Tackling microbial related issues in cultured shellfish via integrated molecular and water chemistry approaches

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Non-Technical Summary

2011/729 Tackling microbial related issues in cultured shellfish via integrated molecular and water chemistry approaches

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PROJECT OBJECTIVES:

1. Using state-of-the-art molecular techniques, rapidly and accurately detect microbial communities in water samples from shellfish farms without the need for laboratory culturing

2. By combining new metagenomic analyses with established hydrochemistry analytical methods, obtain baseline inventories of microbial species associated with abalone and oyster hatchery and grow-out areas in Tasmania

3. Developing a multivariate model by comparing temporal microbial diversity in Tasmanian shellfish farms and identifying correlations between microbial communities and hydrochemistry parameters (under different water profiles)

4. Establishing a proof of concept for a predictive tool for the shellfish sites so that mitigation measures could be put in place to help counteract husbandry issues

OUTCOMES ACHIEVED

This study represents the first in-depth analysis of microbial communities and water chemistry associated with shellfish culture in Australia. As traditional cultivation methods are largely unsuitable for most marine microbes, the microbial characteristics of farm samples were assessed using a combination of new molecular DNA techniques. These state-of-the-art techniques included Next Generation Sequencing (NGS) and Automated Ribosomal Intergenic Spacer Analysis (ARISA). NGS, which determines the order of the four DNA nucleotides (adenine, guanine, cytosine and thymine) in a DNA molecule, was used to identify microbial species and their taxonomy. The ARISA fingerprinting approach was used to assess the diversity of the microbial communities in each sample. In combination, the techniques endorsed the identification of community structure and taxonomy of microbial species found in the water columns from both oyster and abalone farms. The wealth of data generated from these molecular approaches was coupled with the first longitudinal study on *abiotic* and nutrient parameters, measured over a period of 14 months from across multiple locations in Tasmania (including a greenfield/no aquaculture activities site). Regular screening and testing of water quality parameters such as salinity, temperature, dissolved oxygen, ammonium and silicate afforded the project collaborators with information that was not previously recorded on any of the farms.

In order to identify the main factors that influenced microbial community diversity across the five shellfish farms, 497 microbial samples (and accompanying filtered water samples) were collected from two abalone farms (AbTas in Clarence Point and Cold Gold in Dunalley) and three oyster hatcheries (Shellfish Culture - Bicheno, Spring Bay Seafoods in Triabunna and Shellfish Culture - Pipe Clay Lagoon). Microbial DNA extraction of these samples was undertaken with 152 samples analysed by ribosomal tag pyrosequencing for NGS (for taxonomic identification) and 235 samples analysed by 16S-23S rRNA ARISA (for community profiling). Over 2 240

different taxa of marine bacteria were identified from 650 000 tagged sequences including a number of beneficial and pathogenic species that are of significance to shellfish culture. The utility of NGS for detecting algae in the same DNA samples was also shown. ARISA analyses demonstrated farm specific profiles such that the microbial communities in the water samples from each shellfish farm were shown to be significantly different to each other.

We present here substantial evidence to show that metagenomic analyses were effective in resolving taxonomic identity, community profile changes and assessment of microbial abundance from water samples from these shellfish farms without the need for cultivation of microbes within the laboratory. The innovative research undertaken here focused on obtaining baseline information of the microbial communities in the water column. The resulting extensive data set gave insights into microbial community changes over time for each of the specific Tasmanian shellfish farms. While single environmental parameters were not sufficient to establish relationships with microbial composition, higher levels of ammonium and silicate were observed to be significant drivers of the microbial communities, particularly within the abalone farms. To a lesser extent, increased temperature was also a significant driver of the microbial communities. Importantly, significant differences among microbial community profiles were also observed across the geographically separated shellfish farms.

Importantly for the wider aquaculture industry, approaches deployed in this study can be utilised to screen any sourced water – be that from aquaculture farms, grow-out areas, aquarium tanks or blue water sites. These approaches are microbial specific, not aquaculture type specific. Our future research will focus on microbial and algal pathogen detection and the utility of ARISA and NGS for product/farm provenance.

LIST OF OUTPUTS PRODUCED

1. Database and methodologies:
    - protocols for on farm monitoring of water temperature, pH, salinity, dissolved oxygen, ammonium, nitrate, phosphate and silicate (see this report)
    - farm and researcher applicable sampling protocols for water and microbial screening using Isopore filters and Isohelix swabs (see this report) and a stand alone Sterivex unit sampling protocol for on farm use
    - laboratory optimised methods for flow injection analyses of ammonium, nitrate, silicate and phosphate (see this report)
    - laboratory optimised extraction and molecular analysis protocols for microbial DNA (see this report)
    - in house database of microbial taxa and farm specific environmental information from Tasmanian shellfish farms including archival samples
2. Model predicting the cause and agents of events in shellfish production:
    - univariate and multivariate models (alongside statistical pipelines) linking microbial diversity and covariates
    - metagenomic protocols enabling detection of microbial species considered to be significant to shellfish culture
3. Mitigation approaches for the shellfish industry:
    - guidelines for normal safe operating parameters (see this report)
    - fee for service facility enabling ongoing monitoring and surveillance
4. Multi-year dataset and publications:
    - baseline inventories of microbial species associated with abalone and oyster hatcheries/grow-out areas in Tasmania across 14 months of monitoring
    - published articles in general interest industry magazines
    - presentation at AMSA July 2012 conference
    - draft manuscripts in preparation

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Acknowledgments

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1. Introduction

Aquacultured species such as oysters and abalone live their entire lifecycle in seawater. While these eukaryotic organisms are generally cultured in single species systems (at least in Australia), they co-exist with a wide range of innocuous, pathogenic and beneficial microorganisms (Schulze *et al*., 2006) in the aqueous environment. Culture activities such as artificial feeding, use of flow-through or reticulation (and or open sea-water grow-out) and intensive shellfish spawning in land based hatcheries inevitably affect these microbial assemblages in seawater and vice-versa. While we can not see the members of the communities, microbes are known to play essential ecosystem roles through activities such as organic matter cycling, contributions to biological production at higher trophic levels and maintenance of healthy systems (Sakami *et al*., 2007; Zeng *et al*., 2010; Zhu *et al*., 2012). In contrast, microbes also cause important diseases thereby resulting in major effects on production efficiencies and significant economic losses (Schulze *et al*., 2006; Zeng *et al*., 2010; Adams and Thompson, 2011; Shi *et al*., 2012).

To maintain sustainable shellfish aquaculture systems, we believe farms require methods and tools for water monitoring that encompass water chemistry and organic matter testing while also enabling identification (and hence diversity, role and function) of microbial community composition. We believe the identification and documentation of water parameters in a systematic manner helps farmers and managers to identify causal relationships between the physical environment (and chemical parameters) and microbial populations.

In the event of a 'crash' or large scale loss, farmers would then have the tools to determine both the *abiotic* parameters and associated changes in microbial populations that are indicative, thereby identifying conditions that may favour the proliferation of potential causative agents. This information helps to characterise and prevent disease outbreaks and reduce mortalities by increasing the resolution of predictive models for mitigation or enabling timely modifications to husbandry practices before disease can have a major impact. Conversely, the same tools can be used to monitor the beneficial microbial communities, thereby providing farms with ongoing information about the environments in which their shellfish are being grown.

With rapid advances in next generation sequencing (NGS) technologies (Brown *et al*., 2009; Huson *et al*., 2009; Simon and Daniel, 2009; Sun *et al*., 2011), we now have the capability to screen shellfish and water samples for microbial diversity without the requirement to undertake extensive in-laboratory culturing or production of cloning libraries (Simon and Daniel, 2009; Sun *et al*., 2011). Sun *et al*. (2011) have highlighted that up to 90% of bacteria from most environments can not be easily cultured in the laboratory (on standard media – hence the great plate count anomaly, Staley and Konopka, 1985) and for this reason are not available for study. Molecular phylogeny has reinvigorated the study of microbial organisms. Massively parallel tag sequencing (a tool of NGS) offers the ability to determine the microbial assemblages and community structures in more depth (see Stoeck *et al*., 2009; Stoeck *et al*., 2010) and without the bias that could be introduced with culturing (Simon and Daniel, 2009). This is metagenomics, or the study of metagenomes (i.e., genetic material detected in environmental samples). Metagenomics has the ability to access DNA information from microbial genomes while avoiding the discrepancies between direct microscopic counting of microbes and numbers of colonies that can actually be cultured on plates (Aw and Rose, 2012). Importantly, metagenomics enables the assessment of the taxonomic and functional diversity of microbial communities on an ecosystem level (Simon and Daniel, 2009). Deployment of 16S rRNA gene amplification and sequencing allow microbial organisms from the environment to be detected without the need for prior cultivation or isolation (Ma *et al*., 2008; Tamminen *et al*., 2011).

Bacterial community structure and abundance analyses has previously been undertaken for a number of Australian finfish farms using more traditional genomic techniques (e.g., 16S clone libraries) (Edgar *et al*., 2005; Bissett *et al*., 2007; Castine *et al*., 2009) and more widely in other aquaculture industries overseas including abalone and oyster cultivation (e.g., Sandaa *et al*., 2003; Jensen *et al*., 2004; Schulze *et al*., 2006; Ma *et al*., 2008; Pereira *et al*., 2011; Tamminen *et al*., 2011; Trabal *et al*., 2012; Zhou *et al*., 2012). In contrast, the implementation of metagenomic

studies for microbial community analysis in shellfish is a new direction for the Australian shellfish industry (see **Figure 1**).

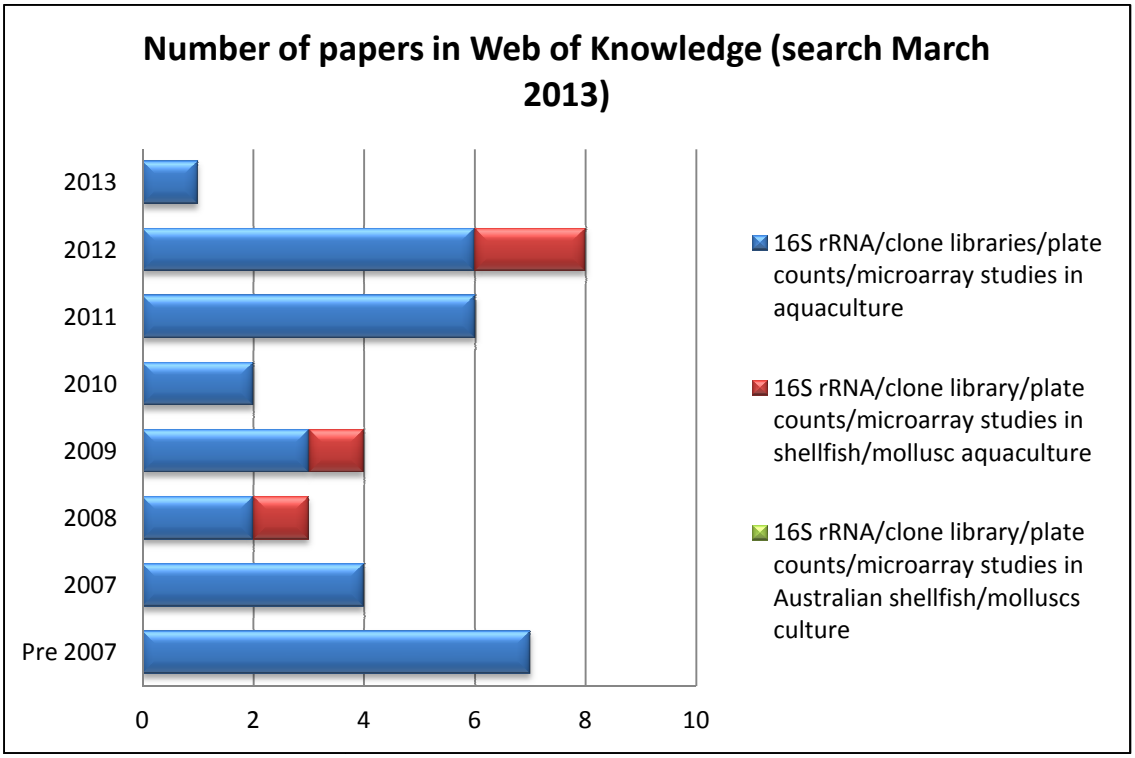


Figure 1 Web of Knowledge search on microbial community analyses and monitoring in aquaculture (does not include studies on pathogen detection, biotechnology, drug discovery or anti-microbial for disease control. Key word search = metagenomics, NGS, bacterial and microbial diversity/communities, monitoring, aquaculture)

Recently, CSIRO developed a suite of new tools based on molecular and NGS methods that enable complex microbial communities in water, sediment or soil samples to be rapidly described without the need for physical culturing of microorganisms on media plates. The identification of both beneficial and harmful microorganisms which are responsive to changes in *abiotic* environmental parameters as well as low abundant microbes (that represent a reservoir for infection), can now be undertaken using highly sensitive, cost effective and high throughput genomics techniques. Furthermore, CSIRO has developed multivariate statistical approaches that are capable of describing links between microbial community profiles and causative factors.

The current project was developed to undertake analyses of metagenomes or genetic material (from microbes) recovered directly from environmental samples from various shellfish farms in Tasmania. Our aim was to provide baseline information about the microbial communities found on the farms and clarification of the environmental conditions that might influence the communities.

1.1 Need

The Australian cultured edible mollusc production for 2009-2010 was worth approximately $A125 million (http://www.abs.gov.au/ausstats/abs@.nsf/Lookup/by%20Subject/1301.0~2012~Main%20Features~Fishing~182). Although conditions and end product use varies considerably among farms and species, all species are cultured in seawater. Here the shellfish co-exist with a range of microorganisms which are variable over temporal and spatial scales. In addition to this, while the industry is generally expanding, over the last few years the shellfish industry has experienced an increase in unpredictable hatchery and settlement rates of abalone and oyster larvae. Batches

crash for no apparent reason despite successful spawning and fertilisation. Additionally, farms experience unexplained stock losses during grow-out periods in both land and sea based culture facilities. More widely, there is increasing evidence to suggest the cause of such events may be microbial in nature (Friedman *et al*., 2000; Elston *et al*., 2008; Romalde and Barja, 2010; Trabal *et al*., 2012).

Recently, the Australian abalone aquaculture industry ranked the 'investigation into the causes of summer mortality and development of management protocols to minimize or prevent losses' as one of the top three priorities for the industry. Summer mortality in land based cultured abalone and more widely in mollusc aquaculture is believed to be caused by *Vibrios* (Hada *et al*., 1984; Romalde and Barja, 2010); however this needs to be confirmed empirically. In France, summer immune depression in *Haliotis tuberculata* has been associated with *Vibrio* (Nicolas *et al., 2002)*. Associations between maturation, spawning ability, immune status and susceptibility to *V. harveyi* were shown where increased temperature was seen as a trigger for vibriosis development and overall depression on molluscan immunity (Handlinger *et al*., 2005; Travers *et al*., 2008; Schikorski *et al*., 2013). *V. parahaemolyticus* has also been identified as the responsible agent for disease outbreaks in Pacific oysters (Comeau *et al*., 2005). In *H. cracherodii*, a recently discovered member of the Rickettsiaceae order, *Candidatus Xenohaliotis californiensis* was identified as the aetiological agent for withering syndrome (a fatal disease) in wild and cultured abalone (Friedman *et al*., 2000).

In oysters, farmers are looking for indicators of potential culture problems (i.e. hatchery failures) (M. Bermudes, pers.comm.). Two of the key issues that affect management of cultured oysters in Tasmania, South Australia and New South Wales are Summer Mortality and unexplained mortality in hatcheries and rearing areas (Leith and Haward, 2010 - http://oysterstasmania.org/downloads/conference-presentations/Peat-Leith-Climate-Adaptation.pdf). Water quality and associated bacterial problems therefore represent a significant issue for the shellfish industry (Adams and Thompson, 2011; Pereira *et al*., 2011) (and aquaculture more widely) with mass mortality events proving costly. For example, severe episodes of vibriosis caused by *V. tubiashii* in shellfish (*Crassostrea gigas*, *C. sikamea* and *Panope abrupta*) hatcheries in North America were associated with the mixing of warmer surface seawater and upwelled cooler nutrient and *Vibrio* enriched seawater (Elston *et al*., 2008).

Importantly, there is no information on the microbial communities present in the water columns from cultured shellfish farms in Australia (albeit there are several international studies in molluscs that can help inform domestic studies e.g., Sandaa *et al*., 2003; Comeau *et al*., 2005; Schulze *et al*., 2006). Therefore, the current research aimed at identifying the microbial diversity present in hatcheries, nurseries and grow-out sites that culture both abalone and oysters in Tasmania. Our aim was to provide shellfish farms with the monitoring tools and capability to help reduce product loss and strengthen husbandry and management techniques, thereby reducing the impacts of disease and increasing farm profitability.

1.2 Objectives

1. Using state-of-the-art molecular techniques, rapidly and accurately detect microbial communities in water samples from shellfish farms without the need for laboratory culturing (*achieved*)

2. By combining new metagenomic analyses with established hydrochemistry analytical methods, obtain baseline inventories of microbial species associated with abalone and oyster hatchery and grow-out areas in Tasmania (*achieved*)

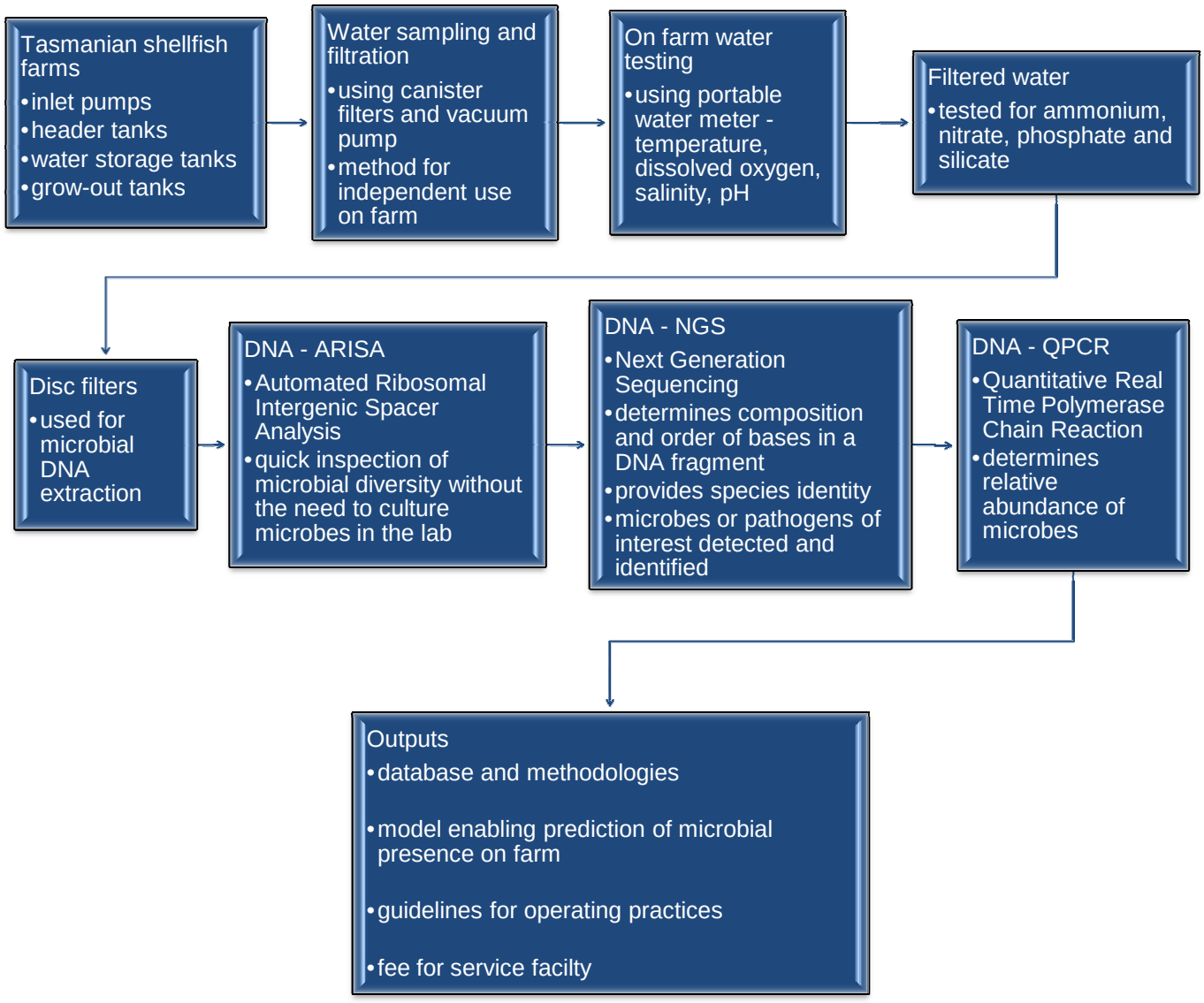
3. Developing a multi-variates model by comparing temporal microbial diversity in Tasmanian shellfish farms and identifying correlations between microbial communities and hydrochemistry parameters (under different water profiles) (*achieved*)

4. Establishing a proof of concept for a predictive tool for the shellfish sites so that mitigation measures could be put in place to help counteract husbandry issues (*achieved*)

The work undertaken during the course of this project satisfies each of the objectives outlined above. In addition to the initial objectives, a number of additional activities have been undertaken during the course of the project. Whilst these additional analyses did not form part of the original Seafood CRC project, they were undertaken after consultation with industry partners who identified a number of areas of particular interest to them. These included sampling on a fifth shellfish farm in Tasmania and a 'greenfield' site, sampling up to five (+) sites per farm, optimising and deploying ARISA analyses, undertaking quantitative polymerase chain reaction (PCR) protocols and using NGS to screen for algae taxa. The results from all of our activities are presented here for scientific completeness as they provide information that would otherwise not have been collected.

2. Methods

An overall outline of the farm sampling, microbial (i.e., metagenomics) and environmental and hydrochemistry (i.e., covariable) data generation and outputs are shown below. These components are then shown in more detail in the following sections.



2.1 Farm sampling

The project commenced in October 2011 and continued to December 2012. Monthly on farm sampling was undertaken across four primary shellfish farms/hatcheries in Tasmania (see Figure 2; Clarence Point (AbTas), Bicheno (ShellfishC-Bicheno), Dunalley (Cold Gold), Pipe Clay Lagoon (ShellfishC-PipeClay)). An additional shellfish hatchery in Triabunna (SpringBay) was sampled during September, November and December 2012 following media coverage of the project. Additionally, water samples were taken monthly from the wharf at Hobart, CSIRO Marine and

Atmospheric Research (CMAR) as this location was considered ‘greenfield’ with no aquaculture activities undertaken in the vicinity (albeit other domestic run-offs were present). Sampling occurred approximately the same time each month, with all locations sampled within two days of each other.

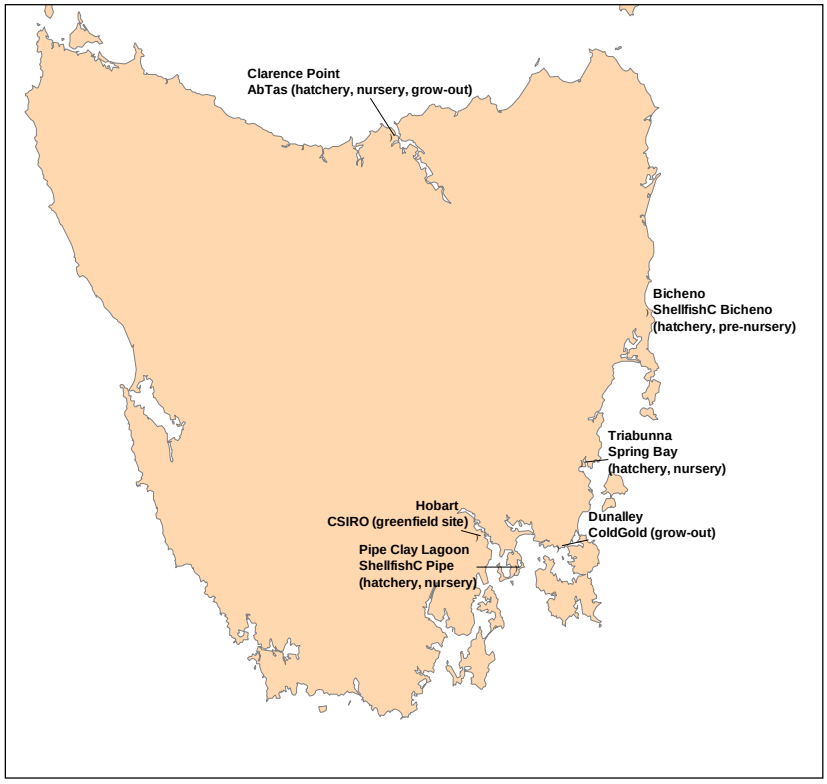


Figure 2 Map of Tasmania highlighting the sampling locations for the project

The Clarence Point and Dunalley farms culture and on-grow greenlip, blacklip and hybrid abalone (*H. rubra*, *H. laevigata* and the interspecies hybrid) while the farms at Bicheno, Pipe Clay Lagoon and Triabunna are Pacific oyster (*C. gigas*) hatcheries and nurseries.

