Final Report

Aquafin CRC

Development of novel methodologies for cost effective assessment of the environmental impact of aquaculture

Maylene G. K. Loo, Kathy Ophel-Keller and Anthony Cheshire (Editors)

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Non Technical Summary

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Objectives

The aim of this project was to test the application of recent advances in DNA assay systems using polymerase chain reaction (PCR) to develop a system for the routine evaluation of marine sediments and macrobenthic infauna for use in environmental impact assessment and monitoring. Key aspects addressed included the development of DNA assays for selected marine organisms in sediments, and the successful application of a SARDI/CSIRO platform technology for DNA extraction from marine sediments.

The project was developed in two phases, an initial “proof of concept” followed by a second “proof of application”.

The specific objectives of the proof of concept phase were:

1. To identify a range of benthic infaunal species that are characteristic of sites ranging from heavily impacted (organically polluted) tuna sea-cages through to non-impacted (pristine) environments.

2. To develop a system for the rapid detection of selected taxa in sediment samples using PCR techniques.

3. To evaluate the extent to which rapid detection systems can be routinely applied to provide quantitative estimates of the relative abundance of indicator taxa or processes in sediments (and therefore of the health of seabed systems).

4. To assess the generality of the technique to other forms of aquaculture (particularly salmon).

Once these had been achieved, additional objectives were added in the "proof of application" phase, specifically:

5. To improve the sensitivity of the assays to levels comparable with those previously developed for terrestrial systems.

6. To develop PCR assays for a total of nine infaunal taxonomic groups[1].

7. To quantitatively calibrate all nine assays[2].

8. To demonstrate the "proof of application" of this methodology in comparison with traditional, manual enumeration methods.

9. To further clarify the phylogenetic relationship in Spionidae and evaluate the need for multiple Spionidae assays.

All of these objectives have been achieved and are reported, in detail, in the body of this report.

[1] In the application to the Aquafin CRC/FRDC this was written as "3 additional assays" given that 2 assays were originally developed through the proof of concept phase. Subsequent work under objective 9 identified the need to have four primers and two probes for the group of infauna known as Spionidae. Further to this, the assay for the group Capitellidae was improved with the development of two primers instead of one and the rewording in this paper "nine assays" reflects these changes of scope.

[2] In the application to the Aquafin CRC/FRDC this was written as "calibrate all five assays" as detailed above the number of assays developed was subsequently increased to nine.

Outcomes Achieved to Date

A complete system has been developed to rapidly assess the "environmental health" of the seabed in the vicinity of southern bluefin tuna sea-cages.

The assay system is based on extraction of DNA from sediments, followed by quantification of key indicator taxa. To provide simple interpretation of results, an Environmental Compliance Scorecard (ECS) system was developed.

The full system comprising the DNA assays and the ECS will improve the monitoring of the environmental performance of sea-cage aquaculture operations and provide better understanding of aquaculture and the environment. With better understanding, productivity of sea-cage aquaculture can be optimised.

The full system from sample collection through to data analysis and interpretation has been implemented for trial by regulatory authorities in South Australia for routine environmental compliance monitoring for southern bluefin tuna aquaculture. This is the first use of DNA-based technology for routine marine environmental monitoring in the world.

The system was also used for environmental assessment in another Aquafin CRC project, which focussed on assessing the feasibility of holding southern bluefin tuna for a longer time period, that is, over two seasons rather than one. Furthermore, a proposal is currently before PIRSA Aquaculture and FRDC to extend this technology to other South Australian finfish aquaculture sectors (specifically yellowtail kingfish and mulloway) to meet Environmental Monitoring Programme (EMP) requirements.

Studies carried out in Australia and overseas have shown that deposition of aquaculture waste from marine finfish farming can result in organic enrichment of the seabed. Traditional approaches to the assessment of environmental impacts of aquaculture often require detailed assessment of sediment organisms (benthic infauna), which is the mainstay of many environmental monitoring programmes for aquaculture over soft sediments. This assessment is both expensive and time consuming, and so severely limits the extent to which the conventional methods can be used for routine environmental monitoring. Therefore, there is a need to develop a system for rapid and cost-effective environmental monitoring of sea-cage aquaculture.

The outcome of the project was a system for the routine evaluation of marine sediments for environmental impact assessment and monitoring using DNA-based assays. A new

approach for reporting environmental assessments, through an Environmental Compliance Scorecard (ECS) system, was also developed. The complete system has the potential to save time (months) in preparing the environmental monitoring assessment reports required by regulators.

The DNA assays identify the quantity of nine groups of small marine organisms that are important indicators of organic enrichment in sediments, by determining the amount of DNA present for each group of organisms. The results obtained from sites near farms (compliance sites) are then compared to results of sites distant from farms.

The method was adapted from technology used by grain growers to assess the risk of soil borne diseases. This technology was developed by the South Australian Research and Development Institute (SARDI) and CSIRO. Transferring this technology to the marine environment required modifications to methods to work with marine sediments. An extensive programme was undertaken to sequence the DNA of the animals in marine sediments and design DNA probes with the required sensitivity and specificity.

The Environmental Compliance Scorecard (ECS) system developed is a method that can be used to summarise the results of environmental monitoring programmes based on the DNA assays in an easily readable format. The ECS system provides an analysis of the DNA data by evaluating a series of pre-determined indices of environmental health. These results are integrated to produce an overall compliance score that is ranked on a scale of 0 (very poor) to 100 (perfect result), which is then displayed as one of five categories: green indicating all is well; yellow/orange, indicating the situation needs watching and red/violet, indicating there is an issue that needs addressing. These categories, roughly matching the colours on a traffic light, provide a very simple message to industry and government environmental managers.

The DNA assay system together with the ECS system can be expanded and applied to other finfish aquaculture and to the assessment of other forms of organic enrichment such as sewage outfalls or meat/fish processors.

Keywords: Environmental Assessment and Monitoring, Southern Bluefin Tuna, Environmental Compliance Scorecard, DNA Assays, Environmental Management

Acknowledgements

The authors would like to thank the funding agencies, the Co-operative Research Centre for the Sustainable Aquaculture of Finfish (Aquafin CRC), the Fisheries Research and Development Corporation (FRDC) and SARDI all of whom provided support for this research. We would also like to acknowledge the support of the members of the Tuna Boat Owners Association of South Australia Inc. In particular, we would like to thank Brian Jeffriess and David Ellis.

We also thank Steven Clarke (FRDC Aquaculture Subprogram Leader) whose unflagging support and hard work made a substantial difference in keeping the project on-track and moving forward. Thank you to Jason Tanner and John Volkman for the substantial feedback.

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This project would not have been possible without the help of all the field and laboratory support we received. A big thank you to everyone. The crew of MRV Ngerin, Neil Chigwidden, Dave Kerr, Chris Small and Ralph Putz for their patience and willingness to do the long hours. Milena Fernandes and Peter Lauer for the many joint field trips that became trying and extended. Russell Burns, Sue Wiebkin and the staff in the Diagnostics group at the Plant Research Centre who were introduced to the wonderful world of marine sediments in this project, and John Curran from CSIRO Entomology who was willing to take a risk and become involved in this project. Ali Bloomfield, Katherine Cheshire, Yvette Eglinton, Michael Gueridian, Magdalena Halt, Matthew Hoare, Ishebel Kerkez, Ian Magraith, Keith Rowling, David Miller, Bruce Miller-Smith, Mandee Theil, Sonja Venema, Rachel Wear and Phillipa Wilson for all their field and laboratory help. Jeff Buchanan, Richard Morrison, Brenton Ebert and Jeremy Barnett for their field support in Port Lincoln and all other staff who helped in one way or another at Lincoln Marine Science Centre and SARDI Aquatic Sciences Centre at West Beach. Thank you also to all those who were unknowingly “pulled” into the laboratory to sort samples and to look for those elusive worms.

Chapter 1 Background

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This chapter will give the background of the project, the need for the research and the specific objectives developed for this project.

Aquaculture of southern bluefin tuna

Aquaculture of southern bluefin tuna (SBT) or *Thunnus maccoyii* has expanded greatly since its beginnings in 1990 to be worth $266.9 million in 2002/03 (Knight *et al.* 2004) and $151 million in 2003/04 (Knight *et al.* 2005) making SBT the largest finfish aquaculture industry in Australia. The success of this industry can be attributed, in part, to a significant economic multiplier from flow-on to other sectors such as manufacturing, trade, business and property services and finance, adding another $218 million in business turnover (EconSearch 2004).

The continued development of the southern bluefin tuna aquaculture industry is dependant upon its capacity to demonstrate the environmental sustainability of the industry, and to support the ongoing production and marketing of products in an increasingly competitive international market. Having a rapid environmental assay that is sensitive to change will provide industry with a management tool that can be used to assess the benefits of alternative management measures designed to reduce environmental interactions. Such information will assist in optimising productivity and reducing operating costs within an ecologically sustainable framework. Achieving these outcomes requires an ability to undertake comprehensive and rapid assessments of the environmental effects of sea-cage farming operations.

Effects of marine aquaculture on the environment

The potential impacts of aquaculture are wide-ranging and include impacts that are both onshore and offshore. For aquaculture in general, the severity of any impacts is scaled depending on a suite of parameters including the species being farmed, the culture methods, feed type, and the size and intensity of the farming operation (Pearson and Black 2001). The characteristics of the receiving environment, in terms of both its physico-chemistry and its ecology will also determine the degree of impact of any given

aquaculture operation. In all cases such impacts can range from aesthetic aspects where operations impact on visual amenity through to direct pollution problems giving rise to eutrophication or organic enrichment of coastal waters and the benthos.

Finfish aquaculture systems typically generate particulate organic wastes (faecal material and uneaten food), soluble organic and inorganic excretory wastes and other by-products (e.g. medication and pesticides) that can have impacts on the environment (Fernandes *et al.* 2001, Carroll *et al.* 2003, Cheshire in press). Of particular concern is the potential for organic inputs to enrich aquatic ecosystems, and through sedimentation of particulate materials to produce a series of changes in the chemical and physical parameters of the sediment, together with effects on the faunal communities in/on the seabed (e.g. Gowen and Bradbury 1987, Weston 1990, Wu 1995, Karakassis *et al.* 1999, Cheshire *et al.* 1996b, Cheshire in press).

Sediments are diverse environments where a whole range of flora and fauna exist in a complex highly interactive matrix. Parameters characterising this matrix include particle size, food (carbon) availability, oxygen concentration and redox potential (Pearson and Black 2001). The biogeochemical processes in marine sediments such as deposition, erosion and oxygen supply, are dominated by hydrography and the net input of carbon into the system. Therefore, any organic enrichment from aquaculture will affect the marine benthic habitats and its processes. The gross effects of wastes from intensive sea-cage aquaculture have been widely studied in northern European and other cool-temperate regions (Brown *et al.* 1987, Weston 1990, Holmer and Kristensen 1992, Hargrave *et al.* 1993, Findlay and Watling 1995, Holmer *et al.* 2001, Wildish *et al.* 2001). Effects include the creation of a chemically reducing environment; hypoxia in the overlying water; increased sulphate reduction and changes in macrobenthic faunal and meiofaunal assemblages. The extent of environmental impact on the seabed is dependent on the local assimilative capacity and the amount of organic waste generated from the aquaculture activities (GESAMP 1996). The nature and extent of impacts associated with southern bluefin tuna farming have been documented in two companion Aquafin CRC projects, the Waste Composition and Mitigation (WMP) project[3] and the Regional Environmental Sustainability (RESA) project[4]. The reader is referred to the final reports of these projects for further details.

[3] Aquafin CRC/FRDC Project 2001/103: Aquafin CRC - Southern Bluefin Tuna Aquaculture Subprogram: Tuna environment subproject - Evaluation of waste composition and waste mitigation strategies.

[4] Aquafin CRC/FRDC Project 2001/104: Aquafin CRC - Southern Bluefin Tuna Aquaculture Subprogram: Tuna environment subproject - Development of regional environmental sustainability assessments for tuna sea-cage aquaculture.

Use of macrobenthic infauna in environmental impact assessment and monitoring of marine aquaculture

Monitoring programmes based on the assessment of chemical water quality parameters have been the most commonly used technique to investigate anthropogenic disturbance and stress in marine environments (Maher and Norris 1990, Connell 1993, Norris and Norris 1995). However, chemical measurements of the water column have limitations in that they give no indication of biological effects or ecosystem responses (Lack and Johnson 1985). As a consequence, methods for measuring effects on both ecosystem structure and function have been extensively developed (Holmer *et al.* 2001, Pearson and Black 2001). These methods encompass comparisons of geophysical, geochemical and biological variables in sediments at impact and reference sites (Hargrave *et al.* 1997, Pearson and Black 2001).

Of these, comparisons of biological variables are most frequently used, in particular, the measurement of benthic communities in environmental assessments. Such assessments of biological variables are preferred because they evaluate biological consequences, integrating the effects of pollutants over time (Maher and Norris 1990, Connell 1993, Warwick and Clarke 1993). Macrobenthic infauna are the most widely known and accepted biological indicators of environmental degradation and restoration in marine sediments (Clarke and Green 1988, Austen *et al.* 1989, Warwick *et al.* 1990, Weston 1990, Warwick and Clarke 1991, Agard *et al.* 1993, Ferraro *et al.* 1994). Macrobenthic infaunal assemblages have been chosen because they encompass a diverse range of species covering a multiplicity of sizes, reproductive strategies, feeding behaviours and life histories. Collectively these communities change in response to a series of parameters including water quality, physico-chemical status of the benthos, and nutrient and organic carbon loading (Bilyard 1987).

Assessing changes in benthic infaunal assemblages using the traditional approach of sorting, enumerating and identifying benthic infauna in sediments is both expensive and time consuming. This is made more so because of the need for specialist technical expertise in the identification of the animals, making this method difficult to apply in a routine manner. There is a need, therefore, to develop new techniques that are less costly both in terms of the turn around time and the labour associated with sample processing.

A number of studies have looked at ways to cut the cost and improve the turn around time of benthic infaunal surveys. These studies have examined processing fewer replicates (Gray *et al.* 1992), using coarser mesh sieves to reduce the number of taxa in samples (Maurer and Nguyen 1996), analysing at lower taxonomic resolutions (Clarke and Warwick 2001a) and using the concept of indicator species (Rygg 1985, Roberts *et al.* 1998). Rygg (1985) identified positive and negative indicator species where positive indicators were pollution tolerant and dominated the macrofauna of low-diversity samples.

Negative pollution indicators were non-tolerant species whose presence indicated little or no impact but which when absent, implied high impact. Indicator taxa may also be selected for their importance in the community (Clements *et al.* 1992), sensitivity to change (Gray and Pearson 1982, Bellan *et al.* 1988) or labour efficiency (Roberts *et al.* 1998).

In Australia, a range of techniques was assessed in relation to suitability and ease of use for industry-based management of sediment conditions for Atlantic salmon (*Salmo salar*) aquaculture in Tasmania (Macleod *et al.* 2004). This completed Aquafin CRC project "Development of novel methods for the assessment of sediment condition and determination of management protocols for sustainable finfish cage aquaculture operations" evaluated sediment recovery based on key physical, chemical and biological criteria. Benthic infaunal evaluation was used as the benchmark against which all other evaluations of recovery were compared. The results showed that some established environmental monitoring techniques were poor indicators of sediment recovery or were too complicated for farm-based monitoring. However, other techniques such as video assessment and benthic photos were simple and effective for industry use (Macleod *et al.* 2004).

Studies assessing the effects of southern bluefin tuna aquaculture in Port Lincoln

In South Australia, the potential use of the recommended techniques from the Atlantic salmon project (Macleod *et al.* 2004) for the environmental assessment and monitoring of the southern bluefin tuna industry was considered. The nine stages defined in the salmon project could not be applied as not all the benthic infauna indicators identified as characteristics of the various stages were found in South Australia. In addition, as indicated by Macleod *et al.* (2004), infaunal community structure can be site specific, depending on characteristics of the site such as substrate type. Consequently, it was quickly recognised that the environment in which the southern bluefin tuna industry operates in South Australia is different from the environment in which the salmon industry operates in Tasmania.

An earlier study (FRDC 1995/091) on the environmental impact of SBT sea-cages in waters off Port Lincoln focussed on impacts on the benthic flora and fauna of Boston Bay (Cheshire *et al.* 1996a, b). This study demonstrated a severe impact within the immediate vicinity of the southern bluefin tuna sea-cages extending to a 20 m radius around each cage with a lesser impact for a further 100 to 150 m from the cages. At a distance of 200 m there was no evidence of an impact relative to control sites situated 1 km away. The spatial scale of the impact was comparable to those described for many salmonid farm sites

and was generally consistent with those described in the Port Lincoln Aquaculture Management Plan (Bond 1993).

Although the work by Cheshire *et al.* (1996a, b) provided some preliminary insights, there was a clear need for more comprehensive research. Importantly, the study identified the need to go beyond characterising and quantifying the spatial extent of impacts from southern bluefin tuna sea-cages and argued for the need to test alternative management systems and technologies in order to reduce the impacts on benthic (and thereby associated) ecosystems. Furthermore, with the relocation of farming operations to outside Boston Bay, much of the previous information, from research carried out inside Boston Bay, was of limited relevance to ongoing farming operations.

To address this change of location and the increases in the size of the industry, an industry wide Tuna Environmental Monitoring Programme (TEMP) was initiated in 1996 to characterise the influence of southern bluefin tuna farming on the environment. Initial monitoring focused on a broad regional approach and a range of indices indicative of the health of pelagic systems (water quality and phytoplankton community structure) as well as the structure of epibenthic and infaunal communities (Clarke *et al.* 1999, Clarke *et al.* 2000). In 2001, the form of the TEMP changed to a farm-site compliance-based monitoring programme, with the methodology based on a synthesis of recommendations by SARDI (Madigan *et al.* 2001) and subsequent negotiations between the regulators, Primary Industries and Resources South Australia (PIRSA) Aquaculture and the Tuna Boat Owners Association of South Australia (TBOASA), representing the industry.

The programme adopted by PIRSA Aquaculture as a licence condition for southern bluefin tuna farming consisted of Farm Management and Benthic Assessments components (PIRSA-Aquaculture 2003). The Benthic Assessment component included a quantitative comparison of the characteristics of the benthic infaunal communities at potentially impacted locations (identified as compliance sites that were located 150m from the lease boundary) of the licence area being monitored and control locations (located at least 1 km from any lease boundaries).

Results from the last three years of compliance-based monitoring by the South Australian Research and Development Institute (commissioned by TBOASA) indicated that to date there have been no impacts detected on epibenthic fauna or sediment infauna at the compliance sites (Loo 2006).

Need

Marine finfish sea-cage culture is an increasingly important economic activity in regional Australia and the longer-term development of these industries requires that they operate in an ecologically sustainable manner. Consequently there is a need to better understand the relationship between farm management practices and the environmental effects of sea-cage aquaculture. In particular, community concerns regarding southern bluefin tuna farming have focussed on impacts to marine ecosystems making the ability to quantify impacts and optimise farm management practices fundamentally important to securing tenure for licence holders. As seabed health also influences water quality in and around farms, an understanding of the relationship between farm management and "souring" of the benthos will also provide significant outcomes for the industry in terms of optimising productivity and product quality. Consequently, both industry and regulators have to balance further development of the aquaculture industry within environmental constraints. This is to ensure acceptability by the general community and the sustainability and productivity of the industry.

Studies carried out in Australia and overseas have shown that the increased deposition of organic material associated with aquaculture waste from marine finfish farming can result in areas of organic enrichment on the sea floor, and this is usually most pronounced in close proximity to the farm (e.g. Wu *et al.* 1994, Findlay and Watling 1995, Naylor *et al.* 2000, Crawford *et al.* 2001, Crawford 2003).

Apart from the direct effects on the environment, there is also potential for substantial negative impact on the stock in the sea-cages. Higher levels of organic waste have been linked to elevated disease rates (see the comprehensive reviews in FAO/NACA 1995). This is also well documented for shrimp ponds where self-pollution is believed to have resulted in disease outbreaks in Thailand, China and India (Phillips 1996). Organic pollution of sites can also have sub-lethal effects through impacting on biological oxygen demand (BOD) and thereby oxygen availability, resulting in reduced feeding activity by fish, which compromises nutrition and growth. Collectively these impacts will result in economic loss either through increased operational costs (disease management and treatment), or through reduced production (increased stock loss and reduced growth rates).

Traditional approaches to the assessment of ecosystem responses to cage culture require detailed assessment and enumeration of benthic infaunal communities that are both expensive and time consuming. This severely limits the extent to which the conventional methods can be used for monitoring and assessment. There is a need therefore, to develop tools which allow for the rapid assessment of ecosystem responses to provide for the cost-effective monitoring of farming systems as well as to test the effectiveness of new

management practices or technologies, and a need for a scientifically defensible, rapid assessment system for evaluating the environmental impacts of sea-cage aquaculture.

DNA monitoring tools

The use of DNA-based testing in medicine and agriculture has increased exponentially over the past 20 years with nucleic acid based techniques having revolutionised diagnostic testing. In environmental studies, nucleic acid technology has been largely applied to the detection of bacteria (Stults *et al.* 2001). However, the use of DNA quantification in environmental samples has traditionally been viewed as difficult, largely because soil and marine sediment samples have high levels of chemical and genetic complexity that are not normally encountered in tissue and/or physiological samples or pure cultures (Sayler and Layton 1990, Stults *et al.* 2001). In addition, the extraction techniques for soils and marine sediments were not considered to be sufficiently robust, and other compounds such as organics, metals and humic acids, which were co-extracted with the DNA, can inhibit *Taq* polymerase, the enzyme which catalyses polymerase chain reaction (PCR) (Wilson 1997, Frostegaard *et al.* 1999, Stults *et al.* 2001). Quantitative PCR-based systems have been developed for the detection of bacteria such as *Geobacter* (Stults *et al.* 2001) and a nanoflagellate (Lim *et al.* 2001) in sediments, but there has been no research to date to develop PCR-based detection of benthic infauna for routine monitoring.

In recent years a DNA-based detection and quantification system has been developed by SARDI and CSIRO Entomology to measure plant pathogen populations in soils before crops are sown (Ophel-Keller *et al.* 1999). This PCR-based technology has been delivered commercially by SARDI as the Root Disease Testing Service (RDTS) since 1998. Aventis Crop Science (a major multinational company, now Bayer Crop Science) was granted a worldwide license to commercialise the technology in agriculture. The RDTS currently measures the levels of nine soil-borne pathogens (nematodes and fungi) in soil samples. A critical feature of this service is its ability to work with soil samples of the same size as that used for manual assessments of pathogen population densities. This system can measure population ranges of three to four orders of magnitude using DNA probes specifically designed to detect target organisms.

Project

The overall aim of this project was to employ these recent advances in techniques for DNA amplification, using polymerase chain reaction (PCR), to develop a system for the routine evaluation of marine sediments for use in environmental impact assessment and monitoring. In the existing plant pathogen quantification systems, DNA probes are generally used to target a particular species (fungi or nematode), but they can be designed

for higher taxonomic categories such as genera or families. There is also the possibility of targeting probes for specific metabolic pathways (such as sulphur reduction or methanogenesis). This was discussed with potential collaborators in this research field, but it was realised that it was beyond the scope of the current project.

The development of DNA assays for selected benthic marine organisms in sediments and the successful application of the RDTS system for DNA extraction to marine sediments would result in a system capable of delivering high throughput, quantitative molecular assays. Such an assay system would provide a capacity to undertake routine evaluation of the environmental effects of southern bluefin tuna aquaculture on sediments. Furthermore, this system may be expanded to cover other forms of aquaculture or other forms of environmental impact in marine systems (such as assessment of the effects of wastewater outfalls). Such a rapid monitoring and assessment system will enable the spatial and temporal scales of impacts to be more effectively determined. Furthermore, the environmental impact of changes in technology (such as alternative waste mitigation strategies), different feeds and husbandry practices can be more efficiently evaluated.

The project was developed in two phases, an initial “proof of concept” (and with this successfully achieved), a second “proof of application”.

The specific objectives of the proof of concept phase were:

1. To identify a range of benthic infaunal species that are characteristic of sites ranging from heavily impacted (organically polluted) tuna sea-cages through to non-impacted (pristine) environments.

2. To develop a system for the rapid detection of selected taxa in sediment samples using PCR techniques.

3. To evaluate the extent to which rapid detection systems can be routinely applied to provide quantitative estimates of the relative abundance of indicator taxa or processes in sediments (and therefore of the health of seabed systems).

4. To assess the generality of the technique to other forms of aquaculture (particularly salmon).

Once these had been achieved, additional objectives were added, specifically:

5. To improve the sensitivity of the PCR assays to levels comparable with those previously developed for terrestrial systems.

6. To develop PCR assays for a total of nine infaunal taxa[5].

7. To quantitatively calibrate all nine assays[6].

8. To demonstrate the "proof of application" of this methodology in comparison with traditional, manual enumeration, methods.

9. To further clarify the phylogenetic relationship in Spionidae and evaluate the need for multiple Spionidae assays.

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[6] In the application to the Aquafin CRC/FRDC this was written as "calibrate all 5 assays" as detailed above the number of assays developed was subsequently extended to nine.

Chapter 2 Exploiting Redundancy in Community Data

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This chapter will address the objective of identifying and validating a range of benthic infaunal species that are characteristic of sites ranging from impacted (organically enriched) southern bluefin tuna sea-cages through to non-impacted environments.

Introduction

Biological assemblages rarely comprise random aggregations of species existing together within a system. Rather, ecological interactions between species act to structure communities and thereby create assemblages in which the relative abundances of the various members are frequently correlated. Relationships between species generally reflect the effects of both physico-chemical variations in systems as well as the direct biological interactions between species, including predation, competition and facilitation.

One consequence of these interactions between species is that quantitative data, collected to describe the structure of these biological assemblages, typically has high levels of redundancy. This redundancy is a result of correlations in the distribution and abundance of species within an assemblage and is a feature that has long been exploited in the development of the multivariate statistical tools typically used to analyse such data (Gauch 1982, Clarke and Warwick 2001c).

Redundancy in community data also has utility in the development of environmental monitoring programmes. In the detection and monitoring of community responses to environmental change, sample relationships are frequently reduced from a high dimensional species-space to a representation that can be illustrated in substantially fewer, typically only two dimensions (Clarke 1993). The extent to which the dimensionality of a multivariate data set can be reduced is in itself an illustration of the redundancy in the data and a demonstration that many species are interchangeable in the way they characterise the samples (Clarke and Warwick 1998). This has driven community ecologists to identify taxonomic groups that are indicators of overall environmental status and to develop multi-metric indices, which satisfy the needs of managers to be able to utilise a reductionist approach in the assessment of habitat quality (Diaz *et al.* 2004). Measurement of these taxonomic groups can reduce the need for more comprehensive (or taxonomically wide ranging) assessments (Peterson 1993). As a consequence, there is broad acknowledgement

of the utility of indicator taxa, particularly in systems where the ecological responses are relatively well understood (March 1996, Bustos-Baez and Frid 2003).

Indicator taxa can be defined as those taxa (or in the case of the current study, phylogenetically related groups of species, defined at the family level) that collectively respond to major environmental influences. In the context of assessing the environmental performance of finfish aquaculture, the major environmental process that is being investigated is organic enrichment of the benthos.

The overall objective of this research was to design a set of DNA assays for a selected subset of taxonomic groups, and thereby provide the basis for the development of a novel, high throughput, quantitative, environmental assessment system for the southern bluefin tuna aquaculture industry. In order to achieve this outcome, it was necessary to validate a set of taxonomic groups for use as indicators of environmental status in relation to the southern bluefin tuna farming operations in Port Lincoln (South Australia).

In identifying suitable indicator taxa the following criteria were utilised:

- Taxa need to represent groups whose biology is sufficiently well understood to allow inferences to be made about the ecological role that they play in the ecosystem, and thereby to interpret differences in relative abundances in terms of the environmental signals that they are being used to measure.

- Taxa need to be relatively abundant, at least within certain environments, so that they provide a strong signal and thereby are relatively easy to detect.

- Taxa need to be relatively consistent indicators within the environment so that their presence or absence can be used as a definitive indication of environmental status.

- Collectively the taxa that are utilised need to allow us to characterise the structure of the community in a manner that is comparable to a more comprehensive community analysis.

The time frame for the research in this project required a decision to be made very early in the process about the taxonomic groups that would be targeted for DNA sequencing and PCR probe design. As a result, a number of taxonomic groups were chosen as targets for assay development at the beginning of the study based on our experience in this field, in particular the Port Lincoln region (South Australia) where this research was being undertaken. The taxonomic groups chosen were Capitellidae, Lumbrineridae, Nephtyidae, Spionidae and Cirratulidae. The rationale for choosing these groups is described in Chapter 3. Many of these taxa would be applicable for use in other areas, although it would need to be confirmed in each case that they represent an adequate group. As an

example, for application to salmon aquaculture in Tasmania, additional taxa or a different suite of taxa may be required. This was realised early in the project during the assessment of the generality of the technique (Objective 4) as differences in benthic infauna between southern bluefin tuna farming region in Port Lincoln and salmon farming in Tasmania were found when comparisons were made (see Macleod *et al.* 2004 for indicator taxa in Tasmania).

The purpose of the work detailed in this chapter therefore was to validate (or otherwise) this choice of taxonomic groups for use in Port Lincoln region for southern bluefin tuna aquaculture. To achieve this, multivariate analyses were undertaken on all available data on benthic infaunal communities from across the southern bluefin tuna farming zone.

Methods

A number of separate data sets were used in this investigation including data collected for the annual Tuna Environmental Monitoring Programme (TEMP) in 2001, 2002 and 2003 and data collected for an assessment of changes in benthic assemblages in response to fallowing. Field collection methods and laboratory processing of samples were similar for all the studies mentioned above except for 2001. These procedures are briefly outlined below with full details given in their respective reports (Madigan *et al.* 2002, Fernandes *et al.* 2003, Madigan *et al.* 2003, Loo *et al.* 2004b).

Field methods

Eight replicate samples were haphazardly taken at each of a number of sites. Haphazard samples are not strictly random, but are taken in a manner that is not influenced by the distribution of what is being sampled and are therefore effectively considered random. A Shipek grab (200 mm by 200 mm) was used for TEMP in 2001 while a HAPS Bottom Corer fitted with a 67 mm diameter sample tube was used for the other studies (Figure 2.1). The grab or the corer was lowered to the benthos using a hydraulic winch. Upon contact with the sea bottom, a counter weight released the sample tube from the corer, which was then forced into the sediment and hence, collected a sample, while the counter weight for the grab releases a scoop (driven by spring tension) to collect a sample. The sampler was retrieved and the sample removed, labelled and preserved in 10% Bennett's solution (a solution of 1:1 propylene glycol and formaldehyde in seawater).

