V. cholerae* O1 El Tor		*V. cholerae* O1 Classical	
			Days Survival	Daily median count (range*)	Days Survival	Daily median count (range*)	Days Survival	Daily median count (range*)
VAS/4	8.1	0.20	15	75 (9.0x10^6-0)	10	4.0x10^5 (3.7x10^6-0)	5	2.2x10^4 (5.0x10^4-0)
VAS/5	8.0	0.11	9	1.7x10^5 (2.9x10^6-0)	29	<50 (3.9x10^4-0)	7	1.7x10^3 (3.4x10^4-0)
VAS/6	8.0	1.20	26	4.4x10^4 (1.6x10^6-0)	<1	N/A	23	<50 (1.4x10^6-0)
VAS/7	7.9	0.29	29	6.8x10^4 (1.0x10^6-0)	1	3.3x10^4 (6.6x10^4-0)	29	1.8x10^4 (7.2x10^5-0)
VAS/8	8.0	3.20	30	9.5x10^3 (2.4x10^6-0)	40	<50 (2.9x10^6-0)	28	<50 (1.2x10^6-0)
VAS/9	N/D	N/D	N/D	N/D (N/D)	N/D	N/D (N/D)	N/D	N/D (N/D)
VAS/10	7.6	2.50	26	2.5x10^2 (1.7x10^6-0)	23	3.4x10^4 (1.9x10^6-0)	23	7.0x10^3 (1.2X10^6-0)
VAS/11	8.2	5.30	34	<50 (5.8x10^5-0)	20	<50 (5.0x10^5-0)	20	<50 (8.6x10^5-0)
VAS/12	8.5	2.50	19	2.7x10^2 (6.5x10^5-0)	14	<50 (1.2x10^6-0)	12	2.0x10^4 (5.5x10^5-0)
VAS/1	8.5	12.00	19	1.4x10^3 (2.2x10^6-0)	32	<50 (1.9x10^6-0)	13	1.5x10^4 (1.4x10^6-0)
Median	8.1	2.5	2.6		2.0		20	
Mean	8.1	3.0	2.3		18.8		17.8	

* The range includes day 0 counts
N/D Not done
N/A Not applicable.

The data of each sampling point were sorted in ascending order of pH value. They were then divided into two equal groups containing the lowest and the highest pH values respectively. These values differed for each sampling point. By means of a one-way ANOVA, the data were analysed in terms of *V. cholerae* survival.

A similar exercise was done on the basis of increasing iron levels. Again, there was no association between survival time and pH or iron levels. This could be attributable to the small sample size and/or the wide variability of the data within each sampling point (total number per sampling point ranged from only 6-10 samples).

As all the sampling points represented polluted surface waters, the data of the 12 points were therefore pooled, yielding a total of 96 samples for re-analysis (table 5.16).

The mean survival times of the El Tor and classical *V. cholerae* strains were the same at 17 days. Mean survival of the non-O1 strain was 24 days which, by means of an ANOVA on the 96 pooled data, was shown to be significantly different from the survival of the El Tor and classical strains of *V. cholerae* ($p = 0.006$ and 0.001 respectively).

Table 5.16 Pooled V.cholerae survival data, sorted by sampling point (n=96).

Sample point	pH	Iron mg/l	Survival of V.cholerae in days		
			Non-01	El Tor	Classical
N7	4.4	17.40	0.5	0.5	0.5
N7	3.6	19.00	0.5	0.5	0.5
N7	4.4	25.00	0.5	0.5	0.5
N7	3.3	20.00	0.5	0.5	0.5
E7	4.6	0.50	0.5	1.0	0.5
E7	6.8	0.25	8.0	12.0	3.0
E7	5.5	0.48	1.0	1.0	1.0
E7	4.6	0.22	42.0	16.0	19.0
E7	4.8	0.41	3.0	2.0	2.0
E7	6.9	3.20	27.0	5.0	7.0
E7	7.2	1.00	26.0	43.0	34.0
E7	5.6	0.75	2.0	1.0	2.0
E7	4.1	0.99	1.0	1.0	1.0
E7	4.5	0.99	1.0	1.0	1.0
E17	4.0	0.10	5.0	5.0	5.0
E17	6.1	0.025	6.0	3.0	2.0
E17	3.6	1.37	0.5	0.5	0.5
E17	5.8	1.00	3.0	3.0	0.5
E17	7.0	0.37	4.0	2.0	2.0
E17	7.2	0.26	16.0	4.0	6.0
E17	6.5	0.61	6.0	9.0	8.0
E17	6.9	0.37	2.0	1.0	1.0
E17	7.3	0.41	1.0	1.0	2.0
V17	7.7	0.025	7.0	4.0	5.0
V17	8.1	0.22	21.0	27.0	27.0
V17	7.1	0.47	38.0	2.0	24.0
V17	8.0	0.06	27.0	22.0	35.0
V17	7.2	0.08	35.0	26.0	26.0
V17	8.1	0.27	27.0	10.0	23.0
V17	8.0	0.39	43.0	29.0	15.0
V17	7.6	1.20	29.0	17.0	16.0
V18	7.7	0.04	21.0	8.0	15.0
V18	7.7	0.025	7.0	7.0	7.0
V18	7.9	0.23	28.0	18.0	23.0
V18	8.1	0.04	24.0	37.0	36.0
V18	6.9	0.74	29.0	33.0	16.0
V18	8.2	0.27	17.0	20.0	22.0
V18	8.1	0.96	23.0	16.0	16.0
V18	8.1	1.00	27.0	16.0	15.0
T1	8.1	0.15	10.0	7.0	7.0
T1	8.2	0.95	54.0	53.0	41.0
T1	8.3	1.20	42.0	24.0	39.0
T1	8.3	2.70	48.0	28.0	29.0
T1	8.7	5.10	45.0	38.0	28.0
T1	8.2	33.00	39.0	21.0	28.0
T1	8.2	26.00	29.0	49.0	34.0

TABLE 5.16 (contd.)

Sample point	pH	Iron mg/l	Survival of V.cholerae in days		
			Non-01	El Tor	Classical
VAS	8.1	0.20	15.0	10.0	5.0
VAS	8.0	0.11	9.0	29.0	7.0
VAS	8.0	1.20	26.0	0.5	23.0
VAS	7.9	0.29	29.0	1.0	29.0
VAS	8.0	3.20	30.0	40.0	28.0
VAS	7.6	2.50	26.0	23.0	23.0
VAS	8.2	5.30	34.0	20.0	20.0
VAS	8.5	2.50	19.0	14.0	12.0
VAS	8.5	12.00	19.0	32.0	13.0
WC	8.3	0.025	11.0	5.0	7.0
WC	9.2	3.00	35.0	0.5	22.0
WC	7.6	2.00	39.0	4.0	42.0
WC	7.7	0.41	34.0	44.0	34.0
WC	8.3	2.10	49.0	22.0	29.0
WC	7.9	0.97	36.0	23.0	23.0
WC	8.2	21.00	32.0	36.0	27.0
WC	8.6	6.40	18.0	26.0	18.0
WC	8.6	3.90	38.0	56.0	20.0
WJ	7.7	0.12	7.0	3.0	7.0
WJ	8.9	1.50	54.0	2.0	0.5
WJ	7.0	1.60	64.0	12.0	22.0
WJ	8.1	0.39	40.0	51.0	44.0
WJ	8.5	2.70	34.0	24.0	26.0
WJ	8.1	0.96	76.0	49.0	47.0
WJ	8.2	0.80	40.0	57.0	47.0
WJ	8.2	1.30	21.0	26.0	14.0
WJ	8.6	4.90	19.0	14.0	19.0
E11	6.8	0.36	30.0	0.5	38.0
E11	6.0	0.44	48.0	1.0	55.0
E11	8.1	1.00	37.0	54.0	44.0
E11	7.0	3.95	6.0	5.0	6.0
E11	5.6	3.10	0.5	1.0	2.0
E11	4.6	3.20	0.5	1.0	1.0
R4	7.9	0.025	13.0	13.0	8.0
R4	7.8	0.025	14.0	8.0	11.0
R4	7.5	0.025	23.0	9.0	13.0
R4	7.9	0.07	14.0	9.0	7.0
R4	8.0	0.69	36.0	13.0	27.0
R4	7.2	0.05	51.0	44.0	15.0
R4	7.6	0.05	20.0	27.0	9.0
R4	7.6	0.025	19.0	14.0	7.0
R4	7.7	0.025	33.0	14.0	7.0
R5	8.0	0.05	15.0	27.0	10.0
R5	7.9	0.06	9.0	5.0	4.0
R5	7.5	0.09	34.0	23.0	15.0
R5	7.5	0.08	37.0	25.0	30.0
R5	7.0	0.05	59.0	52.0	27.0
R5	7.4	0.45	37.0	37.0	14.0
R5	7.4	0.025	52.0	19.0	12.0
R5	7.5	0.025	33.0	14.0	12.0

To evaluate the pooled survival data in relation to pH, they were sorted in order of increasing pH and grouped in 6 batches of 16 values each (table 5.17). For illustrative graphing purposes, the data were also grouped in 12 batches of 8 values each and the 12 survival means were plotted against the pH means (figure 5.3). Survival shows a saddle back type of curve with a peak at pH 7.1 - 7.4 and a second peak at pH >8. Also inserted on this graph were the mean iron concentrations for each batch. It is postulated that the first peak coincides with optimum pH values and the second peak with increasing iron concentrations.

A similar sorting, grouping and graphing procedure (table 5.18) was performed to enable evaluation of the pooled survival data in terms of increasing iron concentrations. The equivalent graph is shown in figure 5.4 which shows the majority of data clustered at the low end of the iron concentration range. That lower end was therefore 'stretched' by using two different scales (figure 5.5) to show more clearly the relationship or, rather, an apparent absence thereof, between iron concentration and survival of the *V. cholerae* strains.

Table 5.17 Pooled V.cholerae survival data, sorted by pH and separated in groups of n=16 each.

I.D.	pH	Iron mg/l	Survival of V.cholerae in days			
			Non-01	El Tor	Class	Mean
N7	3.3	20.00	0.5	0.5	0.5	0.5
N7	3.6	19.00	0.5	0.5	0.5	0.5
E17	3.6	1.37	0.5	0.5	0.5	0.5
E17	4.0	0.10	5.0	5.0	5.0	5.0
E7	4.1	0.99	1.0	1.0	1.0	1.0
N7	4.4	17.40	0.5	0.5	0.5	0.5
N7	4.4	25.00	0.5	0.5	0.5	0.5
E7	4.5	0.99	1.0	1.0	1.0	1.0
E7	4.6	0.22	42.0	16.0	19.0	25.7
E7	4.6	0.50	0.5	1.0	0.5	0.7
E11	4.6	3.20	0.5	1.0	1.0	0.8
E7	4.8	0.41	3.0	2.0	2.0	2.3
E7	5.5	0.48	1.0	1.0	1.0	1.0
E7	5.6	0.75	2.0	1.0	2.0	1.7
E11	5.6	3.10	0.5	1.0	2.0	1.2
E17	5.8	1.00	3.0	3.0	0.5	2.2
Mean	4.6	5.91	3.9	2.2	2.3	2.8
E11	6.0	0.44	48.0	1.0	55.0	34.7
E17	6.1	0.03	6.0	3.0	2.0	3.7
E17	6.5	0.61	6.0	9.0	8.0	7.7
E7	6.8	0.25	8.0	12.0	3.0	7.7
E11	6.8	0.36	30.0	0.5	38.0	22.8
E17	6.9	0.37	2.0	1.0	1.0	1.3
V18	6.9	0.74	29.0	33.0	16.0	26.0
E7	6.9	3.20	27.0	5.0	7.0	13.0
R5	7.0	0.05	59.0	52.0	27.0	46.0
E17	7.0	0.37	4.0	2.0	2.0	2.7
WJ	7.0	1.60	64.0	12.0	22.0	32.7
E11	7.0	3.95	6.0	5.0	6.0	5.7
V17	7.1	0.47	38.0	2.0	24.0	21.3
R4	7.2	0.05	51.0	44.0	15.0	36.7
V17	7.2	0.08	35.0	26.0	26.0	29.0
E17	7.2	0.26	16.0	4.0	6.0	8.7
Mean	6.9	0.80	26.8	13.2	16.1	18.7
E7	7.2	1.0	26.0	43.0	34.0	34.3
E17	7.3	0.41	1.0	1.0	2.0	1.3
R5	7.4	0.03	52.0	19.0	12.0	27.7
R5	7.4	0.45	37.0	37.0	14.0	29.3
R5	7.5	0.03	33.0	14.0	12.0	19.7
R4	7.5	0.03	23.0	9.0	13.0	15.0
R5	7.5	0.08	37.0	25.0	30.0	30.7
R5	7.5	0.09	34.0	23.0	15.0	24.0
R4	7.6	0.03	19.0	14.0	7.0	13.3
R4	7.6	0.05	20.0	27.0	9.0	18.7
V17	7.6	1.20	29.0	17.0	16.0	20.7
WC	7.6	2.00	39.0	4.0	42.0	28.3
VAS	7.6	2.50	26.0	23.0	23.0	24.0
V18	7.7	0.03	7.0	7.0	7.0	7.0
R4	7.7	0.03	33.0	14.0	7.0	18.0
V17	7.7	0.03	7.0	4.0	5.0	5.3
Mean	7.5	0.50	26.4	17.6	15.5	19.8

TABLE 5.17 (contd.)

