A Study of Viable-But-Non-Culturable Pathogens in Water

A Report to the Water Research Commission on the project: Bacterial Pathogens in Groundwater

by

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EXECUTIVE SUMMARY

1. General background and motivation of project

The isolation and identification of microbes in the water industry is performed using conventional assays for a few indicator organisms. However, the use of plate counts as a standard bacteriological method has been criticized because it is not a good estimator of the total number of bacteria present.

Pathogenic bacteria, that may be present in water, enter into a viable-but-non-culturable (VBNC) state as a result of starvation, temperature fluctuations, saline environments or the presence of chemicals. In the VBNC state, the cells remain dormant until environmental conditions become favourable. The ability of pathogenic bacteria to enter the VBNC state means that they may go undetected on laboratory media as they do not form colonies, and therefore pose a potential health risk in untreated water

It is therefore necessary to develop a method that is sensitive and able to detect all viable cells, both culturable and non-culturable. It is also important to study the virulence of VBNC bacteria as a threat to public health.

2. Original objectives of the study

2.1 To determine the fate of pathogenic bacteria in the subsurface environment. Investigations will monitor the transport of pathogens through soil, their movement in aquifer material, and their activity and survival pattern in groundwater;

2.2 To evaluate the application of molecular methods for the detection of VBNC pathogens in surface and groundwater; and

2.3 To assess the health risk posed by VBNC pathogens in surface and groundwater.

3. Final objectives of the study

3.1 To evaluate the application of traditional and molecular methods for the detection of VBNC pathogens in surface and groundwater;

3.2 To determine the fate and survival strategies of VBNC pathogens in surface and groundwater; and

3.3 To assess the virulence properties of VBNC pathogens.

The final objectives are similar to the original objectives, with two changes. Firstly conventional methods will be evaluated in the detection of VBNC pathogens and secondly, virulence properties rather than simply health risks posed by VBNC pathogens will be assessed. These changes make the final objectives more specific in terms of the methods to be used.

4. Methodology

Conventional plate counts and molecular methods were used. The conventional methods used were dilution plate count (DPC), acridine orange direct count (AODC), direct viable count (DVC) and membrane filtration (MF). The molecular methods used were polymerase chain reaction (PCR) and hybridization studies. Also included was sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and proteolytic and haemolytic activity tests.

5. Summary of major findings and conclusions reached

5.1 Factors affecting the transition of culturable pathogens to the VBNC state

It was found that temperature, river water, seawater, saline environment and inoculum concentration affected the transition of bacteria into the VBNC state. The different water sources namely, river water, pond water and seawater were used in the experiment in order to determine the influence of salinity, organic content and pH on the transition of the microorganisms to the VBNC state.
The bacteria tested were ***Salmonella typhimurium***, ***Salmonella typhi***, ***Shigella sonnei***, ***Shigella flexneri*** and ***Escherichia coli.***

5.2 Survival of VBNC bacteria in various microcosms using conventional methods

The AODC, DVC and DPC were used to monitor the change of culturable cells to the VBNC state. The AODC, DVC and DPC methods were used to enumerate the total bacterial population, viable population and culturable population respectively. The VBNC state was confirmed when the DVC < 0.1 cells·ml^{-1}.

It was found that in the pond water tested, no cultures reached the VBNC state after 140 days at either 4°C or 25°C.

In the river water microcosms tested, all cultures except *S. typhimurium* reached VBNC state within 83-145 days at 25°C while at 4°C only *S. typhi* reached VBNC state, which required 138 days.

In the seawater all organisms required between 90 – 111 days to reach VBNC state at 4°C and 25°C except for *S. flexneri* that took 67days at 25°C. *S. sonnei* reached VBNC state in 105 days at 4°C and 110 days at 25°C.

E. coli had a higher survival rate at 4°C than 25°C. The non-culturable state was reached in 105 days at 4°C and 90 days at 25°C. *E. coli* also displayed similar results to *S. sonnei* in the 4°C seawater microcosm where AODC, DVC and DPC yielded showed results from day 1 to day 68. This suggests that the cells were culturable and viable for almost 7 weeks at 4°C.

5.3 Detection of VBNC bacteria in microcosms using molecular methods

Primers specific for *S. typhimurium* were used to detect *S. typhimurium* and *S. typhi* while a primer specific for *E. coli* was used to detect *E. coli*. *S. sonnei* and *S. flexneri*. The concentration of bacterial cells from seawater for PCR was not successful because it involved the use of filters that may have inhibited the PCR reaction and also the presence of salts in seawater may have been inhibitory. Dialysis of seawater microcosm samples, before PCR, resulted in positive amplification in culturable cells for all seawater microcosms.

In the river water samples, only VBNC *S. flexneri* and *S. sonnei* were detected by the PCR/hybridization method.

In the seawater microcosms, only *S. flexneri* and *S. sonnei* were detected by the PCR/hybridization method.

The detection of VBNC bacteria using molecular methods revealed that the culturable bacteria were more easily and consistently detected than their non-culturable forms using the PCR/hybridization method. Also, the quality rather than quantity of DNA was a constraint for respective analyses.

5.4 Potential virulence of VBNC pathogens

In VBNC cells, it was found that the cells changed from rod-shaped, in the culturable state, to small oval cells in the non-culturable state. There was also a decrease in proteolytic and haemolytic activity in the cells and at a concentration of $1x10^5$ cells·ml^{-1} did not infect Wistar rats. Also lipopolysaccharide (LPS) was not found in the VBNC cells. When resuscitated to the culturable state, all these abilities returned to normal in the cells.

5.5 Prevalence and survival strategies of VBNC pathogens in untreated drinking water supplies

Analysis of river water samples near informal settlements indicated a high number of coliforms and faecal coliforms including *Shigella* sp. and *Salmonella* sp. The VBNC cells ranged from 20-50% of the total bacterial counts.

In untreated rainwater samples, collected from storage tanks around schools and clinics in KwaZulu-Natal, the numbers of culturable coliforms and faecal coliforms were higher than the allowable limit also, coliform counts were higher after VBNC cells were resuscitated.

In groundwater samples, from Harding and Hibberdene, a large proportion of VBNC cells were found, the percentage of VBNC cells ranged from 52-87% on diagnostic media. Groundwater is all the water found beneath the surface in a saturated zone of soil and rocks. Groundwater is stored in a water-bearing layer of rock or sand and is a source of water supply for wells and springs.

6. Review of the project in terms of the final objectives

6.1 Evaluation of the application of conventional and molecular methods for the detection of viable-but-non-culturable (VBNC) pathogens in surface and groundwater.

- A combination of the AODC, DVC and DPC methods proved very effective in detecting the presence of VBNC in microcosms. These methods were also able to monitor the transition of culturable cells to the non-culturable state.
- The PCR/hybridization method was inconsistent in detecting the presence of VBNC cells. Nevertheless, it should be noted that this method was very rapid, accurate and consistent in detecting culturable cells.
- Therefore, conventional and molecular methods meet the final objective of detecting VBNC pathogens in surface and ground water.

6.2 Assessment of the fate and survival of pathogenic bacteria in the surface and sub-surface environments.

- Untreated drinking water supplies were unfit for human consumption in many of the areas investigated. A further concern is the survival of significant numbers of pathogens in the VBNC state.
- The findings of this study highlight the fact that bacteria, including pathogens, can survive for long periods in the surface and sub-surface environments.
- Therefore, this final objective was met.

6.3 Determination of the virulence properties of VBNC pathogens.

- The results suggest that although they themselves do not resuscitate in the intestine, VBNC pathogens have the capacity to cause infections and deaths equal to that of normally virulent culturable cells.
- These findings clearly support the hypothesis that VBNC pathogens pose a potential danger to human health, although they themselves do not resuscitate in the intestine.
- This final objective has not been completely met. Therefore, to fulfil this objective, further studies need to be conducted on the conditions which lead to the resuscitation of VBNC pathogens.

7. Recommendations for future research

- Studies should be conducted on whether VBNC bacteria resuscitate in household containers when sufficient nutrients and incubation time are available. This is important in rural areas where people store water for long periods in used containers that may have a high nutrient content.
- Research needs to be done on whether VBNC bacteria are more resistant to chlorination than normally culturable bacteria. This research will help elucidate if normal chlorine-containing household detergents, like JIK, can be used in rural areas to eradicate VBNC bacteria from drinking water.
- The PCR/hybridization method needs to be optimised for the microbiological analysis of untreated water samples. Pre-treatment of the samples may be necessary to remove inhibitors of PCR.

8. Recommendations for technology transfer

- The prevalence of large numbers of potential pathogens in untreated water must serve as an early warning to put into place measures that will prevent a recurrence of water-borne epidemics in our country. Therefore, efforts should be made to transfer the technology of detecting VBNC pathogens to both the formal and

informal sectors of the water industry. This can be implemented via the hosting of technology transfer workshops by researchers. These workshops should make the relevant authorities in the management and supply of untreated water aware of the urgency in including VBNC analyses in routine water testing.

- Innovative strategies are needed for cost-effective testing and treatment of contaminated drinking water supplies, especially in rural areas. As shown in this study, conventional microbiological tests for coliforms and faecal coliforms do not accurately estimate the bacterial load. One strategy should include the resuscitation of VBNC bacteria, which is efficient, economical and most importantly provides results within 24 hours. In rural areas inhabitants should be educated on treating drinking water by first boiling it to eradicate most of the pathogenic bacteria.
- Researchers need to create a database including current information on water-borne diseases, especially in rural areas. This archive should include data on VBNC bacteria and strategies to map the microbiological quality of drinking water supplies. The data must be readily accessible to other researchers and the public via the computer, pamphlets and posters. This initiative should culminate in community workshops hosted by researchers, specifying the prevalence of VBNC pathogens in water supplies.

CAPACITY BUILDING AND CORRECTIVE ACTION

WRC-funding of this project has contributed to both capacity building and corrective action. As a HDI the University of Durban-Westville caters largely for the needs of disadvantaged students. The research initiatives undertaken within the scope of this project have been in keeping with UD-W's Mission Statement as well as our Departmental Mission Statement, i.e., to develop and train scientists from previously disadvantaged communities establishment of a research culture at the University of Durban-Westville

The following were achieved:

- Research culture:

A vibrant and sustainable research culture was developed within the Department of Microbiology. The project facilitated the development and training of disadvantaged students and staff. Furthermore, technology was transferred, from other institutions, and

research collaboration was fostered. This WRC-funded initiative also enabled us to obtain bursaries for postgraduate students within the project. This project has also contributed to increasing the critical mass of our Departmental research team.

- Students:

Postgraduate student numbers have increased as a consequence of the implementation of the project. Many students have successfully completed their studies within this project. Details of these students are included below.

MSc completed:

Ms RM Dwarka

Ms S Naicker

BSc (Hons.) completed

Ms S Naiker

Ms RM Dwarka

Ms K Joseph

Ms TFS Ntlabati

Ms S Naicker

Ms T Duma

Ms Y Naidu

Ms KG Hira

Mr P Nkofo

Ms S Chetty

Ms CS Magagula

MSc current

Ms E Lazarus

Ms Y Naidu

Ms KG Hira

Ms D Seepersad

- Improvements in primary, secondary and higher education:

  Through the Microbiology Student's we have been able to make Microbiology accessible to schools. Pupils, Science teachers and Career Counsellors are invited to the Department and exposed to various aspects of Microbiology (curriculum, entrance requirements, job opportunities and entrepreneurial opportunities.

- Community outreach

  A research programme with relevance to the wider community has been established in Water Microbiology. This will now form one of our focus areas of the Department. Under-developed areas with poor sanitation and contaminated water supplies will be targeted with a view to improving the quality of life for disadvantaged sectors of the population.

PUBLICATIONS AND CONFERENCE PROCEEDINGS

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The Steering Committee responsible for this project consisted of the following persons:

Ms A.P.M. Moolman	Water Research Commission (Chairperson)
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Dr C.A. du Plessis	Billiton
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CONTENTS

GLOSSARY

acridine orange direct counts (AODC)
A basic dye and Flurochrome used e.g., in fluorescence microscopy to distinguish between dsDNA (fluoresces green) and ss nucleic acids (fluoresces orange-red).

anabolism The metabolic reactions by which cell components, extracellular products, etc., are built up from organic and/or inorganic precursors.

anaerobic Refers to an environment in which oxygen is absent.

antibody Any immunoglobulin molecule produced in direct response to an antigen and which can combine specifically, non-covalently, and reversibly with the antigen which elicited its formation.

API system A commercially available system used to identify microorganisms.

Aseptic technique(aseptically)
Precautionary measures taken to prevent the contamination of cultures, sterile media etc., and/or the infection of persons, animals or plants by extraneous microorganisms.

autoclave An apparatus within which objects or materials can be heat sterilised by (air-free) saturated steam, under pressure at temperatures usually in the range 115-134°C.

Bacillus A genus of Gram-positive, strictly aerobic or facultatively anaerobic, typically catalase-positive, rod-shaped, endospore forming bacteria.

bacillus Any rod-shaped bacterial cell, i.e., a cell whose length is ca. two or more times greater than its width.

bacteria Bacteria are a heterogeneous group of single-celled organisms; most have a characteristic type of cell wall, while a few are wall-less.

bacteriophage A virus that infects bacteria and causes the lysis of bacterial cells.

base pair (bp) A pair of bases, each in a separate nucleotide, in which each base is hydrogen-bonded to the other.

biological oxygen demand (BOD) The amount of dissolved oxygen needed for the microbial oxidation of biodegradable matter in an aquatic environment.

bioluminescence Generated by certain microorganisms, by fireflies and by some crustaceans and jellyfish.

bismuth sulphite agar (BSA) The medium is used for the primary isolation of *Salmonella typhi*. *S. typhi* reduces sulphite to sulphide, resulting in black colonies with a metallic sheen.

brain heart infusion agar (BHI) Used for the culture of a range of bacteria and medically important fungi.

brilliant green agar (BGA) A selective medium used for salmonellae which have a broad host range.

capsule A layer of material external to but continuous with the cell wall.

catabolism The metabolic reactions by which exogenous or endogenous organic compounds are degraded to simpler organic or inorganic compounds.

cell envelope The cytoplasmic membrane together with all layers external to it – including the cell wall.

cell wall The structure which forms a (usually rigid) layer external to the cytoplasmic membrane and which is responsible for the shape of the organism and

for protecting it from mechanical damage , osmotic lysis, etc., it may also serve as a permeability barrier, e.g., to antibiotics and other substances. Microbial cell walls differ greatly in structure and composition, according to type and species.

centrifugation The use of a centrifugal field for the sedimentation of fine particulate matter or macromolecules in a liquid medium, or for separating different types of particulate matter, or macromolecules, within a given suspension or solution.

cholera An acute, infectious human disease caused by specific serotypes of *Vibrio cholerae*.

chromosome aberration An abnormality in the number or structure of chromosomes in a cell.

chromosome Found in a prokaryotic cell or in the nucleus of a eukaryotic cell; a structure consisting of or containing DNA which carries genetic information essential to the cell.

cloning An *in vitro* procedure in which a particular sequence of DNA is reproduced in large amounts by inserting it into a suitable replicon, introducing the resulting recombinant molecule into a cell in which it can replicate, and finally growing the cells in culture.

coccus A spherical or near spherical bacterial cell.

coliform Gram-negative, rod-shaped, asporogenous, facultatively anaerobic bacterium which can ferment lactose, with acid and gas formation.

colony forming units (CFU) A single cell or a chain of cells capable of giving rise to a single colony.

colony A number of cells or organisms (of a given species) which, during their growth, have developed as a discrete aggregate or group in which there is commonly direct contact or continuity between the cells.

copiotroph Any organism which grows only in the presence of high concentrations of nutrients.

copy number The number of copies of a given plasmid, per chromosome, in a cell; copy number depends on the replication control system encoded by the plasmid, on the strain or species of the cell in which the plasmid occurs, and on the growth conditions.

counting chamber An instrument used for determining the total cell count, viable cell count, or spore count, etc., of a suspension of cells or spores.

culture A liquid or solid medium on or within which has grown a population of particular type(s) of microorganism or cell as a result of the prior inoculation and incubation of that medium.

cytopathic effect A change or abnormality in cells due to virus infection.

cytoplasmic membrane The lipid and protein containing, selectively permeable membrane which surrounds the cytoplasm in prokaryotic and eukaryotic cells.

deoxycholate-citrate agar (DCA) An agar medium used for the primary isolation of, e.g., *Salmonella* and *Shigella*; most strains of *Escherichia* and of the Proteeae fail to grow on DCA.

deoxyribonucleic acid (DNA) A nucleic acid consisting of deoxyribonucleotides, each of which typically contains one of the bases adenine, guanine, cytosine and thymine.

DNA homology The degree of similarity between base sequences in different DNA molecules (or in different parts of the same molecule).

dormancy The state of an organism or spore which exhibits minimal physical and chemical change over an extended period of time.

electron microscopy Microscopy in which an electron beam interacts with a specimen and subsequently contributes, directly or indirectly, to the formation of an image of the specimen. Electron microscopy can give magnifications within the range ca. 10X to 800,000X.

electrophoresis A procedure by means of which the members of a heterogeneous population of charged particles can be separated by virtue of their dissimilar migration characteristics in an electric field. Electrophoresis is used for the identification or quantification of proteins, nucleic acids, etc., and for molecular weight determinations.

enrichment Any process which increases the proportion of a given microorganism in a mixed population.

enteric 'Enteric bacteria' may refer to any or all bacteria normally found in the (small or large) intestine, or may refer specifically to members of the enterobacteriaceae.

enterobacteriaceae A family of Gram-negative, asporogenous, facultatively anaerobic bacteria.

enteropathogenic Pathogenic to the intestine.

enterotoxin A toxin which, either when ingested or when produced by an organism within the intestine, acts on the intestinal mucosa.

enzyme A protein which acts as a highly efficient and specific biological catalyst.

eosin methylene blue agar (EMB) An agar-based medium used e.g., for the primary isolation of / and for differentiating between enterobacteria.

EPEC Enteropathogenic *Escherichia coli*.

epidemic In a human population within a given geographical region; an outbreak of an infectious disease in which, for a limited period of time, a high proportion of individuals in the population exhibits overt symptoms of the disease; epidemics are characterized by a sudden onset.

epidemiology The study of the interrelationships between a given pathogen, the environment, and groups or populations of the relevant hosts, the object is to investigate the factors and mechanisms which govern the spread of disease within a community or population.

erythrocyte A red blood cell.

eukaryotic A type of cellular organism in which the chromosomes are separated from the cytoplasm by a specialized membrane. Eukaryotic microorganisms include the algae, fungi, lichens and protozoa.

exoenzyme An enzyme which sequentially removes units from one end of a polymer. Sometimes used as a synonym of 'extracellular enzyme'.

exotoxin An extracellular toxin, i.e. a toxin which is secreted by a living cell.

faecal streptococci (FS) Lancefield group D streptococci which occur primarily in human and animal intestines and faeces.

flow cytometry (FCM) A procedure in which individual cells in a suspension are counted, sorted, or otherwise analysed by the use of an apparatus in which the cells pass, individually, through a small hole or tube.

fluorescence The emission of light which occurs when certain substances absorb radiation.

fluorescent antibody (FA) Any procedure in which immunofluorescence is used (e.g., for clinical lab. Diagnosis, for counting or detecting specific microorganisms in water samples, etc.)

fluorochrome Any fluorescent dye, e.g., acridine orange, eosin, fluorescene.

gene amplification The generation of multiple copies of one or more genes in a cell or nucleus.

gene A sequence of nucleotides in a genetic nucleic acid (chromosome, plasmid, etc.,) which codes for a functional polypeptide chain or RNA molecule (rRNA, tRNA).

Gram stain A differential stain by which bacteria are classified as Gram-positive or Gram-negative depending upon whether they retain or lose the primary stain (crystal violet) when subjected to treatment with a decolorizing agent.

haemolysin Any antibody homologous to the surface antigens of red blood cells.

heterologous Derived from or associated with a species different from that being referred to.

homologous Similar in form or structure, but not necessarily in function; homology implies evolutionary relatedness.

hybridization The formation of a double stranded nucleic acid by base pairing between single-stranded nucleic acids derived from different sources.

immunity A state characterized by the tendency of the body to reject, eliminate or otherwise counteract foreign or seemingly foreign materials, or organisms on or within its tissues.

inoculum Material used for the inoculation of a medium, tissue culture, animal etc. ; it typically comprises or contains viable microorganisms.

kilobase (kb) A unit of length equal to 10^3 bases of a DNA or RNA strand.

kilodalton (kDa) 10^3 Daltons.

lipase A type of enzyme which hydrolyses glycerides to free fatty acids and glycerol.

lipopolysaccharide (LPS) Any lipid containing polysaccharide.

MacConkey agar An agar-based medium used, e.g., for the primary isolation of *Salmonella* and *Shigella* from faeces, for the differentiation of lactose-fermenting enterobacteria from non-lactose fermenters.

messenger RNA (mRNA) A RNA molecule which functions as the template for the assembly of amino acids during protein synthesis.

monoclonal antibodies A population of identical antibodies, all of which recognize the same specific determinant on a simple or complex antigen or hapten.

monocyte A large phagocytic leucocyte which contains a single, large, spherical or indented nucleus, and azurophilic peroxidase-containing granules; ca, 200 – 800 monocytes/ml occur in normal adult human blood.

necrosis Localised death and degeneration of tissues in a living organism due to injury or infection.

nucleic acid A polymer of nucleotides in which the 3′ position of one nucleotide sugar is linked to the 5′ position of the next by a phosphodiester bridge.

nucleoside A combination of ribosome for deoxyribose with a purine or pyramidine base.

nucleotide A nucleoside in which the sugar carries one or more phosphate groups.

oedema The abnormal accumulation of fluid in body tissues.

osmotic shock In cells or subcellular organelles sudden changes in osmotic pressure sufficient to cause physical disruption.

pathogen An organism capable of producing a disease.

pentose sugar usually ribose or 2-deoxyribose.

plasmid In many types of prokaryotic and eukaryotic cells, a linear or covalently closed circular molecule of DNA distinct from chromosomal DNA which can replicate autonomously and which is commonly dispensable to the cell.

pleomorphism An inherent variability in size and shape e.g., among the cells in a pure culture or clone of a given organism.

polymerase chain reaction (PCR) An *in vitro* method for enzymatically synthesizing defined sequences of DNA; the reaction uses 2 oligonucleotide primers that hybridize to opposite strands and flank the target DNA sequence that is to be amplified.

polysaccharide Any macromolecule consisting of monosaccharide residues joined by glycosidic linkages.

primer The primer is a short RNA strand which is transcribed on the DNA template at or near the *ori* site.

proteases Enzymes which hydrolyse peptide bonds in proteins and peptides.

psychrophile An organism which grows optimally at or below 15°C, which has an upper limit for growth of ca. 20°C, and which has a lower limit for growth of 0°C or below.

ribonucleic acid (RNA) A nucleic acid consisting of ribonucleotides, each of which contains one of the bases adenine, guanine, cytosine or uracil.

ribosomal RNA (rRNA) Constitutes the major proportion of RNA in the cell and is responsible for ribosome function.

ribosomes A ribonucleoprotein organelle which mediates protein synthesis, ribosomes may either be free or membrane-bound and are usually present in large numbers per cell.

salmonellosis (gastroenteritis) An infection with certain species of the genus *Salmonella*, usually caused by ingestion of food containing salmonellae or their products.

salmonella-shigella agar (SS) SS agar is used as a differential and selective medium for salmonellae and shigellae.

scanning electron microscopy (SEM) SEM is used to examine the surface of the specimen. It can give magnifications of ca. 10X to 100,000X. SEM provides a great depth of field, thus giving valuable three-dimensional images.

septicaemia A particular form of Bacteraemia in which there are clinical symptoms e.g., fever.

shigellosis (dysentery) A disease characterized by inflammation of the intestine, usually the colon.

sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) A specialised form of electrophoresis used for determining the molecular weight of a

protein or for separating mixtures of proteins.

supernatant The liquid over a precipitate or sediment, or the fluid remaining after the removal of the suspended matter.

thiosulphate-citrate-bile salt agar (TCBS) It is used for the isolation of *Vibrio* spp., especially *Vibrio cholerae*. The medium is strongly inhibitory for most faecal enterobacteria, pseudomonads and Gram-positive bacteria.

toxin Any of the various microbial products or components which, when present at low concentrations in cells or tissues of a higher organism, can cause injury by interfering with the structural or functional integrity of those cells or tissues.

transconjugant One bacterium transfers DNA to another while the cells are in physical contact . The recipient which has received DNA from a donor is called a transconjugant.

transformation A type of gene transfer in which a recipient cell acquires a fragment of DNA that is present in free form in the surrounding medium.

transmission electron microscopy (TEM) In TEM an electron beam passes through the specimen which deflects or scatters some of the electrons; electrons whose paths are slightly deviated by the specimen are focused onto a fluorescent screen to form an image of the specimen.

viable-but-non-culturable (VBNC) Under stressful conditions bacteria are known to transit to this stage. The bacteria in this stage are detected by microscopy as they loose the ability to be cultured on media. This stage is similar to dormancy.

vibriod vibrio-like, curved with a twist, i.e., not in one plane, as in an incomplete turn of a helix.

virulence The degree of pathogenicity exhibited by a strain of microorganisms.

LIST OF ABBREVIATIONS

A. hydrophila	*Aeromonas hydrophila*
A. salmonicida	*Aeromonas salmonicida*
AODC	acridine orange direct count
BGA	brilliant green agar
BHI	brain heart infusion
BOD	biological oxygen demand
BSA	bismuth sulfite agar
C. jejuni	*Campylobacter jejuni*
CFU	colony forming unit
COD	chemical oxygen demand
DCA	deoxycholate citrate agar
DNA	deoxyribonucleic acid
DPC	direct plate count
DVC	direct viable count
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
EMB	eosin methylene blue
FA	fluorescent antibody
FC	faecal coliform
FCM	flow cytometry
FD-PCR	fluorescent detection polymerase chain reaction
FS	faecal streptococci
HPC	heterotrophic plate count
KN	Kjeldahl nitrogen
L. pneumophila	*Legionella pneumophila*
LPS	lipopolysaccharide
MAC	MacConkey Agar
MF	membrane filtration
MIPA	magnetic immuno polymerase chain reaction assay
MUG	4-methylumbelliferyl-β-D-glucuronide
NA	nutrient agar
PAGE	polyacrylamide gel electrophoresis

PCR	polymerase chain reaction
rRNA	ribosomal ribonucleic acid
S. dysenteriae	*Shigella dysenteriae*
S. enteritidis	*Shigella enteritidis*
S. flexneri	*Shigella flexneri*
S. sonnei	*Shigella sonnei*
S. typhi	*Salmonella typhi*
S. typhimurium	*Salmonella typhimurium*
SABS	South African Bureau of Standards
SDS	sodium dodecyl sulfate
SEM	scanning electron microscopy
SPF	specific pathogen free
SS	salmonella-shigella
TC	total coliform
TCBS	thiosulfate citrate bile sucrose
TEM	transmission electron microscopy
TSA	tryptic soy agar
V. cholerae	*Vibrio cholerae*
V. harveyi	*Vibrio harveyi*
V. vulnificus	*Vibrio vulnificus*
VBNC	viable-but-non-culturable
VIA	veal infusion agar
XLD	xylose lysine decarboxylase

LIST OF TABLES **Page**

LIST OF FIGURES **Page**

Chapter 3

CHAPTER ONE

SCOPE OF THIS STUDY

1.1 Background and motivation

Currently, the isolation and identification of pathogenic and non-pathogenic microbes in the water industry is largely limited to conventional assays for a small group of indicator organisms. This reliance on current standard bacteriological methods has been increasingly criticized as inefficient estimators of the total number of bacteria. This criticism is linked to the presence of pathogenic bacteria that may enter a viable-but-non-culturable (VBNC) state as a result of sub-lethal stresses, and as a strategy for survival, remain in the dormant form until environmental conditions become more favourable. Therefore, there exists a high priority for the development of reliable and convenient methods which have the sensitivity, specificity and ability to detect and enumerate the total viable cell population, both culturable and non-culturable.

1.2 Objectives

The objectives of this study were: to detect VBNC potentially pathogenic water-borne bacteria by conventional methods, *i.e.*, by acridine orange direct counts, direct viable counts and dilution plate counts (DPC); to detect these VBNC bacteria using molecular techniques; and, to monitor morphological changes associated with the VBNC state of the organisms.

This study aims to elucidate: whether different water sources, temperatures and nutrient levels have varied effects on when the VBNC state is reached; whether stability and concentration of DNA isolated from seawater and river water microcosms has a marked effect on the detection limit of VBNC bacteria via polymerase chain reaction (PCR) / hybridization studies; and whether molecular methods are more accurate than conventional methods in detecting VBNC bacteria in water supplies.

Furthermore, this study was undertaken in order to determine whether extracellular virulence factors such as protease and haemolysins are produced when bacteria transit to a VBNC state; whether cells in the VBNC state are capable of maintaining plasmids associated with virulence; whether there are changes in lipopolysaccharides when bacteria enter a VBNC

state; and, whether *in vivo* studies with rat models would elucidate the ability of VBNC pathogens to cause infection in a susceptible host. Further investigation on resuscitation of VBNC pathogens to the culturable state and increased virulence of such cells should substantiate infectivity of VBNC cells.

1.3 Layout of the Report

The report comprises of 8 chapters and an Executive Summary.

Executive Summary: a short description of the methodology, the original and final objectives, as well as the reasons why the objectives had been amended and an indication as to whether or not the final objectives had been met. Also includes a summary of major findings and conclusions reached.

Chapter One: provides the background, motivation, objectives and a brief layout of the report.

Chapter Two: provides a literature review of the research study undertaken.

Chapter Three: detection of VBNC bacteria by conventional methods viz., acridine orange direct count (AODC), direct viable count (DVC) and dilution plate count (DPC).

Chapter Four: molecular detection of VBNC bacteria using a combination of PCR and gene probe hybridization.

Chapter Five: assessment of the prevalence of VBNC bacteria in various sources of water.

Chapter Six: use of scanning electron microscopy (SEM) for the assessment of the morphological changes associated with the transition of culturable cells to the VBNC state.

Chapter Seven: investigated the expression of certain virulence factors viz., haemolytic and protease activity, lipopolysaccharide (LPS) patterns, and *in vivo* pathogenicity of three species of pathogenic bacteria in the VBNC state as well as their resuscitated form.

Chapter Eight: synopsis of the major findings and conclusions reached.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

In an aquatic environment pathogenic bacteria which are capable of growth in warm-blooded hosts may be stressed by starvation or exposure to sub-optimal temperatures, salinity or toxic chemicals. Microbial cells may enter a viable but non-growing state, as a result of such sub-lethal stresses, and as a strategy for survival, remain in a dormant form until environmental conditions become more favourable (McKay, 1992). Dormancy may result if fluctuations in the natural environment are too severe to permit continuous growth (Stevenson, 1978). Microorganisms have the potential to adjust to environmental changes both structurally and functionally. Phenotypic adaptations allow the uptake and further metabolism of the limiting nutrient even when its concentration is low (Harder and Dijkhuizen, 1983).

The acridine orange direct count (AODC) method has been widely adopted for the detection of non-culturable or non-growing bacteria. It is generally accepted as being reliable for direct enumeration of total bacterial populations.

Kogure *et al.*, (1979) proposed the direct viable count (DVC) for the detection of non-culturable bacteria where the cells can be preincubated with nalidixic acid and yeast extract. This results in the formation of elongated, metabolically active cells in the presence of nutrients. The direct viable count allows for the enumeration of cells that are actively growing, as well as dormant cells that are physiologically responsive.

Isolation of microorganisms, by growth on selective media has been universally applied for the detection of viable microorganisms in water (American Public Health Association, 1989). The reliability of culture methods is of particular importance in testing for the presence of pathogenic microorganisms and microbial indicators of faecal pollution and in monitoring the release and survival of genetically modified microorganisms in water. However, the occurrence of viable-but-non-culturable forms, which fail to grow on selective media under laboratory conditions, calls into question the use of such culture techniques (McKay, 1992).

Saiki *et al.*, (1985) proposed the polymerase chain reaction (PCR) in order to overcome the limited sensitivity of the gene probe detection system, where the amount of target nucleic acid in a given sample will be increased. This is an enzyme-based reaction that can amplify a single DNA molecule by a factor of up to 10^6, thereby greatly facilitating detection by gene probe assays.

2.2 Detection of viable-but-non-culturable bacteria

Determining the number of pathogens and indicator organisms in the aquatic environment is imperative for assessing public health safety. Bacteria entering natural waters are confronted with a variety of temperature, salinity and nutrient concentration changes, with all parameters not necessarily optimal, or even permissive for growth and replication at any given time. Studies employing laboratory microcosms, using *E. coli, Salmonella spp.* and *Shigella spp.* have shown that these organisms persist in a dormant state (Colwell *et al.*, 1985). Cells are incapable of growth in conventional culture media and are said to be VBNC. The quality of water judged acceptable by conventional culture methodology may be potentially hazardous, placing the public health safety in question (Colwell *et al.* 1985).

Considerable attention has been directed at evaluating the survival of *E. coli* in aquatic systems. Plating methods for estimating survival of *E. coli* have severe limitations when used to estimate viable populations of these organisms in the aquatic environment (Xu *et al.*, 1982). In an aquatic environment *E. coli* cells may remain viable but lose the ability to grow on selective media. Microcosm studies with river, estuarine and sea water (Flint, 1987; Xu *et al.*, 1982) have demonstrated VBNC cells which will not grow on non-selective media. Direct culture is routinely used to detect coliform bacteria which act as indicators of faecal contamination. The occurrence of VBNC *E. coli* suggests that results from direct selective culture of water samples must be interpreted with caution (McKay, 1992).

Diaper and Edwards (1994) recommended the use of fluorogenic esters to detect viable bacteria by flow cytometry (FCM). A study of the survival of *Klebsiella pneumoniae* in lake water detected a higher number of viable bacteria both by direct viable counts and FCM than by colony-forming units, suggesting the VBNC state. Duncan *et al.* (1994) suggested luminescence as a means of detecting the activity of starved and VBNC bacteria. *E. coli* and *Pseudomonas fluorescens* had been genetically marked with luminescence from the naturally

occurring luminescent bacterium *Vibrio harveyi*. Luminescence of non-culturable cells of *V. harveyi* and *E. coli* decreased to background levels during starvation when late log phase cells were incubated with yeast extract and nalidixic acid. Changes in light output directly paralleled changes in cell length, as observed during direct viable counting. Signoretto *et al.* (2000) suggested that biochemical changes to the cell wall of VBNC *Enterococcus faecalis* may provide an indirect confirmation of the viability of these cells. Employing a different strategy, Lleo *et al.* (2000) reported on mRNA detection by reverse transcription-PCR for monitoring viability in a VBNC population of *Enterococcus faecalis*.

2.2.1 Conventional methods

2.2.1.1 Acridine orange direct count (AODC) and direct viable count

The AODC is reliable as a direct microscopic count of total bacterial population and has been used for *E. coli* (Byrd and Colwell, 1990), *S. enteritidis* (Roszak *et al.*, 1984), *Legionella pneumophila* (Hussong *et al.*, 1987), *Campylobacter jejuni* (Rollins and Colwell, 1986) and *Vibrio cholerae* (Colwell *et al.*, 1985).

The specificity of action of nalidixic acid was demonstrated by examining its effect on a single round of DNA replication. Under conditions of restricted RNA and protein synthesis, DNA synthesis was inhibited by nalidixic acid without any detectable loss of bacterial viability at least during a 3 h exposure period. The lack of lethality in this system strongly suggested that nalidixic acid does not alter the biological integrity of preformed DNA, but rather prevents only the synthesis of new DNA (Goss *et al.*, 1965). Cells exposed to nalidixic acid for as long as 75 min continue to incorporate thymine after the drug is removed, indicating that it is not firmly bound (Deitz *et al.*, 1966; Goss *et al.*, 1965). Washing the cells may dilute the drug to ineffective intracellular levels permitting resumption of proliferation (Deitz *et al.*, 1966).

2.2.2 Immunological methods

The fluorescent antibody FA technique has been used successfully to detect *V. cholerae* (Colwell *et al.*, 1985) and VBNC *S. dysenteriae* (Islam *et al.*, 1993). Colwell *et al.* (1985) adapted indirect immunofluorescent epifluorescent techniques to environmental samples in

order to obtain a more realistic estimate of population size. The FA procedure utilizes a species-specific antiserum and a fluorescent dye. In 1982, canals and village water sources in Bangladesh were chosen as sampling sites. Conventional culture methods yielded seven positive cultures of *V. cholerae* 01 from a total of 52 water samples collected. Using FA techniques 51 out of the 52 samples were confirmed as positive for *V. cholerae*. All cultures that were positive by culture, were positive by FA staining. Recovery of *V. cholerae* on media was higher at the earlier stages of enrichment incubation and not recoverable at later periods. FA results remained positive throughout.