Designated sampling sites per farm (i.e., inlet pumps and areas, grow-out tanks, water storage tanks) were originally identified in preliminary project discussions with staff at each farm, with the number of sampling points per farm ranging from two at Pipe Clay Lagoon to five sampling points at Clarence Point and Dunalley. Figures 3-7 show examples of the representative sites where water was sampled at each of the locations. Grow-out tanks at Clarence Point and Dunalley were stocked with abalone while water from the storage and header tanks at Bicheno, Triabunna and Pipe Clay Lagoon was used directly in hatchery and nursery facilities.

Figure 3 Sampling site at Clarence Point

Figure 4 Water storage tanks at Bicheno

Figure 5 Grow-out tanks at Dunalley

Figure 6 Inlet site at Pipe Clay Lagoon

Figure 7 Sampling location at the Hobart wharf

Where possible, the same sites at each location were sampled each month so that baseline water chemistry and microbial communities could be compared across temporal samples. Table 1 outlines the sampling that was undertaken across the farms, including tanks and inlet sites.

Table 1 Sampling sites across the five Tasmanian shellfish farms

Sampling***	Bicheno (oysters)**	Clarence Point (abalone)*	Dunalley (abalone)*	Pipe Clay Lagoon (oysters)**	Triabunna (oysters)**
October 2011	wharf, header tank, 1Day# & 3Day storage tanks	A4, A7, C12, D2, inlet pump	A16, C7, D36, E37, inlet pump	inlet area, header tank	
November 2011	wharf, header tank, 0Day & 3 Day storage tanks	A4, A7, C12, D2, inlet pump	A16, C7, D36, E37, inlet pump	inlet area, header tank	
December 2011	wharf, header tank, 4Day storage tank	A4, A7, C12, D2, inlet pump	A16, C7, D36, E37, inlet pump	inlet area, header tank	
January 2012	wharf, header tank, 0Day & 3 Day storage tanks	A4, A7, C12, D1, inlet pump	A16, C7, D36, E37, inlet pump	inlet area, header tank	
February 2012	no sampling due to hatchery renovations	A4, A7, C12, D2, inlet pump	A16, C7, D36, E37, inlet pump	inlet area, header tank	
March 2012	wharf	A4, A7, C12, D3, inlet pump	A16, C8, D36, E37, inlet pump	inlet area, header tank	
April 2012	wharf, header tank	A4, A7, C12, D2, inlet pump	A16, C7, D36, E37, inlet pump	inlet area, header tank	
May 2012	wharf, header tank, 0Day# & 3Day storage tanks	A4, A7, C12, D2, inlet pump	A16, C7, D36, E37, inlet pump	inlet area, header tank	
June 2012	wharf, header tank##	A4, A7, C12, D3, inlet pump##	A16, C7, D36, E37, inlet pump##	inlet area, header tank##	
July 2012	wharf, header tank	A3, A6, C12, D2, inlet pump	A16, C7, D36, E37, inlet pump	inlet area, header tank, 4Day & 4Day on carbon storage	
August 2012	wharf, header tank, 0Day# & 1Day storage tanks	A3, A6, C12, D2, inlet pump	A16, C7, D36, E37, inlet pump	inlet area, header tank	
September 2012	wharf, header tank, 7 Day storage trial#, ##	A3, A6, C10, D2, inlet pump##	A16, C7, D36, E37, inlet pump##	inlet area, header tank##	header tank##
October 2012	wharf, header tank, 7 Day storage trial#, ##	A3, A6, C12, D2	A15, C1, D36, E37, inlet pump	inlet area, header tank	
November 2012	wharf, header tank	A2, A6, C10, D2, inlet pump	A15, C1, D36, E37, inlet pump	inlet area, header tank	header tank##
December 2012	wharf, header tank	A2, A6, C10, D2, inlet pump	A15, C1, D36, E37, inlet pump	inlet area, header tank	header tank##

* sampling points here other than inlet areas/pumps were grow-out tanks that contained abalone; ** sampling points here other than inlet areas/header tanks were water storage tanks containing water that had been heated and filtered and did not contain oysters; *** samples were collected from the CSIRO wharf every month, at approximately the same time as the on farm sampling was undertaken
# water storage tanks representing different length of time of water storage; ## indicates where sampling was undertaken by farm staff using the CSIRO water sampling protocol; all other sampling was undertaken by CSIRO project staff

Our metagenomic approach aimed to identify as yet undescribed microbes present in the water being used for shellfish culture purposes in Tasmania; with a priority to understand the diversity of microbial communities across the shellfish farms and linking these communities with hydrochemistry variates in the water samples. Our sampling design was therefore essentially a block treatment which considered multiple categorical factors:-

- location (each shellfish farm and the Hobart wharf area) represented a different geographical space)
- month (each farm was sampled for 14 months)
- site (multiple sites (e.g., grow-out, header tanks) on each farm were sampled)

Additionally, either oysters or abalone were cultured on the farms (the Hobart location was considered greenfield as there were no aquaculture activities in the vicinity) and sampling was undertaken at either AM (before 12 noon) or PM (after 12pm). The same methods of field measurements and water filtration were undertaken for at least the samples collected by the CSIRO project team. Sampling at each point consisted of filtered water samples, water measurements and microbial samples. For the samples collected by farm staff, filtered water and microbial samples were collected alongside a reduced set of water measurements depending on the water probes available at each farm.

Our sampling design also considered the following multiple covariates (continuous environmental variables):-

- water temperature, pH, salinity, dissolved oxygen
- ammonium, nitrate, silicate, phosphate

While our sampling efforts (termed Primary A samples for this report) were directed at linking water chemistry analyses with microbial assemblages from the water column (from filtered water samples), we also included some *ad hoc* (n = 16) sampling of surface biofilms (e.g., from the floor and side surfaces of grow-out tanks, from concrete hides in grow-out tanks and from individual abalone shells) at the two abalone farms (see Figure 8) using Isohelix DNA Buccal Swabs (Cell Projects, England).

Figure 8 Biofilm sampling from abalone shells and hides

The Isohelix sampling resulted in a set of paired samples from the two abalone farms – each of the 16 paired Isohelix samples had a corresponding Primary A sample (all other sampling conditions were assumed to be the same; the difference in samples here was the type of sampling – i.e., filter and swab).

2.2 Primary A samples, on-farm filtering and water measurements

Where possible, the covariate water measurements were made *in situ* and at each sampling site per location. Temperature, dissolved oxygen, salinity and pH were determined in the field with a Hach HQ 40d field meter (Figure 9, Hach Pacific, Australia).

Figure 9 Hach HQ 40d field meter

Water was then collected in 2L carboys or 1L Nalgene (USA) bottles (Figure 10) and filtered on farm using bench top filtration canisters (Nalgene NAL300-4100, with receiver 1L PSF 500mL) and a vacuum pump (Figure 11). Between 500mL and 1L of water was filtered in each canister, which also contained an Isopore™ Membrane Filter (Merck Millipore, USA) polycarbonate (PC) 45mm 0.2μM filter on which the microbes in the water sample were collected. Two canisters were used per sampling site therefore duplicate PC filters were collected. The water filtrate was poured carefully into nutrient analysis tubes and capped (Figure 12). The PC filters were taken out of the filtration canisters and placed into small 2mL (microfuge) tubes (Figure 13). One filter was used for DNA extraction while the other was later archived in the CMAR freezers. The nutrient and microfuge tubes were transported from the farms on ice back to the CMAR laboratory where the filters were stored at -80℃ until DNA extraction. Water samples were stored at -20℃ until nutrient analyses were undertaken. All data from the field was entered into spreadsheets, with paper copies retained for archival records.

Figure 10 Water sampling using a carboy

Figure 11 Filtration canisters with vacuum pump

Figure 12 Filtered water in nutrient analysis tubes

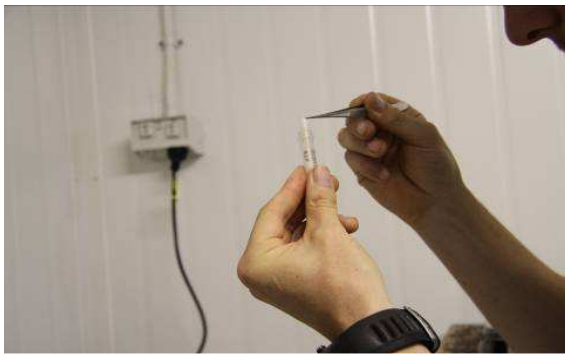


Figure 13 PC filters placed into microfuge tubes

2.2.1 Water filtration using Sterivex filters

While the filtration canisters in combination with the portable vacuum pump worked exceptionally well in our hands, one of our aims was to develop a sampling method which was more amenable for use by farm staff. As part of this, we sent our collaborating farms a questionnaire (see Appendix 3) to garner their available farm resources which they could use for their own water sampling. Based on the farms' responses, we developed our on-farm sampling protocol which can be easily undertaken by farm staff and fits into regular husbandry activities (see Appendix 3). Farm staff used this protocol for their own sampling in June and September 2012, and all samples from the Triabunna farm were sampled using this protocol. All collaborators told us that the on-farm sampling protocol was easy to use and suited their husbandry activities.

Our on-farm sampling protocol was based on capsule filters (Sterivex™ Filter Unit, Merck Millipore (USA), Figure 14) which can be used with either a 60mL syringe or a small peristaltic pump. The Sterivex units enabled filtered water to be collected easily by farm staff. After water was filtered, Sterivex units and water samples were frozen on farm until the CMAR project team transported them back to the laboratory where they were frozen at -80℃ until DNA extraction. Additionally, the Sterivex units can be flooded with a DNA preservation buffer, capped and sent back at room temperature to the labs. In these circumstances, the buffer remains in the Sterivex units; units are frozen at -80℃ until DNA extraction.

Figure 14 Sterivex unit filled by a 60mL syringe

In addition to the Sterivex samples that were collected by the farm staff, throughout the project, we also collected 18 paired samples where each Isopore filter sample was matched with a Sterivex unit sample.

2.3 Water covariate analyses

The following four water covariate measurements were recorded on location (in hardcopy) and in the Hach HQ 40d data log. On arrival back to the laboratory, the measurements were entered into spreadsheets housed on the CMAR servers.

- water temperature - as a commonly measured water quality parameter, temperature influences the chemical and biological processes in the water column and shellfish growth and health responses. Temperature was measured at each site per location and was measured in ℃.

- pH – is the concentration of hydrogen ions in water (i.e., a measure of acidity). pH varies from 1 (highly acidic) to 12 (highly basic) with a pH of 7-8 being suitable for most aquatic animals. Variations outside of this range could be detrimental to the organisms in the system.

- salinity - is the amount of salts dissolved in the water column. We expressed this measurement in parts per thousand (ppt/‰). Salinity also influences other water processes, such as the amount of dissolved oxygen in the water.

- dissolved oxygen (DO) - is a key parameter for assessment in water monitoring with DO the level of oxygen in the water column in molecular form; we report it here as a percentage (between 1-200% saturation).

In addition, the environmental nutrients that were monitored on a regular basis were four hydrochemistry variates - ammonium, nitrate, silicate and phosphate. As previously outlined, water samples were 0.45μM filtered on sample collection and analysed in the CMAR laboratories. Over 1 500 water samples were collected for these analyses. As with all the data collected during the project, the nutrient data was stored on spreadsheets on the servers at CMAR.

Ammonium was determined using an in-house made flow injection analysis (FIA) system (Watson *et al.,* 2005).This involved the separation of ammonium (converted to ammonia) from the sample using a gas-permeable membrane. The ammonia then reacted with ortho-phthaldialdehyde and sulfite with subsequent fluorescence detection. Nitrate, silicate and phosphate results were also obtained using in-house methods on a custom made flow injection analyser (sourced from Monash University, Melbourne). The protocols were based on standard methods for nutrient analyses by flow injection (L. Clementson, pers. comm.). All nutrient concentrations are expressed here as μg L^{-2}. Outlined below are several important considerations for nutrient analyses:-

- in the marine environment, dissolved nutrients such as nitrate and phosphate are important in primary production of phytoplankton. Nitrogen and phosphorus influence the overall biological productivity of an area with nitrogen essential for protein and DNA synthesis in organisms and photosynthesis in plants, and phosphorus critical to metabolic processes (http://sp.uconn.edu/~wwwmsd2/techtxfr/625R02010Chap2.pdf). Silicate is also important for diatom growth. In eutrophic environments, high nutrients can promote algal blooms. The resultant high organic loads decompose, creating hypoxic conditions where the oxygen concentrations are below those required for shellfish to survive. When oxygen is absent (anoxia), such as in or near sediments, ammonium and phosphate is released (Butler *et al.,* 2000). Once released into the water column, ammonium is oxidised by nitrifying bacteria to nitrite and nitrate.
- harmful algal blooms, which impact on shellfish culture, are also associated with nutrient concentration (Hallegraeff, 2010). The blooms of one such species, *Gymnodinium catenatum*, have been associated with the drawdown of nitrate and ammonium in southern Tasmania (Butler *et al.,* 2000; Doblin *et al.,* 2006).
- in aquaculture systems, nutrients are important where the production of shellfish feeds is on-site either as microalgae for filter-feeding bivalves (Knuckey *et al.,* 2002) or the production of biofilms of bacteria and diatoms or of macroalgae such as *Ulva sp*. for feeding abalone (Daume, 2006). Bivalve aquaculture, such as oysters, can improve water clarity as

they filter plankton and detritus from the water but increase dissolved nutrients through excretion (Forrest *et al.,* 2009).

The environmental covariate data (i.e., salinity, temperature, nutrient concentrations etc) were checked first for normality and homogeneity of variances using Q–Q and residual plots in R (http://www.r-project.org/, R-Development-Core-Team, 2010) and Draftsmens Plots in Primer 6 (http://www.primer-e.com/permanova.htm, Plymouth, UK). Data not meeting the assumptions of ANOVA were transformed (square root). Standard Euclidean distance measures (where zero plays no special role, Clarke and Gorley, 2006) were calculated for the covariate data prior to similarity matrices analysis (in Primer 6).

All univariate tests (at α=0.05) and graphing were done using R. Stepwise multiple linear regression was performed to identify the water chemistry parameters that were most predictive for the abundance of different microbial groups. The phylotype abundance was used as a dependent variable whilst water chemistry parameters were used as independent variables. Variables were excluded when they correlated significantly with other variables (see Figure 26). Due to the large number of predictor variables being tested, only the first three variables selected by the model were used so as to avoid over fitting the model. Regression tree analysis was performed to partition the effects of the water chemistry parameters on the abundance of specific microbial species of interest. Regression tree analysis was performed using the Tree package in R.

2.4 Microbial assessment analyses

Microbial assessments were based on a number of genomic analyses - Automated Ribosomal Intergenic Spacer Analysis (ARISA), NGS and Quantitative Polymerase Chain Reaction (qPCR). These techniques enabled a community profile of the temporal and spatial patterns of microbial presence and absence, species identity and assessment of microbial abundance levels in samples from across the shellfish farms. All analyses were studied using culture-independent genomic methods.

Prior to the genomic analyses, high quality microbial DNA was required. DNA was extracted from the various filters and swabs according to the following methods. All extractions were undertaken soon after collection in the field.

2.4.1 Isopore microbial DNA extractions

For the majority of samples, microbial cells had been collected on the Isopore PC filters (average pore size 0.22μM). DNA was extracted from one of the paired Isopore filters (per sampling site) using the following modified spin column extraction method.

The PC filter disc holding the microbial cells was cut into 4-6 pieces, put into a 2mL microfuge tube and covered with a lysis buffer containing lysozyme (see Appendix 3 for recipe). Tubes were vortexed and incubated at 37℃ for at least two hou rs. Following incubation, a series of phenol:chloroform:isoamyl extractions were undertaken, with high speed centrifugation (13 000 rpm) of the supernatant between each extraction. To the resulting aqueous phase, Proteinase K was added and tubes were incubated at 60℃ for 1.5 hours. Following incubation, several chloroform:isoamyl extractions were undertaken on the supernatant. Binding matrix from the FastDNA® SPIN Kit for Soil (MP Biomedicals, USA) was mixed with the cleaned supernatant and the manufacturer's recommendations for the SPIN kit were then followed from this point. DNA was eluted in 100μL of Tris-EDTA (TE) buffer. DNA was quantified on a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Australia) and adjusted to 5-10ng/μL (where possible) for working stock while the undiluted stock was archived at -80℃.

2.4.2 Sterivex microbial DNA extractions

During the on-farm sampling protocol development (where both Isopore filters and Sterivex units were used to filter water) and for June and September during which farm staff undertook their own sampling, a new DNA extraction protocol was developed for use with the Sterivex units. After water filtering in the field, Sterivex units were capped at both ends.

In the laboratory, units were taken out of the -80°C freezer, brought to room temperature and lysis buffer containing lysozyme (as used with the Isopore filters) was added to the inlet end of the unit. The inlet and outlet caps on the unit were tightened and the unit was then attached to a horizontal Vortex-Genie 2 (Mo Bio Laboratories, USA) for at least 60 minutes (during which units were rotated every 30 minutes).

A 3mL syringe was attached to the inlet end of the unit and using back pressure from the syringe, the contents of the unit were syringed into microfuge tubes. Phenol:chloroform:isoamyl was then added to the supernatant, tubes were inverted several times and centrifuged at 13 000rpm for 10 minutes. The resulting aqueous phase was removed and placed into a new microfuge tube to which Proteinase K was added and tubes incubated at 60℃ for 2 hours. A series of chloroform:isoamyl extractions were then undertaken with the resulting aqueous phase added to a 5mL tube. The ST4 buffer from the PowerWater DNA extraction kit (Mo Bio Laboratories) was added to the supernatant and the tubes mixed by inversion. This supernatant:buffer solution was then filtered and washed through the columns in the PowerWater kit according to the manufacturer's instructions. DNA was eluted off the columns using 80*µ*L of TE. DNA was quantified on a Nanodrop and adjusted to 5-10ng/*µ*L (where possible) for working stock while the undiluted stock was archived at -80℃.

2.4.3 Isohelix microbial DNA extractions

The Isohelix swabs were used to sample biofilms from the inside of abalone grow-out tanks or from abalone shells; unlike the Isopore or Sterivex filters, there was no filtered water sampled. However, when the Isohelix swabs were used, they were sampled at the same time as either the Isopore or Sterivex filters. Unlike the Isopore or Sterivex filters which were maintained on ice/frozen in the field and then placed into the -80℃ freezer on arr ival at the laboratory, the LS and PK solutions from the Isohelix DNA isolation protocol were added in the field to the tube containing the swab; these samples were stored at room temperature in the laboratory until DNA extraction.

For extraction, the tube containing the swab (and the LS and PK solutions) was put onto a heat block at 60℃ for up to 3 hours. The liquid was the n transferred to a new microfuge tube, 500*µ*L of CT solution added and vortexed briefly. Tubes were then centrifuged at 13 000rpm for 7 minutes to pellet the DNA. The supernatant was removed, the pellet was air dried for 30-60 minutes and 80*µ*L of TE was added to resolubilise the pellet. Resulting DNA was quantified on a Nanodrop, with working dilutions stored at 4℃ and the undiluted D NA stored at -80℃.

2.5 ARISA

ARISA is a genetic 'fingerprinting' technique that enables the richness and community composition of microbes from environmental DNA to be detected without the need to culture the microbes or produce gene clone libraries (Fisher and Triplett, 1999; Danovaro *et al*., 2006). ARISA distinguishes microbial communities based on differences in fragment length (i.e., length heterogeneity) in the ribosomal intergenic spacer region between the 16S and 23S rRNA genes in the rRNA operon (known as the ITS1 region (Fisher and Triplett, 1999; Danovaro *et al*., 2006; Jones *et al*., 2007). ITS regions are nearly ubiquitous in bacterial genomes and are highly divergent in length and nucleotide sequence (from approximately 150 – 1500 base pairs) (Fisher and Triplett, 1999; Or and Gophna, 2011). ARISA exploits the length heterogeneity of this region in

microbial species. We used this divergence to discriminate between microbial species through molecular analysis by comparing microbial profiles varying in length and peak size depending on the species that were present in the samples.

The amplified fragments in ARISA are assumed to represent microbial composition with each peak representing a specific group of microbes. While we can not assign a specific species to a particular fragment using this technique, the analyses enabled us to directly and rapidly compare the microbial diversity and community composition over time, across different sampling sites within a farm and across farms. Treating the ARISA peaks as operational taxonomic units (OTUs) allowed for microbial community assessments (albeit ARISA provides no specific phylogenetic information). However we need to keep in mind that due to the nature of the analysis, some community members maybe unidentified (Fisher and Triplett, 1999; Yannarell and Triplett, 2005).

2.5.1 ARISA protocol

ARISA was undertaken on at least all Primary A samples from across the 14 month period, across all farms and sampling sites. Following sample extraction, DNA was amplified using universal bacterial primers 16S-1392F (5'-GYACACACCGCCCGT-3') and a 5'HEX labelled version of the reverse primer 23S-125R (5'-GGGTTBCCCCATTCRG-3') which amplified the ITS1 region in the rRNA operon plus approximately 282 bases of the 16S and 23S rRNA (Hewson and Fuhrman, 2004). Two sets of ARISA PCRs per DNA sample were undertaken (in order to minimise stochastic PCR biases from mixed environmental templates, as in Danavaro *et al*., 2006) with the following amplification conditions - in 50*µ*L volumes with 20ng of DNA, 25*µ*L of GoTaq® Green Master Mix (containing Taq DNA polymerase and dNTPs) (Promega, Australia), 2*µ*L of Bovine Serum Albumin (Promega), 1*µ*L of each of the 10*µ*M primers and water. To monitor extraneous contamination, negative controls containing the PCR mix but without any DNA template were run during each PCR.

PCR amplification consisted of an initial denaturation at 94℃ for 3 minutes followed by 35 cycles of denaturation at 94℃ for 1 min, annealing at 55° C for 1 min 30 sec and extension at 72℃ for 1 min 30 sec, with a final extension of 72℃ for 10 m in in a GeneAmp PCR system 9700 (Applied Biosystems, USA) thermocycler. PCR products were checked on 2.5% agarose-Tris-Acetic Acid-EDTA (TAE) gels containing Sybr-Safe (Life Technologies, USA) for DNA staining and visualisation. The two PCR reactions per sample were then pooled together before purification.

The resulting combined PCR products were purified using AMPure™ magnetic beads (Agencourt, Beckman Coulter Life Science, USA) according to the manufacturer's instructions, resuspended in 40*µ*L of TE and then quantified on the Nanodrop.

For each ARISA, approximately 25-40ng of purified amplified reaction was mixed with 0.4*µ*L of internal size standard (GS1200-Liz, Applied Biosystems) in deionised formamide and denatured at 94℃ for 2 minutes. Denaturing capillary electropho resis (on a 50cm capillary array) was carried out for each combined purified PCR using an ABI 3130XL genetic analyzer (Applied Biosystems) in the CMAR laboratory. ARISA profiles were analysed using GeneMapper vers 3.7 (Applied Biosystems). Best fit size calling curves based on second order least squares (which compensates for anomalously running fragments in the internal standard) and the local Southern method were determined for each sample and checked for correlation before proceeding with the automated peak scoring.

