http://researchspace.auckland.ac.nz

ResearchSpace@Auckland

Copyright Statement

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

This thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- Any use you make of these documents or images must be for research or private study purposes only, and you may not make them available to any other person.
- Authors control the copyright of their thesis. You will recognise the author's right to be identified as the author of this thesis, and due acknowledgement will be made to the author where appropriate.
- You will obtain the author's permission before publishing any material from their thesis.

To request permissions please use the Feedback form on our webpage. http://researchspace.auckland.ac.nz/feedback

General copyright and disclaimer

In addition to the above conditions, authors give their consent for the digital copy of their work to be used subject to the conditions specified on the Library Thesis Consent Form.
Effects of contaminants on *Austrovenus stutchburyi* – using biomarkers to detect sublethal stress

Sharon B De Luca-Abbott

Submitted for the degree of Doctor of Philosophy
School of Environmental and Marine Science
University of Auckland

February 2000


Abstract

This thesis examines sublethal stress responses in the common New Zealand estuarine bivalve, Austrovenus stutchburyi (cockle). The approach used throughout this thesis is a refinement of the biological indicator approach of Adams (1990), in which biomarker data are collected primarily at the individual and sub-organisinal levels, but with additional measurements at the population and community levels. In this research several biochemical and physiological biomarkers are used to assess effects of contaminants on energetics, fecundity and growth of cockles.

Adenylate energy charge (AEC) and total adenylate nucleotide pool (TANP) are biomarkers that have previously not been used with A. stutchburyi, and provide new information on energy cycling for this species. RNA concentration has also not previously been used with cockles, and this biomarker is correlated with growth. Glycogen concentration and condition indices are used to indicate reproductive potential and overall health.

In order to fully investigate the utility of this comprehensive suite of biomarkers, their performance in laboratory and field situations was tested. Temporal variability in AEC, TANP and glycogen was analysed over two years for two populations of cockles in a northern harbour. The results indicated seasonal patterns for all biomarkers, with AEC reaching a maximum value in spring, TANP being lowest in winter, and glycogen concentration being high over spring and summer. Variability in biomarker response between cockles sampled at low tide and high tide was analysed in a laboratory
Effects of contaminants on *A. stutchburyi* – using biomarkers to detect sublethal stress

experiment simulating exposure to air during low tide. Adenylate energy charge was found to be significantly lower in cockles after exposure to air for 4 hours, highlighting the importance of standardised collection protocols. These initial patterns, as well as spatial variability in biomarker response, were further investigated at several sites within a second harbour south of Auckland. There was little among site difference in glycogen concentration, and the data suggested that differences in site characteristics, such as sediment quality and type, were driving the mixed response patterns of AEC, TANP and condition.

Because of inherent variability in field conditions, a series of laboratory experiments was then undertaken to assess responses under carefully controlled conditions. Cockles were challenged with PAHs, chlordane and tributyltin at two different doses, and biomarker response (AEC, TANP, glycogen and RNA) measured. One trial examined the effects of a one-off pulse of contaminant, in which sediment containing cockles was dosed at the outset of a 14-day experiment with a high concentration of contaminant. A daily dose of this same concentration of contaminant was supplied to the sediment for 14 days in a second trial to assess effects of a more continuous discharge. There were virtually no significant differences in biomarker response between treatments and controls for either trial, suggesting that the experimental setup may have been causing sublethal stress in some way.

Finally, in order to maximise experimental control whilst maintaining environmental realism, a manipulative field experiment was undertaken in which cockles were transplanted from an uncontaminated site to a series of uncontaminated and contaminated
sites in one of Auckland’s major harbours. Biomarker response (AEC, TANP, glycogen and RNA) was measured in the transplanted and the autochthonous populations two weeks and eight weeks after transplantation. An ability to regulate adenylate nucleotides was detected, as cockles transplanted to contaminated sites had reduced TANP but maintained AEC levels. There were significant differences in glycogen and RNA among sites, with highest levels detected in cockles transplanted to an uncontaminated site.

This suite of biomarkers showed great utility for use in environmental quality assessment. It is recommended that an approach such as that used in the Mussel Watch programme in the United States be employed for cockles, in order to monitor estuarine ecosystem health in New Zealand. The transplantation of cockles to estuaries where there are concerns about environmental quality, and the use of TANP, glycogen and RNA concentration to assess sublethal stress, has potential as a sensitive and cost-effective environmental monitoring technique for estuaries in New Zealand.
Acknowledgements

The completion of my thesis was only made possible through the assistance of large number of people; to all of you I am extremely grateful.

I would like to thank my supervisor Dr Bob Creese for his encouragement, support and faith in my abilities. Especially I thank Bob for his constructive criticism throughout my research. Dr Gillian Lewis co-supervised my work and I am also grateful for her advice and guidance.

My research project was very expensive, and without the funding provided by many groups, it would not have been feasible. Funding was gratefully received from the University of Auckland, Lotteries Grants Board, Resource Management Law Association of New Zealand, Todd Foundation, Grand Lodge of Freemasons, Soroptimists International, Television New Zealand, Auckland Regional Council, Northland Regional Council, Claude McCarthy Fund, Royal Society of New Zealand, School of Environmental and Marine Science, and Norm Thom.

Many people helped me in the field and the laboratory. Megan Stewart cheerfully dedicated an amazing amount of time and effort to my research and for that I am very grateful. Thanks especially for enduring the hail storm at Whangarei Heads! Other field helpers included Carol De Luca, Maxine Heathcote, Chris Clarke, Sarah Spencer, Andrew Berry, John Abbott and the Tollys. Laboratory assistance was also provided by John Abbott, Chris Clarke and Sarah Spencer. Thanks also to Megan Stewart, Karen Tricklebank, Cathie Tollemache, Wendy Kerr and Justine Paterson for proof-reading my chapters.

Three people have patiently answered my many questions and replied to copious emails. The information they provided was crucial. Thanks to Colin Dall (Northland Regional Council), Geoff Mills (NIWA), and Dominic McCarthy (Auckland Regional Council).

Chris Triggs calmed my fears about statistics - thank you for the relaxed and enjoyable meetings. Dianne Brunton, Bob Creese, and Alan Rodrigo also provided statistical advice. Thanks to Beryl Davies for her cheerful help, and to Margaret Lichtwark and Peter Keen for their assistance and friendship. Dr Rufus Wells was extremely helpful teaching me many of the biochemical techniques used in my research.

There are so many people at the Leigh Marine Laboratory who have made my time there memorable. Thanks to Viv, Alan and Sharon for your friendship and for never being grumpy. Thanks to Arthur for counting and juggling all the beans, to Ray, Murray, Tom and Jo for making things and fixing them, to Brady for smiling at my acquisition procedures, and to Brian for telling me I had a tidy lab bench. I am grateful to my office buddies, Sarah Spencer, Kala Sivaguru and Megan Stewart for their friendship. Thanks also to students who provided entertainment; Dibble, Dom, Timmo, Mike T, and Kogs. Special thanks to Karen Tricklebank for being understanding and for giving me advice and encouragement.

Thanks to Mum & Maxine, and Dad & Nolene for the pennies and your faith in me. Support from afar was also given by Davina Boyd, Heather Shepherd and Julie Clothier. Thanks to the Tollys for so many things, mostly for your friendship. Lastly, I thank my husband, John, for his unfailing love, support and encouragement - now I can come to Hong Kong!
Table of Contents

Abstract .................................................................................. i
Acknowledgements .................................................................. iv
Table of Contents ................................................................. v
List of Figures ......................................................................... viii
List of Tables ........................................................................... x
List of Plates ........................................................................... xii
Abbreviations .......................................................................... xiii

CHAPTER 1: General Introduction .......................................... 1
  1.1 Types of Contaminants ....................................................... 2
  1.2 Detection of Contaminant Impacts ..................................... 5

CHAPTER 2: General Methods and Materials ......................... 18
  2.1 Species Studied ................................................................ 18
  2.2 Sampling Sites .................................................................. 21
  2.3 Collection Procedures ..................................................... 21
  2.4 Adenylate Nucleotide Extraction ..................................... 23
  2.5 Adenylate Nucleotide Analyses ....................................... 24
  2.6 Glycogen Analyses .......................................................... 25
  2.7 RNA Analyses .................................................................. 26
  2.8 Condition .......................................................................... 28
  2.9 Statistical Analyses ........................................................... 29

CHAPTER 3: Seasonal Variation of Biomarker Response in Austrovenus stutchburyi from Whangarei Harbour .......... 30
  3.1 Introduction ....................................................................... 30
  3.2 Methods and Materials ..................................................... 36
    3.2.1 Description of study sites and characteristics of the cockle population .............................................. 36
    3.2.2 Seasonal Monitoring of Biomarker Response ................................................................. 39
    3.2.3 Experimental Comparison Between Immersed and Air-Exposed Cockles ....... 40
  3.3 Results ............................................................................... 41
    3.3.1 Population and Community Composition ................................................................. 41
    3.3.2 Sediment Size, Environmental Variables and Condition ........................................... 42
    3.3.3 Biomarker Response – Seasonal ................................................................. 43
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3.4</td>
<td>Immersion vs Emersion Experiment</td>
<td>45</td>
</tr>
<tr>
<td>3.4</td>
<td>Discussion</td>
<td>45</td>
</tr>
<tr>
<td>CHAP 4:</td>
<td>Spatial Variation of Biomarker Response in <em>Austrovenus stutchburyi</em>, within the Mangemangeroa Estuary</td>
<td>63</td>
</tr>
<tr>
<td>4.1</td>
<td>Introduction</td>
<td>63</td>
</tr>
<tr>
<td>4.2</td>
<td>Methods and Materials</td>
<td>68</td>
</tr>
<tr>
<td>4.2.1</td>
<td>Site Characteristics</td>
<td>68</td>
</tr>
<tr>
<td>4.2.2</td>
<td>Adenylate Nucleotides and Glycogen</td>
<td>71</td>
</tr>
<tr>
<td>4.2.3</td>
<td>Condition</td>
<td>71</td>
</tr>
<tr>
<td>4.2.4</td>
<td>Statistical Analyses</td>
<td>72</td>
</tr>
<tr>
<td>4.3</td>
<td>Results</td>
<td>72</td>
</tr>
<tr>
<td>4.3.1</td>
<td>Site Characteristics</td>
<td>72</td>
</tr>
<tr>
<td>4.3.2</td>
<td>Adenylate Energy Charge</td>
<td>76</td>
</tr>
<tr>
<td>4.3.3</td>
<td>Total Adenylate Nucleotide Pool</td>
<td>77</td>
</tr>
<tr>
<td>4.3.4</td>
<td>Glycogen</td>
<td>77</td>
</tr>
<tr>
<td>4.3.5</td>
<td>Body Condition Index</td>
<td>78</td>
</tr>
<tr>
<td>4.4</td>
<td>Discussion</td>
<td>78</td>
</tr>
<tr>
<td>CHAP 5:</td>
<td>Concentration-Response Laboratory Trials</td>
<td>97</td>
</tr>
<tr>
<td>5.1</td>
<td>Introduction</td>
<td>97</td>
</tr>
<tr>
<td>5.2</td>
<td>Methods and Materials</td>
<td>102</td>
</tr>
<tr>
<td>5.2.1</td>
<td>Collection of Sediment and Bivalves</td>
<td>102</td>
</tr>
<tr>
<td>5.2.2</td>
<td>Analysis of Contaminants in Collected Sediment and Shellfish</td>
<td>103</td>
</tr>
<tr>
<td>5.2.3</td>
<td>Experimental Design</td>
<td>103</td>
</tr>
<tr>
<td>5.2.3.1</td>
<td>Tank Set-up</td>
<td>104</td>
</tr>
<tr>
<td>5.2.4</td>
<td>Experimental Procedure</td>
<td>106</td>
</tr>
<tr>
<td>5.2.4.1</td>
<td>Trial A</td>
<td>106</td>
</tr>
<tr>
<td>5.2.4.2</td>
<td>Trial B</td>
<td>107</td>
</tr>
<tr>
<td>5.2.5</td>
<td>Biochemical Analyses</td>
<td>107</td>
</tr>
<tr>
<td>5.2.6</td>
<td>Statistical Analyses</td>
<td>107</td>
</tr>
<tr>
<td>5.3</td>
<td>Results</td>
<td>108</td>
</tr>
<tr>
<td>5.3.1</td>
<td>Polycyclic Aromatic Hydrocarbons</td>
<td>108</td>
</tr>
<tr>
<td>5.3.2</td>
<td>Chlordane</td>
<td>110</td>
</tr>
<tr>
<td>5.3.3</td>
<td>Tributyltin</td>
<td>112</td>
</tr>
<tr>
<td>5.4</td>
<td>Discussion</td>
<td>113</td>
</tr>
<tr>
<td>CHAP 6:</td>
<td>Determination of Sublethal Stress using <em>In-situ</em> Exposure to Contaminated Sediment</td>
<td>137</td>
</tr>
<tr>
<td>6.1</td>
<td>Introduction</td>
<td>137</td>
</tr>
<tr>
<td>6.2</td>
<td>Methods and Materials</td>
<td>141</td>
</tr>
<tr>
<td>6.2.1</td>
<td>Transplant Design</td>
<td>141</td>
</tr>
<tr>
<td>6.2.2</td>
<td>Site Descriptions</td>
<td>142</td>
</tr>
</tbody>
</table>
### 6.2.3 Levels of Contaminants in Sediment
- 143

### 6.2.4 Collection and Tagging of Cockles
- 146

### 6.2.5 Transplantation
- 147

### 6.2.6 Sampling Protocol
- 148

### 6.2.7 Biochemical Analyses
- 148

### 6.3 Results
- 148

#### 6.3.1 Sediment Size
- 148

#### 6.3.2 Biochemical Indices
- 149
  - Adenylate Energy Charge
    - 151
  - Total Adenylate Nucleotide Pool
    - 152
  - Glycogen
    - 153
  - RNA
    - 154

#### 6.3.3 Mortality Estimates
- 155

### 6.4 Discussion
- 156

### CHAPTER 7: General Discussion
- 184

### REFERENCES
- 194

### Appendix I
- 211

### Appendix II
- 234

### Appendix III
- 235
# List of Figures

## Chapter 1:
1.1 Conceptual diagram showing functional and structural links ........................................ 13

## Chapter 2:
2.1 Map showing location of harbours where experiments undertaken .................................. 22

## Chapter 3:
3.1 Map of study sites in Whangarei Harbour ................................................................. 37
3.2 Size-frequency plots for *A. stutchburyi* ................................................................. 50
3.3 MDS plot of community composition ........................................................................... 52
3.4 Community richness, diversity and evenness indices ...................................................... 52
3.5 Adenylate energy charge in *A. stutchburyi* ............................................................... 54
3.6 Total adenylate nucleotide pool in *A. stutchburyi* ....................................................... 56
3.7 Glycogen concentration in *A. stutchburyi* ................................................................. 58
3.8 Adenylate energy charge, total adenylate nucleotide charge, and glycogen for air-exposed and immersed *A. stutchburyi* ........................................................................ 60

## Chapter 4:
4.1 Map of Mangemangeroa, Turanga and Waikopua Estuaries ............................................. 65
4.2 Map of study sites in Mangemangeroa Estuary .............................................................. 69
4.3 Mean cockle density for each season .............................................................................. 87
4.4 Size-frequency for cockles from Site B & C .................................................................... 88
4.5 Size-frequency for cockles from Site D & F .................................................................... 89
4.6 Size-frequency for cockles from Site G ......................................................................... 90
4.7 Mean adenylate energy charge for each season .............................................................. 92
4.8 Mean total adenylate nucleotide pool for each season .................................................. 93
4.9 Mean glycogen concentration for each season .............................................................. 94
4.10 Mean body condition index for each season ............................................................... 95
4.11 Biomarker response averaged across all seasons ......................................................... 96

## Chapter 5:
5.1 Design and composition of individual tubs ...................................................................... 121
5.2 Set-up of laboratory experiments, showing tubs and seawater supply .............................. 121
5.3 Statistical experimental design for laboratory trials ....................................................... 123
5.4 Mean AEC, TANP, glycogen and RNA for PAH Trial A and Trial B ............................ 125
5.5 Semi-interquartile range for AEC, TANP, glycogen and RNA for PAH Trial A and Trial B ........................................................................................................... 128
5.6 Mean AEC, TANP, glycogen and RNA for chlordane Trial A and Trial B .................... 130
5.7 Semi-interquartile range for AEC, TANP, glycogen and RNA for chlordane Trial A and Trial B ........................................................................................................... 132
Effects of contaminants on A. stutchburyi – using biomarkers to detect sublethal stress

5.8 Mean AEC, TANP, glycogen and RNA for organotin Trial A and Trial B... 134
5.9 Semi-interquartile range for AEC, TANP, glycogen and RNA for organotin Trial A and Trial B... 136

Chapter 6:

6.1 Design of transplant experiment................................. 163
6.2 Plan of 22 statistical comparisons for each biochemical analysis ............ 163
6.3 Location of Whangateau and Manukau Harbours and experimental sites... 163
6.4 Median sediment particle size for donor and transplant sites .................. 170
6.5 Plots of mean AEC comparing transplant and autochthonous cockles after 2 and 8 weeks......................................................... 171
6.6 Plot of mean AEC comparing donor and transplant cockles after 2 and 8 weeks................................................................. 172
6.7 Plot of mean AEC for all cockles after 2 and 8 weeks transplanted........... 173
6.8 Plots of mean TANP comparing transplant and autochthonous cockles after 2 and 8 weeks......................................................... 174
6.9 Plot of mean TANP comparing donor and transplant cockles after 2 and 8 weeks................................................................. 175
6.10 Plot of mean TANP for all cockles after 2 and 8 weeks transplanted.......... 176
6.11 Plots of mean glycogen comparing transplant and autochthonous cockles after 2 and 8 weeks......................................................... 177
6.12 Plot of mean glycogen comparing donor and transplant cockles after 2 and 8 weeks................................................................. 178
6.13 Plot of mean glycogen for all cockles after 2 and 8 weeks transplanted....... 179
6.14 Plots of mean RNA comparing transplant and autochthonous cockles after 2 and 8 weeks......................................................... 180
6.15 Plot of mean RNA comparing donor and transplant cockles after 2 and 8 weeks................................................................. 181
6.16 Plot of mean RNA for all cockles after 2 and 8 weeks transplanted.......... 182
6.17 Fate of individuals within each plot and percent mortality for individuals within each plot......................................................... 183
List of Tables

Chapter 3:

3.1 Mean width and density of *A. stutchburyi* .................................................. 51
3.2 Summary of two-way ANOVA for AEC for *A. stutchburyi* .............................. 53
3.3 Summary of two-way ANOVA for TANP for *A. stutchburyi* ............................ 55
3.4 Summary of two-way ANOVA for glycogen concentration for *A. stutchburyi* .......... 57
3.5 Summary of two-way ANOVA for AEC for *A. stutchburyi* from exposure/immersion experiment .................................................. 59
3.6 Summary of two-way ANOVA for TANP for *A. stutchburyi* from exposure/immersion experiment .................................................. 61
3.7 Summary of two-way ANOVA for glycogen concentration for *A. stutchburyi* from exposure/immersion experiment .................................................. 62

Chapter 4:

4.1 Median sediment particle size ............................................................................. 85
4.2 Summary of two-way ANOVA statistics for density ............................................. 86
4.3 Species richness and diversity ............................................................................ 86
4.4 Concentration of copper, lead and zinc in sediment ........................................... 91
4.5 Summary of two-way ANOVA statistics for AEC ................................................. 92
4.6 Summary of two-way ANOVA statistics for TANP ............................................... 93
4.7 Summary of two-way ANOVA statistics for glycogen concentration ..................... 94
4.8 Summary of two-way ANOVA statistics for BCI .................................................. 95

Chapter 5:

5.1 Contaminant data for sediment and shellfish prior to use in trials ...................... 120
5.2 Composition and concentration of PAHs used in trials ........................................ 122
5.3 Summary of one-factor nested ANOVA statistics for PAH trials ......................... 124
5.4 Tissue accumulation of PAHs from Trial A ......................................................... 126
5.5 Tissue accumulation of PAHs from Trial B ......................................................... 127
5.6 Summary of one-factor nested ANOVA statistics for chlordane trials .................. 129
5.7 Tissue accumulation of chlordane from Trial A ................................................... 131
5.8 Tissue accumulation of chlordane from Trial B ................................................... 131
5.9 Summary of one-factor nested ANOVA statistics for organotin trials .................... 133
5.10 Tissue accumulation of organotin compounds from Trial A ............................ 135
5.11 Tissue accumulation of organotin compounds from Trial B ............................ 135

Chapter 6:

6.1 Recently published papers of transplant experiments designed to detect sublethal stress in bivalves .................................................. 162
6.2 Surficial sediment contaminant data for donor and transplant sites ................... 164
6.3 Sample sizes of transplanted cockles recovered for biochemical analyses .......... 169
6.4 Summary of statistics for AEC comparisons........................................... 173
6.5 Summary of statistics for TANP comparisons....................................... 176
6.6 Summary of statistics for glycogen comparisons.................................... 179
6.7 Summary of statistics for RNA comparisons......................................... 182
Effects of contaminants on A. stutchburyi – using biomarkers to detect sublethal stress

List of Plates

1a: Austrovenus stutchburyi ................................................................. 19
1b: Typical habitat of A. stutchburyi .................................................. 19
2a: McLeod Bay in the Whangarei Harbour ....................................... 38
2b: Munroe Bay in the Whangarei Harbour ....................................... 38
3: Mangemangeroa Estuary ................................................................. 66
4: Whangateau Harbour ................................................................. 166
5a: Onehunga study site in the Manukau Harbour ......................... 167
5b: Big Muddy study site in the Manukau Harbour ......................... 167
6a: Takanini study site in the Manukau Harbour ............................. 168
6b: Awhitu study site in the Manukau Harbour ............................. 168
**Effects of contaminants on A. stutchburyi – using biomarkers to detect sublethal stress**

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AEC</td>
<td>Adenylate energy charge</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ARWB</td>
<td>Auckland Regional Water Board</td>
</tr>
<tr>
<td>ASE</td>
<td>Accelerated solvent extraction (Model 2000 Dionex)</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCI</td>
<td>Body condition index</td>
</tr>
<tr>
<td>DDT</td>
<td>Dichlorodiphenyltrichloroethane</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ERL</td>
<td>Effects range low</td>
</tr>
<tr>
<td>ERM</td>
<td>Effects range median</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography with mass selective detection</td>
</tr>
<tr>
<td>GC-MS SIM</td>
<td>Gas chromatography with mass selective detection in the selected ion mode</td>
</tr>
<tr>
<td>GPC</td>
<td>Gel permeation chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>MFO</td>
<td>Mixed function oxygenase</td>
</tr>
<tr>
<td>MDS</td>
<td>Multidimensional scaling</td>
</tr>
<tr>
<td>NIWA</td>
<td>National Institute for Water and Atmosphere</td>
</tr>
<tr>
<td>NOAA</td>
<td>United States National Oceanic and Atmospheric Administration</td>
</tr>
<tr>
<td>PAH</td>
<td>Polycyclic aromatic hydrocarbons</td>
</tr>
<tr>
<td>PCB</td>
<td>Polychlorinated biphenyls</td>
</tr>
<tr>
<td>PEL</td>
<td>Probable effects level</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SIQ</td>
<td>Semi-interquartile range</td>
</tr>
<tr>
<td>TANP</td>
<td>Total adenylate nucleotide pool</td>
</tr>
<tr>
<td>TBT</td>
<td>Tributyltin (organotin)</td>
</tr>
<tr>
<td>TEL</td>
<td>Threshold effects level</td>
</tr>
<tr>
<td>USEPA</td>
<td>United States Environmental Protection Agency</td>
</tr>
</tbody>
</table>
CHAPTER 1

General Introduction

The threat to aquatic habitats from anthropogenic activities is increasing worldwide (Phillips & Rainbow 1993). As technology and science advances, more chemicals and contaminants are produced, which have the potential to affect aquatic habitats when discharged into these environments. Each year, between 200 and 1000 new synthetic chemicals are put on the market (Shane 1994), a rate at which their environmental risks cannot be adequately assessed (Huber 1999). Another significant cause of aquatic habitat degradation is the clearing of vegetation from coastal land for both urban development and rural land use. This practice often results in excessive quantities of sediment being deposited in marine environments, potentially smothering or causing stress to benthic organisms (Norkko et al. 1999, Roper et al. 1989).

In many countries, the recognition of adverse effects on the environment from these activities has brought about the development of new legislation, requiring that toxicity tests be carried out on new chemicals, various discharges be treated, existing effects on habitats be remedied, and the health of ecosystems be monitored for potential adverse effects (Chapman 1995, Eberhardt & Thomas 1991, Phillips & Rainbow 1993, Rand & Petrocelli 1985, Shane 1994, Soule 1987). Thus, research effort has increasingly been put into the development of robust and sensitive techniques for identifying adverse effects on the marine environment.
In New Zealand, the Resource Management Act 1991, requires that adverse effects on the environment be “avoided, remedied or mitigated”. Stemming from the incorporation of components of this Act into regional and district council policy is the requirement that regulatory authorities monitor the quality of their environmental resources (Taylor & Smith 1997). Identifying what aspects of an environmental system to monitor has been recognised as a significant problem, and in light of this, the New Zealand Ministry for the Environment has initiated the development of a suite of environmental performance indicators for sustainable management of ecosystems (Ministry for the Environment 1999). A component of this initiative has been the identification and development of sensitive and cost-effective indicators of environmental health for estuarine systems (Cawthron Institute 1998).

1.1 Types of Contaminants

Contaminants can enter aquatic systems from a variety of sources, including stormwater discharges, sewage outfalls, industrial effluent discharges, dredged sediment disposal, atmospheric fallout, spills, runoff and other nonpoint sources (Donkin et al. 1996, Rand & Petrocelli 1985). Excessive amounts of sediment can have adverse lethal and sublethal effects on marine organisms through smothering (Creese & Cole 1995, Norkko et al. 1999, Roper et al. 1989). In addition, many chemical contaminants in aquatic ecosystems preferentially associate with particulate material, rather than remaining in solution (Williamson et al. 1999b). On settling from the water column they are sequestered in sediments, where they accumulate (Hoffman et al. 1995, Phillips & Rainbow 1993). Contaminants in aquatic systems may be lethal or may impair development, feeding, growth and reproduction of organisms such as fish and shellfish (McDowell et al. 1999).
Xenobiotics, which are of primary concern in coastal aquatic environments, commonly include organic pesticides, petroleum hydrocarbons, and heavy metals (Keenish 1997, Rand & Petrocelli 1985).

Polycyclic aromatic hydrocarbons (PAHs) are composed of carbon and hydrogen, with two or more fused benzene rings (Keenish 1997). Sources of aquatic contamination by PAHs include petroleum spills, urban stormwater, industrial discharges, municipal wastewater outfalls, and incomplete combustion of organic matter (Jackson et al. 1994, Keenish 1997, Hoffman et al. 1995, Phillips & Rainbow 1993). Marine organisms readily accumulate PAHs (Neff 1985) and aromatic hydrocarbons with 4 or more benzene rings are very difficult for organisms to excrete (Hoffinan et al. 1995). Although there is variability in effects both among species and among PAH compounds, generally cellular membrane function and enzyme systems in organisms are affected by PAHs (Keenish 1997, Hoffman et al. 1995, Sole et al. 1994). In addition, many high molecular weight PAHs are known to be carcinogenic to both marine organisms and humans (Ho 1998, Neff 1985, Overton et al. 1994). Organisms with inadequately developed mixed function oxygenase (MFO) capabilities, such as bivalves, do not readily metabolise PAHs (Jackson et al. 1994), and therefore these contaminants can accumulate to very high levels in the tissues, especially lipid-rich tissues, of these organisms (Keenish 1997).

Organochlorine pesticides enter the marine environment from agricultural runoff, urban stormwater, sewage outfalls, and other nonpoint sources (Phillips & Rainbow 1993). Persistence is one of the key characteristics of this group of contaminants, which was made famous by the insecticide DDT (Hoffman et al. 1995). One subgroup of
organochlorine compounds (cyclodienes), were commonly used in New Zealand (Williamson & Wilcock 1994). This subgroup includes insecticides such as andrin, dieldrin, heptachlor, and chlordane (Hoffman et al. 1995). Organochlorines have high solubility in lipids, and are therefore readily accumulated in lipid-rich tissues of marine organisms (e.g. gonads), where they primarily affect molecular receptors (Connell 1995, Hoffman et al. 1995).

The extremely toxic, tri(n-butyl)tin (TBT) or organotin compounds have been used extensively worldwide, and in New Zealand, in marine antifoulant paints. Organotin leaches from antifouling paint rapidly and has a broad range of biological effects, including mortality and reduced growth, of both fouling and non-target species (de Mora et al. 1995, Keenish 1997, Nimmo 1985, Stebbing 1985, Tselentis et al. 1999). For example, body burden of TBT has also been correlated with shell thickening in oysters (King et al. 1989), and the condition known as imposex in whelks (Bryan et al. 1986).

Many countries, including New Zealand, have now prohibited the use of TBT, and although there are declines in the effects of this contaminant on intertidal organisms, open ocean species are still at risk from continued use of TBT on large vessels (Hallers-Tjabbes & Boon 1995).

Many trace metals are important for the functioning of organisms and plants in the natural environment. Often, however, anthropogenic activities cause greater quantities of these metals (e.g. copper, iron, tin and zinc), and other non-essential metals (e.g. lead, cadmium and mercury), to be deposited into marine environments, where they accumulate and can have toxic effects (Chang & Cockerham 1994, Keenish 1997, Leland & Kuwabara 1985).
Sources of anthropogenic inputs cover a range of industrial and agricultural practices such as combustion of fossil fuels, leaching from paint additives, and runoff of fertilisers, pesticides and herbicides. Urban stormwater discharges also often contain high concentrations of metals (Williamson 1993). This group of contaminants has a broad range of toxic effects on marine organisms. For example, zinc intoxication can cause anemia, as it interferes with absorption and utilisation of copper and iron (Underwood 1977) and elicits avoidance behaviour in juvenile bivalves (Roper et al. 1995). Furthermore, copper can cause many different histological abnormalities (Lawson et al. 1995, Leland 1983).

1.2 Detection of contaminant impacts

One approach which can be used to initially ascertain the extent to which a coastal marine habitat is affected by waste discharges, is to analyse water, sediment, or biological tissue for a single, common and easily detectable contaminant. Such a process can highlight areas likely to be contaminated with other pollutants, as well as sites unsafe for the harvesting of edible shellfish. These areas of the coastal habitat could then be more intensively investigated for contaminants and effects on marine organisms. Microorganisms, such as the Enterococci group of bacteria, are common in runoff, stormwater and sewage discharges (Keenish 1997, Snelder & Truman 1995). Techniques for quantifying Enterococci are relatively simple, quick and inexpensive (Donnison 1992). For this reason, initial trials, using Enterococci as an indicator of the presence of other common contaminants in stormwater discharges, were carried out as part of this research (Appendix I).
In that study, spatial and temporal patterns of Enterococci associated with a low volume waste discharge from a small coastal community into the marine environment were documented. Enterococci abundance was used to assess the extent to which cockles (*Austrovenus stutchburyi*), the surficial sediment in which they lived and seawater which passed over them were exposed to toxic contaminants commonly contained in stormwater and runoff discharges. The concentration of Enterococci in shellfish, sediment and water had strong temporal patterns, and was found to be particularly high in winter and after rainfall events (Appendix I). In addition, bacterial levels declined with distance from the point source. This approach helped to estimate the area around this type and size of point source that had the potential to be affected by discharged contaminants. However, as a high abundance of Enterococci only indirectly suggested that cockles might be suffering stress from contaminants, this technique had limited application. My research subsequently focused on the development and application of direct biochemical and physiological measures of contaminant-induced sublethal stress in cockles. The spatial and temporal patterns of Enterococci distribution and concentration identified in the preliminary study also assisted in the design of subsequent field experiments.

Marine organisms have long been used as indicators of environmental health (Römbke & Moltmann 1996, Soule 1987), and there are various definitions and lists of criteria for choice of suitable indicator organisms (Jones & Kaly 1996). The definition of an indicator as used in this thesis is as follows; an organism in which the sublethal effects of chemicals or stressors can be measured in order to provide an early warning of effects of anthropogenic activities on the environment (Jones & Kaly 1996). Some of the commonly cited requirements are that the species is sensitive to contaminants, but
insensitive to handling and laboratory conditions, widely available, a dominant member of
its community, has well understood biology, has the ability to accumulate contaminants,
and is recreationally or commercially important (Boening 1999, Jones & Kaly 1996,
Unfortunately, the ideal aquatic indicator organism is unlikely to exist, and researchers
must proceed with what they believe to be the best alternative (Donkin et al. 1996). Jones
& Kaly (1993) argue that the most important criterion is that the species is ecologically,
economically or culturally important, and that species should not be selected primarily
because they have been used before. The New Zealand cockle, Austrovenus stutchburyi,
fits the criterion of Jones & Kaly (1993) very well. It is an abundant intertidal, soft
sediment bivalve, which is important both economically and culturally, and lives
relatively high on the shore (Morton & Miller 1973) where it is likely to receive a higher
contaminant loading than organisms further down the shore (see also Section 2.1, Chapter
2). Cockles have potential as indicators of aquatic contamination as they are a dominant
species on sheltered or estuarine shores (Morton & Miller 1973), and they are known to
accumulate contaminants (Farrington & Quinn 1973). Because of these characteristics, A.
stutchburyi was chosen as the target indicator organism for my research.

For a contaminant to have an adverse effect on a marine organism, the contaminant, at a
high enough concentration and for an adequate duration, must contact and react with a
receptor site on that organism, i.e. it must be bioavailable (Rand & Petrocelli 1985,
Timperley 1999). Many factors, other than concentration and length of exposure, affect
the toxicity and accumulation of a contaminant in marine organisms, including physical
factors such as distance from point source, dissolved oxygen, pH, and temperature of the
General Introduction

water, and biotic factors such as seasonal cycle, and ability to metabolise and excrete the contaminant (Boening 1999, Rand & Petrocelli 1985 for a review, Stewart 1994).

Stress can be defined as the sum of adaptative physiological responses, involving a diversion of metabolic energy, by which an organism tries to maintain or re-establish normal metabolism in the face of physical and/or chemical changes (adapted from the definitions proposed by Barton & Schreck 1987, and Wedemeyer & McLeay 1981). The stress or effects of the contaminant can be lethal (acute) or sublethal (chronic), with sublethal effects being more difficult to detect, as they are usually initially manifested at low levels of organisation (e.g. suborganismal) (Adams 1990). After effects at these low levels of organisation occur, effects may progressively manifest at higher levels of organisation. For example, growth, reproduction, population structure, community structure, and ecosystem function can subsequently be altered (Adams 1990, McDowell et al. 1999). Estimates of sublethal stress, therefore, have the potential to provide early warning of potential effects at higher levels of organisation. This is often referred to as the bottom-up approach (Adams 1990, Kramer & Botterweg 1991, Munkittrick & McCarty 1995).

Published field studies, however, primarily focus on the identification of sublethal effects of contaminants on marine organisms in areas with known pollution levels. In a recent study conducted in Boston Harbour, Massachusetts, McDowell et al. (1999) showed that Mya arenaria (an infaunal bivalve) from sites with sediment contaminated with PAHs and PCBs had significantly lower gonad index and lipid content, as well as higher incidence of gonadal inflammation and hematopoietic neoplasia than cockles from uncontaminated
reference sites. Incorporation of these types of responses into population models may provide insight into contaminant-induced ecosystem alterations.

As defined by Huggett et al. (1992), biomarkers are biochemical, physiological or histological indicators of sublethal effects of contaminants which manifest at the organismal or suborganismal level. They indicate biological responses to contaminants, and may be used to indirectly detect classes of toxicants or stressors (Cormier & Racine 1992). Biomarkers should be sensitive to contaminants, have low variability in response, exhibit a concentration-response effect, and have ecological relevance (Huggett et al. 1992). Furthermore, biomarker response in a laboratory situation should be validated in field conditions and researchers should be able to distinguish the effect of the contaminant on biomarker response from effects of the natural environment. Even though all these criteria are not often met by a given biomarker (Donkin et al. 1996), this approach has the potential for detecting initial effects on aquatic systems before irreversible damage occurs (Cormier & Racine 1992, McCarthy & Shugart 1990, and see critique by McCarty & Munkttrick 1996, Sastry & Miller 1981). For example, Pridmore et al. (1991), in a manipulative field study in the Manukau Harbour in Auckland, showed that that abundance of juvenile Macomona liliana abundance declined significantly in sediments experimentally dosed with chlordane. Their experiment provides evidence that chlordane can affect bivalve abundance, and that discharges of this contaminant would have implications for the demography of this bivalve species. Some researchers also include population and community characteristics under the definition of biomarkers, although in my research they are generally used to assist with the interpretation of the predictive power of biomarkers at lower organisational level.
A large number of laboratory and field experiments have been undertaken to ascertain the responses of molluscs to natural and contaminant induced stress. In order to establish a dose-response relationship and to satisfy regulatory requirements, researchers have undertaken a range of laboratory experiments and standard toxicity tests where organisms are held in contaminated conditions (e.g. Reish 1987). As laboratory experiments generally have fewer complicating factors (such as predation, competition and seasonal factors) than field experiments the data can be interpreted more easily (Roper & Hickey 1994). However, the laboratory trial approach has been criticised for lacking in environmental realism, in that results cannot readily be extrapolated to the field situation and to higher levels of organisation (Cairns et al. 1993, Underwood 1996). To overcome these criticisms, both these approaches should be employed in concert, as in my study, in order to fully ascertain the response of a given biomarker(s) (Cormier & Racine 1992, Römbke & Moltman 1996, Widdows 1985a).

Physiological and biochemical biomarkers have been employed extensively in aquatic toxicology, in both laboratory and field-based studies (Bayne et al. 1985, Mayer et al. 1992, Mehrle & Mayer 1985). These biomarkers indicate some of the initial effects of contaminants on marine organisms at the organismal and suborganismal level. Biomarkers in this group are very numerous and include various techniques (molecular, chemical and physical) which analyse enzymes, blood chemistry, energetics, and amino acids (see Huggett et al. 1992 for a review, Mayer et al. 1992). Many marine organisms have biochemical and physiological mechanisms that enable them to adapt to environmental stresses such as altered water quality and temperature, diseases, and contaminants. These adaptations, which are often species specific, are generally reflected
in components of their tissue or blood, which can be measured and potentially used as biomarkers. Thus, an understanding of normal, or baseline, measures for biomarkers is essential in order to interpret the significance of alterations and their relationship to the health of the organism (Cormier & Racine 1992, Livingstone 1982, Mehrle & Mayer 1985, Sastry & Miller 1981).