I.D.	pH	Iron mg/l	Survival of V.cholerae in days			
			Non-01	El Tor	Classical	Mean
V18	7.7	0.04	21.0	8.0	15.0	14.7
WJ	7.7	0.12	7.0	3.0	7.0	5.7
WC	7.7	0.41	34.0	44.0	34.0	37.3
R4	7.8	0.03	14.0	8.0	11.0	11.0
R4	7.9	0.03	13.0	13.0	8.0	11.3
R5	7.9	0.06	9.0	5.0	4.0	6.0
R4	7.9	0.07	14.0	9.0	7.0	10.0
V18	7.9	0.23	28.0	18.0	23.0	23.0
VAS	7.9	0.29	29.0	1.0	29.0	19.7
WC	7.9	0.97	36.0	23.0	23.0	27.3
R5	8.0	0.05	15.0	27.0	10.0	17.3
V17	8.0	0.06	27.0	22.0	35.0	28.0
VAS	8.0	0.11	9.0	29.0	7.0	15.0
V17	8.0	0.39	43.0	29.0	15.0	29.0
R4	8.0	0.69	36.0	13.0	27.0	25.3
VAS	8.0	1.20	26.0	0.5	23.0	16.5
Mean	7.9	0.30	22.6	15.8	17.4	18.6
VAS	8.0	3.20	30.0	40.0	28.0	32.7
V18	8.1	0.04	24.0	37.0	36.0	32.3
T1	8.1	0.15	10.0	7.0	7.0	8.0
VAS	8.1	0.20	15.0	10.0	5.0	10.0
V17	8.1	0.22	21.0	27.0	27.0	25.0
V17	8.1	0.27	27.0	10.0	23.0	20.0
WJ	8.1	0.39	40.0	51.0	44.0	45.0
WJ	8.1	0.96	76.0	49.0	47.0	57.3
V18	8.1	0.96	23.0	16.0	16.0	18.3
E11	8.1	1.00	37.0	54.0	44.0	45.0
V18	8.1	1.00	27.0	16.0	15.0	19.3
V18	8.2	0.27	17.0	20.0	22.0	19.7
WJ	8.2	0.80	40.0	57.0	47.0	48.0
T1	8.2	0.95	54.0	53.0	41.0	49.3
WJ	8.2	1.30	21.0	26.0	14.0	20.3
VAS	8.2	5.30	34.0	20.0	20.0	24.7
Mean	8.1	1.06	31.0	30.8	27.3	29.7
WC	8.2	21.00	32.0	36.0	27.0	31.7
T1	8.2	26.00	29.0	49.0	34.0	37.3
T1	8.2	33.00	39.0	21.0	28.0	29.3
WC	8.3	0.03	11.0	5.0	7.0	7.7
T1	8.3	1.20	42.0	24.0	39.0	35.0
WC	8.3	2.10	49.0	22.0	29.0	33.3
T1	8.3	2.70	48.0	28.0	29.0	35.0
VAS	8.5	2.50	19.0	14.0	12.0	15.0
WJ	8.5	2.70	34.0	24.0	26.0	28.0
VAS	8.5	12.00	19.0	32.0	13.0	21.3
WC	8.6	3.90	38.0	56.0	20.0	38.0
WJ	8.6	4.90	19.0	14.0	19.0	17.3
WC	8.6	6.40	18.0	26.0	18.0	20.7
T1	8.7	5.10	45.0	38.0	28.0	37.0
WJ	8.9	1.50	54.0	2.0	0.5	18.8
WC	9.2	3.00	35.0	0.5	22.0	19.2
Mean	8.5	8.00	33.2	24.5	22.0	26.5

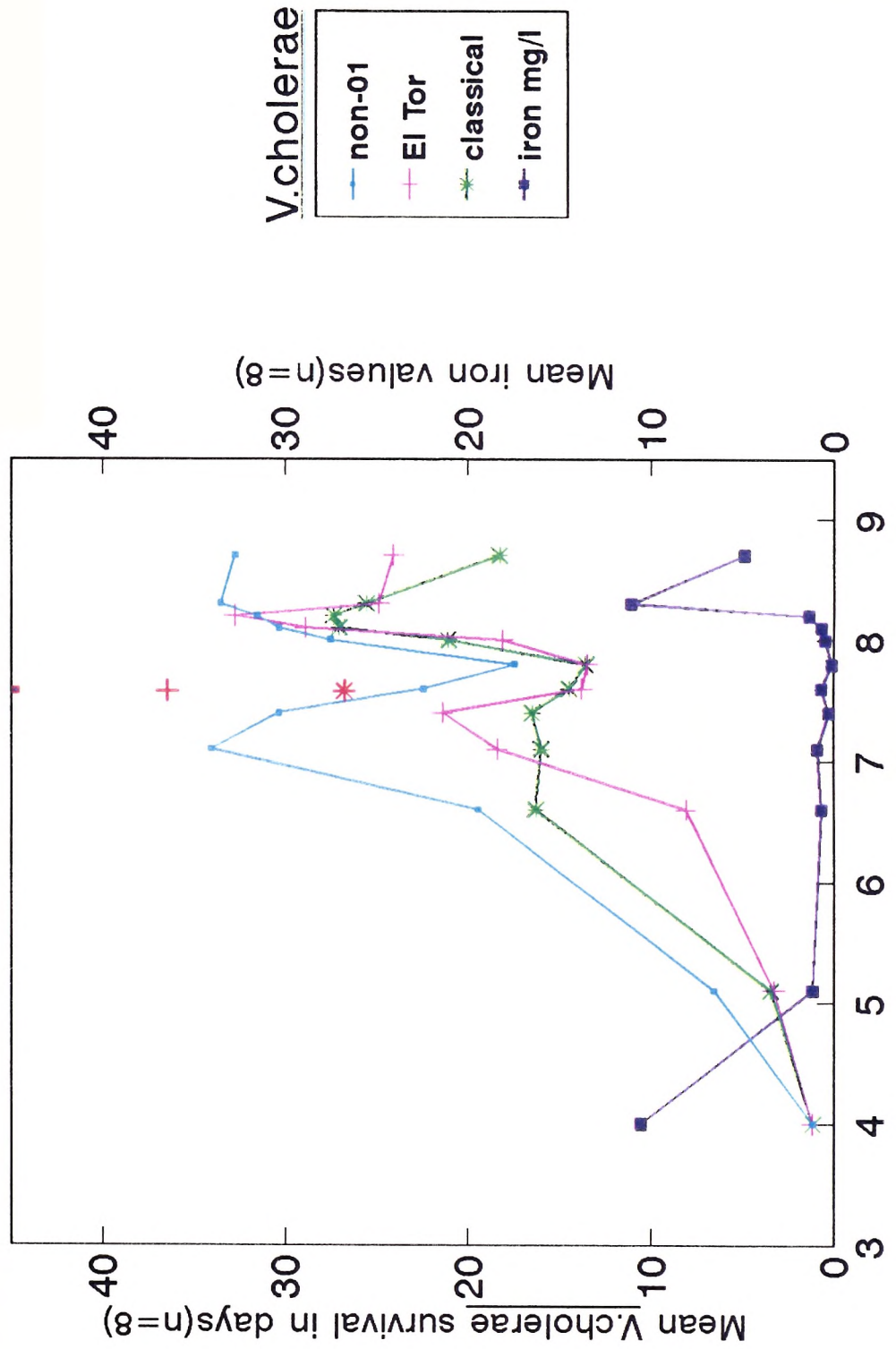

Fig 5.3 Survival of V.cholerae at increasing pH values in effluent polluted water.(all sampling points).Survival in six pH-adjusted samples from point N7 are indicated in red.

Table 5.18 Pooled V.cholerae survival data, sorted by iron and separated in groups of n=16 each.

I.D.	pH	Iron mg/l	Survival of V.cholerae in days			
			Non-01	El Tor	Classical	Mean
E17	6.1	0.03	6.0	3.0	2.0	3.7
R5	7.4	0.03	52.0	19.0	12.0	27.7
R5	7.5	0.03	33.0	14.0	12.0	19.7
R4	7.5	0.03	23.0	9.0	13.0	15.0
R4	7.6	0.03	19.0	14.0	7.0	13.3
V18	7.7	0.03	7.0	7.0	7.0	7.0
V17	7.7	0.03	7.0	4.0	5.0	5.3
R4	7.7	0.03	33.0	14.0	7.0	18.0
R4	7.8	0.03	14.0	8.0	11.0	11.0
R4	7.9	0.03	13.0	13.0	8.0	11.3
WC	8.3	0.03	11.0	5.0	7.0	7.7
V18	7.7	0.04	21.0	8.0	15.0	14.7
V18	8.1	0.04	24.0	37.0	36.0	32.3
R5	7.0	0.05	59.0	52.0	27.0	46.0
R4	7.2	0.05	51.0	44.0	15.0	36.7
R4	7.6	0.05	20.0	27.0	9.0	18.7
Mean	7.6	0.03	24.6	17.4	12.1	18.0
R5	8.0	0.05	15.0	27.0	10.0	17.3
R5	7.9	0.06	9.0	5.0	4.0	6.0
V17	8.0	0.06	27.0	22.0	35.0	28.0
R4	7.9	0.07	14.0	9.0	7.0	10.0
V17	7.2	0.08	35.0	26.0	26.0	29.0
R5	7.5	0.08	37.0	25.0	30.0	30.0
R5	7.5	0.09	34.0	23.0	15.0	24.0
E17	4.0	0.10	5.0	5.0	5.0	5.0
VAS	8.0	0.11	9.0	29.0	7.0	15.0
WJ	7.7	0.12	7.0	3.0	7.0	5.7
T1	8.1	0.15	10.0	7.0	7.0	8.0
VAS	8.1	0.20	15.0	10.0	5.0	10.0
E7	4.6	0.22	42.0	16.0	19.0	25.7
V17	8.1	0.22	21.0	27.0	27.0	25.0
V18	7.9	0.23	28.0	18.0	23.0	23.0
E7	6.8	0.25	8.0	12.0	3.0	7.7
Mean	7.3	0.13	19.8	16.5	14.4	16.9
E17	7.2	0.26	16.0	4.0	6.0	8.7
V17	8.1	0.27	27.0	10.0	23.0	20.0
V18	8.2	0.27	17.0	20.0	22.0	19.7
VAS	7.9	0.29	29.0	1.0	29.0	19.7
E11	6.8	0.36	30.0	0.5	38.0	22.8
E17	6.9	0.37	2.0	1.0	1.0	1.3
E17	7.0	0.37	4.0	2.0	2.0	2.7
V17	8.0	0.39	43.0	29.0	15.0	29.0
WJ	8.1	0.39	40.0	51.0	44.0	45.0
E7	4.8	0.41	3.0	2.0	2.0	2.3
E17	7.3	0.41	1.0	1.0	2.0	1.3
WC1	7.7	0.41	34.0	44.0	34.0	37.3
E11	6.0	0.44	48.0	1.0	55.0	34.7
R5	7.4	0.45	37.0	37.0	14.0	29.3
V17	7.1	0.47	38.0	2.0	24.0	21.3
E7	5.5	0.48	1.0	1.0	1.0	1.0
Mean	7.1	0.38	23.1	12.9	19.5	18.5

TABLE 5.18 (contd.)

I.D.	pH	Iron mg/l	Survival of V.cholerae in days			
			Non-01	El Tor	Classical	Mean
E7	4.6	0.50	0.5	1.0	0.5	0.7
E17	6.5	0.61	6.0	9.0	8.0	7.7
R4	8.0	0.69	36.0	13.0	27.0	25.3
V18	6.9	0.74	29.0	33.0	16.0	26.0
E7	5.6	0.75	2.0	1.0	2.0	1.7
WJ	8.2	0.80	40.0	57.0	47.0	48.0
T1	8.2	0.95	54.0	53.0	41.0	49.3
V18	8.1	0.96	23.0	16.0	16.0	18.3
WJ	8.1	0.96	76.0	49.0	47.0	57.3
WC	7.9	0.97	36.0	23.0	23.0	27.3
E7	4.1	0.99	1.0	1.0	1.0	1.0
E7	4.5	0.99	1.0	1.0	1.0	1.0
E17	5.8	1.00	3.0	3.0	0.5	2.2
E7	7.2	1.00	26.0	43.0	34.0	34.3
V18	8.1	1.00	27.0	16.0	15.0	19.3
E11	8.1	1.00	37.0	54.0	44.0	45.0
Mean	6.9	0.87	24.8	23.3	20.2	22.8
V17	7.6	1.20	29.0	17.0	16.0	20.7
VAS	8.0	1.20	26.0	0.5	23.0	16.5
T1	8.3	1.20	42.0	24.0	39.0	35.0
WJ	8.2	1.30	21.0	26.0	14.0	20.3
E17	3.6	1.37	0.5	0.5	0.5	0.5
WJ	8.9	1.50	54.0	2.0	0.5	18.8
WJ	7.0	1.60	64.0	12.0	22.0	32.7
WC	7.6	2.00	39.0	4.0	42.0	28.3
WC	8.3	2.10	49.0	22.0	29.0	33.3
VAS	7.6	2.50	26.0	23.0	23.0	24.0
VAS	8.5	2.50	19.0	14.0	12.0	15.0
T1	8.3	2.70	48.0	28.0	29.0	35.0
WJ	8.5	2.70	34.0	24.0	26.0	28.0
WC	9.2	3.00	35.0	0.5	22.0	19.2
E11	5.6	3.10	0.5	1.0	2.0	1.2
E11	4.6	3.20	0.5	1.0	1.0	0.8
Mean	7.5	2.07	30.5	12.5	18.8	20.6
E7	6.9	3.20	27.0	5.0	7.0	13.0
VAS	8.0	3.20	30.0	40.0	28.0	32.7
WC	8.6	3.90	38.0	56.0	20.0	38.0
E11	7.0	3.95	6.0	5.0	6.0	5.7
WJ	8.6	4.90	19.0	14.0	19.0	17.3
T1	8.7	5.10	45.0	38.0	28.0	37.0
VAS	8.2	5.30	34.0	20.0	20.0	24.7
WC	8.6	6.40	18.0	26.0	18.0	20.7
VAS	8.5	12.00	19.0	32.0	13.0	21.3
N7	4.4	17.40	0.5	0.5	0.5	0.5
N7	3.6	19.00	0.5	0.5	0.5	0.5
N7	3.3	20.00	0.5	0.5	0.5	0.5
WC	8.2	21.00	32.0	36.0	27.0	31.7
N7	4.4	25.00	0.5	0.5	0.5	0.5
T1	8.2	26.00	29.0	49.0	34.0	37.3
T1	8.2	33.00	39.0	21.0	28.0	29.3
Mean	7.1	13.08	21.1	21.5	15.6	19.4

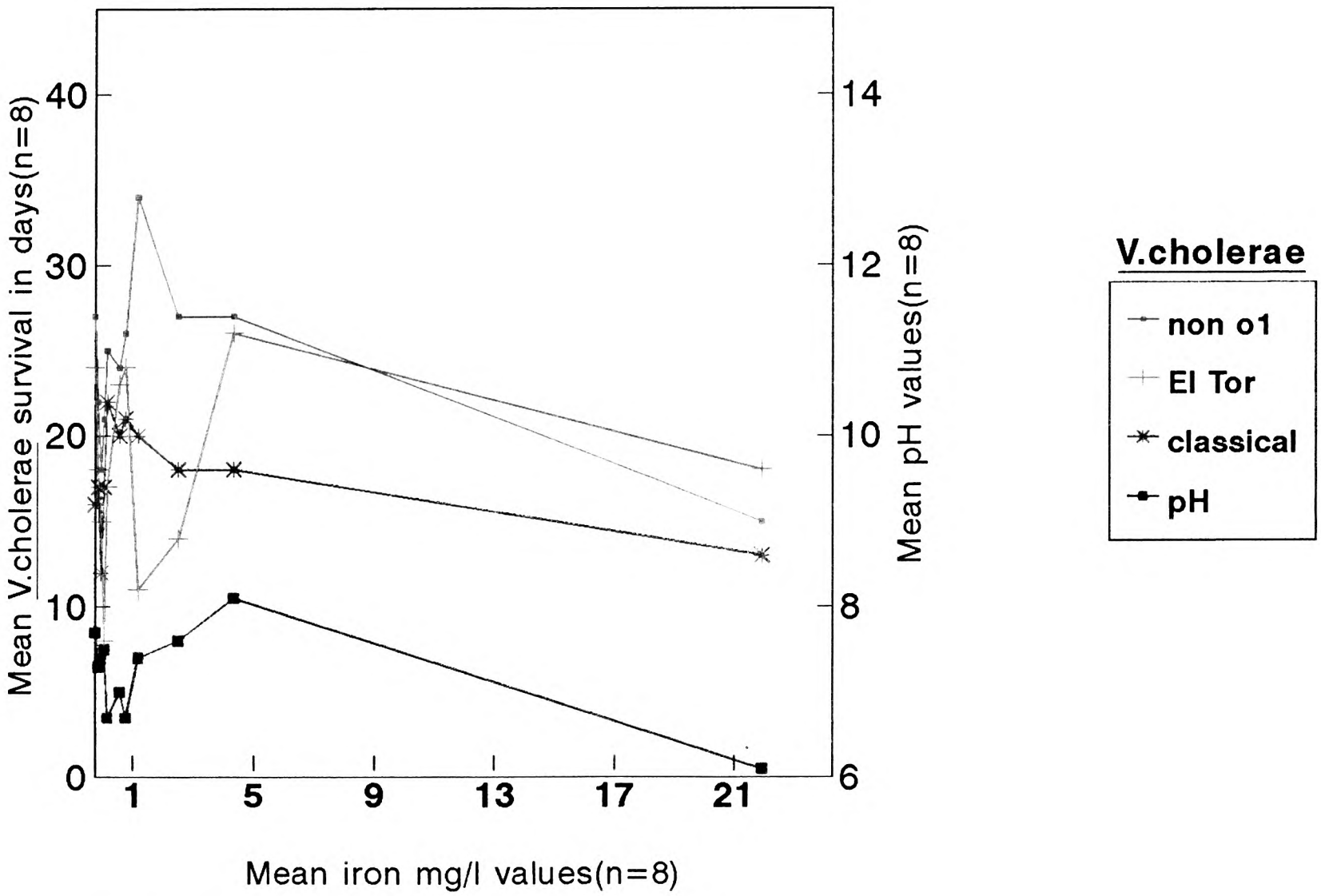

Fig 5.4 Survival of V.cholerae by increasing iron concentration in effluent polluted water.