VBNC *S. dysenteriae* was detected by the FA technique for up to 6 weeks, despite the fact that the cells were non-culturable on solid media (Islam *et al.* , 1993). The VBNC state of *S. dysenteriae* is significant in understanding the epidemiology of shigellosis. People in developing countries like Bangladesh extensively use waters from various surface water sources. Therefore, the non-culturable state of *Shigella spp.* may pose health problems (Islam *et al.* , 1993).

2.2.3 Polymerase chain reaction

PCR permits the *in vitro* replication of defined sequences of DNA whereby gene segments can be amplified. The most obvious application of this technique is to enhance gene probe detection of specific gene sequences. PCR exponentially amplifies a target sequence thus significantly enhancing the probability of detecting rare sequences in heterologous mixtures of DNA (Steffan and Atlas, 1991).

2.2.3.1 Mechanism of PCR

PCR quickly and efficiently produces many copies of specific DNA regions. Greater effort is required to produce similar results with viral or plasmid vectors using standard DNA cloning methods. The main limitations of PCR are the size of the region that can be amplified and the requirements for some knowledge of the sequences flanking the "target" DNA. In most studies a target region of 2 kb or less in length is chosen. Partial knowledge of the sequence of the target is necessary. Molecular systematics relies upon sequence comparisons among known genes. Thus, PCR is ideally suited for this purpose (Giovannoni, 1991).
PCR reactions can be broken down into three steps, which are separated in cycles:

(a) melting of the duplex sample DNA;

(b) annealing of two primers to opposite DNA strands; and

(c) extension of the primers by enzymatic nucleotide additions to produce a copy of the gene.

The specificity of the PCR reaction is governed by the oligonucleotide primers which hybridize to opposite DNA strands at opposite ends of the DNA target and direct the replication of the intervening region. The mechanism of PCR is simply primer-directed DNA synthesis, with the process being repetitive and the number of copies produced increases exponentially. In 30 or 40 cycles, requiring a few hours, the target is amplified a million-fold or more (Giovannoni, 1991; Saiki, 1989; Steffan and Atlas, 1991).

2.2.3.2 Use of PCR to detect microorganisms in the environment

Amplification of a segment of the coding region of *E. coli lac* Z using a PCR primer annealing temperature of 50°C detected *E. coli* and other coliform bacteria (including *Shigella spp.)* but not *Salmonella spp.* and non-coliform bacteria. Also, amplification of a region of *E. coli lam* B by using a primer annealing temperature of 50°C selectively detected *E. coli, Salmonella* and *Shigella spp.* (Bej *et al.*, 1990).

Bej *et al.* (1990) chose as a target for PCR amplification a region of the *mal* B gene that codes for maltose transport protein, because this region includes *lam* B which encodes a surface protein recognised specifically by the *E. coli* bacteriophage. PCR amplification and radiolabelled gene probes detected as little as 1 to 10 fg of genomic *E. coli* DNA and as few as 1 to 5 *E. coli* cells. 100 ml^{-1}. PCR amplification of *lac* Z and *lam* B provides a basis for a method to detect indicators of faecal contamination of water and amplification of *lam* B in particular permits detection of *E. coli* and enteric pathogens *(Salmonella spp.* and *Shigella spp.)* with the necessary specificity and sensitivity for monitoring the bacteriological quality of water so as to ensure the safety of water supplies (Bej *et al.*, 1990).

Bej *et al.* (1991b) examined the ability of the *uid* gene, which codes for the β-glucuronidase enzyme, to serve as a target for PCR-gene probe detection of *E. coli.* Their aim was to develop a PCR amplification gene probe detection method that permits specific detection of target faecal coliform bacteria, using the *uid* target gene, the expression of which forms the basis for the second stage of the Colilert test. The Colilert test is used for monitoring water

quality. It is based on detecting β-galactosidase activity, using a colorimetric reaction and the substrate o-nitrophenyl-β-galactopyranoside for total coliforms, and β-D-glucuronidase activity, using enzymatic transformation of the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide (MUG) to indicate the presence of the faecal bacterium *E. coli*. Amplification and gene probe detection specifically detected *E. coli* and *Shigella spp.* PCR also showed positive amplification of both *uid* A and *uid* R targets for MUG-negative *E. coli* isolated from both clinical and environmental samples which had failed to show positive reactions with the commercially available Colilert system. Multiplex PCR amplification of *lac* Z for total coliforms and *uid* A or *uid* R for faecal coliforms and the development of a non-isotopic gene probe detection technique, such as immobilised capture probes, can permit a rapid and reliable means of assessing the bacteriological safety of water and should provide an effective alternative methodology to conventional viable culture methods (Bej *et al.*, 1991b).

If the PCR gene probe method is to be applied for environmental monitoring of microorganisms, it is essential that even a single target gene be detected in samples of 100 ml or even greater than and equal to 100 gallons (Bej *et al.*, 1991c). While PCR permits detection of single cells in a sample, environmental samples must be concentrated to detect concentrations of microorganisms of < 1 cell. ml^{-1}. Target genes must not be lost or destroyed during the concentration of cells from the environment and substances used in the concentration procedure must not interfere with DNA amplification or detection. Thus, Bej *et al.* (1991c) concentrated the samples by filtration. Successful amplification with the highest sensitivity occurred when Fluoropore (FHLP or FGLP - Millipore) filters with pore sizes of 0.5 μm and 0.2 μm, respectively, were used. A lower sensitivity of detection, 100 to 1000 bacterial cells, was attained when a water wettable Fluoropore (FHLC, pore size 0.5 μm -Millipore) filter was used. Two hydrophobic Durapore membranes, GVHP and HVHP (pore size 0.2 μm and 0.4 μm -Millipore) showed positive PCR amplification at 103 and 102 cells, respectively, after they were treated with 10 x Denhardt's solution. Positive PCR-amplified DNA bands were observed with Durapore or polycarbonate filters only when at least 10^5 to 10^6 or more cells were filtered and amplified using PCR. No PCR amplification was observed when other membranes were used (Bej *et al.* , 1991c).

Toranzos and Alvarez (1992) described PCR carried out on filter membranes as solid phase PCR. Low concentrations of cells containing target sequences were concentrated on membrane filters. Together with nucleic acid probes, the solid phase PCR enhanced the limit

of detection to the level of a single cell. Toranzos and Alvarez (1992) suggested that this technique could be used to detect any bacterium or virus in water or air.

2.2.4 Gene probe technology

The application of nucleic acid probes to bacteria found in environmental samples shows great promise as they have the ability to identify specific nucleic acid sequences and determine the relatedness of similar sequences. Thus, they can be more specific than fluorescent, monoclonal antibodies or fluorochrome stains (Hazen and Jimenez, 1988; Richardson *et al.* , 1991).

The basic principles of gene probe technology include gene probe construction, labelling, hybridization and detection. To construct a gene probe the following must be considered: a microbe's genetic message is written in a four letter code represented by the nucleotides of its nucleic acid, either RNA or DNA; that code is read by determining the sequence of the nucleotide; and, 2 strands of nucleotides are capable of hybridising to form a sequence-specific duplex. The stability of the duplex formed during hybridization depends on the complementarity of the 2 strands and their ability to physically pair by means of hydrogen-bonds between the nucleotides. The stability of a duplex is measured in terms of its melting temperature. Each strand of a duplex will dissociate or denature into individual strands when exposed to temperatures greater than the melting temperature of the duplex. In practical applications of this technology one of the two complementary strands represents the probe and the other strand represents the target (Richardson *et al.*, 1991).

2.2.4.1 Use of gene probe technology to detect microorganisms in the environment

PCR amplification of the target sequences before gene probe detection provides a very sensitive surveillance technique (Steffan and Atlas, 1988). The widespread application of gene probe technology by water utilities is hindered by the requirements for specially skilled personnel. Most gene probes, with the exception of custom synthesised oligonucleotides are not commercially available. Although gene probes are usually obtained from the research institutions where they were developed, the process of gene probe production and labelling then becomes the burden of the water utility laboratory. Molecular techniques required are

not feasible for existing facilities and personnel and can be overcome by the marketing of gene probes by the biotechnology industry. The methodology should become standardised in a manner that will allow for routine application by those who do not have extensive training in this discipline (Richardson *et al.*, 1991).

DNA probes specific for *Salmonella spp.* have been developed by several investigators (Fitts *et al.*, 1983; Gopo *et al.*, 1988 and Rubin *et al.*, 1985). Each of these probes is composed of one or more chromosomal DNA fragments targeted to unique regions of *Salmonella* chromosomal DNA. The probe developed by Fitts *et al.* (1983), a mixture of several cryptic fragments of *S. typhimurium* DNA, is commercially available in a kit for detecting *Salmonella spp.* in food.

Detection methods based on rRNA-directed probes are particularly attractive because of the relatively large copy number of target sites within a cell. Braun-Howland *et al.* (1993) attempted hybridization with pure cultures as well as bacteria isolated from river water. rRNA targeted oligonucleotide probes were used to identify bacteria immobilised on nitrocellulose membranes by colony lifts. Cells could also be transferred by using a vacuum manifold under high salt conditions. Treatment of blotted cells with sodium dodecyl sulphate, urea, formaldehyde or protease had no apparent effect on hybridization signals. Hybridization to rRNA from cells that had been stored at 4°C for 6 days were readily detected. However, five-fold more cells were required to obtain hybridization signals comparable to those generated by cells not subjected to storage.

Gene probe technology may soon provide the water industry with reliable and inexpensive methods for evaluating the microbial quality of water. Further research in the field of gene probe technology should focus on methods for determining the viability of an organism and adaptation of nucleic acid amplification techniques to environmental samples. The commercial availability of gene probes for organisms of environmental interest is now limited and must be increased to permit widespread application of this technology. Gene probe technology may soon make possible widespread monitoring of water supplies in such a manner that assessment of microbial quality will be based on direct examination for potential pathogens and will not rely exclusively on conventional indicator systems (Richardson *et al.*, 1991).

2.3 Virulence factors associated with culturable and non-culturable bacteria

Virulence factors are bacterial products that are required by the organism to cause disease; deletion of any of these factors results in a reduction or loss of virulence. Bacterial virulence determinants include toxins which damage host cells and tissues, capsules and lipopolysaccharides which mask the bacterium from host defences, and enzymes like lipase, and protease which degrade host defence molecules. Bacterial virulence is a multifactorial process and the measurement of a particular virulence factor may give an indication of potential virulence of the organism under investigation (Lye and Dufour, 1993).

2.3.1 Biochemical properties as potential indicators of pathogenicity

The ability of *Salmonella sp.* and enteropathogenic *E. coil* to produce different exotoxins and exoenzymes is often associated with their virulence (Inman *et al.*, 1986; Finlay and Falkow, 1988 and Rahman *et al.*, 1996). Biochemical properties such as the production of lipases, proteases and haemolysins, have been reported as potential indicators of pathogenicity (Santos *et al.*, 1988) because they are putative virulence factors.

Although lipolytic activity has been reported, (Santos *et al.*, 1988; Cahill, 1990) this lipolytic activity was found to be insignificant for differentiation of several aeromonad groups (Pin *et al.*, 1995). Haemolysins are not usually the key virulence factors, however, some bacteria produce single proteins that exhibit exotoxic, enterotoxic, cytotoxic and haemolytic properties (Asao *et al.*, 1984; Potomski *et al.* 1987) which may be useful indicators of putative virulence factors. It is speculated that lipolytic, proteolytic and haemolytic assays may provide an indication of putative virulence characteristics of bacteria when they transit to the VBNC state.

2.3.2 Toxins

The maintenance of virulence by VBNC pathogens is a topic of debate (Madema *et al.*, 1992; Stern *et al.*, 1994) and there are few reports on the ability of VBNC cells to maintain biologically active toxins (Rahman *et al.*, 1996 and Pommepuy *et al.*, 1996). The first report of shiga toxin maintenance by VBNC *Shigella dysenteriae* type I (Rahman *et al.*, 1996) is a finding which has important implications for assessment of the health risk posed by VBNC

S. dysenteriae type I. Although toxin content of VBNC cells may be low, it may pose a significant threat because of the extreme potency of the toxin. Despite the controversy regarding maintenance of virulence by VBNC pathogens, it is important to note that shiga toxin produced by *S. dysenteriae* type I retained its cytopathic effect after cells entered the VBNC state (Rahman *et al.*, 1996).

VBNC *E. coli* cells (H10407) were able to produce heat labile enterotoxin (LT) in rabbit intestinal loops (Pommepuy *et al.*, 1997). The question of whether non-culturable *E. coli* cells producing enterotoxin represent only a few active cells or a total population of slowly metabolising cells was addressed and if a few actively metabolising cells were present in the sample, they should have been recovered on a suitable medium, but no cells were recovered. Thus the public health safety of water must be reassessed if potentially pathogenic bacteria cannot only survive, but retain enteropathogenicity when they enter the VBNC state.

2.3.3 Plasmids

The ability of VBNC cells to remain intact and retain plasmids associated with virulence is an essential factor in evaluating the significance of VBNC bacteria in the environment. van Overbreek *et al.*, (1990) have shown that plasmids present in *Pseudomonas* spp. and *Klebsiella spp.* remain stable when cells become non-culturable in agricultural drainage water. Similarly, Morgan *et al.*, (1991) reported that probes made to 3 different plasmids hybridized with plasmid extracts prepared from non-culturable cells of *A. salmonicida*.

In contrast, Grimes *et al.*, (1986) reported that plasmids harboured by non-culturable cells of *E. coli* H10407 could no longer be detected on direct extraction and electrophoresis. However, when a portion of non-culturable cells were grown in a nutrient broth, plasmid bands were present in culturable cells. Similarly, Grimes and Colwell (1986) have reported that when the cells of the same strain of *E. coli* which had been made non-culturable in natural seawater, were resuscitated in ligated rabbit ileal loops, the same pattern of plasmids as present in the original inocula were evident.

Byrd and Colwell, (1990), found plasmids, pUC 18 and pBR 322 of *E. coli* strains JM101 and JM83, respectively, to be maintained for at least 21 days when non-culturability was induced. The formation of transconjugants from VBNC recipient cells reported by Arana *et al.* (1997)

and the differences detected between the numbers of viable and culturable transconjugants formed during the survival experiments, confirm the importance of plasmid transfer by conjugation in aquatic systems.

Such findings are significant for potential virulence and gene transfer in the environment. Thus, the possibility that such plasmids could remain transferrable to autochthonous bacteria, or resuscitation of non-culturable plasmid-bearing bacteria needs to be addressed.

2.3.4 Nucleic acids

The VBNC state appears to be under genetic control. Molecular detection which involves amplification of DNA by the polymerase chain reaction (PCR), has been widely applied for detection of *Pseudomonas cepacia* (Steffan and Atlas, 1988), *V. cholerae* (Hassan *et al.*, 1992), *S. dysenteriae* (Islam *et al.*, 1993) and *V. vulnificus* (Brauns *et al.*, 1991). Cellular integrity and the presence of nucleic acids, ribosomes and machinery for protein synthesis may be considered necessary but not sufficient for viability. The detection of DNA by PCR or hybridization, the detection of rRNA by hybridization (Amann *et al.*, 1995) and the detection of protein synthesis by means of inducible enzyme activity (Nwoguh *et al.*, 1995) are techniques which identify cells as potentially viable.

An alternative method for assessing viability is the determination of the presence of nucleic acids. *In situ* analysis of nucleic acids revealed that in the cold-induced non-culturable populations of *Vibrio vulnificus*, there are two phases of the VBNC state: the loss of culturability with maintenance of cellular integrity and intact RNA and DNA (and thus possibly viability) and; degradation of RNA and DNA resulting in the loss of viability. Thus, since the majority of long-term, cold-incubated cells of *V. vulnificus* do not contain detectable amounts of intact ribosomes and nucleic acids, they may not be viable. The integrity of ribosomes and nucleic acids, may be maintained in a small fraction of the population and may retain the ability to recover and infect a suitable host (Weichart *et al.*, 1997).

Extensive purification of nucleic acids seems to be necessary for successful PCR amplification of DNA from microorganisms obtained from environmental samples (Steffan and Atlas, 1991; Bej and Mahbubani, 1992). It is possible that as cells enter the VBNC state, some change in the DNA may be occurring which prevents PCR amplification. Several

studies have suggested modifications in DNA composition in VBNC cells (Baker *et al.*, 1983; Hood *et al.*, 1986; Hoff, 1989; Linder and Oliver, 1989; Mayer and Morita, 1989; Brauns *et al.*, 1991). However, no definitive studies in this area have been published.

2.3.5 Lipopolysaccharides

Changes in capsule and membrane composition of cells entering the VBNC state have not been investigated in detail. Linder and Oliver, (1989) found the major fatty acid species (C_{16}, $C_{16:1}$, C_{18}) of VBNC cells of *V. vulnificus* to be decreased almost 60% compared with culturable cells. The percentage of fatty acids with chain lengths less than C_{16} increased as cells became non-culturable and long chain acids (C_{19}, C_{20}, $C_{22:1}$) appeared which were not evident in culturable cells. Morgan *et al.*, (1991) reported similar observations for *Aeromonas hydrophila*. Such changes presumably allow cells to maintain membrane fluidity as they enter the non-culturable state.

In addition, no loss of cell capsules was observed when cells of *V. vulnificus* entered the VBNC state (Linder and Oliver, 1989). However, lipopolysaccharides are important virulence determinants in bacterial pathogens, (such as enteropathogenic *E. coli*, *S. dysenteriae* and *S. typhimurium*). Thus analysis of lipopolysaccharide profiles of VBNC cells is essential in assessing potential virulence of these bacteria.

2.4 Resuscitation of VBNC bacteria

Resuscitation can be defined as a reversal of the metabolic and physiological processes that caused non-culturability resulting in the ability of cells to become culturable on growth media. Thus, resuscitation would appear to be essential to the VBNC state if this state is truly a survival strategy (Kjelleberg *et al.*, 1992). The VBNC state may protect the cell against one or more environmental stresses, while resuscitation to a cell potentially capable of rapid metabolism, would allow the cell to actively compete in the environment.

2.4.1 Resuscitation due to reversal of stress factor

VBNC cells, unlike starved bacteria do not respond quickly to a reversal of the factor which initially induced the non-culturable state. The question as to whether culturable cells appear

following removal of inducing stress are a result of true resuscitation or, of regrowth of a few residual culturable cells, has been debated (Kaprelyants *et al.*, 1993; Nilsson *et al.*, 1991; Oliver *et al.*, 1995; Votyakova *et al.*, 1994).

Roszak *et al.* (1984) were the first to report resuscitation of VBNC *Shigella enteritidis* by nutrient addition. Allen-Austin *et al.* (1984) were only able to achieve "revival" of VBNC *Aeromonas salmonicida* when culturable cells were present in the population. Morgan *et al.* (1991) suggested that regrowth of these few culturable cells resulted in a false resuscitation on non-culturable cells. This conclusion is in agreement with findings of Rose *et al.* (1990).

Roth *et al.* (1988) used a different approach to show that non-culturable cells of *E. coli* were capable of true resuscitation. These cells lost culturability when exposed to an osmotic stress (0.8 M), but could be resuscitated by addition of osmoprotectant betaine. Although nutrient was present, formation of new cells, prevented by addition of chloramphenicol, ruled out the possibility of cell proliferation as the cause of resuscitation. Dukay *et al.* (1997), suggested that recovery of culturability of a hypochlorous acid stressed population of *E. coli* after incubation in phosphate buffer could be due to the growth of the few culturable stressed cells, while there is repair and growth of some mildly injured VBNC cells.

Oliver *et al.*, (1991) pointed out the potential problem with resuscitation studies that rely on nutrient additions to non-culturable cells. In an attempt to circumvent the problem, Nilsson *et al.* (1991) reported resuscitation of *V. vulnificus* to temperature upshifts. A study which has monitored both entry into and exit from a VBNC state by a bacterium in a natural environment suggests that entrance into this state is likely a natural response to adverse environmental factors (Oliver *et al.*, 1995). Further studies suggest that elevated nutrient is in some way inhibitory to cell division in VBNC populations. The mechanism of this inhibition is not yet clear (Whitesides and Oliver, 1997).

2.4.2 *In vivo* resuscitation of VBNC cells

Animal models have been applied to monitor changes in virulence which might accompany entry into VBNC state. The significance of VBNC pathogenic bacteria rests on the capacity of cells to regain the ability to multiply when conditions become favourable within the host.

Several studies have been directed towards the recovery of VBNC bacteria, but reports are fragmentary and conflicting.

VBNC cells of *V. vulnificus* could be resuscitated following passage through clams, thus the ability to detect such an important human pathogen in shellfish is of considerable concern (Birbari *et al.*, 1991). Resuscitation of non-culturable cells when added to murine macrophage culture has also been reported (Simpson *et al.*, 1992).

Colwell *et al.*, (1985, 1990) prepared non-culturable cells of an attenuated *V. cholerae* strain which was then fed to 2 human volunteers at a dosage of 10^{8} cells/ml. Approximately 48 h after the challenge, one volunteer passed culturable *V. cholerae* cells in his stool at a concentration of $3 \times 10^{3}.g^{-1}$. After 5 days the second volunteer passed *V. cholerae* cells. Incubation of non-culturable cells in nutrient media prior to ingestion by volunteers revealed that no culturable cells were present among the non-culturable cells, suggesting that resuscitation of these cells occurred *in vivo*. This supports the hypothesis that non-culturable cells can maintain pathogenic potential and human passage can provide a means of resuscitation of such cells.

Colwell *et al.* (1985) have provided evidence of virulence for both *V. cholerae* and *E. coli* when these non-culturable cells were introduced into ligated rabbit ileal loops. Similarly, injection of chick embryos with non-culturable cells of *L. pneumophila* demonstrated virulence of non-culturable cells. Non-culturable cells of *Campylobacter spp.* in water samples were found to retain infectivity for mice (Rollins and Colwell, 1986). However, VBNC *Campylobacter jejuni* when introduced orally to one-day old chickens, did not colonise the intestine (Rahman *et al.*, 1996). Similar results were obtained in another study in which non-culturable *C. jejuni* were used in an animal model (Hasan *et al.*, 1994).

In addition, initial reports by Linder and Oliver, (1989), suggested that VBNC *V. vulnificus* loses virulence in mice, but further studies revealed that such results may have been the result of low inoculum of non-culturable *V. vulnificus* (Weichert *et al.*, 1992). Later, studies showed that injection of non-culturable *V. vulnificus* which had an infectious dose of less than 0.05 CFU / ml was lethal to mice (Oliver, 1993). Furthermore, it has been suggested that virulence of *V. vulnificus* decreases significantly as cells enter the VBNC state (Oliver

and Bockian, 1995). Pommepuy *et al.*, (1996) showed that VBNC cells of *E. coli* retained pathogenicity, i.e., produced enterotoxin.

VBNC cells of *Shigella dysenteriae* were found to maintain active shiga toxin and the compatibility of adhering to Henle cells. However, the ability to invade these eukaryotic cells was lost (Rahman *et al.*, 1996). The co-incubation of *Acanthamoeba castellani* with VBNC *Legionella pneumophila* resulted in the resuscitation of *L. pneumophila* to a culturable state (Steinert *et al.*, 1997). Reactivated cells retained the capacity for intracellular survival in human monocytes and intraperitoneally infected guinea pigs. However, resuscitation of VBNC legionellae alone was not observed in the animal model.

Thus, maintenance of virulence by non-culturable pathogens varies in different species and is influenced by the conditions applied.

CHAPTER THREE

DETECTION OF VIABLE-BUT-NON-CULTURABLE BACTERIA IN MICROCOSMS USING CONVENTIONAL METHODS

3.1 Introduction

The detection of viable bacteria is a perennial problem in microbiology (Diaper and Edwards, 1994). Conventional methods of culturing are time-consuming and a proportion of the metabolically active bacteria cannot be cultured (Roszak and Colwell, 1987). Thus, there is a need for direct detection of these bacteria. A popular choice amongst conventional methods for the detection of VBNC bacteria has been the use of the acridine orange dye to stain cells. McFeters and co-workers (1991) investigated the acridine orange staining reaction as an index of physiological activity in *E. coli*. They reported that spectrofluorometric observations of purified nucleic acids, ribosomes and the microscopic colour of bacteriophage infected cells stained with acridine orange confirmed the theory that single stranded nucleic acids emit orange to red fluorescence while those that are double-stranded fluoresce green *in vivo*.

Kogure *et al.* (1979, 1980) reported that AODC enumerated the total bacterial population, i.e., cells that stained red and green in colour when stained with acridine orange were counted. AODC were always higher than DVC which was in accordance with other reports (Colwell *et al.*, 1985; Nilsson *et al*, 1991). DVC was carried out using the method of Kogure *et al.* (1979, 1980). Elongated cells that stained red in colour were counted as being viable. All cells that stained green in colour with acridine orange were not counted (Kogure *et al.*, 1979, 1980).

DPC enumerated the culturable population of the microcosms (Colwell *et al.*, 1985). Researchers conducting initial experiments to investigate the VBNC phenomenon made use of DPC, AODC and DVC only. However, with the DPC various media were compared to investigate which agar plates supported growth for the longest period. Xu *et al.* (1982) investigated the survival of *E. coli* on EMB and TSA agar plates. After 10 days, *E. coli* was culturable on both plates. *S. enteritidis* (initial cell concentration 10^5 cells. ml^{-1}) reached zero CFUs. ml^{-1} in two days on veal infusion agar and required three days on XLD agar plates when the VBNC state was investigated by Roszak *et al.* (1984). Colwell *et al.* (1985)

reported that recovery of *E. coli* incubated in microcosms of Chesapeake Bay (initial cell concentration 10^6 cells. ml^{-1}) was not influenced by the growth media. Plate counts on EMB and TSA dropped from 10^6 cells. ml^{-1} on day 1 to 10^3 cells. ml^{-1} on day 14 (Colwell *et al.*, 1985). Islam *et al.*(1993) used MacConkey agar plates to enumerate the VBNC state of *S. dysenteriae* by DPC. Drain water supported the growth of *S. dysenteriae* for the longest period (21 days), whereas river water supported its growth for the shortest period (15 days) on MacConkey agar plates. Comparing the results obtained by the various researchers (above), there was no significant difference in the DPC for the various media. Thus, for the present study BHI agar was the medium of choice since it supported the growth of all five cultures used in this study.

Since there is no recommended standard method to set up microcosms for study of the VBNC phenomenon, the influence of temperature, salinity, shaking and cell concentration were investigated. Microcosm incubation temperatures of 4°C and 25°C were investigated in this study. The 4°C microcosms were equated to the colder months of the year when it is assumed that the sub-optimal temperature enhanced the transition to the VBNC state (Nilsson *et al.*, 1991). The 25°C microcosms were equated to the warmer months of the year. It was assumed that the higher microcosm incubation temperature would promote rapid replication of the cells, thus depleting nutrients and entering the VBNC state. Roszak *et al.* (1984) and Xu *et al.* (1982) used shaking microcosms while Brauns *et al.* (1991); Islam *et al.* (1993) and Nilsson *et al.* (1991) used stationary microcosms to induce the VBNC state.

Since this chapter focussed on the detection of VBNC bacteria by conventional methods AODC, DVC and DPC on BHI agar plates served adequately for the enumeration of total bacterial population, viable population and culturable population, respectively. The various microcosms were evaluated with respect to the induction of the VBNC response in the five bacteria, *Salmonella typhimurium*, *S. typhi*, *Shigella flexneri*, *S. sonnei* and *E. coli*.

3.2 Materials and methods

3.2.1 Bacterial cultures

The following bacterial cultures obtained from the bacterial culture collection of the Department of Microbiology, University of Durban-Westville (UDW) were used to set up

laboratory microcosms: *S. typhimurium* UDW 1 , *S. typhi* UDW 2, *Shigella flexneri* UDW 3, *S. sonnei* UDW 4 and *E. coli* UDW 5.

3.2.2 Growth and maintenance of cultures

The identity of the cultures were verified using the Gram stain, API 20E identification scheme and selective media. Cultures were maintained as working stocks on brain heart infusion (BHI) agar slants at 4°C. For long-term storage broth cultures were stored on microbeads (Microbank^{TM}) at -70°C. In addition, glycerol was added to overnight cultures in BHI broth to a final concentration of 15% and stored at -70°C and -20°C.

3.2.3 Preparation of laboratory microcosms

Initial microcosms were set up with pond water and river water with cell concentrations of 10^7 cells. ml^{-1} and the culturability of the organisms assessed by DPC. These microcosms served to ascertain the time period required to reach the VBNC state. Thereafter, stationary seawater microcosms were set up with cell concentrations of 10^6 cells.ml^{-1}. Although the microorganisms tested were non-marine bacteria, they remained culturable for lengthy periods. Consequently, stationary and shaking river water microcosms with cell concentrations of 10^5 cells.ml^{-1} were set up to investigate if shaking did influence the time taken to reach the VBNC state. The initial cell concentration was decreased in subsequent experiments in an attempt to decrease the incubation time required to induce the VBNC state.

3.2.3.1 Chemical analyses of microcosm water

The following chemical analyses were carried out by Umgeni Water in Pietermaritzburg: alkalinity, nitrate, nitrite, nitrogen (ammonia), phosphate, biological oxygen demand (BOD), chemical oxygen demand (COD), total Kjeldahl nitrogen and total organic carbon.

3.2.3.2 Pre-treatment of microcosm water

The methods of Islam *et al.* (1993), Nilsson *et al.* (1991) and Xu *et al.* (1982) were modified in this study as follows. Water samples were collected aseptically from near the mouth of the Umgeni river (Durban), pond water (from UDW's duck pond) and seawater (South Beach,

Durban). Water samples were first filtered through Whatman No.1 filter, followed by filtration through a 0.45 μm Millipore filter paper and finally autoclaved at 121°C for 15 minutes. Two hundred and fifty ml microcosms were set up in duplicate in one litre acid washed flasks and maintained as follows:

a) river water was maintained at 4°C and 25°C as stationary and shaking microcosms; and

b) pond water and seawater were maintained at 4°C and 25°C as stationary microcosms.

3.2.3.3 Inoculum for microcosms

The methods of Islam *et al.*, (1993), Nilsson *et al.*, (1991) and Xu *et al.*, (1982) were used to prepare the inoculum for the microcosms. Direct counts of the overnight culture grown in BHI broth were carried out using the Weber Scientific International counting chamber. The initial stationary microcosms of river water and pond water were set up with cultures introduced at a final concentration of approximately 10^7 cells.ml^{-1}, unwashed in BHI broth. The stationary seawater microcosms were set up with cultures that were washed three times with sterile seawater to remove excess broth before being introduced into the microcosms at a final concentration of 10^6 cells.ml^{-1}. The final stationary and shaking river water microcosms were set up with cultures that were washed three times with sterile river water before being introduced into the microcosms at a final concentration of approximately 10^5cells. ml^{-1}.

3.2.4 Sampling from microcosms and enumeration of bacteria

3.2.4.1 Dilution plate counts

Cells were enumerated weekly (longer intervals between samplings were also used once it was established that DPC did not change significantly during weekly samplings) on BHI agar plates at 37°C by dilution plate counts (DPC) until the bacteria were no longer culturable. One ml sample from each flask was aseptically removed and 10-fold dilutions were prepared in either sterile distilled water for the pond water and initial river water microcosms, sterile seawater for seawater microcosms or sterile phosphate buffered saline for the final river water microcosms. A 100 µl aliquot from the different dilutions were plated out in duplicate on BHI agar plates and incubated overnight at 37°C. For the 25°C and 4°C microcosms, the diluent and agar plates were maintained at room temperature and 4°C, respectively, prior to plating. When no culturable cells were found after two consecutive samplings from the 10^{-1}

dilution, 0.1ml water was aseptically removed and plated onto BHI agar plates. Also, 10 ml of the microcosm water was filtered through a 0.22 μm (47 mm diameter) Millipore membrane filter to increase the level of detection. When less than 0.1 cells. ml^{-1} of microcosm cells were culturable, the cells were considered to be in the non-culturable state.

3.2.4.2 Acridine orange direct count

The methods outlined by the American Public Health Association (1989) and Hobbie *et al.* (1977) were used for the AODC. Two ml of microcosm water was aseptically removed and mixed with an equal volume of 0.1 % (w/v) acridine orange solution in a sterile test tube, vortexed and incubated in the dark for 3 min. The suspension was then added to a filter tower (Millipore) containing an Irgalan black stained 0.22 μm (25 mm diameter) Nuclepore^R filter. A vacuum of 15 cm of Hg was used to filter the bacterial suspension onto the filter . Two ml of 5 % formaldehyde in phosphate buffer was filtered through to fix the suspension. Two ml of phosphate buffer was also filtered to promote more even cell distribution. The filter was removed, air dried for 1-2 min, and placed on a drop of Cargille immersion oil on a glass slide. A small drop of immersion oil was added to the filter surface and covered with a coverslip. A drop of oil was placed on the coverslip and viewed using a Zeiss microscope fitted with a HBO 50 W mercury lamp. Samples not viewed immediately were stored in the dark at 4°C for several weeks. Ten randomly selected fields were counted using the 100 x oil immersion objective and the average bacterial count of 10 fields was multiplied by a factor of 24416 to obtain the bacterial count. ml^{-1}. This factor is applicable for 1 ml of sample and must be corrected for different sample sizes

3.2.4.3 Direct viable count

The method of Hobbie *et al.* (1977) was used for the DVC. Two ml of microcosm water were enriched with 0.025% (w/v) yeast extract and 0.002% (w/v) nalidixic acid and incubated in the dark at 20°C for 6 h. The suspensions were treated in the same way as for the acridine orange direct count and viewed using a Zeiss microscope fitted with a HBO 50 W mercury lamp. The factor used for enumerating the bacterial cells. ml^{-1} remained the same, i.e., as calculated in 3.2.4.2.

3.3 Results

3.3.1 Chemical analyses of water samples

The following results were obtained for the chemical analyses of water samples:

Table 3.1 Chemical analyses of river water

CHEMICAL ANALYSES	RIVER WATER	
	UNTREATED	AUTOCLAVED
Alkalinity ($mg.l^{-1}$)	82.00	88.60
Nitrate ($mg.l^{-1}$)	4.63	3.56
Nitrite ($mg.l^{-1}$)	<0.05	<0.05
Nitrogen (ammonia) ($mg.l^{-1}$)	0.11	0.62
Phosphate (total -$\mu g.l^{-1}$)	1541.00	5490.00
Biological oxygen demand ($mg.l^{-1}$ oxygen)	2.00	2.10
Chemical oxygen demand ($mg.l^{-1}$)	25.80	27.80
Total Kjeldahl nitrogen ($mg.l^{-1}$)	3.23	<3.00

Table 3.2 Chemical analyses of seawater

CHEMICAL ANALYSES ($mg.l^{-1}$)	SEAWATER	
	UNTREATED	AUTOCLAVED
Alkalinity ($mg.l^{-1}$)	124.00	124.00
Nitrate ($mg.l^{-1}$)	<0.05	<0.05
Nitrite ($mg.l^{-1}$)	<0.05	<0.05
Nitrogen (ammonia) ($mg.l^{-1}$)	0.05	0.03
Phosphate (total -$\mu g.l^{-1}$)	26.00	43.00
Biological oxygen demand ($mg.l^{-1}$ oxygen)	<1.00	<1.00
Chemical oxygen demand ($mg.l^{-1}$)	956.00	794.00
Total Kjeldahl nitrogen ($mg.l^{-1}$)	0.25	0.60
Total organic carbon ($mg.\ l^{-1}C$)	3.05	2.65

3.3.2 Analysis of stationary pond water microcosms

S. typhimurium, S. typhi, S. flexneri, S. sonnei and *E. coli* were introduced into sterile pond water at an initial concentration of 10^7 $cells.ml^{-1}$. Analysis of the pond water microcosms at 4°C and 25°C involved only DPC (Fig. 3.1) since these initial microcosms were set up to determine the time period required to induce the non-culturable state in the laboratory microcosms. After approximately 140 days the five cultures in both the microcosms were still culturable (Fig. 3.1). The organisms in the microcosms are defined as non-culturable when <0.1 $CFU.ml^{-1}$ is detected. After approximately 140 days, between 3.5-6.5 $\log_{10}$ CFUs. ml^{-1} were observed for the 25°C microcosms (Fig. 3.1B) and 6-8 $\log_{10}$ CFUs. ml^{-1} were observed

for the 4°C microcosms (Fig. 3.1A). These experiments were terminated after 140 days. Broth had been introduced into the microcosm as the cells of the initial inoculum were unwashed. This may have provided a source of nutrients to the bacterial cells, thus enabling them to remain culturable for an extended time. The results were also analyzed statistically using the scientific graph system, Sigmaplot 5.1 mathematics function. There was no significant difference between the DPC of *S. typhimurium* in both microcosms. However, there was a significant difference between the DPC of *S. typhi* in both microcosms. There was no significant difference between the DPC of *S. flexneri* and *E. coli* in both microcosms.

3.3.3 Analysis of stationary seawater microcosms

The bacterial cultures were introduced into sterile seawater laboratory microcosms at an initial concentration of 10^6 cells. ml^{-1}. Analysis of the 4°C and 25°C seawater laboratory microcosms involved DPC, AODC and DVC (Fig. 3.2). For seawater microcosms, sterile seawater was used as dilution blanks.