Some of the more common biomarkers used to measure energetic response to contaminants include adenylate energy charge, concentration of total adenylate nucleotides, scope for growth, and oxygen:nitrogen ratio. Biomarkers indicating effects on fecundity include gonad indices, glycogen concentration and condition indices. There are various direct physical measurements of growth which are used as biomarkers, as well as less direct techniques such as RNA concentration, and RNA:DNA ratio. Chapman (1995) contends that in order for responses at the individual and suborganismal levels to be interpreted at higher levels of organisation, biomarkers need to measure endpoints such as energetics, fecundity, and growth. Five biomarkers were selected from the above, at least one from each of Chapman's (1995) categories, based on their successful use in studies of other bivalve species, and availability of resources required to execute the methodology. There were restrictions on choice of biomarkers in my research, mainly due to equipment and logistical constraints, and time limitations for a single researcher. As it was impractical to use all 5 biomarkers for every experiment. At least a common set of 3 were used in each experiment, as well as supplementary indirect measures such as population characteristics and community composition.
General Introduction

It is widely accepted that, in order to better understand effects of contaminants on marine organisms and ecosystems, it is best to use biomarkers at various levels of organisation. This type of approach assists with interpreting the causal relationships between levels (Adams 1990). A new refinement of this approach is used in my research, whereby not only are effects at various levels of organisation simultaneously measured, but several biochemical and physiological biomarkers are used together to assess effects on different critical organism functions and systems i.e. energetics, fecundity, and growth (Figure 1.1). The five main biomarkers used in this study are briefly described below.

**Adenylate Energy Charge**

Adenylate energy charge (AEC) is a measure of metabolic energy instantly available for use by an organism from the adenylate pool (Atkinson 1977, Haya & Waiwood 1983). Its calculation is based on a ratio of concentrations of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP). All organisms use ATP for energy transduction and since this adenylate cannot be stored, any change in its concentration reflects an immediate alteration in capacity for energy production (Newsholme & Crabtree 1986). Consequently, there have been various attempts to develop an instructive index from the adenylate nucleotides based on the theoretical expectation that any environmental stressor can potentially disrupt energy flow. This nonspecific indicator of stress, can therefore, be related to the physiological state of the organism (Mayer et al. 1992). AEC has been extensively employed for the purpose of detecting sublethal stress in bivalves in both laboratory and field experiments (e.g. de Zwaan et al. 1995, Isani et al. 1997, Ivanovici 1980a, Moal et al. 1991, Picado & Le Gal 1990, Wijsman et al. 1976).
Figure 1.1: Conceptual diagram showing the functional links among the five biomarkers used in this study and their relationships with other levels of biological organisation.
General Introduction

Community & Population Characteristics
- Species Richness & Diversity
- Population Structure & Density

Level of Organisation
- Community
- Population
- Individual & Sub-Organismal

Functional Category
- Energetics
- Fecundity
- Growth

Biochemical & Physiological Biomarkers
- Adenylate Energy Charge
- Total Adenylate Nucleotide Pool
- Glycogen
- Condition
- RNA
**Total Adenylate Nucleotide Pool**

Total adenylate nucleotide pool (TANP) is a biomarker closely related to AEC. It represents the sum of the concentration of ATP, ADP and AMP in tissues of the organism, and has been used in several studies of sublethal stress in bivalves in conjunction with AEC (e.g. Ivanovic 1980a, Picado & Le Gal 1990, Wijsman et al. 1976). For example, Picado & Le Gal (1990), in their study on the effect of paper mill effluent on the bivalve *Cerastoderma edule*, concluded that AEC level could be stabilised in situations of stress through a decrease in TANP. The concentration of nucleotides (TANP) can be decreased by degradation of AMP, catalysed by AMP deaminase, which simultaneously creates an increase in AEC.

**Glycogen**

Glycogen is a branched chain polysaccharide, comprising glucose units, which acts as an energy storage substance in many marine organisms, including bivalves (Barber & Blake 1981). A decrease in glycogen concentration can be induced by increased energy demand during conditions of stress (Mayer et al. 1992), and can reflect decreased reproductive potential. Decreases in glycogen concentration attributed to the presence of contaminants has been identified in a freshwater amphipod (Graney & Giesy 1986), fish (Thomas et al. 1981), and crabs (Coglianese & Neff 1982). Although this technique has been used with bivalves (e.g. Cristini 1987), to date, no such relationship has successfully been established between glycogen concentration and contaminants.
**Condition**

Measurement of condition is a simple procedure for estimating the reproductive potential and general health of marine organisms (Crosby & Gale 1990, Roper et al. 1991), which has also been used to indicate physiological stress in marine organisms associated with contaminated habitats (Crosby & Gale 1990, Widdows 1985b). The seasonal pattern of condition in bivalves generally closely follows the reproductive cycle (Marsden & Pilkington 1995, Roper et al. 1991, Savari et al. 1991). A sustained decrease in body condition may be reflected in the long-term viability of bivalve populations; i.e. a decline in recruitment may result from insufficient energy stores to facilitate gametogenesis.

**RNA**

In growing cells, a positive correlation has been identified between protein synthesis and RNA concentration (Brachet 1960). Growth, or replacement of damaged/old cells, can decline when organisms are suffering sublethal stress. In addition, growth tends to decrease with age or size; i.e. larger individuals grow less than small juveniles. Protein synthesis and RNA concentration are also likely to decline in such situations. The relationship of decreased RNA concentration in situations of stress has been shown to occur in many organisms including cockroaches (Sutcliffe 1970), the oyster, *Crassostrea virginica*, (Wright & Hetzel 1985) and fish (Bulow 1987).

There is a need to develop specific indicator species and biomarker protocols for every industrialised country. The aim of my research was to investigate the potential usefulness of the above biochemical and physiological biomarkers to indicate sublethal stress in *Austrovenus stutchburyi*, with a long-term view of incorporation into routine monitoring
of environmental quality and environmental impact by regulatory authorities. At the commencement of this project there was little research being undertaken in New Zealand in this area, and although the biomarker techniques chosen are not new in themselves, their application to a New Zealand estuarine bivalve, especially as a structured group rather than singly, is novel. Whilst recognising that this research can only begin to solve the deficiency of information, the pilot trials undertaken here establish initial data for *Austrovenus stutchburyi* using biomarkers that have been widely embraced by the international scientific community.

The approach used throughout this thesis is that, for an appropriate suite of biomarkers to be employed in the monitoring of environmental quality, natural variability in the response of biomarkers must be fully understood for the indicator species. Furthermore, these responses must be tested in both laboratory and field situations. The initial step to assess the utility of the suite of biomarkers using *A. stutchburyi* was to monitor temporal and spatial variability. In order to ascertain seasonal cycles and to establish baseline values, *A. stutchburyi* from two sites in Whangarei Harbour were sampled over a period of 24 months (Chapter 3). Having gained an appreciation of temporal variation at a limited number of sites, the next step was to investigate spatial variation in biomarker response over a greater range of sites within one harbour (Chapter 4). It was then necessary to ascertain whether biomarker response exhibited a concentration-response characteristic, and in Chapter 5 *A. stutchburyi* are challenged in laboratory trials, with polycyclic aromatic hydrocarbons, chlordane, and organotin compounds to test this. Results from these three studies suggested that field manipulation experiments were likely to be the most profitable approach for testing the utility of the biomarkers. Hence, the
final study used cockles transplanted from a clean environment to field sites where sediment was known to contain a mixture of contaminants (Chapter 6). In Chapter 7 conclusions are drawn about the potential use of these biomarkers for detecting sublethal stress in cockles, and recommendations made about how they can be applied to existing and future scenarios in New Zealand. It is anticipated that further refinement of the techniques developed here will allow early detection of possible degradation of estuarine environments in New Zealand and hence allow remedial action to be taken before serious environmental damage occurs.
CHAPTER 2

General Methods and Materials

2.1 Species Studied

*Austrovenus stutchburyi* (Wood 1828), previously named *Chione stutchburyi* (see Jones 1979), is a venerid bivalve, considered as the ecological equivalent of the European cockle, *Cardium edule*, and North American quahog, *Mercenaria mercenaria* (Grace 1973) (see Plate 1a). *Austrovenus stutchburyi* are generally referred to as the ‘cockle’ in New Zealand, although anatomically *A. stutchburyi* should be classified as a clam and the common term ‘cockle’ is a misnomer. Use of the alternative name “New Zealand Littleneck Clam” has been approved by the US Food and Drug Administration for the purposes of commercial marketing (Belton 1985, Williams *et al.* 1993).

*Austrovenus stutchburyi* are indiscriminate filter-feeders, ubiquitous on soft-shores around New Zealand (Morton & Miller 1973). The cockle is sedentary, buried in the sediment to a depth of approximately 2-5 cm (pers. obs.). Although they thrive in sandy sediment, cockles also survive in mud and gravel/pebbles (Belton 1985) (see Plate 1b). Movement of adults is generally limited, but, Hewitt *et al.* (1996) have observed individuals moving up to 30 cm during one tide. The feeding pattern of cockles is controlled by an endogenous circatidal rhythm of valve movement, with most active pumping occurring for 2-3 hours at high tide (Beenjtes & Williams 1986, Hutchinson 1988). Their diet is predominantly composed of suspended particles, small zooplankton and microalgae (Belton 1985). The overlying water quality is strongly reflected in the gut contents.
Plate 1a: Adult *Austrovenus stutchburyi*. Note the anemone (*Anthropleura* sp.) attached to the shell in the foreground.

Plate 1b: Typical sandy cockle habitat, with empty cockle shells amongst and on top of the sediment, and some live cockles partially exposed above the sediment.
(Pilkington 1992) and the quality of the flesh, with contaminants and microorganisms present in the water being accumulated during the feeding process (Belton 1985).

Cockles are dioecious, and generally reach maturity at 18-20 mm in length (Belton 1985, Stephenson 1981). There is a single breeding season with individuals producing gonad during spring-summer, irrespective of sex and age (Larcombe 1971), and spawning synchronously in summer-autumn (Pilkington 1992). After external fertilisation, the zygote develops into a swimming veliger, which settles 10-15 days later by attaching itself to the substratum with a byssus thread (Belton 1985).

* Austrovenus stutchburyi * are a long-lived species, with some individuals apparently aged over 20 years (Stephenson 1981). Growth is variable, and largely influenced by environmental conditions and height on shore (Belton 1985), with the maximum shell height recorded as 60 mm (Powell 1979, Stephenson 1981). Cockles positioned lower on the shore have a longer feeding period, and consequently grow faster than those located high on the shore, which often suffer restricted growth. Cockles can be considered ecologically important as they often dominate the intertidal community, and densities up to 3000/m² have been recorded (Stephenson & Chanley 1979). Cockles also provide an important substratum for other estuarine species, such as algae of the genera *Ulva* and *Codium*, and anemones (*Anthropleura* sp.) (Larcombe 1971).

*Austrovenus stutchburyi* has both economic and cultural importance in New Zealand. Commercial harvesting of *A. stutchburyi*, for sale in New Zealand and overseas (e.g. to Australia and the USA), predominately occurs in Golden Bay, Otago and on Snake Bank
in the Whangarei Harbour (Morrison & Cryer 1999). Recreational harvest of the cockle is common, and it is a traditional Maori seafood item (Kearney 1999).

2.2 Sampling Sites
Experiments described in this thesis were carried out in the Whangarei Harbour (Northland region), Whangateau Harbour, Mangemangeroa Estuary and Manukau Harbour (Auckland region). The location of these sites is broadly given in Figure 2.1, and described in further detail in the appropriate chapters.

2.3 Collection Procedures
For the adenylate nucleotide and glycogen analyses, *A. stutchburyi* were removed from the sediment, opened with a knife and the foot muscle excised with a scalpel blade. The tissue was wrapped in pre-labeled aluminium foil and immediately frozen in liquid nitrogen. The time lapsed from removal of each animal from the habitat to emersion in liquid nitrogen did not exceed 15 seconds. Samples were transferred to a -75°C freezer and stored until nucleotides were extracted.

Cockles collected for RNA analyses were removed from the sediment, placed in a plastic bag, and transported on ice to the laboratory. Dissection of the foot muscle was carried out using sterile procedures, in order to avoid introduction of RNase enzymes, which hydrolise RNA. Cockles were handled with gloved hands, opened with a sterile knife, and the soft tissue placed into a sterile, plastic petri dish. The foot muscle was excised using a sterile scalpel blade, and placed on a sterile pre-labeled piece of aluminium foil.
Figure 2.1: North Island of New Zealand, showing locations where samples were collected.
General methods and materials
The tissue was wrapped in the aluminium foil and frozen in liquid nitrogen, before storage in a -75°C freezer until analysed further.

For condition analyses, cockles were gathered by hand, placed in a plastic bag, and transported to the Leigh Marine Laboratory on ice, where they were measured and weighed as detailed in section 2.8.

2.4 Adenylate Nucleotide Extraction

The methodology used was a modification of that reported by Carroll & Wells (1995), Lowe (1992) and Ryder (1985). In the laboratory, each sample was individually removed from the freezer, weighed, and chopped into small pieces using a scalpel. A new sterile scalpel blade and petri dish was used for each tissue sample to avoid contamination between samples. The tissue was then added to 1.5 ml of 0.6M perchloric acid, homogenised using a small laboratory blender (Ika®-Ultra-Turrax® Antirieb T25) and extracted on ice for 30 minutes. The aggregate from the blender was thoroughly rinsed in distilled water between samples, in order to minimise between-sample contamination. The homogenate was then transferred to an eppendorf tube and centrifuged for 2 minutes at 11,000 rpm. The supernatant was poured off into a new eppendorf, and the plug of tissue remaining was discarded. Supernatant was neutralised by combining a subsample of 200 µl with a 400 µl 3:1 mixture of Freon-TF (1,1,2 trichlorotrifluoroethane) and tri-n-octylamine. The mixture was then vortexed for 1 minute and centrifuged at 11,000 rpm for 2 minutes, for phase separation. The top aqueous layer, containing the acid-soluble extract, was then pipetted into a new eppendorf tube. Neutralised samples were stored in
a -75°C freezer until processed on High Performance Liquid Chromatograph (HPLC) for adenylate nucleotide separation, or analysed for glycogen content.

2.5 Adenylate Nucleotide Analyses

The HPLC system used for all analyses consisted of two Waters 501 pumps, a Rheodyne model 7125 syringe-loading sample injector, and a Waters 441 absorbance detector. Separations were achieved using a Alltima 5 μm silica RP C18 lichrosorb column (4.6 mm internal diameter × 250 mm long) equilibrated at 30°C. The mobile phase of 0.4M potassium dihydrogen orthophosphate and 0.06M dipotassium hydrogen orthophosphate, dissolved in purified distilled water, was used at a flow rate of 1 ml/minute. Buffer solutions were prepared daily and were vacuum filtered through a 0.45 micron nitrocellulose Millipore filter. Standard adenylate nucleotides were diluted in distilled water as follows; adenosine 5'-triphosphate (Sigma A2383) 7.69 mg/100 ml, adenosine 5'-diphosphate (Sigma A2754) 8.97 mg/100 ml, and adenosine 5'-monophosphate (Sigma A1752) 8.61 mg/100 ml, aliquotted into eppendorf tubes and frozen at -75°C until required. Prior to commencing running a batch of samples on the HPLC, at least 3 injections of the standard preparation were made and, for each adenylate nucleotide, peak areas were compared between runs. If the coefficient of variation was greater than 5%, samples were not run, and the HPLC equipment, mobile phase, guard column, etc. checked for problems. Standard nucleotide preparations were also injected at the beginning and end of each sample batch, and between at least every 10 samples, to ensure no blockages in the column or significant changes in the characteristics of the column had occurred. Each sample (20 μl) was injected into the column and allowed to run for 15 minutes. The injection port was rinsed with distilled water between samples.
ATP, ADP, and AMP peaks were identified by comparison with standard solutions. Peak area was calculated using Maxima 820 software. Adenylate concentration calculations were made by comparison of the individual nucleotide area of the sample with the average for the standard injections made at the beginning and end of the 10 sample run containing the sample being calculated. Adjustments were then made for sample dilution. AEC was calculated using the following formula: \[ \text{AEC} = \frac{\text{ATP} + \frac{1}{2} \text{ADP}}{\text{ATP} + \text{ADP} + \text{AMP}} \] (Atkinson 1977). TANP concentration was the sum of ATP, ADP and AMP.

2.6 Glycogen Analyses

The sample extract was removed from the freezer and thawed (see section 2.3). The Boehringer Mannheim test kit (Cat. No. 207 748) was used for glycogen determination. Amyloglucosidase (100 µl) was added to 50µl of sample (or 50µl of distilled water for the negative control) and incubated at 55°C in a dry water bath for 15 minutes. Triethanolamine buffer (0.5 ml) and 0.5 ml of distilled water were then added and the mixture allowed to stand for 3 minutes before initial absorbance (termed A1) was read at 340 nm on a spectrophotometer (Unicam UV/Vis UV3). Hexokinase (10 µl) was subsequently added and the mixture allowed to stand for 10-15 minutes before a second reading (termed A2) on the spectrophotometer, at absorbance 340 nm.

Glycogen concentration was calculated using the following equation; \[ G = [(A1-A2) - N] \times C \times D, \] where \( G \) was the concentration of glycogen in grams per 1000g of shellfish tissue, \( N \) was calculated as A1-A2 for the negative control, \( D \) was a calculation made for the dilution, and \( C \) was a constant term used to take account of the molecular weight of
glycogen, final volume of the solution, and the characteristics of the light path and absorption. The constant is calculated as \( C = V \times MW/e \times d \times v \times 1000 \), where \( V \) = Final Volume (1.16 ml), \( MW \) = molecular weight of the substance to be assayed (for starch \( MW_{\text{glucose}} - MW_{\text{water}} = 162.1 \text{ (g/mol)} \), \( e \) = absorption coefficient of NADPH at 340 nm = 6.3 (1 x mmol\(^{-1}\) x cm\(^{-1}\)), \( d \) = light path (cm) = 1, \( v \) = sample volume (ml) = 0.5 (Boehringer Mannheim 1993).

2.7 RNA Analyses

Methodology for RNA extraction and quantification was based on Chomczynski (1993) and Life Technologies (1997).

Sterile techniques were used throughout the protocol. Foot tissue (50-100 mg) was weighed, and placed in a sterile petri dish, where it was chopped with a scalpel blade. Tissue was transferred to a sterile plastic tube and homogenised in 1 ml of TRIzol (Life Technologies Product Number 15596-018) for 20 seconds. The homogenate was transferred to a sterile eppendorf tube and centrifuged at 12,000 g for 10 minutes at 4° C. The supernatant was poured into a new sterile eppendorf tube, and in order to separate out the phenol, 200 μl of chloroform (Sigma C2432) was added. The eppendorf tube was shaken by hand for 15 seconds, and left to incubate at room temperature for 3 minutes, before being centrifuged at 12,000 g for 15 minutes at 4° C. Of the three phases formed (clear phenol-free upper phase, white interphase and pink lower phase), the upper clear aqueous phase, containing the RNA, was pipetted off (without penetrating the interphase) and placed into a new eppendorf tube. RNA was precipitated by the addition of 500 μl of isopropyl alcohol (Sigma I9516). The tube was shaken gently, incubated at room temperature for 5 minutes, and then centrifuged at 12,000 g for 10 minutes at 4° C. The isopropyl alcohol was removed by aspirating it, and the RNA was redissolved in deionised H₂O (Sigma 27970).
temperature for 10 minutes, and then centrifuged at 12,000 g for 10 minutes at 4° C, to form the RNA pellet.

The supernatant was pipetted off, leaving the pellet in the base of the eppendorf tube, and 1 ml of 75% ethanol added. High grade ethanol was diluted to 75% in diethylpyrocarbonate (DEPC) water. The DEPC was made up to 0.01% in distilled water (0.01 mg of DEPC in 99.99 ml of water), shaken occasionally, and left overnight, before being autoclaved and cooled prior to use. The tube was then vortexed gently to wash the RNA pellet, centrifuged at 7,500 g for 5 minutes at 4° C, the supernatant was poured off and discarded, and the pellet briefly air dried in an incubator at 25° C (approximately 3 minutes). Care was taken to ensure the pellet did not completely dry.

Sodium dodecyl sulphate (SDS) was diluted to 0.5% in DEPC water (0.5 g in 99.5 ml of DEPC). SDS was added to the eppendorf (1 ml) to dissolve the pellet, and once dissolved, the tube was incubated at 55° C, suspended in a water bath, for 10 minutes. Once cooled to room temperature, the sample was transferred to an acrylic cuvette (Sarstedt 67.740). Absorbance of the solution was read on a spectrophotometer (Unicam UV/Vis UV3).

Prior to reading the absorbance of tissue sample solutions, the spectrophotometer was calibrated to zero, with SDS in both cells, at wavelengths 260 and 280 nm. Samples were read against a reference cell, containing SDS, at both 260 and 280 nm. The ratio of absorbance at 260:280 nm was calculated in order to check for contamination of the sample. A ratio of less than 1:6 indicates contamination, likely to be from incomplete
General methods and materials

phenol removal, or RNA not being fully dissolved. Samples with ratio’s less than 1.6 were excluded from the data set.

RNA concentration was calculated as follows: at 260 nm one absorbance unit is equal to 40μg/ml pure RNA. As tissue samples were a slightly different initial weight (50-100 μg), the final amount of RNA was standardised, relative to the initial weight.

RNA standards (Sigma R7125) (at 20, 40, 60, 80, 100 μg/ml dilutions in SDS) were run with each batch of samples, and a linear relationship between absorbance and RNA concentration was detected at both 260 and 280 nm. The ratio of 260:280 nm was >1.93 for all standard runs.

2.8 Condition

The body condition index (BCI) was used for all estimations of condition (Savari et al. 1991). Cockles were rinsed with tap water in order to remove any sediment from the shell, then shell height, length and breadth were measured using Vernier calipers. The valves of the cockle were then opened by cutting the adductor muscle with a small knife. The soft tissue was removed, positioned in a pre-weighed, labeled tinfoil dish and placed in a drying oven at 50° C for 12 hours, at which stage the tissue was completely dry. The dry tissue was then weighed. BCI was calculated as the weight of the dried soft tissue (g), multiplied by 100, and divided by the volume of shell cavity, where the shell cavity volume was estimated as π/6 multiplied by product of shell height, shell length and shell breadth.
2.9 Statistical Analyses

Experimental designs are presented in each chapter. Standard errors are presented on all graphs as a measure of variation about the mean. Where seasonal collection of samples occurs, this refers to the austral seasons.

Hypotheses were generally tested using univariate analysis of variance (ANOVA) with the SAS statistical package (SAS Institute Inc. 1990). Analyses were two-tailed with a critical probability of 0.05, unless otherwise stated. Homogeneity of variances was tested by plotting residual values against predicted values, and normality tested using the W statistic of Shapiro & Wilk (1965). Where ANOVAs detected significant differences, Tukey’s tests were used \textit{a posteriori} to identify significantly different means in the data sets.

Wilcoxon’s 2-sample tests (Zar 1996) were used where data seriously violated assumptions of homogeneity of variances or normality of distribution. The Bonferroni adjustment was made where several pairs of data were compared, in order to decrease the likelihood of detection of a false significant difference (Miller 1986).

Other procedures, including statistical analyses, were used in particular experiments, and these are described in the appropriate chapter.
CHAPTER 3

Seasonal Variation of Biomarker Response in *Austrovenus stutchburyi* from Whangarei Harbour

3.1 Introduction

Almost half the population of the world lives within 150 km of the coast, generally in large coastal urban centers. Activities associated with these urban areas, such as the clearing of vegetation, discharge of pollutants and land reclamation, impact on coastal environments (Huber 1999, McIntyre 1995). Estuaries are particularly vulnerable as they are often poorly flushed, low-energy environments that retain fine sedimentary particles and any contaminants adhered to them. Historically, estuaries have been viewed by the public as having low environmental value. However, attitudes have changed and, in response to anthropogenic activities, which often adversely affect estuaries, there has been an increasing amount of research into the effects of discharged contaminants on estuarine organisms and habitats (Chapman 1995, Goldberg 1995).

Contamination sources in estuaries can include the discharge of stormwater and industrial effluent, runoff and septic tank leachate. These types of pollution are often characterised by long-term low level or periodic discharges. Stress effects on aquatic organisms from this type of long-term environmental contamination are referred to as chronic. Acute stress occurs in response to short-term perturbations, such as chemical spills.
Seasonal variation of biomarker response

Effects of chronic sublethal stress often first manifest at organism or subcellular levels of biological organisation, before effects at population or community levels manifest (Adams 1990). It is generally recognised that sublethal stress affects subcellular processes, which in turn can impair structural and functional responses, and subsequently affect growth, reproduction and survivorship, with obvious consequences at the population level (see Figure 1.1).

Biochemical techniques have been successfully used to indicate subcellular stress responses, and to predict higher order effects, in several marine bivalves (Leavitt et al. 1990). However, in order to distinguish natural variation and seasonal cycles from contaminant-induced alterations, robust data on background levels are required (Huggett et al. 1992, Jeffries 1972, Livingstone 1982, Sastry & Miller 1981, Widdows 1985a). As this information is often species-specific, data must be gathered for each potential indicator species separately.

Choice of indicator species is often a trade-off between sensitivity and survival. For example, a highly sensitive species is unlikely to be available to sample, whereas a species with low sensitivity to contaminants may not manifest significant alterations until contaminant levels are extremely high. This is especially a problem in estuarine environments, which are characterised by sudden and frequently large-scale changes in environmental factors such as salinity, dissolved oxygen and temperature (Lockwood 1976). In these environments, organisms often have subcellular adaptive compensatory mechanisms or tolerance to stress (Chapman 1995, Lockwood 1976, Russell & Storey 1995). For example, Russell & Storey (1995) showed in a laboratory experiment that the
Seasonal variation of biomarker response

salt marsh bivalve, *Geukensia demissus*, regulates enzymes involved in carbohydrate metabolism during periods of anoxia. Organisms with such tolerances are potentially able to alter their physiology (e.g. energy partitioning) in response to stress. Picado & Le Gal (1990), in a laboratory study of the bivalve *Cerastoderma edule*, demonstrated that 24 hours after dosing with paper mill effluent, *C. edule* instigated a series of regulation processes to ensure a stabilised AEC level. Thus, in estuarine environments, researchers are faced with the dilemma of a vulnerable habitat and often resilient species. In this situation, background or baseline information must be especially clear and robust, and many endpoints must be investigated in order to increase the likelihood of finding an effective suite of biomarkers.

The aim of the sampling described in this chapter was to establish baseline data for the estuarine bivalve, *Austrovenus stutchburyi* (cockle), for three biochemical endpoints; adenylate energy charge (AEC), total adenylate nucleotide pool (TANP) and glycogen. *Austrovenus stutchburyi* is an ideal candidate as a biological indicator of pollution as it is abundant, easily collected and handled and is sedentary (Phillips & Rainbow 1993). In addition, *A. stutchburyi* can be found close to the mean high water mark in many estuaries throughout New Zealand (Belton 1985), where it has the potential to be exposed to a higher concentration of contaminants from discharges and runoff compared to organisms which inhabit sediment further down the shore. The foot muscle of cockles was chosen as the tissue to be analysed as greater alterations in energy reserves have been shown to occur, under stressful conditions, in muscle tissue than in other tissues or whole body homogenates (de Zwaan *et al*. 1995, Isani *et al*. 1997, Wijsman 1976).
Hydrolysis of adenosine triphosphate (ATP) is the primary source of energy for many cellular processes. ATP levels remain relatively constant in muscle tissue, due to a balance between utilisation and formation (Mathews & van Holde 1990). Adenylate energy charge (AEC) is a measure of metabolic energy instantly available for use by the cells of all organisms (Cristini 1987, Huggett et al. 1992). Its calculation comprises a ratio of adenosine triphosphate (ATP) to its breakdown products, adenosine diphosphate (ADP) and adenosine monophosphate (AMP). The theoretical basis for this index is that an organism under stress will use more energy, which is associated with a decrease in the primary biochemical energy source, ATP (Huggett et al. 1992). The calculation is as follows; AEC = ATP + 0.5 ADP/(ATP + ADP + AMP) (Atkinson 1977). This index produces values ranging from 0 to 1, and from the formula it follows that high ATP concentrations will give high AEC ratios. High ratios (0.8-0.9) are thought to occur in organisms found in non-limiting environments, values between 0.5 and 0.7 indicate organisms suffering suboptimal conditions, and animals with AEC lower than 0.5 are thought not to have the capacity to recover from the associated stress (Bayne et al. 1985).

A limitation of this technique for contaminant monitoring studies is that AEC is affected by environmental factors such as decreased salinity, anaerobic conditions and high temperature, as well as contaminants (Haya & Waiwood 1983, Ivanovici 1980a). For example, anoxic conditions have been shown to lower AEC in several marine bivalves (Carroll & Wells 1995, Cristini 1987, Isani et al. 1997, Wijsman 1976). Therefore, in order to assess the effect of collecting samples at low tide, an experiment simulating natural anoxia at low tide (air-exposed) in comparison with oxygenated conditions at high tide (submerged) was undertaken in the laboratory.
Seasonal variation of biomarker response

Total adenylate nucleotide pool (TANP) provides a measure of adenylate capacity within the cells of the organism, and comprises the sum of ATP, ADP and AMP. Previous studies have used this measure in conjunction with AEC. Wijsman (1976), in a study on the mussel *Mytilus edulis*, and Ivanovici (1980b), using the gastropod *Pyrazus ebenimus*, found that TANP concentration did not alter when organisms were placed in suboptimal environmental conditions, although AEC decreased. However, Picado & Le Gal (1990), in their study of paper mill effluent effects on the bivalve *Cerastoderma edule*, determined that TANP decreased in order to stabilise falling AEC. Estimations of TANP concurrently with AEC calculations can provide more information on energy state and adenylate nucleotide exchange and regeneration.

The third biomarker tested in this sampling programme, glycogen, is a branched chain polysaccharide comprising glucose units. It acts as an energy storage substance in many marine organisms including bivalves (Barber & Blake 1981, Lawrence 1989). Glycogen is directly involved in the steps of glycolysis - the metabolism of glucose to produce energy (Withers 1992). Seasonal changes in storage of glycogen in marine bivalves are often closely associated with gonad developmental cycles (reviews by Bayne 1976 and Gabbott 1976). Glycogen is generally stored prior to gametogenesis when energy-rich food is abundant and is subsequently utilised during gamete production (Barber & Blake 1981). However, energy reserves may also be drawn upon during stress response to contaminated environments, such as for the production of metallothionein or stress proteins (see Huggett et al. 1992, Thomas et al. 1981). It might be instructive, therefore, to measure glycogen levels in conjunction with AEC and TANP to allow a more comprehensive assessment of energy state and alterations.
Seasonal variation of biomarker response

Although previous studies have indicated that adenylate nucleotide indices and glycogen levels have potential as biomarkers of sublethal stress in bivalves, lack of field validation data for the indicator species (see review by Huggett et al. 1992) and inadequate experimental design (see Chapter 6, Table 6.1, for examples) have often limited the usefulness of these endpoints. The primary aim of the research described in this chapter is to investigate the possible existence of seasonal cycles in AEC, TANP and glycogen levels for *A. stutchburyi*. These data can then be used to determine whether these 3 biochemical endpoints are sufficiently sensitive as biomarker tools to establish effects of environmental contamination against a background of natural variation.

The sites used for this baseline study of seasonal variation were in the lower reaches of the Whangarei Harbour, approximately 150 km north of Auckland. The area supports a very large population of cockles, which is extensively harvested by traditional, recreational and commercial gatherers. As the Northland Regional Council, the regulatory authority responsible for good water quality in this harbour, was concerned about possible impacts of stormwater and sewage discharges in the general area (Mortimer 1991), the opportunity arose to obtain regular samples of cockles for biochemical analyses. Although this study was not designed as a formal impact assessment, the use of two sites with potentially differing water quality during the trials provided the basis for more extensive investigations of possible links between water quality and stress responses in cockles (described in subsequent chapters).
3.2 Method & Materials

3.2.1 Description of Study Sites and Characteristics of the Cockle Population

Two sites in Whangarei Harbour, Northland, New Zealand were studied over a 3 year period for this research (Figure 3.1). McLeod Bay (35° 49' 00"S, 174° 30' 08"E) has a small urban community with housing predominantly along the shoreline and a history of low-level sewage and stormwater contamination (Mortimer 1991) (Plate 2a). Munroe Bay (35° 47' 26"S, 174° 29' 14") is surrounded by rural land generally used for grazing of sheep and cattle (Plate 2b).

As a large range of both natural and anthropogenic factors affect biochemical indicators, environmental variables and sediment grain size characteristics were monitored. To characterise the sediments in the area, approximately 200 g of sediment was collected from both McLeod and Munroe Bay in May 1996, transported to the Leigh Marine Laboratory and placed in a drying oven at 40°C until completely dry. The sediment was then subsampled and 50-60 g shaken through a stack of graduated sieves (64 mm - <0.063 mm). Each size fraction was weighed and categorised according to Mudroch & Bourbonniere (1994).

On every sampling occasion and at each site, water temperature, dissolved oxygen, salinity and pH were measured using Orion hand-held meters, and chlorophyll a levels were determined using a spectrophotometric technique (Parsons et al. 1985). Condition of cockles was quantified using a gravimetric method described by Crosby & Gale (1990).
Figure 3.1: Location of monitoring sites, McLeod and Munroe Bay, within Whangarei Harbour, Northland, New Zealand.
Seasonal variation in biomarker response

Munroe Bay

McLeod Bay

Bream Head

5 km
Seasonal variation in biomarker response

Plate 2a: McLeod Bay, within the Whangarei Harbour.

Plate 2b: Munroe Bay, within the Whangarei Harbour.
To characterise the biotic environment in which the cockles lived, population and community structure data for *Austrovenus stutchburyi* were collected. At each site, using qualitative sampling, the position on the shore where *A. stutchburyi* were most abundant was identified. This area was determined to begin at approximately 150 m from a fixed point on each foreshore edge, and extend beyond the waters edge at low tide. All subsequent samples for population, community, sediment and biochemical analyses were collected from an area approximately 160 m from the point on the foreshore.

Three 0.0625m² quadrats were haphazardly placed on the sediment at each site and were excavated to a depth of 10 cm. The contents of each quadrat was washed through sieve with a mesh size of 1 mm and retained. In March, May, July, September and November 1996 and January 1997 *A. stutchburyi* were measured and counted, and in September and November 1996 all other macrofauna were also identified and counted. Average width and density were calculated for *A. stutchburyi* and size-frequency plots constructed.

Using the PRIMER software package (Clarke & Warwick 1994), species richness, diversity and evenness were calculated from the community structure data. In addition, a multidimensional scaling (MDS) plot was constructed to examine macrofaunal abundance relationships between sites. Data were 4th root-transformed in order to reduce the effects of dominant occurrences, such as high numbers of *A. stutchburyi* (Clarke & Warwick 1994). Data matrices were produced using Bray-Curtis calculations.

### 3.2.2 Seasonal Monitoring of Biomarker Response

Between 9 and 25 *A. stutchburyi* (15-25 mm shell width) from both sites were sampled at 3-monthly intervals from the summer 1996 to spring 1998 for glycogen analysis and
summer 1997 through to spring 1998 for adenylate nucleotide analyses. Sampling times were based on mean monthly seawater temperature for the previous 3 years; February (summer), May (autumn), August (winter), November (spring). Cockles were collected from a similar distance from the foreshore (c. 160 m), at low tide.

Adenylate nucleotide and glycogen analyses were carried out in accordance with methods described in Chapter 2.

Two-way analyses of variance (ANOVA) were performed, using the SAS software package (SAS Institute 1990), to compare data between sites and among seasons for each biochemical index. Season and location were treated as fixed factors, and replicate cockle tissue samples treated as a random factor. Variance of data were tested by plotting residual versus predicted values and normality checked using the W statistic (Shapiro & Wilk 1965) prior to conducting analyses.

3.2.3 Experimental Comparison Between Immersed and Air-Exposed Cockles

Thirty cockles (widths 15-25 mm) were removed from the sediment at both McLeod and Munroe Bay and transported to the Leigh Marine Laboratory where they were held in seawater aquaria, without sediment, overnight. Fifteen cockles from each site were placed on a tray and exposed to the air for 4 hours under natural daylight conditions in the laboratory (air temperature approximately 20°C), in order to simulate exposure to air at low tide. The remaining 15 cockles from each site were maintained in the aquaria, also in natural daylight conditions with air temperature approximately 20°C. After 4 hours, foot muscle tissue from both the immersed and air-exposed cockles was excised and analysed.
as per the above method. The total time taken to remove the tissue from all animals and place in liquid nitrogen was approximately 25 minutes. Tissues were analysed for adenylate nucleotides and glycogen content.

A two-way ANOVA was performed on the data, using the SAS software package (SAS Institute 1990), to compare immersed and air-exposed cockles, from each site, for each biochemical index. Location and tide (immersed or air-exposed) were treated as fixed factors and the values obtained for each biochemical technique were treated as random factors. Variance and normality were tested as mentioned above.

3.3 Results

3.3.1 Population and Community Composition

The Austrovenus stutchburyi population at McLeod Bay is characterised by predominantly large individuals (10-30 mm width) (Figure 3.2). New, small individuals appeared in the population in July 1996, and these blended with the adult cohort by January 1997. Average width of A. stutchburyi over the entire population, ranged from 13.72 to 18.25 mm (Table 3.1). Mean density was quite variable, ranging from 208 (+/-53.28/m²) in May to 1546.72 (+/-176.32/ m²) in November (Table 3.1).

Munroe Bay cockles had an adult (>18mm width) dominated population in March and May 1996 (see Figure 3.2) (with average widths of 19.54 and 19.34 mm respectively (Table 3.1)). A small recruitment event may have occurred in the autumn, identified by small individuals in the population in July, but due to very low population numbers in following samples it is difficult to trace the recruits through to the adult population. Mean
density in of cockles March was 885.28/m² and 1349.28/m² in May. Density was much lower in subsequent samples, with 85.28/m² recorded in September 1996.

The bivalves Austrovenus stutchburyi, Paphies australis, and Nucula hartvigiana and the gastropods Diloma subrostrata, Zeacumantus lutulentus and Cominella sp. dominated the community structure at both sites. The community at McLeod Bay also included the wedge shell, Macomona liliana, whereas at Munroe Bay the bivalve, Felaniella novaezelandiae, was evident. Multidimensional scaling (MDS) separated samples for the sites, with Munroe Bay samples having greater variability in community composition than those from McLeod Bay (Figure 3.3). This is likely to be due to the large variability in the A. stutchburyi population at Munroe Bay. Margalef’s index (Clarke & Warwick 1994), or species richness, is a measure of the number of species present for a given number of individuals, and is virtually identical for both sites (Figure 3.4). However, Munroe Bay has slightly higher species diversity and evenness than McLeod Bay, reflecting the greater dominance of A. stutchburyi at Munroe Bay (Figure 3.4).

3.3.2 Sediment Size, Environmental Variables, and Condition

The sediment from both sites can broadly be classified as sand. Munroe Bay sediment was marginally coarser than that from McLeod Bay, with the median grain sizes being 0.49 and 0.42 mm respectively. Sediment from Munroe Bay comprised 85.08% sand (0.063-2mm) and 14.92% gravel (>2mm). Sediment from McLeod Bay was found to have 0.88% silt/clay (<0.063mm), 92.24% sand and 7.88% gravel.
Due to non-normality of the data set, non-parametric Wilcoxon two-sample (rank-sum) tests were used to test for differences in the environmental data between sites. Chlorophyll a (p>0.21), dissolved oxygen (p>0.20), pH (p>0.12), seawater temperature (p>0.21), salinity (p>0.21) and condition (p>0.24) were not significantly different between sites. Salinity ranged between 30 and 35 °/oo with higher values in warmer months. Sites had similar trends for seawater temperature and A. stutchburyi condition, with higher values in summer and spring and lower values in colder months. Seawater temperature ranged between 13.4 and 27.1°C, and gravimetric condition index varied between 2.7 and 5.3 for both sites.