Laboratory processing

Samples were returned to SARDI Aquatic Sciences and stored until processed. The samples were gently washed and screened using 1.0 mm sieves. After the fine (<1 mm)

sediment had been washed from the sample, the retained material was washed into a large Petri dish where animals in the retained sediment were picked out with the aid of a stereomicroscope and identified. The common animals were identified mostly to family level, but it was not practicable to identify the less common taxa to this level. Hence these animals were identified to phyla, sub-phyla, class or order. All identified animals were then enumerated and preserved in 70% ethanol for storage.

a) b)

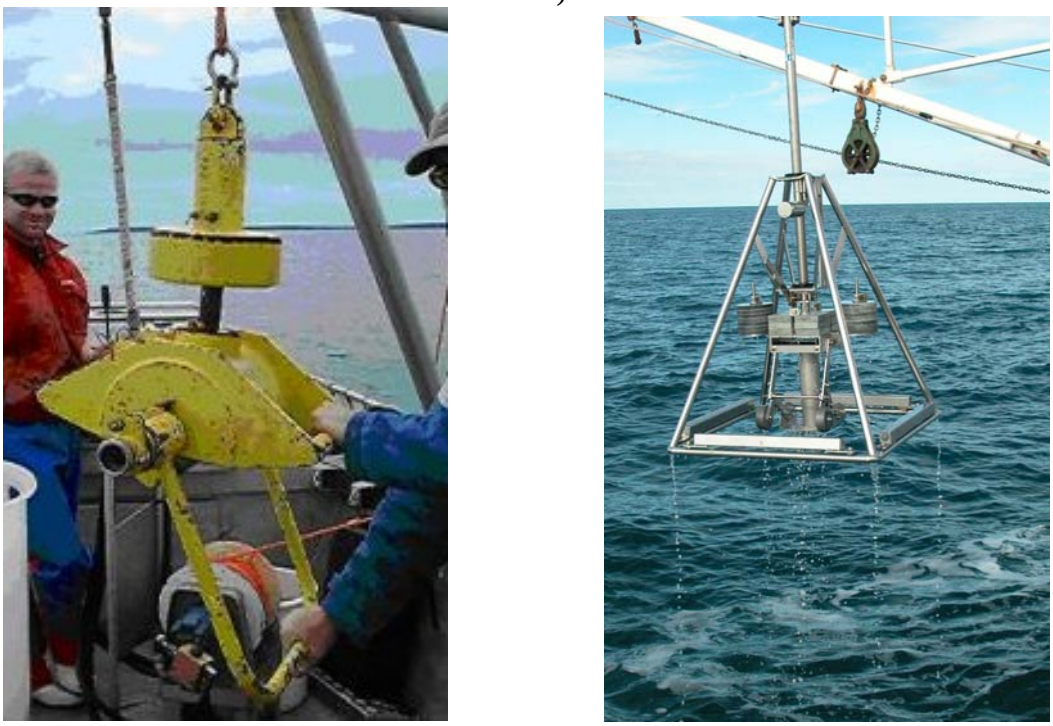

Figure 2.1 (a) Shipek grab and (b) HAPS bottom corer.

Data analysis

Most important species

The information content based on diversity indices and taxa richness calculated from individual taxon abundance was assessed at the scale of both samples and sites. Data on samples comprised an analysis of data on raw counts of abundance from individual cores or grabs whereas site data were calculated by aggregating data across all replicates from each sampling location (in most cases 8 replicate cores or grabs were collected from each site). The analysis presented in the following utilises data from the 2002 TEMP (Tuna Environmental Monitoring Programme; Madigan *et al.* 2003).

For each sample or site, the statistics detailed in Table 2.1 were calculated using PC-ORD version 4 (McCune and Mefford 1999). These indices provide measures of the information content of individual taxonomic groups and thereby give an index of the relative importance of taxa in characterising samples or sites. The indices were calculated on an inverted (R-type) matrix in which the abundance of each taxonomic group at any given site was used as a quantitative characteristic (attribute) for that group[7]. In such an analysis, terms such as mean abundance, richness and diversity (using any given index) need to be redefined (see Table 2.1) to reflect the change in perspective from a quantitative index calculated for a sample to a quantitative index calculated for a species (or as in this study, for a taxonomic group).

These indices have varying utility in the development of a list of candidate taxonomic groups. None of the indices (considered individually) provides a definitive basis for choosing one taxonomic group over another but collectively they provide a balanced view of the relative merits of some taxonomic groups over others.

Taxa were ranked in order of importance (highest value to lowest value) on the basis of the individual scores on each index (Table 2.2). Subsequently a composite score was developed that comprised the summed ranks on all four indices. Results were then reported as a ranked list of taxonomic groups that could be considered as candidates for inclusion in the list of indicators of environmental status.

This analysis was then compared with the outcomes from an analysis across all available data sets (TEMP 2001, 2002, 2003 and a data set collected on recovery of the benthos during fallowing; FALREC) using the "n-most important species" routine in the PRIMER package (Clarke and Warwick 2001b). In this routine the species accounting for > p% of the total score (abundance or biomass) in any one sample are retained (p is chosen to reduce species to the required number). In the PRIMER package, the routine computes an appropriate p to achieve a chosen number of "most important" species (n) to retain.

[7] Traditionally, measures of richness or diversity are calculated using a Q-matrix in which the taxa are defined as attributes of the samples (objects). A Q-matrix can be transposed into an R-matrix in which the samples are defined as attributes of the taxa (objects).

Table 2.1 Four indices were calculated for taxonomic groups across both individual samples and aggregated samples (sites). These statistics comprised the mean abundance and three measures (indices) of diversity (adapted from McCune and Mefford 1999) that were used as a basis for assessing the information content in raw measures of abundance across all taxonomic groups in the analysis.

Index	Description	Calculation	Interpretation
X	Mean abundance	Calculated across all samples/sites.	Average abundance of a taxonomic group calculated across all samples.
S	Richness	For a given taxon, the number of sites at which it found either for an individual sample or across all samples within a site.	Site richness is the total number of sites at which a taxonomic group is found.
H’	Shannon diversity	Calculated across all non-zero taxa within a sample or across all samples within a site (Shannon and Weaver 1949).	Consistency of a taxonomic group across sample units (only non-zero values considered). A quantitative measure, which incorporates both, evenness and site richness (S). A taxonomic group which is found more evenly across many samples will thereby have a higher value for H' than a taxonomic group which is found across the same number of samples but with lower evenness in its distribution.
D’	Simpson’s index of diversity for an infinite population	This is defined by McCune and Mefford (1999) as the complement of Simpson's original index and thereby quantifies the likelihood that two randomly chosen taxa will be from different samples.	Ubiquity of a taxonomic group across sample units (maximal for a taxonomic group that is equally abundant in all sample units).

Factor analysis

Another approach to identifying the key indicator species is through a factor analysis. Factor analysis aims to reduce the dimensionality of a multivariate data set by extracting a series of composite variables, otherwise known as eigenvectors. This approach to dimensional reduction has been likened to taking a photograph of a 3-dimensional object and rendering it as a 2-dimensional print; a good photographic print will retain a lot of the

detail that could be observed in the original object[8]. In general terms, a factor analysis allows one to identify a few key variables that contribute to the major proportion of the variance in a dataset. For this study, principle components analysis or PCA (a form of factor analysis) was run on the TEMP 2002 data and the structure of the eigenvectors was interrogated to identify those taxa, which contributed most strongly to the principle eigenvectors.

Comparative ordination analyses

Once a candidate list of the n-most important taxa was developed, the utility of this selection of taxa was compared with the original comprehensive data set through a series of comparative ordination analyses. Again a range of techniques are available to support this comparison including visual assessments of non-metric Multidimensional Scaling ordination (nMDS) plots or the use of relational statistics including the RELATE routine in the PRIMER package (Clarke and Warwick 2001b). The RELATE test allows an assessment of the similarity between two sets of association measures calculated for matched sets of samples. In this case, the test was run on the Bray-Curtis association matrix calculated across all samples using either all taxa or alternatively only the five most important taxa.

This analysis was undertaken using a number of data sets including the TEMP 2002 data (results not shown) and a data set obtained for a benthic respiration study (undertaken as part of the Waste Composition and Mitigation project[9]). The results from both studies were largely consistent but only the latter have been presented in the following because these data provided a higher degree of heterogeneity between samples (i.e. giving more distinct separation between samples, such as samples from farmed and unfarmed sites). This will therefore provide a better framework for illustrating the analytical process and the associated interpretation.

Results

Most important species

Of the 54 taxonomic groups present in the TEMP 2002 samples, five scored consistently highly on the information content indices (Table 2.2). These five taxonomic groups

[8] The analogy can be taken further if the photograph is black and white (versus a full colour image). Although the photograph is highly simplified (2-D black and white vs. 3-D colour) it can still be used to represent key features of the original object.

[9] Aquafin CRC/FRDC Project 2001/103: Aquafin CRC - Southern Bluefin Tuna Aquaculture Subprogram: Tuna environment subproject - Evaluation of waste composition and waste mitigation strategies.

comprised the families Nephtyidae, Lumbrineridae, Capitellidae, Spionidae and Cirratulidae; these were the five taxonomic groups that were defined *a-priori* as the most likely set of indicators. A number of other taxonomic groups also ranked consistently highly including Eunicidae and Gammaridea but both taxa were ranked below the top five. One taxonomic group, Bivalvia scored highly in terms of mean abundance but ranked consistently much lower on the other indices (Table 2.2).

Table 2.2 Relative importance of taxonomic groups from the TEMP 2002 data based on selected indices (Table 2.1) calculated for each taxonomic group using data from individual samples (i.e. not aggregated to site level). Values represent the rank from 1 most important to n (=total number of taxa) least important. **Bolded** values have a top 5 ranking on at least one index. Taxa that ranked below Bivalvia have been excluded for simplicity.

Taxonomic groups	Mean	S	H'	D	Average
Nephtyidae	**2**	**1**	**1**	**1**	**1.3**
Lumbrineridae	**3**	**3**	**2**	**2**	**2.5**
Capitellidae	**4**	**4**	**3**	**3**	**3.5**
Spionidae	**1**	**2**	**5**	**10**	**4.5**
Cirratulidae	**6**	**5**	**4**	**4**	**4.8**
Eunicidae	7	6	6	6	6.3
Gammaridea	**8**	**7**	**8**	**5**	**7.0**
Nemertea	9	8	7	7	7.8
Ampharetidae	10	9	9	8	9.0
Sabellidae	11	11	10	9	10.3
Terrebellidae	12	12	11	11	11.5
Holothuroidea	13	13	12	12	12.5
Bivalvia	**5**	**10**	**13**	**25**	**13.3**

The top five taxonomic groups, identified in the analysis of TEMP 2002 data, were also shown to feature in the seven most important taxonomic groups when assessed using the "n-most important species" routine (PRIMER) across other data sets available for the southern bluefin tuna farming region of Port Lincoln (Table 2.3). Two other taxonomic groups, Bivalvia and Gammaridea, were included in the top seven (ranked 3rd and 6th respectively).

Table 2.3 Comparative utility of taxonomic groups as indicators of environmental status using PRIMER "n-most important" routine (Clarke and Warwick 2001b). Values represent the rank from 1 most important to n (total number of taxonomic groups) least important. **Bolded** values represent taxa chosen *a-priori* for DNA assay development. Taxa that ranked below Cirratulidae have been excluded for simplicity.

Taxonomic groups	Ranking for taxonomic groups across different data sets					
	TEMP 2001	TEMP 2002	TEMP 2003	FALREC Study	Mean Rank	% Ranked in Top 5
Spionidae	**1**	**1**	**1**	**2**	**1.25**	**100**
Lumbrineridae	**3**	**4**	**3**	**4**	**3.50**	**100**
Bivalvia	8	2	4	1	3.75	75
Capitellidae	**5**	**5**	**9**	**3**	**5.50**	**75**
Nephtyidae	**2**	**3**	**12**	**6**	**5.75**	**50**
Gammaridea	9	8	2	8	6.75	25
Cirratulidae	**13**	**7**	**18**	**5**	**10.75**	**25**

Factor analysis

Analysis of the 2002 TEMP data showed that the five taxonomic groups that had been selected for DNA assay development represented five of the top six taxa that contributed most to the first six eigenvectors obtained through PCA (Table 2.4). The only other taxon that contributed strongly to the definition of the eigenvectors was the Bivalvia.

Table 2.4 Values represent the strength of the contribution by each taxonomic group to the definition of the top 6 eigenvectors. **Bolded** values show the taxonomic group that has the highest weighting on each vector. Data for Eunicidae and Nemertea have been included for comparative purposes; note the much lower weighting on the vectors by these taxa.

Taxa	Vector coefficients					
	Vector 1	Vector 2	Vector 3	Vector 4	Vector 5	Vector 6
Spionidae	**1.00**	0.00	0.05	0.02	0.04	0.00
Bivalvia	0.01	**0.97**	0.12	0.01	0.03	0.03
Lumbrineridae	0.05	0.13	**0.92**	0.21	0.04	0.00
Nephtyidae	0.02	0.00	0.22	**0.91**	0.19	0.23
Capitellidae	0.03	0.06	0.04	0.23	**0.89**	0.19
Cirratulidae	0.00	0.00	0.12	0.11	0.19	**0.85**
Eunicidae	0.03	0.00	0.20	0.08	0.28	0.21
Nemertea	0.00	0.03	0.12	0.06	0.02	0.21

Comparative ordination analysis

Comparative ordinations of data collected for the benthic respiration study, consisting of samples from control sites and at locations adjacent to farms, demonstrated the issue of data redundancy in this system. In this ordination (Figure 2.2), Farm Site 3 represented a stocked farm site where fish had been held for a period of 4 months when sampling was carried out. Farm Site 2 was also a stocked farm site but fish had only been held for 2 months. Farm Site 1 was the SARDI Tuna Research Farm (adjacent to Farmed Site 2) with lower stocking densities. Control Site A and B were sites situated at least 1 km away from any farmed site.

An ordination of the data using all taxonomic groups (58 in total; Figure 2.2) showed a distinct separation of the control and farmed sites. There was also some finer structure to the ordination with the two control sites being distinct from one another and Farmed Site 3 showing some separation from Farm Sites 1 and 2.

Another ordination of the data using only the taxonomic groups selected for DNA assay development (Figure 2.3) demonstrated that much of the information contained in the original data set could still be recovered when using this reduced selection of taxonomic groups. The control and farmed sites could still be differentiated, although there was a loss of resolution in terms of the separation of the two control sites. Farm Site 3 could still be differentiated from the other two farmed sites. Similar results were obtained for analyses of the other TEMP data sets (results not shown).

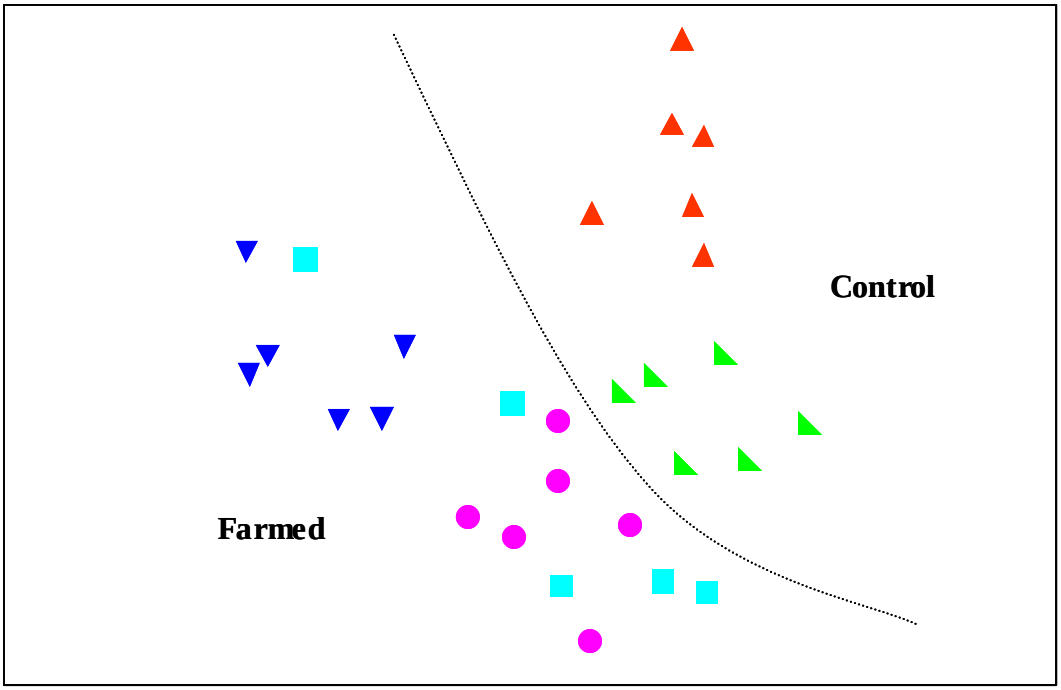

Figure 2.2 nMDS ordination plot (2-dimension, stress = 0.17) of untransformed abundance data from three farmed sites and two control sites. The ordination was undertaken using data for all 58 taxonomic groups identified during manual enumeration of infauna in the samples. ▲ = Control Site A, ◣ = Control Site B, ● = Farm Site 1, ■ = Farm Site 2 and ▼ = Farm Site 3.

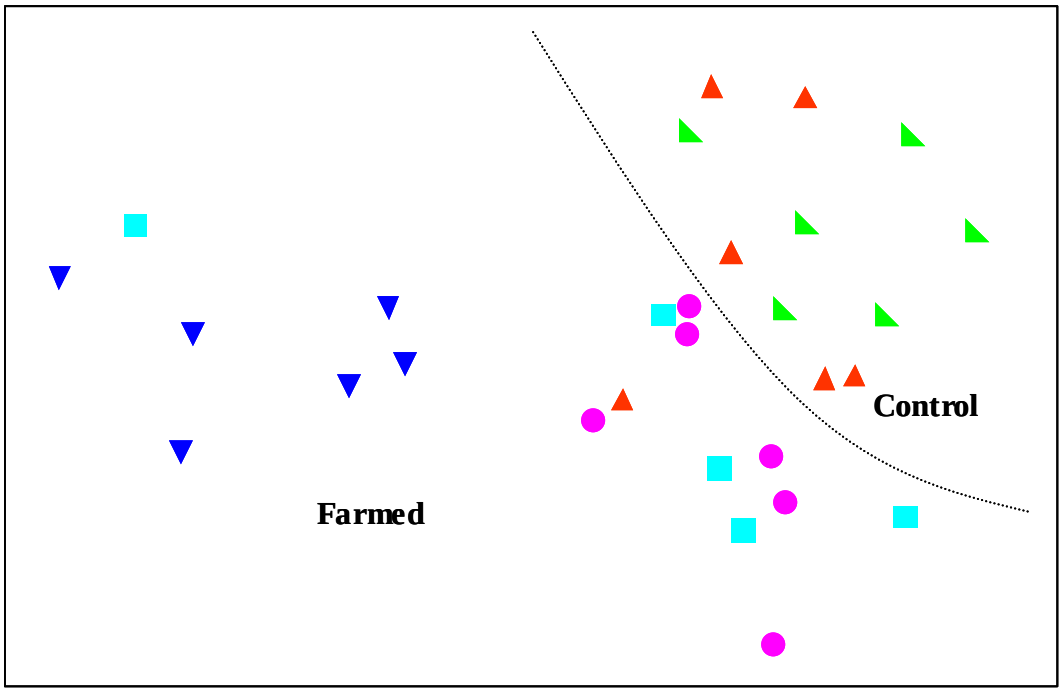

Figure 2.3 nMDS ordination plot (2-dimension, stress = 0.12) of untransformed abundance data from three farmed sites and two control sites. The ordination was undertaken using data for only the taxonomic groups selected for DNA assay development. ▲ = Control Site A, ◣ = Control Site B, ● = Farm Site 1, ■ = Farm Site 2 and ▼ = Farm Site 3).

Discussion

This work has demonstrated that the five taxonomic groups chosen for DNA assay development (Capitellidae, Spionidae, Cirratulidae, Nephtyidae and Lumbrineridae) are representative of the taxonomic groups that are most useful in quantifying the environmental effects of southern bluefin tuna farming on benthic infaunal systems in the Boston and Rabbit Island farming zones. On this basis it is argued that the development of DNA assays for these taxa will provide a reliable tool for the assessment of benthic souring and recovery associated with southern bluefin tuna farming.

All the taxonomic groups that have been identified respond variously to organically enriched sediments in temperate waters (see review in Chapter 3) and particularly in relation to aquaculture systems, not only in South Australia but also in many other locations around the world (Bybee 2001, Bailey-Brock *et al.* 2002, Lee *et al.* 2006). None of the taxa are characteristic of undisturbed sediments (although a number of taxa are indicators of low pollution load) and therefore this tool will not necessarily be useful in providing measures of other farm effects on the system. Not withstanding, all previous studies (see review in Chapter 1) have demonstrated that the principal effect of finfish farming is organic enrichment and on this basis it is believed that the tools developed through this study will provide information to support environmental monitoring and management of the system.

Through this analysis it has also been possible to develop a list of additional taxa for which probes could be developed and which would make the analysis more generally applicable (e.g. extension for use in environmental monitoring of Tasmanian salmon or South Australian yellowtail kingfish industries). These supplementary taxa include Bivalvia, Eunicidae and Gammaridea. Similarly, future work may also focus on additional polychaete taxa which could include members of the Dorvellidae and Ampharetidae both of which have been reported in association with aquaculture and or effluent disposal from locations elsewhere in the world (see for example Lee *et al.* 2006).

Chapter 3 Biology of Selected Indicator Taxa

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This chapter gives the general description and biology of the taxonomic groups chosen and validated through the research discussed in Chapter 2.

Introduction

The class Polychaeta was traditionally grouped together with Oligochaeta (earthworms) and Hirudinea (leeches) in the Phylum Annelida, but more recently this classification has undergone review (Rouse and Fauchald 1995, Rouse and Fauchald 1998, Rouse and Pleijel 2003). However, there was no firm resolution on the systematics of polychaetes although issues were highlighted and discussed. The reviews indicated that most would continue to follow the classification of Rouse and Fauchald (1997), i.e. Annelida is accepted as a valid taxon and comprises two clades, the Polychaeta and Clitellata, but many changes are forthcoming in the future (Rouse and Pleijel 2003). While the class is found in all marine environments, there are few freshwater and virtually no terrestrial polychaetes. Polychaetes are common at all latitudes, and in all marine zones from the intertidal to the abyssal depths. Polychaetes are also found in estuaries with low, high or variable salinities, and may be present in very high numbers and/or diversity in some circumstances (Hutchings 2000b).

Diversity within the class Polychaeta is high. Worldwide, about 13,000 species belonging to 81 families have been described, but many species are yet to be described and the actual number of polychaete species worldwide may be as high as 30,000. Sixty-one families are represented in Australasia, with 1,140 species described from Australian waters (Glasby *et al.* 2000).

Polychaetes fill a wide variety of ecological niches which include being predators, prey, nutrient-recycling detritivores, and modulators of local physical and chemical conditions. There are sessile species that build hard or soft tubes in sediments or on hard surfaces, and free-living varieties that burrow in or crawl across the seafloor as well as pelagic swimming varieties. Polychaetes are the dominant macrofauna in fine marine sediments and some of the highest diversities of polychaetes in the world have been found in the marine soft sediments of southern Australia (Glasby *et al.* 2000). The ubiquity, abundance, diversity and functions of polychaetes in marine habitats and food chains make them an ecologically important group.

Polychaetes are segmented worms, typically consisting of two anterior segments (prostomium and peristomium) with eyes and sensory and/or feeding appendages, followed by a long, segmented trunk with fleshy lateral appendages (parapodia) and chitinous bristles (chaetae), and a pygidium at the end of the body where the anus is located (Figure 3.1).

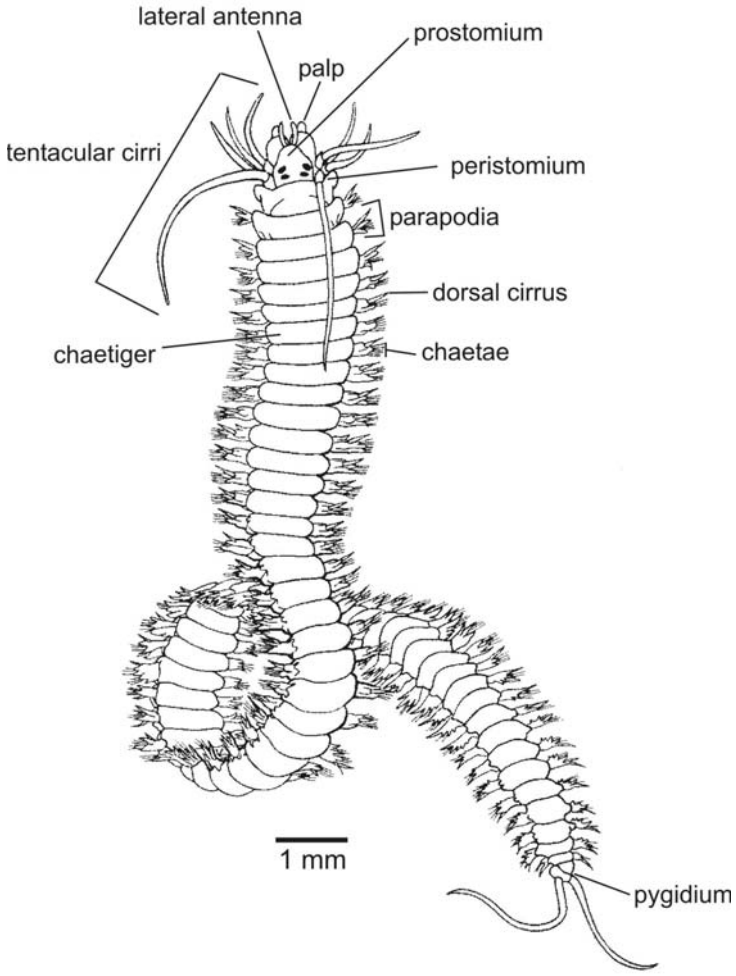

Figure 3.1 A polychaete belonging to the family Nereididae, showing the arrangement of external features in polychaetes (adapted from Wilson 2000b).

Each segment usually has one set of parapodia and associated chaetae on each side, which are used in locomotion. The parapodia typically consist of a dorsal notopodium and a ventral neuropodium. Chaetae vary widely in appearance from hair-like capillaries, to simple or jointed spines and hooks. Dorsal and ventral cirri, which usually have a sensory function, and branchiae, which improve gas exchange, may also be present in association with the parapodia or separately. Characteristics used to define the taxa of polychaetes

include the morphology of the prostomium, peristomium, appendages, eversible pharynx, parapodia and chaetae (Figure 3.1).

Polychaetes as indicators of environmental conditions

The sensitivity of polychaete communities to their environment makes them excellent indicators of the conditions, such as nutrient levels, in local areas (Tomassetti and Porrello 2005, Pearson and Rosenberg 1978). Polychaete species vary widely in their ability to tolerate and exploit biological, physical and chemical changes (Pearson and Rosenberg 1978). An example is organic enrichment, which increases food availability for scavengers but often reduces sediment oxygen levels. As a result, a few tolerant opportunistic scavengers, often in large numbers, will replace many benthic species intolerant of lower oxygen levels. Deposit-feeding polychaetes tend to intensively rework the top few centimetres of sediment, creating an easily re-suspended faecal-rich top layer, a condition that is unfavourable to suspension feeders (Rhoads and Young 1970). Therefore, at sites experiencing organic enrichment, the number and diversity of suspension-feeders tend to decrease as deposit-feeders increase. Table 3.1 gives a summary of the feeding modes and characteristics, which make the selected taxa good environmental indicators, especially of organic enrichment.

The use of infaunal polychaetes as indicators of marine environmental quality is widespread and has been internationally accepted (Pocklington and Wells 1992). However, using polychaetes is time consuming because the animals have to be sorted from the sediment, and requires taxonomic expertise for their identification. A way of reducing the time and expertise required is the use of one (or preferably a few) species as "indicators" of the structure of the entire community and thus of the state of the environment (Carignan and Villard-Marc-Andre 2002). However, studies have shown that there are no truly "universal" indicator species, and the choice of taxa to be used as indicators in an area must be developed from prior knowledge of the infaunal community and its response to the impact of interest (Pocklington and Wells 1992, Bustos-Baez and Frid 2003).

Previous work (Loo *et al.* 2004a, Loo and Drabsch 2005a) has indicated that polychaetes, in particular the five polychaete families chosen, were the most consistent environmental indicators found in the Port Lincoln southern bluefin tuna aquaculture region. While this work was principally used to determine which taxa to use for the development of probes as outlined in Chapter 2, these chosen taxa were supported by various literature to be indicators used in other places worldwide (Pearson and Rosenberg 1978, Bustos-Baez and Frid 2003). Taxa other than polychaetes, such as amphipod crustaceans, have also been used but are cautiously recommended because of the difficulty in identifying amphipods

and the lack of information about their suitability, ecology and distribution, thereby negating their use in much of the world (Thomas 1993), including southern Australia. Reasonably large numbers of some bivalve species were sometimes found near the Port Lincoln southern bluefin tuna sea-cages, but it is suspected that these were cage-fouling organisms that have dislodged and fallen from the cage netting rather than infauna *per se*.

Table 3.1 Summary of feeding modes and features characteristic of selected taxonomic groups.

Taxa	Feeding modes	Characteristics of taxa	Responses of taxa to environmental stress
Capitellidae	Non-selective subsurface and surface deposit feeding	Opportunistic rapid coloniser High tolerance to a variety of environmental conditions	Very commonly found in large numbers in organically enriched or otherwise polluted sites, often the first taxa present
Lumbrineridae	Mostly carnivorous/carrion-feeding, some species are selective deposit feeding or herbivorous	Carnivorous and herbivorous feeding modes suit lower pollution conditions	Associated with “semi-healthy” conditions Indicator of low pollution loads
Cirratulidae	Selective surface deposit feeding	High tolerance to hypoxia	Found commonly in hypoxic conditions Empirical evidence of hypoxia tolerance and physiological adaptations
Nephtyidae	Usually carnivorous, some deposit feeding	Favours conditions where prey abundance is high	Favours first stages of pollution (including organic enrichment) abatement (Indicator of moderate pollution)
Spionidae-polydorids	Selective surface deposit feeding, possibly some supplementary filter-feeding	Opportunistic rapid coloniser Able to populate a variety of substrates	Very commonly found in large numbers in organically enriched sites Often a pioneer coloniser
Spionidae-non-polydorids	Selective surface deposit feeding, possibly some supplementary filter-feeding	Deposit feeding mode	Some species favour heavy pollution, others first stages of pollution abatement

Biology of selected taxonomic groups

CAPITELLIDAE

The family Capitellidae Grube 1862 are common polychaetes that resemble earthworms. Capitellids form burrows and soft tubes in the sediment but are considered motile. They have a long, often red, cylindrical body with distinct segmentation, and a small conical prostomium with an eversible pharynx and sometimes eyespots (Figure 3.2). Antennae and palps are absent. Capitellids have reduced parapodia, so the chaetae seem to arise directly from the body. The body is divided into a thorax that consists of 8-19 segments with capillary and/or hook chaetae, and an abdomen that has hooks arranged in tori. Identifying capitellids to genus or species is difficult due to the small number of variations in body morphology between species. In addition, features used to distinguish species, such as the number of segments with capillary chaetae, can vary with age (Warren 1991). While the taxonomy is still under review, there are 156 species recognised worldwide with at least 37 species in 18 genera present in Australian waters (Hutchings 2000a).

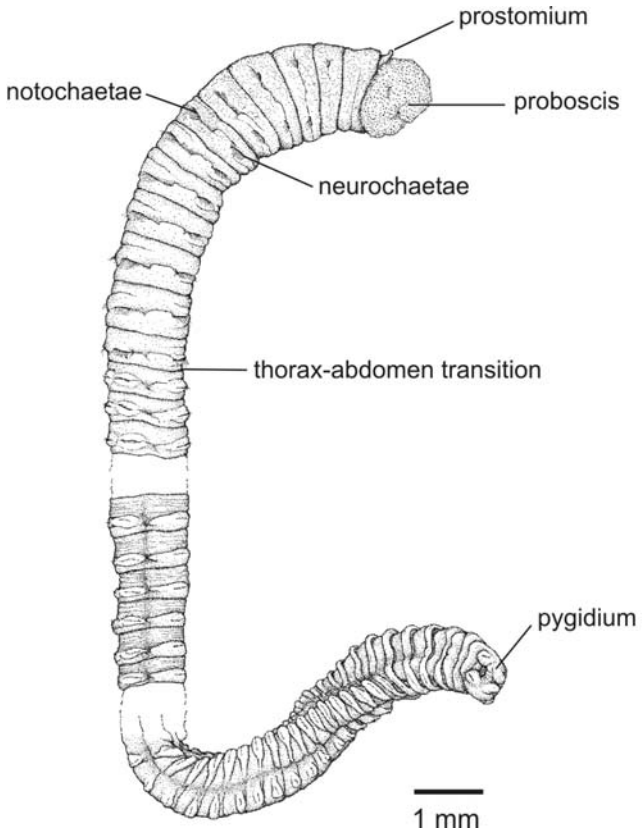

Figure 3.2 *Notomastus torquatus* belonging to the family Capitellidae (adapted from Hutchings 2000a).