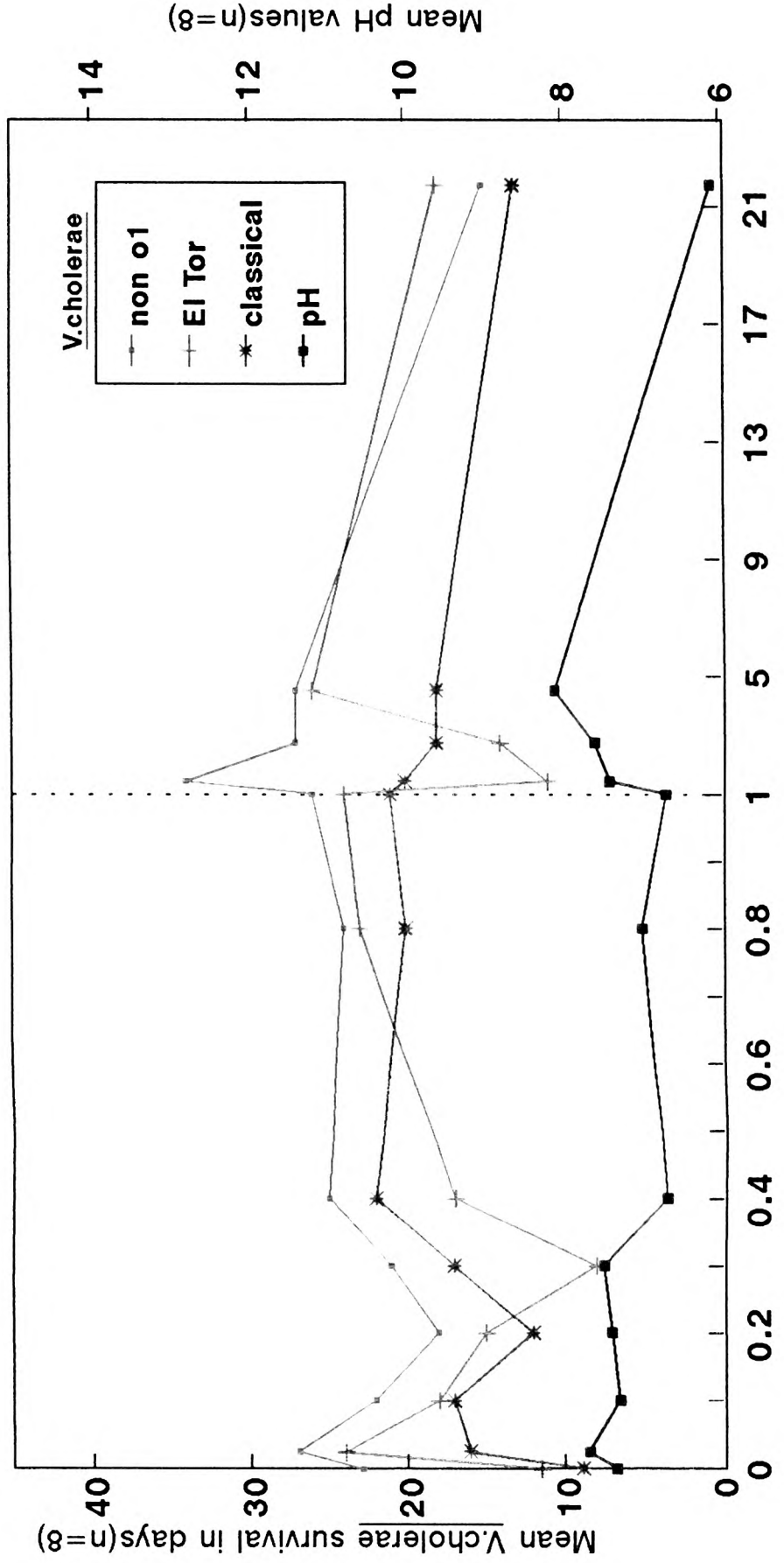

Fig 5.5 Survival of V.cholerae by increasing iron concentration in effluent polluted water - separating the results at iron concentrations below 1 mg/l. Note change of scale at halfway mark of X - axis.

The results of sampling point N7 (table 5.4) attracted attention because of its low pH and high iron levels. The first 4 samples show the highly acidic pH values which are believed, in this case, to have been responsible for the very low survival times (<1 day) of all 3 strains of *V. cholerae*. These results were included in the above analyses. However, six subsequent monthly water samples from this source were used to assess what the survival might have been in the presence of the high iron levels if the pH had been more favourable. The samples were therefore treated with NaOH to raise the pH to a mildly alkaline level of 7.5. The survival results obtained in these samples were among the highest in this study with means of 43 days (non-O1), 37 days (El Tor) and 26 days (classical). These results were superimposed on the graph in figure 5.3.

Table 5.19 presents the ANOVA p values of the survival data at increasing pH. In the case of *V. cholerae* non-O1, the length of survival at a very acidic pH (range 3.3-5.8) is significantly lower ($p < 0.001$) than that at all other pH values studied (A vs B to A vs F). This was done by comparing the survival of *V. cholerae* non-O1 at a mean pH of 4.6 (3.9 days) with the survival at mean pH values of 6.9 (26.8 days), 7.5 (26.4 days), 7.9 (22.6 days), 8.1 (31 days) and 8.5 (33.2 days).

Table 5.19 Analysis of variance (ANOVA) of 96 pooled V.cholerae survival data by pH from 3.3 to 9.2 (the upper half of the table gives the pH, iron and survival times for A,B,C,D,E and F).

Groups compared	pH		Iron mg/l		Survival in days		
	Mean	Range	Mean	Range	Mean	Range	Strain
A:	4.6	3.3-5.8	5.91	0.10-25.00	3.9	0.5-42	NON-01
					2.2	0.5-16	EL TOR
					2.3	0.5-19	CLASSICAL
B:	6.9	6.0-7.2	0.80	0.03- 3.95	26.8	0.5-52	NON-01
					13.2	0.5-52	EL TOR
					16.1	1.0-55	CLASSICAL
C:	7.5	7.2-7.7	0.50	0.03- 2.50	26.4	1.0-52	NON-01
					17.6	1.0-43	EL TOR
					15.5	2.0-42	CLASSICAL
D:	7.9	7.7-8.0	0.30	0.03- 1.20	22.6	7.0-43	NON-01
					15.8	0.5-44	EL TOR
					17.4	4.0-35	CLASSICAL
E:	8.1	8.0-8.2	1.06	0.04- 5.30	31.0	10.0-76	NON-01
					30.8	7.0-57	EL TOR
					27.3	5.0-47	CLASSICAL
F:	8.5	8.2-9.2	8.00	0.03-33.00	33.2	11.0-54	NON-01
					24.5	0.5-56	EL TOR
					22.0	0.5-39	CLASSICAL

Groups compared	Non-01 p =	El Tor p =	Classical p =
A vs B	0.0005	0.01	0.002
A vs C	0.000009	0.00003	0.0002
A vs D	0.00003	0.0002	0.00001
A vs E	0.000004	0.000001	0.000001
A vs F	0.000001	0.000006	0.000001
B vs C	>0.05	>0.05	>0.05
B vs D	>0.05	>0.05	>0.05
B vs E	>0.05	0.007	0.04
B vs F	>0.05	>0.05	>0.05
C vs D	>0.05	>0.05	>0.05
C vs E	>0.05	0.02	0.01
C vs F	>0.05	>0.05	>0.05
D vs E	>0.05	0.01	0.03
D vs F	0.02	>0.05	>0.05
E vs F	>0.05	>0.05	>0.05

Similarly, there is a significant difference ($p = 0.02$) between survival at pH 7.9 and pH 8.5 (D vs F).

The El Tor and classical O1 strains also show significantly shorter ($p \leq 0.01$) survival at the mean pH of 4.6 than at all other pH values (A vs B to A vs F) but, in addition, there is also a significantly longer ($p < 0.05$) survival at mean pH 8.1 than at all mean pH values from 6.9 to 7.9 (B vs E, C vs E and D vs E). These results are not unexpected and are in keeping with known growth characteristics of *V. cholerae* (Sakazaki, R. 1992).

The equivalent results for the data sorted by iron levels are not presented in tabular form as the differences in survival were not significant ($p > 0.05$).

However, the findings suggesting an association between high iron levels and *V. cholerae* survival which is shown clearly as a second peak on the graph in figure 5.3 stimulated a further statistical analysis of the *V. cholerae* survival data focussed around that peak. For this purpose, and again using table 5.17, the data in the last three groups of 16 samples were selected (pH 7.7-9.2). These were then sorted by iron concentration and divided into 6 groups of 8 samples each (for each *V. cholerae* strain), thus obtaining groups with mean pH values that

were more similar to each other. An ANOVA was carried out and yielded the results shown in table 5.20. The p values marked with asterisks denote significant increases in *V. cholerae* survival for water samples which had identical or very similar mean pH values but quite different iron concentrations.

Thus, at pH 8-8.1 and with increasing iron concentrations from 0.04 to 0.52 mg/l the survival time of *V. cholerae* non-O1 increased from 16.8 to 36.6 days, of the El Tor strains it increased from 15.6 to 33.5 days and of the classical strains from 15.8 to 32.4 days. However, with iron levels increasing further from 0.52 to 14.2 mg/l, survival times decreased for all strains.

Although samples with relatively high iron levels formed such a small proportion of the total, making analyses more difficult, it was all the more interesting to find statistically significant survival differences in association with increasing iron levels in the presence of constant pH levels.

Table 5.20 Analysis of variance (ANOVA) of 48 pooled V.cholerae survival data by iron concentration from 0.03 mg/l to 33.0 mg/l (the upper half of the table gives the pH, iron and survival times for A,B,C,D,E and F).

Groups compared	pH		Iron mg/l		Survival in days		
	Mean	Range	Mean	Range	Mean	Range	Strain
A:	8.0	7.7-8.3	0.04	0.03- 0.06	16.8	9.0-27	NON-01
					15.6	5.0-37	EL TOR
					15.8	4.0-36	CLASSICAL
B:	8.0	7.7-8.1	0.17	0.07- 0.27	16.4	7.0-28	NON-01
					14.1	3.0-29	EL TOR
					13.3	5.0-27	CLASSICAL
C:	8.0	7.7-8.2	0.52	0.27- 0.95	36.6	17.0-54	NON-01
					33.5	1.0-57	EL TOR
					32.4	15.0-47	CLASSICAL
D:	8.1	8.0-8.3	1.07	0.96- 1.30	36.0	21.0-76	NON-01
					26.1	0.5-54	EL TOR
					27.6	14.0-47	CLASSICAL
E:	8.5	8.0-9.2	2.70	1.50- 3.90	38.4	19.0-54	NON-01
					23.3	0.5-56	EL TOR
					20.8	0.5-29	CLASSICAL
F:	8.4	8.2-8.7	14.20	4.90-33.00	29.4	18.0-45	NON-01
					29.5	14.0-49	EL TOR
					23.4	13.0-34	CLASSICAL

Groups compared	Non-01 p =	El Tor p =	Classical p =
A vs B	>0.05	>0.05	>0.05
A vs C	0.0005*	>0.05	0.01*
A vs D	0.01*	>0.05	>0.05
A vs E	0.0004	>0.05	>0.05
A vs F	0.01	0.03	>0.05
B vs C	0.0008*	>0.05	0.002*
B vs D	0.01*	>0.05	0.03*
B vs E	0.0006	>0.05	>0.05
B vs F	0.01	0.01	0.03
C vs D	>0.05	>0.05	>0.05
C vs E	>0.05	>0.05	0.05
C vs F	>0.05	>0.05	>0.05
D vs E	>0.05	>0.05	>0.05
D vs F	>0.05	>0.05	>0.05
E vs F	>0.05	>0.05	>0.05

* = Significant survival enhancement in the presence of increasing iron at similar mean pH levels

5.4 CONCLUSIONS

In contrast to the study described in the previous chapter, this study was performed on water samples collected from industrially polluted surface waters, but also addressed the effect of iron and pH on *V. cholerae* survival. The following conclusions were drawn:

- The finding that, at a pH of 8.0-8.1, *V. cholerae* survival time increased significantly with increasing iron levels, suggests that a combination of alkaline pH and high iron levels in surface water bodies is favourable for prolonged survival and persistence of cholera bacilli. This finding has not been reported before.

- *V. cholerae* survival was found to be longest during October. Increased ambient temperature was probably an important factor in this finding.

- *V. cholerae* non-O1 survived significantly longer than O1 strains which was in keeping with the results of other workers. However, the recent finding that non-O1 *V. cholerae* (O139) can cause epidemic cholera, lends new significance to this survival characteristic.

Industrially and agriculturally polluted surface waters are frequently the sole source of domestic water for vast rural populations. Contamination of such water sources with *V. cholerae* may occur. This study showed that, in the presence of high iron and alkaline pH levels, enhanced multiplication and survival of *V. cholerae* may occur, possibly to the extent of precipitating cholera epidemics and/or sustaining endemic persistence of *V. cholerae* in aquatic habitats.

5.5 REFERENCES

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CHAPTER 6

THE EFFECT OF IRON ON THE TOXIGENICITY OF V.CHOLERAE

6.1 INTRODUCTION

Iron is an essential component of many enzymes and other proteins and is required for growth of virtually all bacteria (Lankford, 1973). The environment as well as animal hosts have limited amounts of free iron available for microbial growth since the metal forms insoluble complexes at neutral or alkaline pH and is sequestered in hosts by iron-binding proteins. One iron acquisition system found in some pathogens, including *V. cholerae*, consists of a microbially produced iron-binding compound, or siderophore, and proteins associated with the transport and utilization of chelated iron. According to Sigel and Payne (1982), environmental strains of *V. cholerae* produce more siderophore than clinical isolates.

The iron transport system is related to increased virulence of some invasive pathogens. Low or unavailable iron may trigger virulence factors such as the shiga toxin of *Shigella dysenteriae* (Dubos and Geiger, 1946), diphtheria toxin of *Corynebacterium diphtheriae* (Pappenheimer, 1955),

shiga-like toxin I of enterohaemorrhagic *Escherichia coli* (Calderwood and Mekalanos, 1987) and exotoxin A of *Pseudomonas aeruginosa* (Bjorn *et al.*, 1978; 1979).

No information was found in the literature on the possible effect of iron on toxin production by *V. cholerae*. Since iron is an important industrial pollutant of water, this chapter describes a study of the effect of iron on enterotoxin production by *V. cholerae* outside the human host.

6.2 MATERIALS AND METHODS

Details of the media and solutions used in this study are found in the appendix at the end of this chapter (section 6.7).

