MONASH UNIVERSITY

THESIS ACCEPTED IN SATISFACTION OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

ON..... **3 August 2001** .....

.....

Sec. PhD. and Scholarships Committee

Addendum

Page 4 paragraph 1 line2 Brock and Madigan, (1994) should be Brock and Madigan, (1999)

Page 4 paragraph 2 Add the following sentence to the end of this paragraph. " However, despite their non-living status, it is now recognised that they may play an important role in the growth control of the other groups (Wommack *et al.*, 1998)."

Page 8 New **Aerobe** section

Page 9 New **Anaerobic** and *Nitrate reduction* section

Page 10 paragraph 2 line 4 respiration should be reduction

Page 12 New *Cyanobacteria* section

Page 15 The paragraph at the back of page 14 should be read at the end of section 1.4.4

Page 19 Add the following references (Suttle, 1994; Wommack *et al.*, 1998), and referral See section 1.4.4

Page 25 The paragraph at the back of page 24 should be read before section 1.7.1

Page 27 paragraph 2 line 7 Add reference (Sinton *et al.*, 1998)

Page 27 Add the following sentence and reference to the end of paragraph 3."Recently, human specific bifidobacterium has shown promise as an indicator of diffuse faecal contamination, however further methodological development is still required (Rhodes and Kator, 1999)

Page 34 paragraph 2 line 3 Add reference (Faude and Hoefle, 1997)

Page 36 point (1) line 7 Add reference (Lee and Furham, 1990) at the end of "species composition"

Page 36 point 1 line 8 Add reference (Palmer *et al.*, 1994) at the end of this sentence.

Page 36 point (2) line 6 Add reference (Torsvik, 1990) at the end of this sentence.

Page 38 paragraph 2 line 10 %S should read as 5S

Page 39 paragraph 3 line 9 Add additional references (Chandler *et al.*, 1997; Crump *et al.*, 1999)

Page 39 paragraph 4 line 4 "in section 1.x" should read as "on page 42"

Page 40 point (2) Add the following sentence and references to the end of this point: "A similar approach was also employed to examine populations of sulphate reducing bacteria, methane producers and methane oxidising communities (Edgcomb *et al.*, 1999; Edwards *et al.*, 1999)."

Page 42 The paragraph at the back of page 41 should be read before the section on ***polymerase chain reaction***

Page 42 New paragraph paragraph 3 under ***polymerase chain reaction***

Page 44 Read paragraph (1) at the back of page 43 before ***Denaturing Gradient Gel Electrophoresis***

Page 44 New ***Denaturing Gradient Gel Electrophoresis*** section.

Page 44 Read paragraph (2) at the back of page 43 before ***1.10.4 Flow Cytometry***

Page 45 Read page on the back of page 44 before reading section 1.10.5

Page 46 Read page on the back of page 45 before reading section 1.10.6

Page 53 Paragraph 2 line 4 Sonication was performed at 50% duty, setting 5, for 30 seconds.

Page 54 paragraph 1 line 10 Serial dilution was carried out with the media not containing the agar component.

Page 60 New map

Page 96 Add the following sentence to the end of line 6.."Fermentation processes are not accounted for with CTC method as these organisms under these conditions do not use the electron transport chain. For example, *E. coli* under anaerobic conditions will not reduce CTC even though they are metabolically active.

Page 98 line 23 "underestimate of production shaould read as "overestimate of production"

Page 106 paragraph 1 line 7 "(diluted 1:4 with dH_2O)" should read as "(original stock 20xSSC diluted 1:4 with dH_2O)"

Page 106 paragraph 1 line 10 "The vials" should read as "The decay in the vials"

Page 108 paragraph 1 line 1 "The samples were" should read as "Decay was"

Page 126 Table 2.3 "Production (cells.L-1.hr-1)" should read as "bacterial growth rate (cells.L-1.hr-1)"

Page 148 An extra paragraph has been added at the bottom of this page.

Page 232 New **Reference** section added

Microbial Populations as Indicators of River 'Health'

Jennifer Petronella Driessen B.Sc.(Hons.)

A thesis submitted in total fulfilment of the requirements for the degree of Doctorate of Philosophy

Water Studies Centre and CRC for Freshwater Ecology

Department of Chemistry

Monash University

Clayton, Victoria 3168

Australia

March 2000

Declaration

I Jennifer Driessen, declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other institution and affirms that to the best of my knowledge, the thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Acknowledgments

I would like to thank the following people who, without their help, this thesis would not of been possible.

My supervisor Professor Barry Hart, for being very patient throughout the entire ordeal and for being so good at getting drafts back, even if the comments were sometimes impossible to read.

My co-supervisor Jenny Ross, who's help in the early days was much appreciated.

The following people from the Water Studies Centre: Reshmi Sharma for all her help and advice with SdFFF; Mike Grace who was always willing to help with anything; Peter Pollards enthusiasm and direction with thymidine; Ashley Liang and Sandra Sdrauling for the nutrient analysis; Chris Walsh for the much needed help with statistics; Jason van Berkel; Sophie Bourgues; The morning tea crew - Belinda Lovell, Brett Cole, Claire Sellens, Janelle Boyle.

Sally Bagg and and Rory Nathan at Sinclair Knight and Mertz for their help with the flow data

A very big thank you to Gillian Beattie, who not only was a great lab, office and field work companion but a fantastic housemate and friend. Without her unfailing support, understanding and encouragement when times were bad this thesis probably never would of been completed.

Stephen Cambridge who was a great help with the final editing and finishing touches and who has made me so happy I could burst. I love you.

My wonderful family, (Mum, Dad and Eric) I can never thank you enough for all your love, patience and support, and for always being there for me along this rollercoaster ride of life.

SUMMARY

The protection of aquatic ecosystems is important not only from a conservation view point, but also to ensure the sustainable use of water resources. Waterbodies used for commercial (aquaculture, aquatic foods, agricultural water), recreational (fishing, boating, swimming) and drinking purposes can only be sustained if the ecosystem 'health' is maintained. The management of aquatic ecosystems relies on monitoring and assessment programs that can detect early changes in water quality and identify aquatic systems in need of repair. Early detection of changes in water quality enable the cause to be determined and rectified before the ecosystem is adversely affected. The best measures of aquatic ecosystem 'health' are provided by monitoring the biological communities (biodiversity) and ecological processes (community metabolism).

The microbial community has the potential to provide very good indicators of river 'health'. Their short generation times and metabolic diversity means they can respond rapidly to environmental changes and their large numbers simplifies sampling programs. Studies of the microbial ecology of aquatic systems require the quantification of growth rates, biomass and cell numbers of bacterial populations. The study reported in this thesis evaluated the techniques available to measure these three parameters and also assesses the potential for molecular microbial methods to determine biological diversity. The technique evaluation was field trialed by performing between site comparisons in a small lowland stream (Campbells Creek) polluted by a single point discharge of waste water.

Bacterial cell numbers were easy to measure in aquatic samples, but the information obtained was of little relevance for the assessment of river 'health'. Radio-isotopic methods using tritiated thymidine and leucine, were employed to measure bacterial production. A new tritiated thymidine method was developed after unsuccessful attempts to use the traditional method. This new method will be important in monitoring river 'health'. The relatively new method, Sedimentation Field-Flow Fractionation (SdFFF) was successfully used to measure bacterial biomass.

As expected higher cell concentrations, biomass and growth rates were found to occur downstream of the sewage discharge site. During the 3 year study, cell concentrations upstream ranged from 1.7-5.5x10^9 cells/L compared with a range of 1.8-59x10^9 cells/L downstream of the sewage discharge point. Bacterial growth rates (production) varied from 3-35 µgC/L/hr upstream to 135-523 µg/C/L/hr downstream. The greatest difference (9-fold) in biomass concentration between the two sites was observed in March 1996, with a biomass concentration of 379 µg/L observed downstream and 41 µg/L upstream. Possible factors contributing to this increase in bacterial biomass production and cell concentration are not directly determined in this study. However, differences in nutrient availability, and the large input of allochthonous bacteria from the sewage treatment plant, combined with fluctuations in creek flow, are discussed as likely explanations for the observed increases.

A new method combining SdFFF and bacterial production measurements was also developed during this project. This single method allows measurement of production rates in terms of biomass. Experiments combining SdFFF with the incorporation of tritiated thymidine showed the potential of this new technique to reveal the relationship between cell numbers, biomass and bacterial production.

Unlike traditional microbiological techniques, molecular methods have the ability to provide information on microbial diversity. DNA was extracted from sites upstream and downstream of the sewage discharge site. Polymerase Chain Reaction (PCR) and DNA cross hybridisation experiments were subsequently performed on the extracted DNA. Initial experiments with PCR and DNA cross hybridisation on *E. coli* and DNA extracted from samples collected downstream of the sewage discharge showed the potential of these techniques to reveal changes in bacterial species composition between sites. However, a number of difficulties were encountered when these molecular methods were applied to natural environments. For example, acquiring sufficient quantities of pure DNA from natural samples proved to be a major hurdle which prevented the analysis of microbial diversity when bacterial cell concentrations were low.

Table of Contents

	Page
DECLARATION	i
ACKNOWLEDGEMENTS	ii
SUMMARY	iii
TABLE OF CONTENTS	v
LIST OF FIGURES	x
LIST OF TABLES	xviii

CHAPTER 1

1.1 Introduction 1

1.1.2 Thesis Structure 3

1.2 The role of microorganisms in the aquatic environment 4

1.3 Microorganisms found in freshwater 7

1.3.1 Bacteria 7

1.3 .1.1 Heterotrophic bacteria 8

1.4 Eukarya 13

1.4.1 Algae 13

1.4.2 Fungi 14

1.4.3 Protozoa 14

1.4.4 Viruses 15

1.5 Environmental factors effecting activity and growth 15

1.5.1 Light 16

1.5.2 Temperature 16

1.5.3 Hydrogen ion concentration (pH) 17

1.5.4 Dissolved gases 17

1.6 Bacterial Processes 18

1.6.1 Microbial food web (microbial 'loop') 18

1.6.2 Biogeochemical cycles 19

1.7 Biological assessment of river "health" 23

1.7.1 Microbial populations as indicators of river 'health' 25

1.8 Bacterial indicators of pollution 26

1.8.1 Direct indicators 26

1.8.2 Response Indicators 28

1.8.3 Physiological responses as bioindicators 30

1.9 Indicators of bacterial diversity 30

1.10 Methods in aquatic microbiology 31

1.10.1 Methods for enumerating and identifying bacteria 31

1.10.2 Immunological techniques 33

1.10.3 Molecular microbial methods 34

1.10.4 Flow Cytometry 44

1.10.5 Methods for Determining Microbial Biomass 45

1.10.6 Bacterial productivity measurements 46

CHAPTER 2

2.1 Introduction 48

2.2 Materials and Methods 52

2.2.1 Sampling 52

2.2.2 Determination of total bacterial cell concentrations, coliform counts and heterotrophic plate counts 53

2.2.3 Enumeration of metabolically active bacteria 55

2.2.4 Physico-chemical analysis 55

2.2.5 Statistical analysis 56

2.2.6 Generation of flow data 56

2.3 Results 61

2.3.1 Catchment area and site description....61
2.3.2 Castlemaine sewage treatment plant....66
2.3.3 Flow in Campbells Creek....66
2.3.4 River 'health' assessment....68
2.3.5 Enumeration of bacteria cells....68
2.3.5 Total coliforms and Faecal coliforms....75
2.3.6 Viable cell counts....79
2.3.7 Enumeration of active bacteria....80
2.3.8 Nutrient analysis....82
2.3.9 Physico-chemical analysis....89
2.4 Discussion....91
2.5 Conclusion....96

CHAPTER 3

3.1 Introduction....97
3.1.1 Bacterial production measurements using tritiated thymidine....97
3.1.2 Estimating bacterial production from the incorporation of leucine....99
3.2 Materials and Methods....101
3.2.1 Sampling sites....101
3.2.2 Physico-chemical analysis....101
3.2.3 The thymidine growth assay....102
3.2.4 Method Development....103
3.2.5 Measuring isotope dilution....108
3.2.6 Inhibiting isotope dilution....109
3.2.7 Comparing production upstream and downstream of the sewage outlet using tritiated thymidine....109
3.2.8 Bacterial production measurements using tritiated leucine....110
3.2.9 Statistical analysis....111

3.3 Results **111**

3.3.1 Existing method 111

3.3.2 New method 112

3.3.3 Distribution of radioactivity 118

3.3.5 Bacterial dynamics in Campbells Creek 124

3.3.6 Additional method development 129

3.3.8 Bacterial Production rates with 3H-leucine 142

3.4 Discussion **144**

3.4.1 Method development 144

3.4.2 Bacterial production and river 'health' assessment 145

3.5 Conclusion **149**

CHAPTER 4

4.1 Introduction 151

4.1.1 Bacterial biomass determination using SdFFF 151

4.1.2 Combining SdFFF biomass determination with bacterial production measurements 155

4.2 Materials and Methods 156

4.2.1 Sampling 156

4.2.2 Determination of bacterial cell concentrations 156

4.2.3 SdFFF separation 156

4.2.4 SdFFF combined with 3H-Thymidine incorporation 158

4.3 Results 159

4.3.1 Bacterial cell concentrations 159

4.3.2 SdFFF analysis 160

4.3.3 Biomass distributions 180

4.3.4 SdFFF combined with tritiated thymidine 182

4.4 Discussion **192**

4.4.1 Bacterial biomass 192

4.4.2 Simultaneous biomass and bacterial production measurements 198

4.5 Conclusion **199**

CHAPTER 5

5.1 Introduction 201

5.2 Molecular microbial techniques 202

5.2.1 DNA cross-hybridisation 202

5.2.2 PCR-Clone-Sequence 203

5.3 Materials and methods **204**

5.3.1 Sample collection and filtration 204

5.3.2 Tangential flow filtration 204

5.3.3 DNA extraction 205

5.3.4 Modifications to the DNA extraction procedure 206

5.3.5 DNA extractions on TFF concentrates 207

5.3.6 Polymerase chain reaction (PCR) 208

5.3.7 Agarose gel electrophoresis 209

5.3.8 Dot blot hybridisations 209

5.4 Results and Discussion **211**

5.4.1 Concentrating aquatic bacteria for DNA extractions 211

5.4.2 Extraction of DNA 213

5.4.3 Purification of DNA 214

5.4.4 Dot blot hybridisation of bacterial community DNA 216

5.4.5 Polymerase chain reaction amplification (PCR) of 16s rRNA 219

5.5 Conclusion **222**

SYNTHESIS AND FURTHER RESEARCH **224**

REFERENCES **233**

List of Figures

Figure 2.1 Plot of recorded flows in Campbells Creek (August 1994-November 1995) versus those for the same period in Creswick Creek at Clunes.

Figure 2.2 Victoria catchment map showing the Loddon River catchment area (shaded).

Figure 2.3 Map of Castlemaine, Campbells Creek catchment area and the four chosen sampling sites (A,B,C,D) . Sites A and B are upstream of the STP and C and D downstream.

Figure 2.4a Site A at high flow (September 1995)

Figure 2.4b Site A at low flow (January 1998)

Figure 2.5a Site B at high flow (September 1995)

Figure 2.5b Site C at high flow (September 1995)

Figure 2.6a Site D at high flow (September 1995)

Figure 2.6b Site D at low flow (January 1998)

Figure 2.7 Mean (of replicates) total cell concentration upstream and downstream of Campbells Creek from July 1995 to March 1998. Error bars depict variation between replicates.

Figure 2.8a Mean (of replicates) total coliforms upstream and downstream of Campbells Creek from July 1995 to July 1996. Error bars depict variation between replicates.

Figure 2.8b Mean (of replicates) Faecal coliforms upstream and downstream of Campbells Creek from July 1995 to July 1996. Error bars depict variation between replicates.

Figure 2.9a Filterable reactive phosphorus concentrations (mg/L) upstream and downstream of Castlemaine STP from August 1995 to March 1998.

Figure 2.9b NO_X concentrations (mg/L) upstream and downstream of Castlemaine STP from August 1995 to March 1998.

Figure 2.9c NH3 concentrations (mg/L) upstream and downstream of Castlemaine STP from August 1995 to March 1998.

Figure 2.9d Total phosphorus (TP) concentrations (mg/L) upstream and downstream of Castlemaine STP from August 1995 to March 1998.

Figure 2.9e Total nitrogen (TN) concentrations (mg/L) upstream and downstream of Castlemaine STP from August 1995 to March 1998.

Figure 2.9f Total organic carbon (TOC) concentrations (mg/L) upstream and downstream of Castlemaine STP from August 1995 to January 1996.

Figure 3.1 Steps leading to the determination of bacterial production.

Figure 3.2 TDR incorporation and processing procedure

Figure 3.3 Time course of incorporation of TDR after processing samples with three different methods; TCA method (autoclave or oven incubation) and SSC buffer method

Figure 3.4 Plot of TDR incorporation into DNA over time. Comparison of background levels of TDR between samples stopped with 1 mL of 0.5% unlabelled thymidine and those stopped with formaldehyde only.

Figure 3.5 Time course incorporation of TDR. Samples processed with the Eppendorf method.

Figure 3.6 Time course incorporation of TDR into DNA using a higher specific activity of isotope (35 Ci/mmol). Samples processed with the SSC buffer method.

Figure 3.7 Time course incorporation of TDR (2.0 Ci/mmol of isotope) on 5 mL samples processed with the SSC buffer method.

Figure 3.8 Time course incorporation of TDR (2.0 Ci/mmol of isotope) on 5 mL samples processed with the SSC buffer method. Samples from Site A and site D filtered through GFF filters, the filtrate collected and refiltered through 0.2 µm polycarbonate (PC) filters.

Figure 3.9 The reduction of background radioactivity by pre-soaking GFF filters with ultra-pure water (MQ) or a low concentration solution of thymidine (Tdr). The filters were also washed with either 50 or 100 mL volumes of SSC to see if wash volume influenced background radioactivity.

Figure 3.10 Isotope dilution plots for incorporation of TDR into DNA using the Moriarty and Pollard (1981) method at 3 different concentration of TDR (5 nM, 20 nM & 50 nM).

Figure 3.11 Isotope d. ·tion plots using an alternative method to Moriarty and Pollards (1981) The concentration of isotope in each vial was constant but increasing volumes of sample was used in an attempt to determine at what concentration isotope dilution begins.

Figure 3.12 Isotope dilution plot for site D samples using the SSC buffer method to process samples

Figure 3.13 Plot determining the optimum concentration of TDR required to inhibit isotope dilution in Campbells Creek.

Figure 3.14 Plot of concentration of chlorophyll (mg/L) versus the radioactivity (DPM) of TDR for 0 min incubated samples (blanks)

Figure 3.15 Time course incorporation of TDR into DNA at site A using two different volumes of sample (2 mL and 5 mL).

Figure 3.16a. Filterable reactive phosphorus concentrations (mg/L) in Campbells Creek at site A and D, from December 1997 to March 1998.

Figure 3.16b. NO_X concentrations (mg/L) in Campbells Creek at site A and D, from December 1997 to March 1998.

Figure 3.16c. NH_3 (mg/L) in Campbells Creek at site A and D, from December 1997 to March 1998.

Figure 3.16d. Total phosphorus (TP) (mg/L) in Campbells Creek at site A and D, from December 1997 to March 1998.

Figure 3.16e. Total Nitrogen (TN) (mg/L) in Campbells Creek at site A and D, from December 1997 to March 1998.

Figure 3.17 Time course of incorporation of radiolabelled leucine into protein

Figure 4.1 Comparison of the fractograms produced from the SdFFF separation of samples from the two upstream sites.

Figure 4.2(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Upstream in January 1996.

Figure 4.2(b) Plot of cell concentration for individual SdFFF fractions. Upstream January sample.

Figure 4.3(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Downstream site C in January 1996.

Figure 4.3(b) Plot of cell concentration for individual SdFFF fractions. Site C January sample.

Figure 4.4(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Downstream site D in January 1996.

Figure 4.4(b) Plot of cell concentration for individual SdFFF fractions. Site D January sample.

Figure 4.5(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Upstream in March 1996.

Figure 4.5(b) Plot of cell concentration for individual SdFFF fractions. Upstream March sample.

Figure 4.6(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Downstream in March 1996.

Figure 4.6(b) Plot of cell concentration for individual SdFFF fractions. Downstream March sample.

Figure 4.7(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Upstream in April 1996.

Figure 4.7(b) Plot of cell concentration for individual SdFFF fractions. Upstream April sample.

Figure 4.8(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Downstream site C in April 1996.

Figure 4.8(b) Plot of cell concentration for individual SdFFF fractions. Site C April sample.

Figure 4.9(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Downstream site D in April 1996.

Figure 4.9(b) Plot of cell concentration for individual SdFFF fractions. Site D April sample.

Figure 4.10(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Upstream in August 1996.

Figure 4.10(b) Plot of cell concentration for individual SdFFF fractions. Upstream August sample.

Figure 4.11(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Downstream in August 1996.

Figure 4.11(b) Plot of cell concentration for individual SdFFF fractions. Downstream August sample.

Figure 4.12(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Upstream in November 1996.

Figure 4.12(b) Plot of cell concentration for individual SdFFF fractions. Upstream November sample.

Figure 4.13(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Downstream in November 1996.

Figure 4.13(b) Plot of cell concentration for individual SdFFF fractions. Downstream November sample.

Figure 4.14(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Upstream in December 1996.

Figure 4.14(b) Plot of cell concentration for individual SdFFF fractions. Upstream December sample.

Figure 4.15(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Downstream site C in December 1996.

Figure 4.15(b) Plot of cell concentration for individual SdFFF fractions. Site C December sample. For both graphs, results have been normalised to the same concentration factor and injection volume.

Figure 4.16(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Downstream site D in December 1996.

Figure 4.16(b) Plot of cell concentration for individual SdFFF fractions. Site D December sample.

Figure 4.17(a) Unfractionated sample stained with DAPI (Site C, March 1996). Viewed under UV light at x1250 magnification.

Figure 4.17(b) The cells present in this figure are from the first peak (elution time 8-16 min) of the fractogram (plot of fluorescence versus elution time) shown in figure 4.6a (Site C, March 1996).

Figure 4.17(c) March Site C sample separated by SdFFF and collected into fractions for cell counting. The cells present in this figure are from the second peak (elution time 48-56 min) of the fractogram (plot of fluorescence versus elution time) shown in figure 4.6a. Viewed under UV light at x1250 magnification.

Figure 4.17(d) The cells present in this figure are from the third peak (elution time 64-72 min) of the fractogram (plot of fluorescence versus elution time) shown in figure 4.6a.

Figure 4.18 Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation of two samples, one stained with DAPI and incubated with tritiated thymidine (TDR) and the other stained with DAPI only. Downstream site D in summer 1998.

Figure 4.19(a) Histogram of radioactivity (DPM) from the incorporation of tritiated thymidine (TDR) versus elution time for SdFFF separation. Sample incubated with TDR only, no DAPI staining.

Figure 4.19(b) Histogram of radioactivity (DPM) from the incorporation of tritiated thymidine (TDR) versus elution time for SdFFF separation. Sample incubated with TDR and DAPI stained.

Figure 4.20(a) Histogram of radioactivity (DPM) from the incorporation of tritiated thymidine (TDR) versus elution time for SdFFF separation. Sample incubated with TDR and DAPI stained. Downstream sample from January 1998.

Figure 4.20(b) Histogram of radioactivity (DPM) from the incorporation of tritiated thymidine (TDR) versus elution time for SdFFF separation. Repeat SdFFF run to test reproducibility.

Figure 4.21(a) Plot of fluorescence (DAPI stain) versus elution time for SdFFF separation. Upstream sample incubated with tritiated thymidine from January 1998.

Figure 4.21(b) Histogram of radioactivity (DPM) from the incorporation of tritiated thymidine (TDR) versus elution time for SdFFF separation. Sample incubated with TDR and DAPI stained. Upstream sample from January 1998.

Figure 4.21(c) Downstream site D sample incubated with tritiated thymidine from January 1998. Plot of fluorescence (DAPI stain) versus elution time for SdFFF separation

Figure 4.21(d) Histogram of radioactivity (DPM) from the incorporation of tritiated thymidine (TDR) versus elution time for SdFFF separation. Sample incubated with TDR and DAPI stained. Site D sample from January 1998.

Figure 4.22(a) Histogram of radioactivity (DPM) from the incorporation of tritiated thymidine (TDR) versus elution time for SdFFF separation. Sample incubated with TDR and DAPI stained. Downstream sample from Febuary 1998.

Figure 4.22(b) Plot of cell concentration for individual FFF fractions collected from the SdFFF separation performed on the 4 Febuary 1998. Sample incubated with TDR and DAPI stained

Figure 4.23 Histogram of radioactivity (DPM) from the incorporation of radiolabelled leucine versus elution time for SdFFF separation. Downstream sample from Febuary 1998.

Figure 4.24 Histogram of radioactivity (DPM) from the incorporation of tritiated thymidine versus elution time for SdFFF separation. This graph has the radioactivity present in the void peak included, clearly showing that unincorporated thymidine is eluted earlier than the bacterial cells.

Figure 4.25 Plot of the difference (in fold) in cell concentration between upstream and downstream versus the corresponding difference in biomass observed for the same samples.

Figure 5.1 Ethidium bromide-stained 1.0% agarose gel of total DNA extracted from bacteria collected from site C at Campbells Creek. Lanes1, *HindIII*- digested labda DNA (molecular sizes: 23.1, 9.4, 6.6, 4.4, 2.3, and 2.0 kbp, from top to bottom , repectiely); 2. undigested bacterial DNA from Campbells Creek; 3, undigested bacterial DNA from Campbells Creek.

Figure 5.2 DNA cross hybridisation. X-ray film exposed to nylon filters containing radioactively labelled DNA. Both filters contain immobilised single stranded DNA extracted from; Campbells Creek, Hedgely Dene pond, Maribyrnong River and *E. coli*. A 1:2 serial dilution of each sample was performed. Filter A was probed with single stranded *E. coli* DNA and Filter B with Campbells Creek DNA.

Figure 5.3 Ethidium bromide-stained, 1.0% agarose gel of PCR products obtained from amplifying DNA extracted from site C at Campbells Creek. Lane 1: *HindIII*- digested labda DNA (molecular sizes: 23.1, 9.4, 6.6, 4.4, 2.3, and 2.0 kbp, from top to bottom , repectiely); Lane 2: PCR performed with 1 mmole of $MgCl_2$; Lane 3: 2 mmole of $MgCl_2$; Lane 4: 3 mmole of $MgCl_2$; Lane 5: 4 mmole of $MgCl_2$; Lane 6 & 7: 200 bp control DNA .

List of Tables

Table 2.1 Physico-chemical guideline trigger values for the protection of Lowland river systems (ANZECC & ARMCANZ Australian water quality guidelines 1999)

Table 2.2 Water quality guidelines for recreational and raw drinking waters (ANZECC 1992).

Table 2.3 Flows from gauged catchments regressed against recorded flows in Campbells Creek upstream of the Castlemaine STP

Table 2.4 Generated flows in Campbells Creek (ML/d)

Table 2.5 Mean bacterial cell concentration ($x10^9$ cells/L) and standard deviation for 3-5 replicate samples taken at upstream sites.

Table 2.6 Mean bacterial cell concentration ($x10^9$ cells/L) and standard deviation of 3-5 replicate samples at downstream sites.

Table 2.7 Two-way ANOVA of bacterial cell concentration, total coliforms and faecal coliforms

Table 2.8 Pairwise analysis of bacterial cell concentration upstream and downstream of STP

Table 2.9 Pearson correlation coefficient for cell concentration and daily flow (includes data from 1997-98

Table 2.10 Upstream mean total coliform (TC) and faecal coliform (FC) (CFU/L) and standard deviation of 3-5 replicates

Table 2.11 Downstream mean total coliform (TC) and faecal coliform (FC) (CFU/L) and standard deviation of 3-5 replicates

Table 2.12 Pairwise comparison of total coliforms and faecal coliforms upstream and downstream of STP

Table 2.13 Numbers of active bacteria ($x10^9$ cells/L) upstream (site B) and downstream (site C) of the STP in Campbells Creek

Table 2.14 Nutrient concentration (mg/L) ranges at site A and B from the 10/10/95 to 23/1/97 (n=10)

Table 2.15 Nutrient concentration (mg/L) ranges at site C and D from the 10/10/95 to 23/1/97 (n=10)

Table 2.16 Results of correlation analysis between cell counts and nutrient data SITE A

Table 2.17 Results of correlation analysis between cell counts and nutrient data SITE B

Table 2.18 Results of correlation analysis between cell counts and nutrient data SITE C

Table 2.19 Results of correlation analysis between cell counts and nutrient data SITE D

Table 2.20 Physico-chemical ranges at site A and B from the 10/10/95 to 23/1/97 (n=10)

Table 2.21 Physico-chemical ranges at site C and D from the 10/10/95 to 23/1/97

Table 3.1 Percent DPM in soluble and insoluble hot TCA extraction of five site D replicates

Table 3.2 Bacterial production measurements in Campbells Creek at site A and D from December 1997 to March 1998. Production (cells.L-1.hr-1) calculated using equation 1 and converted to Carbon production using the mean mass of carbon per cell data obtained with SdFFF.

Table 3.3 Pairwise comparisons of bacterial production at site A and D

Table 3.4 Nutrient concentrations (mg/L) at site A and D over the period 2/12/97 to 3/3/98

Table 3.5 Physico-chemical data ranges at site A and D from 2/12/97 to 3/3/98

Table 3.6 Mean bacterial cell concentration ($x10^9$ cells/L) and standard deviation of at least 3 replicate samples

Table 3.7 2-way ANOVA of cell concentration, chlorophyll concentration and bacterial production

Table 3.8 Mean chlorophyll concentration (µg/L) and standard deviation of at least 3 replicate samples

Table 3.9 Site A, Pearson correlations for cell concentration, bacterial production, chlorophyll, flow and physio-chemical results

Table 3.10 Site D, Pearson correlations for cell concentration, bacterial production, chlorophyll, flow and physio-chemical results

Table 3.11 A summary of bacterial production in freshwater river systems

Table 4.1 Mean* bacterial cell concentration (x 10^9 cells/L) and standard deviation at the time of SdFFF runs performed

Table 4.2 Results of biomass measurements in Campbells Creek upstream and downstream of the Castlemaine STP. Biomass concentration was estimated from the total area under biomass distribution curves and knowing the injection volume and concentration factors used in the sample preperation steps.

Table 4.3 Reported biomass concentrations

Table 5.1 Cell concentration after Tangential Flow Filtration concentration

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Since the settlement of Australia by western civilisation over 200 years ago there has been large scale degradation of our waterways. The flow, habitat (especially riparian vegetation) and groundwater hydrology of nearly all our river systems has been altered. Foreign plant and animal species have been introduced and the input of industrial and domestic effluent has adversely affected water quality. In recent years, we have come to realise the long term detrimental social, economic and environmental effects that the abuse of our waterways, has had. Sustainable use of the nation's water resource must involve the maintenance of these aquatic ecosystems. These ecosystems are worthy of protection not only for their own intrinsic value, but for many commercial and recreational benefits (e.g. aquaculture, fisheries, drinking water, irrigation).

The realisation of the importance of conserving our river systems has resulted in the establishment of the 'National water quality management strategy' by the Australia and New Zealand Environment and Conservation Council and the Agriculture and Resource Management Council of Australia and New Zealand. This strategy has designed a framework approach to the long-term management of our water resources involving the following 5 steps:.

(1) Define primary management aims: [e.g. Define waterbody, environmental concerns and level of protection desired].

(2) Determine appropriate water quality guidelines.

(3) Define water quality objectives. i.e specific water quality to be achieved

(4) Establish monitoring and assessment programs based on water quality objectives.

(5) Initiate appropriate management response to attain and maintain water quality objectives.

To assess whether the particular ecosystem is being protected and maintained will require that an appropriate monitoring program able to detect environmental changes is established. This has led to the search for indicators of river 'health'. Biological diversity is

recognised as the best indicator of river 'health' and is increasingly being included in water quality assessment programs. Current biological monitoring focuses on algal and macroinvertebrate populations and ignores the microbial community. To date, most of this monitoring and research (eg AusRIVAS) has concentrated on the structure of population food webs and has ignored the function individual populations play in the ecosystem. Characteristics unique to the bacterial population such as their vast numbers, short generation times and intensive metabolic diversity provides them with the ability to respond rapidly to changes in their environment. Therefore, the bacterial community potentially could be a far more suitable biological indicator than the traditionally used organisms.

In recent years, researchers have acknowledged the important role microbial populations play in river ecosystem functioning. Bacteria are crucial to the recycling of organic and inorganic material and are a significant component of aquatic food webs. However, many difficulties have become apparent when traditional microbial methods have been applied to environmental samples. These difficulties have resulted in only a limited understanding of microbial ecology and a less than complete picture of their role and diversity in river ecosystems. Methodological restrictions have also prevented the use of microbial populations as indicators of river 'health'. However, new developments in molecular microbial technology have provided an opportunity to overcome many of the problems associated with the use of traditional microbiological methods in aquatic environments.

The central theme of this thesis is the use of the microbial population as a tool for assessing river 'health'. The overall aim was to examine the microbial population upstream and downstream of a single point source of sewage effluent and see if changes in bacterial population structure and activity correlated with changes in water quality. However, without appropriate methods to monitor and analyse the bacterial community, their potential as indicator organisms will not be fully utilised. Hence the focus of this study is on method development rather than the ecological significance of its application. The techniques available to examine the four main aspects of aquatic bacterial communities - is bacteria enumeration, biomass, production and diversity, are evaluated for their use and effectiveness

in river environments. The specific aim is to identify inconsistencies and uncertainties of the various methods, if any difficulties are encountered in their environmental application and the usefulness of the results in assessing river 'health'.

It is hypothesised in this thesis that microbial populations will indicate a decline in river 'health' downstream of an input of sewage effluent, with bacterial cell numbers and activity increasing whereas bacterial diversity will be reduced.

1.1.2 Thesis Structure

Chapter Two examines the use of traditional and the more recently developed cell counting methods such as direct counts, viable plate counts, total and faecal coliform counts and active cell enumeration. Water quality (nutrient concentrations and physical conditions) monitoring was also performed and compared with the results obtained from microbial analysis.

Measuring bacterial production from the incorporation of radiolabelled thymidine or leucine are the methods evaluated in the following chapter.

The large amount of error associated with the traditional biovolume:biomass method of biomass determination led to the application of the newly developed Sedimentation Field Flow Fractionation technique to measure bacterial biomass in Chapter 4.

The final chapter, explores the use of molecular microbial techniques to investigate bacterial diversity upstream and downstream of the sewage treatment plant.

1.2 The role of microorganisms in the aquatic environment

The sum of chemical, physical and biological parameters that act on a living organism is given the term environment (Brock and Madigan, 1994). Aquatic environments include both marine (oceans, seas, estuaries, salt marshes) and freshwater (lakes, rivers, ponds, springs) systems which differ greatly chemically and physically. Therefore, it is no surprise that the composition of the microbial community found in these environments also differs. The main focus of this review will be on freshwater river systems, the microbial populations that live in these environments, the role microbes play, their potential as indicators of river 'health' and the techniques used to investigate microbial communities.

The microbial community found in rivers is made up of five very diverse groups of organisms; bacteria, cyanobacteria, fungi, protozoans and algae. Viruses are also present, but are generally considered as non-living because they require living cells of other organisms to replicate and are not capable of independent existence.

Microorganisms are organisms too small (<0.1 mm) to be seen with the naked eye and can only be viewed with the use of a microscope. The cells of microorganisms and all living entities can be differentiated structurally as being either prokaryotic (devoid of an organized nucleus) or eukaryotic (possess a nuclear membrane encompassing an organised nucleus of DNA, RNA and proteins). All higher life forms, and three microbial groups, are constructed from a eukaryotic cell type, including the algae, fungi and protozoa, whereas the bacteria and cyanobacteria have a simpler prokaryotic cell structure. However, this division of cell type does not provide any information on phylogenetic or evolutionary relationships between organisms. In recent years, advances in nucleic acid sequencing has led to the study of ribosomal RNA structure and sequence. These studies have shown that molecular genetics can reveal far more evolutionary information than the traditional phenotype classifications, and have led to the division of life into three distinct groupings - *Bacteria*, *Archae* and *Eukarya* (Brock and Madigan, 1994). The first two groups are both prokaryotic in cell structure and are exclusively microbial in nature, whereas the third group is made up

of micro- and macro- organisms. All three groups, however, are evolutionary distinct from each other, more so even than the differences which separate plant and animal kingdoms (Woese, 1990).

Microbial populations are crucial to the cycling or spiralling of organic and inorganic nutrients in all of the biogeochemical cycles. Microorganisms, and in particular bacteria, have very diverse metabolisms making it possible for many nutritional chains to co-exist and be interrelated. The rapid rate of bacterial metabolism makes bacteria particularly important in biogeochemical cycling. There are 92 naturally occurring elements present on earth, of which 27 are essential to living organisms (but not all organisms require all 27). Six of these elements - carbon, hydrogen, oxygen, nitrogen, phosphorus and sulphur are referred to as key elements because they are the main components of the major biomolecules, proteins, carbohydrates, lipids and nucleic acids, the basic units of all organisms. On a global scale, every one of these elements is cycled through living organisms driven by energy in the form of sunlight (Hurst, 1991). The diverse nature of microorganims allows their involvement in all biogeochemical reactions and for some reactions such as those in the nitrogen cycle, they are the only agents capable of transforming the element into a form suitable for higher organisms.

While most aquatic microorganisms are heterotrophic, playing an important degradative and remineralising role in their environment, many are autotrophic obtaining their energy from sunlight and their carbon from carbon dioxide. Some microrganisms are even chemosynthetic autotrophs, harnessing energy from the oxidation of inorganic materials and carbon from carbon dioxide. Despite our existing knowledge that large numbers of diverse microorganisms live in our river systems there is still a very large gap in our understanding of their role in aquatic ecosystems. This limited information is mainly due to the inapplicability of traditional microbial techniques to aquatic systems.

For many years now, the important role of microorganisms as decomposers and recyclers has been accepted. However, it has only been in the last two decades that bacterial populations have become recognized as significant components of aquatic food webs (Pomeroy, 1974; Azam *et al.*, 1983; Cole *et al.*, 1988). Within every litre of river water

there are billions of microorganism from thousands of different species and their biomass greatly exceeds that of both plant and animal life combined. Bacteria have an incredible ability to rapidly increase in number. Bacterial cells grows until the cell divides in two, a process called binary fission. During this cycle the cellular components of the cell double and two cells are produced from one. The time it takes for this process to occur is referred to as the "generation" time or "doubling" time. Under optimal conditions a cells doubling time may be as short as ten minutes or take as long as 1-2 days depending on the species. However, there are many factors that impact on a cells growth rate, the most influential being nutrient availability. The physico-chemical conditions found in aquatic habitats are rarely optimal, and thus bacterial growth is generally restricted. Unlike cells grown in culture, bacterial doubling times in aquatic environments range from hours to days, a considerably shorter generation time than that of higher organisms found in the same environment.

The microorganisms present in an aquatic habitat may have originally come from sources outside of that particular body of water. Vast numbers of microbes may enter a water body from many different sources including animals, soil, plant material, sewage or other polluting effluents. These 'foreign' bacteria are referred to as allochthonous microorganisms and most of them find it difficult to compete with the normal river flora and die shortly after entering.

Microbial populations respond rapidly to changes in their environment, an ability enhanced by their growth capabilities. This fact along with their vast numbers means that bacterial populations have the potential for an additional role as indicators of river "health", and it is likely that in the future they will play a crucial part in the solving and preempting of environmental problems.

1.3 Microorganisms found in freshwater

1.3.1 Bacteria

The bacterial population found in freshwater habitats correspond morphologically to the basic cell types of spherical (cocci), rod shaped, crescent shaped or spirals. More unusual cell types such as star shaped filamentous and stalked forms may also be observed. The bacterial cell wall structure predominantly stains gram negative and most cells are motile (Rheinheimer, 1991). Bacteria live in the water column as either free entities or attached to particulate matter. Many also exist in biofilms on rocks or debris. There is often a close relationship between the bacterial flora of rivers and the surrounding terrestrial bacteria. The majority of bacteria in rivers are heterotrophic with allochthonous heterotrophs contributing significantly to cell numbers. The autotrophic (photo and chemo) bacteria present in rivers are autochthonous and require only inorganic nutrients for their survival. In general, "true" aquatic bacteria are characterised by their ability to utilise very low concentrations of nutrients and are also a very diverse group from all orders of bacteria.

In the majority of cases, rivers begin as springs or groundwater that are poor in nutrients and microbial diversity. Microbial species such as *Hyphomicrobium, Gallionella, Caulobacter, Achromobacter* and *flavobacterium* are found in such environments. In springs which have an input of iron from the surrounding areas, iron bacteria like *Crenothrix polyspora* or *Gallionella ferruginea* will also be present. Likewise sulphur springs will contain sulphur bacteria and purple bacteria and thermal springs will have thermotolerant species present (Rheinheimer, 1991). As the spring becomes a stream and then perhaps a river the nutrient levels increase and with it the microbial biomass and diversity. Eutrophication of the water results in a decrease in the number of oligotrophic bacteria like those mentioned above and an increase in species from the Pseudomonadaceae, Bacillaceae and Enterobacteriaceae families. Soil bacteria remain an important component and so the numbers of *Azobacter* and *Nitrobacter* bacterium will be high. Other genera likely to be present include; *Vibrio, Spirillum, Thiobacillus, Micrococcus, Sarcina, Norcardia,*

Streptomyces, Cytophaga and some spirochaetes. Physical and chemical conditions can fluctuate quite considerable along a river. Not only may the flow rate, temperature and nutrient levels change from natural phenomena (terrestrial environmental changes or seasonal fluctuations), but input from other polluting sources like sewage or industrial waste will be reflected by a change in the microbial population. Bacteria originating from sewage discharge include the coliform bacteria, which are enteric gram negative rods such as *Eschericia coli* , as well as salmonellae, proteus and clostridium species (Brock and Madigan, 1994). These changes in microbiota as a result of environmental fluctuations are poorly understood and studied, due mainly limitations in the available methods.

1.3.1.1 Heterotrophic bacteria

The majority of bacteria present in rivers are heterotrophic, that is they use organic compounds as their carbon source. Heterotrophic bacteria are found in both the archaea and eubacteria kingdoms of bacteria.

Aerobes

When heterotrophic organisms oxidise organic carbon to produce energy, electrons are produced and the energy source is referred to as an electron donor. The electrons produced must be transferred or disposed of using an electron acceptor, which becomes reduced. For aerobic heterotrophs, molecular oxygen is the terminal electron acceptor and is reduced to carbon dioxide and water. Aerobic heterotrophs present in aquatic environment include the gram negative cocci and rods *(e.g. Pseudomonas, Azobacter, Flavobacterium),* and the motile and spiral cells (e.g. *Spirillum)* (Rheinheimer, 1991). All these organisms have an absolute requirement for oxygen.

Anaerobic

Many microorganisms can survive, and even thrive, in the absence of oxygen by either fermenting or using other molecules as terminal electron acceptors in a process called anaerobic respiration. Organisms that ferment if oxygen is not available are called

facultative aerobes. Enterobacteriaceae (eg. *E. coli*) and Vibrionaceae are facultative aerobes found in freshwater habitats which can ferment if necessary. During fermentation only the partial oxidation of carbon atoms occurs and no externally supplied electron acceptor is required. The fermentation process reduces an intermediate product of substrate catabolism (eg. Pyruvate) and the potential end products, depending on the conditions (ethanol, lactic acid, propionic acid, formic acid, butyric acid, H2, butanol and butandediol) are excreted from the cell and can be used as an identifying feature for particular groups of bacteria. Fermentation in sediments is an important process by which many complex organic molecules such as cellulose are broken down, providing simpler carbon and energy sources for other bacteria.

During anaerobic respiration a number of inorganic ions (e.g. nitrate, sulfate and carbonate), and even some organic compounds such as fumerate, have been used as terminal electron acceptors by a variety of different bacteria. The energy released during respiration is dependent on the redox potential of the final electron acceptor, and because of this, when molecules other than oxygen are used the energy yield is less. Therefore, the order of greatest energy release is oxygen > nitrate > fumerate > sulfate > carbonate > fermentation.

Nitrate Reduction

The most common inorganic electron acceptors used in anaerobic respiration within aquatic habitats are nitrogen compounds. Although other inorganic nitrogen sources have the potential to be electron acceptors, nitrate is by far the most prevalent. Nitrate reduction results in the production of gaseous end products - NO, N_2O and N_2 - in a process called *denitrification*. Denitrification does not occur in the water column because of the presence of oxygen. Denitrification is an interfacial process, relying on the diffussion of NO_3- into suboxic (<10 µm O_2) regions supporting denitrifiers (Knowles, 1982; Paerl and Pickney 1996). Only environments with an external supply of NO_3- have high levels of nitrate reduction, generally denitrification is controlled by nitification (the oxidation of ammonia/ammonium to NO_2- and subsequently NO_3-). The pathway to N_2 is as follows: $NO_3^- \rightarrow NO_2^- \rightarrow NO^- \rightarrow N_2O \rightarrow N_2$. These gaseous products can be easily lost into the atmosphere and with them a source of fixed nitrogen, a growth-limiting nutrient. Denitrification is therefore seen as a detrimental process except in sewage treatment where a

reduction in nitrate is required to retard algal growth in the receiving water. There are many known facultative anaerobes (*Escherichia, Acinetobacter, Pseudomonas, Enterobacter, Vibrio, Flavobacterium Spirillum, Bacillus*) which are known to carry out only the first part of denitrification and so only produce NO_2^- which can be reduced to ammonia by some bacteria (Rheinheimer, 1991). In aquatic habitats there are far more organisms capable of reducing nitrate to nitrite than nitrite to gaseous nitrogen compounds.

Sulphate reduction

Anaerobic respiration using oxidised forms of sulphur as a terminal electron acceptor is wide spread in aquatic environments. During sulphate reduction, H_2S is produced and like nitrogen from denitrification it is lost from the environment. Sulphate reducers are obligate anaerobes that depend entirely on sulphate respiration, an inefficient process in terms of energy yield. Even though there are only a few types of sulphate reducing bacteria (*Desulfovibrio, Desulfomaculum and Desulfomonas*), their role is an important one, with H_2S involved in many biogeochemical processes. In freshwater habitats, the concentration of sulphate is often low, thus restricting the sulphate reducing bacteria. When this is the case, methanogenic bacteria which compete with sulphate reducers for electron donors are more common. Sulphate reduction requires a significant amount of organic matter (electron donor source) and so if a freshwater system becomes polluted with organic matter such as sewage or garbage there is an increased potential for H_2S production. H_2S is toxic to many organisms, as it binds to the iron present in a cells cytochrome system (Hart and Ross, 1995).

Methanogenesis

Methane is produced in anoxic environments as the end product of the anaerobic carbon cycle. Methanogenesis is performed by a group of obligate anaerobic *Archaea* bacteria called methanogens. Methanogens are reliant on a succession of facultative aerobic bacteria to ferment complex organic materials like cellulose, starch, fats and proteins into first, lower molecular weight compounds (organic acids and alcohols) that are then fermented further into acetate, CO_2 and H_2 which are the substrates for methanogenesis. There are at least 4 different trophic levels involved in the anaerobic degradation of organic material and each microbial group (except the hydrolytic bacteria) is in some way dependent

on each other. The organisms at the start of the chain are reliant on the methanogens to remove fermentation end products that would otherwise limit further decomposition. Methanogenesis is common in freshwater environments where there is little competition from sulphate reducers.

The metabolic diversity of the microbial community is centred around its ability to use a variety of different electron acceptors. The use of alternative terminal electron acceptors other than oxygen enables microorganisms to live in anoxic environments, an important requirement for the biogeochemical cycling of elements in aquatic ecosystems.

1.3.1.2 Autotrophic bacteria

Phototrophic

Purple and Green Bacteria

Purple and green bacteria are a group of organisms that have the capability to use light as an energy source to produce ATP via a process called photophosphorylation. Purple and green bacteria are distinct from other phototrophic microorganisms such as cyanobacteria and algae in that they are anoxygenic, posses a number of different types of bacteriochlorophyll and use only one photosystem during photosynthesis (Brock and Madigan, 1994). Purple and green bacteria fix CO_2 using either H_2 or H_2S as the reducing power and most species are also able to fix N_2. The majority of purple and green bacteria are able to anaerobically oxidise reduced sulphur compounds, resulting in the formation of sulphate, an important role in the biogeochemical cycling of sulphur. Purple and Green bacteria are one of the most diverse groups of bacteria known both morphologically (cocci, rods, vibrios, spirals, budding and gliding types) and phylogenetically and are linked mainly by their photophosphorylation capabilities. In addition to their important role as recyclers they often also contribute significantly to primary production, especially in small stratified lakes, ponds and sulphur rich thermal springs. Examples of purple bacteria are *Chromatium, Thiocapsa, Thiopedia, Ectothiorhodospira* and *Rhodospirillium.* Green bacteria include *Chlorobium, prosthecochloris* and *Chloroflexus* (Rheinheimer, 1991).

Cyanobacteria

Cyanobacteria are a large diverse group of phototrophic bacteria. The most distinguishing feature separating them from purple and green bacteria is the fact that they are oxygenic phototrophs. There are both unicellular and filamentous forms of cyanobacteria and they are unique in their fatty acid composition and cell wall structure. Cyanobacteria are widely distributed across aquatic environments. The fact they contain different photosynthetic pigments than that of other phototrophic bacteria enables them to coexist in the same habitats. Unlike all other photosynthetic bacteria cyanobacteria are able to utilise water as a hydrogen donor in the reduction of CO_2. Cleaving of the H_2O molecule results in the evolution of O_2. Like algae cyanobacteria fix CO_2 via the Calvin cycle (Brock and Madigan, 1999) and some have the added ability of nitrogen fixation (Stal *et al* 1981). In large rivers and streams planktonic forms of cyanobacteria are the most common and are often found growing on the surface of rocks and other substrates. Various species of *Pleurocapsa, Hydococcus, Chamaesiphon, Rivularia* , and *Nostoc* are all examples of cyanobacteria found in freshwater rivers and streams (Rheinheimer, 1991). Cyanobacteria can be an important source of primary production, more so though in freshwater lakes and pond then flowing waters. During photosynthesis, cyanobacteria also release a range of dissolved organic carbon compounds that can serve as a source of carbon for heterotropic bacteria (Smith, 1981). Eutrophication of still waters can result in cyanobacterial blooms which can be a serious problem for public health if it occurs in drinking or recreational waters (Carmichael, 1991). *Anabaena* and *Microcystis* are the two most common genera responsible for causing blooms in nutrient rich waters (Rheinheimer, 1991; Carmichael, 1991).

Chemoautotrophic (chemolithotrophs)

The ability to oxidise inorganic material to obtain energy is the common thread which unite chemolithotrophs. Sulphur, nitrogen and iron are all reduced substances utilised by chemolithotrophs. Most chemolithotrophs are able to produce organic matter when provided with CO_2 as their sole source of carbon, but some chemoorganotrophic bacteria are able to supplement CO_2 fixation with organic sources of carbon.

Nitrification

Nitrifying bacteria are widely distributed in both soil and water, however their number are usually low and so are of no great importance in terms of secondary production. Nitrification occurs in aerobic waters and involves the oxidation of ammonia by two separate groups of bacteria. No known organism is able to solely complete the oxidation of ammonia. In nature the process of nitrification occurs sequentially, first ammonia is oxidised to nitrite followed by the oxidation of nitrite to nitrate by the nitrite oxidising bacteria which prevents an accumulation of nitrite. Nitrifying bacteria are generally found in habitats where plenty of ammonia is available such as areas of extensive protein decomposition and sites receiving sewage effluent. In these ammonia rich habitats they reside where reduced and oxygenated phases meet, usually just above the sediment

Sulphur oxidising bacteria

Sulphur oxidising bacteria are found in waters where H_2S and oxygen are present. Only six genera have been consistently isolated which can use reduced sulphur compounds as an energy source; *Thiobacillus, Thiosphaera, Thiomicrospira, Therothrix, Beggiatoa* and *Sulfolobus*. Some of these genera grow at neutral pH while others prefer acidic environments. *Thiobacillus* can be easily mistaken for *Pseudomonas*, since they are morphologically very similar and *Thiobacillus* is capable of growing chemoorganotrophically. Thiobacillus is of particular importance for river 'health' because many of the acidophilic isolates are able to oxidise ferrous iron which is present in coal and metal sulphide ores. Large quantities of acidic water are produced as a result of this oxidation process. Acidic water immobilises mine waste heavy metals, polluting the river and killing many higher life forms.

1.4 Eukarya

1.4.1 Algae

Algae are a group of oxygenic photosynthetic eukaryotic organisms. They can be observed naturally as single cells or in colony formations. Algal cells may be arranged end

to end and when this occurs they are referred to as filamentous algae, branched filamentous formations are also a common occurrence. Like the cyanobacteria, all algae use H_2O as an electron donor and evolve O_2 during photosynthesis. Whilst most algae are obligate aerobes, there are some capable of growing chemorganically, utilising simple carbohydrates or organic acids as an energy source. All algae contain chlorophyll a (also present in plants), but some also posses additional pigments, for example the red algae and brown algae.

1.4.2 Fungi

Fungi are present in many different morphological forms in most water bodies. They are eukaryotic and often produce fruiting bodies. Fungi can be either unicellular or multicellular with large mycelia. All fungi are heterotrophic and play an important role in the decomposition of complex organic material; they break down proteins, starch, sugars, and fats and posses a large range of extracellular enzymes, enabling them to utilise pectins, hemicellulose, cellulose, lignin and chitin. Fungi are present in a wide range of terrestrial and aquatic environments and therefore it is often difficult to distinguish between genuine aquatic fungi and soil varieties surviving aquatic habitats. There are three major groups of aquatic fungi; 1) slime moulds, eg. Labyrinthulomycetes, 2) Lower fungi, eg. Phycomycetes (algal fungi) and 3) higher fungi, eg. Ascomycetes and Basidiomycetes. The phycomycetes are the autochthonous or "true" aquatic fungi completing their whole sexual life cycle in the water. Fungi are generally found growing on some sort of surface but there are some free living organisms. Fungi are usually described as being parasitic in nature, but again there are exceptions with some being almost predatious, catching protozoa and other lower animal life. Fungi are common river inhabitants but are rare in low nutrient waters such as springs and groundwater. Yeasts (unicellular fungi) are also often observed in flowing waters, particularly those polluted with sewage (Rheinheimer, 1992).

1.4.3 Protozoa

Protozoa are single cell eukaryotic microorganisms. They are usually motile and greater in size than prokaryotes. They do not contain cholorophyll (colourless) or a cell wall.

Investigators have also hypothesised that viruses in aquatic microbial communities influence the diversity and clonal composition of bacterial communities (Suttle, 1994). It is proposed that viral infection influences host community diversity by selectively lysing bacterial species that are of high concentration and undergoing fast growth. Research by Wommack *et al.*, (1998) in Cheapeake bay, found viral abundance to be approxiamately 10^7 viruses per mL and that numbers of specific viruses changed dramatically with time and location, supporting the view that viruses play an important role in the control of bacterial diversity.

Protozoa feed by ingesting small organic particles or organisms by either phagocyctoysis where they engulf their food by surrounding it with their cell membrane or by pinocyctosis which is the taking up of fine droplets via a channel formed in the cell membrane. Autochthonous protozoa are important predators in the microbial food web and help to control bacterial and cyanobacterial populations in aquatic systems. Human pathogenic protozoa (*Giardia, Entomoeba* and *Cryptosporidium)* can be introduced into river systems from sewage effluent or other faecal sources. These organisms are a serious health concern, protozoa being the most common cause of diarrhoea in many Australian cities. Protozoa are able to exist as cysts and oocysts giving them the ability to survive for long periods of time (months) in aquatic environments.

1.4.4 Viruses

Viruses are non-cellular and incapable of independent existence. They comprise solely of DNA or RNA encompassed in a protein coat. Viruses are only capable of multiplying within other living cells, taking over the replicative machinery. Bacteriophages are bacteria specific viruses and are the most common viruses found in aquatic habitats. Studies by Bergh *et al.* (1989) found that the concentration of viruses in water systems to be quite high, ranging from 5-15 x 10^6 to 2.5 x 10^8 ml^{-1} and for this reason it is assumed that viruses play a role in the control of bacterial numbers.

1.5 Environmental factors effecting activity and growth

The growth and abundance of microorganisms is affected not only by nutrient availability and also by a variety of other chemical and physical factors such as light, temperature, pH, pressure, salinity and dissolved gases. For an organism to survive and grow it requires certain conditions, these conditions however are not the same for all microorganisms. What may be beneficial for one may be harmful for another. Importantly, environmental factors not only effect growth and composition of a microbial population but they also influence morphology, differentiation, and reproduction.

1.5.1 Light

The visible light spectrum ranges from 320 to 800 nm. Light intensity decreases rapidly with depth, by the square of the distance from the source. The extent to which light is able to penetrate a particular water body is determined by the number and type of suspended particles (turbidity). Light intensity strongly influences the fixation of CO_2 into organic matter via photosynthesis and thus is the energy source for photoautotrophic bacteria and the cyanobacteria. Oxygen-evolving photosynthesis performed by algae and cyanobacteria generally occurs in regions of high light intensity (some cyanobacteria can utilise small levels of light) captured by their cholorophyll *a*. Anoxygenic photosynthesis uses alternative electron acceptors to oxygen and occurs at lower depths where obviously there is less light and oxygen. Higher levels of light intensity can actually have a detrimental effect on these bacteria especially the non-pigmented varieties. Bacteriochlorphylls absorb light in the far end of the spectrum, usually between 720-880 nm, however there is one type that absorbs at 1020 nm (Brock and Madigan, 1994). This region is only minimally absorbed by the water column and there is little competition for this wavelength from other phototrophs.

1.5.2 Temperature

Temperature can greatly effect the growth and composition of a microbial community. As the temperature within an organisms environment rises, the rate of enzymatic reactions in that environment also increases. The doubling time for a bacterial population more than halves with every 10° C rise in temperature, but only up to a certain point (Rheinheimer, 1992). Above this temperature (which varies between bacterial groups), important cellular components (protein, DNA, RNA) will irreversible denature. All organisms therefore have a minimum and maximum temperature for survival with an optimum temperature within this range (called, the cardinal temperature). The extremes at which some bacterial species can survive and even grow are far greater than higher life forms. Bacteria have been found growing at temperatures as low as –10°C and as high as +90°C. In aquatic environments, fluctuations in temperature occur on a daily and seasonal

basis and so aquatic organisms must be able to tolerate a range of temperatures. Changes in water temperature can however cause a transition in the species composition of the microbial population.

1.5.3 Hydrogen ion concentration (pH)

The hydrogen ion concentration or pH of an aquatic system can also effect the growth and life cycle of a microorganism. The pH can effect microbial enzymes and the dissociation and solubility of many molecules, indirectly influencing microbial growth (Atlas and Barth, 1993). The majority of bacteria have an optimum pH between 6.5 and 9, corresponding with the pH range of most water bodies. Acidophilic (acid liking) bacteria can be found in many sulphur rich environments, examples of these genera include *Thiobacillus, Sulfolobus* and *Thermaplasma*.

1.5.4 Dissolved gases

Oxygen, hydrogen, and nitrogen are the main gases found in water. Under certain circumstances other gases such as hydrogen sulphide, carbon monoxide, molecular hydrogen and hydrocarbons may also be present. Oxygen and carbon dioxide are the two most important dissolved gases, oxygen because of its critical role in aerobic respiration as a terminal electron acceptor and carbon dioxide because of its role in primary production by autotrophs and as a pH buffer. The atmosphere and biological processes are sources of oxygen and carbon dioxide in water. The gases reach the water from the air until saturation occurs. Dissolved oxygen concentrations above 100% saturation are found in photic zones where the level of oxygenic photosynthesis exceeds respiration. Regions where O_2 concentration is less than 100% saturation indicate that the rate of microbial respiration is greater than the rate it is being replaced. Obligate aerobic bacteria are only inhibited at very low oxygen levels. Small fluctuations in dissolved oxygen will therefore only have an impact on the microbial population in oxygen poor waters. Dissolved O_2 depletion is usually only a problem in warmer waters where there is a combination of reduced O_2

solubility and an increase in microbial activity. The high solubility of carbon dioxide means that there is rarely a shortage of this gas in aquatic environments.

1.6 Bacterial Processes

1.6.1 Microbial food web (microbial 'loop')

The traditional role of bacteria in aquatic ecosystems as simple decomposers and remineralisers has been modified in recent years to include their important contribution as a food sources for higher organisms. Bacteria convert organic matter into bacterial biomass (secondary production) and this additional biomass is a significant component of aquatic food webs. The return of organic matter to the classic food chain (e.g. phytoplankton - zooplankton - fish) is termed the 'microbial loop' and is an integral part of aquatic food webs (Azam *et al.*, 1983). The microorganisms in the 'microbial loop' include bacteria, algae, protozoa and viruses.

Carbon enters the microbial loop as CO_2 (primary production) or dissolved or particulate organic matter (secondary production) which may have originated from within the waterbody (eg. by phytoplankton) or entered from the surrounding environment. Allochthonous sources of organic matter include; litter fall, groundwater, and flushing from soils. There is only a limited (Table 3.11) amount of information concerning bacterial secondary production in river systems, but it is thought that secondary production greatly exceeds primary production (Findlay *et al.*, 1991).

The fact that bacterial numbers in most aquatic systems remain relatively stable (10^8 -10^{10} cells/L) over time suggests that bacterial biomass and productivity is well regulated. Resources and predatory factors are responsible for the regulation of bacterial biomass and productivity. Resource or *bottom-up* control refers to the limitation of bacteria by carbon and nutrients. Predatory or *top-down* regulation is the limitation of bacteria to levels below which are sustainable by resources alone. Small flagellates and ciliates in the nanoplankton size range (2-20 μm) are considered the predominant grazers of bacteria (Stockner and porter, 1988; Berninger *et al.*, 1993). However, the high number of viruses found in

aquatic systems may also play an important role in controlling the numbers of bacteria (Bergh 1989).

.

1.6.2 Biogeochemical cycles

The cycling of nutrients in rivers and streams is a more complex process than in lakes and oceans. In all aquatic systems, the cycling of materials is influenced by the movement of water. However, in flowing systems these hydrological and hydrodydnamic factors have a far greater affect on the behaviour of materials. The mineralisation and immobilisation of nutrients in a cyclical manner, when coupled with downstream movement is referred to as nutrient spiralling. Spiralling may be quantified in terms of uptake length (S_W) which is the average length a molecule travels before it is immobilised or mineralised (Newbold *et al.*, 1981). This is a useful method to quantify stream ecosystem efficiency, with large S_W values indicating a low capacity for the ecosystem to utilise a particular nutrient and small S_W values indicating an efficient system (Webster and Meyer 1997).

In a typical nutrient spiralling model, a river or stream is conceptualised as consisting of three compartments. The water compartment contains materials in both dissolved and colloidal forms and there may be some interactions between these forms. The seston compartment consists of two components, abiotic (suspended particulate matter) and biotic (eg. bacteria and phytoplankton) both of which are transported by the river. In slow moving large rivers, the phytoplankton will be a larger component of the seston than in faster moving upland streams or highly turbid systems. The third compartment is the benthic compartment and includes the sediments, and attached algae and microbial biofilms. In contrast to large river systems where the majority of biological material is found in the seston compartment of the water column, in fast flowing upland streams the biological community is mainly associated with the benthic compartment (ie. attached algae and microbial biofilms). However, in some large rivers there may be a large concentration of biomass associated with the sediment.

In this basic spiralling model, nutrients and organic matter can enter or leave each water compartment as three different forms - soluble, colloidal and particulate. The

downstream movement of water transports the materials to reactive sites, where exchanges can occur. Such exchanges include chemical transformations, sorption and desorption and biological processes (bacterial or algal uptake, bacterial oxidation and reduction, invertebrate consumption of algae). In small rivers and streams, most of these exchanges occur between the water compartment and the benthic compartment but in larger rivers there may be transfer between the seston and water compartment as well. Materials transferred from the benthic compartment to the water column are carried downstream and the net difference between uptake and release (flow dependent) is referred to as retention (Stream solute Workshop, 1990).

The carbon cycle

All living organisms play a role in the continuos cycling of carbon, the basic element of organic matter. In general, carbon dioxide is the centre point of the carbon cycling process. Autotrophic (photo and chemo) organisms synthesise organic matter from the fixation of CO_2, the organic matter is then degraded by heterotrophic organisms through their oxidative respiration, releasing CO_2 as a by product. In rivers there are two sources of organic matter available to heterotrophs; (1) autochthonous sources, referring to the organic matter which is produced within the stream by bacteria and algae, and (2) allochthonous organic matter which enters streams from the terrestrial environment via runoff and includes substrates such as leaves or bark. Effluent from sewage treatment plants and other types of industrial waste discharged directly into stream are also major sources of allochthonous organic carbon. Organic matter can also be removed via non-biological processes such as sedimentation and washout.

In aquatic environments, the cycling of carbon is strongly influenced by the presence of oxygen, and occurs at its most rapid rate when oxygen is present. In anaerobic sediments the fermentation process can lead to the build up of intermediate products (organic acids, alcohols) and so only part of the organic matter is dissimilated into CO_2. Under these conditions methanogenesis may begin, with most of the methane produced being oxidised by methylotrophs at the sediment-water interface.

Heterotrophic microorganisms are essential to the continual flow of energy and carbon in aquatic habitats, and the proportion of them participating is greater than in terrestrial ecosystems. Heterotrophic microbes are usually the first trophic level to convert particulate and soluble organic matter into more complex forms that can be utilized by other higher heterotrophic organisms.

The nitrogen cycle

Nitrogen is a necessity for primary production, it is a component of protein making it an essential element. Plants are only able to take up nitrogen in the form of ammonia or nitrate, two compounds usually present in waters in only small amounts. For this reason nitrogen is often the limiting nutrient in primary production. Microbial populations are largely responsible for maintaining the availability of nitrogen in aquatic systems by breaking down nitrogen-containing organic compounds, releasing ammonia and through the fixation of atmospheric nitrogen. However, other microbial population are involved in the loss of ammonia from the system in a process called denitrification and so the cycle then continues.

The fixation of nitrogen in aquatic systems is primarily performed by cyanobacteria , although species from other bacterial genera (*eg. Aztobacter, Clostridium, Azospirrilium, Vibrio)* also contribute (Rheinheimer, 1992). Many N_2-fixing microorganisms form symbiotic relationships with plants, with the plant providing a carbon and energy source for the bacterium. Nitrogen fixation is influenced by temperature (optimum 28°C), is dependent on the surrounding concentration of ammonia and nitrate and requires a substantial input of energy in the form of ATP (Atlas and Bartha, 1993).