Capitellids are cosmopolitan polychaetes found around the world in a wide variety of marine zones from intertidal to deep-sea. There are also some estuarine and freshwater varieties. Capitellids are non-selective sub-surface and surface deposit feeders that use their eversible proboscis to pick up particles. Capitellids are found in a broad range of salinities and temperatures, and are more tolerant of hypoxia and toxins than most animals (Grassle and Grassle 1974). This broad range of tolerances has been attributed to short-term natural selection from a range of genotypes available for settlement rather than adaptation in individuals. This was investigated in the laboratory where tests have not revealed unusual ranges of tolerances in individuals to any particular environmental variables (Grassle and Grassle 1974).

Capitellids are excellent opportunists; rapidly colonising and reaching high numbers in organically enriched sites or places that have been defaunated by some disturbance. Capitellids are often the first and one of the few pioneer colonisers of such sites, attributable to their ability to find new opportunities and reach high numbers quickly, as well as their tolerance to a variety of unfavourable conditions such as hypoxic or toxic sediments. Consequently, capitellids, particularly the widespread species *Capitella capitata*, are often used as indicators of pollution (Grassle and Grassle 1974, Pearson and Rosenberg 1978, Karakassis *et al.* 2000, Yokoyama 2002, Brooks *et al.* 2003).

CIRRATULIDAE

The family Cirratulidae Ryckholdt 1851 is a common and relatively well-studied polychaete group with 11 genera recognized worldwide. Eight of these have been reported to be present in Australian waters (Glasby 2000). The family is sub-divided on the basis of the number and origin of palps. They range in length up to 250 mm, and can be vibrant green, orange or yellow in colour (Rouse and Pleijel 2001). Cirratulids have very long filamentous branchiae that are concentrated at the anterior end but often continue along the entire body. There are also multiple palps behind a conical prostomium that lacks appendages but may have a pair of eyespots. Parapodia are either absent or reduced to low lobes; hence the simple chaetae emerge almost directly from the body wall (Figure 3.3).

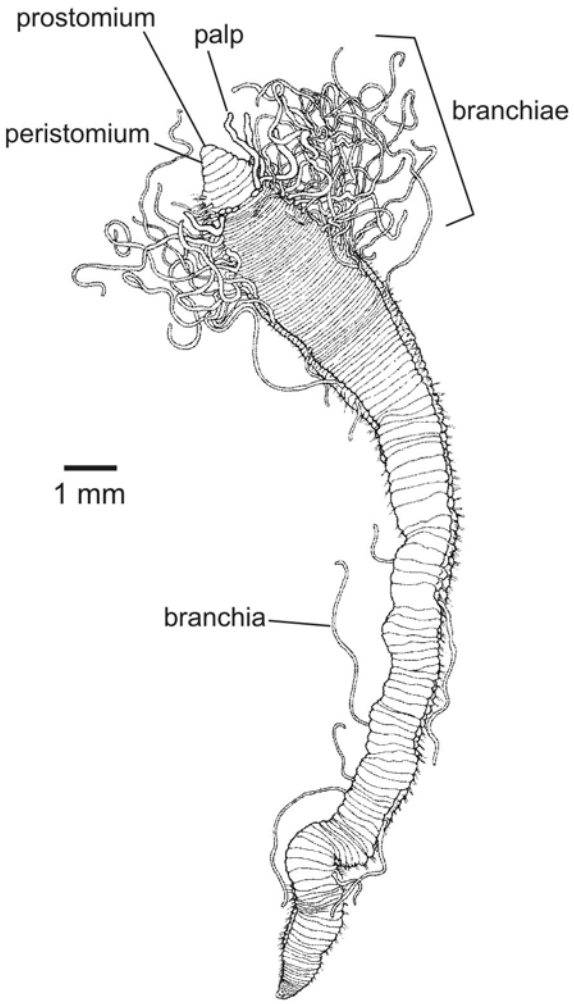

Figure 3.3 *Cirriformia* cf. *filigera* belonging to the family Cirratulidae (adapted from Glasby 2000).

Cirratulids are considered to be surface deposit-feeders, using their long palps and unarmed eversible proboscis to collect food particles. Cirratulids are usually free-living in sediments, rock crevices, algal holdfasts and seagrass, though some species bore into calcareous substrates such as corals and mollusc shells (Glasby 2000). Cirratulids have physiological and behavioural adaptations to survive hypoxia, including haemoglobin with enhanced oxygen-binding capabilities (Dales and Warren 1980). Many species live in anoxic sediments, obtaining oxygen by extending their branchiae into the overlying oxygenated water for gas exchange in a way similar to pneumatophores in mangroves. The ability of cirratulids to inhabit anoxic sediments makes them useful indicators of organic enrichment, which can occur as a consequence of excess fish feed and faeces in marine aquaculture or effluent outfalls (Glasby 2000).

SPIONIDAE

The family Spionidae Grube 1850 is one of the most species-rich polychaete families, found commonly and worldwide with about 1000 described species in 38 genera (Blake 1996, Sigvaldadóttir *et al.* 1997). In Australia, 96 species belonging to 24 genera have been identified (Rouse and Pleijel 2001). Spionids occur from the intertidal zone to the deep sea in a variety of substrates including soft sediments, corals and mollusc shells, often forming dense assemblages (Blake 1996, Glasby *et al.* 2000, Rouse and Pleijel 2001). Spionids are benthic infaunal and epifaunal deposit or suspension-feeders and are often used as indicators of anthropogenic disturbances, including nutrient enrichment.

The family Spionidae can be distinguished by the presence of a pair of large, grooved feeding palps, a posterior prolongation of the prostomium (caruncle), and biramous, foliaceous parapodia (Wilson 2000c, Figure 3.4). Extensive morphological variability has been described for the Spionidae (Blake 1996, Glasby *et al.* 2000, Rouse and Pleijel 2001). Spionids have long, slightly dorso-ventrally flattened cylindrical bodies. The prostomium may be rounded, bilobed or pointed, and may have eyes, a pair of lateral horns and a median antenna. The parapodia are biramous, consisting of a dorsal notopodium and a ventral neuropodium, and contain a variety of chaetae including capillaries, hooded hooks and spines. Variously shaped branchiae also occur dorsally on a differing number of segments. The features used to distinguish the different genera within the Spionidae include modification of one chaetiger, shape and features of the prostomium, type, shape and first appearance of the different chaetae, type of branchiae and on which segments branchiae occur.

The taxonomy of the Spionidae is complex and in need of review, and there are few taxonomic keys to Australian spionids (Read 1975, Blake and Kudenov 1978, Blake and Kudenov 1981, Hutchings and Turvey 1984). The most comprehensive account of south-eastern Australian polydorid spionids dates back to 1978 (Blake and Kudenov 1978). The distribution of spionids in Australia is not well known, except for polydorid species that infect the shells of cultured molluscs such as oysters, abalone, mussels and scallops (Skeel 1979). Studies of closely related species have reported large fluctuations in abundance in a variety of habitats as a result of varying environmental conditions (Zajac 1991).

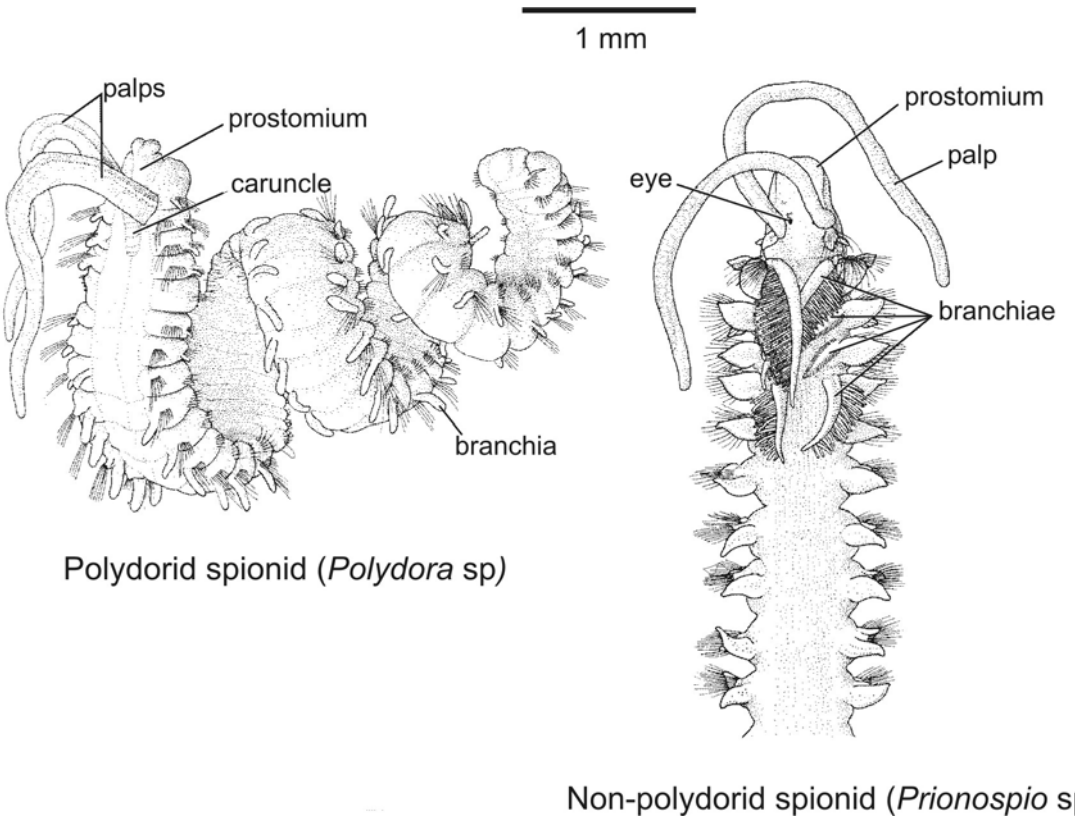

Figure 3.4 *Polydora* sp. and *Prionospio coorilla* belonging to the family Spionidae (adapted from Wilson 2000c).

The family Spionidae includes two distinctive types, loosely called the polydorids and the non-polydorids (Blake 1996, Figure 3.4). Polydorids are characterized by a modified fifth chaetiger (fourth in *Polydorella*), which is enlarged and has specialized thick spines. Non-polydorids lack the modified chaetiger. In Australia, the polydorid complex includes the related genera of *Polydora*, *Dipolydora*, *Pseudopolydora*, *Carazziella*, *Boccardia*, *Boccardiella* and *Polydorella* (Wilson and McDiarmid 2003). There have been 115 species of polydorids described worldwide. However, their small size makes them especially difficult to identify accurately (Blake 1996), as morphological differences between species are often in small-scale details (Glasby *et al.* 2000, Rouse and Pleijel 2001).

In general, polydorids are sessile polychaetes (Haswell 1885, Grassle and Grassle 1974). They are the most common commensal polychaete that bores into mollusc shells, with a low degree of host specificity (Martin and Britayev 1998). In southern Australia, they burrow into the shells of the scallop (*Placopecten merdionalis*) and Pacific oyster (*Crassostrea gigas*) (Haswell 1885), which has an economic impact as it impairs the growth and lowers the market value of these shellfish in cultivation (Wilson 2000c).

Despite this, little is known about the biology of this important group in the southern hemisphere because the majority of information concerning polydorids is based on northern hemisphere species (Read 1975, Blake 1996). However, it is likely that a number of the polydorids found in Australia are exotic species, having been introduced along with their shellfish hosts, or via ship ballast water. In California, the source of some spionids have been traced to the introduction of the Pacific oyster *Crassostrea gigas* from Japan (Blake 1996). Therefore, it is possible that the polydorids found in *C. gigas* in South Australia, which is extensively farmed here, are the same as those found in *C. gigas* in the northern hemisphere.

Non-polydorid spionids are not known to have such notorious habits, but may be useful indicators of pollution. In many studies, a non-polydorid species such as *Scolelepis fuliginosa* has been found, usually in conjunction with *Capitella capitata* (Capitellidae), to be the dominant group in heavily polluted sites (Pearson and Rosenberg 1978). Members of the species-rich genus *Prionospio* have been found in a range of pollution levels, but more often in the second phase of pollution abatement, following the decline of the initial opportunistic colonisers (Pearson and Rosenberg 1978).

As environmental indicators, the polydorid species *Polydora ligni* has been shown to exhibit a regular opportunistic response to disturbance (Zajac and Whitlatch 1982b). Along with the capitellid species *Capitella capitata, Polydora ligni* has been found to be one of the most opportunistic species, rapidly colonizing vacant (defaunated) habitats (Grassle and Grassle 1974). This opportunistic behaviour is attributed to their ability to find new habitats and reach high numbers quickly, and their ability to populate different substrata. Yokoyama (2002) found that the polydorid *Pseudopolydora paucibranchiata* was the second species (after *Capitella* sp.) to colonise sites defaunated by fish farm operations, reaching densities of up to 5670 individuals/m^2.

LUMBRINERIDAE

The family Lumbrineridae Schmarda 1861 has a rounded, bluntly conical prostomium with dorsal nuchal grooves and a ventral muscular pharynx armed with characteristic Eunicida jaws, but without eyes or obvious appendages. The peristomium consists of two rings and lacks appendages. Lumbrinerids have long, cylindrical, undifferentiated bodies that range in size from a few millimetres to half a metre (Rouse and Pleijel 2001). Segmentation is quite distinct, and the epidermis is smooth and sometimes opalescent. Parapodia and chaetae are similar all the way down the body. The parapodia may have a simple neuropodium with capillary chaetae and hooks, may be uniramous (lacking a notopodium altogether), or sub-biramous (having a small notopodial lobe or cirrus but no external chaetae). Most taxa lack branchiae (Figure 3.5).

Lumbrinerids are usually free-living burrowers in the sediments, although some build tubes and others are symbionts (Martin and Britayev 1998). Lumbrinerids are most common in sandy and muddy shelf habitats, but have been found from the intertidal to abyssal depths (Paxton 2000). There are more than 200 species of lumbrinerids in 136 genera worldwide, of which four genera and 12 species are found in Australia (Paxton 2000).

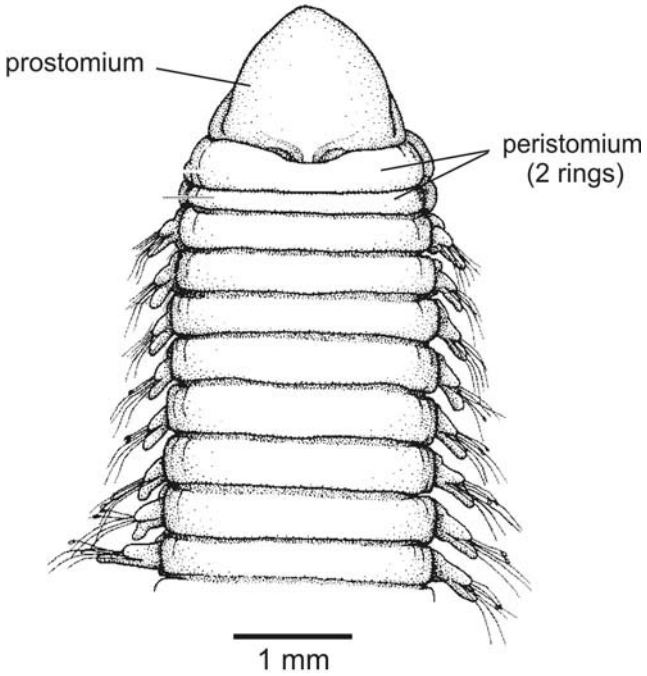

Figure 3.5 *Lumbineris* cf. *latreilli* belonging to the family Lumbrineridae (adapted from Paxton 2000).

Lumbrinerids are mostly carnivores or carrion eaters, but some species have been observed to be herbivores or selective deposit feeders (Fauchald and Jumars 1979). Lumbrinerids may be indicators of pollution in that low numbers of the genus *Lumbrineris* might indicate a large pollution impact (Pocklington and Wells 1992). Pearson and Rosenberg (1978) also listed a number of studies where lumbrinerids were found to be associated with "semi-healthy" zones between highly polluted and pristine areas.

NEPHTYIDAE

The family Nephtyidae Grube 1850 has a distinctive body that is x-shaped in cross-section, with well-developed biramous parapodia. An inter-ramal branchia is present between the noto- and neuropodia of all except the first few segments. The prostomium is relatively small, dorsally located and flattened, and has a pair of anterior antennae and palps. The

first chaetigerous segment is smaller, with parapodia and chaetae directed forward alongside the prostomium. The body tapers gradually to the pygidium (Figure 3.6).

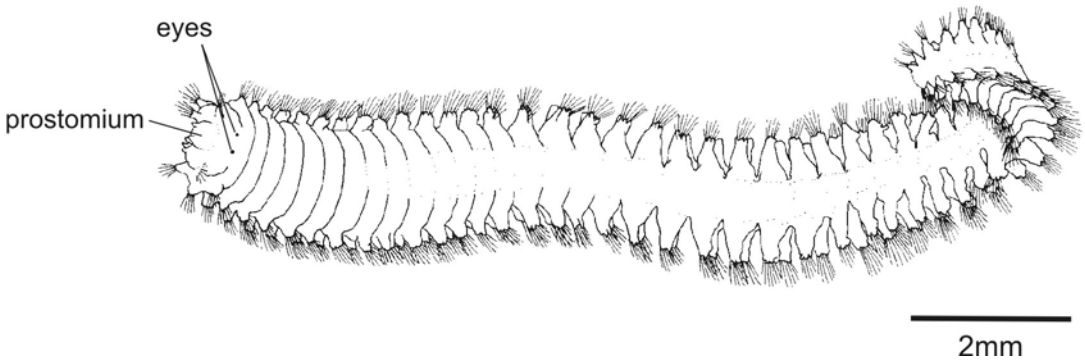

Figure 3.6 *Nephtys inornata* belonging to the family Nephtyidae (adapted from Wilson 2000a).

Nephtyids have very muscular bodies, and burrow and swim powerfully in a lateral sinusoidal movement. Nephtyids are mostly predators, catching other motile invertebrates (mainly crustaceans, polychaetes and molluscs) with their muscular eversible pharynx which is armed with a pair of small jaws (Wilson 2000a). Evidence of subsurface deposit feeding has also been found in some species (Fauchald and Jumars 1979). Nephtyids are accepted as characteristic of early successional phases of pollution abatement (Pocklington and Wells 1992).

Chapter 4 Development of Specific DNA Probes

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This chapter will discuss the development of specific DNA probes. The five taxonomic groups selected for probe development included the polychaete families Capitellidae, Lumbrineridae, Cirratulidae, Nephtyidae and Spionidae. These were initially selected based on experience with previous studies but their choice was validated through the work detailed in Chapter 2 and the rationale discussed in Chapter 3.

Introduction

DNA probes were developed for these taxa based on ribosomal gene sequences. The 18S and 28S subunits of ribosomal DNA (rDNA) gene complex were targeted for sequencing. These regions of rDNA were chosen because they are generally conserved at the species and subspecies level and are therefore suitable targets for probes above species level. Subsequently, the 28S region was chosen as the preferred region because the 28S rDNA region has been extensively used in phylogenetic analyses of major invertebrate groups such as gastropods, arthropods and others (see references in Brown *et al.* 1999). In addition, existing 28S sequence information is available on publicly accessible sequence databases, which allowed for comparisons.

Assays were initially developed using technology that quantifies the amount of specific target sequence amplified at the end of the PCR reaction ("end-point" detection). Later in the project, an alternative technology, "real-time PCR", was used. Real-time PCR is a newer technology, which quantifies the point at which the PCR reaction moves into linear amplification, instead of quantifying the end product of the reaction. The advantage of real-time PCR is that it is faster, requiring only one reaction for amplification and quantification and it has built-in quality assurance parameters, making it more suitable for routine use. The other main advantage of real-time PCR is that it combines sensitivity with excellent linear range. This makes real-time PCR particularly suitable for use on indicator taxa in sediments, allowing accurate quantification from very low numbers/biomass of organisms as it is linear over a range of four- to five-orders of magnitude.

Probe development

Collection of target taxa

An initial broad-scale sampling programme was carried out in March 2002 in order to obtain animals, which were representative of the Port Lincoln region. Sediment samples were collected from sites close to and away from anthropogenic influences, that is, with varying degrees of organic enrichment. The sites included those in proximity to a river mouth, marina, cannery, effluent outfall, port areas, and southern bluefin tuna farms and also sites away from any of these anthropogenic influences (Table 4.1). A Shipek grab (200 mm by 200 mm) was used to collect replicate samples at each site (see Chapter 2). This sampling strategy was used to gain an insight into the infaunal communities characterising the Port Lincoln region. Based on morphology, various organisms were identified to the taxonomic levels of class or family, which included the polychaete families Capitellidae, Cirratulidae, Lumbrineridae, Nephtyidae and Spionidae. DNA was then extracted from these organisms and sequenced.

Table 4.1 Sites and activity descriptions with coordinates (WGS84) in decimal degrees where sediment samples were collected and processed for infauna, which were characteristic in the Port Lincoln region.

SiteID	Site	Site description	Coordinates	
PL01	Proper Bay	Cannery area	S 34.77217 ^{o}	E 135.84272 ^{o}
PL02	Porter Bay	Marina area	S 34.73820 ^{o}	E 135.88623 ^{o}
PL03	Billy Lights Point	Effluent discharge area	S 34.74930 ^{o}	E 135.89535 ^{o}
PL04	Brennan Jetty	Grains loading terminal area	S 34.71802 ^{o}	E 135.86813 ^{o}
PL05	Stinky Creek	River area	S 34.66315 ^{o}	E 135.85845 ^{o}
PL06	Kangaroo Reef	Reef area	S 34.66367 ^{o}	E 135.92642 ^{o}
PL07	Point Boston	Abalone farm area	S 34.64990 ^{o}	E 135.94070 ^{o}
PL08	Louth Bay	Former tuna sea-cage area	S 34.60325 ^{o}	E 135.94410 ^{o}
PL09	River Todd	River mouth	S 34.59430 ^{o}	E 135.91703 ^{o}
PL10	Hayden Point	Channel area	S 34.72953^{o}	E 135.95747 ^{o}
PL11	Rabbit Island	Tuna farming zone	S 34.60830 ^{o}	E 135.99972 ^{o}
PL12	Davidson Rock	Management zone	S 34.67825 ^{o}	E 135.98897 ^{o}
PL13	Picnic Beach	Tuna farming zone	S 34.71527 ^{o}	E 135.96580 ^{o}
PL14	Fanny Bay	Sea-cage holding area	S 34.72613 ^{o}	E 135.94057 ^{o}
PL15	Carcase Rock	Control area	S 34.76713 ^{o}	E 136.01965 ^{o}

DNA extraction of organisms

DNA was extracted from individual organisms using a commercially available DNA extraction kit, MoBio DNA extraction kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA).

Separation of target taxa using 28S rDNA sequences

Universal PCR primers (modified from Brown *et al.* 1999) were used to amplify selected ribosomal gene regions (28S, 18S). Amplified regions were sequenced using direct sequencing. A database of these sequences was developed and used to identify target-specific sequences at the family level. Specific DNA primers for each family group were developed based on these sequences.

28S sequences were placed into a sequence manipulation software program Bioedit[10], and aligned using Clustal W (Thompson *et al.* 1994). A neighbour-joining tree (DNADist by Joseph Felsenstein) was generated using Bioedit default settings, and sequence groups were examined.

There was a good separation of taxa based on family level groups (Figure 4.1), which demonstrated the utility of the 28S gene region for probe development. The sequences obtained were also checked against known sequences in the public domain (GenBank) to ensure their integrity. Genbank is an annotated collection of all publicly available nucleotide and amino acid sequences, maintained by The National Centre for Biotechnology Information (NCBI), a division of the National Library of Medicine (NLM) at the National Institutes of Health (NIH). NCBI has developed databases to deal with molecular data, and facilitates the use of molecular databases by the research and medical community[11].

Good separation of target groups, based on 28S sequences, meant that it was likely that specific gene probes could be developed for target taxa based on this gene region. To develop specific probes, sequences of target taxa and related taxa were aligned and regions of DNA sequence were identified. These areas were checked to ensure that they showed commonality amongst the target taxa but were different from related taxa. Specific primers/probe are then designed to incorporate these regions of unique sequence.

[10] http://www.mbio.ncsu.edu/BioEdit/bioedit.html

[11] http://www.ncbi.nlm.nih.gov/

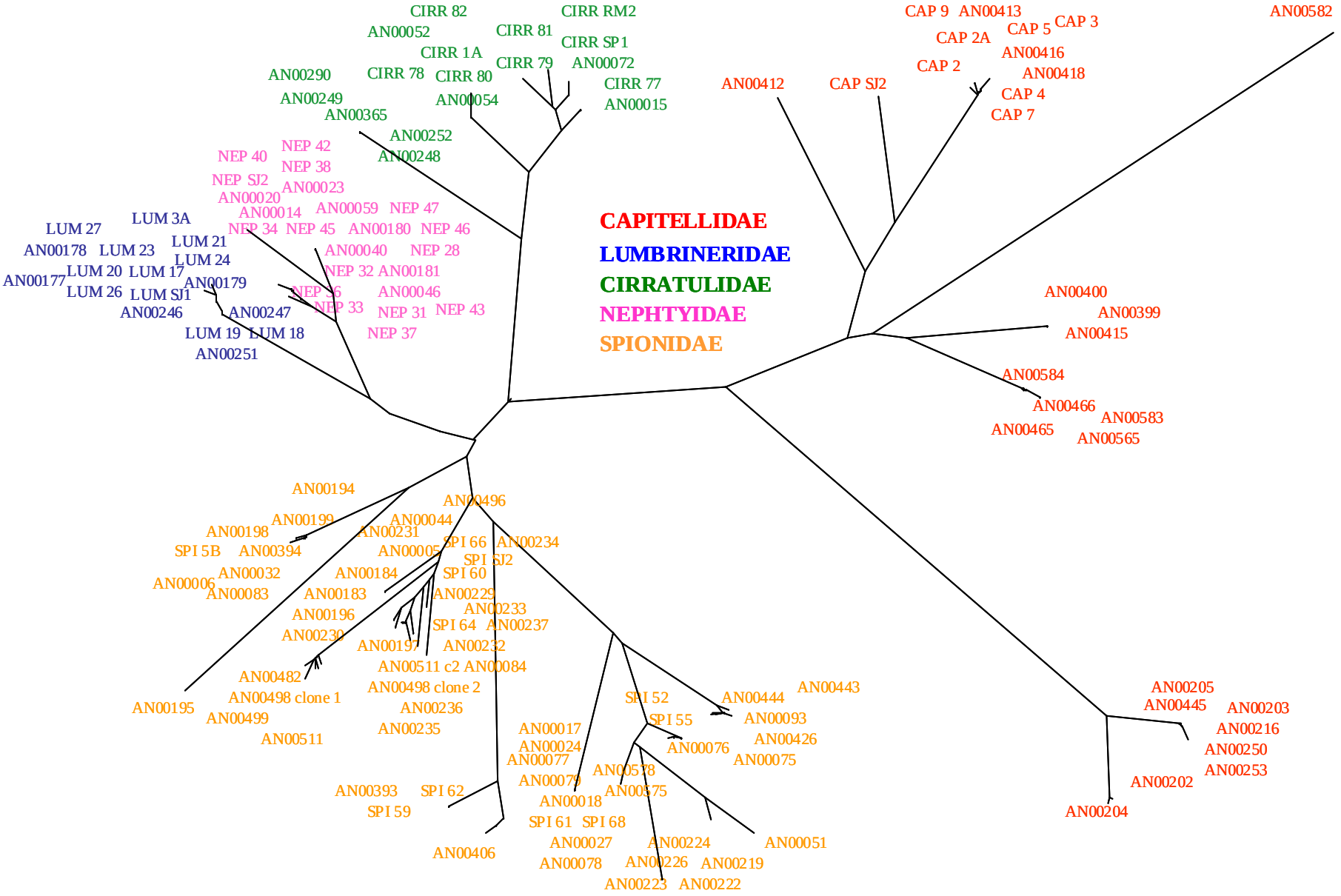

Figure 4.1 Dendrogram of relatedness based on 28s rRNA sequences for collected organisms sequenced.

The need to develop additional Spionidae probes

Initially, the intent was to design a single assay to detect all spionids but it was recognised early on that the family Spionidae is more complex taxonomically than the other target taxa. This complexity is also indicated in the literature, where extensive work had been carried out on spionids of the northern hemisphere; however, information on the taxonomy of spionids in the southern hemisphere is lacking (Read 1975, Blake and Kudenov 1978, Blake 1996). From the work on the development of a single spionid assay, it became apparent that the spionids fell into two clear groups based on the 28S ribosomal DNA sequences. These two groups corresponded to the "polydorid" and "non-polydorid" groups of Spionidae as described in Chapter 3.

Spionids are generally indicative of organically enriched sediments (Pearson and Rosenberg 1978), although they do have different responses to organic enrichment depending on the species (Pearson and Rosenberg 1978, Zajac and Whitlatch 1982a). Non-polydorid spionids are usually found when pollution has started to abate while polydorid spionids tend to be opportunistic species responding to disturbance by rapidly colonizing defaunated habitats (Grassle and Grassle 1974).

On this basis, two separate DNA probes were designed for use in the real-time PCR system. However, subsequent calibration experiments indicated that the assays were still not sufficiently specific or sensitive; hence they were re-designed several times to include the various species of spionids found in Port Lincoln. This resulted in the non-polydorid group being further split into three subgroups (see Chapter 7).

Probe specificity

DNA probes were designed for Capitellidae and Lumbrineridae, and subsequently for Cirratulidae, and Nephtyidae. As detailed above, two Spionidae DNA probes (polydorid and non-polydorid) were developed when specificity testing with the initial single probe did not detect all spionids. The non-polydorid group was subsequently split into three subgroups, each requiring a separate detection assay. Similarly, Capitellidae was split into two subgroups later in this project (see Chapter 7). All assays developed were tested on a collection of 250-300 individually identified organisms to check for specificity.

The potential contamination[12] of specimens used in the specificity testing was also considered, in particular for Capitellidae and Spionidae. Two different approaches were used to investigate this issue; the first involved purging specimens to ensure that they voided any internal or external material and the second was through the cloning of rDNA into bacterial vectors, in order to ensure only single organism DNA was sequenced.

The objective of these experiments was to determine whether individual specimens were contaminated either internally or externally by other taxa. If contamination of the specimens was an issue, then this would explain cross-reactivity of the assays and would also assist in the development of specimen handling protocols for specificity testing in the design of new and additional probes.

Real-time PCR assays

All probes were initially designed as specific PCR primers, with an oligonucleotide hybridisation probe, designed for end-point DNA detection. In the extension phase of the project, all assays were re-designed in the real-time PCR format, which is a generic assay technology, providing superior quantification over a greater linear range (see below). As the Spionidae tests were designed during the extension phase of the project, they were only designed in real-time PCR format.

Specific real-time PCR tests were subsequently designed for all the tests based on the sequence information generated above using the Applied Biosystems TaqMan MGB^{TM} probe system. TaqMan assays were tested initially on a DNA dilution series of target organism DNA to confirm that the tests were quantitative and assay specificity was confirmed as discussed above.

Discussion

Assays for Capitellidae, Lumbrineridae, Cirratulidae and Nephtyidae were initially developed in the end-point detection assay format but all assays were subsequently redesigned in real-time PCR format when the platform became available to the project.