To ensure we detected the maximum number of peaks while excluding background fluorescence, a threshold of 50 fluorescence units (RFUs) greater than the baseline was used for peak detection (see also Jones *et al*., 2007). After individual samples were checked in GeneMapper for accurate size calling, peak sizes, areas and heights were exported to Excel for further analyses. All peaks with less than 50 RFUs and only fragments between 300 and 1250 base pairs were considered. Following filtering on these attributes, peak size and area were used for further analyses with the assumption that cumulative peak area of each peak/OTU was considered a measure of community

presence. Peak size and area output data were further analysed by custom R scripts for Auto and Interactive Binner (Ramette *et al*., 2005; Ramette, 2009).

These programs calculated the relative fluorescence intensity (RFI) of each peak (dividing individual peak areas by the total peak area for each sample) – all peaks with RFI value of <0.09% were not included (as they were assumed to be background peaks) and enabled sensitivity analyses (variability in peak size calling, run to run variations, binning of fragments into different window frames) (Brown *et al*., 2005; Ramette, 2009). For the Primary A sample peak data, a window size of 4 base pairs was chosen as the frame which offered the highest pair-wise similarity among peaks across the samples. The binned ARISA profile data was then analysed in Primer 6 & Permanova+.

We also used the ARISA profiles to identify samples for NGS analysis (see following sections) – those samples that demonstrated greater diversity in peak profiles were chosen for further analyses. NGS does enable us to 'put a name to a face' and therefore using sequencing analyses we are able to understand how the community profiles changed over time and what organisms were present in the samples.

2.6 NGS analysis

DNA sequencing determines the nucleotide composition and order of the four bases (adenine, guanine, cytosine and thymine) of a DNA gene or fragment. Pyrosequencing (or next generation sequencing, NGS) is a new DNA sequencing technology that utilises enzyme-coupled reactions and bioluminescence to monitor the pyrophosphate release accompanying the nucleotide incorporation in real time (Margulies *et al*., 2005; Rothburg and Leamon, 2008; Aw and Rose, 2012). NGS has several advantages over traditional Sanger sequencing including high throughput, unbiased detection of DNA present in environmental samples, production of hundreds of thousands of sequences in a single run, deep sequence coverage (Stoeck *et al.*, 2010) and the ability to detect novel organisms (Hamady *et al*., 2008; Liu *et al*., 2008; Rothburg and Leamon, 2008; Youssef *et al*., 2009).

A parallelized version of pyrosequencing was developed by 454 Life Sciences (now acquired by Roche Diagnostics). The Roche GS-FLX 454 sequencing platform (referred hereafter as 454) is capable of generating one hundred million nucleotides per run of sequences with average length of 400 base pairs (Margulies *et al*., 2005). As part of the platform, DNA is amplified inside water droplets in an oil solution (emulsion PCR); with each droplet containing a single DNA template attached to a single primer coated bead that forms a clonal colony. The 454 platform consists of high density picolitre reactors, with each well containing a single bead and sequencing enzyme (Margulies *et al*., 2005). Pyrosequencing on the 454 then uses luciferase to generate light for detection of the individual nucleotides added to the DNA; the combined data are then used to generate sequence read-outs (Margulies *et al*., 2005).

High throughput 454 sequencing now offers an unprecedented scale of sampling for the molecular detection of microbial diversity (Stoeck *et al*., 2010). Additionally, the platform results in the generation of large genomic datasets derived from environmental samples (Simon and Daniel, 2009) which can be used directly for assessing the phylogenetic diversity of complex microbial assemblages present in environmental samples through analysis of conserved microbial ribosomal RNA (rRNA) gene sequences (Woese, 1987).

We chose to outsource the 454 sequencing for the microbial diversity analyses as there is no microbial pyrosequencing service provided commercially in Australia, and CSIRO does not have the facilities to undertake 454 microbial diversity analyses. We used RTL (Research and Testing Laboratories in Texas, www.researchandtesting.com) for the 454 sequencing. RTL provides specialised bacterial and archaeal NGS analysis, pipeline quantitative control and data analyses of microbial DNA samples (http://www.medicalbiofilm.org/microbial-diversity-services.php). The laboratory specialises in the evaluation of microbial diversity using a TEFAP (tag encoded FLX-amplicon pyrosequencing) approach that was developed by Sun *et al*. (2011).

In TEFAP, a unique tag is encoded within the amplified region of interest (i.e. amplicon) and the tag is unique for each sample. Samples are then combined after labelling with the unique tag, mixed and run together in a single pyrosequencing run (Sun *et al*., 2011). The cost per sample here was approximately $US95 which included up to 3000 individual sequences per sample.

2.6.1 NGS protocol and pipeline analysis at RTL Texas

Following DNA extraction and ARISA analyses (which we used to determine which samples were sequenced), 30μL of undiluted DNA from samples was dried down in a Concentrator Plus (Eppendorf, South Pacific) and sent via express FEDEX post to RTL in Texas USA.

In the RTL laboratories, samples were amplified primarily with the universal Bacterial (also includes archaea) 16S assays (926F (5′-AAA CTY AAA KGA ATT GAC GG-3′) – 1392R (5′-ACG GGC GGT GTG TRC-3′) which is specific for amplifying an approximate 460 base pairs of the V8 variable region of the 16S rRNA gene in bacteria and archaea (Lane *et al*., 1985; Weisburg *et al*., 1991) and an RTL Assay A.1 for Algae (primers commercial in confidence). Initial PCR amplification, PCR for FLX amplicon sequencing and emulsion based clonal amplification and GS FLX sequencing was undertaken as per RTL protocols. On completion of the 454 sequencing of samples, RTL used the following pipeline (Data Analysis Methodology, updated 10/26/2012, http://www.researchandtesting.com/docs/Data_Analysis_Methodology.pdf) to process the data.

Briefly, following next generation sequencing of the samples, RTL provides the first assessment of the data using their data analysis pipeline which consists of quality checking and reads de-noising stage and a diversity analysis stage. For each sample and each gene region, SFF files are produced which are binary files that contain many data about a read in a single file. Over 3000 reads are quality trimmed (using quality scores), clustered, chimera checked and de-noised. The RTL analysis pipeline then takes the resulting FASTA formatted files (that contains a one line descriptor and lines of sequence and quality scores) and quality controls these files (for failed sequence reads, sequences with low quality tags, sequences with short amplicon lengths) thereby producing FASTA reads archives that contain the sequence reads, quality scores and mapping files. The FASTA formatted files are then used in the RTL analysis pipeline to determine taxonomic identification for each sequence read. Sequences are clustered into OTUs with 100% identity using USEARCH. The file is queried against a database of sequences derived from NCBI (using the .NET algorithm and BLASTn+). Based on the BLASTn+ sequence identity percentages, sequences are classified at taxonomic levels (i.e., >97% identify for species level; between 95% and 97% at genus level). The analysis provides the sample's microbial (and or where appropriate, algal) diversity.

We were provided with the raw data files (.SFF), the quality checking and FASTA formatted sequence/quality files (.qual and .fna) and the taxonomic identification files (analysis and .csv organised by taxonomic level).

2.7 ARISA and NGS statistical analyses

The ARISA assemblage data consisted of peak information which consisted of *p* rows (bins, as calculated according to Ramette, 2009) and *n* columns (Primary A samples) where the ARISA data entries were peak areas. The NGS data consisted of *p* rows (bacterial/archaeal taxonomic – species, class and family) and *n* columns (Primary A samples) where the NGS data entries were sequence counts.

The extent to which biological data are treated prior to similarity calculations can generate different outcomes (Clarke and Gorley, 2006). Therefore the binned peak ARISA area data and NGS taxonomic counts were pre-treated in Primer. Data were first standardised (by sample total) and then data transformations were applied to the standardised data set. These transformations act to

down weight the contribution of quantitatively dominant species to the similarities calculated between the samples (Clarke and Gorley, 2006) from the farms. The structural similarity of ARISA and 454 transformed datasets was assessed using the RELATE function to compare the treated Bray-Curtis similarity matrices (Spearman correlation, 9999 permutations) in Primer 6. Relatedness of different profiles for each sample was calculated using Bray-Curtis similarity calculation on all standardised and transformed data (Minchin, 1987).

As it is often difficult to visualise relationships in a similarity matrix (for n(n-1)/2) samples, we needed a robust graphical method of representation. Using CLUSTER (a hierarchical agglomerative clustering algorithm with average group linkage) in Primer 6, distances between pairs of samples reflected their relative dissimilarity (of OTU/species) composition. Statistically significant clusters were tested using permutation tests in SIMPROF (Primer 6). Here we were testing the H_0 that the specified samples which were not *a priori* divided into groups, did not differ from each other in multivariate structure.

We also visualised the ordination of samples as points in low dimensional space by fusing them into larger groups by non-metric multi-dimensional scaling nMDS (Kruskal and Wish, 1978) based on the Bray-Curtis similarity matrices (using between 25 and 100 re-starts). Kruskal's stress value indicated how faithfully the higher dimensional relationships among samples were reported in 2D or 3D space, with points that were close together representing samples that were very similar in composition (in 2D space stress <0.05 = excellent representation; <0.1 good ordination; <0.2 potentially useful 2D dimension space; >0.3 points are arbitrarily placed in ordination space).

The multivariate complexity of the ARISA microbial assemblage and NGS microbial data was then collapsed into single diversity indices for species diversity and evenness of the 16S rRNA gene using the DIVERSE function in Primer 6. Shannon-Weiner diversity index (H') and total number of OTUs/ARISA peaks or species (S) were calculated to give a metric of the diversity of OTUs, with greater values indicating higher diversity. Pielou's evenness (J) was also calculated which is a measure of the distribution of species within the sample with values close to 1 indicating an even community (similar numbers of each OTU) structure, whilst values close to 0 indicate a community dominated by relatively few of its members (Clarke and Gorley, 2006).

- Total species (S) = the number of species in each sample, dependent on sample size
- Shannon Weiner diversity index (H') $H' = -\sum P_i \log(P_i)$
- Pielou's evenness index (equitability) (J) = $H'/\log_e S$

Where the abundance of the $_i$th species is denoted by N_i (I = 1, 2, …S) and divided by their sum (N) is denoted P_i (I = 1, 2, …S) (Clarke and Gorley, 2006).

Pairwise similarity testing of these univariate indices between farms and months was undertaken using SIMPER in Primer.

Non-parametric ANOSIM (analysis of similarity) in Primer 6 was then used to test for differences among the farms (or other) in the multivariate data sets. ANOSIM generates a test statistic R that ranges from -1 to 1 with magnitude of R indicative of degree of separation between samples/groups (large R values = indicative of complete separation of groups; small R = little segregation) (as in Meziti *et al*., 2010). ANOSIM tests pre-defined group structures (e.g., samples from different sites, locations, months) and operates on the Bray-Curtis resemblance matrix. We used the ANOSIM R statistic to indicate if there maybe differences somewhere in the data that warranted further investigation (i.e., if H_0 was rejected, then pair-wise analyses were undertaken). Bonferroni correction to significance values was implemented for multiple comparisons.

As the biotic data (microbial assemblages, microbial identities) was matched by a suite of environmental covariates measured at the same set of locations, we tested the extent to which the physio-chemical data explained the ARISA patterns. BEST in Primer 6 finds the best match (based on rank correlations, Clarke and Gorley, 2006) between the multivariate among sample patterns of the ARISA assemblage and that from the eight environmental variables associated with those

samples (search for a combination that optimises the match). Significant relationships between water chemistry variables and ordination was also determined using Spearman correlation to ordination axes (R>0.5). Significant relationships between ordination and water chemistry variables were plotted as vectors, whereby the vector indicates the direction of the gradient of the variable and the length indicates the correlation between the ordination and the variable.

In addition, spatial (site and farm) and temporal effects (month) were tested using permutation-based testing of multivariate analysis of variation (PERMANOVA) using approaches described previously (Anderson *et al*., 2008). The PERMANOVA model used a three-way factorial design using the factors: Farm (fixed, 5 levels), month (random, 12/14 levels), site (random, 5 levels). Default settings for PERMANOVA were used (Type III sums of squares, 9999 permutations, permutations of residuals under a reduced model). All tests were conducted at $P<0.05$. Pair-wise comparisons were conducted *post hoc* on factors found to be significant in the 'global' test.

Canonical analysis of principal co-ordinates (CAP) (Anderson and Robinson, 2003; Anderson and Willis, 2003) was also undertaken on the Bray-Curtis similarity matrices (ARISA and NGS) to discriminate among *a priori* groups and water chemistry covariates to characterise the differences among the groups in multivariate space (Anderson *et al*., 2008). In CAP, the length of the vectors is proportional to the importance of the environmental variates and the vectors point in the direction of increasing values. CAP was also used to classify samples into location/month groupings based on previous predictions. As both PERMANOVA and CAP test for differences among groups in multivariate space, PERMANOVA was used to test if the between group variation explained a significant proportion of the total variation in the overall systems, while CAP tested for axes in multivariate space that separated the groups (Anderson *et al*., 2008).

2.8 QPCR for bacterial abundance estimates

Quantitative real time PCR (qPCR) is a molecular technique which enables the quantification of DNA targets by monitoring the amplification products during a PCR cycle based on fluorescence detection of the amount of DNA amplified (Aw and Rose, 2012). A DNA binding dye (such as SYBR Green) binds to double stranded DNA in the PCR, which causes fluorescence, with an increase in product during the PCR cycle resulting in increased fluorescence intensity. qPCR offers high sensitivity and specificity, rapid turnaround and requires no post PCR analyses. When PCR conditions are standardised across samples (as in the current study), reasonable comparative analyses can be expected (Sipos *et al*., 2007). qPCR has been shown to be a powerful tool for quantifying microbial (Bacteria and Archaea) abundance in environmental samples, with the application of 16S rRNA genes for the detection and enumeration of bacterial targets well established in microbial ecology studies (Skovhus *et al*., 2004; Morales and Holben, 2009 and refs within). Using absolute quantification PCR, the quantity of a single 16S rRNA target sequence within some Primary A samples was calculated.

An ABI 7500 (Applied Biosystems) Real Time PCR system at the CMAR laboratories was used for the fluorescent based PCR detection of 16S bacterial fragments using dissociation curve analyses. Each 96 well plate included triplicate reactions per DNA sample and the appropriate set of standards. The qPCR standards were generated by PCR amplification of several farm samples which were purified using AMPure beads (Agencourt), quantified and a tenfold serial dilution series (from 10^{-3}ng to 10^{-7}ng) was subsequently used as the standards. Each plate also contained no template negative controls. Real time PCR reactions were prepared with the Power Sybr Green PCR Master Mix (Life Technologies) in 96 well optical PCR plates (Life Technologies).

The PCR primers used here were universal bacterial 16S rRNA. Due to time limitations, not all Primary A samples were amplified here and the qPCR for archaeal 16S was not undertaken. We include the bacterial qPCR here to show the utility of the technique and how microbial abundance can be calculated without the need for plate culturing. The 27F (degenerate, 5'-AGRGTTTGATCMTGGCTCAG-3') (Lane, 1991) and 1492R (5'- GGTTACCTTGTTACGACTT-3') (Turner *et al*., 1999) primers were used for the bacterial qPCR. Assays were performed in a total of 15μL consisting of 1μL each of 10μM forward and reverse primers, 0.15μL of BSA, 7.5μL of Power

Sybr Green PCR mix, 2μL of template DNA (approximately 5-10ng/μL) made up to volume with water. The thermocyling program consisted of an initial 10 min 95℃ step, followed by 45 cycles consisting of 95℃ for 30 sec, 55℃ for 30 sec and 72℃ for 40 sec with a fluorescent acquisition step at 76℃.

Melting curve analysis of the PCR products was conducted following each assay to confirm that the fluorescence signal originated from specific PCR products and not from primer-dimers or other artefacts. All qPCR plates included a 'no template' negative control for each primer set. Results were analysed with Sequence Detection Software ver 1.2.1 (Applied Biosystems).

2.8.1 QPCR statistical analyses

The samples were compared to the standard curve generated in each qPCR run. Quantity values were averaged across the triplicates to calculate the total 16S rRNA bacterial amount in the starting material per sample (and adjusted depending on the amount of water filtered during sample collection). There was no further statistical analyses undertaken on the samples across the farms as the technique and its utility is shown here for information only.

3. Results

The majority of the results and discussion that follows is based on the Primary A samples as these were used to provide baseline information about microbial communities in the Tasmanian shellfish farms and for clarifying/elucidating environmental conditions that may affect the communities. The key hypotheses and questions that we tested from the Primary A data set were:-

- was the microbial presence and community structure homogenous across the six Tasmanian sampling locations (i.e., H_0 = no differences between spatially separated locations)
- can the microbial presence and community structure be considered temporally homogenous (i.e., H_0 = no temporal differences between sampling at locations over 14 months)
- was the microbial presence and community structure related to hydrochemistry/environmental covariates (*abiotic* and nutrient) (i.e., H_0 = there were no specific drivers or causality that affected the community structures)
- what were the key microbial species that were detected in the shellfish farms (innocuous, beneficial)
- detection of possible pathogenic microbes (i.e., were any observed, where were they detected, how do we use this information for husbandry?)

In addition to the Primary A samples and resulting data, we also collected a number of samples as part of a series of *ad hoc* experiments and metagenomic investigations that were important to investigate, but not necessarily part of the Seafood CRC objectives. The results from these experiments will be generally outlined at the end of the results section although not analysed in great detail here.

3.1 Primary A data

At each location, sampling was undertaken from grow-out tanks and from surfaces (i.e. concrete hides, sides and floors of tanks, abalone shells), inlet pumps and areas, header tanks and water

conditioning tanks. Monthly sampling across the farms consisted of between 14-22 samples per month alongside a monthly water sample collected at the CMAR 'green field' site.

Across the 14 months of sampling and using the three sampling protocols, a total of 497 samples were collected. Of these, 241 were Primary A Isopore and Sterivex samples that were directly extracted. These samples also had accompanying environmental covariates recorded and measured. There were 43 Primary A samples collected at Bicheno, 74 samples at Clarence Point, 75 samples at Dunalley, 32 samples at Pipe Clay Lagoon, 3 at Triabunna and 14 at Hobart. In addition to the Primary A Isopore and Sterivex samples that were extracted, we have paired archival filter samples which continue to be stored at -80℃. These samples can be extracted at a later date if required. As outlined above, we also collected other samples on an *ad hoc* basis for experimental optimisations.

Following DNA extraction and quantification from the Primary A samples, 235 samples were analysed with ARISA; 175 samples were analysed with TEFAP 16S rRNA tagged NGS for bacteria and archaea (and 35 of these same samples were also analysed with TEFAP NGS with a set of specifically designed primers for eukaryotic algal identification); and 27 samples were analysed with qPCR.

3.2 Analysis of covariables (*abiotic* water and nutrient parameters)

At each sampling point per location, eight water parameters were measured. The results below are separated into two sections – the first for the *abiotic* water parameters and the second for the nutrient parameters.

3.2.1 Abiotic water parameters

Table 2 lists the overall ranges and averages of the water parameters across all locations, for the 14 months of sampling.

Table 2 Summary of environmental *abiotic* covariates during October 2011 - December 2012

Covariates	Range	Average (± SD)
Temperature (℃)	8.4 - 23.7	16.14 (3.74)
pH (1-14)	7.42 - 8.5	8.10 (2.58)
Salinity (‰)	21.3 - 39.6	29.91 (11.31)
DO (%)	74.0 - 213.4	93.96 (34.14)

The temperature range was wide, with an average of 16.1℃ during the project and a minimum of 8.4℃. The average pH across all areas was 8.10 and salinity averaged approximately 30‰ throughout the project sampling time. Dissolved oxygen was quite variable with a maximum of 213.4% saturation (as shown in Figure 15, this was observed at Dunalley in November 2012).

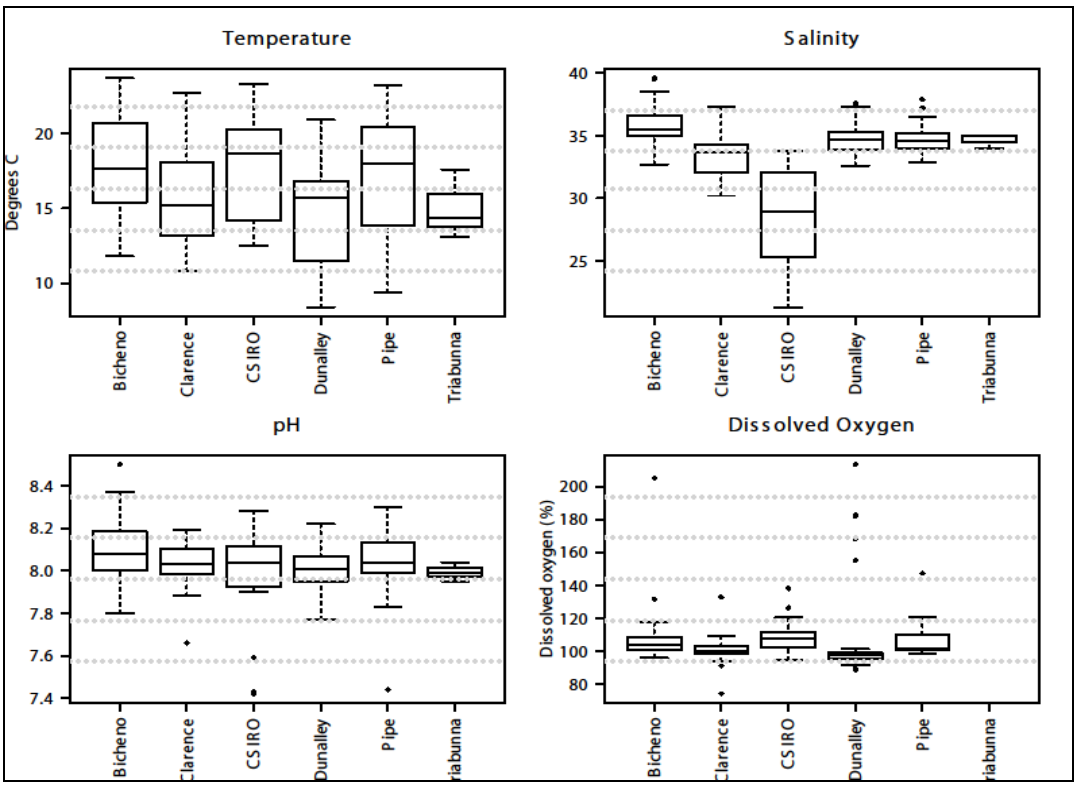


Figure 15 Summary data of *abiotic* water covariates across the six locations

As can be seen in Figure 15, there was a systematic effect of farm on temperature; the variance in temperature among the sampling points per farm also differed across the farms. The shellfish farm with the highest mean temperature was Pipe Clay Lagoon, with the lowest mean temperatures recorded at Triabunna and Clarence Point; Dunalley recorded the lowest temperature across all the farms. Triabunna recorded the smallest variance however sampling was only undertaken at this farm for three months. The water temperatures at Bicheno and Pipe Clay Lagoon are also somewhat inflated, given that the water storage tanks at both farms are artificially heated.

Salinity and pH was generally consistent across the five marine shellfish locations with low variances recorded at all farms (Figure 15) (and little systematic effect across the farms). Salinity levels in the River Derwent were the most variable of the six locations, as a result of freshwater runoff into the river near the CMAR labs and the relatively shallow sampling site. Dissolved oxygen was also consistent across the farms (DO% was not recorded at Triabunna) (Figure 15).

On a month to month basis, the warmest temperature across the shellfish farms was (23.2℃) observed at Pipe Clay Lagoon in December 2012 with the warmest temperatures at all locations observed during November to February. Mid year temperatures (May-August) were the lowest with Dunalley recording the lowest temperature in June of 8.4℃ (Figure 16). Salinity was also relatively consistent across the five marine farms although very different to that observed at the greenfield site in the River Derwent – with a more freshwater profile, salinity is consistently less than 33ppt, depending on water run offs (Figure 17). The break in some tracking data reflects the lack of salinity probe data available in some months. Throughout the sampling period, water pH was generally consistent across month and farm apart from the lower values observed at the CMAR sampling site in January and May/June 2012 (Figure 18). Dissolved oxygen was generally consistent across all months and farms aside from the large variance in values observed in October 2012 and at Dunalley in November and December 2012. Dissolved oxygen at Shellfish C – Pipeclay Lagoon and Bicheno varied the greatest among the farms (Figure 19). With physical aeration in the tanks at Clarence Point and Dunalley, dissolved oxygen was consistent across the sampling points on each abalone farm (with low variance). Dissolved oxygen was not measured at Triabunna and at Hobart it was more variable, depending on the time of year of sampling.