The phosphorus cycle

Phosphorus is also an essential element for all living organisms and like nitrogen it too can be the growth limiting nutrient within a system. Phosphorus is present in living organisms as orthophosphate, a vital component of nucleic acids, cell membranes and the energy storage molecule ATP. Microorganisms and plants take up phosphate in a soluble inorganic form and assimilate it into organic compounds. Although microbial processes are

important in the phosphorus cycle they are not as influential as they are in the nitrogen or sulphur cycles. Microorganisms are responsible for the break down of organic phosphorus compounds, releasing orthophosphate to be utilised by other organisms. The organic acids produced by some bacteria (chemolithotrophs) can also solubilise phosphate from insoluble solid calcium-phosphate compounds (e.g. bones), returning phosphate to the cycle. Within aquatic systems, algae and certain types of bacteria (acintobacter) are known to compete quite effectively for available phosphate and both are able to store phosphorus as polyphosphates in granules for later use if phosphorus becomes scarce. It is now thought that even though algae and bacteria compete for phosphorus, it is not this competition which directly effects their abundance (Currie, 1990). The relationship between algae and bacteria is thought to be more of a "mutualistic" need, the phytoplankton relying on the bacteria to recycle elements and the bacteria needing the phytoplankton for organic carbon.

The exchange of phosphorus between the water column and sediments is controlled by the oxygen content or redox condition in the sediment. When oxygen is present (aerobic) the phosphorus is retained by the sediments as iron or aluminium phosphate. If stratification occurs and the sediment becomes anaerobic, phosphorus is released. Under anaerobic conditions the insoluble iron phosphate is reduced to the divalent soluble forms of ferrous phosphate increasing the phosphate concentration in the water. Bacteria play an important role in the creation of the anaerobic conditions as a result of their aerobic degradation of sediment organic materials. In recent years studies have shown that bacteria may have a more direct role in anaerobic phosphorus release (Gachter *et al.*, 1988; Gachter and Meyer, 1993; Waara *et al.*, 1993). The bacteria actively take up and store phosphorus under aerobic conditions and release orthophosphate under anaerobic conditions as a result of a shift in energy metabolism (Waara *et al.*, 1993).

The sulphur cycle

Sulphur is an essential element for life because it is an important constituent for the amino acids, cysteine and methionine as well as some coenzymes. Sulphur is however, one

of the ten most abundant elements on earth and rarely is it limiting in river systems. When proteolytic bacteria break down proteins the sulphur present in the amino acids is released in the form of H_2S which is toxic. Some chemoautotrophic bacteria oxidise the H_2S and other sulphur compounds making them an important energy source. Microbial populations are also involved in the reduction of sulphate in anaerobic environments where it is used as a terminal electron acceptor. This reaction results in the formation of toxic H_2S, which can be released into the air and lost from the system. Sulphate reduction is more common in marine environments and eutrophic lakes where there is a higher sulphate concentration than in rivers.

1.7 Biological assessment of river "health"

River "health" is a term broadly used to describe its water quality. A so-called "healthy" river must not be overloaded with organic or inorganic nutrients or contain noxious or toxic materials. Faecal contamination should be restricted to prevent them from becoming vehicles of disease transmission, through their use as drinking water or for recreational purposes. A rivers physico-chemical (temperature, dissolved oxygen, turbidity, pH and salinity) makeup should also not be altered significantly. Pollution of river systems can cause a decline in species diversity as a result of the death of some microbial groups, and even fishes and invertebrate animals. Polluted rivers are also a threat to human health if they become disease carriers or toxic. To protect the aquatic ecology and human health it is necessary to employ a warning system that can detect an early change in river health.

Until recently, physico-chemical monitoring of rivers alone was the basis of all assessments of river 'health' in Australia. However, this type of water quality assessment, provides only a partial insight into ecosystem 'health'. There is also the potential with chemical monitoring for changes in river 'health' to go undetected because sampling regimes missed a polluting event, samples were not collected under the right conditions or the wrong parameter was measured. The effects of a changes in water quality may also be long lasting on the aquatic ecosystem even after physico-chemical states have returned to normal.

It is now widely accepted that the ecological consequences of a decline in water quality are best examined by monitoring the biological community as it responds to all types of disturbances, physical and chemical (Bunn 1995, Norris and Norris 1995, EPA 1998). Currently, algae and macroinvertebrates are two groups of aquatic organisms most often recommended for the biological assessment of water quality. In Australia, benthic macroinvertebrates are the most commonly measured group in quantitative biological monitoring programs with the EPA establishing its first program of this type for the Yarra River in 1984. This program using benthic macroinvertebrates developed into the 'rapid bioassessment method' and forms the basis for the national stream monitoring protocol (CEPA 1994).

The rapid bioassessment method involves the collection of samples from both the benthic and littoral habitat of each site. The samples are then live sorted in the field and the animals caught and preserved for later identification. Physico-chemical measurements and habitat descriptions are also performed at each site and are an important component of this method.

The invertebrate data collected is analysed and interpreted using the AUSRIVAS (AUStralian RIVer Assessment System) method, a modelling approach which generates site specific predictions of the macroinvertebrates expected (E) to be present in an undisturbed habitat. The expected fauna are then compared to the observed (O) and the resulting ratio is used to indicate environmental impact. This ratio is referred to as the O/E index and can range from 0 (none of the expected families present) to 1 (the expected number of families found). The derived O/E scores can fall into 5 different bands which represent different levels of biological condition allowing an assessment of the aquatic ecosystem.

The biotic index SIGNAL (Stream Invertebrate Grade Number - Average Level) is another system which has been developed and is recommended to be used in conjunction with AUSRIVAS. SIGNAL is based on the tolerance or intolerance of specific families of aquatic invertebrates to pollution. The aquatic families have been awarded sensitivity scores on their response to various pollutants. The index is then calculated by summing all the scores for the families found at a particular site and dividing by the total number of families,

It is also important to note here, that while a decrease in biodiversity (micro or macro) is generally considered to be the result of a decline in river 'health', biodiversity may also differ within or between systems for a variety of reasons that do not indicate a decline in river 'health'. For example, natural variation in river condition such as organic and inorganic growth requirements, flow, light and temperature can all influence diversity. Temporal or seasonal variation and flood events may also have an affect on river biodiversity.

giving an average grade per family. SIGNAL can vary from 1 to 10 with a typically high water quality site having a score above 6.

Rapid biological assessment however, is not appropriate in all studies of stream impact and has a number of disadvantages. The approach is only suitable when large changes in community structure or composition have occurred. SIGNAL has the limitation that individual species within a single family can vary in their tolerance or intolerance to certain types of pollutants. It is also not known how benthic communities behave over time, therefore changes in macroinvertebrate communities may be the result of temporal variation and not a decline in water quality.

1.7.1 Microbial populations as indicators of river 'health'

Microbial communities have the potential to be very good indicators of pollution. In the past, coliform bacteria such as *E. coli* have been used as indicator organisms. However, no single group of indicator organism will always be suitable (Elliot and Colwell, 1985). It is more appropriate to look at the entire microbial community. There are three main reasons why microorganisms and especially bacteria have the potential to be better indicators of river health than other organisms. (1) They are present in very large numbers making sampling easier and the results more statistically reliable, (2) their role in the recycling of inorganic and organic nutrients as well as being significant components of all food webs is essential to ecosystem function, and (3) their short generation times mean they have the ability to rapidly respond to environmental change more so than any other biological group. It should therefore be possible to evaluate the impact pollution or other environmental disturbances is having on a particular river by monitoring changes in species diversity, population numbers and activity.

1.8 Bacterial indicators of pollution

1.8.1 Direct indicators

Sewage pollution

Sewage discharge can cause many detrimental affects to a river systems, mostly due to an overload of inorganic and organic nutrients. This increase in degradable matter causes a vigorous increase in oxygen consumption due to the increased microbial oxidation. Oxygen depletion will cause the death of many obligately aerobic organisms resulting in a reduction of biological diversity. Many human pathogens are also present in domestic sewage and so there is the possibility of disease transmission where rivers receive inputs of sewage effluent. Traditionally, the presence or absence of certain indicator organisms has been used to determine the presence of faecal matter and assess water quality. These indicator organisms include bacterial groups, such as coliforms (*E. coli*), enterococci and coliphage (coliform infecting viruses) and are not normally problems in their own right but they point to the possibility of pathogenic organisms being present. They are chosen as indicators because of their ease, quickness and harmlessness when it comes to culturing and identifying in the laboratory. More importantly they do not grow or are severely restricted in aquatic environments, are more abundant in faeces than pathogens and survive longer than pathogens as well.

The presence of coliform bacteria can be determined in a number of ways, traditionally, using cell culture and isolation techniques. Currently water samples are membrane filtered and the membranes incubated on special coliform isolation medium (mEndo agar) at 37°C and 44°C. Other mediums have also become available that contain the fluorogen 4-methylumbelliferyl-β-D-galactopyranoside (MUG) and the chromogen indoxyl-β-D-glucuronide and can detect total coliforms and *E.coli* simultaneously (Brenner *et al.*, 1993). These methods are enzyme detection based, a yellow product is seen in the media if β-galactosidase (total coliforms) is present and the *E.coli* enzyme β-glucuronidase will act on the MUG to produce a blue fluorescent product when viewed under U.V light (Edberg *et al., 1990*; Brenner *et al.*, 1996). These tests are commercially available and have been found

to be very quick (<24 hours), sensitive and reliable with only low rates of false positives and negatives (Cowburn *et al.*, 1994; Brenner *et al.*, 1996). Polymerase Chain Reaction has also been employed to identify indicator populations. This method amplifies specific genes such as the β-glucuronidase gene in *E. coli* (Bej *et al.*, 1991).

The use of bacterial groups should not, be relied on as a fool proof method of indicating faecal contamination of a river. It has been reported that coliform bacteria are able to grow in uncontaminated water supplies (LeChevallier *et al.*, 1996) resulting in false positives. Coliform bacteria can also originate from non-human faecal sources, and as a result it is not always possible to distinguish between sewage effluent and farm runoff. These uncertainties can make public health risk assessment and alleviating the source of contamination more difficult (Kreader 1995). More importantly however is the fact that human pathogenic viruses and protozoa (*Cryptosporidium, Giardia)* have been detected in waters that tested negative to faecal indicator bacteria (Badenoch, 1990). Protozoa are amongst the most common causes of gastrointestinal disease and they are life threatening in immunosupressed individuals (eg. AIDS patients). Water contaminated with human or livestock faeces can lead to outbreaks of diarrhoeal disease caused by *Cryptosporidium* and these cysts remain viable in waters for long periods of time (Current and Garcia, 1991; Johnson *et al.*, 1995). Chlorine stressed coliforms are also very sensitive to selective media, the stressed bacterium may become non-culturable but still be viable. This can result in the non-detection or underestimation of their numbers (McFeters *et al.*, 1990)

Many other organisms have been suggested as possible indicator organisms of sewage pollution, including spores of *Clostridium perfringens* and cysts of *Giardia* and *Cryptosporidium* all of which are more robust than coliforms and can reveal faecal contamination after coliforms have disappeared (Rheinheimer, 1992). Molecular microbiological techniques have also been suggested as a means of identifying possible indicator organisms that are otherwise very difficult to isolate. For example, PCR in conjunction with gene probing has been used to detect *Bacteriodes* a fastidious organism which otherwise would be difficult to isolate, but has the potential to be used as a way of distinguishing between human and animal faecal contamination (Kreader, 1995).

Algae and Cyanobacteria

The introduction of high levels of nutrients into oligotrophic or eutrophic waters may result in the increase of cyanobacterial and algal numbers, and in severe cases cyanobacteria and algal blooms that may be toxic. In these circumstances, the algae and cyanobacteria are biological indicators of pollution as well as being pollutants in their own right.

1.8.2 Response Indicators

Organic matter

Large inputs of organic matter can originate from other sources of pollution besides sewage effluent. Refuse from large towns, runoff from modern farms, paper pulp or wastes from industry (eg. Starch from the food industry) can all cause changes in the microflora in both quantity and diversity. As with sewage pollution, the increase in organic matter from these sources results in a large biological oxygen demand as the heterotrophic microorganisms use the oxygen in the degradation of the organic material. If a high biological oxygen demand continues then a depletion of oxygen will occur, leading to a change in microbial species composition. Obligate aerobic organisms will perish and be replaced with facultative aerobes and anaerobes. If nitrate is still present the nitrate reducers and denitrifyers flourish, but if the nitrate disappears, then fermenters and desulphurifiers develop. The presence of suitable organic matter and sulphate in an anaerobic environment leads to the production of H_2S which is a lethal toxic substance for most living organisms. The products from anaerobic processes are very clear indicators of organic pollution.

Thermal pollution

Thermal pollution occurs when industries (eg. electrical and nuclear power stations) acquire large amounts of water for cooling purposes from a water body and then return the water at elevated temperatures to the source. The microbial community responds to (and thus act as indirect indicators) thermal pollution by increasing in numbers for particular genera and an overall reduction in species diversity. The following affects or observations can occur as a result of thermal pollution; (1) an increase in the gram negative rods

Psuedomonas and *Flavobacterium*, normal flora of power station discharge ponds; (2) an increase in fish mortality from microbial pathogens such as myxobacterium (*Chondrococcus columnaris*), vibrios (*vibrio anguillarum*), *pseudomonas* and *Aeromonas* (Mitchell, 1978). and (3) the longer survival of coliforms and human pathogens from other polluting sources in warmer waters. For example, the protozoan pathogen *Naeglaria fowleri* is only found in waters with temperatures of approximately 40°C.

Heavy metals, aromatics and organic pollutants

The metabolic diversity of bacteria provides the microbial community with the ability to utilize a wide range of carbon sources. Amongst this incredible range there is some very surprising substances unique to the microbial population including; chemical pollutants, aromatic compounds and heavy metals. The presence or increased abundance of species using such alternative energy sources could therefore serve as biological indicators of pollution.

A study performed by Lemke and Leff (1997) investigated (with culture based and nucleic acid based methods) the abundance of three species, *Acinetobacter calcoaceticus, Burkholderia cepacia,* and *Pseudomonas putida* as indicators of pollution. All three species utilize a wide range of substrates -*Acinetobacter calcoaceticus* can metabolise aliphatic alcohols, decarboxylic and fatty acids, unbranched hydrocarbons, and aromatic compounds and *Pseudomonas putida* is able to degrade toluene, styrene and naphthalene. The numbers of these species present in an unpolluted stream were compared with those obtained from a stream heavily polluted with inorganic compounds (eg. arsenic cyanide, sulphate, and sodium chloride), metals (mercury, aluminium, chromium, lead and zinc) and organic pollutants (oil, grease, PCBs, organic halogens). The results of these experiments found that *A. calcoaceticus* responded the most to pollution with its numbers being almost nine times higher in the polluted streams suggesting its potential as an indicator organism. *Pseudomonas putida* however is also able to degrade the pollutants that were present in the stream but its numbers actually decreased implying that other site related factors could be affecting its abundance. This is an important point to take into consideration when choosing

an indicator species and interpreting the results. The fact that the concentration of a particular species may not have changed, does not necessarily infer that a pollution event has not occurred or affected the biological population. The findings of Lemke and Leff (1997) suggest the importance of using more than one indicator organism.

1.8.3 Physiological responses as bioindicators

Behavioural responses of microbial populations can also act as bioindicators of various stress causing agents.

Chemotaxis

Bacteria are drawn to many stimuli in their environment. Motile bacteria are able to respond with movement to chemical stimuli such as nutrients. This movement is referred to as chemotaxis. Predation, symbiosis and nutrient location all depend to some degree on chemotaxis and thus it is an important part of microbial ecology. Very low levels of pollutants such as hydrocarbons, pesticides and heavy metals are able to block chemotactic behaviour. The absence of bacterial chemotaxis, could therefore be a behavioural response to indicate the presence of pollutants (Mitchell, 1978).

1.9 Indicators of bacterial diversity

Microbial diversity is of great significance to aquatic ecosystems. A highly diverse population allows for inter-species relationships, an important requirement for the continual cycling of inorganic and organic nutrients. Species diversity helps to maintain the stability of a population. Microbial communities with a high diversity are able to adapt with changes within their environment providing the shift is not to extreme. Microbial communities in environments that are strongly controlled by a particular physical and/or chemical factor (eg. acidic, thermophilic or salty habitats) have a lower population diversity. As already noted in the previous sections, pollution results in a loss of species diversity making biological diversity an important indicator of river 'health.'

Despite the crucial role microorganisms play in aquatic ecosystems, their biodiversity has not been assessed in many studies concerning this area nor has it been accepted as being as important as plant or animal biological diversity. There are a number of reasons why the microbial population is often overlooked and even trivialised in regards to biodiversity studies. The biggest hurdle to overcome is a methodological one. Traditional microbiological techniques (enrichment culture, plating methods) are able to isolate and identify only 1% of aquatic bacteria and even if these methods were capable of performing the task, the time and resources it would require would be out of the reach of most research budgets.

1.10 Methods in aquatic microbiology

The important role of microbial populations in aquatic ecology is accepted throughout the scientific community. However, there still remains many gaps in our knowledge and understanding of microbial ecology especially in the area of species diversity and identification. Research into these areas has been in the past hindered by a lack of techniques suitable for environmental microbiology. The traditional microbial methods that have been used for years in medical, plant, food and industrial microbiology cannot overcome the unique problems that arise when they are applied to environmental microbiology. Despite the setbacks, aquatic microbial ecology has begun to advance rapidly as new molecular biological procedures and new instrumental techniques, such as field flow fractionation and flow cytometry have been developed and applied to the area.

1.10.1 Methods for enumerating and identifying bacteria

Viable counts and enrichment procedures

The number of dividing cells within a sample (viable cells) can be determined by counting the number of cells which are able to form colonies on a solid media containing all the necessary growth requirements. This technique is referred to as plating and is employed to separate individual microbes into distinct colonies. Samples containing viable cells can

also be inoculated into liquid media (broth) which increases (enriches) the concentration of cells. By altering the chemical composition of media and changing incubation conditions, plating and enrichment procedures can be used to select specific bacterial groups on the basis of their diverse nutritional requirements. Once a pure culture has been obtained which can take more than one enrichment and plating procedure, the particular microbe is identified by performing a number of physiological, biochemical and even serological tests.

The pure culture technique can identify thousands of different bacterial species, however research has shown that less than 1% of the total number of bacterial species in nature can be isolated and identified in this way (Torsvik *et al.*, 1990a,b). The reasons why traditional culturing methods are so unsuitable for natural samples are as follows; (1) plating and enrichment procedures will only isolate the organism if it makes up a significant proportion of the microbial population; (2) the technique selects for the dominant microbe, and in order to isolate organisms present in smaller numbers it is necessary to use a growth medium and conditions that strongly favours that organism. Substances that inhibit the growth of other species can also be added to the broth. Problems therefore arise when applying this method to natural samples because a prior knowledge of the species growth requirements is needed and this makes identifying novel organisms very difficult. Many bacterial species in the natural environment are fastidious organisms, unable to grow in the laboratory and so viable counts obtained using culture methods are usually only 0.0001% to 10% of the counts obtained from direct microscopy.

Direct counts

There are a variety of different stains available that can be used to visualize and hence count bacterial cells under a microscope. The two most widely used stains in aquatic microbiology are the fluorochromes – 4',6-diamidino-2-phenylindole (DAPI) (Porter and Feig, 1980) and acridine orange (Hobbie *et al.*, 1977). Water samples are stained, filtered onto a membrane filter and then viewed with epifluorescent microscopy. Both stains bind to the nucleic acids within the cell causing the cell to fluoresce. Direct counts give relatively accurate results but there are a number of disadvantages. Microscope counting is very

tedious and time consuming, the particulate matter found in aquatic samples, often making the differentiation of cells from non-biological material difficult. Poor resolution and the small nature of natural bacteria hinders the identification of morphological characteristics and aggregate formation can prevent the counting of individual cells. Cell concentrations must be higher than 10^6 cells per mL to obtain at least one cell per field of view, with 10 cells per field required for results to be statistically correct. The biggest disadvantage of direct counts is the inability of the technique to distinguish between dead and alive cells.

The Tetrazolium salt iodonitrotetrazolium violet (INT) and the tetrazolium redox dye, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) have been used in conjunction with microscopy to determine the active fraction of the microbial communities in aquatic and soil systems. INT is a non-fluorescent stain that is reduced by an active cells respiration system to INT formazen, a dark red crystal visible with a light microscope. Visualising the non-fluorescent red granules in small cells is often difficult and so CTC is often preferred over INT. CTC is reduced by respiring bacteria to CTC-formazan, it is however easier to visualise because it is viewed with a fluorescent microscope. The main limitation of using either of these stains is the difficulty in determining whether small bacteria contain the red granule (INT more so than CTC), resulting in an underestimation active bacterial numbers. Studies have also questioned the ability of all bacterial species to actually take up and reduce INT or CTC, variations in uptake and reduction within and between populations from different environment could also result in misleading results and requires further investigation (Cook and Garland, 1997).

1.10.2 Immunological techniques

Antibody specificity can be used to detect particular microbial groups in environmental samples. Antibodies attach to specific structures on the surface of cells called antigens. Any structure on the cell wall such as a receptor can be an antigen and there are cell specific, species specific or genus specific antigens. Specific antibodies that bind to antigens on a cell surface are isolated from laboratory animals that have been injected with microbial cells or peptides from the microbial cell. Enzymes can be covalently attached to

the antibody creating a powerful immunological technique. Most of the immunological detection techniques employ some sort of colourimetric or fluorometric system. Fluorescein isothiscyanate, for example is a fluorochrome that can be coupled to antibodies. Peroxidase, and alkaline phosphatase are commonly used antibody linked enzymes that catalyse colour producing reactions.

Immunological techniques have been used to detect a number of aquatic microbes (methanogenic, nitrifiers, acid tolerant, methane oxidisers) that are difficult to identify by traditional bacterial culture methods (Conway de Macario *et al.*, 1982; Howgrave-Graham and Steyn, 1988). Despite the fact that this method can be used to detect organisms that are difficult to grow, it is still restricted to microorganisms that at some time have been grown as pure cultures. There are also factors and problems associated with antibody detection techniques that may affect its successful application; (1) the specificity of some antibodies can be a problem, in some cases they can cross-react with non-target cells, (2) autofluorescence, (3) inconsistent expression of the antigen on the cell surface may occur or the antigen may be unstable under certain environmental conditions, (4) antibodies are expensive because they are time consuming to produce, and (5) the technique doesn't distinguish between live and dead cells and isn't really suitable for quantitative analysis.

1.10.3 Molecular microbial methods

Molecular microbiology is a term used to describe techniques that study the key informational molecules within a cell, nucleic acids and proteins. Molecular methods go directly to the source containing all the information that determines a cells structure and function. Molecular methods can utilise the genetic information encoded with a cells nucleic acids to measure species composition and the abundance of particular groups, thereby circumventing the need to culture microorganisms.

In the last decade, nucleic acid technology has progressed rapidly and there are many examples where molecular analysis has been applied in natural systems, advancing our knowledge of the microbial ecology and providing information unattainable with classical techniques (Holben *et al.*, 1988; Steffan *et al.*, 1988; Giovannoni *et al.*, 1988; Ward *et al.*,

1990; Britschgi and Giovannoni, 1990; Hahn *et al.*, 1992; Voytek and Ward, 1995; Felske and Akkermans, 1998). Molecular microbiology is a very powerful tool. It is however, not without its limitations and these need to be taken into account when applying this technology, especially to environmental samples. The main techniques used in molecular microbiology are discussed below.

Nucleic acid extraction

There are two strategies whereby the total DNA of a microbial population can be obtained and not exclude a large section of the population from analysis like in traditional methods. The first approach is referred to as 'the cell extraction method' and involves the separation of cells from the environmental sample (sediment or soil), followed by cell lysis and nucleic acid purification (Holben *et al.*, 1988; Torsvik *et al.*, 1990). The second approach directly lysis the microbial cells within the environmental sample and then extracts and purifies the nucleic acids (Ogram *et al.*, 1987; Steffan *et al.*, 1988). Furhman and colleges developed a slightly different technique to extract microbial DNA from aquatic samples. Cells are firstly concentrated from the water column by filtration and the DNA is then directly extracted from the filter membrane. Extraction of nucleic acids is generally the initial step in any molecular analysis of microbial ecology, it is very important therefore, that the extraction procedure meet certain criteria to ensure that the DNA is suitable for analysis. The DNA obtained from the extraction procedure must be representative of the total microbial community and any loss of DNA should be non-bias. The sampling strategy employed is also very important. The species composition of the samples collected should depict the natural environment and not change before the extraction procedure takes place. The final product must be of a sufficient quality and quantity for use in other techniques such as PCR, cloning, probing and DNA hybridisations. It is often difficult to obtain high quality DNA from environmental samples, and this is the limiting step for many studies. Contaminants, such as humic substances can interfere and inhibit some molecular techniques, PCR being particularly sensitive. For some samples a simple phenol-chloroform extraction is sufficient to purify DNA (Furhman *et al.*, 1988) but others require

more substantial or additional purification steps. Additional purification procedures available include caesium-chloride-ethidium bromide centrifugation (Holben *et al.*, 1988; Steffan *et al.*, 1988), Hydroxyapatite columns (Torsvik *et al.*, 1990), Sephadex gel columns (Jackson *et al.*, 1997) or the addition of polyvinylpolypyrrolidone (PVPP) which binds to contaminants facilitating their removal by centrifugation (Holben *et al.*, 1988; Steffan *et al.*, 1988; paul *et al.*, 1990).

Once the nucleic acids have been extracted and purified it is ready for analysis. There are a number of direct and indirect molecular approaches available and the method chosen will depend on the information the researcher requires. Four direct procedures have been performed on total microbial community DNA to measure bacterial biodiversity within a particular environment and these are:

(1) DNA cross hybridisation – The microbial DNA from one environment is extracted, denatured (made single stranded) and immobilised on a membrane. Single stranded radiolabelled DNA from another environment is then allowed to re-anneal with the denatured DNA on the membrane. The degree of re-annealing or cross hybridisation that occurs is dependent on the extent of genetic similarity between the two environments. This technique can be used to detect spatial and temporal variations that may exist in species composition. For example, it could be used to make between site comparisons upstream and downstream of a pollution outlet in a river.

(2) The rate of re-annealing – DNA is denatured by temperature and then slowly cooled in a temperature block attached to a spectrophotometer. As the temperature falls, the DNA re-anneals to its homologous partner, the rate at which this re-annealing occurs is measured by the spectrophotometer. The re-annealing rate can be used as an indicator of diversity of a population on the premise that the more diverse the population the longer it takes for DNA to re-anneal.

Both of these methods are capable of detecting changes in species composition within a bacterial community and provide crude estimates of species diversity. They do not however,

provide information regarding individual bacterial groups, which ones have increased in number or declined or perished.

(3) Molecular probes – the DNA or RNA from a microbial community can also be analysed with either domain, kingdom, phyla, genus or species specific probes. Probes can be used to detect changes in the bacterial community as well as providing quantitative and qualitative information concerning particular groups and will be discussed in further detail below.

(4) Electrophoresis – low molecular weight RNA can be separated by gel electrophoresis and the number of bands of different sized RNA molecules present should represent the number of species present.

Hybridisation

Hybridisation is the artificial construction of a double-stranded nucleic acid by the complementary base pairing of two single-stranded nucleic acids. Nucleic acid hybridisation is a powerful tool for studying the genetic relatedness between nucleic acids. It also provides a way for detecting pieces of nucleic acid that are complementary to a single stranded molecule of known sequence, referred to as a probe. For example, a probe that is chemically or radioactively labelled can detect in an unknown mixture a nucleic acid sequence complementary to the probe. Probes can be DNA or RNA of varying lengths from several nucleotides to several kilobases or entire organism genomes. A number or experimental conditions can influence the extent of hybridisation including: salt concentration, contact time, temperature, the degree of mismatch between base pairs, and the length and concentration of the target and probe sequences.

There is a range of methods where the technique of hybridisation can be applied to determine the presence of bacterial DNA or RNA in the environment. Methods include *in situ* hybridisation whereby the labelled probe binds to DNA or RNA within intact cells and individual cells containing the hybridised probe are detected by microscopy. Alternatively, the DNA is extracted from the microbial population, melted (made single-stranded) and

immobilised on a membrane. The single-stranded probe then hybridises to complementary sequences bound on the membrane.

There are two main types of probes that can be employed in hybridisation experiments: (1) rDNA probes are based on the nucleic acid sequences of the ribosomal RNA genes and can vary in their specificity from highly specific species probes to more general group probes such as sulphate reducing to universal probes that bind to all bacteria; (2) functional gene probes, for example heavy metal resistant genes.

Probes directed at the rRNA gene are currently extensively used across microbial ecology and are discussed in more detail below.

Ribosomal RNA techniques

The ribosomal RNA molecule has a number of characteristics which make it particularly useful in the analysis of microbial populations. The ribosome itself is a multienzyme cellular molecule with a very important structural and functional role in protein synthesis. Bacterial cells contain vast numbers of ribosome molecules (*E. coli* contains ~ 20,000 ribosomes).which can contribute as much as 40% of the dry weight of the cell. The rRNA amount to 50-60% of the ribosome mass and subsequently about 20% of the cell dry weight (Pace *et al.*, 1986). In all cell types the ribosome comprises of two subunits, referred to as *small* and *large* subunits. The small subunit of eubacterial cells contains one 16S rRNA molecule (~1500 nucleotides), whereas the large subunits contains 23S rRNA (~2900 nucleotides) and %S rRNA (~ 120 nucleotides).Due to the essential role that ribosomes play in the synthesis of protein they evolved early in evolution, are functionally homologous and highly conserved in structure in all organisms. Different positions in the rRNA sequence have evolved at different rates, providing a means to measure even the most distant phylogenetic relationships (Woese 1987). Much of the nucleotide sequence of rRNA are conserved across kingdoms, in the 16S rRNA sequence as many as 360 out of 1542 nucleotides have remained unchanged between eubacterial, archaebacteria and eukaryotes (Gutell *et al.*, 1985). The conserved regions permit the alignment of variable sequences. When the sequence of 16s rRNA nucleotides of contrasting organisms are compared,

sequences are aligned to the corresponding position in *E.coli* (Gutell *et al.*, 1985). These unique properties of rRNA, researchers have been able to identify virtually any organism or group of related organisms on the basis of their rRNA sequence.

Molecular techniques based on rRNA are very powerful methods of analyses as they are very sensitive, can be employed *in situ* and can be directed at particular species groups, general phylums, or domains of bacteria by the design of specific probes. Currently, there are many specific 16S rRNA probes available for a range of bacterial groups (Giovannoni *et al.*, 1988 & 1990; Ward *et al.*, 1990; Amann *et al.*, 1995).

There are two ways rRNA can be used to study microbial populations which circumvent the need for culturing:

(1) Early attempt at applying rRNA molecular techniques to analyse microbial populations, relied upon the direct extraction, purification and sequencing of 5S rRNA from environmental samples (Pace *et al.*, 1986; Olsen *et al.*, 1986). The short length of the 5S rRNA sequence restricted it use however, in high-resolution phylogenetic analyses. The development of Polymerase Chain Reaction-cloning-sequencing techniques allowed for the analyses of more complex communities using the 16S rRNA molecule (Giovannoni *et al.*, 1990; Ward *et al.*, 1990). In this procedure DNA is directly extracted from the community of interest, primers designed for the conserved regions of the 16S rRNA gene are employed in PCR to amplify the region. The PCR products are cloned in specially designed plasmids, purified and sequenced. The rRNA sequences are then carefully aligned using the conserved regions and compared with published and unpublished sequences available from the 'Ribosomal RNA Database Project' (Olsen *et al.*, 1991).

There are limitations associated with the PCR-cloning-sequencing method that are ....portant and require consideration. Methodological problems include; biases in cell lysis, inefficient recovery of DNA and differential PCR amplification (discussed in section 1.x). Furthermore, bacterial species that are only a minor component of the microbial community are likely to remain undetected unless extensive clone analysis occurs.

(2) The bacterial community in a water sample or a rDNA clone library can be probed with oligonucleotide sequences that will detect specific bacteria or groups. An alternative approach to the time consuming task of sequencing 16S rRNA genes is to probe with a range of phylogenetic probes. For example Rheims *et al.* (1996) extracted DNA from the bacterial community of a peat bog, amplified 16S rRNA genes, and inserted the PCR product into a plasmid vector. By screening the inserts with a number of oligonucleotide probes (*Gram-positive, Planctomycete, Spirochete*, and other taxon specific probes) they were able to screen over 260 clones.

This method of rRNA analyses also has its disadvantages. The reactivity of the oligonucleotide rRNA probes cannot always be predicted. There is the possibility that unknown sequence types may cross-react with a probe compromising its specificity. It is therefore, imperative that the specificity of a probe is adequately tested prior to its application, a process that is very time consuming. Furthermore, the probing of whole cells depends on the *in situ* penetration of oligonucleotide sequences through the cell wall and membrane.

In situ or whole cell hybridisations

In recent years, whole cell *in situ* hybridisation has been used to examine many ecosystems (Amann *et al.*, 1995). The advantage of this technique is that it is a quantitative method where fluorescent or radiolabelled probes specific for phylogenetic groups or functional genes can be used to visually detect bacteria of interest within a mixed population. Multiple probes, labelled with different fluorescent dyes can also be used simultaneously to identify various cell types in the same microscopic field (De Long *et al.*, 1989). There are two methods available to perform whole cell hybridisations:

(1) Bulk dot blot- Water samples are fixed with formaldehyde or glutaraldehyde and vacuum blotted onto a filter membrane. Fixed cells can be stored in this way at -70°C for several months with no loss to their probe hybridising ability. The fixed samples can then be challenged with a range of specific probes (Giovannoni *et al.*, 1988). This method is

most suitable for estimating abundance of different bacterial types within the water sample.

(2) Slide hybridisation - an alternative method for quantifying bacterial groups within a particular community, is hybridisation on microscope slides. In this technique the bacterial cell concentration is firstly determined by epifluorescent microscopy. Cells which contain bound probe are also individually counted microscopically, making it possible to then estimate the contribution of different species or groups to the total population. In this technique because the degree of hybridisation is determined by the counting of the actual cells containing probe and not from the amount of bound probe, the method is not subject to variations in probe binding efficiency. *In situ*-hybridisation can also give an indication of activity levels as 16S rRNA content is proportional to cell activity, non-viable cells contain less rRNA and will have little or no fluorescence (Pace *et al.*, 1986).

There are a number of methodological constraints that exist with whole cell hybridisations. If this method is to be utilised, especially in a quantitative capacity, the influence of factors such as permeability of fixed cells, accessibility of probe to its target and sensitivity must be considered. Cell permeability can be improved with pretreaments such as solvents, acids or enzymes (Zarda *et al.*, 1991; Amann *et al.*, 1992; Hahn *et al.*, 1993), but even then certain cell types with more rigid cell wall structures (eg. gram-positive) may remain relatively resistant to probes (Amann *et al.*, 1992).

Even if cell permeabilization occurs successfully there is still no guarantee that the probe will hybridise to its target sequence. The most likely cause for this anomaly is that the probe is unable to gain access because of strong interactions with ribosomal proteins or secondary structure elements of the rRNA itself. This problem can be highlighted if a strong signal is obtained with a universal probe that has previously been shown to bind to an accessible site. If another probe does not produce a signal in the same cells, then it usually implies inaccessibility of the probe to its target (see Amann *et al.*, 1995 for a list of successfully applied probes).

The disadvantages associated with using rRNA as the target molecule for probes as a quantitative approach to estimating cell numbers (of particular phylogenetic groups) has recently led investigators to examine the feasibility of using rDNAs as an alternative (Edcomb *et al.*, 1999). In this approach total community DNA was extracted and immobilised on a membrane. Hybridisations were performed with probes specific for seven phylogentec groups, including a general bacterial probe and probes designed for most members of the sulphate reducing bacteria. Amounts of DNA detected in each sample was determined by generating a standard curve with hybridiastion signals of known concentrations of DNA. An estimate of the average amount of DNA per cell for the bacterial groups targeted was determined as a conversion factor for converting hybridisation signal to cell numbers (Edcomb *et al.*, 1999). Obviously, there are disadvantages associated with this method as well. Conversion factors generally introduce a source of error and need to be determined for each individual bacterial group examined. The investigators have also assumed that the genome copy per cell is ., because they are environmental samples and hence DNA concentrations per cell should correlalte with genome equivalents and cell numbers. Genome copy per cell varies with growth condition and just because the bacteria being examined have originated from a natural environment where generally, growth rates are not equivalent to those obtained in the laboratory does not mean researchers should assume that genome number per cell is going to remain constant and always low.

Sensitivity of whole cell hybridisation is also a problem associated with the method as small or slow growing cells will not be detected because of their low copy number of cellular ribosomes. A number of approaches have been deployed to try and improve the methods sensitivity including: *in situ* PCR which amplifies 16SrRNA genes within the cell (Hodson *et al.*, 1995); multiple singly labelled probes (Lee *et al.*, 1993); multiply labelled probes, and enzyme linked systems that amplify the signal (Amann *et al.*, 1992).

It has also become apparent as more rRNA sequences have been discovered and collated on sequence database programs that designing specific probes is difficult. This problem obviously, exists for all molecular techniques (eg. PCR) that rely on the specificity of oligonucleotides.

Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a process whereby DNA molecules can be multiplied by up to a billion-fold *in-vitro*., producing large quantities of specific genes for further manipulations (eg. cloning or sequencing). The technique utilises the enzyme DNA polymerase to copy specific sequences or genes, it does however require previous knowledge of a small portion of the nucleotide sequence flanking the gene of interest to allow primers to bind.

There are three basic steps involved in PCR: (1) DNA is denatured (made single stranded) by heating to 94°C, (2) the solution is then cooled, allowing primers to anneal to the target DNA (eg. 16s rRNA), and (3) the primers are extended by nucleotide addition along the length of the gene by the enzyme DNA polymerase. This process is repeated many times, resulting in the exponential increase of the target DNA.

PCR is a very commonly employed tool in environmental microbiology for detecting specific bacterial groups and analysing microbial diversity. It is widely used in the detection of specific bacterial groups, including pathogenic organisms such as toxigenic *E. coli* (Jones *et al.*, 1999), *Cryptosporidium* (Johnson *et al.*, *1995;* Chung *et al.*, 1999) *Pseudomonas* (Leser *et al.*, 1995), *Legionella pneumonia* (Miyamoto *et al.*, 1997), enteroviruses (Reynolds *et al.*, 1998) as well as important functional groups such as

Ammonium-Oxidising bacteria (Voytek and Ward, 1995; Hastings *et al.*, 1998), Sulphate reducing bacteria (Sass *et al.*, 1998) *Nitrobacter* (Degrange and Bardin, 1995) *Mycobacterium chlorophenolicum* (Briglia *et al.*, 1996) and Methanogens (Hales *et al.*, 1996; Edwards *et al.*, 1998).

PCR is an important tool for amplifying genes intended for cloning and sequencing (eg 16S rRNA) when analysing microbial diversity.

PCR has played a pivotal role in advancing our understanding of microbial ecology but it is not without its limitations. First, the technique requires very pure extracts of DNA or RNA, a difficult process for some environmental samples and a knowledge of at least part of the DNA sequence is also required for the design of primers. Preferential amplification of certain sequences has been shown to occur and is thus a source of bias that can affect the results of studies measuring microbial diversity. There is concern that low-abundant sequences and templates with a high G+C ratio are discriminated against (Ward *et al.*, 1992) which could result in the failure to clone some species and their subsequent detection. The generation of chimeric PCR products, where two different sequences become fused from coamplification has also been observed, and this could lead to the reporting of nonexistent novel sequences (Suzuki and Giovannoni, 1996).

Restriction Fragment Length Polymorphism analysis

Restriction fragment length polymorphism analysis is a technique that allows complex microbial communities to be rapidly analysed. Basically, rRNA genes are amplified by PCR using universal primers and the product digested with restriction enzymes that have a four base-pair recognition site. There is the potential with complex bacterial populations that a large number of fragment will result from digestion, therefore resolution of fragments is performed in polyacrylamide gels to obtain community-specific patterns (Martinez-Murcia *et al.*, 1995). Generally, the pattern complexity is indicative of community diversity and a change in this pattern, the result of changes in community structure. Liu and colleges (1997) further developed this method by fluorescently labelling one of the primers so that when the restriction fragments were analysed with an automated DNA sequencer, the sizes of only the terminal restriction fragment could be determined. The technique is referred

(1)

RFLP analysis has been used to analyze changes in the composition and structure of low-biomass, subsurface communites in response to storage time and sample condition (Chandler *.et al.*, 1997). It has also been used to estimate the impact of long term inputs of organic and inorganic pollutants on bacterial diversity within aquatic habitats (Sorci *et al.*, 1999). The advantage of RFLP analysis is that results can be obtained relatively quickly, in comparison to hybridisation/probing experiments and it is a method of analyzing bacterial community diversity and any associated changes.

(2)

Molecular techniques have made it possible to study microbial community structure and diversity within natural habitats that previously was not possible using conventional methods. However, it is important that investigators recognise the many experimantal biases still associated with these molecular techniques. Almost all of the methods discussed above rely on the extraction of community DNA, cell lysis bias and the substantial loss of DNA as a result of the purification steps required with natural samples is a significat problem. Polymerase Chain Reaction introduces a whole range of potenial bias as does the use of probes.

Molecular methods are also only able to give an insight into sequence variations within a population. They do not provide information concerning cell function, growth or activity. Molecular techniques may identify the presence of particular species or uncover novel sequences but they do not reveal how these organisms are contributing to the ecosytem, ie. are they active/inactive? What is the organisms functional role?

to as Terminal Restriction Fragment Length Polymorphisms and it has the added advantage of being able to quantify (by the fluorescence) the fragments.

Denaturing Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (DGGE) is a method that can be used to separate DNA fragments of the same size but of different sequence electophoretically. In this technique, fragments of double-stranded DNA are run through a polyacrylamide gel that has an increasing concentration of a denaturant (eg. urea or formamide). At certain denaturant concentrations, regions of the DNA will denature, slowing the rate of migration through the gel. Different fragments will denature at different points in the gel, allowing two fragments of identical size to be separated from each other. By ensuring that the concentration gradient of the denaturant is only shallow, the technique can be sensitive enough to differentiate DNA fragments that differ by only a single base pair (Sambrook *et al.*, 1989)

DGGE can be applied to analyse 16S rRNA PCR products from environmental samples as the successful resolution of heterogenous PCR products on a polyacrylamide gel can be achieved (Muyzer *et al.*, 1993). Individual bands can be excised from the gel, purified, reamplified and sequenced. Alternatively, the bands can be targeted with a range of oligonucleotide probes, both approaches will help unravel the composition and diversity of the microbial community under investigation. DGGE has been used to identify dominant members of bacterial populations and to analyse the variability in community composition as a function of time, vertical stratification and sampling location (Konopka *et al.*, 1999). DGGE can detect sequence diversity within important functional groups. For example, Speksnijder *et al.*, (1998) discovered sequences of Nitosomonas (ammonia-oxidiser) previously undiscovered in freshwater habitats.

1.10.4 Flow Cytometry

Flow cytometry is a rapid automated technique that can quantitatively measure physical and chemical characteristics of cells. Single cells are sorted at more than 10^3 cells per second on the basis of fluorescence emitted from specifically bound dyes and their light scattering properties The cells flow past a focused light beam in single file. The light source can be either laser based (argon lasers) or a mercury-vapour lamp

The light source can be either laser based (argon lasers) or a mercury-vapour lamp. Flow cytometry can provide data relative to five parametres: forward angle (~10 to 15 °) and side angle (90°) scatter of the incident illumination source and three fluorescence channels (blue, green, and red) (De Leo and Baveye, 1996). A range of different dyes (nucleic acid stains viability stains) and probes can be used to analyse cells and it is possible to use forward and side scatter to distinguish populations and observe changes within populations (De Leo and Baveye, 1996; Wallner *et al.*, 1995; Jepras *et al.*, 1995; Porter *et al.*, 1995; Lebaron *et al.*, 1998). Many of the nucleic acid dyes are available in kit format, however, it has been shown that not all dyes are appropriate for all environments. For example, some dyes are better suited to nonsaline waters, while others are appropriate for only unfixed samples (Lebaron *et al.*, 1998).

Flow cytometry has been used in the enumeration of bacterial cells and has the advantage over direct microscopic counts, in that 50,000 cells in a sample can be quickly and easily counted (De Leo and Baveye, 1996). Technical problems such as cell aggregation in the sample solution that may block the flow cytometer and the dispersal of bacterial cells from particulate matter and aggregates so that cells are not masked and missed in counting are all drawbacks of this method that may interfere with enumeration (De Leo and Baveye, 1996). Despite the technical problems associated with flow cytometry, the method is still considered more accurate than microscopic enumeration because of the higher number of cells counted (Lebaron *et al.*, 1998).

Flow cytometry has also been reported as a potential new technique in the estimation of bacterial biomass. The intensity of forward light scatter is analysed by flow cytometry and used to calculate cell size or dry mass (De Leo and Baveye, 1996; Robertson *et al*, 1998). See section 1.10.5 for microbial biomass estimations.

1.10.5 Methods for Determining Microbial Biomass

Bacterial biomass is a measure of the bacterial mass within a unit area and is the net effect of production, transport and predation (Edwards *et al., 1990*). Biomass measurements are essential for understanding the ecological role of bacteria in aquatic ecosystems as they provide an insight into the bacterial populations potential activity and contribution to the food chain. Measuring bacterial biomass is less straightforward then bacterial enumeration and generally, only crude estimates are obtained.

Biomass from biovolume estimates

The most common conventional method for biomass determination is the estimation of cell biovolume from direct microscopy measurements. Biomass is then calculated by applying biovolume to carbon conversion factors (Norland *et al.*, 1987; Kroer 1994). This method has many sources of error which can result in an overall error of up to 600% (Sharma *et al.*, 1993). Sources of error include inaccurate estimates of biovolume due to the difficulties in enumerating and measuring cell dimensions microscopically and the discrepancies associated with carbon conversion factors. The microbial communities in natural aquatic environments are very complex and the appropriate volume:mass ratio will vary for different cell populations. It has been found (Simon and Azam 1989, Kroer 1994) that bacterial biovolume to carbon conversion factors vary both temporally and spatially with the carbon content per unit volume increasing with decreasing size.

Sedimentation field flow fractionation

In the last decade, Sharma and colleagues (Sharma *et al.*, 1993) have applied a technique called Sedimentation Field Flow Fractionation (SdFFF) to determine bacterial biomass from a diverse range of natural habitats (Sharma *et al.*, 1998). SdFFF uses centrifugal force to separate bacteria on the basis of their buoyant mass. The bacterial cells are first concentrated and stained with a fluorescent dye, 4'6-diamidino-2-phenylindole (DAPI). The sample is then injected into the thin, flat chromatography-like channel of the SdFFF instrument which is spun to produce a centrifugal field. The resulting centripetal

The SdFFF method for biomass determination has many advantages over flow cytometry measurements of biomass. Flow cytometry methods are based on the conversion of fluorescence intensity to dry mass, problems therefore arise when applying this method to natural samples. Samples from natural environments are very heterogenous in composition and fluorescence per cell will vary for different bacterial species. Chemical fixatives such as formaldehyde have also been shown to effect light scatter and as previously mentioned many stains fluoresce at different intensities for different environments (Robertson *et al.*, 1998). In contrast, the SdFFF method measures the buoyant mass of each cell directly. Further research with natural samples examining fluorescence variabilty and the effects of non-biological material on light scatter need to be performed before flow cytometry can be used to measure bacterial biomass in environmental samples.

force causes particles of different effective mass to be pushed to the outer channel wall. After a predetermined "relaxation time", and when the particles have adjusted their position relative to the wall (due to Brownian motion), the carrier flow is started. The empty nature of the SdFFF separation channel causes the carrier fluid to develop a parabolic velocity profile with highest flow rates in the centre and velocities of almost zero towards the channel walls. The parabolic profile of the carrier fluid results in both particles and microbial cells with a small buoyant mass migrating down the channel and eluting at a faster rate than those with a larger biomass. As the cells leave the channel, the cell fluorescence is measured and recorded as a fractogram. The cells are then collected into fractions at desired elution times for counting. The elution time or volume is used to calculate the biomass per cell and total biomass can be determined by measuring the cell concentration in each fraction (See Beckett *et al.*, 1988, 1990, and Sharma *et al.*, 1993, for derivation of relevant equations).

The SdFFF method for biomass determination has many advantages over the conventional biovolume:biomass methods which are discussed in Chapter 4.

1.10.6 Bacterial productivity measurements

Bacterial production is the increase of biomass over a particular period of time, ie the growth rate of a bacterial population. In natural systems biomass can not be used to determine growth rates because different bacteria are in different stages of growth at the same time with many in a starved or dormant condition. The final step in the growth of a bacterial cell is cell division and an increase in numbers and biomass (Moriarty 1986). For natural samples it is not possible to count newly formed cells and determine growth rate directly. However, before cells divide new cellular components such as cell walls, membranes, proteins, RNA and DNA are synthesised and these processes can be measured and used to determine growth. Bacterial production is most commonly measured using radio-isotope incorporation into bacterial cell components (DNA or protein) because they are very specific and highly sensitive (Fuhram and Azam 1980, 1982; Moriarty and Pollard 1981; Simon and Azam 1989; Findley *et al.*, 1991; Kirchman 1992, 1993; del Giogio and Cole 1998).

Thymidine or Leucine incorporation

The tritiated thymidine method for measuring bacterial production is based on the ability of dividing cells to take up and incorporate [^{3}H]-labelled thymidine (TDR) into DNA. This method assumes that DNA synthesis is related to cell division and synthesised in dividing cells at a rate proportional to biomass. Therefore, the rate of TDR incorporation is related to the rate of bacterial production per unit volume or time (Riemann and Bell, 1992). Standard conversion factors from number of cells per mole of incorporated isotope and mass of carbon per cell are then used to calculate growth rate from the thymidine incorporation rate.

An alternative method for estimating bacterial production is the incorporation of [^{3}H]-labelled leucine into cell protein (Kirchman *et al.*, 1985; Chin-Leo and Kirchman 1988). Protein production is considered a good estimate of carbon flux into bacterial biomass because proteins constitute a major stable proportion (approximately 60%) of a cells dry weight.

See Chapter 3 for discussion of the advantage and disadvantages of using thymidine or leucine to measure bacterial production.

This thesis reports on the use of some of the methods available to study microbial diversity (DNA extraction, PCR, DNA hybridisation), the new method of measuring bacterial biomass with SdFFF as well as the more traditional techniques of bacterial enumeration (viable cell counts, DAPI and CTC staining) and measuring bacterial production with radioisotopes (tritated thymidine and leucine). These methods are evaluated as to their usefulness as tools for assessing river 'health' and their ease of application in natural environments.

CHAPTER 2
ASSESSMENT OF RIVER 'HEALTH' USING TRADITIONAL MICROBIAL METHODS AND PHYSICO-CHEMICAL ANALYSIS

2.1 Introduction

An aquatic system is considered 'healthy' when it has the ability to perform all essential ecological processes and can maintain evolutionary potential (i.e preserve future biological diversity) (ANZACC 1992). The ultimate indicator of aquatic ecosystem 'health', is the health and diversity of the biological community. A decline in ecosystem 'health' will result in a reduction of biological diversity and the ability of that system to survive and adapt to change (ANZECC 1992).

In order to assess a particular rivers' health, it is necessary to perform a wide range of physical, chemical and biological analyses. Many of the methods employed t achieve this are termed traditional techniques and have been used in aquatic science for many years and still have an important role today. There are many traditional microbial methods, originating from medical microbiology however, that have major limitations when applied to natural microbial communities and produce unreliable and inaccurate results (Ferguson *et al.*, 1984).

The current techniques used to determine the physico-chemical characteristics of an aquatic system have been well established over the years. Published protocols are available to guide researchers in the most appropriate way to analyse samples (Standard methods APHA-AWWA-WPCF 1995). Accurate results are therefore relatively easy to obtain for these parameters. Water and health authorities have also stipulated certain guidelines and criteria that a water system must meet for it to be considered 'healthy'. The criteria, of course, depend on the intended use of the water body, for example, ecological protection, drinking purposes or recreational use. These criteria for ecosystem protection are outlined briefly Table 2.1.

Table 2.1 Physico-chemical guideline trigger values for the protection of Lowland river systems (ANZECC & ARMCANZ Australian water quality guidelines 1999)

Stressor	Trigger level
Suspended particulate matter	6 mg/L
Dissolved oxygen[1]	90% saturation
Nutrients:	
Total-Phosphorus	37 µgP/L
Total-Nitrogen	1600 µgN/L
pH	6.6-8.0
Salinity	500 µS/cm increase
Turbidity	10 NTU
Temperature	80%ile increase, 20%ile decrease

1. Determined for at least one diurnal cycle

Recommended maximum concentrations are also available for a large range of organic and inorganic toxicants and the importance of biological diversity at all major trophic levels is emphasised as well (ANZAC & ARMCANZ 1999).

Physico-chemical analysis of water samples provides only limited information concerning ecosystem health. There is the potential with this type of monitoring to miss single point polluting events of a short duration, if the sampling has not been taken at the right time, under the correct conditions or the right parameters analysed. There is also the possibility that particular polluting events may adversely affect the 'health' of the aquatic ecosystem long after the physico-chemical characteristics have returned to normal (EPA, 1998). Certain components of the biological community on the other hand, respond to all environmental changes, short and long term. A change in biological health and diversity is

Table 2.2 Water quality guidelines for recreational and raw drinking waters (ANZECC 1992).

Indicators **Recreational purposes**	**Guidelines**
Microbiological	
Primary contact	<150 faecal coliforms/100 mL or < 35 enterococci/100 mL. No pathogenic free living protozoans.
Secondary contact	<1000 faecal coliforms/100 mL or <230 enterococci/100 mL.
Nuisance organisms	No excessive macrophytes, phytoplankton scums, algal mats, sewage fungus. Depending on the species, algal levels should not rise above 15,000-20,000 cells/mL
Physical and chemical	
pH	5.0 -9.0
Temperature	Prolonged exposure, 15-35°C
Toxic chemicals	No toxic substances that cause irritation to the skin or eyes should be present.
Surface films	No visible oil or petrochemical films
Indicators **Raw drinking water**	
Bacteriological	Up to 10 coliforms/100 mL may be accepted on occasions. No coliforms should be detected in 100 mL of 2 consecutive samples. 95% of 100 mL samples should be coliform free. No faecal coliforms at all.
Algal	Up to 5,000 cells/mL will be accepted, but cyanobacterial cells should be less than 2,000 cells/mL

the best indicator of a change in the integrity of the ecosystem. To ensure the future preservation of aquatic systems, protection strategies must be implemented that include monitoring of the biological community.

Up to the present, biological monitoring as a component of water quality assessment studies, has focused on algae and macroinvertebrate populations (Norris and Norris, 1995; Bunn 1995; EPA 1998). The microbial population has been relatively ignored in terms of river 'health' and generally only considered in terms of their potential as a threat to public health. The discovery that disease transmission can occur via drinking water contaminated with pathogenic bacteria, has led to the establishment of strict bacteriological guidelines to protect public health (Table 2.2). Raw waters for drinking purposes obviously, must also meet a long list of criteria for organic and inorganic toxic substances (ANZECC 1992).

Determination of bacterial abundance is often routinely performed during water quality monitoring and in studies of microbial ecology. Abnormal fluctuations in microbial numbers can provide an early insight to changes in the aquatic environment that may indicate a decline in river 'health'. Enumeration of bacterial populations is relatively easy to perform using direct staining procedures and epifluorescent microscopy. Generally, the cells are stained with the fluorochromes -4'6-diamidino-2-phenylindole (DAPI) (Porter, 1980) or acridine orange (Hobbie, 1977), concentrated onto black filters and then viewed and counted with epifluorescent microscopy. It is not possible with this method, however, to distinguish the various microbial groups present, or dead cells from viable cells.

The 'classical' viable count procedure along with other culture-based identification and enumeration techniques have been used in general microbiology to improve public health for decades (Brock, 1994). These methods are unsuitable for use in environmental microbiology as it has not been possible to develop a culture medium that is non-selective for certain groups of organisms, and furthermore, many bacterial species found in aquatic environments are non-culturable. Viable counts obtained with these traditional methods, drastically underestimate bacterial numbers by 90% to 99.9% compared with those obtained from direct microscopy. Selective media is still, however, employed to monitor levels of certain groups of organisms deemed useful as indicator organisms or a threat to public

health. Culture based techniques utilising selective media are approved methods for determining the numbers of coliform bacteria present in water samples. Faecal coliforms are used as indicators of sewage pollution, but even in this limited capacity, traditional microbial methods have problems that can lead to false conclusions. For example, the formation of non-culturable but viable bacteria can lead to underestimations of coliform numbers (Byrd, 1993).

The inabilities of the above methods to estimate active bacterial numbers has led to the employment of alternatives methods. One such method, commonly used to determine the metabolically active portion of the microbial population, is the use of redox dyes such as iodonitrotetrazolium violet (INT) and 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (Rodriguez, 1992; Pyle, 1995). CTC competes with molecular oxygen as an artificial electron acceptor and is thus reduced during respiration. This reduction of CTC by the electron transport chain of active bacteria results in the production of a fluorescent formazan which becomes deposited intracellularly as red crystals. When viewed under long-wave UV light, the CTC-formazan fluoresces red making the active cells easily distinguishable from other inactive cells and abiotic material.

The objective of the work reported in this chapter, was to evaluate the suitability of aquatic microbiological techniques currently available for the identification and enumeration of microorganisms and to use them in conjunction with physico-chemical analysis to determine their relative effectiveness in assessing river 'health'. Campbells Creek in Castlemaine, Victoria was chosen as the study site because it has a single point source of sewage effluent from Castlemaine sewage treatment plant. This made it an ideal location to carry out between-site comparisons upstream and downstream of the effluent release point.

2.2 Materials and Methods

2.2.1 Sampling

Midstream samples were collected from 12/7/1995 until the 3/3/1998 (see table 2.4 for specific dates). Sampling occurred at the same field location and time (0900 to 1100 hr)

for all field trips to minimise spatial and diurnal variation and so between-site comparisons could be performed. All samples were collected in polyethylene bottles, precleaned with detergent (5% Extran) and 20% hydrochloric acid and rinsed with milli-Q H_2O.

2.2.2 Determination of total bacterial cell concentrations, coliform counts and heterotrophic plate counts

Water samples (125 mL) were collected from sites A, B, C, and D (Fig 2.2 & 2.3), and stored on ice until they were transported on ice back to the laboratory. Various volumes (20, 10, 5 and 1 mL) were subsampled from each bottle within 6 hours of collection, and used in coliform counts. Aliquots of 1 mL were also removed from each sample for heterotrophic plate counts. The remaining sample in each container was then fixed with formaldehyde (final concentration 1-3%) and stored at 4°C until cell counting could proceed, generally within 1 week.

Direct cell counts of the entire bacterial community were performed by staining the fixed samples with 4',6-diamidino-2-phenylindole (DAPI) (Porter, 1980). A working stock of 10 µg/mL of DAPI was stored in plastic syringes at -20°C and 3 drops of this solution was used per mL of sample. The samples were sonicated to disrupt cellular aggregates and then vortexed (Vortex-genie, Medos equipment) to ensure they were well mixed. Depending on the concentration of bacterial cells, small volumes (0.3 mL to 2 mL) were added to the filter tower (Millipore, 25 mm filter holder, 15 mL volume) and made up to a volume of 5 mL with sterile deionised water. The DAPI was then added and the cells were left to stain for 2 min. The stained cells were filtered with gentle pressure (7 in/Hg) onto irgalan-black stained 0.2 µm polycarbonate filters (Poretics) and placed on a glass slide with immersion oil and cover slip. The cell tower was washed with sterile deionised water and 20% HCl between samples. UV-excitation epifluorescent microscopy was performed with an Olympus BH-2 microscope equipped with a HBO-100 epifluorescence illuminator, a 100x oil immersion objective (SPlan Apo 100) and exciter-barrier filter sets for UV (334-365 nm) and blue (435-490 nm) excitation. To facilitate counting, one eye piece of the microscope contained an indexed-squared graticule (width 70 µm) divided into 100 squares. Cells were

counted from between 10 to 30 random fields with a minimum of 10 cells per field of view The cell counts per field were converted to cells per litre using the following equation:

$$\text{cells/L} = \frac{\text{(mean cells/field of view) x (area of filter covered by sample)}}{\text{(field of view area) x (dilution factor) x (volume of sample filtered in litres)}}$$

Heterotrophic plate counts were performed using Collins and Willowby (1962) medium. This medium was reported by Holder-Franklin (1981) to support the most varieties of heterotrophic bacteria from aquatic samples. The media comprised the following components: 0.5 g soluble starch (Merk, analytical grade), 0.5 g peptone (Sigma), 0.5 g casein (Townson an Mercer), 1 mL glycerol (AJAX), 0.2 g K_2HPO_4, 0.05 g $MgSO_4$ (BDH, analytical), 4 drops of 0.01% w/v $FeCl_3.6H_2O$, 15 g Agar (DIFCO) and 1 litre of deionised water. The solution was then autoclaved at 121°C for 30 mins and when it had cooled sufficiently (~55°C) it was poured into sterile plates. These plates were freshly preparedthe day before each field trip. When the samples were returned to the laboratory, 1 mL was subsampled from all samples and serially diluted (1:10, 1:100, 1:1000) with media. Aliquots of 100 µL from each dilution were spread onto the heterotrophic plates and incubated at room temperature. The bacterial colonies on each plate were counted and recorded after 7 days of incubation.

For the enumeration of total coliforms and faecal coliforms, a membrane filtration medium was employed, consisting of Membrane endo agar les medium and Membrane -Faecal Coliform (M-FC) medium. Both mediums was acquired from Oxoid and had been prepared according to the Lawrence Experimental Station formulation of McCarthy *et al.*, (1961). Water samples (3 or 4 replicates from each site) were filtered through sterile 47 mm diameter, 0.2 µm membrane filters. The membranes were then placed directly on the agar surface and incubated for 24 hours at either 37°C (total coliforms) or 44°C (faecal coliforms). Colonies that appeared on the M-endo-agar-les medium within 24 hours and had a golden-green metallic sheen were assumed to be coliforms. Dark blue-purple colonies growing on the M-FC medium were assumed to be and counted as faecal coliforms.

2.2.3 *Enumeration of metabolically active bacteria*

The number of active bacteria within the microbial population was determined at each site using the redox dye 5-Cyano-2,3-ditolyl tetrazolium chloride (CTC). The CTC was prepared fresh before each field trip as a 0.04% w/v solution in sterile milli-Q H_2O. The CTC solution was stored on ice, in the dark, until it was required. One mL of the CTC solution was added to 10 mL of sample, contained in a 125 mL polyethylene bottle. The samples were incubated for 2 hours in 0.5 L of creek water held in an insulated cooler. This procedure ensured that the CTC was kept out of direct sunlight and reproduced *in situ* temperatures as closely as possible. Reactions were stopped and the samples preserved with the addition of formaldehyde (1-3%). Samples were stored in the dark at 4°C until the time of counting. Direct counts were performed on the active cells within each sample by, firstly, staining the cells with DAPI and then filtering onto irgalan-black stained filters using the same procedures described above for the direct counts. UV-excitation epifluorescent microscopy was used to count cells containing red crystals from 10-30 random fields at a magnification of x1250.

2.2.4 *Physico-chemical analysis*

For each sampling event, a range of 'traditional' physical and chemical parameters were measured. The pH, conductivity, turbidity, dissolved oxygen and temperature were measured using a HORIBA multi probe, calibrated according to the manufacturers specifications. One litre creek water samples were preserved with sulfuric acid (0.1% final concentration) and analysed (Flow Injection Analysis, Quick Chem, 8000 automated ion analyser) for nitrate/nitrite, ammonia, total phosphorus and total nitrogen. Filterable reactive phosphorus concentrations were determined in smaller 30 mL volumes, filtered in the field through 0.2 µm syringe filters and acidified also with sulfuric acid. The filterable reactive phosphorus is that which passes through the filter and reacts with molybdenum to produce a blue reaction detectable at 690 nm. Glass foiled capped vials that had been fired at 450°C were used to collect 10 mL samples (preserved with 0.1 % HCL) for total organic carbon. Analysis was carried out in the Water Studies Centre (Monash University) analytical

laboratory according to the Standard methods for the examination of water and waste water (APHA-AWWA-WPCF 1995). The following quality controls were performed on each batch of sampes for each nutrient analysed: 2 blanks were analysed; 1 sample was duplicated; 1 sample in each batch was a standard reference material; and one spike or recovery check was carried out.

2.2.5 Statistical analysis

To determine if the differences observed between sites where statistically significant two-way analysis of variance was performed on bacterial cell concentrations, total coliform and faecal coliform bacterial numbers. The data was log(x+1) transformed so it fit the normal distribution for calculation of mean square and significance tests. If the results between sites were significant ($P<0.05$) then questions were asked about particular dates and sites using pairwise analysis. The following hypotheses were tested for each sampling event; site A = B, A and B = C and A and B = D. Pearson correlation Matrices were also generated for log transformed data. Cell count results reported in Chapter 3 were included in the correlation analysis of flow and bacterial cell abundance. Correlation coefficient values were calculated for data collected from each site separately. The data were not grouped for analysis because of the large differences in values upstream and downstream of the treatment plant, resulting in artificially high correlation coefficents.

2.2.6 Generation of flow data

Flows in Campbells Creek were recorded by Serco Australia for a short period between August 1994 and November 1995. The river stage was recorded continuously by a data logger and reported on half hour or hourly intervals over this period. These flows were monitored 1 km upstream of the Castlemaine Sewage Treatment Plant (STP) and therefore, are independent of any effluent release.

Monthly total influent flows for the Castlemaine STP was also received from Serco Australia for January 1994 to February 1999. Based on the assumption that the STP has a short detention time and minimal losses incurr through the system (Wayne Murdoch, Serco

Australia personal communication), effluent flow entering Campbells Creek may be assumed equal to the influent flows.

The data obtained from Serco was converted to a creek flow using the supplied rating table and a daily flow series for the creek calculated. This flow series was regressed against flow data from 8 small gauged catchments in the same region. The flow gauging stations are listed in Table 2.3, along with their corresponding coefficient of determination (R^2).