3.3.3 Biochemical Components – Seasonal

A two-way analysis of variance (ANOVA) was performed on the data for adenylate energy charge data and this indicated a significant season*location interaction term (p<0.0005) (see Table 3.2), which makes interpretation of individual factors problematic. The cause of the interaction can clearly be seen in Figure 3.5a; AEC levels in cockles from Munroe Bay slightly declined between autumn and winter 1997, but AEC in cockles from McLeod Bay increased over this period. Similarly, patterns also diverged between summer and autumn 1998. Although the seasonal trend was not consistent between years (Figure 3.5a), a Tukey’s test identified that spring had the highest AEC value compared to the other seasons (Figure 3.5b). No significant differences between sites were detected (Figure 3.5c), which is emphasised by the frequency of overlapping confidence intervals in Figure 3.5a.
Seasonal variation of biomarker response

An ANOVA revealed significant differences for TANP among seasons (p<0.0001) and locations (p<0.004) (see Table 3.3). Total adenylate nucleotide values ranged from approximately 1.7 to 3.7 mM/g tissue (Figure 3.6a). A Tukey’s test indicated TANP for summer, autumn and spring were not significantly different from each other, but all were different to winter, which had lower average TANP (Figure 3.6b). Although an ANOVA found there to be significant differences between sites, Figure 3.6c indicates that 95% confidence intervals almost overlap, with Munroe Bay having higher values.

Glycogen data was log-transformed in order to overcome non-heterogeneity of variances. An ANOVA was performed on the transformed data and revealed a significant interaction term for season and location (p<0.052). The cause of the interaction can be identified in Figure 3.7a, where patterns in glycogen levels for the two sites diverged between seasons (see between spring and summer 1997, and autumn and winter 1997). Taking the significant interaction into consideration, the ANOVA did however, identify strong differences between seasons (p<0.0001) and locations (p<0.0001) (see Table 3.4). The cycling of glycogen within years is clearly illustrated in Figure 3.7a. A Tukey’s test revealed high glycogen levels in spring and summer, and lower glycogen levels in autumn and winter. Mean glycogen values in summer were not significantly different from those in spring (Figure 3.7b), but autumn values were significantly lower, and glycogen for winter significantly lower still. Although the ANOVA detected significant differences between glycogen values from McLeod and Munroe Bay (p<0.0001), Figure 3.7c suggests they are not greatly different, due to overlapping 95% confidence intervals.
3.3.4 Immersion vs Emersion Experiment

In the experiment testing the effects of tide (simulated in the laboratory with immersed and air-exposed treatments), AEC was found to be similar for cockles sourced from McLeod and those from Munroe Bay (two-way ANOVA, p>0.05). There were significant differences however, between cockles immersed and those air-exposed (p<0.0004) (Figure 3.8a) (see Table 3.5). In the ANOVA analyses for TANP, a significant interaction was identified between location and tide (p<0.0031), which makes it difficult to interpret single factors (see Table 3.6). However, TANP was higher for air-exposed cockles compared to immersed animals for Munroe Bay, but no difference was detected for animals sourced from McLeod Bay (Figure 3.8b) (see Table 3.6). No differences in glycogen, between immersed and air-exposed animals, were detected for either site (Figure 3.8c), although glycogen concentration was higher for Munroe Bay cockles compared to those from McLeod Bay (p<0.0109) (see Table 3.7).

3.4 Discussion

Adults dominated cockle populations at both McLeod and Munroe Bay, although size-structure varied between sampling occasions. This, together with high variability in average density, indicates that cockle distribution at both sites is spatially and temporally patchy. Community structure was dominated by similar species at both sites, although there were differences between sites in the less numerically dominant species, which may be due to small disparities in sediment grain size characteristics between sites. Even though the MDS plot separates sites, this is primarily based on the much greater abundance of cockles at McLeod Bay. Environmental variables were consistent between sites, and both Munroe and McLeod Bay have sandy sediment, are dominated by cockle
Seasonal variation of biomarker response

populations, and have typical soft sediment communities characterised by the presence of *Paphies australis*, *Nucula hartvigiana* and carnivorous gastropods (Morton & Miller 1973).

Few investigations have been undertaken into the effect of season on adenylate energy charge. My study found there was a seasonal cycle in AEC for *A. stutchburyi*, with higher values in spring compared to other seasons. In contrast, Moal *et al.* (1991) detected low AEC values in two species of oyster in a northern hemisphere summer, and high AEC values the following winter. Giesy & Dickson (1981) found AEC values to be highest during winter in the freshwater clam *Corbicula fluminea*. However, they detected maximum AEC in autumn for another freshwater bivalve, *Anodonta imbecillis*. This suggests that seasonal cycles are not consistent for all bivalves, and therefore detailed investigations for each species are warranted. Furthermore, seasonal cycles may not be consistent over time for a particular species, or even a specific population of a species, and this is illustrated in my investigation by the very different patterns in AEC for the autumn and winter sampling occasions between the years 1997 and 1998.

AEC values detected in this study ranged between 0.8 and 0.2 and were often below the 0.5 level, which theoretically is the non-recovery threshold (Bayne *et al.* 1985). These low and variable AEC values may reflect sampling difficulties or potential inaccuracies in executing the methodology. Alternatively, the low AEC values may be an artifact of inherent tolerance to stress in this particular species, suggesting that a range of ‘normal’ AEC values and a non-recovery value need to be established for each species, due to variable stress tolerance among species.
A seasonal cycle was also evident for TANP, with values lower in winter compared to other seasons. Conversely, Giesy & Dickson (1981) found that the freshwater clams, Corbicula fluminea and Anodonta imbecillis, had highest TANP in winter, although their study was only over 1 year and, therefore, it is not possible to say whether this trend was consistent among years.

Several researchers have found lowered AEC in molluscan tissues in both field and laboratory conditions of environmental stress (Carroll & Wells 1995, Cristini 1987, de Zwaan et al. 1995, Isani et al. 1997, Picado & Le Gal 1990). In my study, there was no difference in AEC between sites, but cockles from Munroe Bay had significantly higher TANP than those from McLeod Bay. Adenylate nucleotides are highly regulated biomolecules (Huggett et al. 1992) and this is highlighted in studies of acute contaminant stress where researchers have identified a decrease in TANP in order to maintain AEC levels (Dickson & Giesy 1981, Shofer and Tjeerdema 1998). It may be that AEC was regulated (i.e. maintained) in cockles from McLeod Bay, through a reduction in TANP. As environmental variables were similar between sites, this decrease in TANP may have occurred in response to factors other than natural environmental stress, such as low-level chronic contaminants from runoff and stormwater flows (Mortimer 1991). TANP may potentially have a role as a biomarker of persistent or chronic environmental stress, and AEC may be useful in revealing short-term, more acute perturbations in environmental quality. This possibility is further explored in Chapter 6.

Seasonal cycles in glycogen concentration have been extensively investigated in marine bivalves. Data from my study were similar to the typical pattern described for other
bivalve species (de Zwaan & Zandee 1972, Hickman & Illingworth 1980, Pazos et al. 1997, Wenne & Styczynska-Jurewicz 1987), with maximum values in spring and summer and minimum levels in winter. Commonly, alterations in glycogen concentration relate to gametogenic cycles (Barber & Blake 1981). Energy in the form of glycogen is stored when food is abundant and utilised in the production of gametes (Barber & Blake 1981, Reid 1969). Glycogen concentration in cockles has a similar pattern; a long spawning period in late spring and summer (Larcombe 1971) is associated with a decline in glycogen. Furthermore, minimum glycogen values occur in winter, when energy-rich food is scarce and reserves are depleted by gamete production and metabolic functions. There were no differences in glycogen concentration between sites which suggests that the main influences on glycogen levels, such as diet, reproductive condition, and environmental factors, were similar between sites.

The effect of anoxia on AEC is clearly illustrated in Figure 3.8a. Austrovenus stutchburyi exposed to air for 4 hours showed a similar trend of lowered AEC to that documented by Carroll & Wells (1995) and de Zwaan et al. (1995). TANP was not different between immersed and air-exposed cockles from McLeod Bay, but higher TANP was recorded for air-exposed cockles from Munroe Bay. As anticipated, there was no variation in glycogen content between immersed and air-exposed cockles, but Munroe Bay animals had higher levels than McLeod, reflecting the same pattern as that described in the in-situ seasonal sampling. It is recommended that, as AEC is affected by air-exposure (or anoxia), samples should be collected at a similar state of tide, and if low tide is chosen (for ease of sampling) results should be interpreted with the consideration that AEC values are likely to be depressed compared to samples collected at high tide in oxygenated conditions.
Seasonal variation of biomarker response

This may explain, in part, the low values of AEC obtained in some of the samples collected from the Whangarei Harbour.

This study confirms that *A. stutchburyi*, like most marine bivalves, have cycles of energy storage and utilisation. These cycles are likely to be a consequence of complex interactions among food, environmental factors, reproductive state and habitat condition (Pazos *et al.* 1997). This research describes, for the first time, biochemical cycles for *A. stutchburyi*, and provides baseline data for a species that has potential as a biological indicator in New Zealand estuaries. In addition to the temporal patterns demonstrated during this investigation, spatial comparisons between the two sites suggested that this combination of biomarkers might indeed be able to detect responses of cockles to different environmental stressors. However, to fully investigate this, a more comprehensive understanding of spatial variability within a harbour is required. This could not be done in Whangarei Harbour during 1996/1997, but an opportunity arose in 1998/1999 to confirm the consistency of seasonal patterns over a greater range of sites in a smaller estuary near Auckland. The results of this expanded spatial study are given in Chapter 4.
Figure 3.2: Size-frequency plots for *Austrovenus stutchburyi* populations at McLeod and Munroe Bay. The cockle populations were sampled every 2 months for 1 year.
McLeod Bay

- March 1996 n=133
- May 1996 n=78
- July 1996 n=211
- September 1996 n=209
- November 1996 n=290
- January 1997 n=187

Munroe Bay

- March 1996 n=166
- May 1996 n=253
- July 1996 n=84
- September 1996 n=16
- November 1996 n=69
- January 1997 n=44

Shell Width (mm)
**Seasonal variation in biomarker response**

Table 3.1: Mean shell width and density of *A. stutchburyi* at two sites in the Whangarei Harbour (Munroe Bay and McLeod Bay), sampled every 2 months for one year.

<table>
<thead>
<tr>
<th>Date</th>
<th>Mean Width (mm +/- std error)</th>
<th>Mean Density (individuals per m² +/- std error)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>McLeod Bay</td>
<td>Munroe Bay</td>
</tr>
<tr>
<td></td>
<td>15.1 (0.39)</td>
<td>19.54 (0.37)</td>
</tr>
<tr>
<td>Mar 1996</td>
<td>13.72 (0.49)</td>
<td>19.34 (0.3)</td>
</tr>
<tr>
<td>May 1996</td>
<td>15.7 (0.47)</td>
<td>14.8 (0.57)</td>
</tr>
<tr>
<td>July 1996</td>
<td>18.04 (0.33)</td>
<td>10.25 (1.08)</td>
</tr>
<tr>
<td>Sept 1996</td>
<td>15.98 (0.3)</td>
<td>15.28 (0.6)</td>
</tr>
<tr>
<td>Nov 1996</td>
<td>18.25 (0.3)</td>
<td>17.52 (0.78)</td>
</tr>
</tbody>
</table>
**Figure 3.3:** Multidimensional scaling plot of 4th root transformed community composition data, for McLeod and Munroe Bay. Samples 1-3 and 7-9 were collected in September 1996, and samples 4-6 and 10-12 were collected in November 1996. The circles were added to illustrate the spread of data for each site.

**Figure 3.4:** Plot of community richness, diversity and evenness indices for all community composition data for Munroe and McLeod Bay. Errors are 95% confidence intervals.
Seasonal variation in biomarker response

4th root transformed
stress = 0.08

Richness

Diversity

Evenness
Seasonal variation in biomarker response

Table 3.2: Results of two-way analysis of variance describing AEC in *A. stutchburyi* over four seasons and at two locations within the Whangarei Harbour. DF=degrees of freedom.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Season</td>
<td>3</td>
<td>0.56</td>
<td>27.67</td>
<td>0.0001</td>
</tr>
<tr>
<td>Location</td>
<td>1</td>
<td>0.03</td>
<td>1.28</td>
<td>0.2601</td>
</tr>
<tr>
<td>Season*Location</td>
<td>3</td>
<td>0.13</td>
<td>6.19</td>
<td>0.0005</td>
</tr>
<tr>
<td>Residual</td>
<td>204</td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Figure 3.5a:** Plot of adenylate energy charge detected in *A. stutchburyi* foot tissue at Munro and McLeod Bay each season from summer 1997 to spring 1998 (+/- 95% confidence intervals).

**Figure 3.5b:** Plot of adenylate energy charge detected in *A. stutchburyi* foot tissue for each season (data combined for sites and years) (+/- 95% confidence intervals). Different letters designate significant differences detected with Tukey’s test.

**Figure 3.5c:** Plot of adenylate energy charge detected in *A. stutchburyi* foot tissue for each site (data combined for seasons and years) (+/- 95% confidence intervals). Different letters designate significant differences identified with Tukey’s test.
Table 3.3: Results of two-way analysis of variance describing TANP in *A. stutchburyi* over four seasons and at two locations within the Whangarei Harbour. DF=degrees of freedom.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Season</td>
<td>3</td>
<td>8.40</td>
<td>14.67</td>
<td>0.0001</td>
</tr>
<tr>
<td>Location</td>
<td>1</td>
<td>7.45</td>
<td>13.01</td>
<td>0.0004</td>
</tr>
<tr>
<td>Season*Location</td>
<td>3</td>
<td>0.40</td>
<td>0.7</td>
<td>0.553</td>
</tr>
<tr>
<td>Residual</td>
<td>203</td>
<td>0.57</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.6a: Plot of total adenylate nucleotide pool (mM/g foot tissue wet weight) detected in *A. stutchburyi* at Munroe and McLeod Bay each season from summer 1997 to spring 1998 (+/- 95% confidence intervals).

Figure 3.6b: Plot of total adenylate nucleotides (mM/g foot tissue wet weight) detected in *A. stutchburyi* for each season (data combined for sites and years) (+/- 95% confidence intervals). Different letters designate significant differences detected with Tukey’s test.

Figure 3.6c: Plot of total adenylate nucleotides (mM/g foot tissue wet weight) detected in *A. stutchburyi* for each site (data combined for seasons and years) (+/- 95% confidence intervals). Different letters designate significant differences identified with Tukey’s test.
Table 3.4: Results of two-way analysis of variance describing glycogen concentration in *A. stutchburyi* over four seasons and at two locations within the Whangarei Harbour. DF=degrees of freedom.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Season</td>
<td>3</td>
<td>7.36</td>
<td>178.94</td>
<td>0.0001</td>
</tr>
<tr>
<td>Location</td>
<td>1</td>
<td>0.60</td>
<td>14.7</td>
<td>0.0001</td>
</tr>
<tr>
<td>Season*Location</td>
<td>3</td>
<td>0.18</td>
<td>4.31</td>
<td>0.052</td>
</tr>
<tr>
<td>Residual</td>
<td>381</td>
<td>31.74</td>
<td>0.0001</td>
<td>0.052</td>
</tr>
</tbody>
</table>
Figure 3.7a: Plot of glycogen concentration (g/1000g foot tissue wet weight) detected in *A. stutchburyi* at Munroe and McLeod Bay each season from summer 1996 to spring 1998 (+/- 95% confidence intervals).

Figure 3.7b: Plot of glycogen concentration (g/1000g foot tissue wet weight) detected in *A. stutchburyi* for each season (data combined for sites and years) (+/- 95% confidence intervals). Different letters designate significant differences detected with Tukey’s test.

Figure 3.7c: Plot of glycogen concentration (g/1000g foot tissue wet weight) detected in *A. stutchburyi* for each site (data combined for seasons and years) (+/- 95% confidence intervals). Different letters designate significant differences identified with Tukey’s test.
Table 3.5: Results of two-way analysis of variance describing AEC for air-exposed and immersed *A. stutchburyi* from Munroe and McLeod Bay within the Whangarei Harbour. DF=degrees of freedom, E=air-exposed, I=immersed.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location</td>
<td>1</td>
<td>0.003</td>
<td>0.63</td>
<td>0.4324</td>
</tr>
<tr>
<td>Tide (E/I)</td>
<td>1</td>
<td>0.06</td>
<td>13.99</td>
<td>0.0004</td>
</tr>
<tr>
<td>Location*Tide (E/I)</td>
<td>1</td>
<td>0.002</td>
<td>0.39</td>
<td>0.5352</td>
</tr>
<tr>
<td>Residual</td>
<td>54</td>
<td>0.004</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.8a: Plot of adenylate energy charge (+/- 95% confidence intervals) for *A. stutchburyi* from Munroe and McLeod Bay at experimentally simulated high and low tide (ie. air-exposed and immersed). Pink symbols indicate cockles exposed to air (low tide) and blue symbols indicate cockles immersed (high tide).

Figure 3.8b: Plot of total adenylate nucleotide concentration (mM/g foot tissue wet weight) for *A. stutchburyi* from Munroe and McLeod Bay at experimentally simulated high and low tide (ie. air-exposed and immersed). Colour codes as above.

Figure 3.8c: Plot of glycogen concentration (g/1000g foot tissue wet weight) for *A. stutchburyi* from Munroe and McLeod Bay at experimentally simulated high and low tide (ie. air-exposed and immersed). Colour codes as above.
Seasonal variation in biomarker response

AEC
(± 95% CI)

TANP
(mMg wet weight ± 95% CI)

Glycogen
(g/1000g tissue ± 95% CI)

Munroe Bay

McLeod Bay

Site
Table 3.6: Results of two-way analysis of variance describing TANP for air-exposed and immersed *A. stutchburyi* from Munroe and McLeod Bay within the Whangarei Harbour. DF = degrees of freedom, E = air-exposed, I = immersed.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location</td>
<td>1</td>
<td>5.29</td>
<td>13.59</td>
<td>0.0005</td>
</tr>
<tr>
<td>Tide (E/I)</td>
<td>1</td>
<td>2.02</td>
<td>5.18</td>
<td>0.0268</td>
</tr>
<tr>
<td>Location*Tide (E/I)</td>
<td>1</td>
<td>3.74</td>
<td>9.61</td>
<td>0.0031</td>
</tr>
<tr>
<td>Residual</td>
<td>54</td>
<td>0.39</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.7: Results of two-way analysis of variance describing glycogen concentration for air-exposed and immersed *A. stutchburyi* from Munroe and McLeod Bay within the Whangarei Harbour. DF=degrees of freedom, E=air-exposed, I=immersed.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location</td>
<td>1</td>
<td>265.32</td>
<td>6.88</td>
<td>0.0109</td>
</tr>
<tr>
<td>Tide (E/I)</td>
<td>1</td>
<td>7.55</td>
<td>0.20</td>
<td>0.6596</td>
</tr>
<tr>
<td>Location*Tide (E/I)</td>
<td>1</td>
<td>35.89</td>
<td>0.93</td>
<td>0.3382</td>
</tr>
<tr>
<td>Residual</td>
<td>63</td>
<td>38.54</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 4

Spatial Variation of Biomarker Response in *Austrovenus stutchburyi*, within the Mangemangeroa Estuary

4.1 Introduction

Regulatory authorities around the world are charged with the responsibility of monitoring and protecting their environmental resources (Taylor & Smith 1997). As a consequence of this onus, an increasing amount of research has been directed towards the development and implementation of sensitive, robust, simple and cost-effective techniques and the identification of indicator organisms in order to assess environmental health (Cawthron Institute 1998, Huggett *et al.* 1992, Osenberg & Schmitt 1996). In aquatic environments, sedentary organisms, such as bivalves, are often used as indicator organisms (Römbke & Moltmann 1996, Soule 1987), and in the New Zealand context, the cockle *Austrovenus stutchburyi* is being examined as a potential environmental indicator.

As a preliminary investigation, three biomarkers (adenylate energy charge (AEC), total adenylate nucleotide pool (TANP) and glycogen) were monitored in cockles at two sites in the Whangarei Harbour at 3-monthly intervals for over two years. This provided information on the temporal patterns exhibited by these biomarkers in populations of *Austrovenus stutchburyi* (Chapter 3). A seasonal pattern was established for AEC (with highest levels in spring) and TANP (characterised by low levels in winter). Glycogen levels followed a strong seasonal cycle with high levels in spring and summer, and low levels in autumn and winter. These patterns were generally consistent between the two sites, but one biomarker (TANP) showed significant differences which might have been
due to differences in water quality i.e. lower TANP at McLeod Bay corresponding to reputedly poorer water quality. Because there was no replication of putative impact sites, however, a more parsimonious explanation would be that the two sites differed slightly in their natural environmental conditions, with McLeod Bay being a slightly less favourable habitat for cockles than Munroe Bay. To further examine among site variability, there is a need to sample a much wider range of sites than was possible in the lower Whangarei Harbour.

The Mangemangeroa Estuary (36° 55' 00"S, 174° 56' 50"E) is situated on the boundary between the metropolitan limits of south-east Auckland and rural land to the south (Figure 2.1). This estuary forms part of a larger intertidal aquatic ecosystem which also contains the Turanga and Waikopua Estuaries (Figure 4.1). These three estuaries drain into a large shared embayment. As urban Auckland spreads, this catchment is increasingly under threat from effects associated with urban development (see Plate 3).

Intensification of development in the Mangemangeroa catchment may have detrimental effects on the estuarine ecosystem (Stewart & De Luca 1999). Activities such as the clearing of vegetation to enable earthworks are likely to increase sediment loading in the receiving coastal environment (Creese & Cole 1995). In addition, a change in land use from rural to urban means runoff of contaminants such as toxic chemicals, nutrients and pathogens may be increased, all of which have potential effects on estuarine organisms such as cockles which are is a dominant component of the macrobenthic marine community in the Mangemangeroa Estuary. As a further consequence, human health may be endangered if contaminated shellfish are consumed (Cockerham & Shane 1994).
Figure 4.1: Location of Mangemangeroa, Turanga & Waikopua Estuaries in southeast Auckland.
Spatial variation of biomarker response
Plate 3: Mangemangeroa Estuary at low tide, showing the surrounding land use, and the proximity of urban Auckland.
It is generally recognised that biomarker response to spatial variation in natural environmental conditions needs to be formally addressed before biomarkers can be employed in ongoing pollution monitoring (e.g. Phillips & Segar 1986). In estuarine systems, where there are large variations in environmental factors such as salinity, sediment type, and period of submergence (Lockwood 1976), it is likely that there is sufficient variability in biomarker response to require that several sites be monitored within an estuary. Thus, within the structure of a wider collaborative investigation of the health of the Mangemangeroa Estuary, a sampling exercise was undertaken to test whether position in an estuary consistently results in predictable responses in a range of biomarkers.

The sub-organismal biomarkers tested in Chapter 3 are further tested here, along with a further biomarker at the individual level; body condition index (BCI). This additional index also has the potential to signal effects at higher levels of organisation. A sustained decrease in body condition may be reflected in bivalve populations long term; i.e. a decline in recruitment may result from insufficient energy stores to facilitate gametogenesis. BCI was also included in this experiment to potentially assist with interpretation of results at the sub-organismal level, and provide baseline information for ongoing monitoring in this estuary. Adams (1990) recommends this type of tiered approach, rather than investigating a single level of biological organisation, because it enables assessment of a wider range of sensitivity (or tolerance) to stress, and can better assist with identification of functional linkages between levels of organisation. It is the establishment of these relationships between responses at lower levels of organisation and responses at higher levels of organisation which has the potential to predict effects of
Spatial variation of biomarker response

ext ecological relevance; a common criticism of biomarkers at the subcellular and organism
level when used on their own.

4.2 Methods and Materials

4.2.1 Site Characteristics

Aerial photographs and qualitative distributional data for *Austrovenus stutchburyi* assisted
with selection of sampling sites within the Mangemangeroa Estuary. Six sites (three pairs
of two) were selected along a downstream gradient, in order to ascertain potential stress
on cockles in different microhabitats. This gradient was considered important for site
location, as sites in the upper reaches of estuaries often have higher deposition of silt and
contaminants, and sites located near harbour entrances are generally better flushed by
changes in the tide. The main cockle population is located on a sandy area in the main
bay, opposite the entrance to the Mangemangeroa Estuary, which is exposed at low tide
(Figure 4.2). *Austrovenus stutchburyi* within the estuary can be considered as being on
the edge of cockle distribution, where they may be under greater pressure from natural
stress (salinity and oxygen changes) and anthropogenic stress (siltation and
contaminants).

At the mouth of the estuary, Site B was located on the point of a moderately elevated sand
spit (Figure 4.2). Site G was situated on the opposite side of the channel to Site B, where
the sediment was muddy. Site C was adjacent to a cluster of housing along the foreshore,
in an area where fine mud had accumulated, and Site F, on the opposite side of the
channel, was characterised by having patches of muddy sediment between outcrops of
rocky reef (Figure 4.2). Upstream in the estuary, Site D was located at the fringe of a
Figure 4.2: Location of sampling sites within the Mangemangeroa Estuary.
Spatial variation of biomarker response

Main A. stutchburyi population

B  G
C  F
D  E
Spatial variation of biomarker response

mangrove stand in fine mud (Figure 4.2). Site E, on the opposite side of the channel, was also characterised by fine mud and stands of small mangroves. As the Mangemangeroa Estuary is relatively small, on each sampling occasion samples could be collected from all sites over one low tide. This enabled physical factors, such as weather and temperature, to be considered similar among sites.

As with the preliminary study in Whangarei Harbour, sediment grain size, cockle population characteristics and macrofaunal community composition were analysed in order to characterise each site. Approximately 100 g of sediment was collected from each site in September 1998, and frozen until grain size was analysed, using a Galai WCIS100 laser particle analyser, and median grain size calculated. On each of the four sampling occasions; (June (autumn), September (winter), December (spring) 1998, and February 1999 (summer)), five quadrats (0.0625m²) were haphazardly placed at each site, and the contents excavated to a depth of 10 cm. Sampling was abandoned at Site E during the autumn collection period, however, due to the extremely low abundance of cockles. The contents of each quadrat were washed through a box sieve (mesh size 2 mm) and all cockles retained were measured and counted. Size frequency and density calculations were made. In December 1998, five quadrats (0.0625m²) were haphazardly positioned at each site, and the sediment excavated to a depth of 10cm. The sediment and fauna contained within them were washed in a box sieve (mesh size 2 mm) and the large macrofauna retained were identified and counted. Species richness (Margalef's index) and species diversity (Shannon-Weiner) were calculated using the statistical software package PRIMER (Clarke and Warwick 1994).
Five of the original six sites (C, D, E, F, G) were further characterised using measures of sediment metal contamination (Tricklebank unpublished data). No data on metal contaminants were collected from Site B. In August 1999, twelve replicate scoops of surficial sediment (top 2 cm) were collected from an area of approximately 20 m x 20 m at each site. The replicate sediment samples were combined to create a single sample for each site, weighing approximately 400 g. Samples were refrigerated and sent to a professional testing laboratory (R.J. Hill Laboratories Ltd) for contaminant analyses. The protocol used to recover metals from the sediment involved nitric/hydrochloric acid digestion followed by inductively coupled plasma mass spectrometry (USEPA 200.2).

4.2.2 Adenylate Nucleotides and Glycogen

Ten cockles (shell width 15-25 mm) were collected from each site in June, September and December 1998, and February 1999. Collection, handling and analyses for adenylate energy charge (AEC), total adenylate nucleotide pool (TANP) and glycogen were carried out in accordance with the protocols described in Chapter 2.

4.2.3 Condition

Fifteen cockles (shell width 15-25 mm) were collected from each site in September and December 1998, and February 1999, transported to the Leigh Marine Laboratory and held in flow-through seawater aquaria overnight. The following morning, cockles were measured and soft tissue dried to a constant weight at 50°C for 12 hours in a drying oven. The dry tissue was then weighed and the body condition index (BCI) (Savari et al. 1991) calculated.
BCI = \frac{\text{dry tissue weight (g)} \times 100}{\text{internal shell cavity volume}}

Where shell cavity volume = \frac{\pi}{6} \times (\text{shell height} \times \text{length} \times \text{breadth})

4.2.4 Statistical Analyses

Two-way analyses of variance (ANOVA) were carried out on adenylate energy charge, total adenylate nucleotide pool, glycogen, condition and density data using the SAS software package (SAS Institute 1990). For each biomarker, comparisons were made among sites and seasons. The factors site and season were both treated as fixed for the analyses. Prior to conducting analyses, homogeneity of variances in the data set were tested by plotting residual values against predicted values, and normality analysed using the W statistic (Shapiro & Wilk 1965).

Pearson's correlations were undertaken, using the SAS statistical package (SAS Institute 1990), in order to ascertain whether patterns of community composition, cockle density or contaminant levels were associated with sediment grain size trends.

4.3 Results

4.3.1 Site Characteristics

Sediment grain size analyses revealed that Site B had the coarsest sediment (median grain size 161.24 μm), and Site D the finest sediment (median grain size 70.46 μm). Sites C, E, F and G had a similar median grain size (100-125 μm) (see Table 4.1).
Spatial variation of biomarker response

A two-way ANOVA, performed on cockle density data, identified a significant interaction term for location and season (Table 4.2) suggesting cockle distribution is patchy at the spatial and temporal scales which samples were collected. Among site patterns are reasonably clear however (Figure 4.3); Site F had the highest density of cockles in all seasons except summer (February 1999), and Site G the lowest cockle density. At Site B cockle density was similar to that at Site C and Site D for all seasons, although the density of cockles at Site D was always higher than that at Site C (Figure 4.3). No clear seasonal trends were discernable (Figure 4.3).

The structure of the cockle populations differed among sites within the estuary. Size-frequency histograms indicated a bimodal *A. stutchburyi* population at Site B in June 1998 (Figure 4.4a). By February 1999, however, the smaller individuals had largely joined the adult population (Figure 4.4b-d). Site C had a unimodal cockle population, with an average shell width of approximately 20 mm (Figure 4.4e-h). There was a small cluster of juvenile cockles evident in histograms for Site D (Figure 4.5a-d), which were largely combined with the adult population by February 1999. The average shell width at Site D was approximately 23 mm. The cockle population distribution at Site F was unimodal in June 1998 (Figure 4.5e-h), but was bimodal in subsequent sampling occasions, due to a recruitment event. The average size of cockles in the adult size-frequency peak at this site was similar to that at Site D (i.e. 23 mm). It is difficult to interpret the size-frequency histograms for Site G due to the low abundance of cockles (Figure 4.6a-d). However, there were few juveniles present at this site, and adult cockles were generally large (> 25 mm shell width).
Species richness, a measure of the number of species present for a given number of individuals, was greatest at Site G (5.28) and lowest at Site C (3.54). Shannon-Weiner diversity index was also highest at Site G and lowest at Site C (Table 4.3). Site B had similar species richness to Site G, but species diversity at Site B was similar to Site C. Richness and diversity were similar at Sites D and F (Table 4.3).

On an international scale, contaminant levels at all sites were low, significantly below the minimum biological effects concentrations defined in sediment quality guidelines proposed by Long et al. (1995) and Smith et al. (1996) (Appendix II). The presence of copper, zinc and lead at low levels indicate minor impact from urban and rural land use practices in the catchment surrounding the Mangemangeroa (Geoff Mills (NIWA) pers. comm.).

The concentrations of copper, lead and zinc were highest in sediment collected from Site F and lowest at Site G (Table 4.4). Lead, copper and zinc levels were similar among Sites C, D and E. In the context of sediment quality guidelines proposed by Smith et al. (1996) and Long et al. (1995), the concentration of metals in sediment, at all sites in the estuary, was below the level at which biological affects have been identified in previous studies i.e. below the Threshold Effects Level (TEL) (Smith et al. 1996) and the Effects Range Low (ERL) level (Long et al. 1995) (see Appendix II). The highest copper level detected in the Mangemangeroa sediment samples (14.3 µg/g) was approximately 77% of the TEL, and less than 43% of the ERL concentration. The highest lead concentration of 13.0 µg/g was 43% of the TEL concentration and 28% of the ERL, whereas zinc concentration at Site F was 41% of the TEL level and 34% of the ERL level (Table 4.4, and Appendix II).
Data were not available for Site B, but it is likely that contamination is lower at this site compared to other sites, as it has coarser sediment, is located at the estuary mouth where contaminants are likely to be better flushed and diluted by the changing of the tide, and is in a position within the estuary similar to Site G, which had low levels of contaminants.

Pearson's correlation coefficients were calculated in order to examine relationships among median sediment grain size and species richness, species diversity, cockle density, copper concentration, lead concentration and zinc concentration. No significant correlations were detected ($\alpha=0.05$). Only 64% of the variation in species richness ($r^2=0.64$, $p=0.24$) and 3.8% of the variation in species diversity ($r^2=0.038$, $p=0.95$) could be explained by sediment size. Cockle density was also found to be unrelated to sediment grain size ($r^2=0.12$, $p=0.85$). Of the contaminant data, 78% of the variability in zinc ($r^2=0.78$, $p=0.22$), 67% of the variability in lead ($r^2=0.67$, $p=0.33$) and 55% of the variability in copper ($r^2=0.55$, $p=0.45$) data could be explained by sediment grain size.

Site characterisation data can be useful in the interpretation of biomarker variability among sites. In this experiment, sites were characterised by sediment grain size, cockle population structure and density, community composition and heavy metal contamination of surficial sediment. Although median sediment grain size was different among sites, there were no significant correlations between sediment grain size and community composition, cockle density or contaminant levels. Therefore, other unknown factors must be influencing community composition and the concentration of heavy metal contaminants in sediment.
Although site selection was designed as a gradient, the spatial patterns were complex and primarily influenced by small scale geomorphological characteristics. To summarise, Site B was characterised by coarse sandy sediment with medium cockle density, high species richness, but low species diversity. Site C was located in a deposition area, and the cockle population here had a unimodal distribution which was dominated by larger individuals. Species richness, species diversity and the concentration of metals in the sediment were low at this site. Upstream in the estuary, Site D had low levels of metals in its fine sediment, and a bimodal cockle size distribution. Site E was primarily characterised by its lack of cockles. The concentration of metals was highest at Site F, which was an erosion area with parts of the bedrock exposed, but this site had a high density of cockles. Site G had the highest species richness and diversity, but the lowest density of cockles and the lowest concentration of metals in the sediment.

4.3.2 Adenylate Energy Charge

A two-way ANOVA identified a significant interaction term for season and location (Table 4.5). However, there is evidence of some strong site patterns. A Tukey's test identified that AEC was higher at Sites F, G and B compared to Site C, and AEC was different between Sites F and D (Figure 4.7). In June, September and February AEC was higher at Site F compared to Site D (Figure 4.7). However, the reverse was true in December. Apart from June, when Site D had a mean AEC of approximately 4.5 and Site C had an AEC value of 6, Site C had lower mean AEC than all other sites (Figure 4.7). Adenylate energy charge (AEC) was highest in September (winter) at all sites (Figure 4.7).
4.3.3 Total Adenylate Nucleotide Pool

A two-way ANOVA revealed significant differences both among sites and seasons (Table 4.6). Among sites, a Tukey’s test identified TANP to be higher at Sites F and B compared to Sites C and D. In addition, Site G had significantly higher TANP than Site D. Generally, Sites C & D had lower TANP than the other sites. Sites had different trends in TANP among the four seasons (Figure 4.8). There is an increase in TANP at all sites from June to September, but from September to December two sites had a decrease in TANP (B and D), two sites had an increase (F and G) and TANP at Site C remained at a similar level (Figure 4.8). Patterns were similarly mixed between December and February, with the TANP of cockles increasing at three sites, but decreasing at two. Variability in the data is unusually high in February for Site D and Site F. Total adenylate nucleotide pool (TANP) was lowest in June (autumn) for all sites except Site C.

4.3.4 Glycogen

A two-way ANOVA revealed significant differences in glycogen concentration among seasons, but failed to detect differences among sites (Table 4.7). A Tukey’s test determined June and February to have significantly lower glycogen levels than September and December (Figure 4.9). Concentration of glycogen was similar for June and February, and for September and December. All sites followed a similar pattern over the seasons; moderate levels in autumn (June), an increase in winter (September), high levels in spring (December) and a decrease in late summer (February) (Figure 4.9).
4.3.5 Body Condition Index

Data for body condition index (BCI) were only available for three seasons: winter (September), spring (December) and summer (February). A two-way ANOVA detected a significant interaction term for site and season, which is most likely due to diverging trends between seasons for some sites (Figure 4.10, Table 4.8). For example; there is an increase in BCI at Site G between September and December, whereas BCI for all other sites declines over this period. Sites C and D had lowest BCI in December, and were close to the lowest value in September and February (Figure 4.10). Condition appears to be highest in winter and lowest in summer (Figure 4.10).

Biomarker values averaged across all seasons for each site indicate that Site C had the lowest values for AEC, TANP, and BCI (see Figure 4.11). Site D also had low AEC, TANP and BCI values. In contrast, these two sites had slightly higher mean glycogen concentration than Sites B and F. Sites B, F and G had very similar AEC, TANP and BCI, and Site B had the lowest glycogen levels (Figure 4.11). However, given the clear seasonal glycogen patterns evident at each site (Figure 4.9), an average of glycogen values across all seasons may not be particularly instructive nor biologically significant.

4.4 Discussion

Estuaries are highly variable ecosystems, in terms of environmental factors. They are often characterised by large temporal and spatial variations in salinity, temperature, dissolved oxygen, sediment type and contaminant levels (Lockwood 1976). Because of this intrinsic variability, it is realistic to expect that monitoring only one part of an estuary will not adequately reflect or predict effects across the entire system. Therefore, several
representative sites, including areas most likely to be affected by anthropogenic activities, should be monitored in order to establish a reliable picture of the health of the whole estuarine ecosystem.

The differences in density of *A. stutchburyi* among sites in other estuaries has been attributed to physical factors such as sediment size (Clarke 1997). From studies in the nearby Tamaki Estuary, Clarke (1997) concluded that cockle abundance was negatively related to silt content and positively related to sand content. If this were the case, Site B in the Mangemangeroa Estuary should have the highest density of cockles, as sediment at this site is comprised of coarse sand. However, this hypothesised relationship does not strictly hold as sediment at Site B, because of its location at the tip of a sandy spit, may be prone to shifting due to the changing of the tide and storm events.

Sediment at Site C, however, is predominantly comprised of fine mud, and larger cockles may sink into the sediment and become smothered. Population density at this site may be reduced as the reproductively mature component of the population (individuals >18 mm) (Larcombe 1971) may have lower survivorship in this type of microhabitat. Cockles at Site D have a shorter feeding period due to a shorter submergence time at high tide, which may restrict population growth. This site is also likely to have a higher rate of siltation as it is in the upper reaches of the estuary. Siltation has been documented to impinge on cockle survival (Bioresearchers 1989) and affect recruitment (Martin 1984). Harvesting has been observed at Site G (pers. obs.) which may affect the number of larger individuals at this site. Site F had the highest density of *A. stutchburyi*, and this is likely to be due to cockles being retained within pockets of sandy mud within the surface bedrock. As
Spatial variation of biomarker response

anticipated, no seasonal pattern was detected for cockle density, and fluctuations may be attributed to the patchy distribution of cockles in this estuary.