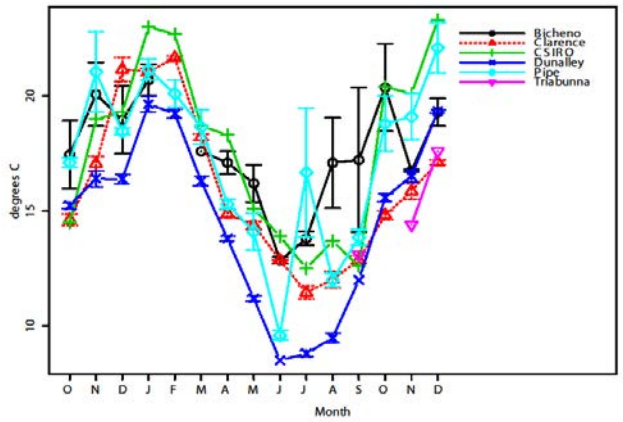


Figure 16 Temperature trend data

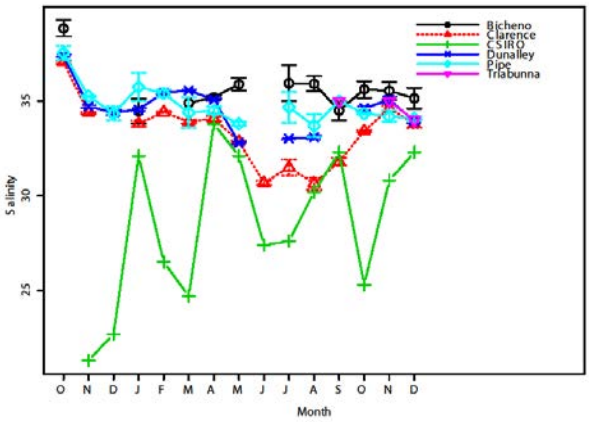


Figure 17 Salinity trend data

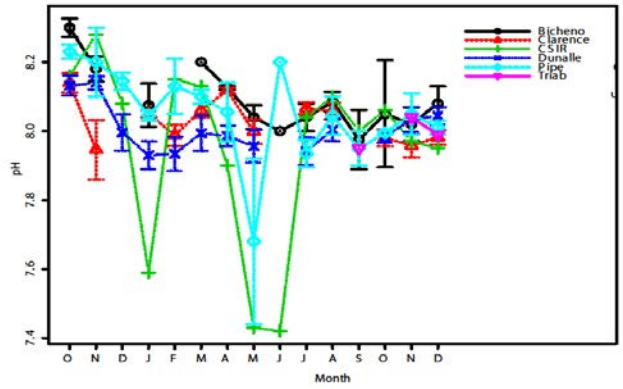


Figure 18 pH trend data

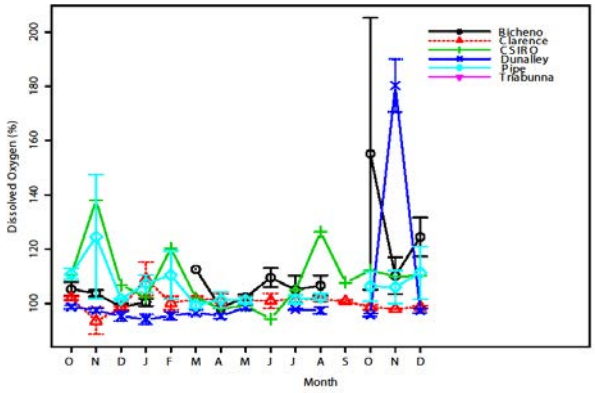


Figure 19 Dissolved oxygen trend data

3.2.2 Nutrient parameters

Table 3 lists the overall ranges and averages of the nutrient parameters across all areas, for the 14 months of sampling.

Table 3 Summary of environmental nutrient covariates during October 2011 – December 2012

Covariates	Range	Average (± SD)
Ammonium (μg L^{-2})	0.00 – 108.00	20.30 (20.40)
Nitrate (μg L^{-2})	0.00 – 159.80	7.63 (14.12)
Phosphate (μg L^{-2})	0.00 – 200.30	21.60 (11.21)
Silicate (μg L^{-2})	0.00 – 1202.00	179.04 (160.14)

The average ammonium level across the study was 20.30mg/L and was most variable at the Clarence Point and Dunalley abalone farms. Nitrate levels were generally low at Dunalley, Pipe Clay Lagoon and Triabunna with the greenfield site recording the highest variance. Phosphate levels across the farms during the study were consistent while there was a systematic effect of site on silicate levels; again the greenfield site showed the highest mean value (in comparison to the shellfish farms) and was the most variable (Figure 20).

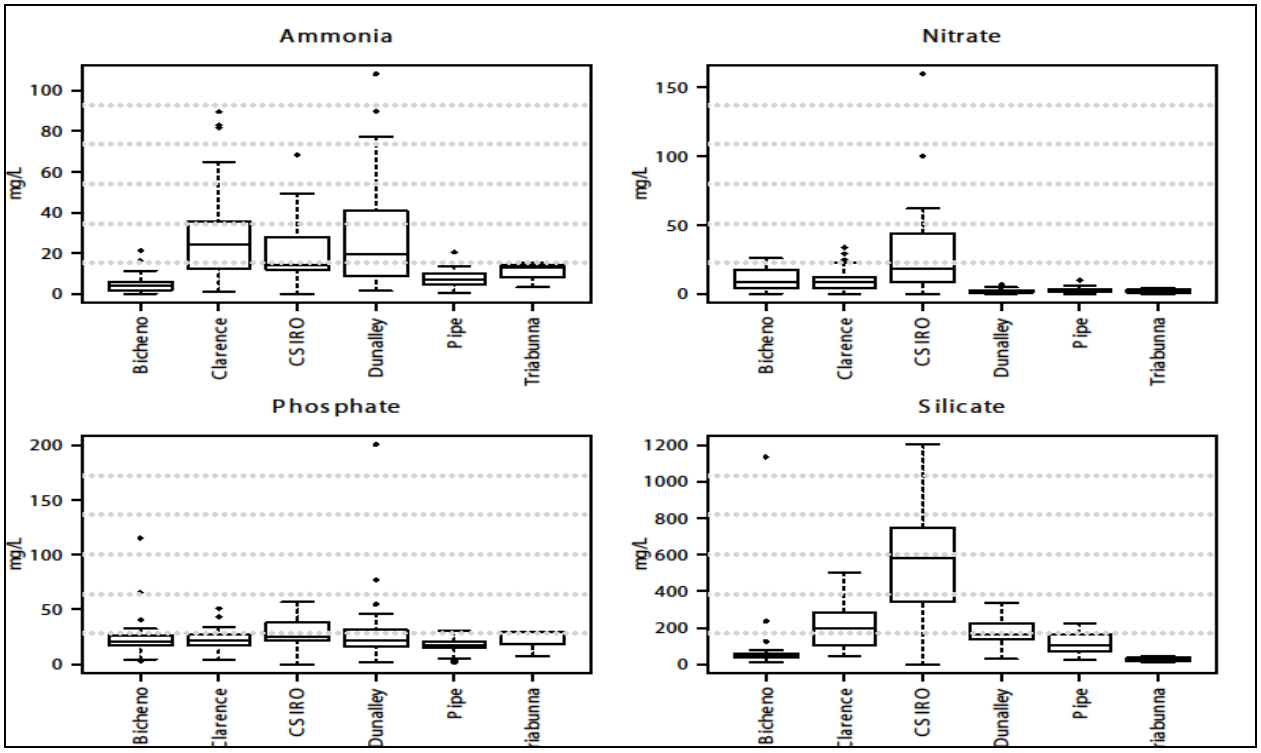


Figure 20 Summaries of nutrient covariate data across the six locations

In comparison to temperature, we can see that there is also a systematic effect of farm on the ammonium levels. Perhaps not unsurprisingly, the highest median values (and variance) for ammonium were observed across the tanks at Clarence Point and Dunalley in all months; these grow-out tanks contain abalone whereas there are no animals being on-grown in the water storage tanks and inlet areas at the three oyster farms. The highest ammonium levels were observed at Dunalley during February 2012 and Clarence Point in January 2012. Ammonium levels at Bicheno and Pipe Clay Lagoon were very similar and were generally less than 15mg/L throughout the 14 months of sampling (Figure 21). There was a large spike in the ammonium levels at the CSIRO wharf in September 2012, which was accompanied by high nitrate levels. The sampling area at the CSIRO wharf was also the most variable for nitrate across the 14 months of sampling (Figure 22). The shellfish farms generally showed low nitrate levels (with slightly higher levels observed at Bicheno during the later half of 2012) that did not vary greatly. Phosphate levels were generally consistent among the farms during each of the 14 sampling months, with the exceptions of the August sampling at Dunalley and the December sampling at Bicheno when phosphate levels over 80mg/L were observed (Figure 23). Of the five shellfish farms, water silicate varied the most at Clarence Point, particularly in the later half of 2012 and the wharf site in Hobart was highly variable throughout the year (Figure 24).

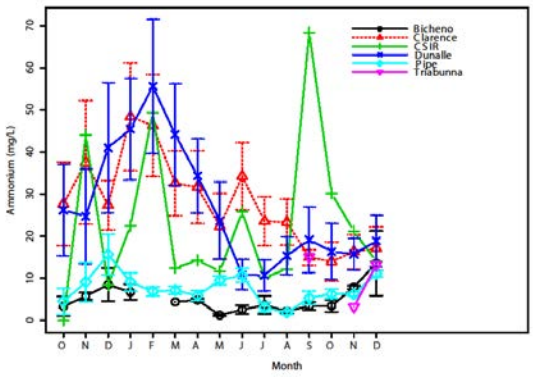


Figure 21 Ammonium trend data

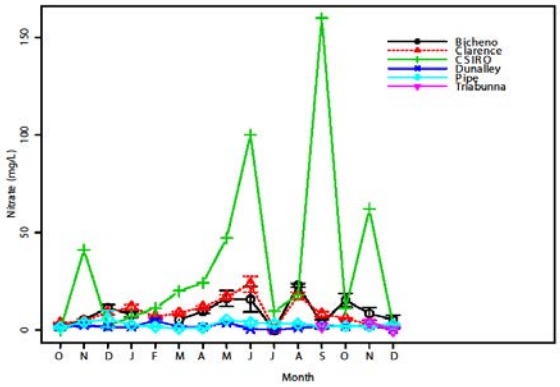


Figure 22 Nitrate trend data

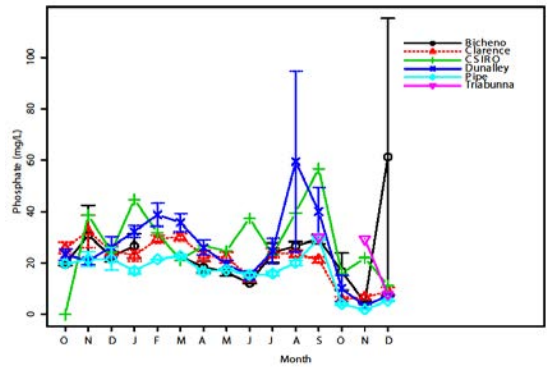


Figure 23 Phosphate trend data

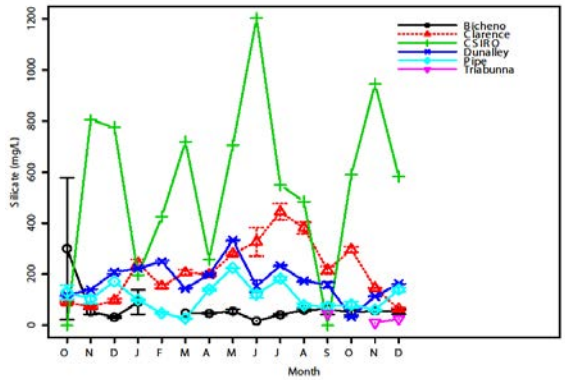


Figure 24 Silicate trend data

3.2.3 Data transformations

The Q-Q plots, which are scatter plots of the quantile function of the dataset versus the quantile function of the distribution (and to which a straight line slope has been fitted) are shown in Figure 25. From these plots it can be seen that several of the water parameters were not normally distributed (e.g. pH, ammonium) with several outliers in each of the parameters (e.g. silicate). This lack of normality does have biological implications, for example, pH and salinity were not normally distributed (with both tails of the distribution above the reference line – indicating distributional asymmetry) however this is not surprising, given the relatively narrow distribution ranges of these parameters in the marine environment. For subsequent analyses (including transformations) however, we did not remove these outliers.

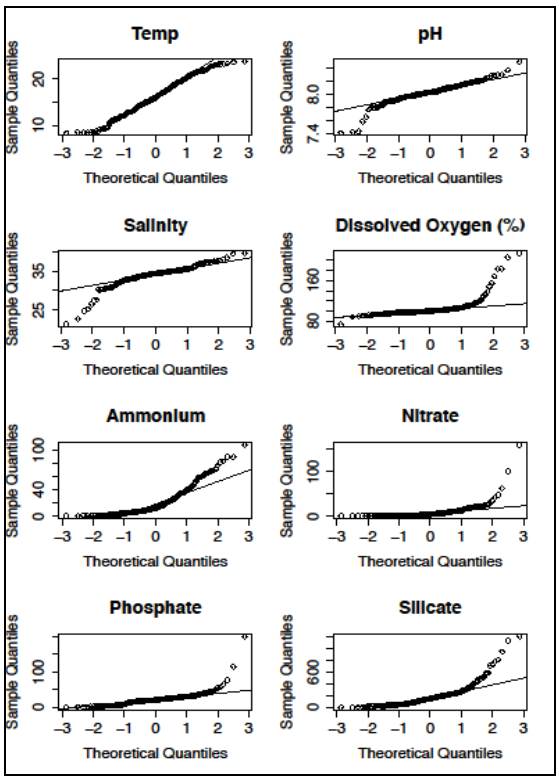


Figure 25 Q-Q plots for each of the water covariates (across all locations) prior to transformations

Figure 26 shows the water covariate data distributions following individual parameter transformations (√ transformations) where required. The transformed covariate data set was used all in subsequent analyses.

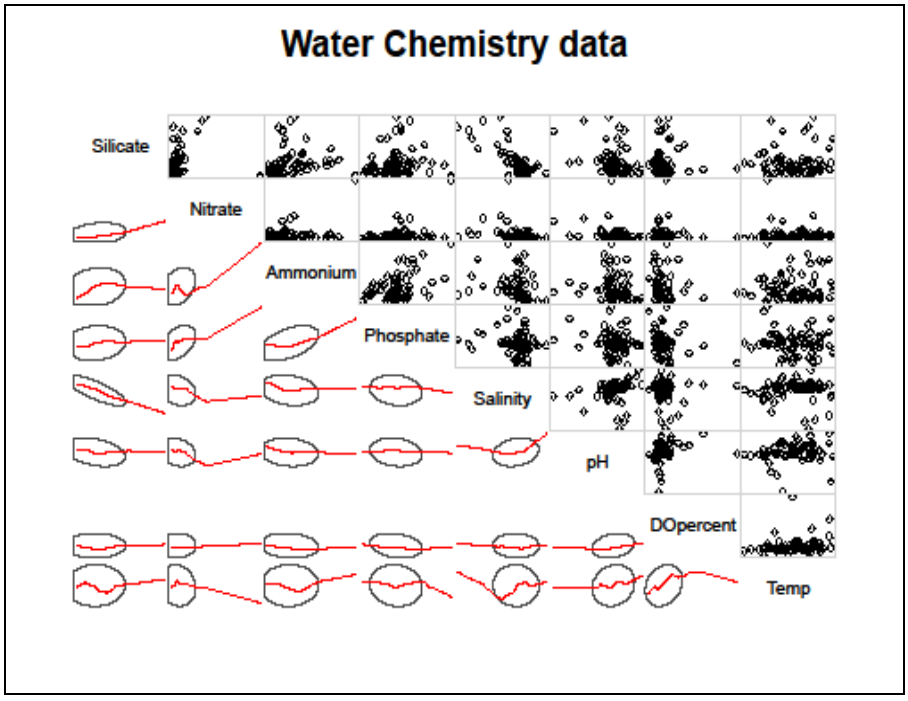


Figure 26 Corrolelogram plot demonstrating the relationship between water quality parameters, used to identify potential co-varying environmental variables (R^2>0.7)

3.3 ARISA community assemblage results

The results presented here are based on the ARISA amplified community profiles per sample across the locations. While community patterns are outlined and analysed (and each peak is assumed to represent a 'species' or OTU), due to the nature of the ARISA analyses, there is no information given here on actual taxonomic identifications. The following section (3.4) presents the NGS results and actual taxonomic findings.

We successfully amplified 235 primary A samples (Bicheno = 40 samples; Clarence = 74 samples; Dunalley = 74 samples; Pipe Clay = 31 samples; Triabunna = 3; Hobart = 13 samples) using the ARISA protocol with products electrophoresed on the CMAR 3130XL DNA Autosequencer. Comparisons to internal standards enabled accurate peak calling and area calculations (e.g., see Figure 27 and Figure 28 for examples of ARISA amplification products shown on an agarose gel and on the 3130XL Autosequencer).

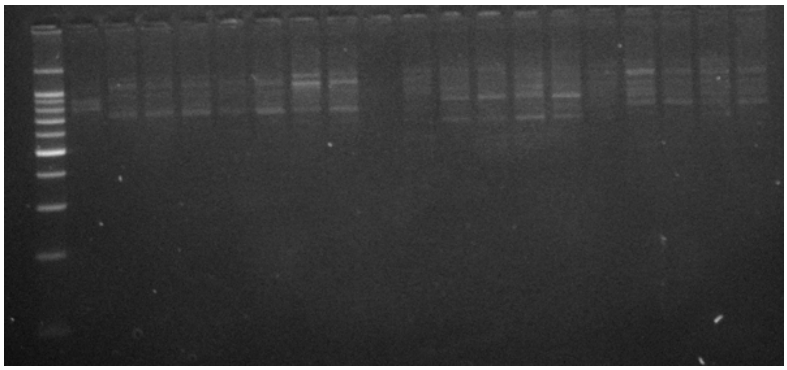


Figure 27 2.5% agarose gel of ARISA fragments from DNA extracted from November 2011 samples. Gel run at 120V for 1.5hrs, stained with Sybr Safe. Lanes 1 = PCR 100bp ladder; Lane 2 = Bicheno sample; Lanes 3-7 = Dunalley samples; Lanes 8& 9 = Pipe Clay Lagoon samples; Lane 10 = blank control; Lane 11 = Hobart sample; Lanes 12-16 = Clarence samples; Lanes 17-20 = Bicheno samples

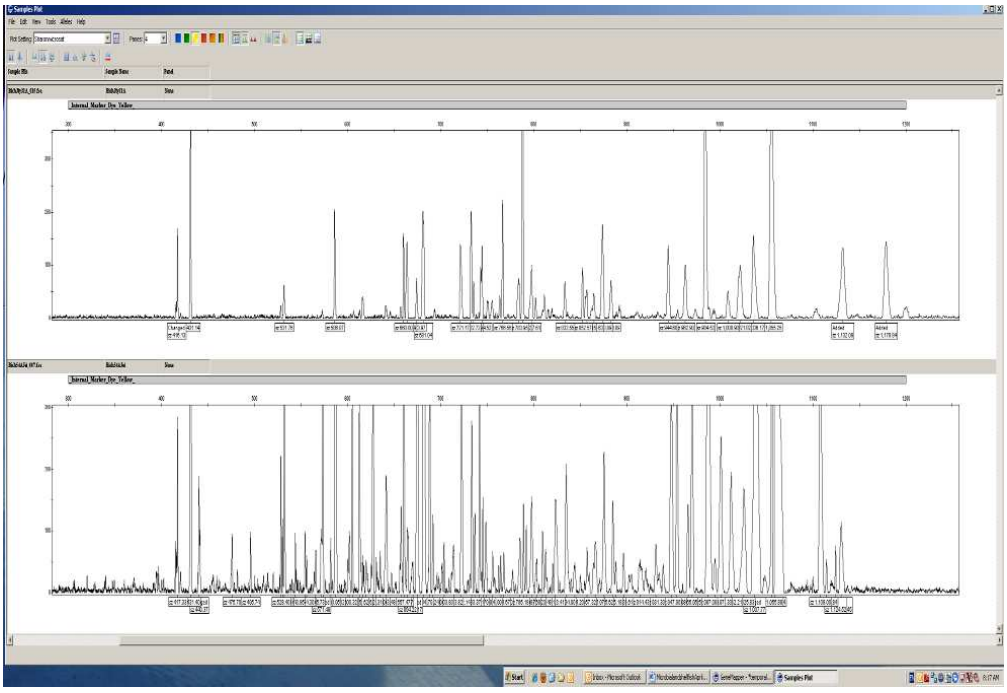


Figure 28 ARISA electropherograms from Bicheno samples 16S-23S rRNA DNA amplicons. Top panel - community profile from November 2011; bottom panel – community profile from January 2012

Following ARISA genotyping and binning, 221 OTUs (i.e. taxa) each comprising >0.09% of the total amplified DNA were observed across the Primary A samples (in the size range from 300 base pairs – 1208bp). The biological assemblage data therefore consisted of 221 bins/rows across 235 samples/columns with biological information represented as peak areas.

Prior to Mantel tests of the Spearman r rank correlation (between resemblance matrices based on different data treatments), data was standardised. Testing then demonstrated that the square root transformation on the standardised data gave the highest correlation to untreated data ($\rho = 0.953$, $P<0.001$, number of permutations = 999). For the ARISA assemblage data, all subsequent analyses were based on the standardised, transformed data.

Similarity permutation tests across all samples demonstrated that the ARISA farm assemblage data was significantly different at each farm in multivariate structure ($\pi = 3.718$, $P<0.001$). There was also significant differences observed across the assemblage profiles across the sites at each of the farms (with the exception of Triabunna) and the greenfield site across the 14 months of sampling (Bicheno $\pi = 5.431$, $P<0.001$; Clarence $\pi = 3.852$, $P<0.001$; Dunalley $\pi = 4.415$, $P<0.001$; Pipe Clay $\pi = 3.81$, $P<0.001$; Hobart $\pi = 2.507$, $P<0.001$; Triabunna $\pi = 1.07$, $P>0.05$). The Triabunna farm was only sampled for three months towards the end of 2012. Figure 29 shows a dendogram representation of the 235 samples, with linkages in hierarchical groups based on Bray-Curtis similarity between the farm clusters.