Table 2.3 Flows from gauged catchments regressed against recorded flows in Campbells Creek upstream of the Castlemaine STP

Number	Station Name	R^2
406208	Campaspe River at Ashbourne	0.06
406216	Axe Creek at Sedgwick	0.53
406235	Wild Duck Creek u/s Heathcote-Mia Mia Rd	0.45
407214	Creswick Creek at Clunes	0.81
407217	Loddon River at Vaughan, d/s Fryers Creek	0.51
407221	Jim Crow Creek at Yandoit	0.53
407230	Joyces Creek at Strathlea	0.40
407246	Bullock Creek at Marong	0.48

Clearly, the best regression with the available recorded flows in Campbells Creek is with 407214, Creswick Creek at Clunes (R^2=0.81). This relationship is shown in Figure 2.1. The regression equation was then applied to recorded flows at 407214 providing data for Campbells Creek covering the required period of interest (July 1995 to March 1998).

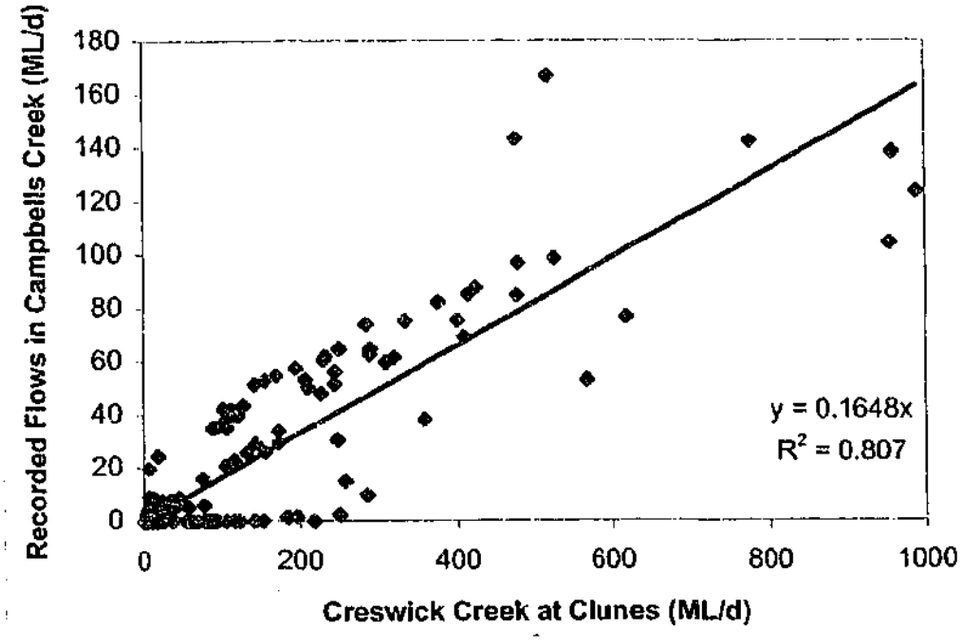


Figure 2.1 Plot of recorded flows in Campbells Creek (August 1994-November 1995) versus those for the same period in Creswick Creek at Clunes.

Downstream flows were estimated by disaggregating the monthly STP influent recorings to daily influents based on the number of days in the month of interest, it was therefore assumed that the influent flow was constant throughout the month. These influent flows, assumed to be equivalent to the STP effluent, were then added to the upstream flows, to provide a time series of flows downstream of the STP. Given that the local catchment area at site D is approximately 2% of the total area at site B, any additional catchment inflows would be small and therefore were not included in the downstream flow estimate.

Figure 2.2 Victoria catchment map showing the Loddon River catchment area (shaded).

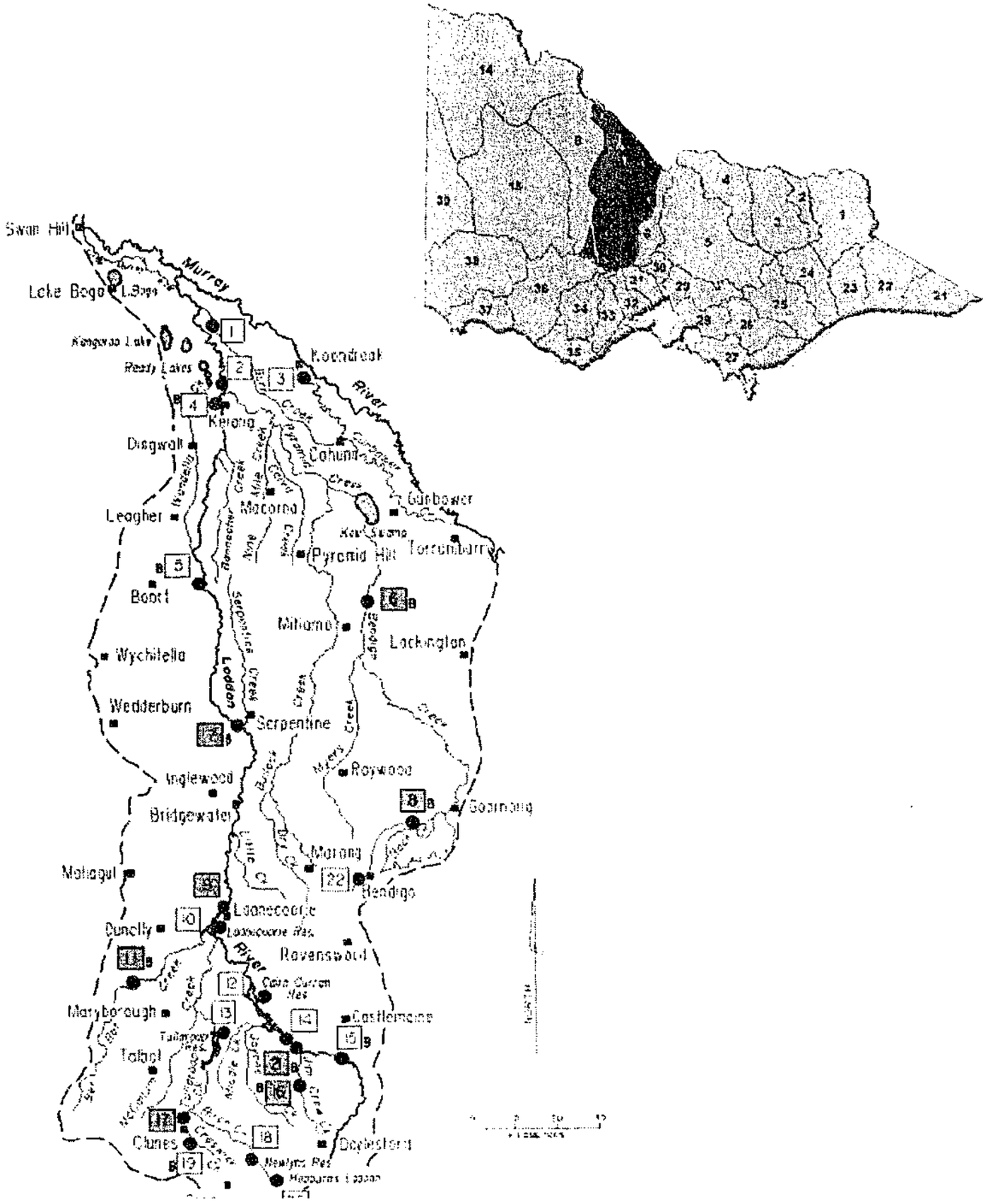
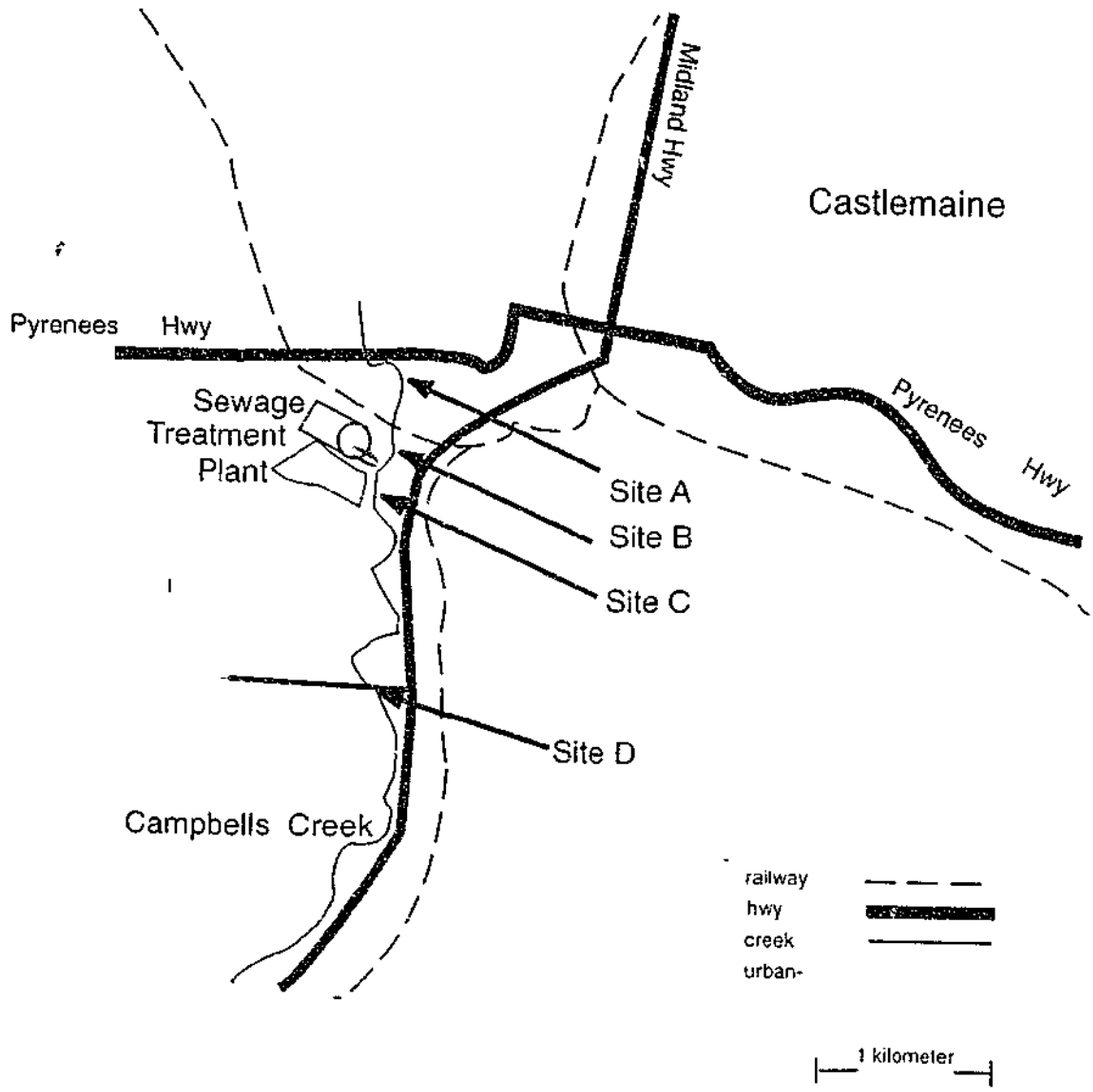


Figure 2.3 Map of Castlemaine, Campbells Creek catchment area and the four chosen sampling sites (A,B,C,D) . Sites A and B are upstream of the STP and C and D downstream.



2.3 Results

2.3.1 Catchment area and site description

The chosen field site for this study was Campbells Creek, Castlemaine, Victoria, Australia. It is part of the upper Loddon River catchment area which covers 405,000 hectares in north-eastern Victoria (Fig 2.2) The majority (80%) of the Loddon River catchment has been cleared for farming purposes, although some forested areas still remain between Daylesford and Castlemaine (City of Maryborough, 1994).

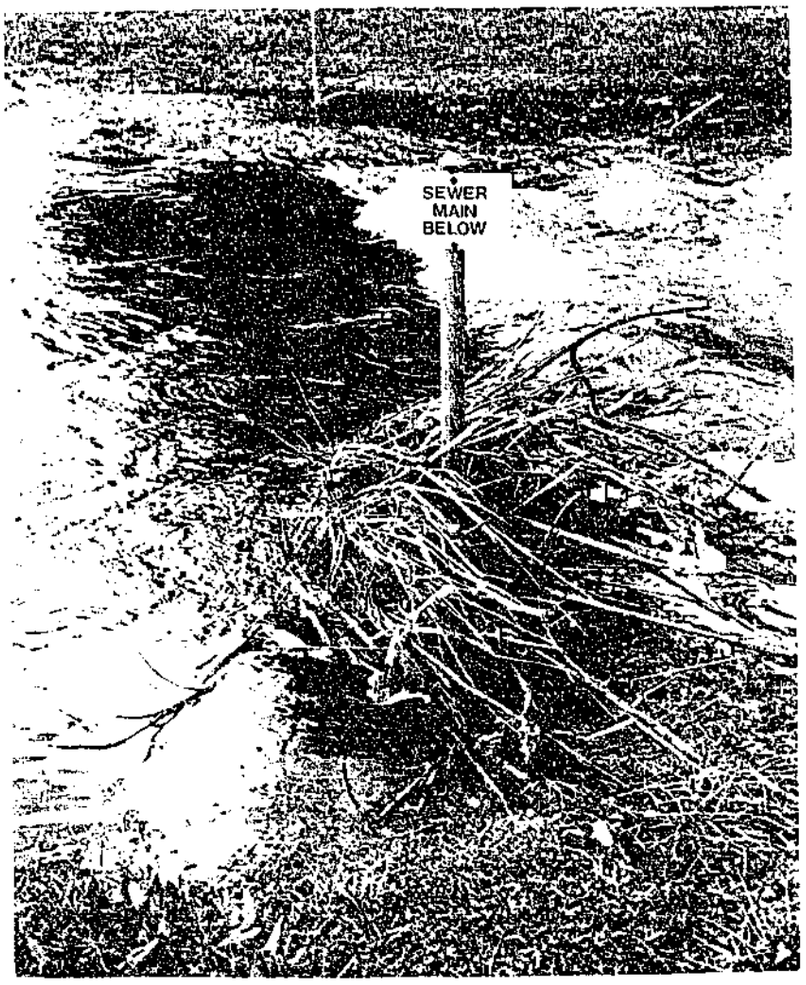
Campbells Creek is an urban stream which has undergone a regime of continual pollution and degradation. The creek water quality, hydrology and surrounding riparian condition have all been adversely affected by urbanisation. The catchment area of Campbells Creek collects flows from both Barkers Creek and Forest Creek catchments. The catchment is overlaid by the Harcourt and Poverty Gully channel systems, that deliver water from the Coliban Main Channel to the Bendigo and Castlemaine area. The flow of Campbells Creek is strongly influenced by terrestrial rainfall run-off, by discharge water from the Castlemaine sewage treatment plant and upstream releases of water from Barkers Creek reservoir. In summer, releases from the reservoir are made to closely 'mimic' natural summer conditions and during the winter months, flows are released at a more or less constant rate (although no actual measurements are taken). In the spring time, however, creek flows are held to fill the reservoir.

Four sites were chosen along Campbells Creek on the basis of their location and ease of access (Fig. 2.3). Site A is approximately 1 km upstream of the Castlemaine sewage treatment plant and is cleared of all riparian vegetation (Fig. 2.4). The second upstream site, site B is located 50 metres from the point of effluent release (Fig. 2.5a). The two downstream sites, site C and D are found 50 metres and 1 km, respectively, downstream of the discharge point (Fig. 2.5b & 2.6). The banks on both sides of site B, C and D are heavily weeded (blackberries, thistles, willows) and contain overhanging trees. The location of sites upstream and downstream of the sewage release point allowed a two-way ANOVA to be set up to test the effect of the sewage effluent. The catchment area of the upstream and

downstream sites is 140 km^2 and 143 km^2, respectively (calculated from a 1:100 000 topographical map).

(a)

(b)



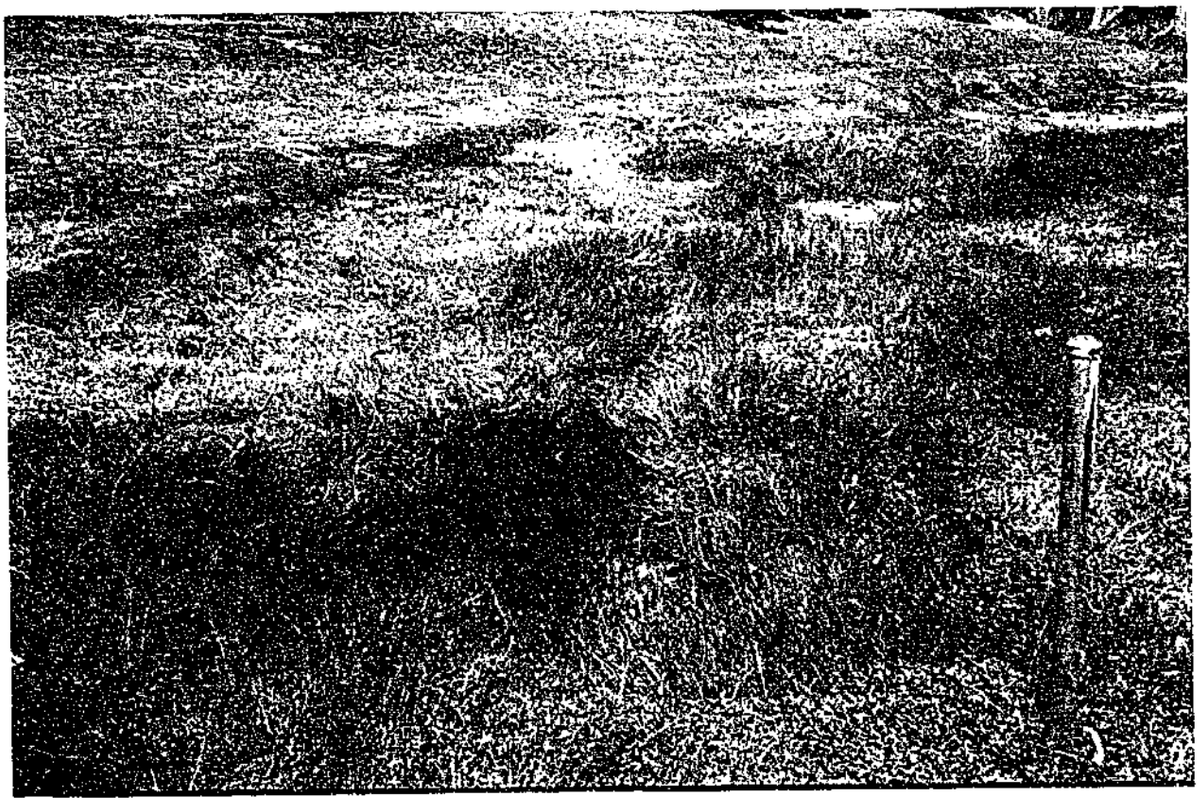
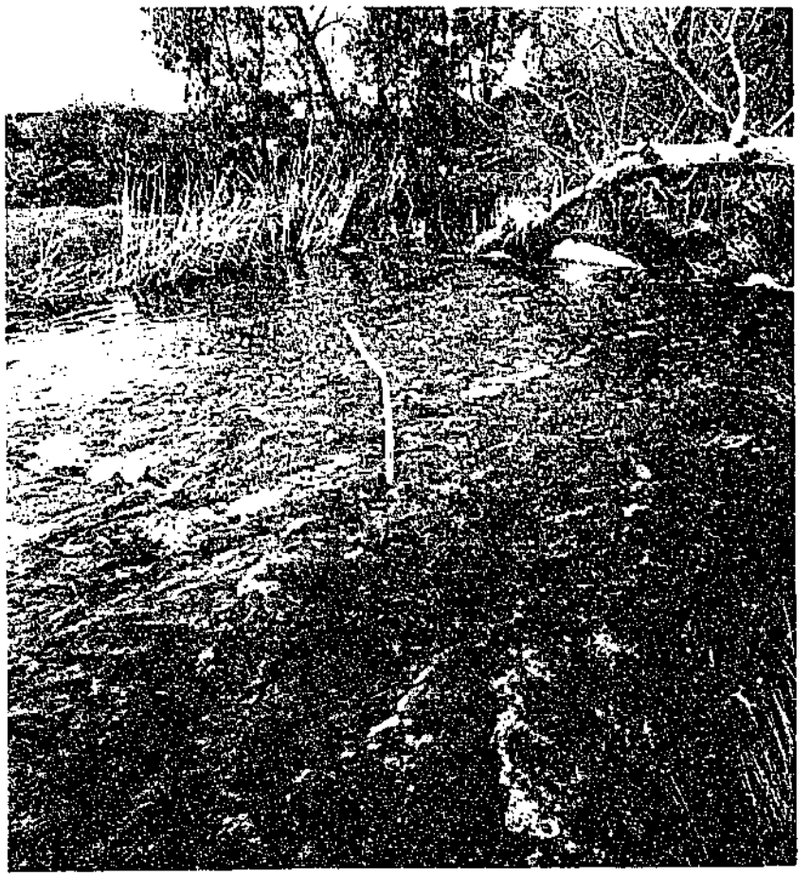


Figure 2.4a Site A at high flow (September 1995)

Figure 2.4b Site A at low flow (January 1998)

(a)



(b)

Figure 2.6a Site D at high flow (September 1995)

Figure 2.6b Site D at low flow (January 1998)

2.3.2 Castlemaine sewage treatment plant

The wastewater received at Castlemaine sewage treatment (STP) plant undergoes the following processing before it is discharged into Campbells Creek: mechanical screening, primary sedimentation, secondary treatment using biological filtration and disinfection by chlorination/maturation lagoons. Chlorination of effluent occurs at the outlet of the clarifier as it is released into the lagoons and the levels of residual chlorine are not permitted to exceed 1 mg/L (Environment Protection Act 1970). On average, the plant releases, from outlet weirs on the final lagoon, 1095 ML/y of treated effluent into Campbells Creek, 110 ML in summer and 985 ML in winter (City of Maryborough 1994). Industries such as Castlemaine Bacon contribute 45% of the total wastewater volume, up to 70% of the total N load and 65% of the total P load, thus explaining the high concentrations of total P (15 mg/L) and total N (30 mg/L) typically found in the effluent (City of Maryborough 1994).

2.3.3 Flow in Campbells Creek

Two sets of flow time series were produced for sites A and D, between January 1994 and April 1998. This was achieved by using data available for Campbells Creek from August 1994 to November 1995 to generate a time series and then regressing this against regional information for 8 small gauged catchments in the Campaspe and Loddon Basins (Table 2.3).

Downstream flows were determined by adding the STP influent flows to the upstream flow. The monthly influent recordings were uniformly disaggregated into daily values based on the number of days in each month. Obviously, this procedure eliminates any day to day variability in STP discharges. The generated flows for the upstream and downstream sites for the days sampling events occurred are presented in Table 2.4

Table 2.4 Generated flows in Campbells Creek (ML/d)

Date	Site B (u/s STP)	Site D (d/s STP)
12/7/95	28.7	32.7
14/8/95	23.5	26.7
10/10/95	2.0	4.7
28/11/95	0.6	3.2
18/12/95	0.2	2.4
31/1/96	0.2	2.5
31/3/96	0.7	3.1
18/4/96	0.9	3.4
23/5/96	0.5	3.1
16/7/96	42.3	45.9
19/9/96	383	386
21/11/96	1.8	4.2
18/12/96	0.2	2.5
23/1/97	0.3	2.3
2/12/97	0.2	2.8
9/12/97	0.1	2.6
16/12/97	0.1	2.6
6/1/98	0.1	1.8
13/1/98	0.1	1.9
20/1/98	0.1	1.8
28/1/98	0.2	1.9
4/2/98	0.1	2.1
17/2/98	0.2	2.2
3/3/98	0.1	2.4

It is obvious from these results that the STP effluent is contributing substantially (up to 95%) to the downstream flow for the majority of the year, with the exception of the wetter months between July and September.

2.3.4 River 'health' assessment

Conventional methods of water analysis were used to measure bacterial numbers, coliform and faecal coliform concentrations, viable cells, nutrient levels and physical characteristics in Campbells Creek, upstream and downstream of the sewage treatment plant. This routine monitoring of Campbells Creek was carried out between June 1995 and January 1997 on 14 occasions.

2.3.5 Enumeration of bacteria cells

Bacterial populations in freshwater aquatic systems exist as free-living and particle associated cells. To accurately measure the abundance of cells within collected samples it was necessary to disrupt cellular aggregates with sonication. The cell concentration was determined for 3-5 replicates from each site, by counting DAPI stained cells microscopically. The variation between replicates was small (s.d $< 0.6 \times 10^9$ cells/L). The results of these counts are presented in Figure 2.7.

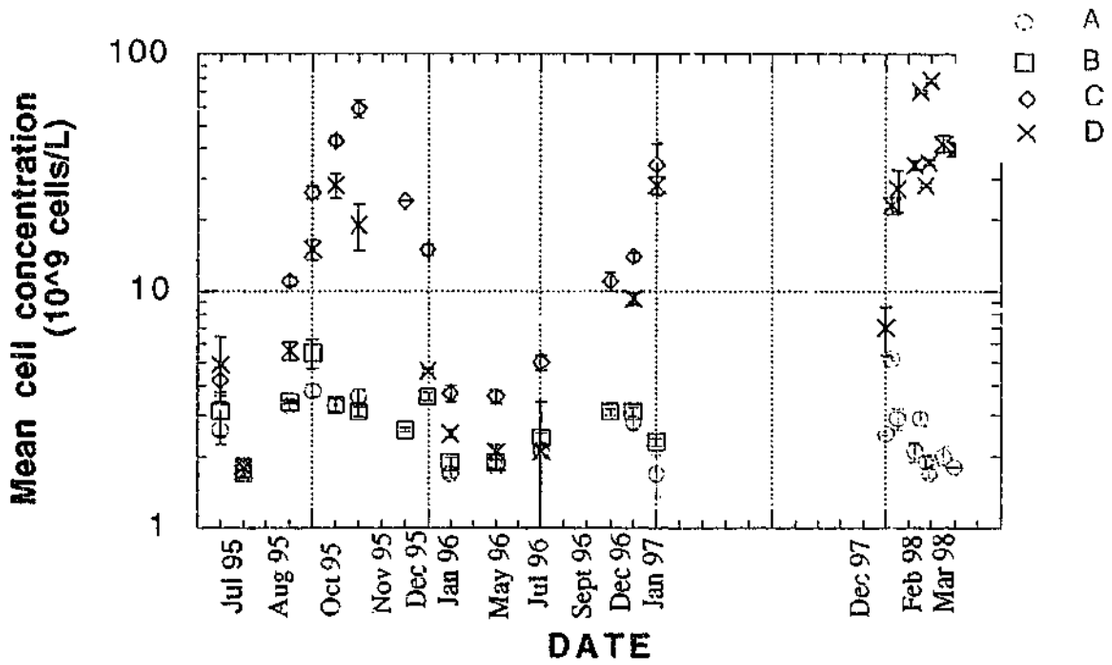


Figure 2.7 Mean (of replicates) total cell concentration upstream and downstream of Campbells Creek from July 1995 to March 1998. Error bars depict variation between replicates.

Regardless of the time of year, the bacterial cell concentration varied little between the two upstream sites, A and B. Statistically, no significanct difference (Pairwise analysis, $P<0.05$) was observed between the two upstream sites on all dates analysed (Table 2.8). The total cell numbers at the upstream sites ranged from 1.7×10^9 cells/mL in January 1997 to 5.5×10^9 cells/mL, in November 1995 (Table 2.5). This range is relatively small as can be seen from Figure 2.7. Therefore, it is not surprising that only a weak relationship was observed between flow and cell concentration upstream of the STP (Table 2.9). The cells observed microscopically were mainly small rods and cocci.

Table 2.5 Mean bacterial cell concentration ($x10^9$ cells/L) and standard deviation for 3-5 replicate samples taken at upstream sites.

Date	Site A		Site B	
	mean	s.d	mean	s.d
12/7/95	2.6	0.34	3.1	0.66
14/8/95	1.9	0.07	1.7	0.09
10/10/95	3.3	0.12	3.4	0.06
28/11/95	3.8	0.24	5.5	0.80
18/12/95	3.3	0.18	3.3	0.18
31/1/96	3.6	0.29	3.1	0.15
26/3/96	ND	ND	2.6	0.05
18/4/96	ND	ND	3.6	0.15
23/5/96	1.7	0.08	1.9	0.09
16/7/96	1.8	0.30	1.9	0.13
19/9/96	2.1	0.14	2.4	0.11
28/11/96	ND	ND	3.1	0.1
18/12/96	2.8	0.17	3.1	0.14
23/1/97	1.7	0.34	2.3	0.09

ND= Not determined

Table 2.6 Mean bacterial cell concentration ($x10^9$ cells/L) and standard deviation of 3-5 replicate samples at downstream sites.

Date	Site C		Site D	
	mean	s.d	mean	s.d
12/7/95	4.2	0.59	4.9	1.5
14/8/95	1.8	0.16	1.8	0.15
10/10/95	11	0.48	5.6	0.48
28/11/95	26	1.6	15	1.4
18/12/95	43	1.8	28	3.4
31/1/96	59	5.2	19	4.2
26/3/96	24	0.1	ND	ND
18/4/96	15	0.76	4.6	0.10
23/5/96	3.7	0.29	2.5	0.09
16/7/96	3.6	0.24	2.1	0.15
19/9/96	5.0	0.39	2.1	1.3
28/11/96	11	1.1	ND	ND
18/12/96	14	0.56	9.3	0.52
23/1/97	34	8.2	28	2.5

ND= Not determined

The overall bacterial abundance observed downstream of the STP was significantly greater than that at the upstream sites (ANOVA $P<0.05$, Table 2.7). At site C, the lowest bacterial concentration was 1.8×10^9 cells/mL in August 1995 and the highest 59×10^9 cells/mL was observed in January 1996 (Table 2.6). The range at site D was a little less than at site C, fluctuating from 1.8×10^9 cells/mL to 28×10^9 cells/mL (Table 2.6). Cell abundance at site C and D was found to be strongly related to flow, with a correlation coefficient (r^2) of 0.7 obtained for both sites. The results of the pairwise analysis (Table 2.8) reveal that the difference in cell concentration between site C and D was significant on 7 out of the 11 occasions samples were collected at both sites. Cell counts were also

performed on samples collected during bacterial production determination experiments in the summer of 1997-98. For this period, counts were only conducted at site A and D and were therefore, statistically analysed separately, but have been included with the data from 1995-97 for graphical presentation and flow correlation analysis. The cell concentrations and results of the statistical analysis for the 1997-98 sampling events are presented and discussed in Chapter 3 .

The downstream samples, that comprised mainly of small rods and cocci, also had some large rods and crescent shaped cells contributing to the total counts. Algal cells were more numerous in samples obtained from site C and site D, particularly at warmer times of the year, and were distinguished from bacterial cells on the basis of their morphology and by the presence of photopigments seen under blue excitation. Suspended particulate matter was present in all samples and at times (high flow and recent rain fall) caused problems with the filtering and cell count procedure. Compared however, with samples from other freshwater systems such as the Darling River (turbidity readings of up to 2000 NTU during flood events, (Dr Mike Grace, Water Studies Centre, personal communication), Campbells Creek samples were relatively low in suspended particular matter (average turbidity readings 9.0 NTU). All samples contained autofluorescing particles which sometimes made cell counting difficult. Autofluorescing *Pseudomonas* species are often found in the microbial community of sewage effluent (Mitchell, 1978).

Table 2.7 Two-way ANOVA of bacterial cell concentration, total coliforms and faecal coliforms.

Effects	df	mean-square	F-ratio	P
Cell conc.				
Date	10	1.1	6112	0.0000*
Site	3	3 3	1861	0.0000*
Date x Site	30	2.2	124	0.0000*
Residuals	149	0.0		
Total colif.				
Date	7	1.7	53	0.0000*
Site	3	0.1	4.2	0.0074*
Date x Site	21	0.2	6.8	0.0000*
Residual	95	0.0		
Faecal Colif.				
Date	6	0.9	21.9	0.0000*
Site	3	0.3	7.8	0.0001*
Date x Site	18	0.2	5.1	0.0000*
Residuals	81	0.0		

*Significant P<0.05

df= degrees of freedom

Table 2.8 Pairwise analysis of bacterial cell concentration upstream and downstream of STP

Date	Site A vs Site B	Site C vs Site D	Site A & B vs C	Site A & B vs D
12/7/95	0.3794	0.4638	0.0134*	0.0010*
14/8/95	0.6532	0.9080	0.9818	0.9119
10/10/95	0.8498	0.0000*	0.0000*	0.0007*
28/11/95	0.0372	0.0001*	0.0000*	0.0000*
18/12/95	0.9299	0.0030*	0.0000*	0.0000*
31/1/96	0.4852	0.0000*	0.0000*	0.0000*
23/5/96	0.6978	0.0554	0.0002*	0.1118
16/7/96	0.8267	0.0158*	0.0005*	0.4823
19/9/96	0.5912	0.0001*	0.0000*	0.5062
18/12/96	0.5866	0.0182*	0.0000*	0.0000*
23/1/97	0.1850	0.2653	0.0000*	0.0000*

* Significant ($P<0.05$)

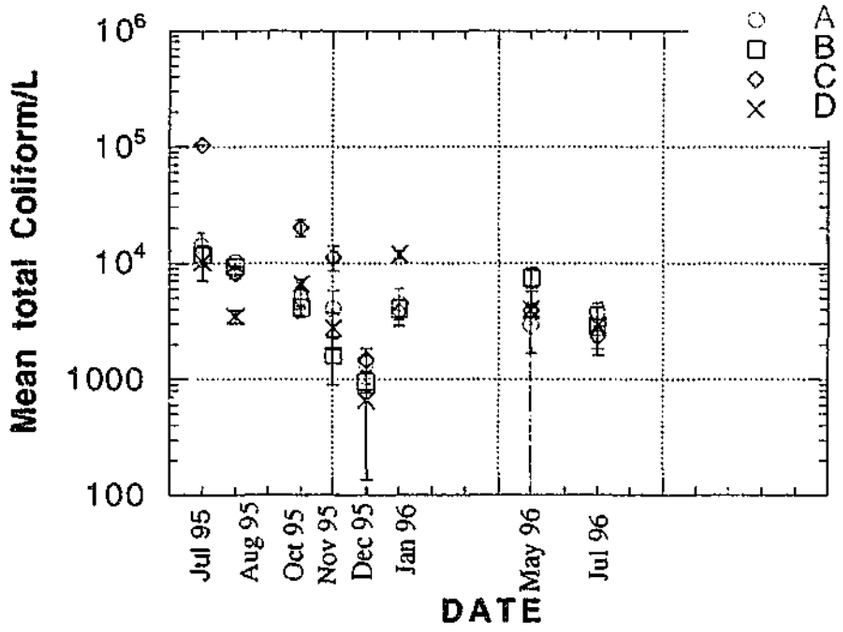
Table 2.9 Pearson correlation coefficient for cell concentration and daily flow (includes data from 1997-98)

Site	Coefficient
Site A	-0.1
Site B	-0.4
Site C	-0.7
Site D	-0.7

2.3.5 Total coliforms and Faecal coliforms

Microbial water quality has been, and often still is, described only in terms of the presence or absence of certain 'indicator' bacteria. The most common bacterial group utilised for this purpose are coliform bacteria (eg. *Escherichia coli*). The numbers of total coliforms and faecal coliform was determined at all four sites using conventional plate counts on selective media. Samples from July 1995 until July 1996 were filtered directly onto membrane filters and placed on M-Endo agar les or M-faecal coliform plates. The coliform concentration at site A and site B ranged from 400 colony forming units (CFU)/L to 20,000 CFU/L (Table 2.10). The range was slightly higher at site C (600 CFU/L to 22,000 CFU/L) and the lowest concentrations were observed at site D, 340 CFU/L to 15,000 CFU/L (Table 2.11). As expected, the faecal coliform concentration is considerably lower (4 to 10-fold) than the total coliform counts at all four sites (Table 2.10 and 2.11). When the results of both counts are presented in a graphical format (Fig 2.8 a &b), no trends in terms of time of year or site appear to be evident. Statistical analysis reveals that significant differences exist between the sites for some dates for both total and faecal coliform counts. On the particular dates when significant differences were observed between the sites, these differences were not always observed for both bacterial groups. A higher degree of variation in the total counts was seen between the replicates for the Faecal coliforms than the Total coliform counts.

(a)



(b)

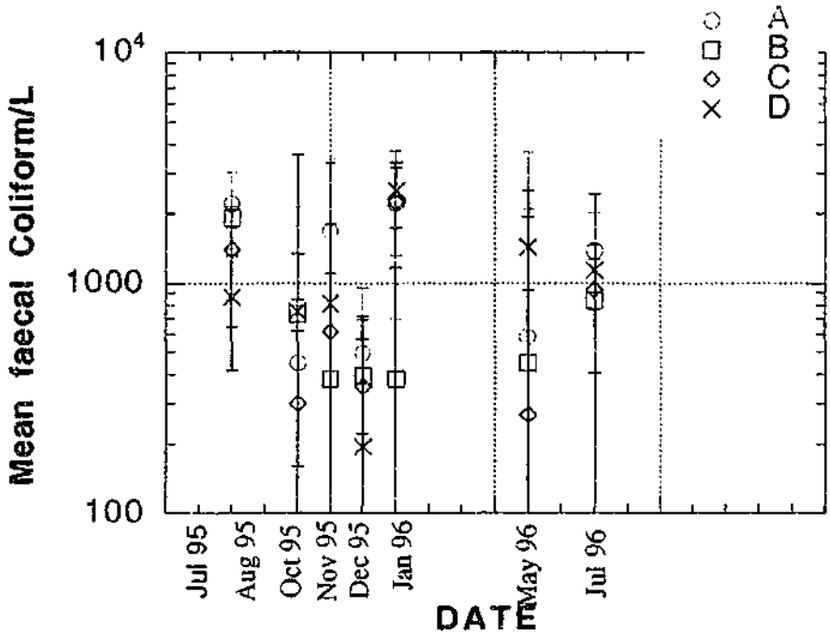


Figure 2.8a Mean (of replicates) total coliforms upstream and downstream of Campbells Creek from July 1995 to July 1996. Error bars depict variation between replicates.

Figure 2.8b Mean (of replicates) Faecal coliforms upstream and downstream of Campbells Creek from July 1995 to July 1996. Error bars depict variation between replicates.

Table 2.10 Upstream mean total coliform (TC) and faecal coliform (FC) (CFU/L) and standard deviation of 3-5 replicates

Date	Site A				Site B			
	TC mean	**TC s.d**	**FC mean**	**FC s.d**	**TC mean**	**TC s.d**	**FC mean**	**FC s.d**
12/7/95	14200	4024	ND	ND	11800	2490	ND	ND
14/8/95	10200	800	2220	225	9330	176	1920	29
10/10/95	5070	1700	450	312	4130	115	733	462
28/11/95	4040	1780	1680	781	1600	712	383	220
18/12/95	1370	466	495	58	950	174	396	57
31/1/96	4450	1540	2230	86	4000	783	382	33
23/5/96	2950	3130	583	290	7400	1641	448	240
16/7/96	3900	622	1380	574	2800	432	833	159

ND=Not determined

Table 2.11 Downstream mean total coliform (TC) and faecal coliform (FC) (CFU/L) and standard deviation of 3-5 replicates

Date	Site C				Site D			
	TC mean	**TC s.d**	**FC mean**	**FC s.d**	**TC mean**	**TC s.d**	**FC mean**	**FC s.d**
12/7/95	10330	577	ND	ND	10250	3200	ND	ND
14/8/95	8100	755	1400	200	3470	451	867	202
10/10/95	20000	3300	300	141	6600	589	750	574
28/11/95	11200	2680	613	412	2760	974	813	388
18/12/95	1470	336	357	42	656	521	195	60
31/1/96	3800	938	2250	741	11850	806	2530	1280
23/5/96	3900	2242	267	164	3950	503	1433	241
16/7/96	2350	526	933	313	2900	1290	1133	278

ND=Not determined, TC=Total Coliforms, FC=Feacal Coliform

Table 2.12 Pairwise comparison of total coliforms and faecal coliforms upstream and downstream of STP

Date	Site A vs B		Site C vs D		Site A & B vs C		Site A & B vs D	
	TC	FC	TC	FC	TC	FC	TC	FC
12/7/95	0.5267	0.4153	0.9042	0.6638	0.4675	0.6638	0.3358	0.6638
14/8/95	0.9777	0.0145*	0.9190	0.6638	0.9190	0.0312*	0.9190	0.1154
10/10/95	0.6253	0.4153	0.0004*	0.0751	0.0000*	0.2134	0.1651	0.4560
28/11/95	0.0021*	0.0000*	0.0000*	0.1217	0.0000*	0.1409	0.6247	0.7780
18/12/95	0.3017	0.5317	0.0004*	0.0521*	0.2935	0.4615	0.0044*	0.0045*
31/1/96	0.8379	0.0000*	0.0002*	0.9640	0.7239	0.0076*	0.0001*	0.0088*
23/5/96	0.0000*	0.4818	0.3413	0.0000*	0.4348	0.0266*	0.7496	0.0060*
16/7/96	0.2684	0.2447	0.5728	0.5537	0.1747	0.6868	0.4766	0.7786

*Significant P<0.05

2.3.6 Viable cell counts

The number of viable cells within a sample was determined for each site by counting the number of cells capable of forming colonies on agar medium (Collins and Willowby 1962). It is not possible to draw any conclusions from these plate counts since the results were highly variable between the replicates and were prone to domination by single colony types. The variable generation times (hrs to days) for natural aquatic bacteria means that it can take from a few days to a week for some species to grow sufficiently to form a visible colony. Problems then arise because the more vigorous bacterial species take over the plate before the other slower species get a chance to form colonies. The plates, because of these reasons, were very difficult to count and are therefore, inaccurate and only a fraction of the cells counted by direct microscopy, a finding also reported by other researchers (Ferguson *et al.*. 1984).

2.3.7 Enumeration of active bacteria

The inaccuracy of the plate counts coupled with the fact that total direct counts provide no information on the viability or activity of bacterial populations, led to the employment of the redox dye 5-Cyano-2,3-ditolyl tetrazolium chloride (CTC) to detect respiring bacteria. A number of researchers (Rodriguez, 1992; Pyle, 1995) have successfully used this method to enumerate active bacteria in a range of different environmental samples. The incubation of samples with CTC was performed at *in situ* temperatures to limit the possibility of cells becoming active or inactive due to a change in their environment. Samples were also counter stained with DAPI prior to filtration so that the inactive cells could be counted. From microscopic observation, it was noted that for large rod shaped bacteria it was possible to identify the red crystalline deposit within the DAPI stained cell. Smaller cells however, appeared entirely red, making it difficult to be certain they were cells.

Initial experiments were performed at incubation times of 2 and 4 hours. No significant increase in fluorescent cells was observed with the longer incubation time (Table 2.13). The main objective of the CTC experiments was to examine the effectiveness of this method for determining active bacterial cell numbers in a polluted lowland stream. For this reason and the fact that it was found to be extremely difficult tp distinguish small active cells, CTC incubations were performed on only six sampling occasions from November 1995 until September 1996. The CTC was also very expensive and so only 2 samples from site B and site C were enumerated for active bacteria Results are presented in Table 2.13.

Table 2.13 Numbers of active bacteria ($x10^9$ cells/L) upstream (site B) and downstream (site C) of the STP in Campbells Creek

	Site B			Site C		
Date	**Total cells**	**Active cells**	**% active**	**Total cells**	**Active cells**	**% active**
Nov 1995	5.5	0.4	7	26	3.7	14
Dec 1995	3.4	0.4	12	43	2.7	6
Jan 1996	3.1	0.8 (0.7)*	26	60	4.8 (5.0)*	8
May 1996	1.9	0.4	21	3.7	1.1	30
June 1996	1.9	0.9	47	3.6	1.3	36
Sept 1996	2.4	0.4	17	5.0	0.9	18
Mean			21			19
Range			7-47			6-36

Total Cells- Average of direct cell counts (10^9 cells/L) presented in Table 2.5 & 2.6

* Results in brackets from incubating samples for 4 hours. All other experiments performed with 2 hour incubations

The percentage of metabolically active cells in Campbells Creek upstream of the sewage treatment plant ranged from 7 to 47% (mean = 21%). At site C, below the sewage treatment plant, the absolute number of active cells was greater, but appeared to make up a low percentage being 6-36%. These results are consistent with those observed (5-36%) by other researchers in freshwater environments (Zimmermann, 1978; Rodriguez, 1992). Unfortunately, there is no information regarding active cell numbers in Australian lowland streams.

2.3.8 Nutrient analysis

There were very minor differences in nutrient concentration, between the two upstream sites (Table 2.14). The extent of variation appeared to depend upon the particular nutrient. For example, only a 2-fold range in TOC concentration was observed upstream, whereas a 32-fold range in NO_X was seen. In contrast with the upstream results, differences were observed between sites C and D in the concentration range of the various nutrients analysed, but the median values were very similar between the two sites (Table 2.15). As expected the nutrient concentrations at the downstream sites were much larger than those at the upstream sites (Fig. 2.9 a-f). The greatest difference (1000-fold based on median values) was observed in FRP concentration, followed by a 300-fold difference in NH_3, 250-fold in total P, 20-fold in total N, 19-fold in NO_X and only 1.5 in total organic carbon.

The higher nutrient concentration observed at the downstream sites are attributable to the input of sewage effluent from the Castlemaine sewage treatment plant. The range of concentrations observed for the various nutrients correlate with the fluctuations in creek flow, with the greatest values being obtained at times of low flow.

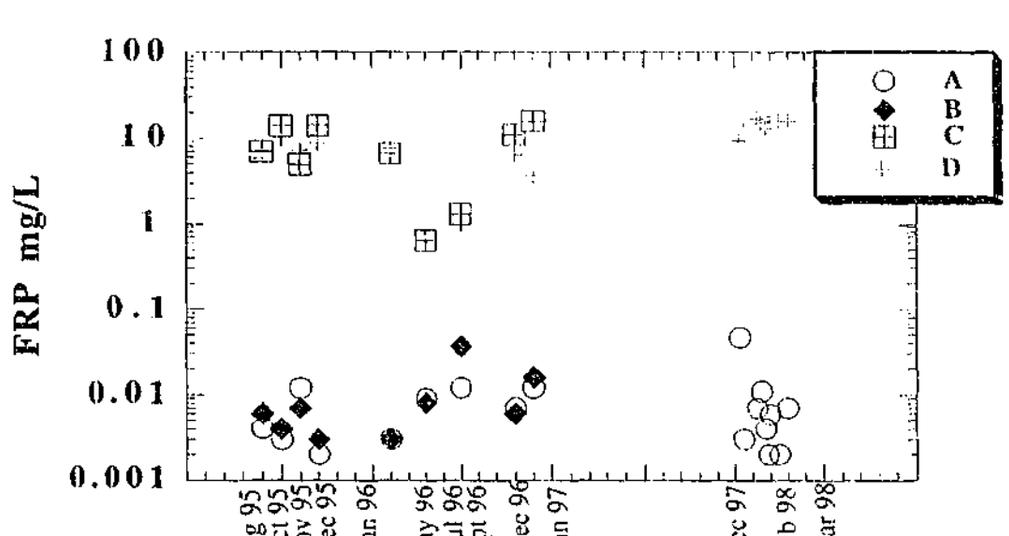
Table 2.14 Nutrient concentration (mg/L) ranges at site A and B from the 10/10/95 to 23/1/97 (n=10)

	Site A		Site B	
	range	median	range	median
FRP	0.002-0.012	0.007	0.003-0.036	0.006
NO_x	0.005-0.16	0.05	0.006-0.15	0.05
NH_3	0.006-0.088	0.028	0.02-0.19	0.03
TP	0.018-0.12	0.03	0.017-0.12	0.03
TN	0.46-0.97	0.60	0.38-1.0	0.62
TOC	8.0-15	12	8.0-16	12

Table 2.15 Nutrient concentration (mg/L) ranges at site C and D from the 10/10/95 to 23/1/97 (n=10)

	Site C	Median	Site D	Median
FRP	0.64-16	7	1.0-10	6.4
NOx	0.13-2.7	0.94	0.71-2.3	0.96
NH3	1.6-18	10	0.71-12	7.5
TP	0.65-16	7.8	0.70-11	7.6
TN	2.5-28	12	2.3-18	11
TOC	13-27	18	12-18	17

(a)



(b)

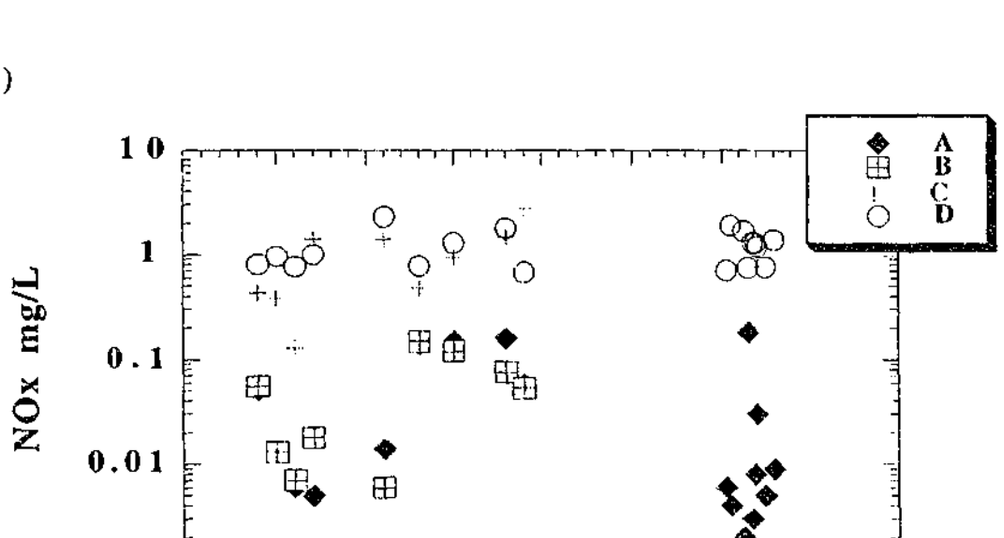
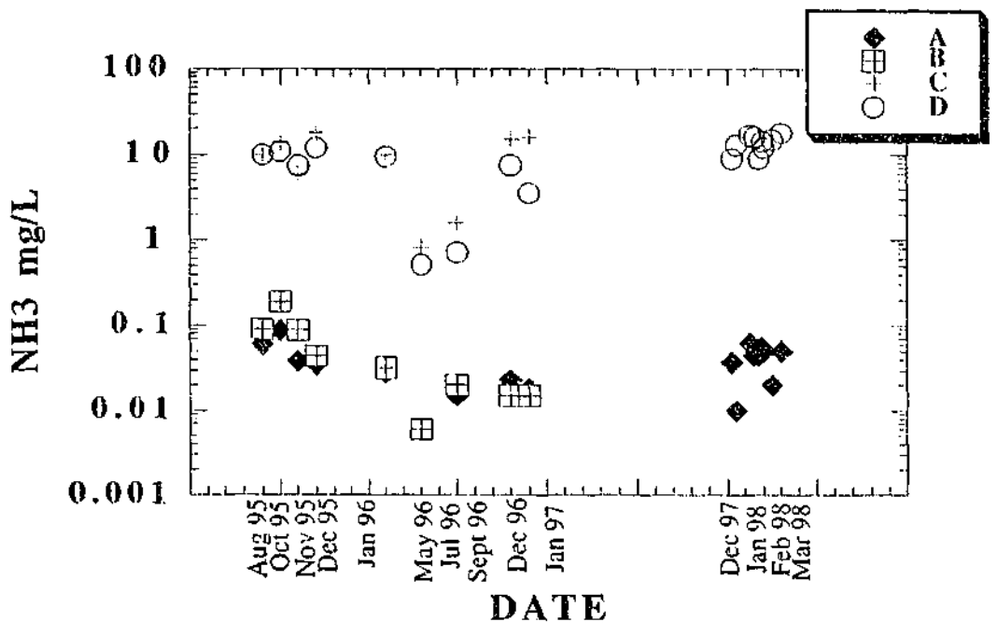


Figure 2.9a Filterable reactive phosphorus concentrations (mg/L) upstream and downstream of Castlemaine STP from August 1995 to March 1998.

Figure 2.9b NO_X concentrations (mg/L) upstream and downstream of Castlemaine STP from August 1995 to March 1998.

(c)



(d)

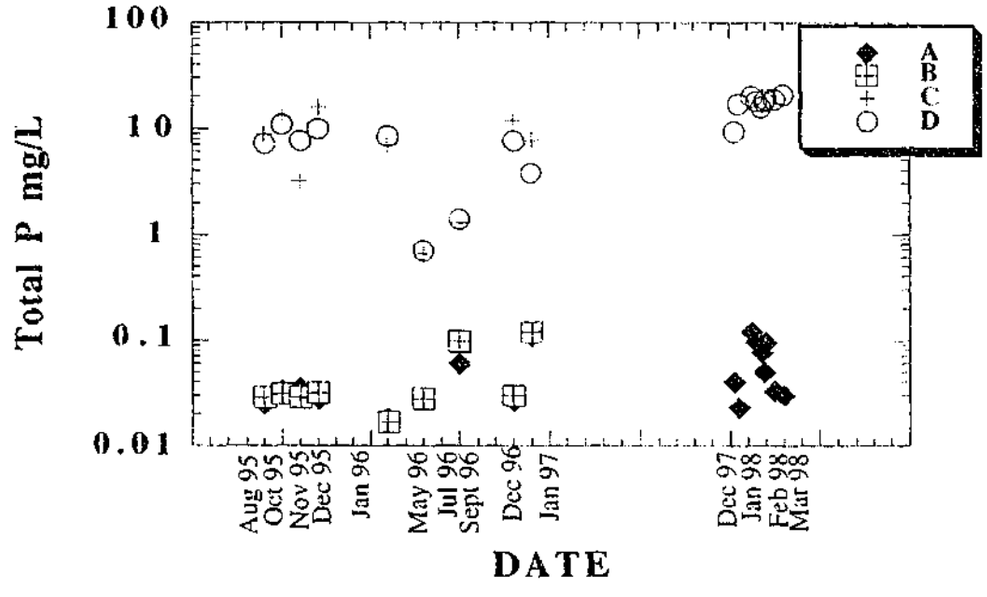
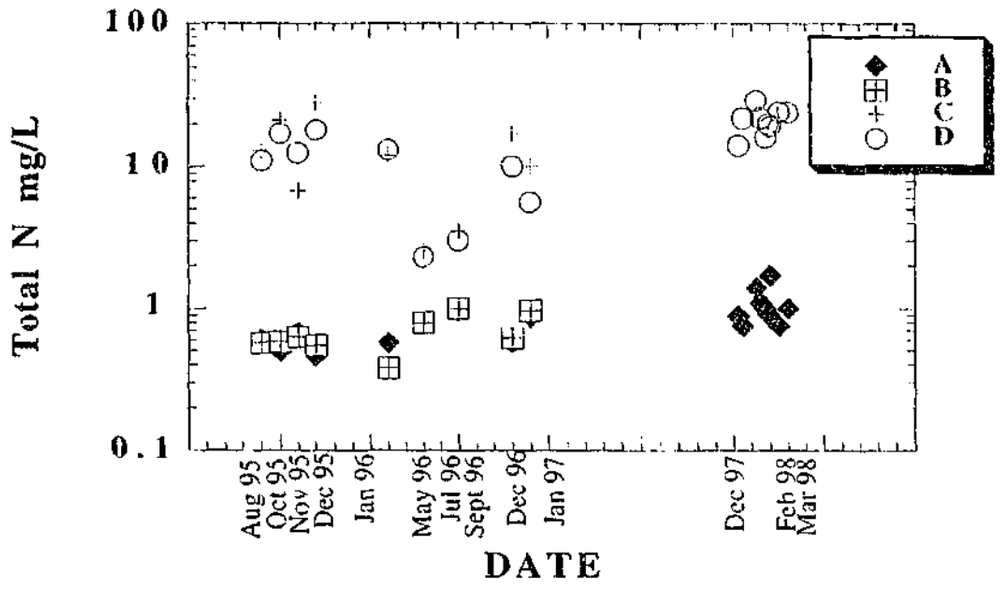


Figure 2.9c NH3 concentrations (mg/L) upstream and downstream of Castlemaine STP from August 1995 to March 1998.

Figure 2.9d Total phosphorus (TP) concentrations (mg/L) upstream and downstream of Castlemaine STP from August 1995 to March 1998.

(e)



(f)

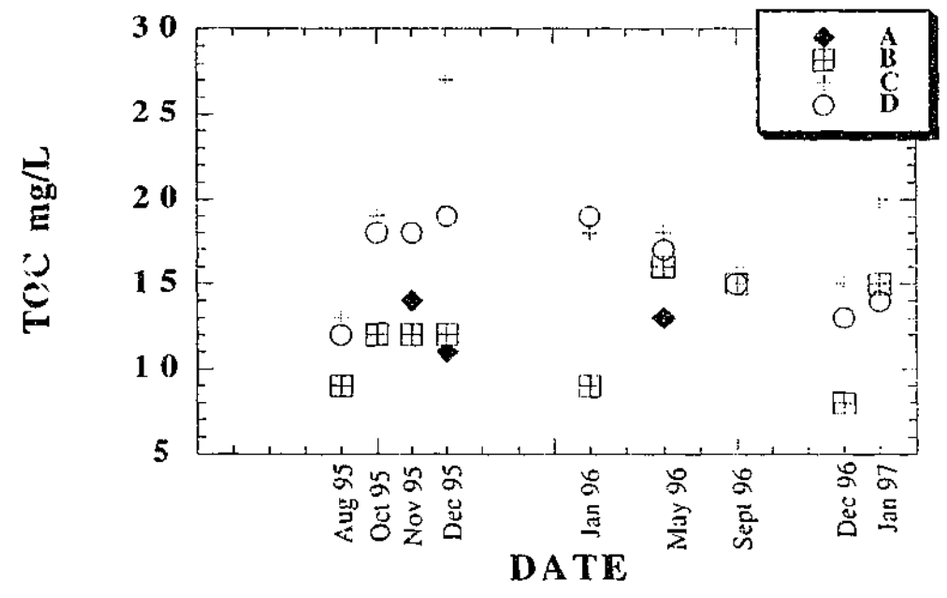


Figure 2.9e Total nitrogen (TN) concentrations (mg/L) upstream and downstream of Castlemaine STP from August 1995 to March 1998.

Figure 2.9f Total organic carbon (TOC) concentrations (mg/L) upstream and downstream of Castlemaine STP from August 1995 to January 1996.

Table 2.16 Results of correlation analysis between cell counts and nutrient data SITE A

	Flow	Cell conc.	Total Coliform	Feacal Coliform	Active Cell
Cell conc.			0.9	0.4	0.9
Total Col.	0.3	0.9		0.2	0.9
Feacal Col.	-0.03	0.4	0.2		-0.12
NH_3	-0.5	0.7	-0.1	-0.6	0.8
NO_X	0.8	-0.3	0.6	0.2	0.5
FRP	0.4	-0.3	0.2	-0.1	0.3
TOC	0.3	-0.2	-0.2	0.2	0.3
Total N	0.7	-0.6	0.3	-0.03	0.2
Total P	0.02	-0.3	0.4	0.1	-0.14

Table 2.17 Results of correlation analysis between cell counts and nutrient data SITE B

	Flow	Cell conc.	Total Coliform	Feacal Coliform	Active Cell
Cell conc.			-0.6	-0.6	-0.6
Total Col.	-0.4	0			0.5
Feacal Col.	0.08	-0.	0.3		0.5
NH_3	-0.3	0.9	-0.6	-0.6	-0.7
NO_X	0.8	0.5	0.2	0.6	0.8
FRP	0.7	-0.3	-0.4	0.4	0.3
TOC	0.6	-0.3	-0.3	-0.1	0.7
Total N	0.6	-0.9	-0.4	0.1	0.6
Total P	0.3	-0.25	-0.5	-0.5	0.3

Table 2.18 Results of correlation analysis between cell counts and nutrient data SITE C

	Flow	Cell conc.	Total Coliform	Feacal Coliform	Active Cell
Cell conc.			0.3	0.2	0.8
Total Col.	0.3	0.3		0.1	0.5
Feacal Col.	0.4	0.2	0.1		0.4
NH_3	-0.8	0.7	0.3	0.2	0.6
NO_X	-0.3	0.2	-0.4	0.1	-0.7
FRP	-0.7	0.7	0.3	0.3	0.7
TOC	-0.2	0.4	-0.1	0.7	-0.05
Total N	-0.6	0.6	0.3	0.4	0.6
Total P	-0.7	0.6	0.4	0.4	0.6

Table 2.19 Results of correlation analysis between cell counts and nutrient data SITE D

	Flow	Cell conc.	Total Coliform	Feacal Coliform	Active Cell
Cell conc.			0.5	0.3	0.5
Total Col.	0.2	0.5		0.9	-0.9
Feacal Col.	-0.3	0.3	0.9		-0.9
NH_3	-0.7	0.4	0.4	-0.2	-0.9
NO_X	-0.05	-0.45	0.2	0.5	-0.9
FRP	-0.8	0.5	0.1	-0.3	-0.9
TOC	-0.1	0.1	-0.2	0	-0.9
Total N	-0.7	0.5	0.2	-0.3	-0.9
Total P	-0.8	0.5	0.1	-0.3	0.9

Nutrient analysis was also conducted from December 1997 to March 1998, during the bacterial production studies (Chapter 3.) at sites A and D. The results from this analysis is presented in Fig 2.9 and Table 3.4. From the graphical presentation of these results, the nutrient concentrations across this period appear to be slightly higher than those from June 1995 to January 1997. The higher nutrient concentrations in the summer of 1997-98 are most likely caused by the lower flow in Campbells Creek for this same period.

Correlation analysis was performed to determine if any relationship existed between the various nutrients analysed and bacterial cell concentration, total coliforms, feacal coliforms and active cell concentrations (Table 2.16, 2.17, 2.18, 2.19). The results of this analysis reveal a strong positive relationship between bacterial abundance and NH_3, FRP, total N and total P concentration downstream of the sewage effluent, with slightly lower correlation coefficients observed for site D. However, this strong correlation was not observed at either of the upstream sites. Nutrient concentrations were found to be negatively correlated with flow downstream of the STP, with the exception of NO_X, but positively correlated with flow at the upstream site.

2.3.9 Physico-chemical analysis

Physico-chemical parameters (pH, conductivity, turbidity, dissolved oxygen and temperature) were measured at each site (Table 2.20 and 2.21). The main difference between the upstream and downstream sites was observed for dissolved oxygen (DO) and conductivity. The median DO concentration was lower at site C and D, 7.5 and 4.9 mg/L, respectively, compared to 8.3 and 7.9 at A and B. The lower dissolved oxygen concentrations observed downstream, are most probably caused by the higher bacterial production (oxygen consuming process) rates at sites C and D. Two factors are likely to be contributing to the lower median DO at site D compared with site C. First, site C is very close to the discharge pipe and therefore there has been insufficient time (1.2 min in high flow to 20 min in low flow) for the bacterial population to have an influence on the dissolved oxygen. Second, the large numbers of algae likely to be present in the STP ponds will be

producing oxygen as a by product of their photosysnthesis and oygenating the water before it is released into Campbells Creek.

Table 2.20 Physico-chemical ranges at site A and B from the 10/10/95 to 23/1/97 (n=10)

	Site A		Site B	
	range	median	range	median
pH	6.4-7.8	7.2	6.7-7.2	7.1
Cond.(mS/cm)	0.46-3.9	0.73	0.48-4.13	0.72
Turbidity (NTU)	1-10	6.5	2-8	5
D.O (mg/L)	4.1-13	8.3	3.2-11	7.9
Temp (°C)	6-18	13	6-18	13

Table 2.21 Physico-chemical ranges at site C and D from the 10/10/95 to 23/1/97

	Site C		Site D	
	range	median	range	median
pH	7.1-7.6	7.2	6.9-7.7	7.1
Cond. (mS/cm)	0.72-4.22	1.6	0.8-4.1	1.5
Turbidity (NTU)	2.0-8.0	5.0	3.0-9.0	5.0
D.O (mg/L)	5.2-11	7.5	1.6-12	4.9
Temp (°C)	6-20	13	6.0-20	13

2.4 Discussion

During this study, the concentration of various nutrients was determined in Campbells Creek for two reasons. First, one of the main aims of this part of the project was to evaluate traditional methods of water quality assessment samples and the analysis of nutrient concentrations is used in most investigations of water quality. Secondly, it was of interest to determine if a relationship existed between changes in the microbial population (numbers, production, diversity) and the physico-chemical indicators (e.g. nutrient concentrations).

Although nutrients are non-toxic, they do stimulate the growths of plants, algae and cyanobacteria (blue-green algae) which can cause very serious long term damage to an ecosystem. In eutrophic rivers and streams, periphyton and macrophytes can cause problems, such as a fluctuation of dissolved oxygen and pH, two conditions which can cause stress and possible elimination of some sensitive species. The excessive growth of periphyton and macrophytes can also reduce habitat quality for fish and invertebrates by covering stream beds and reducing the penetration of light. The detrimental effects (foul tasting, odour and toxic) to freshwater environments caused by cyanobacterial blooms are also well documented (ANZACC 1992). Nutrient analysis can play a relevant role in identifying the sources of these nutrients. The extremely high levels of phosphorus and nitrogen found in Campbells Creek make a significant contribution to the high concentration of these nutrients found in the Loddon river and subsequently in Cairn Curran reservoir (City of Maryborough 1994). For example, it has been reported that Campbells Creek contributes from 16 to 22 tonne of phosphorus per annum to the upper London Catchment which is 50 to 66% of the total load (City of Maryborough 1994). The algal bloom problems in Cairn Curran reservoir have been largely attributed to the input of high concentrations of nutrients from the Loddon River. It is therefore, important to monitor nutrient concentrations and limit their concentrations in river and streams as a water quality management strategy.

While the importance of managing and attaining decreases in the inputs of nutrients into rivers and streams is not disputed, its value as a tool for assessing water quality in

freshwater river systems is limited. There is a large variation in nutrient concentration in Australian rivers, with some systems seemingly able to cope with high nutrient loads with no excessive plant or cyanobacterial growth (ANZECC 1992). The negative effects of high nutrient concentrations may only become evident in downstream lakes, resevoirs or weir pools. For example, the sites studied along Campbells Creek were mostly free from algal blooms despite the excessive nutrient concentrations (compared with recommended guidelines in ANZECC 1992), but algal blooms are a regular occurrence in Cairn Curran reservoir. Analysis of nutrient concentration therefore provides little insight into the effect, if any, that changes in nutrient levels may be having on the health and diversity of the biological community within the creek.