S. typhimurium reached the non-culturable state in 105 days at 4°C and in 104 days at 25°C. Statistical analysis showed that DPC of *S. typhimurium* at both temperatures were significantly different from the AODC and DVC. However, there was no significant difference between the AODC and DVC of *S. typhimurium* (Fig. 3.2A, 3.2B). There was no significant difference between the DPC of *S. typhimurium* at both temperatures. At 25°C there was a slight decrease in culturability from day 30 to day 90 where the DPC dropped from 4.20 log to 3.89 log. The AODC was 5.75 log and 5.52 log for 4°C and 25°C, respectively, when the VBNC state was attained. There were significant differences between the AODC and the DVC at both temperatures.

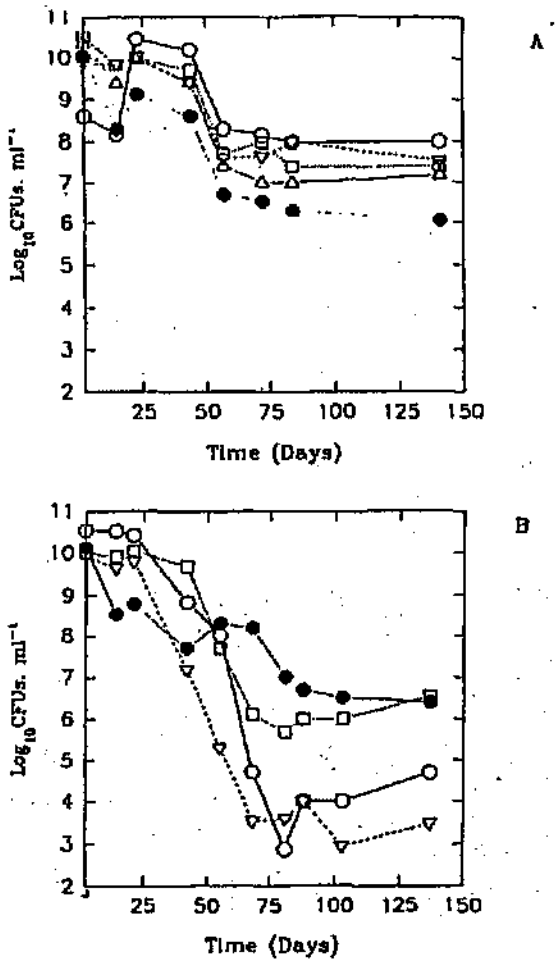

Fig. 3.1 DPC of *S. typhimurium* (o), *S. typhi* (∇), *S. flexneri* ( ), *S. sonnei* (Δ) and *E. coli* (•) from pond water microcosms at (A) 4°C and (B) 25°C.

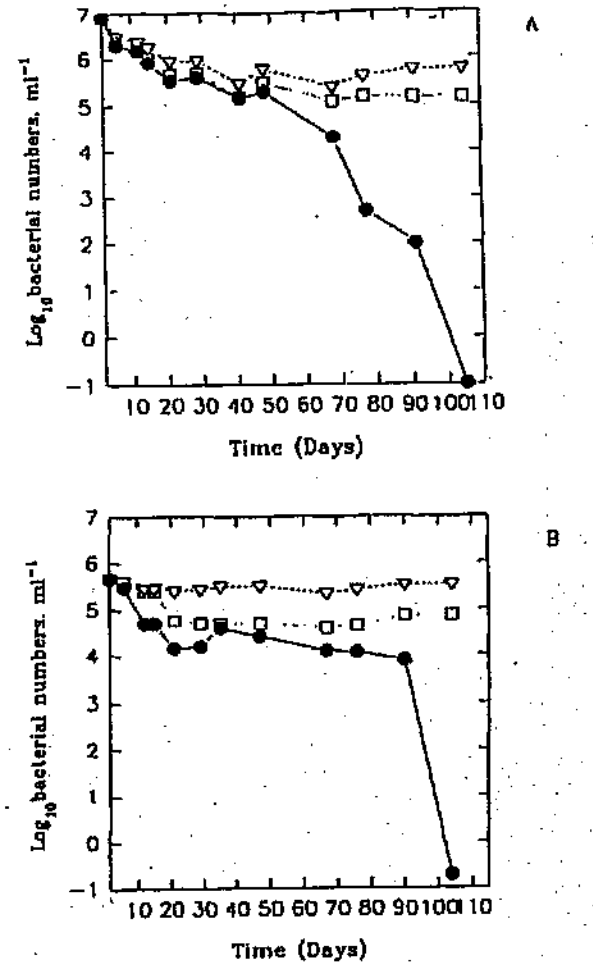

Fig. 3.2 DPC(•), AODC (v) and DVC ( ) of *S. typhimurium* from seawater laboratory microcosms at (A) 4°C and (B) 25°C.

S. typhi attained the non-culturable state in 105 days at 4°C and 111 days at 25°C (Fig. 3.3). There was a steady decrease in culturability of *S. typhi* at 4°C where the DPC decreased from 5.80 log to -1.00 log. The DPC of *S. typhi* is significantly different from the AODC and the DVC at 4°C and 25°C.

There was no significant difference between the DPC of *S. typhi* at both temperatures. At 4°C the AODC and DVC were similar from day 1 to day 40, suggesting that all the cells were viable (Fig. 3.3A). There was a significant difference between the AODC and DVC at both temperatures. However, the AODC was not significantly different at both temperatures, whereas the DVC differed significantly.

S. flexneri reached the non-culturable state in 105 days at 4°C compared to 67 days at 25°C (Fig. 3.4). There was no significant difference between DPC at both temperatures.

At 25°C (Fig. 3.4 B) there was a decrease in culturability of *S. flexneri* from day 1 through to day 67 when non-culturability was attained. This was the shortest period in which any of the organisms reached the VBNC state in the seawater microcosms. There was a significant difference between the DPC and AODC of *S. flexneri* at both temperatures. However, there was no significant difference between the DPC and DVC at both temperatures. There was a significant difference between the AODC and DVC at both temperatures. The DVC decreased from day 1 to day 15 at 25°C, thereafter remaining virtually constant (Fig. 3.4B). There was no significant difference between the AODC at both temperatures. However, there was a significant difference between the DVC at both temperatures.

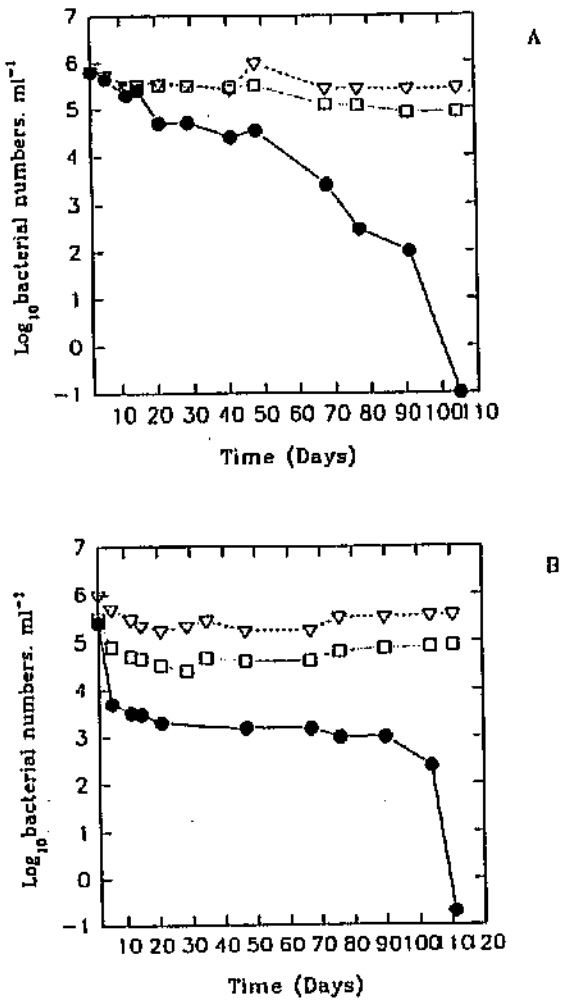

Fig. 3.3 DPC (•), AODC (▽) and DVC ( ) of *S. typhi* from seawater laboratory microcosms at (A) 4°C and (B) 25°C.

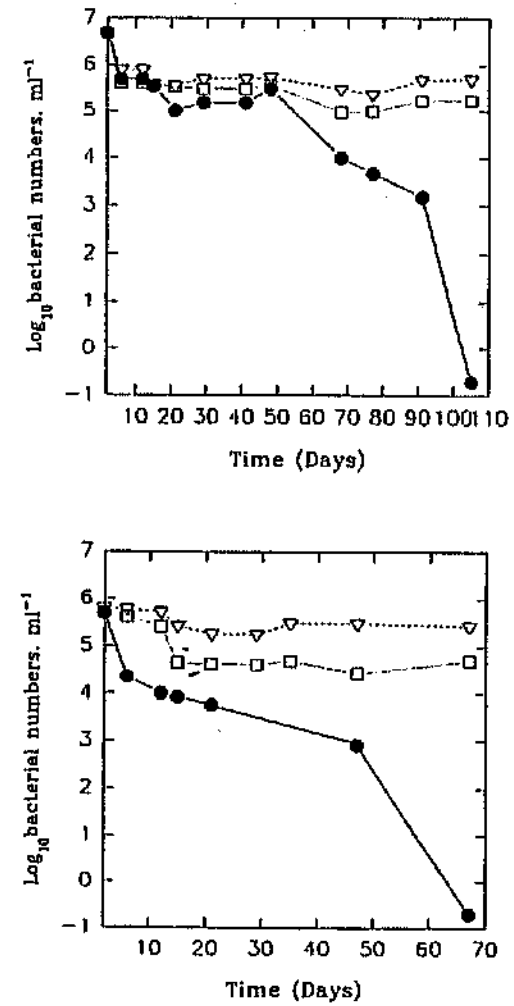

Fig. 3.4 DPC (•), AODC (▽) and DVC ( ) of *S. flexneri* from seawater laboratory microcosms at (A) 4°C and (B) 25°C.

S. sonnei exhibited the non-culturable state in 105 days at 4°C compared to 110 days at 25°C (Fig. 3.5). There was no significant difference between DPC and AODC for the 4°C microcosm (Fig. 3.5A), whereas there was a significant difference between the DPC and AODC at 25°C (Fig. 3.5B). The same statistical analysis was obtained for the DPC and DVC for both temperatures. There was no significant difference between the DPC at both temperatures.

S. sonnei at 4°C (Fig. 3.5 A) proved to be an interesting example where the AODC, DVC and DPC were similar from day 1 through to day 68 suggesting that the entire population of bacterial cells were culturable and viable. This was followed by a constant decrease in culturability. Partial death of the microcosm population at 25°C indicated by the decrease in AODC in the first 20 days could have enriched the microcosm water, thus providing nutrients for the growth of the bacterial population present. However, statistically the AODC and DVC differed significantly at 25°C. There is also a significant difference between the AODC and DVC at both temperatures (Fig. 3.5 A,B).

E. coli attained the non-culturable state in 105 days at 4°C (Fig. 3.6A) and 90 days at 25°C (Fig. 3.6B). The effect of temperature was marked in the transition of *E. coli* to the VBNC state. A similar observation was made for *S. flexneri* (Fig.3.4). *E. coli* showed (Fig. 3.6A) a similar pattern to *S. sonnei* at 4°C (Fig. 3.5A) where there was a negligible difference among the AODC, DVC and DPC from day 1 through to day 68. Statistically there was a significant difference between the DPC at 4°C and 25°C. DPC differed significantly from AODC and DVC at 25°C. However, DPC did not differ significantly from the AODC and DVC at 4°C. There was a significant difference between the AODC and DVC at both temperatures. Partial death of the *E. coli* population in the microcosm at 25°C seemed to have increased the viability of the population but not the culturability as DPC decreased constantly through the experiment. There was also a significant difference between the AODC at both temperatures. This phenomenon was also evident for the DVC at both temperatures for *E. coli*.

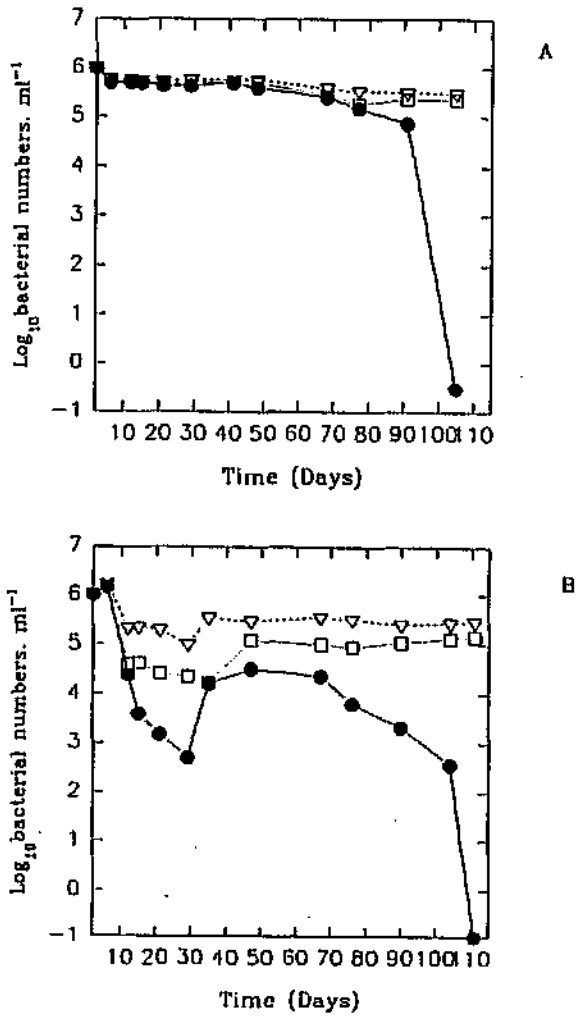

Fig. 3.5 DPC (•), AODC (▽) and DVC ( ) of *S. sonnei* from seawater laboratory microcosms at (A) 4°C and (B) 25°C.

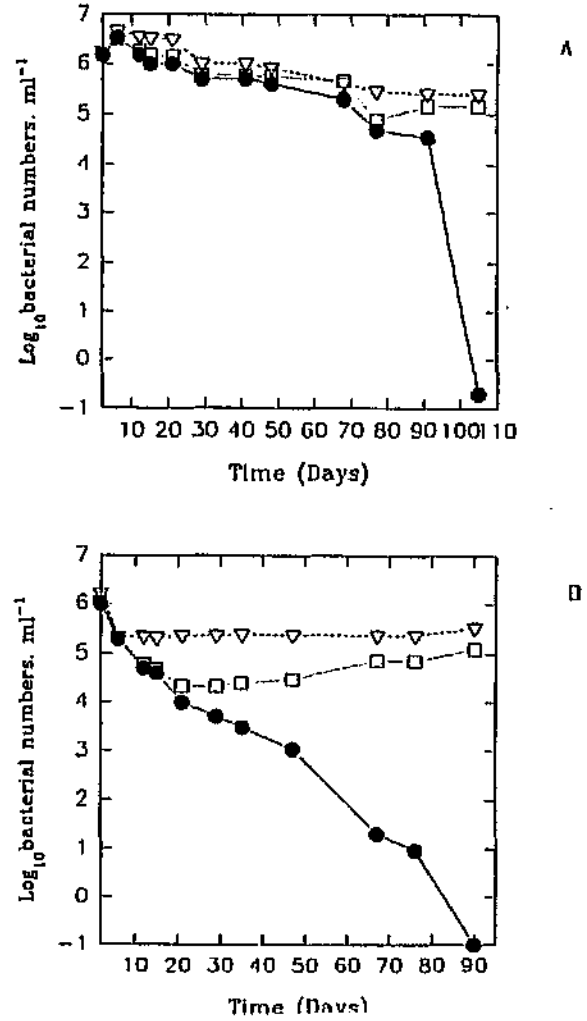

Fig. 3.6 DPC (•), AODC (▽) and DVC ( ) of *E. coli* from seawater laboratory microcosms at (A) 4°C and (B) 25°C.

3.3.4 Analysis of stationary and shaking river water microcosms

3.3.4.1 Initial stationary river water microcosms (initial cell concentration 10^7 cells. ml^{-1})

The five bacterial cultures examined previously (pond and seawater) were also investigated in these microcosms. The initial stationary river water microcosms, like the pond water microcosms, were set up to determine the time period involved in reaching the non-culturable state. At 4°C the culturability of the five bacterial cultures were very erratic from day 1 through to day 84. However, the non-culturable state was not induced for the five bacterial cultures at 4°C (Fig. 3.7A). The DPC were in the region of 6.00 log and higher indicating that the entire population was still culturable. Statistically, there were no significant differences between the DPC of *S. typhimurium* and *S. typhi* at both temperatures. At 25°C, *S. flexneri* was the only culture that accomplished the non-culturable state in 68 days (Fig. 3.7B). At 25°C the culturability of the five bacterial cultures did decrease. However, after approximately 140 days the DPC were in the region of 4.00 log and higher at 25°C (Fig. 3.7B). This indicated that the bacterial cultures were in a better state of preservation at 4°C where they were able to maintain their culturability. Also, DPC of *S. sonnei* and *E. coli* did not differ significantly at both temperatures. Sampling in these microcosms like the pond water microcosm were terminated after 140 days.

3.3.4.2 River water microcosms at 4°C (initial concentration 10^5 cells. ml^{-1})

A second attempt was made to induce the non-culturable state in river water microcosms. The initial concentration was decreased from 10^7 cells. ml^{-1} to 10^5 cells. ml^{-1}. The cells were also washed three times with sterile river water before being introduced into the microcosms. A comparison of stationary and shaking microcosms at 4°C and 25°C was also included in these experiments.

S. typhimurium in the stationary (Fig. 3.8A) and shaking (Fig. 3.8B) river water microcosms at 4°C did not reach the non-culturable state. The DPC were 3.62 log and 3.00 log after 138 days for the stationary and shaking microcosms, respectively.

S. typhi in the stationary and shaking river water microcosms entered the non-culturable state in 138 days (Fig. 3.9A,B). The DPC fluctuated in both microcosms (Fig. 2,9A,B). Statistically, there was a significant difference between the DPC and AODC for both microcosms. The same held true for DPC and DVC for both microcosms. However , the DPC of *S. typhi* in both microcosms does not differ significantly. *S. typhi* at 4°C in the stationary (Fig. 3.9A) and shaking (Fig. 3.9B) microcosms was the only culture that attained the non-culturable state.

S. flexneri in the stationary and shaking river water microcosms did not reach the non-culturable state. DPC were 3.30 log and 4.00 log for the stationary and shaking microcosm, respectively, (Fig. 3.10 A,B) after 138 days. Thus, keeping the microcosms shaking or stationary did not contribute significantly to any change in the culturability of *S. flexneri.*

S. sonnei in the stationary and shaking river water microcosms failed to reach the non-culturable state after 138 days (Fig. 3.11 A,B). DPC were 3.30 log and 3.88 log for the stationary and shaking river water microcosms, respectively. There was no significant decrease in culturability for both microcosms.

E. coli in the stationary and shaking river water microcosms did not reach the non-culturable state after 138 days (Fig. 3.12 A,B). There was no significant change in the culturability of *E. coli* in the stationary and shaking microcosms. DPC was significantly different from the AODC and DVC in both microcosms.

Thus, it could be concluded that four of the five cultures maintained their culturability for up to 140 days at 4°C in the stationary and shaking river water microcosms.

3.3.4.3 River water microcosms at 25°C (initial concentration 10^5 cells. ml^{-1})

S. typhimurium in stationary and shaking river water microcosms did not reach the non-culturable state (Fig. 3.13 A,B). This was the only culture in the 25°C microcosms that did not reach the non-culturable state. The DPC after 145 days were 5.03 log and 4.99 log for the stationary and shaking microcosms, respectively.

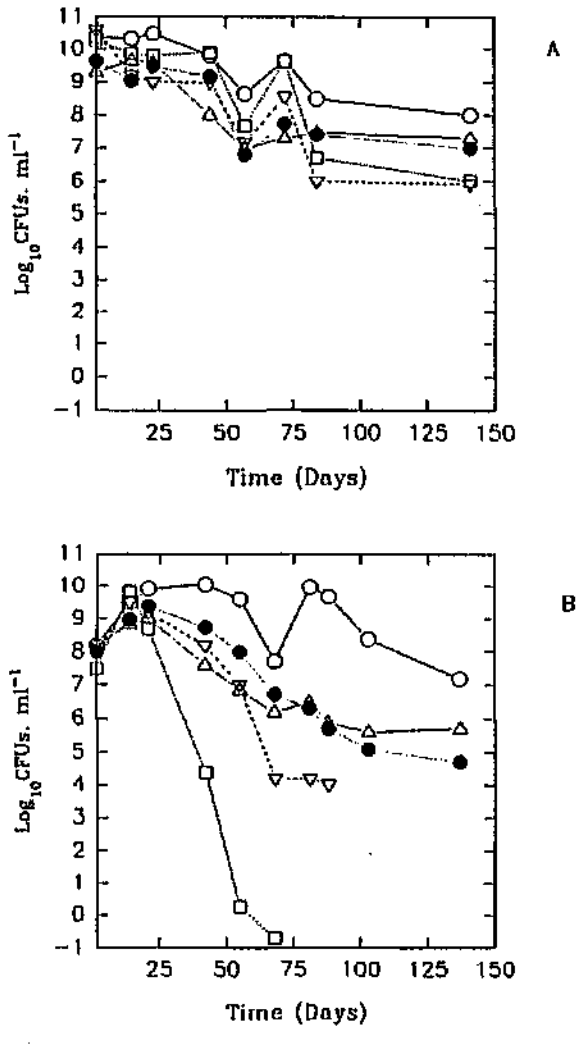

Fig. 3.7 DPC of *S. typhimurium* (o), *S. typhi* (▽), *S. flexneri* ( ), *S. sonnei* (Δ) and *E. coli* (•) from initial river water microcosms (10^7 cells.ml^{-1}) at (A) 4°C and (B) 25°C.

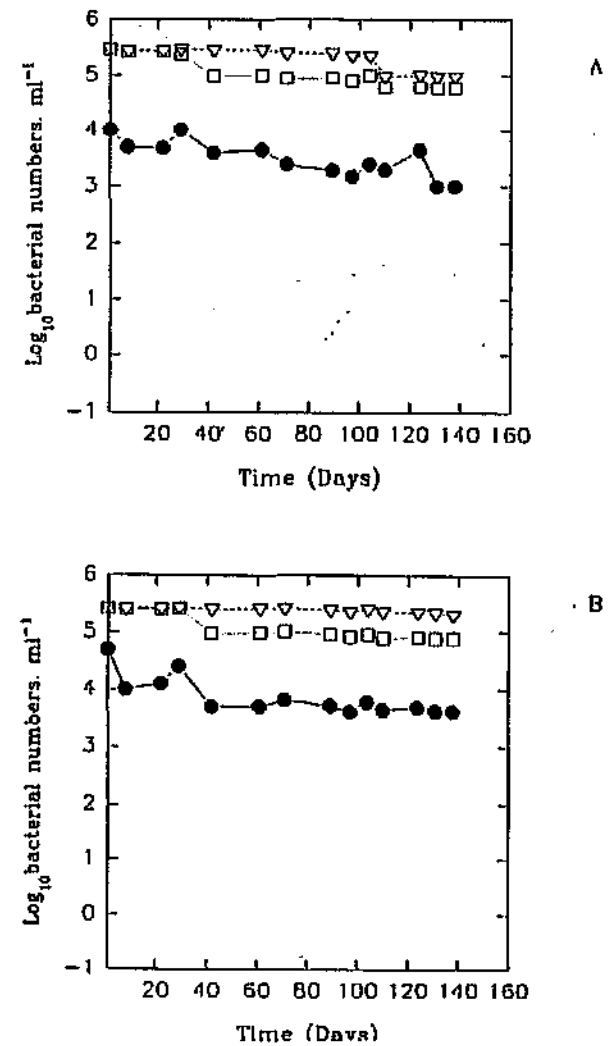

Fig. 3.8 DPC (•), AODC (▽) and DVC ( ) of *S. typhimurium* from river water microcosms at 4°C (A) stationary and (B) shaking.

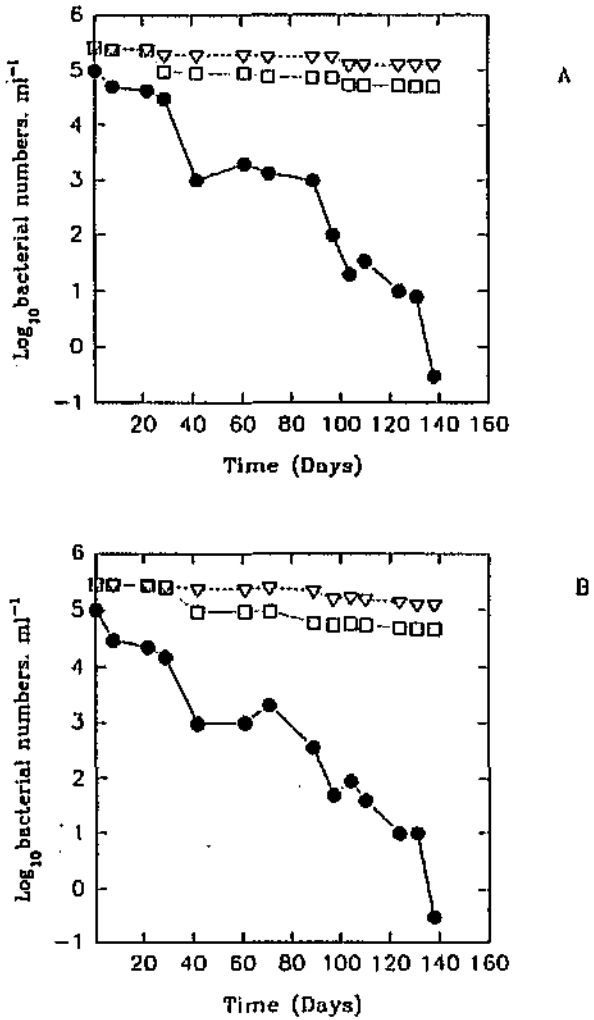

Fig. 3.9 DPC (•), AODC (∇) and DVC ( ) of *S. typhi* from river water microcosms at 4°C (A) stationary and (B) shaking.

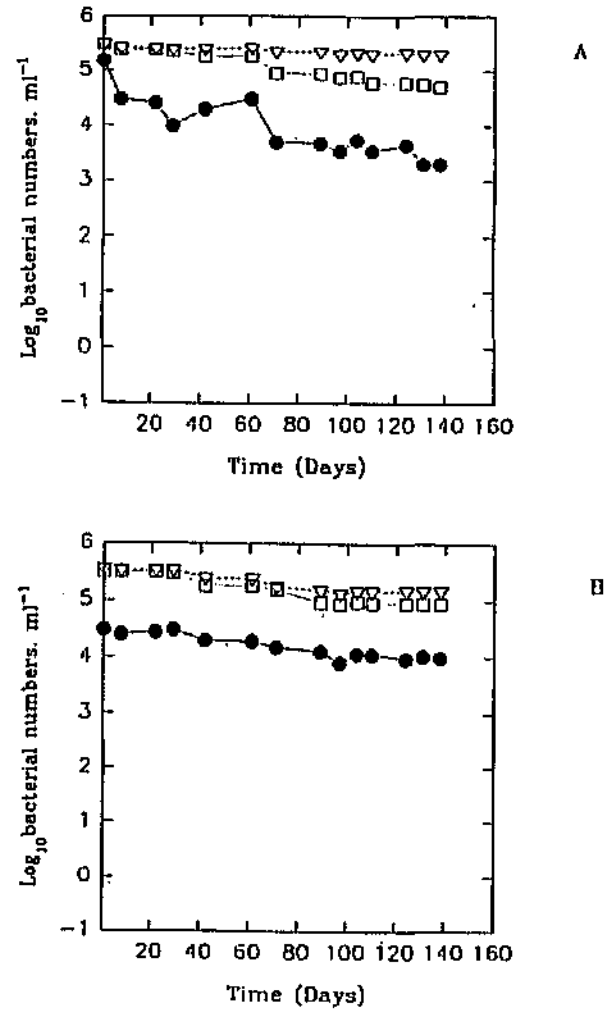

Fig. 3.10 DPC (•), AODC (∇) and DVC ( ) of *S. flexneri* from river water microcosms at 4°C (A) stationary and (B) shaking.

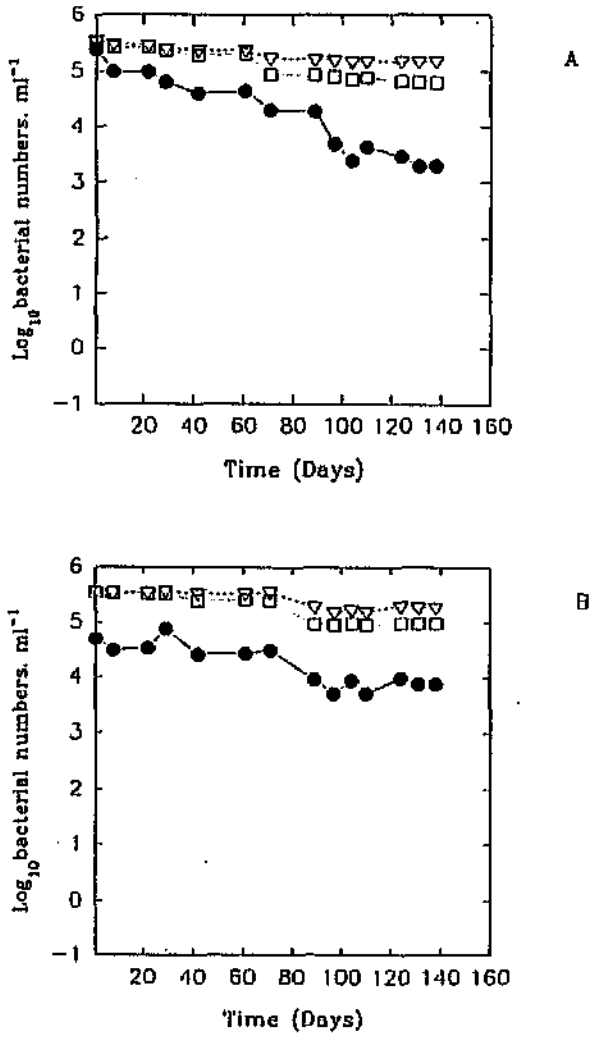

Fig. 3.11 DPC (•), AODC (▽) and DVC ( ) of *S. sonnei* from river water microcosms at 4°C (A) stationary and (B) shaking.

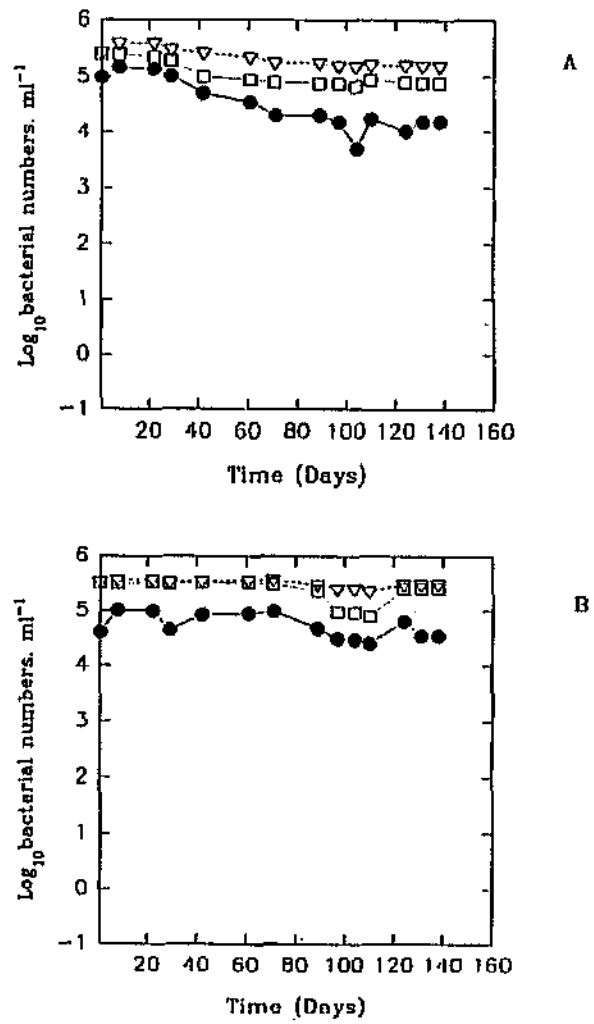

Fig. 3.12 DPC (•), AODC (▽) and DVC ( ) of *E. coli* from river water microcosms at 4°C (A) stationary and (B) shaking.

S. typhi entered the non-culturable state after 83 days in the stationary microcosm (Fig. 3. 14A) compared to 89 days in the shaking microcosm (Fig. 3.14B). There was a steady decrease in culturability for the stationary river water microcosm (Fig. 3.14A). Also, there was a steady decrease in culturability for the shaking microcosm except from day 30 to day 54 where the DPC remained virtually constant at 3.00 log (Fig. 3.14B). DPC differed significantly from the AODC and DVC for both microcosms. However, there was no significant difference between the DPC for both microcosms.

S. flexneri exhibited the non-culturable state in 138 days in the stationary river water microcosm (Fig. 3.15A) compared to 89 days for the shaking microcosm (Fig. 3.15B). Thus, shaking decreased the culturability of *S. flexneri* at a rapid rate in the river water microcosm (Fig. 3.15B). The DPC differed significantly from the AODC and DVC for both microcosms. However, the DPC did not differ significantly when the two microcosms were compared.

S. sonnei entered the non-culturable state in 83 days and 145 days for the stationary (Fig. 3. 16A) and shaking (Fig. 3. 16B) river water microcosms, respectively. *S. sonnei* attained the non-culturable state faster in the stationary microcosm than the shaking microcosm.

DPC was significantly different from the AODC and the DVC for both microcosms. However, the DPC does not differ significantly when the two microcosms were compared. Also, the AODC differed significantly from the DVC for both the microcosms. However, there was no significant difference between the DVC for the two microcosms.

E. coli accomplished the non-culturable state in 124 days and 145 days for the stationary (Fig. 3.17A) and shaking (Fig. 3.17B) river water microcosms, respectively. There was a steady decrease in culturability for both microcosms except for a slight increase in culturability from 4.74 log to 5.15 log from day 33 to day 42 for the shaking river water microcosm. There was a significant difference between the DPC and the AODC and DVC for both microcosms. However, there were no significant differences between the DPC for both microcosms. Also, the AODC and DVC for the shaking river water microcosms differed significantly . There was a significant difference between the AODC for both temperatures, however the DVC for both microcosms did not differ significantly .

Thus, it could be concluded that the culturable state of *S. typhimurium* was persistent at 4°C and 25°C. The stationary river water microcosms at 25°C facilitated entry of *S. typhi*, *S. flexneri* and *E. coli* into the non-culturable state. However, the shaking river water microcosm induced non-culturability faster for the *S. flexneri* culture. Table 3.3 summarizes the time period required to induce the non-culturable state at both temperatures.

Table: 3.3 Time taken to reach the VBNC state in seawater, pond water and river water laboratory microcosms

		Time (Days)			
		Pond water	Sea water	River water	
	Temperature	Stationary	Stationary	Stationary	Shaking
S. typhimurium	4°C	>140	105	>145	>145
	25°C	>140	104	>145	>145
S. typhi	4°C	>140	105	138	138
	25°C	>140	111	83	89
S. flexneri	4°C	>140	105	>145	>145
	25°C	>140	67	138	89
S. sonnei	4°C	>140	105	>145	>145
	25°C	>140	110	83	145
E. coli	4°C	>140	105	>145	>145
	25°C	>140	90	124	145

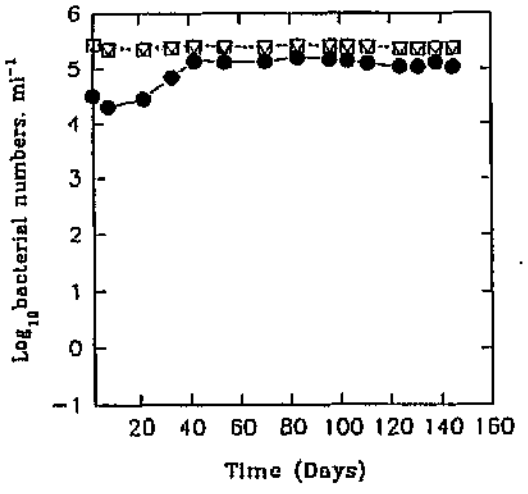

Fig. 3.13 DPC (•), AODC (∇) and DVC ( ) of *S. typhimurium* from river water microcosms at 25°C (A) stationary and (B) shaking.