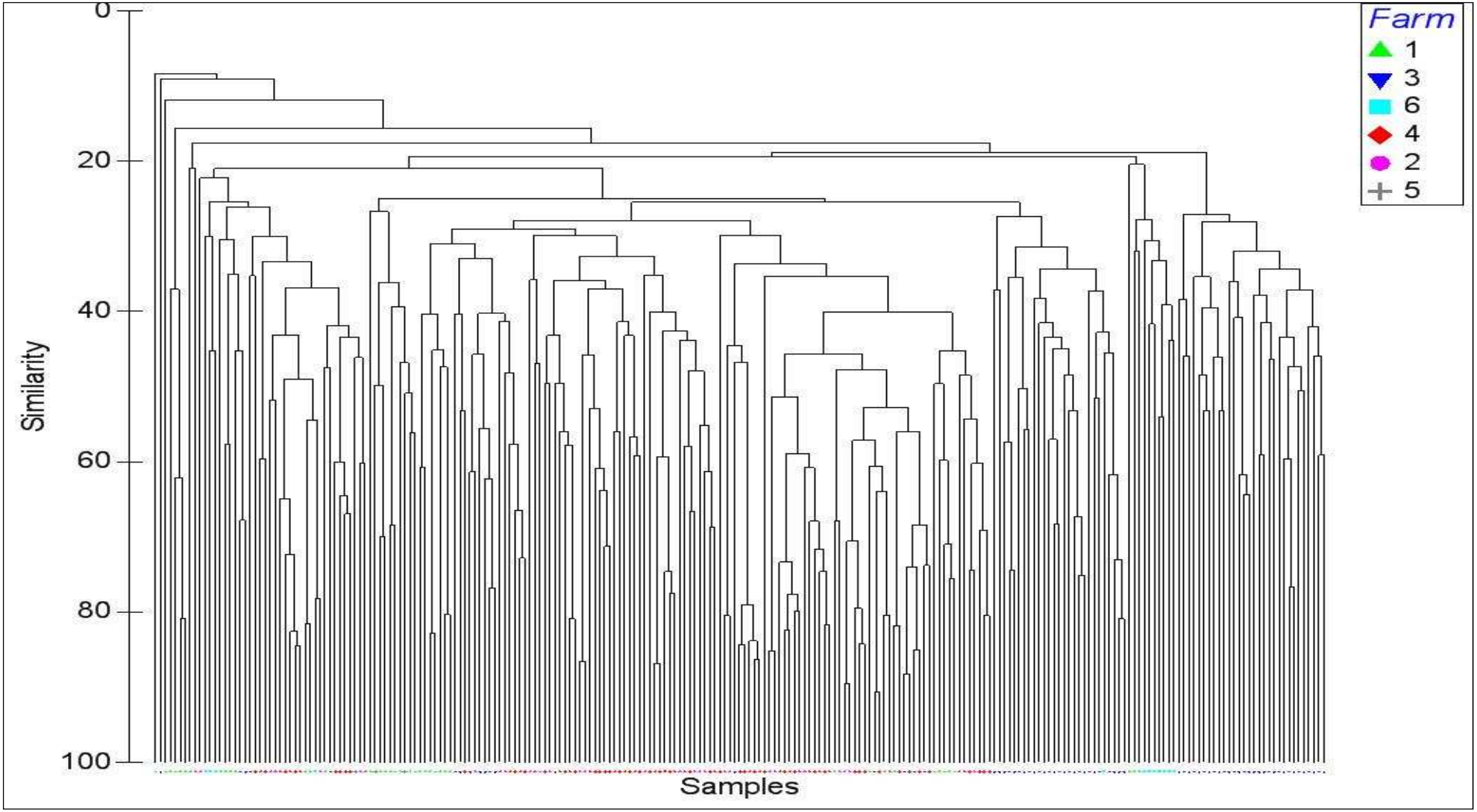


Figure 29 Dendogram of hierarchical clustering (Bray-Curtis similarities from transformed ARISA peak area assemblage data) of Primary A samples across the six locations (1 = Bicheno; 2 = Pipe Clay; 3 = Clarence; 4 = Dunalley; 5 = Triabunna; 6 = Hobart)

The ARISA data was also represented in two and three dimensional space. Two dimensional MDS plots showed a stress level of 0.26; this level was reduced further when the third dimensional ordination was fitted (stress = 0.19). The 3D nMDS ordination (Figure 30) showed potentially useful separation of the ARISA similarity data among the five farms and the greenfield site.

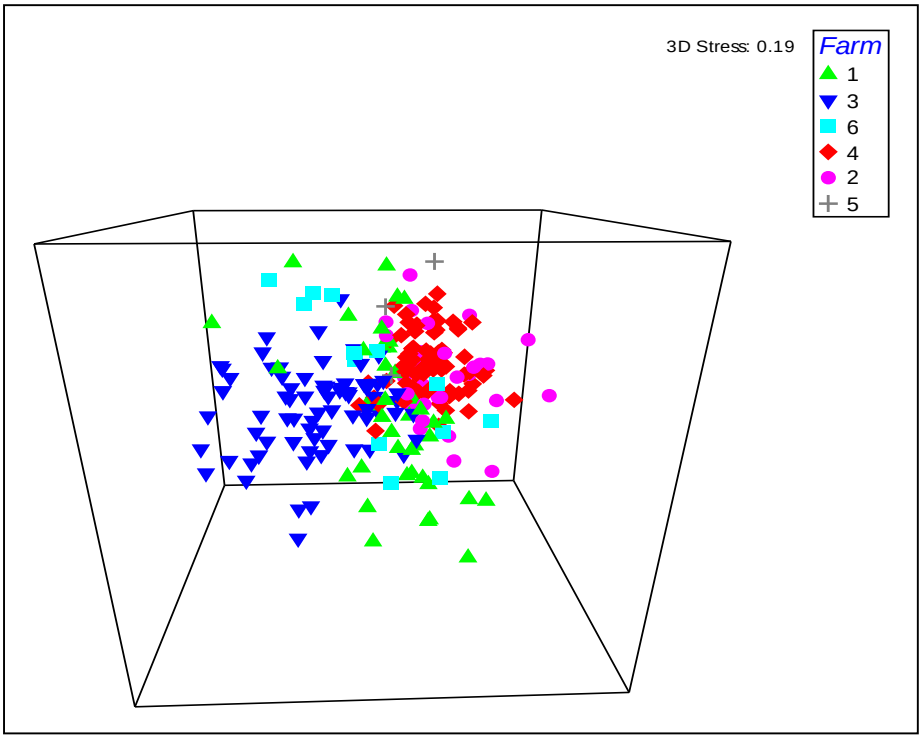


Figure 30 nMDS ordination plot of Bray-Curtis similarities from the transformed ARISA peak area assemblage data for the six locations (1 = Bicheno; 2 = Pipe Clay; 3 = Clarence; 4 = Dunalley; 5 = Triabunna; 6 = Hobart)

The estimated univariate ARISA diversity and richness averages for the farms and the greenfield site are shown in Table 4. Total OTUs ranged from 28.3 (in Triabunna) to 36.5 (in Clarence Point) indicating that the greatest number of microbial species (i.e. OTUs) were observed at the farm in the north of Tasmania. The Shannon-Weiner *H'* diversity estimates were variable across the farms (although not significantly) with diversity ranging from 3.134 to 3.433. Species evenness ranged from 0.966 to 0.976; as these estimates were all close to one, it was considered that the microbial communities at each of the sites were not dominated by just a few members. There were no significant pair-wise comparisons for either the *H' or J* estimates among the farms. This indicated that while the univariate diversity and evenness indices were high, overall there were no differences among the locations based on these estimates (*H'* pair-wise *P* among the locations ranged from non-significant 6-94%; *J* pair-wise *P* among the locations ranged from non-significant 5-95%).

Table 4 Average univariate measures for microbial diversity and evenness obtained from ARISA peak data. Significance levels are from one-way ANOVA testing

Location (total species *S*)	*Shannon Weiner diversity (H')(loge)*	Peilou (*J*) evenness
Bicheno (34.2)	3.361	0.971
Clarence (36.5)	3.404	0.970
Dunalley (33.0)	3.309	0.966
Pipe Clay (31.7)	3.286	0.967
Triabunna (28.3)	3.134	0.967
Hobart (35.4)	3.433	0.976
Global R	-0.006	-0.007
P	>0.61	>0.64

S is OTU richness

While the average univariate indices demonstrated no significant differences among the farms for overall microbial diversity or evenness, the multivariate analyses showed a different outcome. The multivariate analyses are richer in data points as the information per location was not collapsed into single index measurements. As measured by ARISA, the microbial communities were not homogenous across the locations (R = 0.320, *P*<0.001). Large pair-wise R values between some locations (e.g., Triabunna and Dunalley R = 0.462, *P*<0.001; Triabunna and Clarence Point R = 0.414, *P*<0.001) indicated differences among the farms were worth further examination (Table 5). Interestingly, the marine farms that were closer in spatial distance were not significantly different and or had smaller R values (cf. Bicheno and Triabunna; Pipe Clay and Dunalley v Pipe Clay and Clarence).

Table 5 Pair-wise ANOSIM comparisons among farms (below diagonal R value; above diagonal *P* value, following 999 permutations) (significant *P* values shown in bold)

Location	Bicheno	Clarence	Dunalley	Pipe Clay	Triabunna	Hobart
Bicheno	-----	<**0.001**	<**0.001**	<**0.001**	0.387	0.085
Clarence	0.284	-----	<**0.001**	<**0.001**	<**0.002**	<**0.001**
Dunalley	0.391	0.398	-----	<**0.002**	<**0.001**	<**0.001**
Pipe Clay	0.120	0.357	0.144	-----	0.026	<**0.002**
Triabunna	0.039	0.414	0.462	0.324	-----	<**0.002**
Hobart	0.111	0.290	0.486	0.270	0.399	-----

The one way ANOSIM Global test based on multivariate samples across the months was also significant (R = 0.179, *P*<0.001). This was a result of high R values between Autumn/Winter and Spring/Summer months (e.g., January and September R = 0.488, *P*<0.001; January and June R = 0.565, *P*<0.001; January and May R = 0.404, *P*<0.001). A one way ANOSIM test also indicated differences among the sites across the farms (e.g., grow-out tanks containing abalone v water conditioning tanks with no animals present) (R = 0.112, *P*<0.001), with the largest differences observed between grow-out tanks and water storage tanks (R = 0.234, *P*<0.002) and the smallest value between inlet areas and header tanks (R = 0.004, *P*>0.400).

Similarity testing indicated there was a low but significant effect of the type of culture (abalone v oyster v no culture) albeit this global R value was much smaller than that observed for the spatial (farm) or temporal (month) factors (R = 0.13, *P*<0.001). Perhaps not surprisingly, the comparison between the ARISA profiles from samples taken from water at the oyster hatcheries and the Derwent River (neither of these sampling sites contained any animals) showed there was no significant differences in the assemblages (R = 0.067, *P*>0.100). The most significant comparison in this respect was that between microbial communities in water taken from sites in which abalone were grown and the Derwent River (R = 0.164, *P*<0.02) – indicating a significant difference in community structure amongst these sites. The time at which the sampling was undertaken at the locations (i.e. AM v PM) contributed little to the overall large differences among the samples with a small but significant R value (R = 0.042, *P*<0.001).

When a two way analysis was undertaken on month nested within farm, there was significant heterogeneity observed across the months (R = 0.461, *P*<0.001) and slightly smaller, but still significant R value of 0.263 (*P*<0.001) when farms were used as the group factor. Similarly, sites nested within farm also indicated there was significant differences in the ARISA profiles among the various sites between the farms (R = 0.417, *P*<0.001) but microbial diversity as measured by ARISA was observed to be homogenous across the sites per farm (R = 0.088, *P*>0.200).

As the biotic data (ARISA assemblage information) was matched by a suite of environmental multi-variates measured at the same sites and locations, and as the initial one way ANOVAs had indicated *a priori* that there was differences (among the locations, months and sites), we also tested to what extent the *abiotic* and nutrient data (taken either singularly, or in combination) matched the ARISA community structures.

Microbial diversity as measured by ARISA was not significantly explained by the covariate data (ρ = 0.078, *P*>0.100) although the single *abiotic* variable which best groups the ARISA profiles was phosphate (ρ = 0.073), and the next best was silicate (ρ = 0.070). However, since we would not expect a single environmental variable to provide a successful match, the best multi-variable combination was observed to be ammonium, nitrate and phosphate (ρ = 0.078). There was no single variable that provided a successful match to this same level; the poorest match was with temperature on its own (ρ = -0.003) (Table 6).

Table 6 ARISA assemblage data and combinations of the eight covariates, taken *k* at a time, with the best match of biotic and environmental matrices for each *k*, as measured by Spearman rank correlation

k	Best variable combinations (ρ_w)
1	Temperature (-0.003) pH (0.034) Salinity (0.061) DO% (0.043) Ammonium (0.053) Nitrate (0.047) Phosphate (0.073) Silicate (0.070)
2	Amm, Phos (0.078)
3	Amm, Nit, Phos (0.078)
4	pH, Amm, Nit, Phos (0.073)
5	pH, Amm, Nit, Phos, Sil (0.073)
6	Temp, pH, Amm, Nit, Phos, Sil (0.072)

We then tested the data further for differences among the groups (locations and months) in multivariate space. PERMANOVA tested whether the between group variance explained a significant proportion of the total variation in the system as a whole, and CAP tested if there were axes in multivariate space that separated the groups. As Table 7 demonstrates, there was significant effect of the location groupings (pseudo-*F* = 3.497, √VA = 18.520) and month groupings (pseudo-*F* = 2.790, 29.487) on the observed multivariate variability (based on the Bray-Curtis resemblance measure).

Table 7 PERMANOVA ARISA results across the six locations (farms/locations = 6 levels; months = 12; sites = 5, *P* values obtained by 999 permutations)

Source of variation	df	Sum of Squares (Type III)	√source variation	Pseudo-*F*	*P* (permutated)
Farms	5	73899	18.520	3.497	0.001
Months (Farms)	56	2.867E5	29.487	2.790	0.001
Residual	173	3.175E5	42.841		
Total	234	6.8906E5			

Individual pair-wise *t*-tests demonstrated most of the locations differed from one another ($P<0.005$ for most comparisons) (Table 8) however the evidence for heterogeneity was weakest in comparisons of Triabunna with all other locations (*t* values ranged from 1.193 – 1.461, $P = 0.026$ – 0.124). There were three ARISA samples for Triabunna whereas all other locations had a larger number of samples.

Table 8 Pair-wise tests among locations based on ARISA profiles (below diagonal, t value; above diagonal *P* value following 999 permutations. Bolded values are significant after Bonferroni correction)

Location	Bicheno	Pipe Clay	Clarence	Dunalley	Triabunna	Hobart
Bicheno	-----	0.006	**0.001**	**0.002**	0.124	**0.001**
Pipe Clay	1.539	-----	**0.001**	0.441	0.036	0.003
Clarence	1.819	2.157	-----	**0.001**	0.041	**0.001**
Dunalley	1.944	0.995	2.366	-----	0.026	**0.001**
Triabunna	1.193	1.334	1.461	1.425	-----	0.007
Hobart	1.570	1.666	1.859	2.003	1.337	-----

Pair-wise comparisons also demonstrated that while there were no significant differences among the months (once *P* values were corrected), there was some evidence to indicate that the ARISA profiles across the locations were most similar in the adjacent cooler months (e.g., May and July $t = 0.890$, $P = 0.640$; July and August $t = 1.164$, $P = 0.242$) and warmer months (e.g., January and February $t = 0.943$, $P = 0.548$; September and November $t = 1.133$, $P = 0.264$) as compared to months that were more temporally separated (January and August $t = 2.014$, $P = 0.01$; January and April $t = 2.664$, $P = 0.004$).

The CAP analyses demonstrated that ammonium and nitrate were the vectors that maximised location groups, with 82.2% of the samples correctly classified according to their location (Total correct: 193/235, Mis-classification error: 17.87%). The samples from Clarence and Dunalley were clearly located in the top half of the cloud (Figure 31). Of the locations with more than 10 samples (i.e., leaving out the Triabunna samples), this CAP analyses demonstrated 80% of Bicheno samples, 93% of Clarence, 77% of Hobart, 86% of Dunalley and 58% of Pipe Clay samples were successfully classified to their correct groups (i.e., location) within the multivariate cloud space.

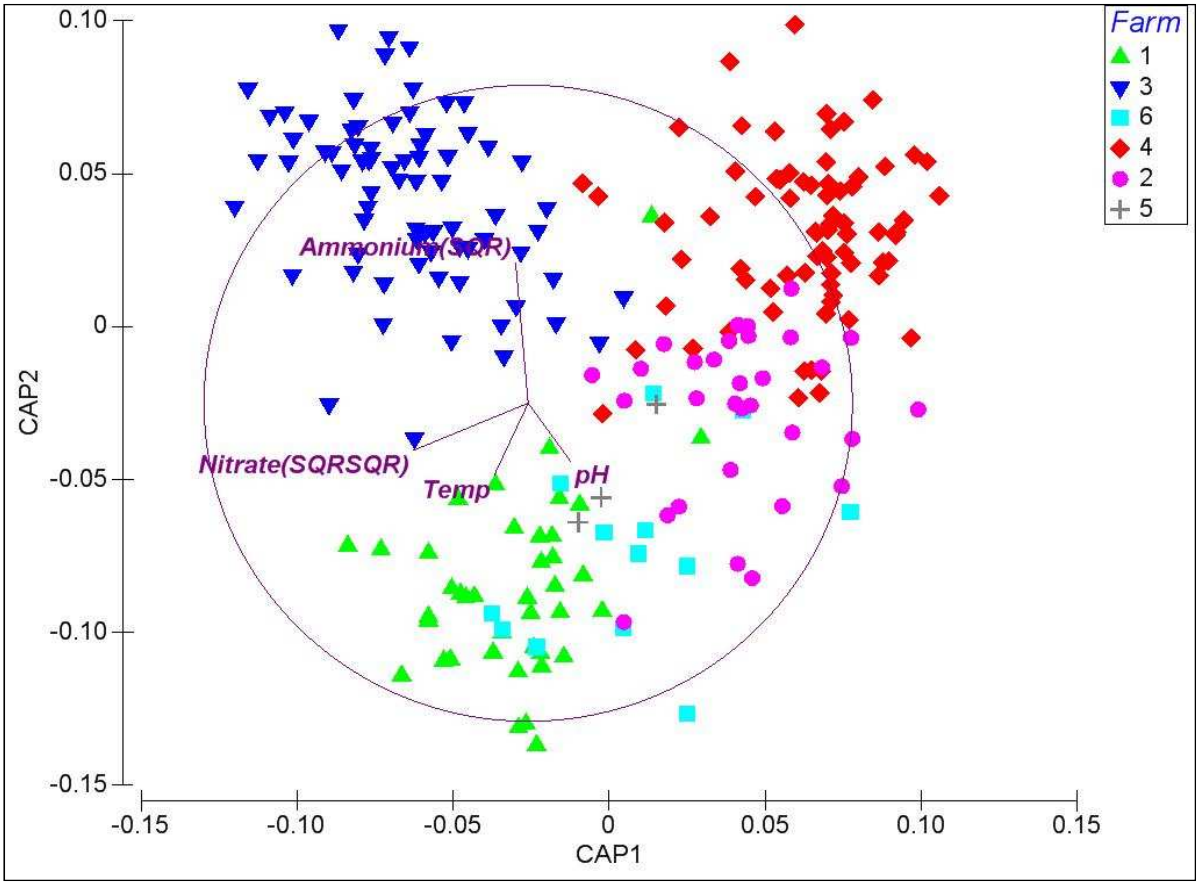


Figure 31 CAP ordination of ARISA data from the six sampling locations, with environmental CAP axes restricted to those having lengths of >0.2 (1 = Bicheno; 2 = Pipe Clay; 3 = Clarence; 4 = Dunalley; 5 = Triabunna; 6 = Hobart)

As can be seen in Figure 32 however, when month was used as the factor of interest in the CAP analysis, temperature and ammonium were the axes that maximised the groupings however successful classification was lower than that with location (75.7%, Mis-classification error: 24.3%). Samples from April (93%), January (88%) and March (86%) were successfully classified to the correct group (i.e. month) while samples from May were only correctly classified approximately half the time (53%).

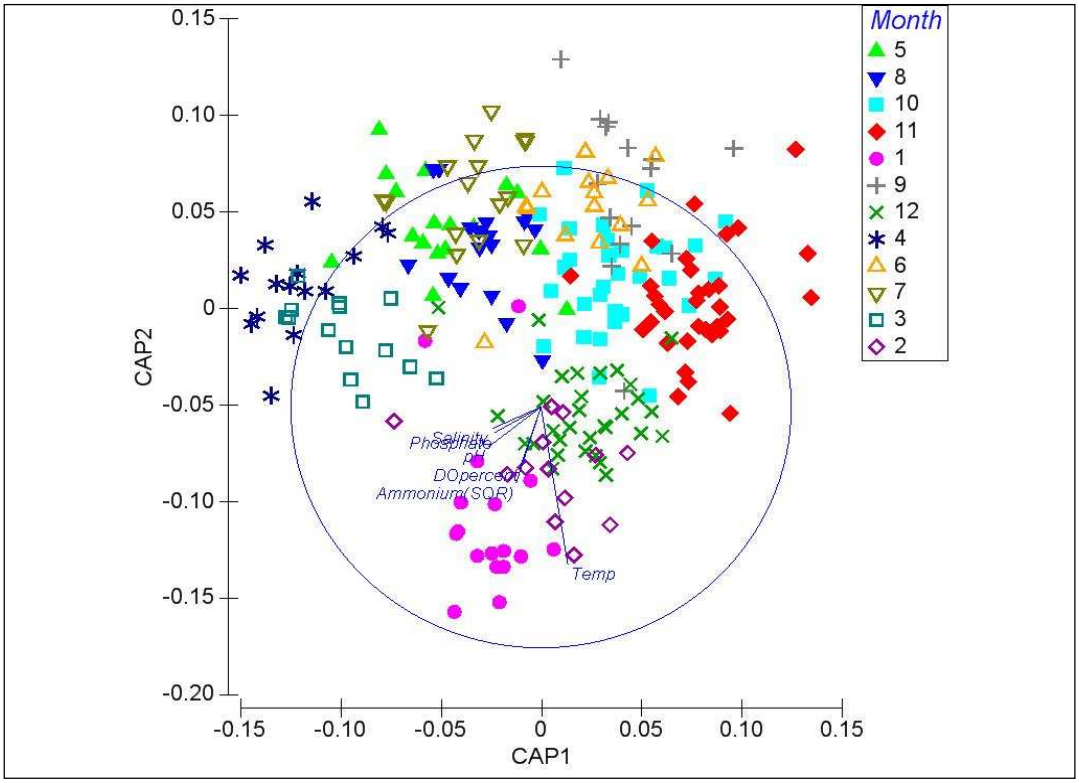


Figure 32 CAP ordination of ARISA data from the temporal sampling period, with environmental CAP axes restricted to those having lengths of >0.2 (Months 1-12 = Jan – Dec)

3.4 NGS results – taxonomic identifications

By sequencing the V6-V8 region of the 16S rRNA gene, we were able to identify specific microbial organisms that were present in each of the samples. Following quality control, we obtained sequence data from 152 Primary A samples (Bicheno = 23 samples; Clarence = 48 samples; Dunalley = 43 samples; Pipe Clay = 21; Triabunna = 3 samples; Hobart = 14 samples) and detected gene sequences from 2 243 microbial species (based on 97% similarity equating to species identity).

3.4.1 Microbial identifications across locations

We observed a wealth of information from the NGS bacterial analyses (over 650 000 sequences, not including archaea, eukaryota or plantae sequences). As a large diversity of microbial species (across the six locations) was detected, the results presented here are primarily based on microbial Class and Family information. Given the nature of the shellfish farms (and previously reported pathogens of interest from the literature), we also present here results specific to pathogens of interest (to the species level where possible) and observations of various *Vibrios* across the farms.

In the first instance, the species were grouped into higher level taxa, thereby reducing the complexity of the dataset for initial observation. Figure 33 shows the summary heat map of bacterial Class which is a graphical representation of the sequence data, where count values contained in the taxa matrix are shown as colours. As can be seen, the microbial diversity as classified according to class averages is relatively simple with the α-Proteobacteria, γ-Proteobacteria and Flavobacteria classes dominating most locations (see Figure 33, left most vertical panel (α-Proteobacteria) and two right panels (γ-Proteobacteria and Flavobacteria). Members of the Mollicutes, Cyanobacteria, Fusobacteria, Actinobacteria and β-Proteobacteria groups were also observed in some samples. While it is difficult to discern the sample names in

Figure 33 (as there are approximately 150 samples on the RHS y axis), as the PERMANOVA and ANOSIM results will show, there was strong structuring of the samples according to location.