[12] In this context contamination refers to the potential for other taxa to interfere with the specificity testing. The most obvious mechanism by which such contamination may occur is when one organism (e.g. a polydorid spionid) eats another (e.g. a capitellid). When polydorid spionids are extracted from the sediment and assayed for DNA specificity there may well be a cross-reaction because some capitellid DNA is contained in the gut of the organism under investigation. Such contamination would make it difficult to infer whether or not the test was really non-specific (i.e. the capitellid probe responds to polydorid DNA rather than capitellid DNA that was contained in the gut of the polydorid).

The real-time PCR assay has advantages over the end-point detection assay in that it maintains linear quantification over a greater range of biomass and is faster to run with at least a day less time required for processing. In addition, real-time PCR technology is simpler to transfer to other laboratories if the opportunity arises.

Although not developed further in this study, the information obtained about taxonomic relationships within the Spionidae provides fertile grounds for potential taxonomic studies on this family in the future. The 28S rDNA separates the family into two clear groups; consistent with the morphologically based separation of this family into the "polydorid" and non-polydorid groups (Blake 1996). Several attempts were made to design tests that would detect all non-polydorid species at Port Lincoln, but the non-polydorid group is taxonomically diverse and this was not possible.

The DNA assays developed in this project had good specificity in detecting organisms within the target taxa. However, specificity testing did reveal occasional anomalies. These anomalies were subsequently found to be related to the difficulties in obtaining pure specimens of some taxa and was further addressed by the work detailed in Chapter 7.

Chapter 5 DNA Extraction and Quantification

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This chapter will begin to address the objective of developing a system for the rapid detection of selected taxa in sediment samples using the specific DNA probes developed as discussed in Chapter 4.

Introduction

Worldwide, the extraction of clean DNA from a broad range of soil and sediments has been a major limitation to the development of DNA assays for soil-borne organisms. A system for extracting DNA from sediments, in a cost-effective manner, which can supply reasonable throughput of samples, and yield clean DNA from a wide range of sediment types, is an essential precursor to any routine monitoring system based on DNA assays.

The South Australian Research and Development Institute (SARDI) has developed a DNA extraction system, which is now used routinely on a broad range of soil samples, for the purpose of quantifying soil-borne fungi and nematodes. This system was evaluated for use on marine sediments in this research. Issues of quantification of target DNA in different sediment types and impact of sample handling were examined to determine how robust and reliable the assays were for routine monitoring.

The possibility that contaminants such as humic acids and phenolic compounds found in soils are also present in marine sediments was considered. If present, contaminants may be concentrated with the DNA during extraction. These contaminants can inhibit *Taq* polymerase activity, affecting hybridisation specificity and thereby inhibit PCR (Picard *et al.* 1992, Wilson 1997).

Materials and methods

Initial work focused on a comparison of SARDI's "in-house" DNA extraction method (RDTS method) from soil and a commercially available soil DNA extraction kit (MoBio Laboratories Inc., Carlsbad, CA, USA). The yield and amplification of the two extraction methods were compared and the extraction processes were measured against DNA recovery and purity.

DNA yield

To evaluate the MoBio and RDTS DNA extraction systems, contrasting marine sediment samples were collected from a subtidal site off Christies Beach in Gulf St Vincent and an intertidal site off Snowden's Beach along the Port River; all near Adelaide, South Australia. Soil samples to act as controls were collected from Waikerie, South Australia. Samples collected from the Snowden's Beach site, along the Port River waterline at low tide, were expected to have high levels of organic and inorganic inputs from surrounding activities.

DNA was extracted using MoBio and RDTS methods, with varying sample: buffer ratios. *Metarhizium* DNA was added to extracted sediment DNA samples prior to polymerase chain reaction (PCR) amplification. *Metarhizium* is a fungus and DNA from this taxon is unlikely to be present in marine sediments. *Metarhizium* DNA was detected after PCR via end-point detection. The *Metarhizium* DNA was used as a control to detect any differences in PCR efficiency that may suggest the presence of a contaminant in the sediment DNA extract.

DNA recovery and purity

The RDTS DNA extraction system was tested on a range of sediments collected from both South Australia and Tasmania. The sediment samples were collected from sites close to and away from anthropogenic influences (i.e. with varying degrees of organic enrichment). South Australian sites were similar to the ones given in Table 4.1 and the Tasmanian sites included those adjacent to and distant from a salmon farm.

The extraction process was measured against two criteria, DNA recovery and purity. DNA recovery was measured by the addition of a reference organism (brewers yeast) to sediment samples prior to DNA extraction. Total DNA was then extracted from the sample and the yeast quantified by DNA assay. DNA purity is a measure of PCR inhibition, that is, inhibition of the enzyme (*Taq* polymerase) which catalyses the PCR reaction. DNA purity was measured by the addition of exogenous fungal DNA (*Metarhizium*, as described above) directly into the PCR reaction. This exogenous fungal DNA was then quantified after amplification and compared to reference sediment, known not to contain substances inhibitory to *Taq* polymerase.

Quantification of target DNA in sediments

DNA extracted from target taxa was quantified and a dilution series added to sediment. The purpose of this experiment was to demonstrate that the assays were able to accurately

quantify target DNA in a background of sediment DNA. DNA in the range of 0-1000 fg/PCR reaction for Capitellidae and 0-2000 fg/PCR reaction for Lumbrineridae were added to sediments and then quantified.

Impact of sediment type on added DNA

One of the problems encountered in DNA quantification from environmental samples is the impact of background soil or sediment type on the results. The reason for this is that soils, sediments and water samples contain varying amounts of organic compounds, which can inhibit the chemistry of PCR.

Calibration was undertaken with a range of sediment types (from clay to sandy sediment) to ensure that quantification was reliable regardless of sediment type. Both capitellids and lumbrinerids were used in experiments designed to determine the impact of sediment type. Frozen capitellids/lumbrinerids were added to the range of sediment samples, after which DNA was extracted from the spiked sediments. A dilution series was made of the extracted DNA and quantification assays were carried out using the specific assays developed.

Results

DNA yield

For both extraction systems (RDTS and MoBio), the total yield increased with increasing sample:buffer ratio. However, the RDTS method produced 1.5 to 2 times more DNA than the MoBio extraction kit.

The Christies Beach samples had a lower concentration of PCR inhibitors than the Snowden's Beach samples. This is indicated by the more efficient amplification of the *Metarhizium* DNA added to the PCR mixture (data not shown). However, the effect of inhibitors in the Snowden's Beach samples on PCR efficiency was minimised by reducing the sample:buffer ratio (10 g sediment:20 ml buffer) for DNA extraction and by diluting the DNA prior to PCR. The decreased inhibition was measured by comparing the efficiency of PCR amplification of added *Metarhizium* DNA to the reference soil (from Waikerie), which had no known inhibition (Table 5.1).

Table 5.1 Effect of RDTS sample:buffer ratio and DNA dilution on PCR inhibition.

Sample	Sediment Weight (g)	Buffer (ml)	DNA Yield (pg/µl)	*Metarhizium* reading DNA Dilution 1:2	1:10
Control Soil (Waikerie)	10	20		15983	42262
Snowden Beach #2	10	20	4574	9623	36202
Snowden Beach #3	10	20	3408	13511	45332
Snowden Beach #4	10	20	4433	10458	33530
	10	10	8641	5878	15589

DNA recovery and purity

Using the optimised RDTS extraction protocol, the added yeast was recovered equally well from all samples of all sites, indicating that organically enriched sediments did not interfere with DNA extraction (Figure 5.1). The quantification of exogenous fungal DNA was the same for all sediment samples and the reference soil, with no significant PCR inhibition, indicating that DNA purity was the same for sediment samples in comparison with the reference soil (Figure 5.2). Therefore, DNA extracted using the RDTS protocol was considered to be suitable for use in quantitative PCR to assess levels of target organisms.

Quantification of target DNA in sediments

The assays designed to detect each of the target taxa were capable of quantitatively detecting DNA of those taxa in a background of sediment DNA. The amount of capitellid DNA recovered was strongly correlated to the amount added over the range of 0 fg/PCR reaction to 1000 fg/PCR reaction (r^2 = 0.9826, Figure 5.3). This was similarly observed in added and detected Lumbrineridae DNA (r^2 = 0.9938, Figure 5.4) in background sediment DNA.

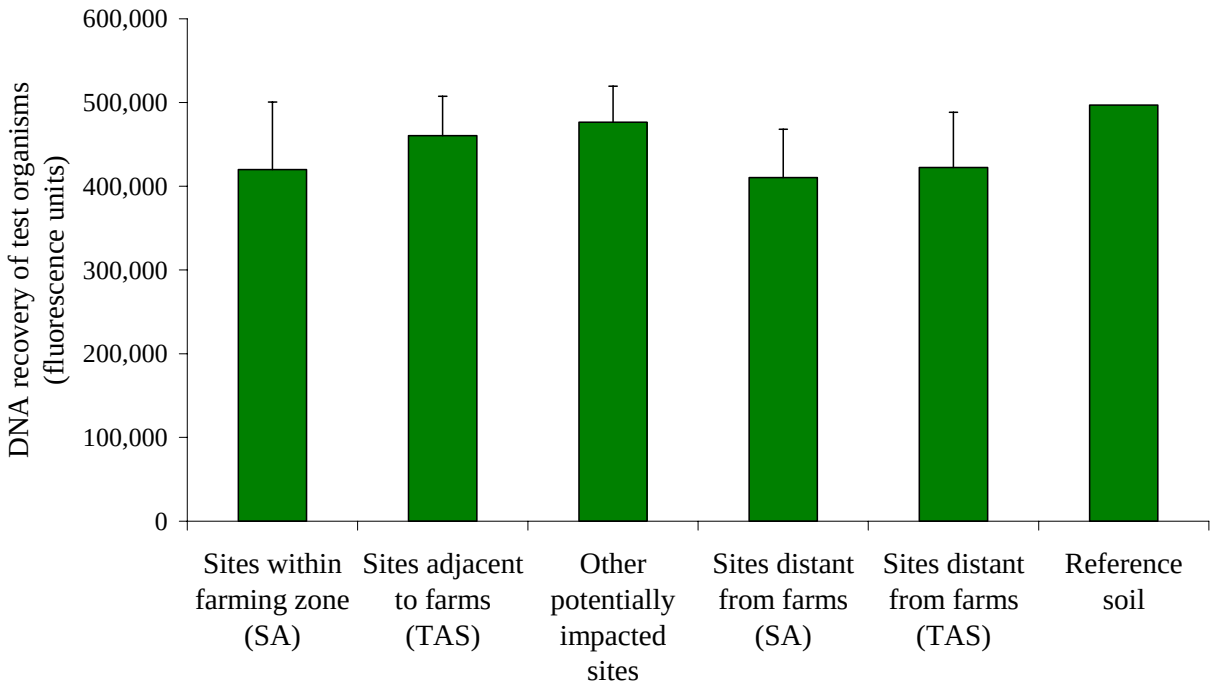

Figure 5.1 DNA recovery of test organisms for samples collected from different sites in South Australia (SA) and Tasmania (TAS) with varying degrees of organic enrichment, compared to reference soil.

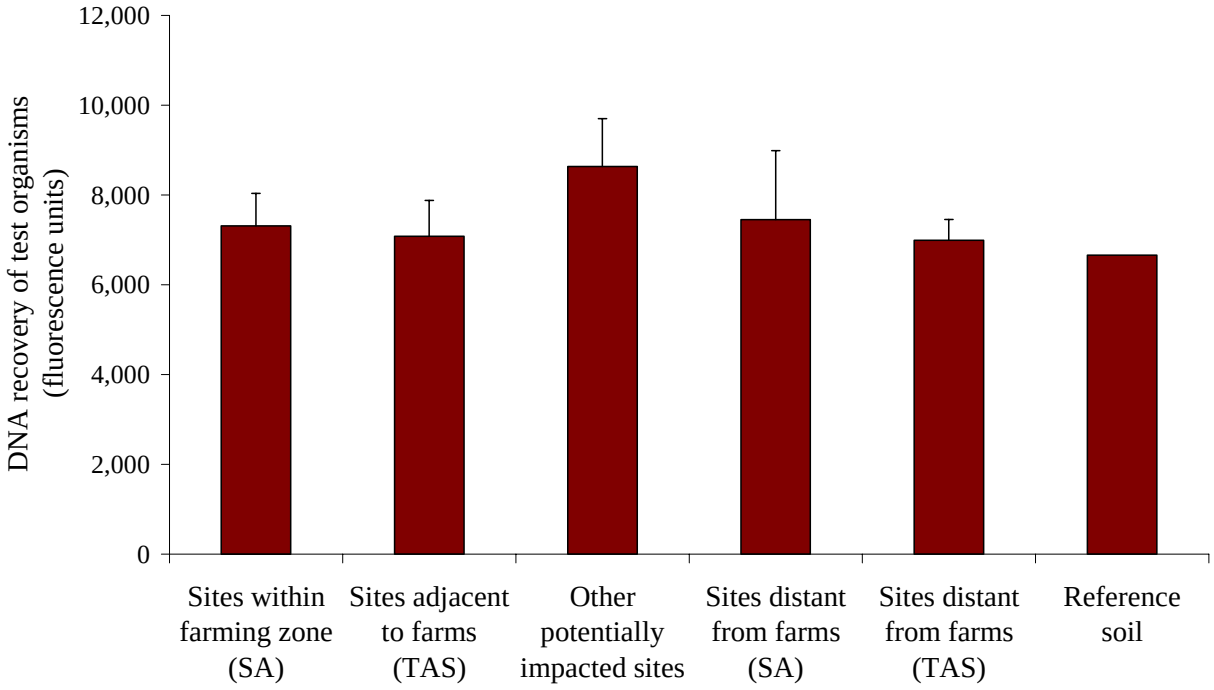

Figure 5.2 DNA purity of samples collected from different sites in South Australia (SA) and Tasmania (TAS) with varying degrees of organic enrichment, compared to reference soil.

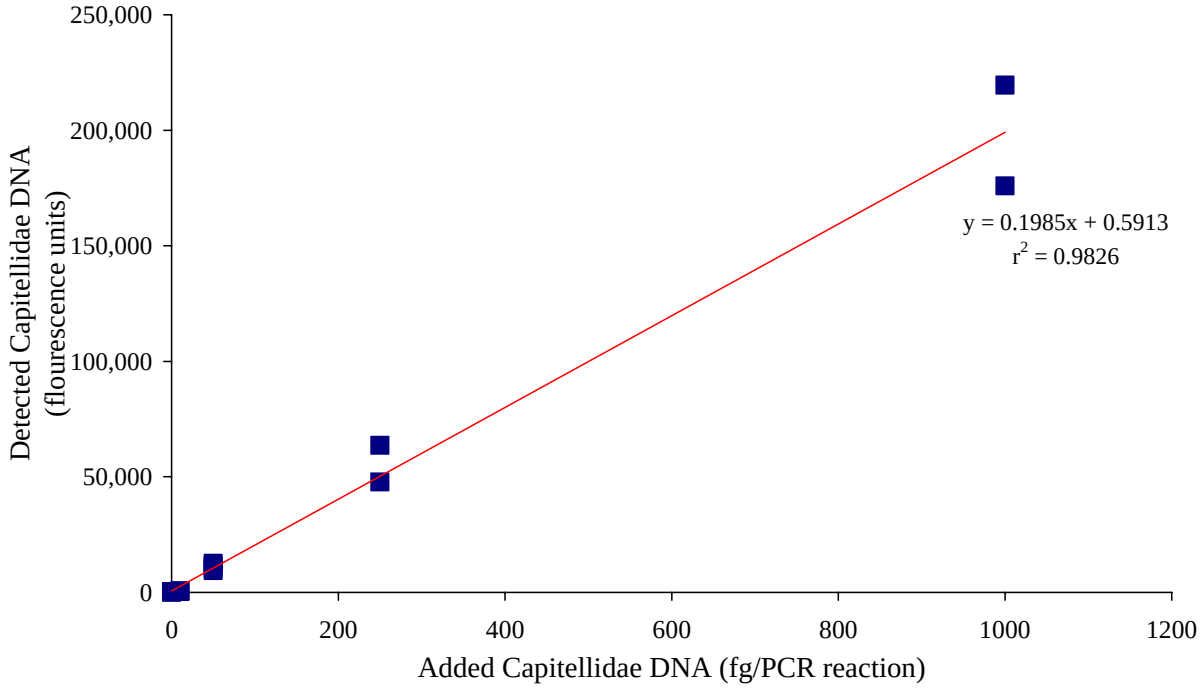

Figure 5.3 Quantification of Capitellidae DNA (0-1000 fg/PCR reaction) in extracted sediment DNA.

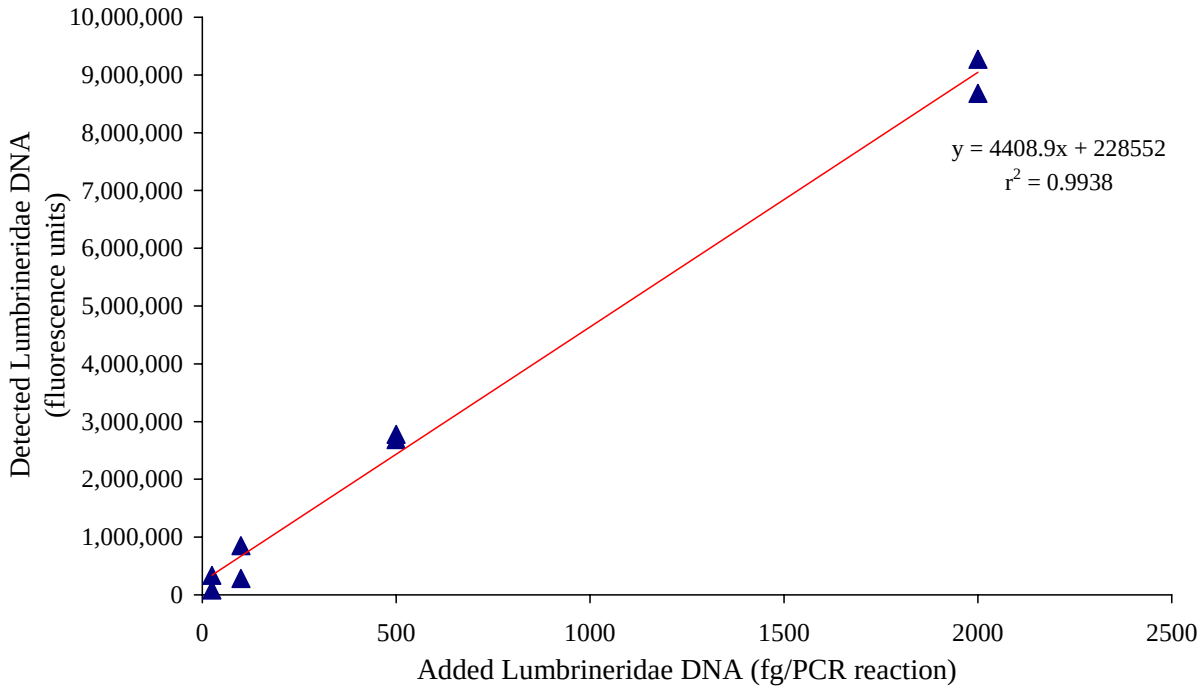

Figure 5.4 Quantification of Lumbrineridae DNA (0-2000 fg/PCR reaction) in extracted sediment DNA.

Impact of sediment type on added DNA

Linear calibrations were achieved for added versus detected Lumbrinerid DNA for three sediment types although differences existed in the slopes obtained for each dilution series (Figure 5.5). These differences in slopes may be attributable to differences in the sizes of organisms between dilution series or differences in DNA extraction efficiency from different sediment types.

If DNA extraction efficiency differed between sediment types, this level of assay inhibition can be overcome by the use of an internal control. The internal control corrects for this relatively minor DNA inhibition and/or minor differences in extraction efficiency and such a control is used in the RDTS soil analysis system.

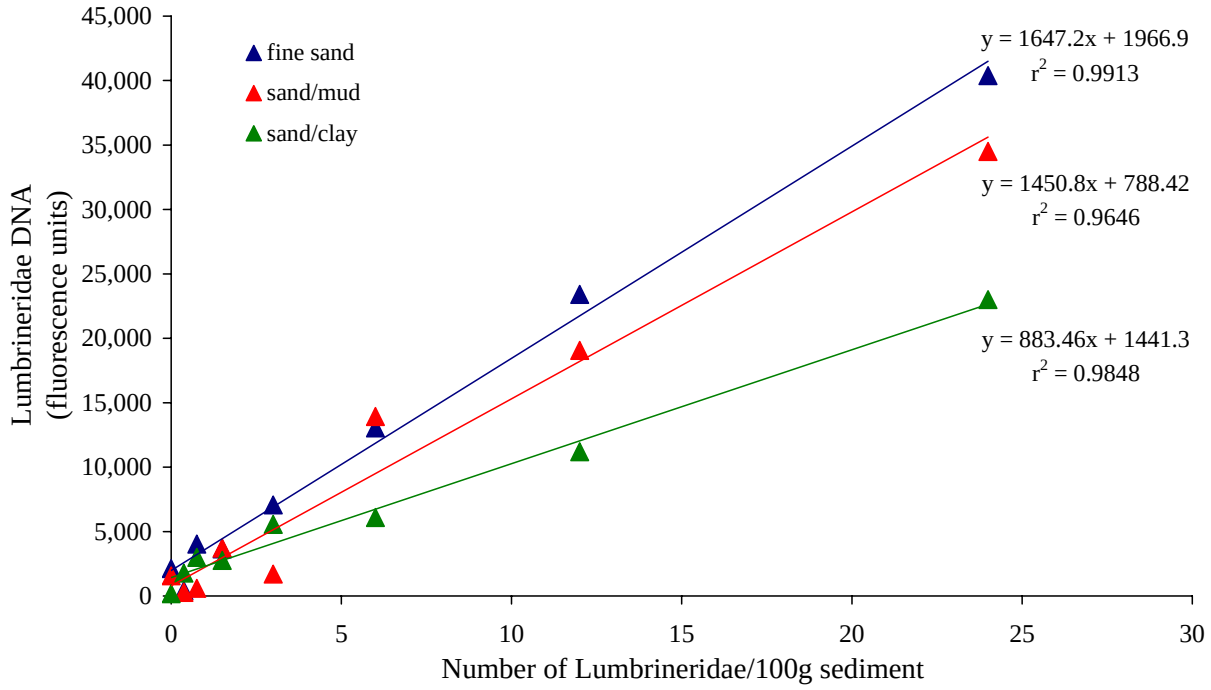

Figure 5.5 Quantification of Lumbrineridae DNA in three different sediment types.

Discussion

In order to develop a system for the rapid detection of selected taxa in sediment samples using specific DNA probes, the extraction and quantification of DNA using the RDTS protocol used for soil had to be optimised for marine sediments. Sediment DNA was therefore assessed for yield by measurement of total extracted DNA, and for purity by

measurement of quantitative amplification of a known “spiked” target organism. Initial results indicated that DNA yield was low from sediments; however, several parameters were subsequently optimised in the extraction process, which were then successful in increasing DNA yield for all sediment types. Furthermore, to achieve quantitative DNA amplification, conditions for PCR amplification from extracted DNA had to be optimised and it was also essential to remove inhibitory substances such as humic and fulvic acids which co-extracted with DNA.

The RDTS method was found to be satisfactory for achieving quantitative DNA extraction and amplification. The problem of potential inhibitors could also be addressed by the method and low-level inhibition could be corrected with the implementation of internal PCR controls. Therefore the RDTS method can be used for routine analysis of marine sediments.

Chapter 6 Proof of Concept – Calibration of DNA Tests

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Having developed specific DNA assays to quantify DNA of the target organisms in a background of sediment DNA (Chapter 4 and Chapter 5), this chapter will address the objective of evaluating the extent to which this system could be routinely applied to provide quantitative estimates of the relative abundance of indicator taxa.

Introduction

The assays developed were validated using sediment samples "spiked" with known numbers of target organisms to determine the sensitivity and linear range of the assays. If a good correlation between the DNA readings and added numbers of organisms under controlled conditions was established, the assessment of the correlations to samples collected from the field, where there are more variables could then proceed.

One concern expressed about the use of the DNA-based assays is that they potentially detect dead organisms, which may cause overestimation of biomass. Experiments were therefore undertaken to examine the persistence of DNA in recently dead organisms in sediment samples over time. The move from an end-point detection system to a real-time platform was also evaluated when this technology was available part way through the project.

Materials and Methods

Quantification of added target organisms

Calibrations were carried out by adding known numbers of target organisms to sediment samples. Target animals were obtained from multiple sediment samples collected from either Barker Inlet or Port Lincoln. These sediment samples were processed immediately after collection and live target animals were sorted, identified and individually weighed. The animals were kept either alive or frozen and added to individual frozen sediment samples (Table 6.1).

Table 6.1 Treatment of target organism and sediment for quantification experiment.

Target taxa	Treatment of organism	Treatment of sediment
Capitellidae	Frozen	Oven-dried frozen
Lumbrineridae	Frozen	Oven-dried frozen
Cirratulidae	Frozen	Oven-dried frozen
Nephtyidae	4^0C	Oven-dried frozen
Spionidae	Frozen	Oven-dried frozen

Different numbers of animals (0, 1, 2, 5, 10, 15 or 20 individuals) were added to a known amount of sediment where possible and three replicates of each treatment were used. After drying, DNA was extracted from each spiked sediment sample, and DNA quantified using the specific assays developed. For the cirratulid and lumbrinerid spiking experiments, DNA was quantified using both end-point detection and real-time PCR assays. Spiking experiments for nephtyids, capitellids and spionids were run using real-time PCR assays only. Correlation analyses were carried out between DNA of target taxa with number of added target taxa. Similar correlation analyses were also carried out between DNA and biomass of target taxa.

Quantification of live versus dead organisms

To ensure the validity of the DNA-based assays developed, it was important to determine whether there was over-estimation as a consequence of the persistence of DNA from dead organisms in sediments. Persistence of DNA in soil had been studied by various laboratories (see Herdina *et al.* 2004 for references). The results from these studies showed that DNA persistence in soil varied with different soil types and environmental conditions, but generally DNA degraded very quickly. The work by Herdina *et al.* (2004) on persistence of DNA of a fungus in soil as measured by DNA-based assay also concluded that the rate of breakdown of DNA of the fungus (in particular dead fungus) was very high and DNA from dead fungus probably contributed little to the total DNA extracted.

To investigate if these results would be obtained with the target organisms in this research, experiments were carried out to compare the detection of live cirratulid polychaetes with results of killed cirratulids added to sediments. Detection of killed cirratulids added to sediments was also monitored over a three-week period to determine whether the DNA assays continued to detect dead organisms over time, or whether DNA degraded over time.

Cirratulids were killed by incubation at -20ºC for 60 min. Five cirratulids were added to each 250 g sediment sample and incubated at 15ºC for 0, 2, 4, 7, 14 or 21 days. Control sediment samples with no cirratulids added were incubated at the same time, and another control with five live cirratulids/250 g sediment (at day 0 only) was included in the experiment. Four replicates of each treatment were used.

Quantification of biomass using real-time PCR versus end-point detection assay

The assays for Lumbrineridae, Cirratulidae and Nephtyidae were initially developed in the end-point detection format. With the availability of real-time PCR to the project, there was the opportunity to evaluate this newer technology. Experiments were therefore set up to compare the two assay formats, end-point detection and real-time PCR. This was carried out using DNA extracted from the sediment samples spiked with a known biomass of lumbrinerids. The experiment was then repeated using cirratulids but not with nephtyids as it was difficult to obtain sufficient numbers of nephtyids for spiking.

Results

Quantification of added target organisms

Number of organisms

Results from the quantification of added target organisms demonstrated a positive correlation of DNA readings to added number of Lumbrineridae (r^2 =0.6975, Figure 6.1) and also for number of Cirratulidae individuals (r^2 = 0.7712, Figure 6.2) in real-time detection assays. However, it was evident that the correlation was very dependent on the size of organism added to sediment. For Cirratulidae, individual organisms were sorted by size visually and added in three separate series where each replicate was composed of a series of small, medium or large organisms. When correlations were made separately to each series of cirratulids (Figure 6.3), the correlation coefficients were much higher with r^2 values ranging from 0.8091 to 0.9820. Similar quantification results were obtained with the other target groups (data not shown).

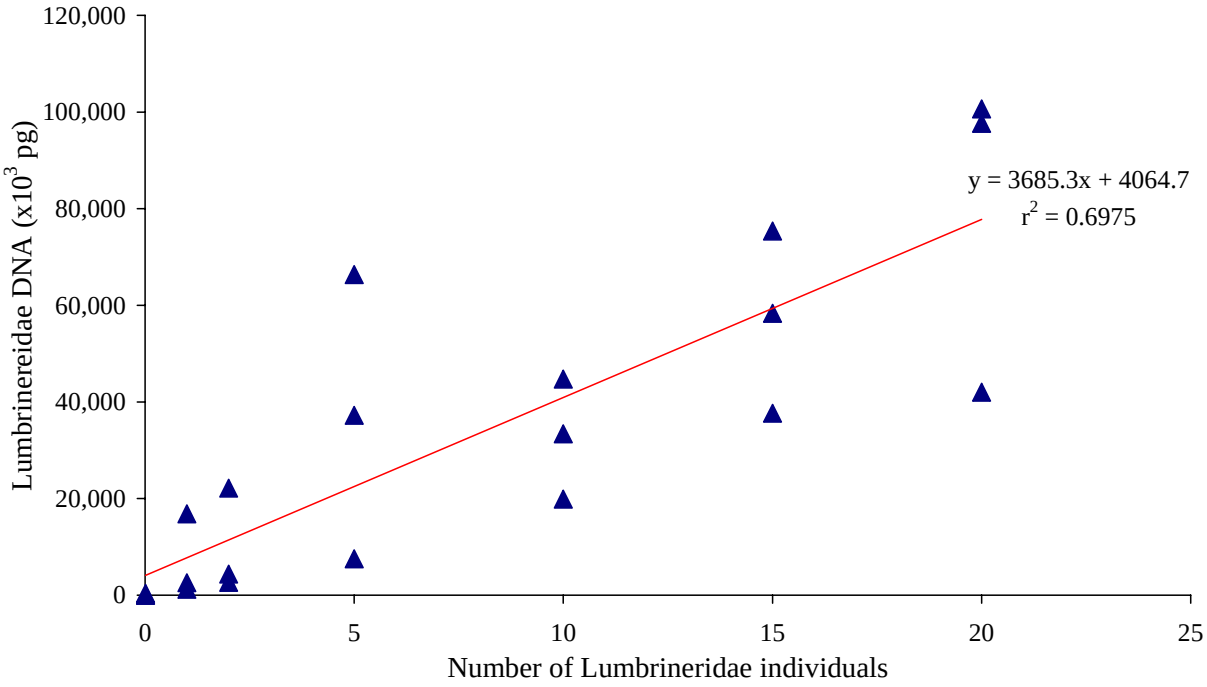

Figure 6.1 Relationship between number of Lumbrineridae individuals added to sediment and Lumbrineridae DNA extracted using real-time PCR assay showing positive correlation (r^2 = 0.6975). Line does not intercept at zero to account for background Lumbrineridae DNA in sediment used.