As concluded from Chapter 3, adenylate nucleotides are highly regulated in bivalves, in order to maintain a high ratio of ATP to ADP and AMP. In this experiment, in all but two cases, mean AEC levels were above the threshold value of 0.5 (Bayne et al. 1985), suggesting ability to maintain an adequate energetic balance. As heavy metal concentration is low at all sites, differences in AEC among sites are likely to be due to variations in environmental factors among sites, such as period of submergence and sediment size, rather than concentration of heavy metal contaminants.

Data from Chapter 3 suggested relatively simple spatial patterns in AEC in cockles from Whangarei Harbour. However, the results from the Mangemangeroa Estuary presented in this chapter suggest a more complex situation. It is difficult to isolate the reason that Site C had lower AEC than other sites. Fine particles accumulate at this site, as it is located on the outside of a bend in the main channel (Figure 4.2), and is buffered by the sandbank on which Site B is located. The relatively fine sediment (median grain size approximately 100 μm) at this site is not the optimal habitat for cockles (Belton 1985). Indeed, Clarke (1997) concluded that cockle abundance in the nearby Tamaki Estuary was negatively correlated with percentage of silt in the sediment. These factors may, in some way, affect the energetics of cockles at this site. Adenylate energy charge at all other sites (excluding the outlying AEC values for Site D in June, and Site C in February) was within expected levels for cockles sampled at low tide, based on data discussed in Chapter 3.
The seasonal pattern of higher AEC levels in winter is consistent with that previously found in oysters (Moal et al. 1991) and in the freshwater clam *Corbicula fluminea* (Giesy & Dickson 1981). However, in populations of *Austrovenus stutchburyi* in the Whangarei Harbour, highest AEC was identified in spring (see Chapter 3). This disparity in seasonal pattern between populations in the Whangarei Harbour and the population in the Mangemangeroa Estuary suggests that AEC patterns may differ among harbours. Total adenylate nucleotide pool (TANP), which showed potential in Chapter 3 for detecting chronic sublethal stress, was generally found to be lower at Sites C and D compared to other sites in the Mangemangeroa Estuary. *Austrovenus stutchburyi* at these sites may hydrolyse AMP, in order to increase AEC, thereby reducing their TANP. This may be necessary at Sites C and D for a variety of reasons, such as shorter period of submergence, less food availability, or lower sediment quality. The seasonal trend is obscured by diverging trends between seasons, and large variability in some samples (especially those collected in February), but generally TANP was lowest in autumn (June). The trend is different to that detected in populations in the Whangarei Harbour, where TANP was lowest in winter.

Differences in glycogen concentration among sites were minimal, suggesting that factors influencing glycogen levels were similar throughout the estuary. The range of glycogen concentration detected over the four seasons were similar to those identified in populations at Whangarei Heads, and the seasonal pattern was also comparable. Other researchers have also found glycogen levels in bivalves to be highest in summer and spring (see Pazos et al. 1997, and Hickman & Illingworth 1980). Glycogen is insensitive to the spatial scale at which *Austrovenus stutchburyi* samples are collected and, as
Spatial variation of biomarker response

glycogen concentration closely follows the reproductive cycle in bivalves, this biomarker can provide useful information regarding the reproductive potential and functioning of populations.

Measurement of condition is a commonly used, simple technique for estimating the general health of marine organisms (Crosby & Gale 1990, Roper et al. 1991). Such techniques have also been used to indicate physiological stress from pollution (Crosby & Gale 1990, Widdows 1985b). Condition is also affected by factors such as availability of food, population density, and sediment quality (Gabbott & Walker 1971, Lawrence & Scott 1982). Marsden & Pilkington (1995) concluded that salinity affected condition of Austrovenus stutchburyi in their study of populations in the Avon-Heathcote Estuary, Christchurch, but Gardner (1992) failed to detect differences in A. stutchburyi condition between polluted and unpolluted sites in the Manukau Harbour. This type of condition index was successfully used to separate polluted and clean sites when used with the oyster, Crassostrea gigas, in this same harbour (Pridmore et al. 1990). In the present study there were no noteworthy differences in condition among sites, although Sites C and D had some of the lowest values on every sampling occasion. This may be due to a reduced period of submergence and hence feeding time, or low salinity. In addition, these sites have fine sediment which may be a limitation for the functioning and survival of cockles (Clarke 1997). The seasonal pattern of condition indices in bivalves generally closely follows the reproductive cycle (Marsden & Pilkington 1995, Roper et al. 1991, Savari et al. 1991). Condition is usually high in winter, and decreases after spawning in spring/summer. However, only cockles from Site F showed a large decrease in condition between winter (September) and summer (February), which may indicate that only
cockles from this site spawned. However, a more likely explanation is that a large decline in condition was not detected because sampling was only carried out every three months. Cockles may have lost and regained condition between the sampling events.

Among-site differences, for all biomarkers, were small. However, cockles from Site C, located in an area of fine sediment accumulation, and Site D situated in the upper reaches of the estuary, generally had somewhat different biomarker responses compared to the other sites. These two sites had the smallest median sediment grain size and may be exposed to air for a longer period at low tide. In some way, these factors may have caused a depression of AEC, TANP and BCI in cockles. Furthermore, there may be pollutants, other than the heavy metals analysed here, present in sediment at these sites which may have caused sublethal stress in cockles, thereby affecting biomarker response. However, it is not clear why cockles from these two sites should have the highest glycogen levels.

The adenylate nucleotide indices (AEC and TANP) have the greatest potential for indicating initial sublethal stress in cockles in the Mangemangeroa Estuary as responses of glycogen, condition and density, would most likely become apparent after there were measurable effects on energy fluxes. However, these data suggest that site-specific environmental characteristics are important in determining the complex spatial patterns in biomarker response in the Mangemangeroa Estuary. These patterns are more complex than initially predicted from results obtained in the Whangarei Harbour (Chapter 3). Whangarei Harbour is very large and, although the two sites studied are several kilometers apart, their physical characteristics are similar. In the Mangemangeroa
Estuary, however, there is a pronounced estuarine gradient over a small area, superimposed on a complex geomorphology. Therefore, although sites in this estuary are relatively close, i.e. less than 1 km apart, they have different characteristics due to microhabitat effects. Hence, there is a need for greater sophistication in the application of these biomarkers, and a refinement of the techniques. In order to better assess the utility of biomarker response in cockles and to overcome problems of high spatial variability, sediments can be standardised and environmental conditions controlled in laboratory-based experiments (see Chapter 5). Manipulative field experiments are also needed to examine how this suite of biomarkers behaves in response to environmental stress in-situ (see Chapter 6).
Spatial variation of biomarker response

Table 4.1: Median sediment particle size for sites in the Mangemangeroa Estuary, collected December 1998, and analysed using a Galai-WCIS100 particle analyser.

<table>
<thead>
<tr>
<th>Site</th>
<th>Median Sediment Grain Size (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>161.24</td>
</tr>
<tr>
<td>C</td>
<td>103.71</td>
</tr>
<tr>
<td>D</td>
<td>70.46</td>
</tr>
<tr>
<td>E</td>
<td>124.37</td>
</tr>
<tr>
<td>F</td>
<td>116.71</td>
</tr>
<tr>
<td>G</td>
<td>124.32</td>
</tr>
</tbody>
</table>
Table 4.3: Indices of community composition at sites in Mangemangeroa Estuary, collected December 1998. MargarleF's index for species richness, and Shannon diversity index are presented. The error (95% confidence interval) for all estimates of species richness was 1.48, and for estimates of diversity 0.24.

<table>
<thead>
<tr>
<th>Site</th>
<th>Species Richness</th>
<th>Species Diversity</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>5.06</td>
<td>0.29</td>
</tr>
<tr>
<td>C</td>
<td>3.54</td>
<td>0.21</td>
</tr>
<tr>
<td>D</td>
<td>4.03</td>
<td>0.35</td>
</tr>
<tr>
<td>F</td>
<td>3.94</td>
<td>0.37</td>
</tr>
<tr>
<td>G</td>
<td>5.28</td>
<td>0.54</td>
</tr>
</tbody>
</table>
Figure 4.3: Mean density of *Austrovenus stutchburyi* (no./0.0625 m² +/- s.e.) at each site, collected in June 1998 (autumn), September 1998 (winter), December 1998 (spring) and February 1999 (summer).

Table 4.2: Results of two-way analysis of variance describing density for *Austrovenus stutchburyi* collected from 5 sites within the Mangemangeroa Estuary, Auckland. DF=degrees of freedom, SS=sum of squares.
Spatial variation of biomarker response

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Season</td>
<td>3</td>
<td>1,049.46</td>
<td>2.5</td>
<td>0.0656</td>
</tr>
<tr>
<td>Site</td>
<td>4</td>
<td>30,526.20</td>
<td>72.65</td>
<td>0.0001</td>
</tr>
<tr>
<td>Season*Site</td>
<td>12</td>
<td>1,702.57</td>
<td>4.05</td>
<td>0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>80</td>
<td>420.16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.4: Size-frequency histograms for *Austrovenus stutchburyi* populations at Site B (a-d) and Site C (e-h), sampled at 3-monthly intervals, in the Mangemangeroa Estuary.
Spatial variation of biomarker response

Site B

- **June 1998**
  - n=410

- **September 1998**
  - n=171

- **December 1998**
  - n=278

- **February 1999**
  - n=252

Site C

- **June 1998**
  - n=346

- **September 1998**
  - n=28

- **December 1998**
  - n=187

- **February 1999**
  - n=223

Shell Width (mm)
Figure 4.5: Size-frequency histograms for *Austrovenus stutchburyi* populations at Site D (a-d) and Site F (e-h), sampled at 3-monthly intervals, in the Mangemangeroa Estuary.
Spatial variation of biomarker response

Site D

June 1998
n=481

Site F

June 1998
n=781

September 1998
n=245

December 1998
n=276

February 1999
n=380

Shell Width (mm)
Figure 4.6: Size-frequency histograms for *Austrovenus stutchburyi* population at Site G (a-d), sampled at 3-monthly intervals, in the Mangemangeroa Estuary.
Spatial variation of biomarker response

Site G

a) June 1998
   n=33

b) September 1998
   n=46

c) December 1998
   n=77

d) February 1999
   n=25
Table 4.4: Estimates of metal concentrations in sediment collected in August 1999 from some of the sampling sites in the Mangemangeroa Estuary in August 1999 (Tricklebank unpublished data). Samples were analysed by R.J. Hill Laboratories Ltd, Hamilton. DW=dry weight.

<table>
<thead>
<tr>
<th>Site</th>
<th>Copper (µg/g DW)</th>
<th>Lead (µg/g DW)</th>
<th>Zinc (µg/g DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>9.0</td>
<td>10.0</td>
<td>41.5</td>
</tr>
<tr>
<td>D</td>
<td>7.7</td>
<td>10.5</td>
<td>40.0</td>
</tr>
<tr>
<td>E</td>
<td>9.7</td>
<td>11.5</td>
<td>41.8</td>
</tr>
<tr>
<td>F</td>
<td>14.3</td>
<td>13.0</td>
<td>51.1</td>
</tr>
<tr>
<td>G</td>
<td>5.6</td>
<td>8.1</td>
<td>33.7</td>
</tr>
</tbody>
</table>
**Figure 4.7**: Mean adenylate energy charge (AEC) (+/- s.e) in *Austrovenus stutchburyi* foot tissue, collected at each site, in June 1998 (autumn), September 1998 (winter), December 1998 (spring) and February 1999 (summer).

**Table 4.5**: Results of two-way analysis of variance describing AEC for *Austrovenus stutchburyi* collected from 5 sites within the Mangemangeroa Estuary, Auckland. DF=degrees of freedom, SS=sum of squares.
Spatial variation of biomarker response

Adenylate Energy Charge (+/- s.e.)

Month

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Season</td>
<td>3</td>
<td>0.26</td>
<td>26.01</td>
<td>0.0001</td>
</tr>
<tr>
<td>Site</td>
<td>4</td>
<td>0.10</td>
<td>10.19</td>
<td>0.0001</td>
</tr>
<tr>
<td>Season*Site</td>
<td>12</td>
<td>0.04</td>
<td>4.37</td>
<td>0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>170</td>
<td>0.01</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Figure 4.8:** Mean total adenylate nucleotide pool (TANP) (mM/g wet weight +/- s.e) in *Austrovenus stutchburyi* foot tissue, collected at each site, in June 1998 (autumn), September 1998 (winter), December 1998 (spring) and February 1999 (summer).

**Table 4.6:** Results of two-way analysis of variance describing TANP for *Austrovenus stutchburyi* collected from 5 sites within the Mangemangeroa Estuary, Auckland. DF=degrees of freedom, SS=sum of squares.
Spatial variation of biomarker response

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Season</td>
<td>3</td>
<td>4.40</td>
<td>4.51</td>
<td>0.0045</td>
</tr>
<tr>
<td>Site</td>
<td>4</td>
<td>5.76</td>
<td>5.90</td>
<td>0.0002</td>
</tr>
<tr>
<td>Season*Site</td>
<td>12</td>
<td>1.75</td>
<td>1.79</td>
<td>0.0529</td>
</tr>
<tr>
<td>Residual</td>
<td>170</td>
<td>0.98</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Figure 4.9:** Mean glycogen concentration (g/1000g tissue +/- s.e.) in *Austrovenus stutchburyi* foot tissue, collected at each site, in June 1998 (autumn), September 1998 (winter), December 1998 (spring) and February 1999 (summer).

**Table 4.7:** Results of two-way analysis of variance describing glycogen concentration for *Austrovenus stutchburyi* collected from 5 sites within the Mangemangeroa Estuary, Auckland. DF=degrees of freedom, SS=sum of squares.
Spatial variation of biomarker response

Glycogen (g/1000g muscle tissue +/- s.e.)

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Season</td>
<td>3</td>
<td>997.94</td>
<td>24.38</td>
<td>0.0001</td>
</tr>
<tr>
<td>Site</td>
<td>4</td>
<td>71.26</td>
<td>1.74</td>
<td>0.1431</td>
</tr>
<tr>
<td>Season*Site</td>
<td>12</td>
<td>23.31</td>
<td>0.57</td>
<td>0.8645</td>
</tr>
<tr>
<td>Residual</td>
<td>172</td>
<td>40.93</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.10: Mean body condition index (BCI) (+/- s.e.) for *Austrovenus stutchburyi*, collected at each site, in September 1998 (winter), December 1998 (spring) and February 1999 (summer).

Table 4.8: Results of two-way analysis of variance describing body condition index for *Austrovenus stutchburyi* collected from 5 sites within the Mangemangeroa Estuary, Auckland. DF=degrees of freedom, SS=sum of squares.
Spatial variation of biomarker response

![Graph showing body condition index (BCI) for different months with error bars for each data point.]

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Mean Square</th>
<th>F Value</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Season</td>
<td>2</td>
<td>585.95</td>
<td>14.27</td>
<td>0.0001</td>
</tr>
<tr>
<td>Site</td>
<td>4</td>
<td>77.04</td>
<td>1.88</td>
<td>0.1159</td>
</tr>
<tr>
<td>Season*Site</td>
<td>8</td>
<td>91.43</td>
<td>2.23</td>
<td>0.0268</td>
</tr>
<tr>
<td>Residual</td>
<td>210</td>
<td>41.07</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.11: Biomarker response (AEC, TANP, Glycogen, and BCI) averaged across all seasons for each site (+/- std error).
Spatial variation of biomarker response

Mean AEC (+/− s.e.)

Mean TANP (mM/g WW +/− s.e.)

Mean Glycogen (g/1000g tissue +/− s.e.)

Mean BCl (+/− s.e.)

SITE

B C D F G
CHAPTER 5

Concentration-Response Laboratory Trials

5.1 Introduction

One of the many requirements for bioindicator species and biomarker techniques is that responses to contaminants show a dose-response relationship (McCarty & Munkittrick 1995, Widdows 1985b); i.e. response is more acute as dose increases. In order to establish this relationship, researchers have undertaken an enormous range of laboratory experiments where various organisms are subjected to contaminants at an assortment of concentrations (Connell et al. 1999, and see Huggett et al. 1992 and Reish 1987 for reviews). In the laboratory situation, environmental conditions such as temperature, photoperiod, pH, dissolved oxygen concentration, and chemical composition of substrate can be relatively easily controlled and reproduced (Connell et al. 1999). Because of these advantages, researchers have been better able to establish concentration-response relationships due to a reduction in the extent of background variability found in the real environment, such as that found in biomarker response in cockles from Mangemangeroa Estuary (Chapter 4). Due to the virtual elimination of synergistic, cumulative and antagonistic effects of mixtures of contaminants, and interactions between environmental factors and contaminants in a controlled laboratory situation, the power or reliability of results from these types of experiments are often thought to be greater than experiments undertaken in-situ.
Extrapolating from data obtained in laboratory experiments to the prediction of effects in the natural environment and effects at different levels of organisation can be useful if the realism of the contrived experiment is high (Cairns & Pratt 1989). Often, laboratory trials are a poor mimic of the real world, however, and data from these experiments have the potential to be misleading (Cairns et al. 1993, Connell et al. 1999, Spies 1989). Connell et al. (1999) warn that it is virtually impossible to predict effects in the field based on laboratory trial data, although laboratory data can assist with the interpretation of field data and vice versa. Furthermore, Cairns & Pratt (1989) argue that laboratory bioassay experiments often suffer from high variability in biomarker response. Hence, in order to make the conservative extrapolations from laboratory derived data to effects in the natural environment, the experimental design and actual set-up of laboratory trials should aim to be as realistic as possible (Römbke & Moltmann 1996).

Bioassays, toxicity tests and laboratory trials have their place in ecotoxicology, and they will continue to be extensively used because they are simpler to interpret than experiments in the natural environment, can be standardised and therefore readily compared, and are relatively inexpensive (Römbke & Moltmann 1996, Cairns & Niederlehner 1987, Chapman & Long 1983). Furthermore, such experiments are widely regarded as being major improvements on earlier ecotoxicological techniques, which relied solely on chemical/physical measurements to provide information on environmental health (Cairns & Pratt 1989, Connell et al. 1999).

The choice of species to use in laboratory trials depends on many criteria. Often, sensitivity to contaminants is the primary concern (Gray 1989). Also of importance is
Concentration-response laboratory trials

availability of the species, tolerance of the species to laboratory conditions, availability of information on the biology of the animal, and convenience of size (Reish 1987, Phillips & Segar 1986). One of the problems with using a species which is chosen primarily for its sensitivity is loss of ecological relevance. In fact, the main criticism of aquatic toxicology, especially where sensitive species or biomarkers at the suborganismal level are used, is that there is little ecological relevance (Caims & Pratt 1989, Römbke & Moltmann 1996). This is frequently referred as the “So What?” syndrome. For example, it can be difficult to understand extrapolations from effects of a contaminant on Daphnia sp., a sensitive genus of zooplankton commonly used in acute toxicity tests, in a laboratory situation, to functionally distant organisms (such as fish and shellfish) or ecosystems.

If we establish ecological relevance as one of the primary criteria for choice of species, then many species of marine bivalves (e.g. oysters such as Crassostrea virginica, mussels such as Mytilus edulis, or infaunal clams such as Mercenaria mercenaria) fit almost all of the above requirements (Kramer & Botterweg 1991, Reish 1987). Many researchers have considered sensitivity to toxicants as the primary criteria for choice of indicator species, however, and as marine bivalves often show relatively high resistance to contaminants, they have tended to be passed over in favour of zooplankton, amphipods and early life stages of many marine organisms (Caims & Niederlehner 1987, Reish 1987).

Applied aquatic toxicology in New Zealand is in its infancy in comparison to countries such as the U.S.A.. Furthermore, the literature suggests that the level of contamination is generally lower in New Zealand’s aquatic environments compared to more industrialised

Scientists at New Zealand's largest, government-sponsored aquatic research organisation, the National Institute of Water and Atmosphere (NIWA) have carried out a significant number of aquatic toxicity tests (Snelder & Truman 1995). Their efforts have primarily focused on acute toxicity testing (using the marine amphipod Paracorophium excavatum), bioaccumulation experiments with various marine molluscs and polychaetes, and sublethal changes in behaviour and physiological condition (using a range of marine bivalves including Macomona liliana, Austrovenus stutchburyi and Crassostrea gigas) (ARWB 1990, Hickey & Roper 1992, Pridmore et al. 1991, Roper et al. 1991, Snelder & Truman 1995, Wilcock et al. 1993). However, in New Zealand there are large gaps in our information and understanding of the effects of contaminants on indigenous marine organisms, including bivalves.

In order to address some of these gaps in the literature, experiments were designed to ascertain the sensitivity of a ubiquitous, indigenous bivalve to some common marine contaminants. In this series of laboratory experiments, the estuarine bivalve Austrovenus stutchburyi was challenged with three contaminants, and responses of the following biomarkers analysed; adenylate energy charge, total adenylate nucleotide pool, glycogen and RNA concentration. The three contaminants chosen for the experiments were a group of PAHs, the organochlorine pesticide chlordane, and the active constituent in marine antifouling paints, tributyltin. These contaminants were selected because they occur in
many marine environments in New Zealand (de Mora et al. 1995, Williamson & Wilcock 1994) and have been shown to affect marine bivalves in other countries (see Hoffman et al. 1995, Huggett et al. 1992, and Chapter 1).

Due to criticisms in the literature that laboratory experiments lack realism and relevance, these experiments were designed to not only have controlled conditions for contaminant challenges, but to also be as environmentally realistic as was feasible. Given these design requirements, cockles were provided with clean marine sediment to burrow into, and a simulated high and low tide regime. In addition, cockles were held at a density similar to that found in many New Zealand harbours (Stewart 1999).

The concentration of contaminants used in the experiments approximated some of the highest concentrations found in local environments (de Mora et al. 1995, Williamson et al. 1999b). PAH and chlordane concentrations were calculated using the Effects Range Median (ERM) concentrations of chemicals from the sediment quality guidelines proposed by Long et al. (1995), which are based on biological effects data from North America (see Appendix II). Data from de Mora et al. (1995) and Hall (1999) provided the information for the choice of tributyltin concentration used in the experiments. These laboratory trials were carried out in order to assess biomarker response in an experimental situation where spatial and temporal variability in sediment and site characteristics could be better controlled.
5.2 Methods and Materials

5.2.1 Collection of Sediment and Cockles

Cockles and sediment for the laboratory trials were collected from the Whangateau Harbour where cockles abound (see Kearney 1999, Stewart 1999). They were transported to the Leigh Marine Laboratory where they were stored separately in flow-through seawater aquaria. Sediment was taken from an area within the harbour which was well flushed by tidal movements, and where cockles were abundant. The sediment was coarse-sieved through a 5 mm mesh, before being placed in a concrete tank with continuous seawater flow. Macrofauna, which were retained in the sieve, were returned to the field. Samples of the stored sediment were completely dried in a drying oven (at 70°C) for 24 hours and passed through a series of graduated sieves in order to determine the grain size distribution. It was established that the sediment comprised 96% sand and 4% silt, which is suitable for Austrovenus stutchburyi inhabitation (see Chapter 4).

The organic content of sediment in the storage tank was analysed prior to the commencement of each laboratory trial, to ensure consistency between experiments, because sediments with a higher level of organic matter tend to accumulate higher concentrations of contaminants, thereby reducing the amount of contaminant which is bioavailable (Cockerham & Shane 1994). With large shell material removed, 3 replicate 10 g samples of wet sediment were digested in 20 ml of 10% nitric acid for 30 minutes. The nitric acid was then removed and sediment dried at 46°C for 24 hours. A subsample of 5 g of dry sediment was taken from each replicate and placed in a muffle furnace for 1 hour at 500°C. Organic matter content was calculated as the difference in weight between dry and incinerated sediment.
5.2.2 Analysis of Contaminants in Collected Sediment and Shellfish

In order to ensure sediment and animals were uncontaminated prior to starting the laboratory experiments, two sediment and two composite *A. stutchburyi* samples were sent to a professional testing laboratory (R.J. Hill Laboratories Ltd) for analyses. Samples were analysed for polycyclic aromatic hydrocarbons, organochlorine pesticides and tributyltin oxide. The protocols used by R.J. Hill Laboratories Ltd were as follows. Measurements of tributyltin oxide levels in sediment and shellfish flesh were obtained using methanol/acetic acid microwave extraction, followed by ethylation and gas chromatography using mass selective detection (GC-MS). Levels of polycyclic aromatic hydrocarbons were calculated using accelerated solvent extraction (USEPA Method 3545), followed by GC-MS selected ion monitoring quantitation (USEPA 3540 and 3630). Organochlorine pesticides were measured using ASE extraction, GPC cleanup, and capillary gas chromatography, using mass selective detection in selected ion mode (GC-MS SIM). The analyses revealed that the concentrations of all contaminants were below the method detection level for both sediment and shellfish samples (see Table 5.1). Because the sediment and shellfish flesh samples were found to be as void of contaminants as possible, the stored sediment and cockles held in aquaria were considered suitable for the laboratory trials.

5.2.3 Experimental Design

Two types of trials were carried out to examine the response of cockles to each contaminant: one simulating a single contamination event (Trial A), and the other simulating an ongoing discharge of contaminants, or chronic exposure (Trial B). Each trial type was undertaken separately for each of the three contaminants (polycyclic...
aromatic hydrocarbons, chlordane, and tributyltin), i.e. six individual experiments were run.

Trial A involved spiking surficial sediment with a contaminant before the sediment was added to the experiment, whereas contaminant was added directly to the sediment (at “low tide”) each day for 14 days in Trial B. Prior to each trial being undertaken, 9 cockles were randomly selected from the stock population being maintained in flow-through seawater tanks, and foot tissue for each individual was analysed for adenylate energy charge, total adenylate nucleotide pool and glycogen (see Chapter 2 for description of methodology). Foot tissue samples from a further 9 cockles were analysed for RNA content (see Chapter 2 for protocol). This provided initial “pre-trial” values for each biomarker. Laboratory trials were carried out in the following order; PAH Trial A (May 1998), PAH Trial B (July 1998), chlordane Trial B (August 1998), chlordane Trial A (December 1998), organotin Trial B (January 1999), organotin Trial A (February 1999). The sediment used for the first experiment (PAH Trial A), which had been recently obtained from the field and had not aged, was likely to have higher organic content than the other trials.

5.2.3.1 Tank Set-up

The configuration of the experiments involved the use of 22 round plastic tubs (230 mm high x 200 mm diameter) (see Figure 5.1). In order to collect the contaminated wastewater from the experiment, the tubs were placed within two adjacent plastic tanks, with each tank holding eleven tubs (see Figure 5.2). Each tub had two 10 mm diameter holes drilled 20 mm above the base, and a further two holes 20 mm below the top (Figure
5.1). The holes near the base allowed water to seep through the sediment out of the tubs. These holes had squares of 0.5 mm x 0.5 mm plastic mesh fixed over them on the inside of the tub, in order to minimise sediment loss. The holes near the top of each tub provided an overflow for excess seawater.

Sediment was added to each tub to a depth of approximately 150 mm. Tubs were placed in the large plastic tanks, and a plastic pipe frame was positioned over them (Figure 5.2). Small holes drilled in the pipe frame allowed a gentle stream of seawater to trickle down the inside edge of each tub. Seawater was provided from the laboratory system, which draws its supply from the adjacent marine reserve. Water accumulated in the tubs to a depth of approximately 60 mm above the sediment surface, before overflowing through the holes positioned 20 mm from the top of the tubs, thereby simulating high tide. Seawater was added constantly for 24 hours in order to allow the sediment to settle.

At the start of each experiment, twelve cockles (shell width 30-40 mm) were randomly selected from the storage aquaria and placed in each tub. The seawater supply was then altered to deliver water constantly for 6 hours. Then followed a 6 hour period where no water was supplied. This procedure simulated the alternating high and low tides that would be experienced in the natural environment. Cockles were left, under this regime, for 7 days to burrow and adjust to their new habitat. After this period, 7 tubs, predominantly those where cockles had not burrowed adequately, were selected from the total of 22, and removed from the experiment. Five replicate tubs were then randomly allocated to each of the three treatments: contaminant added, solvent only added, and control (i.e. only seawater added). In order to ensure that there were no disparities in
Concentration-response laboratory trials

Environmental variables at the beginning of each experiment, temperature, dissolved oxygen and salinity were measured in the seawater in each tub, at the simulated high tide, using Orion handheld meters.

Waste seawater from the tanks was collected and passed through an activated carbon filter in order to remove the organic contaminants. The wastewater was then discharged through a subterrain leach field. At the completion of each laboratory trial the sediment from each tub and the activated carbon from the filter was collected and sent to a waste treatment company where they were incinerated.

5.2.4 Experimental Procedure

5.2.4.1 Trial A

For each of the three contaminant trials, 15 surficial sediment samples (70g each) were taken from the storage aquarium, and placed in sterile plastic petri dishes. To five of these (hereafter referred to as ‘contaminated sediment’) the target contaminant was added and mixed. Polycyclic aromatic hydrocarbons (PAH) were added to each of the 5 ‘contaminated sediment’ replicate petri dishes at a concentration of 25.5 mg/kg (diluted in a 1:1 mix of methanol and dichloromethane) (see Table 5.2). Chlordane at a concentration of 60 μg/kg (diluted in methanol), and tributyltin (as Sn) at a concentration of 10.95 μg/L (diluted in methanol) were added to their respective ‘contaminated sediment’ replicates. To a further five petri dishes containing sediment samples (referred to ‘solvent control sediment’), an identical quantity of the appropriate carrying solvent was added and mixed. The same quantity of seawater was added and mixed into the remaining five sediment samples (referred to as ‘control sediment’). Petri dishes
Concentration-response laboratory trials

5.2.4.2 Trial B

The experimental configuration was identical to the 1-dose trials, but instead of spiking the sediment prior to addition to the tubs, the same treatment dose (i.e. contaminant or solvent or seawater) was added each day to the sediment surface in each tub, for 14 days, at the beginning of a simulated low tide. This was to simulate a scenario of continued contaminant input rather than a one-off pulse. After 14 days cockles were sacrificed for the biomarker analyses.

5.2.5 Biochemical Analyses

At the conclusion of each trial, 3-4 animals were sacrificed for adenylate nucleotide and glycogen analyses, and a further 3-4 for RNA analyses (see Chapter 2 for description of methodology). In addition, tissue from the remaining cockles in the contaminated treatment was extracted, and sent to R.J. Hill Laboratories Ltd for contaminant analyses.

5.2.6 Statistical Analyses

Initially, a two-factor design was carried out (Figure 5.3), but as there was no significant difference among tubs (the nested factor) in every case, these data were pooled in order to increase the power for detecting differences among treatments. For each contaminant trial, treatments were compared using one-way analysis of variance, with the statistical
package SAS (SAS Institute 1990). Homogeneity of variance was tested by plotting the residuals against predicted values, and normality was tested by examining stem & leaf, and normal probability plots. Where significant differences among treatments were detected, Tukey's tests were used to identify where differences existed.

The semi-interquartile range was compared for each contaminant trial and treatment, using the pooled data as described above. This calculation (see below) gives an estimate of dispersion, which is not biased by extreme values (Zar 1996). This measure was used because variance in estimates of a biomarker response may be greater when organisms of a population are stressed.

semi-interquartile range = \( \frac{Q_3 - Q_1}{2} \)

Where; \( Q_3 = 75^{th} \) percentile, \( Q_1 = 25^{th} \) percentile.

5.3 Results

5.3.1 Polycyclic aromatic hydrocarbons

There were no significant differences among treatments for adenylate energy charge or total adenylate nucleotide pool, for either Trial A or Trial B (Table 5.3). However, across all three treatments AEC was higher (approximately 0.5 in Trial A and 0.7 in Trial B) and TANP lower (approximately 3.8 mM/g in Trial A and 2.8 mM/g in Trial B), in Trial B compared to Trial A (Figure 5.4 a,b,e,f).
Glycogen concentrations were not different among treatments for Trial A, but a significant difference was detected among treatments for Trial B (Table 5.3). A Tukey’s comparison revealed that in Trial B cockles dosed with PAH had significantly higher glycogen than cockles from the solvent control. Cockles from the untreated control did not have significant different glycogen levels to either the contaminated or the solvent control treatments (Figure 5.4c,g). Over all treatments, glycogen levels were higher in Trial A (approximately 10 g/kg) compared to Trial B (approximately 5 g/kg). RNA in cockles showed no significant difference between treatment and either control group in both trials (Table 5.3). Average RNA for both experiments was between 0.4 and 0.55 µg/g (Figure 5.4d,h).

Formal statistical comparisons can not be made between cockles sampled before the experiment from the storage aquaria (B), and cockles from treatments in the trials (X, S, C) as the cockles were not treated in the same manner. However, there were no large disparities in biochemical indices between animals used in the PAH trials and those sampled prior to each experiment.

Cockle tissue from tubs receiving PAHs had a total PAH body burden of 35.65 ng/g for Trial A, whereas those from Trial B had a body burden of 155.5 ng/g total PAH (i.e. 4.4 times greater) (Table 5.4, 5.5). A comparison of accumulation of individual PAH compounds in cockle tissue between trials revealed that in contrast to complex PAH compounds, those with fewer carbon rings accumulated to a concentration in Trial B many times more than the concentration in Trial A. For example; acenaphthylene (2 ring)
was 8 times more concentrated in cockle tissue in Trial B compared to Trial A, whereas benzo[a]pyrene (5 ring) was only 2.4 times higher.

Semi-interquartile range (SIQ) calculations failed to indicate higher variability in data from PAH 'contaminated' treatments compared to 'solvent control' or 'control' treatments (Figure 5.5). However, for both TANP and RNA, SIQ was higher for all treatments in Trial B compared to Trial A. No clear trends for SIQ were identified for AEC or glycogen.

The organic content of sediment used for Trial A was 3.26 +/- 0.37%, and for Trial B was 0.35 +/- 0.038%. The decrease in organic content between trials is likely to be due to natural aging of sediment and degradation of organic matter.

5.3.2 Chlordane

Statistical analyses failed to detect any significant differences among treatments for any of the biochemical indices, both for Trial A and Trial B (Table 5.6, Figure 5.6a-h). However, in a comparison of biomarker response between trials, AEC across all treatments was slightly lower in Trial B (approximately 0.6 in Trial B and 0.7 in Trial A) and TANP slightly lower in Trial A (approximately 2.8 mM/g in Trial B and 2.2 mM/g in Trial A) (Figure 5.6 a,b,e,f). Glycogen levels were lower for Trial A compared to Trial B (approximately 2 g/kg in Trial A and 5 g/kg in Trial B) (Figure 5.6c,g). Levels of RNA across all treatments were lower for Trial B than Trial A (approximately 0.6 µg/g in Trial A and 0.4 µg/g in Trial B), although within each trial no significant difference among treatments was detected (Figure 5.6d,h).
Mean AEC values, prior to commencement of Trial A, were similar to those detected at the completion of this experiment for all treatments (approximately 0.7) (Figure 5.6a). However, TANP was lower for cockles in the experiment (approximately 2.1 mM/g), compared to cockles sampled before the experiment (approximately 2.9 mM/g) (Figure 5.6b). Cockles in Trial B had lower AEC (approximately 0.6) compared to cockles sampled prior to the trial (approximately 0.9), whereas TANP was higher in the cockles used in the experiment (approximately 3.0 mM/g) compared to cockles sampled before the experiment (approximately 2.0 mM/g) (Figure 5.6e,f).

Glycogen levels calculated before each chlordane trial were similar to the values identified across treatments for each trial (Figure 5.6c,g). RNA levels, however, were lower prior to Trial A (approximately 0.42), but higher prior to Trial B (approximately 0.55) (Figure 5.6d,h). The chlordane body burden of cockles from the contaminated treatment in Trial A was below the detection limit of 2.0 ng/g, whereas animals from the contaminated treatment in Trial B had a body burden at least 2.85 times higher with 5.7 ng/g in tissue (Tables 5.7 and 5.8).

Unexpectedly, the semi-interquartile range (SIQ) for AEC was higher for the ‘solvent control’ compared to ‘control’ and ‘contaminated’ treatments for both Trial A and Trial B (Figure 5.7). There was no clear trend in SIQ for TANP, or RNA. However, the SIQ for glycogen revealed higher variability in the ‘contaminated’ treatment for Trial B compared to the ‘solvent control’ and ‘control’ treatments.
Organic matter content of sediment was similar between the Trial A and Trial B, with values of 0.48 +/- 0.012% and 0.42 +/- 0.023% respectively.

### 6.3.3 Tributyltin

No significant differences were detected among treatments for any of the four biochemical indices for either Trial A or Trial B (Table 5.9, Figure 5.8). Although no effects on biochemical indices were detected, organisms were coming into contact with the contaminant, as the body burden was 4.2 ng/g in Trial A, and 3.5 times greater in Trial B (14.7 ng/g) (Tables 5.10, 5.11). AEC was higher in cockles sampled prior to the commencement of Trial B (approximately 0.8), compared to cockles within the experiment (approximately 0.6) (Figure 5.8). A similar pattern was evident for both glycogen trials, with cockles sampled before the experiments having almost double the amount of glycogen compared to cockles sampled from the experiment. There were no apparent differences in TANP or RNA among treatments or between cockles sampled before the experiments and those from the experiments.

There was a weak trend of greater variability in the AEC data for tributyltin ‘contaminated’ treatments compared to the ‘control’ and ‘solvent control’ treatments (Figure 5.9). However, there was no consistent trend for TANP, glycogen or RNA. The organic content of sediment was found to be similar between Trial A and Trial B; 0.45 +/- 0.03 % and 0.48 +/- 0.01% respectively.
5.4 Discussion

The effects of contaminants on organisms are the result of interactions between the chemical and the biochemical receptors within the organism (Hoffman et al. 1995). The use of biochemical biomarkers should, therefore, provide an early indication of potential effects at higher levels of organisation.

Although PAHs, chlordane and tributyltin have been found to cause sublethal effects in marine organisms in other research (see Huggett et al. 1992), there was virtually no evidence of effects on adenylate energy charge, total adenylate nucleotide pool, glycogen and RNA levels in these laboratory experiments. There was only one instance where a significant difference was detected. In this case, cockles from the 'contaminated' treatment had higher glycogen levels than the 'solvent control' cockles in Trial B of the PAH experiments. However, based on the large natural variability in glycogen concentration identified in Chapter 3 and 4, this relatively small difference in glycogen level between treatments may not be biologically significant.