6.2.1 Cholera toxin titration

Cholera toxin titration was done by diluting concentrated (1 μg/μl) purified toxin (ICN Biochemicals, Cleveland, Ohio) to 10 ng/ml using phosphate buffered saline (PBS). This was further diluted through five, two-fold (doubling) dilutions. Each dilution was subjected to the GM_1-ELISA described in table 6.1 (microtitre ganglioside enzyme-linked immunosorbent assay). The entire procedure was

TABLE 6.1 Micro titre ganglioside ELISA method used for determination of cholera toxin concentration (Sack et al, 1980).

Procedure	Diluent	Concentration	Vol per well (µl)	Incubation time	Incubation temp
1. Precoat plate with GM_1 ganglioside	PBS	1µg/ml	100	Overnight	Room temp
2. Wash 3 X with PBS-Tween			200	3 min	Room temp
3. Add test sample	Undiluted or diluted PBS-Tween with 1% FCS		100	Overnight	Room temp
4. Wash 3 X with PBS-Tween			200	3 min	Room temp
5. Add guinea pig anti-CT	PBS-Tween 1% FCS	1:2500	100	1 h	37° C
6. Wash 3 X with PBS-Tween			200	3 min	Room temp
7. Add enzyme-labeled sheep guinea pig globulin	PBS-Tween with 1% FCS	1:1000	100	1 h	37° C
8. Wash 3 X with PBS-Tween			200	3 min	Room temp
9. Add substrate	10% DEA buffer	1 mg/ml	100	45 min	37° C
10. Stop reaction with NaOH		3 M	25		
11. Read OD at 405nm in colorimeter					

PBS - Phosphate-buffered saline;
FCS - Fetal calf serum
DEA - Diethanolamine
CT - Cholera toxin
OD - Optical density

repeated 10 times and the results which were obtained in the form of optical densities, were analysed using linear regression to generate a standard curve for measurement of cholera toxin production by test organisms.

6.2.2 Bacterial cultures

Ten toxin and non-toxin producing strains of *V.cholerae* were used (table 6.2). The cultures were maintained in semi-solid agar at room temperature.

6.2.3 Procedure used to compare toxin production in YEP (Yeast extract peptone) and CAYEG (Casamino acid yeast extract glucose) media

V. cholerae cultures planted on blood agar plates were incubated at 37°C for 24 hours. A loopful of culture was then inoculated into 10ml alkaline peptone water (APW) and incubated at 37°C for 16 hours.

Sterile glass tubes (150 mm x 15 mm) containing 10 ml of YEP (yeast extract peptone) medium were inoculated with 1 ml of the incubated APW culture and incubated at 37°C in a stationary position (Iwanaga *et al.*, 1986). After 4 hours, cultures were transferred into 50 ml sterile flasks and further incubated at 37°C on a rotary shaker at 150 rpm for

TABLE 6.2 Description of V.cholerae strains used in this study.

Origin and Ref. no.	Biotype	Serotype	Toxigenic
ATCC 25870[a]	Classical	01 Inaba	Yes
ATCC 9459[a]	Classical	01 Inaba	Yes
ATCC 9458[a]	Classical	01 Ogawa (rough)	Yes
ATCC 582[a]	El Tor	01 Inaba	Yes
SAIMR 22453[b] (ex-patient)	El Tor	01 Ogawa	Yes
SAIMR 20856[b] (ex-sewage)	El Tor	01 Ogawa	Yes
ATCC 51394[c] (M045)	-	0139	Yes
V315[c]	-	Non-01 untypable	Yes
0469[c]	-	05	No
SG2[c]	-	060	No

[a] By courtesy of the CDC, Atlanta, Georgia, USA
[b] By courtsey of the SAIMR, Johannesburg, RSA
[c] By courtesy of Dr G B Nair, NIC & ED - WHO CDDRT, Calcutta, India.

16 hours. The cultures were centrifuged at 5000 rpm for 30 minutes. From the supernatant, cholera toxin was quantitated using a microtitre GM_1 ganglioside ELISA (enzyme-linked immunosorbent assay) (Sack *et al.*, 1980). The experiment was done once with each strain of *V. cholerae* and was also repeated using CAYEG, an organic nutrient medium.

6.2.4 Preparation of bacterial inoculum and culture medium titration

Cultures planted on blood agar plates were incubated at $37^{o}C$ for 24 hours. A loopful of culture was then inoculated into 50 ml CAYEG. After 16 hours incubation at $37^{o}C$, 5 ml of above culture was subcultured in another 50 ml of CAYEG and further incubated at $37^{o}C$ for 4 hours. Approximately 15 ml of the culture was centrifuged at 4000 rpm for 20 minutes. The sediment was washed twice with ¼ strength Ringers solution and the final sediment reconstituted in 5 ml of the same solution. The count of organisms in the final suspension was always found to be approximately 10^{8} organisms per ml.

The ingredients of the CAYEG medium used may have contained small, unspecified amounts of iron which could have affected the experiments in which the consistency of

carefully measured iron concentrations was very important. Such 'contaminating' iron was therefore eliminated by a process of deferration based on a calcium chloride mediated iron precipitation as described by Bjorn *et al* (1978)(see appendix 6.7.1).

A series of 25 flasks of 25 ml capacity was prepared with ferric oxide (Fe_2O_3) after which the flasks were autoclaved. Aliquots of 25 ml sterile distilled water containing deferrated CAYEG, were then added aseptically to the flasks. The amounts of ferric oxide and CAYEG added to the flasks were calculated to yield final concentrations as shown in the following checkerboard scheme.

ferric oxide g/l \ CAYEG g/l	0	0.14	0.28	0.56	1.12
10.0					
1.0					
0.1					
0.01					
0					

One such set of 25 flasks with relatively low concentrations (0.14 - 1.12 g/l) of CAYEG was prepared for each cholera strain under study. During the course of the experiments it was found necessary (See 6.3.3 below) to use also a high CAYEG concentration of 5.6 g/l. Each flask was inoculated with 100μl of the final suspension of the appropriate cholera strain and incubated at 37°C, on a shaker at 150 rpm, for 16 hours. Counts of *V.cholerae* were done on each flask using a microdilution technique (Kramer J., 1977), with minor modifications (Patel and Isaäcson, 1994), detailed in section 4.2.1.

6.2.5 Procedure to determine the effect of iron on toxin production by *V. cholerae*

Five 25 ml flasks were prepared with 0, 0.01, 0.1, 1.0 and 10.0 g/l Fe_2O_3 respectively, and autoclaved. To each flask, 25 ml of sterile distilled water containing deferrated CAYEG was added aseptically to give a final concentration of 5.6 g/l. One such set of 5 flasks was prepared for each cholera strain studied. The flasks were inoculated and incubated as described above. After 16 hours incubation the cultures were centrifuged at 5000rpm for 30 minutes. The presence of cholera toxin was then assayed in the supernatants using the microtitre GM_1-ELISA and Y_1 mouse adrenal cell line techniques. This experiment

was replicated five times with all ten strains of *V. cholerae*.

6.2.6 Microtitre ganglioside ELISA assay

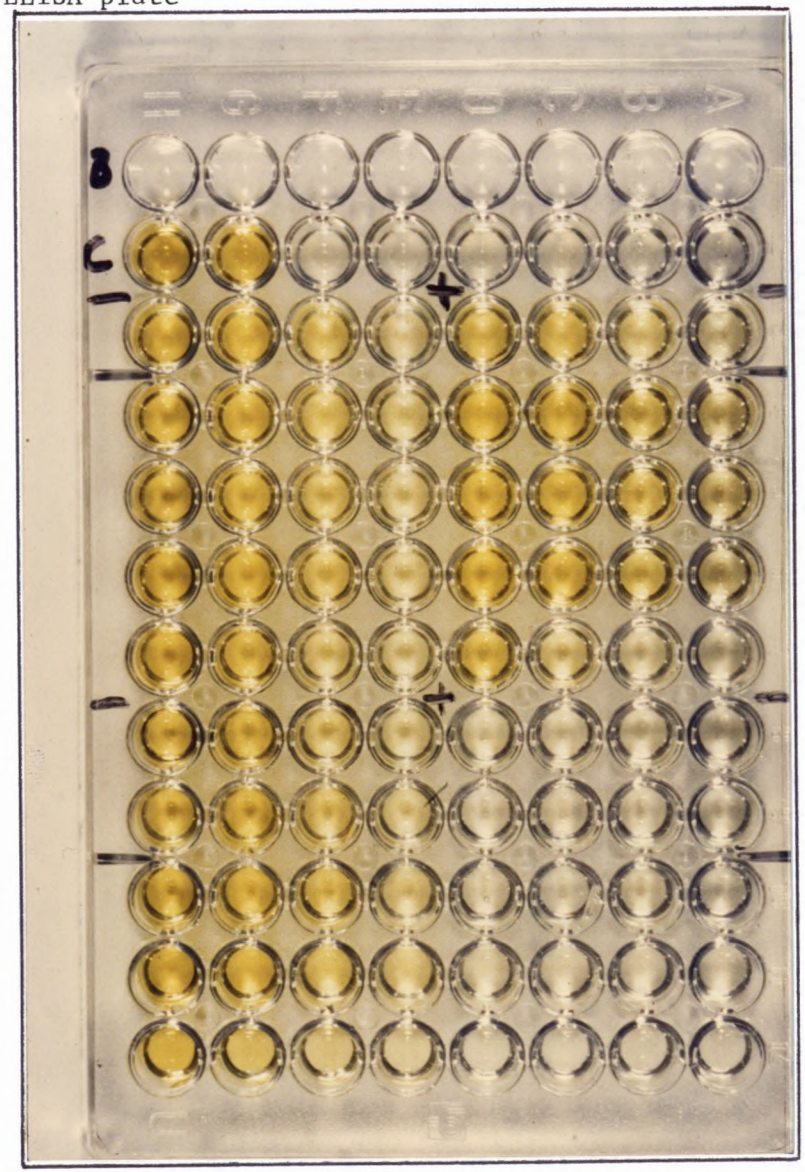
Polyvinyl microtitre "U" plates (Wellcome Reagents Limited, S.A) were used for all ELISA procedures. GM_1 ganglioside (Matreya Inc., Pleasant, GAP PA, USA), which is supplied as a chloroform methanol suspension, was allowed to stand uncovered to enable complete drying by evaporation of the solvent. It was then diluted in PBS to 1 μg/ml.

To obtain cholera antitoxin, guinea pigs were immunised subcutaneously with 10 μg of cholera toxin (CT) in Freund's complete adjuvant on day 0, with 10 μg of CT in Freund's incomplete adjuvant on day 21 and with 10 μg of CT in saline on day 36. Blood was collected on day 50 and centrifuged at 2000 rpm for 15 minutes. The serum was separated, dispensed into 1 ml aliquots and frozen at -20°C until required. Sheep anti-guinea pig globulin conjugated with alkaline phosphatase (Boehringer Mannheim Biochemica), p-nitrophenyl phosphate (Sigma Chemicals), and purified cholera toxin (ICN Biochemicals, Cleveland, Ohio) were also used in the ELISA assay.

This assay involved the consecutive addition of reagents (at different time intervals) to the wells of the microtitre plates as outlined in table 6.1 (Sack *et al.*, 1980) and washings were performed to remove unbound material. Cholera toxin and PBS-Tween as well as uninoculated culture medium with and without iron were included on each plate as positive and negative controls respectively. The optical density (OD) of the colour reaction was measured in a colorimeter (ELISA reader, Dynatech) at 405nm wave length (figure 6.1). As the scanner can read OD efficiently only up to 2.0, the samples were always diluted (double dilutions) in the microtitre plates and the readings were multiplied by the dilution factors. The OD of negative controls never exceeded 0.1. In accordance with the practice of Sack *et al.* (1980) an OD of ≤ 0.2 was therefore considered as a negative result.

The results of the microtitre GM_1-ELISA were analysed for their significance by multiple regression analysis. This was done instead of linear regression because of the former's ability to show significant concentrations of iron in terms of toxin production.

Fig 6.1 GM1-ganglioside microtitre ELISA plate



Blank - No fluid

Controls-H&G-Positive
F&E-Negative

CT production: 25870-H-undiluted
$G\frac{1}{2}$, $F\frac{1}{4}$, $E\frac{1}{8}$ Replicate 1

Replicate 2

Replicate 3

Replicate 4

Replicate 5

Ct production: 9458-H-undiluted
$G\frac{1}{2}$, $F\frac{1}{4}$, $E\frac{1}{8}$ Replicate 1

Replicate 2

Replicate 3

Replicate 4

Replicate 5

Blank - No fluid

D, C, B, A - No samples

9459 - D undiluted, $C\frac{1}{2}$, $B\frac{1}{4}$, $A\frac{1}{8}$
Replicate 1

Replicate 2

Replicate 3

Replicate 4

Replicate 5

No samples

No samples

No samples

No samples

No samples

6.2.7 Y_1 mouse adrenal cell line assay

6.2.7.1 Cell culture storage, recovery and subculture

Aliquots of 1 ml Y_1 adrenal cells in RPMI (Roswell Park Memorial Institute) 1640 (Highveld Biologicals, S.A) complete growth medium with 7.5% vol/vol dimethyl sulfoxide (DMSO) containing $5x10^6$ cells/ml were frozen and stored at -70^oC (Schroy and Todd, 1976). DMSO was used as a cryopreservative.

The cells were recovered by vigorously agitating the vial in a 37^oC water bath. The cells were established in 20 ml complete RPMI 1640 in a 75 cm^2 tissue culture flask and incubated at 37^oC in a humidified atmosphere containing 95% air and 5% CO_2. The medium was replaced after 4 hours incubation i.e after attachment of cells to the surface of the flask.

The culture medium was changed every 48 hours until 80% confluency of cells was obtained when the medium was changed again. After a further 16 hours incubation, the cells were dissociated using 0.05% trypsin and 1:3000 wt/vol EDTA in a Ca^{++} and Mg^{++} free Hanks' balanced salt solution (1:1) (Highveld biologicals, S.A) and incubated at 37^oC for 20 minutes. The cells were separated by

centrifuging the suspension at 1000 rpm for 5 minutes and were resuspended in complete RPMI 1640 medium. Some cells were utilized for the experiment and some were passaged into fresh tissue culture flasks (Maneval *et al.*, 1980).

6.2.7.2 Toxin assay

Two flasks, one without Fe_2O_3 and one with Fe_2O_3, 10 g/l, were otherwise prepared, inoculated, incubated and centrifuged as described under 6.2.5 above. One such pair of flasks was used for each cholera strain to be studied. The culture supernatants were filtered through 0.45 μm disposable filters. Samples (100 μl) of the culture filtrates, serially diluted in Hanks' balanced salt solution, were added to each well of 96-well tissue culture plates (Nunc, Weil Organisation Pty Ltd). A 200 μl amount of the cell suspension (10^4 cells/well) was added to each well of the plates which were incubated at 37°C in a 5% CO_2 humidified incubator (Ramamurthy *et al.*, 1993). Cytotonic and cytotoxic changes in 50% of the cells were recorded at 48 hours. The uninoculated culture medium and pure cholera toxin were used as negative and positive controls respectively. The experiment was replicated three times on each cholera strain under study.

6.3 RESULTS

6.3.1 Titration of known cholera toxin (CT) concentrations.

Table 6.3 shows the optical densities of 10-0.312 ng CT/ml, read at 405nm. These results were used to plot a regression line which is presented in figure 6.2. CT concentrations were arbitarily chosen to range from 10 ng/ml to 0.312 ng/ml for the construction of regression slope. The finding that certain *V. cholerae* strains produced toxin in quantities larger than 10 ng/ml, was only obtained subsequently. The formula used to calculate *the* concentration of CT in unknown samples is as follows:

CT in ng/ml = (a x OD) - b where:

a (the slope of the line) = 4.777

b (X-intercept) = 0.071

This formula allowed calculation of toxin levels with an accuracy of two decimal points. The OD results are similar to those obtained on cholera toxin by other workers such as Sack *et al* (1980). The lowest anount of toxin accurately measured by this technique was 0.625 ng/ml. For the purpose of this study measurements below this amount were considered negative.