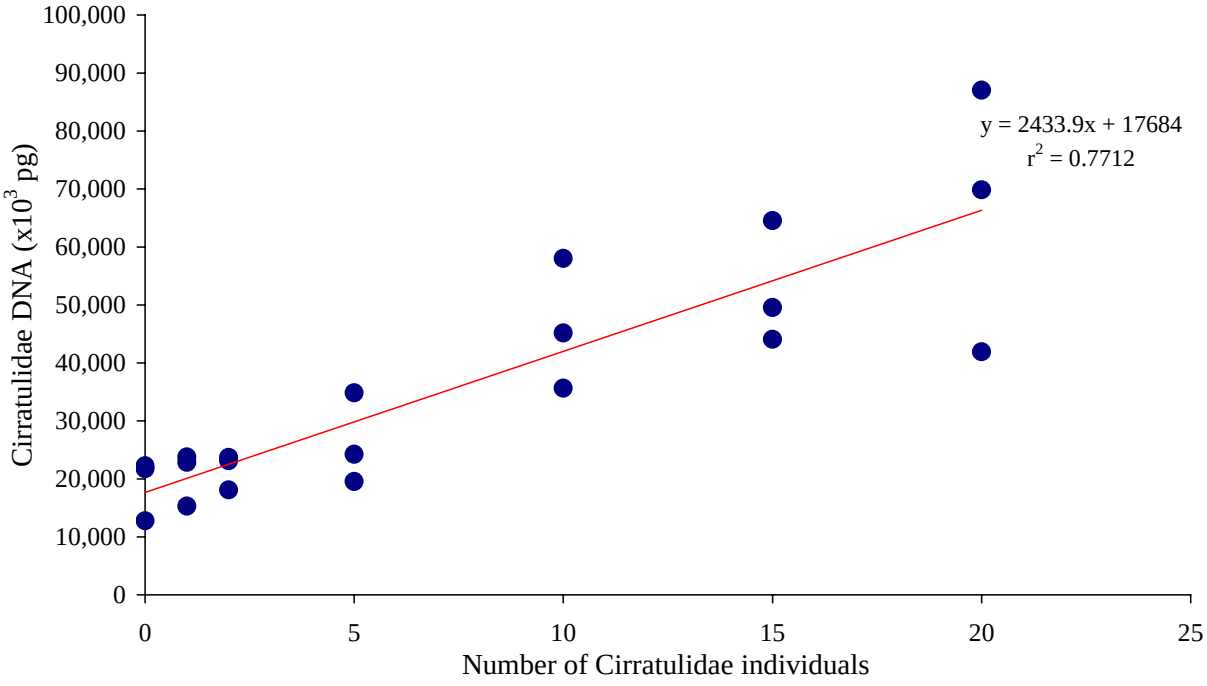

Figure 6.2 Relationship between number of Cirratulidae individuals added to sediment samples regardless of size ranges and Cirratulidae DNA extracted using real-time PCR assay showing positive correlation (r^2 = 0.7712). Line does not intercept at zero to account for background Cirratulidae DNA in sediment used.

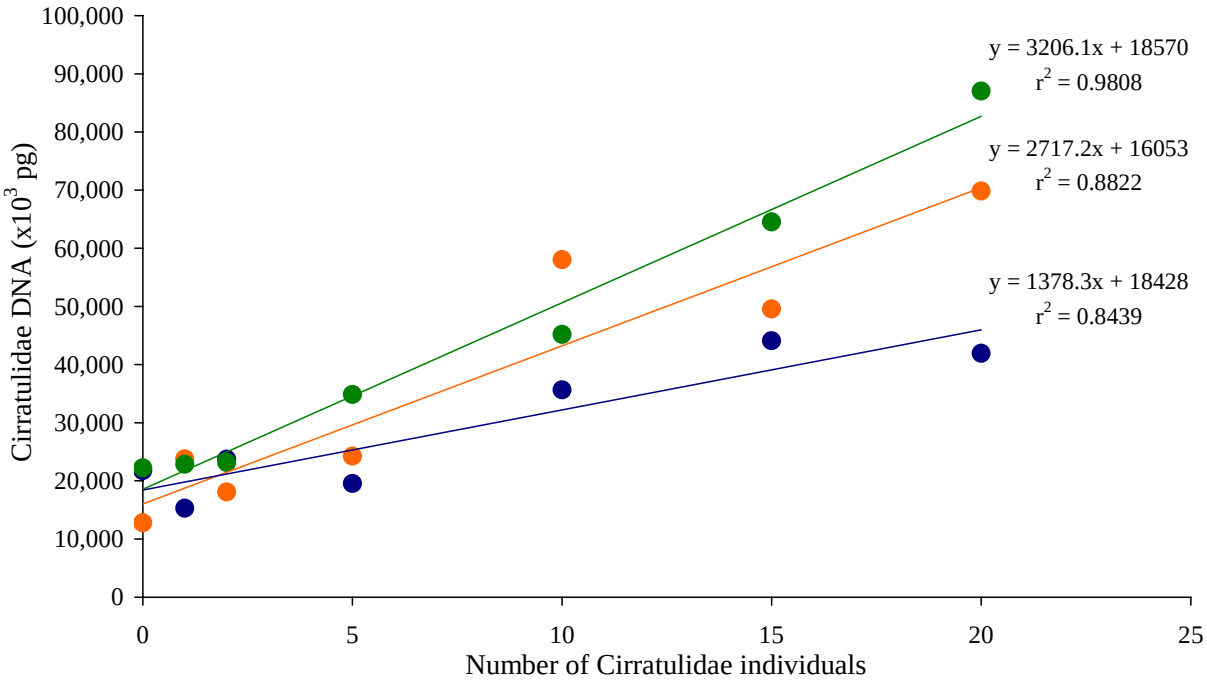

Figure 6.3 Relationship between number of Cirratulidae individuals of different size ranges added to sediment samples and Cirratulidae DNA extracted using real-time PCR assay showing positive correlations. Average biomass of individuals for the three size ranges are: ● = 0.042g, ● = 0.028g and ● = 0.011g. Line does not intercept at zero to account for background Cirratulidae DNA in sediment used.

Biomass

Correlation analyses were repeated using individually weighed target organisms. Results showed a much stronger correlation of DNA readings and biomass of added organisms over the same range. The r^2 value for correlation of DNA to biomass of Cirratulidae was 0.8778 (Figure 6.4) as compared to 0.7712 for numbers of Cirratulidae (Figure 6.2). Correlation of DNA and biomass of Lumbrineridae also gave a higher positive r^2 value of 0.8482 (Figure 6.5) as compared to numbers of Lumbrineridae (Figure 6.1). This difference in correlation with abundance and with biomass was probably due to the high degree of variation between the sizes of individual organisms obtained from the sediment samples as shown in Figure 6.3.

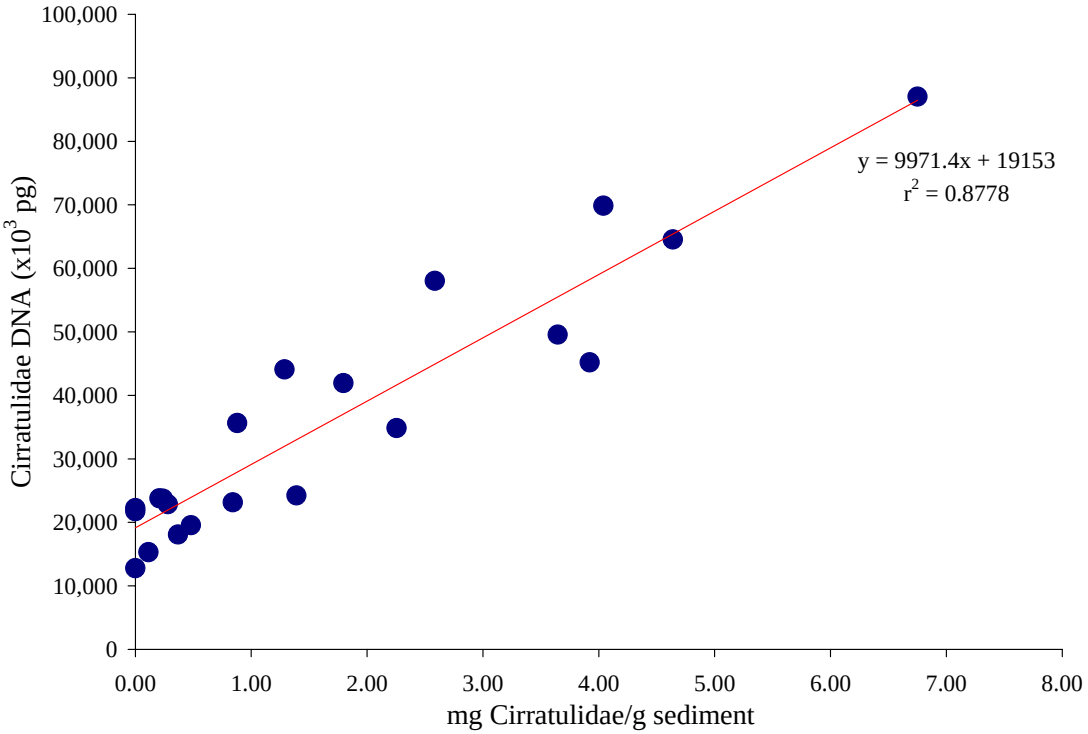

Figure 6.4 Relationship between biomass of Cirratulidae added to sediment samples and Cirratulidae DNA extracted using real-time PCR assay showing positive correlation (r^2 = 0.8778). Line does not intercept at zero to account for background Cirratulidae DNA in sediment used.

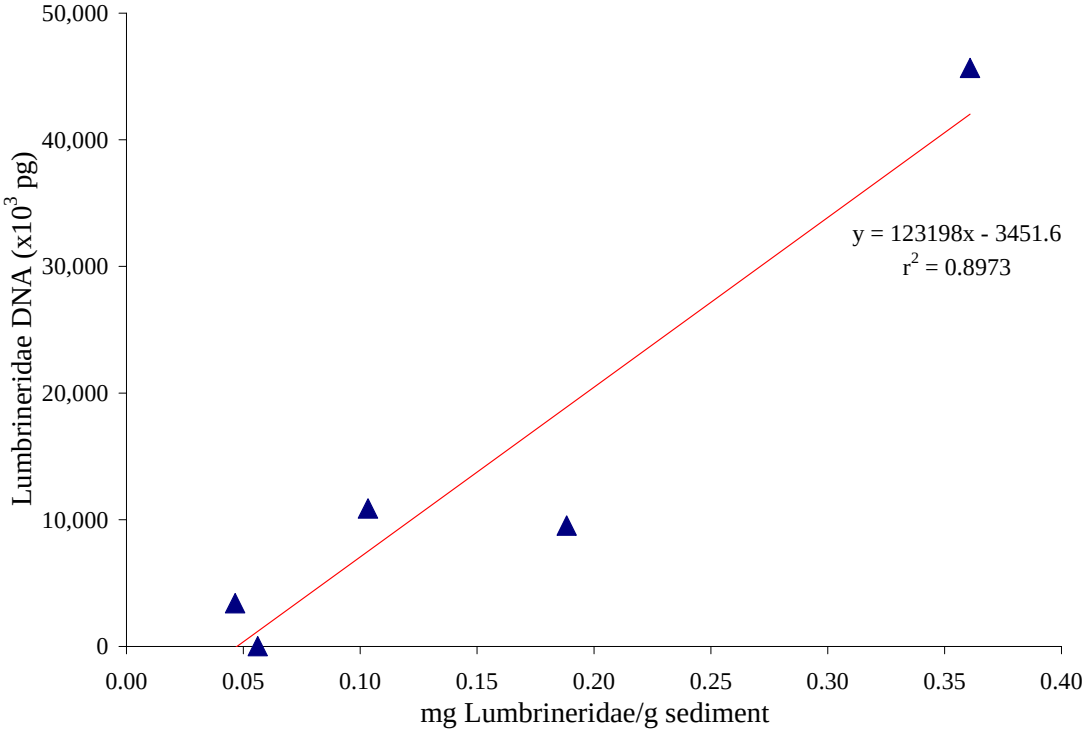

Figure 6.5 Relationship between biomass of Lumbrineridae added to sediment samples and Lumbrineridae DNA extracted using real-time PCR assay showing positive correlation (r^2 = 0.8973). Line does not intercept at zero to account for background Lumbrineridae DNA in sediment used.

Quantification of live versus dead organisms

The mean DNA reading for sediments spiked with live cirratulids compared to freshly killed cirratulids was different at Day 0. For sediment spiked with five live cirratulids, 438,842 pg Cirratulidae DNA/g sediment (or 4059 pg Cirratulidae DNA/g Cirratulidae added) was recovered while for sediments spiked with five freshly killed cirratulids, recovery was only 342,940 pg Cirratulidae DNA/g sediment (or 2734 pg Cirratulidae DNA/g Cirratulidae added, Figure 6.6). This difference in recovered DNA, although not significant, between samples spiked with freshly dead cirratulids and samples spiked with live cirratulids, indicated that the DNA of killed animals started to decline almost immediately.

By Day 14, DNA of added killed cirratulids had declined to background levels (Figure 6.6). This indicated that, although the DNA assay will detect intact dead organisms, they degraded over relatively short periods of time. The sediments had natural background levels of cirratulids. Therefore the sediment with no added cirratulids also showed a decline in DNA detection over the same period (Figure 6.6). This decline is probably due to death resulting from the incubation conditions of the experiments.

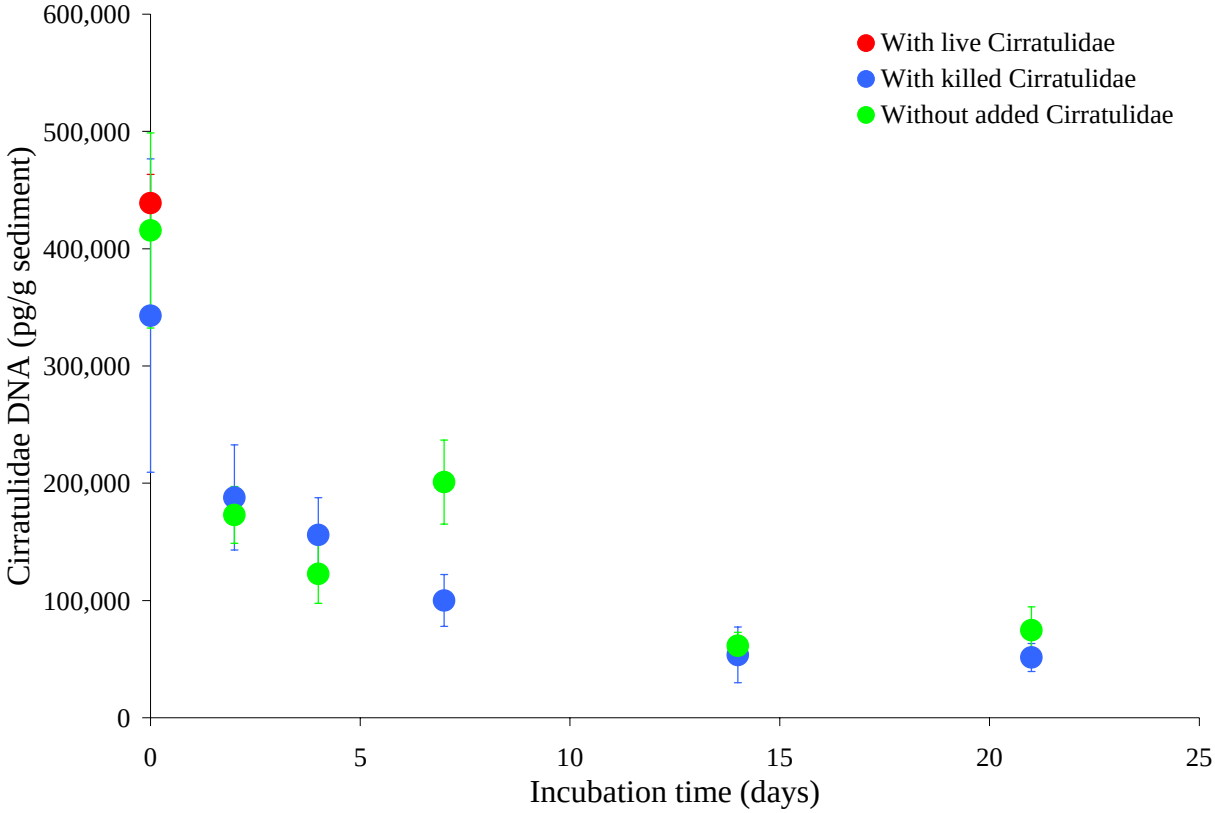

Figure 6.6 Detection of DNA over time of live and killed Cirratulidae added to sediment and also for no added Cirratulidae.

Quantification of biomass using real-time PCR versus end-point detection assay

Results from the comparison of end-point detection assays and real-time PCR demonstrated that the end-point detection assays reached a plateau at the upper end of the scale. End-point detection assay was unable to quantify accurately levels above 4.0 mg of added cirratulids/g sediment (Figure 6.7), while the real-time PCR assay had excellent linear correlation over the entire range of added cirratulids (Figure 6.8). A similar result was also obtained when sediment were spiked with Lumbrineridae (Figure 6.9 and Figure 6.10). Levels above 0.2 mg of added lumbrinerids/g sediment could not be quantified accurately by the end-point assay while the real-time PCR assay gave linear correlation for the range of added lumbrinerids.

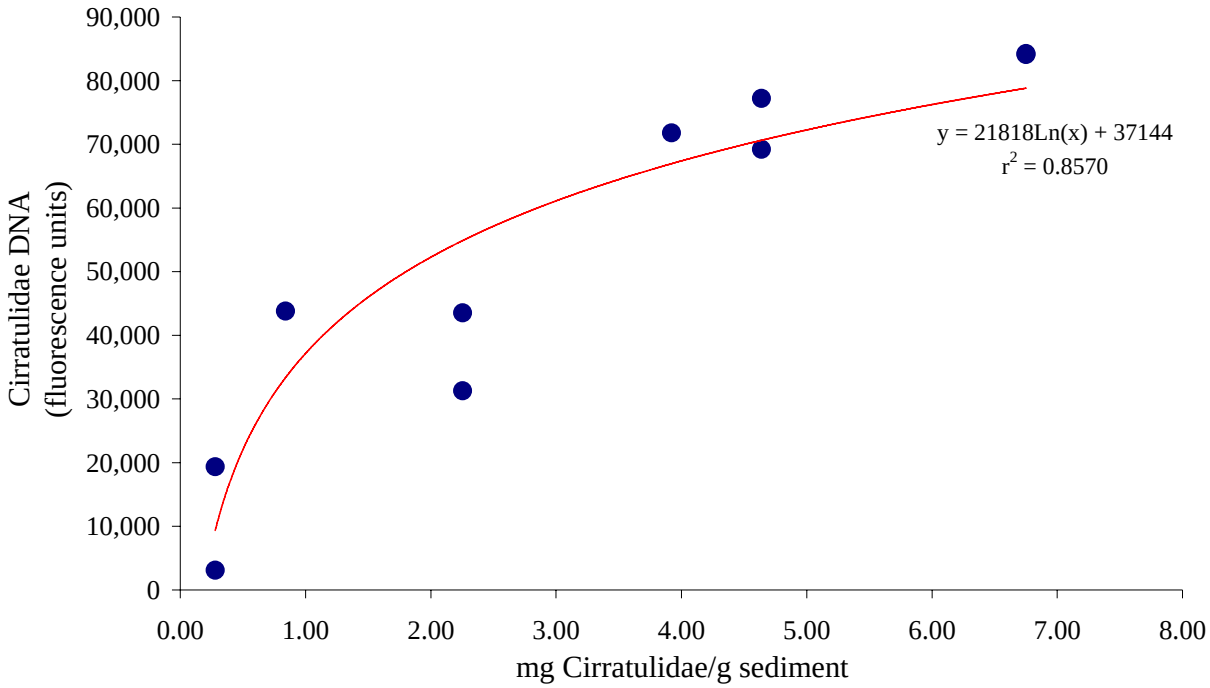

Figure 6.7 Quantification of Cirratulidae DNA using end-point detection assay showing inability to detect high levels of target organisms.

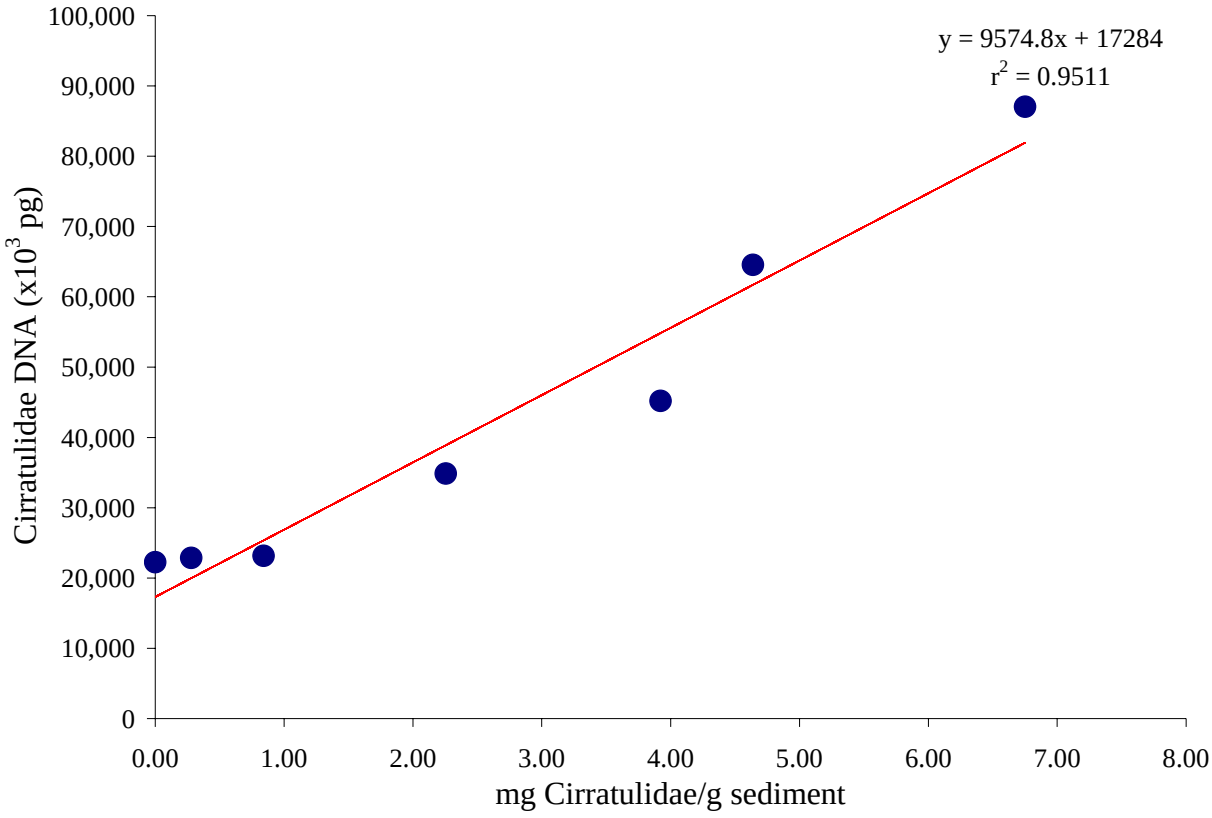

Figure 6.8 Quantification of Cirratulidae DNA using real-time PCR assays showing greater linear range as compared to end-point assay.

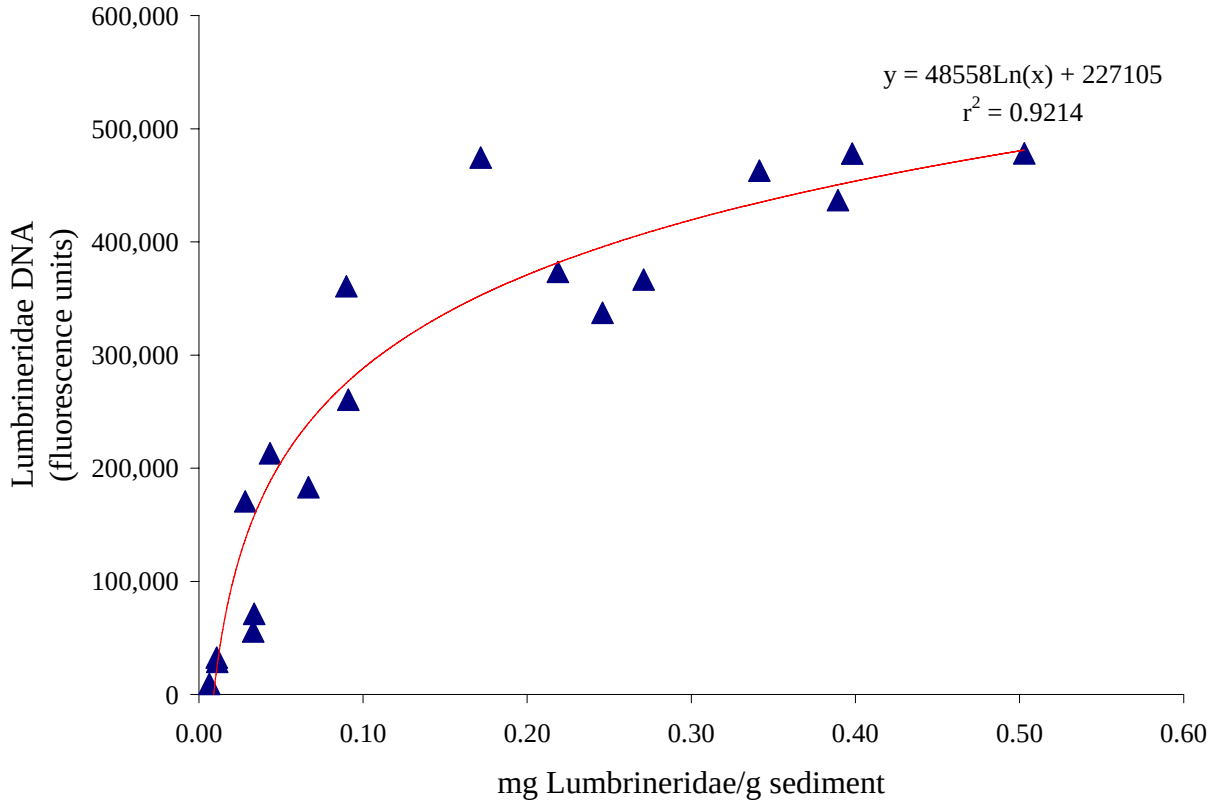

Figure 6.9 Quantification of Lumbrineridae DNA using end-point detection assay showing inability to detect high levels of target organisms.

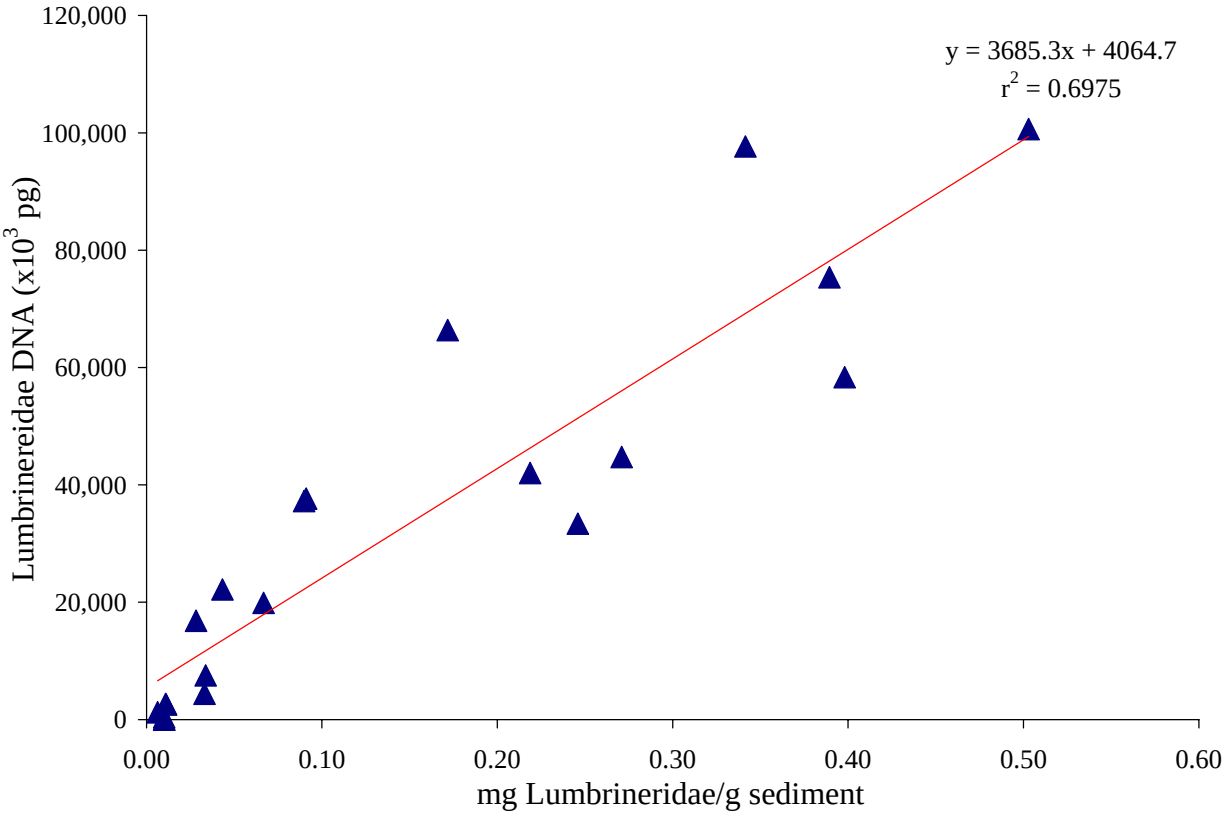

Figure 6.10 Quantification of Lumbrineridae DNA using real-time PCR assays showing greater linear range as compared to end-point assay.

Discussion

In evaluating the extent to which this system could be routinely applied, the quantification of added target organisms showed that there was a high degree of variability between individual organisms due to variation in size. The work also demonstrated that the correlation of DNA results to biomass was more valid than the correlation to number of individuals because DNA assays measure total DNA content of organisms, which relates to biomass rather than to numbers of organisms.

Concerns about the persistence of DNA from recently deceased organisms in sediments was addressed and this work showed that the DNA assays would detect recently deceased organisms but degradation of DNA was almost immediate and rapid. These results were comparable to the work in other studies (e.g. Herdina *et al.* 2004, McKay *et al.* 2006). In

particular, the study by McKay *et al.* (2006) on development and assessment of quantitative DNA assays for plant root growth, he showed that the DNA assays developed were sensitive and able to detect treatment responses more quickly than changes in root dry weights (traditional method), indicating that DNA degrades quickly in dying cells. Consequently, the contribution by dead organisms to the total DNA extracted would be small and/or negligible.

Furthermore, at any point in time, there may be a proportion of freshly dead organisms in any sample, but in most situations, these would not have a major impact on quantification of target organisms in sediments as dead organisms will not ordinarily persist in sediments over time. Through the calibration and validation work reported in Chapter 7, DNA of deceased organisms was not a problem for samples taken from the southern bluefin tuna farming zone. The results from the DNA assays are to give an index to compare between sites and assuming similar life histories, a similar proportion of animals will be dead at any one site and the index is an integrative measure of organic enrichment and not an instantaneous measure. As such, the detection of dead organisms in samples would not be a major problem.

With the availability of real-time PCR to the project, the opportunity to evaluate this newer technology showed that the real-time assay format proved to be superior to end-point detection, giving a much better linear quantification over the range of organisms added. This was not surprising as this is the major strength of the real-time assay platform.

The proof of concept phase of the project was completed where a range of benthic infaunal species, which are indicator taxa of organic enrichment were identified. A rapid DNA assay system was then developed and evaluated for the potential to be routinely applied for the environmental assessment and monitoring of southern bluefin tuna aquaculture.

Chapter 7 Proof of Application

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Having achieved the objectives in the proof of concept phase (Chapter 2, Chapter 4, Chapter 5 and Chapter 6), this chapter will address the next phase of the project, which was to demonstrate the application of this technology for environmental monitoring.

Introduction

To achieve the proof of application, DNA assays were compared with traditional processing and enumeration techniques. Statistical analyses of the data were carried out and the degree of concordance was used to quantify the relative ability of the DNA assays to provide results that were comparable with the assessments of environmental effects (characterised by manual sorting and enumeration of the key indicator taxa). These analyses were carried out using samples collected for the 2003 Tuna Environmental Monitoring Programme (TEMP). In the course of the "proof of application" phase, several issues were identified which needed to be addressed before the technology could be used routinely. These included having to collect new samples from farmed and control sites and also several experiments to address sample-handling issues.