Although there was little evidence of statistically significant difference among treatments, there were some interesting patterns in the data sets worth highlighting. Based on the results for cockles monitored at two sites at Whangarei Heads each season for several years (see Chapter 3) the adenylate energy charge and total adenylate nucleotide pool values, which were measured in cockles from all of the control treatments in the laboratory trials, were within the range of values detected in cockles from the field. Cockles in PAH Trial B had higher AEC levels and lower TANP levels than cockles in PAH Trial A. It is likely that cockles in Trial B utilised their adenylate nucleotide pool in
order to maintain high AEC levels, which simultaneously decreased the total concentration of adenylate nucleotides (TANP). This pattern was consistent among treatments and may have occurred in response to a difference in environmental variables between trials, such as reduced water temperature in Trial B as it was carried out in the cooler month of July, whereas Trial A occurred in May. AEC levels were maintained at a high level in chlordane Trial A, whereas levels were low in Trial B. However, there was no clear suggestion in the data that TANP was affected as a consequence of alterations in AEC levels in either of these trials. In the tributyltin experiments, AEC in cockles sampled before Trial B was higher than that measured in any of the treatments, which may indicate that the experimental setup itself caused stress to the cockles in some way. Trial B treatments had slightly lower AEC, but higher TANP compared to that for Trial A, which again suggests some regulation of adenylate nucleotides in order to maintain AEC levels. Regulation of adenylate nucleotides such as that suggested here has been documented in other marine organisms (Dickson & Giesy 1981, Schöttler 1978, Shofer & Tjeerdema 1998, Verschraegen et al. 1985), and is investigated further in the following chapter.

Effects of season may account for cockles in PAH Trial B having lower glycogen levels compared to cockles in PAH Trial A. Glycogen levels in cockles in both chlordane trials were among the lowest values measured in cockles throughout this research (see Chapters 3 & 4). Chlordane Trial B was carried out in winter (August) which corresponds with low glycogen levels previously measured in natural populations, but Trial A was undertaken in late spring (December) which is generally when glycogen levels are at their maximum in natural cockle populations (Chapter 3). This gives some supporting evidence for the
hypothesis that the cockles stored in the holding aquaria may have been suffering some degree of nutritive stress, i.e. they may not have been getting sufficient microalgae (either in quantity or quality) through the laboratory seawater system. This is further suggested by the fact that in all trials some cockles did not burrow into the sediment when placed in the tubs. Glycogen levels were somewhat higher in tributyltin Trial B compared to Trial A, although levels in both experiments were lower than that generally measured in natural populations at this time of year (see Chapter 3 and 4). Season may have had a complicating influence on the level of glycogen in cockle tissue in these laboratory experiments, making the results difficult to interpret. In an ideal laboratory setup, a series of experiments such as these should be run simultaneously, in order to control any effect of season, but that was simply not logistically possible in this research.

RNA levels were relatively constant throughout all trials and across all treatments. Values ranged from approximately 0.3 to 0.6 μg/g, and there was no obvious relationship between concentration of RNA in cockle tissue and the time of year experiments were conducted. This biomarker requires testing on natural populations, and will be further investigated in the following chapter.

In my experiments, sediments were dosed to levels which have been found by other researchers to induce effects, contaminants were bioavailable, and cockles accumulated all contaminants in both Trial A and Trial B. It is difficult to determine why no significant effects on the biomarkers were detected. It may be that Austrovenus stutchburyi is indeed a very tolerant species, too tolerant for use in laboratory-based contaminant trials of this nature. Alternatively, it may be that cockles are very sensitive
to the disruption they suffered when they were removed from the field and placed in the storage aquaria. This may have caused sublethal stress in the cockles, which obscured any effects from the contaminants in the trials. Because the animals bioaccumulated the contaminants, the possibility that the chemicals were either diluted out of the experimental tubs, or were irreversibly bound to the sediment or organic particles (i.e. not bioavailable) can be ruled out.

Römbke & Moltmann (1996) listed three reasons for uncertainty in laboratory tests on single species: (i) interactions with other species are not considered, (ii) the influence of physical environmental conditions on dose-response relationship and mechanism of exposure are not taken into account, and (iii) only one stage in the life cycle is analysed. These criticisms are valid for many laboratory experiments, including those analysed in this chapter. However, laboratory experiments often fall into a “catch 22” situation. Data from such experiments can be more powerful than those collected from field experiments because of the simplicity of their design and the ability of the researcher to reduce complicating factors (such as interactions among species). Laboratory trials are also commonly criticised as lacking in realism or complexity. However, it is this very complexity which makes the results from field trials less clear and difficult to interpret (e.g. Chapter 4). Römbke & Moltmann (1996) also conclude that biomarker response for a given organism is not always more sensitive in laboratory experiments compared to field experiments, i.e. in some circumstances, field experiments give more sensitive results than laboratory trials. It is argued that uncertainties with laboratory experiments often stem from lack of realism, and additional stress placed on the organism from being held under unnatural conditions in aquaria and in the experimental set-up itself (Hoffman
Concentration-response laboratory trials

eq al. 1995). Cairns & Niederlehner (1987) also recognise that the environmental requirements of a species are very rarely met in laboratory trials, and the stress of the contaminant may be masked or overridden by the stress of the experimental conditions themselves. There is some evidence that this may have occurred in some of my experiments i.e. the experimental setup itself may have induced sublethal stress. For example, in chlordane Trial B AEC and RNA were lower, and TANP higher, in cockles sampled from all treatments compared to cockles sampled from the storage tank.

In these experiments, care was taken to ensure test organisms were of high quality and that the holding aquaria were clean, well supplied with water and oxygen, and not overstocked with cockles. The experiment was designed to mimic the natural environment as much as possible; the sediment used had an appropriate grain size distribution, cockles were not positioned too densely, and high and low tide conditions were simulated. However, there may well have been a lack of other natural environmental features which caused stress. This would account for the fact that AEC and glycogen levels were higher in cockles prior to the commencement of many of the contaminant trials compared to levels in ‘control’ treatment cockles at the conclusion of the experiments. Possible deficiencies in the experimental setup which may have inadvertently caused stress to the cockles could potentially have included poor food quality and quantity as mentioned above, inadequate mimicking of tidal cycle, and lack of sunlight.

On face value, my experiments show that even high levels of the contaminants tested do not cause a significant response in these biomarkers in cockles. One may be tempted to
Concentration-response laboratory trials

extrapolate from this to conclude that naturally occurring cockles would not be affected by the chemicals used here at quite high concentrations. This series of experiments however, highlights the fact that laboratory experiments may give misleading results, as the literature abounds with studies where many other marine organisms have been found to suffer adverse affects in the natural environment from these chemicals at much lower concentrations than those used in these experiments (see Cockerham & Shane 1994, Hoffman et al. 1995, Huggett et al. 1992).

A further consideration is whether investigating the dose-response of a single chemical is environmentally relevant (Hoffman et al. 1995). Generally, in the natural environment, a target species will be challenged with a complex mix of contaminants. In addition, individual chemicals often behave differently in mixtures than when isolated (Chapman 1995). Although no effect of contaminants was demonstrated in these laboratory trials, it may be that cockles are affected sublethally in their natural environment by a combination of chemicals. This is investigated using a transplant experiment in the field, which is described in the following chapter.

The aim of the laboratory experiments carried out in this chapter was to resolve some of the problems with variability encountered with the field sampling in Chapters 3 and 4. However, cockles do not seem to be robust to the laboratory situation. In fact, it can be concluded that cockles are not an ideal laboratory species as they do not appear to adapt well to being held, without sediment, in aquaria with flow-through seawater supply, nor do they adapt to being held in small tubs with sediment and low flow of seawater. It may be the case that cockles are useful indicator species in field situations only. The following
chapter advances from this point to a more refined application of biomarker techniques to cockles in a manipulative field experiment.
Table 5.1: Contaminant data for two samples of sediment and two composite tissue samples from *Austrovenus stutchburyi* held in aquaria prior to being used in laboratory contaminant trials. Analyses were carried out by R.J. Hill Laboratories Ltd. DW=dry weight, WW=wet weight (whole animal tissue from 10 individuals, as received by laboratory).
## Concentration-response laboratory trials

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>Sediment 1 (ng/g)DW</th>
<th>Sediment 2 (ng/g)DW</th>
<th>Shellfish 1 (ng/g)WW</th>
<th>Shellfish 2 (ng/g)WW</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tributyltin oxide</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monobutyltin oxide</td>
<td>&lt;2.0</td>
<td>&lt;2.0</td>
<td>&lt;2.0</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td>Dibutyltin oxide</td>
<td>&lt;2.0</td>
<td>&lt;2.0</td>
<td>&lt;2.0</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td>Tributyltin oxide</td>
<td>&lt;2.0</td>
<td>&lt;2.0</td>
<td>&lt;2.0</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td>Triphenyltin oxide</td>
<td>&lt;2.0</td>
<td>&lt;2.0</td>
<td>&lt;2.0</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td><strong>Polycyclic aromatics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naphthalene</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>&lt;2.0</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td>Acenaphthylene</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>&lt;0.5</td>
<td>&lt;0.4</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>&lt;0.5</td>
<td>&lt;0.4</td>
</tr>
<tr>
<td>Fluorene</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>&lt;0.5</td>
<td>&lt;0.4</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>&lt;0.5</td>
<td>&lt;0.4</td>
</tr>
<tr>
<td>Anthracene</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>&lt;0.5</td>
<td>&lt;0.4</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>&lt;0.5</td>
<td>&lt;0.4</td>
</tr>
<tr>
<td>Pyrene</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>&lt;0.5</td>
<td>&lt;0.4</td>
</tr>
<tr>
<td>Benzo[a]anthracene</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>&lt;0.5</td>
<td>&lt;0.4</td>
</tr>
<tr>
<td>Chrysene</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>&lt;0.5</td>
<td>&lt;0.4</td>
</tr>
<tr>
<td>Benzo[b]fluoranthene</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>&lt;0.5</td>
<td>&lt;0.4</td>
</tr>
<tr>
<td>Benzo[k]pyrene</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>&lt;0.9</td>
<td>&lt;0.9</td>
</tr>
<tr>
<td>Indeno(1,2,3-c,d)pyrene</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>&lt;2.0</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td>Dibenzo[a,h]anthracene</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>&lt;2.0</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td>Benzo[g,h,i]perylene</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>&lt;0.9</td>
<td>&lt;0.9</td>
</tr>
<tr>
<td><strong>Organochlorine pesticides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alpha-BHC</td>
<td>&lt;0.4</td>
<td>&lt;0.3</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Beta-BHC</td>
<td>&lt;0.4</td>
<td>&lt;0.3</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Gamma-BHC (Lindane)</td>
<td>&lt;0.4</td>
<td>&lt;0.3</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Delta-BHC</td>
<td>&lt;0.4</td>
<td>&lt;0.3</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Heptachlor</td>
<td>&lt;0.4</td>
<td>&lt;0.3</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Heptachlor epoxide</td>
<td>&lt;0.4</td>
<td>&lt;0.3</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Aldrin</td>
<td>&lt;0.4</td>
<td>&lt;0.3</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Dieldrin</td>
<td>&lt;0.4</td>
<td>&lt;0.3</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Endrin</td>
<td>&lt;0.4</td>
<td>&lt;0.3</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Endrin aldehyde</td>
<td>&lt;0.4</td>
<td>&lt;0.3</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Endosulfan I</td>
<td>&lt;0.4</td>
<td>&lt;0.3</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Endosulfan II</td>
<td>&lt;0.4</td>
<td>&lt;0.3</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Endosulfan sulphate</td>
<td>&lt;0.4</td>
<td>&lt;0.3</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>2,4-DDE</td>
<td>&lt;0.4</td>
<td>&lt;0.3</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>2,4-DDD</td>
<td>&lt;0.4</td>
<td>&lt;0.3</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>2,4-DDT</td>
<td>&lt;0.4</td>
<td>&lt;0.3</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>4,4’-DDE</td>
<td>&lt;0.4</td>
<td>&lt;0.3</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>4,4’-DDD</td>
<td>&lt;0.4</td>
<td>&lt;0.3</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>4,4’-DDT</td>
<td>&lt;0.4</td>
<td>&lt;0.3</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Chlordane ((cis+trans)*100/42)</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Methoxychlor</td>
<td>&lt;0.4</td>
<td>&lt;0.3</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
</tbody>
</table>
Figure 5.1: Diagrammatic representation of individual tubs in contaminant challenge experiments (not to scale). Blue dashed lines indicate seawater flow routes, and green dots symbolise individual cockles within the sediment. The diameter and height of tubs was 200 mm and 230 mm respectively.

Figure 5.2: Diagram showing the experimental set-up. Eleven tubs were located within two large tanks. The dotted lines across the tubs indicate the plastic pipe frame which supplied seawater. Seawater entered the frame where indicated by an arrow, passed through the plastic pipe, and entered each tub through a small hole. The waste seawater exited the base of each tank and was collected and treated.
Concentration-response laboratory trials

Diagram showing a setup for laboratory trials involving cockles and seawater. The diagram includes a container labeled 'Seawater' at the top and another labeled 'Seawater' at the bottom, with a central area labeled 'Sediment' containing a layer of cockles. The diagram also indicates a 550 mm dimension and a 850 mm dimension.
Table 5.2: Composition of polycyclic aromatic hydrocarbon (PAH) solution used for contaminant trials. Methanol and dichloromethane were the solvents used to dissolve and carry the PAHs.

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>No. of carbon rings in compound</th>
<th>Amount (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acenaphthylene</td>
<td>2</td>
<td>0.0295</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>3</td>
<td>0.0320</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>4</td>
<td>0.0311</td>
</tr>
<tr>
<td>Benzo[a]pyrene</td>
<td>5</td>
<td>0.0318</td>
</tr>
<tr>
<td>Dibenz[a,h]anthracene</td>
<td>6</td>
<td>0.0297</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>0.1541</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Solvent</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td></td>
<td>30.00 ml</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td></td>
<td>30.00 ml</td>
</tr>
</tbody>
</table>

Therefore, contaminant concentration = 154.1 mg in 60 ml solvent.

Target concentration in sediment = 245 mg/kg (=10 x ERM of 24.5 mg/kg);

Therefore, 9539 µl/kg were needed (i.e. 667.73 µl/70g surficial sediment).
Figure 5.3: Experimental design used to investigate the effect of three treatments on adenylate energy charge, total adenylate nucleotide pool, glycogen and RNA in *Austrovenus stutchburyi* foot tissue. X=contaminated sediment, S=solvent control sediment, C=control sediment.
### Concentration-response laboratory trials

<table>
<thead>
<tr>
<th>Factor</th>
<th>Fixed/Random</th>
<th>Orthogonal/Nested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>X S C</td>
<td>Fixed</td>
</tr>
<tr>
<td>Tubs</td>
<td>1 2 3 4 5</td>
<td>Random</td>
</tr>
<tr>
<td>Replicates</td>
<td>3 cockles taken from each tub</td>
<td>Random</td>
</tr>
</tbody>
</table>
Table 5.3: Summary of one-factor nested ANOVAs for PAH Trial A and Trial B, describing differences between experimental treatments (Contaminated, Solvent Control, Control) (n=15). Degrees of freedom 2,12. MS = mean square, F = F ratio, p = probability, S = significant and NS = non significant at p<0.05, N/A = not applicable.

<table>
<thead>
<tr>
<th>Variable</th>
<th>MS</th>
<th>F</th>
<th>p</th>
<th>Significance</th>
<th>Tukey's</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trial A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEC</td>
<td>0.038</td>
<td>2.55</td>
<td>0.091</td>
<td>NS</td>
<td>N/A</td>
</tr>
<tr>
<td>TANP</td>
<td>0.288</td>
<td>1.01</td>
<td>0.373</td>
<td>NS</td>
<td>N/A</td>
</tr>
<tr>
<td>Glycogen</td>
<td>13.499</td>
<td>0.70</td>
<td>0.502</td>
<td>NS</td>
<td>N/A</td>
</tr>
<tr>
<td>RNA</td>
<td>0.008</td>
<td>1.70</td>
<td>0.199</td>
<td>NS</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Trial B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEC</td>
<td>0.002</td>
<td>0.21</td>
<td>0.812</td>
<td>NS</td>
<td>N/A</td>
</tr>
<tr>
<td>TANP</td>
<td>0.466</td>
<td>2.08</td>
<td>0.144</td>
<td>NS</td>
<td>N/A</td>
</tr>
<tr>
<td>Glycogen</td>
<td>17.547</td>
<td>5.48</td>
<td>0.009</td>
<td>S</td>
<td>Contaminated&gt; Solvent Control</td>
</tr>
<tr>
<td>RNA</td>
<td>0.011</td>
<td>0.57</td>
<td>0.573</td>
<td>NS</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Figure 5.4: PAH Contamination Trials. Biomarker response (mean +/- std error) in *Austrovenus stutchburyi* for the three treatment groups (contaminated sediment (X), solvent control sediment (S) and control sediment (C)) in Trial A and Trial B (n=15). Biomarker values for *A. stutchburyi* before addition to the experiment (shown in red) (B) are given as a comparison, although they cannot be formally compared.
Concentration-response laboratory trials

Trial A

- AEC
- TANP (mM/g)
- Glycogen (g/kg)
- RNA (µg/g)

Trial B

- AEC
- TANP (mM/g)
- Glycogen (g/kg)
- RNA (µg/g)
Table 5.4: Concentration of polycyclic aromatic hydrocarbons (PAH) in *Austrovenus stutchburyi* tissue from Trial A. The PAH compounds added to the experiment are shown in bold. The Total PAH figure is sum concentration of the PAH compounds added to the experiment. WW= wet weight of composite whole animal tissue as received by R.J. Hill Laboratories Ltd.

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>Tissue sample (ng/g WW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Napthalene</td>
<td>&lt;4.0</td>
</tr>
<tr>
<td><strong>Acenaphthylene</strong></td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Fluorene</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td><strong>Phenanthrene</strong></td>
<td>10</td>
</tr>
<tr>
<td>Anthracene</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td><strong>Fluoranthene</strong></td>
<td>21</td>
</tr>
<tr>
<td>Pyrene</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Benzo[a]anthracene</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Chrysene</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Benzo[b]fluoranthene</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Benzo[k]pyrene</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td><strong>Benzo[a]pyrene</strong></td>
<td>4.1</td>
</tr>
<tr>
<td>Indeno(1,2,3-c,d)pyrene</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td><strong>Dibenzo[a,h]anthracene</strong></td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Benzo[g,h,i]perylene</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td><strong>Total PAH</strong></td>
<td>35.65</td>
</tr>
</tbody>
</table>
**Table 5.5:** Concentration of polycyclic aromatic hydrocarbons (PAH) in *Austrovenus stutchburyi* tissue from Trial B. The PAH compounds added to the experiment are shown in bold. The Total PAH figure is sum concentration of the PAH compounds added to the experiment. WW= wet weight of composite whole animal tissue as received by R.J. Hill Laboratories.

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>Tissue sample (ng/g WW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Napthalene</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td><strong>Acenaphthylene</strong></td>
<td><strong>4.0</strong></td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Fluorene</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td><strong>Phenantherene</strong></td>
<td><strong>52</strong></td>
</tr>
<tr>
<td>Anthracene</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td><strong>Fluoranthene</strong></td>
<td><strong>88</strong></td>
</tr>
<tr>
<td>Pyrene</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Benzo[a]anthracene</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Chrysene</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Benzo[b]fluoranthene</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Benzo[k]pyrene</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td><strong>Benzo[a]pyrene</strong></td>
<td><strong>10</strong></td>
</tr>
<tr>
<td>Indeno(1,2,3-c,d) pyrene</td>
<td>0.6</td>
</tr>
<tr>
<td><strong>Dibenzo[a,h]anthracene</strong></td>
<td><strong>1.5</strong></td>
</tr>
<tr>
<td>Benzo[g,h,i]perylenne</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td><strong>Total PAH</strong></td>
<td><strong>155.5</strong></td>
</tr>
</tbody>
</table>
Figure 5.5: Semi-interquartile range for biomarker response in *Austrovenus stutchburyi* for the three treatment groups (contaminated sediment (X), solvent control sediment (S) and control sediment (C)) in PAH contamination Trial A and Trial B.
Concentration-response laboratory trials

Trial A

- AEC
- TANP
- Glycogen
- RNA

Trial B

X S C

Values:
- AEC: 0.00, 0.04, 0.08
- TANP: 0.1, 0.2, 0.3
- Glycogen: 0.1, 1.0, 2.0
- RNA: 0.00, 0.04, 0.08
Table 5.6: Summary of one-factor nested ANOVAs for chlordane Trial A and Trial B, describing differences between experimental treatments (Contaminated, Solvent Control, Control), n=15. Degrees of freedom 2,12. MS = mean square, F=F ratio, p=probability, S=significant and NS=non significant at p<0.05.

<table>
<thead>
<tr>
<th>Variable</th>
<th>MS</th>
<th>F</th>
<th>p</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trial A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEC</td>
<td>0.049</td>
<td>2.7</td>
<td>0.083</td>
<td>NS</td>
</tr>
<tr>
<td>TANP</td>
<td>0.143</td>
<td>0.43</td>
<td>0.652</td>
<td>NS</td>
</tr>
<tr>
<td>Glycogen</td>
<td>0.792</td>
<td>0.86</td>
<td>0.435</td>
<td>NS</td>
</tr>
<tr>
<td>RNA</td>
<td>0.018</td>
<td>1.11</td>
<td>0.352</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Trial B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEC</td>
<td>0.012</td>
<td>1.08</td>
<td>0.354</td>
<td>NS</td>
</tr>
<tr>
<td>TANP</td>
<td>0.105</td>
<td>0.26</td>
<td>0.7721</td>
<td>NS</td>
</tr>
<tr>
<td>Glycogen</td>
<td>25.466</td>
<td>2.91</td>
<td>0.070</td>
<td>NS</td>
</tr>
<tr>
<td>RNA</td>
<td>0.017</td>
<td>2.48</td>
<td>0.102</td>
<td>NS</td>
</tr>
</tbody>
</table>
Figure 5.6: Chlordane Contamination Trials. Biomarker response (mean +/- std error) in *Austrovenus stutchburyi* for the three treatment groups (contaminated sediment (X), solvent control sediment (S) and control sediment (C)) Trial A and Trial B (n=15). Biomarker values for *A. stutchburyi* before addition to the experiment (shown in red) (B) are given as a comparison, although they cannot be formally compared.
Concentration-response laboratory trials

Trial A

- AEC
- TANP (mM/g)
- Glycogen (g/kg)
- RNA (μg/g)

Trial B

- AEC
- TANP (mM/g)
- Glycogen (g/kg)
- RNA (μg/g)
Concentration-response laboratory trials

**Table 5.7:** Concentration of chlordane compounds in *Austrovenus stutchburyi* tissue from Trial A. WW = wet weight of composite whole animal tissue as received by R.J. Hill Laboratories Ltd. Chlordane calculated from the cis & trans isomers (42% of total) x 100/42.

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>Tissue sample (ng/g WW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cis-Chlordane</td>
<td>&lt;0.4</td>
</tr>
<tr>
<td>trans-Chlordane</td>
<td>&lt;0.4</td>
</tr>
<tr>
<td>Chlordane ((cis+trans)*100/42</td>
<td>&lt;2.0</td>
</tr>
</tbody>
</table>
Figure 5.7: Semi-interquartile range for biomarker response in *Austrovenus stutchburyi* for the three treatment groups (contaminated sediment (X), solvent control sediment (S) and control sediment (C)) in chlordane contamination Trial A and Trial B.
Concentration-response laboratory trials

Trial A

- a) AEC
- b) TANP
- c) Glycogen
- d) RNA

Trial B

- e) AEC
- f) TANP
- g) Glycogen
- h) RNA
Table 5.9: Summary of one-factor nested ANOVAs for tributyltin Trial A and Trial B, describing differences between experimental treatments (Contaminated, Solvent Control, Control), n=15. Degrees of freedom 2,12. MS = mean square, F=F ratio, p=probability, S=significant and NS=non significant at p<0.05.

<table>
<thead>
<tr>
<th>Variable</th>
<th>MS</th>
<th>F</th>
<th>p</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trial A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEC</td>
<td>0.017</td>
<td>1.05</td>
<td>0.363</td>
<td>NS</td>
</tr>
<tr>
<td>TANP</td>
<td>0.014</td>
<td>0.10</td>
<td>0.904</td>
<td>NS</td>
</tr>
<tr>
<td>Glycogen</td>
<td>0.086</td>
<td>0.14</td>
<td>0.867</td>
<td>NS</td>
</tr>
<tr>
<td>RNA</td>
<td>0.012</td>
<td>2.66</td>
<td>0.088</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Trial B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEC</td>
<td>0.001</td>
<td>0.05</td>
<td>0.953</td>
<td>NS</td>
</tr>
<tr>
<td>TANP</td>
<td>0.605</td>
<td>1.31</td>
<td>0.285</td>
<td>NS</td>
</tr>
<tr>
<td>Glycogen</td>
<td>0.103</td>
<td>0.23</td>
<td>0.794</td>
<td>NS</td>
</tr>
<tr>
<td>RNA</td>
<td>0.005</td>
<td>1.20</td>
<td>0.316</td>
<td>NS</td>
</tr>
</tbody>
</table>
Figure 5.8: Organotin Contamination Trials. Biomarker response (mean +/- std error) in *Austrovenus stutchburyi* for the three treatment groups (contaminated sediment (X), solvent control sediment (S) and control sediment (C)) in Trial A and Trial B (n=15). Biomarker values for *A. stutchburyi* before addition to the experiment (shown in red) (B) are given as a comparison, although they cannot be formally compared.
Concentration-response laboratory trials

Trial A

- AEC
- TANP (mM/g)
- Glycogen (g/kg)
- RNA (ug/g)

Trial B

- AEC
- TANP (mM/g)
- Glycogen (g/kg)
- RNA (ug/g)

Legend:
- B
- X
- S
- C
Table 5.10: Concentration of organotin compounds in *Austrovenus stutchburyi* tissue from Trial A. WW= wet weight of composite whole cockle tissue as received by R.J. Hill Laboratories Ltd.

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>Tissue sample (ng/g WW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dibutyltinoxide (as Sn)</td>
<td>1.0</td>
</tr>
<tr>
<td>Tributyltinoxide (as Sn)</td>
<td>2.9</td>
</tr>
<tr>
<td>Triphenyltinoxide (as Sn)</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>Total (Sn)</td>
<td>4.2</td>
</tr>
</tbody>
</table>

Table 5.11: Concentration of organotin compounds in *Austrovenus stutchburyi* tissue in Trial B. WW= wet weight of composite whole cockle tissue as received by R.J. Hill Laboratories Ltd.

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>Tissue sample (ng/g WW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dibutyltinoxide (as Sn)</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>Tributyltinoxide (as Sn)</td>
<td>13.0</td>
</tr>
<tr>
<td>Triphenyltinoxide (as Sn)</td>
<td>&lt;0.7</td>
</tr>
<tr>
<td>Total (Sn)</td>
<td>14.7</td>
</tr>
</tbody>
</table>
Figure 5.9: Semi-interquartile range for biomarker response in *Austrovenus stutchburyi* for the three treatment groups (contaminated sediment (X), solvent control sediment (S) and control sediment (C)) in organotin contamination Trial A and Trial B.
Concentration-response laboratory trials

![Graphs showing concentration-response for Trial A and Trial B](image-url)
CHAPTER 6

Determination of Sublethal Stress Using In-situ Exposure to Contaminated Sediment

6.1 Introduction

Biochemical and physiological responses to natural and contaminant-induced sublethal stress are frequently documented in marine molluscs, both in field and laboratory trials (see Chapter 5). Field experiments are commonly used to investigate ecological impact of pollutants, whereas laboratory trials are undertaken predominantly to assess toxicity (Connell 1999). Investigations of the effects of contaminants, which are undertaken on the same selected species, and have both a laboratory and field component, provide additional information on the validity of extrapolating from laboratory data to effects in the field (Connell 1999, Cormier & Racine 1992, Römbke & Moltman 1996, Widdows 1985a). This approach of complimenting laboratory toxicity trials with manipulative field experiments also has the potential to add environmental realism and ecological relevance to ecotoxicological investigations. Underwood (1996) concurs and goes even further to argue persuasively that, in order to interpret actual environmental alterations, appropriately designed field experimentation with real impacts should be used.

The concept of transplanting shellfish within the marine environment for the purposes of monitoring accumulation rates or subsequent effects of contaminants was primarily established by the United States National Oceanic and Atmospheric Administration.
Determination of sublethal stress using in-situ exposure to contaminated sediment

(NOAA), with the large-scale Mussel Watch Programme, using transplanted *Mytilus edulis*, which started in 1986 (O'Connor 1992). There are many situations in which epifaunal filter-feeding bivalves, such as oysters and mussels, do not naturally occur, and therefore transplanting such organisms into that environment might cause 'artefactual' stress. For these situations, such as estuaries with extensive intertidal sediment flats, it would be preferable to use a 'model organism' more suited to the environment, and in the New Zealand context, cockles are ideal for the reasons given in Chapter 1. Infaunal species, such as *Austrovenus stutchburyi*, filter-feed at the water-sediment interface and are potentially exposed to contaminants from both sediment and water sources (Boening 1999).

Bivalve transplantation experiments have been carried out in order to ascertain physiological differences between populations (Worrall & Widdows 1983), to provide information on the basis of life history traits (Sato 1999) and ecosystem functioning (Essink & Bos 1985, Martin 1984), to determine the impact of invasive species on community structure (Creese et al. 1997), and for the purposes of resource enhancement (Beal & Kraus 1991, Dobbinson et al. 1989, Hendrickson et al. 1988, Stewart 1999). There are numerous published accounts of experiments where bivalves are transplanted in order to ascertain levels of bioaccumulation, or exposure to, contaminants (e.g. King & Davies 1987, Langston 1984, Riedel et al. 1995, Zorba et al. 1992). Sublethal effects of contaminants on marine organisms can also be determined using such approaches, and one advantage of transplanting and caging bivalves for this purposes is that it provides a combination of the experimental control of laboratory bioassays with the environmental realism of field monitoring (Salazar & Salazar 1997). The premise for transplanting soft-
Determination of sublethal stress using in-situ exposure to contaminated sediment

sediment, estuarine organisms from "clean" environments to those containing contaminants, is that the individuals have not previously had the opportunity to regulate their physiology in order to withstand contaminant stress. This gives the opportunity to estimate the stress response (lethal and/or sublethal) and potentially correlate this to levels of measurable contaminants.

There are few well designed studies in which molluscs are transplanted to habitats with different contaminant levels in order for initial sublethal stress responses to be identified. In one of the first such studies, Ivanovic (1980b) transplanted the estuarine gastropod *Pyrazus ebeninus* to field sites where sediment was known to be contaminated with hydrocarbons, and also to sites where sediment was devoid of hydrocarbons. Organisms at contaminated sites had significantly lower adenylate energy charge (AEC) levels compared to those transplanted to "clean" sites. In a similar study, Cristini (1987) compared both glycogen levels and adenylate nucleotides of caged, transplanted bivalves (*Mya arenaria*) with natural, uncaged populations of this species, at sites with various levels of contaminants. She concluded that AEC was lower in bivalves transplanted from uncontaminated to contaminated sites, compared to indigenous bivalves at the contaminated site. As bivalves from natural populations were not treated in the same way as transplanted organisms, however, between-treatment comparisons could not be unambiguously interpreted in that study; i.e. it is not possible to determine whether the reduction in AEC was due to the presence of contaminants or due to stress associated with the transplantation procedure. With the exception of Couillard et al. (1995), and some experiments carried out by Bowmer et al. (1991), there are no other published accounts of bivalve transplant experiments of this type which do not have experimental design.
Determination of sublethal stress using in-situ exposure to contaminated sediment

deficiencies (i.e. where inappropriate controls make interpretation difficult due to possible confounding effects) (see Table 6.1).

In New Zealand, there have been no published studies of soft-sediment bivalves transplanted to contaminated areas for the purposes of identifying biochemical or physiological sublethal stress as a result of a contaminated habitat. However, *A. stutchburyi* have been transplanted for experimental investigation of behaviour and growth responses (e.g. Blackwell 1984, Dobbinson *et al.* 1989, Stewart 1999, Thrush *et al.* 1996), and these studies indicate that *A. stutchburyi* is a suitably robust species to allow experimental manipulation using transplantation.

Many investigations have concluded that sublethal stress can reduced the surplus of energy over and above that which is required for maintenance and functioning of the animal, at the expense of somatic growth and fecundity (Bayne *et al.* 1985, review by Huggett *et al.* 1992, Phillips & Rainbow 1993). AEC and total adenylate nucleotide pool (TANP) provide measures of immediately available metabolic energy, the amount of stored glycogen gives an indication of reproductive potential, and the level of RNA provides an estimate of growth. These analyses were selected for their sensitivity and ecological relevance (see Chapters 3, 4 and 5).

Manipulative experiments in this chapter were undertaken in order to establish whether cockles, transplanted from a site with low levels of contaminants in sediment, to a range of sites with higher levels of sediment-bound contaminants, suffer sublethal stress and
whether they regulate their biochemistry and physiology with increased period of exposure to the contaminated sediment. These experiments have the potential to indicate whether environmental quality monitoring programmes, using a transplantation (or "Mussel Watch") approach, may be able to be used with cockles in New Zealand estuaries.

6.2 Methods and Materials

6.2.1 Transplant Design

The design of the transplant experiment, and treatment of cockles, was carefully developed in order to allow justified comparisons of cockle response both within sites and among sites. *Austrovenus stutchburyi* were collected from a relatively unimpacted site, in the Whangateau Harbour, and transplanted to four sites in the Manukau Harbour (see section 6.2.2 for detailed site descriptions) with levels of heavy metals and polycyclic aromatic hydrocarbons (PAH) in sediment ranging from uncontaminated to contaminated (contamination level assessment is described in section 6.2.3). A schematic representation of the transplant design and experimental design are presented in Figure 6.1 and Figure 6.2 respectively. In order to compare cockles transplanted from the Whangateau Harbour and autochthonous cockles at each site in the Manukau Harbour, cockles were also collected from each Manukau Harbour site, treated in an identical manner as those collected from the Whangateau Harbour, and transplanted back to their site of origin (Figure 6.1). This procedure insured against differences in biomarker responses between transplanted and undisturbed autochthonous cockles at each Manukau Harbour site being regarded simply as a transplant effect. Cockles from the Whangateau Harbour were also transplanted back to their site of origin, in order to compare biomarker
Determination of sublethal stress using in-situ exposure to contaminated sediment

responses of transplanted cockles at the Manukau Harbour sites with those at their site of origin.

6.2.2 Site Descriptions

*Austrovenus stutchburyi* were transplanted from Lews Bay in the Whangateau Harbour (36°19.5'S, 174°45'E) to 4 sites within the Manukau Harbour (37°02'S, 174°41'E) (Figure 6.3). The Whangateau Harbour is 9.2 km² in area (Larcombe 1968), has extensive intertidal sandflats, and is surrounded by semi-urban and rural land use. Discharges into this harbour are likely to include low-level urban stormwater, farm runoff and septic system leachate (see Appendix I). The Whangateau Harbour is well flushed, losing up to 99% of its volume with the ebb tide (Larcombe 1968). Lews Bay was chosen as the donor site as it has extensive cockle beds (Stewart 1999) (Plate 4), and is relatively uncontaminated, as defined by contaminant analyses described in section 6.2.3 (see also Appendix I).

The Manukau Harbour covers an area of 344 km² and, during spring tides, almost half this area is exposed as intertidal sand/mudflats (Williamson & Wilcock 1994). The harbour receives sewage, urban stormwater, industrial wastes and farm discharges (see Williamson & Wilcock 1994 for a historical review). Contaminants reaching the harbour commonly include PAH, chlordane, DDT, Pb, Zn, Cu, Ni, Cr, and Fe (Snelder & Trueman 1995, Williamson & Wilcock 1994).

An initial survey was undertaken in order to ensure that potential transplant sites within the Manukau Harbour supported natural cockle populations that could be used as within
site controls, and 4 sites were selected (Table 6.2). Onehunga is located in the northeastern arm of the harbour (36°56′20″ S, 174°51′40″ E) (Plate 5a), where heavy industry was historically centered (Williamson & Wilcock 1994). Sites in this part of the harbour have high levels of sediment-bound contaminants (Williamson & Wilcock 1994). In addition, this part of the harbour is sheltered from strong tidal currents and wave action, enabling considerable sediment deposition and retention to occur (Williamson & Wilcock 1994). Big Muddy (36°59′10″ S, 174°37′30″E) is a site with medium density urban housing on the southern side, and native bush and reserve land on the northern side (Plate 5b). The Takanini site (37°03′45″ S, 174°51′40″ E), within the Pahurehure Inlet, on the southern shores of the harbour, is surrounded by medium to high density housing, with a major motorway close by, bisecting the harbour (Plate 6a). Both these sites have lower levels of contaminants than Onehunga. Awhitu is located within a regional park, near the headwaters of the harbour (37°05′15″ S, 174°39′00″ E), and has no permanent housing nearby (Plate 6b). This site is the least contaminated, although the adjacent land supports some stock grazing.

### 6.2.3 Levels of Contaminants in Sediment

Sediments from Onehunga, Big Muddy and Takanini were collected in 1998 by the Auckland Regional Council (ARC) and analysed for contaminants by the National Institute for Water and Atmosphere (NIWA) (Williamson et al. 1999a). As part of this experiment, sediment was collected from Awhitu and Lews Bay in early 1999, following an identical protocol as that used by the ARC, and these samples were analysed in the same manner by NIWA. At each site, fifty scoops of sediment, from the top 2 cm, were taken from a 20 m x 50 m area on the intertidal flat (containing the area in which my
Determination of sublethal stress using in-situ exposure to contaminated sediment

experiments were undertaken, but not containing channels, banks, mangrove plants or pneumatophores). The fifty sediment samples were condensed to 5 samples, each containing a composite of 10 replicate scoops. Metal concentrations were analysed in the mud fraction (< 63 µm), in order to reduce among site variation caused by changes in sediment grain size composition. PAH concentration was analysed on coarse-sieved sediment (< 500 µm), and normalised with concentration of total organic matter (Williamson et al. 1999a).

The reactive fraction of metals was extracted using hydrochloric acid, and extracts analysed for copper, lead and zinc by atomic absorption spectrometry using flame atomisation (Perkin Elmer 3100). PAHs were extracted by accelerated solvent extraction, and quantitative analysis carried out by capillary gas chromatography, using mass selective detection in selected ion mode (GC MS SIM) (Williamson et al. 1999a). There was estimated to be a between-run precision of ±5% (Geoff Mills (NIWA) pers. com.). Sediment contaminant data for Awhitu and Lews Bay were obtained following an identical protocol, although sediments were air dried, instead of freeze-dried. Due to low levels of contaminants in all samples, the different drying methods would not have resulted in any differences in accuracy (Geoff Mills (NIWA) pers. com.).

Standardised sediment quality guidelines, as proposed by Long et al. (1995) and Canadian guidelines (Smith et al. 1996) are presented to provide context and comparison with the known levels of contaminants in the Manukau Harbour (Appendix II). Both recommended guidelines are based on data from modeling, laboratory and field studies performed in marine and estuarine sediments. Long et al. (1995) established a biological
Determination of sublethal stress using in-situ exposure to contaminated sediment

effects database for sediments incorporating chemical and biological data from various studies throughout North America. The data they had gathered from these studies was then arranged in order of ascending concentration, and the distribution analysed and categorised. The Effects Range Low (ERL) was determined for each chemical as including the significant biological effects data in the lower 10th percentile of all the ordered effects data, and the Effects Range Median (ERM) was identified as concentrations of chemicals at the 50th percentile. The Canadian guidelines were calculated using a similar, but more conservative method. Their data comprised two sets; one where significant biological effects were detected and another where no significant effects were identified. For each contaminant, the lower value, or threshold effect level (TEL), was calculated as the geometric mean of the lower 15th percentile concentration of the 'biological effects' data set and the 50th percentile concentration of the 'no biological effects' data set. The probable effect level (PEL) was identified as the geometric mean of the 50th percentile concentration of the 'biological effects' data set and the 85th percentile concentration of the 'no biological' effect data set (Smith et al. 1996).