TABLE 6.3 Optical densities (at 405nm) of ten cholera toxin titrations using the GM_1 ganglioside ELISA.

Titration no.	Optical density of toxin concentrations (ng/ml)					
	10.0	5.0	2.5	1.25	0.625	0.312
1	1.714	0.996	0.508	0.281	0.165	0.110
2	1.708	0.829	0.420	0.238	0.140	0.084
3	1.737	0.918	0.464	0.237	0.123	0.078
4	1.819	0.986	0.478	0.300	0.106	0.060
5	1.842	0.933	0.522	0.253	0.135	0.082
6	2.274	1.013	0.474	0.215	0.150	0.089
7	2.246	1.145	0.632	0.346	0.192	0.140
8	2.013	1.011	0.456	0.283	0.158	0.098
9	2.350	1.382	0.734	0.394	0.232	0.152
10	2.604	1.341	0.786	0.498	0.277	0.162

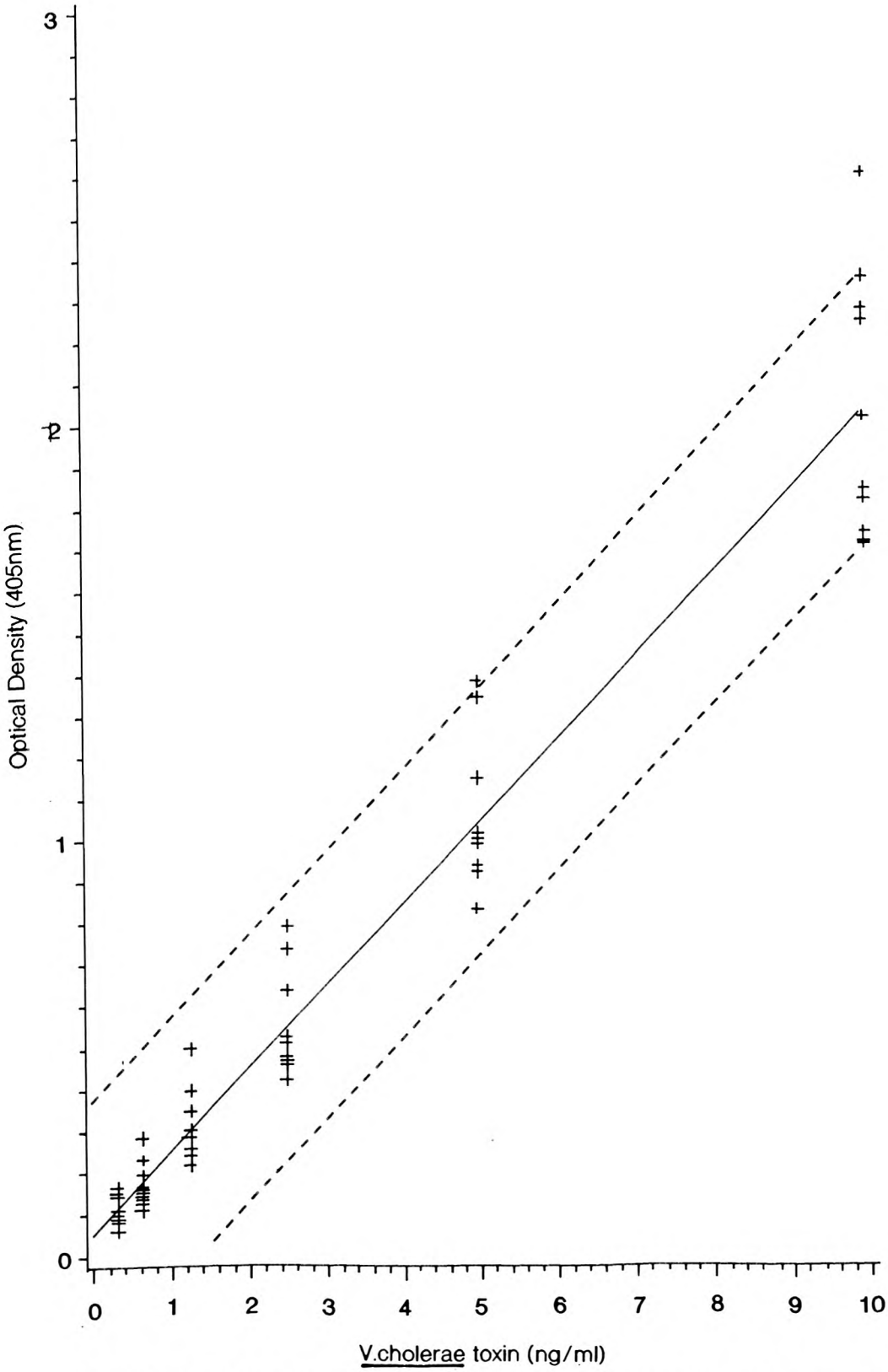


Fig 6.2 Titration slope using linear regression for cholera toxin values obtained by GM1 ganglioside micro – ELISA. Ten OD readings, taken for each CT concentrations are represented by + symbols and the interrupted lines represent 95 % confidence intervals.

6.3.2 Cholera toxin production in YEP medium.

The results of CT production by *V. cholerae* in YEP and CAYEG media are shown in table 6.4. Of the 8 known toxigenic strains tested, those of El Tor produced the least amount of toxin. The toxigenic non-O1 isolates produced more toxin than El Tor, but considerably below the very high levels yielded by classical *V. cholerae*. The latter produced more toxin in YEP than in CAYEG medium. The known non-toxigenic non-O1 strains (negative controls) yielded OD readings below 0.2.

Although toxin production was better in YEP (1.82-2850.91 ng/ml) than in CAYEG (1.42-121.69 ng/ml), the actual growth of bacteria was much better (in terms of visual turbidity) in the latter. CAYEG was therefore used as the nutrient source.

6.3.3 Effect of iron on toxin production by *V. cholerae* as demonstrated by the microtitre GM_1-ELISA technique.

To demonstrate the effect, if any, of iron on the toxigenicity of *V. cholerae*, it was also necessary to eliminate enhanced bacterial growth giving a false impression of enhanced toxigenicity. All the cholera strains were therefore initially incubated in deferrated

TABLE 6.4 Production of cholera toxin by ten strains of V.cholerae in YEP and CAYEG medium.

Culture	Strain	Cholera toxin ng/ml	
		YEP	CAYEG
V.cholerae O1 classical	25870	320.77	14.10
	9458	2850.91	147.20
	9459	1146.66	121.69
V.cholerae O1 El Tor	582	1.82	1.42
	22453	2.96	2.25
	20856	3.29	3.88
V.cholerae non-O1 toxigenic	M045	7.59	7.79
	V315	5.55	4.90
V.cholerae non-O1 non toxigenic	0469	0.58	0.44
	SG2	0.75	0.83

CAYEG media containing CAYEG in rising concentrations from 0-1.12 g/l with added iron ranging from 0-10 g/l. Bacterial counts steadily increased with increasing iron. In an attempt to overcome this growth-stimulating effect of iron a higher concentration of 5.6 g CAYEG/l was then selected arbitrarily. It was found that this high CAYEG concentration yielded maximal bacterial growth which was not boosted by the addition of iron in any concentrations of up to 10 g/l. This CAYEG concentration was therefore selected for use as the medium of choice in which to demonstrate the effect of increasing iron levels on toxigenicity without interference by iron-enhanced bacterial growth.

6.3.3.1 Results with the microtitre GM_1-ELISA technique.

The effects of increasing iron concentrations on the toxigenicity of V. cholerae are given as individual optical density readings in Appendix 6.7.2 (tables 6.5b, 6.6b and 6.7b) whereas tables 6.5a, 6.6a and 6.7a are summaries to show, for each cholera strain, the OD ranges, their means, standard deviations, conversions to toxin levels in ng/ml and the statistical significance of any differences in the findings at the various iron levels. Means and standard deviations are also shown in figures 6.3, 6.4 and 6.5. Probability values were calculated separately for each

TABLE 6.5a Summarised results: Effect of iron (Fe_2O_3) on cholera toxin production by V.cholerae 01 classical strains. Toxin measured by GM_1 micro-ELISA (A405).

V.cholerae strain no.	Iron concentration g/l	Optical density			Toxin level ng/ml	p value
		Range (n=5)	Mean	SD*		
25870**	0	0.755-1.192	0.906	0.172	34.05	baseline
	0.01	0.886-1.493	1.134	0.230	42.76	0.17
	0.1	0.962-1.115	1.027	0.063	38.67	0.46
	1.0	1.537-1.875	1.777	0.139	67.33	<0.01
	10.0	1.750-2.710	2.172	0.457	82.43	<0.01
9458	0	0.721-1.242	0.928	0.216	4.36	baseline
	0.01	0.607-1.355	0.999	0.288	4.70	0.80
	0.1	0.678-1.396	0.978	0.345	4.60	0.86
	1.0	1.227-2.661	1.770	0.542	8.38	<0.01
	10.0	2.055-3.729	2.870	0.611	13.64	<0.01
9459	0	1.403-1.903	1.650	0.188	7.81	baseline
	0.01	1.503-2.080	1.713	0.233	8.11	0.73
	0.1	1.375-2.242	1.847	0.314	8.75	0.29
	1.0	2.149-3.148	2.735	0.417	12.99	<0.01
	10.0	2.518-3.076	2.923	0.235	13.89	<0.01

* Standard deviation.

** The optical density was measured at ⅛ dilution and adjusted.

TABLE 6.6a Summarised results: Effect of iron (Fe_2O_3) on cholera toxin production by V.cholerae O1 El Tor strains. Toxin measured by GM_1 micro-ELISA (A405).

V.cholerae strain no.	Iron concentration g/l	Optical density			Toxin level ng/ml	p value
		Range (n=5)	Mean	SD*		
582	0	0.108-0.249	0.178	0.060	0.78	baseline
	0.01	0.150-0.353	0.253	0.088	1.14	0.19
	0.1	0.138-0.359	0.250	0.097	1.12	0.21
	1.0	0.221-0.440	0.305	0.090	1.39	<0.05
	10.0	0.230-0.475	0.411	0.103	1.89	<0.01
22453	0	0.616-0.767	0.684	0.064	3.20	baseline
	0.01	0.601-0.711	0.664	0.046	3.10	0.91
	0.1	0.510-0.816	0.698	0.119	3.26	0.94
	1.0	0.866-1.632	1.116	0.334	5.26	<0.05
	10.0	2.051-3.446	2.815	0.512	13.37	<0.01
20856	0	0.169-0.295	0.249	0.052	1.12	baseline
	0.01	0.192-0.294	0.242	0.045	1.08	0.84
	0.1	0.187-0.288	0.239	0.042	1.07	0.77
	1.0	0.176-0.338	0.274	0.066	1.24	0.45
	10.0	0.403-0.547	0.455	0.055	2.10	<0.01

* Standard deviation.

TABLE 6.7a Summarised results: Effect of iron (Fe_2O_3) on cholera toxin production by V.cholerae non O1 strains. Toxin measured by GM_1 micro-ELISA (A405).

V.cholerae strain no.	Iron concentration g/l	Optical density			Toxin level ng/ml	p value
		Range (n=5)	Mean	SD*		
M045	0	0.269-0.399	0.347	0.064	1.59	baseline
0139	0.01	0.288-0.464	0.378	0.079	1.73	0.41
	0.1	0.390-0.482	0.424	0.036	1.95	0.05
toxigenic	1.0	0.445-0.515	0.492	0.027	2.28	<0.01
	10.0	0.523-0.705	0.585	0.072	2.72	<0.01
V315	0	0.786-0.865	0.818	0.031	3.84	baseline
	0.01	0.775-0.800	0.787	0.009	3.69	0.31
toxigenic	0.1	0.701-0.879	0.826	0.072	3.87	0.78
	1.0	1.105-1.180	1.150	0.029	5.42	<0.01
	10.0	1.017-1.180	1.115	0.060	5.25	<0.01
0469	0	0.157-0.185	0.177	0.012	0.77	N/A
non-	0.01	0.149-0.188	0.167	0.017	0.73	N/A
toxigenic	0.1	0.164-0.184	0.172	0.008	0.75	N/A
	1.0	0.146-0.174	0.160	0.011	0.69	N/A
	10.0	0.151-0.176	0.163	0.011	0.71	N/A
SG2	0	0.103-0.169	0.124	0.026	0.52	N/A
non-	0.01	0.098-0.131	0.109	0.014	0.45	N/A
toxigenic	0.1	0.095-0.130	0.108	0.016	0.41	N/A
	1.0	0.099-0.126	0.109	0.011	0.45	N/A
	10.0	0.100-0.116	0.107	0.008	0.44	N/A

* SD - Standard deviation
N/A - Not applicable

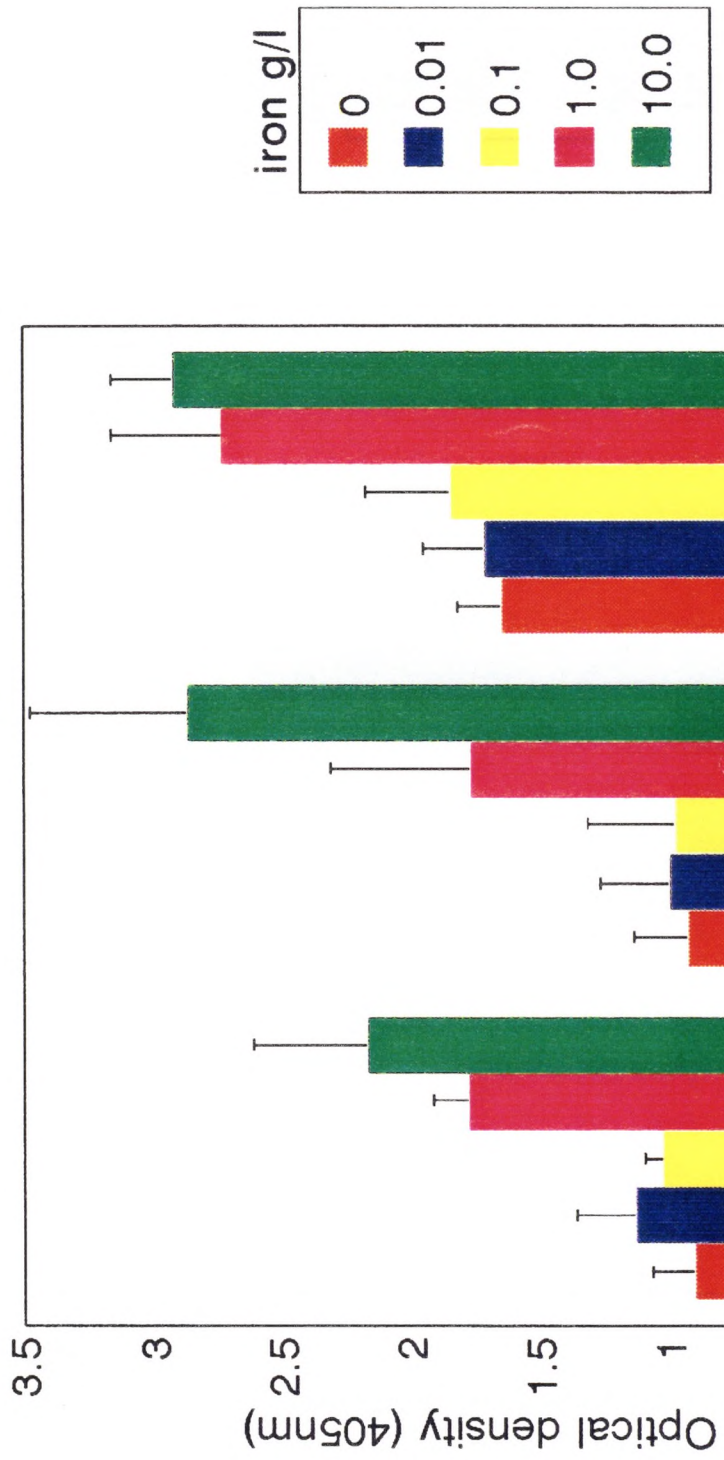


Fig 6.3 Cholera toxin production by V.cholerae 01 classical in response to increasing iron concentrations.For strain 25870, which had very high toxin levels,the ODs are plotted at 1/8 dilution.