Initial comparative analyses

During the 2003 Tuna Environmental Monitoring Programme (TEMP), duplicate samples were collected at each of the compliance and control sites (see Chapter 2 for details). For all the samples collected, one set was manually sorted and enumerated while the duplicate set was analysed using the DNA assays.

Initial analysis of the TEMP samples used the original end-point detection assays for Capitellidae, Lumbrineridae, Cirratulidae and Nephtyidae and real-time assays for Spionidae. The comparison of the data from the traditional manual technique and DNA assay technique showed that the DNA assays appeared to substantially under-estimate the relative biomass of target taxa, especially when levels of these taxa were low. Correlations at higher abundance appeared to be better. To illustrate this, mathematical transformations were applied to the data to investigate the extent of these differences between the manual

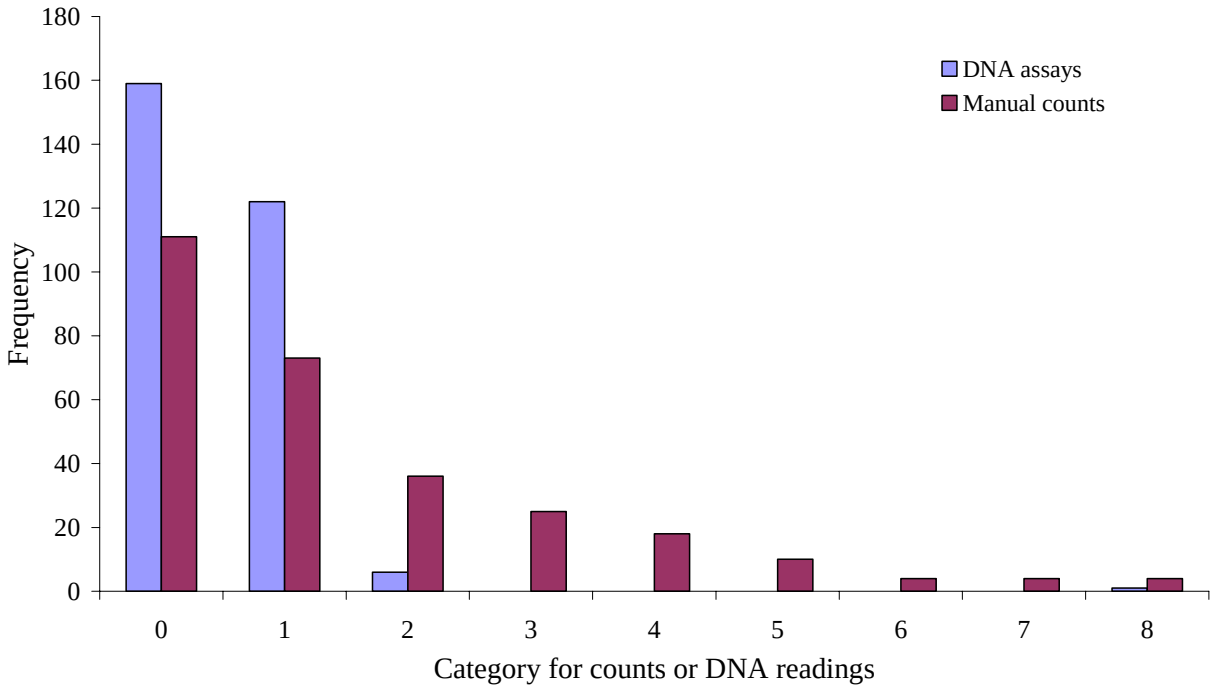
count data and the DNA data and histograms were plotted to show the distribution of counts and DNA readings. A 5^{th} root transformation of the DNA data was required (based on a best fit analysis using a linear regression model) in order to get concordance between the count data and those obtained using the DNA assays (Figure 7.1a versus b).

This result suggested that a number of key issues needed to be resolved particularly in relation to the sensitivity of the DNA assay system. In broad terms three issues were identified:

- Sample handling.
- Suitability of TEMP samples for comparative work.
- Cross-reaction of some tests with non-target taxa.

Work presented in this chapter summarises the results of the final series of experiments, conducted to resolve these issues.

(a)



(b)

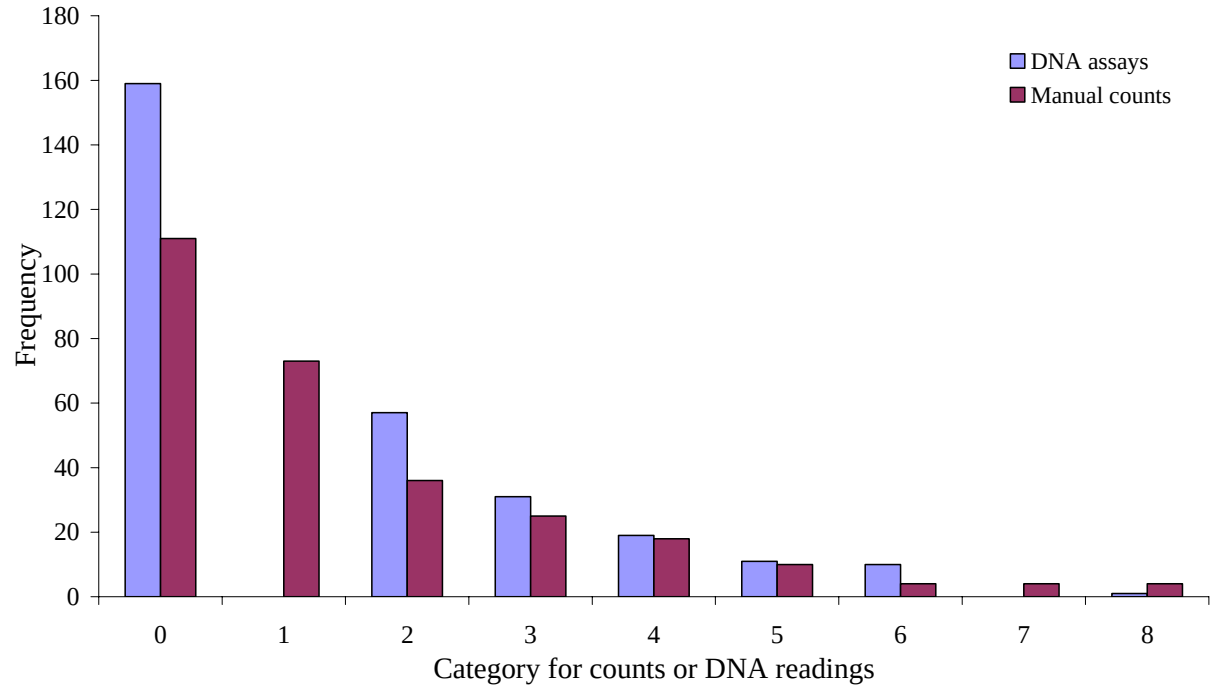


Figure 7.1 The frequency distribution of sample abundances of Lumbrineridae obtained from 288 samples. Abundances obtained by manual counting (■) or DNA assays (■). DNA assays were standardised using (a) linear interpolation based on the assumption that the maximum count corresponded with the highest DNA reading (categories represent evenly spaced bins between 0 and the highest value) or (b) a 5^{th} root transformation of the DNA readings. The 5^{th} root transformation was obtained by trial and error using goodness-of-fit criteria.

Sample handling

Given that previous work had demonstrated the sensitivity of assays using samples spiked with low levels of target taxa, it was considered likely that the sample handling and preparation methods may be resulting in a reduced sensitivity of the DNA assays. Two aspects of sample handling and preparation were examined; the effect of sieving manually counted samples and the impact of sediment drying on sensitivity.

Sample sieving

In the traditional manual technique of processing sediment samples, the samples were washed through a 1 mm sieve. This mesh size was chosen as it is the most commonly used for studies of marine macrofauna (Eleftheriou and Holme 1984, Kingston and Riddle 1989). The retained sediment was then transferred to a tray, sorted, and the infauna identified and enumerated. In the DNA assays developed in this project, the samples were processed whole, without any washing through sieves. Consequently, in calibrating the DNA assays, experiments were carried out to investigate the effect of sieving samples on the quantification of infauna using the DNA assay system[13].

Thirty-two sediment samples were washed through a 1 mm sieve with the portion going through the sieve being retained in a bucket. The sediment retained on the 1 mm sieve was then transferred into a container and frozen. The portion that went through the sieve, which included water and fine sediment, was poured through a fabric 34 μm sieve, washed and the retained sediment was placed into a separate container and frozen. This mesh size was chosen because it would retain all sources of macroinvertebrate DNA, including polychaete eggs which are generally 50 μm or larger. The new sub-samples were then labelled as the A series (sediments retained on 1 mm sieve) and the B series (sediments retained on the 34 μm sieve).

DNA for all 32 samples was extracted and assays were carried out for four of the indicator taxa; results for the Lumbrineridae (Figure 7.2) were typical. Of the 32 samples extracted, 27 samples had detectable Lumbrineridae DNA (Figure 7.2). In general the amount of DNA extracted from the A-series samples (> 1 mm mesh size) was three to four orders of

[13] It may be anticipated that the sieving process would remove material from the samples and thereby reduce the manual counts relative to the DNA assay procedures. An alternative hypothesis is that the sieving process may remove material that would interfere with the DNA assay and that sieved samples would therefore have higher DNA results than non-sieved samples.

magnitude higher than the B-series samples (< 1 mm mesh size). For some samples the DNA assay was higher for the B-series samples; these were mostly for samples that had much reduced readings (for A-series or B-series). Only 1 sample (Sample 10; Figure 7.2) had a higher reading from the B-sample and a high reading overall.

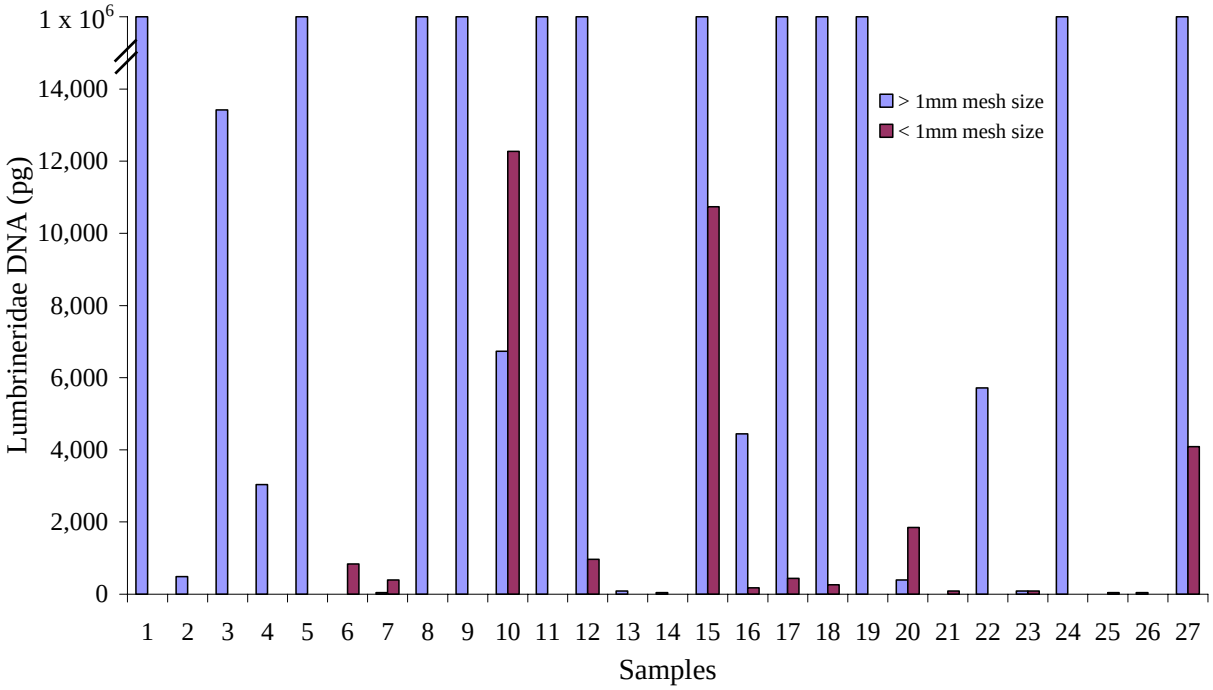


Figure 7.2 DNA assay for Lumbrineridae showing huge differences between samples retained on 1mm mesh size and samples that went through 1mm mesh size (note split scale on the y-axis).

Similar results were also obtained for the other assays, which confirmed that most of the DNA being detected was attributable to material retained on the > 1 mm sieve. On this basis it was concluded that the DNA assays were detecting indicator taxa comprising a size fraction comparable to those being enumerated using the manual technique. Indeed, this result confirmed our belief that the DNA assay should be giving higher readings than the manual enumeration because in some cases there was additional material being detected in the < 1 mm fraction that would be contributing to the DNA signal but that would not have been found on a manual count.

Sample drying

The impact of sample drying on DNA levels of target taxa was then investigated. In the initial analysis of the TEMP samples, sediments were received frozen and thawed at 40^0C. This process took several days and led to the hypothesis that the low sensitivity of DNA assays may be due to sample degradation (with resultant decomposition of DNA) during the thawing and drying process. Two separate experiments were undertaken to examine the effects of the sample drying process on DNA levels.

The first experiment involved investigating the effects of the process of thawing frozen samples and drying them at 40^oC. Twenty replicate samples were randomly assigned to one of two treatment groups. In treatment group 1, the ten replicate samples were spiked with capitellids and then frozen, thawed, dried and DNA was extracted. In treatment group 2, the ten replicate samples were frozen (no spiking with capitellids), thawed, dried, spiked with capitellids and then the DNA was extracted. In summary:

- Spiked – frozen – thawed – dried – extracted
- Frozen – thawed – dried – spiked – extracted

Two additional sets of 10 samples were assigned as control groups but were spiked with yeast instead of capitellids.

The results showed that recovery of added capitellid DNA from sediment samples spiked after the samples were frozen and dried was much higher than recovery from samples spiked before the drying process (Table 7.1). However, recovery of added yeast did not show any such difference between the treatments of spiking before or after drying, indicating that there was substantial loss (degradation) of capitellid DNA during the thawing and drying process.

Table 7.1 Results from the experiment to investigate the effects of the process of thawing and drying frozen samples on DNA recovery, showing a 10 fold reduction in yield of capitellid DNA during the freezing, thawing, drying process; this was not observed for samples spiked with yeast DNA.

Treatment - Addition of target organisms	**DNA (pg)**	
	Samples spiked with Capitellidae (x 10^3 ± SE)	**Samples spiked with yeast DNA (x 10^6 ± SE)**
Added **before** freezing, thawing, drying	0.602 ± 0.227	5,769 ± 515
Added **after** freezing, thawing, drying	5,848 ± 1,388	6,592 ± 493

A second experiment was undertaken to investigate if this degradation of DNA during the drying process could be minimised by freeze-drying the samples. Sediment samples were collected from an area known to have very few capitellids. Three sets of 12 replicate sediment samples were set up with each sample weighing approximately 150g wet weight. One set of samples was spiked with capitellids while another set was spiked with yeast DNA and the third set was left as the control without any spiking. All samples were then frozen before freeze-drying. Another three sets of samples set up similarly as above were frozen and dried at 40^{o}C.

Results indicated that recovery of added yeast DNA was comparable between frozen and freeze-dried sediments (Figure 7.3). This implied that there was no significant difference (ANOVA, $F_{1,22}$ = 0.3084, p = 0.5843) in the DNA extraction process with respect to sample handling prior to extraction. However, there was a significant difference (ANOVA, $F_{1,22}$ = 15.4628, p = 0.0007) in the recovery of Capitellidae DNA from freeze-dried sediments, with a mean 10-fold increase in recovery (Figure 7.4). This indicated that the process of oven-drying frozen sediments led to degradation of organisms prior to DNA extraction, resulting in a very significant decrease in DNA detection.

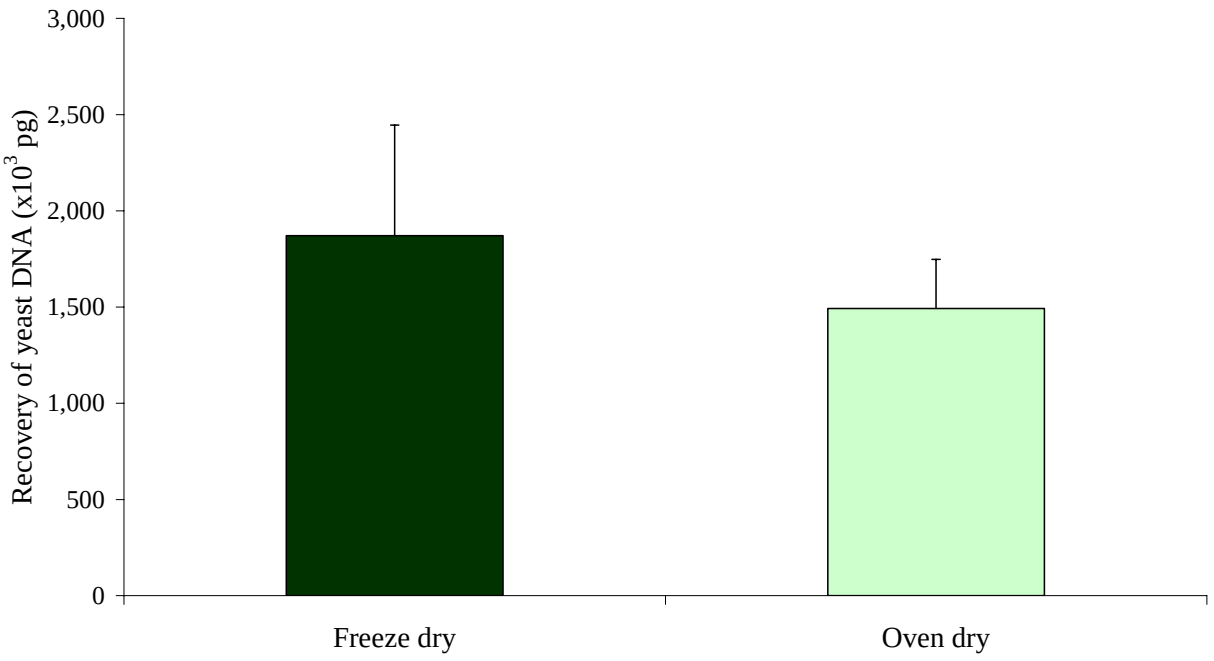


Figure 7.3 Recovery of yeast DNA (x10^3 pg ± SE) showing no significant difference ($p > 0.05$) between freeze dried and oven dried samples.

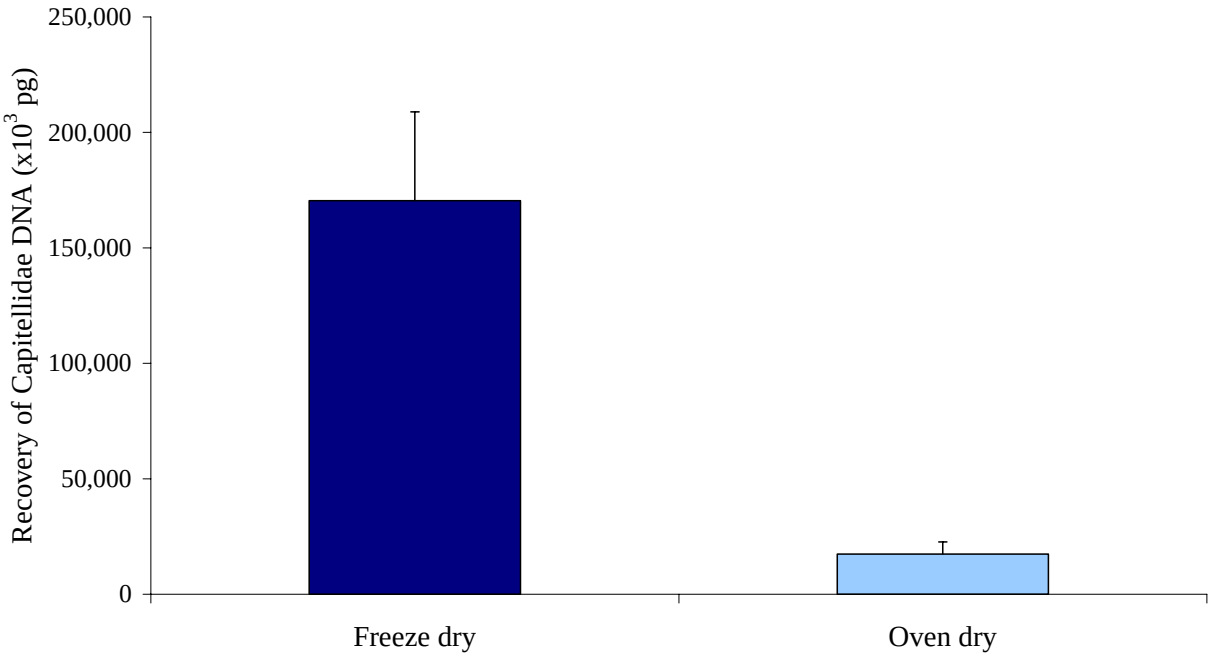


Figure 7.4 Recovery of Capitellidae DNA (x10^3 pg ± SE) showing significant difference ($p < 0.05$) between freeze dried and oven dried samples.

The above experiments were repeated using Cirratulidae and the recovery of DNA again showed a difference between oven-dried samples and freeze-dried samples. These results indicated that there was degradation of cirratulids in the oven drying process but the difference was less significant (ANOVA, $F_{1,8} = 3.658$, $p = 0.0922$). Detection of spiked Cirratulidae DNA in freeze-dried sediment was approximately 1.5 times higher than in oven-dried sediment (Figure 7.5). This may be because Cirratulidae are larger organisms, less fragile or release fewer cell degrading enzymes during the drying process and are therefore less prone to degradation in the warm, moist conditions experienced in the early part of the drying process.

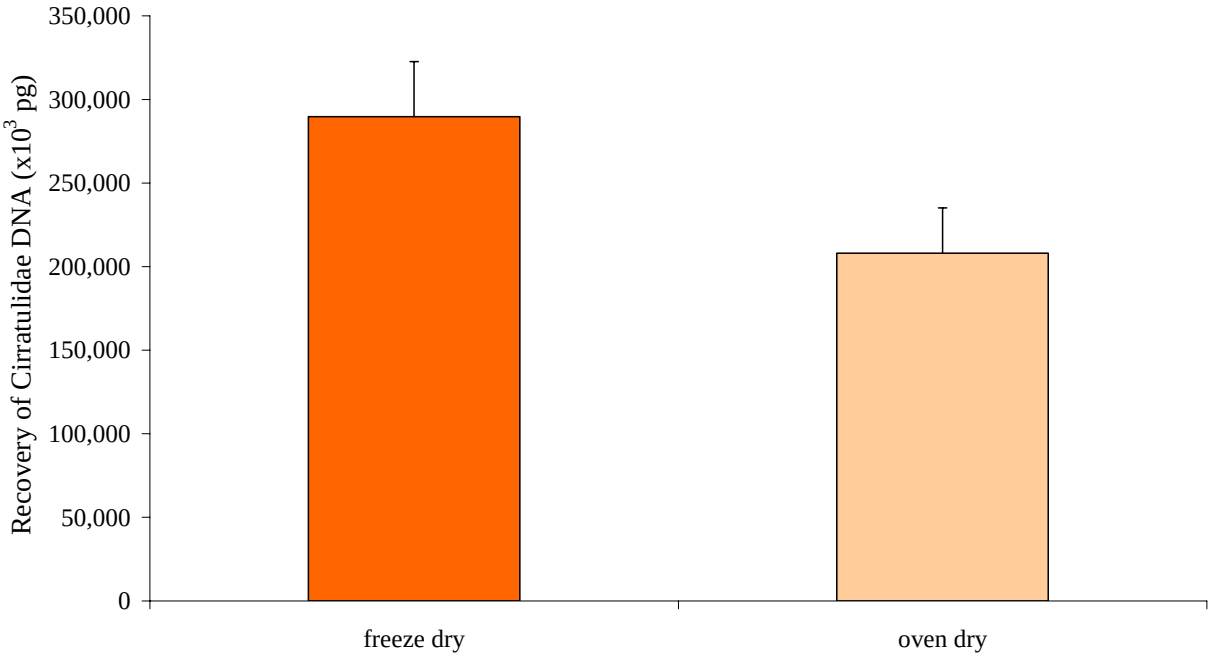


Figure 7.5 Recovery of Cirratulidae DNA ($x10^3$ pg ± SE) showing significant difference ($p < 0.1$ but not $p < 0.05$) between freeze dried and oven dried samples.

Analysis of samples from farmed versus control sites

The initial comparative analyses of TEMP 2003 samples indicated that the assays developed were not able to detect any DNA in samples with naturally low abundances and this problem was exacerbated by the deterioration of DNA during the drying process. Consequently, new samples were collected, which had more obvious differences in animal numbers, (i.e. samples were collected from sites adjacent to sea-cages during the farming

season and control sites located at least 1 km away). The new samples for manual enumeration of infauna were taken from the benthic respiration experiment of the Waste Composition and Mitigation Project[14] (WMP) and additional samples were collected at the same time from the same sites for DNA assays. The samples were taken from two control sites and three farmed sites, located in the Rabbit Island and Boston Island East Farming Zones (Table 7.2).

Table 7.2 Control and farmed sites from the Rabbit Island and Boston Island East Farming Zones.

Sites	**Farming Zones**
Control A	Rabbit Island
Control B	Boston Island East
Farmed 1	Boston Island East
Farmed 2	Boston Island East
Farmed 3	Rabbit Island

Samples from the benthic respiration experiment were manually sorted and enumerated, in the same way as the TEMP samples were processed (details given in Chapter 2) while the additional samples were analysed using DNA assays. Multivariate techniques were used to analyse the two sets of data. Similarity matrices using the Bray-Curtis similarity coefficient were computed with untransformed data. Non-metric multidimensional scaling analyses (MDS ordination, Kruskal and Wish 1978) were carried out on the Bray-Curtis similarity matrices. Goodness-of-fit in the MDS ordination plots was measured as stress with Kruskal's stress formula I. The multivariate analyses were carried out using the various routines in the software package PRIMER (Plymouth Routines in Multivariate Ecological Research) (version 5.2.9, Clarke and Warwick 2001a)

The MDS ordination plot shown in Figure 7.6a is for manually sorted and enumerated samples and the analysis is based on all 58 taxonomic groups. The ordination indicated that there was separation between control and farmed sites where the control sites are grouped to the right of the plot while the farmed sites are on the left. In addition there were separations between the two control sites and also for the individual farmed sites, especially between Farmed Site 3 and the other two farmed sites (Figure 7.6a). Farmed Site 3 represented a stocked farm site where fish had been held for a period of 4 months

[14] Aquafin CRC/FRDC Project 2001/103: Aquafin CRC - Southern Bluefin Tuna Aquaculture Subprogram: Tuna environment subproject - Evaluation of waste composition and waste mitigation strategies.

when sampling was carried out. Farmed Site 2 was also a stocked farm site but fish had only been held for 2 months. Farmed Site 1 was the research farm (adjacent to Farmed Site 2) with much lower stocking densities. Control Sites A and B were sites situated at least 1 km away from Farmed Site 3 and 2 respectively.

Another ordination was carried out using only the six polychaete groups, which were chosen for the development of DNA assays. Again, there was separation between control and farmed sites; however, the control sites were not as distinctly separated as the previous analysis, indicating a loss of resolution (Figure 7.6b), but Farmed Site 3 was still separated from the other two farmed sites.

In order to compare the results of the manual enumeration technique with the DNA assays, the data were range standardised to ensure consistency in the treatment of the different data sets. Range standardisation has been used because it puts differently scaled variables on the same footing, thereby eliminating any signal other than relative amounts (McCune and Mefford 1999). This was done to reduce the effect of the differences in quantification methodology of the two methods particularly the change from estimates of abundance (manual count) to estimates of biomass (DNA assay).

Subsequent multivariate analysis of the range standardised abundance data showed that there was further loss of resolution in the relative separation of sites, although there was still separation between farmed and control sites and also between Farmed Site 3 and the other two farmed sites (Figure 7.6c). The range standardised ordination of the DNA data using the six polychaete groups showed even greater loss of resolution. There was no true separation of farmed sites from control sites; furthermore, the three farmed sites were not clearly separated (Figure 7.6d).

These results indicated that there were additional issues associated with the sensitivity of the DNA assays. Consequently, further experiments were carried out to resolve these issues. Sequences for the probes and primers were re-examined and further specificity testing was carried out.

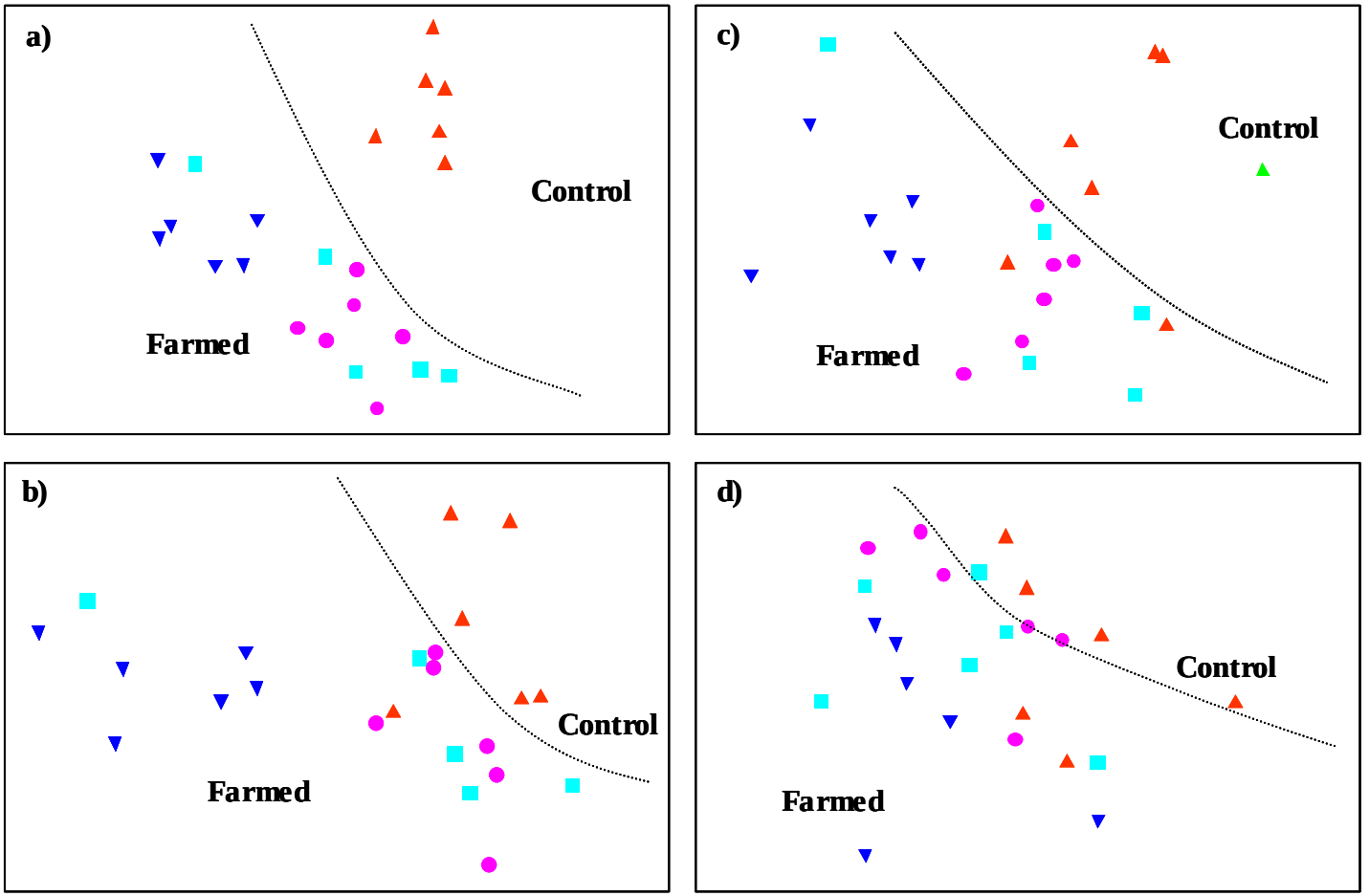


Figure 7.6 Two-dimensional nMDS ordination plot of untransformed a) abundance data for 58 taxonomic groups (stress = 0.17), b) abundance data for six polychaete groups (stress = 0.12), c) range standardised abundance data for six polychaete groups (stress = 0.15), d) range standardised DNA data for six polychaete groups (stress=0.14) showing varying separation of control sites (▲ = Control Site A and ◣ = Control Site B) from farmed sites (● = Farmed Site 1, ■ = Farmed Site 2 and ▼ = Farmed Site 3).