PAH levels at sites used for this transplant experiment are low in terms of these international guidelines (Table 6.2 and Appendix II); PAH concentration was highest at the Onehunga site, but even this value is an order of magnitude lower than the ERL value, and only 30% of the TEL concentration. However, the concentration of metals at some sites were close to, and occasionally in exceedance of, the ERL and TEL guidelines. Zinc levels at Awhitu and Lews Bay were very low compared to the guidelines, whereas those at Big Muddy and Takanini were approximately 50% of the TEL concentration. Sediment from the Onehunga site had zinc, copper and lead concentrations in exceedance of both
Determination of sublethal stress using in-situ exposure to contaminated sediment

the ERL and TEL, but below the ERM and PEL concentrations. Copper and lead levels at both Lews Bay and Awhitu were very low. Sediment from Big Muddy had a copper concentration which was 30% of the ERL, and 54% of the TEL, whereas Takanini sediment had a concentration which was 40% and 74% of the ERL and TEL concentration respectively. Lead concentration in sediment at both Big Muddy and Takanini was approximately equal to or less than one third of the ERL and TEL concentrations.

6.2.4 Collection and tagging of cockles

A total of 500 cockles (15-25 mm shell width) were collected at low tide from Lews Bay (28/3/99). One hundred cockles of a similar size (15-25 mm) were also collected from each site in the Manukau Harbour (27/3/99). The 15-25 mm size range (measured as shell width) was chosen as it was representative of the individuals dominating populations at the four sites in the Manukau Harbour. Cockles were transported to the Leigh Marine Laboratory where they were stored in seawater aquaria.

Tagging, as opposed to caging, was chosen as the method for identification and subsequent retrieval. A major disadvantage with caging is that cockles can occasionally enter or leave cages and confuse the experiment (Dobbinson et al. 1989). In addition, cages, especially when placed intertidally, can attract attention from the public, and tampering with the experiment may occur. Cages themselves may impede cockle movement, burrowing or feeding, and this could potentially cause sublethal stress. Furthermore, hydrological and sediment characteristics may be altered within the cage, and therefore additional controls are required in order to be able to eliminate these effects from final interpretation of the data. Tag and recapture has been shown, however, to be
an effective identification method for *A. stutchburyi*. For example, Stewart (1999) in a study of cockles in the Whangateau Harbour, transplanted double-tagged cockles between bays, and among shore levels, and clearly demonstrated that a high proportion of adult cockles remained within experimental transplant plots, without the need for caging. In order to tag the cockles, they were removed from the aquaria and patted dry with paper towels. A dot of enamel paint (diameter approximately 5 mm) was then placed on one valve of each shell. In order to differentiate cockles in the field, green paint was placed on cockles from Lews Bay and blue paint on cockles from the Manukau Harbour. In addition, a square of aluminium (5 mm x 5 mm x 1 mm) was glued to the shell with 2-part epoxy resin (Araldite brand), to enable later relocation using a metal detector (Stewart & Creese 2000). In their study, Stewart & Creese (2000) found that this method of double-tagging had no adverse effects on cockle behaviour and survival. Cockles were held in aquaria for less than 48 hours before replanting in the field. Placement of cockles in the field was undertaken at all sites, at low tide, on 29/3/99.

6.2.5 Transplantation

At each site in the Manukau Harbour, 100 cockles previously collected from that site (autochthonous cockles), plus 100 cockles from Lews Bay (transplant cockles), were transplanted at low tide into separate 25 cm x 25 cm plots i.e. one plot contained the autochthonous cockles, and a second plot contained the transplanted cockles. The plots were excavated to a depth of 2 cm, the cockles added and sediment placed on and around the cockles. Plots were at similar heights on the shore at each site. As a control, 100 cockles from Lews Bay were returned to the donor site, and placed a single 25 x 25 cm plot, adjacent to the site they were originally collected from.
A sediment sample (approximately 100g), from the top 2 cm of the sediment, was taken from each site and frozen for later grain size analyses using a Galai WCIS100 laser particle analyser. Median sediment particle size was obtained for each sample.

6.2.6 Sampling protocol

Two weeks after transplantation, 20 cockles from each plot were collected at low tide. The soft-tissue was extracted, and the foot muscle was removed and frozen in liquid nitrogen for biochemical analyses (see Chapter 2 for detailed description of collection protocol). Ten of the twenty individuals collected from each plot were sacrificed for adenylate nucleotide and glycogen analyses, and a further ten for RNA analyses. In addition, shells from dead cockles were collected and counted. After a further 6 weeks, a second set of samples was collected in the same manner. However, due to mortality and missing individuals, it was not possible to collect ten individuals for each technique at some sites (Table 6.3).

6.2.7 Biochemical analyses

Adenylate energy charge, total adenylate nucleotide pool, glycogen and RNA concentration analyses were undertaken on all cockle samples (see Chapter 2 for detailed methodology).

6.3 Results

6.3.1 Sediment Size

Median sediment particle size was highest for Awhitu (229 μm) and lowest for Takanini (85 μm) (Figure 6.4). According to the Wentworth (1922) scale, sediment from Lews...
Bay, Awhitu and Big Muddy can broadly be classified as fine sand, whereas sediment from Takanini and Onehunga is classed as very fine sand. Sediment particle sizes were approximately normally distributed for all sites, apart from Takanini which was strongly skewed towards fine silt. Cockle populations were present at all sites, indicating that differences in sediment particle sizes among sites did not prevent the establishment or subsequent survival of some cockles.

6.3.2 Biochemical Indices

The design of the analyses undertaken for each biochemical index is shown diagrammatically in Figure 6.2. There were several sets of comparisons for each index which were of interest in this experiment. Firstly, at each site in the Manukau Harbour, transplanted and autochthonous cockles were compared at 2 weeks and 8 weeks. There were 4 comparisons for the 2 week data set, and 3 for the 8 week data set, due to the loss of transplanted individuals at Takanini. The second group of comparisons was between the control plot at Lews Bay and transplanted plots at each Manukau Harbour site. Again, there were 4 comparisons at 2 weeks, and 3 comparisons at 8 weeks. The last group of comparisons was for each plot, at each site, between 2 and 8 weeks, of which there were 8 comparisons.

Data were tested for equal variance (tested by plotting residuals versus predicted values) and normality (tested using the Shapiro-Wilk (1965) W statistic). Many of the data sets failed tests for equal variance or normality (45% of comparisons for AEC, 28% for TANP, 41% for RNA, and 18% for glycogen). Because the data had these characteristics,
occasionally combined with unequal sample sizes, conclusions drawn from parametric tests may be biased (Zar 1996). Therefore, the non-parametric Wilcoxon 2-sample test was used for all comparisons.

The Bonferon adjustment (Miller 1986) was also used because, with the high number of comparisons being made, there was a greater chance of incorrectly finding a significant result.

**Bonferoni adjustment** \[ 1 - 0.05 = (1-\alpha)^M \]

where 0.05 = significance level originally chosen

\[ \alpha = \text{new significance level} \]

\[ M = \text{number of comparisons} \]

Therefore; for 3 comparisons;
\[ 1 - 0.05 = (1-\alpha)^3 \]
\[ \alpha = 0.013 \]

for 4 comparisons;
\[ 1 - 0.05 = (1-\alpha)^4 \]
\[ \alpha = 0.017 \]

for 8 comparisons;
\[ 1 - 0.05 = (1-\alpha)^8 \]
\[ \alpha = 0.0064 \]
6.3.2.1 Adenylate Energy Charge (AEC)

Two weeks post transplantation comparisons between transplanted and autochthonous plots at each site revealed significant differences only at Onehunga. Here, autochthonous cockles had significantly higher AEC (p<0.0003) compared to transplanted cockles (Table 6.4). Although not statistically significant at the adjusted 0.013 significance level, autochthonous cockles at Big Muddy also had higher AEC compared to transplanted animals (p=0.0233) (Figure 6.5a). There was also no significant difference in AEC between autochthonous and transplanted cockles at Awhitu or Takanini (p=0.0892 and p=0.7959 respectively).

AEC levels for autochthonous cockles were not significantly different from transplanted groups at sites within the Manukau Harbour at the 8 week sampling period (excluding Takanini, where comparisons could not be made due to loss of transplanted cockles) (Figure 6.5b). Transplanted cockles at Big Muddy had a significant increase in AEC from 0.55 at 2 weeks to 0.72 at 8 weeks (p=0.0011). There was a similar pattern at the Onehunga site, although the increase in AEC for transplanted animals from 0.55 at 2 weeks to 0.63 at 8 weeks was not statistically significant (p=0.4318) (Table 6.4).

A comparison of transplanted cockles at each site in the Manukau Harbour with cockles transplanted back to Lewis Bay (control plot) revealed no significant differences between sites at 2 weeks or at 8 weeks (all p-values >0.2) (Figure 6.6a,b). The biggest changes in AEC between sampling periods, and between treatments, were at the more contaminated sites, as summarised in Figure 6.7.
6.3.2.2 Total Adenylate Nucleotide Pool (TANP)

At the 2 week sampling period, average TANP was higher for autochthonous cockles compared to transplanted cockles at all sites (Figure 6.8a). Differences were however, only statistically significant at Awhitu (p<0.0037) and Takanini (p<0.0068). Mean TANP of transplanted cockles was 2.28 mM/g compared to 2.9 mM/g for autochthonous cockles for Awhitu, 2.25 and 3.12 for Big Muddy, 2.3 and 3.06 for Takanini and 1.92 and 2.31 for Onehunga respectively (Table 6.5). At Awhitu, after 8 weeks, mean TANP for transplanted cockles increased above the autochthonous cockles, a rise from 2.3 mM/g at two weeks to 3.2 mM/g (Figure 6.8b). At both Big Muddy and Onehunga, there was a decrease in mean TANP for transplanted cockles between the 2 and 8 week sampling period; 2.25 to 1.68 mM/g and 1.92 to 1.19 mM/g respectively (Figure 6.8b and Table 6.5). TANP of autochthonous cockles remained at similar levels for the 2 and 8 week sampling period at these sites. Autochthonous cockles at Big Muddy had significantly higher TANP than transplanted cockles after 8 weeks (p<0.0005). Little can be concluded regarding the Takanini plots due to loss of transplanted animals at week 8.

After two weeks, the mean TANP of cockles from the transplanted plots and the control plot at Lews Bay were very similar (Figure 6.9a). However, after 8 weeks, sites were separated, with TANP of Awhitu transplanted animals greatly increased and significantly different from that of the control plot at Lews Bay (p=0.0021) (Figure 6.9b). TANP of cockles from all plots (autochthonous, transplant and control) did not alter significantly between sampling periods (Figure 6.10).
6.3.2.3 Glycogen

Two weeks after transplantation, glycogen levels were higher for autochthonous cockles compared to transplanted cockles at Takanini, Big Muddy and Onehunga, although not statistically significant at Big Muddy and Onehunga (Table 6.6). These differences were especially apparent at Big Muddy and Takanini with mean glycogen for autochthonous cockles being 9.98 g/kg and 10.68 g/kg respectively and glycogen for transplanted cockles at both sites being 50% less (Figure 6.11, Table 6.6). In contrast, the transplanted cockles at Awhitu had higher, but not statistically different, glycogen levels compared to autochthonous cockles (p>0.0147).

After 8 weeks, the pattern was similar for Awhitu, Onehunga and Big Muddy (Figure 6.11b). However, autochthonous cockles at Big Muddy increased glycogen concentration from 9.98 to 13.2 g/kg (Table 6.6). Glycogen levels in autochthonous cockles at Takanini decreased between the 2 and 8 week sampling period, from 10.68 to 4.98 g/kg, but no conclusions can be drawn regarding Takanini transplanted cockles due to mortality.

At the first sampling period, glycogen levels for transplanted cockles at Big Muddy, Takanini and Onehunga were very similar to the control cockles at Lews Bay (Figure 6.12a). In contrast, transplanted animals at Awhitu had significantly higher glycogen levels. This pattern was relatively consistent at 8 weeks (Figure 6.12b). There were no significant increases or decreases in glycogen levels between the 2 week and 8 week sampling period for individual plots (Figure 6.13). It is clear from this graph though, that transplanted cockles at the uncontaminated site (Awhitu) had higher glycogen levels than
those at the more contaminated sites. In addition, autochthonous cockles at these sites generally had higher glycogen levels than the transplanted animals (Figure 6.13).

**6.3.2.4 RNA**

RNA levels were significantly higher in transplanted cockles at Awhitu compared to autochthonous cockles at 2 weeks \((p=0.0007)\), whereas levels were similar between transplanted and autochthonous cockles at the remaining 3 transplant sites \((p>0.05)\) (Table 6.7 and Figure 6.14a). At 8 weeks, the pattern was very similar, although RNA levels were no longer statistically significantly different between autochthonous and transplanted cockles at Awhitu (Figure 6.14b).

After 2 weeks, transplanted cockles had higher RNA than cockles from the control plot at Lews Bay, at Awhitu \((p=0.00001)\), Big Muddy \((p=0.0015)\) and Takanini \((p=0.012)\) (Figure 6.15a). Although not statistically significant, transplanted cockles at Onehunga also had higher RNA levels than those at Lews Bay. Again, the pattern is similar at 8 weeks, where transplanted cockles at Awhitu and Big Muddy had higher RNA levels than those from the control plot, although only statistically significant at Awhitu \((p=0.0142)\) (Figure 6.15b). RNA levels were virtually identical for Onehunga transplanted cockles and Lews Bay control plot cockles.

There were no significant alterations in RNA levels between the 2 and 8 week sampling period for any specific plot (Figure 6.16). However, there was a weak trend of transplanted cockles having higher RNA at Awhitu and Big Muddy compared to
Determination of sublethal stress using in-situ exposure to contaminated sediment

autochthonous animals, whereas levels were similar between treatments at Takanini and Onehunga.

6.3.3 Mortality Estimates

In order to assess survival, the 100 cockles originally transplanted were categorised according to fate: those that were sacrificed for the analytical procedures, those known to have died during the first two weeks, and during the 2-8 week period, those alive at the end of the experiment, and those which could not be accounted for (Figure 6.17a). Known mortality could be accurately measured because of the presence, in the experimental plots, of tagged, but empty, cockle shells. Therefore, a mortality range was calculated for each plot, making two different assumptions about the fate of the missing individuals. The lower mortality figure was calculated assuming the missing individuals were alive, and the higher mortality estimate assumed that the missing individuals had died.

Lews Bay control plot cockles had the lowest mortality (<5%) (Figure 6.17b). At Awhitu, transplanted cockles had lower mortality than autochthonous cockles, primarily due to the loss of 60% of the autochthonous cockles from this plot, most likely due to human interference or harvesting, between the 2 and 8 week sampling periods. Mortality was similar for autochthonous and transplanted plots at Big Muddy, with approximately 50% mortality. Takanini transplanted cockles suffered high mortality (approximately 80%), whereas autochthonous cockles had 45-65% mortality. Empty valves of transplanted cockles were found approximately 30 cm beneath the sediment surface, indicating that these individuals had sunk into the fine mud at this site. Transplanted cockles at
Onehunga had approximately 65-70% mortality, but autochthonous cockles fared better with 45%. These unpredicted high mortalities at some sites meant that in some biochemical analyses sample sizes were compromised.

6.4 Discussion

Estuarine bivalves are useful for manipulative studies such as the transplant experiments undertaken in this study (Cristini 1987, Ivanovici 1980c, Veldhuizen Tsoerkan et al. 1991, Zorba et al. 1992). Transplantation of organisms from non-limiting environments to contaminated habitats overcomes problems associated with physiological regulation and detoxification mechanisms masking initial stress in naturally-occurring individuals living in contaminated habitats. Furthermore, transplanting can enable the contaminant body burden to be calculated over a defined exposure period (Salazar & Salazar 1997).

Results for the adenylate nucleotide indices indicate that AEC in cockles transplanted to contaminated sites may be regulated in order to maintain a high ratio, and that this may be at the expense of TANP. At Onehunga and Big Muddy, sites with relatively high levels of contaminants, the AEC of transplanted cockles increased between the 2 and 8 week period, but TANP declined; i.e. a simultaneous degradation of AMP increased AEC. Picado & Le Gal (1990) found a similar trend in their study of the bivalve Cerastoderma edule, which was challenged with paper mill effluent. A decrease in TANP in response to contaminants, while AEC is maintained, has been documented to also occur in oligochaetes (Schöttler 1978), polychaetes (Verschraegen et al. 1985), abalone (Shofer and Tjeerdema 1998) and crustaceans (Dickson & Giesy 1981). At Awhitu, the uncontaminated site, both transplanted and autochthonous cockles maintained AEC values.
Determination of sublethal stress using in-situ exposure to contaminated sediment

between 0.6 and 0.7, which are typical levels based on data in previous chapters. Whilst maintaining AEC levels, transplant cockles at Awhitu were able to increase TANP significantly. At all sites, autochthonous cockles showed no change in AEC or TANP during the experiment, which supports the hypothesis that these cockles had already energetically compensated for their environmental conditions.

Transplanted cockles, originally from the same donor population at Lews Bay, fared differently among sites in the Manukau Harbour. A comparison of AEC across sites indicated that among-site differences evident at 2 weeks were reduced at 8 weeks. TANP showed a contrasting pattern, with very similar values among sites at 2 weeks. However, a significant increase at the clean site and decreases at contaminated sites were evident after 8 weeks. These patterns strongly suggest that transplanted cockles at all sites were able to regulate the concentrations of their individual adenylate nucleotides in order to regain or maintain AEC values of between 0.6 and 0.7, but the effect of this on TANP levels was considerable. TANP values separated sites according to contaminant levels, with Awhitu transplant cockles achieving the highest levels of TANP, whereas cockles at Big Muddy and Onehunga had lowered TANP. These results point to a potential role for TANP as a biomarker of persistent environmental stress. Conversely, AEC may only be useful in revealing short-term perturbations in environmental quality.

Glycogen has been used in studies of environmental effects as an indicator of reproductive capacity (Huggett et al. 1992, Trueman & Snelder 1995). In this study, glycogen levels clearly separated the uncontaminated site from the contaminated sites. If we assume that glycogen levels were similar for all transplant cockles at the beginning of
the experiment (a reasonable assumption because they are from the same population, are of a similar size and have been subjected to identical environmental conditions), then it is clear that cockles transplanted to Awhitu experienced environmental conditions which enabled the accumulation of glycogen, whereas those moved to the contaminated sites were in glycogen limiting conditions. Furthermore, after 8 weeks, the three transplant plots that could be analysed are clearly separated on the basis of contaminant levels, with Onehunga cockles having the lowest levels, followed by Big Muddy, and Awhitu with the highest. Glycogen serves as an ecologically relevant indicator as there is clear evidence that cockles transplanted to Awhitu would have greater reproductive capacity than those at the contaminated sites (see Chapter 3, and Barber & Blake 1981).

There are few other transplantation experiments where glycogen has been analysed as a measure of environmental stress in bivalves. Cristini (1987) however, concluded that Mya arenaria transplanted to contaminated sites were able to maintain their glycogen levels and therefore their gametogenic cycle. However, in her study, sampling was undertaken monthly and short-term perturbations, such as those identified after 2 weeks in cockles transplanted to sites in the Manukau Harbour, may have been obscured using this temporal sampling scale. Huggett et al. (1992) review studies where glycogen has been shown to decrease in response to metals in fish and organic contaminants in fish, crabs and an amphipod. Glycogen levels may also be affected by environmental conditions such as food supply, which may be different among sampling sites. Comparisons of biomarker response between autochthonous and transplanted cockles within sites overcome potential disparities in habitat conditions, as both treatment groups are subject to identical environmental conditions. This research recognises glycogen as having
Determination of sublethal stress using in-situ exposure to contaminated sediment

potential as a biomarker of sublethal stress in A. stutchburyi, with the added advantage of having direct ecological relevance.

RNA levels, and therefore protein synthesis (Brachet 1960), decreased with increased contaminant levels at the transplant sites in the Manukau Harbour. Transplanted animals at the uncontaminated site (Awhitu) had highest RNA, whereas those at the contaminated Onehunga site had the lowest. These data suggest that potential growth is higher at the uncontaminated site compared to the contaminated sites. It is unknown why control cockles at Awhitu and Big Muddy had lower RNA levels than the treatment cockles. Growth, and therefore RNA concentration, is also associated with food supply, and there may have been among site differences in the availability of food. However, as within each site autochthonous and transplanted cockles are subject to identical environmental conditions, comparisons can be made between these two groups without the suggestion of confounding effects, such as potential differences in food supply.

Other studies have shown similar trends in RNA levels, with the waterflea Daphnia magna (Knowles & McKee 1987), fathead minnows (Barron & Adelman 1984), and cockroaches (Sutcliffe 1970), but application to bivalves has been limited. In their laboratory study using the oyster Crassostrea virginica, Wright & Hetzel (1985) found significant correlations between RNA:DNA ratios and condition and RNA:DNA ratios and growth. However, there is a paucity of experiments where RNA level is used as an indicator of contaminants in molluscs.
Determination of sublethal stress using in-situ exposure to contaminated sediment

Mortality was high for cockles, regardless of site of origin, at all sites within the Manukau Harbour. It is unknown why a large number (>50%) of autochthonous cockles at Awhitu were missing at the completion of the experiment, and it is likely that there was interference with this experimental plot by harvesters. Public interference is one of the disadvantages of using manipulative field experiments. At Takanini, empty valves of cockles transplanted from Lews Bay were found in the mud, and it may be that they sunk into the very fine sediment at this site. Autochthonous cockles did not suffer the same fate. Cockles in both plots were a similar size, but autochthonous cockle shells may have been thinner and therefore lighter. This conclusion remains speculative, however, as cockles were not weighed prior to placement in the experimental plots. Mortality of transplanted cockles at both Takanini and Onehunga was marginally higher than that for autochthonous cockles, which may indicate autochthonous cockles have tolerance or adaptation to local conditions.

The experiment discussed in this chapter effectively showed that the energetics of the New Zealand cockle, Austrovenus stutchburyi, is affected by environmental sublethal stress. The stress responses detected suggest that reproductive potential and growth may be reduced in limiting conditions, such as those created by contamination of sediment. This experimental approach worked well for Austrovenus stutchburyi, although it is recommended for future studies that a greater number of individuals be transplanted in order to compensate for mortality or movement. Further, if sufficient research funds are available, contaminant body burden should also be analysed to provide direct evidence of exposure to contaminants. Biomarker response in this experiment was shown to be associated with sediment contaminant levels and exposure period. In addition, the
biomarkers used have the advantage of being clearly related to the population and ecology of *Austrovenus stutchburyi*. The suite of biomarkers used in this experiment has potential as a monitoring tool for statutory authorities charged with "avoiding, remedying or mitigating" adverse environmental effects.
Table 6.1: Summary of published accounts in the past 15 years of experiments where bivalves were transplanted for the purposes of detecting sublethal stress effects due to contaminants in the transplanted environment.

<table>
<thead>
<tr>
<th>Bivalve Species</th>
<th>Exposure Method</th>
<th>Experimental Design</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Andara granosa</em></td>
<td>Not stated</td>
<td>No transplant controls</td>
<td>Din &amp; Ahamad 1995</td>
</tr>
<tr>
<td><em>Arca zebra</em></td>
<td>Suspended in water column</td>
<td>No transplant controls</td>
<td>Widdows <em>et al.</em> 1990</td>
</tr>
<tr>
<td><em>Cerastoderma edule</em></td>
<td>Not stated</td>
<td>No transplant controls</td>
<td>Picado &amp; Le Gal 1990</td>
</tr>
<tr>
<td><em>Dreissena polymorpha</em></td>
<td>Suspended in water column</td>
<td>No within-site transplant controls, but adequate between-site transplant controls</td>
<td>Bowmer <em>et al.</em> 1991</td>
</tr>
<tr>
<td><em>Mya arenaria</em></td>
<td>Positioned on benthos</td>
<td>No transplant controls</td>
<td>Cristini 1987</td>
</tr>
<tr>
<td><em>Mytilus edulis</em></td>
<td>Suspended in water column</td>
<td>No transplant controls</td>
<td>Phelps <em>et al.</em> 1981</td>
</tr>
<tr>
<td><em>Mytilus edulis</em></td>
<td>Suspended in water column</td>
<td>No transplant controls</td>
<td>Nelson 1987</td>
</tr>
<tr>
<td><em>Mytilus galloprovincialis</em></td>
<td>Suspended in water column</td>
<td>No transplant controls</td>
<td>Salazar &amp; Salazar 1997</td>
</tr>
<tr>
<td><em>Pyganodon grandis</em></td>
<td>Positioned on benthos</td>
<td>Adequate transplant controls</td>
<td>Couillard <em>et al.</em> 1995</td>
</tr>
</tbody>
</table>
**Figure 6.1:** Design of transplant experiment. Each arrow represents the transplantation of 100 *Austrovenus stutchburyi*. Lews Bay is the donor site, and Awhitu, Big Muddy, Takanini and Onehunga are the transplant sites. The red arrows represent cockles transplanted from Lews Bay to the transplant sites (termed transplant cockles for this experiment), the blue arrows represent cockles originating from the transplant sites and transplanted back to those sites (termed autochthonous cockles for this experiment), and the green arrow represents cockles originating from the donor site and returned to that site (termed control cockles for this experiment).

**Figure 6.2:** Plan of the 22 comparisons of interest (represented by arrows) for each biochemical analysis (AEC, TANP, Glycogen, RNA). **A** = autochthonous cockles transplanted back to each site in the Manukau Harbour, **T** = cockles transplanted from Lews Bay (donor site) to each site in the Manukau Harbour, **C** = control plot of cockles from donor site (Lews Bay) transplanted back to Lews Bay, 2 = 2 week sampling period, 8 = 8 week sampling period. The missing T cell represents absence of cockles transplanted from Lews Bay to Takanini (within the Manukau Harbour) at 8 weeks.
**Table 6.2:** Surficial sediment contaminant data for donor and transplants sites. Due to potential loss of lower weight compounds (e.g. naphthalene) during sampling and drying, total PAH is conservatively based on the following compounds: anthracene, phenanthrene, benz[a]anthracene, benzo[a]pyrene, dibenzo[a,h]anthracene, fluoranthene, and pyrene. Contaminant levels which exceed the Effects Range Low (ERL) (Long et al. 1995) are shown by an *, and those which also exceed the Threshold Effects Level (TEL) (Smith et al. 1996) are shown in bold (see Appendix II). Data for Onehunga, Big Muddy and Takanini is from Williamson et al. (1999), and data for Lewis Bay and Awhitu was collected by this researcher (see section 6.2.3).

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>LEWS BAY</th>
<th>ONEHUNGA</th>
<th>BIG MUDDY</th>
<th>TAKANINI</th>
<th>AWHITU</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total PAH</strong> (total ng/g DW)</td>
<td>80.3</td>
<td>201.6</td>
<td>125.1</td>
<td>68.3</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>Zn</strong> (total µg/g DW)</td>
<td>12.2</td>
<td>**180.5 ***</td>
<td>61.5</td>
<td>64</td>
<td>4.6</td>
</tr>
<tr>
<td><strong>Cu</strong> (total µg/g DW)</td>
<td>1.6</td>
<td>**43.8 ***</td>
<td>10.1</td>
<td>13.8</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>Pb</strong> (total µg/g DW)</td>
<td>1.1</td>
<td>**47.1 ***</td>
<td>9.6</td>
<td>6.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>
Figure 6.3: Location of Whangateau and Manukau Harbours, and the experimental sites within each harbour.
Determination of sublethal stress using in-situ exposure to contaminated sediment

North Island, New Zealand

Lews Bay

Omaha Bay

Whangateau Harbour

Onewhunga

Big Muddy

Manukau Harbour

Takanini

Awhitu

10 km

3 km

0 1 2
Plate 4: Lews Bay within the Whangateau Harbour.
Determination of sublethal stress using in-situ exposure to contaminated sediment

Plate 5a: Onehunga study site within the Manukau Harbour.

Plate 5b: Big Muddy study site within the Manukau Harbour.
Determination of sublethal stress using in-situ exposure to contaminated sediment

Plate 6a: Takanini study site within the Manukau Harbour.

Plate 6b: Awhitu study site within the Manukau Harbour.
Table 6.3: Sample sizes of transplanted cockles recovered for biochemical analyses from 5 sites (see Figure 6.1). Control cockles are those originating from Lews Bay and moved back to Lews Bay, autochthonous cockles are those from each site in the Manukau Harbour planted back to each site, transplant cockles are those originating from Lews Bay which were transplanted to sites in the Manukau Harbour. Samples with fewer than 10 cockles are shown in bold.

<table>
<thead>
<tr>
<th>TYPE OF ANALYSIS</th>
<th>AEC, TANP &amp; GLYCOGEN</th>
<th>RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time of collection of cockle tissue samples after start of experiment (29/3/99)</td>
<td>2 weeks</td>
<td>8 weeks</td>
</tr>
<tr>
<td>Lews Bay</td>
<td>Control</td>
<td>10</td>
</tr>
<tr>
<td>Awhitu</td>
<td>Autochthonous</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Transplant</td>
<td>10</td>
</tr>
<tr>
<td>Big Muddy</td>
<td>Autochthonous</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Transplant</td>
<td>10</td>
</tr>
<tr>
<td>Takanini</td>
<td>Autochthonous</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Transplant</td>
<td>10</td>
</tr>
<tr>
<td>Onehunga</td>
<td>Autochthonous</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Transplant</td>
<td>10</td>
</tr>
</tbody>
</table>
**Figure 6.4:** Median sediment particle size, for the donor site at Lews Bay and for the 4 sites in the Manukau Harbour to which cockles were transplanted.
Determination of sublethal stress using in-situ exposure to contaminated sediment

Median particle size (um)

- Lewis Bay
- Awhitu
- Big Muddy
- Takanini
- Onehunga

Site
Figure 6.5:

a) Plot of mean adenylate energy charge (AEC) +/- std error, for transplant (T) and autochthonous (A) groups at 4 transplant sites in the Manukau Harbour after two weeks. Yellow dots represent cockles collected from Awhitu, dark blue dots represent those from Big Muddy, pink dots represent those from Takanini, and light blue dots represent those from Onehunga.

b) Plot of mean AEC +/- std error, for transplant (T) and autochthonous (A) groups at 4 transplant sites in the Manukau Harbour after eight weeks. Colour codes are as above.

* indicates statistically significant.
Determination of sublethal stress using in-situ exposure to contaminated sediment

![Graph showing mean adenylate energy charge (+/− s.e.) for different treatments and sites over 2 weeks and 8 weeks.]

- **Awhitu**
- **Big Muddy**
- **Takanini**
- **Onchunga**

**Treatment/Site**
Figure 6.6:

a) Plot of mean AEC +/- std error, for control group at Lews Bay and transplant groups from sites in the Manukau Harbour after 2 weeks.

b) Plot of mean AEC +/- std error, for control group at Lews Bay and transplant groups from sites in the Manukau Harbour after 8 weeks.
No data available for Takanini site due to mortalities.

* indicates statistically significant.
Determination of sublethal stress using in-situ exposure to contaminated sediment

**2 weeks**

**8 weeks**

Mean Adenylate Energy Charge (+/- s.e.)

<table>
<thead>
<tr>
<th>Site</th>
<th>Lews Bay</th>
<th>Awhitu</th>
<th>Big Muddy</th>
<th>Takanini</th>
<th>Onehunga</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 weeks mean</td>
<td>0.6</td>
<td>0.6</td>
<td>0.7</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>8 weeks mean</td>
<td>0.6</td>
<td>0.7</td>
<td>0.7</td>
<td>0.6</td>
<td>0.6</td>
</tr>
</tbody>
</table>
Table 6.4

Summary of statistics for adenylate energy charge comparisons.

T = cockles transplanted from Lewis Bay to sites in the Manukau Harbour.
A = autochthonous cockles transplanted back to sites of origin in the Manukau Harbour.
C = cockles from Lewis Bay transplanted back to Lewis Bay.

Significant differences are shown in bold.

Figure 6.7:

Summary plot of mean AEC +/− std error, for transplant (T), autochthonous (A) and control plots at all sites, after 2 and 8 weeks. Colour codes are as per descriptions for Figure 6.5 and Figure 6.6.
Determination of sublethal stress using in-situ exposure to contaminated sediment

<table>
<thead>
<tr>
<th>AEC</th>
<th>Comparison</th>
<th>p value (Wilcoxon test)</th>
<th>α Level</th>
<th>Significance (i.e. p&lt;α)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Awhitu</td>
<td>T2 vs A2</td>
<td>0.0892</td>
<td>0.013</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T8 vs A8</td>
<td>1.0000</td>
<td>0.017</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T2 vs T8</td>
<td>0.3527</td>
<td>0.0064</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>A2 vs A8</td>
<td>0.7679</td>
<td>0.0064</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T2 vs C2</td>
<td>0.7197</td>
<td>0.0130</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T8 vs C8</td>
<td>0.7197</td>
<td>0.0130</td>
<td>No</td>
</tr>
<tr>
<td>Big Muddy</td>
<td>T2 vs A2</td>
<td>0.0233</td>
<td>0.0130</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T8 vs A8</td>
<td>0.0630</td>
<td>0.0170</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T2 vs T8</td>
<td><strong>0.0011</strong></td>
<td>0.0064</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>A2 vs A8</td>
<td>0.0433</td>
<td>0.0064</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T2 vs C2</td>
<td>0.3154</td>
<td>0.0130</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T8 vs C8</td>
<td>0.8421</td>
<td>0.0170</td>
<td>No</td>
</tr>
<tr>
<td>Takanini</td>
<td>T2 vs A2</td>
<td>0.7959</td>
<td>0.0130</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>A2 vs A8</td>
<td>0.5622</td>
<td>0.0064</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T2 vs C2</td>
<td>0.2110</td>
<td>0.0130</td>
<td>No</td>
</tr>
<tr>
<td>Onehunga</td>
<td>T2 vs A2</td>
<td><strong>0.0003</strong></td>
<td>0.0130</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>T8 vs A8</td>
<td>0.0653</td>
<td>0.0170</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T2 vs T8</td>
<td>0.4318</td>
<td>0.0064</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>A2 vs A8</td>
<td>0.7984</td>
<td>0.0064</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T2 vs C2</td>
<td>0.3510</td>
<td>0.0130</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T8 vs C8</td>
<td>0.4376</td>
<td>0.0170</td>
<td>No</td>
</tr>
<tr>
<td>Lews Bay</td>
<td>C2 vs C8</td>
<td>0.2581</td>
<td>0.0064</td>
<td>No</td>
</tr>
</tbody>
</table>

Mean Adenylate Energy Charge (+/- s.e.)

Site/Treatment

Least contaminated → Most contaminated

173
Figure 6.8:

a) Plot of mean total adenylate nucleotide pool (TANP) +/- std error, for transplant (T) and autochthonous (A) groups at 4 transplant sites in the Manukau Harbour after two weeks.

b) Plot of mean TANP +/- std error, for transplant (T) and autochthonous groups at 4 transplant sites in the Manukau Harbour after eight weeks.

Colour codes are as per Figure 6.5.

* indicates statistically significant.
Determination of sublethal stress using in-situ exposure to contaminated sediment

![Graph showing total adenylate nucleotides (mM/g wet weight ± s.e.) for different treatments and exposure times.](image)

- **2 weeks**
  - Treatment: Awhitu, Big Muddy, Takanini, Onchunga
  - Significant difference marked with "*

- **8 weeks**
  - Treatment: Awhitu, Big Muddy, Takanini, Onchunga
  - Significant difference marked with "*"
Figure 6.9

a) Plot of mean TANP +/- std error, for control group at Lews Bay (C) and transplant (T) groups from transplant sites after 2 weeks.

b) Plot of mean TANP +/- std error, for control group at Lews Bay and transplant (T) groups from transplant sites after 8 weeks.
No data available for Takanini site due to mortalities.

Colour codes as per described in Figure 6.6

* indicates statistically significant.
Determination of sublethal stress using in-situ exposure to contaminated sediment

(a) 2 weeks

(b) 8 weeks

Total Adenylate Nucleotides (mM/g wet weight +/- s.e.)

Site

Lews Bay  Awhitu  Big Muddy  Takanini  Onehunga
Table 6.5:

Summary of statistics for total adenylate nucleotide pool comparisons.
T = cockles transplanted from Lews Bay to sites in the Manukau Harbour.
A = autochthonous cockles transplanted back to sites of origin in the Manukau Harbour.
C = cockles from Lews Bay transplanted back to Lews Bay.
Significant differences are shown in bold.

Figure 6.10:

Summary plot of mean TANP +/- std error, for transplant (T), autochthonous (A), and control groups at all sites after both 2 and 8 weeks. Colour codes are as per descriptions for Figure 6.5 and Figure 6.6.
**Determination of sublethal stress using in-situ exposure to contaminated sediment**

<table>
<thead>
<tr>
<th>TANP</th>
<th>Comparison</th>
<th>p value (Wilcoxon test)</th>
<th>α Level</th>
<th>Significance (i.e. p&lt;α)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Awhitu</td>
<td>T2 vs A2</td>
<td>0.0037</td>
<td>0.0130</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>T8 vs A8</td>
<td>0.5135</td>
<td>0.0170</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T2 vs T8</td>
<td>0.0089</td>
<td>0.0064</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>A2 vs A8</td>
<td>0.9530</td>
<td>0.0064</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T2 vs C2</td>
<td>0.7197</td>
<td>0.0130</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T8 vs C8</td>
<td><strong>0.0021</strong></td>
<td>0.0130</td>
<td>Yes</td>
</tr>
<tr>
<td>Big Muddy</td>
<td>T2 vs A2</td>
<td>0.0147</td>
<td>0.0130</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T8 vs A8</td>
<td><strong>0.0005</strong></td>
<td>0.0170</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>T2 vs T8</td>
<td>0.0630</td>
<td>0.0064</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>A2 vs A8</td>
<td>0.7959</td>
<td>0.0064</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T2 vs C2</td>
<td>0.6038</td>
<td>0.0130</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T8 vs C8</td>
<td>0.4002</td>
<td>0.0170</td>
<td>No</td>
</tr>
<tr>
<td>Takanini</td>
<td>T2 vs A2</td>
<td><strong>0.0068</strong></td>
<td>0.0130</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>A2 vs A8</td>
<td>0.2635</td>
<td>0.0064</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T2 vs C2</td>
<td>0.7802</td>
<td>0.0130</td>
<td>No</td>
</tr>
<tr>
<td>Onehunga</td>
<td>T2 vs A2</td>
<td>0.1893</td>
<td>0.0130</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T8 vs A8</td>
<td>0.0673</td>
<td>0.0170</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T2 vs T8</td>
<td>0.0480</td>
<td>0.0064</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>A2 vs A8</td>
<td>0.5054</td>
<td>0.0064</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T2 vs C2</td>
<td>0.5525</td>
<td>0.0130</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T8 vs C8</td>
<td>0.8981</td>
<td>0.0170</td>
<td>No</td>
</tr>
<tr>
<td>Lews Bay</td>
<td>C2 vs C8</td>
<td>0.1615</td>
<td>0.0064</td>
<td>No</td>
</tr>
</tbody>
</table>

**Total Adenylate Nucleotides (mM/g wet weight ± s.e.)**

- **Least contaminated**
- **Most contaminated**
Figure 6.11:

a) Plot of mean glycogen concentration +/- std error, for transplant (T) and autochthonous (A) groups at 4 transplant sites in the Manukau Harbour after two weeks.

b) Plot of mean glycogen concentration +/- std error, for transplant (T) and autochthonous (A) groups at 4 transplant sites after eight weeks.