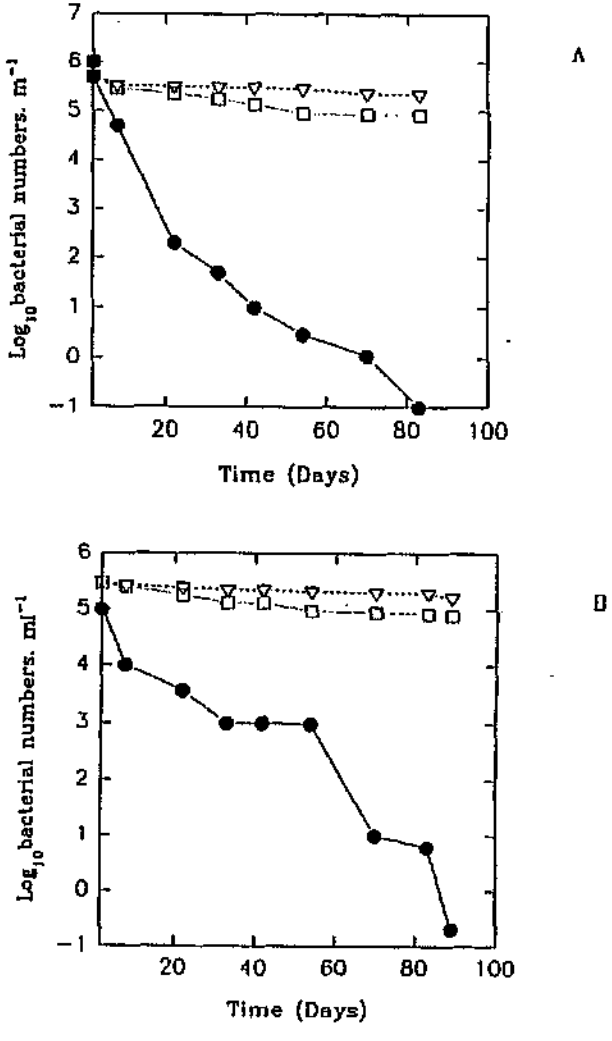

Fig. 3.14 DPC (•), AODC (∇) and DVC ( ) of *S. typhi* from river water microcosms at 25°C (A) stationary and (B) shaking.

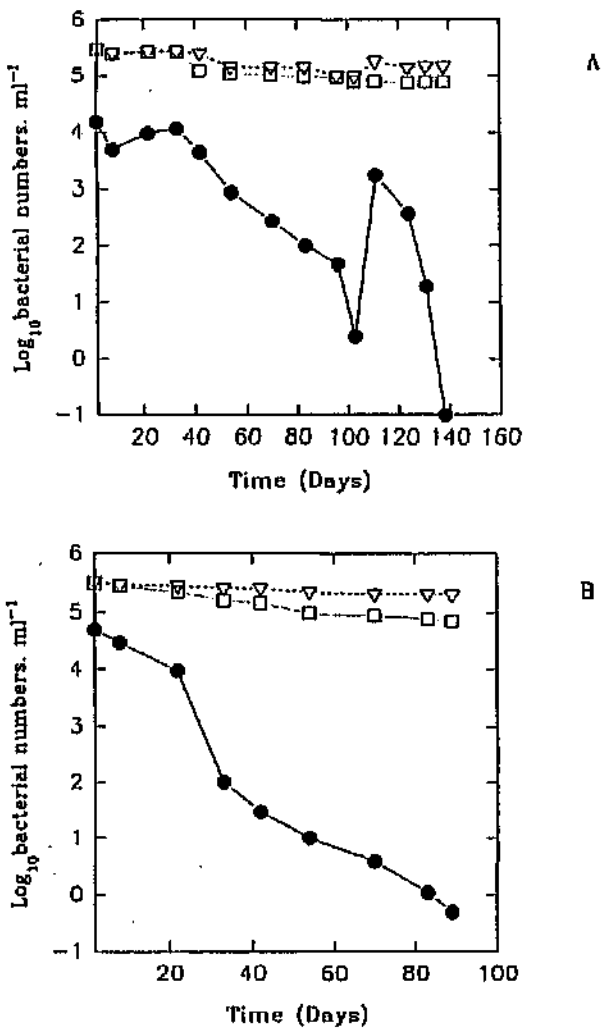

Fig. 3.15 DPC (•), AODC (▽) and DVC ( ) of *S. flexneri* from river water microcosms at 25°C (A) stationary and (B) shaking.

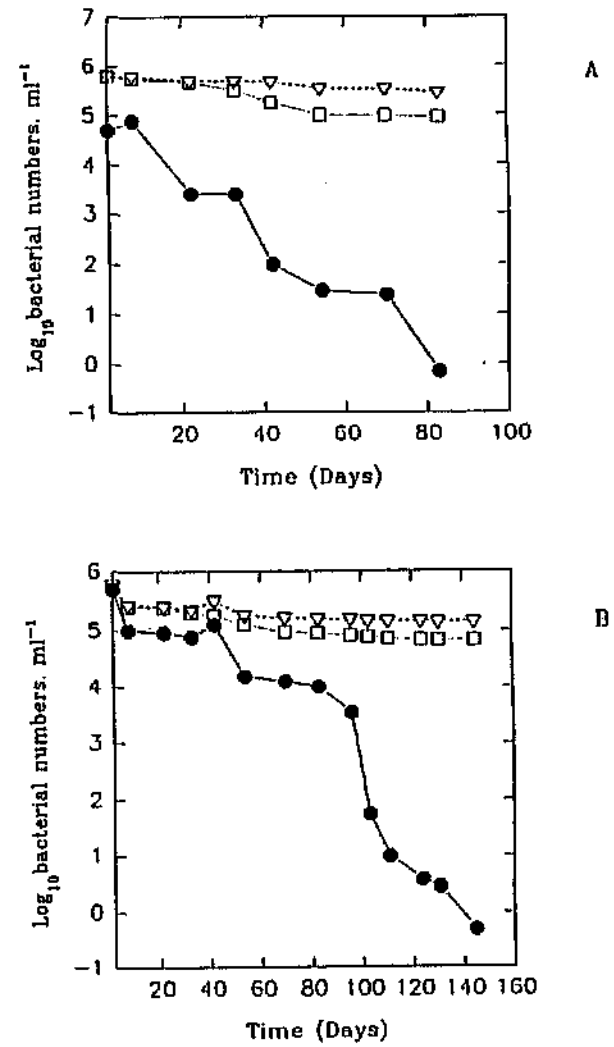

Fig. 3.16 DPC (•), AODC (▽) and DVC ( ) of *S. sonnei* from river water microcosms at 25°C (A) stationary and (B) shaking.

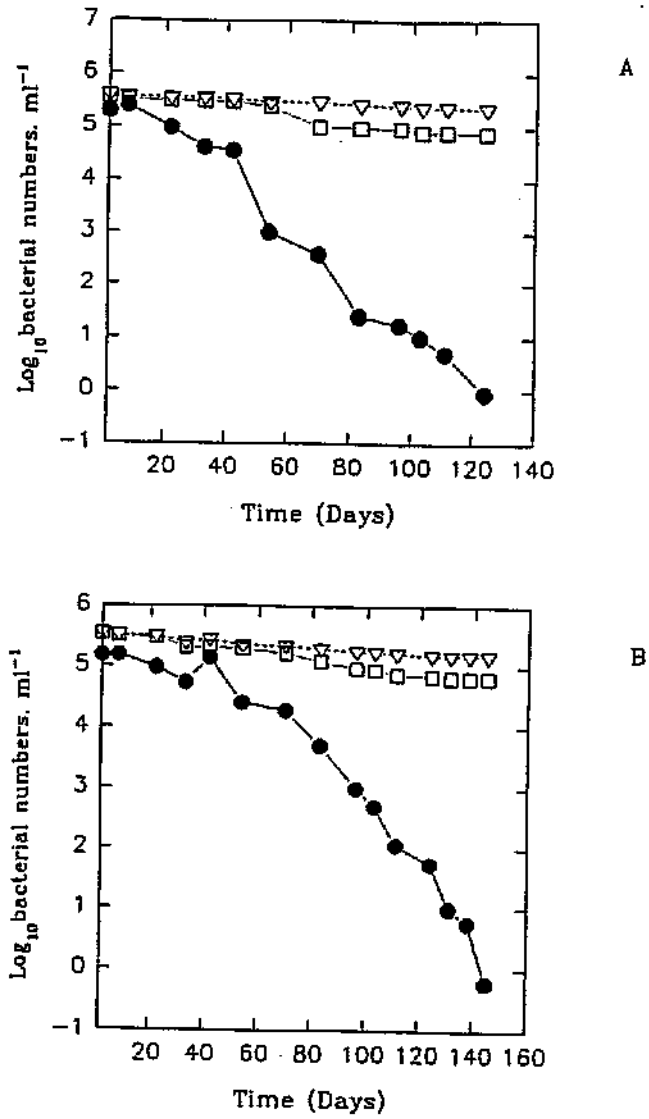

Fig. 3.17 DPC (•), AODC (▽) and DVC ( ) of *E. coli* from river water microcosms at 25°C (A) stationary (B) shaking.

3.4 Discussion

Chemical analyses of river and seawater allowed one to ascertain the quality of the water used for the microcosms. There was no significant difference between untreated and filtered autoclaved river water analyses. The exception was in the total phosphate content of 5490 μg. l^{-1} in autoclaved river water compared to 1541 μg. l^{-1} in untreated river water. According to Dr P.L. Kempster, (Institute for Water Quality Studies-personal communication) the results obtained were fairly typical of river water with moderate amounts of nutrients. Nitrate (4.63 mg.l^{-1}) was lower than the recommended limit in drinking water, *i.e.*, 26 mg.l^{-1} as NO_3 or 6 mg.l^{-1} as N. The total Kjeldahl nitrogen (KN) values confirmed the presence of low levels of organic matter . Spring waters have a KN value of less than 0.1 mg.l^{-1}.

Total phosphate (1541 μg.l^{-1}) was relatively high. In unpolluted waters the phosphate content is normally less than 100 μg.l^{-1} and the nitrate less than 0.1 mg.l^{-1}. The high phosphate content would strongly stimulate the growth of green algal plants in the water. Harder and Dijkhuizen (1983) studied the growth of a marine strain of *Rhodotorula rubra* under phosphate limitation. *R. rubra* showed a high substrate affinity by forming a phosphate transport system.

The chemical oxygen demand (COD) of 25.8 mg.l^{-1} and 27.8 mg.l^{-1} for untreated and autoclaved river water, respectively showed that moderate amounts of organic matter were present. In unpolluted water COD is less than 5 mg.l^{-1} (Dr P.L. Kempster, Institute for Water Quality Studies-personal communication).

The results obtained for seawater were typical. However, the COD values were meaningless as COD cannot be determined in seawater because of the high interference from the chloride salts in seawater. Nitrate (0.05 mg.l^{-1}) and nitrite (0.05 mg.l^{-1}) content were typical for seawater which has a range of 0.001-0.7 mg.l^{-1} for nitrate and 0 mg.l^{-1} for nitrite. Nitrogen as ammonia was 0.05 mg.l^{-1} and 0.03 mg.l^{-1} for untreated and autoclaved seawater, respectively, which were within the reference range of 0.005-0.05 mg.l^{-1}. There was a higher total phosphate content in autoclaved seawater (4.3 μg.l^{-1}) than untreated seawater (26 μg.l^{-1}), however, both are within the reference range of 1-100μg. l^{-1}.

The reference range for organic carbon is 1.2 -3.0 $mg.l^{-1}$ for seawater. The organic carbon content of the untreated and autoclaved seawater were 3.05 $mg.l^{-1}$ C and 2.65 $mg.l^{-1}$ C, respectively, which were within the reference range. A condition of carbon-substrate limitation is characterised by a high carbon conversion efficiency in which, most organisms, diversion of substrate carbon into extracellular products is minimized. Although rates of catabolism and anabolism may be adequately tuned to a particular condition of nutrient constraint, organisms will frequently catabolize at a higher rate any excess substrate suddenly added (Harder and Dijkhuizen, 1983). However, the organic carbon content. of seawater in the present study was within the reference range.

The KN was 0.25 $mg.l^{-1}$ and 0.60 $mg.l^{-1}$ for untreated and autoclaved seawater, respectively. The reference range is 0.03-0.2 mg. l^{-1}, thus the KN of autoclaved seawater was outside the recommended range. Autoclaving does not cause major changes in the chemical composition of the water. However, the difference between the untreated and autoclaved samples could partially be due to the inhomogeneities in the samples themselves.

The initial microcosms that were set up with pond and river water (Fig. 3.1 and Fig. 3.7) had cell concentrations of 10^7 cells. ml^{-1}. As indicated in the results all the cultures failed to reach the non-culturable state at 4°C and 25°C except for *S. flexneri* in stationary river water microcosm at 25°C. Addition of BHI broth into the microcosms in the form of unwashed cell suspensions could have provided the necessary additional nutrients required for growth and survival. The death of part of the bacterial population could have also provided additional nutrients for survival. All cultures seemed to be in a better state of preservation at 4°C in pond and river water. DPC for both (Fig. 3.1A and Fig. 3.7A) were in the region of 6.00 log and higher. Although *S. flexneri* entered the non-culturable state in 68 days in the river water microcosm at 25°C, the same culture had a very high DPC of 6.00 log at 68 days for pond water. Thus the theory that different sources of water could influence bacterial cultures differently (Tempest and Neijssel, 1978) holds true.

The pond and river water influenced the bacterial cultures differently at both 4°C and 25°C. *S. typhimurium* had the highest culturability rate, i.e., the highest CFU. ml^{-1} in pond water at 4°C followed by *S. typhi, S. flexneri* and *S. sonnei. E. coli* had reached the lowest CFU. ml^{-1} after approximately 140 days (Fig. 3.1A). In the river water microcosm at 4°C, *S. typhimurium* also had the highest CFU. ml^{-1} followed by *S. sonnei, E. coli* and *S. flexneri,* while *S. typhi* had the

lowest CFU. ml^{-1} (Fig. 3.7A). Again it had been demonstrated that different water sources and temperatures influence bacterial cultures differently. A similar phenomenon was observed for the 25°C microcosm for both water sources with *S. flexneri* and *E. coli* having the highest CFU. ml^{-1} in pond water at 25°C compared to *S. typhimurium* in the river water microcosm. However, *S. typhi* had the lowest CFU. ml^{-1} in both water samples at 25°C (Fig. 3.1B, Fig. 3.7B).

Non-culturability was also investigated using stationary seawater microcosms at 4°C and 25°C. The initial bacterial inoculum was decreased 10 fold to 10^6 cells. ml^{-1} and washed with sterile seawater before being introduced into the microcosms. It was speculated that the nutrient-free cells and a lower cell concentration would aid in attaining the non-culturable state in a shorter period of time.

An attempt was also made to investigate stationary and shaking microcosms using river water samples. The initial cell concentration was decreased further to 10^5 cells. ml^{-1}.

The VBNC state was induced over a much longer period in the present study than previously reported (Colwell *et al.*, 1985, Islam *et al.*, 1993, Nilsson *et al.*, 1991, Xu *et al.*, 1982). *S. typhimurium* and *S. typhi* exhibited the VBNC state in 105 days in the seawater microcosms at 4°C. *S. typhimurium* and *S. typhi* attained the VBNC in 105 days and 110 days in the seawater microcosms at 25°C. *S. typhimurium* in the stationary and shaking river water microcosms at 4°C did not reach the VBNC state, as DPC were 3.62 log and 3.00 log, respectively, after 138 days (Fig. 3.8). *S. typhimurium* was the only culture that did not reach the VBNC at 25°C (Fig. 3.13). *S. typhi*, on the other hand was the only culture that did reach the VBNC state at 4°C in the river water microcosm. At 25°C *S. typhi* reached the VBNC state in 83 days and 89 days for the stationary and shaking river water microcosms, respectively. In comparison, the *S. typhi* used in this study had taken a longer time to reach the VBNC state in the river water microcosms at 4°C compared to the seawater at the same temperature. However, *S. typhi* exhibited the VBNC state much sooner at 25°C in the river water microcosm compared to the seawater. Thus, each water source and temperature had a different effect on the *Salmonella spp.* used in this study.

Roszak *et al.* (1984) reported that *S. enteritidis* entered the VBNC state in 3 days on veal infusion agar (VIA) and 2 days on xylose lysine decarboxylase agar (XLD). The water source

was the Potomac river and the initial cell concentration was 10^5 cells. ml^{-1}. The agar plates used also were of different nutritional content than the agar plates used in the present study. The different water source, culture and agar plates could account for the different time periods in which the *Salmonella spp.* entered the VBNC state. Despite the different parameters used by Roszak *et al.* (1984), the *Salmonella spp.* used in this study required a very lengthy incubation period in the microcosms before the VBNC state was exhibited.

Roszak *et al.* (1984) also investigated resuscitation of *S. enteritidis.* Four days after the VBNC state was accomplished, i.e., when plate counts were zero, supplementation of the starved cultures with various strengths of BHI broth produced culturable CFUs. ml^{-1}. After 21 days of incubation, however, resuscitation failed to produce culturable cells. Therefore, there seems to be a linear sequence of responses by bacterial cells to conditions of nutrient depletion at the end of which an inactive (dormant-like) but viable cell results. The first response of non-culturability could be reversed by resuscitation of the cells with rich broth medium. However, protracted nutrient limitation requires more than a simple addition of nutrients, since the DVC was stable for 60 days (Roszak *et al.*, 1984).

In the seawater microcosm at 4°C *S. flexneri* and *S. sonnei* attained the VBNC state at 105 days and in 67 days and 110 days, respectively, at 25°C. *S. flexneri* and *S. sonnei* failed to reach the VBNC state in both the stationary and shaking river water microcosms at 4°C, where DPC were 3.30 log and 4.00 log, respectively, after 138 days. However, both cultures did reach the VBNC state at 25°C in the river water microcosms. *S. flexneri* exhibited the VBNC state faster in the stationary seawater at 25°C taking 67 days compared to 138 days in the stationary river water microcosm at the same temperature. *S. sonnei,* however, entered the VBNC state faster in the stationary river water microcosm, taking 83 days compared to 110 days in the seawater microcosm. *S. flexneri* was the only culture that reached the non-culturable state faster in the shaking river water microcosm at 25°C taking 89 days to reach the VBNC state in the shaking microcosm compared to 138 days in the stationary microcosm. *S. sonnei* on the other hand entered the non-culturable state faster in the stationary microcosm at 25°C taking 83 days compared to 145 days for the shaking microcosm. Thus, shaking and stationary microcosms had different effects on the survival of the two *Shigella spp.* used in this study.

Colwell *et al.* (1985) reported that *S. sonnei* exhibited the VBNC state in 21 days on tryptic soy agar (TSA) and 14 days on MacConkey agar. These researchers set up microcosms in Chesapeake Bay water *(salinity-15%)* with an initial concentration of 10^6 cells. ml^{-1}. AODC remained at the initial concentration (6.50 log) throughout the experiment. However, in the present study the AODC, DVC and DPC concurred from day 1 through to day 68 for the seawater microcosm at 4°C. At 25°C the AODC and DVC decreased initially but levelled off in the region of 5.00 log.

Colwell *et al.* (1985) also investigated the survival *S. flexneri* in Chesapeake Bay water. This organism entered the non-culturable state three times faster than *S. sonnei.* In the present study, *S. flexneri* and *S. sonnei* accomplished the VBNC state in 105 days in the seawater microcosm at 4°C. *S. flexneri* entered the VBNC state in 67 days at 25°C compared to 110 days for *S. sonnei.* Thus, *S. flexneri* did reach the VBNC state faster than *S. sonnei* for the seawater microcosms, however, the time taken was not three times faster as reported by Colwell *et al.* (1985). However, with the river water microcosms at 25°C, a different result was obtained (Table 3.3). *S. flexneri* reached the VBNC state faster than *S. sonnei* in the shaking microcosm taking 89 days compared to 145 days for *S. sonnei.* On the other hand, *S. sonnei* attained the VBNC state faster (83 days) than *S. flexneri* (138 days) for the stationary microcosm at 25°C (Table 3.3).

Islam *et al.* (1993) investigated the VBNC state of *S. dysenteriae* in pond water (Institute of Public Health, Mohakhali, Dhaka, Bangladesh), lake water (Dhanmandi Lake, Dhaka), Tongi river and drain water samples (Paramedical Institute, Dhaka) .These researchers found that *S. dysenteriae* exhibited the VBNC state in 15 days for river water, 17 days for pond and lake water and 21 days for drain water when sampled on MacConkey agar plates. The results obtained by Islam *et al.* (1993) were comparable to that of Colwell *et al.* (1985) but not to the present study with regard to time taken to induce the VBNC state. Even with the different water sources and different *Shigella spp.* , there seemed to be a similar pattern in which the VBNC state was entered in both cases. The persistence of *S. dysenteriae* cells in laboratory microcosms had been observed by fluorescence microscopy for up to six weeks, despite the fact that at six weeks the cells cannot be cultured on solid medium (Islam *et al.*, 1993). Direct viable counting showed that these cells retained viability at six weeks.

According to Islam *et al.* (1993) that there was circumstantial evidence suggesting that *S. dysenteriae* survived for extended periods in natural aquatic environments after deposition by humans attributable to indiscriminate defaecation in countries like Bangladesh, because water sources have been implicated in the outbreaks of shigellosis. Thus, a parallel could be drawn to the present study where seawater was obtained from the South beach surfing area, located in close proximity to a waste disposal pipeline. Thus, the water source could have been rich in nutrients (BOD: < 1.00 $mg.l^{-1}$ oxygen, total phosphate: 43µg. l^{-1} and total organic carbon: 3.65 $mg.l^{-1}$) to support the growth of all the bacterial cultures used in the present study, contributing to the extended period in which the bacteria were culturable. Similarly, informal settlements along the Umgeni river banks could account for the enriched nutrients (BOD: 3.10 $mg.l^{-1}$ oxygen, COD: 27.80 $mg.l^{-1}$ and total phosphate 5490 $\mu g.l^{-1}$) present in river water, thus contributing to the long-term survival of all the organisms in the river water.

E. coli entered the VBNC state in 105 days at 4°C and in 90 days at 25°C in the seawater microcosms. However, in the stationary and shaking river water microcosms at 25°C, *E. coli* took a longer period of time to reach the VBNC state. The culture accomplished the VBNC state after 124 days in the stationary microcosm and after 145 days in the shaking microcosm. Thus, it could be speculated that the river water microcosms were rich in nutrients, and therefore, supported growth for a longer period of time. General comparison of the chemical analyses of autoclaved river water (Table 3.1) and seawater (Table 3.2) indicated that river water was nutritionally richer than seawater. In comparison, *E. coli* in the stationary and shaking river water microcosms at 4°C did not reach the VBNC state. A possible reason for this phenomenon is that the possible enriched water and the low temperature served to keep the *E. coli* cells in a well preserved culturable state. Although the culturability of *E. coli* decreased at different rates for seawater and river water microcosms at 25°C, AODC and DVC of these microcosm cultures at the end of the experiment differed negligibly. AODC for the stationary and shaking river water microcosms at 25°C was 5.36 log and 5.18 log, respectively, compared to 5.52 log for the seawater microcosm at 25°C. The DVC for the stationary and shaking river water microcosms (25°C) were 4.89 log and 4.82 log, respectively, compared to 5.09 log for the seawater microcosm at 25°C.

Colwell *et al.* (1985) investigated the VBNC state of *E. coli* H10407 in Chesapeake Bay water in laboratory microcosms with an initial concentration of 10^7 cells. ml^{-1}. The AODC remained

at the initial concentration of 7.00 log throughout the 19 day period. Direct viable counts declined only 0.50 log. Plate counts on Tryptic soya agar (TSA) and Eosin methylene blue agar (EMB) dropped from 10^6 cells. ml^{-1} on day 1 to 10^4 cell. ml^{-1} on day 7 and then to 10^3 cells. ml^{-1} on day 14 but did not go completely to the VBNC state. In comparison *E. coli* (present study) in the seawater laboratory microcosm (Table 3.3) and river water laboratory microcosm (Table 3.3) at 25°C entered the VBNC state after a prolonged period of incubation.

In studies carried out using membrane chambers with the same *E. coli* strain H10407 a different result was obtained (Colwell *et al.*, 1985). *In situ* experiments were set up in the semi-tropical waters of Bimini, Bahamas (salinity 38 %, mean temperature, 25°C) with an initial concentration of 10^6 cells. ml^{-1}. The fluorescent antibody (FA) and FA-DVC remained constant at 10^5 cells. ml^{-1}. Plate counts on BHI agar plates decreased from 10^4 cells. ml^{-1} at zero hour to less than 1 CFU.ml^{-1} at 13 h and remained as such for the term of the experiment. *E. coli* attained the VBNC state in the membrane chambers over a very short period of time. The reason given by Colwell *et al.* (1985) was that the conditions were much harsher than the laboratory microcosms. Thus, one could suggest that the same organism can behave differently in different bodies of water, i.e., there is inherent variability of natural aquatic systems.

Linder and Oliver (1989) also reported the long-term survival of *E. coli* in laboratory microcosms. These experiments were set up with artificial seawater at 25°C, unlike bay water used by Colwell *et al.* (1985). Linder and Oliver (1989) found *E. coli* to be culturable on BHI and EMB agar plates after 32 days compared to 19 days on TSA and EMB by Colwell *et al.* (1985) and 138 days on BHI agar plates in river water at 4°C for the present study. In all these cases the DVC and AODC were higher that the DPC. Linder and Oliver (1989) commented that the survival of a non-marine organism, *E. coli*, in seawater for a long time (32 days), surprising. *E. coli* in the present study in seawater, however, did reach the VBNC state but required a lengthy incubation period. A study by Dave and Penrose (1978) concluded that the rate of injured coliform cells in seawater was very high but survival was high as well. They speculated that while seawater injures coliforms, it actually protects debilitated cells from death.

Xu *et al.* (1982) also investigated the VBNC state of *E. coli* in Chesapeake bay water (5% and 25% salinity). At both salinities, temperatures of 10°C and 25°C were investigated. Culturability decreased in both cases, however, the total population did not reach the non-culturable state. After 24 h at 10°C in 5% salinity, the total population (AODC) was 5.19 log ± 0.11, the active population (DVC) was 5.06 log ± 0.12 and the culturable population was 4.64 log ± 0.18. After 24 h at 25°C in 5 %, AODC was 5.57 log ± 0.05, DVC was 5.52 log ± 0.06 and the culturable population was 5.04 log ± 0.08. Similar results were obtained for the 25 % salinity microcosms. It was suggested that the portion of bacteria that cannot be cultured have entered the VBNC state.

Prabu and Mahadevan (1992) investigated the survival of *E. coli* in the coastal waters off Madras, India. These researchers found that the death of *E. coli* in unfiltered seawater was three fold higher than in the filtered seawater and the death rate was higher when incubated in the light than in the dark. Thus, this could account for the extended period that *E. coli* survived in seawater in this study. CFUs of *E. coli* declined significantly with time when unfiltered seawater was compared to filtered seawater. CFUs declined from 10^7 cells. ml^{-1} to 10^3 cells. ml^{-1} in 10 days for unfiltered water, but remained at 10^6 cells. ml^{-1} for filtered seawater after 10 days. The decline in the survival was less in the unfiltered microcosm compared with other types. Filtered seawater and incubation of seawater microcosms in the dark (present study) could account for the extended period of survival of *E. coli* in seawater used.

Survival of bacteria in aquatic environments is known to be affected by a variety of parameters, including salt concentration, presence of heavy metals, nutrients levels and temperature. The viability of such bacteria has routinely been estimated by plating techniques using solid media and enumeration of the resulting colony forming units. It is evident that such plating methods may be misleading and underestimates the number of viable cells in marine and non-marine habitats (Linder and Oliver, 1989). The findings of this study support this sentiment.

Summary and conclusion

Conventional AODC, DVC and DPC on BHI agar plates served adequately for the enumeration of total bacterial population, viable population and culturable population respectively. However temperature and nutrient levels also effect the survival of bacteria in aquatic environments.

S. typhimurium had the highest CFU.ml^{-1} in pond water at 4°C, followed by *S. typhi*, *S. flexneri*, and *S. sonnei*, while *E. coli* reached the lowest CFU.ml^{-1} after approximately 140 days. In river water microcosm at 4°C, *S. typhimurium* also had the highest CFU.ml^{-1} followed by *S. sonnei*, *E. coli* and *S. flexneri*, while *S. typhi* had the lowest CFU.ml^{-1}. At 25°C *S. flexneri* and *E. coli* had the highest CFU.ml^{-1} in pond water, while *S. typhi* had the lowest CFU.ml^{-1}.

In seawater microcosms at 25°C *S. typhi* and *S. sonnei* attained VBNC state in 110 days. *S. typhimurium*, *E. coli* and *S. flexneri* reached the VBNC state in 105, 90 and 67 days, respectively. In seawater microcosms at 4°C all bacterial cultures exhibited VBNC state in 105 days. *S. typhi* was the only bacterium that reached VBNC state in stationary and shaking river water microcosms at 4°C. At 25°C *S. flexneri* and *S. typhi* reached VBNC state in shaking river water microcosms in 89 days. At 25°C *S. sonnei* and *E. coli* attained VBNC state in shaking river water microcosms in 145 days. At 25°C *S. typhi* and *S. sonnei* achieved VBNC state in stationary river water microcosms in 83 days. At 25°C *S. flexneri* and *E. coli* reached VBNC state in 138 and 124 days respectively. At 25°C *S. flexneri* was the only culture to reach VBNC state faster in shaking river water microcosms than stationary river water microcosms. Thus, different water sources and temperatures had varying effects on when VBNC state was attained. The prolonged culturability of the bacterial cultures could also be attributed to the high nutrient content of the water sources used.

CHAPTER FOUR

DETECTION OF VIABLE-BUT-NON-CULTURABLE BACTERIA USING THE POLYMERASE CHAIN REACTION AND DNA HYBRIDIZATION PROBES

4.1 Introduction

Saiki and colleagues (1985) introduced the polymerase chain reaction (PCR) for gene amplification which has the qualities of a great scientific discovery: technical simplicity, yet having the remarkable power and versatility to cause rapid transformations in many fields (Giovannoni, 1991). PCR permits the *in vitro* replication of defined sequences of DNA whereby gene segments can be amplified. The most obvious application of this technique is to enhance gene probe detection of specific gene sequences. PCR exponentially amplifies a target sequence thus significantly enhancing the probability of detecting rare sequences in heterologous mixtures of DNA (Steffan and Atlas, 1991). PCR is also rapidly gaining favour as a technique for the detection of bacterial pathogens from the environment.

The application of nucleic acid probes to bacteria found in environmental samples shows great promise as they have the ability to identify specific nucleic acid sequences and determine the relatedness of similar sequences. Thus, they can be more specific than fluorescent, monoclonal antibodies or fluorochrome stains (Hazen and Jimenez, 1988; Richardson *et al.*, 1991).

The basic principles of gene probe technology include gene probe construction, labelling, hybridization and detection. The stability of the duplex formed during hybridization depends on the complementarity of the two strands and their ability to physically pair by means of hydrogen-bonds between the nucleotides. The stability of a duplex is measured in terms of its melting temperature. Each strand of a duplex will dissociate or denature into individual strands when exposed to temperatures greater than the melting temperature of the duplex. In practical applications of this technology one of the two complementary strands represents the probe and the other strand represents the target (Richardson *et al.*, 1991).

In this study the molecular detection of the VBNC bacteria involved a combination of PCR and gene probe hybridization. A serial dilution of DNA isolated from pure cultures and

microcosm cultures was used as template DNA. DNA from the pure cultures was diluted to the equivalent concentration of DNA isolated from the microcosms and also served as a positive control for amplification of microcosm DNA. Thus, an entire range of DNA dilution series was tested for amplification by PCR and hybridization. PCR with equivalent concentrations of DNA from pure culture and microcosm cultures was attempted to determine the limit of detection in each case. Also, hybridization studies were carried out to assess if the probes increased the level of detection, i.e., a positive hybridization signal would be observed for a particular concentration of DNA. However, a PCR product would not be visible on the gel.

4.2 Materials and methods

In the present study primers specific to the *uid* R region of *E. coli* were used to amplify the DNA of *Shigella spp.* and *E. coli* by PCR as the *uid* R gene is present in both species. Primers specific to the origin of replication of *S. typhimurium* were used to amplify the DNA of the *Salmonella spp.* Hybridization studies were carried out using the Enhanced Chemiluminescent (ECL) Kit^{R} (Amersham). The amplified PCR-products of *S. typhimurium* and *E. coli* served as probes for hybridization.

4.2.1 PCR of pure cultures and microcosm cultures

4.2.1.1 Isolation of genomic DNA

Total DNA was isolated using three methods, viz., boiling (Dwarka, 1995), freeze-thaw (Bej *et al.*, 1991) and lysozyme and proteinase K treatment (Islam *et al.*, 1993). Salts from seawater microcosm water were removed by dialysis against 3 x 2 h changes of sterile distilled water at 4°C with stirring. Template DNA for PCR was quantified using the Genequant^{R} (Pharmarcia).

4.2.1.2 PCR mixture and conditions

The modified methods of Bej *et al.* (1991c) and Widjojatmodjo *et al.* (1991) were used. Amplification primers (shown below) specific to the origin of replication were used for *S. typhimurium* and *S. typhi.*

1: [5'-TTATTAGGATCGCGCCAGGC-3'] ; 2: [5'-AAAGAATAACCGTTGTTCAC-3']
Amplification primers specific to the *uid* R regulatory region (shown below) were used for *S. flexneri*, *S. sonnei* and *E. coli* URL-301 : [5' -TGTT ACGTCCTGT AGAAAGCCC- 3 '] ; URR-432: [5'-AAAACTGCCTGGCACAGCAATT-3'] .

The following were added to a 0.5 ml Eppendorf tube on ice: x µl sterile deionised water, y µl template DNA (DNA dilution series of pure cultures and microcosm cultures or DNA from microcosm cultures filtered onto fluoropore filters), 1 U *Taq* polymerase, 5 µl *Taq* buffer, 1µl (50 ng) of each primer, 1 µl (200 µM) dNTP's (total vol.= 50 µl). Contents were vortexed and centrifuged for 5 s at 14 000 x *g* in a microfuge. The mixture was overlaid with 30 µl of mineral oil and placed in the heating block of the Hybaid^{R} thermal cycler. The thermal cycling (35 cycles) conditions for *S. typhimurium* and *S. typhi*: were as follows : 94°C for 1 min denaturation of template DNA, 50°C for 1 min primer annealing, and 72°C for 1 min primer extension . The same conditions were used for *S. flexneri*. *S. sonnei* and *E. coli*, except for an increase in the annealing temperature to 60°C.

Eppendorf tubes were placed on ice once the 35 cycles were complete. Ten µl (pure cultures) or 25 µl (microcosm cultures) of the reaction mixture was separated on a 1.5 % agarose gel in 0.5 x TBE at 80 V for 2.5 h. The gels were stained with ethidium bromide and viewed using a transilluminator (UVP, Inc.). The gels were photographed on Ilford FP4 Plus black and white film using a Minolta camera fitted with a UV filter and a Vivitar number 25 (red) filter.

4.2.2 Hybridization studies of pure cultures and microcosm cultures

Hybridization and signal detection of pure cultures and microcosm cultures were carried out using the Enhanced Chemiluminescent Kit^{R} (ECL^{R} -Amersham).

4.2.2.1 Isolation of probes

Probes were purified using the Geneclean^{R} II Kit, centrifugal membrane filtration and the Qiagen^{R} Kit (Qiagen Inc.).

4.2.2.2 Southern blot

Two pieces of Whatman 4MM filter paper were cut to the size of the gel and the nylon membrane was cut 4-5 mm bigger than the gel. The filter papers and nylon membrane were pre-wet in deionised water for 15-20 min. Two pieces of pre-wet filter papers were placed on the support screen of the vacuum blotting unit. The gel was carefully positioned over the nylon membrane and a seal formed around the gel by using parafilm. A vacuum of 15 cm of Hg was used during the entire transfer process. Transfer solution was regularly poured to about twice the depth of the gel to prevent the gel from drying out and left on for 60 min.

At the end of the transfer, the vacuum pump was turned off and the gel and filter paper removed. The position of the wells were marked onto their corresponding positions on the nylon membrane which was then air dried under the laminar flow. DNA was crosslinked onto the membrane by exposure to UV light for 3 min. The nylon membrane was sealed in a plastic bag and stored at 4°C.

4.2.2.3 Labelling of probes, hybridization and detection

Labelling of probes, hybridization and detection were performed using the ECL^{R} Kit according to the manufacturers instructions.