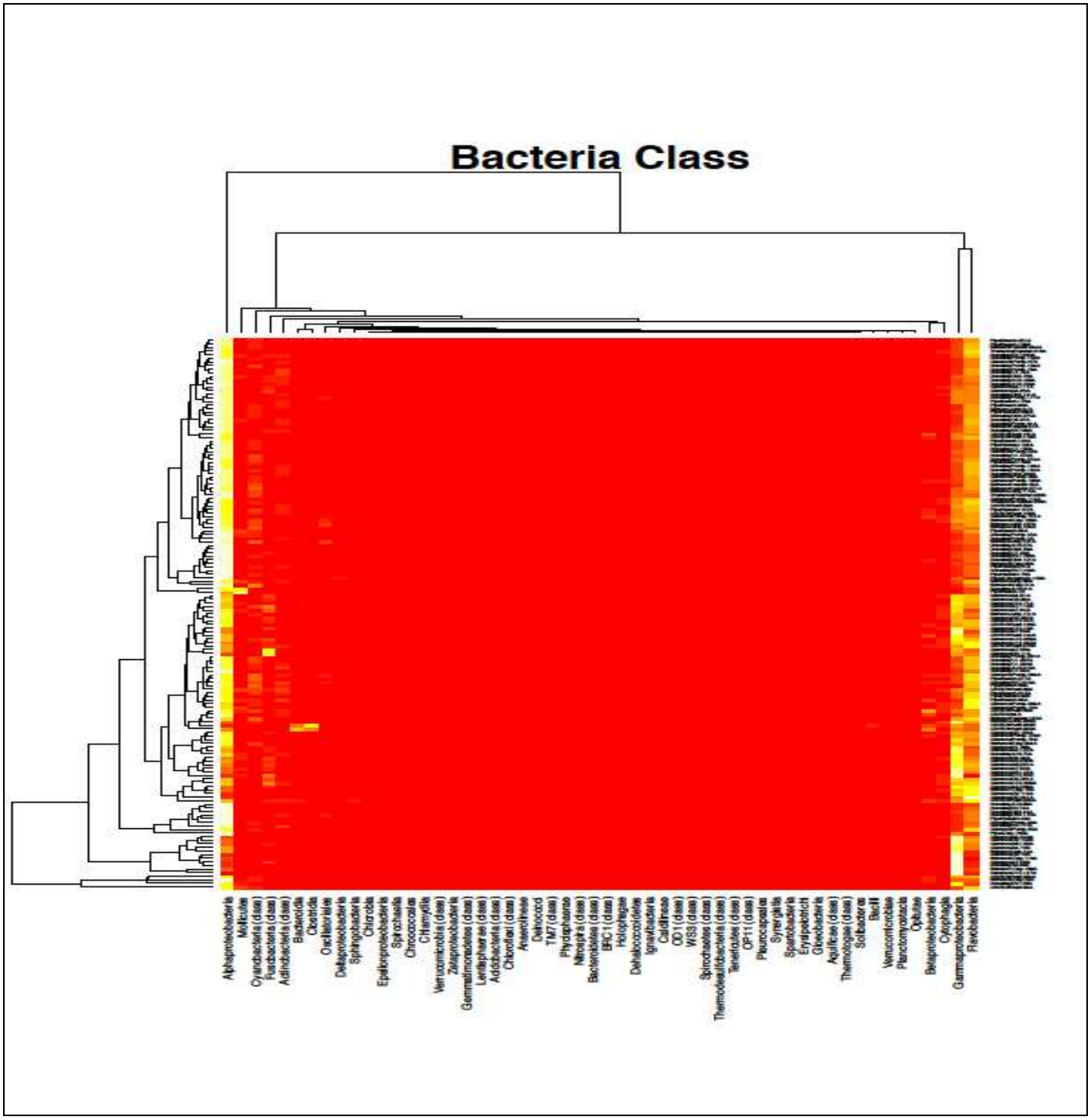


Figure 33 Bacterial Class heat map, based on microbial count averages per location (the 152 samples that were sequenced are listed on the right hand side y axis; Bacterial Class is listed on the x axis). The coloured squares (yellows/oranges/whites/reds indicate an increased number of observations for that Class). The dendogram on the left indicates grouping of samples based on similarity.

Further taxonomic investigation of these samples indicated that in many instances, the microbial grouping (averaged across locations) were classified only to genus (i.e., identified to genus level but not species, hence classification ends in '*sp*'; e.g. *Roseobacter sp*/*Vibrio sp*) (Table 9). This shows that the majority of the bacteria identified have no closely related cultured representatives which have been sequenced with resulting information provided to the public databases. This indicates that traditional systematics is not very useful, as many of the organisms present in the sea water are uncultivable.

Table 9 Most abundant NGS sequences assigned to species (across the six locations). Numbers represent % of total sequences assigned for that location

Name	Bicheno	Clarence Point	Dunalley	Pipe Clay	Triabunna	Hobart
Candidatus Pelagibacter ubique	7.46	5.66	14.48	12.61	16.31	6.09
Flavobacterium sp	8.37	7.29	9.04	11.15	13.03	10.71
Roseobacter sp	4.02	4.71	3.76	7.59	13.20	7.29
Pseudoalteromonas sp	0.81	13.00	2.73	0.52	1.37	1.18
Neptuniibacter sp	19.67	0.05	0.07	0.93	0.14	0.01
Synechococcus sp	3.67	1.11	2.59	3.33	1.37	1.77
Nisaea nitritireducens	0.85	0.88	2.58	2.33	1.72	1.44
Glaciecola sp	1.28	3.01	1.60	1.40	3.92	0.94
Marinimicrobium sp	1.52	0.32	0.98	2.00	2.41	1.24
Vibrio sp	0.42	3.10	2.18	0.20	2.95	1.91
Mycoplasma sp	0.40	1.02	1.86	3.40	0.29	1.26

The NGS data was useful in identifying the presence and relative abundance of key groups of microbes for shellfish aquaculture. As Table 9 shows, there is a substantial degree of variability between locations for many of the groups including *Candidatus Pelagibacter ubique*, *Roseobacter sp* and *Vibrio sp*. It should also be highlighted that the majority of the organisms identified here are typically observed in marine environments.

We interrogated the data for observation of pathogenic/disease causing microbes. There were no DNA sequences detected from organisms listed on the Australian National list of Reportable Diseases of Aquatic Animals (Oct 2011 list, http://www.daff.gov.au/animal-plant-health/aquatic/reporting/reportable-diseases). However, from the 152 Primary A samples, we did detect DNA from the following organisms which have previously been reported to cause disease in either abalone or oysters (Table 10).

Table 10 Significant known bacterial pathogens in shellfish culture and observations within the current study

Name	Disease	Organism affected	Detected in Survey	Reference
Candidatus Xenohaliotis californiensis	Withering Syndrome	Abalone	No	Andree *et al*., 2000
Vibrio fluvalis II	Pustule disease	Abalone	Yes	Li (1998)
Vibrio harveyi		Abalone	Yes	various
Clostridium lituseberense		Abalone	No	
Vibrio parahaemolyticus		Abalone	Yes	
Vibrio alginolyticus		Abalone	Yes	
Shewanella alga		Abalone	No	Cai *et al*., (2006)
Klebsiella oxytoca		Abalone	No	Cai *et al*., (2008)
Nuadamonas halioticida		Abalone	No	Doce *et al*., (2008)
Nuadamonas abalonii		Abalone	No	Doce *et al*., (2008)
Vibrio carchariae		Oysters	No	Nicolas *et al*., (2002)
Vibrio tubiashi		Oysters	Yes	Hada *et al*., (1984); Elston *et al*., 2008; Hasegawa *et al*., (2008)
Vibrio pectinicida		Oysters	Yes	Sandlund *et al*., (2006)
Vibrio anguillarum		Oysters	No	
Vibrio splendidus	Summer Mortality	Oysters Abalone	Yes	Lacoste *et al*., (2001); Handlinger *et al*., (2005)
Pseudomonas sp		Bivalves	Yes*	

*detection is not necessarily associated with the pathogenic organisms as organisms assigned this taxonomy are distant from described species and may comprise a number of divergent taxa

We also observed several detections of *V. cholerae* and as shown in Table 9, *Flavobacterium sp*. were observed at all locations. Flavobacterium 'like' bacteria have been observed in the literature as pathogens of molluscs (Handlinger *et al*., 2005) although there are approximately 20 species found in this genus with not all causing disease.

The majority of sequences detected were however from innocuous microbes which form part of the overall marine environment and are expected in the water samples. Additionally, at all locations we detected sequences from *Pseudolateromonas* sp. and *Phaeobacter gallaeciensis* which may have potential probiotic properties and proffer beneficial properties of inhibition against other pathogenic bacteria in aquaculture systems respectively (Romalde and Barja, 2010).

3.4.2 Spatial and temporal results

As with the ARISA community data, the species count data was standardised and transformed prior to the calculation of the Brays-Curtis similarity matrix. Similarity tests across the 152 samples also demonstrated here that species presence data was significantly different among the locations in multivariate structure (π = 4.984, P<0.001). There was significant species presence differences observed across the sites at each of the locations (with the exception of Triabunna π = 1.009, P>0.100) (other location results not shown here, although very similar to that observed for the ARISA community data). Figure 34 shows the dendogram representation of the 152 samples, with linkages in hierarchical groups based on Bray-Curtis similarity between the farm clusters.

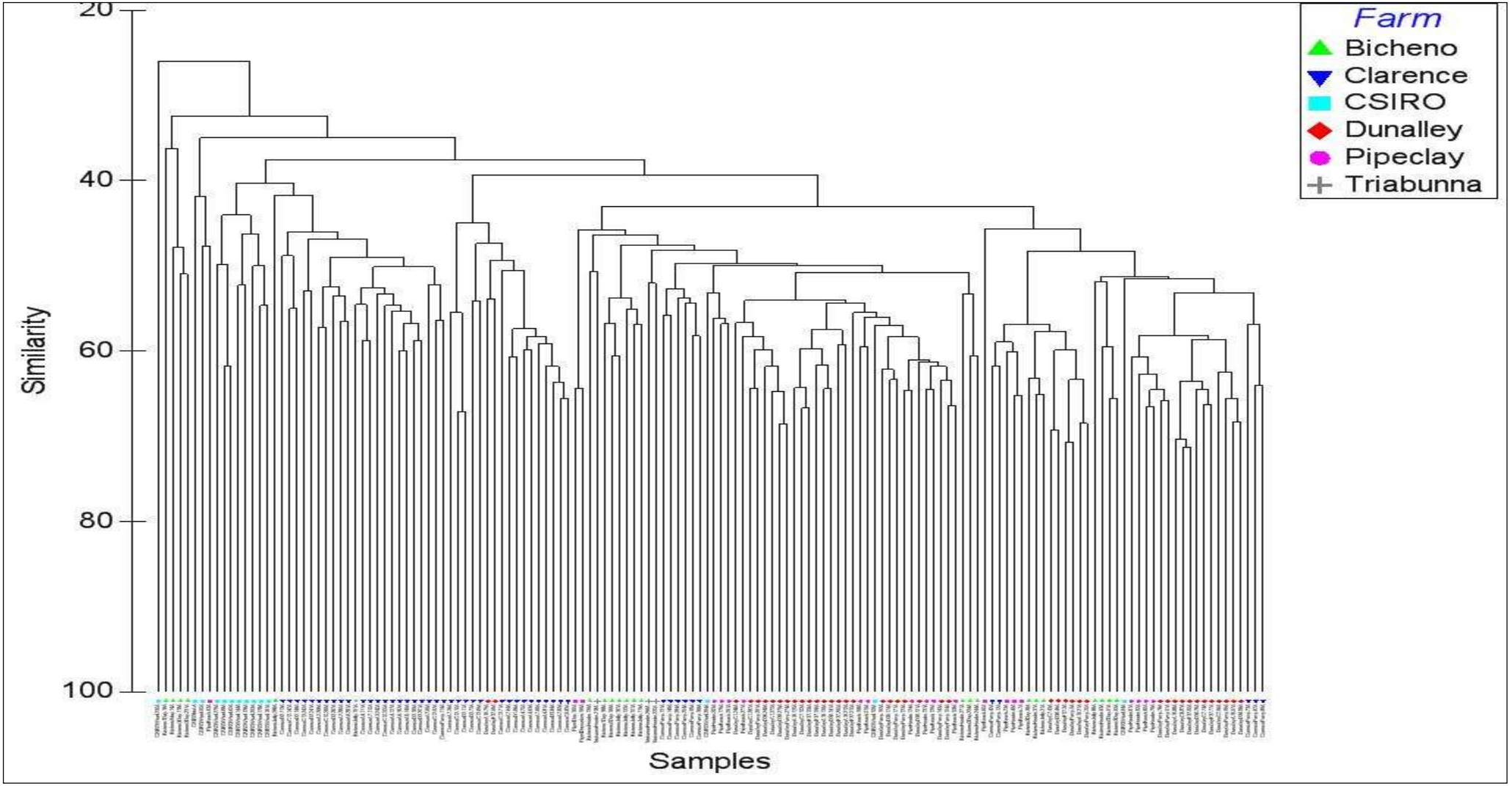


Figure 34 Dendogram of hierarchical clustering (Bray-Curtis similarities from transformed species presence data) of Primary A samples (n=152, shown on the x axis) across the six locations.

The NGS species data was represented in two and three dimensional space at lower stress levels than that observed with the ARISA data (2D = 0.16 and 3D = 0.12, Figure 35) indicating potentially very useful separations of the NGS species data among the locations existed in the data set.

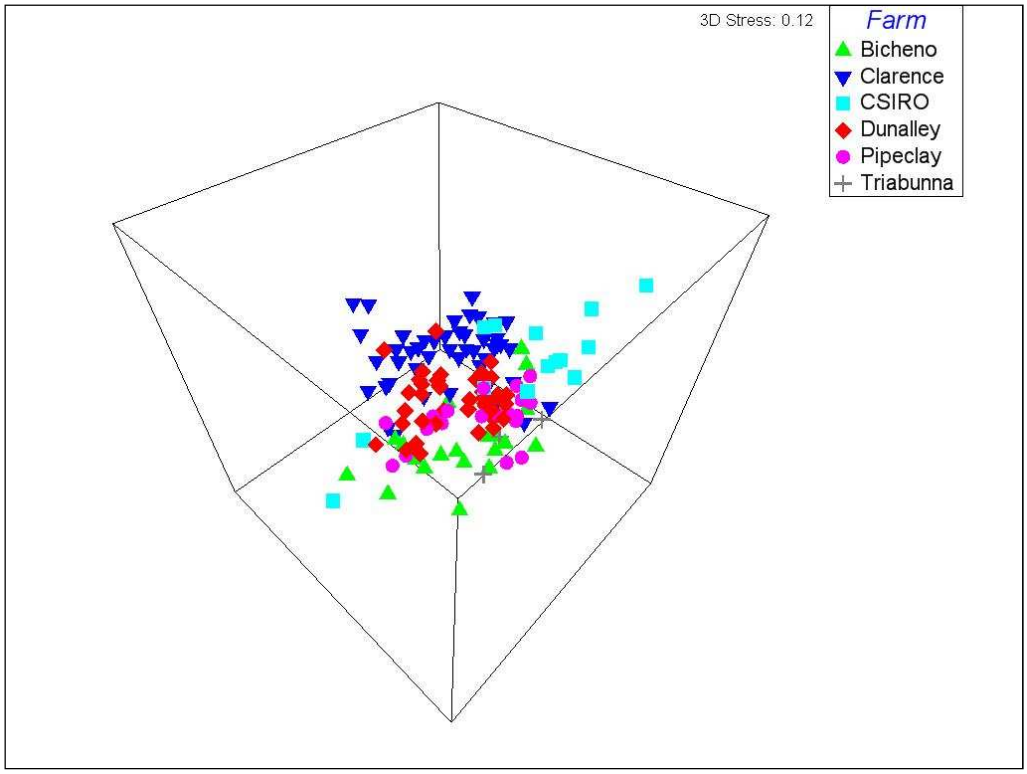


Figure 35 nMDS ordination plot of Bray-Curtis similarities from the transformed species presence data for the six locations

Species data was then collapsed into univariate diversity and evenness indices for the locations. As Table 11 shows, total species ranged from 140 (in Dunalley) to over 280 in the Derwent River (Hobart). The Shannon-Weiner *H'* diversity estimates were significantly variable across the farms with diversity ranging from 4.790 to 5.441 and the microbial species at all locations were not dominated by just a few species. The significant univariate indices were mainly driven by differences observed between Hobart and Dunalley (R = 0.432, *P*<0.001) and Hobart and Pipe Clay (R = 0.186, *P*<0.002).

Table 11 Average univariate measures for microbial diversity and evenness obtained from species presence data. The significance levels are from one-way ANOVA testing

Location (total species *S*)	*Shannon Weiner diversity (H')(loge)*	Peilou (*J*) evenness
Bicheno (156.7)	4.847	0.981
Clarence (181.35)	5.037	0.983
Dunalley (140.12)	4.790	0.980
Pipe Clay (157.71)	4.834	0.981
Triabunna (160.3)	4.965	0.982
Hobart (287.21)	5.441	0.988
Global R	0.090	0.124
P	<0.001	<0.001

S is OTU richness

Species detections were not homogenous across the locations (R = 0.403, *P*<0.001). Large pair-wise R values between some farm locations (e.g., Triabunna and Dunalley R = 0.582, *P*<0.001; Bicheno and Dunalley R = 0.484, *P*<0.001) indicated differences among the farms were worth further examination. As with the ARISA data, the marine farms that were closer in spatial distance were not significantly different (at least according to species presence) and or had smaller R values (e.g. Bicheno and Triabunna; Pipe Clay and Dunalley v Pipe Clay and Clarence). The river site at Hobart was significantly different to all locations except Triabunna (Table 12). While samples from the two Shellfish Culture farms were significantly different, the R value between them was much smaller than that between the oyster and abalone farms.

Table 12 Pair-wise ANOSIM comparisons among the locations (below diagonal, R value; above diagonal *P* value, significant values shown in bold)

Locations	Bicheno	Clarence	Dunalley	Pipe Clay	Triabunna	Hobart
Bicheno	-----	**<0.001**	**<0.001**	**<0.001**	0.414	**<0.001**
Clarence	0.427	-----	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Dunalley	0.484	0.430	-----	**<0.001**	**<0.001**	**<0.001**
Pipe Clay	0.168	0.430	0.253	-----	0.066	**<0.001**
Triabunna	0.028	0.446	0.582	0.235	-----	0.082
Hobart	0.384	0.493	0.718	0.384	0.300	-----

The one way ANOSIM Global test based on multivariate samples across the months was also significant (R = 0.354, *P*<0.001). This was a result of high R values between Autumn/Winter and Spring/Summer months (e.g., December and April R = 0.969, *P*<0.001; August and December R = 0.964, *P*<0.001).

As with the ARISA data, we tested for variance differences among the groups (locations and months), in multivariate space and tested if there were water parameter axes that separated these groupings. As Table 18 demonstrates, there was significant effect of 'location' groupings (pseudo-*F* = 3.850, √VA = 16.576) and 'temporal' groupings (pseudo-*F* = 2.190, 23.206) on the variance of species presence across the locations.

Table 13 PERMANOVA Species presence results across the six locations (farms/locations = 6 levels; months = 14; sites = 5, *P* values obtained by 999 permutations)

Source of variation	df	Sum of Squares (Type III)	√source variation	Pseudo-F	*P* (permutated)
Farms	5	36355	16.576	3.850	0.001
Months (Farms)	63	1.362E5	23.206	2.190	0.001
Residual	83	81966	31.425		
Total	151	2.635E5			

Individual pair-wise *t*-tests again demonstrated most of the locations differed from one another (*P*<0.008 for most comparisons) – locations that were closer in distance to each other (e.g., Bicheno and Triabunna) were not significantly different (*t* = 1.103, *P* = 0.264). Interestingly, the Clarence and Dunalley farms both cultured abalone, however the species comparisons between these two farms were the most different, thereby attributing the most to the overall variance (t = 2.557, *P* = 0.001) (see Table 14).

Table 14 Pair-wise tests among locations based on microbial species counts (below diagonal, t value; above diagonal *P* value following 999 permutations. Bolded values are significant after Bonferroni correction)

Location	Bicheno	Pipe Clay	Clarence	Dunalley	Triabunna	Hobart
Bicheno	-----	0.014	**0.001**	**0.001**	0.264	**0.001**
Pipe Clay	1.539	-----	**0.001**	0.028	0.137	**0.002**
Clarence	1.961	2.367	-----	**0.001**	0.054	**0.001**
Dunalley	1.873	1.456	2.557	-----	0.024	**0.001**
Triabunna	1.103	1.161	1.382	1.366	-----	0.034
Hobart	1.895	1.876	2.128	2.397	1.267	-----

Pair-wise comparisons also demonstrated that while there were no significant differences among the months (once *P* values were corrected), there was some evidence to indicate that the species counts across the locations were most similar in adjacent cooler months (e.g., April and May t = 0.795, P = 0.663; May and July t = 1.049, P = 0.276) and warmer months (e.g., October and November t = 0.999, P = 0.475; November and December t = 0.980, P = 0.653) as compared to months that were more temporally separated (January and July t = 1.781, P = 0.082; December and August t = 2.027, P = 0.106). The largest t value observed was between January 2012 and December 2012 (t = 2.322, P = 0.237, 11 months apart).

CAP analyses (Figure 36) demonstrated that ammonium, silicate and temperature were the vectors that maximised location grouping of the species data, with 94.7% of the samples correctly classified according to their location (Total correct: 144/152, mis-classification error: 5.26%). With actual species identifications in hand, the samples from all locations (aside from Bicheno and including the greenfield site) were clearly located in the top half of the cloud with the Bicheno samples clearly separated. Of the locations with more than 10 samples (i.e., leaving out the Triabunna samples), this CAP analysis demonstrated 100% of Bicheno, Clarence and Dunalley samples were correctly classified to their locations, with 86% of Hobart and Pipe Clay samples successfully allocated within the multivariate cloud space. Temporal classification according to month was less successful with only 62.5% of all samples assigned to their correct temporal groups. The samples in March 2012 demonstrated the highest correct percentage of allocation at 84.6% while the samples in September 2012 had the lowest allocation rate (only 33.3%).

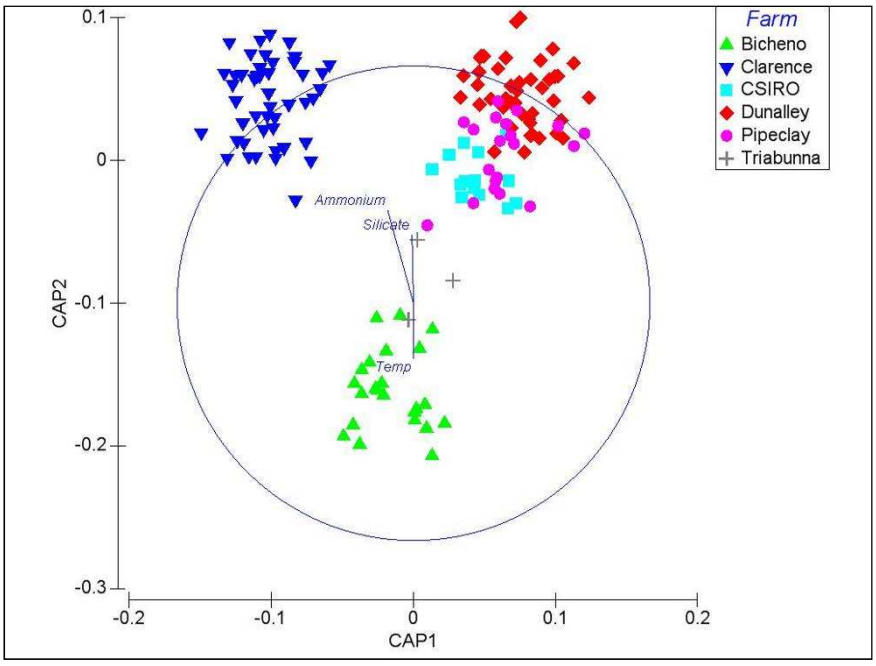


Figure 36 CAP ordination of species presence data from the spatially separated locations, with environmental CAP axes restricted to those having lengths of >0.2

3.4.3 Specific bacterial species of interest

Recent studies of the role of bacterial species in shellfish disease and aquaculture have identified a number of significant agents in abalone and oysters (Table 10). Examination of the results of DNA sequencing from this study indicated that a number of these agents were present in water samples taken from the different locations. These organisms include a number of species of the genus *Vibrio* as well as unclassified members of the genus *Pseudomonas*. The identification of members of the genus *Vibrio* in samples of seawater is not unexpected as this genus represents a group of common marine organisms, only a few of which are considered pathogenic. Of the samples that were sequenced using NGS, *Vibrio* and *Pseudomonas* species were identified at all times and locations across the study. Whilst a number of the organisms identified as belonging to the genus *Vibrio* were identified only to genus level, a number were identified to species level using pyrosequencing. These included *V. splendidus*, *V. harveyi* and *V. parahaemolyticus.*

The data presented in Figure 37 demonstrates the variation in the relative abundance of the different *Vibrio* species detected during the study. For example, the relative abundance of *V. splendidus* was low (<0.2% of all sequences) during the first four months of the study but then doubled in relative abundance across most of the farms in the subsequent months. Additionally, the presence of the various *Vibrio* species was not homogenous among the locations (Table 15).