Colour codes as per described in Figure 6.5.

* indicates statistically significant.
Determination of sublethal stress using in-situ exposure to contaminated sediment

2 weeks

8 weeks

Glycogen g/1000g (+/- s.e.)

T A T A T A T A

Awhitu Big Muddy Takanini Onchunga

Treatment
Figure 6.12:

a) Plot of mean glycogen concentration +/- std error, for control group at Lews Bay and transplant groups from sites in the Manukau Harbour after 2 weeks.

b) Plot of mean glycogen concentration +/- std error, for control group at Lews Bay and transplant groups from sites in the Manukau Harbour after 8 weeks. No data available for Takanini site due to mortalities.

Colour codes as per described in Figure 6.6.

* indicates statistically significant.
Determination of sublethal stress using in-situ exposure to contaminated sediment
Table 6.6:

Summary of statistics for glycogen comparisons.
T = cockles transplanted from Lews Bay to sites in the Manukau Harbour.
A = autochthonous cockles transplanted back to sites of origin in the Manukau Harbour.
C = cockles from Lews Bay transplanted back to Lews Bay.
Significant differences are shown in bold.

Figure 6.13:

Summary plot of mean glycogen concentration +/- std error, for transplant (T),
autochthonous (A) and control groups at all sites after both 2 and 8 weeks. Colour codes
are as per descriptions for Figure 6.5 and Figure 6.6.
## Determination of sublethal stress using in-situ exposure to contaminated sediment

### Glycogen

<table>
<thead>
<tr>
<th>Location</th>
<th>Comparison</th>
<th>p value (Wilcoxon test)</th>
<th>α Level</th>
<th>Significance (i.e. p&lt;α)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Awhitu</td>
<td>T2 vs A2</td>
<td>0.0147</td>
<td>0.0130</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T8 vs A8</td>
<td>0.1255</td>
<td>0.0170</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>T2 vs T8</td>
<td>0.5622</td>
<td>0.0064</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>A2 vs A8</td>
<td>0.3710</td>
<td>0.0064</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>T2 vs C2</td>
<td><strong>0.0000</strong></td>
<td>0.0130</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T8 vs C8</td>
<td><strong>0.0004</strong></td>
<td>0.0130</td>
<td>No</td>
</tr>
<tr>
<td>Big Muddy</td>
<td>T2 vs A2</td>
<td>0.0892</td>
<td>0.0130</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T8 vs A8</td>
<td>0.0274</td>
<td>0.0170</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>T2 vs T8</td>
<td>0.8421</td>
<td>0.0064</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>A2 vs A8</td>
<td>0.4083</td>
<td>0.0064</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>T2 vs C2</td>
<td>0.9401</td>
<td>0.0130</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>T8 vs C8</td>
<td>0.9422</td>
<td>0.0170</td>
<td>Yes</td>
</tr>
<tr>
<td>Takanini</td>
<td>T2 vs A2</td>
<td><strong>0.0052</strong></td>
<td>0.0130</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>A2 vs A8</td>
<td>0.0553</td>
<td>0.0064</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T2 vs C2</td>
<td>0.8421</td>
<td>0.0130</td>
<td>No</td>
</tr>
<tr>
<td>Onehunga</td>
<td>T2 vs A2</td>
<td>0.3930</td>
<td>0.0130</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T8 vs A8</td>
<td>0.4318</td>
<td>0.0170</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T2 vs T8</td>
<td>0.0553</td>
<td>0.0064</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>A2 vs A8</td>
<td>0.6691</td>
<td>0.0064</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>T2 vs C2</td>
<td>0.6038</td>
<td>0.0130</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T8 vs C8</td>
<td>0.2398</td>
<td>0.0170</td>
<td>No</td>
</tr>
<tr>
<td>Lews Bay</td>
<td>C2 vs C8</td>
<td>0.2581</td>
<td>0.0064</td>
<td>No</td>
</tr>
</tbody>
</table>

### Glycogen g/1000g (+/- s.e.)

- **Lews Bay**: Least contaminated
- **Awhitu**: Slightly contaminated
- **Big Muddy**: Moderately contaminated
- **Takanini**: Considerably contaminated
- **Onehunga**: Most contaminated

---

179
Figure 6.14:

a) Plot of mean RNA +/- std error, for transplant (T) and autochthonous (A) groups at 4 transplant sites in the Manukau Harbour after two weeks.

b) Plot of mean RNA +/- std error, for transplant (T) and autochthonous (A) groups at 4 transplant sites in the Manukau Harbour after eight weeks.

Colour codes as per described in Figure 6.5.

* indicates statistically significant.
Determination of sublethal stress using in-situ exposure to contaminated sediment

RNA ug/ml (+/- std error)

2 weeks

8 weeks

Treatment

Awhitu  Big Muddy  Takanini  Onehunga
Figure 6.15:

a) Plot of mean RNA +/- std error, for control group at Lews Bay and transplant groups from sites in the Manukau Harbour after 2 weeks.

b) Plot of mean RNA +/- std error, for control group at Lews Bay and transplant groups from sites in the Manukau Harbour after 8 weeks. No data available for Takanini site due to mortalities.

Colour codes as per described in Figure 6.6.

* indicates statistically significant.
Determination of sublethal stress using in-situ exposure to contaminated sediment

![Graph showing RNA ug/ml (+/- s.e.) at 2 weeks and 8 weeks for different sites.](graph.png)
Table 6.7:

Summary of statistics for RNA comparisons.
T = cockles transplanted from Lewis Bay to sites in the Manukau Harbour.
A = autochthonous cockles transplanted back to sites of origin in the Manukau Harbour.
C = cockles from Lewis Bay transplanted back to Lewis Bay.
Significant differences are shown in bold.

Figure 6.16:

Summary plot of mean RNA +/- std error, for transplant (T), autochthonous (A) and control groups at all sites after 2 and 8 weeks. Colour codes are as per descriptions for Figure 6.5 and Figure 6.6.
Determination of sublethal stress using in-situ exposure to contaminated sediment

<table>
<thead>
<tr>
<th>RNA</th>
<th>Comparison</th>
<th>p value (Wilcoxon test)</th>
<th>α Level</th>
<th>Significance (i.e. p&lt;α)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Awhitu</td>
<td>T2 vs A2</td>
<td>0.0007</td>
<td>0.0130</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>T8 vs A8</td>
<td>0.1455</td>
<td>0.0170</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T2 vs T8</td>
<td>0.5490</td>
<td>0.0064</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>A2 vs A8</td>
<td>0.9091</td>
<td>0.0064</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T2 vs C2</td>
<td>0.0000</td>
<td>0.0130</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>T8 vs C8</td>
<td>0.0142</td>
<td>0.0130</td>
<td>Yes</td>
</tr>
<tr>
<td>Big Muddy</td>
<td>T2 vs A2</td>
<td>0.0553</td>
<td>0.0130</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T8 vs A8</td>
<td>0.0315</td>
<td>0.0170</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T2 vs T8</td>
<td>0.7802</td>
<td>0.0064</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>A2 vs A8</td>
<td>0.5360</td>
<td>0.0064</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T2 vs C2</td>
<td>0.0015</td>
<td>0.0130</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>T8 vs C8</td>
<td>0.0315</td>
<td>0.0170</td>
<td>No</td>
</tr>
<tr>
<td>Takanini</td>
<td>T2 vs A2</td>
<td>0.5787</td>
<td>0.0130</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>A2 vs A8</td>
<td>0.4002</td>
<td>0.0064</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T2 vs C2</td>
<td>0.0115</td>
<td>0.0130</td>
<td>Yes</td>
</tr>
<tr>
<td>Onehunga</td>
<td>T2 vs A2</td>
<td>0.7802</td>
<td>0.0130</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T8 vs A8</td>
<td>0.8939</td>
<td>0.0170</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T2 vs T8</td>
<td>0.7679</td>
<td>0.0064</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>A2 vs A8</td>
<td>1.0000</td>
<td>0.0064</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T2 vs C2</td>
<td>0.0753</td>
<td>0.0130</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>T8 vs C8</td>
<td>1.0000</td>
<td>0.0170</td>
<td>No</td>
</tr>
<tr>
<td>Lews Bay</td>
<td>C2 vs C8</td>
<td>0.4470</td>
<td>0.0064</td>
<td>No</td>
</tr>
</tbody>
</table>
Figure 6.17:

a) Fate of individuals within each plot. L = Lews Bay control group, AT = Awhitu transplant, AA = Awhitu autochthonous, BT = Big Muddy transplant, BA = Big Muddy autochthonous, TT = Takanini transplant, TA = Takanini autochthonous, OT = Onehunga transplant, OA = Onehunga autochthonous.

b) Percent mortality range for individuals within each plot. Site and treatment codes are as above.
Determination of sublethal stress using in-situ exposure to contaminated sediment

a)

<table>
<thead>
<tr>
<th>Site/Treatment</th>
<th>L</th>
<th>AT</th>
<th>AA</th>
<th>BT</th>
<th>BA</th>
<th>TT</th>
<th>TA</th>
<th>OT</th>
<th>OA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Individuals</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Green: Taken for Analyses
- Yellow: Mortality at 2 weeks
- Dark Blue: Mortality at 8 weeks
- Pink: Remaining Alive
- Light Blue: Missing

b)

% Mortality Range

Site/Treatment
CHAPTER 7

General Discussion

Regulatory authorities around the world are charged with the responsibility for protecting the environmental resources in their area. However, often there is insufficient information available for these authorities to assess the health of such resources (Cawthron Institute 1998, Osenberg & Schmitt 1996). In New Zealand, the Ministry for the Environment is attempting to establish a set of indicators that regulatory authorities can use to assess environmental performance in estuaries and tidal inlets (Cawthron Institute 1998). The research presented in this thesis was designed to assist with this process. By assessing the response of cockles (*Austrovenus stutchburyi*) to sublethal stress, this work has sought to ascertain whether biochemical and physiological biomarkers could be used with this organism to indicate estuarine ecosystem health. This is the first such application of many of these biomarker techniques to an estuarine species in New Zealand. Furthermore, it is the first such case where several biomarkers have been used concurrently. The data obtained from these experiments confirm that these techniques can be powerful tools for indicating sublethal stress.

Fairweather (1999) indicates a sequence of steps necessary for the scientific development of a biomarker. Initially there should be a perceived need for the biomarker based on public concern or deficiencies in scientific data/knowledge. Fairweather (1999) further states that there should be an hypothesised relationship between the biomarker and environmental perturbations, which should subsequently be tested in laboratory trials and
in field situations. Once incorporated into monitoring programmes, further evaluation and on-site assessment can then assist with refining the application and interpretation of the biomarker. In the New Zealand context, there is a clear need for the establishment of biomarkers of aquatic perturbations, and in accordance with the sequence of steps outlined by Fairweather (1999), five biomarkers were tested in several field and laboratory conditions and relationships between biomarkers and perturbations analysed in my study.

The hypothesis tested in this research is that *A. stutchburyi* suffer sublethal stress due to the presence of contaminants in their environment, and that this stress can be measured, using sensitive biochemical and physiological biomarkers, and related to the functioning of this species. Furthermore, biomarkers of sublethal stress may, after refinement, be used to identify initial habitat contamination and warn of potentially lethal effects if remediation of the environment does not occur.

Use of a suite of biomarkers, such as that used in this research, as opposed to a single technique helps to minimise interpretation errors associated with obtaining false positive or negative results (Ringwood & Keppler 1998). The approach used in this study, where the utility of 5 biomarkers was investigated in a range of field and laboratory situations, supported by site characterisation data (such as sediment type, population structure of the indicator species, and community composition) provided a comprehensive basis for the assessment of sublethal stress in *Austrovenus stutchburyi*. This approach enabled the assessment of three of the main organism functions, i.e. energy cycling (AEC and TANP), growth (RNA) and reproductive potential (glycogen and condition).
An organism’s response to its natural environment is complex. Therefore, it is likely that an organism’s response to anthropogenic perturbations of the natural environment are even more complex (Sastry & Miller 1981). The successful use of biochemical and physiological biomarkers, however, hinges on the ability to distinguish an organism’s response to natural alterations in the environment from those responses brought about by contaminants (Sastry & Miller 1981). In my research, natural temporal variability in the response of three biomarkers was tested at two sites in the Whangarei Harbour, and spatial variability was investigated at several sites within the Mangemangeroa Estuary. In a controlled laboratory situation, biomarker response was further tested, with cockles challenged with different types of contaminants individually. Finally, manipulative field experiments were used to assess adaptative responses in cockles, with natural populations being compared with transplanted cockles.

The adenylate nucleotide biomarkers (AEC and TANP) showed seasonal trends in cockle populations from Whangarei Harbour. AEC was highest in spring, and TANP was lowest in winter. The AEC pattern was in contrast to other researchers who found that other bivalve species had highest levels in winter and autumn (Giesy & Dickson 1981, Moal et al. 1991). The variability in AEC values among years, at both sites, was significant in my study, which suggests that factors other than season, such as the discharge of contaminants and physical disturbance, affect this biomarker. There was suggestion from the data that lower levels of TANP at one site in the Whangarei Harbour were correlated with low-level contamination at this site. Spatial variability was further investigated in the Mangemangeroa Estuary. There were no clear spatial patterns in AEC and TANP in cockles from the various sites in this estuary, although cockles located at a site furthest up
the estuary generally had the lowest values for these two biomarkers. It transpired from this experiment that AEC and TANP levels might have been influenced by the topographic characteristics at each site, which created quite distinct microhabitats within the estuary. Thus, the energetic status was not homogenous for the entire cockle population. Rather energetic responses were site specific on a scale of 100’s of metres. This has important implications for the use of the biomarkers in estuarine management. It means, for example, that there may not be great utility in comparing biomarker response in cockles between sites within an estuary. Furthermore, in order to compare biomarker response in cockles over time, samples should be collected at identical sites, and comparisons made within sites. Laboratory experiments assessing the affect of anoxia on these biomarkers clearly indicated a decline in AEC when cockles were exposed to air for 4 hours, but there was no significant affect on TANP. This experiment confirmed that the collection of samples should be undertaken at a similar tidal state on each sampling occasion. These qualifications do not mean that the adenylate nucleotide biomarkers are too complicated to be used in estuarine management, but rather that monitoring programmes should be carefully designed and executed, in order to avoid known confounding factors.

Cockles in laboratory trials, challenged with PAHs, chlordane and tributyltin showed no significant difference in AEC or TANP among the control and contaminated treatments. The apparent tolerance to these contaminants, although associated with a significant contaminant tissue burden, was not anticipated. Cockles have been documented to be tolerant to environmental stress (Wilcock et al. 1993), as indeed are many estuarine species faced with the extremes in environmental conditions (Lockwood 1976), but the
lack of response in adenylate nucleotides was interesting. Indicator species ideally should have balanced sensitivity; i.e. they should be sensitive to contaminants and not hypersensitive to test conditions (Ringwood & Keppler 1998), which does not appear to be the case for A. stutchburyi. It was concluded that a manipulative field experiment should be undertaken to determine whether cockles were tolerant of contaminants in their natural environment.

This experiment aimed to provide information on whether the laboratory experiment was in some way inadequately designed, or whether cockles were indeed tolerant to contaminants in any situation. The results suggested that cockles were sensitive to quite rapid changes in the quality of their environment as they were able to respond energetically. Cockles were transplanted from an uncontaminated harbour to several sites in another harbour, some of which were contaminated. When transplanted to a contaminated site, cockles showed an ability to regulate their adenylate nucleotide pool, through the degradation of AMP, in order to regain “normal” AEC levels. TANP in cockles transplanted to these sites fell as a result of the decrease in AMP concentration, whereas cockles transplanted to an uncontaminated site achieved high levels of TANP. These results strongly suggest that AEC may be useful for indicating initial sublethal stress response, as AEC levels declined and subsequently recovered in cockles transplanted to contaminated sites (see also Appendix III). It is likely that there is greater utility in measuring AEC at a temporal scale of weeks rather than seasons, as used in Chapter 3 and 4. Furthermore, TANP may have utility as an indicator of chronic environmental perturbations, as cockles transplanted to uncontaminated sites had a significantly higher concentration of adenylate nucleotides than cockles transplanted to
contaminated sites. Because the response of adenylate nucleotide biomarkers in cockles is relatively site-specific, their greatest utility in environmental monitoring may be in the detection of changes in biomarker response at particular sites, rather than a single measurement that is compared to theoretical guideline values, such as that for AEC described by Bayne et al. (1985).

Seasonal cycles in glycogen concentration were clearly detected in cockles at both sites in the Whangarei Harbour and also at sites in the Mangemangeroa Estuary. These trends correspond to seasonal patterns detected in many other bivalve species (de Zwaan & Zandee 1972, Hickman & Illingworth 1980, Pazos et al. 1997) and relate to the reproductive cycle. There was little spatial variability in glycogen levels among sites in the Mangemangeroa Estuary, which suggests that this biomarker may have limited utility for indicating subtle differences in environmental quality. As with the adenylate nucleotide biomarkers, no significant differences in glycogen concentration were detected in any of the laboratory trials. However, glycogen concentration in cockles showed an unanticipated plasticity in the transplant experiment. Cockles transplanted to an uncontaminated site were able to vastly increase glycogen concentration, but cockles at contaminated sites suffered a decrease in glycogen levels. These results suggest that glycogen concentration may be useful for distinguishing among sites that have gross differences in environmental quality. Furthermore, changes in glycogen concentration in repeated samples at a particular site have great potential to indicate alterations in environmental quality.
Condition was analysed in cockles from the Mangemangeroa Estuary, and no clear spatial or temporal patterns in this biomarker emerged from the experiment. Body condition of bivalves has been used in many monitoring programmes and in some situations of extreme environmental perturbation may give a “rough and ready” response (Roper et al. 1991, Pridmore et al. 1990, Widdows 1985b). This biomarker is unlikely to be as sensitive to subtle alterations in environmental quality as perhaps the adenylate nucleotide biomarkers and is unlikely to show rapid response demonstrated by glycogen. Consequently, it was not used in subsequent experiments.

RNA concentration was tested in cockles in the laboratory contaminant experiments and in the manipulative field experiment. Although no effect of contaminants on RNA levels was apparent from the laboratory trials, there was a clear pattern in the response of RNA concentration in cockles transplanted to sites in the Manukau Harbour. RNA was reached a higher concentration in cockles transplanted to the uncontaminated site than in cockles transplanted to contaminated sites. This biomarker, along with glycogen concentration, showed great potential for distinguishing between sites that have measurable differences in environmental quality.

Manipulative field studies are instructive as organisms living in a contaminated environment can show some resistance to certain contaminants, and this may lead to the non-detection of some effects in these organisms (Klerks & Weis 1987). For example, Luoma et al. (1983) found that the clam *Macoma balthica* from a site with high copper levels in the sediment had elevated copper tolerance when subjected to copper in solution in laboratory trials. However, these somewhat tolerant organisms may suffer effects that
are not often directly measured, such as reduced viability of gametes, and these organisms are more likely to be affected by other secondary stressors, such as disease (Anderson et al. 1981). In addition, organisms from contaminated environments, though appearing unstressed, may have high body burdens of contaminants, which present a potential risk if consumed by other organisms (including humans). Therefore, in order to measure realistic levels of sublethal stress it is necessary to transplant organisms from an uncontaminated habitat to contaminated, or "at risk", habitats, rather than sampling autochthonous organisms.

Other researchers have concluded that adult shellfish are generally too tolerant of contaminants to be useful in biomonitoring. Stiles et al. (1991) concluded from experiments at both contaminated and uncontaminated sites in Long Island Sound (New York, USA), in which the reproductive success of the clam Mercenaria mercenaria was investigated, that adult shellfish are relatively insensitive to contaminants. However, conclusions from the manipulative field experiments carried out with cockles in the present research suggest otherwise, as adult A. stutchburyi showed sensitive sublethal stress responses. Field experiments which measure sublethal stress responses in organisms, such as those undertaken in my research, can be used to indicate effects of contaminants, and these effects can be related to the ecology of the habitat. However, it is recognised that this type of approach does not enable the identification of specific contaminants, nor does it establish a cause and effect relationship between the sublethal stress responses in the organism and the contaminants present in the environment.
Degradation of environmental quality in coastal aquatic ecosystems is likely to continue to occur in New Zealand, and worldwide, as urban boundaries are continuously extending outwards, and as our rural land use practices change. In a perfect world, regulatory authorities would foresee every potential risk to the environment, and avoid any detrimental effects. However, in reality protection of the environment is somewhat of a catch-up game, in which environmental problems are identified (sometimes before chronic and persistent effects occur) and in some situations, these problems are remedied. There is a need therefore, to monitor the quality of ecosystems, in order to forewarn of the likelihood of severe adverse effects.

A similar monitoring programme to the Mussel Watch programme used in the United States could be established using cockles in New Zealand’s “at risk” estuaries, or incorporated into Environmental Effects Assessments to monitor the effects of new coastal developments. Biomarker response in cockles transplanted from an uncontaminated habitat to new monitoring sites could be used to indicate whether ecosystems are suffering environmental stress and also if the ecosystem is too vulnerable to be able to withstand potential effects of catchment development. This approach would take advantage of the sensitive biochemical and physiological responses shown to occur in cockles in my research when transplanted to new habitats. It is recommended that TANP, glycogen and RNA concentration should be used in such a monitoring programme, as three of the main functions of cockles can be assessed using this suite. It is suggested that, due its hypersensitivity to small-scale changes in environmental quality, AEC should not be used in such monitoring programmes.
Some of these techniques can be expensive and require specialised equipment, although once set up, the costs of consumables and labour may be significantly less than the high costs associated with repeated analyses of contaminant concentrations in shellfish tissue, which is presently the common monitoring alternative. Of the these three biomarkers, the methodology for measurement of TANP requires the greatest input of time, specialised equipment and technical expertise, followed by the protocol for RNA calculation. Glycogen is the most straightforward and least time consuming of these three biomarker techniques. However, as it is recognised that as aquatic environments receive a mixture of contaminants, which often have many different toxic effects, no one single biomarker can adequately assess all the environmental effects of contaminants on marine organisms (Donkin et al. 1996). Consequently, use of a greater number of biomarkers, such as the three recommended, measuring different organism responses, will provide a more comprehensive assessment of the effects of contaminants.

The overall conclusion from this thesis is that Austrovenus stutchburyi has significant utility as an in-situ indicator of environmental degradation in New Zealand estuaries. The biomarkers TANP, glycogen and RNA used together have the greatest utility for assessment of sublethal stress in cockles. Furthermore, manipulative field experiments can provide unequivocal biomarker response information and thereby highlight sublethal stress in A. stutchburyi that may forewarn of more serious ecosystem damage, and also identify vulnerable ecosystems.


References


References


References


References


References


References


References


References


References


Phelps, D.K., Galloway, W., Thurberg, F.P., Gould, E. & Dawson, M.A., 1981. Comparison of several physiological monitoring techniques as applied to the blue mussel, *Mytilus edulis*, along a gradient of pollutant stress in Narragansett Bay,


Riedel, G.F., Abbe, G.R. & Sanders, J.G., 1995. Silver and copper accumulation in two estuarine bivalves, the eastern oyster (Crassostrea virginica) and the hooked mussel (Ischadium recurvum) in the Patuxent River estuary, Maryland. Estuaries, 18 (3), 445-455.
References


References


References


TEMPORAL AND SPATIAL DISTRIBUTION OF ENTEROCOCCUS IN SEDIMENT, SHELLFISH TISSUE, AND WATER IN A NEW ZEALAND HARBOUR

S. DE LUCA-ABBOTT, G. D. LEWIS, AND R. G. CREESE

Leigh Marine Laboratory
School of Environmental and Marine Science
University of Auckland
PO Box 349
Warkworth, New Zealand

RUNNING TITLE: Enterococcus in Sediment, Shellfish and Water

KEY WORDS: stormwater, enterococci, Austrovenus stutchburyi, cockle, Macomona liliana.
ABSTRACT Enterococci, a group of faecal bacteria commonly found in stormwater discharges, were used to trace the spatial and temporal impact of waste streams from an outfall in the Whangateau Harbour, northeastern New Zealand. A seasonal trend in levels of enterococci in two infaunal bivalves, Austrovenus stutchburyi (Gray in Wood 1828) and Macomona liliana (Iredale 1915) was detected, with maximum contamination correlating with high winter rainfall. Rainfall events were also shown to affect stormwater and harbour water significantly. Median enterococci levels in A. stutchburyi were higher at the putative impact site compared to the reference site, and were higher than M. liliana at both sites. Bacterial levels in surficial sediment and A. stutchburyi tissue declined with distance from the stormwater outfall and the stormwater channel. Enterococci were effective for determining the spatial and temporal patterns of stormwater discharge in this harbour, and may have general applicability as an indicator of such discharges.

INTRODUCTION

There is a paucity of literature concerning temporal and small-scale spatial effects of stormwater discharges on the bacteriological quality of the marine environment adjacent to small coastal settlements in New Zealand. Typically, these settlements comprise dwellings along harbour or estuary fringes, with stormwater flows discharged directly onto the intertidal zone, which is inhabited by many edible shellfish species. Long-term monitoring of bacterial contamination is needed to establish average or background levels, which in turn assist in the interpretation of short-term event-driven episodes. It is also important to ascertain the distribution of bacterial contamination across the adjacent foreshore, in order to identify areas of potential high risk. Such information would highlight areas likely to be contaminated with other stormwater pollutants, as well as sites unsafe for harvesting of edible shellfish resources and areas unsuitable for shellfish restoration efforts.

Snelder and Williamson (1997) define stormwater as rainwater that runs off impervious surfaces and is usually discharged as a point source into waterways such as estuaries and harbours. When discharged into high-energy receiving environments there is immediate dilution, whereas sheltered habitats such as harbours and estuaries accumulate particulate matter contained in such discharges (Snelder and Williamson 1997). The main contaminants found in urban stormwater include heavy metals, polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides, hydrocarbons, nutrients,
Appendix I: Journal of Shellfish Research, in press.

suspended solids, and microorganisms (Snelder and Truman 1995, Snelder and Williamson 1997). All have the potential to compromise the sustainability of marine environments (Morrisey 1997).

Stormwater discharges often affect the bacteriological quality of the receiving water (Gannon and Busse 1989). Of concern to environmental managers is the potential for microorganisms contained in waste discharges to affect human health adversely through bathing or the consumption of affected shellfish (Kebabjian 1994). Water and seafood contaminated with sewage have been reported to transmit gastrointestinal disorders, hepatitis, cholera, and a range of eye, ear, nose, and throat infections (McIntyre 1995). The spread of disease via contaminated water and seafood has prompted the use of indicator microorganisms as a warning of unsafe conditions (Elliot and Colwell 1985). These indicator microorganisms are not themselves pathogenic. They generally occur in high concentrations in the same environments as pathogenic microbes but are simpler to detect and quantify (Fattal et al. 1984). Therefore, they can be used to indicate a potential risk of disease (Elliot and Colwell 1985).

Survival of both pathogenic and indicator bacteria in marine waters is affected by many physical, chemical, and biological factors, including temperature, sunlight, salinity, predation, adsorption, sedimentation, and dilution (Borrego et al. 1983, Nicholson 1988). Biological parasitism and predation are also responsible for destruction of faecal bacteria entering the marine environment in untreated sewage (Roper and Marshall 1974). Survival characteristics of indicator bacteria and the pathogens they are modelling should be similar.

The coccoid bacteria Enterococcus, which naturally inhabits the gut of humans and warm-blooded animals, has gained favour in recent years over another bacterium, Escherichia coli, as an indicator bacteria, as they survive longer in seawater, and have good correlation with gastrointestinal symptoms (Miesciar and Cabelli 1982, Elliot and Colwell 1985, Donnison 1992, Sinton et al. 1993).

Shellfish have the ability to concentrate bacteria and other contaminants from water or sediment via their mode of feeding, and are therefore useful tools for investigating faecal pollution (Ayres et al. 1978, Nicholson 1988, Prieur et al. 1990). Most bivalves are filter-feeders, passing large volumes of water across their gills to obtain food and oxygen. Microorganisms and food particles in suspension are trapped in mucus on gill, mantle, and labial palp surfaces and then transported by ciliary action to
the mouth (Perkins et al. 1980, Cook 1991). Deposit-feeding bivalves feed by ingesting surficial sediment, which may have considerably higher levels of bacteria than the water column (Elliot and Colwell 1985, Kueh 1987). Accumulation of bacteria in sediments is due to their sorption to particles suspended in water, which then settle out onto the surficial sediment (Davies et al. 1995). Whether ingested via filter-feeding or deposit-feeding, digestive processes are not thought to inactivate all microorganisms (Hedstrom and Lycke 1964). Depuration occurs through the discharge of faeces and from the pumping of water through the mantle cavity (Perkins et al. 1980). Factors affecting feeding, and therefore accumulation of bacteria, include temperature, turbidity, salinity, and physiological condition of shellfish (Bonadonna et al. 1990). Filtration rate may decline when these factors are suboptimal and therefore less indicator and pathogenic microorganisms would be accumulated (Ayres et al. 1978, Nicholson 1988). Retention of microorganisms by bivalves is a function of spacing of gill filaments, water flow through the mantle cavity, and filtering behaviour, all of which add to the variability in accumulation of bacteria among species (Bonadonna et al. 1990).

This research aims to identify the effects of stormwater discharges on enterococci levels in bivalve shellfish, sediment, and water. Seasonal and year-to-year bacterial levels are investigated in experiment A, and event-driven episodes such as rainfall events are analyzed in experiment B. The spatial distribution of bacterial levels along the foreshore is presented in experiment C. This work uses relatively simple, inexpensive microbiological techniques to trace and determine spatial and temporal patterns associated with low volume waste discharges from small coastal communities into marine environments.

METHOD AND MATERIALS

Shellfish Description

Experiments in this study focus on the cockle, Austrovenus stutchburyi (Gray in Wood 1828), and the wedge shell, Macomona liliana (Iredale 1915). These two shellfish have overlapping distributions throughout many estuaries and harbours in New Zealand (Larcombe 1968, Marsden and Pilkington 1995). Both are abundant and widespread in the Whangateau Harbour. They have contrasting feeding modes (A. stutchburyi is a filter-feeder, and M. liliana is a deposit-feeder) and therefore different exposure routes to bacterial contamination.
Site Description

Three experiments were carried out in Whangateau Harbour, northeastern New Zealand (Fig 1). This harbour is typical of many New Zealand estuaries, and is fringed by several small, rural communities, with a combined population of c. 1,400. Low levels of waste enter the harbour from these communities (e.g., Klein and Gowing 1993). Samples were taken from an area adjacent to a stormwater outfall at the putative impact site (Point Wells). This is a small residential community, of 324 people in 137 dwellings, with houses close to the foreshore and open stormwater drains. All homes have on-site sewage disposal systems (septic tanks). The comparison site at Lewis Bay, across the harbour, has only three residences close to the shore.

Sample Collection and Processing (All Three Experiments)

Shellfish

Bivalves were collected at low tide, placed in labelled plastic bags, and transported to the laboratory on ice, where they were processed within 6 hours of collection. Shellfish were opened using aseptic techniques, and the whole animal was extracted and placed in sterile stomacher bags. For each sample, sufficient individual shellfish were used to provide a sample weight of between 10 and 40 g. Generally, 15 A. stutchburyi from both sites, 15 M. liliana from Lewis Bay, and 7 M. liliana from Point Wells were used. Fewer M. liliana were required from Point Wells to achieve the desired sample weight, as they were larger than those from Lewis Bay. Bags containing samples were individually placed in a Seaward Stomacher laboratory blender (model 400) and macerated at normal speed for 2 minutes. Samples were diluted tenfold with Geldrech phosphate buffer (WHO 1982). A five-tube Most Probable Number (MPN) series of azide dextrose broth, as described by Donnison (1992), was set up, with double-strength medium used in the first row. Tubes were inoculated with 10 ml, 1 ml, and 0.1 ml of diluted sample, according to the MPN series, and then incubated in water baths at 35°C for 48 hours. Positive tubes (turbid) were plated onto membrane filters, which had been divided into six segments (one for each of the five tubes within a dilution and one control segment). Filters were placed onto Mac agar plates, which had been brought to room temperature. Plates were inverted, placed in a sealed plastic box and incubated for 48 hours at 41 ± 0.5°C. Filters were aseptically transferred to esculin iron agar (EIA) plates (which had been equilibrated at room
temperature), and incubated at 41 ± 0.5°C for approximately 20 minutes. Streaks of growth that were pink-to-red with a brownish-black precipitate underneath were scored as positive. MPN scores were determined from standard MPN tables and bacteria per 100 g of shellfish tissue calculated.

**Sediment**

Surficial sediment samples (approximately 50 g) were collected by scraping a sterile stainless steel scoop across the sediment at low tide, capturing the top centimeter of sediment. The scoop was washed and flamed in alcohol prior to the collection of each sample in order to ensure no cross-contamination. Sediment was placed in sterile plastic bags and transported on ice to the laboratory and processed within 6 hours of collection.

Sediment samples were prepared according to the enterococci MPN technique described by Donnison (1992). Approximately 10 g of each sample were weighed out and placed in a sterile plastic bag. A tenfold dilution was made using sterile phosphate buffered diluent (WHO 1982). Diluted samples were shaken by hand for 2 minutes to release bacteria from the sediment into suspension. Diluted samples were then used as the inoculum for a five-tube MPN series and processed as for shellfish.

**Water**

Water samples were collected in sterile glass bottles and transported to the laboratory on ice and away from sunlight. The mE/EIA membrane filter technique was used to enumerate enterococcus concentration (APHA 1992, Donnison 1992). Three replicates of 10 ml and three replicates of 50 ml from each water sample were filtered through a Sartorius 0.45-μm cellulose nitrate filter paper, which was aseptically transferred to mE agar plates (previously equilibrated at room temperature). The plates were inverted, placed in a sealed plastic box, and incubated for 48 hours at 41 ± 0.5°C. Filter papers from plates showing positive growth were transferred to EIA agar plates, and incubated for a further 30 minutes. Colonies that were pink-to-red with a brownish-black precipitate underneath were scored as enterococcus. The mean number of colonies per 100 ml of sample was calculated.

Rainfall data were derived from daily information collected at the University of Auckland, Leigh Marine Laboratory, approximately 15 km from sampling sites.

*Experimental Protocols*
Appendix I: Journal of Shellfish Research, in press.

Experiment A

The first experiment investigated the seasonal and year-to-year variability of enterococci levels in the body tissue of cockles and wedge shells. Three replicate composite samples were collected from each site, 50 m from the foreshore, every 2 months during 1996–1998, and analyzed according to the schedule above. Median MPN of enterococci per 100 g shellfish flesh were plotted against maximum daily rainfall over the preceding 4 days.

Experiment B

The long-term monitoring in experiment A revealed high variability in enterococci levels, and peaks of enterococci appeared to be related to high rainfall. This hypothesis was examined in more detail in this second experiment, which investigated the effect of specific rainfall events on the microbiological quality of shellfish tissue, sediment, and water. Samples were taken from late April to early May for a 14-day period in 1996 and a 12-day period in 1997. Each day at low tide a single sample each of sediment, *A. stutchburyi*, and *M. liliana* was taken from the sites at Point Wells and Lews Bay. Sampling sites were 50 m from the foreshore at both sites. A stormwater sample and a harbour water sample (from Big Omaha Wharf, see Fig. 1) were also collected daily. Processing and analyses were carried as detailed above. Median number of enterococci/100 ml water were plotted against daily rainfall and compared with MPN enterococci/100g of shellfish flesh and sediment. Median enterococci concentrations for harbour water, sediment, and both shellfish species were calculated using data from experiments A, B and C, and plotted with results of this experiment (B) to provide a comparison with typical levels.

Experiment C

Data from experiment B regarding the temporal patterns of bacteria in shellfish and sediment prompted investigation into the spatial patterns of bacterial levels in sediment and tissue of *A. stutchburyi* around a stormwater outfall at Point Wells. To delineate the effects a suite of experiments was carried out using three 80 m transects extending seaward from the stormwater drain outfall. The first transect was placed perpendicular to the foreshore. The other two transects were placed on either side of the first transect, at 45° and 135° to the foreshore (see Fig. 2). *Austrovenus stutchburyi* samples were taken in July and August 1998 every 10 m from 10 to 80 m along the central transect
and from 20 to 80 m along the two radials. In July 1998, sediment samples were taken at 0 m, 5 m, and then every 10 m out to 60 m on the central transect, and 2.5 m, 5 m, and every 10 m out to 60 m on the radial transects. MPN enterococci/100g shellfish flesh and sediment were plotted against distance from the stormwater drain.

RESULTS

Long-term temporal variability in enterococci levels in *A. stutchburyi* and *M. liliana* tissue plotted against maximum daily rainfall for the preceding 4 days is shown in Fig. 3 a,b. Bacterial levels of 20 MPN/100g were at or below the detection limit of the procedure, which is shown as a horizontal dotted line on each graph. Peaks in the levels of enterococci occurred over the winter months (June to October) (Fig. 3 a,b), and these winter peaks approximately corresponded with highest rainfall. Bacterial levels were also raised in February and April for *A. stutchburyi* at Point Wells. Only three results over the entire sampling period at this site for *A. stutchburyi* were below the detection limit of 20 MPN/100g. Bacterial levels were below detection in most months for *A. stutchburyi* at Lewis Bay, and for *M. liliana* at both sites (Fig. 3 a,b). During the sampling program, enterococci levels in *A. stutchburyi* were above the detection limit 77% of the time at Point Wells, compared with 24% at Lewis Bay. The detection limit was exceeded 41% of the time for *M. liliana* at both sites.

The effect of a rainfall event on stormwater and harbour water quality was specifically investigated in the first period of the second experiment. During intense monitoring in the autumn of 1996, rainfall of 40 mm on day 5 resulted in an approximate 100-fold increase in enterococci on day 5 for stormwater and on day 6 for harbour water (Fig. 4a). Enterococci levels then declined and returned to background levels (< 100/100 ml) by day 8. Enterococci levels in sediment showed a similar pattern (Fig. 4b), with increased concentration on day 6 (the day after the rainfall event) to approximately 1,000 MPN/100g, followed by a slower decline over the subsequent few days to background levels of less than 100 MPN/100g. Analyses of tissue from *A. stutchburyi* showed the same pattern (Fig. 4c), reflecting harbour water quality at 10,000 MPN/100g on day 6. Bacterial depuration by this bivalve was very rapid, with levels declining to approximately 100 MPN/100g by day 7. *Macomona liliana* were affected to a lesser extent, with a slight rise above 100 MPN/100g on days 6 and 7, remaining elevated until day 8 (Fig. 4d). These patterns suggest that enterococci levels
Appendix I: Journal of Shellfish Research, in press.

in *A. stutchburyi* closely reflect the bacterial levels in water, whereas those for *M. liliana* more closely mimic levels in sediment.