4.3 Results

4.3.1 Amplification and detection of pure cultures by PCR

The DNA of the five cultures used to set up the microcosms were amplified by the PCR to verify the expected amplification products for their respective primers. The supernatant of boiled cells of overnight cultures served as template DNA. *S. typhimurium* and *S. typhi* yielded a PCR product of 163 bp with the primer sequences specific to the origin of replication of *S. typhimurium. S. flexneri, S. sonnei* (Fig. 4.1) and *E. coli* yielded a 154 bp PCR product using primer sequences specific to the *uid* R gene of *E. coli.* Also, specificity of the probe was tested where the amplified product of *S. typhimurium* was used as a probe against itself and the amplified product of *E. coli.* Similarly, the amplified product of *E. coli*

was used as a probe against itself and the PCR product of *S. typhimurium*. The *S. typhimurium* probe hybridized to the PCR-amplified product of itself and not to the PCR-amplified product of *E. coli*, thus confirming that the probe was specific for the detection of *S. typhimurium*. Similarly, the *E. coli* probe hybridized to the PCR-amplified product of itself and not to the amplified product of *S. typhimurium*, thus confirming that the probe was specific for the detection of *E. coli*. Specificity of the PCR-amplified products of *S. typhimurium* and *E. coli* as probes were only tested, as these served as probes for hybridization studies.

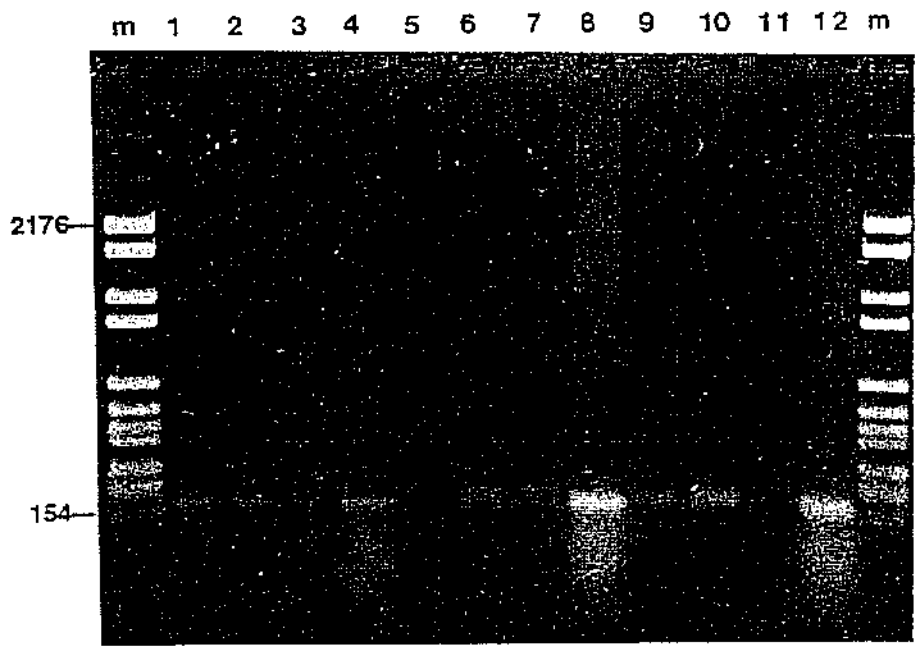

Fig. 4.1 **Agarose gel of amplified PCR-products of *S. flexneri* (lanes 9, 10, 11: 0.32, 0.20, 0.10 ng.µl^{-1}, respectively of template DNA), *S. sonnei* (lanes 5, 6, 7: 0.96, 0.60, 0.29 ng.µl^{-1}, respectively of template DNA) and *E. coli* (lanes 1, 2, 3: 3.28, 2.05, 0.98 ng.µl^{-1}, respectively of template DNA) from seawater microcosm at 4°C; lanes 4, 8, 12: pure culture of *E. coli*, *S. sonnei* and *S. flexneri*, respectively; m: Boehringer molecular weight marker VI.**

4.3.2 Amplification and detection of DNA isolated from seawater microcosms

No PCR product was obtained with DNA from the seawater microcosms (4°C and 25°C) by concentrating the cells on polycarbonate and fluoropore filters and extracting DNA by freeze-thawing. The method of Islam *et al.* (1993) also did not yield a PCR product. It was established that the salt concentration of seawater was interfering with the PCR reaction. When the supernatant of cultures dialysed against sterile distilled water (3 x 2 h changes) were boiled, a positive PCR amplification was achieved. Thus, all seawater samples were dialysed to remove excess salts before DNA isolation and PCR were attempted.

Table 4.1 provides a summary of the results obtained after PCR amplification and hybridization of the 4°C and 25°C seawater microcosm cultures. Neither amplification products nor hybridization signal were obtained for the microcosm cultures of *S. typhimurium*, *S. typhi* and *E. coli*. However, positive amplifications as well as specific hybridizations were obtained for the microcosm cultures of *S. flexneri* (Fig. 4.2) and *S. sonnei*. It should be pointed out that the best results for the microcosms were obtained with *S. flexneri* where the level of detection (0.004 ng.μl^{-1}) matched that of the control cultures.

4.3.3 Amplification and detection of DNA isolated from river water microcosms

Tables 4.2 and 4.3 provide a summary of the results obtained for the river water microcosms at 4°C and 25°C, respectively. Neither amplification products nor hybridization signals were obtained for *S. typhimurium*, *S. typhi* in the river water microcosms at 4°C and *E. coli* was only detected by the PCR/hybridization method in the 25°C shaking river water microcosm.

At DNA concentrations of 0.30 ng.$\mu l^{-1}$and 0.48 ng.μl^{-1} a positive PCR product was obtained for the *S. flexneri* stationary microcosm (4°C) and a positive hybridization signal was obtained at 0.14 ng.μl^{-1}, 0.30 ng.μl^{-1} and 0.48 ng.μl^{-1}whereas in the shaking microcosm DNA concentrations of 0.20 ng.μl^{-1} and 0.32 ng.μl^{-1} produced positive PCR products and a hybridization signal was obtained at DNA concentrations of 0.10 ng.μl^{-1}, 0.20 ng.$\mu l^{-1}$and 0.32 ng.μl^{-1}.

Table 4.1: Summary of PCR amplification and DNA hybridization results obtained for stationary seawater microcosms

S. typhimurium	DNA concentrations (ng/µl)		0.07	0.22	0.44	0.89	1.85	2.96
	4°C	PCR	-	-	-	-	-	-
		Hybridization	-	-	-	-	-	-
	DNA concentrations (ng/µl)		0.07	0.22	0.44	0.89	1.85	2.96
	25°C	PCR	-	-	-	-	-	-
		Hybridization	-	-	-	-	-	-
S. typhi	DNA concentrations (ng/µl)		0.07	0.21	0.42	0.84	1.75	2.80
	4°C	PCR	-	-	-	-	-	-
		Hybridization	-	-	-	-	-	-
	DNA concentrations (ng/µl)		0.01	0.04	0.07	0.14	0.30	0.48
	25°C	PCR	-	-	-	-	-	-
		Hybridization	-	-	-	-	-	-
S. flexneri	DNA concentrations (ng/µl)		0.01	0.02	0.05	0.10	0.20	0.32
	4°C	PCR	-	+	+	+	+	+
		Hybridization	-	+	+	+	+	+
	DNA concentrations (ng/µl)		0.004	0.01	0.02	0.05	0.10	0.16
	25°C	PCR	+	+	+	+	+	+
		Hybridization	+	+	+	+	+	+
S. sonnei	DNA concentrations (ng/µl)		0.02	0.07	0.14	0.29	0.60	0.96
	4°C	PCR	-	-	+	+	-	+
		Hybridization	-	-	+	+	-	+
	DNA concentrations (ng/µl)		0.004	0.01	0.02	0.05	0.10	0.16
	25°C	PCR	-	-	-	-	+	+
		Hybridization	-	-	-	+	+	+
E. coli	DNA concentrations (ng/µl)		0.08	0.25	0.49	0.98	2.05	3.28
	4°C	PCR	-	-	-	-	-	-
		Hybridization	-	-	-	-	-	-
	DNA concentrations (ng/µl)		0.002	0.006	0.12	0.02	0.05	0.08
	25°C	PCR	-	-	-	-	-	-
		Hybridization	-	-	-	-	-	-

In the 25°C *S. flexneri* shaking microcosm a positive PCR product and hybridization signal was obtained at a DNA concentration of 0.48 ng.µl^{-1}. *S. sonnei* 4°C stationary and shaking microcosms produced positive PCR products and hybridization signals at DNA concentrations above 0.05 ng.µl^{-1}and 0.006 ng.µl^{-1}whereas for the 25°C stationary microcosm positive PCR products were obtained for concentrations of 1.25 ng.µl^{-1}and 2.00 ng.µl^{-1}and the shaking microcosm produced products at 0.43 ng.µl^{-1}, 0.90 ng.µl^{-1}and 1.44 ng.µl^{-1}DNA. Hybridization signals for the *S. sonnei* stationary and shaking microcosms were obtained at DNA concentrations above 0.30 ng.µl^{-1}and 0.22 ng.µl^{-1}, respectively.

E. coli. was only detected by the PCR/hybridization method in the 25°C shaking river water microcosm at DNA concentrations of 0.75 and 1.2 ng.μl^{-1}.

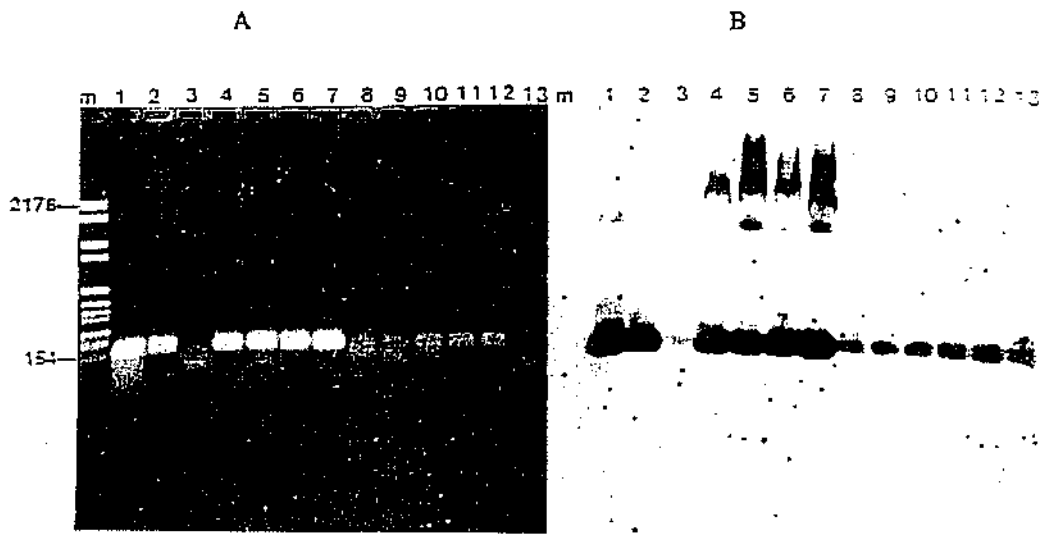

Fig. 4.2 **(A) Agarose gel electrophoresis and (B) southern hybridization of amplified PCR-products of *S. flexneri*: control DNA (lane 1); pure culture (lanes 2-7); seawater microcosm at 25°C (lanes 8-13); m: Boehringer molecular weight marker VI; template DNA concentrations were as follows: lanes 2 & 8: 0.004 ng.μl^{-1}, lanes 3 & 9: 0.01 ng.μl^{-1}, lanes 4 & 10: 0.02 ng.μl^{-1}, lanes 5 & 11: 0.05 ng.μl^{-1}, lanes 6 & 12: 0.10 ng.μl^{-1}, lanes 7 & 13: 0.16 ng.μl^{-1}.**

4.4 Discussion

The present study was successful in simulating conditions which induce the VBNC state in certain instances. Conventional methods of detecting the VBNC bacteria, i.e., use of DPC, AODC and DVC were also successful. However, conventional methods lack the sensitivity and specificity of molecular detection. Thus, an attempt was made to detect these bacteria at a molecular level using PCR and gene probe technology to increase the sensitivity and specificity of detection.

Table 4.2: Summary of PCR amplification and DNA hybridization results obtained for river water microcosms (4°C)

S. typhimurium	DNA concentrations ($ng.\mu l^{-1}$)		0.04	0.11	0.22	0.43	0.90	1.44
	Stationary	PCR	-	-	-	-	-	-
		Hybridization	-	-	-	-	-	-
	DNA concentrations ($ng.\mu l^{-1}$)		0.03	0.10	0.20	0.41	0.85	1.36
	Shaking	PCR	-	-	-	-	-	-
		Hybridization	-	-	-	-	-	-
S. typhi	DNA concentrations ($ng.\mu l^{-1}$)		0.014	0.04	0.08	0.17	0.35	0.56
	Stationary	PCR	-	-	-	-	-	-
		Hybridization	-	-	-	-	-	-
	DNA concentrations ($ng.\mu l^{-1}$)		0.30	0.08	0.156	0.31	0.65	1.04
	Shaking	PCR	-	-	-	-	-	-
		Hybridization	-	-	-	-	-	-
S. flexneri	DNA concentrations ($ng.\mu l^{-1}$)		0.01	0.04	0.07	0.14	0.30	0.48
	Stationary	PCR	-	-	-	-	+	+
		Hybridization	-	-	-	+	+	+
	DNA concentrations ($ng.\mu l^{-1}$)		0.008	0.02	0.05	0.10	0.20	0.32
	Shaking	PCR	-	-	-	-	+	+
		Hybridization	-	-	-	+	+	+
S. sonnei	DNA concentrations ($ng.\mu l^{-1}$)		0.02	0.05	0.10	0.19	0.40	0.64
	Stationary	PCR	-	+	+	+	+	+
		Hybridization	-	+	+	+	+	+
	DNA concentrations ($ng.\mu l^{-1}$)		0.002	0.006	0.12	0.02	0.05	0.08
	Shaking	PCR	+	+	+	+	+	+
		Hybridization	-	+	-	+	+	+
E. coli	DNA concentrations ($ng.\mu l^{-1}$)		0.02	0.06	0.01	0.24	0.50	0.80
	Stationary	PCR	-	-	-	-	-	-
		Hybridization	-	-	-	-	-	-
	DNA concentrations ($ng.\mu l^{-1}$)		0.02	0.06	0.01	0.24	0.50	0.80
	Shaking	PCR	-	-	-	-	-	-
		Hybridization	-	-	-	-	-	-

Various techniques were used in an attempt to successfully isolate DNA from the microcosms. Concentrating the bacterial cells of the seawater microcosms on polycarbonate filters and subsequent lysis of the cells by freeze-thawing was unsuccessful. Since polycarbonate filters inhibits PCR (Bej *et al.*, 1991c), its removal from the PCR resulted in the loss of DNA as the DNA was bound to the filter. Use of fluoropore filters, which were recommended by Bej *et al.* (1991c), also did not yield any PCR products in the seawater microcosms. Bej *et al.* (1991c) used fluoropore filters to concentrate a dilution series of bacterial cells (101, 102, 103, 106 cells. 100 ml^{-1}) in declorinated potable water and to extract DNA by freeze-thawing, which resulted in positive amplifications.

Table 4.3: Summary of PCR amplification and DNA hybridization results obtained for river water microcosms (25°C)

S. typhimurium	DNA concentrations ($ng.\mu l^{-1}$)		0.04	0.12	0.24	0.48	1.00	1.60
	Stationary	PCR	-	-	-	-	-	-
		Hybridization	-	-	-	-	-	-
	DNA concentrations ($ng.\mu l^{-1}$)		0.06	0.19	0.37	0.74	1.55	2.48
	Shaking	PCR	-	-	-	-	-	-
		Hybridization	-	-	-	-	-	-
S. typhi	DNA concentrations ($ng.\mu l^{-1}$)		0.03	0.10	0.20	0.41	0.85	1.36
	Stationary	PCR	-	-	-	-	-	-
		Hybridization	-	-	-	-	-	-
	DNA concentrations ($ng.\mu l^{-1}$)		0.04	0.11	0.23	0.46	0.95	1.52
	Shaking	PCR	-	-	-	-	-	-
		Hybridization	-	-	-	-	-	-
S. flexneri	DNA concentrations ($ng.\mu l^{-1}$)		0.04	0.11	0.22	0.43	0.90	1.44
	Stationary	PCR	-	-	-	-	-	-
		Hybridization	-	-	-	-	-	-
	DNA concentrations ($ng.\mu l^{-1}$)		0.01	0.04	0.07	0.14	0.30	0.48
	Shaking	PCR	-	-	-	-	-	+
		Hybridization	-	-	-	-	-	+
S. sonnei	DNA concentrations ($ng.\mu l^{-1}$)		0.05	0.15	0.30	0.60	1.25	2.00
	Stationary	PCR	-	-	-	+	+	+
		Hybridization	-	-	+	+	+	+
	DNA concentrations ($ng.\mu l^{-1}$)		0.04	0.11	0.22	0.43	0.90	1.44
	Shaking	PCR	+	-	-	_	+	+
		Hybridization	-	-	+	+	+	+
E. coli	DNA concentrations ($ng.\mu l^{-1}$)		0.06	0.18	0.36	0.72	1.50	2.40
	Stationary	PCR	-	-	-	-	-	-
		Hybridization	-	-	-	-	-	-
	DNA concentrations ($ng.\mu l^{-1}$)		0.03	0.09	0.18	0.36	0.75	1.20
	Shaking	PCR	-	-	-	-	+	+
		Hybridization	-	-	-	-	+	+

Thus, it was assumed that the salt concentration of seawater interfered with the PCR. DNA isolation from the seawater microcosm cultures by the method of Islam *et al.* (1993) using lysozyme and proteinase K also yielded no PCR products. Thus, dialysis of microcosm samples against sterile distilled water prior to boiling the cells resulted in positive amplification products for all the seawater microcosms. However, dialysis was not necessary for the river water microcosms.

Once DNA was successfully isolated from the microcosms, specific primers had to be chosen for PCR. Primers specific to the regulatory region of the *uid* A gene of *E. coli* (Bej *et al.*,

1991a, 1991c) were used to amplify the DNA of *E. coli*. These primers were also specific for *Shigella spp.* (Bej *et al.*, 1991c) and were used to amplify the DNA of *S. flexneri* and *S. sonnei*. Primers unique to *Shigella spp.* have not yet been reported. Positive amplifications resulting in the 154 bp product were achieved in all three isolates. Primers specific to the origin of replication of *S. typhimurium* (Widjojatmodjo *et al.*, 1991) were used to amplify a 163 bp product in *S. typhimurium* and *S. typhi*. Widjojatmodjo *et al.* (1991) used the primers specific to the origin of replication of *S. typhimurium* to amplify DNA from a serial dilution of *S. typhimurium* cells. Also, the amplified product of *S. typhimurium* was used as a probe to detect the amplified products obtained from the serial dilution of *S. typhimurium* cells. It was found that the PCR products from 10^7 cells to 10^2 cells. ml^{-1} were detectable on the agarose gel. However, the probe detected 10 CFUs of *S. typhimurium*, thus increasing sensitivity. Widjojoatmodjo *et al.* (1991) also reported that this set of primers amplified various other *Salmonella spp.* However, *S. typhi* was not used as an example in their studies. In this study it was shown that the 163 bp product was produced for *S. typhi* with the above set of primers. Thus, in this study it was decided to use the amplified product of *S. typhimurium* as the probe to detect the DNA dilution series of *S. typhimurium* and *S. typhi*. This enabled the detection limit as well as the homology of the probe to the PCR product of *S. typhi* to be established.

Similarly, the amplified product of *E. coli* was used as a probe against *S. flexneri, S. sonnei* and itself. The probes (amplified PCR products of *E. coli* and *S. typhimurium)* were purified using the Gene Clean^R II kit, Qiagen^R kit and the Ultra-free centrifugal membrane filtration method. However, recovery by all three methods was very low, possibly due to their low molecular weight. Thus, the PCR products were labelled without being purified after amplification. Widjojotmodjo *et al.* (1991) labelled the *S. typhimurium* probe by incorporating digoxigenin-11-dUTP in the PCR and detection of hybridization signal was achieved with the DIG^R kit. This could not be achieved with the ECL^R kit as the labelling reagent in this kit is used to label the probes after the PCR is complete and cannot be used to label probes during the PCR.

The specificity of the probes had to be assessed with pure cultures before hybridization studies with the amplified product of the microcosms could be attempted. Amplification products from *S. typhimurium* and *E. coli* also served as positive controls. The amplified product of *E. coli* was hybridized against the PCR products of *S. typhimurium* and itself. Similarly, the amplified product of *S. typhimurium* was hybridized against the PCR product

of *E. coli* and itself. The probe hybridized to the amplified product of its DNA, thus indicating that the probe was specific under the conditions employed.

Once specificity of the primers and probes for PCR and hybridization were tested for pure cultures, an attempt was made to detect the VBNC bacteria from the microcosms. DNA isolated from all five pure cultures were diluted to the same DNA concentration as the microcosm cultures and subjected to PCR. Positive amplification was achieved for the entire DNA dilution series for all of the pure cultures.

S. typhimurium and *S. typhi* DNA from the seawater and river water microcosm cultures at 4°C and 25°C yielded no PCR product. $MgCl_2$ concentration is very important in the PCR reaction. Mg^{2+} influences Taq polymerase activity, and forms soluble complexes with dNTPs to produce actual substrate that the Taq polymerase recognises. Therefore, one could speculate that seawater, possibly contained compounds that complexed with Mg^{2+}. This could have in turn prevented Mg^{2+} from forming soluble complexes with dNTPs. Thus, there was no substrate for Taq polymerase to recognise. Therefore, no PCR product was formed in the seawater microcosms at 4°C and 25°C.

There have been limited reports on the detection of the VBNC state of *Salmonella* spp. from water. Thus, a detailed comparison to the present study could not be achieved. Roszak *et al.* (1984) reported on the detection of the VBNC bacterium, *S. enteritidis*. However, since detection was by conventional methods, a comparison at a molecular level was not possible. Widjojoatmodjo *et al.* (1991) reported the magnetic immuno PCR assay (MIPA) where culturable *S. typhimurium* cells were extracted by monoclonal antibodies bound to the magnetic particles. The magnetic particles had to be removed for successful PCR. However, there was no loss of PCR product with the removal of the magnetic particles. Thus, the MIPA assay could serve as an alternative method to increase DNA isolation from the microcosm for PCR.

The fluorescent detection polymerase chain reaction (FD-PCR) assay in microwell plates was used by Cano *et al.* (1993) to screen for *Salmonella* in foods. The level of detection of the assay was as low as 1-10 CFUs. This assay could be advantageous when detecting low concentrations of bacteria from natural environments. Also, when the VBNC state was reached the CFU was in the region of 1. Thus the FD-PCR assay could increase the level of

detection. The FD-PCR assay could serve to further increase the level of detection of VBNC bacteria from the microcosms used in the present study.

PCR and hybridization studies detected *S. flexneri*, *S. sonnei* and *E. coli* in seawater and river water microcosms. In seawater microcosms at 4°C *S. flexneri* DNA was detected within the concentration range of 0.02-0.32 ng.μl^{-1} and at 25°C within the range 0.004-0.16 ng.μl^{-1}. *S. flexneri* reached the VBNC state in the seawater microcosm at 25°C in the shortest period of time compared to the other seawater microcosms. This VBNC culture yielded the most distinct PCR product.

In the stationary river water microcosms (4°C) of *S. flexneri* a positive PCR product was detected at concentrations of 0.30 ng.$\mu l^{-1}$and 0.48 ng.$\mu l^{-1}$and a positive hybridization signal was obtained at 0.14 ng.μl^{-1}, 0.30 ng.$\mu l^{-1}$and 0.48 ng.μl^{-1} whereas the shaking microcosm DNA concentrations of 0.20 ng.$\mu l^{-1}$and 0.32 ng.μl^{-1} produced positive PCR products and a hybridization signal was obtained at 0.10 ng.μl^{-1}, 0.20 ng.$\mu l^{-1}$and 0.32 ng.μl^{-1}.

In the 25°C *S. flexneri* shaking river water microcosm a positive PCR product and hybridization signal were obtained at a DNA concentration of 0.48 ng.μl^{-1}.

S. sonnei 4°C stationary and shaking microcosms produced positive PCR products and hybridization signals at DNA concentrations above 0.05 ng.$\mu l^{-1}$and 0.006 ng.μl^{-1}, respectively, whereas for the 25°C stationary microcosm positive PCR products were obtained for concentrations of 1.25 ng.μl^{-1} and 2.00 ng.μl^{-1}. The shaking microcosm produced products at 0.43 ng.μl^{-1}, 0.90 ng.μl^{-1} and 1.44 ng.μl^{-1} DNA. Hybridization signals for the *S. sonnei* stationary and shaking microcosms were obtained at DNA concentrations above 0.30 ng.$\mu l^{-1}$and 0.22 ng.μl^{-1},respectively.

Comparison at a molecular level between the present study using *S. flexneri* and *S. sonnei* and that of Colwell *et al.* (1985) where the same organisms were used, could not be made as detection of the VBNC state of *S. flexneri* and *S. sonnei* was carried out by conventional methods and not PCR. Islam *et al.* (1993) used PCR to detect the VBNC state of another

Shigella spp. viz, *S. dysenteriae.* However, these researchers used primers specific to the invasion plasmid antigen gene *(ipa* H) which generated a 700 bp PCR product. The DNA extraction protocol proposed by Islam *et al.* (1993) where lysozyme and proteinase K was used for DNA isolations from pond, river, drain and lake water, was not successful with DNA isolations from seawater microcosms in this study. A possible reason for this phenomenon was that the salt from the seawater was still present and thus inhibited the PCR reaction. These sets of primers could not be used for the specific detection of *Shigella spp.* as the invasion plasmid antigen gene *(ipa* H) has been found to be unique to shigellae and enteroinvasive *E. coli.*

In the present study *E. coli* was only detected by the PCR/hybridization method in the 25°C shaking river water microcosm at DNA concentrations of 0.75 $ng.\mu l^{-1}$ and 1.2 $ng.\mu l^{-1}$. Detection of the VBNC state of *E. coli* by PCR and hybridization could not be compared to that of Colwell *et al.* (1985) and Bej *et al.* (1990) who tested various primer annealing temperatures for the *lac* Z and *lam* B genes. Amplification using primers specific to the *lac* Z and primer annealing of 40°C produced positive PCR products for both coliforms and non-coliforms. However, the amplified PCR product of *Citrobacter* was larger than *E. coli.* Increasing the temperature to 50°C to increase stringency eliminated all non-coliforms and some coliforms as these researchers only used conventional methods in detection. However, Bej *et al.* (1990) proposed the use of PCR and gene probes for the detection of coliform bacteria.

DNA concentration can have a marked effect on the amplification product formed. Brauns *et al.* (1991) reported the detection of VBNC *V. vulnificus* using PCR. They found that PCR was able to detect 72 pg of DNA from the culturable *V. vulnificus.* For the detection of non-culturable *V. vulnificus,* 4.2 µg of DNA resulted in positive amplification, only after an additional 40 cycles were completed. Thus, different VBNC bacteria may require different PCR conditions to adequately amplify their DNA. In the present study the number of cycles used for PCR for DNA isolated from pure cultures and microcosm cultures was the same. In most cases the entire DNA dilution series of the pure culture resulted in positive amplification. However, the entire DNA dilution series of the microcosm cultures did not yield positive amplification. This showed that the concentration of DNA isolated from the microcosm must be high for positive amplification. When stable DNA was isolated from the

microcosms, positive amplifications resulted. Thus, there was no interference from other inhibitory substances in the microcosms.

4.5 Summary and conclusion

Unlike conventional AODC, DVC and DPC methods, PCR and gene probe technology offers increased sensitivity and specificity in detecting VBNC bacteria. Primers specific to the regulatory region of the *uidA* gene of *E. coli* amplified a 154 bp product in *E. coli*, *S. flexneri* and *S. sonnei*. Primers specific to the origin of replication of *S. typhimurium* amplified a 163 bp product in *S. typhimurium* and *S. typhi*. The 163 bp amplification product of *S. typhimurium* was used as a probe to ascertain its detection limit against a DNA dilution series of *S. typhimurium* and *S. typhi*. Similarly, the 154 bp amplification product of *E. coli* was used as a probe against *E. coli*, *S. flexneri* and *S. sonnei*.

PCR and hybridization studies detected *S. flexneri*, *S. sonnei* and *E. coli* in seawater and river water microcosms. In sea water microcosms at 4°C *S. flexneri* DNA was detected within the range 0.02-0.32 ng.μl^{-1} , and at 25°C within the range 0.004-0.16 ng.μl^{-1}. Compared to other seawater microcosms at 25°C, *S. flexneri* reached the VBNC state fastest and yielded the most distinct PCR product.

In the 4°C stationary river water microcosms of *S. flexneri* a positive PCR product was detected at DNA concentrations of 0.3 ng.μl^{-1} and 0.48 ng.μl^{-1}, and a positive hybridization signal was obtained within the range 0.14 - 0.48 ng.μl^{-1}. In the 4°C shaking microcosm of *S. flexneri* DNA concentrations of 0.2 ng.μl^{-1} and 0.32 ng.μl^{-1} produced positive PCR products and a hybridization signal was obtained within the range 0.10 - 0.32 ng.μl^{-1}. In the 25°C *S. flexneri* shaking river water microcosm a positive PCR product and hybridization signal was obtained at a DNA concentration of 0.48 ng.μl^{-1}.

At 4°C the *S. sonnei* stationary and shaking river water microcosms produced positive PCR products and hybridization signals at DNA concentrations above 0.05 ng.μl^{-1} and 0.006 ng.μl^{-1} , respectively. For the *S. sonnei* stationary river water microcosm at 25°C positive PCR products were obtained for DNA concentrations of 1.25 ng.μl^{-1} and 2 ng.μl^{-1}. The shaking *S. sonnei* river water microcosm at 25 °C produced PCR products at DNA concentrations within the range 0.43 - 1.44 ng.μl^{-1}.

In the present study *E. coli* was only detected by the PCR/hybridization method in the 25°C shaking river water microcosm at DNA concentrations of 0.75 $ng.\mu l^{-1}$ and 1.2 $ng.\mu l^{-1}$.
Thus, the stability and concentration of DNA isolated from the microcosms has a marked effect on the detection limit of VBNC bacteria using PCR/hybridization studies.

CHAPTER FIVE

ASSESSMENT OF THE PREVALENCE OF VBNC PATHOGENS IN ENVIRONMENTAL SAMPLES USING CONVENTIONAL METHODS

5.1 Introduction

Worldwide it is estimated that 80% of all diseases is attributable to inadequate water treatment and sanitation (Bitton, 1994). In South Africa it is estimated that intestinal infections are responsible for 20% of deaths of one to five-year old children. The US Centre for Disease Control and Prevention estimates that each year in the United States up to 900 000 cases of illness and possibly 900 deaths occur as a result of waterborne microbial infections (CDC, 1999).

Microbiological quality of water refers to the presence or absence of pathogenic microorganisms in the water. Water quality standards require that the water delivered for human consumption must be free of any pathogens. Therefore, the water has to be tested for these organisms. It would be far too complex to try to detect all of these on a routine basis, and in any case, many of the pathogens may only be present in very small numbers or not at all. It is therefore normal practice to look for "indicator bacteria". Conventionally, indicator bacteria have been used to determine the possible presence and to estimate the amount of faecal contamination in water, foods and other samples. The detection of indicator bacteria is preferred over direct pathogen detection because the former are considered to be normal, non-pathogenic intestinal inhabitants that are present in faeces and wastewater in much higher numbers than pathogenic microorganisms and because they are technically easier to detect and enumerate than pathogens. Present standards for the sanitary quality of water, foods and other materials, with respect to faecal contamination, are expressed in terms of concentrations of indicator bacteria (DeZuane, 1991).

Untreated water, like any other natural water, will contain many bacteria. Nearly all these bacteria will not be of any health significance and their presence in the water is quite irrelevant to public health. In testing an untreated water supply therefore, it is necessary to look for bacteria of definite faecal origin. The faecal coliforms are most commonly used for this purpose, but

other groups of bacteria (such as faecal streptococci) would also serve this purpose (WHO, 1993).

The objective of this study was to assess the prevalence of VBNC bacteria in various sources of water. In order to achieve this objective the standard methods for the detection of indicator bacteria were compared to the resuscitation method of Kogure *et al.*, 1979.

5.2 Materials and methods

Water samples used in this study were placed into three broad categories:

- River, dam and tap water;
- Rain water storage tanks and small-scale water supply projects; and
- Groundwater.

The sources of the water samples analysed in this study are listed in Table 5.1. A total of twenty-two sites from various areas in Kwa-Zulu Natal were sampled. The water samples were collected aseptically in sterile Schott bottles.

5.2.1 Membrane filtration (MF) test.

From the samples collected 100ml, 1ml and 0.1ml volumes were passed through the membrane filtration unit by applying a vacuum pump. Using sterile forceps, the millipore filters were then placed onto the various selective agar plates, viz., m-Endo for coliforms; m-FC for faecal coliforms; Brain Heart Infusion Agar (BHI) for fastidious and non-fastidious microorganisms; Thiosulfate Citrate Bile Sucrose (TCBS) and *Vibrio* Diagnostic Agar for *Vibrio spp*; Deoxycholate Citrate Agar (DCA) and Xylose Lysine Desoxycholate (XLD) for *Shigella spp.*; and Brilliant Green Agar (BGA) and Bismuth Sulfite Agar (BSA) for *Salmonella spp.* Except for the m-FC agar plates which were incubated at 44.5°C, all other plates were incubated at 37°C for 24 hours (American Public Health Assoc. 1989).

Table 5.1: Sampling sites

LOCATION	NEAREST TOWN/CITY	POPULATION	SOURCE OF SUPPLY
Enkanyisweni School	Umkomaas	894	Rainwater
Isifisosethu Senior Secondary School			
Nyuswa Clinic	Umvoti	600	Rainwater
Montobelo Project	Umvoti	50 out-patients per day	Rainwater
Emayisweni Project	Umvoti	600	Unknown
Mbeka O.P School	-	100	Unknown
St. Christopher Primary School	Cato Ridge	400	Rainwater
Mpumelelo Clinic	Cato Ridge	775	Rainwater
Tshelabantu School	Umvoti	50 out-patients per day	Rainwater
Umzumbe	Tugela	850	Rainwater
Enkulo	Hibberdene	No data available	Groundwater
Tantuta	Qwabe	No data available	Groundwater
Khabingwe	Qwabe	No data available	Groundwater
Nqoleni	Babanango	No data available	Groundwater
Mnqadayi (a)	Babanango	No data available	Groundwater
Nomiyata	Babanango	No data available	Groundwater
Kabingwe Section 1	Babanango	No data available	Groundwater
Nomyaca	Babanango	No data available	Groundwater
Mnqadayi (b)	Babanango	No data available	Groundwater
Hammersdale	Babanango	No data available	Groundwater
Harding	Hammersdale	No data available	Groundwater
Hibberdene	Harding	No data available	Groundwater
	Hibberdene	No data available	Groundwater

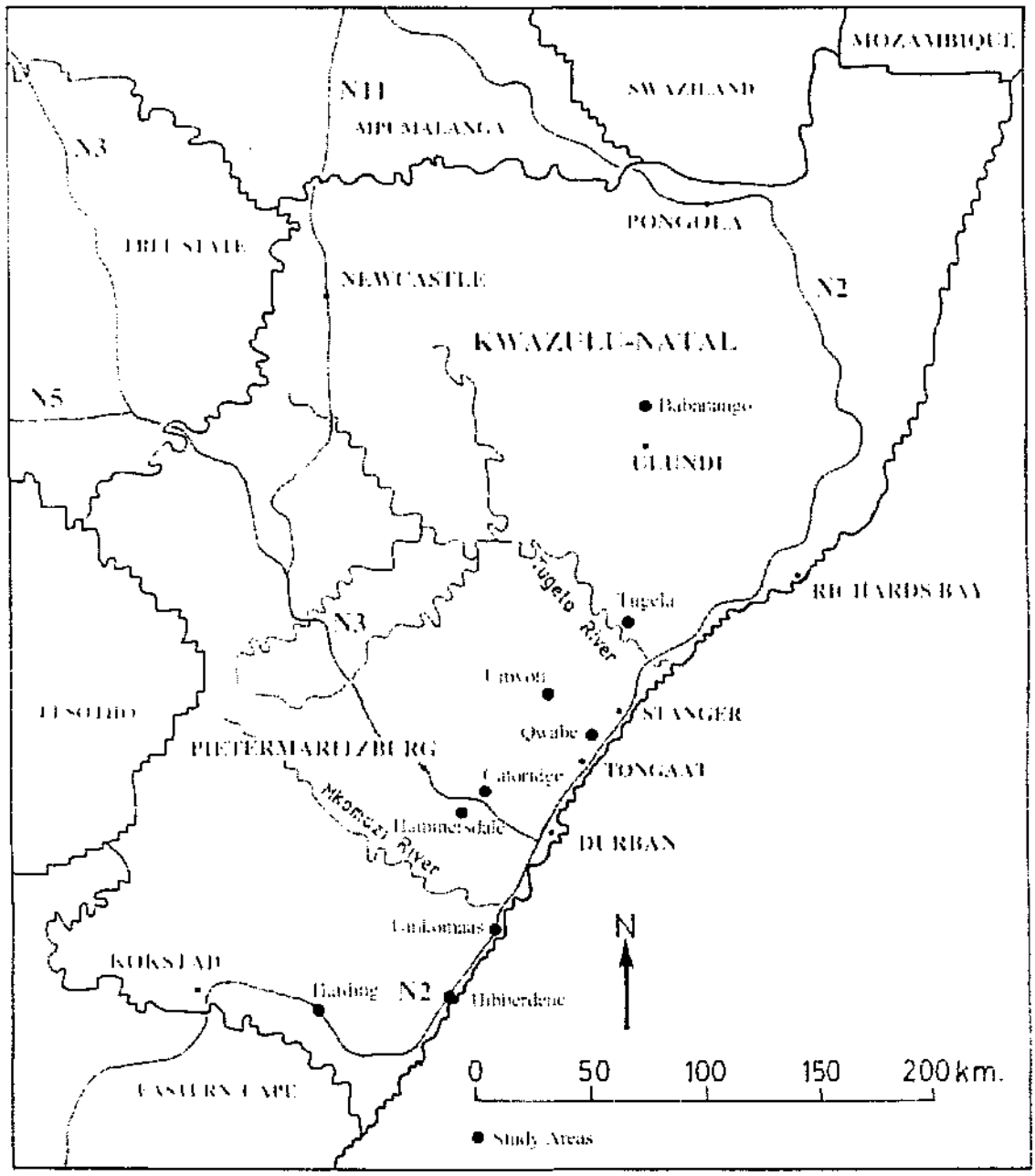

Fig. 5.1 Map showing the various sampling sites mentioned in Table 5.1.