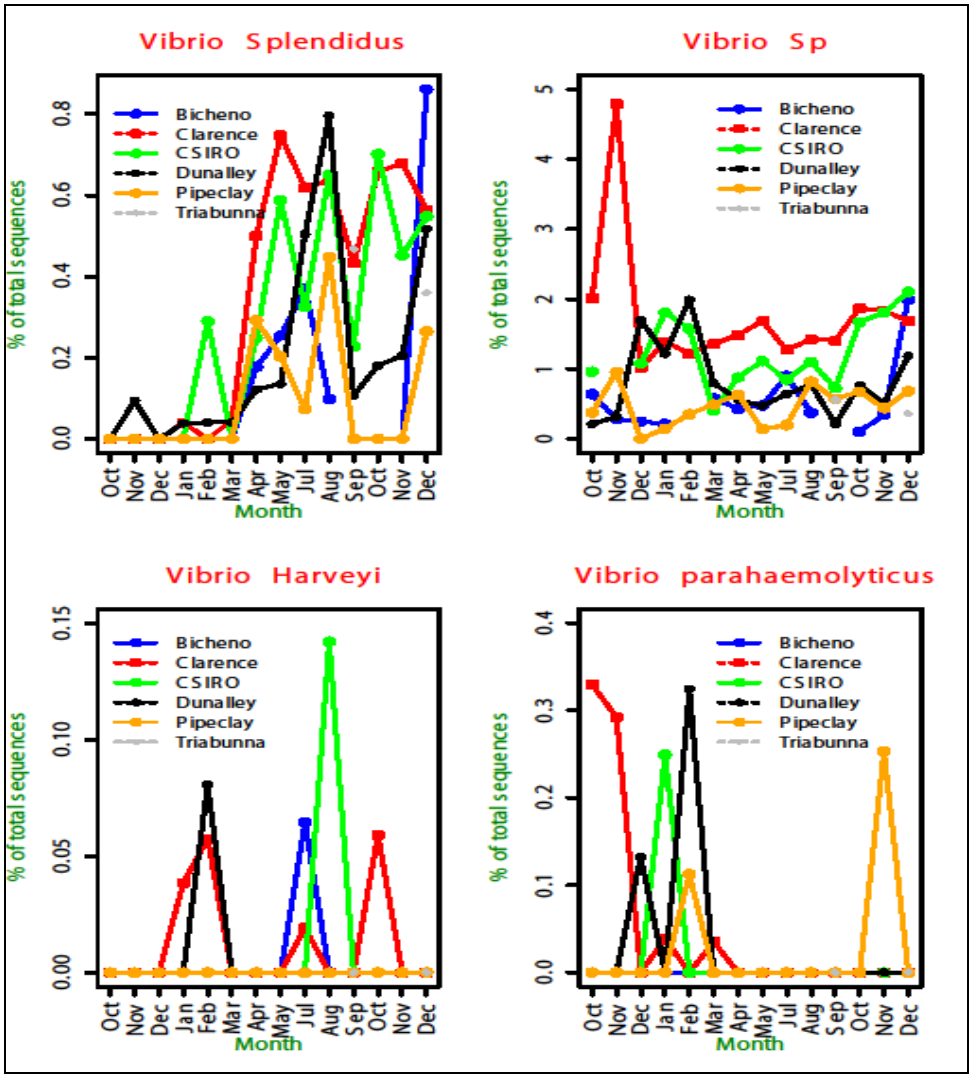


Figure 37 NGS sequences assigned to the genus *Vibrio* based on spatial and temporal sampling points. Numbers represent % of total sequences assigned for that location

Table 15 PERMANOVA of *Vibrio* species presence results across the six locations (farms/locations = 6 levels; months = 14; sites = 5, *P* values obtained by 999 permutations)

Source of variation	df	Sum of Squares (Type III)	√source variation	Pseudo-F	*P* (permutated)
Farms	5	33770	21.745	4.373	0.001
Months (Farms)	62	95763			
Residual					
Total	67	1.295E5			

Examination of the relative abundance of *Vibrio* sp, *Pseudomonas sp*. and specifically *V. splendidus* using multiple linear regressions (Table 16) was undertaken to attempt to identify the factors that may be related to the proliferation or relative abundance of these species of interest.

Table 16 Results of multiple linear regression analysis of individual species community abundance with water chemistry parameters. Models were limited to 3 or less predictor variables to avoid over fitting of models. Regression tree analysis was performed on samples marked * as shown in Figures 38 and 39.

Location	*Vibrio sp.*		*Pseudomonas sp*		*Vibrio splendidus*	
	Predictors	R^2	Predictors	R^2	Predictors	R^2
Hobart	Temperature + DO	0.62	Temperature + DO	0.31	Temperature + DO	0.27
Bicheno	Temperature + DO + Ammonium *	0.72	Salinity	0.39	Ammonium + DO	0.56
Clarence	Nil	<0.2	Temperature + Silicate	0.24	Temperature + Silicate*	0.38
Dunalley	Nil	<0.2	Temperature + Salinity	0.21	Salinity + Temperature	0.24
Pipe Clay	DO + Silicate	0.37	Temperature + Silicate	0.41	Temperature + Phosphate	0.37

Whilst there were no trends that applied across all locations, examination of the six locations individually revealed significant location-specific trends. As shown in Table 16, up to 72% of the variation in relative abundance of individual *Vibrio* species was able to be explained by water chemistry parameters at individual locations (e.g. Bicheno - temperature, dissolved oxygen and ammonium).

In order to further study the relationship between the water chemistry (*abiotic* and nutrient parameters) and relative abundance of selected bacterial species, regression tree analyses were performed (Figure 38 and Figure 39). This analysis serves to partition the effects of the different water chemistry factors, shown to be significant, in a way that allows a prediction of the response variable given certain conditions. As Figure 38 shows, the mean abundance of *Vibrio* sp in samples at Bicheno with DO >108.35% is 1.33% of total sequence reads, whilst samples with DO <108.35%, temperature >16.65℃ and ammonium >5.35 $\mu g/L^{-2}$ is 0.163% of total sequence reads.

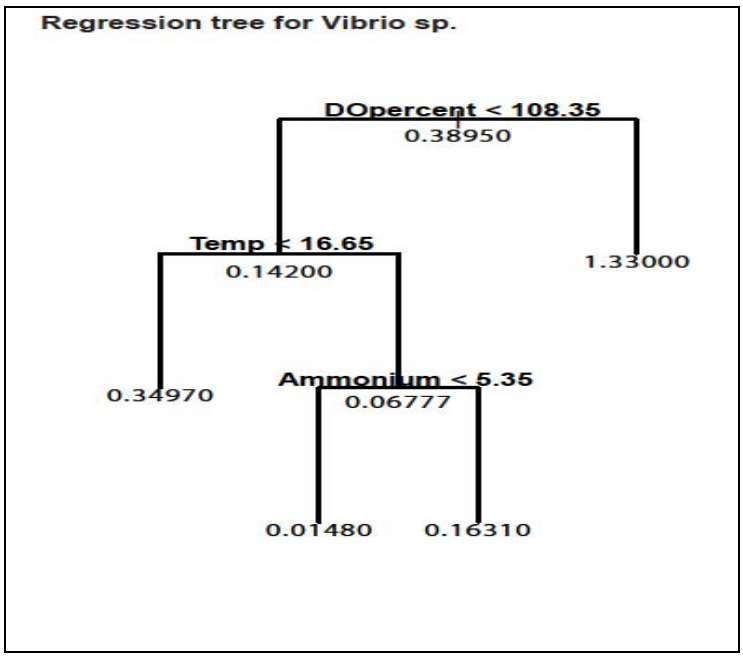


Figure 38 Regression tree of demonstrating the partitioning of variance between water chemistry parameters in predicting *Vibrio* sp. Relative abundance at Bicheno shown as an example. Variable values in bold represent the mean value for the subset at the decision point and the numbers below the node are the mean value for that group

In Figure 39, we can see that the mean abundance of *V. splendidus* in samples at Clarence with silicate > 317$\mu g/L^{-2}$ is 0.862% of total sequence reads, whilst samples with silicate < 317.5 $\mu g/L^{-2}$ and temperature <17.5℃ is 0.279% of total sequence reads.

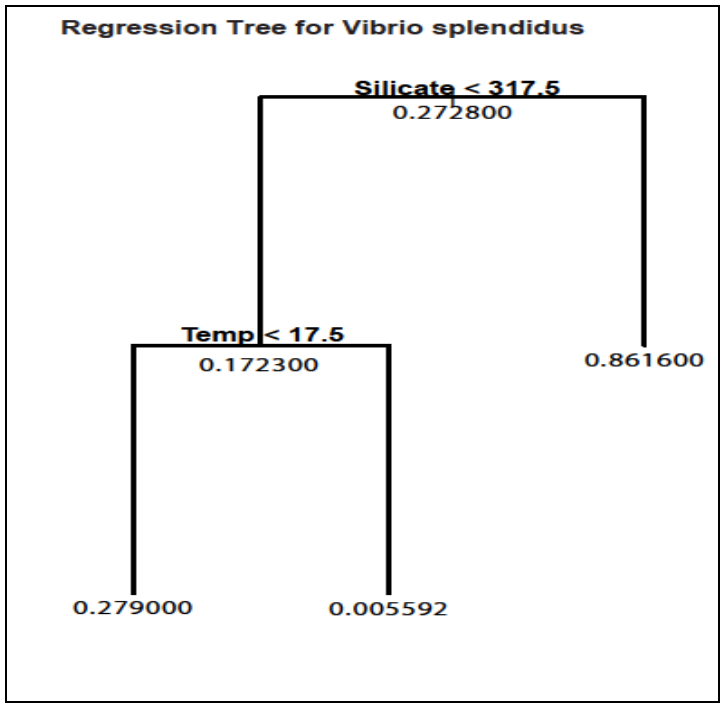


Figure 39 Regression tree of demonstrating the partitioning of variance between water chemistry parameters in predicting *V. splendidus* relative abundance at Clarence. Variable values in bold represent the mean value for the subset at the decision point and the numbers below the node are the mean value for that group

The examples shown in these Figures are based on a limited dataset, but it is reasonable to expect that a greater resolution of data (more samples analysed using purely quantitative approaches such as qPCR) would provide for a robust framework for the identification of conditions that may represent a significant hazard to the individual farms.

3.4.4 Farm specific report cards

While it is beyond the scope of this report to outline individual results for each farm on a month by month basis, we have this data in hand and if required by the farms, a 'farm specific report card' (e.g., see Figure 40) can be organised. We have water parameter data, ARISA profiles and microbial taxonomic information for each site (i.e., grow-out tanks, header tank, inlet area etc) from each farm on a monthly basis. Water parameter and bacterial abundance averages per farm can also be presented, alongside bacterial abundance (and trends) once the qPCR results are finalised. The ARISA profiles give an instant visual indication of the variability across the farm's sites and can also be presented on a site or monthly basis thereby showing any spatial or temporal changes (see Figure 28).

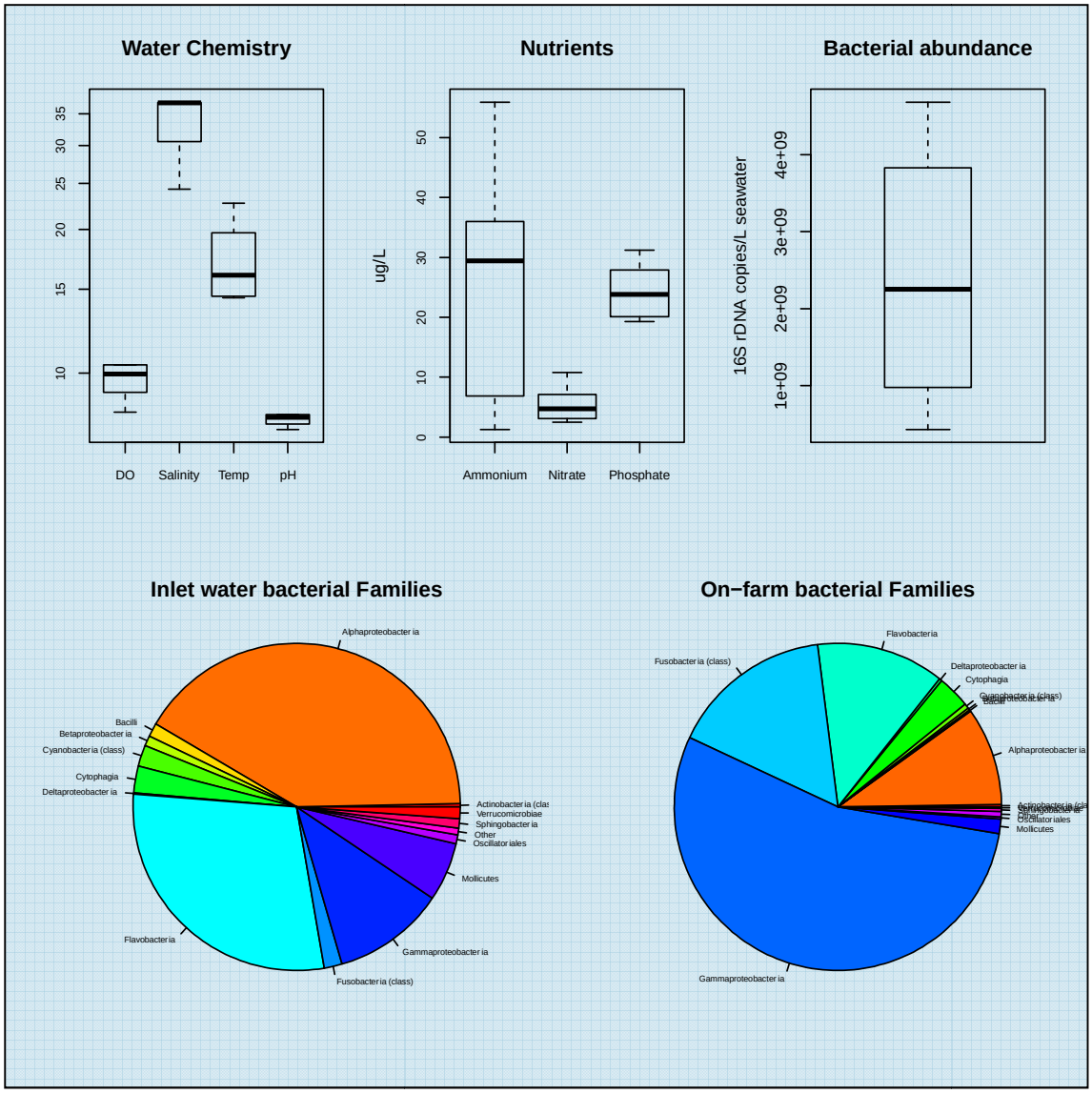


Figure 40 Example of a farm specific 'report card'

3.5 Additional experimental data collected

In addition to the Primary A samples and resulting data, as part of our broader interest in the application of metagenomic approaches for the shellfish industry, we also collected samples as part of the following *ad hoc* experiments:-

- total bacterial abundance per sample
- Bicheno 7 day water storage experiment (two sets of 7 day water storage samples)
- paired microbial information between Isohelix and Isopore samples from the Dunalley and Clarence Point tanks, hides and abalone shells
- utility of algal (eukaryotic) primers deployed in NGS

3.5.1 Total bacterial abundance

The calculation of bacterial and archaeal abundance in the water samples was not originally an objective of the current project. However, given the real time PCR cycler at the CMAR labs, and as specific 16S rRNA primers for bacteria (27F and 1492R) were in hand, we used qPCR to quantify the amount of total bacterial abundance in the samples. In Table 17, the results from the first two months of sampling (October 2011 and November 2011) are given. As can be seen, the abundance varies considerably depending on the source water; we would expect higher levels of bacteria within grow-out tanks (due to the presence of shellfish) as compared to header tanks or pump inlets.

Table 17 Bacterial abundance as detected by 16S rRNA qPCR for the October and November 2011 samples. Samples labelled a-e represent different sites on each location

Sample	Quantity detected from bacterial qPCR	Concentration of sample (ng/μL)	Volume of filtrate (L)	Bacterial 16S copies per L water
DunalleyOctober 2011 - a	24554.01	10.8	0.5	53036.7
DunalleyOctober 2011 - b	185923.47	8.7	0.5	323507
DunalleyOctober 2011 - c	94448.25	11.5	0.5	217231
DunalleyOctober 2011 - d	23311.99	8.2	0.5	38231.7
DunalleyOctober 2011 - e	24957.32	20.7	0.5	103323
PipeClayOctober 2011 - a	12622.23	11.7	0.5	29536
PipeClayOctober 2011 - b	2654.17	42	0.5	22295
ClarenceOctober2011 - a	14055.95	5.5	0.5	15461.5
ClarenceOctober2011 - b	51672.25	7	0.5	72341.2
ClarenceOctober2011 - c	32408.4	12.2	0.5	79076.5
ClarenceOctober2011 - d	24910.82	7.9	0.5	39359.1
ClarenceOctober2011 - e	603.6	6.5	0.5	784.68
BichenoOctober2011 - a	1823.85	7.6	0.5	2772.25
BichenoOctober2011 - b	26893.39	14.3	0.5	76915.1
BichenoOctober2011 - c	331.05	8.3	0.5	549.543
BichenoOctober2011 - d	3010.8	21.4	0.75	8590.82
DunalleyNovember2011 - a	38641.89	14.88	0.5	114998
DunalleyNovember2011 - b	16348.93	5.09	0.5	16643.2
DunalleyNovember2011 - c	30429.29	7.93	0.5	48260.9
DunalleyNovember2011 - d	54502.04	15.98	0.75	116126
DunalleyNovember2011 - e	27562.28	10.9	0.5	60085.8
PipeClayNovember2011 - a	15092.97	13.72	0.5	41415.1
PipeClayNovember2011 - b	10756.11	11.09	0.5	23857.1
HobartNovember2011 - a	147353.33	21.96	0.5	647176
ClarenceNovember2011 - a	38877.41	8.53	0.5	66324.9
ClarenceNovember2011 - b	25578.76	5.41	0.5	27676.2
ClarenceNovember2011 - c	232013.4	7.07	0.5	328067

We continue to undertake qPCR analysis for the rest of the Primary A samples; given the short time frame of the project (and as it was not a priority of the sampling), the results given here show the utility of qPCR. It should be remembered however that these are not definitive and will be more informed following completion of the qPCR experiments.

3.5.2 Bicheno seven day water storage experiment

The data from these replicated experiments are not presented here as they were separate experiments in their own right. During the 14 months of sampling, the CMAR team also collected samples from the various water storage tanks at Bicheno where available as part of the monthly sampling; this data will be used for comparisons at a later stage.

Over two separate weeks in October and September 2012, farm staff at Bicheno collected filtered water samples and Sterivex units from a series of water conditioning tanks (Day 0 – Day 7) that were heated and treated with carbon filtration. Staff collected temperature, pH and salinity data for each daily sample and both nutrient and microbial community analyses have been undertaken on these samples. During the seven day water storage experiments, temperatures and pH in the tanks varied little from 22.1℃ to 23.9℃ and 7.8 – 7.9 respectively, salinity was between 34-36‰, ammonium ranged from 0.0 – 0.4$\mu g/L^{-2}$, nitrate from 2.3-20.2$\mu g/L^{-2}$, phosphate from 21.4-32.4 $\mu g/L^{-2}$ and silicate from 33-268$\mu g/L^{-2}L$ - all of which were within the variance observed at Bicheno for these parameters across the 14 months of sampling.

DNA was successfully extracted from the 16 Sterivex units with DNA ranging from 1700ng DNA/L to 6260ng DNA/L filtered, given 500mL of water had been filtered through the Sterivex units. Of the 16 DNA samples, five did not amplify successfully for ARISA and these samples had generally lower levels of extracted DNA in the first instance. The Sterivex units were also supplied to the CMAR labs still full with sea water, despite the instructions in the sampling protocol 'after filtering, remove as much of the remaining liquid as possible in the Sterivex filter unit by pushing through a syringe volume of air into the Sterivex unit. Then cap the Sterivex unit with the provided inlet and outlet cap'. This may have impacted on the quality of the extracted DNA for the ARISA screening. Two of these five samples were successfully screened with NGS. Of the 16 samples, 11 were sequenced using NGS; we have just not had time to process the results from these experiments as yet. The results and experimental findings from these samples will be presented and discussed with the Bicheno farm at a future date.

3.5.3 Paired microbial sampling, biofilm study

During the 14 months of sampling, we collected 16 sets of paired Isopore and Isohelix samples from the two abalone farms. By using the Isopore filters, we were able to filter water for nutrient analyses however the Isohelix swabs only sampled biofilms on surfaces, without subsequent water samples. The swab samples sampled the biofilm on hides (n = 3), abalone shells (n = 5) and tank floors (n = 8). The DNA extraction method for the Isohelix swabs was not microbial specific and the extracted DNA from these swabs was quite high (and samples were not pre-filtered), ranging from 1700ng DNA to over 12 000ng DNA. However it should be highlighted here that this DNA if from a different community to that found in the water column. The high DNA concentrations are also a reflection of a wide range of eukaryota DNA in addition to microbial DNA in the samples. The 0.2μM Isopore filters in comparison selectively sampled the organisms in the water column prior to DNA extraction.

Of the 16 Isohelix samples, five of these earlier samples did not successfully amplify for ARISA despite multiple trials. The ARISA profiles from the other 11 samples will be analysed alongside their matching Isopore samples for peak profile concordance at a later date. Four of the Isohelix samples were sent for NGS analysis and we have good bacteria Class, Family and species data in hand for all four which will be compared with the comparative NGS sequencing from the Isopore samples.

3.5.4 Algal screening using NGS

In two of the four rounds of NGS screening, we also trialled the algae specific primer set offered by RTL. While RTL's speciality is the evaluation of microbial diversity using tagged pyrosequencing (the 16S rRNA Assay that we deployed was specific for Bacteria and Archaea kingdoms) they also have a number of assays available including Fungi (18S, ITS1-4), Mycobacterium and Algae. In these instances, a sub-set of the DNA samples that were sent for microbial 16S rRNA TEFAP screening were also screened with the algal primers (in the third and final NGS runs, 106 DNA samples were sent for 16S pyrosequencing, 35 of these same DNA samples were pyrosequenced with the algae primers).

Using these primers, DNA from Bacteria, Eukaryota and Plantae were detected. The algal sequencing results demonstrated DNA from over 10 Algal/Eukaryota Classes including Silicofilosea, Florideophyceae (red algae), Bacillariophyceae (diatoms), Compsopogonophyceae (red algae), Pavlovales and Bangiophyceae (red algae) consisting of over 90 Algal/Eukaryota species including *Paulinella chromatophora*, *Nitzschia sp*, *Ganonema samaense* and *Chondrophycus undulatus*. DNA from over 25 Plantae species was also detected, including *Trebouxia gelatinosa*, *Rosenvingiella radicans* and *Scenedesmus sp*. As with the tagged 16S pyrosequencing, we have an extremely large amount of sequencing data from these 35 samples - these have not been analysed fully given the time frame of the current project. These data however are a priority for us and we will be analysing them in the near future.

4. Discussion

In natural marine, freshwater and estuarine environments, we can expect microbial diversity to be quite high (Jensen *et al*., 2004). Marine microbes are in fact the most numerous group of organisms on the planet (Kirchman, 2008). Nonetheless, we actually have very little knowledge of the microbial communities in the marine waters that are used for aquaculture.

To start to resolve this situation, and in order to establish a baseline of microbial communities from shellfish farms in Tasmania, we sampled five farms (and a greenfield/no aquaculture activities location) on a monthly basis for 14 months. We did not destructively sample any oysters or abalone in the current study, preferring to focus on the microbiota associated in the water column so that comparisons could be made across locations and geographical areas. We implemented three different types of microbial and two alternate water sampling methods. In total, 497 microbial samples were collected, 241 from the primary data set (this does not include the large number of archival samples that are maintained at -80℃) and over 1 500 water samples were tested for nutrients using flow injection analyses.

While we have shown here that single environmental parameters were not sufficient to establish significant causative relationships with microbial composition, higher levels of ammonium and silicate were observed to be significant drivers of the microbial communities, particularly within the abalone farms. To a lesser extent, increased temperature was also a significant driver of the communities. Highly significant differences among microbial community profiles were also observed across the geographically separated shellfish farms

In answer to the hypotheses and questions raised in Section 3, we found that:-

- *microbial community structure was significantly variable across the six sampling locations*
- *microbial presence and community structure varied on a temporal scale albeit not as significantly as at spatial scales*

- *in addition to spatial and temporal factors that affected the community structure across the locations, ammonium (and to a lesser extent nitrate), silicate and temperature were considered drivers of the community structures. Overall, microbial communities were most significantly associated with location and water quality*

- *based on over 650 000 bacterial DNA sequences, the key groups detected from the five shellfish farms belong to over 2 240 microbial taxa from across 50+ α-Proteobacteria, γ-Proteobacteria and Flavobacteria Classes*

- *there were no reportable or listed pathogenic microbes detected in the current study; however DNA from a number of bacterial pathogens identified with abalone and oyster culture in Australia and elsewhere was detected at various time points including V. harveyi, V. splendidus, V. parahaemolyticus and members of Pseudomonas sp. Nonetheless the majority of bacteria detected across the five farming locations are considered innocuous and consistent with a healthy marine environment*

Following here is a detailed discussion of these results and outcomes for on-farm husbandry.