The second intensive monitoring period, in 1997, was during a period where rainfall did not exceed 4 mm on any day. Enterococci levels in stormwater and harbour water were not above 100 MPN/100 ml during the sampling period, with a small rise above background levels in harbour water due to factors other than rainfall (Fig. 5a). Levels in sediment did not rise above 100 MPN/100 g and were only marginally higher than median background levels at any time (Fig. 5b). Bacterial levels in *A. stutchburyi* were more variable, with most results ranging between 100 and 1,000 MPN/100 g (Fig. 5c), often above background levels. Again, enterococci levels remained relatively constant at or below 100 MPN/100 g for *M. liliana*, with small increases above background levels occurring (Fig. 5d).

The first in the suite of experiments investigating the spatial distribution of enterococci around a stormwater outfall at Point Wells was carried out after 4 days of very heavy rainfall (daily maximum 118.9 mm) in July 1998. There was little change in bacterial levels with increased distance from the origin (i.e. the stormwater drain) (Fig. 6 a–c). Along the central transect enterococci density declined from 300 MPN/100 g at 0 m to 70 MPN/100 g at 70 m (Fig. 6a). A similar pattern is evident along the two radial transects, with levels dropping from 230 to 270 MPN/100 g at 20 m to 20–90 MPN/100 g at 80 m respectively (Fig. 6b,c). This experiment was repeated during a moderate rainfall event in August 1998; the maximum daily rainfall for the preceding 4 days was 13.2 mm. The overall pattern was different on this occasion, with levels nearest to the origin not being the maximum for each transect (Fig. 7a–c). Results for the left-hand transect (Fig. 7b), which is directed away from the flow of the stormwater stream (see Fig. 2), showed a relatively clear pattern of lower bacterial levels with increased distance from the stormwater outfall. However, bacterial levels along the right-hand transect reached a maximum at 30 m (Fig. 7c), which is the point where the stormwater stream crosses this transect (Fig. 2). Levels are high for the full extent of this transect. The central transect's lowest bacterial levels are at the origin and highest levels at the 80 m mark, with high variability among points (Fig. 7a).

Enterococci levels in sediment declined from 9,000 MPN/100 g at 0 m to 270 MPN/100 g at 60 m along the central transect (Fig. 8a). The pattern is similar for the left-hand transect (Fig. 8b),
Appendix I: Journal of Shellfish Research, in press.

although the first sample at 2.5 m (1,100 MPN/100 g) is lower than that at 5 m (3,000 MPN/100 g). However, the density of enterococci declines to 340 MPN/100 g at 60 m. Figure 8c indicates a decline in bacterial levels with increased distance. Daily maximum rainfall for the preceding 4 days was 46.3 mm.

DISCUSSION

There is a seasonal nature to the patterns of enterococci levels in shellfish in the Whangateau Harbour. As both species at both sites exhibited peaks during the wetter winter months, it is likely that these increases in bacterial levels are due to the cumulative effects of many stormwater outfalls, nonpoint source runoff, and leaching from on-site sewage systems. Soils often become saturated during winter months due to high rainfall and flooded effluent leach fields associated with on-site wastewater treatment can cause the discharge of untreated wastewater into the marine environment (Gover 1993). Other researchers have found similar seasonal effects. For example, Paille et al. (1987) detected a peak in enterococci levels in oysters in Louisiana during late spring-early summer plus a peak in early winter. However, as no rainfall figures are given, it is difficult to ascertain the cause. LeMay et al. (1995) found that in the first few months of winter, when there was high rainfall, the levels of enterococci in marine water samples in California were correspondingly high. Levels dropped to a background level of around 100 Colony Forming Units (CFU)/100 ml in the later winter months.

A confounding factor in winter is decreased water temperature, as lower temperatures generally affect survival and detection of bacteria (Ayres et al. 1978). Inhibition due to sunlight can also decrease bacterial survivorship in summer (Borrego et al. 1983). Austrovenus stutchburyi at Point Wells exhibited high enterococci levels in winter and summer months, and it is likely that A. stutchburyi at this site have higher background levels overall. Macomona liliana at Point Wells did not exhibit high levels in summer, and this may be related to both their location 10–15 cm below the sediment surface, where they are more removed from waste discharge flows, and to different feeding methods. The summer enterococci peak detected in A. stutchburyi may be due to the influx of holiday visitors to this coastal settlement during the warmer months, which would place a strain on the ability of on-site sewage systems to adequately treat wastewater. This may lead to leach fields becoming
overloaded and surface or groundwater seepage into the open stormwater drains or directly into the harbour. It can be concluded that factors other than simply winter rainfall affect the microbiological quality of cockles at Point Wells. Further, in terms of human health risk, wet summers, where high rainfall and increased human population occur concurrently, may represent the "worst case" scenario.

The experiments which investigated the effect of rainfall events on water, sediment, and shellfish bacterial levels unequivocally indicated that rainfall is associated with increased levels of enterococci. This immediate effect on enterococci levels in stormwater with increased rainfall (Fig. 4a-d) is intuitive, as stormwater is defined as rainwater directed from impervious surfaces and stormwater is known to generally contain high levels of fecal bacteria. Indeed, Pitman (1995) found > 10,000 fecal bacteria per 100 ml in stormwater runoff samples taken in Goleta, California. The delayed effect on the harbour waters found during our study is likely to be due to dilution of the input from many stormwater drains and runoff from adjacent roads and farmlands, with the cumulative effect not being detected until the day following the rain event. However, LeMay et al. (1995) concluded that during wet weather levels of enterococci in marine waters increased dramatically and subsequently fell to background levels within 2–3 days. The one-day delay in accumulation of bacteria by A. stutchburyi, M. liliana, and sediment is also expected, as shellfish would take at least one tidal cycle to accumulate high levels of bacteria, and as sediment samples were taken at low tide, it is unlikely that bacteria from the stormwater outfall would have sorbed to surficial sediment sampled on day one. Similar increases in bacterial levels following heavy rainfall have been found in sediments (Goyal et al. 1977) and shellfish (Paillé et al. 1987, Pitman 1995).

Samples taken during a 12-day period of little or no rainfall give an indication of the background variability in enterococci levels for A. stutchburyi in the Whangateau Harbour (Fig. 5c). It is clear that A. stutchburyi shows greater variability in enterococci levels than M. liliana, and this could be due to its proximity to the sediment surface (and therefore the discharge flow) and filter-feeding mechanism.

The delineation experiments (Figs. 6–8) have complex interpretations. Transects sampled after very heavy rainfall (Fig. 6) give the anticipated pattern of decreased enterococci levels with increased distance from the stormwater outfall. However, the levels overall are relatively low, and this is likely
to be due to the flushing effect of high rainfall (LeMay et al. 1995). After a more moderate rainfall event (an order of magnitude lower than the peak levels experienced) the pattern is unclear. Enterococci levels along the central transect increase to a maximum at 70–80 m, and this may be due to pooling of water in this area, which can cause bacteria to be retained in water from which cockles feed. The left transect, extending away from the direction of the stormwater channel shows the more expected decline in bacterial levels with increased distance from source. Enterococci levels are high for the length of the right transect, and at the 30 m mark, due to the transect crossing the stormwater channel, an increase is detected. It is likely that levels remain high along this transect due to its proximity to the stormwater channel, which overflows onto the adjacent "flood-plains" and crosses the path of the transect.

Enterococci in sediment decline in an anticipated almost linear pattern with increased distance along the central and right transect (Fig. 8). Again the pattern along the left transect is different, with distant samples also having high levels. The cause is likely to be overflow from the stormwater channel at low tide, as the daily maximum rainfall for the 4 days prior to this particular sampling exercise was high (46 mm).

The identification of ecological impacts in harbours and estuaries is problematic due to natural temporal and spatial variability in populations (Snelder and Williamson 1997). A better approach may be to focus on stormwater treatment rather than identification of effects. In New Zealand, mitigation of effects of stormwater discharges is based on the "Best Practicable Option" as defined by the Resource Management Act 1991. Treatment generally involves either sedimentation in ponds or filtration, both of which still allow some contaminants to enter the receiving environment (Snelder and Williamson 1997). However, Treworgy and Garrett (1989) believe loss of coastal resources due to effects of waste discharges often must incur a financial hardship in order to justify restoration costs. A negative impact on the economy of the Ria Formosa area in Portugal was attributed to an increase in anthropogenic discharges and the associated decrease in bivalve production (Bebianmo 1995). However, it is generally very difficult to calculate the financial cost of contaminated marine environments, and it may be this factor that hinders remediation and mitigation processes.
We conclude that enterococci can be used to trace the spatial and temporal extent of stormwater discharges from small, urban coastal communities. This information, once combined with knowledge of the common toxic contaminants in such discharges, can be used to identify coastal areas suitable for shellfish restoration programmes, as well as detect and model potential effects on the marine environment and shellfish resources.

ACKNOWLEDGMENTS

Thanks goes to the following for funding assistance for this research: Northland Regional Council, Lotteries Grants Board, Todd Foundation, Resource Management Law Association of New Zealand, Grand Lodge of New Zealand, Freemasons, Soroptimist International, and TVNZ Group. In addition, we would like to thank Christopher B. Clarke and Megan Stewart for field and laboratory assistance.

LITERATURE CITED


Appendix I: Journal of Shellfish Research, in press.


Appendix I: *Journal of Shellfish Research, in press.*


Figure 1. Location of sampling sites within the Whangateau Harbour.
Figure 2. Position of temporal shellfish and sediment sampling sites and transect design for spatial sampling around stormwater outfall at Point Wells.
Figure 3. Temporal pattern of enterococci levels in body tissue of (a) *A. stutchburyi* and (b) *M. liliana* at Point Wells and Lews Bay.
Figure 4. Daily enterococci levels, over 14 days in April and May 1996, in (a) stormwater and harbour water, (b) sediment, (c) *A. stutchburyi* and (d) *M. liliana* tissue. Median (background) enterococci levels are shown as a dashed line.
Figure 5. Daily enterococci levels, over 12 days in April and May 1997, in (a) stormwater and harbour water, (b) sediment, (c) *A. stutchburyi* and (d) *M. liliana* tissue. Median (background) enterococci levels are shown as a dashed line.
Figure 6. Enterococci levels in *A. stutchburyi* tissue around stormwater outfall at Point Wells, following heavy rainfall (27/7/98).
Figure 7. Enterococci levels in *A. stutchburyi* tissue around stormwater outfall at Point Wells, following moderate rainfall (28/8/98).
Figure 8. Enterococci levels in surficial sediment around stormwater outfall at Point Wells (13/7/98).
Sediment Quality Guidelines


The **effects range low** (ERL) represents the threshold of possible adverse effects. The **effects range median** (ERM) represents the concentration above which adverse effects would probably occur frequently.


**Threshold effects level** (TEL) is the concentration below which adverse effects are expected to rarely occur. **Probable effects level** (PEL) is the concentration above which adverse effects are predicted to occur frequently.

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>ERL (ng/g DW)</th>
<th>ERM (ng/g DW)</th>
<th>TEL (ng/g DW)</th>
<th>PEL (ng/g DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total PAH(_i)</td>
<td>2,344.7</td>
<td>13,760.0</td>
<td>569.4</td>
<td>5,272.0</td>
</tr>
<tr>
<td>Zn (μg/g DW)</td>
<td>150.0</td>
<td>410.0</td>
<td>124.0</td>
<td>271.0</td>
</tr>
<tr>
<td>Cu (μg/g DW)</td>
<td>34.0</td>
<td>270.0</td>
<td>18.7</td>
<td>108.0</td>
</tr>
<tr>
<td>Pb (μg/g DW)</td>
<td>46.7</td>
<td>218.0</td>
<td>30.2</td>
<td>112.0</td>
</tr>
</tbody>
</table>

Adenylate energy charge and total adenylate nucleotide pool as biomarkers of sublethal stress in the cockle, *Austrovenus stutchburyi*.

Sharon De Luca-Abbott, Robert G. Creese, Gillian D. Lewis, and Rufus M.G. Wells

1Leigh Marine Laboratory, School of Environmental and Marine Science, University of Auckland, PO Box 349, Warkworth, New Zealand.

2School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand.
Running Head: Adenylate nucleotides and sublethal stress in cockles.

ABSTRACT

Measurements of adenylate energy charge (AEC) and total adenylate nucleotide pool (TANP) were identified as potential techniques to indicate sublethal stress in the estuarine cockle, Austrovenus stutchburyi. Cockles from a site with low-level urban contamination were compared with cockles from an uncontaminated site. There were no clear differences in AEC between sites and no obvious seasonal trends. However, TANP was significantly lower at the uncontaminated site and relatively higher during summer than in winter months at both sites. These results suggest that AEC may be regulated, but that TANP is reduced in response to the discharge of contaminants. The concept was further tested by transplanting cockles from an uncontaminated site to four sites varying in the level of contaminants in the substratum. Transplanted cockles showed consistently lower AEC after 2 weeks compared with autochthonous cockles, but by 8 weeks appeared energy compensated except for the mortality of transplants at a heavily contaminated site. TANP values however, were similar or slightly lower for transplanted cockles at 2 weeks, but by 8 weeks TANP had significantly decreased in cockles from contaminated sites. The results suggest a potential role for TANP as a biomarker of chronic environmental stress, and for AEC in revealing short-term perturbations in environmental quality.

Keywords: cockles, adenylate nucleotides, contaminants, New Zealand, transplant.
INTRODUCTION

Over the last 50 years, scientists have recognised that anthropogenic activities often negatively affect the well-being of marine organisms (Goldberg 1995). Many of these problems have arisen from increasing human settlement along the coasts and the use of estuaries and oceans for waste disposal (McIntyre 1995). In response, there has been increasing research into the adverse effects of discharged contaminants on aquatic organisms and the environment (Chapman 1995).

Many investigators have emphasised the need for biomarkers that may be used to evaluate organism health under conditions of contaminant-induced sublethal stress. All organisms use adenosine triphosphate (ATP) for energy transduction, and since this adenylate cannot be stored, any change in its concentration reflects an immediate change in the capacity for energy production (Newsholme and Crabtree 1986). Accordingly, there have been various attempts to devise an instructive index from the adenylate pool based upon the theoretical expectation that any environmental stressor can potentially disrupt energy flow. Adenylate energy charge (AEC) is a ratio of ATP to the total adenylate nucleotide pool (TANP) (Atkinson 1977) and has been promoted as a useful tool in the assessment of the physiological condition of organisms subjected to environmental insults (Ivanovici 1980a,b). This index gives values that range between 0 and 1 (Atkinson 1977). High AEC values (0.8-0.9) are thought to occur in organisms found in non-limiting environments, values between 0.5 and 0.7 indicate organisms suffering sub-optimal conditions, and animals with AEC lower than 0.5 are thought not to have the capacity to recover from the associated stress (Bayne et al. 1985).
Application of AEC both to controlled laboratory studies and to field populations of marine bivalves has proved equivocal. Cristini (1987) found AEC to be a sensitive indicator in field studies of the clam *Mya arenaria*, as did Picado and Le Gal (1990) for the cockle, *Cerastoderma edule* and Ivanovici (1980a) for *Trichomya hirsuta*. Sub-optimal environmental conditions were shown to result in reduced AEC under laboratory conditions for the mussel, *Mytilus provincialis* (Isani *et al.* 1997), and other intertidal bivalves (Carroll and Wells 1995, de Zwaan *et al.* 1995). Conversely, other researchers have concluded that AEC has limited use as a biomarker of environmental stress (Rainer *et al.* 1979, Savari *et al.* 1989, Shofer and Tjeerdema 1998). As pointed out by Haya and Waiwood (1983) and Ivanovici (1980b), contaminant monitoring studies are often confounded by environmental factors such as salinity, temperature, and oxygen content. Moreover, fluctuations in AEC reflecting seasonal changes in energy flow have been reported (Giesy and Dickson 1981, Moal *et al.* 1991).

Low and variable values of AEC may however, simply reflect sampling difficulties. Organisms that are handled or collected at low tide may have exhausted arginine phosphate reserves and have switched from oxidative phosphorylation to glycolysis as the oxygen-limiting, and less efficient ATP-generating mechanism. One way to overcome this difficulty is to monitor the total adenylate nucleotide pool (TANP) that includes the hydrolysed products of ATP. Wijsman (1976), with the mussel *Mytilus edulis*, and Ivanovici (1980c) with the gastropod *Pyrazus ebeninus*, noted greater stability in the TANP index compared to AEC. Seasonal changes in TANP were correlated with the reproductive status in *Corbicula fluminea* and *Anodonta imbecillis*, with high values detected over winter months (Giesy and Dickson 1981). Furthermore, reduction in TANP while AEC is maintained occurs in polychaetes.

(Verschraegen et al. 1985) and abalone (Shofer and Tjeerdema 1998) in response to pollutants.

Regulatory authorities and government agencies in New Zealand have recognised a need to establish sensitive biomarkers of estuarine contamination. The literature revealed adenylate nucleotide indices had potential as biomarkers of sublethal stress in bivalves, although flawed experimental design and lack of long-term monitoring data for the species being studied often limited their usefulness. This research aims to establish background values for AEC and TANP for an intertidal, estuarine bivalve and then, using manipulative experiments, determine whether these indices can be used as biomarkers of environmental contamination. The cockle, Austrovenus stutchburyi, was the species selected as it can be found close to the mean high water mark in many estuaries, and therefore, has the potential to be affected by contaminant discharges and runoff. In addition, A. stutchburyi is widespread on many soft shores of New Zealand, is relatively abundant and has cultural significance as a food item. The foot muscle was chosen as the tissue to be analysed as it has contact with potentially contaminated sediment, is simple to dissect, and is of an adequate size for the biochemical techniques proposed.

MATERIALS AND METHODS

Long-term monitoring experiment

Cockle foot tissue samples were collected from McLeod Bay and Munroe Bay in Northland, New Zealand (Figure 1). McLeod Bay has a small urban community with housing predominantly along the shoreline and a history of low-level sewage and stormwater contamination (Mortimer 1991). Munroe Bay is surrounded by rural land,
which is generally used for grazing of sheep and cattle. Between 9 and 25 cockles (15-25 mm shell width) from each site were collected at 3-monthly intervals from autumn 1996 through to spring 1998. Cockles were collected from similar heights on the shore at low tide. In addition to tissue samples, environmental variables were also measured. Water temperature, dissolved oxygen, salinity and pH were measured using hand-held meters, chlorophyll a levels determined using a spectrophotometric technique and condition quantified using a gravimetric method described by Crosby and Gale (1990).

Transplant experiment

In late summer 1999, cockles were transplanted from a relatively uncontaminated site at Lews Bay in the Whangateau Harbour (De Luca et al. 1999), to sites varying in levels of contamination in the Manukau Harbour (Figure 1). Data on concentration of contaminants (metals and PAHs) in sediments were already available (Williamson et al. 1999) for 3 sites (Onehunga, Takanini, and Big Muddy Creek) in the Manukau Harbour. Using an identical protocol sediment samples (approximately 200 g) were also collected from Awhitu and the source site at Lews Bay. These samples were sent to the same laboratory that had analysed the sediments for Williamson et al. (1999) (R.J. Hill Laboratories, Hamilton, New Zealand). This procedure allowed accurate comparison of contaminant levels among all 5 sites (Table 1).

A total of 500 cockles (15-25 mm shell width) were collected from Lews Bay and transported to the Leigh Marine Laboratory where they were stored in seawater aquaria. From each site in the Manukau Harbour, 100 cockles of the same size range were also collected, transported to the Leigh Marine Laboratory and stored in
seawater aquaria. Cockles were subsequently patted dry with paper towels, and treatments colour coded with a dot of enamel paint placed on the shell. In addition, a square of aluminium (5 mm x 5 mm x 1 mm) was glued to the shell with 2-part epoxy resin (Araldite), for later relocation using a metal detector (Stewart and Creese 1999). Cockles were held for less than 48 hours before replanting in the field.

At each site in the Manukau Harbour the 100 animals previously collected, plus 100 from Lews Bay were transplanted into separate 25 x 25 cm plots. Cockles were placed at similar heights on the shore at each site. As a control plot, 100 animals from Lews Bay were returned to the collection site. Water temperature, dissolved oxygen, salinity and chlorophyll a concentrations were measured at each site, excluding Takanini where logistical difficulties were encountered. Sediment samples were also taken, and frozen for later grain size analyses using a Gali WCIS100 particle analyser. Median sediment particle size for each site was determined.

Sample collection and extraction of nucleotides

*Austrovenus stutchburyi* were extracted from the sediment, opened and the foot muscle removed. The sample was wrapped in pre-labelled tinfoil and immediately frozen in liquid nitrogen. Time taken from removal of each animal from the habitat to emersion in liquid nitrogen did not exceed 15 seconds. Samples were transferred to a -75°C freezer and stored until nucleotides were extracted. In the laboratory, each sample was individually removed from the freezer, weighed, and chopped into small pieces using a scalpel. The tissue was then added to 1.5 ml of 0.6M perchloric acid, homogenised using a small laboratory blender, and extracted on ice for 30 minutes. The sample was then transferred to an eppendorf tube and centrifuged for 2 minutes at

11,000 rpm. The supernatant was poured off into a new eppendorf, leaving behind a plug of tissue. Samples were neutralised by mixing 200 µl of supernatant with a 400 µl 3:1 mixture of Freon-TF (1,1,2 trichlorotrifluoroethane) and tri-n-octylamine. The mixture was then vortexed for 1 minute to neutralise the extract and centrifuged at 11,000 rpm for 2 minutes for phase separation. The top aqueous layer, containing the acid-soluble extract, was then pipetted into a new eppendorf tube. The neutralised samples were stored in a -75°C freezer until processed on High Performance Liquid Chromatograph (HPLC).

A Waters HPLC was used for all analyses. Separations were achieved using a Alltima 5µm silica RP C18 lichrosorb column (4.6 mm internal diameter x 250 mm long) equilibrated at 30°C. The mobile phase of 0.4M potassium dihydrogen orthophosphate and 0.06M dipotassium hydrogen orthophosphate dissolved in purified distilled water was used at a flow rate of 1 ml/minute. Buffer solutions were prepared daily and were vacuum filtered through a 0.45 micron Millipore filter. Standard nucleotide preparations were injected at the beginning and end of each sample run and between at least every 10 samples, to ensure no significant changes in the characteristics of the column, or blockages in the column had occurred. 20µL of each sample was injected into the column and allowed to run for 15 minutes. The injection port was rinsed with distilled water between sample runs.

Peaks were identified by comparison with standard adenylate nucleotide solutions. Peak area was calculated using Maxima 820 software. Adenylate concentration calculations were made by comparison of the nucleotide area of the sample with that of standards, and adjustments made for sample dilution. Adenylate energy charge (AEC)
was calculated using the following formula: \( AEC = \frac{1}{2} \frac{ADP}{(ATP + ADP + AMP)} \) (Atkinson, 1968). TANP concentration was the sum of ATP, ADP and AMP.

Statistical analyses

Statistical comparisons of mean values among seasons and sites, for the long-term monitoring experiment, were made using a two-way analysis of variance, followed by Tukey's test for multiple comparisons (SAS statistical package, version 6.12). For the transplant experiment, one-way analyses of variance were used to compare mean AEC and TANP of transplant and autochthonous treatments within sites, and source and transplant treatments for all sites (Sigmastat statistical package, version 1.0).

RESULTS

Long-term monitoring experiment

Mean AEC values varied over the entire sampling period from as low as 0.2 to a maximum of approximately 0.8 (Figure 2). A two-way ANOVA revealed no significant difference in mean AEC between the two sites \( p>0.05 \). In addition, no clear seasonal pattern was evident, although high AEC values are associated with spring sampling (Figure 2). Mean TANP was found to be significantly higher at Munroe Bay (rural land use) compared to McLeod Bay (urban land use) using a two-way ANOVA \( p=0.0001 \). Again it is difficult to determine a clear seasonal pattern, but values were consistently lower in winter (Figure 3). However, a significant interaction exists between season and site, complicating the interpretation of the data. Variability was similar for each site and sampling period.
For each site, Spearman’s rank correlation tests were carried out between both adenylate indices and the 5 environmental variables measured in order to ascertain associations. The only significant association detected was a positive relationship between TANP and salinity for both sites (Table 2).

Transplant experiment

Most environmental variables (where data are available) were relatively similar among sites (Table 3). Salinity and water temperature were lower at Onehunga than at the other sites in the Manukau Harbour, as the water sample was taken some distance from the sampling site where a freshwater lagoon flows into the harbour. Water temperature was lower at Lews Bay compared to the Manukau Harbour sites as samples were taken earlier in the day. Sediment size was smallest at Takanini and largest at Awhitu.

A one-way ANOVA revealed that two weeks after transplantation AEC was similar for transplanted and autochthonous cockles at both Takanini and Awhitu (p>0.05). Transplanted cockles had significantly lower AEC at Big Muddy (p<0.05) and Onehunga (p<0.001) compared to respective autochthonous cockles (Figure 4). No significant difference in AEC was detected between cockles from the plot at Lews Bay and those transplanted to site in the Manukau Harbour. Although not statistically significantly, mean AEC for Big Muddy and Onehunga transplants were lower than those from Awhitu and Takanini (Figure 4).

After 8 weeks, mean AEC of transplanted cockles at Big Muddy Creek, Onehunga and Awhitu was not significantly different from that of autochthonous cockles (p>0.05). Mean AEC of transplanted cockles at these three sites increased between the 2 and 8 week sampling occasions (Figure 4), although this increase was only statistically significant at Big Muddy Creek (p=0.001). Again, a one-way ANOVA detected no significant difference in AEC between Lews Bay cockles and transplants (p>0.05). Cockles transplanted to Takanini suffered 100% mortality at some time between the 2 week and 8 week sampling, and therefore no AEC or TANP data are available for this plot.

Two weeks after cockles were transplanted, TANP was significantly higher for autochthonous cockles from Awhitu (p=0.001), Big Muddy Creek (p=0.01) and Takanini (p=0.007) compared to respective transplanted cockles, but TANP was not significantly different for plots at Onehunga (p>0.05) (Figure 5). In addition, TANP of transplanted cockles was not significantly different to cockles from the Lews Bay source site. After 8 weeks, TANP of transplanted cockles at Awhitu had increased significantly (p=0.01) from 2.3 to 3.3 mM/g. Mean TANP of transplanted cockles from Awhitu had increased between the 2 and 8 week sampling occasions and at 8 weeks was not significantly different from the control cockles at Lews Bay (Figure 5). Transplanted cockles at Big Muddy Creek and Onehunga had significantly lower TANP than control cockles at Lews Bay (p<0.05). TANP at these two sites decreased between the 2 and 8 week sampling periods, although these differences were not statistically significantly.
DISCUSSION

Seasonal cycles in AEC and TANP have previously been reported in bivalve tissue. Moal et al. (1991) detected low AEC values in summer and high values in winter for two species of oyster. In addition, Giesy and Dickson (1981) found TANP to be high in winter, but samples in their study were only taken over 1 year and it is not clear that this trend would be similar every year. However, in our study, no consistent seasonal trends in either AEC or TANP were evident over the 3 years. Variability within sample groups was not greatly different among seasons, indicating no single optimal season for using these techniques.

TANP being consistently higher at Munroe Bay than at McLeod Bay suggests an effect from stormwater and septic tank contaminants on the energetics of cockles. These types of contaminants are periodically at high levels at McLeod Bay (Mortimer 1991). As environmental factors were found to be similar between sites, these factors are unlikely to be the cause of these differences detected in adenylate nucleotides between sites.

Changes in AEC can occur over small temporal scales e.g. over one tidal cycle (De Luca unpublished data), and it is concluded that seasonal sampling of AEC may not provide useful environmental monitoring information. As many variables that affect the energetics of cockles also change on small temporal scales (such as salinity, temperature, tidal height, and contaminant discharges), it is thought that seasonal sampling may not accurately represent variations in adenylate nucleotides. For example, a day of heavy rain that lowered salinity at low tide could (based on
laboratory experiments by Rainer et al. (1979) and Ivanovici (1980c) cause a short-term depression of AEC in intertidal molluscs. Short-term variations in AEC such as this could readily mask any underlying longer-term patterns (e.g. seasonal variation). Future studies may overcome these problems by sampling shellfish tissue intensively each season, and interpretation using detailed environmental data may better elucidate natural temporal changes in adenylate nucleotides.

Transplant experiments often show effects where monitoring surveys may not, as organisms appear to regulate adenylate nucleotides in limiting environments (Cristini 1987). In our experiment, AEC was found to be lower in transplanted cockles compared to indigenous cockles at sites with the highest PAH levels in sediment. Other literature confirms that hydrocarbons can affect adenylate energetics of bivalves (Ivanovici 1980b, Haya and Waiwood 1983). AEC levels of transplanted cockles at Onehunga was significantly lower than for autochthonous cockles, and it is this site, which has both the highest PAH concentration and the highest heavy metal concentrations in sediment. PAHs (not heavy metals) may be causing this effect, as at Big Muddy Creek (where the second highest PAH levels were found) AEC is also depressed, but heavy metal concentrations at this site are similar to those at Takanini (Table 1) where no significant difference in AEC between transplanted and autochthonous cockles was detected.

As there was no initial significant difference in AEC between cockles from the source site at Lewis Bay, and those transplanted to various sites in the Manukau Harbour, AEC of transplants may not have varied during the initial 2 weeks. However, as discussed above, this explanation is unlikely because AEC can vary on time scales less
than 14 days (see Carroll and Wells 1995, de Zwaan et al. 1995). After 8 weeks, AEC levels in cockles transplanted to the Manukau Harbour and those at Lews Bay remain similar. However, AEC of cockles transplanted to Onehunga and Big Muddy Creek increased (from 0.55 to 0.63 and 0.55 to 0.72 respectively) and became more similar to AEC levels of autochthonous cockles. These cockles appear to be regulating adenylate nucleotide concentration. Further, cockles in contaminated habitats may have had to initially breakdown more ATP in order to release energy for critical functions, causing a decrease in AEC, whereas after 8 weeks AEC is maintained at higher levels by a decrease in ADP and AMP, reflected in lower TANP (as discussed below). The complete mortality of transplants at Takanini suggests a catastrophic event. It may be that transplanted cockles were not able to withstand the very fine sediment and became smothered, as empty valves, still with aluminium and paint tags, were found approximately 30 cm below the sediment surface.

TANP is thought to be insensitive to environmental modifications (Ivanovici 1980a), and this is the likely hypothesis for TANP being similar among Lews Bay cockles and those transplanted to the Manukau Harbour. After 2 weeks, TANP of autochthonous cockles were consistently higher than those of transplanted cockles, suggesting TANP alters over longer time scales. However, a different pattern begins to emerge after 8 weeks, with TANP of transplanted cockles at the uncontaminated site (Awhitu), increasing from 2.3 to 3.3 mM/g. TANP of cockles transplanted to Big Muddy Creek and Onehunga was significantly lower compared to autochthonous cockles, with a slight lowering of TANP in transplanted cockles. No conclusions can be drawn regarding Takanini due to total mortality of transplanted cockles at this site. No significant difference in TANP existed among Lews Bay control cockles and
transplanted cockles at Big Muddy and Onehunga. However, TANP of cockles transplanted to Awhitu was significantly higher. It may be that the contaminants at Big Muddy Creek and Onehunga were restricting the ability of cockles to accumulate adenylate nucleotides, whereas the lack of contaminants at Awhitu leaves animals able to take advantage of non-limiting environmental conditions.

Other transplant experiments with bivalves have been undertaken to test differences in AEC and TANP concentrations in response to pollution (Ivanovici 1980a, Cristini 1987, Picado et al. 1988, Picado and Le Gal 1990). Cristini (1987) transplanted Mya arenaria from clean to polluted sites and analysed a large number of environmental variables, biochemical indices and adenylate nucleotides in order to test energetic adaptation. Significantly lower AEC was detected in transplanted animals at the polluted site, but as indigenous animals were not treated in the same way as transplants, no clear conclusions can be drawn from this work. However, we believe it is this type of design (with appropriate controls in place) that has potential for detecting altered energetics in bivalves in response to pollution.

CONCLUSIONS

Researchers have stated that AEC may be used as a general indicator of stress, and have compared levels from studies with other species, using them to interpret their findings as normal, or outside the normal range of AEC values. We believe this interpretation is flawed, as AEC and TANP are likely to not only be species-specific, but also vary between hydrodynamic systems and with natural environmental changes. Indeed, the commonly used generalised interpretation of values (Bayne et al. 1985) may be misleading. Therefore, when designing a monitoring or transplant programme,
baseline information on AEC and TANP for the species and habitat to be used must first be established in order to ascertain "normal" and "stressed" AEC ranges.

Adenylate nucleotide indices have strong potential as biomarkers of contamination in bivalves. However, because bivalves regulate the concentrations of adenylate nucleotides depending on environmental conditions, transplant experiments must be used. Normal variability must be established for each species and each habitat investigated. In addition, detailed environmental data should be collected simultaneously, to assist with the interpretation of findings. When these requirements are undertaken and rigorous experimental designs used, adenylate nucleotide indices can be a powerful monitoring tool.

ACKNOWLEDGEMENTS

Field assistance from M. Stewart, C. De Luca, M. Heathcote, and J. Abbott is gratefully acknowledged. Financial assistance was provided by Lotteries Grants Board, Northland Regional Council, University of Auckland, Resource Management Law Association of New Zealand, Todd Foundation, Television New Zealand, Grand Lodge of New Zealand Freemasons, and Soroptimist International.

REFERENCES


---


### Table 1: Sediment contaminant concentrations.

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>Lews Bay</th>
<th>Awhitu</th>
<th>Big Muddy</th>
<th>Takanini</th>
<th>Onehunga</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PAH</strong></td>
<td>129.4</td>
<td>1.4</td>
<td>258.11</td>
<td>146.82</td>
<td>472.13</td>
</tr>
<tr>
<td>(total ng/g DW)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Zn</strong></td>
<td>12.2</td>
<td>4.6</td>
<td>61.5</td>
<td>64</td>
<td>180.5</td>
</tr>
<tr>
<td>(total µg/g DW)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cu</strong></td>
<td>1.6</td>
<td>0.3</td>
<td>10.1</td>
<td>13.8</td>
<td>43.8</td>
</tr>
<tr>
<td>(total µg/g DW)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pb</strong></td>
<td>1.1</td>
<td>0.5</td>
<td>9.6</td>
<td>6.5</td>
<td>47.1</td>
</tr>
<tr>
<td>(total µg/g DW)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2: Spearman’s correlation co-efficients ($r_2$) and p-values (p) for environmental variables and adenylate nucleotide indices (AEC and TANP) for two sites in the Whangarei Harbour.

<table>
<thead>
<tr>
<th>FACTOR</th>
<th>Munroe</th>
<th>McLeod</th>
<th>Munroe</th>
<th>McLeod</th>
<th>Munroe</th>
<th>McLeod</th>
<th>Munroe</th>
<th>McLeod</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r_2$</td>
<td>$p$</td>
<td>$r_2$</td>
<td>$p$</td>
<td>$r_2$</td>
<td>$p$</td>
<td>$r_2$</td>
<td>$p$</td>
</tr>
<tr>
<td>Temperature</td>
<td>0.0</td>
<td>1.0</td>
<td>-0.1</td>
<td>1.0</td>
<td>0.7</td>
<td>0.2</td>
<td>0.9</td>
<td>0.1</td>
</tr>
<tr>
<td>Dissolved Oxygen</td>
<td>0.3</td>
<td>0.8</td>
<td>-1.0</td>
<td>0.3</td>
<td>0.6</td>
<td>0.3</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>pH</td>
<td>-0.8</td>
<td>0.1</td>
<td>0.4</td>
<td>0.4</td>
<td>-0.1</td>
<td>0.8</td>
<td>-0.1</td>
<td>0.9</td>
</tr>
<tr>
<td>Salinity</td>
<td>0.1</td>
<td>0.9</td>
<td>0.2</td>
<td>0.7</td>
<td>0.9</td>
<td>0.0</td>
<td>0.9</td>
<td>0.0</td>
</tr>
<tr>
<td>Chlorophyll a</td>
<td>-0.1</td>
<td>0.8</td>
<td>-0.1</td>
<td>1.0</td>
<td>-0.6</td>
<td>0.2</td>
<td>-0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>Condition</td>
<td>0.2</td>
<td>0.7</td>
<td>0.1</td>
<td>0.8</td>
<td>-0.3</td>
<td>0.7</td>
<td>0.7</td>
<td>0.1</td>
</tr>
</tbody>
</table>
Table 3: Environmental factors for seawater and sediment characteristics at cockle donor site (Lews Bay) in the Whangateau Harbour and transplant sites in the Manukau Harbour.

<table>
<thead>
<tr>
<th>Site</th>
<th>Temperature (°C)</th>
<th>Dissolved Oxygen (mg/L)</th>
<th>Salinity (ppt)</th>
<th>Chlorophyll a (µg/L)</th>
<th>Median Sediment Grain Size (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lews Bay</td>
<td>16.2</td>
<td>8.8</td>
<td>34.6</td>
<td>1.33</td>
<td>182.1</td>
</tr>
<tr>
<td>Awhitu</td>
<td>22.3</td>
<td>11.6</td>
<td>34.5</td>
<td>n/a</td>
<td>236.2</td>
</tr>
<tr>
<td>Big Muddy</td>
<td>22.2</td>
<td>9.3</td>
<td>33.8</td>
<td>1.38</td>
<td>164.6</td>
</tr>
<tr>
<td>Takanini</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>89.2</td>
</tr>
<tr>
<td>Onehunga</td>
<td>18.8</td>
<td>7.1</td>
<td>19.6</td>
<td>n/a</td>
<td>115.6</td>
</tr>
</tbody>
</table>
Figure 1: Location of sampling sites. The seasonal sampling exercise was carried out in Whangarei Harbour, and the transplant experiments were undertaken in the Whangateau and Manukau Harbours.

Figure 2: Mean adenylate energy charge (AEC) (+/- s.e.) in cockle sampled each season at Munroe and McLeod Bay in the Whangarei Harbour. McLeod Bay is the putative contaminated site.

Figure 3: Mean total adenylate nucleotide pool (TANP) (mM/g wet weight (WW) +/- s.e.) in cockle sampled each season at Munroe and McLeod Bay in the Whangarei Harbour. McLeod Bay is the putative contaminated site.

Figure 4: Mean adenylate energy charge (AEC) (+/- s.e.) in cockles from Lews Bay, those transplanted to plus autochthonous cockles from sites in the Manukau Harbour. Awhitu is the least contaminated transplant site, and Onehunga the most contaminated.

Figure 5: Mean total adenylate nucleotide pool (TANP) (mM/g wet weight (WW) +/- s.e.) in cockles from Lews Bay, those transplanted to plus autochthonous cockles from sites in the Manukau Harbour. Awhitu is the least contaminated transplant site, and Onehunga the most contaminated.
The graph shows the total adenylate nucleotides (mM/g WW +/- s.e.) at different sites over 2 and 8 weeks. The sites include Lews Bay, Awhitu, Awhitu, Big Muddy, Big Muddy, Takanini, Takanini, Onehunga, and Onehunga.

- **2 Weeks**
  - Lews Bay (n=9)
  - Transplants (n=8)
  - Controls (n=7)

- **8 Weeks**
  - Lews Bay (n=9)
  - Transplants (n=5)
  - Controls (n=6)

The graph indicates that there are significant differences in adenylate nucleotides between different sites and time periods.