There was a strong relationship between flow and nutrient concentration in Campbells Creek. The relationship varied upstream and downstream of the STP, with the relation found to be a positive upstream of the STP but strongly negative downstream. This result suggests that nutrient concentrations upstream of the STP are influenced primarily by runoff from the surrounding environment. Thus, the greater the inflow the higher the nutrient concentrations in the creek becomes. In contrast, the high nutrient concentrations observed downstream are the result of large inputs of sewage effluent that have a much higher concentration of nutrients than the creek water. In times of high flow (winter and spring), the sewage effluent, nutrients are diluted more by the creek water than in times of low flow (summer and autumn).

A weaker relationship was obtained between downstream flow and NO_X than for the other nutrients analysed. The difference in median NO_X concentration between upstream and downstream was also less (20-fold compared with 1000-fold for FRP) than that observed for the other nutrients and this is the likely cause for the weaker correlation between flow and NO_X. The smaller difference in NO_X concentration between the upstream and downstream sites means that the dilution of NO_X released into the creek from the STP by upstream waters will be less pronounced. A possible explanation for the smaller differences in NO_X concentration (compared with the other nutrients analysed), is its reduction in the sewage treatment ponds by *denitrification* (see Chapter 1).

A strong positive correlation was obtained between NH_3, FRP, total N and total P concentrations and cell concentrations which could be interpreted to mean that bacterial numbers are controlled or strongly influenced by nutrient concentrations. However, there are two facts that call into question this interpretation. First, the relatively high concentration of nutrients upstream suggests that microbial growth in Campbells Creek is unlikely to be nutrient limited, and second, the strong relationship between nutrient concentration and cell concentration was only observed at the downstream sites. There was a strong relationship between flow rate and biological and physico-chemical parameters in Campbells Creek. It is therefore, more probable that flow determines the concentration of both the nutrients and bacterial cells and only a weak relationship exists between the biological and abiotic factors. Therefore, nutrient concentrations were found to be of little value in assessing the impact of sewage pollution on the ecological health of Campbells Creek. But it is a useful tool for identifying potential problems, e.g. those caused by high concentrations of nutrients or high dissolved organic carbon (DOC) concentrations. It is then up to the researcher to examine the biological community to assess the affects of these high nutrient or high DOC concentrations.

It was hypothesised that cell abundance would be greater at the sites downstream of the Castlemaine sewage treatment plant because of the input of effluent into Campbells Creek. To test this hypothesis, bacterial cell enumeration was performed at all sites. As expected, cell concentrations were generally higher at sites C and D. However, large fluctuations in cell concentrations were observed at sites C and D over this study period, and it appears that bacterial numbers are influenced by when the sampling occurred (Fig.2.7). Despite the fact that the downstream cell concentration was overall significantly greater than upstream, on some particular dates no significant difference in cell concentration between the sites was observed. At times of high flow (winter months), there was little difference in bacterial abundance between upstream and downstream sites. In contrast, samples collected during low flow periods (summer and autumn) contained bacterial numbers 5 to10-fold greater at the downstream sites, compared with those at the upstream sites. The greater cell concentrations observed at downstream sites in times of reduced creek flow, are likely to be

the result of large inputs of bacteria from the sewage treatment plant. During periods of low rainfall (summer and autumn), creek flow is significantly reduced (Table. 2.4) and as a result the sewage effluent contributes a greater proportion (up to 95%) to the downstream flow, thus elevating the cell concentration. However, many of these introduced bacteria are unlikely to be able to compete with the natural microbial community and some will already be dead from the chlorination treatment. Dead cells, together with those bacteria unable to survive in natural systems, will disappear from the water column as a result of their decomposition during the continual recycling and remineralisation of organic matter (microbial loop) in the sediments and water column. This removal of dead cells is the most probable explanation for the lower cell counts observed at site D.

The direct enumeration of bacterial cells was a relatively easy and accurate technique and revealed differences in cell concentration upstream and downstream of the treatment plant. The method was unable to provide information on the bacterial groups present, their origin (autochthonous or allochthonous), or if the increase in cell numbers observed downstream was due to live or dead cells. Despite these disadvantages, direct counts are useful and can provide early insights into adverse changes occurring in the environment. However, if this technique is to be used as a tool in river health assessment, it is important that changes in cell concentration be distinguished from natural fluctuations.

The results of the total and faecal coliform counts were generally unexpected. Both coliform and faecal cell concentrations were relatively high upstream and downstream of the sewage discharge point on all occasions. Campbells Creek water quality failed to meet the recommended criteria for raw drinking water and for recreational on most occasions. This type of bacterial analysis is therefore useful for indicating the suitability of a water body for human consumption or recreational activity, but provides little information on the biological 'health' of the ecosystem or the impact of the sewage effluent on Campbells Creek. The high numbers of coliform bacteria present both upstream and downstream would suggest contamination is occurring from the surrounding terrestrial environment. The Campbells Creek catchment includes urban areas of Castlemaine and cleared farm land. Thus, the coliform bacteria present in Campbells Creek most probably originate from urban pollution

and domestic livestock within the catchment area. It seems the sewage treatment plant is contributing very little to the overall concentration of coliform bacteria, indicating that the chlorination treatment of effluent is successfully terminating a large proportion of these bacteria.

The inability of the above techniques to provide information on the viable cell numbers or the activity of the microbial population has resulted in the development of other methods to obtain this information. Redox dyes such as CTC are commonly used to detect respiring (active) bacteria.

The difficulties and uncertainties encountered when the CTC method was trialed in Campbells Creek (see results section 2.3.6) resulted in only a limited data set being obtained. Therefore, it is not possible to make conclusive statements about the level of activity in Campbells Creek upstream and downstream of the sewage effluent discharge point. However, the results suggest that at both sites there are less active cells (as a percentage of the total population) in the summer months. This was particularly the case at site C with only 6% of the population active in December and 8% in January, compared with 12% and 26% at site B for the same times. There are a number of possible reasons that may explain the slightly lower percentage of active cells downstream during the summer months. First, it is possible during times of low flow, when the sewage effluent makes up a larger proportion of the Creek flow, that the large numbers of introduced sewage bacteria are mainly inactive (or even dead from chlorine disinfection treatment), therefore reducing the percentage of the total bacterial population that is actively respiring. It may also be possible that under low flow conditions, residual chlorine in the sewage effluent is high enough to inactivate some of the natural bacteria in Campbells Creek. It has also been discovered that a number of factors may affect the performance of the CTC assay (Pyle, 1995). For example, CTC reduction by certain bacteria decreases in the presence of elevated concentrations of orthophosphate and at pH values above 6.5. Thus, the high phosphorus concentrations observed in Campbells Creek, particularly at site C, may be inhibiting CTC reduction resulting in an underestimation of the number of metabolically active bacteria. Another uncertainty about the CTC method is the possibility that variations may exist in the ability of certain cells to take up and reduce

CTC, both within a particular bacterial population and between populations from different environments (Cook and Garland 1997). It is not known if all bacterial species take up and reduce CTC with the same efficiency or even if all cell types are capable of this, a factor that must be considered when interpreting results. Besides pH and phosphorus, other potentially inhibiting substances may be present in natural environments, and the sensitivity of the technique has also been brought into question for environments that are nutrient limited. The effects of nutrient supplementation on starved populations significantly increases the numbers of active cells in a population (Rodriguez *et al.*, 1992; Cook and Garland 1997). Limitation of N and P on CTC sensitivity should not be a concern in Campbells Creek, but the possible absence of other essential micronutrients is not known.

2.5 Conclusion

In conclusion, there are many disadvantages and limitations with all of the above mentioned methods (physico-chemical analysis, direct cell counts, total and feacal coliform counts, CTC) as tools for evaluating river 'health'. Cell counts may provide an early insight to possible detrimental changes in the environment, but it is not possible to determine with this technique the cause of significant changes in cell numbers, where the additional cells have originated from (in cases where numbers have increased), or even if these cells are alive or dead. Additionally, there is a need for further research to investigate the ability of different bacterial species to take up and reduce CTC before the CTC method can be used reliably. There is also the added problem with the CTC method of accurately counting small cells and in distinguishing them from abiotic material. However, Despite these limitations, the CTC method, provides a more accurate representation of microbial community activity then viable cell counts which are entirely unsuitable for natural environments. Conventional methods, such as coliform counts, provide specific results that can inform on the suitability of a particular water body for human consumption or recreational use, but provide little information on biological health or the ecological impact of a polluting event. Since biological monitoring has now been acknowledged as a necessity for assessing and protecting river health, it is apparent that new techniques and methods need to be developed.

CHAPTER 3
MEASUREMENT OF BACTERIAL PRODUCTION IN CAMPBELLS CREEK

3.1 Introduction

It now well established that microbial populations play a key ecological role in marine and freshwater ecosystems. They are critical in the cycling and transformation of organic matter and nutrients as well as being an important component of aquatic food webs. Three aspects are crucial in any microbial ecology investigation: bacterial numbers, biomass and production (or growth rate). The pros and cons of measuring bacterial abundance in natural environments was discussed in Chapter 2, and biomass determination is evaluated in Chapter 4. Both these parameters play an important role in microbial ecology and can be useful in assessments of river 'health'. They do not, however, give any insight into the activity or rate of growth of the bacterial population. It is therefore desirable that heterotrophic bacterial production be measured to help understand and unravel ecosystem function. Bacterial growth rates can be determined by measuring cellular processes such as DNA and protein synthesis.

Measurement of bacterial production in Campbells Creek was initially attempted using the tritiated thymidine method described by Moriarty (1986). The sample processing component of this method was found to be unsuitable for Campbells Creek samples, making necessary an extensive re-development of the method. This Chapter reports the application of the new modified method (SSC buffer method) on environmental samples and evaluated for its potential use in river 'health' assessment. The SSC Buffer method for measuring bacterial production was also compared with radiolabelled leucine incorporation.

3.1.1 Bacterial production measurements using tritiated thymidine

Methods involving the incorporation of tritiated thymidine (TDR) into DNA are currently the most common techniques employed for measuring bacterial production. They have been in use in various formats since 1967 (Brock 1967), but did not appear regularly

until Fuhram and Azam's work in the early 1980s (Fuhram and Azam 1980, 1982). The methods have now been applied to many different environments in both fresh and marine waters (Cole *et al.*, 1988) and this probably explains the absence of a standard protocol.

The tritiated thymidine assay is based on the ability of dividing cells to take up exogenously supplied thymidine and convert it to the nucleotide triphosphate thymine, one of the four bases of DNA. This is known as the 'salvage pathway' (O'Donovan, 1978). The basic underlying premise of the tritiated thymidine method is that (a) DNA synthesis is related to cell division and is synthesised in growing cells at a rate proportional to biomass, and (b) if cell division occurs in the presence of radioactive thymidine the new cells produced will be radiolabelled. The main advantage of the thymidine method is that neither the key enzyme involved in the salvage pathway, thymidine kinase, or the transport mechanism for thymidine incorporation into DNA have not been found in other microbial groups (microalgae, cyanobacteria or fungi). The enzyme has been found in protozoans, but these organisms are not considered to have efficient uptake mechanisms for the substrate and therefore this is not a problem as long as the incubations are of a short duration and a relatively low concentrations of TDR are used.

There are thrree major uncertainties involved with the thymidine method of bacterial production measurement (see Robarts and Zohary, 1993 for review).

(1) Non-specific labelling of other macromolecules such as protein may occur if thymidine is degraded within a cell by thymidine phosphorylase and the ^{3}H methyl group enters other biochemical pathways. It is therefore essential that the proportion of radiolabel present in DNA and protein be determined, to ensure that the radiolabel is actually being incorporated into the DNA and avoid underestimates of production. The problem of non-specific labelling can be kept to a minimum by ensuring that only short incubation periods, called 'pulses' are used (<30 minutes). Pulse labelling reduces the extent to which thymidine can be degraded by thymidine phosphorylase and hence decreases the possibility of the ^{3}H methyl group entering other biochemical pathways.

(2) The specific activity of radiolabelled thymidine can be diluted by extracellular and intracellular sources of deoxyribonucleotides, ie. other 'non-labelled' TDR (Pollard and

Moriarty 1984). If dilution occurs then not all of the newly synthesised DNA will contain radiolabel and thus production will be underestimated. The most common dilution occurrence is from the biosynthetic *de novo* pathway, where thymidine monophosphate is synthesised from deoxyuridine monophosphate. To avoid under-estimation of DNA synthesis it is vital that the *de novo* pathway is blocked. Pollard and Moriarty (1984) have shown that the *de novo* synthesis of dTMP can be inhibited by high concentrations of radiolabelled thymidine.

(3) Not all species of heterotrophic bacteria have the ability to utilise tritiated thymidine - the genus Pseudomonas is one such example (Pollard and Moriarty, 1984). The limited information available for anaerobic bacteria suggests caution when using the 3H-TDR method in anaerobic environments (e.g. sediments) (Gilmour *et al.*, 1990). In addition, the ability of bacteria with specialised nutrient requirements, such as chemolithotrophs, to incorporate 3H-TDR is not well understood. Despite these short comings many researchers still prefer to use the thymidine method.

3.1.2 Estimating bacterial production from the incorporation of leucine

An alternative method for estimating bacterial production is measuring the incorporation of radiolabelled leucine into bacterial proteins. Kirchman *et al.* (1985) demonstrated that nanomolar concentrations of leucine were incorporated into proteins and this occurred virtually exclusively by the bacterial population. The observed increases in leucine incorporation correlated with corresponding increases in bacterial cell numbers and protein content. Protein production is considered a good estimate of total bacterial production and energy consumption because proteins constitute a large fraction (approximately 60%) of the dry weight of bacterial cells and their synthesis requires a great proportion of the cells energy (Kirchman 1993).

Proteins are the main cellular component that are not soluble in hot trichloroacetic acid. Leucine incorporation into proteins can therefore be measured by performing a hot trichloroacetic acid extraction of the protein fraction. The radioactively labelled leucine is essentially (at least 90%) incorporated into proteins and it is not degraded re-incorporated

into other amino acids. The proportion of leucine in protein remains relatively constant at 0.073, meaning that any increases in leucine incorporation is not a result of changes in the ratio of leucine in protein (Kirchman 1993).

The major advantage of the leucine incorporation method is that it is possible to calculate bacterial production without the need for information regarding cell sizes and the per-cell carbon content. The method does not require highly variable and controversial carbon conversion factors because there is a constant carbon:protein ratio in bacterial cells (Simon and Azam 1989).

To avoid errors in the estimation of production of new cells, there are two cellular processes that must be considered when using the leucine bacterial production method:

1) Like the thymidine method, isotope dilution can also occur if there is synthesis of the substrate from other compounds. Addition of sufficient concentrations of radiolabelled leucine will help minimise, but not entirely inhibit the *de novo* synthesis of intracellular leucine (Simon and Azam 1989). High concentrations of leucine cannot be used to overcome isotope dilution as some of the isotope may diffuse into or be taken up by other microorganisms such as phytoplankton (Kirchman 1993). Using two independent methods, Simon and Azam (1989) found that there was a 2-fold intracellular isotope dilution when 10 nM of leucine was used. Other researchers (Kirchman 1992) have also discovered that isotope dilution is generally around this value and because of the difficulties involved in the methodology of measuring leucine dilution a value of 2 is widely used by most researchers.
2) Protein turnover may lead to the overestimation of bacterial production. Bacterial cells are able to degrade and synthesise some proteins independent of overall growth. This process has the potential to cause an overestimation of production especially in environments where the production rates are low. Although Kirchman (1989) found during his experiments that protein turnover was not significant, it is important that this possibility is not ignored when interpreting leucine uptake results.

The leucine method is an order of magnitude more sensitive than the thymidine method because 10-times more leucine is required in the synthesis of a new bacterial cell than

thymidine (Riemann and Bell 1990). The thymidine and leucine methods complement each other in that they measure different aspects of bacterial metabolism. Bacterial growth in natural habitats is rarely balanced and for this reason the ratio between leucine incorporation and thymidine incorporation may vary on short time scales. It has been shown that over larger time scales of days to weeks, covering a number of bacterial doubling times the two methods do give very similar results (Simon and Azam 1989; Riemann and Bell 1990; Kirchman 1992). It is beneficial when determining bacterial production to use two independent methods simultaneously. Concordant results from the two methods indicates that variation in production rates is a real result of changes in bacterial production and not a methodological problem. Daneri *et al.* (1994) have shown that using both the thymidine and leucine methods produces the most reliable results for bacterial production rates.

This chapter presents a modified version of the Moriarty method (1986) for TDR incorporation measurements which overcomes the problems of extreme variability between replicate samples and high background counts found in preliminary studies (reported in section 3.2.4). It is being successfully used to measure bacterial production in the Goulburn, Murray and Yarra Rivers and Campbells Creek, all in south east Australia.

3.2 Materials and Methods

3.2.1 Sampling sites

Samples were collected from Campbells Creek and the Hedgelely Dene Pond during the Summer and Autumn of 1996. Campbells Creek and the site locations have been described in Chapter 2. Samples were collected at site A and D and stored in an insulated container until their return to the laboratory. Hedgely Dene pond is a small productive urban pond in the Melbourne Metropolitan area, Victoria.

3.2.2 Physico-chemical analysis

For each sampling event a range of physical and chemical parameters were measured as outlined in Chapter 2. Chlorophyll a concentration was measured in four water samples

(40 ml for site A and 30 ml for site D) from each site. The samples were filtered through 45 mm GFF filters using a syringe filter in the field to measure Chlorophyll levels. The filters were kept on ice in 10 ml centrifuge tubes until they were returned to the laboratory where they were frozen at -20°C until further analysis. Chlorophyll was extracted by soaking the filters overnight in 6 ml of 90% acetone at 4°C. The tubes were vortexed the following day and centrifuged at 2000 rpm for 10 mins to remove filter debris from the acetone solution. One ml of the acetone was removed from each tube without disturbing the pellet and transferred to a new tube to prevent resuspension of the pellet. The chlorophyll concentration was measured downstream using spectrophotometry. Absorbance was read at the following wavelengths: 750 nm (turbidity), 664 nm (chlorophyll *a*), 647 nm (chlorophyll b) and 630 nm (chlorophyll *c*). The turbidity reading at 750 nm was subtracted from all the other wavelength readings and the equations of Jeffrey and Humphrey (1975) were applied. A fluorometer (Fluorspec, Hitachi) was used to read absorbance for upstream samples that had low concentrations of chlorophyll. The Fluorospec was calibrated using an extracted field sample whose chlorophyll concentration had been pre-determined spectrophotometrically. This calibrating sample was subsequently serially diluted to produce a linear calibration plot

The bacterial cell concentration was determined for each site and field trip. Volumes of 10 ml were collected in blood vials (Bio-Lab) and fixed with formaldehyde (1-3% final concentration). Direct cell counts were performed on each sample using DAPI staining and epifluorescence microscopy (see Chapter 2)

3.2.3 The thymidine growth assay

Initially the 'cold trichloroacetic (TCA)' procedure described by Moriarty (1986) was used to measure bacterial growth. The following procedure was followed for the initial time course experiments: Samples were collected and 25 ml aliquot's dispensed into clean 150 ml polyethylene bottles. 50 µl of 2 Ci/mmol methyl-3H Thymidine (ICN) was added to each sample (final concentration 1 µM) and incubated for 5, 10, 15, 20 and 25 minutes. At the end of each time period, 1 ml of 1% non-radioactive thymidine was added resulting in a

swamping of the 3H-thymidine (1/1500 radioactive:non-radioactive thymidine) and a cessation in the uptake of 3H-TDR. The thymidine also exchanges with the radioactive thymidine which has non-specifically bound to any particles. After a further 5 minutes, 250 µl of 37% formaldehyde solution was added (final concentration 0.37%) to preserve the sample before filtering and processing. Samples were refrigerated until filtration. Samples were filtered through 47mm diameter 0.2 µm polycarbonate membranes. As soon as filtration was completed 5 ml of ice cold 80 % ethanol was used to rinse the sides of the filter holder and filter. This cold ethanol rinse was repeated once. Four washes with 2.5 ml of ice cold 3 % TCA (ICN, analytical grade) then followed. The filter was removed, placed in a vial and if necessary stored at 3°C. To each vial, 2 ml of 3 % TCA was added, making sure that the entire filter was covered. The vials were then heated at 90°C for 30 minutes. After the vials had cooled to room temperature, a 0.5 ml aliquot was removed and placed in a 6 ml scintillation vial, 4 ml of liquid scintillation cocktail (Sigma) was added and the vials counted in a Beckman scintillator counter.

3.2.4 Method Development

Unsuccessful attempts (no linear increase in TDR incorporation) with the cold TCA method described above, led to different processing procedures being trialed (Fig. 3.1). Sample collection and TDR incorporation procedure remained the same as section 3.2.3. Volume optimisation experiments were performed to determine the required concentration of TDR to inhibit isotope dilution-10, 20, 40, 50, 60, and 80 ml samples were incubated for 15 minutes with 50 µl of isotope (2.0 Ci/mmol). After the first set of experiments and volume optimisation, smaller 5 ml samples were used in 10 ml blood vials.

Samples were filtered within 48 hours of TDR incorporation taking place. The samples were processed using either (a) a modified cold TCA method, (b) "eppendorf/centrifugation " procedure or (c) the SSC (Sodium chloride and sodium citrate) buffer method; SSC buffer was made up as 20X SSC solution, containing 175.3 g of NaCl

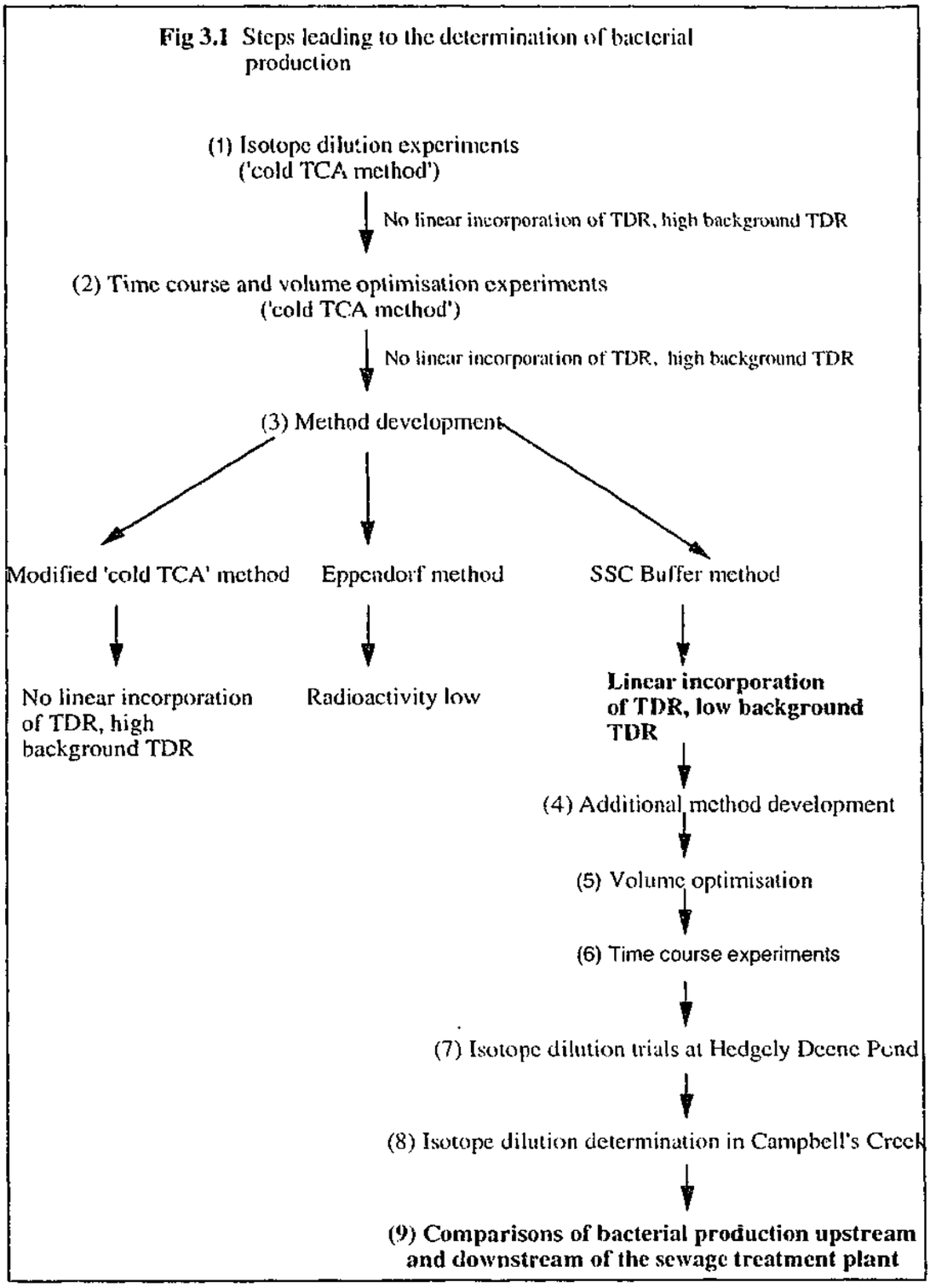
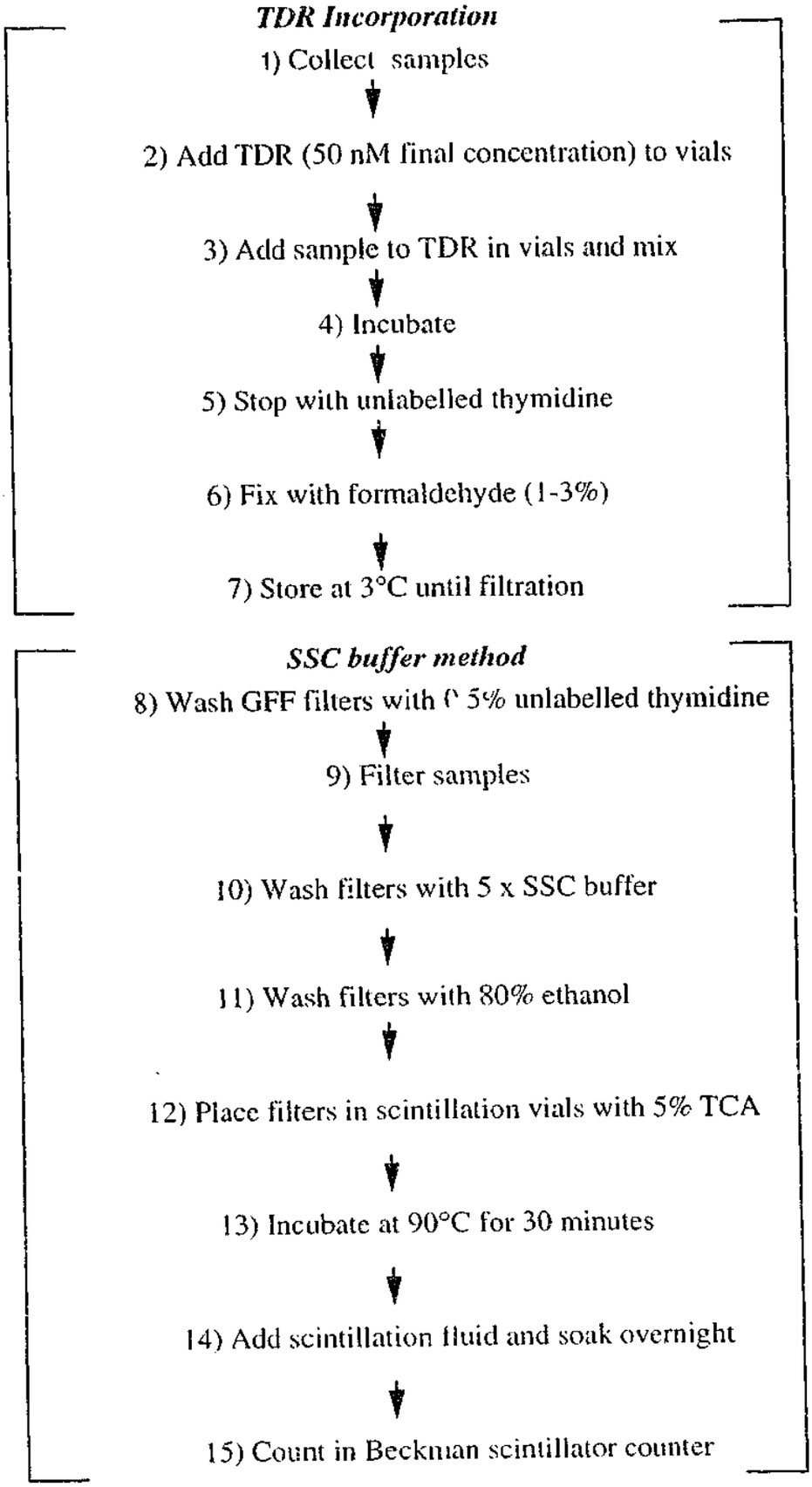


Fig 3.1 Steps leading to the determination of bacterial production

Fig. 3.2 TDR incorporation and processing procedure



and 88.2 g of sodium citrate in 1 L of deionised H_2O, pH 7.0 (Sambrook *et al.*, 1989). In the modified cold TCA method, the hot TCA extraction step was completed in an autoclave (121°C, 60 minutes) in an attempt to more effectively extract the soluble fraction (containing ^{3}H-labelled DNA) from the filter. The "eppendorf/centrifuge" procedure involved removing 3 ml of fixed sample (after TDR incorporation) and centrifuging (Beckman, Avanti™ 30 centrifuge) for 10 minutes at 13,000 x g. The resulting pellet was then washed by resuspending it in 1 ml of 5 X SSC (diluted 1:4 with dH_2O) buffer and the centrifuge step repeated. 0.5 ml of 5% TCA was then added to the pellet and autoclaved (121°C, 60 Min). After the hot extraction was cooled, it was transferred to a scintillation vial (sigma) and 4 ml of scintillant was added. The vials were counted in a Beckman Scintillator counter.

SSC Buffer method: Prior to filtration of the sample a pre-filtration step, involving the washing of the filter, was incorporated into the protocol (see Fig. 3.2 for summary of protocol). Three filter washing treatments were tested; 5 mls of Milli-Q water, 5 mls of 0.5% non-radioactive thymidine or no pre-filtration (dry filter). Samples were filtered onto GF/F filters (Whatman) in a 12 place Millipore 1225 sampling manifold. To determine the proportion of counts being 'lost' by passing through the GF/F filters, the filtrate was collected in 10 ml polycarbonate test tubes and refiltered through 0.2 µm polycarbonate filters. Both sets of filters were then processed using the SSC buffer procedure and counted separately. Filtering samples through 0.2 µm polycarbonate filters alone was found to take too long to be practical and at times when samples were more turbid, not possible as the filters would block. Unincorporated radioactive thymidine was washed from the sample with repeated washes of 5 X SSC buffer using continual gentle suction. Immediately after the samples were filtered, the filters were washed with either 10 X 10 mls or 5 X 10 mls of 5 X SSC. To ensure the entire sample was filtered, the first wash was added to the empty vial and then poured into the filter cup. Each wash was allowed to pass through the filter before the next was added. The filters were then again washed with 4 X 5 ml volumes of 80 % ethanol. Filtration was ceased as soon as the filters appeared dry. The filters were placed in scintillation vials and 2 mls of 5% TCA was added. The vials were placed in an oven at

90°C for 30 minutes, they were then allowed to cool before 4 ml of scintillant was added.

The samples were counted for 10 minutes in a Beckman counter at least 12 hours after the scintillation fluid was added It was found that the counts were considerably lower and more variable amongst the replicates if the counting was performed earlier than this.

The distribution of the radiolabel in DNA, which is soluble from the filter in hot TCA, and protein which is insoluble in the same solution was estimated by the following procedure: Succeeding the oven incubation (90°C for 30 minutes) 1.5 ml of TCA extract was transferred to an eppendorf tube and centrifuged at 10,000 X g for 10 minutes. A small volume (1 ml) of the supernatant containing the dissolved radiolabelled DNA was removed by pipetting to a new scintillation vial. The remaining supernatant was carefully decanted from the tube and the pellet containing the protein was resuspended in 1ml of 5% TCA and also transferred to a new scintillation vial. Scintillation fluid was added to both sets of vials and mixed thoroughly with the TCA sample. Once again the radioactivity in each sample was measured in a scintillation counter at least 12 hours after the addition of the scintillation fluid.

3.2.5 Measuring isotope dilution

In the summer of 1997-98, isotope dilution in Campbells Creek was measured. One litre samples were collected in clean polypropylene bottles, the contents of the bottles kept well mixed by shaking, and 2 ml volumes were subsampled from them for the assay. The concentration of ^{3}H-TDR in all samples was 50 nM and the concentration of cold thymidine in each tube was either 2.5 mmol, 5 mmol, 10 mmol, 15 mmol, 20 mmol or 10,000 mmol. The hot and cold thymidine was added to sterile 10 ml blood vials prior to sample addition to ensure the two were well mixed and to facilitate mixing with the sample. It is much easier to ensure that the thymidine is dispersed throughout the sample when the sample, being a large volume (5ml) is added to the smaller volume of thymidine. The action of the sample leaving the pipette under pressure causes swirling and better mixing within the tube then when sample addition is performed first and eliminates the need to recap and shake before the stopping procedure. Uptake was allowed to proceed for 15 mins before it was stopped by swamping with cold thymidine and then 250 µl of formaldehyde. The experiment was

performed in triplicate at the field site and samples were processed back in the laboratory using the SSC buffer method (as previously described).

The isotope dilution experiments described above were initially performed on samples taken from Hedgely Dene garden to test out the isotope dilution procedure before it was applied to samples from Campbells Creek, Castlemaine. During these experiments at Hedgely Dene pond, the optimal order was determined for adding the reagents (sample, ^{3}H-TDR and ^{1}H-TDR) in terms of lowest backgrounds and variation between replicates. The following alternatives for adding the reagents were examined;

a) ^{1}H-TDR, sample and then ^{3}H-TDR,

b) ^{1}H-TDR, sample, mixed well and left for a couple of minutes to ensure the ^{1}H-TDR was well dispersed and then the ^{3}H-TDR added, and

c) ^{1}H-TDR, ^{3}H-TDR, mix, sample and then the solution was mixed again.

3.2.6 Inhibiting isotope dilution

The following concentrations of ^{3}H-TDR were trialed to determine the concentration required to limit isotope dilution and ensure optimum incorporation of isotope: 5 nM, 20 nM, 50 nM, 100 nM, 150 nM and 200 nM. These inhibition experiments were performed simultaneously with the isotope dilution experiments at Campbells Creek (section 3.2.5) and the same sample volume, incubation time, stopping and processing procedure were used.

3.2.7 Comparing production upstream and downstream of the sewage outlet using tritiated thymidine

After determination of the optimum concentration of isotope, optimum sample volume and estimation of isotope dilution, measurement of bacterial production upstream and downstream of the sewage treatment plant was performed Initially, three 1 litre samples were collected from site A, C and D. After the first field trip, samples were no longer collected from site C and the number of samples per site was increased from 3 to 4 so that there was more replicates for statistical analysis. Time series (1, 5, 10, 15, and 20 minutes) experiments were carried out on each 1 litre sample; 2 ml volumes were subsampled and

added to 10 ml blood vials already containing the ^{3}H-TDR to give a final isotope concentration of 50 nM. Reactions were stopped and samples filtered as described in section 3.2.4.

3.2.8 Bacterial production measurements using tritiated leucine

Once the modified thymidine method (SSC buffer method) was well established and between site comparisons underway, it was decided to also use ^{3}H-Leucine incorporation into bacterial proteins as a way of measuring bacterial production. A modified procedure described by Kirchman (1993) was followed. Sample volumes of 5 ml (subsampled from the same one litre bottles as the ^{3}H-TDR samples) were incubated for 10, 20, 30, 40, and 50 minutes with ^{3}H-Leucine, specific activity 114 Ci/mmol at a final concentration of 10 nM. The high rate of leucine incorporation into proteins at a concentration of 10 nM means that all the added Leucine doesn't need to be radioactive, a mixture of 1.0 ^{3}H-Leucine to 9.0 nM ^{1}H-Leucine is sufficient (Kirchman, 1993). The reaction was stopped by adding 50% TCA to give a final concentration of 5.0% TCA. Samples were kept at 4°C until processing which was performed in the following way: Samples were heated to 80°C for 15 mins and then allowed to cool. The solution was then filtered through GFF filters and washed 3 times with 5XSSC buffer and twice with cold 80% ethanol. The filters containing the extracted proteins were then placed in scintillation vials and 1 ml of 5% TCA was added. The vials were incubated at 90°C for 30 minutes and allowed to cool to room temperature before scintillation fluid was added. The filters were left to soak overnight to enable the scintillant to soak completely through the filters and for the radioactivity to be dispersed evenly through the scintillant. Samples were counted on a Beckman scintillation counter.

Biomass production was calculated using the theoretical approach (Kirchman, 1993) according to the following equation:

$$\text{Production} = \text{Leu}^{*}\ 131.2 \times (\%\ \text{Leu})^{-1} \times (\text{C/protein}) \times \text{ID}$$

where: Leu* is the rate of Leu incorporation (mol/L/h),

Formula weight of Leu = 131.2

% Leu = Fraction of Leu in protein = 0.073

C/Protein = Ratio of cellular carbon to protein = 0.86

ID = Isotope dilution = 2

3.2.9 Statistical analysis

A two-way analysis of variance was performed on bacterial cell concentrations, chlorophyll levels and bacterial production using the computer program 'Systat', to determine if the differences observed between sites were statistically significant. Data were log(x+1) transformed for calculation of mean square and significance tests. If the results were significant ($P<0.05$) then pairwise analysis was performed for each sampling event. A correlation matrix between chlorophyll, cell concentration and productivity against the physico-chemical parameters was also performed.

3.3 Results

3.3.1 Existing method

The ^{3}H-thymidine (TDR) incorporation method described by Moriarty (1986) was employed to measure bacterial production in Campbells Creek, upstream and downstream of the Castlemaine sewage treatment plant.

In the preliminary studies, time series experiments were performed in conjunction with isotope dilution experiments. A plot of TDR incorporation should be linear over at least the first 15 minutes and extrapolating this plot back to time zero is the best estimate of background as it takes into account non-specific binding and short initial lag periods before thymidine is incorporated. The results obtained from these early experiments were highly variable and showed high levels of background incorporation of TDR (Fig. 3.3). As a result, no linear increase in thymidine incorporation could be seen when the radioactivity was either plotted against volume or time, masking the ability to detect any bacterial growth.

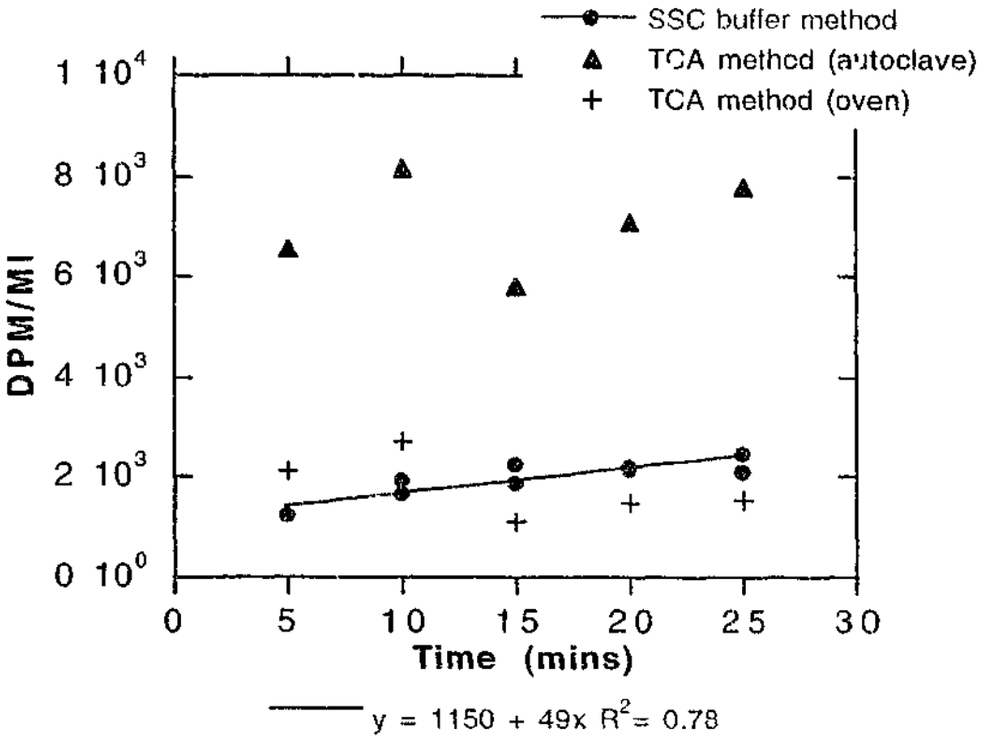


Figure 3.3 Time course of incorporation of TDR after processing samples with three different methods; TCA method (autoclave or oven incubation) and SSC buffer method

3.3.2 New method

The very high levels of background TDR and variability seen in the initial production determination experiments performed at Campbells Creek suggested that the method (Moriarty 1986) used in processing (filtering and washing the samples) may be the cause of the inconsistent results and high levels of background which were hiding any linear increase in TDR incorporation. It was therefore decided to try a number of alternative processing methods (Modified Moriarty, Eppendorf and SSC Buffer) to remove the unincorporated TDR and decrease the background level. Once again time series assays were performed. The reactions were also stopped with non-radioactive thymidine prior to the addition of formaldehyde. This swamping of TDR with "cold" thymidine helped in the reduction of background TDR by exchanging with any radioactive thymidine which was non-specifically bound to biotic or abiotic particles. The results of including a 5 minute unlabelled thymidine incubation step, prior to formaldehyde addition can be seen in Figure 3.4. Although there

was minimal incorporation of radioactivity, the background levels of TDR and variation between the replicates were reduced by a factor of 7-10.

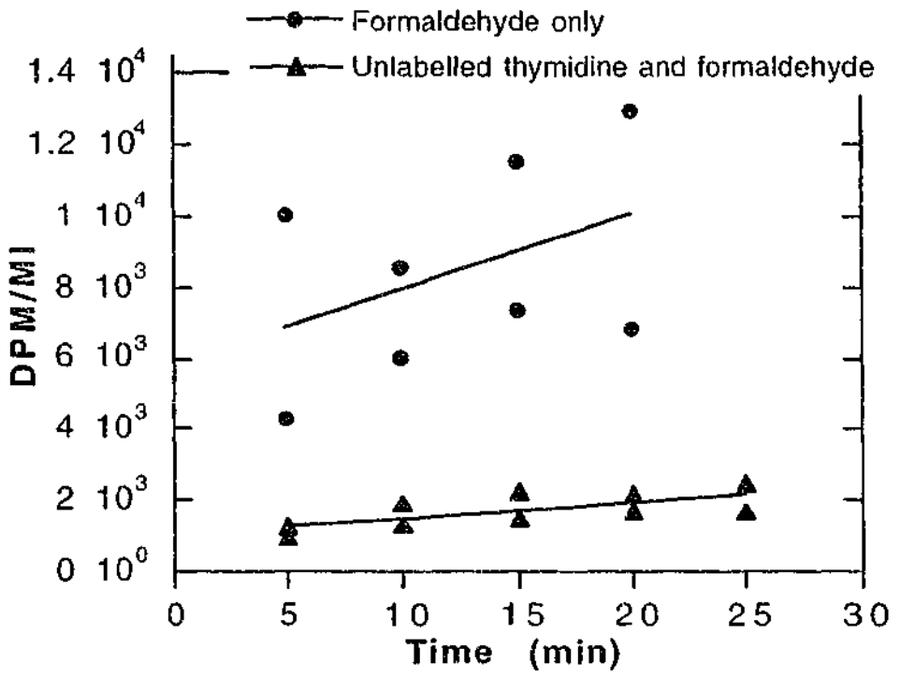


Figure 3.4 Plot of TDR incorporation into DNA over time. Comparison of background levels of TDR between samples stopped with 1 mL of 0.5% unlabelled thymidine and those stopped with formaldehyde only.

The autoclave modification of the Moriarty method resulted in higher radioactive counts being observed (5500-8000 DPM/ml) but background levels were still high and plotting of the data against time was not linear (r^2= 0.05, Fig. 3.3). On the other hand however, a linear rate of ^3H incorporation (r^2= 0.75) was obtained with the Eppendorf method, but the radioactive counts were very low with the DPM/ml ranging from only 250 to 700 (Fig. 3.5). This would lead to an underestimation of bacterial production. The low counts observed with this method were most probably due to the loss of some of the DNA pellet during the washing procedure.

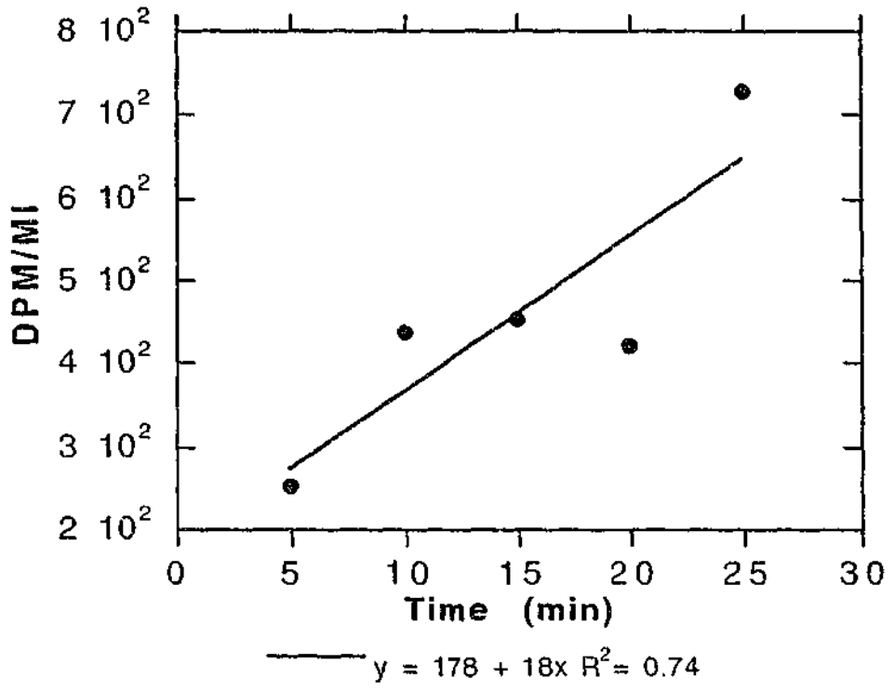


Figure 3.5 Time course incorporation of TDR. Samples processed with the Eppendorf method.

The SSC buffer method produced the most encouraging results and all further experiments used method. The radioactive counts were of a similar magnitude to the traditional cold TCA Moriarty method. However, the variability and background counts were reduced and it was possible to detect growth shown by a linear relationship (r^2=0.78) between time and ^{3}H TDR incorporation (Fig. 3.4). A time series experiment using a higher specific activity of isotope were also conducted (Fig 3.6). As expected, the counts were higher using 35 µCi/mmol with a DPM/ml range of 2000 to 12000 compared with only 800-2700 for 2 µCi/mmol. More importantly though, the slope of the graph and hence rate of TDR incorporation was greater with 2.0 Ci/mmol. This is due to the higher concentrations of thymidine present in the assay when a lower specific activity isotope is used. Lower specific activity of isotope (i.e 2.0 Ci/mmol) is obtained by diluting tritiated thymidine with

unlabelled thymidine. Therefore, the concentration of total (labelled and unlabelled) thymidine is greater when 2.0 Ci/mmol is used than when 35 Ci/mmol. The higher concentration of thymidine in 2.0 Ci/mmol is likely to inhibit isotope dilution more effectively than with 35 Ci/mmol, resulting in the greater incorporation of TDR into the DNA of dividing cells.

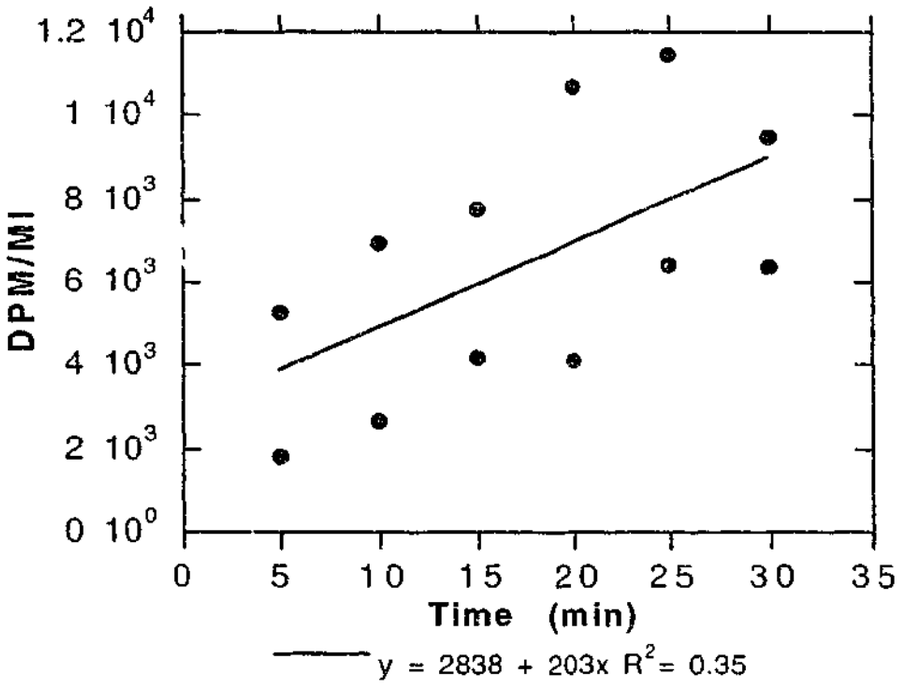


Figure 3.6 Time course incorporation of TDR into DNA using a higher specific activity of isotope (35 Ci/mmol). Samples processed with the SSC buffer method.

Volume experiments (see section 3.2.4) performed at the same time as the time series incubations, and processed using the SSC buffer method, suggested that the optimum volume of sample to incubate with 50 µl of 2.0 Ci/mmol was less than 10 ml. In other words the concentration of thymidine in the sample needed to be higher than 2.5 µM to inhibit the *de novo* synthesis of dTMP. Time course incubations performed with 5 ml volumes of samples were repeated using TDR at a specific activity of 2.0 Ci/mmol (Fig.3.7).

Samples were again processed using the SSC buffer method with much improved results compared with those obtained previously using the cold TCA method. The DPM/ml obtained when a sample volume of 5 ml was used increased to a range of 800-8000 with a good linear rate (with time) of incorporation (r^2= 0.88). Filtering samples onto GFF filters did result in the loss of counts, probably because a small percentage of the bacterial cells are able to pass through these filters (Fig 3.8). However, filtering through 0.2 µm polycarbonate filters alone is extremely slow and not practical for processing large numbers of samples. From the results, it is therefore recommended that a two step (GFF, 0.2 µm) filtration of samples be used for at least one set of replicates to gauge the activity in the GFF filtrate.

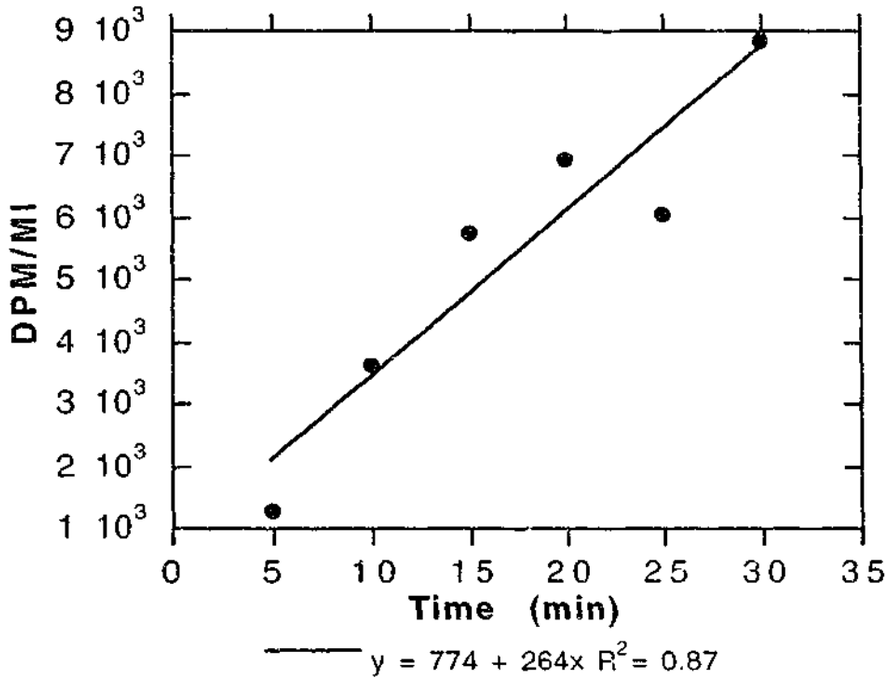


Figure 3.7 Time course incorporation of TDR (2.0 Ci/mmol of isotope) on 5 mL samples processed with the SSC buffer method.

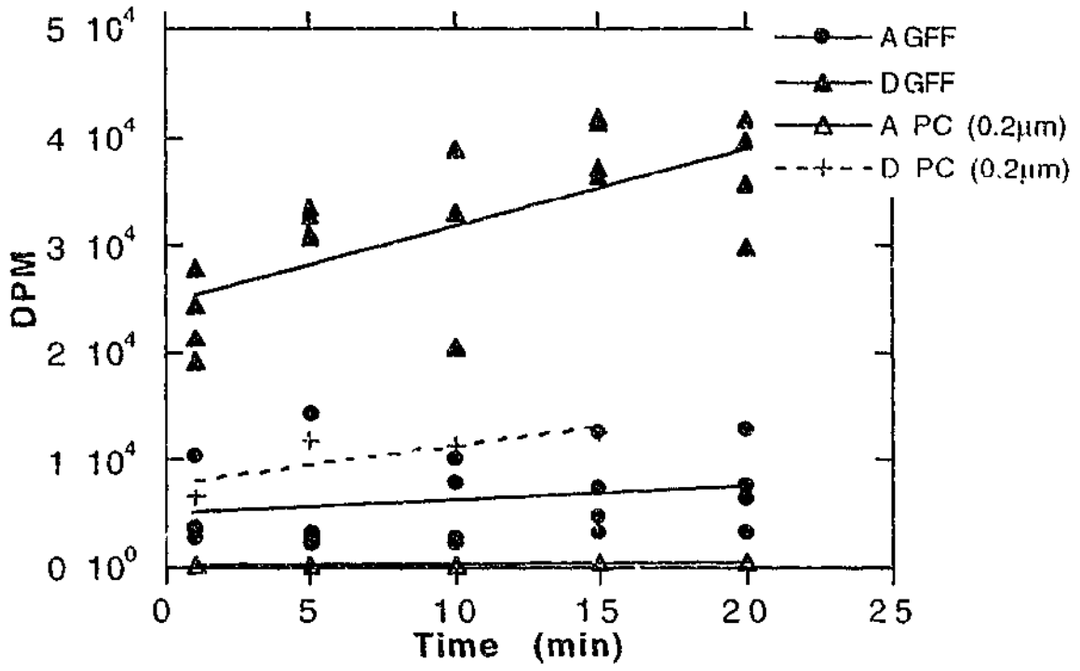


Figure 3.8 Time course incorporation of TDR (2.0 Ci/mmol of isotope) on 5 mL samples processed with the SSC buffer method. Samples from Site A and site D filtered through GFF filters, the filtrate collected and refiltered through 0.2 µm polycarbonate (PC) filters.

Ending the thymidine growth assays with unlabelled thymidine 5 minutes before the cells were fixed with formaldehyde decreased the variability amongst replicates, decreased background counts and hence helped improve the detection and measurement of bacterial growth. The effect of not adding unlabelled thymidine to the reactions is clearly demonstrated in Fig 3.4 with a resulting r^2 value of only 0.184. Additional experiments showed that the background TDR levels could be further reduced by two orders of magnitude if the GFF filters were soaked with ultra-pure water (Milli-Q) or a low thymidine concentration (0.5% w/v) solution prior to filtration. Pre-filtering with unlabelled thymidine solution gave slightly lower and less variable background values than pre-filtering with ultra-pure water alone (Fig. 3.9).

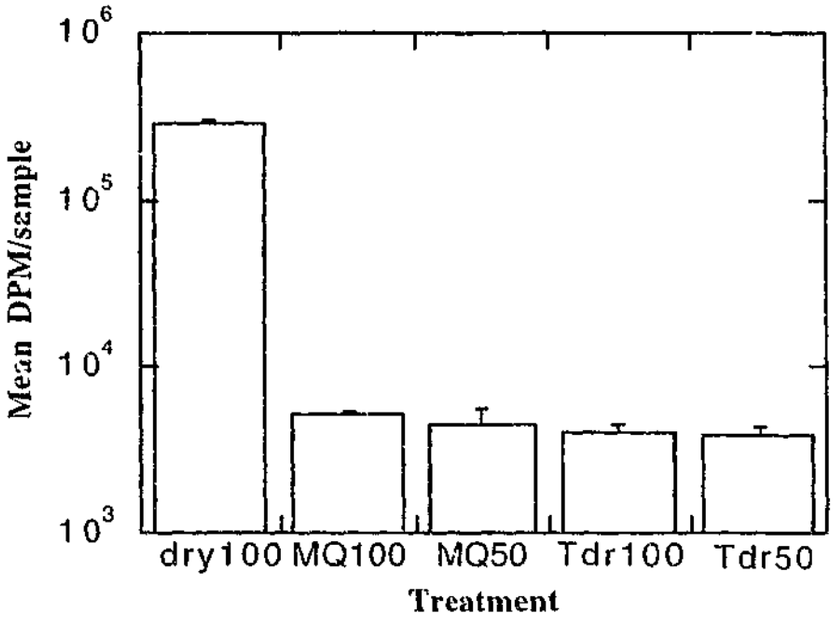


Figure 3.9 The reduction of background radioactivity by pre-soaking GFF filters with ultra-pure water (MQ) or a low concentration solution of thymidine (Tdr). The filters were also washed with either 50 or 100 mL volumes of SSC to see if wash volume influenced background radioactivity.

3.3.3 Distribution of radioactivity

The amount of TDR incorporated into the DNA and other macromolecules (protein) was examined using acid hydrolysis as described in section 3.2.4. The hot TCA soluble fraction (containing the hydrolysed DNA) of the SSC buffer method yielded greater than 98% of all the extracted counts with less than 1.5% of counts in the hot TCA insoluble fraction (Table 3.1). The results show that at least 98% of the TDR was being incorporated into the DNA of dividing cells and hence non-specific labelling of other macromolecules was not significantly affecting bacterial production measurements.

Table 3.1 Percent DPM in soluble and insoluble hot TCA extraction of five site D replicates

Soluble Fraction	Insoluble Fraction
99.3	0.7
99.3	0.7
98.6	1.4
98.9	1.1
99.0	1.0

3.3.4 Isotope dilution and volume optimisation experiments

The first requirement for the use of TDR in the determination of bacterial production, is to estimate the extent of isotope dilution from intracellular and extracellular deoxyribonucleotide pools. Intracellular dilution from the biosynthetic *de novo* pathway, where bacteria synthesise thymidine monophosphate (dTMP) from dUMP is the most common form (Pollard and Moriarty, 1984). In preliminary studies, the procedure suggested by Moriarty and Pollard (1981) was employed to measure isotope dilution. This method involved the incubation of samples with a fixed concentration of radioactively labelled thymidine and various concentrations of unlabelled thymidine. When the reciprocal of isotope (DPM/ml) incorporation is plotted against the concentration of added unlabelled thymidine a linear graph should result. Where the line of best fit crosses the x-axis represents the isotope dilution occurring in each sample.

Attempts to use the Moriarty and Pollard (1981, 1986) method in conjunction with the cold TCA method of processing were unsuccessful. The results were variable and had high background levels of incorporation (Fig 3.10). As a linear graph was not produced when the results were plotted, it was not possible to determine whether isotope dilution was occurring. Using the 'cold TCA' method (Moriarty 1986), a volume optimisation approach

was also employed to try and determine the required concentration of TDR to inhibit isotope dilution. The volume optimisation method maintains the concentration of labelled thymidine added to each vial constant but increases the volume of sample (and hence the number of actively growing bacteria). The amount of TDR incorporation should increase with increasing volume until there is insufficient thymidine for that quantity of bacteria and so isotope dilution will begin. A plot of growth rate versus the sample volume will peak at this point. It is then possible to see where dilution began and any sample volume below this point will contain bacteria in which the *de novo* pathway for thymidine synthesis is inhibited. The results from these volume experiments were variable, had high levels of background radioactivity and it was not possible to see at what volume isotope dilution was occurring (Fig. 3.11). These initial unsuccessful attempts at measuring isotope dilution were attributed to the processing and washing procedure. Further attempts to measure isotope dilution were therefore suspended until a suitable processing method had been developed.

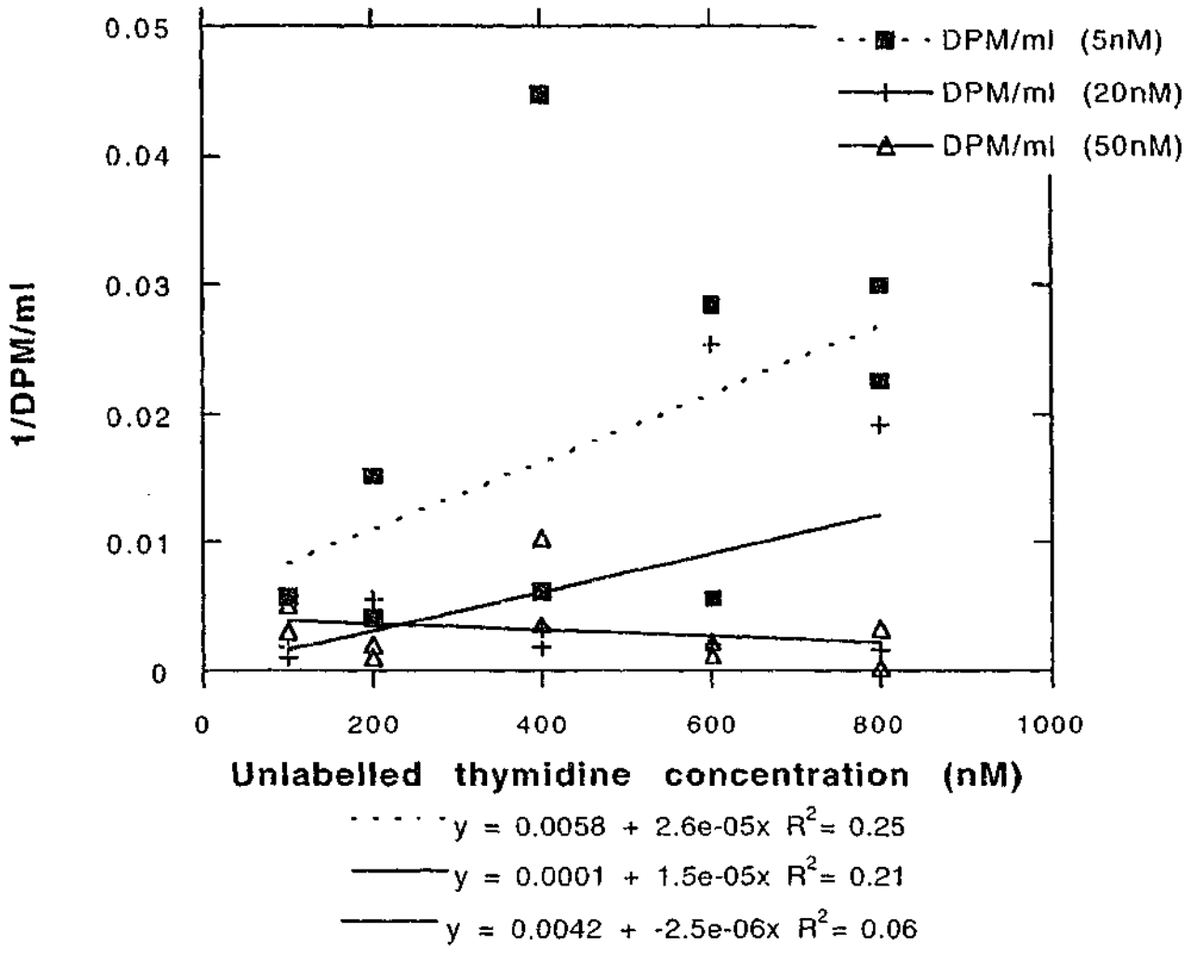


Figure 3.10 Isotope dilution plots for incorporation of TDR into DNA using the Moriarty and Pollard (1981) method at 3 different concentration of TDR (5 nM, 20 nM & 50 nM).

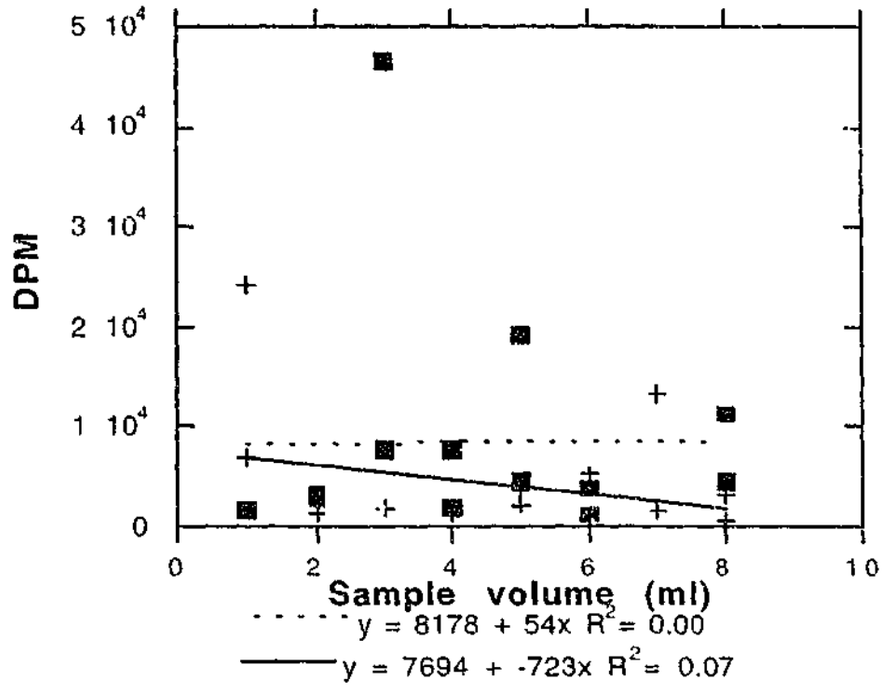


Figure 3.11 Isotope dilution plots using an alternative method to Moriarty and Pollards (1981) The concentration of isotope in each vial was constant but increasing volumes of sample was used in an attempt to determine at what concentration isotope dilution begins.