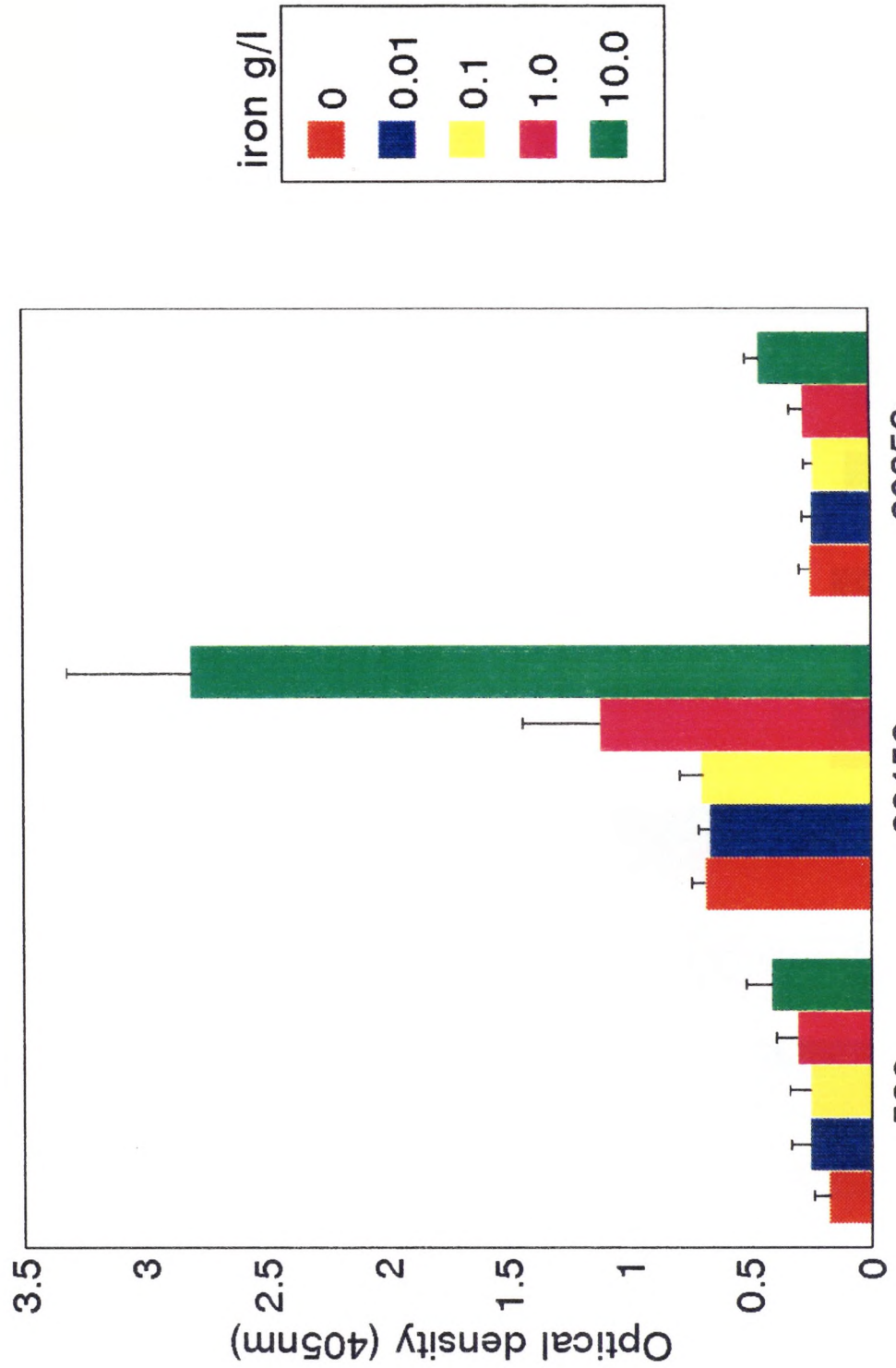


Fig 6.4 Cholera toxin production by V.cholerae O1 E1 Tor in response to increasing iron concentrations.

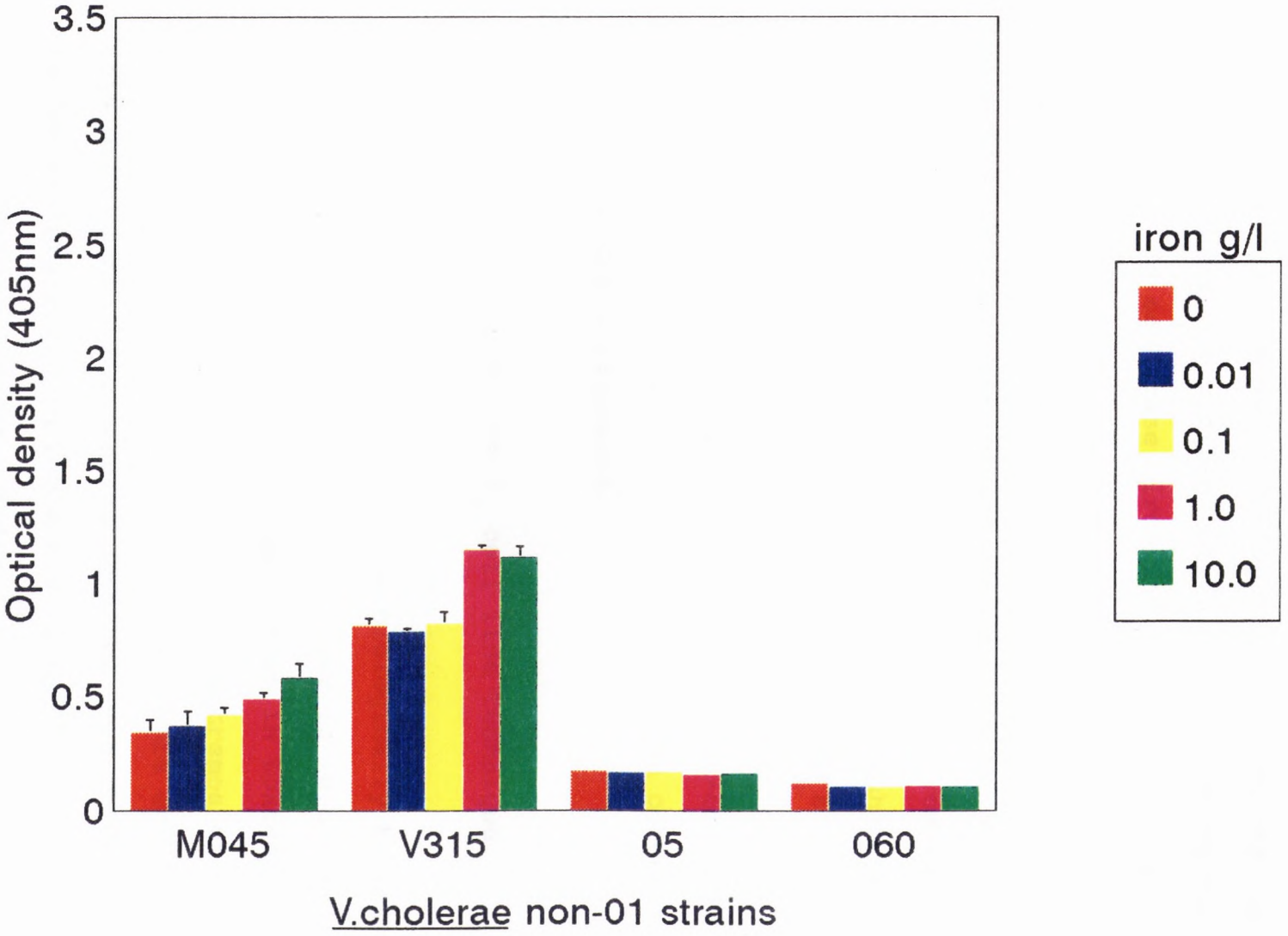


Fig 6.5 Cholera toxin production by V.cholerae non-01 in response to increasing iron concentrations.

bacterial strain by means of a multiple regression analysis, using the SAS computer programme (SAS, 1988). Iron concentrations were entered into the computer as variables and the toxin production OD readings as the responses. In each case, iron was found to be the significant factor in the toxin production of the strain concerned. The data were further analysed to identify the iron concentration/s of significance in this context. The results of this analysis showed that, when iron levels were increased to 1 g/l and higher, significant enhancement of toxigenicity occurred with all the toxigenic strains except one El Tor isolate (no. 20856). The OD readings for the non-toxigenic strains remained below the cut-off point of 0.2 and were not increased by increased iron concentrations in their growth environment.

6.3.3.2 Results with the Y_1 cell line technique.

Toxin production was also assayed biologically by using the Y1 cell line method. As this technique is less sensitive for the detection of slight differences in toxigenicity, only the low (zero) and high (10 g/l) extremes of the iron exposure range used in the ELISA study, were employed in this experiment. The results obtained with three separate series are presented in table 6.8 as the highest dilution showing cytotoxic and/or cytotonic effects.

TABLE 6.8 Effect of iron (Fe_2O_3) on cholera toxin production by V. cholerae. Toxin measured by Y_1 mouse adrenal cell line method.

Strain	Fe_2O_3 g/l	Positive titre		
		replicate 1	replicate 2	replicate 3
25870	0	1:128	1:64	1:128
	10	>1:2048	>1:2048	>1:2048
9458	0	1:512	1:64	1:64
	10	>1:1024	1:1024	1:1024
9459	0	1:256	1:256	1:64
	10	>1:1024	>1:1024	1:1024
582	0	1:64	1:64	1:64
	10	1:1024	1:1024	1:512
22453	0	1:8	1:16	1:4
	10	1:64	1:32	1:256
20856	0	1:8	1:4	1:8
	10	1:64	1:64	1:32
MO45	0	1:8	1:4	1:4
	10	1:128	1:64	1:64
V135	0	1:32	1:64	1:16
	10	1:512	1:256	1:32

A greater than fourfold increase in titre was obtained with 87.5% of cultures exposed to 10 g/l iron. The remainder showed a doubling of the titre. In view of the findings with the ELISA tests, which were negative for the non-toxigenic strains (05 and 060), it was not considered necessary to include these 2 strains in the less sensitive cell-line study. The results of the ELISA and the Y1 cell line techniques respectively showed that iron enhanced the immunological effect as well as the biological properties of CT.

6.4 DISCUSSION

According to the typing system developed by Sakazaki and Shimada (1977) at least 139 different O groups of *V. cholerae* are now recognized. Until recently, it was generally held that epidemic cholera was caused exclusively by O1 strains of *V. cholerae*, in particular its two biotypes, classical and El Tor. Conversely, non-O1 strains of *V. cholerae*, epidemiologically, were thought to behave quite differently in that they were not known to cause explosive, rapidly spreading epidemics whilst, clinically, they caused a diarrhoeal disease that was usually milder than cholera. The recent emergence of *V. cholerae* O139, also known as the Bengal strain, as a significant cause of epidemic, potentially pandemic spread of cholera

contradicted these traditional beliefs. Another classification system, based on enzyme electrophoretic patterns, was developed by Momen and Salles (1985). By means of this system epidemic *V. cholerae* strains could be grouped into two closely related zymovars. Thus, O1 classical strains of *V. cholerae* were shown to belong to zymovar 13 whilst the O1 El Tor and O139 (Bengal) strains were classified under zymovar 14. The O139 strain is therefore thought to be very closely related to, and perhaps derived from O1 El Tor.

The studies covered in this thesis included two known non-toxigenic strains of non-O1 *V. cholerae*. These strains were included principally to control the procedures used to test for toxin production and gave results that were consistently below the defined cut-off point.

All of the known toxigenic strains used, O1 and non-O1, in this study showed increased enterotoxin production on exposure to high ferric oxide concentrations. In keeping with the findings of other workers (Iwanaga and Kuyyakanand, 1987; Turnbull *et al.*, 1985) the El Tor isolates studied produced much lower levels of toxin than the classical strains.

Of great interest was the finding that moderate increases of iron (up to 0.1 g/l) had little effect on toxin production. However, iron concentrations greater than 0.1 g/l markedly stimulated the toxigenicity of some strains, notably the classical strains and the clinical isolate of El Tor Ogawa (figures 6.3 and 6.4). In this regard, it is also of interest to note that, during the current epidemic in South America, the initial cholera waves in many epidemic regions were caused by the Inaba serotype, but an Inaba-to-Ogawa shift occurred and gave rise to later outbreaks. Such shifts can be caused by several genetic mechanisms (Stroeher *et al.*, 1992). The causative Inaba strain involved in the Inaba-to-Ogawa shift during the recent Latin American cholera epidemic had been shown, by biochemical analyses and RNA restriction fragment length polymorphism, to be unique to Latin America (Manning *et al*, 1994). The emerging Ogawa strain in that region was shown to be identical to the local Inaba strain in all other respects and it was suggested that a serotype conversion had occurred, possibly as a result of immune pressure. Not surprisingly, in view of the electrophoretic findings (Momen and Salles, 1985) which grouped the O139 (Bengal) and the O1 El Tor strains of *V. cholerae* into the same zymovar, the responses of these strains to iron in this study were also similar.

The highest iron-associated increases of toxin production were observed with two of the three known Ogawa strains (table 6.5a, strain no. 9458 and table 6.6a, strain no. 22453). Are serological shifts, observed during cholera epidemics, associated with increased toxigenicity? The 'evolution of virulence' theory (Ewald, 1993) would agree with this in that it presents the view that waterborne pathogens tend to increase in virulence with time.

Another question that arises from these studies, although not addressed in this thesis, concerns the possibility that environmental factors (in addition to host factors such as serotype-specific immunity) may be instrumental in the serotype shifting observed during the course of cholera epidemics.

Chemical water monitoring data collected over many years on numerous surface waters, were kindly made available for this study by Rand Water. These data showed that total iron concentrations in waters that are not subject to significant industrial pollution, with rare exceptions, were below 1 g/l.

Iron, which constitutes some 5% of the earth's crust, is an essential requirement for most living organisms. It is, for example, a vital component in biological electron

transport mechanisms (Neilands, 1974).

Accumulation of iron in the natural environment where it may persist and exert toxic effects on local ecosystems (Friberg et al., 1986) has received much attention (Stumm and Morgan, 1980), especially in recent years. Several studies in different parts of the world have shown an association between industrial and other water pollution and raised levels in water bodies of metals such as lead, nickel, copper, cadmium, manganese, zinc and iron. Iron concentrations are usually not very high in surface waters but accumulation of the metal occurs to very high levels in sediments, aquatic vegetation and zooplankton which consists mainly of small crustaceans and fish larvae. All of the studies cited below were carried out to investigate the relationship between the excessive presence of iron (and other metals) and their potential adverse effect on ecosystems.