5.2.2 Dilution plate count (DPC)

A serial dilution of up to 10^{-4} using sterile distilled water was performed on the water samples. From the dilutions 0.1 ml of water sample was plated onto Eosin Methylene Blue Agar (EMB), BHI, TCBS, DCA, Salmonella-Shigella Agar (SS), BGA, MacConkey Agar (MAC) and Nutrient Agar (NA). The plates were then incubated for 24 hours at 37°C.

5.2.3 Resuscitation of VBNC bacteria.

Resuscitation was carried out using a modified DVC method (Kogure *et al.*, 1979). Samples were resuscitated using 0.002% nalidixic acid and 0.025% yeast extract. The sample was then incubated at 20°C for 6 hours. Following incubation MF and DPCs were performed and plated onto the selective media which were then incubated at 37°C for 24 hours.

5.3 Results

5.3.1 Rainwater and small-scale water supply projects

5.3.1.1 Dilution plate counts

The number of bacterial colonies present in the water samples collected from the water schemes, schools and rainwater tanks ranged from 117 to > 100 000 CFUs.100ml^{-1} for organisms on BGA plates, 0 to 2300 for organisms on DCA plates and 4 to >18 million for those organisms growing on BHI agar plates (Table 5.2).

Table 5.2: Comparison of CFUs of presumptive total heterotrophs *Shigella* and *Salmonella* species on selective agar before and after resuscitation

AREA	BHI (Total heterotrophs)		DCA (*Shigella*)		BGA (*Salmonella*)	
	Unresuscitated (CFU\100ml)	Resuscitated (CFU\100ml)	Unresuscitated (CFU\100ml)	Resuscitated (CFU\100ml)	Unresuscitated (CFU\100ml)	Resuscitated (CFU\100ml)
Nyuswa Clinic	>50000	100000	148	30000	735	2500
Isifisosethu School	No data	No data	3	0	117	0
Montobelo Project	>50000	120000	700	107000	2350	103000
Emayisweni Project	2800	91000	350	0	No data	No data
Mbeka OP School	No data	No data	0	0	No data	No data
St. Christopher Primary School	22000	10200000	59	2500	No data	No data
Mpumelelo Clinic	50850	18300000	56	1000	429	6500
Tshelabantu School	>50000	3070000	2300	3000	No data	No data

Bacterial numbers obtained after resuscitation with nalidixic acid and yeast extract were higher than the original dilution plate counts. This indicates that a large proportion of the bacteria present in the water samples were in a VBNC state, shown in Table 5.2.

5.3.2 Groundwater

5.3.2.1 Membrane filtration

Results obtained from groundwater samples using MF showed an overall increase in CFU's following resuscitation (Table 5.3). Following resuscitation a 100% increase in CFU's on *Vibrio* diagnostic agar was obtained from the sample isolated from Mnqadayi (b). A 10-fold increase in bacterial numbers were observed in water from Kabingwe Section I. There was a 120% increase in the number of colonies on BSA agar isolated from Mnqadayi (b) following resuscitation. The greatest increase, 45% on XLD agar, was isolated from Mnqadayi (b). The Khabingwe area showed a 36% increase in CFU.100ml^{-1} on m-Endo. The area showing the greatest increase in CFU's following resuscitation on BHI agar was Kabingwe Section I, where 661% was recorded.

Table 5.3: Comparison of CFUs of presumptive total heterotrophs enteropathogenic *E. coli*, *Shigella*, *Salmonella* and *Vibrio* species on selective agar using MF before and after resuscitation

AREA	BHI (Total heterotrophs)		m-ENDO (Enteropathogenic *E. coli*)		XLD (*Shigella*)		BSA (*Salmonella*)		*Vibrio* Diagnostic (*Vibrio*)	
	Unresuscitated (CFU\100ml)	Resuscitated (CFU\100ml)	Unresuscitated (CFU\100ml)	Resuscitated (CFU\100ml)	Unresuscitated (CFU\100ml)	Resuscitated (CFU\100ml)	Unresuscitated (CFU\100ml)	Resuscitated (CFU\100ml)	Unresuscitated (CFU\100ml)	Resuscitated (CFU\100ml)
Umzumbe	138	1680	1	83	35	100	8	30	111	10
Enkulo	136000	210000	17100	37200	77000	270000	95000	85000	6400	16300
Tantuta	188000	>8200000	24050	121000	49000	114000	65000	109000	180000	227000
Khabingwe	928000	>8200000	283	4000	640000	2480000	133	11866	800	14400
Nqoleni	167000	262000	400	14500	300	3600	600	10100	900	42000
Mnqadnyi (a)	664000	2100000	1000	4000	4000	34000	4000	12000	1000	2500
Nomiyata	55000	207000	No growth	No growth	11000	39000	No growth	No growth	16000	448000
Kabingwe Section I	134000	8200000	108000	960000	287000	2760000	187000	7260000	81500	990000
Nomyaca	189000	1470000	3600	93000	53000	430000	9300	720000	3300	353000
Mnqadayi (b)	41000	1330000	No growth	No growth	5000	228300	6000	756300	No growth	35000

Table 5.4: **Comparison of CFUs of presumptive total heterotrophs *Salmonella, Shigella, E. coli* and lactose fermenters from groundwater supplies using DPCs before and after resuscitation**

AREA	BHI (Total heterotrophs)		BGA (*Salmonella*)		SS (*Shigella*)		EMB (*E. coli*)		MAC (lactose fermenters)		NA (Total heterotrophs)	
	Unresuscitated (CFU\100ml)	Resuscitated (CFU\100ml)	Unresuscitated (CFU\100ml)	Resuscitated (CFU\100ml)	Unresuscitated (CFU\100ml)	Resuscitated (CFU\100ml)	Unresuscitated (CFU\100ml)	Resuscitated (CFU\100ml)	Unresuscitated (CFU\100ml)	Resuscitated (CFU\100ml)	Unresuscitated (CFU\100ml)	Resuscitated (CFU\100ml)
Hammersdale (B1)	210	347	57	107	35	43	9	26	199	657	298	657
Hammersdale (B2)	231	447	23	129	25	43	No growth	6	54	105	129	775
Harding (C1)	110	447	7	70	No growth	30	No growth	15	50	258	301	934
Harding (C2)	10	447	No growth	No growth	No growth	No growth	No growth	No growth	13	42	45	260
Hibberdene (D1)	230	1120	330	245	12	30	40	515	50	140	310	950
Hibberdene (D2)	200	112	33	45	2	3	4	9	50	140	430	1150
Hibberdene (D3)	210	1120	No growth	5	No growth	No growth	40	15	50	60	110	780

5.3.2.2 Dilution plate counts

Groundwater samples that were analysed using DPCs also showed an increase in CFUs following resuscitation (Table 5.4). A 45% increase in CFUs following resuscitation on BHI agar was observed from water obtained from Harding (C2). Following resuscitation a 100% increase in CFUs were noticed on BGA (Hibberdene D3), SS Agar (Harding C1) & EMB agar (Hammersdale B2, Harding C1). A 5 fold increase was observed on MAC agar after resuscitation from the Harding C1 area. Samples from Hibberdene D3 showed a 7 fold increase in CFUs on NA after resuscitation.

5.3.3 River, dam and tap water

Table 5.5 lists the various sources and locations of the samples analysed. Tap water samples were collected from domestic households in Clare Estate, Chatsworth, Umbilo and Westville.

Table 5.5 Key to sampling sites for the various water samples in the Durban Metro area

Sample no.	Source	Location
1,9	Palmiet River	University of Durban-Westville
2, 5, 14	Palmiet River	Varsity Drive
3, 4, 10	Umbilo River	Umbilo
6, 11, 17	Pond water	Umbilo Park
7, 8, 15, 16	Umgeni water	Blue Lagoon river mouth
12, 18	Umhlatuzana River	Marianhill
13	Dam water	Durban Heights

The results obtained followed a similar trend for all samples viz., higher bacterial counts were obtained after resuscitation. Tap water samples were negative for all indicator organisms except heterotrophic bacteria (results not shown). No pathogenic species were detected in any of the tap water samples with all the methods used. The numbers of pathogens fluctuated among the various river and dam water samples examined (Table 5.6-5.7).

Total coliform counts increase up to 4-fold from the Palmiet River samples (Table 5.6). In general the total coliform counts were high for all the samples tested. Faecal coliform counts, which pose a greater health hazard, were detected at dangerously high levels (Table 5.6). In particular, the Umbilo Pond water exceeded 120000 CFU's.100ml^{-1}, while the Umbilo River sample exceeded 90000 CFU's.100ml^{-1}. These represent post-resuscitation counts which increased more than 2-fold from the standard plate counts.

As expected, heterotrophic plate counts were high in all samples (Table 5.6). The highest counts were obtained from the Palmiet River samples. Faecal streptococcal numbers exceeded 80000 CFU's.100ml^{-1} in samples from Palmiet and Umgeni Rivers, and the Durban Heights dam (Table 5.6). High counts of *Vibrio* spp., *Salmonella* spp., *Shigella* spp. and *Legionella* spp. were observed for samples from the Palmiet and Umgeni River samples (Table 4.7).

Table 5.6: Comparison of CFU's of presumptive total coliforms, faecal coliforms, heterotrophic plate counts and faecal streptococci in environmental water supplies before and after resuscitation

	Total Coliform		Faecal Coliform		Heterotrophic Plate Count		Faecal Streptococci	
	Unresuscitated (CFU/100ml)	Resuscitated (CFU/100ml)	Unresuscitated (CFU/100ml)	Resuscitated (CFU/100ml)	Unresuscitated (CFU/100ml)	Resuscitated (CFU/100ml)	Unresuscitated (CFU/100ml)	Resuscitated (CFU/100ml)
1	9 000	15 000	10 000	17 500	80 000	460 000	85 000	98 500
2	17 000	28 000	15 000	19 000	90 000	375 000	56 000	67 000
3	62 000	110 000	31 000	82 000	250 000	345 000	3 500	4 000
4	43 000	87 000	37 000	52 000	220 000	330 000	9 000	11 000
5	35 000	117 000	21 000	37 500	180 000	250 000	13 000	19 000
6	15 000	27 000	5 000	40 000	245 000	360 000	1 000	2 000
7	26 000	54 000	18 000	21 000	170 000	170 000	20 000	34 000
8	10 000	34 000	10 000	22 000	100 000	165 000	6 500	7 500
9	51 000	76 000	20 000	23 000	75 000	135 000	12 000	19 500
10	5 000	9 000	3 000	9 000	60 000	65 000	5 000	7 500
11	80 000	116 000	59 000	117 000	160 000	197 000	1 500	2 000
12	50 000	109 000	52 000	73 500	50 000	51 000	2 000	4 500
13					100 000	215 000	45 000	85 000
14					90 000	155 000	36 000	52 500
15							72 000	19 500
16							90 000	117 000
17							3 500	5 000
18							8 500	9 700

Table 5.7: **Comparison of CFU's of presumptive *Vibrio, Salmonella, Shigella* and *Legionella* species in environmental water supplies before and after resuscitation**

	Vibrio species		*Salmonella* species		*Shigella* species		*Legionella* species	
	Unresuscitated CFU/100ml	Resuscitated CFU/100ml	Unresuscitated CFU/100ml	Resuscitated CFU/100ml	Unresuscitated CFU/100ml	Resuscitated CFU/100ml	Unresuscitated CFU/100ml	Resuscitated CFU/100ml
1	60 000	180 000	80 000	150 000	80 000	123 000	23 000	38 000
2	125 000	175 000	115 000	145 000	91 000	182 000	52 000	90 000
3	1 000	5 000	2 000	2 000	7 500	9 000	1 000	1 700
4	12 500	30 000	2 500	25 000	18 700	26 000	7 500	33 000
5	145 000	275 000	115 000	160 000	43 000	65 000	20 000	35 000
6	12 500	25 000	50 000	120 000	6 200	8 000	2 000	2 500
7	12 000	20 000	20 000	50 000	5 000	15 000	3 000	7 000
8	2 000	15 000	2 000	3 000	25 000	40 000	3 500	6 500
9	0	1 000	0	2 000	20 000	25 000	21 500	62 000
10	1 000	3 000	1 000	3 000	9 000	30 000	37 000	58 500
11	12 000	25 000	25 000	75 000				
12	1 000	2 000	1 000	2 000				
13	2 500	12 000	1 000	2 000				
14	25 000	40 000	1 000	3 000				
15	70 000	225 000	21 000	77 000				
16	32 000	50 000	120 000	27 500				
17	2 000	2 000	1 000	2 000				
18	0	0	0	0				

5.4 Discussion

Bacteria in water experience stressful conditions caused by a variety of physical and chemical factors. These influence not only the size and composition of the microbial population, but also the morphology and physiology of individual bacteria (Rheinheimer, 1980). In natural environmental waters, such factors include lack of nutrients, temperature, pH, light, salt concentrations, pressure, and water stress. In treated waters there is also the influence of disinfectants.

Injured or stressed bacteria undergo a morphological change from rod to coccoid shaped cells (Roszak and Colwell, 1987). These are said to have entered the VBNC state. They are unable to grow on selective media and may lead to inaccurate water quality results.

Samples tested in this investigation included river, tap, dam, pond and groundwater. Indicator organisms investigated were Total Coliforms (TC), Faecal Coliforms (FC), Faecal Streptococci (FS), and Heterotrophic Plate Count (HPC) bacteria. Pathogenic species included *Vibrio, Salmonella, Shigella*, enteropathogenic *E. coli* and *Legionella* species.

All tap water samples tested were free of any indicators of faecal contamination. The only plate counts that showed positive results for tap water samples were HPC and the numbers ranged between >1 and 60 colony forming units per 100 ml with the standard method. These were within the SABS specifications. With the resuscitation procedure the numbers were as high as 70 colony forming units per 100 ml. The environmental samples tested showed a variety of results, but they were all positive for the indicators of faecal contamination and at least one of the pathogenic species.

No specific relationships were observed between pathogens and indicator organisms in the environmental samples. Pathogenic colony forming units varied with each sampling site. This can be attributed to the specific environmental conditions of each pathogen and also to the presence or absence of sources of contamination at each sampling point. Resuscitation of VBNC bacteria yielded significantly higher numbers of colony forming units than the standard methods, both for the quantification of indicator bacteria and the pathogenic species.

The procedure for resuscitation of VBNC bacteria involves incubation of cultures in a dilute solution of yeast extract and nalidixic acid. The yeast extract provides nutrients for the recovery of stressed organisms. Nalidixic acid is an anti-microbial agent that is used to prevent cell division during recovery; it ensures that there is no over-estimation of colony forming units due to replication. This is especially important when the cells are suspended in solution. Nalidixic acid is bacteriostatic and not bactericidal and this ensures that no cells are killed during the resuscitation of VBNC bacteria.

To prevent over-estimation of colony forming units, the membrane filtration method was used. The cells were immobilised on the filter membrane, so that cell division during recovery did not result in an increase in colony forming units but encouraged formation of larger colonies.

When transferred onto selective media, all bacteria undergo a lag phase during which they adapt to the specific medium. This slows down the process of division and colony formation and is a direct cause of the delay in observing results for water quality testing. Bacteria take longer to produce visible colonies.

The significance of VBNC bacteria in water is two-fold and somewhat contradictory. Injured or VBNC bacteria may be present but not detectable because they do not develop in selective media. This means that they are a potential hazard because they are capable of repair and regaining their virulence. Therefore, every effort must be made to improve techniques for detection of injured or VBNC organisms.

The VBNC bacteria are especially important in recreational waters where the environmental factors are continuously changing. The nutrient content of the water also plays an important role in their recovery and virulence. In recreational waters nutrients are introduced into the water by a variety of factors including swimmers. These aid the recovery of VBNC bacteria and might cause infection.

VBNC bacteria are also important in rural areas and informal settlements where untreated water is used for drinking and domestic purposes. It is especially important where water is kept at temperatures that are conducive to re-growth and resuscitation.

According to the South African Bureau of Standards (SABS) the maximum allowable limit for coliforms is 5 per 100 ml and the total faecal coliform count should be zero. However, from the results obtained in this study it is clearly visible that most of the water supply systems are vastly inadequate when it comes to providing a healthy drinking source. Children attending primary schools are forced to drink water containing up to 500 times more coliforms than what is termed acceptable. It is therefore not surprising that some areas experienced outbreaks of diarrhoea which can only be attributed to the poor quality of drinking water. At the Mpumelelo Clinic, outside Stanger, water is stored outdoors in large metal rainwater tanks. This clinic sees an average of about 50 patients per day, many of them babies. The water is used to wash down medication, to clean wounds and to wash areas of skin before administering an injection or vaccine. The coliform count from this water sample revealed a total of 195 colonies.ml^{-1}. This is 39 times the accepted level; the faecal coliform count showed 13 colonies.100 ml^{-1}. These conditions are clearly unacceptable.

DPCs revealed potentially pathogenic bacterial concentrations in certain areas. This is especially of concern to human health as many diseases like dysentery, shigellosis and cholera are water-borne.

Given the results obtained, the present analysis of water does not accurately reflect the microbiological quality of the water. This can be observed in water samples where bacteria were resuscitated by the addition of nalidixic acid (which inhibits DNA synthesis and prevents cell division) and yeast extract. Increases in bacterial numbers of between 70-98% were observed on BGA and 23-98% on DCA were observed. There was a 97-99% increase in the number of colonies on BHI.

The increase in the number of CFUs with respect to the *Vibrio* diagnostic agar is especially important due to the recent outbreak of cholera in Kwa-Zulu Natal. An estimated 85000 cases have been reported with the number of deaths listed as 189.

This study has brought to light the disturbing conditions under which a large number of the population is living. Inadequate water supplies, storage facilities and water treatments are glaringly obvious. It is easy to impose safety levels on drinking water but most people living in the rural areas do not have the ways or the means to analyse the water. Health inspectors need to be sent out to schools, clinics and water schemes in rural areas where they can collaborate with researchers on the analysis and treatment of water. Communities also need to be educated to the fact that simply boiling their drinking water can kill the majority of bacterial pathogens. In rural areas people should be taught that using Jik or household bleach can help eliminate most of the pathogens from drinking water. Reference to the Assessment Guide **(Quality of Domestic Waste Supplies-Series),** should be made in helping these rural communities.

A number of studies have been conducted to investigate the efficiency of water quality testing methods, however, no significant changes have been implemented in the standard methods used by water treatment companies. The main concern is that minimal time should be taken to produce results. A more sensitive issue of concern is the cost of performing the tests. Standard methods that are currently used require at least 24 hours before results are produced. The use of

resuscitation can improve the efficiency of these methods, at minimal extra costs and also providing results within 24 hours.

This study has also shown that the standard microbiological tests for coliforms and faecal coliforms are inadequate for detecting the total number of bacteria present. Tests, which include detection of pathogens and the resuscitation of bacteria, need to be incorporated into the standard methods for water quality testing in order to achieve an accurate estimation of bacterial load.

5.5 Summary and conclusion

Tap and environmental water samples were subjected to TC, FC, FS and HPC tests for bacteria. Pathogenic bacterial species included *Vibrio*, *Salmonella*, *Shigella*, enteropathogenic *E. coli* and *Legionella* species. Tap water tested positive for HPC bacteria ranging between 1 and 60 CFU.100ml^{-1}, and 70 CFU.100ml^{-1}after resuscitation. All environmental water samples were positive for the indicators of faecal contamination and at least one of the pathogenic species. According to the SABS the maximum allowable limit for coliforms is 5 colonies.100ml^{-1} and the total faecal coliform count should be zero. However, results from water supply systems tested exceed the SABs limits, deeming these drinking sources unhealthy. In the primary school tested children are forced to consume water containing up to 500 times more coliforms than what is termed acceptable. The water samples from the Mpumelelo Clinic revealed a TC count of 195 bacterial colonies.ml^{-1}, which is 39 times the accepted limit, and a FC count of 13 colonies.100 ml^{-1}.

When bacterial cultures from the microcosms were resuscitated, an increase in cell numbers of 70 - 98% on BGA, 23 - 98% on DCA and 97 - 99% on BHI was observed. On *Vibrio* diagnostic agar there was an increase in the number of CFUs. This is important due to the recent outbreak of cholera in Kwa-Zulu Natal, where an estimated 85 000 cases with 189 deaths have been reported. To curtail similar cases involving potentially pathogenic VBNC bacteria, communities must be educated on killing the majority of bacteria by first boiling their drinking water.

Conventional microbiological tests for coliforms and faecal coliforms do not accurately estimate bacterial load. However, resuscitation of VBNC bacteria is more efficient, economical and most importantly provides results within 24 hours. Therefore, tests which include detection of pathogens and the resuscitation of VBNC bacteria, need to be incorporated.

CHAPTER SIX

MORPHOLOGICAL STUDY OF CULTURABLE AND NON-CULTURABLE BACTERIA BY SCANNING ELECTRON MICROSCOPY (SEM)

6.1 Introduction

The scanning electron microscope is one of the most versatile and widely used tools of modern science. Its utilisation extends from the world of the physicist and the engineer to the biologist, with many disciplines in between. The most critical part of scanning electron microscopy (SEM) is adequate preparation of the specimen (Postek *et al.*, 1980).

SEM was the method of choice to investigate the morphological changes associated with VBNC bacteria. It was superior to simple light microscopy as the cultures could be observed at higher magnifications, thus providing a clear definition of the morphology of the cells. Since the present study was specifically to observe morphological changes specifically SEM was most appropriate.

Initial preparation was carried out by fixing the cells in Karnovsky's fixative (Karnovsky, 1965) and osmium tetroxide. However, this resulted in collapsed and extreme clumping of the bacterial cells and was not used for further preparation. The sample preparation that proved successful was that recommended by Smit *et al* (1975). Bacterial cells (used in the present study), fixed by glutaraldehyde at 0°C, resulted in clearly defined rod-shaped cells, typical of the morphology of gram-negative bacteria. Also, coating the coverslips with poly-L-lysine improved the adhesion of the bacterial cultures to the coverslips. When the above method was used for the preparation of the microcosm samples, only a few cells were observed since the bacterial concentration was very dilute. Therefore, the microcosm cultures had to be concentrated before preparation for SEM.

The method of Todd and Kerr (1972), proved more appropriate for the preparation of microcosm cultures used in the present study. These researchers examined several pure cultures to establish the feasibility of scanning electron microscopy for *in situ* observation and calibration of biomass. Two types of membrane filters were utilized by Todd and Kerr (1972), *viz.*, MF Millipore filters and polycarbonate filters (Nuclepore^{R}). It was observed that

cells concentrated on filters resulted in minimum distortion of cells and that polycarbonate filters proved superior. With Millipore filters the bacterial suspension aggregated and thus clear morphology could not be seen (Todd and Kerr, 1972). Thus, for SEM preparations of the microcosm cultures in the present study, the cultures were concentrated onto polycarbonate filters and fixed as recommended by Todd and Kerr (1972). Distinct morphology of the bacteria was observed when microcosm cultures were concentrated on polycarbonate filters in the present study.

The objective of this study was to assess the morphological changes associated with the transition of culturable cells to the VBNC state by the use of scanning electron microscopy.

6.2 Materials and methods

6.2.1 SEM preparations of pure bacterial cultures

The method of Karnovsky (1965) was used to prepare the pure bacterial cultures for SEM.

6.2.2 SEM preparations of pure bacterial cultures (initial inoculum)

The method of Smit *et al.* (1975) was the best method used to prepare the pure cultures for SEM.

6.2.3 SEM preparations of microcosm cultures

The microcosm cultures were prepared by the method of Todd and Kerr (1972).

6.3 Results

6.3.1 Scanning electron microscopy (SEM) of *S. typhimurium*

Fig. 6.1A shows the typical characteristic of *S. typhimurium*, i.e., the gram-negative rod shaped bacteria. This was the morphology of the pure culture of *S. typhimurium* when introduced as initial inoculum to set up all microcosms.

In the seawater microcosm at 4°C, the morphology of the bacterial changed to rounded forms with the onset of non-culturability, a phenomenon associated with non-culturable bacteria (Fig. 6.1B). Although *S. typhimurium* in the seawater microcosm at 25°C (Fig. 6.1C) did show a tendency to round up, the cells did not demonstrate the typical cocci-shaped cells that were observed at 4°C.

In the river water microcosm there was a tendency for *S. typhimurium* to form aggregates (Fig. 6.1D, E, F). *S. typhimurium* in stationary and shaking river water microcosms at 4°C showed a change of morphology from rod-shaped to rounded forms. This change of morphology was observed although *S. typhimurium* did not reach the VBNC state after 138 days at which stage the experiment was discontinued (Fig. 6.1D, E). *S. typhimurium* in shaking river water microcosm displayed aggregates of cells (Fig. 6.1E). *S. typhimurium* in stationary and shaking river water microcosms at 25°C also showed the typical rounded morphology of cells (Fig. 6.1F,G). This was the only culture at 25°C in the river water microcosms that did not reach the non-culturable state after 145 days at which stage the experiment was terminated.

6.3.2 Scanning electron microscopy (SEM) of *S. typhi*

Typical gram-negative rod shaped bacteria of the initial microcosm inoculum are shown in Fig. 6.2A. With the onset of the VBNC state in the seawater microcosm at 25°C, *S. typhi* showed characteristic rounding of cells (Fig. 6.2C). In the seawater microcosm at 4°C there was a tendency for some cells to round up, while others remained as rods (Fig. 6.2B).

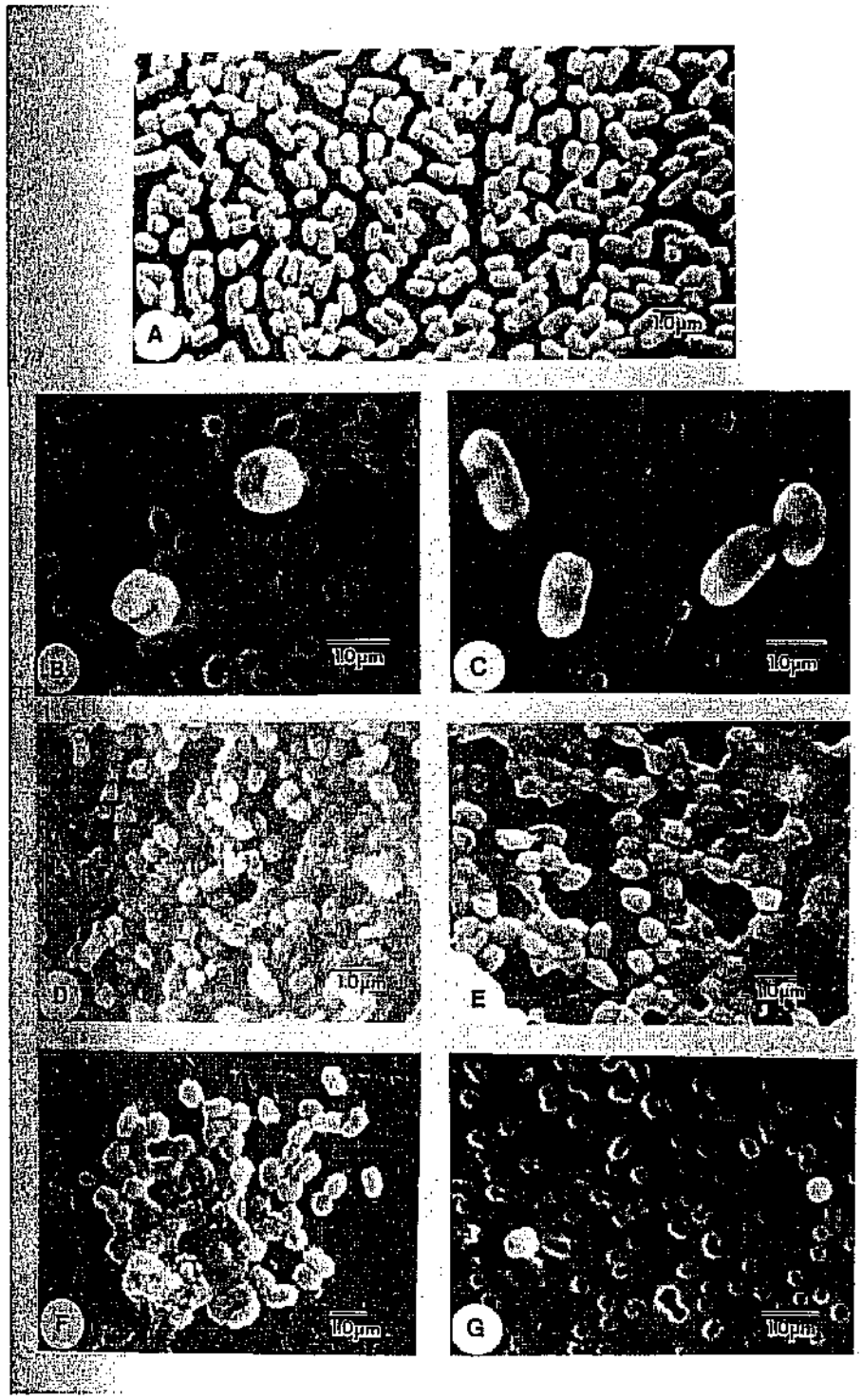

Fig. 6.1 Scanning electron micrographs of *S. typhimurium*:
- (A) pure culture
- (B) seawater microcosm at 4°C
- (C) seawater microcosm at 25°C
- (D) stationary river water microcosm at 4°C
- (E) shaking river water microcosm at 4°C
- (F) stationary river water microcosm at 25°C
- (G) shaking river water microcosm at 25°C

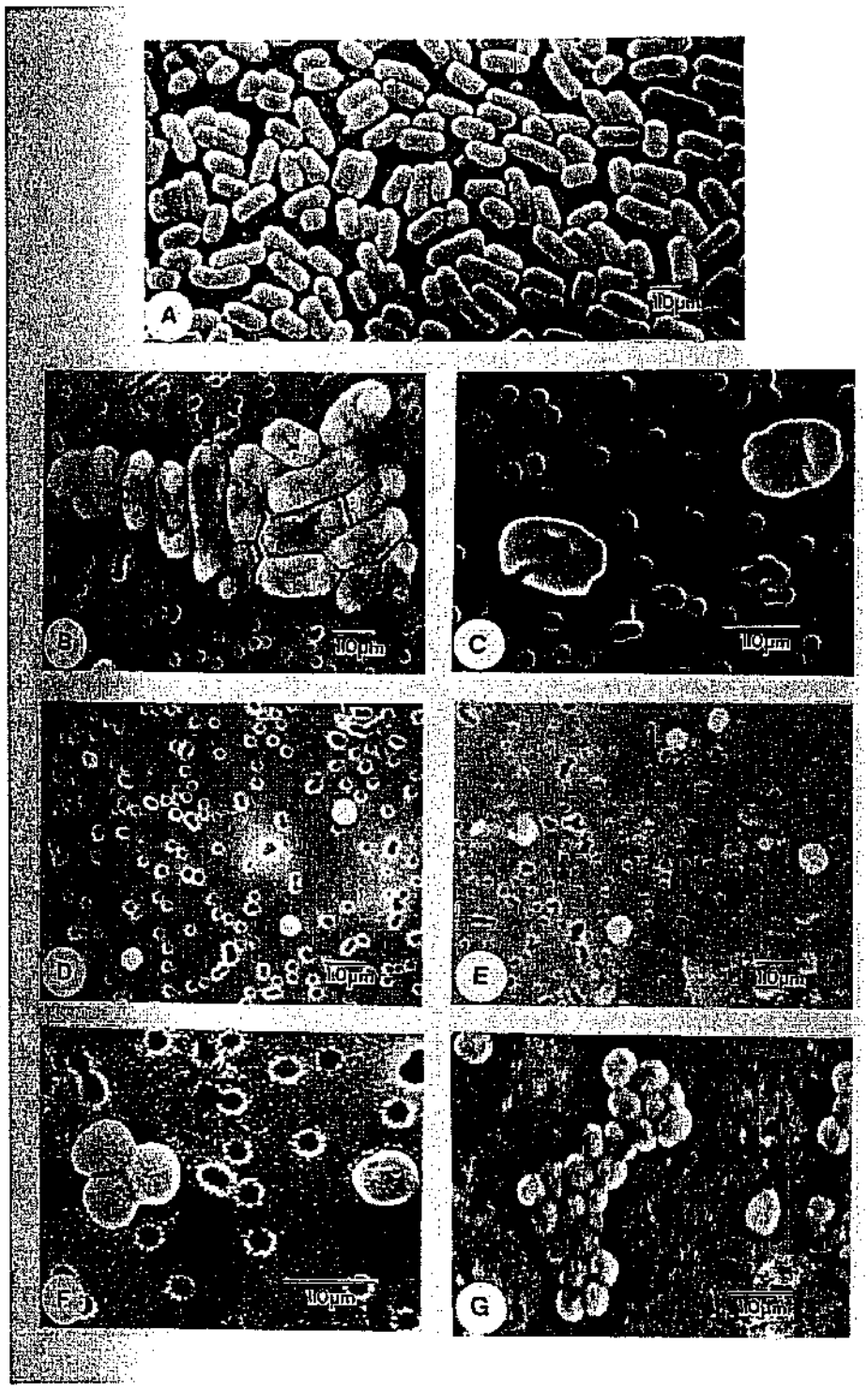

Fig. 6.2 Scanning electron micrographs of *S. typhi*

(A) pure culture
(B) seawater microcosm at 4°C
(C) seawater microcosm at 25°C
(D) stationary river water microcosm at 4°C
(E) shaking river water microcosm at 4°C
(F) stationary river water microcosm at 25°C
(G) shaking river water microcosm at 25°C

The morphology of *S. typhi* in the stationary and shaking river water microcosms at 4°C changed from the typical rod-shaped cells to rounded cells. There were dense aggregates of bacterial cells present in the river water microcosms at 4°C (Fig. 6.2D, E). *S. typhi* displayed rounding of cells once the VBNC state was attained in the river water microcosms at 25°C. The cells had rounded to the extent that their morphology was typically coccoid. Although aggregation was observed with these river microcosms at 25°C (Fig. 6.2F, G), the cells were not as tightly packed as seen at 4°C (Fig. 6.2D,E).

6.3.3 Scanning electron microscopy (SEM) of *S. flexneri*

S. flexneri, one of the *Shigella spp.* used as initial inoculum to set up the microcosms displayed the typical rod-shaped morphology associated with the pure culture (Fig. 6.3A). *S. flexneri* (Fig. 6.3B,C) in seawater microcosms displayed a tendency to round up once the VBNC state was attained. However, it was not a typical characteristic. A percentage of the population was rod-shaped even though the VBNC state was reached. Aggregates of cells formed in the 4°C and 25°C (Fig. 6.3B,C) seawater microcosms.

S. flexneri in the river water microcosms displayed definite rounding of cells (Fig. 6.3D,E,F, G). Rounding of bacterial cells was also observed for the stationary and shaking river water microcosms at 4°C (Fig. 6.3 D,E), even though the VBNC state was not reached. One could therefore infer that these bacterial cells possess the potential to reach the VBNC state. Aggregates of cells were also observed in the river water microcosms at 25°C (Fig. 6.3F,G).

6.3.4 Scanning electron microscopy (SEM) of *S. sonnei*

S. sonnei was the other *Shigella spp.* used to set up microcosms. The pure culture used as initial inoculum displayed the typical rod-shaped morphology (Fig. 6.4A). In the seawater microcosms at 4°C and 25°C some cells displayed rounding up once the VBNC state was attained. A percentage of cells was still rod-shaped, although the culture had entered the VBNC state (Fig 6.4B, C).