Once the new SSC buffer processing procedure had been established isotope dilution determination resumed. The isotope dilution method was initially tested at the Hedgeley Dene pond. The experiments with Hedgeley Dene samples revealed the importance of the order of reagent addition. Large variation between the replicates was observed unless the 'hot' and 'cold' thymidine was mixed together in the tube before the sample was added. Using the SSC buffer method of processing the isotope dilution experiments performed at site D, Campbells creek were successful (linear decrease in the incorporation of TDR with increasing concentrations of unlabelled thymidine) and indicated that a small amount (1.92 nmol) of dilution was occurring when a final concentration of 50 nM of isotope was used (Fig. 3.12). Isotope dilution inhibiting experiments using the following final concentrations of ^{3}H-TDR: 5 , 20, 50, 100, 150 and 200 nM were carried out simultaneously with the isotope dilution determination. It is clearly shown in Figure 3.13 that the optimum

concentration of TDR to ensure maximum incorporation of isotope and limit isotope dilution is around 50 nM. This optimum concentration of isotope does not mean that all of the isotope is available for uptake by the cells, some of it will be non-specifically bound to the outside of other biotic and abiotic material. It is, however, the concentration required to saturate non-specific binding sites and still leave sufficient TDR available to inhibit isotope dilution. Concentrations greater than 50 nM did not result in a significant increase in observed counts (DPM) and concentrations of 5 nM and 20 nM produced counts 5-fold and 2-fold less, respectively.

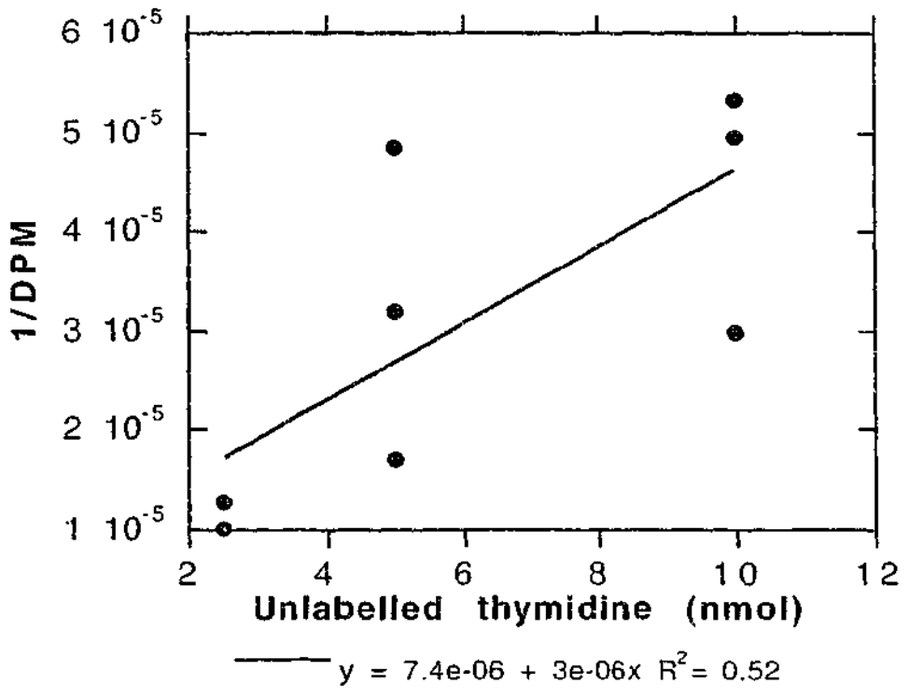


Figure 3.12 Isotope dilution plot for site D samples using the SSC buffer method to process samples

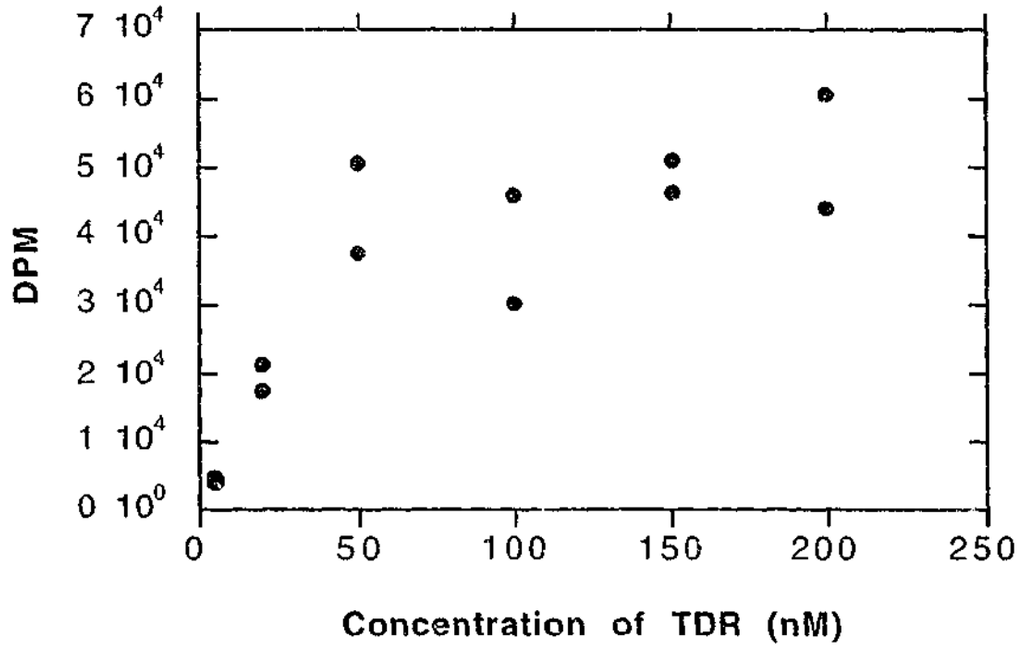


Figure 3.13 Plot determining the optimum concentration of TDR required to inhibit isotope dilution in Campbells Creek.

3.3.5 Bacterial dynamics in Campbells Creek

Bacterial production upstream and downstream of the sewage discharge point

The new SSC buffer method was used to measure bacterial production upstream and downstream of the sewage discharge in Campbells Creek. Bacterial production was measured at both site A and site D, some 10 times over the period December 1997 to March 1998 (Table 3.2). Time course experiments were performed on either 3 or 4 replicates at each site. The results of these assays were plotted as DPM/ml versus time and the slope of the graph was used to calculate bacterial production at each site. The rate of thymidine incorporation was converted to moles of ^{3}H-TDR per unit time and volume using the following equation (Bell 1993);

(Equation 1)

$$\text{moles thymidine ml}^{-1}\ \text{min}^{-1} = \left(\frac{\text{slope x Ci/DPM}}{\text{SA}}\right) \times 10^{-3}$$

Where: slope = slope of the graph (time series experiments)

Ci/DPM = number of curies per DPM = 4.5×10^{-13}

SA = specific activity of TDR in curies per mmol

10^{-3} = mmol per mole

The commonly used empirical thymidine conversion factor of 2×10^{18} cell/mol was employed to convert incorporated moles of thymidine to cells produced per unit time (Smits and Riemann, 1988; Ducklow and Carlson, 1992; Bell 1993).

To obtain productivity in the more useful term of units of carbon, a further calculation is required using another and more uncertain carbon conversion factor. Since the carbon content of cells is dependent on cell volume (Simon and Azam 1989, Kroer 1994), and the carbon content per unit volume increases with decreasing size, the mean cell volume is not universal across all environments and use of a single carbon conversion factor is not possible. For this reason it was decided to use the average (from >80% of the bacterial population as determined from the cell concentration of the SdFFF fractions, (see chapter 4) mass of organic matter per cell data obtained from biomass determination with SdFFF to calculate carbon production (µg/L) upstream and downstream. This information can then be easily converted to the more common term of carbon content using the generally accepted assumption that organic carbon is approximately 50% of a cells dry weight (Luria 1960).

Using this procedure, the mean mass of carbon per cell upstream and downstream of the sewage treatment plant was found to be 6.2 fgC/cell and 7.5 fgC/cell, respectively (Table 3.2). These values are somewhat less than the literature values of 10.0 fgC/cell and 20.0 fg/cell recommended for oligotrophic and eutrophic environments, respectively (Bell 1993). Indeed values as high as 28 fg C/cell have been used as conversion factors (Findlay *et al.*, 1991).

Table 3.2 Bacterial production measurements in Campbells Creek at site A and D from December 1997 to March 1998. Production (cells.L-1.hr-1) calculated using equation 1 and converted to Carbon production using the mean mass of carbon per cell data obtained with SdFFF.

Site	Date	r^2	Sample Vol (mL)	Slope (dpm ml-1 min-1)	Production (cells.L-1.hr-1)	Carbon production µg C.L-1.hr-1
A	2.12.98	0.86	2	1122.00	3.0E+10	188
A	2.12.98	0.23	2	438.00	1.2E+10	74
A	2.12.98	0.88	2	806.50	2.2E+10	135
A	9.12.98	0.45	2	42.67	1.2E+09	7
A	9.12.98	0.40	2	129.00	3.5E+09	22
A	9.12.98	0.72	2	194.50	5.3E+09	33
A	16.12.97	0.54	2	1289.00	3.5E+10	216
A	16.12.97	0.45	2	327.00	8.8E+09	55
A	16.12.95	0.46	2	189.27	5.1E+09	32
A	6.1.98	0.64	2	42.63	1.2E+09	7
A	6.1.98	0.84	2	65.95	1.8E+09	11
A	6.1.98	0.68	2	265.86	7.2E+09	45
A	13.1.98	0.91	2	574.00	1.5E+10	96
A	13.1.98	0.59	2	451.00	1.2E+10	76
A	13.1.98	0.09	2	103.00	2.8E+09	17
A	20.1.98	0.65	2	80.50	2.2E+09	14
A	20.1.98	0.70	2	269.00	7.3E+09	45
A	20.1.98	0.16	2	75.50	2.0E+09	13
A	28.1.98	0.47	2	122.50	1.7E+09	10
A	28.1.98	0.39	2	112.00	3.0E+09	19
A	28.1.98	0.42	2	299.22	4.0E+09	25
A	28.1.98	0.64	2	324.00	4.4E+09	54
A	4.2.98	0.96	2	294.50	4.0E+09	25
A	4.2.98	0.42	2	43.00	5.8E+08	3.6
A	4.2.98	0.95	2	37.00	5.0E+08	3.1
A	17.2.98	0.07	2	22.00	5.9E+08	3.7
A	17.2.98	0.07	2	27.84	7.5E+08	4.7
A	17.2.98	0.03	2	6.50	1.8E+08	1.1
A	20.2.98	0.83	5	688.80	1.9E+10	116
A	20.2.98	0.24	5	314.40	8.5E+09	53
A	20.2.98	0.24	5	433.80	1.2E+10	73
A	20.2.98	0.84	2	497.50	1.3E+10	84
A	20.2.98	0.98	2	810.50	2.2E+10	136
A	20.2.98	1.00	2	554.50	1.5E+10	93
A.P.C	20.2.98	0.97	5	55.60	1.5E+09	9.3
A.P.C	20.298	1.00	5	51.00	1.4E+09	8.6
A.P.C	20.2.98	0.96	5	42.40	1.1E+09	7.1
A.P.C	20.2.98	0.98	2	43.00	1.2E+09	7.2
A	3.3.98	0.39	2	41.00	1.1E+09	6.9
A	3.3.98	0.14	2	-107.00	-2.9E+09	-18.0
A	3.3.98	0.30	2	-50.50	-1.4E+09	-8.5
A	3.3.98	0.53	5	1147.40	3.1E+10	193
A	3.3.98	0.65	5	34.20	9.2E+08	5.7
A	3.3.98	0.44	5	94.00	2.5E+09	16
A.P.C	3.3.98	0.58	5	4.20	1.1E+08	0.7

Table 3.2 continued

Site	Date	r^2	Sample vol (mL)	Slope (dpm ml-1 min-1)	Production (cells.L-1.hr-1)	Carbon production µg C.L-1.hr-1
D	2.12.98	0.00	2	89.50	2.4E+09	18
D	2.12.98	0.09	2	-312.00	-8.4E+09	-63
D	2.12.98	0.46	2	737.50	2.0E+10	148
D	2.12.98	0.00	2	16.50	4.5E+08	3.3
D	9.12.98	0.96	2	732.00	2.0E+10	147
D	9.12.98	0.85	2	968.50	2.6E+10	195
D	9.12.98	0.87	2	592.50	1.6E+10	119
D	9.12.98	0.97	2	952.00	2.6E+10	191
D	16.12.98	0.17	2	415.00	1.1E+10	83
D	16.12.98	0.05	2	-256.50	-6.9E+09	-52
D	16.12.98	0.02	2	89.00	2.4E+09	18
D	16.12.98	0.15	2	368.50	9.9E+09	74
D	6.1.98	0.51	2	1258.50	3.4E+10	253
D	6.1.98	0.71	2	1770.50	4.8E+10	356
D	6.1.98	0.63	2	1725.00	4.7E+10	347
D	6.1.98	0.77	2	1133.00	3.1E+10	228
D	13.1.98	0.23	2	259.00	7.0E+09	52
D	13.1.98	0.20	2	-521.00	-1.4E+10	-105
D	13.1.98	0.02	2	-370.50	-1.0E+10	-75
D	13.1.98	0.00	2	-11.00	-3.0E+08	-2.2
D	20.1.98	0.54	2	270.00	7.3E+09	54
D	20.1.98	0.30	2	213.50	5.8E+09	43
D	20.1.98	0.48	2	448.50	1.2E+10	90
D	20.1.98	0.64	2	493.00	1.3E+10	99
D	28.1.98	0.41	2	2138.50	2.9E+10	215
D	28.1.98	0.76	2	2447.50	3.3E+10	246
D	28.1.98	0.84	2	2508.00	3.4E+10	252
D	28.1.98	0.74	2	3321.00	4.5E+10	334
D	4.2.98	0.82	2	2174.50	2.9E+10	219
D	4.2.98	0.81	2	1866.50	2.5E+10	188
D	4.2.98	0.84	2	3508.00	4.7E+10	353
D	4.2.98	0.59	2	1257.00	1.7E+10	126
D	17.2.98	0.42	2	368.50	9.9E+09	74
D	17.2.98	0.58	2	1367.50	3.7E+10	275
D	17.2.98	0.47	2	956.50	2.6E+10	192
D	3.3.98	0.69	2	1213.00	3.3E+10	244
D	3.3.98	0.60	2	4235.50	1.1E+11	852
D	3.3.98	0.90	2	2413.50	6.5E+10	485
D	3.3.98	0.89	2	4655.50	1.3E+11	936
D	3.3.98	0.86	2	917.50	2.5E+10	185
D	3.3.98	0.82	2	1961.50	5.3E+10	395

Bacterial production measurements using the ^{3}H-TDR incorporation method revealed that bacterial growth at site D site was overall significantly greater (ANOVA $P<0.05$) than at site A, but not for all dates (Table 3.3).

Pairwise analysis of site and date was therefore performed to determine the times when dates bacterial production differed significantly between the upstream and downstream sites. No significant differences were observed on three separate field trips (16/12/97, 13/1/98 and 20/1/98), and production was actually found to be greater on one occasion (2/12/97) at site A. It is possible, however, that the lack of a significant difference obtained on these occasions is the result of an inability to detect growth at site D. It was noted that on these three particular dates, the background levels of tritiated thymidine were high and that large variations existed between replicates. These results prompted further method development which is discussed in section 3.3.5. When high background levels of radioactivity were observed, it is very difficult to obtain a reliable estimate of bacterial production or to conclusively state that there were no significant differences in bacterial production between the sites.

For all remaining field trips, bacterial production was found to be significantly greater downstream of the discharge point (Table 3.3), with mean production ranging from 135 µgC/L/hr (17/2/98) to 523 µgC/L/hr (28/1/98). In contrast, production at site A when significant differences were observed ranged from only 3.0 µgC/L/hr (17/2/98) to 35 µgC/L/hr.

Table 3.3 Pairwise comparisons of bacterial production at site A and D

Date	P value
2/12/97	0.0255*
9/12/97	0.0486*
16/12/97	0.2067
6/1/98	0.0007*
13/1/98	0.0537
20.1.98	0.2066
28/1/98	0.0085*
4/2/98	0.0004*
17/2/98	0.0068*
3/3/98	0.0000*

*Significant P< 0.05

3.3.6 Additional method development

During the bacterial production monitoring experiments further methodology developments were continually taking place. It was observed that on 3 occasions (16/12/97, 13.1.98 and 20.1.98) when bacterial production was not significantly greater at site D, the backgrounds levels of radioactivity had risen, which resulted in no linear increase in TDR incorporation with time, making it difficult to detect growth When background levels of radioactivity are high it is very difficult to measure increases in TDR incorporation per unit time and volume. Background radioactivity also seems to be higher when chlorophyll concentrations and turbidity levels are high. A positive, although weak, relationship was observed when the radioactive counts for the first time point in the time series, were plotted against chlorophyll (Fig. 3.14). This increase in background radioactivity may be the result of the TDR non-specifically bound to particulate matter within the sample not effectively exchanging with the unlabelled thymidine when higher concentrations of chlorophyll were present.

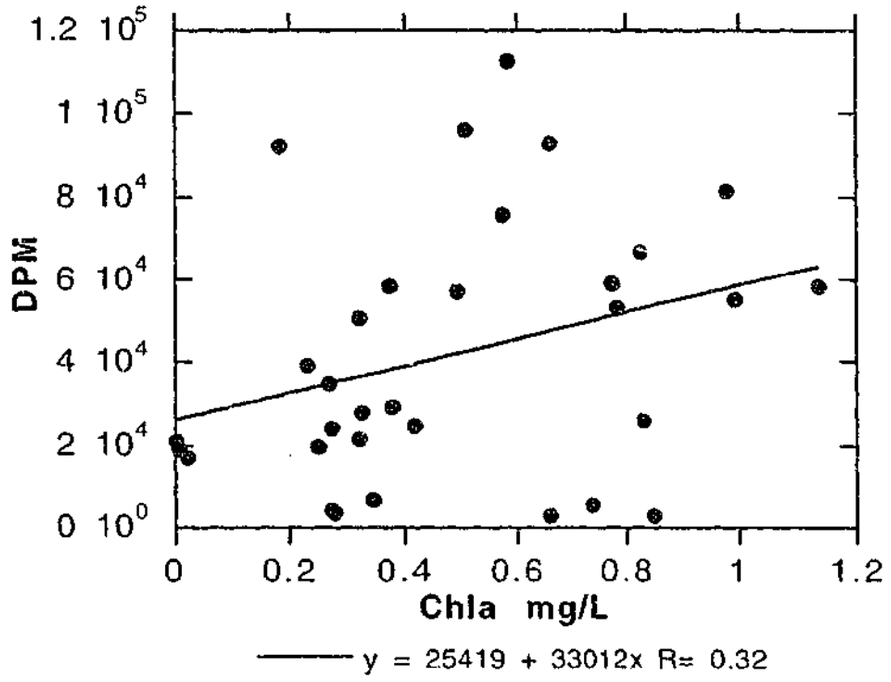


Figure 3.14 Plot of concentration of chlorophyll (mg/L) versus the radioactivity (DPM) of TDR for 0 min incubated samples (blanks)

The concentration of non-radioactive thymidine added to the sample during the stopping process was increased from 0.5% to 5.0% for 3 replicate time course experiments to try to counteract this effect,. Another 3 replicates were stopped with the original 0.5% solution. The increase in non-radioactive thymidine concentration had a small affect on the slope of the time course graph (5241 for 0.5% and 5525 for 5%) but more importantly it improved the variation between replicates. The r^2 value for the graph increased from 0.25 to 0.6. These results suggest that a higher concentration of cold thymidine should be used in the stopping process for samples with a relatively high level of non-bacterial matter.

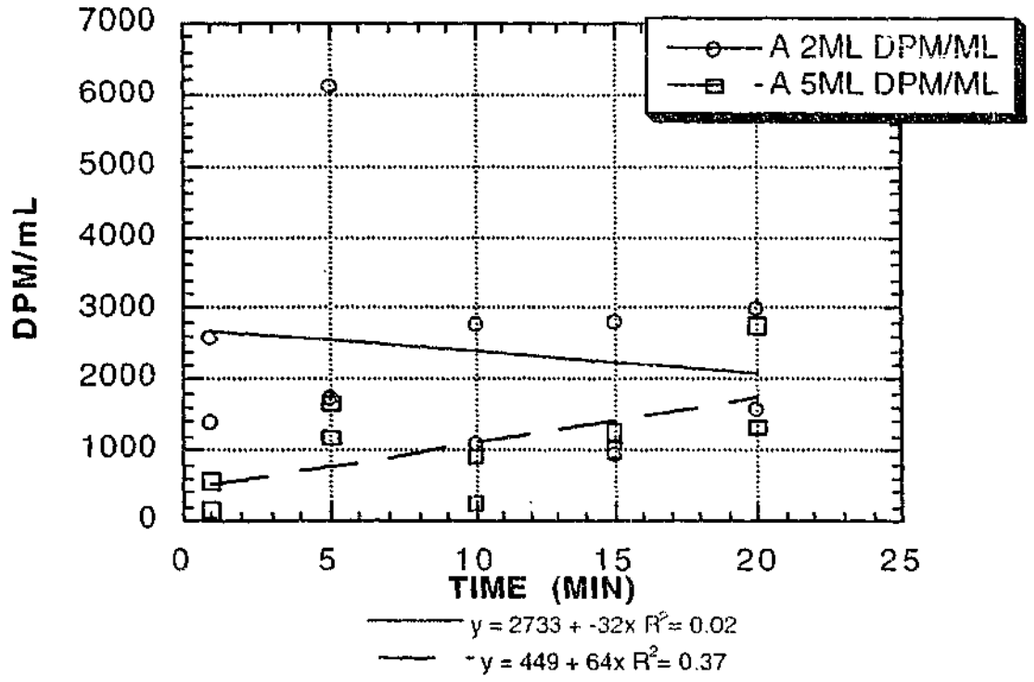


Figure 3.15 Time course incorporation of TDR into DNA at site A using two different volumes of sample (2 mL and 5 mL).

The lower radioactive counts observed at upstream site A, suggest that the rate of bacterial production at this site is lower than that upstream. On some occassions the counts were so low that it was difficult to detect production and the variation between replicate time courses was greater than at site D . For this reason the sample volume at site A was increased from 2 mL to 5 mL. The increased sample size did improve variation between the replicates; for example, on 3/3/98 the r^2 value for the replicates increased from 0.16 for the 2 mL time courses to 0.6 for the 5 mL samples and the slope of the graph went from indicating no production (m= - 32) to detecting an increase in TDR incorporation (m= 64) (Fig. 3.15). Obviously, larger sample sizes mean an increase in bacterial cells, resulting in less variation and more statistically reliable results. The disadvantage is the increase in expense caused by the larger quantities of isotope required to maintain the final concentration of tritiated thymidine at 50 nM.

3.3.7 Bacterial abundance, chlorophyll concentration, physico-chemical and nutrient Analysis

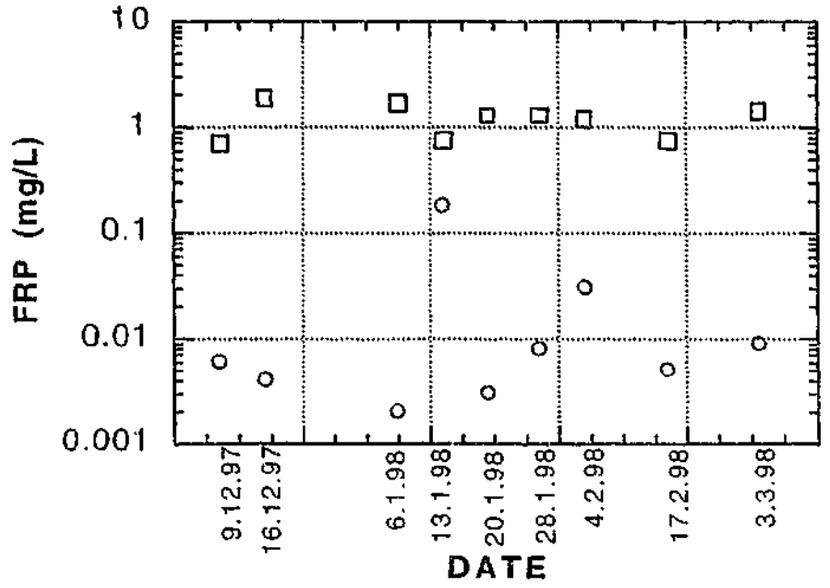
For each sampling event involving bacterial production measurements, a range of other parameters (bacteria and chlorophyll concentrations and physical and chemical factors) were also measured.

Nutrient analysis was performed to determine the concentration of nitrogen, soluble reactive phosphorus, nitrate/nitrite and ammonia in Campbells Creek upstream and downstream of the sewage discharge point at the same time that bacterial production experiments were performed. Table 3.4 and Figure 3.16 a-e clearly show the enormous differences in nutrient concentration at the two sites. The median concentration of total nitrogen at the downstream site D was 22-fold greater than upstream, the total phosphorus was 360-fold greater, the ammonia 300-fold greater, the nitrate/nitrite 216-fold, and the soluble reactive phosphorus 2,600 times higher.

Table 3.4 Nutrient concentrations (mg/L) at site A and D over the period 2/12/97 to 3/3/98

	Site A range	Median	Site D range	Median
FRP	0.002-0.046	0.006	9.3-16	16
NOx	0.002-0.18	0.006	0.71-1.9	1.3
NH3	<0.01-0.062	0.05	9-18	14
TP	0.023-0.12	0.05	9.3-21	18
TN	0.76-1.7	1	14-24	22

(a)



(b)

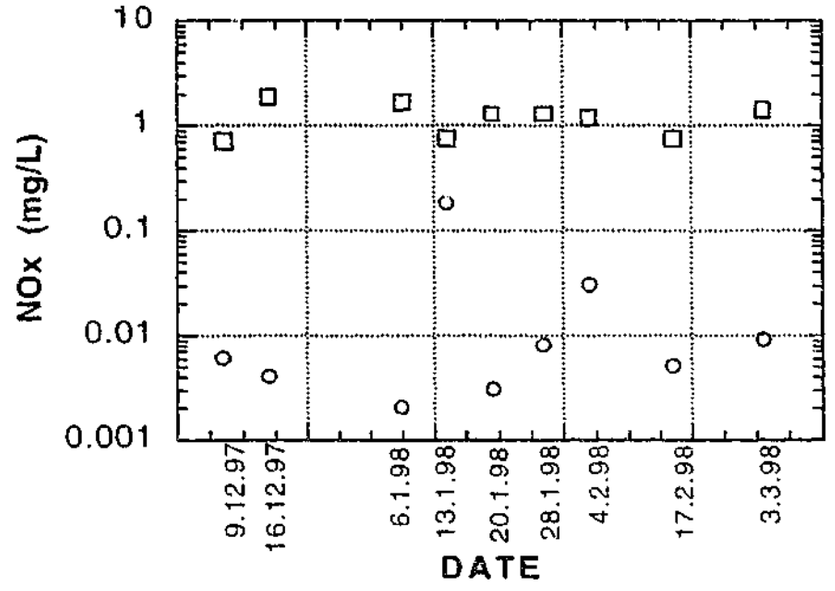
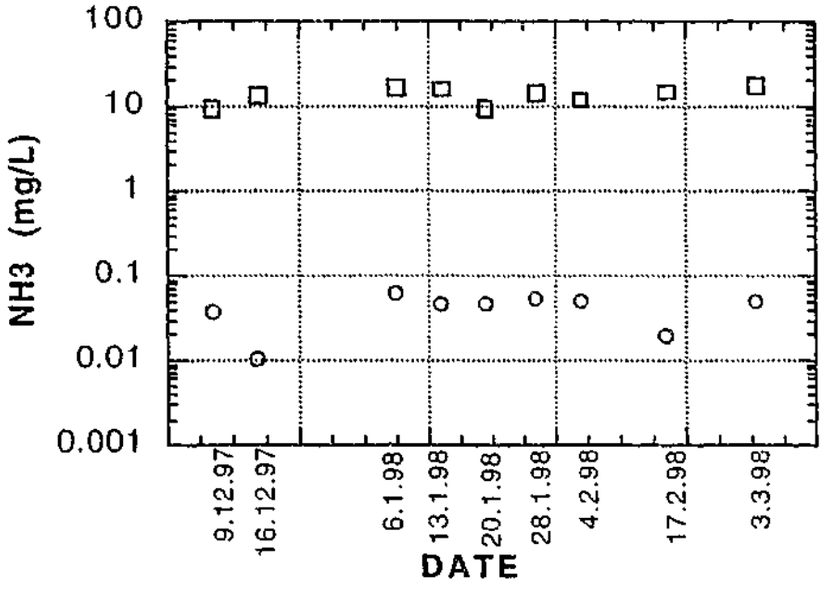


Figure 3.16a. Filterable reactive phosphorus concentrations (mg/L) in Campbells Creek at site A and D, from December 1997 to March 1998.

Figure 3.16b. NO_X concentrations (mg/L) in Campbells Creek at site A and D, from December 1997 to March 1998.

(c)



(d)

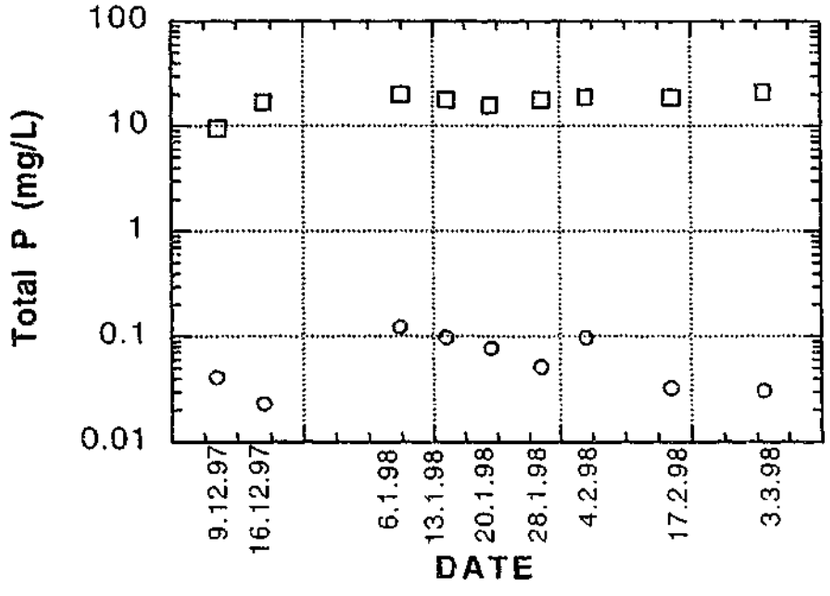


Figure 3.16c. NH_3 (mg/L) in Campbells Creek at site A and D, from December 1997 to March 1998.

Figure 3.16d. Total phosphorus (TP) (mg/L) in Campbells Creek at site A and D, from December 1997 to March 1998.

(e)

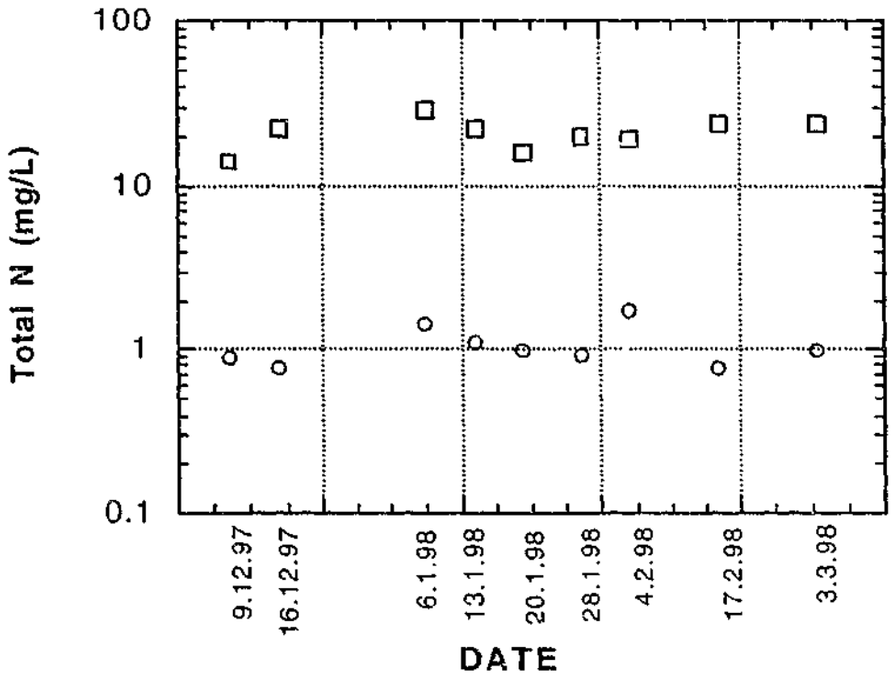


Figure 3.16e. Total Nitrogen (TN) (mg/L) in Campbells Creek at site A and D, from December 1997 to March 1998.

According to Pearsons correlation analysis, FRP concentrations were found to have a stronger positive influence (0.9) on bacterial numbers at site A than at site D (0.6), and no other positive correlations were observed between nutrient levels and cell concentration. On the other hand, a positive relationship was observed between Chlorophyll concentration and Total P (0.6), Total N (0.7), NH_3 (0.5), NO_X (0.5) and SRP (0.6) at site D but not site A (Table 3.9 & 3.10). During this period of sampling (summer 1997-98), the nutrient concentrations at site A were found to be negatively correlated with flow, which is the opposite finding to that discussed in Chapter 2 where positive correlations were observed. The most likely explanation for this is that the data analysed in Chapter 2 included high and low flow periods and therefore incorporated results from samples collected when run off with higher nutrient concentration than the creek contributed substantially to flow. In times of low flow, rainfall is obviously lower and hence run off is contributing little to the nutrient concentration. In these instances, flow is further reduced by evaporation resulting in an increase in nutrient concentration.

Physico-chemical (pH, conductivity, turbidity, dissolved oxygen, temperature and salinity) data were also collected for between-site comparison (Table 3.5). The most significant differences were observed with the turbidity readings; these ranged from 1 NTU to 30 NTU upstream and 20 NTU to 84 NTU downstream. The higher flow rates and chlorophyll concentrations observed downstream, are the likely causes for these higher turbidity readings. The conductivity range is higher at site A than at site D suggesting that the effluent must have a relatively low conductivity. The higher dissolved oxygen concentrations (and in particular the 15.9 mg/L concentration) observed at site D are most likely the result of greater oxygen production from higher algal numbers as indicated from the higher chlorophyll concentrations downstream (Table 3.8).

Table 3.5 Physico-chemical data ranges at site A and D from 2/12/97 to 3/3/98

n=10

	Site A	Median	Site D	Median
pH	7.0-7.7	7.3	7.5-8.1	7.7
Cond.(mS/cm)	1.37-2.59	1.7	0.066-1.99	1.6
Turbidity (NTU)	1-30	3.5	20-84	65
D.O. (mg/L)	2.4-8.8	5.4	5.1-15.9	7.9
Temp °C	17.9-24.7	22.5	17.7-24	20.4

Bacterial cell concentrations were an order of magnitude greater downstream of the sewage discharge site, with the exception of one sample collected on the 2/12/97 when the difference was only 3-fold (Table 3.6). As expected, on all sampling dates, the difference in cell concentration was significantly greater at site D, most probably due to the input of bacterial cells from the Castlemaine sewage effluent (Table 3.7). At site A the highest cell counts (5.1×10^9 cells/L) were observed on the 9/12/97 and the lowest (1.7×10^9 cells/L) on the 28/1/98. In contrast with upstream, the downstream D site showed a greater range of cell concentrations; 7.0×10^9 cells/L (2/12/97) to 78×10^9 cells/L (4/2/98).

Table 3.6 Mean bacterial cell concentration ($x10^9$ cells/L) and standard deviation of at least 3 replicate samples

Date	Site A mean	Std.Dev	Site D mean	Std. Dev.
2/12/97	2.5	0.04	7.0	1.6
9/12/97	5.2	0.1	23	2
16/12/97	2.9	0.3	27	5
6/1/98	2.1	0.2	34	2
13/1/98	2.9	0.2	70	1
20/1/98	1.9	0.1	28	1
28/1/98	1.7	0.1	35	1
4/2/98	1.9	0.1	78	2
17/2/98	2.0	0.2	42	3
3/3/98	1.80	0.01	40	3

Table 3.7 2-way ANOVA of cell concentration, chlorophyll concentration and bacterial production

Effects	df	Mean-square	F-ratio	P
Cells				
(10^9/L)				
Date	9	0.1	67	0.0000*
Site	1	19.6	10285	0.0000*
Date x site	9	0.2	100	0.0000*
residuals	40	0.0		
Chla				
(mg/L)				
Date	8	0.0	17	0.0000*
Site	1	0.4	4.9	0.0000*
Date x site	8	0.0	17	0.0000*
residuals	46	0.0		
Production(				
μgC/L/hr)				
Date	9	0.7	1.9	0.0658
Site	1	8.8	23	0.0000*
Date x site	9	2.4	6.4	0.0000*
residuals	55	0.4		

*Significant P<0.05

df= degrees of freedom

Significant differences were also observed in the chlorophyll concentrations between the upstream and downstream sites (Table 3.7). The highest mean chlorophyll concentration obtained downstream was 857 µg/L compared with 40.0 µg/L upstream (Table 3.8). The effluent discharge into Campbells Creek from the sewage treatment plant is the most likely source of these large chlorophyll concentrations observed at site D. Environmental conditions (light, nutrients, flow velocity) in the treatment plant retention ponds are ideal for supporting algal growth, and some of these organisms are most probably washed into Campbells Creek as part of the effluent discharge. Light may also be a growth limiting factor for algal cells at site A, as the site is heavily choked with weeds in the warm low flow months. Thick benthic algal mats were also present at site A and site D.

Correlation analysis was performed on the bacterial cell concentration, production and chorophyll a results obtained from Site A and Site D, but no relationship was observed between any of the three measurements (Table 3.9 & 3.10).

Table 3.8 Mean chlorophyll concentration (µg/L) and standard deviation of at least 3 replicate samples

Date	Site A mean	Std.dev	Site D mean	Std.Dev
9/12/97	7	1	13	10
16/12/97	14	7	850	281
6/1/98	2.1	1.1	860	80
13/1/98	6.1	0.8	580	60
20/1/98	1.1	0.2	330	70
28/1/98	0.7	0.3	280	90
4/2/98	0.9	0.3	300	50
17/2/98	15	3	760	80
3/3/98	40	44	300	40

Table 3.9 Site A, Pearson correlations for cell concentration, bacterial production, chlorophyll, flow and physio-chemical results

	Cell concentration	Bacterial Production	Chlorophyll concentration	Flow
Production	0.2			
Cell conc.		0.2		
Chlorophyll	0.2	-0.4		
Flow	-0.4	-0.4	0.3	
Ph	0.4	0.7	-0.5	-0.6
Conductivity	-0.5	-0.04	-0.4	-0.2
Turbidity	0.3	0.5	0.03	-0.4
DO	0.5	-0.3	0.2	-0.02
Temp	-0.3	0.5	-0.5	-0.5
FRP	0.9	0.1	0.1	-0.4
NO_X	0.2	0.3	0.05	-0.3
NH_3	-0.3	0.4	-0.7	-0.4
TP	-0.2	0.3	-0.6	-0.6
TN	-0.2	-0.02	-0.5	-0.5

Table 3.10 Site D, Pearson correlations for cell concentration, bacterial production, chlorophyll, flow and physio-chemical results

	Cell concentration	Bacterial Production	Chlorophyll concentration	Flow
Production	0.1			
Cell conc.	-0.2			
Chlorophyll	-0.2	-0.2		
Flow	-0.7	-0.04	-0.2	
Ph	0.3	-0.5	0.4	0.2
Conductivity	-0.3	-0.2	0.3	0.6
Turbidity	0.1	-0.1	0.6	0.2
D.O	0.6	-0.2	0.6	-0.2
Temp	-0.12	-0.01	-0.4	0.4
FRP	0.3	0.4	0.6	-0.1
NO_X	0.6	-0.02	0.5	-0.2
NH_3	0.2	0.5	0.5	0.01
TP		0.3	0.6	0.9
TN		0.3	0.7	0.8

3.3.8 Bacterial Production rates with ^{3}H-leucine

Bacterial production was also determined using radiolabelled leucine incorporation into proteins. These experiments were performed simultaneously with the ^{3}H-TDR time course experiments on four field trips. The leucine method was employed to evaluate its effectiveness at determining bacterial production in Campbells Creek and as a way of independently checking the thymidine method.

The leucine method results were generally less variable between replicates than those obtained with the thymidine method (Fig 3.17 for r^2 value). Background levels of ^{3}H-

leucine were also less of a problem in that radioactive counts for the blanks were always significantly less then the samples and did not hinder our ability to detect growth (Fig.17).

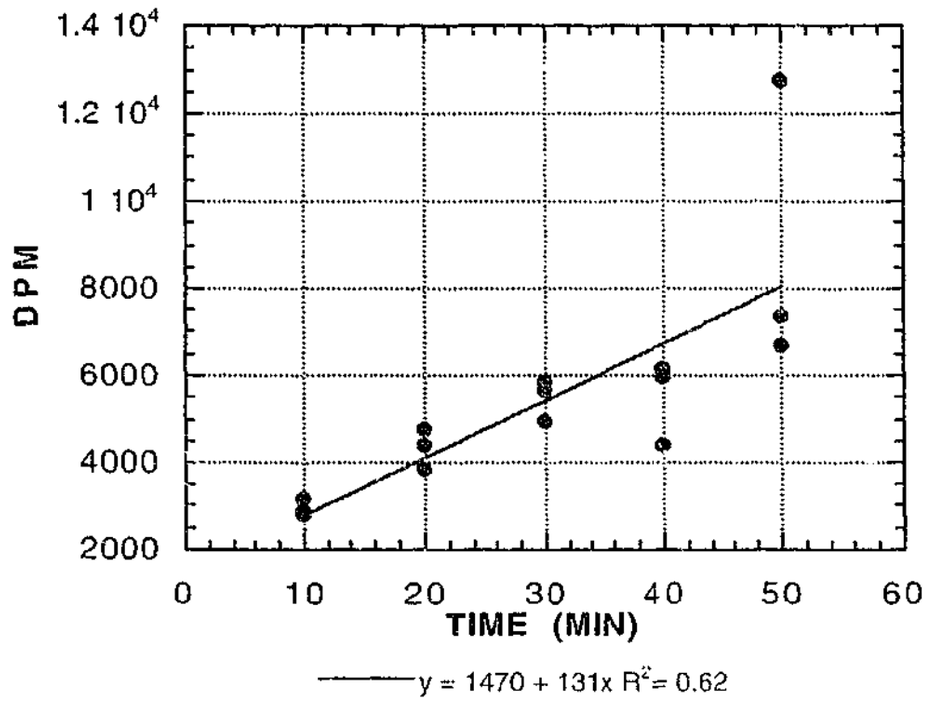


Figure 3.17 Time course of incorporation of radiolabelled leucine into protein

3.4 Discussion

3.4.1 Method development

A new SSC buffer method for processing samples to measure the amount of TDR incorporation into the DNA of growing bacteria has given significantly better results than the cold TCA method (Moriarty 1986). For two Australian inland waterways the variability amongst replicates using the cold TCA method has been successfully reduced using a gentle but effective washing regime. Background levels of unincorporated TDR were significantly reduced by including a 5 minute unlabelled thymidine incubation prior to formaldehyde fixation and filtrating with 5 ml of thymidine before sample filteration. Dramatic reduction of background counts by use of pre-soaked GFF filters also resulted in far greater discrimination of the low bacteria growth rates typically found in natural aquatic systems.

Two main problems were encountered with the cold TCA method. First, the hot oven incubation where TCA hydrolysis of DNA occurs does not completely extract the radiolabelled DNA from the filter and into solution. Secondly, the washing of unincorporated 3H-thymidine from the filter is inadequate. These problems have resulted in high variability between replicates and high background levels of ^{3}H compared with sample values making the detection of bacterial growth very difficult, particularly when productivity is low. It is also very important when using method described by Moriarty (1986) that the TCA and the filtration manifold is kept ice cold to ensure the DNA is precipitated onto the filter and not lost in the filtrate. This is very difficult to do in warm laboratories especially during the summer months and therefore we recommend that the processing should be performed in a cold room. The fact that we did not have access to a cold room may have contributed to our unsuccessful attempts at using this method. The advantage of using multiple washes with SSC is that it is not necessary to keep the manifold or the SSC very cold, it is less toxic then using acid solutions and it produces more reliable, less variable results.

3.4.2 Bacterial production and river 'health' assessment

Bacterial production in Campbells Creek at site A (3-35 µgC/L) was found to be of a similar magnitude to that observed in other freshwater systems (Table 3.11). In contrast to these production rates, bacterial production at site D (135-523 µgC/L) is significantly greater than at site A and many other freshwater systems during this period of sampling. This increase in bacterial production, compared with other aquatic systems, at site D would suggest that the sewage effluent is influencing production rates.

Table 3.11 A summary of bacterial production in freshwater river systems

Site	Bacterial Production µgC/L/hr	Reference
Campbells Creek site A	3-35	This study
Campbells Creek site D	135-523	This study
Hudson River	10	Findlay *et al.*, (1991)
Amazon River	1.16	Brenner *etal.*, (1995)
River Seine	30	Servais and Garnier 1993
Murray River	2-11	Beattie (personal comm.)*
Ovens River	16	Beattie (personal comm.)*
Yarra River	16-54	Grace (pesonal comm.)*
Goulburn River	4	Beattie (personal comm.)*

*Dr Michael Grace and Gillian Beattie, Monash University

Chlorophyll concentrations were also significantly greater at site D than site A. The concentrations observed at site A (1.1-40µg/L) during the summer of 1997/98 were similar to those found in other lowland rivers. For example, chlorophyll concentrations in the Goulburn River generally fluctuate around 3 µg/L, but can be as high as 20 µg/L and chlorophyll concentrations in the Ovens River are 2-9 µg/L and on some rare occasions as high as 1000 µg/L (Gillian Beattie personal communication. Monash University). The

chlorophyll concentrations at site D (13-860 µg/L) are somewhat higher than those observed in the Goulburn and Ovens Rivers, most likely from the higher nutrient concentrations and algae entering Campbells Creek from the sewage treatment plant. Obviously, these results are applicable to only this period of study (summer 1997/98) and such high concentrations of chlorophyll at site D will not always be the case.

It was expected that there would be some evidence of a relationship between bacterial production and cell numbers in Campbells Creek. According to the correlation analysis this was not the case at either site A or Site D on the dates examined (Table 3.9 & 3.10). In contrast to the large fluctuations in productivity observed at site A and D, relatively little variation was observed in cell concentration during this period of sampling. This would suggest (as expected) that not all bacterial cells are actively growing and that there are factors strongly influencing the activity of the bacterial population resulting in large variations in bacterial secondary production.

The correlation analysis also showed no relationship between water column chlorophyll concentration and bacterial production. This would suggest that the bacterial population is supported by either allochthonous sources of carbon or epilithic and epiphytic algae, and not phytoplankton primary production like in many autotrophic systems. The high nutrient levels and low turbidity readings observed in Campbells Creek are conditions conducive to the production of benthic algae. Epilithic algae were clearly evident at sites A and D, with thick algal mats covering most creek rocks. The lack of correlation between chlorophyll concentration and secondary bacterial production at site D is not surprising given the large amount of carbon present in sewage effluent. It is however, possible that bacterial production is not limited by carbon supply, but rather by some other factor, such as a toxicant or micronutrient (this possibility is discussed in more detail further in the discussion). No significant relationship was evident either, between chlorophyll and bacterial cell numbers. The lack of such a relationship has also been reported by Findlay *et al* . (1991) who proposed that the factors controlling algal abundance were not having the same influence on bacterial abundance.

The development of this modified thymidine method enabled bacterial production to be evaluated as a specific indication of how the microbial communities were responding to a pollution source, and a more general indicator of river 'health'. The results from this study have shown that in periods of low flow, bacterial production was significantly greater at site D than at site A, and considerably greater than observed in other freshwater environments. This would suggest that bacterial secondary production in Campbells Creek at site D is strongly affected by sewage effluent when released into low flow situations. The main aim of this project was to investigate various microbiological techniques as tools in microbial-based river health assessment. For this reason, long-term studies of bacterial production and the relationship with other factors (primary production, sources of organic carbon, cell numbers, chlorophyll concentration or nutrients levels) were not examined. During the time frame investigated the environment in Campbells Creek was one of low flow and warm temperatures. It is therefore, possible to only draw conclusions regarding bacterial production for these particular conditions and points in time.

Statistical analysis of the results showed that bacterial production at site A and D was not related to cell abundance, chlorophyll concentration or nutrient levels. Fluctuations in bacterial production at site D were far more pronounced then at site A and the results suggest that these fluctuations are unlikely to be nutrient related. It is a possibility that bacterial production may have been influenced by other micronutrients or from a combination of increases in effluent release and a decrease in the effectiveness of chlorine treatment. An increase in wastewater discharge or ineffective chlorination could result in the greater input of viable and active 'foreign' bacteria, which would result in higher bacterial production rates. An increase in viable or active bacteria would not be evident from the cell count data as it is not possible with this technique to distinguish between inactive and active cells. Bacterial production therefore, will not correlate with total cell counts, but most probably relates to the percentage of active cells, an observation also made by Giorgio and Bird (1997) (refer to Chapter 2 regarding the difficulties in determining active cell numbers). Bacterial production measurements can, therefore, reveal environmental changes that are undetectable from total cell counts. It has been observed in other freshwater systems that perturbations,

such as floods, can cause peaks in secondary production. Flood events cause an increase in the input of terrestrial bacteria and sources of organic carbon. The fluctuations in waste water release may be having a similar effect on production rates as these flood pulses.

The leucine method for measuring bacterial production did not cause the same problems in regards to high background levels of radioactivity and large variations between replicates as those observed with the tritiated thymidine method. The question may therefore be asked, why most of the bacterial production experiments were performed using the tritiated thymidine method when fewer problems were encountered with the leucine method. There are two main reasons for this: first, tritiated thymidine has become the more widely used method for determining bacterial production and thus it became an objective to develop a protocol suitable for natural aquatic systems with characteristics similar to Campbells Creek. The extensive method development undertaken took up much of the project time. Secondly, the main aim of the project was to evaluate various microbial techniques as tools in river 'health' assessment, and not to seek answers to broader ecological questions.

As discussed in section 3.1.2, there are advantages of using the leucine method of measuring bacterial production over the thymidine method. This study also found that fewer methodology problems (high background radioactivity, variation between replicates) were encountered with leucine incorporation. Possibly, the lower background levels of radiation observed with the leucine method could be caused by a lower affinity of leucine for particulate matter than thymidine. It is therefore recommended, that if researchers intend to use bacterial production measurements in river 'health' assessment studies, that they consider radioactive leucine incorporation into proteins and not simply choose the tritiated thymidine method because it is the more traditional and widely accepted method.

Ideally, if funding and time permits it is suggested that both methods are used to measure bacterial production as the two methods measure different aspects of bacterial metabolism. Thymidine is only incorporated into the DNA of new cells and therefore, is a more direct measure of cell division. In contrast, leucine is incorporated into proteins and is a measure of metabolic activity. When these two methods are used together a more reliable estimate of bacterial production is obtained. Each method can give a different interpretation of ecosystem 'health', useful information when trying to interpret environmental impacts or ecosystem processes

3.5 Conclusion

Bacterial production is an important parameter and provides the researcher with an insight into the energy fluxes and dynamics of a particular aquatic system. Other investigations in freshwater systems have found that bacterial production is relatively stable over short time scales with some seasonal variation and is generally significantly related to primary production, chlorophyll a, or bacterial numbers. The results from Campbells Creek do not agree with these findings. The significant increase in bacterial production downstream of the sewage treatment plant, implies that the single pollution point source is affecting the microbial production and thus the flow of carbon through the aquatic food web. Bacterial production measurements could therefore be an important component of studies involving river 'health' assessment, particularly for between-site comparisons.

Environmental microbiology and the potential use of microbial populations as indicators of river 'health' is still a very new area. It is not yet possible to set guideline values for bacterial production rates for 'healthy' systems, especially for the relatively unstudied Australian rivers. High bacterial production rates can have a detrimental effect on the aquatic ecology by decreasing the concentration of dissolved oxygen. Sensitive fish and invertebrate populations may be lost as a result of reduced oxygen availability. However, extensive research is required before specific bacterial production rates could be recommended in river 'health' protection guidelines.

If guidelines are to be produced, they must be developed from research undertaken on a wide variety of Australian river systems. For example, production rates for freshwater upland streams are probably not applicable to lowland rivers. Research would need to be conducted across the country and incorporate regulated, polluted lowland rivers and streams, relatively unpolluted lowland rivers, polluted

and pristine highland steams. However, bacterial production measurements do provide useful information concerning energy sources for higher organisms, and this study has

shown such measures can be successfully employed to quantify the affect a sewage discharge can have on the downstream aquatic ecology.

CHAPTER 4
MEASUREMENTS OF BACTERIAL BIOMASS BY SEDIMENTATION FIELD-FLOW FRACTIONATION

4.1 Introduction

The enumeration of bacteria, and the quantification of bacterial production and biomass are fundamental parameters which need to be measured in almost any study of microbial populations. There are now well developed methods for the first two of these; for example, measurement of bacterial cell concentrations using epifluorescence microscopy and bacterial production using the method discussed in the previous chapter. However, the methods currently employed to estimate bacterial biomass are less straight forward and imprecise (Sharma *et al.*, 1993).

This chapter reports the development of a new technique called Sedimentation Field Flow Fractionation (SdFFF) as an alternative to traditional methods for determining bacterial biomass in aquatic environments. We also report a new method combining SdFFF with the method for measuring bacterial production (Chapter 3) to provide production rates in terms of biomass.

The aim of the work recounted in this chapter was to utilise SdFFF to determine bacterial biomass in a freshwater lowland creek, upstream and downstream of a single point source of sewage pollution and to evaluate the potential of SdFFF determination of bacterial biomass as a tool in the assessment of river health.

4.1.1 Bacterial biomass determination using SdFFF

The most common conventional method for biomass determination is the estimation of cell biovolume from direct microscopy measurements. Biomass is then calculated by applying biovolume to carbon conversion factors (Norland *et al.*, 1987; Kroer 1994). This method has many sources of error which can result in an overall error of up to 600% (Sharma *et al.*, 1993).

In recent years, Sharma and colleagues (Sharma *et al.*, 1993) have applied a technique called Sedimentation Field Flow Fractionation (SdFFF) to determine bacterial biomass in samples taken from a diverse range of natural habitats (Sharma *et al.*, 1998). SdFFF is a subtechnique of the Field Flow Fractionation (FFF) separation method which is a class of technique where selective migration occurs perpendicular to the direction of fluid flow and it has been applied to a number of different macromolecules and collodial materials (Beckett and Hart 1993). Like chromatography, sample elution occurs which allows for the collection of fractions during the run procedure (Giddings 1984).

SdFFF is both a sizing and separation method for colloidal samples in the size range 0.05-50 μm. The collecting of fractions during the separation of a sample permits further analysis, for example the characterisation of particles with electron and epifluorescent microscopy or quasi-elastic light scattering. SdFFF uses centrifugal force to separate particles on the basis of their buoyant mass. The separation occurs in thin open channels (generally with dimensions of 90 x 2 x 0.025 cm) which has a continuos flow of carrier fluid under centrifugal force. The carrier liquid passes through rotating seals that transfer the eluted samples from the channel to the detector.

The SdFFF technique separates particles on the basis of particle mass alone. Therefore, with the necessary experimental alterations, particle mass, size or density can be measured.

To measure bacterial biomass within a water sample using SdFFF, the bacterial cells are first concentrated and stained with a fluorescent dye, 4'6-diamidino-2-phenylindole (DAPI). The sample is then injected into the thin, flat chromatography-like channel of the SdFFF instrument which is spun to produce a centrifugal field. The resulting centripetal force pushes the bacterial cells and other particles to the outer wall of the channel. After a predetermined "relaxation time", and when the particles have formed an equilibrium cloud (due to Brownian motion), the carrier flow is started. The parabolic profile of the carrier fluid results in both particles and microbial cells with a small buoyant mass migrating down the channel and eluting at a faster rate than those with a larger biomass. As the cells leave the channel, the cell fluorescence is measured and recorded as a fractogram. The cells are

then collected into fractions at desired elution times for counting. The elution time or volume is used to calculate the biomass per cell and total biomass can be determined by measuring the cell concentration in each fraction (see Beckett *et al.*, 1988, 1990, and Sharma *et al.*, 1993, for derivation of relevant equations).

To calculate biomass from the SdFFF fractogram, literature values for the average density of cell dry matter are used.

The basic retention equation for constant field normal mode FFF separation is:

$$R = V^o/V_r = 6\lambda[\coth(1/2\lambda) - 2\lambda]$$

where R is the retention ratio, V^o the channel void volume, V_r the sample elution volume, λ is the dimensionless cloud thickness (l/W) (l being the average cloud thickness and W the channel thickness), and coth is a standard abbreviation for the hyperbolic cotangent. At any given point of a SdFFF run, the retention parameter λ is dependent on the bouyant mass of the sample particles, (Equation 4.1).

$$\lambda = \kappa T/ (Gw\ \Delta m_p) \qquad (4.1)$$

where:

w = width of the channel, κ = Boltzman constant, T = absolute temperature, Δm_p = effective particle mass, and G = centrifugal field strength (acceleration).

The water content of the cell is not dependent on λ, as long as the density of the cellular water is not altered from its normal bulk value. Therefore, in equation 4.1, the effective mass (Δm_p) is equivalent to the cellular buoyant mass (Δm_{om}).

$$\Delta m_p = \Delta m_{om} \qquad (4.2)$$

The dry biomass per cell is calculated using an estimation of the average density of cellular organic matter, which translates as the density difference between the organic matter and the carrier fluid ($\Delta\rho_{om}$), so

$$m_{om} = \Delta m_{om}(\rho_{om}/\Delta\rho_{om}) \qquad (4.3)$$

By substituting equations 4.2 and 4.3 into equation 4.1 we obtain the following;

$$m_{om} = \kappa T \rho_{om}/(\omega^2 r\, w\, \Delta\rho_{om}\lambda) \qquad (4.4)$$

Following Sharma (1994), we used literature values of the average content and density of cellular constituents to estimate the mean density of organic matter or the ρ_{om} value (1.6 g/mL) for all calculations in this thesis.

The SdFFF fractograms can be transformed to a biomass distribution curve with the total area under this curve giving the biomass concentration of the sample (Sharma *et al.*, 1998). The biomass per cell and total biomass of the sample is determined using the cell concentration values for each collected fraction. These independently obtained cell counts (epifluorescence microscopy) are then multiplied by the average organic matter per cell in the increments of organic matter along the x-axis. The y-axis of the histogram is then produced by dividing these values by the organic matter per cell values for that bar or column (Sharma *et al.*, 1998). Therefore, it is possible to estimate the dry weight biomass for a particular organic matter per cell range by calculating the area under that portion of the histogram. Relative biomass is calculated as follows;

$$\text{Rel. Biomass} = [(\text{Cells/L})\ (\text{Total volume of fraction})\ (\text{Ave.}m_{om}/\text{cell})]/\Delta m_{om}$$

The total area under the histogram represents the biomass of the injected sample.

The SdFFF method for biomass determination has many advantages over the conventional biovolume to biomass ratio methods. It eliminates the errors involved in microscopically measuring cell dimensions and the use of conversion factors which can vary up to 11-fold (Norland *et al.*, 1987). The microbial communities in natural aquatic environments are very complex and the appropriate volume:mass ratio will vary for different cell populations. It has been found that bacterial biovolume to carbon conversion factors vary both temporally and spatially with the carbon content per unit volume increasing with decreasing size (Simon and Azam 1989, Kroer 1994). This problem can be overcome with SdFFF because the buoyant mass is measured directly and is unrelated to the water content of the cell (Sharma *et al.*, 1993). SdFFF relies on only one piece of independent information from the literature, the density of cell organic matter, which at most could

introduce a 6-fold source or error (Sharma *et al.*, 1993). SdFFF is also far less tedious and time consuming then conventional biomass determination methods.

4.1.2 *Combining SdFFF biomass determination with bacterial production measurements*

Bacterial biomass and production measurements are crucial to the evaluation of the bacterial populations potential activity in a particular environment, and its importance as a food source for higher organisms in the food web. The combination of bacterial production measurements with SdFFF should allow the determination of bacterial production in terms of biomass. If successful, this new method would provide useful information and save researchers valuable time. For example, it should be possible to determine the amount of carbon produced per unit time and volume by a known quantity of biomass. Also, the new procedure could potentially determine differences in production between cells of different organic masses. SdFFF separates cells on the basis of buoyant mass which can be calculated to organic matter per cell. If the cell fractions of a particular buoyant mass (or organic matter per cell) are collected, and the cell concentration of the fraction determined (by direct cell counts), the biomass of the fraction can be calculated. Furthermore, if the cells were incubated with ^{3}H-thymidine prior to SdFFF separation, then the production for a particular fraction can also be determined.

Bacterial growth in natural habitats is rarely balanced, and for this reason it is beneficial when performing production determination experiments to use ^{3}H-TDR and ^{3}H-leu incorporation techniques. Both radiolabelled leucine and thymidine incorporation were therefore combined with SdFFF. Results from earlier SdFFF runs (section 4.3.2) have shown that algal cells with their larger buoyant mass, elute later than bacterial cells. This separation is easily confirmed by microscopic examination of the separated fractions. The lack of radioactive counts in these algal fractions, would be further evidence that algal cells do not incorporate significant levels of ^{3}H-leucine in pulse labelling reactions.

4.2 Materials and Methods

4.2.1 Sampling

Between January 1996 and December 1996, water samples were collected from 4 sites along Campbells Creek near Castlemaine, in southeast Australia. Campbells Creek is a small lowland stream with a single point source of sewage effluent from the Castlemaine sewage treatment plant. The first sampling site (Site A) was approximately 1 km upstream of the sewage treatment plant, Site B was situated about 50 metres upstream of the discharge pipe, Site C, 50 metres downstream of the effluent release point and site D a further 1 km downstream (see Fig. 2.3 for site map). One litre samples were collected in polyethylene bottles and fixed with formaldehyde (final concentration of 1-3% vol/vol). Samples from downstream D (site D) were only collected for the January, April, and December 1996 field trips. Samples (125 mL) were also collected and preserved (with formaldehyde) to perform direct total cell counts. Samples were kept on ice on their way back to the laboratory where they were stored at 4°C until analysis took place (< 1 month).

4.2.2 Determination of bacterial cell concentrations

Direct cell counts were performed on raw samples (3x125 mL replicates from each site) and on fractions collected from the SdFFF analysis. Samples were stained with DAPI and filtered onto irgalan-black stained filters. UV-excitation epifluorescent microscopy was used to count cells (10-30 random fields per slide) at a magnification of x1250 with an Olympus BH-2 microscope. Cell concentrations of raw samples are presented in Table 4.1.

4.2.3 SdFFF separation

Prior to SdFFF analysis, samples were prepared in the following way. Tetrasodium pyrophosphate (SPP, $Na_4P_2O_7.10H_2O$) was added to give a final concentration of 0.001 M and left overnight. SPP helps to break up bacterial aggregates and detach cells from particulates. After this the samples were sonicated (Cole Parmer 4710 Ultrasonic homogenizer) and then filtered (Whatman GF/D glass fibre filter 2.7 μm) to remove the

larger particulate matter which can interfere with SdFFF and direct cell counting. The low concentration of aquatic bacteria made it necessary to concentrate the filtrate (Table 4.2). This was achieved by centrifugation of samples at 20,000 g for 30 minutes and resuspending the resulting pellet in 1 mL of SPP (0.01 M) solution.

A detailed description of the SdFFF system used in this study is given by Beckett *et al.* (1988, 1990) and Sharma *et al.* (1993, 1998). The basic SdFFF system (FFFractionation Inc., Salt lake City, Utah) used in this research had a channel radius of 15.1 cm which was wrapped inside a centrifuge basket. The channel dimensions were as follows: tip to tip length (L) = 89.1 cm; thickness (w) = 0.0289 cm; breath (b) = 2.0 cm; Channel void volume (V^o) = 4.8 mL (estimated from the elution volume of an unstained species, sodium benzoate).

The speed of centrifugation was regulated with computer programs (FFF fractionation Inc) run on a Epsom PC and microcomputer.

Twenty four hours prior to SdFFF analysis the cell suspensions (pellet and SPP) were stained with DAPI (Sigma Chemical) and stored at 4°C. Before the SdFFF runs took place the stained samples were sonicated for 5 to 10 seconds to produce an aggregate-free cell suspension. Then 70-200 µL of sample was loaded into the channel, and the sample left to relax for 20 minutes at 1200 rpm before the carrier flow (0.0001 M SPP in cell free water with 0.2% formaldehyde) was started. The carrier fluid was transported from a 2 L side arm flask with a Gilston Minipills 2 peristaltic pump and DAPI stained cells were detected leaving the channel with a Waters 470 scanning fluorescence detector (excitation maximum, 350 nm; emission maximum, 460 nm; and band width, 18 nm). The detector response was recorded with a Hewlett-Packard 3394A integrator or an Omniscribe chart recorder (Houston Instruments).

Natural bacterial populations are heterogenous and contain a large range of cell sizes. All SdFFF runs were therefore performed using a field decay program (Sharma *et al.*, 1998). Run times were approximately 100 minutes (+ 20 minutes relaxation) per sample. Fractions of 16 mL were collected (ISCO Retriever 500) for direct cell counts with epifluorescent microscopy.

4.2.4 SdFFF combined with ^{3}H-Thymidine incorporation

Water sample volumes of 80 mL were collected from site A and site D for use in experiments where bacterial biomass and production determination was combined. ^{3}H-TDR (2 mL) with a specific activity of 6.7 Ci/mmol (ICN) was added to one bottle from each site (final concentration 50 nM) and incubated for 10 minutes. The ^{3}H-TDR incorporation was stopped with the addition of 10 mL of 5% Thymidine. The samples were left to stand for 5 min to allow the exchange of ^{3}H-TDR non-specifically bound to particles with the unlabelled thymidine. Formaldehyde (final concentration of 1-3%) was used to fix the samples and a second 80 mL sample from each site was also fixed as a control. Samples were kept at 4°C until required.