In contrast, the possible impact of excess iron on the ecology and epidemiology of cholera, which was the main objective of this thesis, was not an objective of investigations by these other workers. Their findings and conclusions, however, I believe to have important implications in the interpretation of the toxigenicity findings detailed above. Moreover, these same workers

have produced ample evidence of high iron levels in sediments of industrially polluted water levels and in aquatic plants and animals. The high iron levels demonstrated by these workers overlap with the iron concentrations found in this study to enhance cholera toxin production.

Tiwary and Dhar (1994), for example, found that iron and other heavy metals were more heavily concentrated in the sediment than in the water of the Damodar, a river which runs through a coal mining region in India (table 6.9). In Bulgaria, Yurukova and Kochev (1994) obtained monthly data on heavy metals in swamp water and sediments and found that the presence of iron was 10 times higher than that of other metals. They also showed a marked difference between iron levels in water as compared to those in the sediment (table 6.9). The same workers recorded a similar phenomenon in aquatic vegetation in that iron levels in emergent plants ranged from 43 to 322 mg/kg whereas in floating and submerged flora it ranged as high as 2154-8631 mg/kg. In the Transvaal, South Africa, Steenkamp and colleagues (1993) also found iron levels in sediments to be greatly in excess of those in the water of aquatic ecosystems which were heavily polluted by mining activities and sewage (table 6.9). They also examined the fresh water crab, *Potamonautes warreni*, in these habitats and found that

TABLE 6.9 Range of published iron concentrations in surface waters and sediments.

	Surface water iron mg/l	Sediment iron mg/kg
Damodar river, India[a]	2 - 5	80 - 400
Swamp, Bulgaria[b]	0.01 - 0.08	3045 - 8040
Natalspruit, RSA[c]	1.24 - 4.78	8250 - 164000
Bronkhorstspruit, RSA[c]	2.01 - 4.43	6650 - 65950
Nooitgedacht Dam, RSA[c]	1.84 - 2.25	12550 - 42600

[a] Tiwary and Dhar (1994)
[b] Yurukova and Kochev (1994)
[c] Steenkamp et al. (1993)

concentration of iron of up to 3000 mg/kg and greater occurred in some of their tissues and organs. In a similar context, Du Preez and Steyn (1992) studied the presence of heavy metals in the tigerfish (*Hydrocynus vittatus*) of the industrially polluted Olifants River in the Kruger National Park. The iron concentration in this river's surface water ranged from 1.83-2.14 mg/l. In the tigerfish, however, iron levels ranged from 15-2620 mg/kg with the highest values having been obtained from the liver (500-2620 mg/kg).

Tiwary and Dhar (1994) as well as Yurukova and Kochev (1994) observed that iron tended to be taken up and concentrated more heavily than the other metals included in their studies. Several workers also pointed out that, although industrial and other forms of man-made pollution are important sources of the high iron levels in certain aquatic environments, the metal's abundant presence in the earth's crust is an additional, albeit natural source.

Evidence accumulated over the past two decades by Colwell *et al* (1977; 1994) indicates that *V. cholerae* is an autochthonous inhabitant of brackish water and estuarine systems. These workers have shown that a close association between plankton and *V. cholerae* affects the latter's survival. The survival of this organism between cholera

epidemics and its isolation from such waters, even in non-epidemic regions, can be explained on the basis of its persistance in a viable, though often non-culturable state.

It has been observed that the largest concentrations of water hyacinths (*Eichhornia crassipes*) are found in waters enriched by sewage and industrial effluent or by run-off from fertilised farm lands (figure 6.6) (Anonymous, 1980). Studies establishing attachment of *V. cholerae* to fresh-water plants, such as water hyacinth (Spira *et al*, 1981), phyto and zooplankton (Huq *et al*, 1990; Tamplin *et al*, 1990) and green algae (Islam *et al*, 1989; Islam 1990) were reviewed in chapter 2. Previous work by Patel and Isaäcson (1994) showed that iron contributed to the enhancement of both the survival and multiplication of cholera bacilli.

Several factors, therefore, render the environment ideal for the survival and growth of *V. cholerae* which, in their turn, are important factors in the epidemiology and pathogenesis of cholera. Some of these studies also addressed the organism's toxigenicity, an important virulence factor. Thus, Tamplin and Colwell (1986) showed, that a salinity of 2-2.5% was optimal for cholera toxin production. These workers used microcosm cultures which are artificially produced bacterial growth media designed to simulate the natural aquatic environment. Also in

Fig 6.6 Illustrating different views of water hyacinths. Note the extensive coverage of water surface by this plant. Courtesy of Dr. C. J. Cilliers, Institute for Plant Protection, Research institute Biological Control, Pretoria, South Africa.



a. Oudewerfspruit, Parys 18-01-1990



b. Buffels River, E. Cape 05-06-1994



c. In the laboratory 1991



d. Thornhill Dam, Hectorspruit 17-12-1985

microcosm Miller *et al* (1986) showed toxigenicity to be quite a stable property. On the other hand, Islam (1990) demonstrated increased cholera toxin production by *V. cholerae* O1 when it was associated with *Rhizoclonium fontanum*, a green alga. Islam's findings, therefore, suggest that cholera toxin production can be affected by environmental factors.

The study reported in this chapter showed that, in an artificial aquatic environment, iron concentrations of 1-10 g/l can significantly enhance cholera toxin production. This finding may have important epidemiological and pathogenetic implications. From the pathogenesis point of view, the infectious dose needed for development of clinical disease is usually quite high, $\geq 10^6$ organisms. In healthy volunteers, the infectious dose was found to be in excess of 10^8 cholera bacilli (Bennish, 1994). The infectious dose for cholera due to *V. cholerae* with an enhanced virulence, in this case iron-mediated, may be considerably lower than 10^6. Other potential clinical consequences of enhanced toxigenicity could include a shorter incubation period and/or more severe disease. All of these sequelae can in their turn affect the epidemiology of the causative agent in that more sick individuals with more rapid onset of illness and more severe illness are likely to result in heavier contamination of the

environment by *V. cholerae*. Without appropriate intervention a vicious cycle can be envisaged.

Other workers (chapter 2 section 2.4.1) showed that chitin-attached cholera bacilli have a markedly increased acid resistance whilst McCarthy and Miller (1994) demonstrated that they are also associated with an increased resistance to disinfectants. Chemical contamination and acidic pH frequently occur in industrially polluted waters and, contrary to expectation, may promote rather than inhibit the survival and multiplication of *V. cholerae* and, as shown above in the case of iron, even enhance their toxigenicity. As discussed in Chapter 4 (Section 4.4) the successful persistence of coliform bacteria in 'corrosion tubercles' has been demonstrated in iron pipes carrying clean, chlorinated water supplies. It is likely that *V. cholerae* will likewise survive in similar circumstances where the increased iron concentration may then further enhance its enterotoxigenicity.

6.5 CONCLUSIONS

In vitro and in vivo studies were conducted to assess the response of cholera toxin (CT) production to increasing iron concentrations in an aquatic environment. As a result it was found that:

- CT production by 7 of 8 *V. cholerae* strains tested, including the Bengal strain (0139), was significantly enhanced in the presence of iron concentrations of 1.0 and 10 g/l. The exception (El Tor Ogawa) had a significant CT response only in the presence of 10 g iron/l. CT enhancement also occurred with iron concentrations below 1.0 g/l, but not to a statistically significant degree (tables 6.5a, 6.6a and 6.7a).

High iron concentrations of the order found here to have stimulated CT production, have been described by others in association with sediments, water plants and chitinous fauna. Yet other workers have shown a predilection by *V. cholerae* to attach to these sites in the aquatic environment.

The findings in this chapter have also not been reported before. It is suggested that their importance may relate to the risk of developing clinical disease after ingestion of *V. cholerae*. Provided that iron-enhanced CT production is maintained after ingestion, a lower infectious dose for the development of clinical cholera may be required than might be the case with the same strain without prior iron exposure. Therefore, more susceptible persons may develop cholera sooner, thus increasing and intensifying the pool of *V. cholerae* excretors, and thereby precipitating or accelerating the evolution of an epi/pandemic.

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6.7 APPENDIX

6.7.1 Recipes

1. Yeast Extract Peptone Medium (YEP)

Bacto peptone.....	1.5%
Yeast extract.....	0.4%
NaCl.....	0.5%

Dissolve the ingredients in distilled water; adjust the pH to 7.4 - 7.6. Distribute 10 ml aliquots in 150 mm x 15 mm tubes with caps and autoclave at 121°C for 15 minutes.

2. Casamino Acid Yeast Extract Glucose Medium (CAYEG)

Casamino Acids.....	3.0%
Yeast extract.....	0.3%
Glucose.....	0.2%

Dissolve the ingredients in distilled water. Adjust the pH to 8.4. Distribute 50 ml aliquots into glass bottles and autoclave at 121°C for 15 minutes.

3. Deferrated Organic Nutrient or CAYEG

Dissolve the ingredients in 100 ml of distilled water. Adjust the pH to 8.5. Add 0.2 ml 50% $CaCl_2$ and boil the medium for 5 minutes in a waterbath with constant stirring. Remove the precipitates by filtering. Add $CaCl_2$ again and repeat the procedure. Sterilize the final medium by filtration using a 0.45 μm filter.

4. Blood Agar

See Appendix of chapter 4 page 109

5. ¼ Strength Ringers solution

See Appendix of chapter 4 page 110

6. Semisolid Agar

See Appendix of chapter 4 page 109

7. Alkaline Peptone Water

See Appendix of chapter 4 page 110

8. Phosphate Buffered Saline (PBS)

NaCl.....	8.0 g
KH_2PO_4.....	0.2 g
Na_2HPO_4 $12H_2O$.....	2.9 g
KCL.....	0.2 g
Distilled water.....	1000 ml

9. PBS-Tween

Prepare PBS as above, add 0.5 ml of Tween 20 and mix. Store at 4^oC.

10. Diethanolamine Buffer (DEA)

Diethanolamine.....	97 ml
Distilled water.....	800 ml
NaN_3.....	0.2 g
$MgCl_2$ $6H_2O$.....	0.1 g

Mix and dissolve the ingredients. Adjust the pH to 9.8 using 1M HCl. Add more distilled water to a final volume of 1 litre. Store at 4^oC in the dark. Remove sufficient amount 1-2 hours before use and allow to warm to room temperature.

11. 3M NaOH

NaOH..... 120 g

Distilled water..... 1000 ml

Dissolve the ingredient and store at room temperature.

12. RPMI 1640 Complete Growth Medium

Add 100 ml of foetal bovine serum to 1000 ml sterile RPMI 1640 aseptically. Mix and store at $4^{o}C$.

6.7.2 Tables

Table 6.5b. Full results: Effect of iron (Fe_2O_3) on cholera toxin production by *V. cholerae* O1 classical strains.

Table 6.6b. Full results: Effect of iron (Fe_2O_3) on cholera toxin production by V. cholerae O1 El Tor strains.

Table 6.7b. Full results: Effect of iron (Fe_2O_3) on cholera toxin production by *V. cholerae* non-O1 strains.

TABLE 6.5b Full results: Effect of iron (Fe_2O_3) on cholera toxin production by V.cholerae O1 classical strains. Toxin measured by GM_1 micro-ELISA (A405).

Strain	Iron concentration g/l	Optical densities				
		1	2	3	4	5
25870*	0	1.192	0.913	0.755	0.787	0.884
	0.01	0.886	1.181	1.493	1.111	0.997
	0.1	0.968	1.043	0.962	1.046	1.115
	1.0	1.875	1.835	1.861	1.776	1.537
	10.0	1.874	2.626	1.750	2.710	1.902
9458	0	1.056	0.721	0.788	0.835	1.242
	0.01	0.607	0.890	1.355	0.950	1.191
	0.1	0.754	1.311	0.753	0.678	1.396
	1.0	1.798	1.667	1.227	1.499	2.661
	10.0	2.736	2.055	3.729	3.103	2.726
9459	0	1.903	1.403	1.567	1.747	1.632
	0.01	1.537	1.503	2.080	1.660	1.786
	0.1	1.917	1.375	2.242	1.783	1.920
	1.0	3.148	3.121	2.695	2.149	2.560
	10.0	2.922	3.074	3.076	2.518	3.026

* - Optical density measured at ⅛ dilution.

TABLE 6.6b Full results: Effect of iron (Fe_2O_3) on cholera toxin production by V.cholerae 01 El Tor strains. Toxin measured by GM_1 micro-ELISA (A405).

Strain	Iron concentration g/l	Optical densities				
		1	2	3	4	5
582	0	0.188	0.127	0.108	0.217	0.249
	0.01	0.209	0.217	0.150	0.337	0.353
	0.1	0.186	0.226	0.138	0.343	0.359
	1.0	0.221	0.228	0.297	0.337	0.440
	10.0	0.230	0.453	0.429	0.475	0.468
22453	0	0.621	0.616	0.701	0.713	0.767
	0.01	0.711	0.601	0.707	0.645	0.655
	0.1	0.659	0.757	0.816	0.749	0.510
	1.0	1.632	0.866	1.274	0.942	0.867
	10.0	3.446	2.051	2.856	3.036	2.687
20856	0	0.169	0.295	0.237	0.295	0.249
	0.01	0.192	0.207	0.235	0.294	0.282
	0.1	0.187	0.235	0.288	0.274	0.212
	1.0	0.305	0.313	0.240	0.338	0.176
	10.0	0.547	0.442	0.403	0.430	0.454

TABLE 6.7b Full results: Effect of iron (Fe_2O_3) on cholera toxin production by V.cholerae non 01 strains. Toxin measured by GM_1 micro-ELISA (A405).

Strain	Iron concentration g/l	Optical densities				
		1	2	3	4	5
M045	0	0.383	0.397	0.399	0.269	0.286
0139	0.01	0.399	0.304	0.437	0.288	0.464
toxigenic	0.1	0.390	0.419	0.429	0.400	0.482
	1.0	0.515	0.500	0.498	0.445	0.504
	10.0	0.705	0.586	0.578	0.523	0.534
V315	0	0.830	0.865	0.786	0.794	0.815
toxigenic	0.01	0.785	0.775	0.791	0.800	0.786
	0.1	0.865	0.879	0.701	0.834	0.853
	1.0	1.180	1.169	1.142	1.105	1.152
	10.0	1.017	1.121	1.135	1.123	1.180
0469	0	0.185	0.182	0.157	0.185	0.177
nontoxigenic	0.01	0.172	0.152	0.176	0.188	0.149
	0.1	0.178	0.168	0.164	0.169	0.184
	1.0	0.152	0.161	0.174	0.167	0.146
	10.0	0.176	0.164	0.171	0.151	0.155
SG2	0	0.169	0.126	0.103	0.111	0.113
nontoxigenic	0.01	0.131	0.115	0.103	0.099	0.098
	0.1	0.130	0.095	0.119	0.097	0.097
	1.0	0.126	0.109	0.113	0.099	0.101
	10.0	0.102	0.113	0.100	0.116	0.100

CHAPTER 7

SUMMARY AND CORRELATION OF RESULTS: A PROPOSED MODEL FOR THE ECOLOGY AND EPIDEMIOLOGY OF CHOLERA IN THE CONTEXT OF ENVIRONMENTAL POLLUTION.