Modification of tests

From the results discussed above, it was recognised that several of the assays used in the initial comparative studies might have cross-reacted with non-target taxa or alternatively were inadequate in their capacity to detect all sub-groups within the critical indicator taxa chosen. Additional specimen collection and sequencing were therefore undertaken to test these assays even more broadly. The specificity of assays was re-examined to optimise sensitivity, and existing assays were re-designed to reduce cross-reactions with non-target taxa.

Redesign of Capitellidae and Spionidae Assays

It became apparent from the analysis of TEMP samples and the "farmed versus control" samples that the assays for Capitellidae and Spionidae needed redesigning to increase sensitivity and to decrease potential cross-reaction with non-target taxa. The redesign was also referenced to the taxonomic complexity of the various groups (see e.g. Figure 4.1). It was clear on a close analysis of Figure 4.1 (and the underlying data) that two groups (Capitellidae and Spionidae) were taxonomically more diverse (in terms of the 28S rDNA sequence data) than the other taxonomic groups. On this basis, the probes and primers for these two families were substantially redesigned to provide assays for specific subgroups that could be used to more accurately quantify these families.

In the final system, nine different quantitative 'real-time PCR' assays were developed including assays for two subgroups of Capitellidae, four subgroups of Spionidae and the assays that had already been developed for Lumbrineridae, Nephtyidae and Cirratulidae.

Final Analysis

At the conclusion of the specificity and sensitivity testing, a set of quantitative 'real-time PCR' assays had been developed for Lumbrineridae, Nephtyidae and Cirratulidae, with additional assays for two subgroups of Capitellidae and four subgroups of Spionidae.

With this set of assays it was possible to undertake a comparison between the data from the final DNA assay system and the traditional processing and manual enumeration technique. The DNA extracted from the farmed versus control samples (as described above) were tested using the new improved nine assay system. The data obtained from this were then compared to data from manual enumeration of samples and also to data from the earlier

(non-optimised) DNA assays. The comparisons included the analysis of five different datasets:

1. Count-All-6: analysis of data from manual enumeration for all taxa using 6 replicates per site,

2. Count-6-6: analysis of data from manual enumeration for the 6 taxonomic groups (initially targeted taxa for PCR probe development), using 6 replicates per site,

3. DNA-6-6-old: analysis of data from the original DNA assay system (non-optimised) for 6 taxonomic groups using 6 replicates per site,

4. DNA-9-6-new: analysis of data from the optimised DNA assay system for 9 taxonomic groups using 6 replicates per site,

5. DNA-9-8-new: analysis of data from optimised DNA assay system for 9 taxonomic groups using 8 replicates per site.

The comparative analysis across the various datasets was undertaken using Bray Curtis similarity/dissimilarity measures obtained for farmed (F) and control (C) sites. Comparisons were made graphically where the similarity/dissimilarity measures were plotted using a radar plot. The shape of the plot for any given analysis was used as a comparator with any other analysis to examine the concordance of the two analyses (Figure 7.7).

In Figure 7.7 the five axes show:

- F vs C – Dissimilarity scores for farmed versus control sites. Most analyses identify a high level (around 70-75%) of dissimilarity between farmed and control sites. This is expected as these represent those sites, which have been perturbed due to organic enrichment (farmed) and those in relatively undisturbed condition (control).

- C vs C – Dissimilarity scores for comparison between control sites. Three assay systems (DNA-9-8, DNA-9-6 and Count-all-6) all identified higher levels of dissimilarity (differences) between the two control sites. The other assay systems were less able to differentiate control sites.

- F vs F – Dissimilarity scores for comparison between farmed sites. The Count-6-6 assay system was less able to differentiate between farmed sites than the other systems.

- F’ – Similarity measures within farmed sites – assay systems give mixed but generally intermediate results. Overall the manual enumeration approaches indicated higher levels of similarity within sites than the DNA assay systems.

- C’ – Similarity measures within control sites – as with farmed sites results are mixed but generally intermediate. Overall the manual enumeration approaches indicated higher levels of similarity within sites than the DNA assay systems.

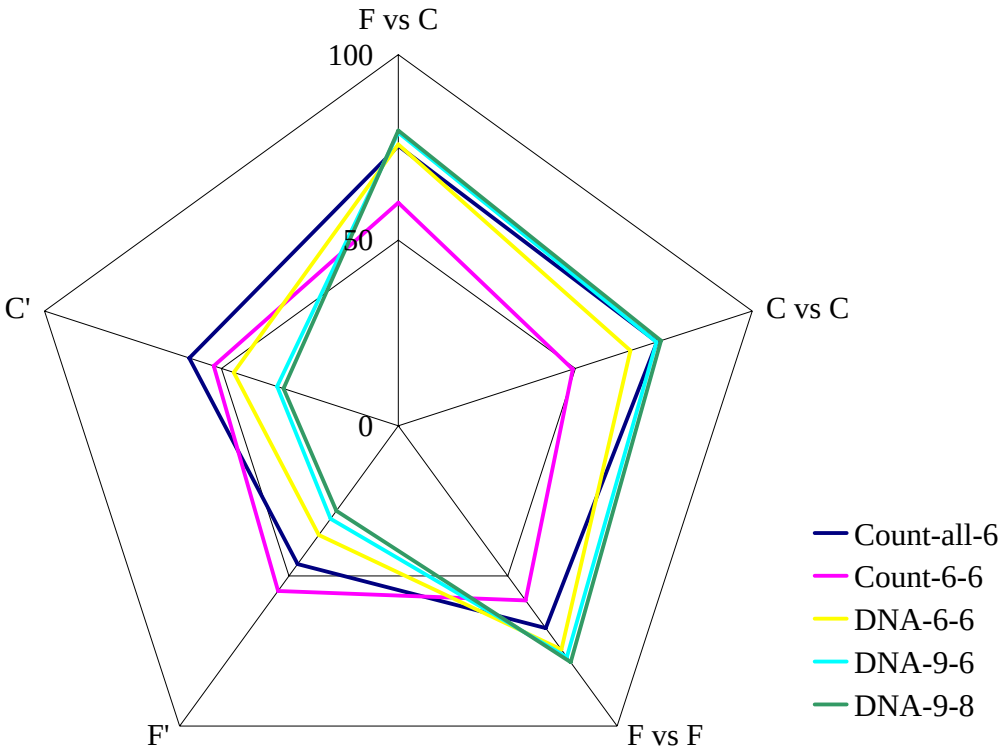


Figure 7.7 Radar plot showing comparative measures of dissimilarity (axes F vs C, C vs C and F vs F; 0 is least dissimilar and 100 is most dissimilar in any given analysis) or similarity (axes C’ and F’; 0 is least similar and 100 is most similar).

The Count-all-6 analysis utilised data from a larger number of taxa (54 in total) most of which were not incorporated into the other analyses. The results from this analysis strongly supported the conclusions from Chapter 2 that there is scope for further refinement of the DNA assays to incorporate additional taxa such as Gammaridea and Ampharetidae, both of which characterise differences in natural (non-impacted) communities.

Having established a general picture of how the various assay systems performed, a second analysis was conducted to assess differences between farmed and control sites using the

"Analysis of Similarities" (ANOSIM, a routine in PRIMER version 5.2.9) randomisation/permutation test (Clarke and Green 1988, Clarke 1993, Clarke and Warwick 2001a). This is a non-parametric method based on rank similarities among all samples with pairwise comparisons for all sites. The ANOSIM results indicated significant differences (all $p < 0.05$) between farmed and control sites for all datasets and the pairwise comparisons are summarised in Table 7.3.

Table 7.3 Analysis codes are as given above and sites sharing similar letters are not significantly different. The "Resolution score" represents the extent to which any given analysis can differentiate major groups of sites from one another. A low score (e.g. 2) indicates a poor capacity for differentiation of sites; a high score (e.g. 4) indicates a greater capacity to differentiate sites using the given analysis.

Analysis	Site					Resolution score
	Control A	Control B	Farm 1	Farm 2	Farm 3	
Count-all-6	a	b	c	c	d	4
Count-6-6	a	a	b	ab	c	3
DNA-6-6-old	a	a	ab	ab	b	2
DNA-9-6-new	a	a	ab	b	c	3
DNA-9-8-new	a	b	abc	c	d	4

In broad terms, the Count-all-6 analysis should be considered as the "gold standard" for this assessment because it utilised the best possible taxonomic resolution to characterise individual sites and thereby had the highest capacity to differentiate major site groupings. In the analysis of the farm versus control data (Figure 7.6a), the Count-all-6 analysis was able to differentiate four site groups. Control Site A, Control Site B and Farm Site 3 each formed single site groups and Farm Site 1 and Farm Site 2 fell out together as the fourth group (Table 7.3). Only one other analysis was able to differentiate four groups from the data and this was the DNA-9-8-new analysis (which is the optimised DNA analysis using 8 replicates per site; Table 7.3). This analysis was able to differentiate Control Site A, Control Site B, Farm Site 2 and Farm Site 3 from one another with Farm Site 1 (the research site with low stocking density) being intermediate in character (with similarities to each of Control Site A and B and Farm Site 2). A similar analysis to that of the farm versus control data was applied to the DNA-9-8-new data. The ordination plots showed that the plot for the DNA-9-8-new data was comparable to the Count-all-6 data (Figure 7.8a and b). Although not as distinct, there was still separation of Control Site A, Control Site B and Farm Site 3 forming single site groups with Farm Site 1 and Farm Site 2 intermixed (Figure 7.8b).

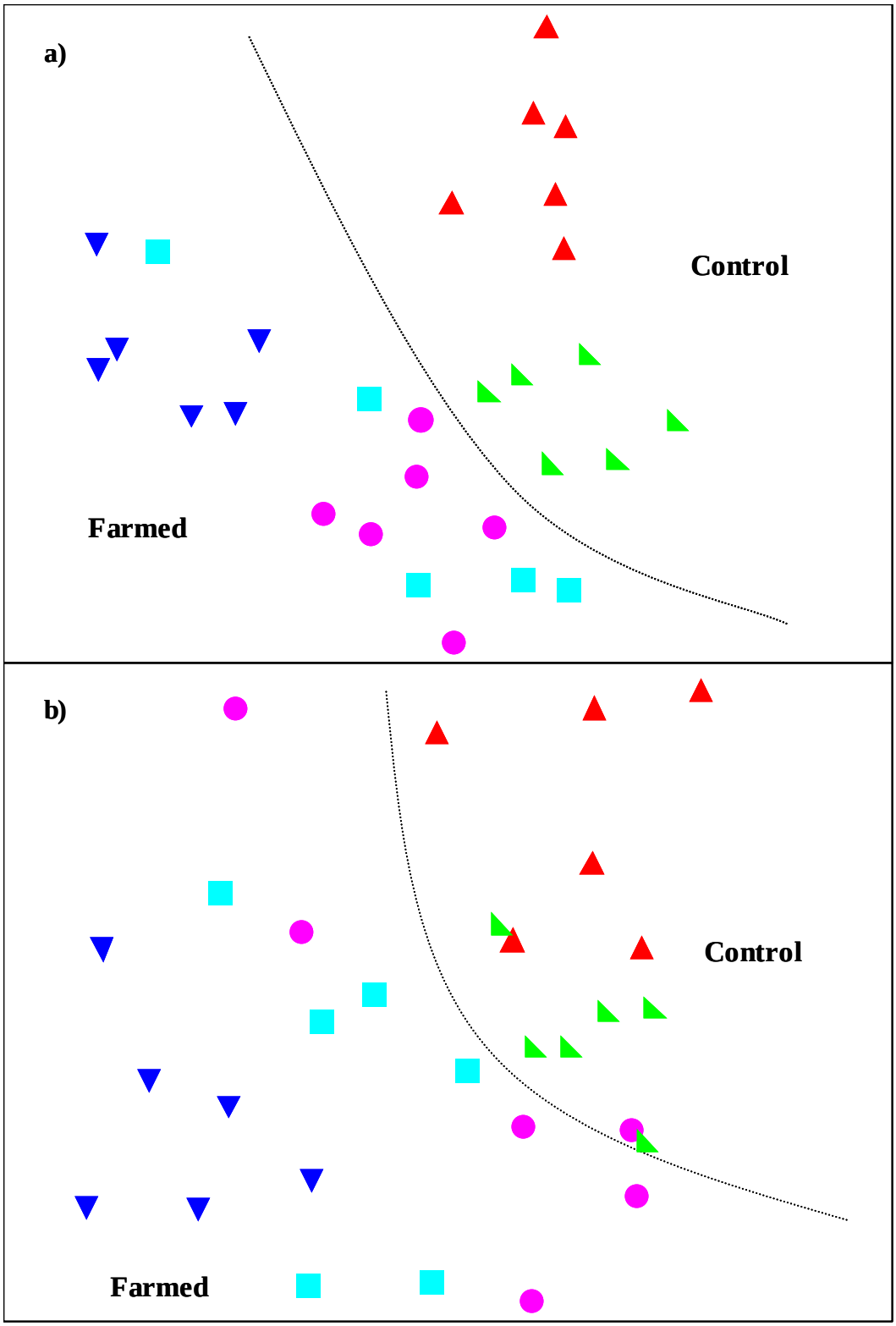


Figure 7.8 Two-dimensional nMDS ordination plot of untransformed a) abundance data for 58 taxonomic groups (stress = 0.17), b) range standardised DNA data for nine taxonomic groups (stress = 0.18) showing separation of control sites (▲ = Control Site A and ◣ = Control Site B) from farmed sites (● = Farmed Site 1, ■ = Farmed Site 2 and ▼ = Farmed Site 3).

The other assay systems were only able to differentiate two or three site groups, with Control Sites A and B (the two control sites) being distinct from Farm Site 3 (the farm site which had been stocked for the longest period of time). Farm Sites 1 and 2 were generally intermediate in character and not clearly differentiated from the other sites (Table 7.3).

Conclusions

Overall the results of the "proof of application" work demonstrated that the optimised DNA assay system consisting of the following nine assays,

- Lumbrineridae
- Nephtyidae
- Cirratulidae
- Capitellidae (two subgroups)
- Spionidae (four subgroups)

provided a suitable alternative to the manual enumeration method and may be used for routine environmental assessment and monitoring of southern bluefin tuna aquaculture.

It was recognised that this DNA assay system developed for rapid detection and quantification of indicator taxa in sediment samples could be expanded and applied to environmental assessment of other forms of organic enrichment such as sewage outfalls or meat/fish processors and also to other finfish aquaculture sectors. For each of these other applications, the indicator taxonomic groups may be different from what has been developed for southern bluefin tuna farming in Port Lincoln, South Australia. However, many of the taxa listed above would be applicable for use in other areas (see Chapter 3 for selection of taxa), but this would need to be confirmed for each case. The technology developed and used in this research can be applied in the development of any additional DNA assays and an expanded system would increase the resolution of the DNA assay system for environmental assessment and monitoring (see Chapter 9, Further Development).

Chapter 8 Developing a Balanced Scorecard Approach for Environmental Compliance in the Southern Bluefin Tuna Aquaculture Industry

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Having developed the DNA assay system, there was then the need to develop a method for analysing, integrating and reporting the DNA results. This chapter details the development of the Environmental Compliance Scorecard (ECS), which provided a basis for analysis and integration of data, development of a compliance score, establishment of business rules for the application of regulatory arrangements and communication of recommendations for appropriate management responses to ECS results.

Environmental monitoring of southern bluefin tuna farming

Organic enrichment associated with southern bluefin tuna farming has the potential to impact on the environment around the farms. In order to address the potential for environmental harm, state regulators have required farm operators to undertake environmental monitoring within the farming zone on a periodical basis as part of their licence conditions (PIRSA-Aquaculture 2004). The results of this monitoring programme have been published as a series of TEMP (Tuna Environmental Monitoring Programme) reports over the period 1996 to present (Clarke *et al.* 1999, Clarke *et al.* 2000, Madigan *et al.* 2002, Madigan *et al.* 2003, Loo *et al.* 2004b, Loo and Drabsch 2005b).

Environmental monitoring serves a number of purposes; it helps to allay the fears held by many that southern bluefin tuna farming impacts on the environment, and also provides data to support the contention that the industry is ecologically sustainable. Nevertheless, the current process of monitoring and assessment is slow and lacks the capacity to provide a rapid evaluation of the industry's performance. The timeframe for evaluation and the cost of traditional environmental monitoring were two of the key factors that justified the development of the current project, which aimed to provide a rapid turn around of environmental assessments and thereby allow industry to use the information more effectively for management rather than simply in a regulatory compliance role (the full benefits of the outcomes of this project are discussed in Chapter 9).

One challenge in making effective use of environmental monitoring data is to present the results of a monitoring programme in such a way that they are clearly articulated and easily

understood by all stakeholders including farm owners, managers, regulators and the community. To this end, this project has developed a scorecard system, which can be implemented using data obtained from the DNA assays to provide a simple statement of results using a robust and defensible analytical framework to produce a single line "Compliance rating". The following provides a summary of the background to the use of environmental scorecards and outlines how this system has been developed for application in the southern bluefin tuna industry.

Application of balanced scorecards to environmental monitoring

The idea of using a Balanced Scorecard to improve business management is not novel and has been a key strategy adopted by many major commercial enterprises for well over a decade (Kaplan and Norton 1992, 1996a, b). In developing the approach, Robert Kaplan argued that the use of a balanced scorecard has a direct "… payoff [through better] strategic relationships with partners" and goes on to argue that the key strengths of the approach are in the accessibility of the information to all partners (clients, customers, employees and regulators).

In more recent times, the approach has been extended to consider the role of balanced scorecards to integrate environmental performance of enterprises into the overall management strategy (Dyllick and Hamschmidt 2000):

> "It shows that so far ***environmental sustainability remains largely separated from the traditional core business strategies and management systems,*** which are geared nearly solely towards financial performance indicators. … Accordingly management tools are needed that help to overcome this gap between EMS and business management systems. Firms need to integrate financial, environmental and social management systems."

For any industry sector the willingness to move to a balanced scorecard will be influenced by the perceived benefits relative to the cost of implementation. Not withstanding the relative benefits and costs, in areas such as environmental performance reporting, the use of a scorecard approach has a number of attractions, not least of which is the potential to use the scorecard as a tool for communicating otherwise complex issues both within the industry sector as well as to government, regulators and the community.

One of the major challenges for any industry sector therefore, is to effectively integrate reporting on its environmental performance into its overall management reporting systems. One way of achieving this is to standardise environmental reporting obligations into a

scorecard system and to use this scorecard to communicate with stakeholders both internally and externally.

Environmental scorecards

Environmental scorecards are intended to provide a system for representing the outcomes of environmental assessments in an easy to understand and standardised format that is accessible to both the specialist and the general reader. In broad terms, they provide a simplified, ideally single line or pictorial, summary of the system under consideration. Having a quantitative and/or statistical basis, environmental scorecards have applications in various reporting frameworks, including the National ESD (Ecologically Sustainable Development) framework for aquaculture and wild capture fisheries (Fletcher *et al.* 2002, Fletcher *et al.* 2004), the Triple Bottom Line reporting, which focuses on the economic, social and environmental aspects of corporate activities to achieve cost savings and improve environmental performance, and Environmental Management Systems (EMS), which is a methodical approach for continuous improvement in planning, implementation and review of an organisation's efforts to manage its impacts on the environment[15].

In Australia, there are a growing number of examples of the development and application of environmental scorecards (see review by Auricht 2004), which reflect the growing acceptance of this approach to environmental reporting. One of the best examples of the use of an environmental scorecard in South Australia is the system that has been adopted by the Environment Protection Authority (SA) for reporting on the status of water quality along the Adelaide Metropolitan coast[16]. This system provides an integrated assessment of water quality parameters and then uses a simple traffic light system to report on results:

- GREEN indicates all is well,
- ORANGE indicates that some parameters have breached (or are close to breaching) acceptable levels (e.g. high *E. coli* counts), and
- RED indicates that the status of the system is outside acceptable management bounds and that remedial action is required.

[15] http://www.environment.sa.gov.au/sustainability/triple_bottom_line.html, http://www.deh.gov.au/settlements/industry/corporate/ems.html

[16] http://www.epa.sa.gov.au/nrm_map.html

Aims and objectives

In this chapter we report on the application of an environmental scorecard approach to simplify the reporting and use of information collected during the environmental monitoring of the southern bluefin tuna aquaculture industry (Tuna Environmental Monitoring Programmes). The scorecard system provides a method for readily interpreting the results of assessments thereby giving information that is directly relevant to managers and regulators. Importantly, the system provides a tool for extending the results of the work presented in this study and will have general applicability to other environmental monitoring programmes. This tool has been codified into a spreadsheet template to provide an accessible system for reporting on the outcomes of the benthic infaunal component of the environmental monitoring programme that has been adopted by the southern bluefin tuna industry.

Although not in the original proposal, this system was developed to allow the DNA assay technology, developed in this project, to be delivered as a routine tool for use in compliance-based monitoring of the environmental performance of the southern bluefin tuna industry. In particular the objectives were:

1. To provide an outline of an environmental compliance scorecard system that has been developed for reporting on the environmental monitoring of the southern bluefin tuna aquaculture industry in SA.

2. To demonstrate the application of a spreadsheet-based template that has been developed to support the analysis, integration and reporting of the results from the infaunal component of the periodical TEMP assessments.

3. To review the establishment of compliance rules, business rules and management advice based on the evaluation delivered through the scorecard system.

Development of an environmental compliance scorecard (ECS) for the southern bluefin tuna industry

An Environmental Compliance Scorecard (ECS) was developed in this project, which provided a system for analysing and evaluating the results obtained from the mandated compliance-based environmental monitoring programmes for the southern bluefin tuna industry. The ECS provides a packaged set of statistical and mathematical routines to analyse, integrate and summarise results into a format that can be used to support decision making in relation to environmental compliance. In broad terms, the ECS system engages scientists in the identification of environmental status indicators and the calculation of quantitative indices of performance. It engages regulators in establishing business rules for

the assessment of environmental performance and farm managers in the development of appropriate management responses.

Presuming that an agreed system about what to monitor currently exists (which is the case for the southern bluefin tuna aquaculture industry) the ECS system comprises four modules:

1. Data analysis module - delivers a quantitative analysis of the assay data and evaluates a series of pre-determined indices of environmental status.

2. Compliance score module – integrates the results from the data analysis to produce an overall environmental compliance score that is ranked on a scale of 0 (very poor performance) to 100 (perfect result).

3. Business rules module – uses a series of business rules to determine whether the compliance score is within acceptable limits (as determined by the regulatory authorities). Results are presented using a 5-tiered system[17] where GREEN, the highest level, indicates all is well and VIOLET, the lowest level, indicates that the result is in breach of the compliance limits (note that the system can have as many levels as desired but in this report, we only refer to the use of a five tiered reporting system).

4. Management response module – identifies an appropriate management response in relation to the application of the business rules. In general terms these comprise; GREEN – business as usual through to VIOLET – take immediate remedial action.

To a large extent the design of these modules reflects the disposition of responsibility in arriving at the constituent rules or acting on the information presented. In particular;

- the decisions about how to analyse the raw data should be based on the scientific judgement of relevant technical specialists;

- the method for aggregating these data into an overall score needs to be developed collaboratively between technical specialists, regulators and industry;

- the decisions about threshold values for assessing environmental compliance is largely one for regulators; and

[17] During presentations to industry it was requested that the system be implemented with five bands (Green, Yellow, Orange, Red and Violet) instead of the original three (Green, Orange and Red) recommended. This has been done for the final version of TEMPEST that has been released and the text in this report reflect this change.

- the nature of management responses to the outcomes is largely a responsibility for industry given the constraints and opportunities within their operations.

Environmental data collection

The collection and processing of environmental samples to provide a base set of data on environmental performance in the southern bluefin tuna industry had been the subject of a number of previous studies (see in particular the work by Madigan *et al.* 2001). This work established the utility of benthic infaunal assessments as a robust indicator of the health of benthic systems and this subsequently established the rationale for the study presented in this report.

For compliance purposes, the southern bluefin tuna industry in South Australia collected two sorts of data from 2001 to 2004. Firstly, data were collected on the composition of benthic infaunal communities and secondly video data was obtained to assess the status of associated epibenthic systems. However, since 2004, there has been no collection of video data because no effects of aquaculture were discernable from the video recordings for the previous three years and a universal lack of confidence in the sensitivity of this technique in the particular environment occupied by the southern bluefin tuna farms. This was in contrast to the work by Macleod *et al.* (2004) for salmon aquaculture in Tasmania where video assessments were found to be simple and reliable for routine farm-based monitoring by industry themselves.

The development of an ECS in this project only used the benthic infaunal data because it is in this field that the new DNA assay systems have been developed. However, there is every opportunity to expand the system to incorporate other sorts of data including video data or even data on sediment and water column chemistry.

Importantly the system described in this chapter has been developed to allow application where benthic infauna have been quantified either using the traditional manual enumeration method or where the samples were processed using the rapid-throughput DNA assays developed in the present project (i.e. the system could be used for an analysis of historical data sets).

Data analysis

For the purposes of environmental monitoring of southern bluefin tuna farming, data needed to be analysed to develop a quantitative picture of the extent to which compliance samples[18] differed from a selection of control samples[19]. For the southern bluefin tuna industry in Port Lincoln there was already agreement about the process by which these samples were collected and this was part of the reporting requirements for licensees (PIRSA-Aquaculture 2004) and was broadly consistent with the outline detailed in Madigan *et al.* (2001).

These data comprised counts of infaunal taxa present in sediments quantified on a per unit area basis. From this dataset, a range of different indices could be derived, including measures of species richness, relative abundance and community composition (the latter typically described using multivariate indices such as dispersion or similarity/dissimilarity between either sites or samples; see for example the comparative analyses of different assay systems presented in Chapter 7 of this report).

The current monitoring programme utilises measures of both taxon richness and total abundance to determine whether a farming operation is compliant. These indices have been adopted because they are considered to reflect the basic assumption that increased levels of organic enrichment (associated with farming) will either reduce the number of species (richness) and/or increase the abundance (numbers of individuals) of benthic infauna in samples from the compliance site when compared with those from the control sites. Both of these indices were evaluated using count data from benthic samples.

The move to the PCR technology makes measurements of taxon richness largely inappropriate because the DNA assay system only provides quantification of a small subset of target taxa. Given that most of these were likely to be present in almost every sample (albeit at very different levels of relative abundance), calculation of a richness index would yield little useful information. Multivariate descriptions of community composition on the other hand, and particularly the characterisation of changes associated with organic enrichment, are well-established as indices of environmental status and as discussed in previous chapters (see Chapter 2 and Chapter 7), the DNA assay system has been shown to provide a sensitive tool for quantifying these changes.

On this basis we propose that the richness index be dispensed with (as it is only applicable to count data where all taxa are assessed) and a multivariate index that aims to quantify the relative dissimilarity between control and compliance samples be incorporated into the

[18] Taken from specific locations adjacent to farm sites (Madigan *et al.* 2001).

[19] Taken at agreed locations distant from farm sites (Madigan *et al.* 2001).

data analysis protocols (recognising that this analysis could be conducted on either count or DNA assay data and was therefore suitable for farms regardless of which data processing protocol they choose to use).

In developing the indices two key issues were considered; firstly the extent to which the indices could be routinely calculated, without recourse to analytical tools which might not be in the public domain (e.g. the requirement to use specialist statistical packages) and secondly the extent to which changes in these indices could be interpreted in the context of environmental performance.

Total abundance of infauna as an index of environmental performance

Total abundance was incorporated into the original TEMP system because it was relatively simple to measure (albeit manual counting was slow and tedious) and it was known that the most frequent response to organic enrichment was an overall increase in the number of organisms in the sediment (see review by Pearson and Rosenberg 1978). In simple terms the relationship could be described on nutritional grounds as:

more organic inputs = more food => more organisms

Following from this we would conclude that if there were a substantial increase in the abundance of benthic infauna (summed across all of the taxonomic groups that have been enumerated) then this would provide a measure of organic enrichment. Total abundance is therefore considered to be a reasonably robust indicator of change that can be interpreted in the context of an organic enrichment gradient. A potential problem with this assumption is that more food might result in the development of a community characterised by a smaller number of much larger organisms. Such a change would confound our traditional manual enumeration method because such a circumstance could potentially produce an overall reduction in the number of organisms even though there might be an increase in the biomass. Arguably the shift to the DNA assay makes this assumption more robust because the DNA assay measures the increase in biomass as compared to abundance and is not influenced by how that biomass is packaged (i.e. many small or fewer large animals).

In the existing TEMP system, a site is considered to be non-compliant if the overall abundance of infauna at that compliance site is four or more times greater than the average abundance of all the control sites. In the ECS system, we are proposing to replace the overall calculation of abundance by a composite score that evaluates the percentage of taxa that have a significantly greater abundance/biomass than the average measure for that taxa across all control sites. This percentage is calculated in the following way (see worked example):

1. The abundance/biomass of each taxon is measured for each control sample and the mean and standard deviation is calculated for each taxon.

2. The abundance/biomass for each taxon is measured for the compliance sample and the value reported[20].

3. The value for the compliance sample is compared to the upper 95% confidence limit (CL) of the mean of the control samples. If the value is greater than the upper 95% CL then the taxon scored 0, if lower it scored 1.

4. The process described in 3 is repeated for all taxa measured at the compliance site.

5. A total is calculated where the number of taxa not exceeding the 95% CL of the control mean is summed across all taxa and this total is then divided by the total number of taxa (non-zero values only) to give a percentage of taxa that are within the range for the controls. A value of 100% means that the abundance/biomass of all taxa is within the expected range as defined by the controls; a value of 0% means that all taxa exceed the expected range. In simple terms the higher the value the better the result.

6. The process can be repeated using a different threshold value (e.g. the 99% CL of the control mean). In general it is expected that the result for the test undertaken using a *higher* CL of the control means will be *less* sensitive to variations in compliance samples.

[20] For this set of analyses, samples were aggregated within sites so there was only one sample for each compliance site and therefore distributional statistics could not be calculated. It is recommended that this be addressed in a future version of the system so that the statistical treatment of the data is more robust.

The worked example below shows how the calculation can be performed for any given taxon; once this has been done then the overall result can be calculated as a percentage across all taxa. For example, if 12 of 18 taxa are within the prescribed range then each will score 1 to give a total value of 12 out of a possible of 18; the final score being 66.7%.

Worked example – Demonstration using two taxa. This worked example evaluated whether the abundance at the compliance site was greater than the 95% CL of the mean for the control sites. In this example the abundance of Ampharetidae at the compliance site was 11 compared to the mean across all control sites of 9.1 with a 95% CL of 2.8. This meant that there was a 95% chance that the real mean for all control sites is less than 11.9 (9.1+2.8)[21]. In this case the abundance measured at the compliance site was within the statistical bounds of the mean for the control site and it was assumed to be not different (scored 1). However, for Capitellidae, the abundance at the compliance site was 14 which was above the upper 95% CL of the mean for all control sites (10.4) so it is assumed that the abundance at the compliance site was inflated relative to the natural background levels (scores 0).