S. sonnei in the river water microcosms at 25°C displayed typical cocci formation once the VBNC state was exhibited (Fig. 6.4 F,G) *S. sonnei* in the river water microcosms at 4°C

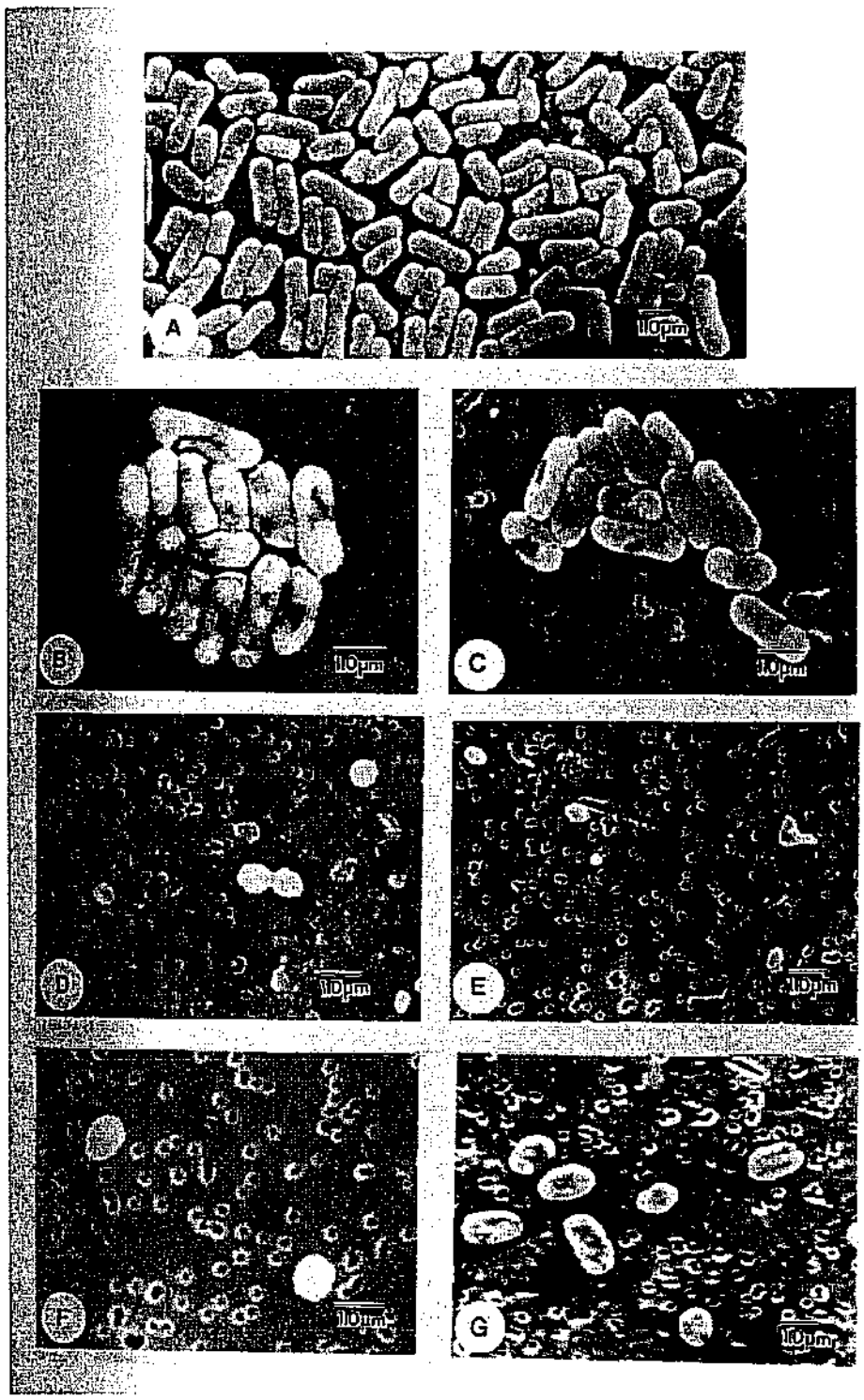

Fig. 6.3 Scanning electron micrographs of *S. flexneri*:

- **(A)** pure culture
- **(B)** seawater microcosm at 4°C
- **(C)** seawater microcosm at 25°C
- **(D)** stationary river water microcosm at 4°C
- **(E)** shaking river water microcosm at 4°C
- **(F)** stationary river water microcosm at 25°C
- **(G)** shaking river water microcosm at 25°C

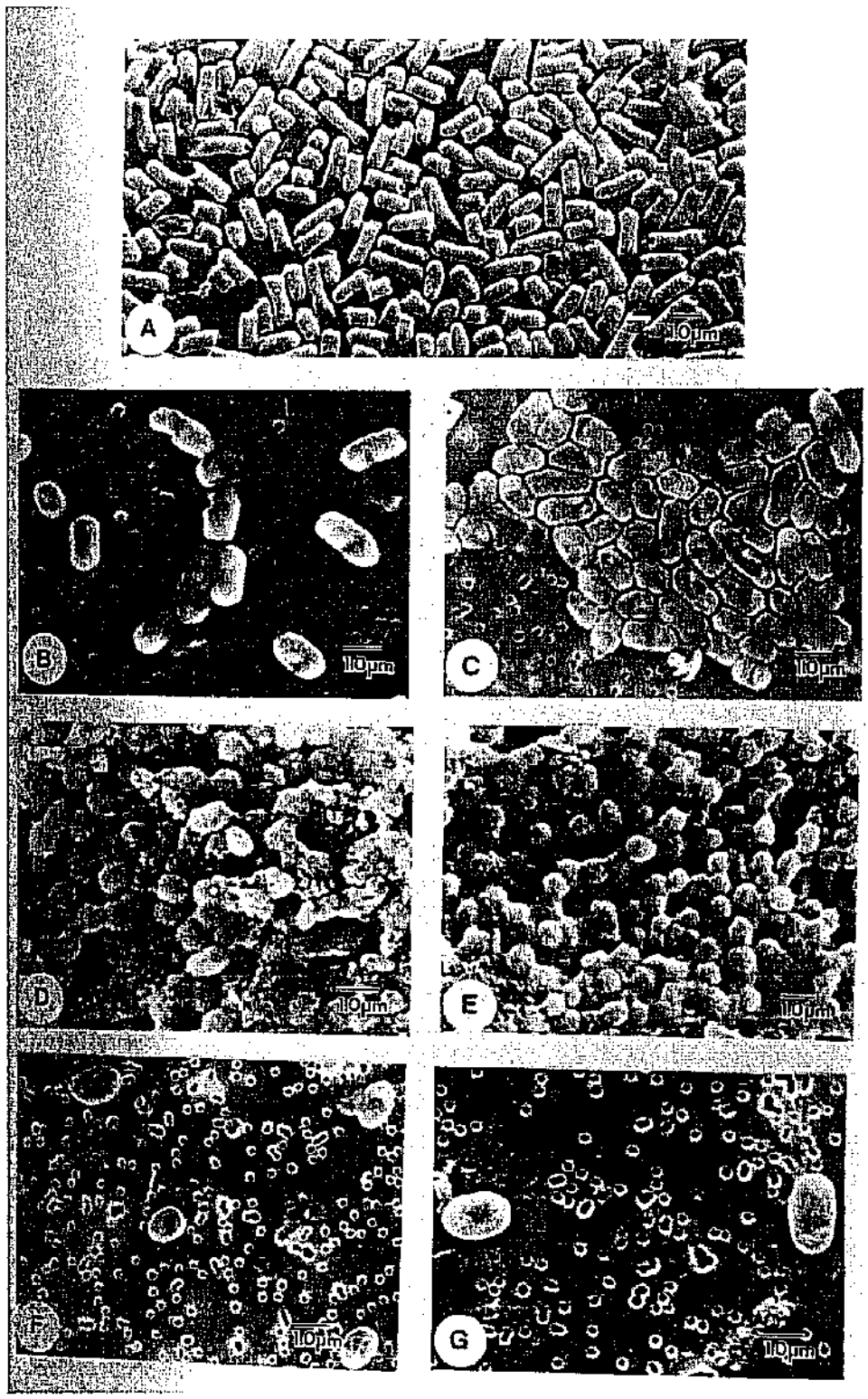

Fig. 6.4 Scanning electron micrographs of *S. sonnei*:

- (A) pure culture
- (B) seawater microcosm at 4°C
- (C) seawater microcosm at 25°C
- (D) stationary river water microcosm at 4°C
- (E) shaking river water microcosm at 4°C
- (F) stationary river water microcosm at 25°C
- (G) shaking river water microcosm at 25°C

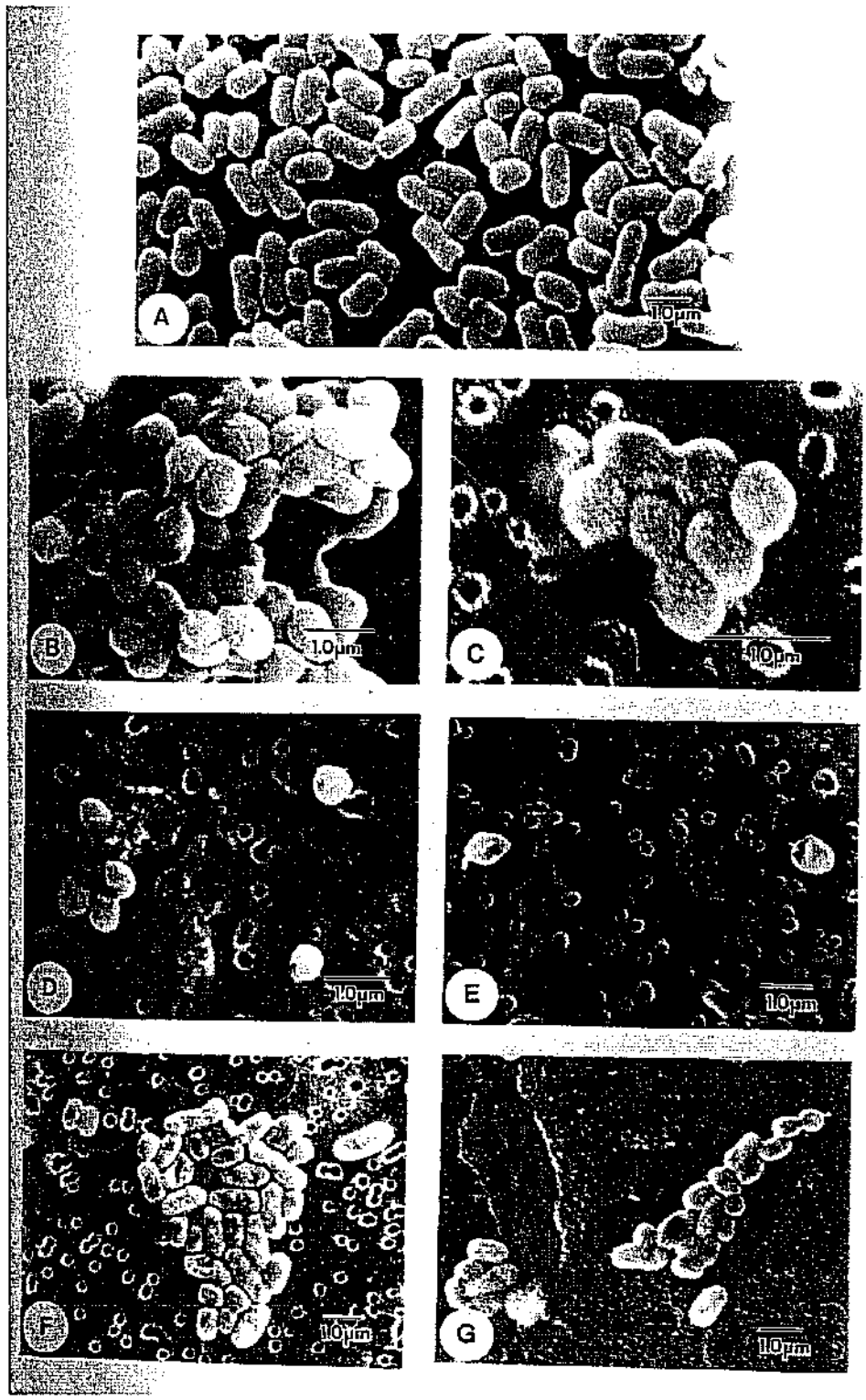

Fig. 6.5 Scanning electron micrographs of *E. coli*:

- (A) pure culture
- (B) seawater microcosm at 4°C
- (C) seawater microcosm at 25°C
- (D) stationary river water microcosm at 4°C
- (E) shaking river water microcosm at 4°C
- (F) stationary river water microcosm at 25°C
- (G) shaking river water microcosm at 25°C

displayed rounding up of the cells although these microcosm cultures did not reach the VBNC state (Fig. 6.4 D,E). Thus, one could infer that these cells have the potential to reach the VBNC state. Aggregates of the rounded cells resulted in the river water microcosms at 25°C (Fig. 6.4F,G) once non-culturability was reached.

6.3.5 Scanning electron microscopy (SEM) of *E. coli*

The typical rod-shaped *E. coli* cells used as initial inoculum for the microcosms are shown in Fig. 6.5A. This was the shape of the initial bacterial cells used to set up the microcosms. *E. coli* in the seawater microcosm at 4°C and 25°C showed the typical rounding of cells associated with the VBNC state. The cells also formed aggregates (Fig. 6.5 B,C).

E. coli bacterial cells in the river water microcosms formed rounded cells once the VBNC state was attained at 25°C (Fig. 6.5 F,G). These cells also displayed the rounding phenomenon in the 4°C stationary and shaking river water microcosm although the VBNC state was not attained (Fig. 6.5D,E). The culture in the 4°C shaking river water microcosm tended to form aggregates (Fig 6.5E).

6.4 Discussion

In a given natural environment bacteria present may or may not find conditions optimal for growth and reproduction. Some of the favourable factors are an excess or lack of water, acidity, oxygen or suitable hydrogen acceptor. However, the dominant factor is the lack of specific energy yielding substrates for the various physiological types of bacteria (Morita, 1982). When unfavourable conditions prevail, it has been reported that bacteria undergo a morphological change from rod-shaped cells to coccoid shaped cells (Linder and Oliver, 1989; Nilsson *et al.*, 1991; Novitsky and Morita, 1976). Changes initiated at the onset of starvation are associated with the depletion of cellular reserves and the nature of the marked decrease in volume is a function of the process of scavenging non-essential cellular components and material (Morita, 1982).

To study the morphological change associated with the VBNC state, the best method for SEM sample preparation had to be established. Pure cultures were initially prepared for SEM viewing by fixing in Karnovsky's fixture and osmium tetroxide. Once the suspensions were

spread onto coverslips, freeze-drying, critical point drying and air-drying of the sample were assessed. The critical point dried bacterial culture resulted in the best preparation for SEM. However, the method of Smit *et al.* (1975), where the bacterial suspension in 3% glutaraldehyde in 0.1 M phosphate buffer was fixed on ice for 30 min, proved superior and was used for all future preparations of pure cultures for SEM. This method of sample preparation was also attempted to prepare microcosm cultures for SEM. However, the microcosm cultures were dilute, thus this method could not be used. The best method of preparation for microcosm cultures was the method recommended by Todd and Kerr (1972) where the microcosm cultures were concentrated and fixed on 0.22 μm polycarbonate filters.

Novitsky and Morita (1976) investigated the various pore sizes of filters which showed the cell size distribution during starvation. Size reduction was most rapid during the first two days of starvation. After two weeks of starvation 100% of the viable cells were able to pass through 3.0 μm and 1.0 μm filters. After three weeks of starvation 100% of the viable cells were able to pass through 0.8 and 0.6 μm filters. However, at no time during the starvation were any cells able to pass through a 0.2 μm polycarbonate (Nuclepore^R) filter. Thus, polycarbonate (Nuclepore^R) filters (0.22 μm) were the filters of choice in the present study to view the non-culturable cells using SEM.

Once the best method of sample preparation was established, the morphology of the initial inoculum could be compared to the VBNC state in the microcosm. *S. typhimurium* in the seawater microcosms at 4°C displayed defined cocci-shaped cells (Fig. 6.1B). In the 25°C seawater microcosm there was a tendency to round up. However, well-defined cocci were not present (Fig. 6.2C). *S. typhimurium* in the river water microcosms gave rise to cocci-shaped cells. However, the microcosms (stationary and shaking) at 4°C and 25°C did not reach the non-culturable state but displayed rounding of cells (Fig. 6.1D, E, F. G). One could infer that although the cells are culturable, the miniaturization increases the cell surface/volume ratio. Hence its ability to scavenge energy yielding substrates from its environment will be increased (Morita, 1982).

S. typhi in seawater microcosms at 4°C and 25°C also displayed rounding up of cells once the VBNC state was attained (Fig. 6.2B, C). However, some bacterial cells tend to remain slightly elongated especially in the seawater microcosm at 4°C. In the river water microcosm

all cells rounded up, even at 4°C where the VBNC state was not attained after 138 days. Thus, the *Salmonella spp.* used as initial inoculum did not display the characteristic rounding of cells associated with the VBNC state in all microcosms. Each microcosm had influenced the bacterial culture differently. Aggregate formation was a prominent feature in the river water microcosms. The aggregate formations could have served as an advantage to the microcosm cultures at 4°C as all cultures, with the exception of *S. typhi* did not reach the VBNC state. Thus, one could suggest that the aggregates could scavenge for substrate more efficiently compared to single cells. Morita (1982) proposed that the basis for substrate capture was the organism's binding proteins that permitted the organism to capture substrate on its surface. The bacteria are embedded in a polysaccharide matrix.

A comparison of the morphological change of *Salmonella spp.* observed in the present study to other research reports on the same organism could not be made. There have been no reports dealing with the morphological change of *Salmonella spp.* observed in the VBNC state. However, Novitsky and Morita (1976) reported the rounding up of a psychrophilic marine *Vibrio* Ant 300. These researchers carried out studies of light microscopy and transmission electron microscopy. Light microscopy studies were carried out at one week and six weeks after the starvation period. A reduction in size was observed at one week and gradually progressed to the coccoid form after six weeks of starvation. In the present study, morphology of the cells was assessed only after the VBNC state was reached. However, from the above study one could infer that the change observed in this study was also a gradual change and not a drastic change from rod-shaped to coccoid-shaped.

Linder and Oliver (1989) also reported on the rounded forms of another non-culturable *Vibrio spp., viz., V. vulnificus* from artificial seawater microcosms. Transmission electron microscopy showed that membrane integrity was maintained. Although the polysaccharide external layer was retained, loss of virulence of *V. vulnificus* in the non-culturable state was observed in the mouse model. Thus, these results indicate that even though virulent (encapsulated) cells are present, virulence is greatly decreased in these cells. This suggests that the loss of virulence was due to factors other than loss of the antiphagocytic capsule. Colwell *et al.* (1985), however, showed that non-culturable *E. coli* and *V. cholerae* were recovered from ligated ileal rabbit loops in which entertoxigenicity was exhibited. Thus, it would be an interesting phenomenon to investigate virulence using the cultures in the present study. Of particular interest would be cultures in the river water microcosms at 4°C that

display coccoid shapes although still culturable. One could expect these cells to be virulent as they are still culturable. However, their reduced energy state could decrease their ability to display virulence as reported by Linder and Oliver (1989) with respect to *V. vulnificus*. On the other hand, a rich energy source could be found in the ileal rabbit loop as reported by Colwell *et al.* (1985) which could influence the bacteria to display virulence. Thus, for a definite conclusion regarding the virulence of the VBNC or long surviving bacteria in the present study, virulence has to be investigated using the cultures employed in the present study.

Nilsson *et al.* (1991) also investigated the non-culturable state of *V. vulnificus* at 5°C in artificial seawater microcosms. Their research involved resuscitation after the VBNC state was accomplished. *V. vulnificus* gradually decreased in size finally displaying the characteristic coccoid shaped cells once the VBNC state was attained. After 48 h of resuscitation at room temperature the cocci changed to rod-shaped cells. Roszak *et al.* (1984) added various concentrations of BHI broth to non-culturable *S. enteritidis* to resuscitate the cells compared to only a temperature increase used by Nilsson *et al.* (1991). Upon resuscitation of *V. vulnificus* the plate counts and AODC increased slightly compared to initial values. The larger cells observed after 48 h of resuscitation undergo reductive cell division under starvation conditions present in the microcosm.

In the present study resuscitation was not investigated because of the lengthy period required to induce the VBNC state. Nilsson *et al.* (1991) reported two cycles of non-culturability and resuscitation. The non-culturable state was reached in approximately three days. Since the cultures used in the present study displayed long survival rates in river and seawater microcosms, these cultures could pose a problem for resuscitation by temperature upshift only. All energy resources would be depleted, thus successful resuscitation would not be achieved. However, addition of a certain percentage of nutrients as broth as suggested by Roszak *et al.* (1984) may serve to resuscitate the cultures in the microcosms. Also, one could speculate that the cultures in the river water microcosm at 4°C would give rise to rod-shaped cells upon temperature upshift only. These cells are culturable although their DPC are low and displayed coccoid shape. Thus, their reduced size would have served to minimise their maintenance requirements.

The VBNC state was also investigated using *S. flexneri* and *S. sonnei*. These cultures in seawater (4°C and 25°C) and river water (25°C) entered the VBNC. The cultures in the seawater did not display distinct coccoid morphology as compared to the river water microcosms. Although Colwell *et al.* (1985) reported that *S. flexneri* and *S. sonnei* entered the VBNC state in salt water, a morphological study was not carried out. Thus, a comparison based on morphological changes could not be made. Islam *et al.* (1993) also reported that *S. dysenteriae* entered the VBNC state, however no morphological studies were carried out.
In an attempt to explain the cocci-bacilli shaped bacteria observed in certain seawater microcosms one could draw on the study of MacDonell and Hood (1982), who reported the isolation and characterisation of ultramicrobacteria from estuarine waters. Water from Perdido Bay, USA was filtered through 0.2μm polycarbonate filter. It was observed by fluorescent microscopy that various cocci and coccibacilli were present. Of the isolates 89% were initially incapable of growth in several full strength nutrient broths. A diluted broth was initially used to promote growth. The cultures underwent nutrient conditioning and finally adapted to the nutrient rich environment. The size of the bacteria also increased. Thus, the occurrence of coccibacilli in some seawater microcosms in the present study was not an unusual feature. Some of the ultramicrobacteria isolated by MacDonell and Hood (1982) in a nutrient stressed environment were coccibacilli which failed to grow in a nutrient rich environment were coccibacilli which failed to grow in a nutrient rich environment. Thus, the cocci-bacilli shape is associated with nutrient stressed cells.

Torella and Morita (1981) investigated the use of microculture technique and time lapse to follow the division of individual cells by phase-contrast photomicrography. A certain number of marine bacteria increased in size upon inoculation onto a nutrient rich agar surface. Other bacteria were identified as very small marine bacteria (ultramicrobacteria) which had a slow growth rate when inoculated on nutrient rich agar surface. The ultramicrobacteria appear as cocci cells when viewed through an optical microscope, but with transmission electron microscopy the ultramicrobacteria appear not only as cocci but also as vibrios, bacilli, horseshoes and sigmoid forms (Torella and Morita, 1981). Thus, the formation of cocci-bacilli shaped cells once the VBNC state was attained could be compared to ultramicrobacteria. From this research one could infer that viewing the ultramicrobacteria under high magnification was important to enable one to clearly distinguish morphology. In the present study SEM proved efficient in viewing the VBNC bacteria at high magnifications which enabled the clear and precise morphological study of VBNC bacteria.

The VBNC state can be associated with exogenous dormancy. Certain environmental stresses, e.g., starvation can apparently coerce bacteria to decrease in both size and activity. Release of this stress at any point would permit "normal development" (Stevenson, 1978). Thus, these bacteria were capable of considerable pleomorphisms. Cultures of marine bacteria would not divide in seawater containing limiting substrate concentrations. Below certain levels of substrate concentrations, cultures were surviving but "inactive". Dormancy may result if fluctuations in the natural environment were too severe to permit continuous growth and this would permit survival so that proliferation could ensure upon the reappearance of favourable conditions (Stevenson, 1978).

Kjelleberg *et al.* (1982) reported the effect that interfaces could have on starved marine bacteria. The copiotropic marine *Vibrio spp.* strain DW1 formed viable small cells with low endogenous respiration at interfaces, i.e., at the air water interface. A definite growth response was found at the air water interface with small starved *Vibrio spp.* It was clear that the bacteria rapidly exhausted the nutrients confined to the surface, since they exhibit a size decrease after the initial growth response. Small, starved cells need to increase their size twelve times before entering log phase of the growth curve. The surviving cells need to increase cell volume to attain the cell mass necessary to commence active growth. From this study one could perceive the importance of swirling the microcosm flasks before sampling was attempted. A false indication of the viability of cells would be achieved if sampling was carried out from the surface of the microcosm water as the bacteria at the surface rapidly exhaust the nutrients found here.

Transmission electron microscopy (TEM) study could complement SEM study. TEM preparations of pure and VBNC state of *V. vulnificus* was examined by Linder and Oliver (1989) where it was reported that the number of ribosomes in the VBNC state was reduced which was expected as the cells were in a dormant form. The nuclear region was less distinguishable. The cell wall was convoluted and pulled from the cell membrane. This was attributed to the continuous size reduction and decreasing volume of the cells.

This study showed that all bacteria in the VBNC state do not necessarily display the cocci-shaped cells conventionally associated with the VBNC state. Also, the VBNC bacteria

displayed aggregate formation, a phenomenon that has not been associated with the VBNC state.

6.5 Summary and conclusion

Under unfavourable conditions certain bacteria are reported to undergo a morphological change from culturable rod-shaped to the well-defined cocci-shaped VBNC state. In the present study SEM proved efficient in viewing the VBNC bacteria at high magnifications which enabled the clear and precise morphological study of VBNC bacteria. *S. typhimurium* in the seawater microcosms at 4°C displayed well-defined cocci-shaped cells. In the 25°C seawater microcosm, and stationary and shaking river water microcosms at 4°C and 25°C, *S. typhimurium* displayed culturable rounded cells. One could infer that although these cells are culturable, their miniaturization increases their cell surface/volume ratio and ability to scavenge energy yielding substrates from the environment. *S. typhi* in seawater microcosms at 4°C and 25°C also displayed rounding of cells once the VBNC state was reached. However, some *S. typhi* cells tended to remain elongated especially in the seawater microcosms at 25°C. In the river water microcosms all cells rounded up, even at 4°C where the VBNC state was not attained after 138 days. The cultures in the seawater did not display distinct coccoid morphology as compared to the river water microcosms.

Aggregate formation was a prominent feature in river water microcosms. *S. flexneri* and *S. sonnei* entered the VBNC state in seawater (4°C and 25°C) and river water microcosms at 25°C. The aggregate formation could have served as an advantage to the microcosms at 4°C as all cultures, with the exception of *S. typhi*, did not reach the VBNC state. Thus one could suggest that the aggregates could scavenge for substrate more efficiently compared to single cells. This study showed that VBNC bacteria form aggregates and are not necessarily coccoid-shaped.

CHAPTER SEVEN

POTENTIAL VIRULENCE OF VIABLE-BUT-NON-CULTURABLE BACTERIA IN WATER

7.1 Introduction

Environmental conditions impose a plethora of stresses on bacteria. Non-sporulating bacteria may enter a state where they become non-culturable on standard media but retain certain features of living cells such as respiratory activity and substrate uptake. This condition is now generally referred to as the "viable-but-non-culturable" (VBNC) state. It has been hypothesized that the VBNC state represents a survival strategy, adopted notably by gram negative bacteria, in response to adverse environmental conditions (Baker *et al.*, 1983; Oliver *et al.*, 1995 and Weichart and Kjelleberg, 1996). Bloomfield *et al.* (1998) proposed that the production of excessive superoxides and free radicals which cannot be readily metabolised result in cell death explaining why these cells cannot be cultured on standard rich media. Others propose that the majority of bacteria are dead but there are a few viable cells which are undetectable by standard culture techniques at least in part due to the inaccuracies of the method (Barer *et al.*, 1998; Bogosian, 1998; Bogosian *et al.*, 1998). These undetected viable cells are responsible for the effects attributed to VBNC cells (Barer *et al.*, 1998; Bogosian *et al.*, 1998; Kell *et al.*, 1998).

It is beyond the scope of this chapter to argue the merits of what is commonly known as the VBNC state. It is accepted that there is a population of cells, be they dormant or dying, which are not detected by conventional DPC techniques. It is established that using the correct techniques, at least a portion of these cells can be induced to undergo division and multiply in numbers. Furthermore, several authors have suggested that VBNC cells may resuscitate *in vivo* and are potentially pathogenic (Colwell *et al.*, 1985; Colwell *et al.*, 1996; Jones *et al.*, 1991; Oliver and Bockian, 1995; Rahman *et al.*, 1996).

The transition to non-culturability may not indicate a loss of potential virulence. If so, pathogenic VBNC bacteria may pose an under estimated public health threat. Several reports have implied that VBNC cells are potentially pathogenic (Colwell *et al.*, 1985; Hussong *et al.*, 1987; Oliver and Bockian, 1995; Rahman *et al.*, 1996; Steinert *et al.*, 1997). However, when VBNC cells were administered to animals and human volunteers conflicting results

were obtained (Beumer *et al.*, 1992; Colwell *et al.*, 1996; Hussong *et al.*, 1987; Medema *et al.*, 1992; Rose *et al.*, 1990 ; Steinert *et al.*, 1997).

In South Africa and many other developing countries, a large proportion of the rural population has no source of treated potable water but rely on natural water sources. Many of these dry to a trickle during the dry season and the lack of runoff water means low levels of nutrient input. Conditions such as these may well favour the transition of bacteria to the VBNC state. Furthermore, the surrounding area may be contaminated with dried faecal material which may pollute the water during rainfall. A preliminary survey carried out in this laboratory suggested that many local water bodies had significant numbers of bacteria in the VBNC state.

It is important, therefore, to investigate the potential pathogenicity of bacteria in the VBNC state. The present study is an investigation of the expression of certain virulence factors, viz., haemolytic and protease activity, lipopolysaccharide (LPS) patterns, and *in vivo* pathogenicity of three species of pathogenic bacteria in the VBNC state as well as their resuscitated form.

7.2 Materials and methods

7.2.1 Bacterial cultures

S. typhimurium and *S. dysenteriae* were obtained from the University of Natal Medical School, Durban and an enteropathogenic *E. coli* (strain O157 H:7) from the University of Western Cape. Colonies derived from single cells were cultured in BHI broth at 37°C with shaking. Culture supernatant was recovered by sedimenting cells at 15 000 x g for 30 min. and filtering the supernatant through a 0.45 µm filter. Pelleted cells were used as required.

7.2.2 Preparation of laboratory microcosms

Microcosms were prepared in sterile distilled water. Cells were washed twice and suspended in 250 ml sterile distilled water at final concentrations of 10^7 and 10^8 cells/ml. Microcosms were incubated at 28°C. Bacterial populations were monitored by Acridine Orange Direct Counts (AODC) (Hobbie *et al.*, 1977), Direct Viable Counts (DVC) (Kogure *et al.*, 1979) and

Dilution Plate Counts (DPC) (American Public Health Association, 1989). Microcosm supernatant was prepared by centrifugation of the whole microcosm at 10 000 x g.

7.2.3 Resuscitation of VBNC bacteria

VBNC bacteria were resuscitated by a modification of the published methods of Hobbie *et al.* (1977) and Kogure *et al.* (1979). Cells from a 250 ml microcosm were harvested and resuspended to a value of 2.5 ml of 0.025% yeast extract, 0.1mM NaOH and 10 mg/ml nalidixic acid. The suspension was incubated at 20°C for six hours in the dark after which the contents of the tube were inoculated into 100 ml BHI broth and incubated with shaking at 37°C for two days. Cells and supernatant were prepared as described above.

7.2.4 Proteolytic activity

Proteolytic activity was determined by the azocasein method of Pin *et al.* (1995). One ml of the supernatant was added to one ml of 0.8% azocasein in 2 M Tris-HCl, pH 7.0 and incubated at 37°C for 24 h. The reaction was stopped by addition of trichloracetic acid (final concentration 0.2%), filtered and absorbance of the filtrate measured at 440 nm. A unit of proteolytic activity was defined as the enzyme activity that produced an increase in the absorbance of the filtrate measured at 440 nm in one ml.

7.2.5 Haemolytic activity

The method of Datta-Roy *et al.* (1986) was modified to determine haemolytic activity. One ml of 1% whole rabbit blood in phosphate buffered saline (100mM sodium phosphate, 0.85% NaCl, pH7.2) was added to a mixture of 750 ml of microcosm supernatant and 250 ml saline to give a final NaCl concentration of 0.45%. This concentration was selected empirically as no lysis resulted from osmotic shock but there was sufficient osmotic stress to ensure that lysis could readily be determined. The supernatant from cultures was used without addition of saline. The mixture was incubated for one hour at 37°C and cells removed by gentle centrifugation (200 x g, two minutes). The amount of lysis was determined by measuring the absorbance of the supernatant at 540 nm. Results were expressed as the percentage of lysis occurring in an identical erythrocyte suspension lysed in a saline concentration of 0.425%.

7.2.6 Isolation of lipopolysaccharides

The methods of Hitchcock and Brown (1983) and Inzana (1983) were modified for the isolation of LPS. Cells from two ml of culture or 200 ml of a microcosm were washed twice with cold 0.9% NaCl and suspended in 500 ml of hot (65°C) 90% aqueous phenol, adjusted to 0.5 M NaCl and precipitated with 10 volumes of 95% ethanol. The precipitate was dissolved in 0.05 M NaCl and reprecipitated. The pellet was finally dissolved in 50 ml water and stored at -20°C.

7.2.7 SDS polyacrylamide gel electrophoresis

LPS were analysed by SDS-PAGE according to Laemmli (1970) in 15% acrylamide containing 2.7% bis-acrylamide. Samples were heated in 1% SDS and 1% mercaptoethanol and separated by electrophoresis at 25 V overnight. Separated LPS were visualized by silver staining (Manniatis, 1982).

7.2.8 *In vivo* animal studies

Three week old Wistar male rats, born of SPF (specific pathogen free) reared parents and raised in a "minimal disease area", were used in this study. Animals were free of infections of *S. dysenteriae, S. typhimurium* and enteropathogenic *E. coli*. Cells from microcosms were harvested and a sample resuscitated as described to determine the number of VBNC cells. Only those cells which elongated in the presence of nalidixic acid and fluoresced orange (RNA) following staining with acridine orange were regarded as VBNC.

Rats in sets of six were orally inoculated with one ml of a 5% solution of sodium bicarbonate followed by one ml of a bacterial suspension containing 10^5 cells. *S. dysenteriae, S. typhimurium* and *E. coli* were each inoculated in the culturable, VBNC and resuscitated forms. The control group received one ml physiological saline solution in place of the cell suspension. Those animals which showed no apparent sign of disease were euthanased seven days following inoculation. Internal organs were removed, fixed in 10% formalin and sent for histopathological examination (Veterinary Diagnostix, Pietermaritzburg, RSA). Animal experimentation was approved by the University of Durban-Westville Ethics Committee (Ref. 97175).

7.3 Results

The survival pattern in microcosms with different initial inoculum concentrations of bacteria were similar except that the microcosms with the lower cell concentrations reached equilibrium slightly sooner. Viability and/or activity as measured by DPC, AODC and DVC decreased rapidly in all microcosms. Decrease in DPC was more rapid than total cell counts (AODC) and active cell counts (DVC). Fifty percent DPC was reached within four, 11 and 13 days for *S. dysenteriae*, *E. coli* and *S. typhimurium* respectively. The VBNC state was accepted as a DPC of <0.1 cell/ml and was reached after 114 days for both *S. dysenteriae* and *E. coli* and 128 days for *S. typhimurium* (Fig. 7.1). The VBNC cells could be resuscitated using the method described to develop into apparently normal healthy colonies.

Haemolytic and proteolytic activities were present in the supernatant of normal cultures in all three bacterial species tested. Similar activity was also present in the microcosm supernatants but decreased with time (Fig. 7.2). By day 44 neither haemolytic nor proteolytic activity was detectable in any of the microcosms. However, resuscitated VBNC cells demonstrated both proteolytic and haemolytic activities comparable with that of normal cells (Fig. 7.3).

LPS were readily isolated from culturable and resuscitated VBNC bacteria. As expected the electrophoretic profiles of the different species differed but the LPS profiles of normal cultured cells and the resuscitated cells were visually indistinguishable (Fig. 7.4). However, no LPS could be detected in extracts of the VBNC bacteria despite gross overloading of the gels.

Transmission electron microscopy was performed on the microcosm pellet to ensure that the cells had reached the VBNC state. The results (not shown) revealed the gross changes in morphology of VBNC cells reported by other authors (Linder and Oliver, 1989; Rollins and Colwell, 1986 and Hood *et al.*, 1986). There was a change to a coccoid form and decrease in cell volume as well as the loss of the electron dense envelope. This indicated that the cells had indeed entered the VBNC state.