The survival and enterotoxin production of *V. cholerae* in sterile water without added nutrients (except for iron in the form of ferric oxide)(chapter 4), and in filtered or autoclaved industrially polluted water (chapter 5) were studied with special reference to the effect of pH and added iron. The effect of iron on the production of cholera enterotoxin (chapter 6) was also investigated. Figure 7.1 is a chart presenting a composite of some of the findings. Logarithms of iron concentrations (mg/l) are reflected on the X-axis, *V. cholerae* survival time on Y1-axis and the toxin production on Y2-axis. In each study category the results of the various strains of *V. cholerae* used were pooled for greater clarity (polluted water study: table 5.20; clean water study: tables 4.3, 4.4 and 4.5; toxin study: tables 6.5a, 6.6a and 6.7a). This was feasible since the patterns of behaviour by the different strains had been shown to be similar.

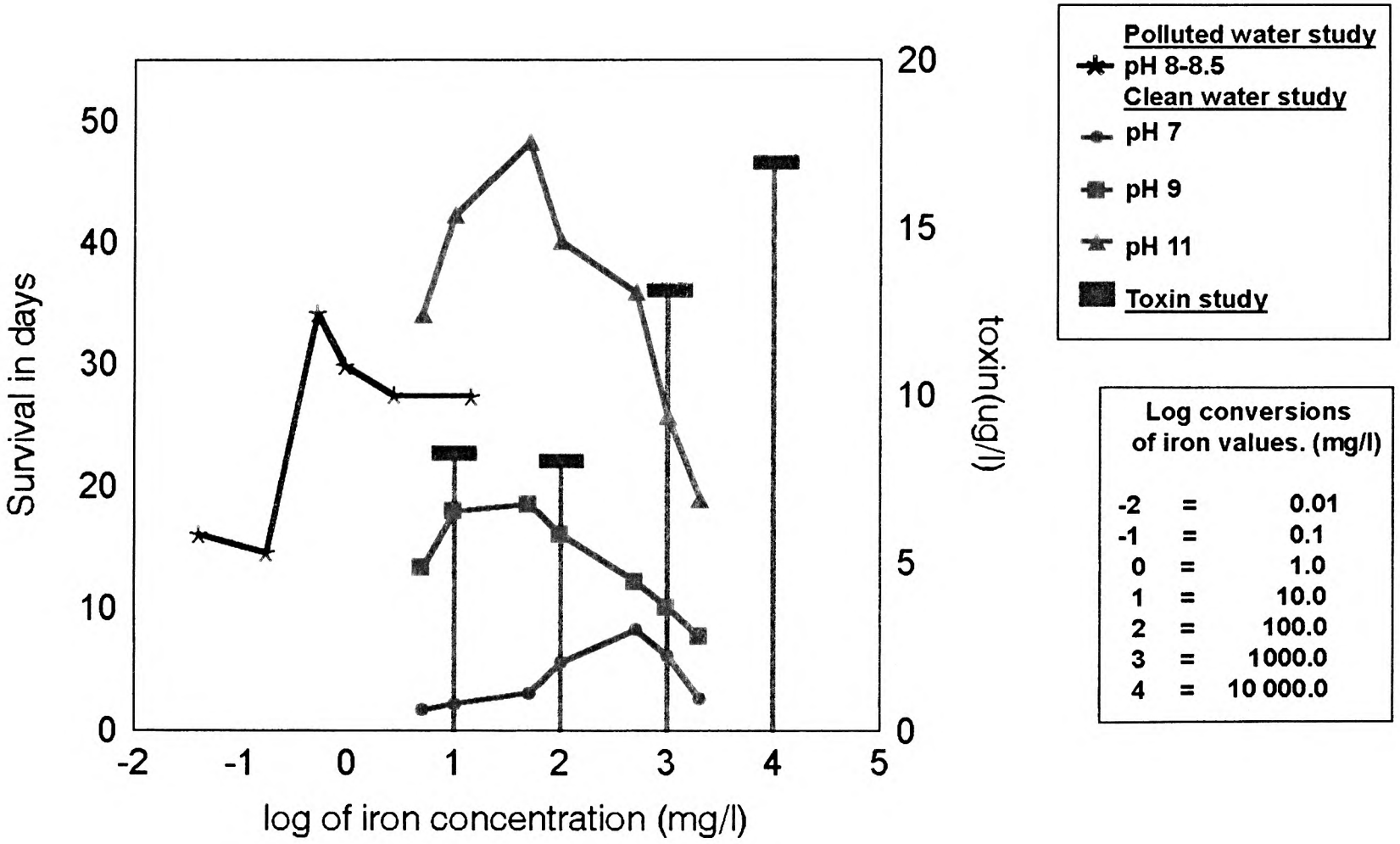


Fig 7.1 The effect of iron on the survival and toxin production of V.cholerae

Polluted water:

The mean cholera survival results, ranging from 15 to 34 days, (table 5.20) obtained in samples of water polluted with effluent were plotted. The mean pH values of these samples ranged from 8 to 8.5 and their mean iron concentrations from 0.04 mg/l to 14.2 mg/l).

Clean water:

Also plotted in figure 7.1, but in this case separately for each pH value (7, 9 and 11), were the pooled cholera survival results (means ranging from 2-48 days) obtained in the clean water experiments (chapter 4). In this study the iron concentrations were designed to range from 5-2000 mg/l which are higher than those found in surface waters, but are in accordance with the levels found in ground waters contaminated by leachate, in water obtained via corroded hand pumps or delivered by corroded water distribution systems, and also in sediments.

Toxigenicity study:

The third component of the chart is formed by the cholera toxigenicity results in µg/l. This work (chapter 6) was carried out in the presence of an iron concentration range of 10-10000 mg/l. For better contrast with the cholera survival graphs these findings were charted in the form of vertical bars.

The chart illustrates several conclusions:

1. At a pH of 8-8.5, despite the lower iron concentration, the survival of *V. cholerae* in polluted water was generally longer than in clean water at a pH of 7-9. This is probably due to the presence of various other nutrients in the polluted surface water samples whilst all nutrients other than iron had been excluded in the clean water study. As confirmed by statistical analyses in chapters 4 and 5, increased iron improved cholera survival in both polluted and clean water, but the concentration of iron needed to do this is shown to be lower in polluted water. In both types of water there seems to be an optimal iron concentration above which it may become inhibitory.

2. The clean water findings are especially relevant to installed water supplies obtained by pumps and/or conveyed by the older type of distribution system in which iron is a component that, when corrosion takes place, results in increased ferric oxide levels in the water. This, as shown in this work, may stimulate survival of *V. cholerae* and is likely to apply also to other enteric bacterial pathogens. As shown by others, the products of corrosion in water distribution systems not only provide shelter to microorganisms but also counteract the antimicrobial effects of chlorine, one of the commonest chemicals used

for water purification. Clearly, therefore, the combined effects of harbourage, disinfectant neutralisation and bacterial growth stimulation by iron favour the persistence and reproduction of *V. cholerae* in domestic water supplies. Whilst other workers have shown that *V. cholerae* is capable of survival in brackish sediments, the studies carried out in fresh water and described here may well provide a mechanism enabling such enhanced survival in both fresh and brackish water.

3. In polluted surface water, the survival of *V. cholerae* was also significantly longer at higher iron concentrations. Since such water is a common and often the sole source of drinking water for vast rural populations, the potential implications of industrial pollution have here been shown to range far beyond the well recognised association with chronic diseases such as malignancies, cardiac disease, toxic hepatic disease etc. In contrast, these studies show iron to be capable of mediating the occurrence of an acute, epidemic infectious disease.

4. At even higher iron concentrations which, according to the published literature, have been found in association with sediments and with elements that tend to accumulate and concentrate iron such as water plants, fish and crabs, survival of *V. cholerae* tended to decrease but this effect

is seen to overlap with a marked increase in toxin production by toxigenic strains of *V. cholerae*. This effect was unexpected and it is tempting to regard it tentatively as a survival mechanism for the organism in view of the findings by Lacey (1995). This author, whilst reviewing the literature on cholera, found that cholera toxin itself can alter sodium output by other aquatic organisms, thus enhancing its own survival by increasing local levels of salinity. If the iron-induced toxin enhancement of *V. cholerae*, found in this study, is maintained after ingestion, this effect could also cause lower infectious doses being capable of causing illness of a severity usually associated only with high infectious doses of a cholera strain of 'normal' toxigenicity.

Natural as well as artificial means of dissemination abound for the world wide dissemination of *V. cholerae*. Natural means include migratory aquatic birds (Ogg *et al.*, 1989; Lee *et al.*, 1982) and an ever increasing mass of ever faster travelling humans. Artificial means include, for example, coastal discharges of contaminated ballast from ships. This was shown to be a major factor in the onset of the recent cholera epidemic in Peru (Epstein *et al.*, 1993). Once disseminated, environmental conditions at destination are not always found to be favourable by *V. cholerae* for its survival. Other workers have shown its ability to

contract up to 85% (Baker *et al.*, 1983) and to reduce its metabolic rate to a point where it attains a state of 'hibernation' in which it tolerates adverse changes in salinity, pH, temperature and available nutrients (Dawson *et al.*, 1981). In this state the organism may not be demonstrable by routine culture techniques.

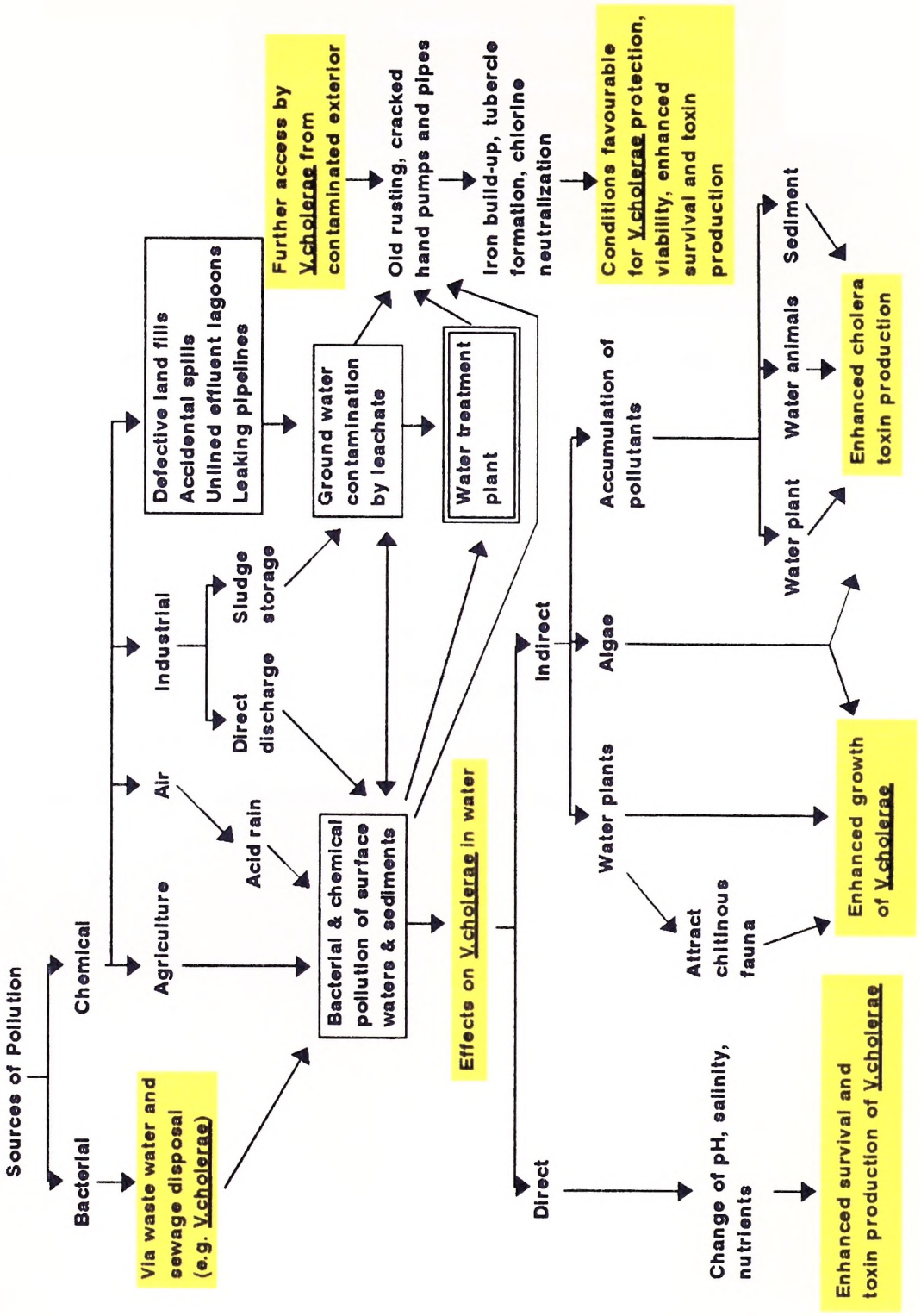
In both surface and ground waters the growth and survival of *V. cholerae* are directly and indirectly related to pollution. The adverse effects of human activities only become obvious when these result in a degree of pollution in excess of the natural coping capacity of an ecosystem. The various components of the environment are not isolated from each other and the effect of pollution on one component inevitably affects the others. Water is especially vulnerable in this respect. Thus, air pollutants gain access to surface waters via rainfall whereas pollutants of the soil, via a process of leaching, also in due course reach water bodies.

Excessive, poorly planned urbanisation, accompanied as it often is by industrialisation and over-fertilisation of agricultural land may result in eutrophication, i.e. a process of excessive enrichment by organic and mineral nutrients. Industrial effluents may be treated prior to discharge but frequently are dumped untreated and

indiscriminately into near-by rivers or dams. Leaching of highly concentrated chemicals may also occur from sites where industrial sludge is stored. These and many other direct and indirect water polluting mechanisms have resulted in changes in the aquatic ecology. Thus, some aquatic flora is destroyed and other plants, e.g. water hyacinths (Spira *et al.*, 1981) *Eichhornia crassipes* and common duckweed (Islam *et al.*, 1990a) on which *V. cholerae* has been shown to survive, Kariba weed (*Salvinia molesta*), common duckweed and several other species have proliferated out of all proportion to the normal flora. Many of these plants support the growth of chitinous fauna which, in its turn, supports the growth and survival of *V. cholerae*. Moreover, eutrophication tends to lead to algal overgrowth. The blue-green alga, *Anabaena variabilis*, was shown (Islam *et al*, 1990b) to support *V. cholerae* for more than a year inside its mucilaginous sheaths and the cholera season in Bangladesh is related to the seasonal algal bloom formation. Similarly, *Rhizoclonium fontanum*, a green alga, also supports *V. cholerae* growth (Islam *et al.*, 1989) and toxin production (Islam, M.S., 1990c).

What is the effect of these and many other consequences of pollution on the ecology of *V. cholerae*? The flow diagram in figure 7.2 attempts to provide a few of the answers to this question.

Fig 7.2 Model for potential impact of environmental pollution on the ecology and epidemiology of V.cholerae.



Human faecal contamination, directly or indirectly, is the chief mechanism by which *V. cholerae* is introduced into domestic water sources. Chemical contamination of water is, however, postulated here to be an important mechanism in the ability of *V. cholerae* to persist, multiply and survive in the aquatic environment.

It is suggested that the 7th cholera pandemic, due to *V. cholerae* El Tor, which spread from Sulawesi, Indonesia, in 1961 is, at least in part, a consequence of extensive and severe chemical water pollution caused by rapid and often uncontrolled urbanisation and industrialisation during the post World War II era. It has been shown in this study that iron, one of the major polluting chemicals, plays an important role in this scenario, not only by enhancing the survival of *V. cholerae*, but also by increasing its enterotoxin production.

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