	Taxonomic grouping		**Explanation**
	AMPHARETIDAE	**CAPITELLIDAE**	
Compliance site			
Abundance	**11**	**14**	Value measured at the compliance site
N	1	1	Number of compliance sites
Control sites			
Mean abundance	**9.1**	**8.6**	Mean of values across all control sites
Standard deviation	4.1	2.7	Standard deviation across all control sites
N	8	8	Number of control sites
Standard error	1.5	0.9	Standard error across all control sites
95% CL of mean	**2.8**	**1.8**	Magnitude of 95% CL of mean
Upper 95% bound of mean	**11.9**	**10.4**	There is a 5% chance that the real mean (cf. sample mean) is above this value
Compliance < Upper 95% CL	1	0	Is the compliance value less than the upper 95% CL? (1 = yes, 0 = no)

[21] Using a 1-tailed test.

Differences in the composition of benthic infaunal communities

A second index that can be applied to the assessment of environmental performance was derived through a quantitative assessment of the differences in the composition of the infaunal communities at the compliance site in comparison with the communities at the control sites. This analysis involved the calculation of the Bray-Curtis index of similarity (or dissimilarity). There are two questions that can be asked using this analysis:

1. Is there a significant difference between the infaunal community at the compliance site and the average communities across all control sites? This question is illustrated graphically (Figure 8.1) and was tested by calculating whether the average dissimilarity between all pairs of control sites was lower than the average dissimilarity between the control and compliance sites[22]. If the answer was yes then this implied that the compliance sites had quantitatively dissimilar communities and by implication that they had been impacted by the adjacent farming operation.

2. Assuming that a significant difference was detected through the first analysis then the second question is: How big is the difference between the compliance site and the control sites? This was tested by comparing the ratio of the average dissimilarity between the compliance point and each control point with the average dissimilarity between all pairs of control points. The difference was then scored as small (< 1.5), moderate (1.5 – 3.0) or large (> 3.0). The objective of this test was to determine the magnitude of the differences between the compliance and control sites.

[22] In graphical terms the dissimilarity is essentially a measure of the distance between two points (e.g. a control and compliance point) in the multi-dimensional space represented by the abundance/biomass data for each taxa measured at any given site. If two points are close to one another, they have very similar infaunal communities and are said to have a low dissimilarity. Conversely if the samples have very different communities (difference relative to absolute abundances of infaunal taxa) they will be further apart in the multivariate space and have a concomitantly higher dissimilarity.

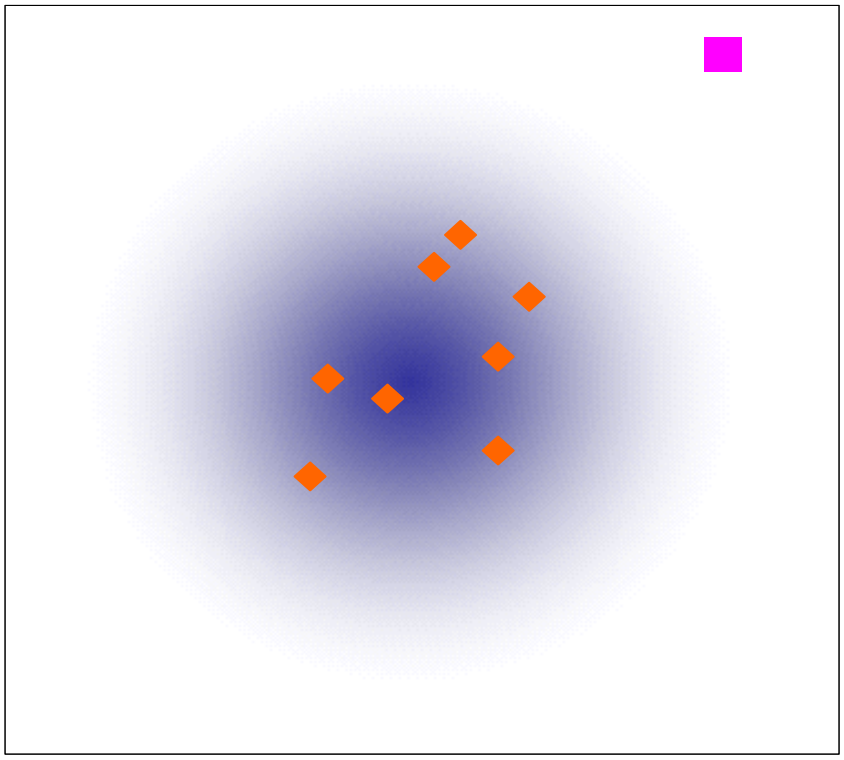

Figure 8.1 Hypothetical ordination of a multivariate dataset in two dimensions. ◆ represent control sites; these points are relatively close to one another because the samples they represent have relatively similar communities. ■ is a hypothetical compliance site that is separated from the control sites because it has a quantitatively different community composition. The graded blue background represents a probability density function of where one would expect to find control points, more in the deeper blue areas and fewer in the lighter areas. In a statistical context, the fact that the compliance point is outside the shaded area, indicates that there is a very low probability that the compliance point is part of the group represented by the control points (i.e. it is significantly different – see question 1). The distance of the compliance point from the centre of the shaded area, when compared to the average distance of each control point to the centre, is a measure of the relative dissimilarity of the compliance sample relative to the control samples (i.e. there is a moderate-high difference of around 3 times – see question 2).

Compliance rules

Compliance rules were established to aggregate the scores from the various tests. This aggregation involved a simple numerical addition of the various test scores. Individual test scores have been weighted so that those tests, which are deemed more important, have been given a higher weighting in calculating the final score. The simplest form of weighting is to give all scores equal weight but there might be cases (e.g. see Table 8.2) where the same test was used repeatedly but with a different threshold value. In such cases a differential weighting can be applied so that those tests with the more stringent thresholds had a higher weighting than the less rigorous tests.

In developing the scorecard for the TEMP data, the tests and weighting scheme shown in Table 8.1 were used. This system was calibrated using data from previous years where the outcomes (derived from the application of the existing compliance analyses) were already known.

In moving to develop a system for interpretation of the TEMP data we propose that the compliance rules outlined in Table 8.1 should be adopted with the weighting system as shown. This provides for a system whereby any individual farm can be scored on a scale of 0 (dreadful outcome) to 100% (superlative outcome). In general terms, intermediate values could be expected but based on an analysis of previous data it is apparent that most would score in the region of 75% to 95%.

Having outlined the compliance rules and aggregation principles, an integrative analysis of all compliance and control data was undertaken. The general principles underlying this analysis are presented in Table 8.2, which illustrates the results for a hypothetical dataset; in this instance the compliance score is relatively low because tests 1, 2 and 3 have resulted in a fail and tests 5 and 6 have low scores.

Table 8.1 Compliance rules recommended for the TEMP. Note that for the purposes of illustration, paired rules that are serially more rigorous (1 versus 2, 3 versus 4, 5 versus 6) have been used to provide some scaling of the results. In this system, any given compliance site may pass tests 2, 4 and 6 but fail tests 1, 3 and 5.

Rule	Text description	Calculate	Test	Test level	Score	Weighting
1	Compliance point is not significantly different to controls	*p*	>	0.05	1	10.0%
2	Compliance point is not significantly different to controls	*p*	>	0.01	1	20.0%
3	Compliance point is less similar to controls than controls are to one another	BC[Compliance-Control]/ BC[Control-Control]	<	1.5	1	10.0%
4	Compliance point is less similar to controls than controls are to one another	BC[Compliance-Control]/ BC[Control-Control]	<	3.0	1	20.0%
5	Number of compliance taxa with abundance not exceeding the upper 99% CL of control mean abundance	n	/	N	n/N	13.3%
6	Number of compliance taxa with abundance not exceeding upper 95% CL of control mean abundance	n	/	N	n/N	26.7%
Total score						100.0%

Table 8.2 Hypothetical test results for TEMP data.

Rule	Calculated value	Test	Level	Test result	Test score	Weighting	Overall score
1	0.00	>	0.05	**Fail**	0	10%	**0.0%**
2	0.00	>	0.01	**Fail**	0	20%	**0.0%**
3	2.34	<	1.50	**Fail**	0	10%	**0.0%**
4	2.34	<	3.00	**Pass**	1	20%	**20.0%**
5	11.00	/	18.00	**0.61**	0.61	13.3%	**8.1%**
6	10.00	/	18.00	**0.56**	0.56	26.7%	**14.9%**
Giving a compliance rating of							**43.0%**

Business rules

Once a system for calculating a compliance score has been agreed upon, the next step is to establish business rules that determine how the compliance score will be interpreted. In simple terms this means agreeing on a series of threshold values that will be used to trigger management or regulatory responses. It should be noted that when adopting this system, the maximum and minimum score for any given rating are set at the discretion of the *regulatory agencies* and the values incorporated into the table are purely illustrative. These thresholds may be set at higher or lower levels based on the environmental standards that have been established *a priori* (and this may even be varied from one area to another depending on things such as the conservation status of the relevant farming zone). Furthermore, managers may choose to implement a different number of ratings (e.g. five bands as opposed to the three that were originally recommended[23]).

Therefore the system we now recommend for TEMP data uses a simple five-tiered system. This provides a system of Green, Yellow, Orange, Red and Violet indicators that gave simple but clear messages to both farm managers and regulators (Table 8.3).

[23] During presentations to industry it was requested that the system be implemented with 5 bands (Green, Yellow, Orange, Red and Violet). This has been done for the final version of TEMPEST that has been released and the text in this report reflect this change.

Table 8.3 Proposed business rules for the TEMP scorecard.

Rating	Min. Score	Max. Score	Score Interpretation	Practice Interpretation
Green	80%	100%	A green rating means that control and compliance samples are not different	Current environmental management practices are working well
Yellow	60%	80%	A yellow rating demonstrates a situation where, although there are some differences between control and compliance points, the magnitude or significance of the difference is relatively low	Environmental management practices are not consistent with industry best-practice
Orange	40%	60%	An orange rating demonstrates a situation where, although there is a significant difference between control and compliance points, the magnitude or significance of the difference is low	Environmental management practices need to be changed to prevent further deterioration of the supporting environment
Red	20%	40%	A red rating demonstrates a situation where there is a significant difference between compliance and control sites and where this difference has a magnitude that warrants immediate remedial action	This outcome is technically in breach of licence conditions and immediate changes should be made to farming practices to prevent further environmental harm.
Violet	0%	20%	A violet rating demonstrates a situation where there is a substantial difference between compliance and control sites and where this difference has a magnitude that warrants immediate remedial action.	This outcome is in breach of licence conditions. Farming should cease on this site to prevent further environmental harm.

Management response

The management response to any given scorecard result should be worked out in collaboration with industry but in general terms the scope for action within any given farming system needs to be considered. For the southern bluefin tuna farming industry there are relatively simple management responses to any breach of compliance conditions including:

- Reduce feed wastage through better management of feeding – better management of staff and operational practices.

- Move farms to locations with deeper water or better current flow – negotiate access to a better lease site.

- Reduce stocking densities – consider balance between stocking density and holding time.

- Better manage net fouling – work to reduce net cleaning during periods of low current flow.

Environmental assessments in any industry can only invoke prospective management responses. Any changes that are made will affect future outcomes and not past performances. In this context, the five-tiered system should provide farmers who run the risk of being non-compliant with a warning. This will allow them to modify their management, review the best practice options and thereby improve outcomes in future years.

TEMPEST© – a spreadsheet template for evaluating environmental performance

In order to facilitate the application of the DNA assay system for routine compliance-based environmental monitoring, a spreadsheet template has been developed which automates the entire analysis and reporting system. The template application is entitled TEMPEST© and can be used not only for analysis of the DNA assay data but may also be used for manually enumerated count data. TEMPEST© has been set up so that the compliance rules and business rules are clearly articulated and easily changed.

TEMPEST© has been tested extensively by using manually enumerated count data from the Tuna Environmental Monitoring Programme (TEMP) collected over the period 2001 to 2004. From a total of 72 datasets tested (comprising data from every compliance site sampled each year), 11 sites scored between 80% and 90% and 60 sites scored between 90% and 100% (Figure 8.2). Only one site scored less than 50% (indicating a non-compliant result), and this finding was consistent with the result for that site determined from the previous approach to analysis of compliance performance (Madigan *et al.* 2001).

This site did not comply with the criteria set by PIRSA Aquaculture, because the mean abundance of infauna was more than four times the mean of the associated control sites. Subsequent investigations by PIRSA Aquaculture indicated that the licensee had incorrectly translated the licence coordinates from one geographical projection system to another. Consequently, a number of sea-cages were located outside the prescribed

boundaries of the licensed farming area and were in much closer proximity to the compliance site than would be prescribed under normal compliance monitoring.

Opportunities for improvement of TEMPEST©

In formulating the compliance score used in TEMPEST©, a number of the compliance tests were based on threshold responses. In such cases the results are all or nothing (scoring 1 or 0) and as a consequence most of the compliance scores were greater than 80%. Arguably, although making it easier to determine whether a site is compliant, more value would be provided to farmers if the results were graded more evenly across the scale (0 to 100). In order to test the effect of grading sites (rather than using threshold values) the TEMPEST© spreadsheet was reformulated to provide a graded score for all tests. When run across the 72 datasets (as detailed above) there is a substantial change in the frequency distribution relative to the original analysis (Figure 8.3).

In the second analysis none of the sites scored above 90% with most of the sites (41) scoring between 70 and 80%. Fifteen sites scored 80 to 90% and ten sites scored between 60 and 70%. Five sites were close to the 50% mark and the one site that scored poorly in the former analysis was scored even lower on this system (29% versus 43%).

It is arguable that this graded scale is more useful for informing managers by providing a more highly resolved view of environmental status at any point in time. It is reasonable to expect that this will thereby allow a more timely response, as farmers will be able to respond when environmental effects first start to become apparent rather than when they fail the compliance tests. Similarly, regulators will be able to develop a better sense of the performance of farms over time and thereby be better positioned to work with managers to ensure that they remain compliant.

In summary, we would recommend that the TEMPEST© application should be used in the graded mode, which is more useful in supporting an adaptive management approach for the industry. Not withstanding that the system was originally developed to operate in the threshold mode (and this is how it has been presented to industry and regulators), we believe that there would be substantial merit to revising this strategy.

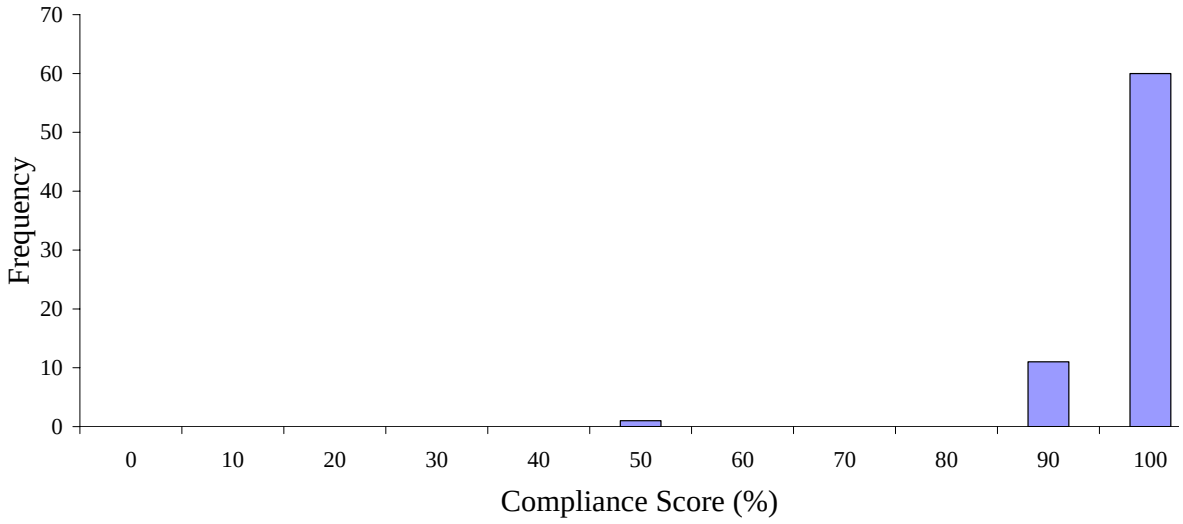

Figure 8.2 Frequency distribution of compliance scores for manually enumerated count data from TEMP 2001 to 2004 using the application template, TEMPEST©.

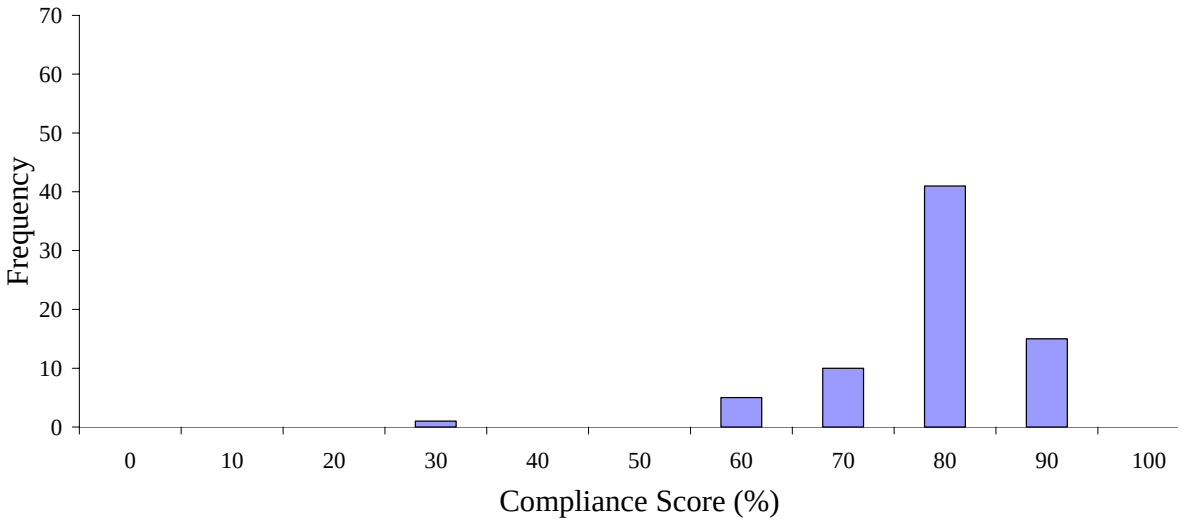

Figure 8.3 Frequency distribution of compliance scores for manually enumerated count data from TEMP 2001 to 2004 using TEMPEST© to produce a graded scale for each of the component scores.

Conclusions

In order to deliver the DNA assay system for use as a tool in routine environmental assessment and monitoring there was a need to develop a method for analysing and presenting the results from the assays. An Environmental Compliance Scorecard (ECS) has been developed that provides a system to facilitate the analysis and reporting of these results.

The ECS system (implemented through the TEMPEST© application) can be used to support decision-making in relation to environmental compliance. In broad terms, the ECS system engages scientists in the identification of environmental status indicators and in the calculation of quantitative indices of performance; regulators in establishing business rules for the assessment of environmental performance and farm managers in the development of appropriate management responses. The ECS system comprises four modules:

1. Data analysis module - delivers a quantitative analysis of the assay data and evaluates a series of pre-determined indices of environmental status.

2. Compliance score module – integrates the results from the data analysis to produce an overall environmental compliance score that is ranked on a scale of 0 (very poor performance) to 100 (perfect result).

3. Business rules module – uses a series of business rules to determine whether the compliance score is within acceptable limits (as determined by the regulatory authorities). Results are presented using a five-tiered system where green indicates the achievement of best-practice performance, yellow indicates a good result, orange is a warning to managers that environmental performance is below industry standards, red is a technical breach of compliance alerting managers and regulators of the need for immediate changes in management practices and a violet result indicates a clear breach of compliance requiring immediate remedial action.

4. Management response module – identifies an appropriate management response in relation to the application of the business rules.

The ECS system was developed as a spreadsheet application (TEMPEST©), which will be available under licence from the Aquafin CRC.

Future work

The TEMPEST© application was designed to facilitate the interpretation of data from the TEMP system. In many ways however it is a work in progress. In particular the analytical routines that underpin TEMPEST© were designed specifically to deal with the data

obtained through the TEMP system and this limits its more general application to other farming systems.

In particular, there is a need to re-design the analysis to deal with sample level versus site level data[24]. This requires a reprogramming of TEMPEST© to allow the user to enter multiple rows of data for each site (including the compliance and all the control sites). Similarly, provision is needed for uneven sample numbers (per site) to allow for situations when samples are accidentally lost, destroyed or contaminated.

TEMPEST© could be further improved through a revision of some of the statistical procedures. For example, the current approach of only enumerating the number of taxa for which the abundance exceeds the controls could be modified to calculate the number where the abundance is different to the controls (i.e. greater or less). This would have the advantage of dealing with situations where organic enrichment has gone to an extreme level making the environment unsuitable for any taxa to survive in elevated numbers.

[24] TEMP data generally comprise eight samples per site. Samples are collected at one compliance site and eight control sites. Sample results are aggregated at the site level and this provides a dataset comprising a multivariate matrix with nine sites (eight control and one compliance) and n taxonomic groups (n=9 for DNA assay data, n is variable – typically 50-90 – for manual count data).

Chapter 9 Conclusions

Benefits and Adoption

The direct benefit of this project is the application of both the DNA assay system together with the Environmental Compliance Scorecard system to the Tuna Environmental Monitoring Programme (TEMP). TEMP is a farm-site compliance-based environmental monitoring programme required as a South Australian State Government licence condition for southern bluefin tuna farmers. The entire system has been trialled as part of the 2005 Tuna Environmental Monitoring Programme.

The DNA based system developed enables the rapid assessment of environmental performance for individual farms at a reduced cost. Furthermore, there is the potential for the routine compliance monitoring of environmental impacts to be delivered with further cost reductions than current monitoring techniques if scale-based efficiencies can be realised due to an increase in the number of samples processed on a "batch" basis.

The time required for the laboratory processing (without data analysis and reporting) of the samples using this new DNA assay system was approximately a quarter of the time required using the traditional technique of manual sorting and enumeration (20 days versus 90 days). Similarly the cost to industry (assuming the field component of sample collection was equal) has been reduced by **11%** (TEMP 2005 - $353 per sample cf. TEMP 2004 $397 per sample) using the DNA assay system. It should be noted however that there were fewer samples processed in 2005 and if the number of samples had been the same, the cost of TEMP 2005 would have been $284 per sample representing a **28%** reduction (not including an inflation rate of 3% to 4%) in the cost of monitoring. This is because the cost of DNA laboratory processing, which uses high throughput systems, is scale-based and currently ranges from $200 per sample (1 – 200 samples), $150 (201 – 500 samples) to $130 (> 500 samples). If the DNA assay system is extended to other finfish aquaculture sectors (see Further Development below), there may also be cost savings in the annual set-up fee, which is for the maintenance of DNA standards and quality assurance of the DNA assay system, as this may be able to be shared across sectors.

The original proposal identified that 90% of the benefits of this project would be to the commercial sector in South Australia, with the remaining 10% being to non-fisheries beneficiaries. This is a good representation of the flow of benefits. The primary beneficiary in the commercial sector will be the southern bluefin tuna industry, however, the outcomes from this project have the potential to be applied to other marine finfish sectors in South Australia, as well as other states/territories in Australia.

Further Development

The DNA assay system developed for rapid detection and quantification of indicator taxa in sediment samples could be expanded and applied to the assessment of other forms of organic enrichment such as sewage outfalls or meat/fish processors.

Currently, a proposal is before PIRSA Aquaculture and the Fisheries Research and Development Corporation to extend the DNA assay system and the Environmental Compliance Scorecard system to other finfish aquaculture sectors (specifically yellowtail kingfish and mulloway) to meet Environmental Monitoring Programme (EMP) requirements in South Australia. It is anticipated that additional DNA assays will need to be developed for application to other finfish aquaculture. For application to salmon aquaculture in Tasmania, additional assays or a different suite of assays may be required. This was realised early in the project during the assessment of the generality of the technique (Objective 4) as differences in benthic infauna between southern bluefin tuna farming region in Port Lincoln and salmon farming in Tasmania were found when comparisons were made. However, the development of any additional assays would increase the resolution of the DNA assay system for environmental assessment and monitoring. The application of this expanded system would benefit not only other finfish aquaculture but also southern bluefin tuna aquaculture.

There is also the opportunity to cost effectively screen sediment samples collected as a component of EMPs to address other issues for which DNA assays have been developed. An example is the prevalence of various disease organisms with DNA assays having recently been developed for blood fluke (*Cardicola forsterii*) and gill fluke (*Hexostoma thyni*) by the Aquafin CRC/FRDC project (2004/085), "Detection of SBT pathogens from environmental samples".

The improvement of TEMPEST© through a revision of some of the statistical procedures as discussed in Chapter 8, Future work, would be useful for more general application to other farming systems such as other finfish aquaculture.

Intellectual property for potential commercialisation is outlined in Appendix I. Licensing or assignment of the intellectual property will be determined by the Board of Aquafin CRC subject to approval by the Australian Government.

Planned Outcomes

The outcomes pertaining to this project as agreed between the Commonwealth of Australia and Aquafin CRC participants for the environment programme were:

- Improved monitoring of the environmental performance of cage aquaculture operations.
- Better community understanding of aquaculture and the environment.
- Increased productivity of cage aquaculture.

The three planned outcomes given in the original project proposal were:

- A system for quantitatively assessing the impact from alternative farming processes that will provide certainty in planning and management and thereby help to secure tenure for aquaculture industries in marine environments.
- An ability to rapidly determine the status of marine sediments in the vicinity of cages will allow for more efficient waste management and a greater ability to identify potential risks that will reduce mortality and improve feed conversions.
- Other research, requiring data on benthic infaunal abundances, can sample more intensively with this cheaper and faster option. This will allow for greater statistical power and thus improve the quality of results.

The DNA assay system developed provides the ability to rapidly assess the status of the seabed in the vicinity of sea-cages, which will enable the spatial and temporal scales of impacts to be more effectively determined. This technology will then provide a tool to ensure optimal production rates, minimal disease rates, optimisation of the size and configuration of lease sites, sea-cage fallowing and rotation strategies and optimal stocking densities. Furthermore, the environmental impacts of changes in technology (such as alternative waste mitigation strategies), different feeds (including manufactured feeds) and husbandry practices can be evaluated much more efficiently.

Applications of this technology include the periodical compliance assessments of lease sites (required as a state government licence condition for southern bluefin tuna farmers) and the evaluation of alternative farming strategies such as long-term holding of fish in sea-cages.

In addition, a new approach has been developed and proposed for the reporting of environmental assessments through the development of an Environmental Compliance Scorecard (ECS). This system will help facilitate the uptake of this technology across both industry and regulatory environments.

Conclusions

All specified objectives were met over the two phases of the project. In the proof of concept phase, benthic infaunal species that are characteristic of sites ranging from organically enriched to non-enriched were identified. A DNA assay system for the detection of these selected taxa in sediment samples was then developed. This assay system was calibrated and extensively tested for sensitivity and specificity to evaluate its application for quantitative estimation of indicator taxa. The generality of the DNA assay system was assessed, in particular for salmon aquaculture, and it was realised early in the project that additional assays or a different suite of assays might need to be developed for application to other forms of aquaculture in other locations.

In the second phase of the project, nine DNA assays were finally developed, calibrated and sensitivity improved to demonstrate the proof of application of this assay system in comparison with the traditional infauna manual enumeration technique. Although not in the original project proposal, a new approach was developed and proposed for analysing and presenting the results from the DNA assays. The development of an Environmental Compliance Scorecard (ECS; implemented through the TEMPEST© spreadsheet software package) will help facilitate the uptake of this technology across both industry and regulatory environments.

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Appendix I Intellectual Property

The technology to deliver this DNA-based environmental monitoring system

1. Methods of sampling to detect changes in the infauna of marine sediments related to organic enrichment;
2. Methods of processing large samples (200-800 g) for DNA-based analysis;
3. Method of analysing samples using real-time PCR (RT-PCR) to measure biomass of specified infauna;
4. Probes and primers, based on selected DNA sequences, for use in RT-PCR as indicated in item 3;
5. Methods for interpreting RT-PCR readings so as to determine changes in biota and their significance as a measure of ecosystem change;
6. Evidence of performance of the above methods in comparison to slower conventional methods;
7. Environmental Compliance Scorecard (TEMPEST©) for presenting results in a meaningful framework.

The technology developed in the course of the CRC project

From the above section, items 1, 4, 5, 6 and 7 are achievements of the CRC project.

In addition, in item 2, a method for extracting DNA from soil samples has been refined and demonstrated to be effective for marine sediments, as a further achievement of the CRC project.

Background intellectual property that is available to the CRC

In item 2 above, the original method of processing large soil samples for analysis using DNA probes is the joint property of SARDI and CSIRO, and was established independently of the CRC. CSIRO must give formal approval for commercial delivery of this technology in this field of application.

In item 3 above, the use of TaqMan^{TM} chemistry analysis is the property of Applied Biosystems. PCR Royalty must be paid to Applied Biosystems for commercial testing. This is incorporated into the retail price and paid by the diagnostic laboratory of SARDI.

Appendix II Data Register

Location of data	Topic(s)	Author/custodian	Access to data
Lab notebooks and files – located at SARDI Aquatic Sciences, Room B2.17	Notes of meetings, discussions and experiments Infauna identification and abundance	M. Loo and S. Drabsch	Confidential
Lab notebooks and files – located at Plant Research Centre, Diagnostics Office, Plant and Soil Health Unit	DNA quantification and amplification results	K. Ophel Keller, A. McKay and Herdina	Confidential
Database – located at SARDI Aquatic Sciences, Room B2.17 on Computer IT07615 in folder D:\TUNA ENVIRONMENT and on J:\ SARDI Aquatic Sciences, Room B2.17 on Computer IT07615 in folder D:\TUNA ENVIRONMENT and on J:\Aquapcr on 'Pirsa\Pirsa_met\Grenfell\ Servers\Pirsaf01_user3\ Sharedat' (backup copy on external Maxtor hard drive)	Infauna identification and abundance, DNA assay results, calibration and specificity testing results, reports	M. Loo and K. Ophel Keller	Confidential
Database – located on G:\RDTS\RDTS Commercial	Raw DNA results on processed sediment samples	A. McKay	Confidential
Files of DNA analysis of experimental work – located on G:\RDTS\Aquatics. Hard copies in locked files in Plant Research Centre, Diagnostics Office, Plant and Soil Health Unit	Summaries of DNA analysis-specificity testing, spiked samples and test calibration	K. Ophel Keller and Herdina	Confidential
Database – located at CSIRO Entomology, Canberra	Sequence data of specimens	D. Hartley	Confidential

Appendix III Project Staff

Main Project Staff

SARDI Aquatic Sciences	Dr Maylene Loo (Principal Investigator) Prof Anthony Cheshire (original Principal Investigator) Mr Steven Clarke Ms Sharon Drabsch
SARDI Crops	Dr Kathy Ophel-Keller Dr Alan McKay Dr Herdina
CSIRO Entomology	Dr John Curran Ms Diana Hartley

Casual Project Staff

SARDI Aquatic Sciences	Ms Katherine Cheshire Ms Yvette Eglinton Mr Michael Gueridian Mr Matthew Hoare Ms Sandra Leigh Mr Ian Magraith Mr Keith Rowling Mr David Miller Mr Bruce Miller-Smith Ms Mandee Theil Ms Sonja Venema Ms Rachel Wear Ms Phillipa Wilson
SARDI Crops	Mr Russell Burns Dr Sue Wiebkin