Prior to the SdFFF runs, 800 µL of 0.1 M SPP was added to the radioactive samples and controls and incubated overnight at 4°C. The following day the samples were sonicated for 2 hours in a sonicating bath and subsequently filtered through 2.7 µm GFD filters (Whatman). The filtrate was concentrated by centrifugation (25,000 x g, 30 min) and the resulting pellet resuspended in 1 mL of the supernatant for site D samples and 0.5 mL for site A. The concentrate (400 µL) was removed to a clean eppendorf tube, to which 50 µL of DAPI was added and the solution left to stain overnight at 4°C. The remaining concentrate was stored at 4°C and used in SdFFF runs with no DAPI staining or stained and used in subsequent runs. SdFFF runs were performed on radioactive and non-radioactive samples with and without DAPI staining.

The SdFFF run conditions and procedures were the same as previous runs (see section 4.2.3) with Campbells Creek samples except that radioactive samples could not be sonicated with the sonicating probe. For these samples, the eppendorf tube containing the stained concentrated cells was placed in a 50 mL beaker with a small amount of tap water and the surrounding water was sonicated for 5 minutes. After fractionation in SdFFF, fractions were collected every 5 min (10 mL volumes) for each run. Volumes of 2 mL were removed from each fraction and placed in a scintillation vial with 4 mL of scintillant (ICN) and counted on a Beckman scintillation counter. Direct cell counts were also performed on some fractions using the same procedure described earlier.

^{3}H-Leucine uptake and SdFFF was also combined in one experiment. A sample of 80 mL was collected and incubated for 40 minutes with tritiated leucine. The ^{3}H-leucine solution was made up as a 50 nM concentrate with a ratio of 1 labelled leucine to 9 unlabelled leucine. Of this radioactive leucine solution ,16 mL was added to the 80 mL sample. The reaction was stopped and fixed with formaldehyde (1-3% final concentration). The same protocol as ^{3}H-TDR was then followed.

4.3 Results

4.3.1 Bacterial cell concentrations

Direct counts were performed on samples taken from Campbells Creek both upstream and downstream of the Castlemaine sewage treatment plant. The cell concentrations of upstream samples collected for SdFFF analysis ranged from $1.6x10^{9}$ to $5.5x10^{9}$ cells/L (Table 4.1). The bacterial cell concentrations at site C, downstream of sewage discharge were higher and showed a greater variation in cell numbers ($1.9x10^{9}$ cells/L in July 1996 to 60 x 10^{9} cells/L in January 1996) than for upstream. For the 3 field trips when samples where collected from site D, the cell concentration was found to be higher than the upstream site but less than at site C (Table 4.1).

Table 4.1 Mean* bacterial cell concentration ($x10^9$ cells/L) and standard deviation at the time of SdFFF runs performed

Date (1996)	Site A	Site C	Site D
January	3.1 (0.2)	59 (5.2)	19 (4.2)
March	2.6 (0.05)	24 (0.1)	ND
April	3.6 (0.2)	15 (0.8)	4.6 (0.1)
July	1.9 (0.1)	3.6 (0.2)	2.1 (0.2)
November	3.1 (0.1)	11 (1.1)	ND
December	3.1 (0.1)	14 (0.6)	9.3 (0.5)

* Average of 3 separate samples

ND-Not determined

4.3.2 SdFFF analysis

The concentration of bacterial cells found in natural environments are usually well below the detection limit of SdFFF, and because of this the samples must first be concentrated. In Campbells Creek, however, the original cell concentrations were higher than for most freshwater environments and samples only required concentrating by a factor of 100 to 160.

The fractograms produced from the two upstream sites were almost identical (Fig 4.1) and, because of the time and resources required to perform a run and count the bacterial numbers in each fraction, it was decided to collect samples from only one upstream site. Samples taken upstream and downstream of the sewage outlet resulted in quite different SdFFF fractograms (Fig. 4.2-4.16), with upstream samples having two distinct peaks, and downstream samples having an additional third peak (60-80 min elution time).

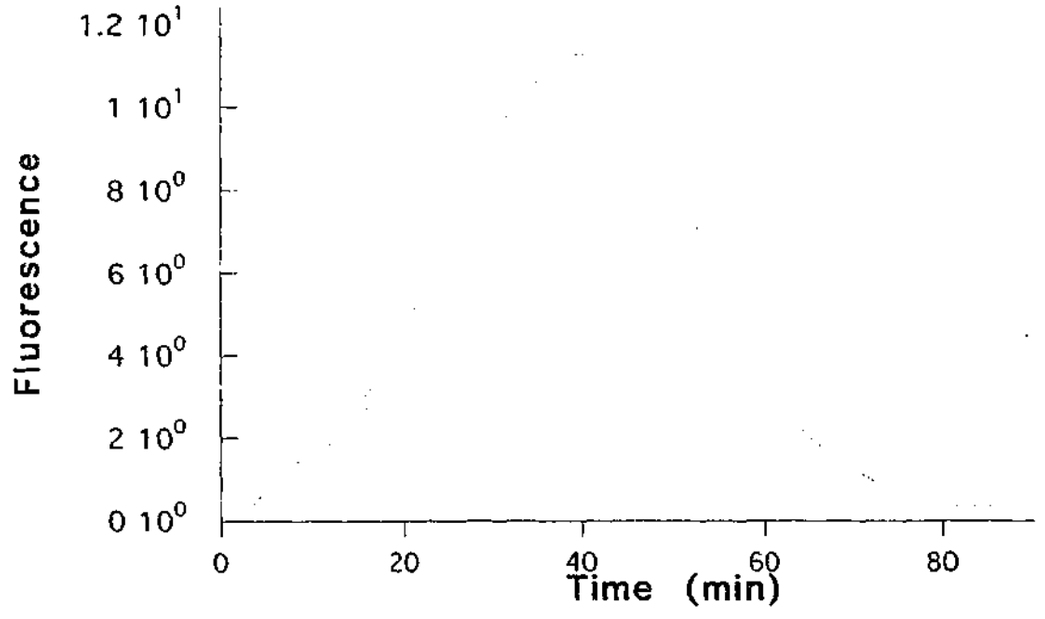
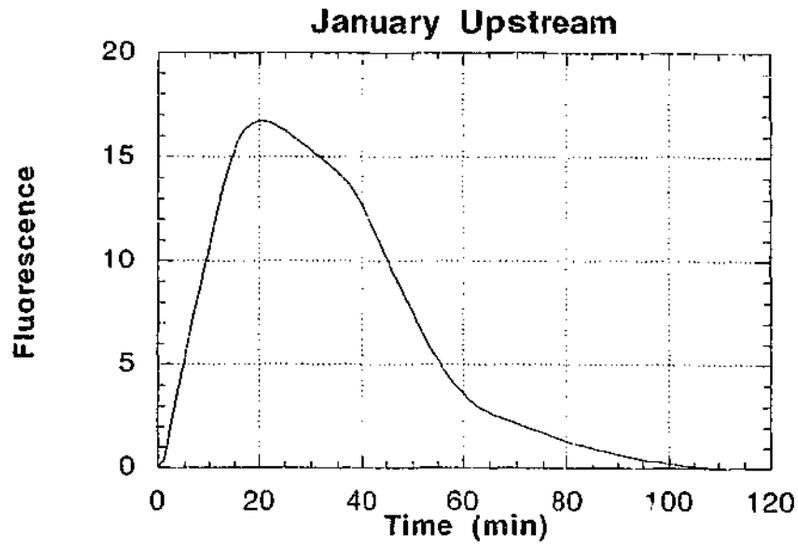


Figure 4.1 Comparison of the fractograms produced from the SdFFF separation of samples from the two upstream sites.

(a)



(b)

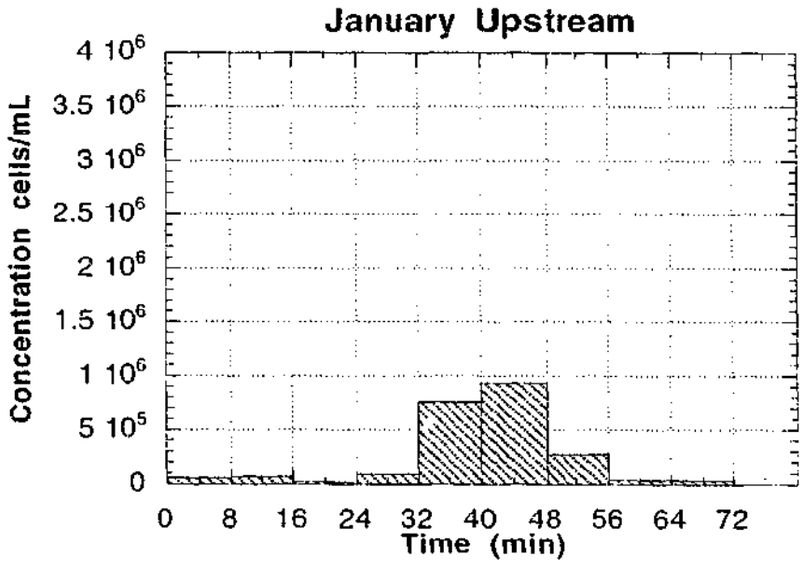


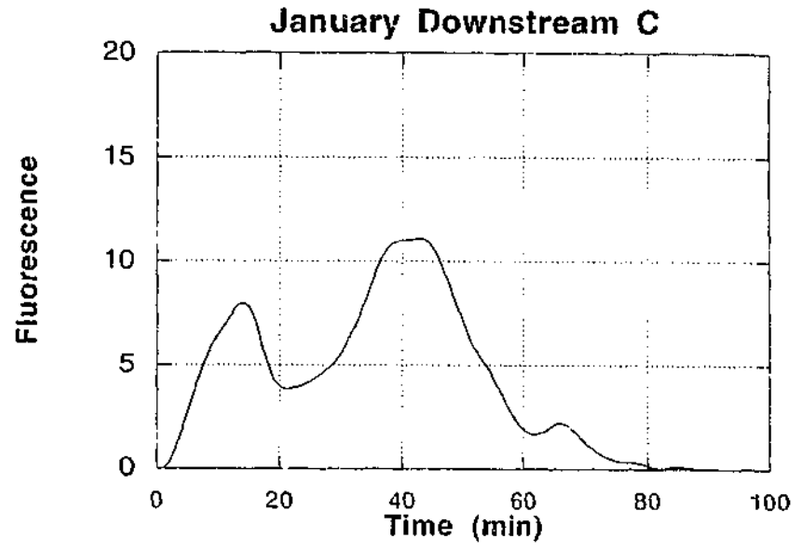
Figure 4.2

(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Upstream in January 1996.

(b) Plot of cell concentration for individual SdFFF fractions. Upstream January sample.

For both graphs, results have been normalised to the same concentration factor and injection volume.

(a)



(b)

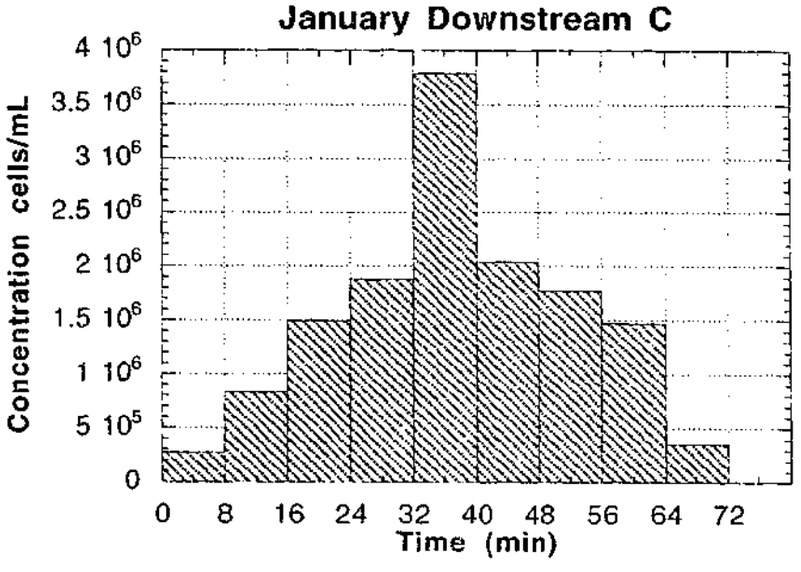


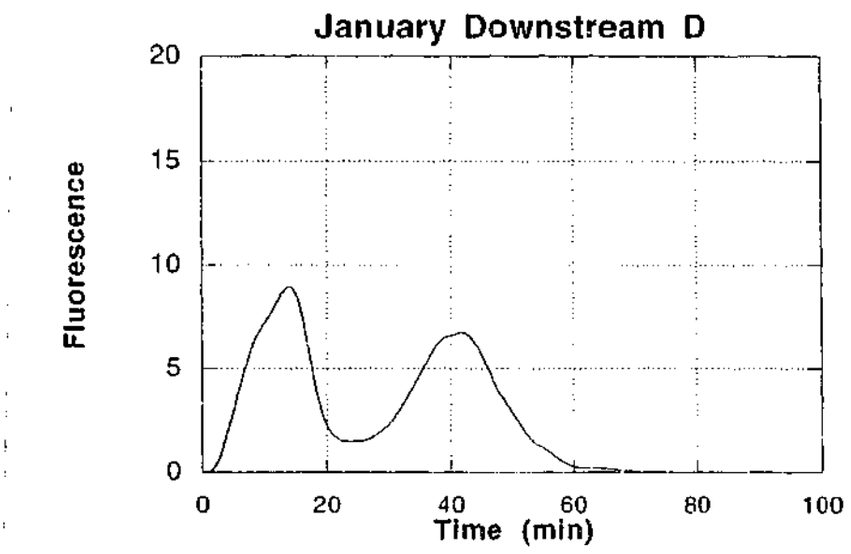
Figure 4.3

(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Downstream site C in January 1996.

(b) Plot of cell concentration for individual SdFFF fractions. Site C January sample.

For both graphs, results have been normalised to the same concentration factor and injection volume.

(a)



(b)

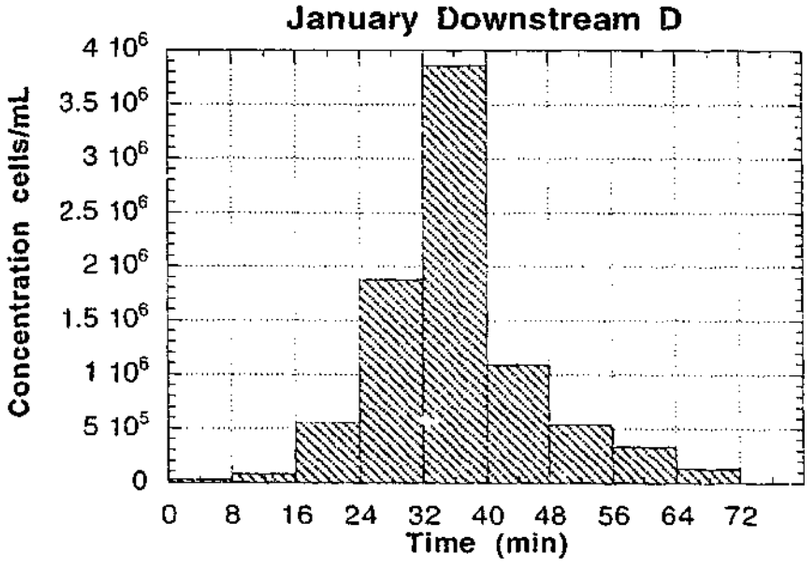


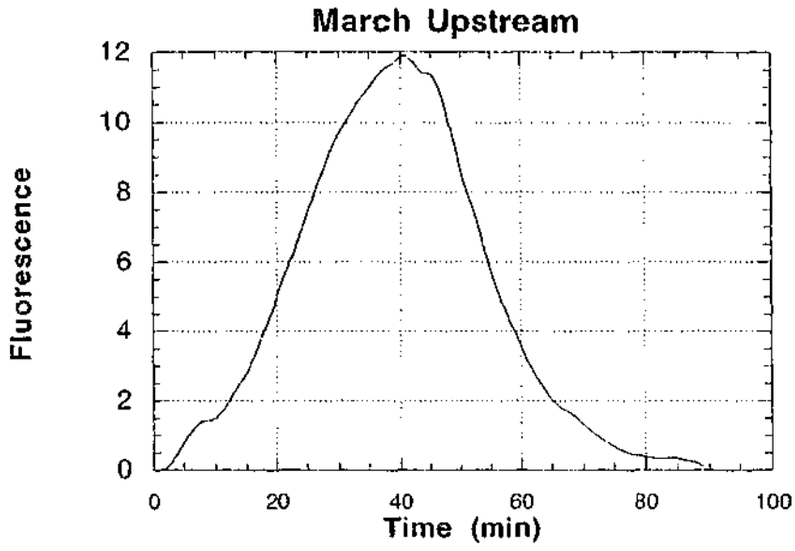
Figure 4.4

(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Downstream site D in January 1996.

(b) Plot of cell concentration for individual SdFFF fractions. Site D January sample.

For both graphs, results have been normalised to the same concentration factor and injection volume.

(a)



(b)

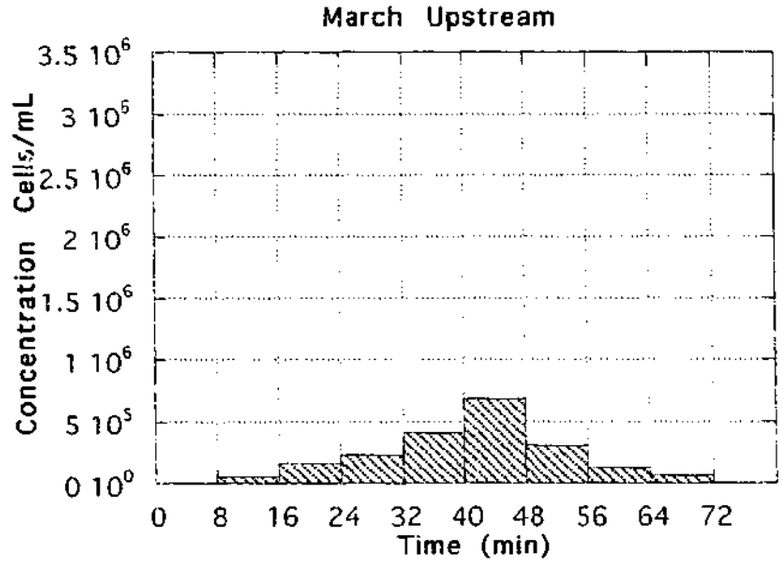
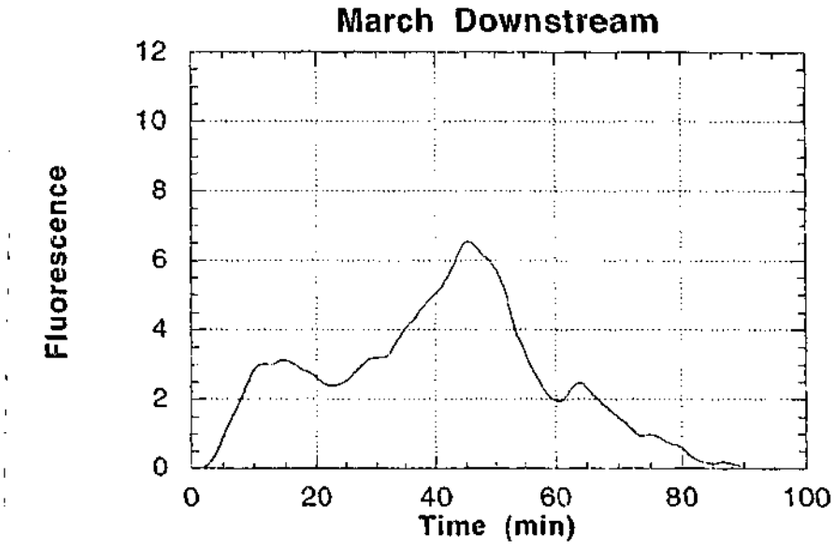


Figure 4.5

(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Upstream in March 1996.

(b) Plot of cell concentration for individual SdFFF fractions. Upstream March sample. For both graphs, results have been normalised to the same concentration factor and injection volume.

(a)



(b)

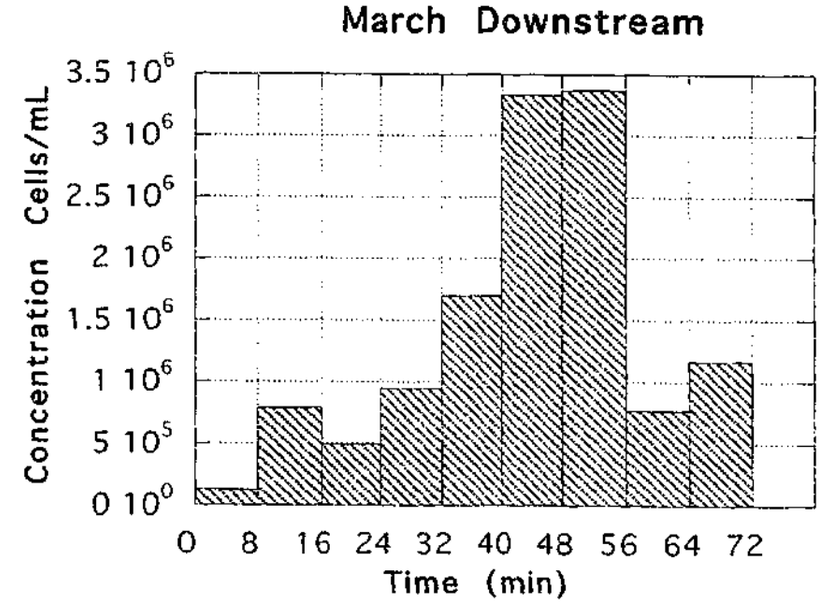
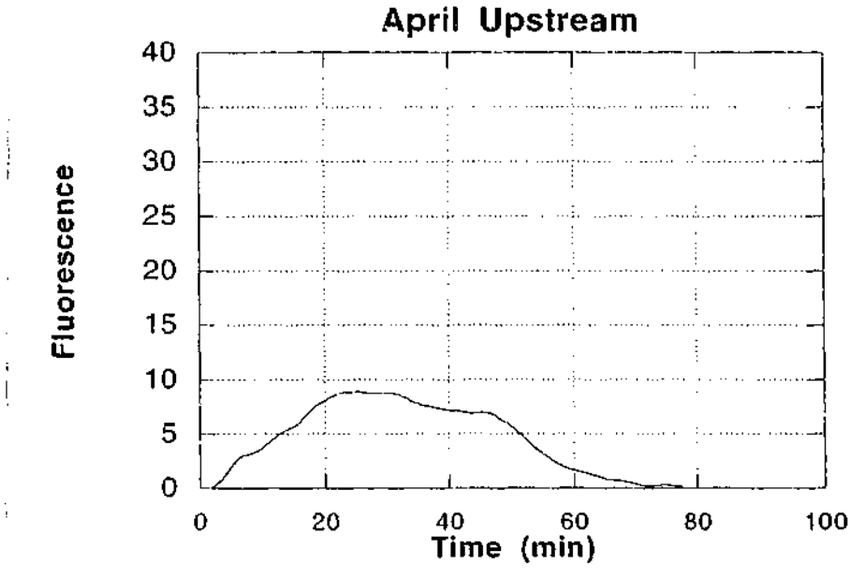


Figure 4.6

(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Downstream in March 1996.

(b) Plot of cell concentration for individual SdFFF fractions. Downstream March sample. For both graphs, results have been normalised to the same concentration factor and injection volume.

(a)



(b)

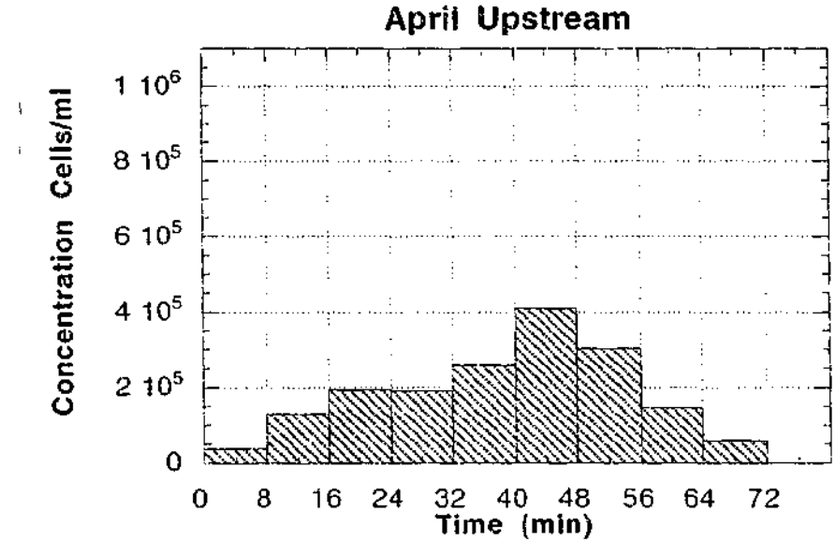


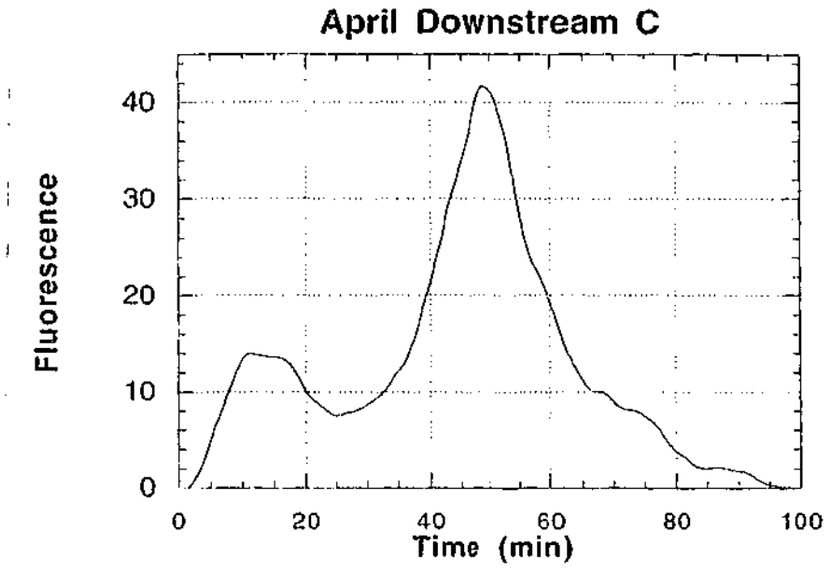
Figure 4.7

(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Upstream in April 1996.

(b) Plot of cell concentration for individual SdFFF fractions. Upstream April sample.

For both graphs, results have been normalised to the same concentration factor and injection volume.

(a)



(b)

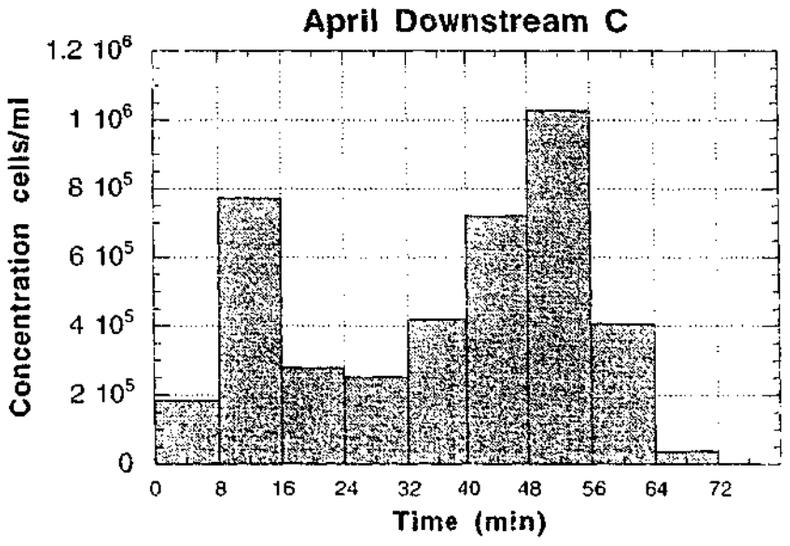


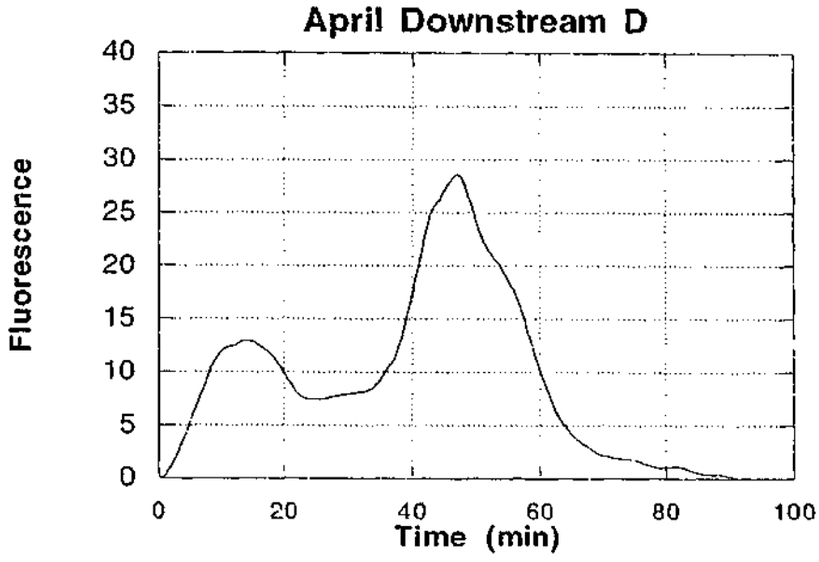
Figure 4.8

(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Downstream site C in April 1996.

(b) Plot of cell concentration for individual SdFFF fractions. Site C April sample.

For both graphs, results have been normalised to the same concentration factor and injection volume.

(a)



(b)

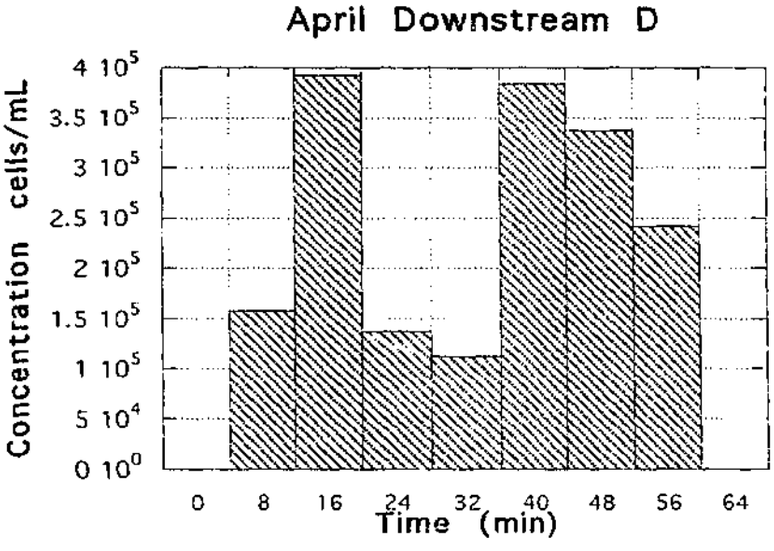


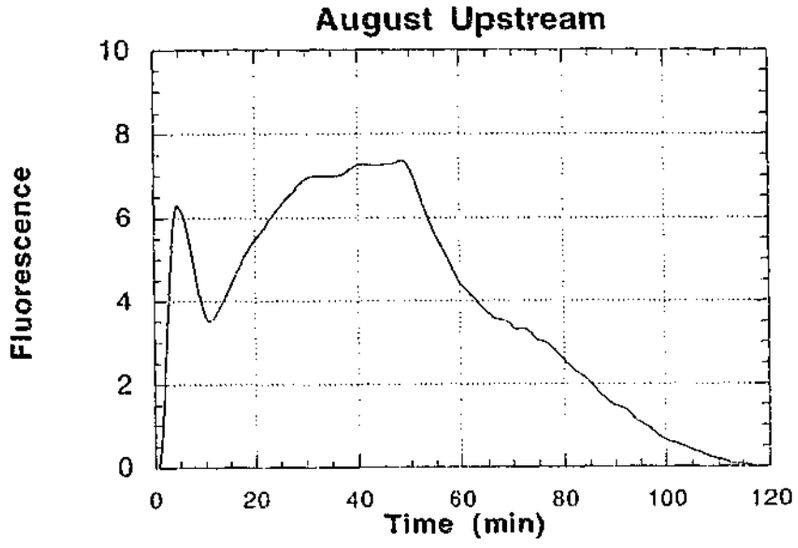
Figure 4.9

(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Downstream site D in April 1996.

(b) Plot of cell concentration for individual SdFFF fractions. Site D April sample.

For both graphs, results have been normalised to the same concentration factor and injection volume.

(a)



(b)

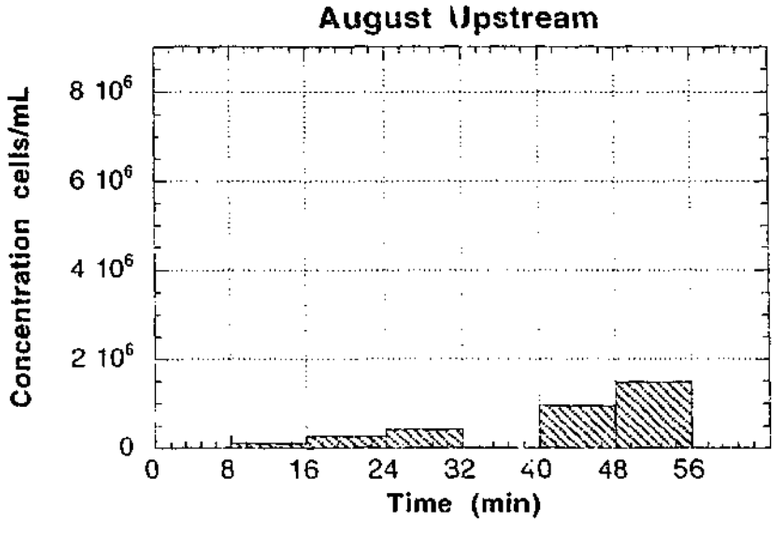
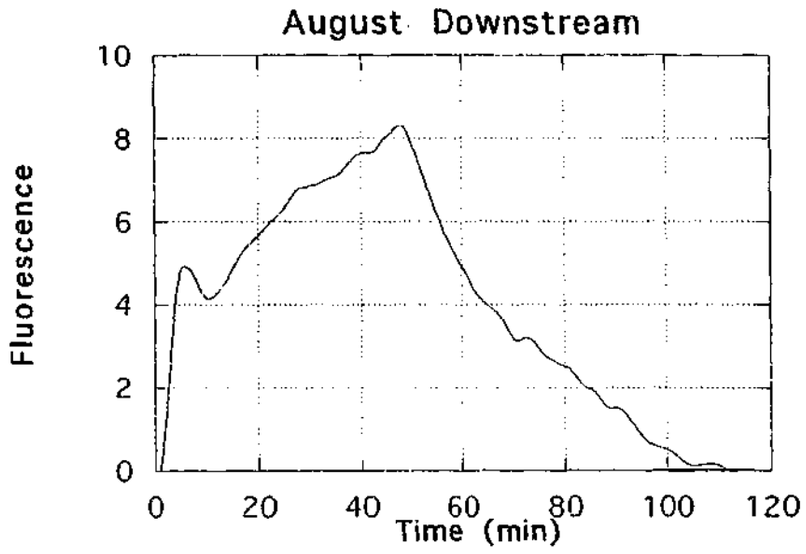


Figure 4.10

(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Upstream in August 1996.

(b) Plot of cell concentration for individual SdFFF fractions. Upstream August sample. For both graphs, results have been normalised to the same concentration factor and injection volume.

(a)



(b)

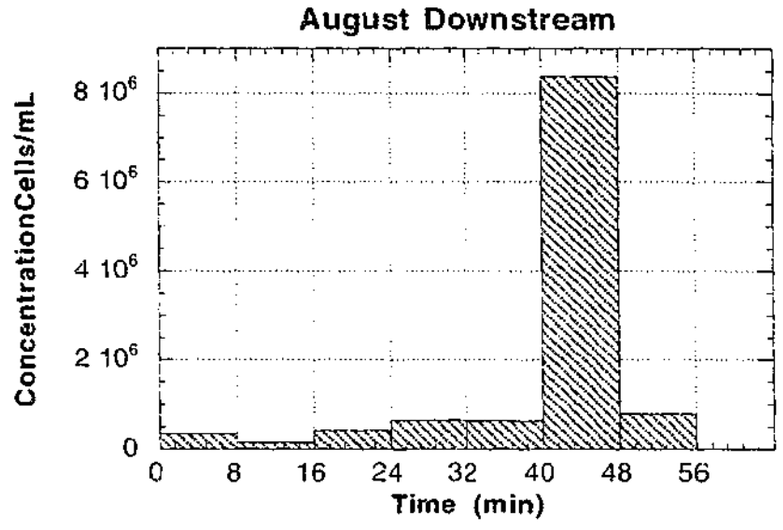
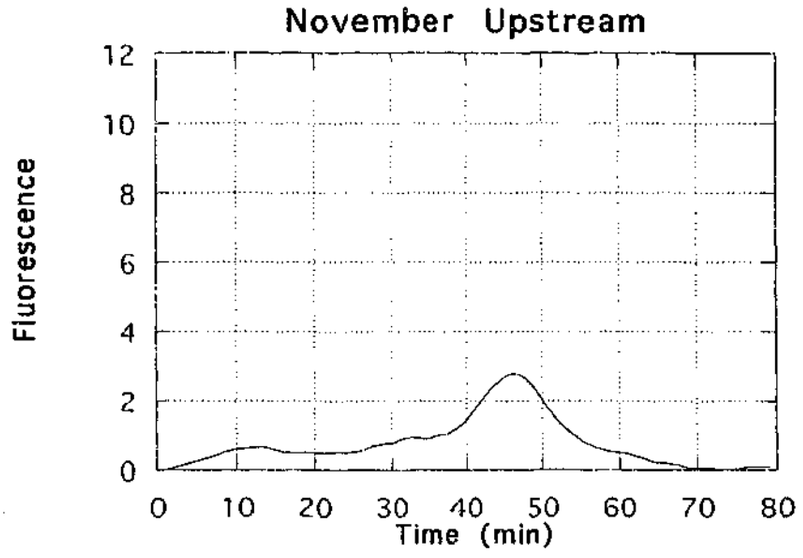


Figure 4.11

(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Downstream in August 1996.

(b) Plot of cell concentration for individual SdFFF fractions. Downstream August sample. For both graphs, results have been normalised to the same concentration factor and injection volume.

(a)



(b)

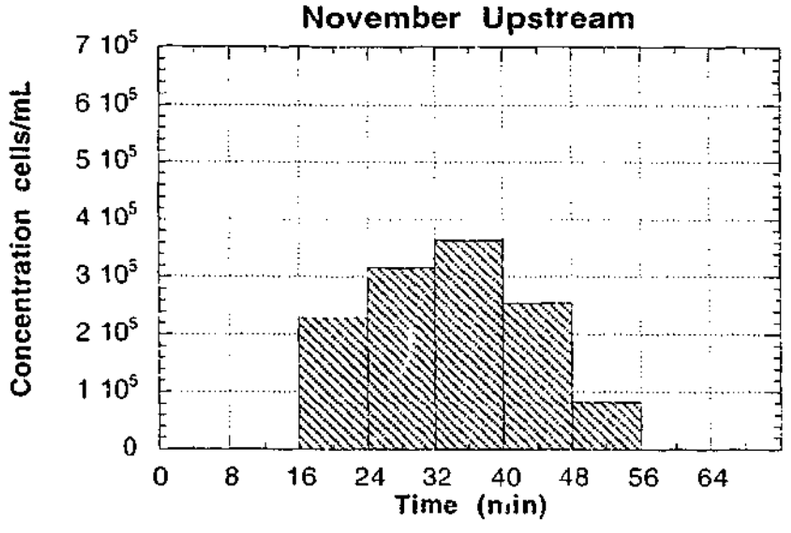
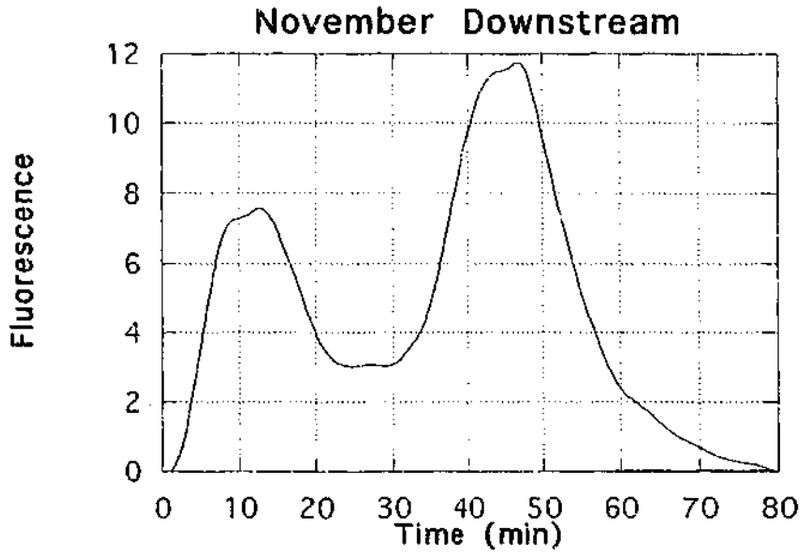


Figure 4.12

(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Upstream in November 1996.

(b) Plot of cell concentration for individual SdFFF fractions. Upstream November sample. For both graphs, results have been normalised to the same concentration factor and injection volume.

(a)



(b)

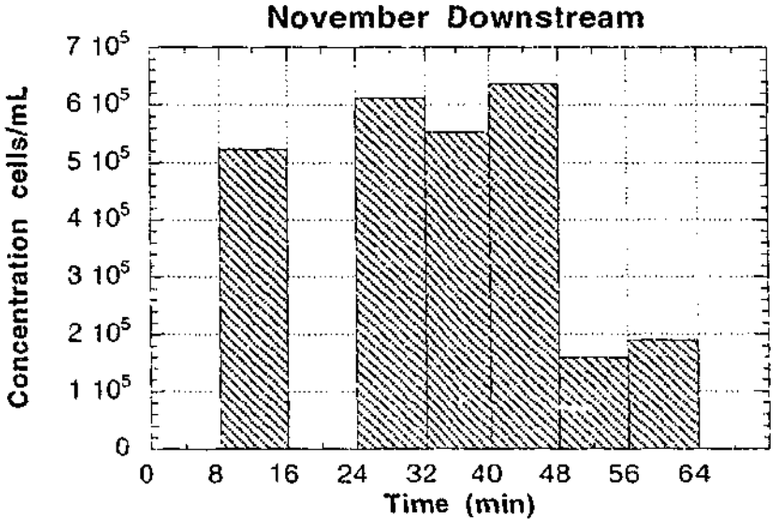


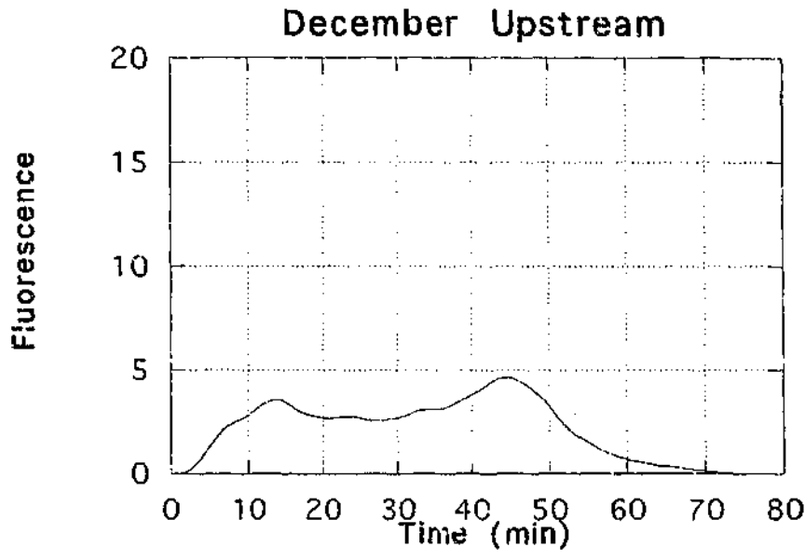
Figure 4.13

(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Downstream in November 1996.

(b) Plot of cell concentration for individual SdFFF fractions. Downstream November sample.

For both graphs, results have been normalised to the same concentration factor and injection volume.

(a)



(b)

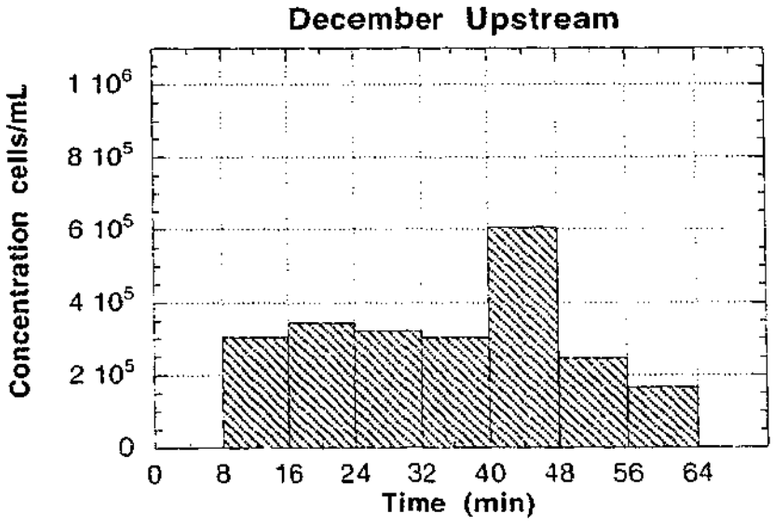
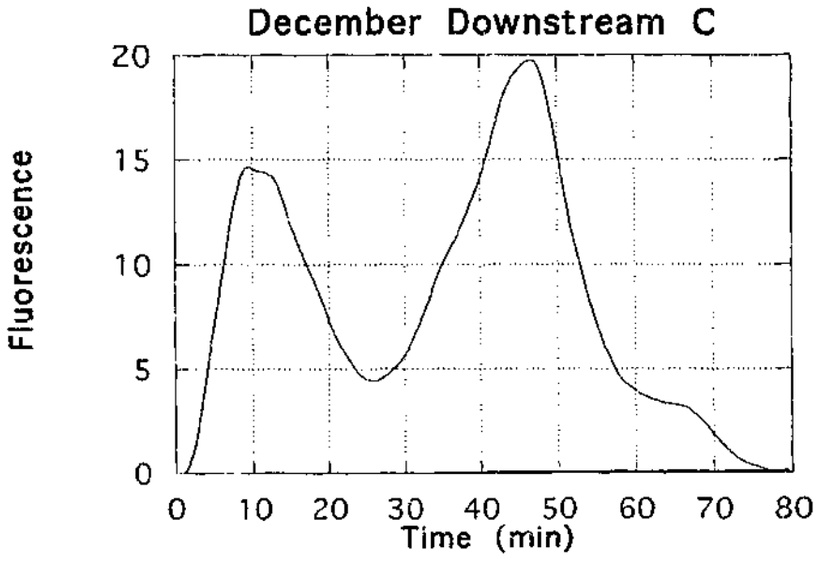


Figure 4.14

(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Upstream in December 1996.

(b) Plot of cell concentration for individual SdFFF fractions. Upstream December sample. For both graphs, results have been normalised to the same concentration factor and injection volume.

(a)



(b)

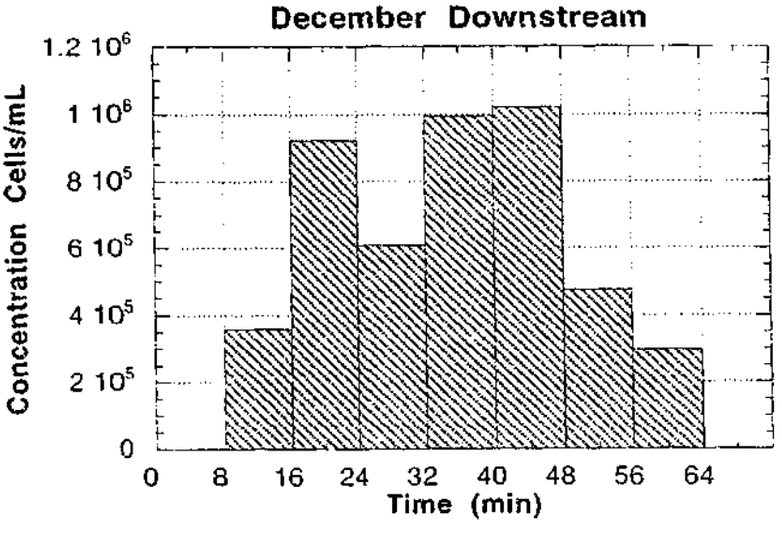
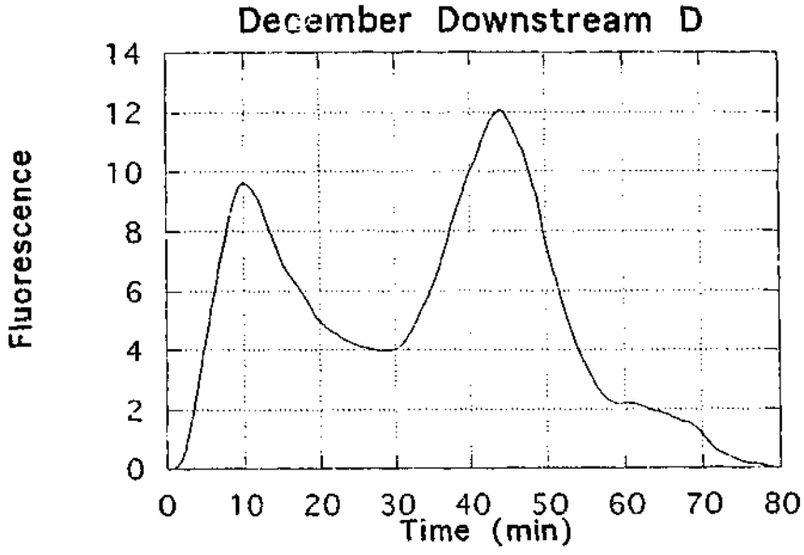


Figure 4.15

(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Downstream site C in December 1996.

(b) Plot of cell concentration for individual SdFFF fractions. Site C December sample. For both graphs, results have been normalised to the same concentration factor and injection volume.

(a)



(b)

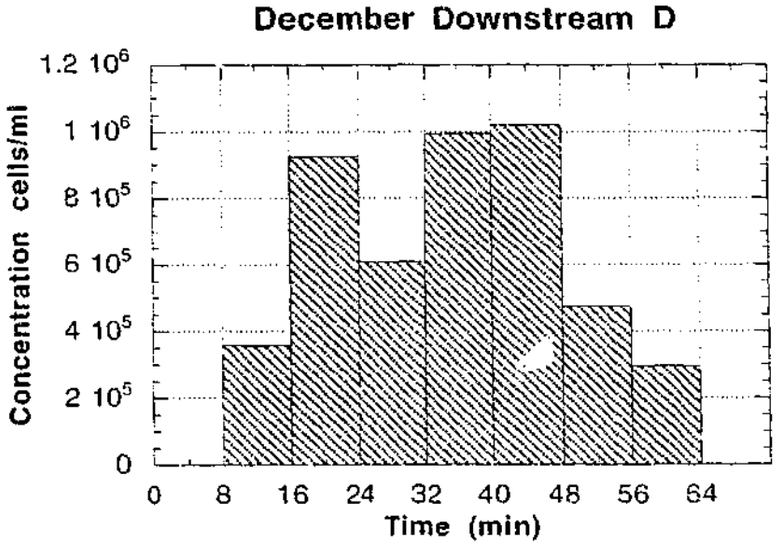


Figure 4.16

(a) Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation. Downstream site D in December 1996.

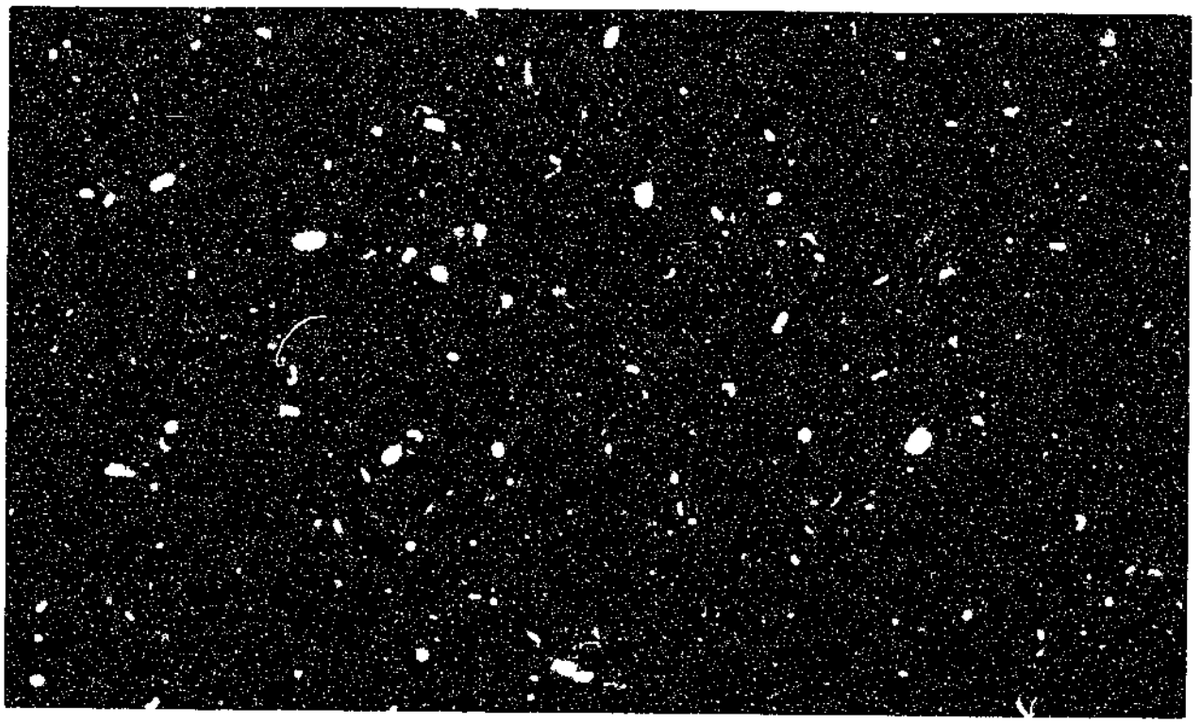
(b) Plot of cell concentration for individual SdFFF fractions. Site D December sample. For both graphs, results have been normalised to the same concentration factor and injection volume.

Photographs of the 3 peak fractions taken with a camera attached to the microscope, show clearly the ability of SdFFF to separate bacterial cells and to also separate them from algal cells. Figure 4.17a is an unfractionated sample showing bacterial cells of all shapes and sizes. In contrast, Fig. 4.17b-d are photographs taken from each peak of the fractogram produced from site C samples collected in March 1996. The cells in Figure 4.17b (peak 1, elution time 10-15 min) are uniformly small and cocci shaped, whereas the cells present in the second peak (Fig 4.17c) are larger and a mix of rods and cocci. The third peak (Fig 4.17d), contains very few bacterial cells, being made up of mainly small algal cells. From these observations it appears that SdFFF is able to separate bacterial cells into a series of size classes, and additionally can separate bacterial cells from algal cells.

A larger fluorescence signal was recorded for all downstream samples, with the exception of the March 1996 samples. The magnitude of the difference between the sites appeared to depend upon the time of year the samples were collected. For example, in August 1996 (Fig. 4.10 & 4.11) the fluorescence signal was not significantly different between sites. However, in April, November and December 1996, site C was 4-fold higher than the upstream samples, and site D was 3-fo'd higher than upstream (April and December 1996). Taking into account differences in concentration factor and injection volume, the variation at the downstream sites was from 8.5 (arbitrary units) observed in August 1996, to 47 in April 1996 and 9 in January 1996 to 29 in April 1996, for sites C and D respectively. In contrast to the large fluctuations in fluorescent signal observed between samples, the overall pattern and shape of the fractogram varied little between sites.

Fractions were collected during the SdFFF runs and the cell concentration of each fraction was determined using epifluorescence microscopy. The cell count data was obtained independently by direct counting of DAPI stained cells and not using a fluorescence detector, because of the large variations in the average fluorescence per cell between different bacterial species and at various stages of their growth cycle (Ross *et al.*, 1996). In common with most natural samples, those used in these experiments also contained autofluorescing particles. The majority of these autofluorescing particles are sediment and humic material which have a similar excitation emission spectra to that of the DAPI-DNA compound

(a)



(b)

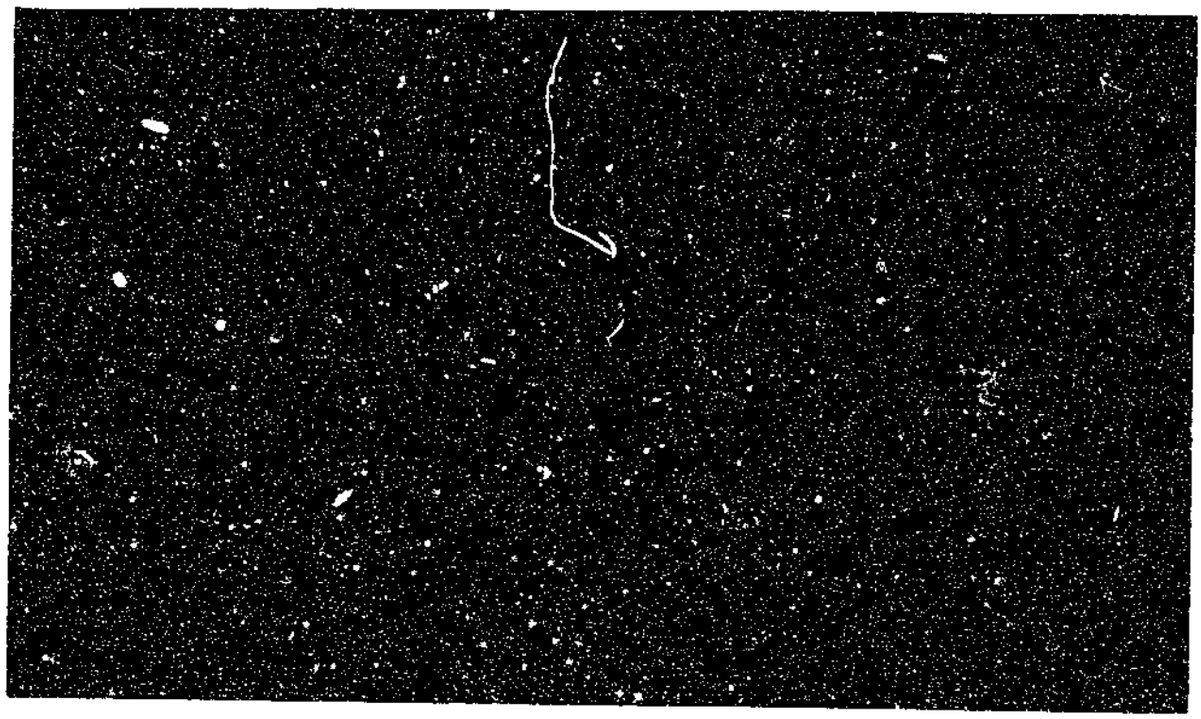
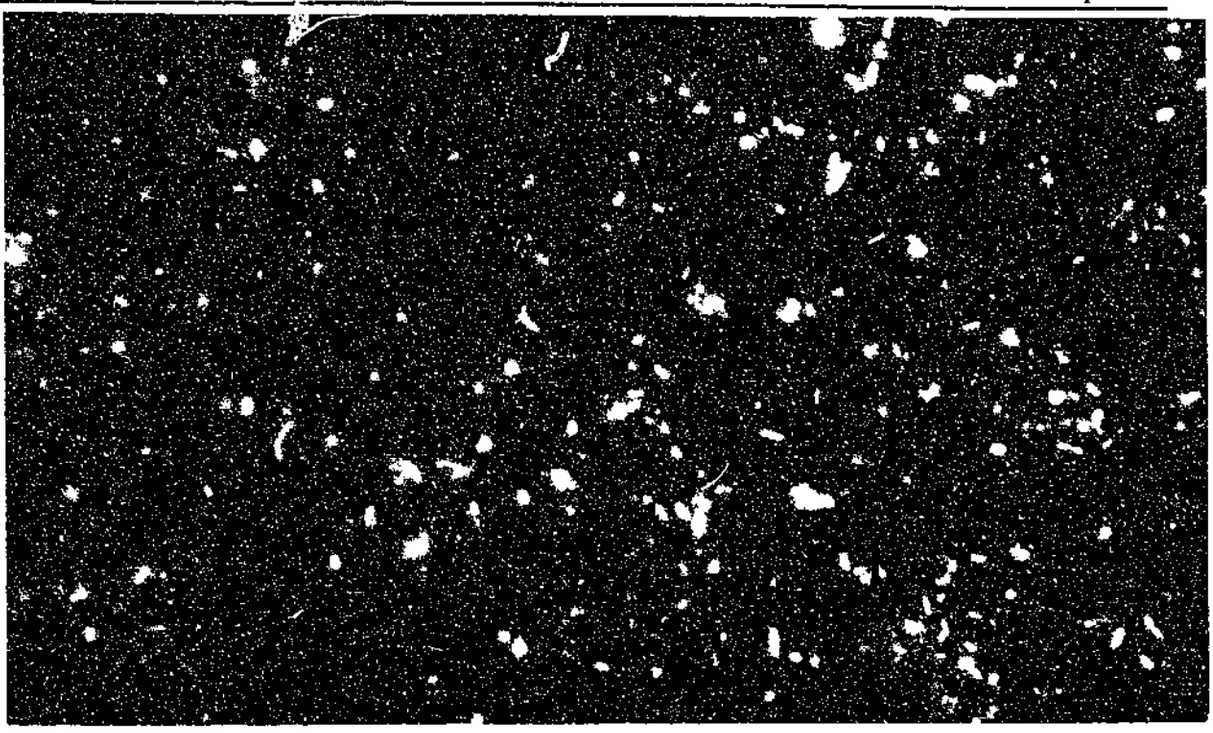


Figure 4.17a Unfractionated sample stained with DAPI (Site C, March 1996). Viewed under UV light at x1250 magnification.

(b) The cells present in this figure are from the first peak (elution time 8-16 min) of the fractogram (plot of fluorescence versus elution time) shown in figure 4.6a (Site C, March 1996).

(c)



(d)

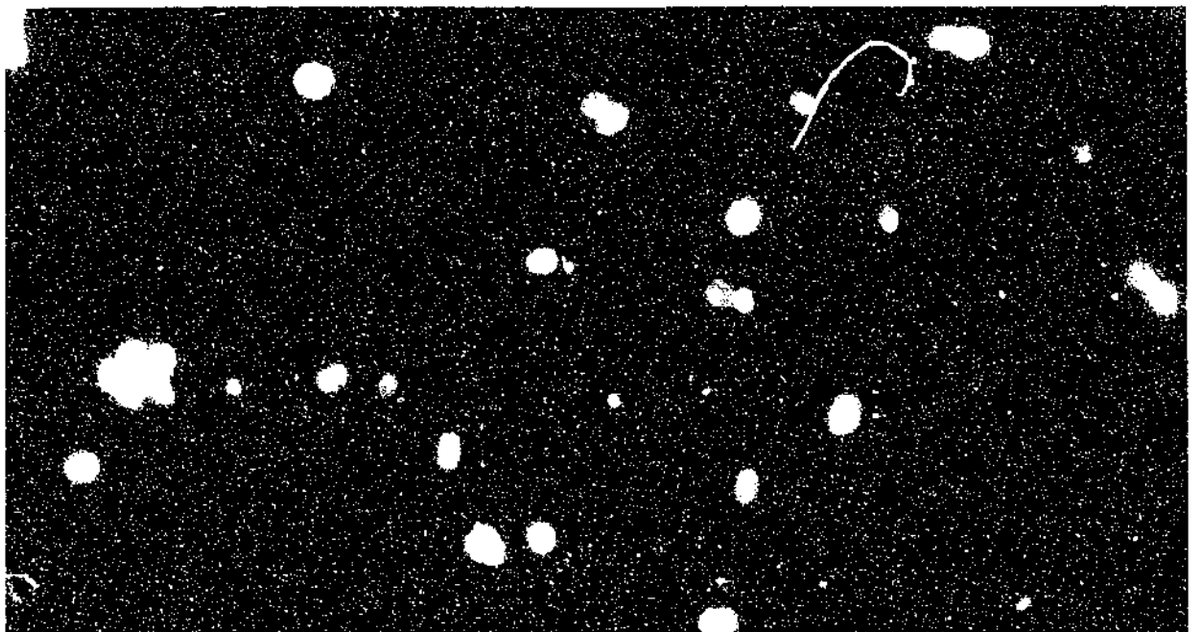


Figure 4.17

(c) March Site C sample separated by SdFFF and collected into fractions for cell counting. The cells present in this figure are from the second peak (elution time 48-56 min) of the fractogram (plot of fluorescence versus elution time) shown in figure 4.6a. Viewed under UV light at x1250 magnification.

(d) The cells present in this figure are from the third peak (elution time 64-72 min) of the fractogram (plot of fluorescence versus elution time) shown in figure 4.6a.

(Müller-Wegener, 1977). Direct cell counts are far more labour intensive than when a fluorescence detector is used, but necessary for natural samples.

The result of these counts are presented in Figure 4.2-16 as graphs of concentration (cells/mL) verses elution time. The cell counts obtained for each fraction closely follow the fractograms for both upstream and downstream samples, with the exception being January 1996. In January, the large amount of suspended particulate matter seen in the first fraction may be the cause of this discrepancy. The small autofluorescing sediment particles and humic material may have produced this "false" peak at 20 min elution time instead of 32-48 minutes as the cell counts suggests. Histograms containing no cell count results for a particular fraction does not necessarily indicate that no cells are present in that fraction. It probably means that the concentration of cells for that fraction were below the detectable and statistically reliable levels (approx 0.03 x 10^9 cells/L for a 16 mL fraction) for epifluorescent microscopy.

4.3.3 Biomass distributions

The SdFFF fractograms were transformed to a biomass distribution curve, with the total area under the curve giving the biomass concentration of the sample. The results of this analysis are provided in Table 4.2. As expected, the biomass (µg/L) was found to be greater at the downstream sites for all field trips, with biomass at site C always greater than at site D. The range of biomass concentrations was also found to vary more at site C, with a 15-fold difference between the lowest biomass concentration (25 µg/L) in November 1996 and the highest (380 µg/L) in March 1996. It is not known whether the same amount of variation would have been observed for site D, because SdFFF runs were not performed for March 1996, July 1996 and November 1996 for this site. The variation in biomass concentration observed at site C is 3 times greater than the 5-fold difference in biomass concentration observed over the same time period upstream of the sewage treatment plant outlet.