Table 7.1 shows the percentage of rats developing infection following inoculation with 10^5 cells of each of the three bacterial species. Normally cultured and resuscitated cells were highly pathogenic. *Post mortem* histological studies (results not shown) revealed multifocal

colonic epithelium erosion (Fig. 7.5) with mucosal oedema and submucosal hyperplasia together with renal tubular nephrosis in rats (Fig. 7.6) inoculated with culturable and resuscitated *S. dysenteriae*. Rats inoculated with culturable and resuscitated *S. typhimurium* demonstrated *inter alia* erosion of small and large intestinal mucosa and multifocal hepatic necrosis consistent with *S. typhimurium* septicaemia. Similarly, *E. coli* in normally culturable and resuscitated forms caused intestinal lesions. This pathology is consistent with infection by *S. dysenteriae*, *S. typhimurium* and enteropathogenic *E. coli* (Veterinary Diagnostix, Pietermaritzburg, RSA). Control rats and animals inoculated with VBNC cells had no visible pathology on histological examination.

Table 7.1 Survival of rats infected after ingestion of (A) culturable, (B) VBNC and (C) resuscitated VBNC bacteria.

Organism	Mortality (%)	Morbidity (%)	Tot. no. of rats infected (%)
S. dysenteriae			
A	66.7	16.7	83.3
B	0.00	0.00	0.00
C	50.0	33.3	83.3
S. typhimurium			
A	83.3	0.00	83.3
B	0.00	0.00	0.00
C	50.0	33.3	83.3
E. coli			
A	33.3	33.3	66.7
B	0.00	0.00	0.00
C	16.7	50.0	66.7
CONTROL	0.00	0.00	0.00

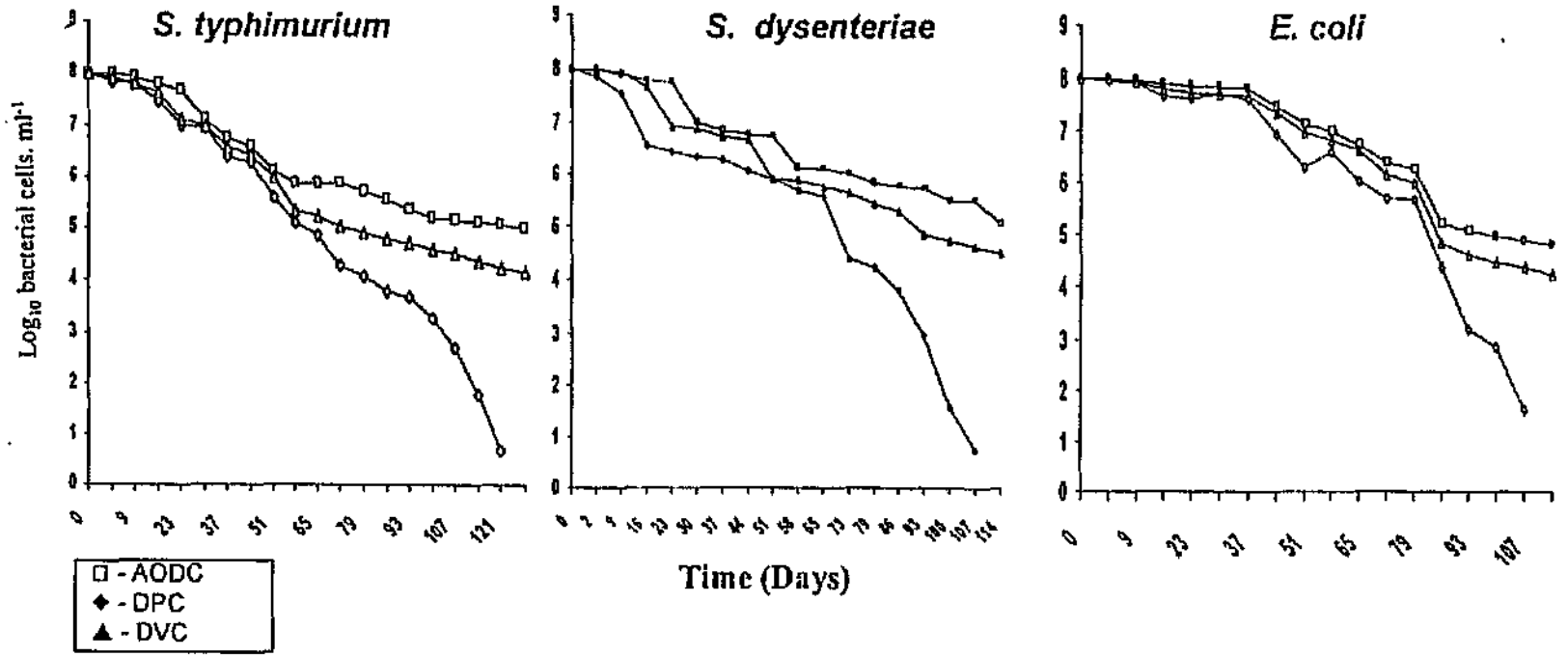

Fig. 7.1 Survival of bacteria in microcosms

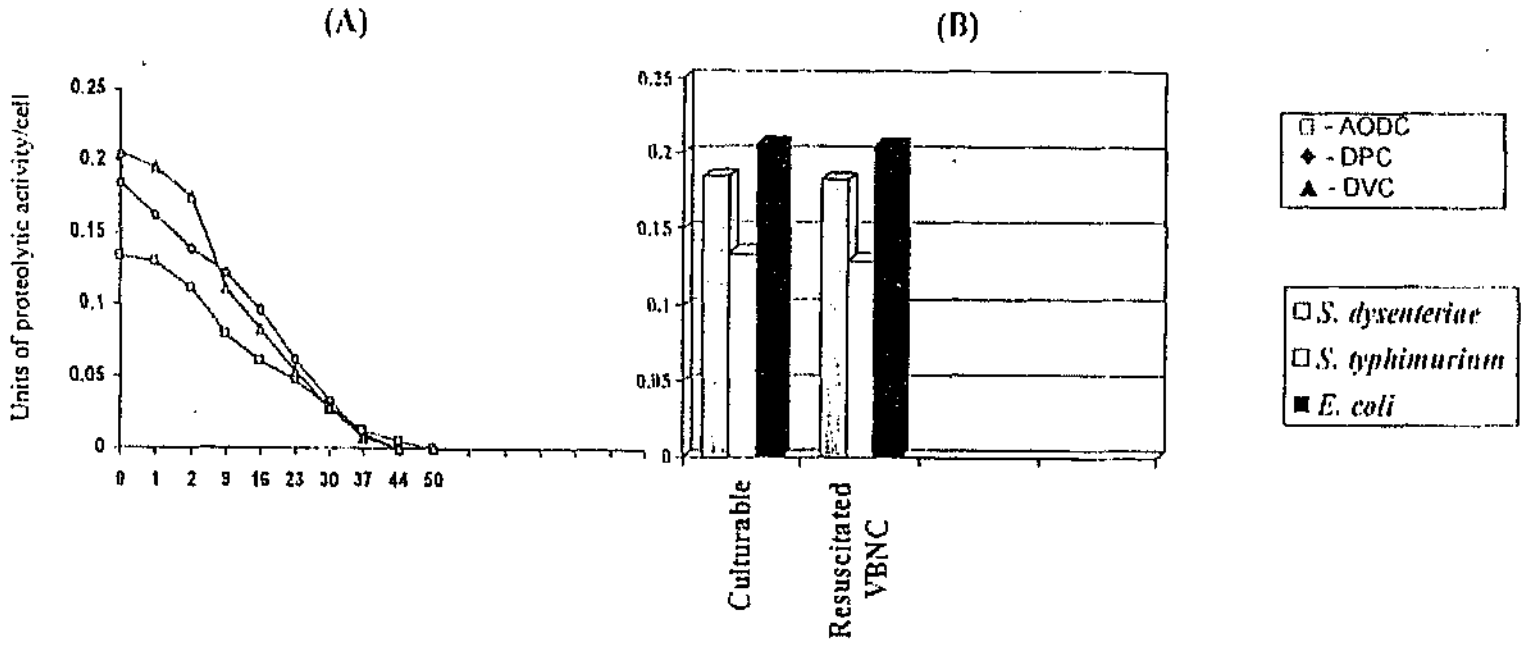

Fig. 7.2 Proteolytic activities of bacteria during (A) transition to the VBNC state and (B) culturable and resuscitated VBNC

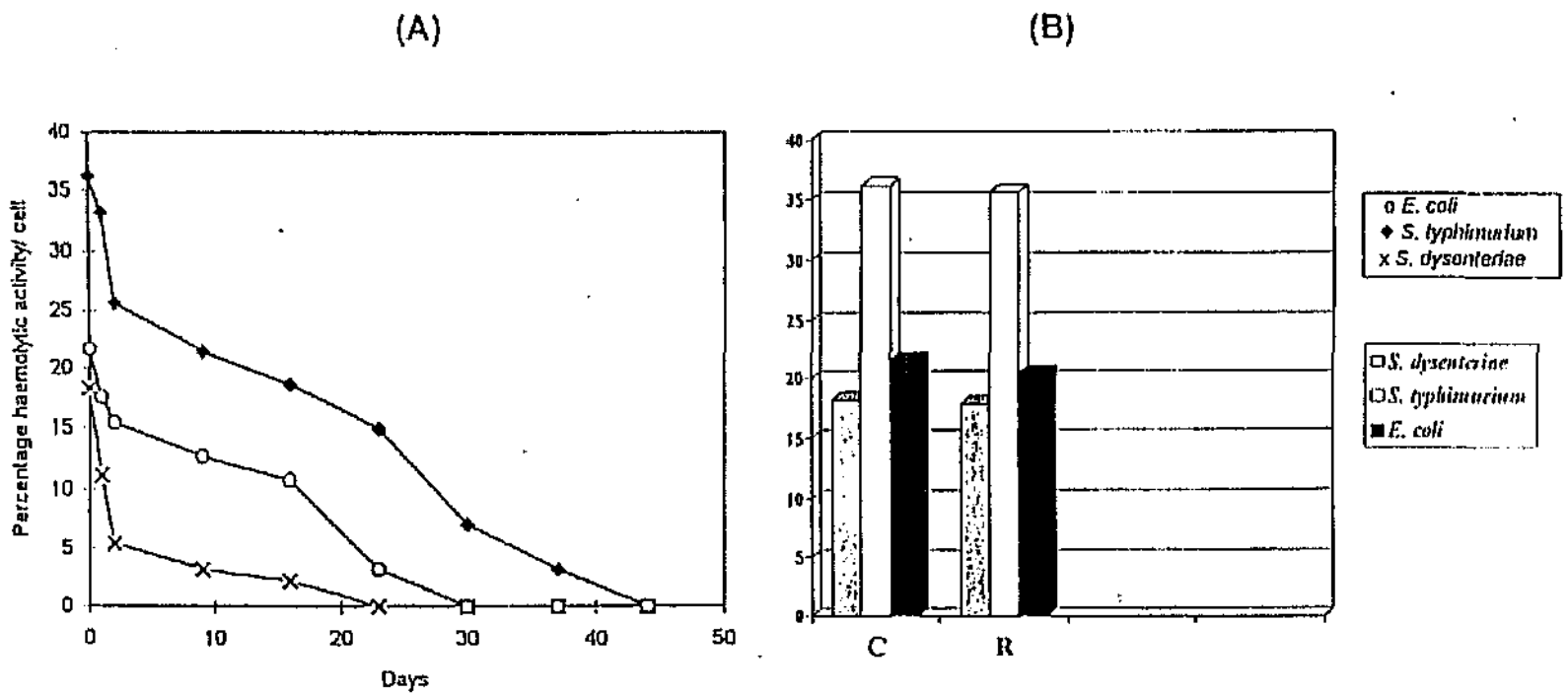

Fig. 7.3 Haemolytic activities of bacteria during (A) transition to the VBNC state and (B) culturable and resuscitated bacteria

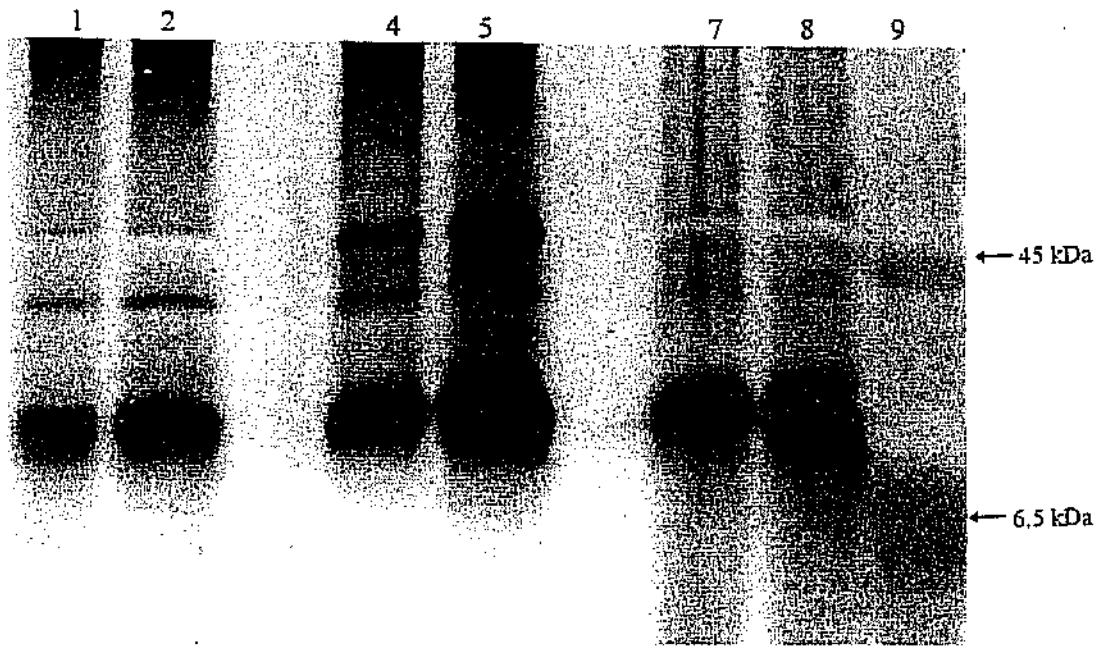

Fig. 7.4 **LPS profiles of culturable (lanes: 1, 4 & 7), and resuscitated VBNC cells (lanes: 2,5 &8) of *S. dysenteriae, S. typhimurium* and enteropathogenic *E. coli*, respectively; wide range Sigma coloured marker C3437 (lane 9: 116kDa/66kDa/45kDa/29kDa/20.1kDa/14.2kDa /6.5kDa)**

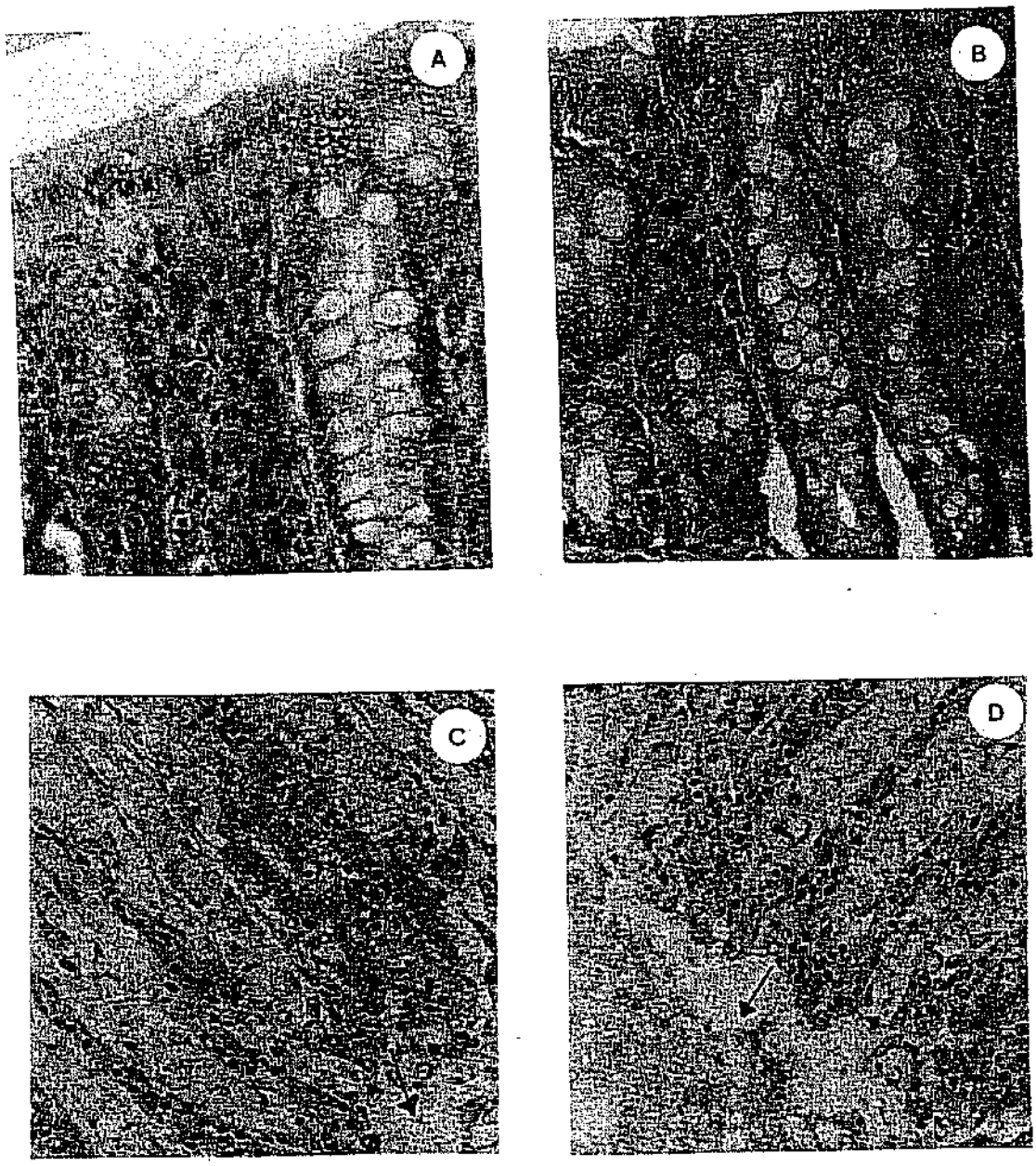

Fig. 7.5 **Large intestine of rats administered with *S. dysenteriae* indicating colonic erosion (shown by arrows) caused by culturable and resuscitated VBNC cells. (A) control, (B) VBNC cells, (C) culturable cells and (D) resuscitated VBNC cells (40x)**

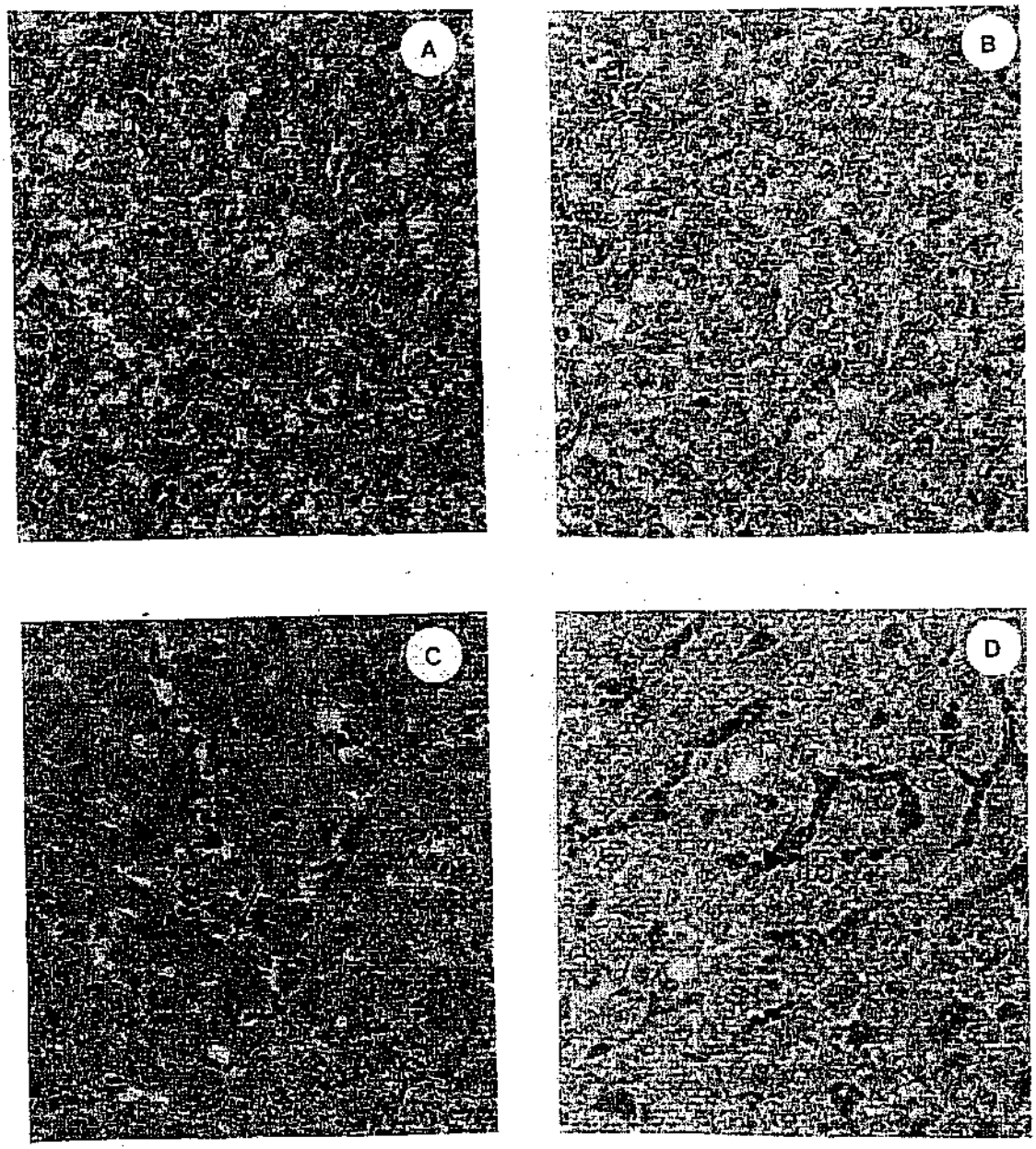

Fig. 7.6 **Liver of rats administered with *S. dysenteriae* indicating hepatic degeneration (shown by arrows) caused by culturable and resuscitated VBNC cells. (A) control, (B) VBNC cells, (C) culturable cells and (D) resuscitated VBNC cells (40 x).**

7.4 Discussion

It is now well established that bacteria maintained in microcosms enter a state where they cannot be readily cultured using standard conditions, i.e., the VBNC state. However, the true meaning of this state remains unclear.

Preliminary results from this laboratory suggest that the VBNC population of local natural water sources are a high proportion (up to 25%) of the total population. Some pathogenic bacteria also enter the VBNC or dormant state and, from a practical point of view, it is important to know the implications for public health. Certain non-specific indicators of virulence (haemolytic and proteolytic activity and the LPS) during the development of the VBNC state has been followed. Haemolytic and proteolytic activity declined rapidly paralleling the fall in DPC. LPS could not be detected by conventional extraction (phenol) and analysis (SDS PAGE and silver staining) after the cells had reached the VBNC state. It would appear therefore that these virulence factors are a feature of normally culturable cells, suggesting that VBNC *S. typhimurium*, *S. dysenteriae* and *E. coli* are non-pathogenic. This was confirmed by the administration of these cells to rats. At the dose used, normally culturable cells, resulted in 60 to 90% infection, causing severe morbidity and 30 to 80% mortality. At the same dose VBNC cells had no apparent effect and histological examination revealed no aberration of the internal organs. No culturable cells were recovered from the stools or intestinal organs of VBNC inoculated animals.

The findings of the study are in keeping with other authors who were unable to cause morbidity with VBNC cells. VBNC *Aeromonas salmonicida* caused no infection in salmon (Rose *et al.*, 1990). *Campylobacter jejuni* VBNC cells administered to day old chicks had no apparent effect and culturable cells were not recovered from the caecum (Medema *et al.*, 1992). Beumer *et al.*, (1992) were unable to induce normal culturability in, or pathogenicity of, VBNC *C. jejuni* administered to human volunteers.

However, these results are in sharp contrast to those of other laboratories. When VBNC *V. cholerae* and *E. coli* were introduced into rabbit ileal loops, normally culturable cells were recovered (Colwell *et al.*, 1985). Mortality in chick embryos was reported following inoculation with VBNC *Legionella pneumophila* (Hussong *et al.*, 1987). Administration of attenuated *V. cholera* 01 VBNC cells to two human volunteers resulted in diarrhoea in one,

and both subjects passed normally culturable *V. cholerae* in their stools. However, with an older preparation of VBNC cells, none of seven volunteers developed diarrhoea or passed detectable normally culturable *V. cholerae* in their stools (Colwell *et al.*, 1996).

Much of the work on resuscitation and particularly the work by Colwell *et al.* (1996) has been criticised on the basis that there could be undetected normally culturable cells in the inoculum used (Barer *et al.*, 1998; Bogosian, 1998). In this study the selection of VBNC cells for inoculating rats was based on the ability of cells to elongate and take up acridine orange in the presence of nalidixic acid. When resuscitated cells were inoculated into broth in lower numbers than given to the rats, normally culturable cells would develop and could be grown in broth and on agar plates. Rats were inoculated with cells which demonstrated all the potential to return to the normally culturable state but no morbidity was detected. We do not believe that this was due to insufficient normally culturable cells in the inoculum but rather to the inability of the VBNC cells to resuscitate in the rat intestine.

The results of the study suggest that pathogenicity is associated with culturability following resuscitation from the VBNC state. These findings support previous reports in which laboratory resuscitation of VBNC cells has been commonly demonstrated in numerous organisms with many indications of potential pathogenicity (Colwell *et al.*, 1985; Colwell *et al.*, 1996; Jones *et al.*, 1991; Oliver and Bockian, 1995; Rahman *et al.*, 1996). Using the nalidixic acid technique it has been confirmed and extended this observation. Proteolytic and haemolytic activity were regained to normal levels and the LPS profile returned to an apparently normal state after resuscitation. In animal studies resuscitated cells had the equivalent pathogenicity to normally culturable cells. In all accounts then the resuscitated cells appear to regain their original state.

Recently, Mukamolova *et al.* (1998) have suggested that "rather strictly defined parameters" may be required for the successful resuscitation of bacteria. Research by other authors suggest that these conditions include temperature shifts (Whitesides and Oliver, 1997) and low levels of nutrient (Colwell *et al.*, 1985). In arid areas nutrients may be supplemented by run off after rainfall. Conditions such as these may trigger the resuscitation of VBNC cells, be they dying or dormant. While it appears that VBNC cells *per se* are non-toxic, these results suggest that VBNC cells of at least *S. dysenteriae*, *S. typhimurium* and enteropathogenic *E. coli* have the potential to resuscitate and become infective. In many

developing countries of the world, the high levels of poverty and lack of education render many communities vulnerable to this potential threat. Further studies need to be conducted on the conditions which lead to the resuscitation of VBNC pathogenic bacteria.

7.5 Summary and conclusion

Since some pathogenic bacteria also enter the VBNC state this may raise important health concerns. VBNC *S. typhimurium*, *S. dysenteriae* and *E. coli* were administered to rats. At the dose used, normally culturable cells resulted in 60 to 90% infection, causing severe morbidity and 30 to 80% mortality. At the same dose VBNC cells had no apparent effect and histological examination revealed no aberration of the internal organs. No culturable cells were recovered from the stools or intestinal organs of rats inoculated with VBNC bacteria. The VBNC state cells administered to rats did not return to culturability because of their inability to resuscitate in the intestine. This, together with the result that the LPS of the cells in the VBNC state could not be detected by SDS-PAGE, confirmed that these VBNC state cells were non-pathogenic.

However, pathogenicity was associated with culturability following laboratory resuscitation of VBNC bacteria. Proteolytic and haemolytic activity and LPS profiles returned to normal after laboratory resuscitation. When inoculated into rats, laboratory resuscitated VBNC cells had the equivalent pathogenicity effects as normally culturable cells. Although VBNC cells *per se* are non-toxic, VBNC cells of at least *S. typhimurium*, *S. dysenteriae* and enteropathogenic *E. coli* have the potential to resuscitate and cause infection. Thus, further studies need to be conducted on conditions which induce resuscitation of VBNC pathogenic bacteria.

CHAPTER EIGHT

CONCLUSIONS AND RECOMMENDATIONS

8.1 Summary of major findings and conclusions reached

8.1.1 Factors affecting the transition of culturable pathogens to the VBNC state

- The modified methods of Islam *et al.* (1993), Nilsson *et al.* (1991) and Xu *et al.* (1982) that were used to set up laboratory microcosms, successfully induced the VBNC state in *Salmonella typhimurium*, *Salmonella typhi*, *Shigella flexneri*, *Shigella sonnei*, and *Escherichia coli*. It was established that temperature, river water, seawater, salinity and inoculum concentration had a differential effect on the transition of the individual bacterial pathogens into a VBNC state.

8.1.2 Survival of VBNC bacteria in various microcosms using conventional methods

- The acridine orange direct counts (AODC), direct viable counts (DVC) and direct plate counts (DPC) were reliable and accurate in monitoring the transition of culturable cells to the VBNC state. AODC, DVC and DPC methods were complementary in enumerating the total bacterial population, viable population and the culturable population, respectively. The VBNC state of the cultures was confirmed when the DVC was <0.1 cells.ml^{-1}.
- None of the cultures reached the VBNC state in pond water microcosms after 140 days at either 4°C or 25°C.
- In river water microcosms four cultures did not reach the VBNC state at 4°C, the exception being *S. typhi*, that required 138 days. At 25°C all cultures, with the exception of *S. typhimurium*, reached the VBNC state within 83-145 days.

- In seawater microcosms incubated at 4°C and 25°C all 5 organisms required between 90-111 days to attain the non-culturable state. The only exception to this was *S. flexneri* that reached the VBNC state in only 67 days at 25°C. *S. sonnei* was observed to be non-culturable in 105 days at 4°C compared to 110 days at 25°C. There was no significant difference ($P<0.05$) between the DPC and DVC at both temperatures.
- *E. coli* displayed a higher survival rate at 4°C than at 25°C; the VBNC state was reached in 105 days at 4°C and in 90 days at 25°C. An interesting observation for both *S. sonnei* and *E. coli* in the 4°C seawater microcosm was that the AODC, DVC and DPC yielded similar results from day 1 through to day 68. This suggests that the entire total population of cells were culturable and viable for almost 7 weeks in the microcosm at 4°C.

8.1.3 Detection of VBNC bacteria in microcosms using molecular methods

- Primers specific to the origin of replication of *S. typhimurium* were used to successfully amplify a 163 bp product in culturable cells of *S. typhimurium* and *S. typhi*. The amplification product of *S. typhimurium* exhibited specificity as a hybridization probe for the detection of both *S. typhimurium* and *S. typhi* DNA. No hybridization signal was obtained for this probe against the DNA of *E. coli, S. sonnei and S. flexneri*.
- Primers specific to the regulatory region of the *uid* A gene of *E. coli* successfully amplified this gene in culturable cells of *E. coli, S. sonnei and S. flexneri*. The amplified product of *E. coli* exhibited specificity as a hybridization probe for the detection of *E. coli, S. sonnei and S. flexneri* DNA. No hybridization signal was obtained for this probe against the DNA of *S. typhimurium* and *S. typhi*.
- The use of polycarbonate and fluoropore filters in concentrating bacterial cells from seawater microcosms for PCR was unsuccessful.

This was probably due to the fact that PCR is inhibited by some filters as well as the salts present in seawater.

- Dialysis of seawater microcosm samples prior to being subjected to PCR resulted in positive amplification in culturable cells for all seawater microcosms.
- Only VBNC *S. flexneri* and *S. sonnei* were detected by the PCR/hybridization method in shaking and stationary river water microcosms.
- In seawater microcosms only *S. flexneri* and *S. sonnei* were detected by the PCR/hybridization method.
- In general, the culturable bacteria were more easily and consistently detected than their non-culturable counterparts by the PCR/hybridization method.
- Results indicate that the detection of DNA by the PCR/hybridization method was constrained by the quality rather than the quantity of DNA in the respective analyses.

8.1.4 Potential virulence of VBNC pathogens

- The transition of pure microcosm cultures of *S. typhimurium*, *S. typhi*, *S. flexneri*, *S. sonnei*, and *E. coli* to the non-culturable state was accompanied by a change from rod-shaped cells to small oval cells. A concomitant decrease in proteolytic and haemolytic activity to undetectable levels accompanied this transition. In addition, LPS was not detected in the VBNC bacteria. When VBNC cells, at a concentration of $1x10^5$ cells.ml^{-1}, were inoculated into Wistar rats they were found to be non-pathogenic.
- However, when resuscitated to the culturable state VBNC cells regained both proteolytic and haemolytic activities and the LPS profiles were normal. Moreover, these resuscitated VBNC bacteria were infective in rats to the same extent as that of the culturable controls.

8.1.5 Prevalence and survival strategies of VBNC pathogens in untreated drinking water supplies

- Analysis of river water samples in the vicinity of informal settlements indicated the presence of high numbers of coliform, faecal coliforms, *Shigella* spp., and *Salmonella* spp. The VBNC population ranged between 20-50% of the total bacterial counts.
- Untreated rain water supplies at schools and clinics in KZN exceeded the maximum allowable limit for culturable coliforms as well as faecal coliforms. These counts were relatively higher following resuscitation of the non-culturable bacteria, including *Salmonella* spp. and *Shigella* spp.
- Groundwater samples from Harding and Hibberdene demonstrated the presence of a very high proportion of VBNC bacteria. The VBNC population ranged from 52- 87% on various diagnostic media.

8.2 Review of the project in terms of the final objectives

8.2.1 Evaluation of the application of conventional and molecular methods for the detection of VBNC pathogens in surface and groundwater

- A combination of the AODC, DVC and DPC methods proved very effective in detecting the presence of VBNC in microcosms. These methods were also able to monitor the transition of culturable cells to the non-culturable state.
- The PCR/hybridization method was inconsistent in detecting the presence of VBNC cells. Nevertheless, it should be noted that this method was very rapid, accurate and consistent in detecting culturable cells.
- Therefore, conventional and molecular methods meet the final objective of detecting VBNC pathogens in surface and ground water.

8.2.2 Assessment of the fate and survival of pathogenic bacteria in the surface and sub-surface environments

- Untreated drinking water supplies were unfit for human consumption in many of the areas investigated. A further concern is the survival of significant numbers of pathogens in the VBNC state.
- The findings of this study highlight the fact that bacteria, including pathogens, can survive for long periods in the surface and sub-surface environments.
- Therefore, this final objective was met.

8.2.3 Determination of the virulence properties of VBNC pathogens

- The results suggest that although they themselves do not resuscitate in the intestine, VBNC pathogens have the capacity to cause infections and deaths equal to that of normally virulent culturable cells. These findings clearly support the hypothesis that VBNC pathogens pose a potential danger to human health, although they themselves do not resuscitate in the intestine.
- This final objective has not been completely met. Therefore, to fulfil this objective, further studies need to be conducted on the conditions which lead to the resuscitation of VBNC pathogens.

8.3 Recommendations for future research

- Studies should be conducted on whether VBNC bacteria resuscitate in household containers when sufficient nutrients and incubation time are available. This is important in rural areas where people store water for long periods in used containers that may have a high nutrient content.
- Research needs to be done on whether VBNC bacteria are more resistant to chlorination than normally culturable bacteria. This research will help elucidate if normal chlorine-containing household detergents, like JIK, can be used in rural areas to eradicate VBNC bacteria from drinking water.

- The PCR/hybridization method needs to be optimised for the microbiological analysis of untreated water samples. Pre-treatment of the samples may be necessary to remove inhibitors of PCR.

8.4 Recommendations for technology transfer

- The prevalence of large numbers of potential pathogens in untreated water must serve as an early warning to put into place measures that will prevent a recurrence of water-borne epidemics in our country. Therefore, efforts should be made to transfer the technology of detecting VBNC pathogens to both the formal and informal sectors of the water industry. This can be implemented via the hosting of technology transfer workshops by researchers. These workshops should make the relevant authorities in the management and supply of untreated water aware of the urgency in including VBNC analyses in routine water testing.
- Innovative strategies are needed for cost-effective testing and treatment of contaminated drinking water supplies, especially in rural areas. As shown in this study, conventional microbiological tests for coliforms and faecal coliforms do not accurately estimate the bacterial load. One strategy should include the resuscitation of VBNC bacteria, which is efficient, economical and most importantly provides results within 24 hours. In rural areas inhabitants should be educated on treating drinking water by first boiling it to eradicate most of the pathogenic bacteria.
- Researchers need to create a database including current information on water-borne diseases, especially in rural areas. This archive should include data on VBNC bacteria and strategies to map the microbiological quality of drinking water supplies. The data must be readily accessible to other researchers and the public via the computer, pamphlets and posters. This initiative should culminate in community workshops hosted by researchers, specifying the prevalence of VBNC pathogens in water supplies.

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