The total biomass at each site can be converted to the more common term of carbon content, using the general assumption that organic carbon makes up approximately 50% of

Table 4.2 Results of biomass measurements in Campbells Creek upstream and downstream of the Castlemaine STP. Biomass concentration was estimated from the total area under biomass distribution curves and knowing the injection volume and concentration factors used in the sample preperation steps.

DATE 1996	SITE	Lowest OM 10^-16g/Cell	Highest OM 10^-13g/Cell	AVERAGE BIOMASS Peak Maximum Mom/cell g	AREA UNDER HISTOGRAM Biomass in grams	CONCENTRATION FACTOR	BIOMASS CONCENTRATION grams/L	BIOMASS CONCENTRATION µg/L	CARBON µg/L
January	Site A	0.2	11.4	1.9E-14	6.6E-07	160	2.73E-05	27	13
	Site C	0.2	10.7	9.4E-15	5.2E-06	160	2.18E-04	218	109
	Site D	0.2	11.6	9.4E-15	2.1E-06	160	8.92E-05	89	44
March	Site A	0.2	11.0	1.5E-14	6.2E-07	100	4.13E-05	41	20
	Site C	0.2	10.9	3.0E-14	5.7E-06	100	3.79E-04	379	189
April	Site A	0.2	2.7	1.5E-14	6.3E-07	100	4.23E-05	42	21
	Site C	0.2	10.4	3.0E-14	1.2E-06	100	1.70E-04	170	85
	Site D	0.2	10.4	1.5E-15	6.0E-07	100	5.97E-05	60	29
August	Site A	0.2	9.1	1.3E-14	8.4E-07	114	3.70E-05	37	18
	Site C	0.2	8.8	1.3E-14	2.2E-06	114	1.31E-04	131	65
November	Site A	0.2	9.7	6.4E-15	1.4E-07	114	8.45E-06	8.4	4.2
	Site C	0.2	2.0	1.3E-14	4.4E-07	114	2.55E-05	26	13
December	Site A	0.2	1.8	1.5E-14	4.9E-07	114	2.87E-05	29	14
	Site C	0.2	3.4	1.5E-14	2.0E-06	114	1.17E-04	117	58
	Site D	0.2	3.4	1.5E-14	9.4E-07	114	5.50E-05	55	27

the cell dry weight (Luria 1960). Biomass values in terms of carbon are also presented in Table 4.2.

4.3.4 SdFFF combined with tritiated thymidine

Preliminary experiments were undertaken to investigate the potential of combining bacterial production and biomass measurements into the one technique. First it was necessary to test the possibility that ^{3}H-TDR interfered in some way with the DAPI staining of cells and the subsequent fluorescent fractogram produced by the SdFFF. This was done by performing a control SdFFF run (DAPI only, no ^{3}H-TDR) and comparing its fractogram with that obtained from a sample that had been incubated with ^{3}H-TDR and stained with DAPI. The results (Figure 4.18) show there is little difference between the two fractograms in either intensity (peak maximum 9.5 for DAPI and ^{3}H-TDR and 11 for DAPI only) or shape. The variation amongst natural samples most probably accounts for the small differences observed between the two fractograms.

The reverse scenario, DAPI interfering with the radioactive counts obtained for each fraction, was also investigated. This was examined by dividing the 1 mL concentrate produced from centrifugation of the 80 mL samples incubated with ^{3}H-TDR (see materials and methods) into two 500 µL volumes. The cells from one sample were stained with DAPI and the other sample was not stained. The same distribution of counts for the two samples was observed, the highest counts were obtained at an elution time of 45 to 50 min, followed by 50 to 55 min and then 45 to 50 min (Fig. 4.19a &b). The radioactive counts were slightly higher (< 20%) in the DAPI stained sample, however this difference, is no greater than that observed between replicate samples in ^{3}H-TDR incorporation assays performed in the traditional way.

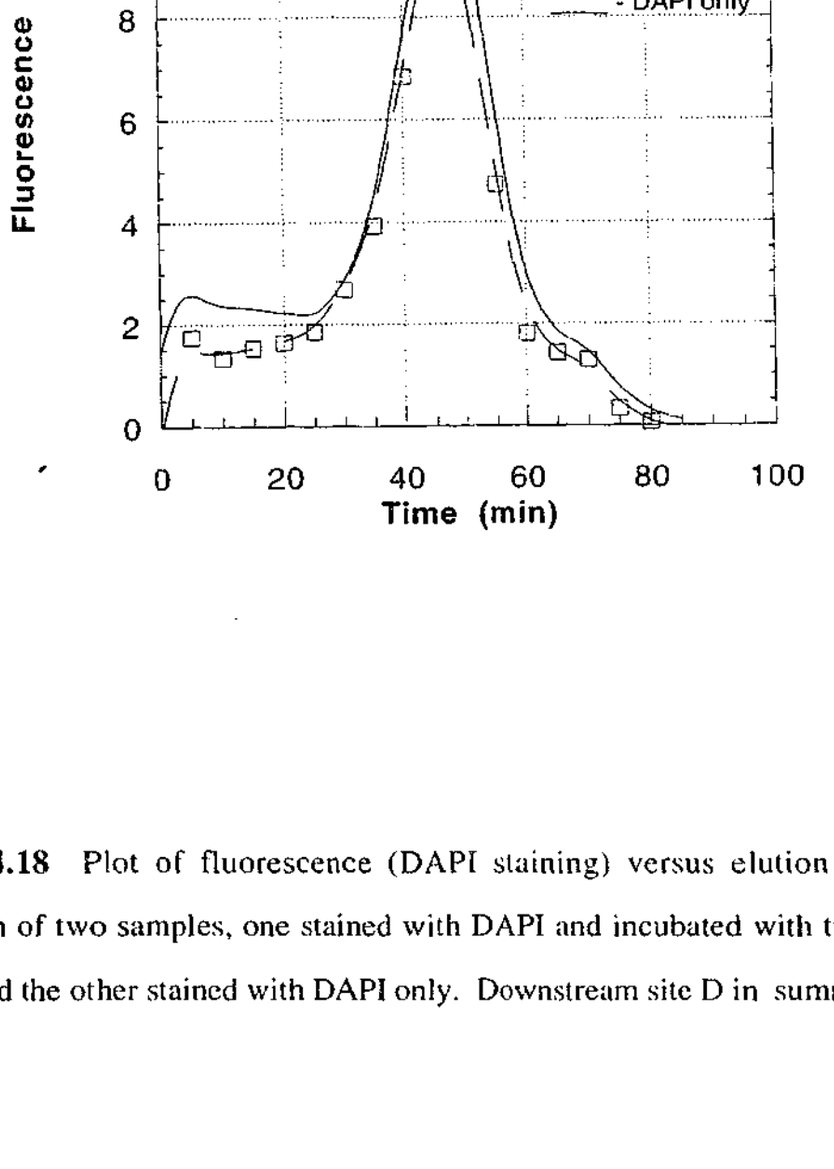
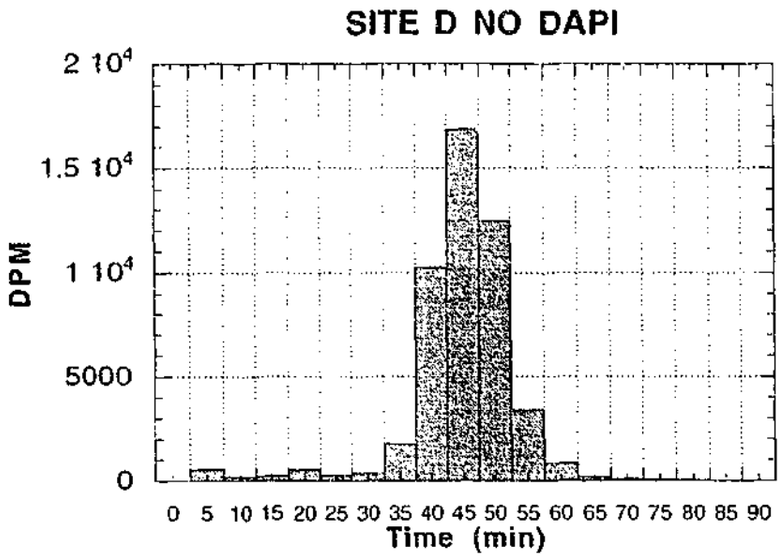


Figure 4.18 Plot of fluorescence (DAPI staining) versus elution time for SdFFF separation of two samples, one stained with DAPI and incubated with tritiated thymidine (TDR) and the other stained with DAPI only. Downstream site D in summer 1998.

(a)



(b)

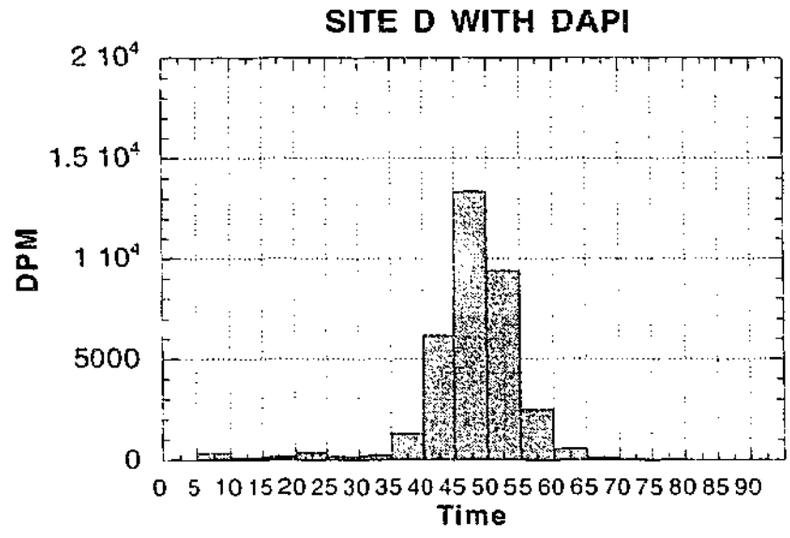


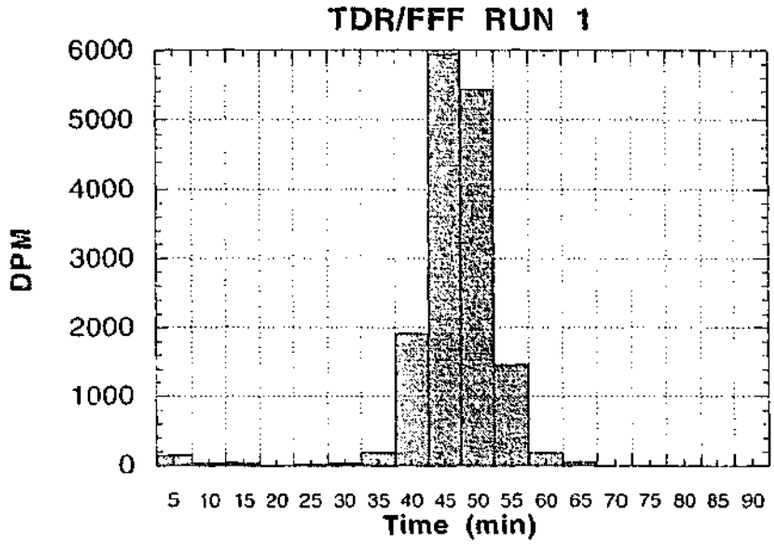
Figure 4.19 Histogram of radioactivity (DPM) from the incorporation of tritiated thymidine (TDR) versus elution time for SdFFF separation.

(a) Sample incubated with TDR only, no DAPI staining.

(b) Sample incubated with TDR and DAPI stained.

Downstream site D in summer 1998.

(a)



(b)

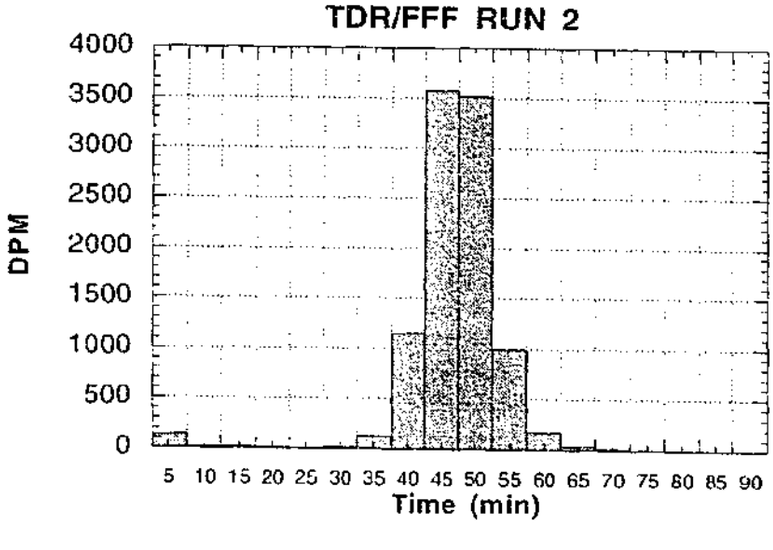


Figure 4.20 Histogram of radioactivity (DPM) from the incorporation of tritiated thymidine (TDR) versus elution time for SdFFF separation.

(a) Sample incubated with TDR and DAPI stained. Downstream sample from January 1998.

(b) Repeat SdFFF run to test reproducibility.

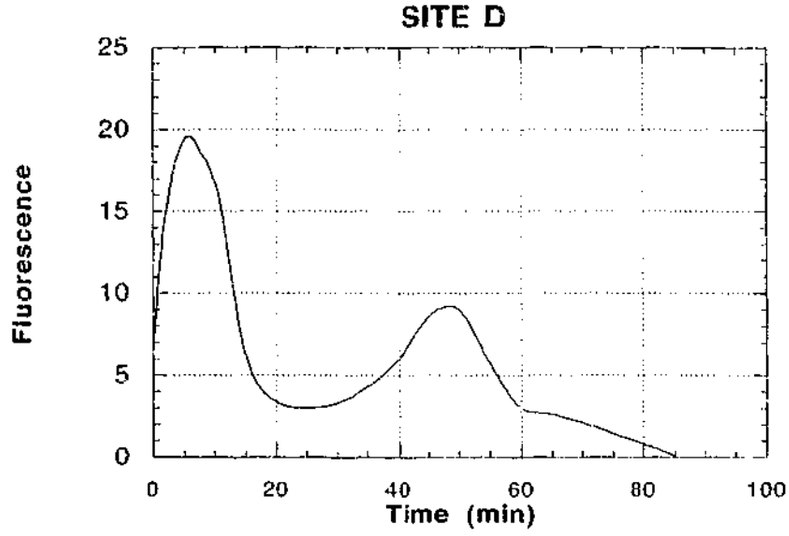
Two SdFFF runs on the same concentrate were performed to test the reproducibility of this technique, time constraints prevented additional runs. The radioactive counts were distributed in the same way across the fractogram for both SdFFF runs (Fig 4.20a and b), The highest counts were observed in the 45 min to 50 min elution time, followed by the 50-55 min and the 40-45 min fraction. The counts obtained from the first SdFFF run was however, 1.5 times greater than those from the second. It is likely that this difference is due to bacteria clumping together in the time between the first and second runs. Past practice, if more than one run was to be performed on the same day on the same concentrate, has been to only sonicate before the first run and then store the sample at 4°C until required. A sonicating probe was not available for radioactive samples and so for all samples sonication occurred in a sonicating bath. This method of sonication is not as affective as with a probe. If all the bacterial or bacteria and particulate aggregates had not been successfully disrupted into single cells then this may also cause discrepancies between SdFFF runs on the same sample and produce inaccurate results. Obviously, this can be overcome by using a sonicating probe available for radioactive samples.

The new method combining SdFFF with ^{3}H-TDR was performed on one sample from each of site A and site D on the 28 January 1998. The distribution of counts across the fractogram and the magnitude of counts differed substantially between the upstream and downstream sample (Fig. 4.21). The highest radioactive counts for site A was observed in the 50-55 min fraction (250 DPM) and for the downstream site D sample at 45-50 min with a peak count of 13,000 DPM.

The new SdFFF/^{3}H-TDR procedure was again used for the site D sample collected on the 4 February 1998 (Figs 4.22). The cell concentration of each fraction was also determined for these runs which enabled the biomass to be determined. The radioactive counts observed for each fraction did not correlate directly with the cell concentration, inferring that the radioactivity of the fraction is not determined solely by the number of cells present. The fraction collected at 45-50 min had the largest concentration of bacterial cells (1.1×10^{7} cell/mL), although the maximum radioactive count (19,000 DPM) was observed

in the fraction collected at 50-55 min. These results suggest that the cells in some fractions are much more productive than the cells in other fractions.

(a)



(b)

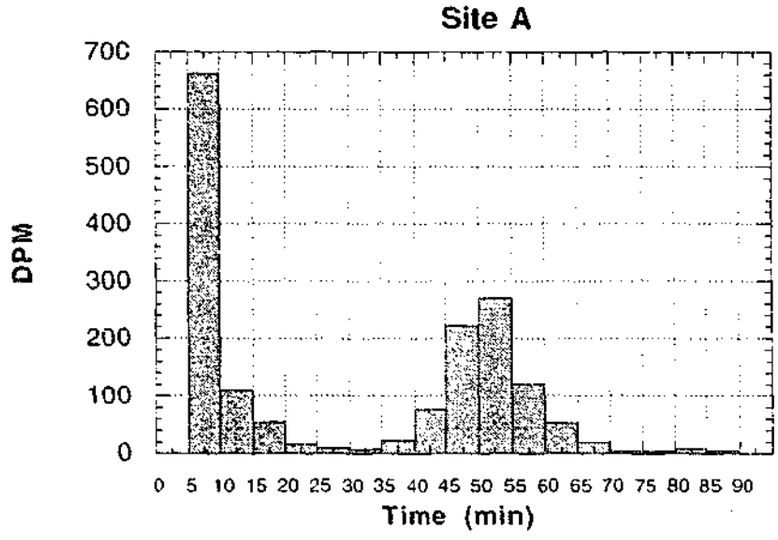
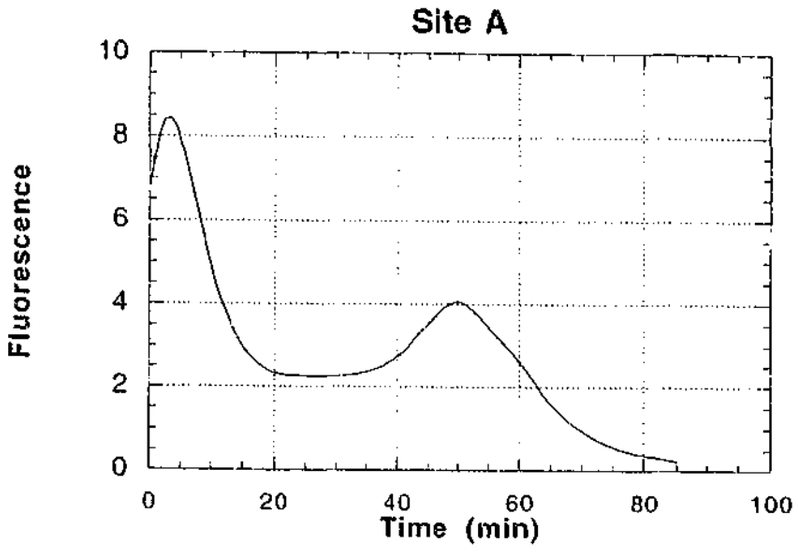


Figure 4.21

(a) Plot of fluorescence (DAPI stain) versus elution time for SdFFF separation. Upstream sample incubated with tritiated thymidine from January 1998.

(b) Histogram of radioactivity (DPM) from the incorporation of tritiated thymidine (TDR) versus elution time for SdFFF separation. Sample incubated with TDR and DAPI stained. Upstream sample from January 1998.

(c)



(d)

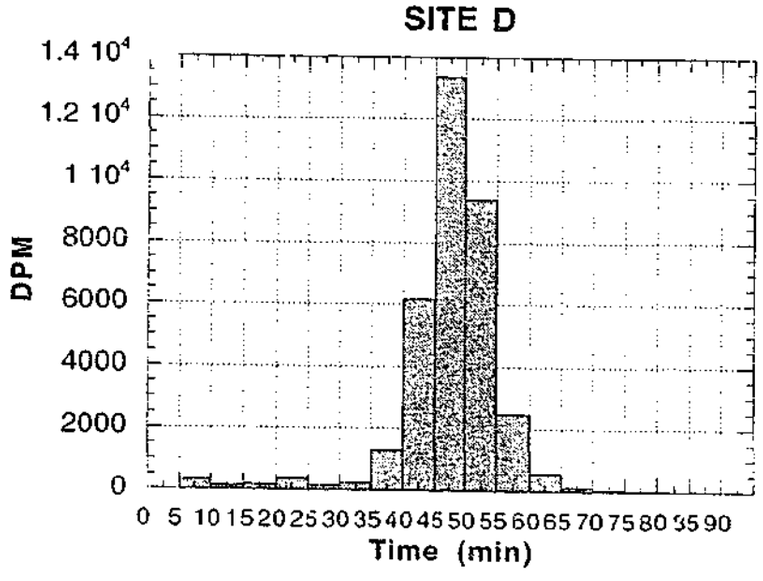
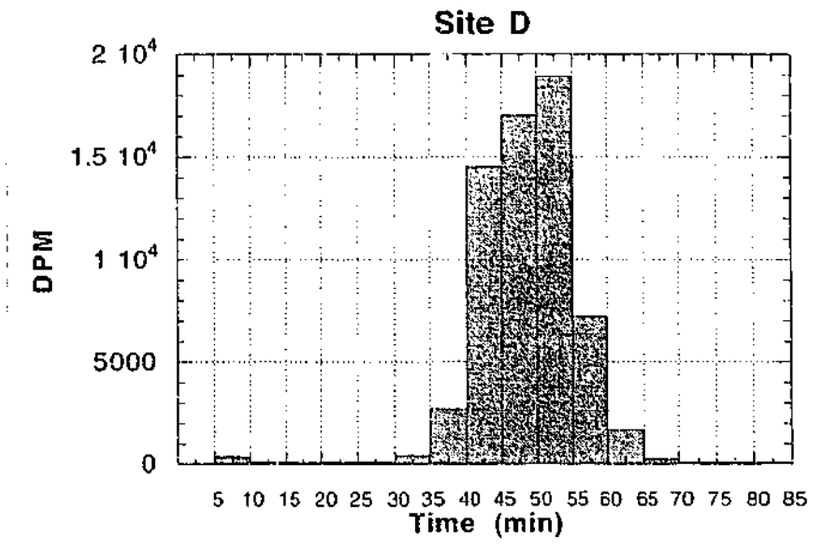


Figure 4.21

(c) Downstream site D sample incubated with tritiated thymidine from January 1998. Plot of fluorescence (DAPI stain) versus elution time for SdFFF separation

(d) Histogram of radioactivity (DPM) from the incorporation of tritiated thymidine (TDR) versus elution time for SdFFF separation. Sample incubated with TDR and DAPI stained. Site D sample from January 1998

(a)



(b)

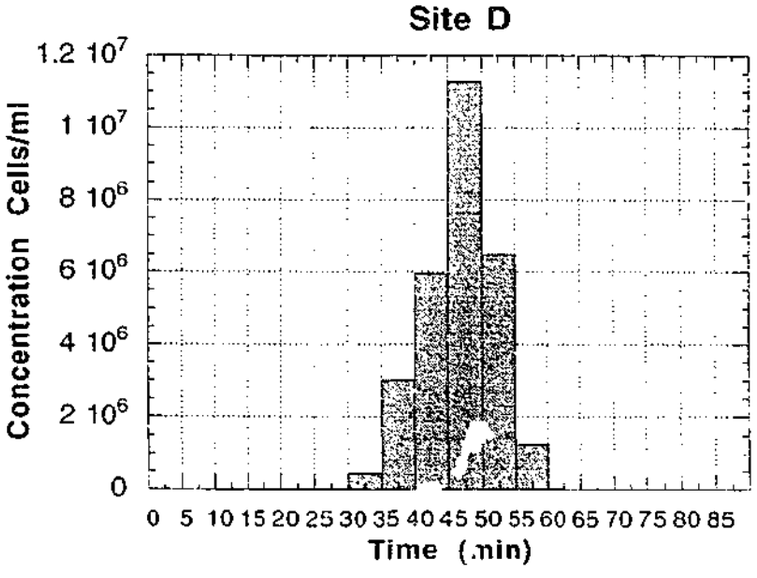
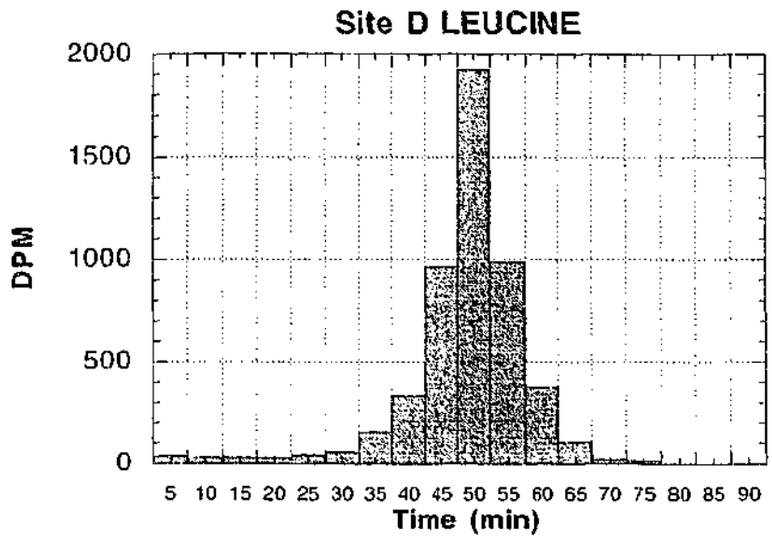


Figure 4.22

(a) Histogram of radioactivity (DPM) from the incorporation of tritiated thymidine (TDR) versus elution time for SdFFF separation. Sample incubated with TDR and DAPI stained. Downstream sample from Febuary 1998.

(b) Plot of cell concentration for individual FFF fractions collected from the SdFFF separation performed on the 4 Febuary 1998. Sample incubated with TDR and DAPI stained

(4.24)



(4.25)

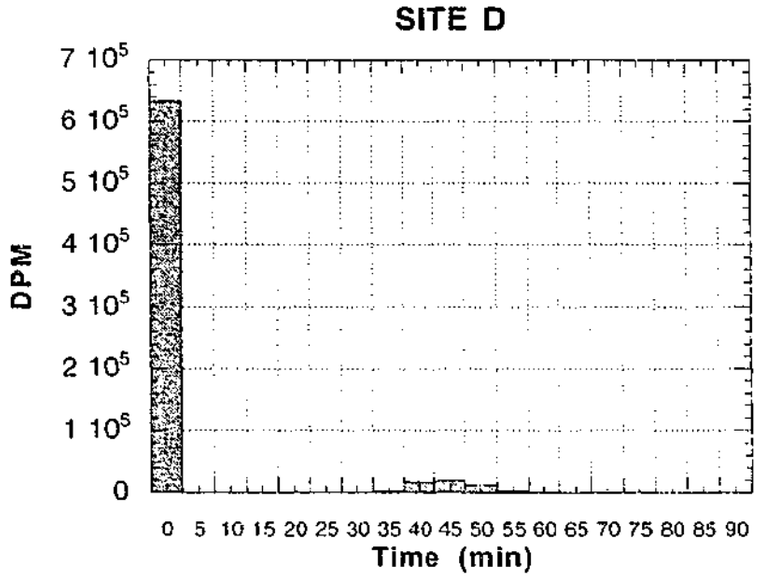


Figure 4.24 Histogram of radioactivity (DPM) from the incorporation of radiolabelled leucine versus elution time for SdFFF separation. Downstream sample from Febuary 1998.

Figure 4.25 Histogram of radioactivity (DPM) from the incorporation of tritiated thymidine versus elution time for SdFFF separation. This graph has the radioactivity present in the void peak included, clearly showing that unincorporated thymidine is eluted earlier than the bacterial cells.

4.4 Discussion

4.4.1 Bacterial biomass

The overall pattern or shape of the SdFFF fractogram obtained for samples taken upstream and downstream of the sewage discharge changed little between the six field trips, which encompassed all four seasons. However, the intensity of the fluorescence signal did fluctuate considerably between samples. These fluctuations in fluorescence generally corresponded to changes in bacterial cell concentrations, with the bacterial cell counts more variable at the downstream site. The cell count data presented in Table 4.1 clearly demonstrates the variation in bacterial cell concentrations for each field trip. Differences in fluorescence signal can also result from variations in the average fluorescence per cell for different samples. For example, the cell count (obtained by DAPI staining) for March 1996 was higher at the downstream sites even though the signal fluorescence was less than that observed at the upstream site. This may be caused by differences in the bacterial community structure at each site at this given point in time. It has been shown by Ross *et al.* (1996) that different bacterial species may vary by up to 500-fold in specific fluorescence. Such variations in fluorescence between cells are due to two major factors: (a) the amount of DNA per cell, and (b) the base composition of the DNA. As DAPI binds to the adenine-thymine bases in double stranded DNA, the cellular DNA content will greatly influence the fluorescent intensity of a given cell. The DNA content of a cell is determined by the genome size and the number of genome copies present in the cell, which can change according to the cells growth cycle. Taxonomic differences such as a species ratio of guanine to cytosine in their DNA (G+C ratio), are also important factors affecting the specific fluorescence of cells (Ross *et al.*, 1996).

The bacterial biomass was consistantly greater at the downstream sites C and D. In general, these differences in biomass can be attributed to differences in cell concentration. However, the results suggest that bacterial biomass is also affected by other factors, most likely chemical and physical parameters such as time of year, temperature and nutrient availability. Interestingly, although the lowest cell concentration was observed at site C in

July 1996 ($1.9x10^9$ cells/L), the biomass concentration (130 µg/L) at this site was still significantly greater than even the highest biomass values obtained at the upstream site. For example, at site A the higher cell concentrations, $3.7x10^9$ cells/L in April 1996, had a biomass of only 42 µg/L.

The biomass values were converted into the more commonly used carbon per litre term, to allow comparisons to be made with other researchers results. The biomass concentrations obtained upstream (4.2-21 µg C/L) and downstream (13-190 µg/L) of the sewage outlet fall within the range reported in the literature (Table 4.3).

Table 4.3 Reported biomass concentrations

Site	Biomass concentration µgC/L	Reference
Hudson River estuary	28-250 µg C/L	Findlay *et al.*(1991)
Billabong (oxbow lake)	225	Sharma (1998)
Port Phillip bay (marine)	17	Sharma (1998)
Templestowe lake (freshwater)	11	Sharma (1998)
Latrobe Valley sewage system	1800	Sharma (1998)
Campbells Creek upstream STP	4.2-21	This study
downstream STP	13-190	This study

There are a number of different factors which can contribute to differences in bacterial biomass. Nagata and Watanabe (1990) found that a large variability in mean cell volume and biomass C per litre (45.3 - 4,350 µg C/L) resulted when they grew bacteria under a range of different temperatures, dissolved organic carbon and nutrient concentrations. However, when these changes in biomass occur in natural environments it is not possible to use SdFFF or conventional methods of biomass measurement to determine

whether the differences are due to a change in species composition or a change in cell volume within the same species.

Two reasons can be offered to explain the higher biomass values consistently obtained downstream of the sewage outlet. First, it is possible that the differences in the nutritional status of the creek between the two sites (Chapter 2) may have caused the increase in biomass. Other researchers have noted such increases in response to an increase in nutrients (Nagata and Watanabe, 1990). Second, and more probable, is the close proximity (50 m) of the downstream sampling site to the sewage outlet which would mean that large numbers of allochthonous bacteria from the sewage effluent would be added to this site and increase the biomass. The relatively short time (approx 1.2 min at high flow to 20 min at low flow) it takes for the creek to flow from the discharge point to the sampling site is not sufficient for changes to occur in either bacterial species composition or cell volume. The generation time or doubling time of bacteria varies considerably from as little as 10 minutes to more than two days depending upon the species and its growth conditions (Brock and Madigan, 1994).

The biomass values calculated for Campbells Creek downstream of the discharge point are not as high as those obtained from sewage effluent alone from other waste treatment plants. Sharma *et al.* (1998) have reported biomass values of 1800 μg C/L for the bacteria from the Latrobe Valley sewage system. Values even higher than this have been recorded in other effluent discharge (Rheinheimer, 1992) suggesting that a "dilution effect" may have occurred at the downstream Campbells Creek site. This would certainly be possible as the high biomass effluent mixes with low biomass upstream water, resulting in the overall biomass within the creek downstream of the discharge site being higher than that upstream of the discharge site, but lower in relation to the sewage effluent alone. The actual differences would obviously be highly dependant on the flow rate of the creek at the time of sampling, and this probably explains why greater differences in biomass are seen between the two sites during the summer and autumn months (Table 4.2). Obviously, the sewage effluent will make a greater contribution to the downstream biomass when the creek flow rate is low, eg. during summer.

During low flow periods, site D is approximately 7 hours flow time from site C, and although 3 hours is sufficient time for many bacteria to divide and produce a new generation, the fact that it is a natural habitat and the physico-chemical conditions are far from optimal for most bacterial groups, means that it is unlikely that many of them would divide within this time. The most probable explanation for the lower bacterial biomass at site D is that the allochthonous bacteria cannot compete with the natural bacterial populations in the creek and perish. This explanation is also supported by the lower cell concentrations at site D (Table 4.1).

It would be necessary to determine the bacterial biomass at a number of sites further downstream from the sewage outlet pipe to establish whether the sewage discharge is increasing the bacterial biomass of the creek by affecting the mean cell volume of the natural microbial community. By sampling further from the discharge point, the travelling or flow time involved for the bacteria to move downstream from the treatment plant to the sampling site would increase, and thus allow more time for the bacterial community to increase in mean cell volume over the future generations. The distance downstream from the sewage point source required to allow sufficient doubling time for most of the bacterial population would depend on the flow rate of the creek at the particular time of sampling. Generally, it would need at least 2 days in flow time downstream for a significant change to be observed. However, this would not provide information regarding any changes in species composition, but only changes in the mean cell volume of the bacterial population.

The changes in biomass concentration observed downstream of the Castlemaine sewage treatment plant cannot be solely attributed to fluctuations in cell concentrations. When only small differences in cell concentration were observed (< 2-fold difference) between site A and site C, the increase in the corresponding biomass concentration was greater (3.5 or 4 fold greater). As the differences in cell concentration between the upstream and downstream sites increased to between 5 and 10 fold, the increase in biomass concentration was only 4 and 9 fold, respectively. However, when the greatest differences in cell concentrations were observed between site A and site C (20-fold in January and 15-fold in December 1996), the corresponding increase in biomass concentration was less than

half (8-fold and 4 fold for January and December, respectively). The changing relationship between cell concentration and biomass concentration is illustrated in Figure 4.23. Although the observations from this study suggest that when cell concentrations downstream are very high, the mean cell biovolume is decreased, it is not possible to make any conclusive statements concerning the relationship between cell concentration and biomass in Campbells Creek with the limited data set available.

A number of reasons can be advanced to explain the apparent reduction in cell density occurring at high cell concentrations at the downstream site. First, the decrease in mean cell biovolume could be caused by an increase in cell numbers for a particular bacterial group with a small cell biovolume, i.e. a shift in species composition. Such an effect could only be determined using molecular microbial analysis. An increase in bacterial abundance with smaller cell biovolumes could be occurring in the sewage treatment process (e.g. retention ponds) or within Campbells Creek itself. The second possible explanation may be related to the fact that the greatest differences in cell concentration are observed for low rainfall periods (summer months) when the sewage effluent makes up a larger proportion of the creek flow. The effluent at the Castlemaine sewage treatment plant is chlorinated to kill pathogenic bacteria before discharge into Campbells Creek. The dead bacterial cells would slowly deteriorate, resulting in a decrease in the mean cell biovolume. During periods of low flow, the dead cells with a reduced biomass would contribute substantially more to the overall cell concentration present at site C. Since DAPI staining does not distinguish between live and dead cells, dead cells would all be included in the total cell counts The samples collected for SdFFF runs are preserved with formaldehyde, maintaining the cells integrity and structure at the time of sampling for both alive and dead cells. Thus, these preserved dead cells are also included in the biomass calculations.

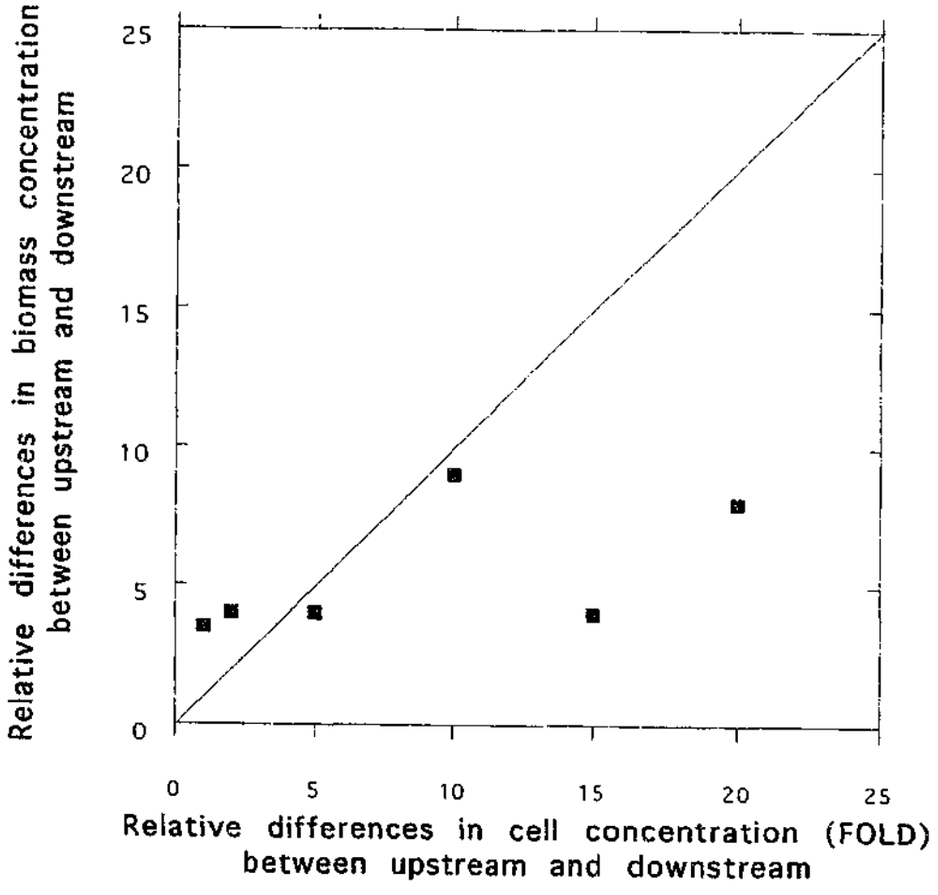


Figure 4.23 Plot of the difference (in fold) in cell concentration between upstream and downstream versus the corresponding difference in biomass observed for the same samples.

4.4.2 Simultaneous biomass and bacterial production measurements

The incorporation of tritated thymidine into cells did not follow directly the concentration of cells in the eluted fractions. This result, suggests that more productive cells exist within some fractions. This is a very important finding. SdFFF separates the cells into fractions on the basis of their buoyant mass which is related to the mass of organic matter of the cell. It appears that production varies for cells with different masses of organic matter (for this particular sample at least). The nature of SdFFF separation means that cells are rather crudely separated on the basis of cell type, but obviously with some overlap between fractions, suggesting that production varies amongst the different cell types and hence species. This work has shown that SdFFF combined with ^{3}H-TDR incorporation can provide insight into the variation in production amongst the different bacterial cell types present at a particular sampling site.

SdFFF was also combined successfully with the ^{3}H-Leucine method of bacterial biomass determination (Fig 4.24). Unfortunately, this was performed for only one run because of time and funding restrictions. Further experiments will be needed to validate this method.

Initially, there were two main concerns regarding combining SdFFF and bacterial production methods. First, it was possible that unincorporated label would be present in the SdFFF fraction, and second, because no traditional ethanol washes are performed during the technique, non-specific labelling of other cellular components and particulate matter could occur, resulting in artificially high counts. To help prevent non-specific labelling, the reactions were incubated with 20 mL of 5% unlabelled thymidine, where exchange should occur between any unincorporated label non-specifically bound and the unlabelled thymidine in solution. Sonication should also disrupt any label physically bound to particulate matter. These two precautions were carried out prior to concentration by centrifugation, resulting in much of the unincorporated label being removed with the supernatant. The remaining unincorporated label was separated from the cells during the SdFFF run shown by the substantial amount eluted in the void peak (Fig. 4.25). Unincorporated thymidine has a much smaller buoyant mass to labelled cells and so will elute from the SdFFF much earlier.

The small volume (150 µL) of sample injected into the SdFFF is carried with 2 litres of carrier fluid that acts in some ways as a washing step. Microscopic examination of the fractions revealed that very few counts (<50 DPM) are present in fractions containing no cells, with the exception of the void peak, and cells were always present in fractions with high radioactivity. These results strongly support the conclusion that the radioactivity present in each fraction is from cells containing ^{3}H incorporated in their DNA.

4.5 Conclusion

Biomass is a very important component in the study of microbial ecology. Changes in biomass of the bacterial community can have important ramifications to other higher organisms within the food web which rely on bacteria as a carbon source. If increases in biomass do not correspond with increases in cell concentration then this could be an early indicator of a change within the bacterial community and would provide important information concerning river 'health'.

This study has shown that differences in both bacterial numbers and biomass exist upstream and downstream of the sewage discharge site. The results suggest that the increase in biomass concentration observed downstream is not simply due to a corresponding increase in cell numbers, and that other factors seem to be contributing to the changes in biomass concentration. The fact that the differences in biomass concentration are not entirely the result of changes in bacterial cell concentration implies that a change in the bacterial population has occurred. These changes are most probably due to the introduction of 'foreign' sewage bacteria from the treatment plant.

The study has also provided further evidence of the potential of the new SdFFF method when coupled with epifluorescent microscopy to directly determine the bacterial biomass of a natural aquatic sample. It is a more useful and precise method of bacterial biomass determination than traditional procedures. SdFFF could be employed with other microbial techniques to study the effect pollution may be having on a particular aquatic environment by quantifying changes in bacterial biomass.

Perhaps the most useful result, was the successful combination of SdFFF with bacterial production measurements. The application of this technique to environmental samples will not only save the researcher valuable time, but also provides previously unattainable information concerning production in terms of biomass. The new method also has the ability to reveal if cells of different buoyant masses are more productive. Results from the use of this technique could have important implications for river health assessments. For example, in cases where between site comparisons are being made, if cells from different fractions and hence different buoyant masses, where found to have different production rates than this would suggest that some sort of change, either in the community composition or production rates of certain cell types, had taken place. This type of change in the microbial community may be an indication of declining in river 'health'. The combination of SdFFF and bacterial production determination, therefore provides additional information to demonstrate that a particular polluting event is having an affect on the microbial population and most probably the aquatic ecosystem as a whole.

CHAPTER 5
BACTERIAL COMMUNITY DIVERSITY

5.1 Introduction

The use of microbial populations as potential indicators of river 'health' has so far, focused on the determination of cell numbers, bacterial biomass and bacterial growth. Bacterial biomass and growth experiments allow the evaluation of the bacterial populations activity in a particular environment, essential information for studies concerning biogeochemical cycling. Bacterial biomass concentrations are critical in assessing the role of the bacterial population as a carbon source for higher trophic levels or examining the relationship between primary and secondary production. In these instances, the microbial population can be viewed as an indicator of ecosystem function. The information gained from such studies can then be extended to the measurement of ecosystem health.

While the techniques involved in measuring bacterial numbers, biomass and growth are paramount for the evaluation of ecosystem function, they do not permit the assessment of biodiversity. Biodiversity is also an important indicator of river 'health'. Generally, a decline in diversity is considered to be the result of decline in the health of the ecosystem (Hart *et al.*, 1996). Given the fact that microorganisms are the most diverse of all biological groups, are present in large numbers and respond rapidly to changes in their environment, they have the potential to be very good indicators of diversity. In the past, limitations in microbiological techniques have prevented the use of microbial populations in this role. Traditional cell culture and isolation techniques permit only a small fraction (0.1-10%) of the bacterial population, visible by epifluorescent microscopy, to be cultivated (Ferguson *et al.*, 1984). However, developments in molecular microbial ecology have circumvented the need to culture isolates and can provide insight into the composition and diversity of a microbial community. Molecular microbiology utilises the information contained within a microbial communities nucleic acids to identify bacterial groups and analyse species diversity.

A variety of molecular microbial methods exist to obtain information regarding microbial diversity. In general, the first step in molecular microbial ecology is the extraction of nucleic acids from the microbial community. Once this has been achieved, the DNA can be used in either Polymerase Chain Reaction (PCR)-cloning-sequencing procedures, electrophoretic analysis, directly hybridised with probes, used in DNA hybridisation experiments (see Chapter 1 for a review of each technique). The choice of technique will depend on the information desired and available resources. However, they all give insight into the diversity or species composition of the microbial community. As discussed in Chapter 1, these techniques are not without their limitations and disadvantages that need to be considered before undertaking any study using them.

The aim of this part of the project was to investigate the use of the microbial diversity as an indicator of ecosystem 'health' in Campbells Creek. Molecular microbial techniques were used to perform between site comparisons of genetic diversity, upstream and downstream of the Castlemaine sewage effluent discharge point. Microbial community DNA was extracted from each site and its similarity measured with DNA cross-hybridisation. PCR was employed to amplify the 16s rRNA gene of the extracted DNA with the intention of cloning and sequencing the fragments to identify the bacterial groups present.

5.2 Molecular microbial techniques

5.2.1 DNA cross-hybridisation

The method of DNA cross-hybridisation exploits the specificity of DNA hybridisation to determine the degree of identical or very closely related bacterial strains that two different microbial communities have in common. In this procedure DNA extracted from one environment is radioactively labelled, denatured, and then allowed to reassociate with denatured DNA from a different environment. The level of cross-hybridisation (reassociation) represents the genetic similiarity of the two bacterial communities (Lee and Fuhrman 1990). The fraction of identical DNA shared is estimated by comparing the radioactive signal obtained from cross-hybridisation with that from self hybridisation.

The application of cross hybridisation to natural bacterial assemblages allows for the comparison of bacterial communities and can therefore be employed to reveal shifts in bacterial composition that may have occurred between two sites. If a source of pollution or some other environmental degradative event exists between sites then DNA cross-hybridisation has the potential as a tool for uncovering changes in microbial populations that represent a decline in river 'health'. If DNA-cross hybridisation is to be an appropriate method for estimating the similarity of bacterial communities then it is important that different strains do not cross-hybridise. Studies have shown that generally this is not a major concern with total DNA rarely cross-hybridising (from <1 to 11%) with the species tested (Lee and Fuhrman 1990). This, however, could become a problem in situations where the two communities being compared, contained very closely related bacterial strains which were a major component of the population (Lee and Fuhrman 1990). Because of the specificity of DNA hybridisation and its ability to discriminate down to the species level, it is accepted that cross-hybridisation of more than 70% represents a single bacterial species. Therefore, two communites that have greater than 70% of their DNA in common are the same at the species level.

5.2.2 PCR-Clone-Sequence

PCR-cloning and sequencing are methods that can be used to analyse the diversity of naturally occurring microbial populations. DNA extracted from environmental samples is subjected to PCR amplification using primers specific for the 16S rRNA gene. This particular gene is most commonly chosen because it contains regions (or sequences) that are universally conserved at the 5' and 3' ends which allows for the amplification and recovery of nearly complete 16S rRNA genes. Within these conserved regions of DNA there are also sequences which are specific or unique to particular microbial groups (Genus, species and strain). The sequencing of amplified 16S rRNA genes, therefore, permits the identification of bacteria.

PCR products can be cloned into commercially available plasmids designed specifically for sequencing purposes resulting in the production of a clone library.

Sequencing the cloned PCR product is facilitated by the presence of universally conserved sequences along the length of the 16s rRNA gene which allow the design of primers for the complete sequencing of the molecule. The sequences obtained from the clone library can be analysed by specialised computer programs, and the diversity of the microbial community determined.

PCR can also be applied to directly detect individual bacterial species (eg. *E. coli*) against a background of ambient bacteria by designing primers specific for sequences unique for that particular species.

5.3 Materials and methods

5.3.1 Sample collection and filtration

Water samples were collected from sites A, B, C and D (Fig 2.3) to perform total DNA extractions of the entire microbial community. Five litre composite samples were collected (as 5 x 1 L subsamples) from the middle of the creek.

The water samples from each site were pre-filtered through 90 mm glass fibre filters (Bonnet Equipment) with a nominal pore size of 3.0 µm, to remove most eukaryotic organisms and to speed up filtration through the final filter. The entire bacterial community was collected by pressure filtration onto 0.2 µm cellulose acetate or polycarbonate membrane filters (Bonnet Equipment). Generally, 1 L volumes were collected on each filter before they became blocked, this would take approximately 1 hour (a little less for upstream samples). All filtration was completed on the day of collection. The filters were stored at -20°C until the extraction procedure could take place. Bacterial numbers were determined in unfiltered water and filtrates by epifluorescence microscopy with DAPI staining within one month of collection (see section 2.2.2).

5.3.2 Tangential flow filtration

Tangential flow filtration (TFF) was trialed in an attempt to increase the number of bacterial cells, and hence DNA from each site and to increase the speed of filtration. For

these experiments the volume of water collected from each site at Campbells Creek was increased to 10 litres. The water was first prefiltered through 3.0 µm pore size glass fibre filters as described above. The TFF system concentrates all particles within a sample above a certain size, the TFF used in these experiments was a Minitan-S Ultrafiltration System supplied by Millipore. The size of the particles concentrated depends on the size of the filters used in the system. In this instance, 0.2 µm filters (GVLP Minitan plate, Durapore, Millipore) were used and so all matter (biological and non-biological) larger than 0.2 µm was concentrated. The degree of concentration can vary, but generally samples were concentrated from 10 L volumes to 150-250 mL. It is very difficult to concentrate samples more than this as sufficient concentrate is required to wash the filters at the end of filtration and some sample is also retained within the system. Once the filtration was complete, the TFF instrument was dismantled and the filters cleaned carefully in the concentrate. A substantial amount of particles and cells are caught on the filters, and to ensure that potential DNA is not lost, as much of the material must be removed from the filters as possible. This was achieved by carefully rubbing the filters with a finger (wearing sterile gloves) in the filtrate. Small aliquot's (100 µL) of the concentrate were collected to perform direct cell counts, using DAPI staining and epifluorescent microscopy. The remaining concentrate from each site was stored in 85 mL centrifuge tubes at -20°C.

To prevent cross contamination between samples, two TFF systems and sets of filters were used in the filtration process, one for upstream samples and the other for downstream samples.

5.3.3 DNA extraction

The DNA was extracted from the filters according to the procedure described by Furhman *et al.* (1988). The frozen filters were thawed and cut into small pieces with a sterile razor blade. The filter pieces were suspended in 4 ml of STE buffer (10mM Tris hydrochloride pH 8, 1mM EDTA, 100 mM NaCl) and vortexed briefly. An equal volume of 10 % sodium dodecyl sulfate (SDS) was added and mixed gently by inverting the tube. The tubes were placed in a boiling water bath for 5 minutes. The liquid was poured into a

clean centrifuge tube. The filter pieces were rinsed with 1-2 ml of STE buffer which was then poured into the centrifuge tube. The liquid was centrifuged for 10 minutes (10,000 x g) at 15 °C to remove cellular debris.

The DNA was purified from this solution by ethanol precipitation; 1.5 ml of 10.5 M ammonium acetate plus 14 mL of ice cold 100% ethanol were added and precipitation proceeded for a minimum of 2 hrs at -20°C. The DNA was pelleted by centrifugation (20 min, 14,000 x g, 4°C) and the resulting pellet air dried. The DNA was resuspended in 0.5 ml of TE buffer (10 mM Tris hydrochloride [pH 8], 1 mM EDTA) and transferred to a 1.5 mL centrifuge tube (eppendorf). Phenol (0.5 mL, pH 8) was added and the contents mixed by inverting the tube a number of times. The solution was subsequently centrifuged at 15,000xg for 2 min to separate the organic and aqueous phases. The lower organic phase containing contaminants was removed. The remaining interface and aqueous phase were re-extracted with 0.6 ml of phenol-chloroform (3:1). The final extraction was with 0.5 Ml of chloroform and this time the interface was removed. The DNA was precipitated with 120 µL of 10.5 M ammonium acetate and 1 Ml of ice-cold ethanol for at least 1 hour at -20°C. To pellet the DNA, the cold solution was centrifuged at 4°C for 10 minutes (15,000xg). The pellet was dried under vacuum and resuspended in 300 µL of TE buffer.

5.3.4 Modifications to the DNA extraction procedure

The pellet obtained after DNA extraction, using the above method, was often still contaminated with humic material. Acid-washed polyvinylpolypyrrolindone (PVPP) was trailed to help purify the DNA. PVPP has been used by many researchers to help purify DNA extracted from soils or sediments (Holben *et al.*, 1988; Steffan *et al.*, 1988). PVPP (75 g) was suspended in 3 M hydrochloric acid for 12-16 hours. The suspension was then filtered and resuspended in 1 L of 20 mM potassium phosphate buffer. This process was repeated until the pH of the PVPP had reached 7.0. The acid washed PVPP was used in a DNA extraction protocol referred to as Direct Lysis Alkaline method (Atlas 1993).

A membrane filter containing a filtered sample was cut into small pieces and submerged in 4 mL of 10% SDS. The filter and SDS was vortexed thoroughly. The lysed

mixture was decanted into a clean tube and the filter pieces were washed with 1 mL of 10% SDS to remove any DNA bound to the filter. Acid washed PVPP (1 g) was added to the lysed cell mixture to help remove humic material. The solution was centrifuged for 5 Min at 10,000xg to pellet and separate the PVPP from the DNA. The supernatant was poured into a fresh centrifuge tube. To recover any remaining DNA, 2 mL of 0.12M sodium phosphate buffer (pH 8.0) was added to the PVPP pellet. The solution was again centrifuged for 5 Min at 10,000xg. This washing step was repeated twice more and all the supernatant's were pooled. NaCl was then added to the DNA suspension to give a final concentration of 0.5M.

When PVPP was used in the extraction procedures 0.5 volumes of 50% weight/volume of polyethylene glycol (PEG) was used to precipitate the DNA at 4°C for the first precipitation step instead of ethanol. Other contaminants were removed from the DNA by performing one phenol and two phenol-chloroform-isoamyl alcohol (25:24:1) extractions. For samples that were particularly dirty, PVPP was also added after the phenol/chloroform purification steps. Potassium acetate was added to achieve a final concentration of 0.5 M and the samples were incubated on ice for 2 hours. Less than 0.5 g of PVPP was then added and the mixture was incubated for 15 minutes (room temperature) with intermittent swirling. The PVPP was then separated from the DNA solution by centrifugation (10,000xg, 5 min). The DNA was precipitated from solution by adding ethanol and incubating overnight at -20°C. The DNA was pelleted by centrifugation and resuspended in TE buffer as mentioned earlier.

In some instances the extracted DNA was further purified by putting it through a Promega wizard DNA clean up column according to the instructions acquired with the kit.

5.3.5 DNA extractions on TFF concentrates

A modified procedure to the one described in section 5.2.3 and 5.2.4 was used to extract the DNA from the TFF concentrates because because the samples were not concentrated onto filters. The concentrates were thawed in a 70°C water bath for 1 hour with 0.5 g of SDS added to them. The concentrates were regularly mixed to facilitate the thawing process and to dissolve the SDS into solution. The samples were allowed to cool

before 1 g of acid washed PVPP was added. The PVPP containing the contaminants was removed from solution by centrifugation (10,000xg, 5 min). The supernatant was poured into a clean 85 mL centrifuge tube and the PVPP was washed with 0.12 M sodium phosphate buffer (pH 8.0) to recover any remaining DNA. Enough 0.12M sodium phosphate buffer was added so that it would make up 50% (vol/vol) of the solution containing the DNA. The centrifugation step was repeated. The supernatant's were pooled, mixed and then separated into two tubes if the volumes were to large for one. NaCl was added to the DNA suspension to give a final concentration of 0.5 M. The DNA was precipitated by adding 0.5 volume of PEG 8000, mixing very well and incubating overnight at 5°C. The procedure then continued as the direct lysis alkaline method described above.

5.3.6 Polymerase chain reaction (PCR)

Polymerase chain reaction was employed to amplify bacterial rRNA genes. The sequence of the primers used in the reaction were as follows (DeLong *et al.*, 1993):

5'-AGA GTT TGA TCC TGG CTC AG-3'

5'-GGT TAC CTT GTT ACG ACT T-3'

The primers were synthesized at the Microbiology Department, Monash University. The primers were resuspended in sterile deionised water and diluted to a concentration of 12.0 pmole/μL. The PCR reactions were made up in 50 μL volumes which were set up on ice. The thermal cycling was performed in a MJ-Research PCR machine (PTC-100™). The amplification of the 16 S rRNA gene was originally performed on purified *E. coli* DNA to optimise the reaction conditions. The reagents (Promega) were added to the PCR tube in the following order: RBx10 (reaction buffer, 5 μL per tube) DNTPs (25 mM, 2.5 μL per tube), MgCl (1mM, 2mM, 3mM, 4mM, final concentrations trailed), primers (25 pmole), DNA (1 μL), and sterile deionised water to bring the final volume up to 50 μL. The tubes were then pulsed centrifuged to ensure the reagents were well mixed. The Taq DNA Polymerase (0.5 μL per tube) was added last, just before the tubes were placed in the cycler. Mineral oil was overlayed over the reaction mix to prevent evaporation. Four different cycle programs were trailed during the PCR experiments and these were;

Cycle (1)

95°C 1.5 Min, 55°C 1.5 Min, 72°C 1.5 Min for 30 cycles

Cycle (2)

95°C 1.5 Min, 50°C 1.5 Min, 72°C 3 Min for 30 cycles

Cycle (3)

94°C 5 Min, 55°C 1.75 Min, 72°C 3 Min for 1 cycles

94°C 1.5 Min, 55°C 1.75 Min, 72°C 3 Min for 28 cycles

94°C 1.5 Min, 55°C 1.75 Min, 72°C 7 Min for 1 cycles

Cycle (4)

94°C 1 Min, for 1 cycle

94°C 1 Min, 54°C 30 sec, 72°C 30 sec for 25 cycles

5.3.7 Agarose gel electrophoresis

To visualise extracted DNA and PCR products, small aliquots were separated on agarose gels by electrophoresis (Sambrook *et al.*, 1989). Agarose (0.5 g) was added to 50 Ml of 1xTAE buffer (0.04M-Tris-acetate, 0.01M EDTA) and heated until the solution had boiled and all agarose had dissolved. A drop of ethidium bromide (10 mg/Ml stock solution) was added to the molten agarose which was left to cool to approximately 55°C before it was poured into the electrophoresis tray. When the gel had set, 5 µL volumes of sample were mixed with 1 µL of blue gel loading buffer (Promega) and added to the wells. Lambda *HindIII* markers were ran alongside the samples in order to estimate, in base pairs, the size of the DNA. The known concentrations of Lambda *HindIII* DNA were also used to estimate the concentration of DNA on the gel. The ethidium bromide stained DNA was visualised by UV fluorescence.

5.3.8 Dot blot hybridisations

The nylon membrane (Nitrobind) for the immobilisation of DNA, was firstly cut to shape and soaked in distilled water, followed by 5xSSC (20XSSC buffer; 3M NaCl, 0.3M trisodium citrate, pH 7.0) buffer for 5 minutes. Once the membrane had been washed, it

was placed in the dot blot vacuum manifold (Schleider & Schuell, Minifold II) and the samples were prepared. The target DNA (0.5 ng-500 µg) was denatured by heating it for one hour at 60°C in NaOH (final concentration 0.3 M). The samples were cooled to room temperature and ammonium acetate was added to a 1M concentration. The DNA was subsequently boiled for 5 minutes in a water bath and then placed immediately on ice. Serially diluted (1:2) target DNA was dot blotted onto the membrane under vacuum (15 psi) and immobilised by baking at 80°C for 1.5 hours.

The DNA probes used in hybridisation experiments were prepared from bacterial genomic DNA, extracted from the microbial population in Campbells Creek. The DNA was radioactively labelled using a Prima-Gene labelling kit (Promega) according to its following instructions. The DNA intended as a probe was firstly linearized by using the restriction enzyme *EcoR1*. The restriction digest procedure described by Sambrook *et al.* (1989) was employed. Sterile deionised water was added to the DNA to give a final volume of 18 µL. Reaction buffer (2µL) was added to the DNA solution followed by 0.5 µL of the restriction enzyme, *EcoR1*. Digestion was allowed to proceed at 37°C for 1 hour when it was then terminated by heating the digest mixture to 65°C for 10 Min.

The linearized DNA was radioactively labelled with ^{32}P (3000 Ci/mmol, 10 µCi/µL). The DNA (25 ng) was dissolved in 32 µL volume with sterile water in a 0.5 ml PCR tube. The DNA was denatured by boiling in the heat block of a PCR machine. The DNA was cooled rapidly on ice and the other reagents were added in the following order: buffer (10 µL), dNTPs (2 µL), bovine serum albumin (2 µL), ^{32}PdCTP (3 µL) and lastly the Klenow fragment (1 µL). The solution was mixed gently and incubated at room temperature for 1 hour. The reaction was terminated by heating the tube for 2 minutes in a boiling water bath. To ensure the DNA remained denatured the labelled probe was chilled rapidly on ice and EDTA was added to give a final concentration of 20 mM. The probe was then either used immediately in a hybridisation reaction or stored at -20°C.

Before the radioactive probe can be added to the membrane containing the single stranded target DNA, the membrane must firstly be prehybridised. This process involved the incubation of the membrane at 68°C for 6 to 8 hours in 50 Ml of; 6xSSC, 5xDenhardts

solution (50x Denhardts-1% Ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin) and 1 mg of denatured calf thymus DNA. The prehybridisation solution helps prevent non-specific binding of the probe to the membrane. Once prehybridisation has occurred, the probe is added to the prehybridisation solution and incubated for 24 hours at 68°C. The prehybridisation and hybridisation steps were performed in 100 ml cylindrical bottles (Schott) that fitted into a rotating apparatus in the incubator. The membranes were rolled into cylinder shapes and placed in the bottles, the prehybridisation solution was then poured in and the rotating ensures that the solution is well mixed and coating the membrane. This method is a lot easier and cleaner, with less chance of leakage of radioactive material then heat sealing plastic.

The following day after the hybridisation step had concluded the membranes were washed twice in; 2xSSC (5 Min at room temperature), 0.5% SDS in 2xSSC (30 Min at 68°C) and 0.1% SSC (5 Min at room temperature). The membranes were then allowed to dry at room temperature and subsequently sealed in plastic. The sealed membranes were then placed in a autoradiography cassette with a piece of autorad film (Kodac) and left to expose at -20°C. The films were developed using Kodac developing and fixative solution.

5.4 Results and Discussion

5.4.1 Concentrating aquatic bacteria for DNA extractions

The first step in the use of molecular microbial techniques as a tool to study aquatic bacterial communities is extraction of the genomic DNA. To extract the DNA, the bacteria need to be in a concentrated form. There are many techniques available to concentrate the microbial fraction of water including filtration and centrifugation. The numbers of bacteria in natural aquatic environments are very low (10^8 to 10^{10} cells/L), and as a result large quantities of water need to be concentrated so that a reasonable quantity of DNA can be acquired. The water samples must be filtered immediately (12-24 hrs) after collection to limit the possibility of the microbial community composition altering from the change in environmental conditions (Furhman, 1988). The species composition can alter from: (a) the

possible dying off of certain bacterial groups; (b) other bacterial groups increasing in number as a response; or (c) if the samples are kept for too long any bacteria that may have been introduced into the sample during collection will have had time to multiply and contribute to the composition. If this latter was to occur, the DNA extracted from the samples would not be a true representation of the natural microbiota.

Concentrating large volumes of river water can be a problem. The equipment required to filter large quantities of water (>20 litres) is expensive and therefore not always available, depending on the laboratories resources. There are a number of possible techniques or pieces of equipment that can be used to undertake the task of concentrating the bacterial population from large volumes of water. Centrifugation is one such method. However, a high speed centrifuge (>10,000xg) that can spin large buckets (1 L) is required so that the samples can be processed relatively quickly. Investigators (Fuhrman, 1988) have also found difficulties in obtaining a visible pellet of bacterial cells and many bacterial cells were lost. An initial pre-filtration step may also be necessary to remove organic and inorganic matter larger than 3.0 µm from contaminating the DNA. Various filter holders are available that can hold filters from 25 mm to 120 mm in diameter. Filtering substantial volumes of natural water samples through a 0.2 µm filter is very slow and tedious, particularly if the sample is very turbid. Obviously, the larger the filter, the more rapid the filtration process. Stirred cell filtration systems are quicker, but are expensive particularly for systems that can hold more than 1L. There are also a range of more complicated concentrating equipment available such as Tangential Flow Filtration systems. Whatever the choice of equipment it is important that filtration is performed in less than 24 hours and that as little of the microbial community is lost (cells adhering to filters or failing to pellet during centrifugation) as possible. It is also preferable that the equipment can be sterilised or well cleaned in suitable detergent and acid to limit the possibility of cross contamination between samples.

The filtration equipment that was available for this project (2 X 120 mm diameter and 2x90 mm diameter filter holders) allowed 5 litres from each site (20 litres total) to be pre-filtered and the filtrate collected onto 0.2µm filters in the recommended time (12-24hrs).

Tangential flow filtration (TFF) was trialed but it was found that 40-90% of the cells were lost in the process, most probably from the ineffective removal of bound cells from filters (Table 5.1). It was also difficult with TFF to prevent samples from being contaminated during the cleaning of the cells off the filters into the concentrate.

Table 5.1 Cell concentration after Tangential Flow Filtration concentration

Cell numbers in TFF concentrate	Expected cell numbers	% cells collected after TFF concentration
6.9×10^9	1.6×10^{10}	43
9.6×10^9	1.6×10^{10}	60
2.8×10^{10}	2.1×10^{11}	18
9.8×10^{10}	1.4×10^{11}	17
5.6×10^9	1.8×10^{10}	31
1.7×10^9	1.5×10^{10}	11
1.3×10^{11}	6.0×10^{11}	21
2.1×10^{10}	9.5×10^{10}	22

5.4.2 Extraction of DNA

Bacterial abundance in the water samples ranged from a 1.6-5.5 x 10^9 cells per litre upstream of the sewage outlet and 1.3-59 x 10^9 cells per litre downstream. Direct counts of the filtrate after pre-filtration through glass fibre filters showed that less than 10% of the population was removed during this step. Despite this preventable loss of bacteria, pre-filtering was still necessary to substantially reduce the number of other organisms and abiotic material in the sample and to increase filtration speed. Assuming a DNA/cell value of 5.7 fg (approximation of DNA in marine bacterium), the five litre of samples (10^9 cells/L) collected from each site could potentialy yield 11-37 µg of DNA upstream and 51-1800 µg downstream. A 100% recovery is, however, not achievable. Furhman *et al.* (1988) found that with their extraction procedure approximately 23 to 45% of the total bacterial DNA in a

sample was recovered. This significant loss of DNA during the extraction process reduces the quantity of DNA obainable from each site to less than 5-16 µg upstream and 23-800 µg downstream.

The difficulties encountered in recovering nucleic acids from environmental samples did not stop at the initial filtration step. DNA extraction procedures on environmental samples are not as straight forward as those used on pure cultures. The extraction method must be non-selective and recover as much of the total community DNA as possible. The ability to achieve this is hindered by the fact that some microbial groups are more readily lysed then others (Head *et al.*, 1998). Spores and Gram positive cells are more resistant to lysis then vegetative and Gram negative cells, respectively (Head *et al.*, 1998). Direct cell counts performed after the initial lysis step revealed that a lysis efficiency of greater than 90% could be obtained with the DNA extraction procedures used in this study. These results are only representative of the suspended bacteria, and it is not known if the bacterial cells on the filter lysed at the same efficiency.

5.4.3 Purification of DNA

The second problem encountered when extracting DNA from natural samples is purifying the DNA. Removing humic matter and fine particulate matter from the sample is very difficult as they have a similar size and weight as DNA, and therefore complete removal by centrifugation or filtration is not possible. DNA of sufficient purity for use in molecular experiments can be achieved with the use of humic-binding substances such as polyvinylpolypyrrolindone (PVPP), enabling their removal by centrifugation and DNA purification columns. Additional phenol/chloroform extraction steps were also included for some samples in an attempt to remove contaminating substances. The disadvantage of including extra purification steps is that the recovery efficiency of DNA is further reduced. Obtaining reasonable quantities (> 5 µg) of clean DNA proved to be very difficult.

Electrophoresis in a 1% agarose gel revealed that the majority of the DNA extracted from sites C and D was larger than 23,000 base pairs (23-kbp), indicating that the extraction procedure produced mainly high molecular weight DNA (Fig. 5.1). It also revealed,

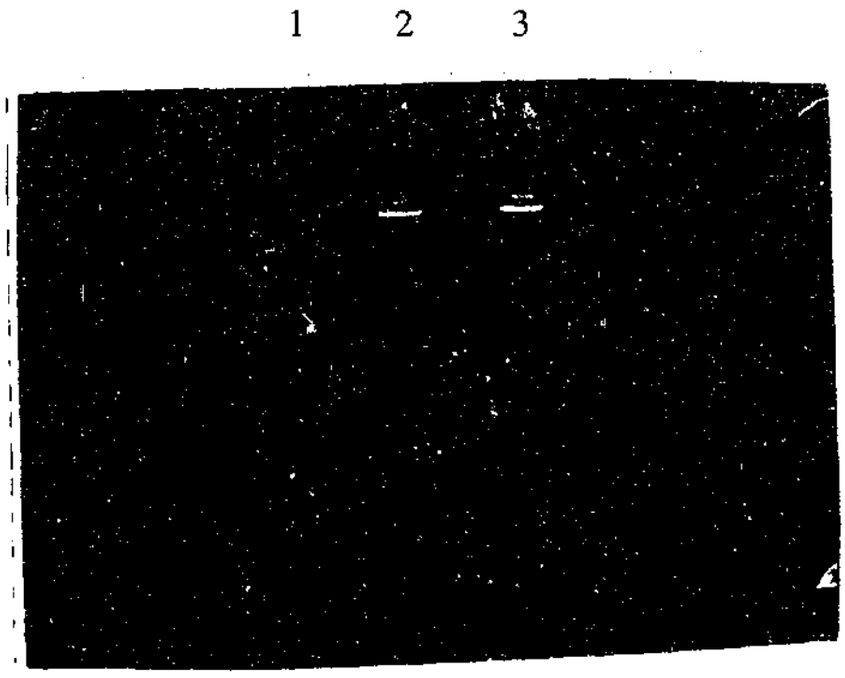


Figure 5.1 Ethidium bromide-stained 1.0% agarose gel of total DNA extracted from bacteria collected from site C at Campbells Creek. Lanes1, *HindIII*- digested labda DNA (molecular sizes: 23.1, 9.4, 6.6, 4.4, 2.3, and 2.0 kbp, from top to bottom , repectiely); 2, undigested bacterial DNA from Campbells Creek; 3, undigested bacterial DNA from Campbells Creek.

however, by comparing the DNA illumination (from ethidium bromide staining) on the gel with that from a known concentration of standard (lambda DNA cut with the restriction enzyme *Hindi III*) that substantial losses of DNA were occurring. The combined losses from pre-filtration, extraction and purification resulted in a low recovery of the total DNA. Only 1 litre volumes of sample could be filtered onto each 0.2 μm filter before the filters became blocked. No DNA was evident when 10μl (10% of the total extraction volume) volumes from extractions performed on upstream or winter downstream samples were separated on an agarose gel. The sensitivity of ethidium bromide-electrophoresis visualisation of DNA is approximately 80 ng, and so, at most, only 0.8 μg of DNA was recovered from a 1 litre sample containing an estimated 7.0 μg of DNA. In other words, 90% of the total DNA was lost during the filtration, extraction and purification of the natural samples.

With the equipment available, it was only possible to visualise DNA by electrophoresis for the downstream samples where the concentration of bacteria was very high (10^{10} cells/L). The recovery of DNA could have been improved if larger volumes of water could have been filtered. Unfortunately, with the equipment available this would have exceeded the time required to maintain sample integrity. The loss of DNA experienced during the extraction and purification steps is considerably greater than the 23-45% estimated by Furhman (1988). Most likely, the cause of this greater loss was the additional purification procedures required to obtain a clean DNA pellet from the Campbells Creek samples. Furhman's (1988) experiments were performed in marine environments which contain less contaminating particulate matter, and where intensive purification steps were not required.

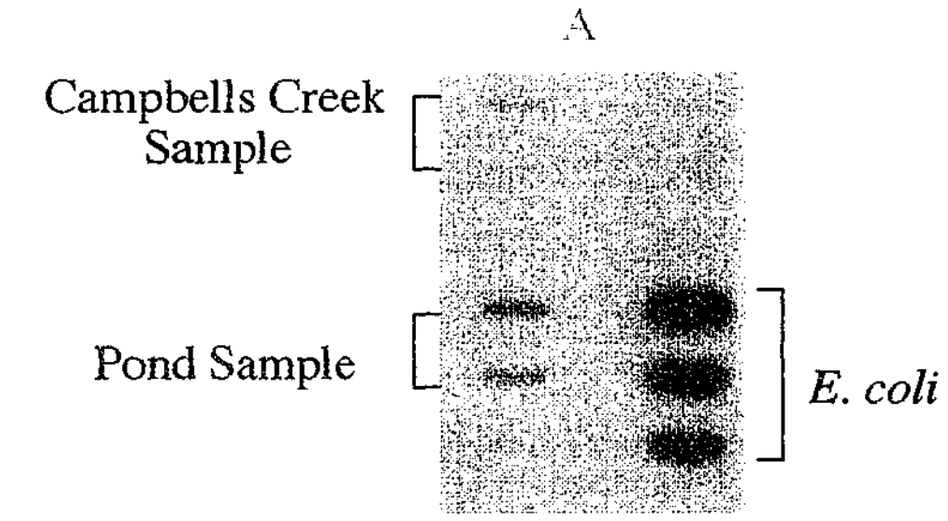
5.4.4 Dot blot hybridisation of bacterial community DNA

Dot blot hybridisation of DNA is a technique that can be employed to examine community DNA similarity, this being the proportion of identical DNA sequences that two communities share. DNA extracted from one community is irreversibly bound to nylon membrane and is cross-hybridised (or probed) with DNA extracted from another

community. The extent of hybridisation (determined by the band intensity) is compared with that obtained from self-hybridisation (ie. DNA extracted from one particular site probed with its own DNA) and represents the level of similarity between the two communities. For example, if the band intensity (autoradiography) observed from cross-hybridisation is only 50% of that from self-hybridisation then the two communities have 50% identical DNA. This method can be applied, in river 'health' assessment to uncover any changes that may have occurred in species composition as a result of a reduction in microbial diversity.

It was possible to obtain sufficient quantities of DNA from a small number of downstream samples from Campbells Creek to trial the DNA-DNA hybridisation technique. The hybridisation method was successfully applied to these Campbells Creek samples, along with DNA extracted from Marybinong river samples, Hedgley Deene pond and *E. coli* DNA. Figure 5.2 shows two hybridisation membranes, Fig 5.2.a contains immobilised DNA from all four samples and has been probed with *E. coli* DNA. As expected the most intense bands on the filter are with the *E. coli* DNA, this is referred to as self hybridisation and is the control and the reference point for the other samples. The less intense bands obtained for Campbells Creek and Hedgley Deene pond indicate that *E. coli* is present in these samples, but obviously in lower concentrations. The second filter in this figure (2.b) has been probed with DNA extracted from site C, Campbells Creek. Once again, the self hybridisation of DNA from site C produced the strongest band. The results from this cross-hybridisation experiment also show that the Marybyrnong River, Hedgley Deene pond and Campbells Creek have a number of bacterial species in common.

The observation that band intensity is greater when the more complex DNA from Campbells Creek is used as the probe against *E. coli* DNA than with the reciprocal hybridisation, concurs with the findings of Lee and Fuhrman (1990) that the hybridisation of a mixture DNA probe to a single-strain DNA target often results in the overestimation of similarity. This is caused by the complexity of DNA. For example, if the mixture probe (Campbells Creek in this instance) contains 10% *E. coli* DNA, a sample target of 100% *E. coli* would have 10 times the binding sites for the *E. coli* DNA than that of the control (Campbells Creek mixture) target which contains only 10% of *E. coli*..



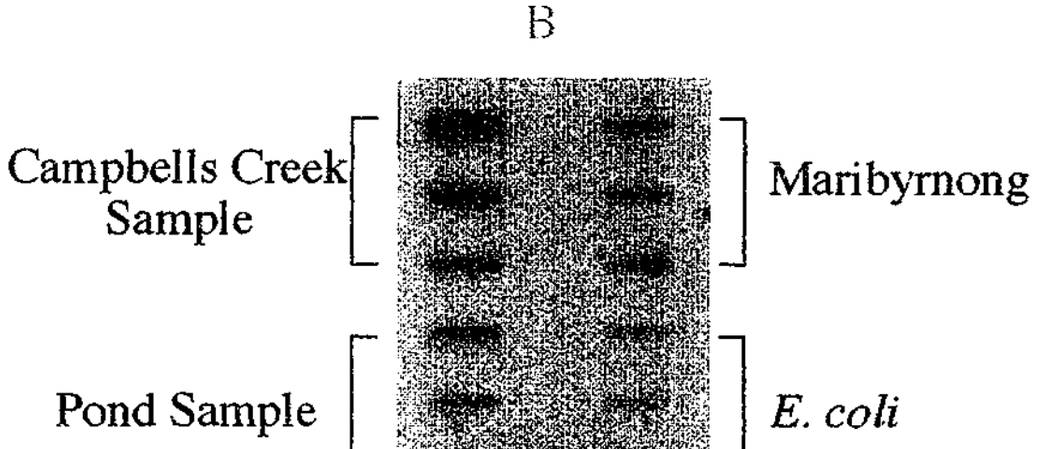


Figure 5.2 DNA cross hybridisation. X-ray film exposed to nylon filters containing radioactively labelled DNA. Both filters contain immobilised single stranded DNA extracted from; Campbells Creek, Hedgely Dene pond, Maribyrnong River and *E. coli*. A 1:2 serial dilution of each sample was performed. Filter A was probed with single stranded *E. coli* DNA and Filter B with Campbells Creek DNA.

The results of the dot blot DNA hybridisation with DNA extracted from three different aquatic environments (Campbells Creek, Marybyrnong River, Hedgeley Deene Pond) has highlighted the potential of this method to provide information on species variations between sites. The differences observed between the three samples used in these experiments are as expected given the very different environments they are from. The variation in band intensity observed after the cross hybridisation of DNA from each site, implies that there are differences in DNA sequences, and these differences are likely the result of variations in species composition between the three communities. By revealing the degree of similarity between sites, this technique can therefore, also demonstrate how microbial populations adapt to a range of environments by varying their species composition.

The inability to obtain sufficient quantities of purified DNA significantly hindered attempts to use molecular microbial techniques to examine microbial diversity and to subsequently use this information to assess river 'health'. With such low concentrations of DNA recovered from upstream samples and winter downstream samples, it was not possible to perform the necessary auxilliary hybridisation experiments. The problems encountered in recovering DNA from environmental samples prevented the application of this technique to samples from site A or B. Comparisons of community DNA upstream and downstream of the Castlemaine sewage treatment plant as a method of determining bacterial community similiarity therefore could not be performed.

5.4.5 Polymerase chain reaction amplification (PCR) of 16s rRNA

The technique of PCR was employed in an attempt to identify bacterial species present in Campbells Creek. The aim was to amplify a 1 kb segment of 16s rRNA DNA using primers specific for regions of the gene which have been conserved across all bacterial species. The amplified product can then be cloned into commercially designed plasmids for sequencing. The origin of the 16s rRNA DNA can be determined by comparing the obtained sequence with those on extensive gene bank programs. This method of species

identification can be used to identify novel gene sequences, indicating a possible new strain or species. PCR and gene sequencing can also reveal differences in community composition that may exist between different sites or environments. However, ensuring that a library of sequences truly represents a particular bacterial community is an extremly labor intensive task and not without many uncertainities (see Chapter 1).

The amplification of the 16s rRNA gene was originally performed on a DNA extracted from pure *E. coli* culture using primers (see section 5.2.6) obtained from the literature (DeLong *et al.*, 1993). The amplification cycle used by DeLong *et al.* (1993) was not successful at producing a 1 Kb PCR product in the thermal cycler available for this study. Four various programs were trialed before a 1 Kb PCR product was obtained (see section 5.2.6). The successful cycle program was as follows; 94°C 1 minute, for 1 cycle, 94°C 1 minute, 54°C 30 seconds, 72°C 30 seconds for 25 cycles. The optimum $MgCl_2$ concentration for these PCR reactions was found to be 3 mmoles (Fig. 5.3).

Once PCR amplification of 16s rRNA DNA had been successfully applied to *E. coli* DNA, it was trialed on DNA extracted from site C, Campbells Creek. A 1 Kb PCR product was obtained, however the yield was very low and was not improved with additional amplification cycles. The most likely cause for the low concentrations of PCR product obtained with DNA extracted from environmental samples, is the presence of contaminating substances that are acting as PCR inhibitors. All future attempts at using PCR in conjunction with DNA sequencing to identify bacterial species present in Campbells Creek were abandoned once it became evident that obtaining sufficient quantities of purified DNA was not possible with the resources available for this project. Unfortunately, because adequate amounts of pure DNA from Campbells Creek could not be obtained, further optimisation of reaction conditions, PCR amplification of 16S rRNA and its subsequent further manipulation (cloning-sequencing, Restriction Fragment Polymorphism analysis) were not possible. It should be noted that aquiring sufficient quantities of pure DNA, is not the only limitations associated with PCR. Additional concerns such as template bias and chimeric template formations (Chapter 1) must also be considered.

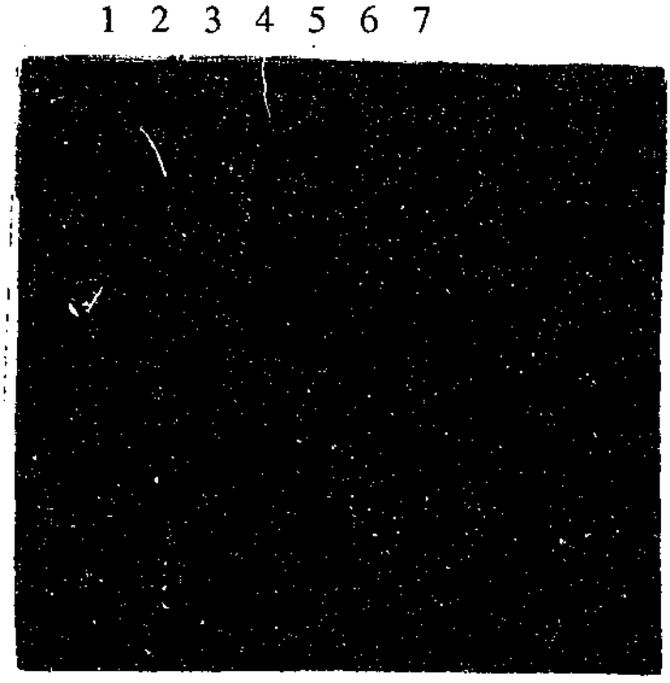


Figure 5.3 Ethidium bromide-stained, 1.0% agarose gel of PCR products obtained from amplifying DNA extracted from site C at Campbells Creek. Lane 1: *HindIII*- digested labda DNA (molecular sizes: 23.1, 9.4, 6.6, 4.4, 2.3, and 2.0 kbp, from top to bottom , repectiely); Lane 2: PCR performed with 1 mmole of $MgCl_2$; Lane 3: 2 mmole of $MgCl_2$; Lane 4: 3 mmole of $MgCl_2$; Lane 5: 4 mmole of $MgCl_2$; Lane 6 & 7: 200 bp control DNA .

5.5 Conclusion

Microbial populations have potential as indicators of biological diversity, a critical factor in maintaining and monitoring river 'health'. Presently, the only techniques available to examine bacterial species composition, and hence biological diversity of aquatic communities, are molecular microbial methods. The successful application of these techniques to environmental samples has important implications in studies of river 'health'. Molecular methods utilise the unique differences in genetic sequences between bacterial species and eliminate the necessity to culture isolates. Molecular microbial techniques are powerful tools that can provide environmental researchers with invaluable information concerning biological diversity that was previously unobtainable (Giovannoni *et al.*, 1990; Ward *et al.*, 1990) . The use of bacterial diversity and changes in bacterial species within and between communities in river 'health' assessment relies on molecular microbial methods. These methods have been largely developed on pure culture isolates, and many obstacles must be overcome before they can be successfully used in natural aquatic environments.

The first problem encountered in this study of microbial diversity in Campbells Creek, was in concentrating the microbial fraction in the water column. In contrast to bacterial cultures, cell concentrations in natural aquatic systems are low and therefore must be concentrated prior to manipulation with molecular microbial techniques. Although this sounds trivial, concentrating large quantities of creek water within a short period of time (<24 hrs) can be a difficult task. The equipment and labor required to concentrate sufficient quantities of aquatic bacteria for molecular analysis is costly, and must be considered thoroughly prior to undertaking studies of microbial diversity.

The poor yield of sufficiently uncontaminated DNA, obtained after extraction and purification of DNA from the microbial community was also a fundamental problem. The intensive purification steps required, combined with the loss of cells and DNA during the filtration and extraction procedure resulted in only 10% of the potential DNA being

recovered. This substantial loss of DNA and the inability to concentrate larger volumes of water thwarted attempts to analyse the microbial diversity in Campbells Creek.

Molecular microbiology has the potential to provide insights into microbial diversity/ecology and therefore river 'health', in habitats where conventional methods have failed. However, before these methods can be routinely used in assessments of river 'health', futher research is required to address the problem of acquiring sufficent quanitities of pure DNA that is a true representative of the microbial community. There are other difficulties with some molecular methods, such as PCR, where preferential amplification of some sequences and the formation of chimeric PCR products are a major concern.

It has been found in higher trophic levels (e.g. macroinvertebrates) that biological diversity generally decreases as a result of reductions in water quality (Norris and Norris 1995). However, the lack of appropriate techniques have left the area of diversity relatively under researched in microbial communities. Because of the limited information concerning microbial diversity in natural aquatic systems, there exists the possiblity that microbial diversity may not alter as a result of a decline in river 'health'. Instead of a shift in species composition, the contribution of certain bacterial groups in terms of activity, biogeochemical cycling or biomass production may change and go undectected by molecular microbial methods.

Even if the problems associated with the application of molecular microbiology to environmental samples were overcome, the important role of classical microbiology in studies should not be overlooked. Studies based soley on sequence analysis allow assessments of diversity to be made but do not consider the role or function (or even if it has one) of that particular bacterial group in its environment. Ideally, to gain the most from investigations of the microbial community as indicators of river 'health', diversity analysis should be combined with experiments assigning function to form (i.e determining the role a particular bacterium plays in an ecosystem) and determining the abundance of biogeochemically important groups.

SYNTHESIS AND FURTHER RESEARCH

Conserving the 'health' and ecological integrity of Australian rivers has been recognised by government bodies and the community as a major need. Along with an increase in large scale projects assessing river health, there has been a shift in the strategies used to accomplish the task of river 'health' conservation. The acknowledgment that biological diversity is the best indicator of river 'health' has led to the incorporation of biological parameters, along side traditional physico-chemical parameters, into water quality assessment programs. Most of the biological monitoring currently in use is focused on algal and macroinvertebrate populations. Other biological communities, including fish and bacteria, have been suggested as possible alternative indicators of river 'health'. Bacterial communities in particular, have a number of advantages over macroinvertebrates as indicator organisms: they are present in greater numbers which simplifies sampling regimes and their considerably shorter generation times gives them the ability to respond more rapidly to changes in their environment.

However, difficulties in applying traditional microbiological methods to environmental samples has left many gaps in our knowledge of microbial ecology and has restricted the use of bacterial communities as indicators of river 'health'. Without appropriate methods to investigate microbial populations it is not possible to use them as indicators of river 'health' and so the focus of the study reported in this thesis was methodological rather than ecological. The main objective was to examine the techniques available to investigate river microbial populations and to evaluate the use and effectiveness of these methods to provide information on aquatic ecology. The results of this research have implications for the future direction of microbial-based river 'health' assessment. Methods for measuring bacterial abundance (total, coliform and active cell counts), biomass, production and diversity have been evaluated by conducting between site comparisons of the microbial populations upstream and downstream of a single point source of sewage effluent, the Castlemaine STP. Traditional physico-chemical monitoring was also undertaken and compared with the microbial measurements. These between site comparison experiments

enabled the following three methodological aspects to be examined: (a) Inconsistencies and uncertainties associated with the results and their interpretation, for example unacceptable variation between replicate samples or the questionable accuracy of the technique; (b) The difficulties encountered when applying the various methods in aquatic environments where bacterial numbers are usually low and particulate matter concentration is high; and (c) the usefulness of the results in assessing 'health' of a riverine system.

The of physico-chemical water quality data (eg. nutrient concentrations, dissolved oxygen, conductivity, flow rate) was found to be more useful in identifying large differences between the upstream and downstream sites. However, the value of this data in assessing ecosystem 'health' was very limited.

The DAPI staining method of bacterial cell enumeration was performed on samples taken from Campbell's Creek upstream and downstream of the Castlemaine sewage treatment plant. Cell concentration was found to be greatly influenced by flow and was generally higher downstream, with the greatest cell numbers observed in times of low flow. The most probable cause for the higher cell concentration at the downstream sites, particularly at low flows, is the large input of bacterial cells with the sewage discharge. The direct count method of bacterial cell enumeration was easy to perform and resulted in no significant variations between replicate samples. Thus, direct counts of bacterial cells proved to be a useful tool to indicate changes in the microbial population that may occur as a result of adverse changes in water quality. However, fluctuations in bacterial abundance does occur naturally and is strongly influenced by river flow. This is an important consideration when interpreting results and correlating a change in numbers with a possible decline in river 'health'. Disadvantages associated with the direct count method include:

(a) the method is laborious and tedious,

(b) it can be difficult to perform when large amounts of autofluorescing particulate matter are present;

(c) the method does not provide information on the origin of bacterial cells at a particular site. Direct counts cannot reveal if increases in cell concentration have been caused by an

increase in cell division of autochthonous species or from increased inputs of allochthonous cells;

(d) it is not possible to distinguish between the various bacterial groups present and thus fluctuations in cell numbers could be caused by particular species or by the entire bacterial community;

(e) the technique is incapable of distinguishing between live and dead bacterial cells. Direct counts on their own, are an inadequate tool in river 'health' assessment.

Traditional viable cell counts and total and faecal coliform counts are of little use for assessing ecosystem 'health'. The results from viable cell counts were inaccurate and only a fraction of those obtained from direct counts. The only relevance of total and faecal coliform counts is as indicators of poor drinking water quality. After the failure of the traditional viable count method to determine active cell numbers, the CTC method was trialed. However, difficulties and uncertainties were also encountered with the CTC method of active cell enumeration, which calls into question its suitability for use in natural environments. It is, however, far more accurate than viable cell counts and in the absence of a more dependable method it can provide an estimate of active cell numbers.

Use of the traditional tritiated thymidine method described by Moriarty (1986) for measuring bacterial production was unsuccessful. We found high background levels of radioactivity and unacceptable levels of variation between replicates for samples from Campbell's Creek. Extensive modifications to the method overcame these problems and resulted in the development of a new protocol (SSC buffer method) suitable for use in river systems that have high loads of particulate matter. The new SSC buffer method was used to successfully measure bacterial production in Campbell's Creek upstream and downstream of the Castlemaine sewage discharge point.

Significantly higher bacterial production rates were observed at the downstream site (135-523 µg C/L/hr compared with 3.0-35 µgC/L/hr at site A) suggesting that the sewage input affected production. The initial success of the SSC buffer method in measuring differences in bacterial production rates between sites, suggest it as a possible tool for assessment of river 'health', particularly in situations where between site comparison are

required. However, these preliminary data have not covered the range of bacterial production rates one would expect from 'healthy' and 'polluted' aquatic systems. Further research on this aspect is required before bacterial production estimates can be used as an indicator of river 'health'.

The use of microbial populations as indicators of river 'health' is still a new concept and currently there is insufficient data on natural bacterial production rates in Australian rivers to provide information on the range for a 'normal' or 'healthy' system. Studies presently underway on the Goulburn, Murray, Ovens and Yarra rivers will assist in providing much of the required information. However, there is a long way to go before ecosystem protection guidelines can be recommended. Further research on bacterial production is greatly needed in a wider range of river systems (pristine highland streams, regulated and unregulated lowland rivers) and the results compiled into a reference base.

A more intensive investigation into bacterial production upstream and downstream of the sewage discharge site would provide a greater insight into the effect of the sewage effluent. Bacterial production measurements of the sewage effluent itself and at more creek sites, across a wider range of physical conditions (flow and temperature) may help reveal if the observed increase in bacterial production downstream of the sewage discharge is a result of an input of sewage bacteria that are growing more rapidly than the natural bacteria, or if the polluting effluent is increasing the growth rate of the 'natural' bacteria. It is also possible, that a combination of both scenarios is occurring or that there has been a shift in species composition to those that prefer the changed creek conditions and have a higher production rate. Changes in the bacterial community composition could only be revealed with molecular microbial techniques such as DNA cross-hybridisation, Polymerase Chain Reaction-cloning-sequencing, Restriction Fragment Polymorphism analysis or Denaturing Gel electrophoresis.

The leucine method for measuring bacterial production also revealed a higher rate of bacterial production downstream of the sewage input. The problems of high background radioactivity and large variation between replicates that had to be overcome with the tritiated thymidine method were not encountered with the use of radiolabelled leucine. As identified

(in Chapter 3), the leucine incorporation method has a number of advantages over tritiated thymidine. Many researchers still prefer to use the thymidine method as it is a direct measure of cell division, while leucine incorporation is a measure of protein synthesis which is converted to biomass production using the assumption that protein is a relatively constant fraction (60%) of biomass. The fact that tritiated thymidine incorporation was the original method developed for measuring bacterial production, also contributes to its wider use. There appears to be a general reluctance by researchers to use alternative methods when they have invested considerable time and money into a particular technique.

Biomass was the third aspect of the microbial community that was investigated as a possible tool for gaining insight into the effect of sewage pollution on the receiving aquatic ecosystem. Bacterial biomass is a vital component of aquatic food webs, since changes in biomass can have important ramifications to organisms higher in the food chain. In this project, bacterial biomass was measured using the newly developed SdFFF technique, and when coupled with epifluorescent microscopy, provided a considerably more accurate method for measuring biomass than traditional procedures. SdFFF analysis of samples from Campbell's Creek showed an increase in biomass concentration at the downstream sites. However, this increase in biomass could not be attributed solely to an increase in bacterial cell concentration, implying that changes in the bacterial population had occurred as a result of the input of sewage effluent. Bacterial biomass measurements on the sewage effluent itself and at sites even further downstream, would have aided our understanding of the cause of the higher biomass concentrations observed at site D, but were prevented by time and funding restrictions. In particular, these extra studies would give a greater insight into the dynamics of Campbell's Creek and would help in identifying whether the fluctuations in biomass concentration is the result of an input of sewage bacteria with a larger buoyant mass, or the upstream bacteria increasing in cell density because of additional nutrients added via the sewage. Biomass measurements of the sewage effluent, together with measures of the creek flow, would help show if a simple 'dilution effect' of the effluent bacterial biomass by the upstream water is occurring. Dilution is thought to be the most likely explanation for the lower biomass values observed downstream compared with effluent itself, but it is

possible that some of the upstream bacteria have increased their cell density, or a shift in the bacterial population composition to species with a greater cell biomass has occurred.

The successful use of the SdFFF method in detecting changes in the bacterial biomass of Campbell's Creek suggests that this method may be a useful tool for measuring a decline in river 'health'. However, much still remains to do before this method can be applied routinely. In particular, application of SdFFF to aquatic systems which have lower bacterial cell concentrations and higher particulate loads still needs to be assessed.

One of the most promising results to emerge from this work was the potential for combining SdFFF determination of bacterial biomass with bacterial production measurements. The successful development of a protocol which incorporated both methods would aid in the further advancement of microbial ecology by showing if cells of different organic masses exhibit different production rates. The simultaneous measurement of bacterial production and biomass was undertaken in Campbell's Creek with some very promising results. Time limitations however, prevented the further refinement of this new technique. There is the need for additional experiments to examine the reproducibility of the method and the variability in natural samples. For example, the variation in the radioactivity in individual fractions collected from SdFFF runs on the same sample needs to be compared with that observed from runs performed on replicate samples collected from the same site. After sorting out any methodological problems, the technique could then be trialed in a range of natural systems to ascertain variations in biomass and production in the microbial community.

Maintaining biological diversity is a critical aspect of managing many rivers; a reduction in biodiversity is generally assumed to indicate a decline in river 'health'. The use of microbial population diversity to indicate the overall 'health' of an ecosystem has been suggested, but the use of bacteria for this purpose has been hindered by an inability to apply many microbiological techniques in natural environments. Molecular microbial methods have been suggested as the new wonder tools that will overcome the inadequacies of traditional culture-based methods to examine bacterial diversity.

The investigations undertaken in this project found two major problems in the application of molecular methods to measure bacterial diversity upstream and downstream of the Castlemaine sewage treatment plant.

(a) There were major problems experienced in concentrating large volumes of the water sampled in a short period of time (<12 hours) prior to extraction of the DNA . Equipment capable of concentrating large volumes is available, but is quite expensive; and

(b) poor yields of uncontaminated DNA were obtained after the extraction and purification steps. The need to remove humic contaminants from DNA extractions on real samples required additional purification steps that resulted in substantial losses of DNA. If molecular microbial methods are to be successfully applied to environmental samples, additional research will be required to develop extraction and purification procedures that result in high yields of DNA. There is already some concern about how representative is the DNA extracted from natural microbial communities, and if losses of DNA occur during the extraction and purification processes this will further erode confidence in the application of molecular techniques to natural aquatic environments.

The difficulties encountered in concentrating large volumes of water samples could potentially be overcome by analysing the microbial community from other parts of the aquatic environment, such as the sediments or biofilms. Generally, higher concentrations of bacterial cells are found in these habitats circumventing the need to filter large sample volumes. However, disaggregating and separating the bacterial cells from sediment and biofilm material present additional problems. Purifying DNA extracted from these sample types is also more difficult than that experienced with the water column.

However, despite the setbacks encountered when trying to extract genomic DNA representative of the bacterial communities in Campbells Creek, some successful outcomes where achieved using Polymerase Chain Reaction (PCR) and dot blot hybridisation. A PCR product assumed to be from the amplification of the 16s rRNA gene was obtained with DNA extracted from site D samples. Dot blot hybridisation was also successfully used to compare the similarities of the bacterial population present in Campbell's Creek and two other water bodies - Maribyrnong River and Hedgeley Dene pond. Unfortunately, it was not possible to

use the PCR and dot blot hybridisation techniques to compare bacterial diversity upstream and downstream of the Castlemaine sewage input, primarily because of an inability to acquire sufficient DNA from the bacterial communities.

While, molecular microbial methods may have the power to unravel the complexity of microbial community composition and its level of diversity, and thus to indicate the 'health' of an ecosystem, our present understanding of microbial processes in aquatic systems is sparse. Assigning function to form (i.e. the biological processes carried out by particular bacterial groups) is an area that warrants further research and should be one of the future directions of microbial ecology. Molecular methods can play an important role in these type of experiments. They can be applied to measure specific microbial processes such as nitrogen fixation, sulphate reduction, methanogenesis, methane oxidation, nitrification and denitrification. Currently, these processes are so under-researched that it is not possible to relate measurement specific processes with the 'health' of an ecosystem, or to determine whether changes in these processes are the result of a decline in river 'health' or simply 'normal' temporal and spatial variation.

In summary, this thesis has focused on the development of microbial methods for assessing river 'health'. One natural system - Campbells Creek was used to test whether these methods worked. However, there is still much to learn about the spatial and temporal changes that occur in the microbial populations in both polluted and unpolluted systems, before the microbial tools developed here can be used to assess river 'health'. Of all the methods investigated bacterial counts were the easiest and quickest to perform, but they did not provide very useful information on the functioning and 'health' of the ecosystem. On the other hand, bacterial production measurements are relatively straight forward to undertake, and can play a useful role in river 'health' assessments. Biomass measurement using SdFFF can be laborious, mainly due to the necessity of performing direct cell counts for each individual fraction. Advancements in automated cell counting will improve efficiency. Whilst molecular microbial techniques may possess the greatest potential for future studies of ecosystem functioning and 'health', they are the most time consuming with many problems associated with the use of these techniques in natural aquatic environments still to overcome.

REFERENCES

Amann RL, Zarda B, Stahl DA & Scheifer K-H (1992) Identification of individual prokaryotic cells by using enzyme-labeled, rRNA-targeted oligonucleotide probes. Applied and Environmental Microbiology 58(9): 3007-3011.

Amann R, Ludwig W & Schleifer K-H (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbial Reviews 59: 143-169.

ANZECC & ARMCANZ (1999) Australian and New Zealand Guidlines for Fresh and Marine Water Quality, Australia and New Zealand Environment and Conservation Council; Agriculture and Resource Management Council of Australia and New Zealand.

APHA-AWWA-WPCF (1998) Standard methods for the examination of water and waste water. American Public Health Association, Washington

Atlas RM & Bartha R (1993) Microbial Ecology: Fundamentals and Applications. The Benjamin/Cummings Publishing Company, Inc, New York

Atlas RM (1993). Extraction of DNA from soils and sediments. Handbook of methods in aquatic microbial ecology. P. F. Kemp, B. F. Sherr, E. B. Sherr and J. J. Cole. Ann Arbor, Lewis Publishers: 261-266.

Azam F, Fenchel, T., Field, J. G., Gray, J. S., Meyer-Reil, L. A. and Thingstad, F. (1983) The ecological role of water-column microbes in the sea. Marine Ecology Progress Series 10: 257-263.

Baath E (1992) Thymidine incorporation into macromolecules of bacteria extracted from soil by homogenisation-centrifugation. Soil Biology and Biochemistry 24: 1157-1165.

Badenoch J (1990) Cryptosporidium in water supplies. HMSO, London

Beckett R, Nicholson G, Hart BT, Hansen M & Giddings JC (1988) Separation and size characterization of colloidal particles in river water by SdFFF. Water Research 22: 1535-1545.

Beckett R (1989) Some Applications of FFF Techniques to Environmental Studies. FFF Symposium (Utah) .

Bej AK, Mahbubani MH & Atlas RM (1991) Detection of viable Legionella pneumophila in water by polymerase chain reaction and gene probe methods. Applied and Environmental Microbiology 57: 597-600.

Bell RT (1993). Estimating Production of heterotrophic bacterioplankton via incorporation of tritiated thymidine. Handbook of methods in aquatic microbial ecology. P. F. Kemp, B. F. Sherr, E. B. Sherr and J. J. Cole. Ann Arbor, Lewis Publishers: 495-503.

Bergh O, Borsheim KY, Bratbak G & Heldal M (1989) High abundance of viruses found in aquatic environments. Nature 340: 467-468.

Berninger U-G, Wickham SA & Finlay BJ (1993) Trophic coupling within the microbial food web: a study with fine temporal resolution in a eutrophic freshwater system. Freshwater Biology 30: 419-432.

Bradley G, Carter J, Gaudie D, King C, Stewart-Tull DES, Dennis PJ & Godfree AF (1999) Distribution of the human faecal bacterium Bacteroides fragililis, its bacteriophages and their relationship to current sewage pollution indicators in bathing water. Journal of Applied Microbiology 85: 90S-100S.

Brenner KP, Rankin CC, Roybal YR, Stelma GN, Scarpino PV & Dufour AP (1993) New medium for the simultaneous detection of total coliforms and Escherichia coli in water. Applied and Environmental Microbiology 59: 3534-3544.

Brenner R, Opsahl S, Chin-Leo G, Richey JE & Forsberg BR (1995) Bacterial metabolism in the Amazon River system. Limnology and Oceanography 40: 1262-1270.

Brenner KP, Rankin CC, Sivaganesan M & Scarpino PV (1996) Comparison of the recoveries of Escherichia coli and total coliforms from drinking water by the MI agar method and the U.S Environmental Protection Agency-approved membrane filter method. Applied and Environmental Microbiology 62: 203-208.

Briglia M, Eggen RIL, deVos WM & vanElsa JD (1996) Rapid and sensitive method for the detection of Mycobacterium chlorophenolicum PCP-1 in soil based on 16S rRNA gene-targeted PCR. Applied and Environmental Microbiology 62: 1478-1480.

Brock TD (1967) Bacterial growth in the sea: Direct analysis by thymidine autoradiography. Science 155: 81-83.

Brock TD & Madigan MT (1999) Biology of Microorganisms. Prentice Hall, New Jersey

Bunn SE (1995) Biological monitoring of water quality in Australia: WOrkshop summary and future directions. Australian journal of ecology 20: 220-227.

Byrd J & Colwell R (1993) Long-term survival and plasmid maintenance of *E. coli* in marine microcosms. FEMS Microbiology Ecology 12: 9-14.

Carmichael WW & DesMarais DJ (1991) Toxic freshwater blue-green algae (cyanobacteria): an overlooked health threat. Health and Environment Digest 5: 1-4.

CEPA (1994) River Bioassessment manual, Commonwealth Environment Protection Agency, Department of Environment, Sports and Territories, Canberra.

Chandler DP, Brockman FJ & Frederickson JK (1997) Use of 16S rDNA clone libraries to study changes in microbial community resulting from ex situ perturbation of a subsurface sediment. Bachofen 20: 217-230.

Chin-Leo G & Kirchman DL (1988) Estimating bacterial production in marine waters from the simultaneous incorporation of thymidine and leucine. Applied and Environmental Microbiology 54: 1934-1939.

Chung E, Aldom JE, Carreno RA, Chagla AH, Kostrzynska M, Lee H, Palmateer G, Trevors JT, Ungers S, Xu R & Grandis SA (1999) PCR Based quantification of Cryptosporidium parvum in Municipal water samples. Journal of Microbial Methods 38: 119-130.

Cole JJ, Findlay S & Pace ML (1988) Bacterial production in fresh and saltwater ecosystems: a cross-system overview. Marine Ecology Progress Series 43: 1-10.

Collins VG & Willoughby LG (1962) The distribution of bacterial and fungal spores in Blenham Tarn with particular references to an experimental overturn. Arch. Microbiol. 43: 294-297.

Cook KL & Garland JL (1997) The relationship between electron transport activity as measured by CTC reduction and CO2 Production in mixed microbial communities.

Microbial Ecology 34: 237-247.

Council) AAaNZEaC (1992) Australian Water Quality Guidlines For Fresh and Marine Waters.

Crump BC, Armbrust EV & Baross JA (1999) Phylogenetic analysis of particle attached and free-living bacterial communities in the Columbia River, its estuary, and the adjacent coastal ocean. Applied and Environmental Microbiology 65: 3192-3204.

Current WL & Garcia LS (1991) Cryptosporidiosis. Clinical Microbiology Review 4: 325-358.

Daneri G, Riemann B & Williams PJL (1994) In situ bacterial production and growth yield measured by thymidine, leucine and fractioned dark oxygen up-take. Journal Plankton Research 16: 105-113.

Degrange V & Bardin R (1995) Detection and Counting of Nitrobacter Populations in Soil by PCR. Applied and Environmental Microbiology 61: 2093-2098.

DeLeo PC & Baveye P (1996) Enumeration and biomass estimation of bacteria in aquifer microcosm studies by flow cytometry. Applied and Environmental Microbiology 62: 4580-4586.

DeLong EF, Wickham GS & Pace NR (1989) Phylogenetic Strains: Ribosomal RNA-Based probes for the identification of single cells. Science 234: 1360-1363.

Ducklow HW & Carlson CA (1992) Oceanic Bacterial Production. Advances in Microbial Ecology 12: 113-181.

Edberg SC, Allen MJ, Smith DB & Kriz NJ (1990) Enumeration of total coliforms and Eschericia coli from water defined substrate technology. Applied and Environmental Microbiology 56: 366-369.

Edgcomb VP, Donald JHM, Devereux R & Smith DW (1999) Estimation of bacterial cell numbers in humic acid-rich salt marsh sediments with probes directed to 16S ribosomal DNA. Applied and Environmental Microbiology 65: 1516-1523.

Edwards RT, Meyer JL & Findlay SEG (1990) The relative contribution of benthic and suspended bacteria to system biomass, production and metabolism in a low-gradient

blackwater river. Journal of the Nrth American Benthological Society 9:.

Edwards C, Hales BA, Hall GH, McDonald IR, Murrell JC, Pickup R, Rlechie DA, Saunders JR, Simon BM & Upton M (1998) Microbiological processes in the terrestrial carbon cycle: Methane cycling in peat. Atmospheric Environment 32: 3247-3255.

Elliot EL & Colwell RR (1985) Indicator organisms for estuarine and marine waters. FEMS Microbiology Reviews 32: 61-79.

Faude UC & Hoefle MG (1997) Development and application of monoclonal antibodies for in situ detection of indigenous bacterial strains in aquatic ecosystems. Applied and Environmental Microbiology 63: 4534-4542.

Felske A & Akkermans ADL (1998) Spatial homogeneity of abundant bacterial 16S rRNA molecules in grassland soils. Microbial Ecology 36: 31-36.

Ferguson RL, Buckley EN & Palumo AV (1984) Response of marine bacterioplankton to differential filtration and confinement. Applied and Environmental Microbiology 47: 49-55.

Findlay S, Pace M, Lints D, Cole J, Caraco N & Perierls B (1991) Weak coupling of bacterial and algal production in a heterotrophic ecosystem: The Hudson River estuary. Limnol. Oceanogr. 36: 268-278.

Fuhrman JA & Azam F (1980) Bacterioplankton secondary production estimates for coastal waters of British Columbia, Antartica and California. Applied and Environmental Microbiology 39: 1085-1095.

Fuhrman JA & Azam F (1982) Thymidine incorporation as a measure of heterotrophic bacterioplankton production in marine surface water: Evaluation and field results. Mar. Biol. 66: 109-120.

Fuhrman JA, Comeau DE, Hagstrom A & Chan AM (1988) Extraction from Natural Planktonic Microorganisms of DNA Suitable for Molecular Biological Studies. Applied and Environmental Microbiology 54(6): 1426-1429.

Gachter R, Myer JL & Mares A (1988) Contribution of bacteria to release and fixation of phosphorus in lake sediments. Limnology and Oceanography 33: 1542-1558.

Gachter R & Meyer JS (1993) The role of microorganisms in mobilization and fixation of phosphorus in sediments. Hydrobiologia 253: 103-121.

Giddings JC & Caldwell KD (1984) Field-Flow Fractionation : Choices in programmed and non-programmed operation. Anal. Chem. 56: 2093.

Gilmour CC, Leavitt ME & Shiaris MP (1990) Evidence against incorporation of exogenous thymidine by sulphate reducing bacteria. Limnology Oceanography 35: 1401-1409.

Giorgio PAd, Prairie YT & Bird DF (1997) Coupling between rates of bacterial production and the abundance of metabolically active bacteria in lakes, enumerated using CTC reduction and Flow Cytometry. Microbial Ecology 34: 144-154.

Giorgio PAd & Cole JJ (1998) Bacterial growth efficiency in natural aquatic systems. Annu. Rev. Ecol. Syst. 29: 503-541.

Giovannoni SJ, DeLong EF, Olsen GJ & Pace NR (1988) Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. Journal of Bacteriology 179(2): 720-726.

Giovannoni SJ, Britschgi TB, Moyer CL & Field KG (1990) Genetic diversity in Sargasso Sea bacterioplankton. Nature (London) 345: 60-63.

Gutell RR, Weiser B, Woese CR & Noller HF (1985) Comparative anatomy of 16S-like ribosomal RNA. Progress of Nucleic Acid Research and Molecular Biology 32: 155-216.

Hahn D, Amann RI, Ludwig W, Akkermans ADL & Schleifer K-H (1992) Detection of micro-organisms in soil after in situ hybridisation with rRNA-targeted, fluorescently labelled oligonucleotides. Journal of General Microbiology 138: 879-887.

Hahn D, Amann RI & Zeyer J (1993) Detection of mRNA in *Streptomyces* cells by whole-cell hybridization with digoxigenin-labeled probes. Applied and Environmental Microbiology 59(8): 2753-2757.

Hart BT, Ross J & Veal D (1995) Microbial Indicators of River Health, Water Studies Centre, Monash University.

Hastings RC, Saunders JR, hall HH, Pickup RW & McCarthy AJ (1998) Application of

molecular biological techniques to a seasonal study of ammonia oxidation in a eutrophic freshwater lake. Applied and Environmental Microbiology 64: 3674-3682.

Head IM, Saunders JR & Pickup RW (1998) Microbial Evolution, Diversity, and Ecology: A Decade of Ribosomal RNA Analysis of Uncultivated Microorganisms. Microbial Ecology 35: 1-21.

Hellawell JM (1986) Biological indicators of freshwater pollution and environmental management. Elsevier, London

Hobbie JE, Daley RJ & Jasper S (1977) Use of nucleopore filters for counting bacteria by fluorescence microscopy. Applied and Environmental Microbiology 33: 1225-1228.

Hodson RE, Dustman WA, Garg RP & Morgan MA (1995) In situ PCR for visualization of microscale distribution of specific genes and gene products in prokaryotic communities. Applied and Environmental Microbiology 61: 4074-4082.

Holben WE, Jansson JK, Chelm BK & Tiedje JM (1988) DNA probe method for the detection of specific microorganisms in the soil bacterial community. Applied and Environmental Microbiology 54(3): 703-711.

Holder-Franklin MA, Thorpe A & Cormier CJ (1981) Comparison of numerical taxonomy and DNA-DNA hybirdization in diurnal studies of river bacteria. Canadian Journal of Microbiology 27: 1165-1184.

Howgrave-Graham AR & Steyn PL (1988) Application of the fluorescent-antibody technique for the detection of *Sphaerotilus natans* in activated sludge. Applied and Environmental Microbiology 54: 799-802.

Hurst CJ (1991) Modeling the Environmental Fate of Microorganisms. American Society for Microbiology, Washington

Jackson CR, Harper JP, Willoughby D, Roden EE & Churchill PF (1997) A simple, Efficient method for the seperation of humic substances and DNA from Environmental samples. Applied and Environmental Microbiology 63: 4993-4995.

Jeffrey SW & Humphrey GF (1975) New Spectrophotometric Equations for determining Chlorophylls a, b, c_1 and c_2 in higher plants, algae and natural phytoplankton.

Biochemistry Physiology Pflanzen 167: 191-194.

Jepras RL, Carter J, Pearson SC, Paul FE & Wilkinson MJ (1995) Development of a robust flow cytometric assay for determining numbers of viable bacteria. Applied and Environmental Microbiology 61: 2696-2701.

Johnson DW, Pieniazek NJ, Griffin DW, Misener L & Rose JB (1995) Development of a PCR protocal for sensitive detection of Cryptosporidium oocycts in water samples. Applied and Environmental Microbiology 61: 3849-3855.

Jones K, Obiri-Danso K, Stewart-Tull DES & Godfree A (1999) Non-compliance of beaches with the EU directives of bathing water quality: evidence of non-point sources of pollution in Morecambe Bay. Journal of Applied Microbiology 85: 101S-107S.

Kirchman D, Kiness E & Hodson R (1985) Leucine incorporation and its potential as a measure of protein synthesis by bacteria in natural aquatic systems. Applied and Environmental Microbiology 49(3): 599-607.

Kirchman DL (1992) Thymidine and leucine incorporation in the subarctic Pacific: measuring bacterial production. Mar. Ecol. Progr. Ser. 82: 301.

Kirchman DL (1993). Leucine Incorporation as a measure of biomass production by heterotrophic bacteria. Handbook of methods in aquatic microbial ecology. P. F. Kemp, B. F. Sherr, E. B. Sherr and J. J. Cole. Ann Arbor, Lewis Publishers: 509-512.

Kirchman DL & Ducklow HW (1993). Estimating Conversion factors for the thymidine and leucine methods fro measuring bacterial production. Handbook of methods in aquatic microbial ecology. P. F. Kemp, B. F. Sherr, E. B. Sherr and J. J. Cole. Ann Arbor, Lewis Publishers: 513-517.

Knowles R (1982) Denitrification. Microbial Reviews 46: 43-70.

Konopka A, Bercot T & Nakatsu C (1999) Bacterioplankton community diversity in a series of thermally stratified lakes. Microbial Ecology 38: 126-135.

Kreader C (1995) Design and evaluation of *Bacteroides* DNA probes for the specific detection of human fecal pollution. Applied and Environmental Microbiology 61: 1171-1179.

Kroer N (1994) Relationships between biovolume and carbon and nitrogen content of bacterioplankton. FEMS Microbial Ecology 13: 217-224.

Lebaron P, Parthuisot N & Catala P (1998) Comparison of blue nucleic asid dyes for flow cytometric enumeration of bacteria in aquatic systems. Applied and Environmental Microbiology 64: 1725-1730.

LeChevallier MW, Welch NJ & Smith DB (1996) Full-scale studies of factors related to coiform regrowth in drinking water. Applied and Environmental Microbiology 62: 2201-2211.

Lee S & Furman J (1990) DNA hybridisation to compare species compositions of natural Bacterioplankton assemblages. Applied and Environmental Microbiology 56: 739-746.

Lee S, Malone C & Kemp PF (1993) Use of multiple 16S rRNA-targeted fluorescent probes to increase signal strength and measure cellular RNA from natural planktonic bacteria. Marine Ecology Programme series 101:.

Lee S, Malone C & Kemp PF (1993) Use of multiple 16S rRNA-targeted probes to increase signal strength and measure cellular RNA from natural planktonic bacteria. Marine Ecology Progress Series 101: 193-201.

Leff LG & Lemke MJ (1998) Ecology of Aquatic Bacterial Populations: Lessons from Applied Microbiology. Journal of North American Benthological Society 17: 261-271.

Lemke MJ, Brown BJ & Leff LG (1997) The response of three bacterial populations to pollution in a stream. Microbial Ecology 34: 224-231.

Lesser TD, Boyle M & Hendriksen NB (1995) Survival and activity of Pseudomonas sp. strain B13(FR1) in a marine microcosm determined by quantitative PCR and an rRNA-targeting probe and its effect on the indigenous bacterioplankton. Applied and Environmental Microbiology 61: 1201-1207.

Liu W, Marsh TL, Cheng H & Forney L (1997) Characterization of Microbial Diversity by Determining Terminal Restriction Fragment Length Polymorphisms of Genes Encoding 16S rRNA. Applied and Environmental Microbiology 63: 4516-4522.

Luria SE (1960). The bacterial protoplasm: Compostion and organization. The bacteria:

Structure. I. C. G. a. R. Y. Stanier. New York, Academic Press Inc. I.

Maasdam R & Smith DG (1994) New Zealand's national river quality network, 2. Relationships between physico-chemical data and environmental factors. New Zealand Journal of Marine and Freshwater Research 28: 37-54.

Macario ECCd, Wolin MJ & Macario AJL (1982) Antibody analysis of relationships among methanogenic bacteria. Journal of Bacteriology 149: 316-319.

Martinez-Murcia AJ, Acinas SG & Rodriguez-Valera F (1995) Evaluation of prokaryotic diversity by restrictase digestion of 16S rDNA directly amplified from hypersaline environments. FEMS Microbiology Ecology 17: 247-256.

Maryborough Co (1994) Nutrient sources for the upper Loddon River catchment area, upstream of Laanecoorie reservoir, City of Maryborough.

McCarthy JA, Delaney JE & Grasso RJ (1961) Measuring Coliforms in water. Water and Sewage Works 108: 238-243.

McFeters GA (1990). Enumeration. occurrence and significance of injured indicator bacteria in drinking water. Drinking Water Microbiology. G. A. McFeters. New York, Springer-Verlag: 478-492.

Medema GJ, Schets FM, Ketelaars H & Boschman G (1998) Improved detection and vital staining of Cryptosporidium and Giardia with flow cytometry. Health Related Water Microbiology 61-65.

Mitchell (1978) Water Pollution Microbiology. Wiley Inter-Sciences, New York

Miyamoto H, Jitsurong S, Shiota R, Maruta K, Yoshida S & Yabuuchi E (1997) Molecular determination of infection source of a sporadic Legionella pneumonia case associated with a hot spring bath. Microbiology and immunology 41: 197-202.

Monteith RW (1975) Waste Licence, Environment Protection Authority.

Moriarty DJW & Pollard PC (1981) DNA synthesis as a measure of bacterial productivity in seagrass sediments. Marine Ecology and Progress Series 5: 151-156.

Moriarty DJW (1986) Manual of techniques for estimating bacterial growth rates, productivity and numbers in aquaculture ponds.". Bacterial growth rates and productivity.

Moriarty DJW (1986). Measurement of bacterial growth rates in aquatic systems form rates of nucleic acid synthesis. Advances in Microbial Ecology. K. C. Marshall. New York, Plenum. **9:** 245-292.

Moyer CL, Dobbs FC & Karl DM (1995) Phylogenetic Diversity of the Bacterial Community from a Microbial mat at an Active, Hydrothermal Vent System, Loihi Seamount, Hawaii. Applied and Environmental Microbiology 61: 1555-1562.

Muller-Wegener U (1977) Fluorescence spectroscopic investigations of natural humics. Chemie der Erde 36: 352-354.

Muyzer G, Waal Ed & Uitterrlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology 59: 695-700.

Muyzer G, Waal ECD & Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of Polymerase Chain Reaction-amplified genes coding for 16D rRNA. Applied and Environmental Microbiology 59: 695-700.

Nagata T & Watanabe Y (1990) Carbon and nitrogen ratios of bacterioplankton grown under different nutritional conditions. Applied and Environmental Microbiology 56: 1303-1309.

Newbold JD, Elwood JW, O'Neill RV & Van Winkle W (1981) Measuring nutrient spiralling in streams. Can. J. Fish. Aquatic Sci. 38: 860-863.

Norland S, Heldal M & Tumyr O (1987) On the relation between dry matter and volume of bacteria. Microbial Ecology 13: 95-101.

Norris RH & Norris KR (1995) The need for biological assessment of water quality: Australian perspective. Australian journal of ecology 20: 1-6.

O'Donovan GA (1978). Thymidine metabolism in bacteria (and "How, or how not, to label DNA"). DNA synthesis: Present and Future. I. Molineux and M. Kohiyama. New York, Plenum Press: 219-253.

Ogram A, Sayler GS & Barkay T (1987) The extraction and purification of microbial DNA from sediments. Journal of Microbial Methods 7: 57-66.

Olsen GJ, Lane DJ, Giovannoni SJ & Pace NR (1986) Microbial ecology and evolution: a ribosomal RNA approach. Annual Review of Microbiology 40: 337-365.

Olsen GJ, Larsen N & Woese CR (1991) The ribosomal RNA database project. Nucleic Acids Research 19: 2017-2021.

Pace NR, Stahl DA, Lane DJ & Olsen GJ (1986). The analysis of natural microbial populations by ribosomal RNA sequences. Advances in Microbial Ecology. K. C. Marshall. New York, Plenum Press. **9:** 1-55.

palmer SE, Niederlehner BR, Cairns J & Munawar M (1994) Assessment of pollution impact on aquatic microbial communities based on DNA hybridisation and protozoan identification: Preliminarty method development and comparison. Journal of Aquatic Ecosystem Health 3: 35-44.

Paul JH, Jeffrey WH & Cannon JP (1990) Production of dissolved DNA, RNA and protein by microbial populations in a Florida reservoir. Applied and Environmental Microbiology 56: 2957-2962.

Pearl HW & Pickney JL (1996) A mini-review of microbial consortia: Their roles in aquatic production and biogeochemical cycling. Microbial Ecology 3: 225-247.

Pollard PC & Moriarty DJW (1984) Validity of the titrated thymidine method for estimating bacterial growth rates : Measurement of isotope dilution during DNA synthesis. Applied and Environmental Microbiology 33: 1076-1083.

Pomeroy LR (1974) Cycles of essential elements. Dowden, Hutchinson, Ross, Stroudsburg, Penn

Porter KG & Feig YS (1980) The use of DAPI for identifying and counting aquatic microflora. Limnology Oceanography 25: 943-948.

Porter J, Diaper J, Edwards C & Pickup R (1995) Direct measurements of natural planktonic bacterial community viability by flow cytometry. Applied and Environmental Microbiology 61: 2783-2786.

Pyle BH, Broadway SC & McFeters GA (1995) Factors Affecting the Deteremination of Respiratory Activity on the Basis of Cyanoditolyl Tetrazolium Chloride Reduction with Membrane Filtration. Applied and Environmental Microbiology 61: 4304-4309.

Pyle BH, Broadaway SC & McFeters GA (1995) A rapid, direct method for enumerating respiring enterohemorrhagic *Escherichia coli* 0157:H7 in water. Applied and Environmental Microbiology 61: 2614-2619.

Reyesenbach A, Giver LJ, Wickham GS & Pace NR (1992) Differential amplification of rRNA genes by polymerase chain reaction. Applied and Environmental Microbiology 58: 3417-3418.

Reynolds KA, Roll K, R SF, Gerba CP & Pepper IL (1998) Incidence of enteroviruses in Mamaia Bay, Hawai using cell culture and direct polymerase chain reaction methodologies. Canadian Journal of Microbiology 44: 598-604.

Rheims H, Rainey FA & Stackebrandt E (1996) A molecular approach to search for diversity among bacteria in the environment. Journal of Industrial Microbiology 17: 159-169.

Rheinheimer G (1991) Aquatic Microbiology 4th Edition. John Wiley and Sons, West Sussex

Rheinheimer G (1992) Aquatic Microbiology. John Wiley and Sons, Chichester

Rhodes MW & Kator H (1999) Sorbito-fermenting bifidobacteria as indicators of diffuse human faecal pollution in estaurine watersheds. Journal of Applied Microbiology 87: 528-535.

Riemann B & Bell RT (1990) Advances in estimating bacterial biomass and growth in aquatic systems. Archives Hydrobiology 118: 385-402.

Riemann B & Bell RT (1992) Advances in estimating bacterial biomass and growth in aquatic systems. Archives of Hydrobiology 118: 385-402.

Robarts RD & Zohary T, Eds. (1993). "Fact or fiction - bacterial growth rates and production as determined by [methyl-3H]-thymidine?". Advances in Microbial Ecology. New York, Plenum Press.

Robertson BR, D KB & Koch AL (1998) Determination of the biomass of small bacteria at low concentrations in a mixture of species with forward light scatter measurements by flow cytometry. Applied and Environmental Microbiology 64: 3900-3909.

Rodriguez GG, Phipps D, Ishiguro K & Ridgway HF (1992) Use of a Fluorescent Redox Probe for Direct Visualization of Actively Respiring Bacteria. Applied and Environmental Microbiology 58(6): 1801-1808.

Ross J, Boon PI, Sharma R & Beckett R (1996) Variations in the fluorescence intensity of intact DAPI-stained bacteria and their implications for rapid bacterial quantification. Letters in Applied Microbiology 22: 283-287.

Sambrook J, Fritsch EF & Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York

Sharma RV, Edwards RT & Beckett R (1993) Physical characterization and quantification of bacteria by sedimentation field-flow fractionation. Applied and Environmental Microbiology 56(6): 1864-1875.

Sharma R (1994) Physical characterisation, quantification and biomass determination of bacteria using sedimentation field-flow fractionation., Chemistry Department, Monash University.

Sharma RV, Edwards, R. T. and Beckett, R. (1998) Analysis of bacteria in aquatic environments using sedimentation field-flow fractionation: (II) Physical characterization of cells. Water Research 32: 1508-1514.

Sharma RV, Ross JL, Boon PI & Beckett R (1998) Sedimentation field-flow fractionation as a technique to separate bacterial cells on the basis of cell biomass: Physical and biochemical analysis, ans suitability for probing with 16S rRNA oligonucleotide probes. J Microbiol. Methods 32: 1497-1507.

Simon M & Azam F (1989) Protein content and protein synthesis rates of planktonic marine bacteria. Marine Ecology, Progress Series 51: 201-213.

Sinton LW, Finlay RK & Hannah DJ (1998) Distinguishing human from animal faecal contamination in water: a review. New Zealand Journal of Marine and Freshwater

Research 32: 323-348.

Smith AJ (1981). Cyanobacterial contributions to the heterotrophc connection. Microbial growth on C-1 compounds. H. Dalton. London, Heyden: 122-130.

Smits JD & Riemann B (1988) Calculation of cell production from [^{3}H]Thymidine incorporation with freshwater bacteria. Applied and Environmental Microbiology 54: 2213-2219.

Sorci JJ, Paulauskis JD & Ford TE (1999) 16S rRNA restriction fragment length polymorphism analysis of bacterial diversity as a biomarker of ecological health in polluted sediments from New Bedford harbor, Massachusetts, USA. Marine Pollution Bulletin 38: 663-675.

Speksnijder AGCL, Kowalchuk GA, Roest K & Laanbroek HJ (1998) Recovery of a Nitrosomonas-like 16S rDNA sequence group from freshwater habitats. Sytematic and Applied Microbiology 21: 321-330.

Stal L, Grossberger S & Krumbein W (1984) Nitrogen Fixation associated with the cyanobacterial mat of a marine laminated micronial ecosytem. Marine Biology 82: 217-224.

Steffan RJ, Goksoyr J, Bej AK & Atlas RM (1988) Recovery of DNA from soils and sediments. Applied and Environmental Microbiology 54(12): 2908-2915.

Stockner JG & Porter KG (1988). Microbial food webs in freshwater planktonic ecosystems. Complex Interactions in Lake Communities. S. R. Carpenter. Heidelberg, Springer Verlag: 69-83.

Stream Solute Workshop (1990) Concepts and methods for assessing solute dynamics in stream ecosystems. Journal North American Benthological Society 9: 95-119.

Suttle CA (1994) The significance of viruses to mortality in aquatic microbail communities. Microbial Ecology 28: 237-243.

Suzuki MT & Giovannoni SJ (1996) Bias Caused by Template Annealing in the Amplification of Mixtures of 16S rRNA by PCR. Applied and Environmental Microbiology 62: 625-630.

Tiller D & Metzeling L (1998) Rapid Bioassessment of Victorian Stream, The Approach and Methods of the Environment Protection Authority, Environment Protection Authority.

Torsvik V, Goksoyr J & Daae FL (1990) High diversity in DNA of soil bacteria. Applied and Environmental Microbiology 56: 782-787.

Torsvik V, Salte K, rheim RS & yr JG (1990) Comparsion of phenotypic diversity and DNA heterogeneity in a population of soil bacteria. Applied and Environmental Microbiology 56: 776-781.

Trebesius KR, Amann R, Ludwig W, Muhlegger K & Schleifer K-H (1994) Identification of whole fix d bacterial cells with non-radioactive rRNA-targeted transcript probes. Applied and Environmental Microbiology 60: 3228-3235.

Voytek MA & Ward BB (1995) Detection of Ammonium-oxidising bacteria of the Beta-subclass of the class Proteobacteria in Aquatic samples with the PCR. Applied and Environmental Microbiology 61: 1444-1450.

Waara T & Janson M (1993) Phosphorus composition and release in sediment bacteria of the genus Pseudomonas during aerobic and anaerobic conditions. Hydrobiol. 253: 131-140.

Wallner G, Erhart R & Amann R (1995) Flow cytometric analysis of activated sludge with rRNA-targeted probes. Applied and Environmental Microbiology 61: 1859-1866.

Ward DM, Weller R & Bateson M (1990) 6S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature 345: 63-65.

Ward DM, Bateson MM, Weller R & Ruff-Roberts AL (1992) Ribosomal RNA analysis of microorganisms as they occur in nature. Advanced Microbial Ecology 12: 219-286.

Webster JR & Meyer JL (1997). Organic matter budgets for streams: a sysnthesis. Stream organic matter budgets. J. R. Webster and J. L. Meyer.

Woese CR (1987) Bacterial Evolution. Microbial Reviews 51(2): 221-271.

Woese CR, Kandler O & Wheelis ML (1990) owards a natural system of organisms: Proposal for the domains Archaea, Bacteria, and Eucarya. PNAS 87: 4576-4579.

Wommack KE, Ravel J, Hill RT & Colwell RR (1998) Hybridisation analysis of Chesapeake Bay virioplankton. Applied and Environmental Microbiology 65: 241-250.

Zarda B, Amann R, Wallner G & Schleifer K-H (1991) Identification of single bacterial cells using digoxigenin-labelled rRNA-targeted oligonucleotides. Journal of General Microbiology 137: 2823-2830.

Zimmermann R, Iturriaga R & Becker-Birch J (1978) Simultaneous determination of the total number of aquatic bacteria and the number thereof involved in respiration. Applied and Environmental Microbiology 36: 926-935.