4.1 *Abiotic* water and nutrient parameters

The use of hand held water probes was essential for us in obtaining real time information on temperature, pH, salinity and dissolved oxygen. As part of best practice husbandry activities, we recommend the use of such probes for all aquaculture activities so that changes and impacts on the culture environment brought about by new practices or culture modifications can be tracked over time. While water probes were used by all farms, some did not have the facility to measure salinity, dissolved oxygen or pH. While our results indicated that pH of marine water did not vary considerably irrespective of location or time of the year, there was some indication to suggest that monitoring of salinity and dissolved oxygen should be considered moving forward. If farms were in the market for a new probe, we would recommend purchasing a multi-probe instrument that measures at least temperature, salinity and dissolved oxygen.

During the 14 months of sampling, we did not observe any periods of grow-out or culturing that used water with less than optimal *abiotic* and nutrient parameters – while there were seasonal fluctuations in temperature that were expected, salinity and pH were relatively stable across the marine farms. High levels of dissolved oxygen were observed at all locations throughout the sampling period, thus it was never considered a limiting factor for culture.

We successfully deployed flow injection in house methods to analyse the nutrient data. These analyses were based on filtered water, with results produced in a timely fashion. As the results demonstrated, several of the nutrient parameters were variable. Higher feeding rates in the abalone tanks lead to higher ammonium levels (and consistently so) in the grow-out tanks. The greenfield location near the CMAR wharf in Hobart also had higher ammonium levels. In contrast, nitrate and phosphate was relatively consistent with silicate fluctuating according to sampling period. Ammonium and silicate were observed to be significant drivers of the microbial communities, particularly within the abalone farms. Nitrate and phosphate are also important nutrient parameters to monitor if possible, although nitrate is coupled to ammonium. However, unlike the measurement of *abiotic* factors such as temperature and salinity, outside of this project, the provision of nutrient analyses may not be readily accessible for all farms. In the section following, we present some information on the fee for service facility that CMAR can provide for microbial community analyses however at this point in time this does not include nutrient analyses.

As shown in the current study, single environmental parameters are not usually sufficient to establish relationships with microbial composition (Trabal *et al*., 2012). We observed that ammonium (and nitrate), temperature and silicate were significant drivers of the communities. We

therefore recommend that ongoing monitoring of these parameters continues in some form, preferably monthly or at least quarterly.

4.2 Molecular analyses – taxonomic identifications

Given that there is no published information in the scientific literature on what constitutes a 'healthy microbial community' for Australian abalone and oyster grow-out areas and hatcheries/nurseries (and as there was no large scale mortality event observed during the project at any location) – we consider the microbial communities detected here from a longitudinal perspective, are constituents of a healthy shellfish culture environment.

The taxonomic information provided by the NGS allowed us to review the types of microbial organisms present in the water samples. 454 sequencing of the V6-V8 region of the 16S rRNA enabled us to screen a large number of samples cost effectively for specific microbial presence. It needs to be kept in mind that the pyrosequencing of the Primary A samples resulted in massive amounts of sequence data. This microbial diversity information is readily classified into various taxonomic levels, starting with Kingdom, Phylum, Class, Order, Family, Genus and Species. At each subsequent level, more information is obtained on the microbial diversity, such that while our results highlighted that there were less differences in the microbial communities at the Class (n = 54) level, species presence across the locations was highly variable with over 2 240 species detected. That is not to say that this large number of bacterial species was detected on any particular farm, rather NGS was successful in detecting temporal diversity across the six sampling locations. We would not have been able to detect this many different bacterial taxa if we had relied on in-laboratory culturing or production of clone libraries.

Using NGS, we determined that the majority of microbes detected in our study were primarily from the alpha (α)-Proteobacteria (including Roseobacter and *Pelagibacter ubique*), gamma (γ)-Proteobacteria (*Vibrios*, *Pseudoalteromonas*, *Marinomonas*) and Flavobacteria classes (http://microbewiki.kenyon.edu/index.php/MicrobeWiki; Kirchman, 2008).
The α-Proteobacteria (within the Roseobacter lineage, which was detected at all locations in this study) occurs readily in seawater, making up to 20% of coastal microbial water communities. This group is particularly important for the transformation of sulphur compounds in the water column, participating in marine biogeochemical cycles and, importantly, processes carbon in the marine environment. *P. ubique* is one of the smallest, self-replicating free living cells and is part of the SAR11 clade. The γ-Proteobacteria (including the *Vibrios*) is also readily observed in marine waters and aquaculture areas. The Flavobacteria are a single order class of environmental bacteria which are both commensal and opportunistic pathogens. We also observed Bacteroidetes (which are a highly diverse group, thought to play a role in organic material degradations) and Cyanobacteria (which possess chlorophyll-*a* and perform oxygenic photosynthesis).

In context, our findings were similar to that observed in other international aquaculture studies which have also found a prevalence of alpha and gamma Proteobacteria in fish and shellfish farms (Sandaa *et al*., 2003; Jensen *et al*., 2004; Tanaka *et al*., 2004; Arias *et al*., 2006; Ma *et al*., 2009; Zhu *et al*., 2012). β-Proteobacteria (also observed in the current study) has been observed in commercial culture of *C. gigas* and *C. corteziensis* across different growth phases (Trabal *et al*., 2012). In comparison to our study, Ma *et al*. (2008) found high bacterial genetic diversity in abalone pond water, with microbes clustering to the alpha and gamma Proteobacteria and Flavobacteria however they found *Vibrio* sp to be the most abundant members within the γ-Proteobacteria group. Ma *et al*. (2008) found α-Proteobacteria, γ-Proteobacteria, Flavobacteria, Acidobacteria and uncultured candidate of division TM7 when they sampled settlement substrates of *H. supertexta* – but not *Vibrios*. In great scallops (*Pecten maximus*), 53% of sequences from scallop larvae and water samples were shown to have similar 16S rRNA gene sequences to γ-Proteobacteria. Unlike the current study, Sandaa *et al*. (2003) observed differences in bacterial profiles between samples taken from inlet tanks and water pipes used for culturing *P. maximus*, indicating a change in community composition as the water passed through the pipes (Sandaa *et al*., 2003). Based on denaturing gradient gel electrophoresis, Zeng *et al*. (2010) demonstrated that while there were

substantial differences in microbial community composition across various aquaculture ponds that were adjacent to each other, the dominant groups they identified were as in the current study:- α-Proteobacteria, γ-Proteobacteria, Bacteroidetes and Actinobacteria.

Recent studies of the role of bacterial species in shellfish disease and aquaculture have identified a number of significant agents. Members of the genera *Vibrio*, *Nocardia*, *Aeromonas* and *Streptococcus* (all of which are very common in the marine environment) are known contaminants of aquatic organisms (Shi *et al*., 2012). Of these, we detected DNA sequences from *Vibrio*, extremely low levels of *Nocardia* and *Streptococcus* at the marine locations and low levels of *Aeromonas* primarily in the Derwent River. While we detected DNA from several bacteria that have been shown as the causative disease agent in oyster and abalone mortalities, the relative health of the environments on all five farms was reflected in the zero occurrences of any large scale mortalities, spawning irregularities or batch failures during the period of the study.

Many of the *Vibrio* detected sequences were unclassified to species. Identification of *Vibrio* to the species level based on 16S rRNA sequences is known to be difficult due to the presence of closely related sister species (in the species complex of *V. alginolytics*, *V campbellii*, *V. harveyi*, *V. rotiferianus*, *V. natriegens* and *V. parahaemolyticus* (Gomez-Gil *et al*., 2004; Cano-Gomex *et al*., 2009; Thompson *et al*., 2009; Shi *et al*., 2012)) with these species sharing nearly identical 16S sequences. The ability to discriminate among any of the species (not just in the *Vibrio* genus) also depends on the sequence information that is present in databases to which the NGS data is matched against for taxonomic identification.

It is not unusual to detect *Vibrio* as part of aquaculture activities given *Vibrio*s are part of the natural marine environment. Sakami *et al*. (2008) suggested that intensive culture and feed input affects bacterial communities, with *Vibrio* observed as the dominant bacteria in shrimp ponds. While we also detected approximately 50 *Vibrio* taxa, they were not the dominant bacteria at any of the locations, representing about 2% of the total sequences we detected in the filtered water samples (with the exception of higher counts of *Vibrio* sp in November 2011 at Clarence Point (approximately 4.8% of total sequences)).

Of particular relevance to abalone and oyster culture, we detected a bi-modal presence (based on % of total sequences) of *V. harveyi* at Dunalley and Clarence Point (during the warmer summer months of 2011/2012, at Bicheno and Clarence (in February and July and October 2012) and at Hobart (in August 2012) however detection levels were all less than 0.15% of total sequences. This bacterial species is a known pathogen of abalone which has been implicated in summer mortality in abalone (N. Savva and M. Wing pers. comm.) on several occasions (from screening of animals submitted by Clarence Point and Dunalley). It is known as an economically significant pathogen for the aquaculture industry. Animal Health Laboratory (Tasmania) reports (from routine screening and checks of abalone) provided to us by Clarence Point and Dunalley also indicated that *Vibrio spp* (and specifically *V. harveyi*) are the cause of abalone Blister Disease observed in abalone following increased stress. *V. harveyi* has also been identified as a known pathogen in *H. tuberculata* in French cultured stocks (Schikorski *et al*., 2013) having been involved with mortality events following increased water temperatures.

We also detected DNA from *V. splendidus* (which has been implicated in summer mortality in oysters) and several other pathogenic *Vibrio* species (such as *V. parahaemolyticus* - at all locations except for Bicheno and Triabunna and *V. cholerae* was detected at Clarence albeit at very low levels). Other observed Vibrios of interest were *V. fluvalis* II, *V. alginolyticus*, *V. tubiashi* and *V. pectinicida*, detected at different abundance levels across the locations and months. Significant location specific *Vibrio* trends were observed in the data however there should be no correlation drawn here between the detected DNA from these bacteria and the onset of disease. They are highlighted here primarily as an indication that they exist in the environment and if other stresses were present (such as increased warm water temperatures, increased levels of silicate (often associated with changes in salinity), overcrowding in grow-out tanks), they could become causative agents of disease. Trends in the relative abundance of these organisms were not consistent across the farms as illustrated by Figure 37 and Table 16, indicating that the cause of

increased proliferation of these organisms is multi-factoral and may be driven by different factors at each location.

In an attempt to account for these factors we have performed preliminary analysis on the existing dataset using regression tree analysis. These analyses, illustrated in two examples (Figures 38 and 39) indicated the relationship between relative abundance of target organisms and water quality parameters at individual farms. Our analyses showed that in combination, some factors such as lower dissolved oxygen, higher temperatures and increased ammonium are related to and may be linked to an increased abundance of *Vibrio sp*. while the abundance levels of *V. splendidus* at Clarence was related to increased silicate levels at higher temperatures. This suggests that for these locations, as water temperatures increase alongside increasing salinity and or silicate, pathogenic *Vibrios* may become an issue. Whilst it is important to stress that these analyses are based on a small dataset and are only indicative of associated trends, they do illustrate the potential predictive power of a larger dataset that may, ultimately, be used to develop guidelines for the monitoring of water chemistry with a mind to minimising the risk of overgrowth of potentially problematic bacterial species.

Whilst we have made some attempts to focus on potentially deleterious bacterial species associated with shellfish culture, the list of organisms that are associated with shellfish mortality worldwide, is growing. To this end the data collected during this study will form the basis of an important baseline study. In the event of the discovery of new shellfish pathogens, we will be afforded the opportunity to retrospectively interrogate the data arising from this study to determine if these organisms are already present in Tasmanian shellfish farms and if so, at what levels they exist asymptomatically and which conditions may be associated with their proliferation.

Despite the detection of potentially deleterious bacterial species, the majority of sequences detected in the study were from innocuous microbes. Interestingly, we detected *Pseudoalteromonas* sp and *Phaeobacter gallaeciensis* which may have potential probiotic/beneficial properties against other pathogenic bacteria (Romalde and Barja, 2010).

4.3 Molecular analyses - spatial and temporal effects

We used the ARISA and NGS analyses to compare the microbial profiles and community composition patterns both spatially and temporally and the NGS analyses to taxonomically identify microbial constituents. The amplified ARISA fragments were assumed to represent microbial composition with each peak representing a specific group of microbes; although using this qualitative analysis we were not able to assign a specific species (in contrast to NGS) to a particular fragment. Rather, ARISA enabled a direct and rapid comparison of the microbial diversity and community composition prior to species specific information being obtained from sequencing. Once DNA was extracted and the ARISA PCR undertaken, ‘fingerprinting’ the microbial profiles on the CMAR Autosequencer was quick and valuable for making decisions on the fly. In comparison, the turnaround time for NGS results from the provider in the USA was approximately three weeks, using their priority service. The challenge therefore still remains to obtain NGS sequence information in an extremely short period of time so that on-farm husbandry could be modified real time if required.

Highly repeatable ARISA community profiles were obtained. Very few samples were re-run; in the small number of instances where this was required, the issue was usually a less than satisfactory run on the DNA 3130XL Autosequencer, not the actual PCR amplification of the ITS ARISA region. Additionally, while ARISA may have underestimated the diversity because related microorganisms may have ITS regions of identical length (hence fingerprinting the resulting fragment peaks does not enable species discrimination (Fisher and Triplett, 1999)), by using both ARISA and direct NGS methods to analyse the microbial communities, we were able to somewhat overcome the limits of any one method (to detect observed microbial diversity based on primer bias). To further reduce the bias in the ARISA PCRs, each sample was independently amplified twice, with the resulting PCR products combined and analysed on the DNA Autosequencer.

Next generation sequencing enabled the extensive taxonomic identification of microbial species without the need for in-laboratory culturing. As highlighted in the results section, we also detected Eukaryota and Plantae taxa when a different set of tag sequencing primers was used (in addition to the universal 16S rRNA microbial specific set). We consider NGS a relatively cost effective technique given the very large data sets of sequencing information that resulted. Nonetheless, we believe ARISA offers a cost effective technique to analyse a large number of samples simultaneously in real time. ARISA confirmed the uniqueness of the farm samples and differentiated among the samples from different shellfish locations as effectively as did NGS. As highlighted by Arias *et al*. (2006), we recommend using ARISA to screen ongoing/future microbial DNA samples from the shellfish farms (thereby monitoring changes in the microbial communities over time). We suggest using NGS for more quantitative assessment of taxonomic information. As the ARISA community profiles and NGS species abundance data showed strong concordance, and as Brown *et al*. (2005) demonstrated that ARISA resolution is 'near' to the species level, we believe that ARISA profiling is a suitable proxy for microbial diversity assessment, particularly if absolute taxonomic information is not required.

The composition of microbial communities associated with shellfish is known to be affected by many factors including characteristics of the host, diet and environmental conditions (Trabal *et al*., 2012). We observed clear differences between the microbial communities at each spatial location irrespective of the type of species specific shellfish culture being undertaken. Our research demonstrated that the microbial diversity (be that reflected in ARISA community profiles or in specific bacterial species counts) varied significantly among the spatially located farms with the communities detected in the water from marine farms closer in spatial distance (e.g. Bicheno and Triabunna, Dunalley and Pipe Clay Lagoon) more similar to each other. We also observed significant differences in farm profiles at different times of the year (and across farms). As multiple sites per farm were sampled, our analyses indicated that the presence of individuals (e.g. abalone) in grow-out tanks has an effect on the microbial communities in those tanks (i.e., on-farm water) as compared to inlet water sources (i.e. off-farm/source water). The microbial diversity and structure was different among the farms and different to the more freshwater environment of the Derwent River.

There were high similarities (homogeneity) in the community composition between water samples taken each month at each location – although the communities were somewhat dynamic with differences in monthly samples per location indicating fluctuating communities across different time periods. For example, the five sampling locations at Dunalley in June 2012 were more similar to each other than samples from the Pipe Clay Lagoon sampled during the same period.

4.4 Outcomes for husbandry

As microbial community structure across the farms was dependent on the spatial location of the farms (as in Sakami *et al*. (2008)), these location specific profiles may be used as biological indices for evaluating healthy water constituents in the water columns and for 'profiling' or allocating water samples to each marine shellfish location. Indeed, the canonical analyses of principal co-ordinates (CAP) demonstrated that based on the NGS bacterial abundance data, up to 100% of the samples at Dunalley, Bicheno and Clarence were successfully identified to their farm group (ARISA enabled 82% of samples to be classified to location successfully).

Ammonium, silicate and temperature were identified as the major drivers of the communities on the farms such that water from the abalone farms was significantly different to that at the oyster farms due to the increased level of ammonium in the tanks associated with culturing abalone (and the microbial communities within the tanks reflected this). Our analyses indicated that the effects of different water chemistry at the various locations were variable. Examination of the relative abundance of *Vibrio* sp, *Pseudomonas sp*. and specifically *V. splendidus* using multiple linear regressions was undertaken to attempt to identify the factors that may be related to the proliferation or relative abundance of these species of interest. This analysis indicated that in

combination, some factors such as lower dissolved oxygen, higher temperatures and increased ammonium may lead to an increased abundance of *Vibrio* species. Whether these changes in abundance are related to seasonal or site specific factors were difficult to test given the relatively short study period. Moreover, general lack of an overall relationship between individual species and water chemistry as well as the different predictor variables that were identified for each location, suggests that the proliferation or abundance of these important aquaculture microbial groups is driven by different factors at each of the sites. This is not surprising given the range of conditions and different water-masses that apply to each of the different locations.

A more in-depth longitudinal study (over multiple years) would help to resolve this. For example, we only sampled across the locations in December (at the start of the warmer summer temperatures) twice – in December 2011 and December 2012, only in one 'winter' period (June-August 2012) and once in a high 'summer' period (January 2012 and February 2012) as the field sampling for the project finished in December 2012. This time frame was not long enough to establish whether a cyclical pattern (observed on a yearly basis) actually existed in the communities, however the baseline information that we collected is a robust foundation on which to build ongoing monitoring and surveillance.

Fortunately, there was no large scale deleterious event that affected multiple abalone or oyster stocks, spat or grow-out tanks across all five farms during the 2011/2012 sampling period. Given the hydrochemistry parameters, nutrient analyses and taxonomic information collected during the sampling period, we now have a multivariate model which can be used to analyse the links between microbial community profiles and causative factors for non-deleterious events. By archiving sample DNA, we also have the ability to screen the samples retrospectively if specific diseases do become an issue for any of the five farms. As Adams and Thompson (2011) highlight, we believe ongoing monitoring of the environmental conditions that abalone and oysters are being grown in via the sampling protocols that we have established, enables action to be readily taken if and when pathogens are first detected and before pathogens become a major issue.

Our sampling questionnaire to the four primary farms (Clarence Point, Bicheno, Dunalley and Pipe Clay Lagoon) demonstrated to us that the shellfish farms also believe ongoing water sampling (particularly for the hydrochemistry parameters e.g. ammonium, temperature and salinity) is necessary for farm success (particularly given that baseline information on water parameters and constituent microbial communities had not previously been collected). We would therefore recommend monitoring on a monthly basis, unless conditions on farm changed rapidly or deteriorated with increased monitoring during spawning, larval rearing and water conditioning due to the dynamic nature of these activities (and that often larval or spat stages/rearing are higher risk activities). The farms agreed that monitoring at this frequency would suit their on-farm husbandry activities. Additionally, the oyster farms were particularly interested in chlorophyll and other pigment analyses, presumably for algal identifications and or monitoring. While it was beyond the scope of this study to take samples specifically for pigment analyses, using NGS (and commercial in confidence primers for algae and plants, ttp://www.researchandtesting.com/microbial-diversity-services.php) applied to the extracted DNA from the filtered water samples, we detected taxa belonging to over 10 algal classes and over 100 algal/eukaryota species (including *Paulinella chromatophora*, *Nitzschia* sp*, Ganonema samaense* and *Neoizziella divaricata)* aside from the bacteria and archaea. Given the utility of NGS for the detection of microbes and algae, this should be a route that is considered for future uptake, in addition to the more traditional methods of pigment analyses, if algal investigations are needed on-farm.

The five Tasmanian shellfish farms have resources on farm that enable water sampling and sample storage at either 4℃ or -20℃. By deploying our water sampling protocol using Sterivex filter units (see Appendix 3), all farms successfully undertook their own sampling of the microbial communities and filtered water for nutrient analyses. We developed water sampling protocols which would easily suit day to day husbandry activities and farms do not need special equipment aside from their own water meters/probes, fridge or freezers and a water bucket or carboy alongside the sampling kit. We sent each farm the sampling protocol and accompanying consumables (including the Sterivex filter unit and 60mL syringe) and can do so for any

aquaculture farms/interested farmers who would like to undertake their own sampling. If filtered water for nutrient analyses is taken, then farms need to freeze the water at -20℃ until nutrient analyses can be undertaken either at CMAR or elsewhere, while a room temperature preservation buffer can be supplied in the sampling kit so that the Sterivex unit does not need to be frozen. We have not looked into whether the preserved Sterivex units can then be domestically posted back to the CMAR labs but we see no foreseeable reason why this could not be arranged, given that the units are supplied with tight fitting screw inlet and outlet caps.

5. Benefits and Adoption

This project was co-funded by the Seafood CRC under its R&D Innovation Funds initiative. We believe we have successfully developed and implemented innovative strategies for scientific discovery for the cultured Tasmanian oyster and abalone industries. We have demonstrated great potential (if up taken further) for significant step-wise changes in the way these industries could undertake their water monitoring and surveillance.

We highlight the following benefits for our shellfish farm collaborators here. Importantly, we consider that many of these benefits would be applicable to other aquaculture industries culturing organisms in either sea or fresh water. Our protocols and results demonstrate the utility and application to the wider aqueous environment.

1. increased information and knowledge of the environment in which Tasmanian shellfish farms operate
    - there was no baseline information on microbial communities or water chemistry parameters prior to this research

2. the deployment of innovative and state-of-art analyses presented here for assessment of microbial diversity and nutrient parameters constitutes invaluable tools for study and monitoring of the aquaculture environment
    - expensive, time consuming and ineffective in-laboratory microbial culturing is not required
    - tag encoded pyrosequencing, ARISA and qPCR accurately evaluated bacterial (and preliminary algal) communities in samples from the Tasmanian shellfish farms. These approaches enable the evaluation of microbial diversity in almost any environment without extensive microbial culturing
    - flow injection nutrient analyses provide timely and informative results for parameters that are highly important for successful farm culturing but which are not often considered

3. farm staff were trained on the job and upskilled in water sampling protocols
    - staff now have a better appreciation and understanding of the importance of ongoing water monitoring and screening
    - farm staff can independently (without needing CSIRO staff) undertake their own on-farm sampling

4. CSIRO staff were trained on the job and upskilled in bioinformatics and molecular analyses
    - team members benefited by gaining a wider understanding and appreciation of the Tasmanian shellfish industry and aquaculture in general

5. the Tasmanian shellfish industry now has the tools for ongoing monitoring and a strengthened ability to detect disease causing bacteria before they proliferate to disease levels, including monitoring of healthy systems so that change can be determined
    - Tasmanian shellfish farms can focus on preventative aspects of culture via sample screening rather than applying treatments after an occurrence

6. water sampling protocols and metagenomic analyses outlined here are applicable for any type of aquaculture activity thereby providing benefits to the wider sector. The optimised protocols and techniques for microbial assessments can be deployed, irrespective of the aquaculture organism under culture
    - as other aquaculture industries recognise the benefits of obtaining microbial community baselines for their sites (and or if green water sites are being considered for new activities), our research could easily be uptaken by other industries such as Atlantic salmon or ocean trout aquaculture. We can send out our Sterivex sampling kits with the microbial analyses subsequently undertaken at the CMAR laboratories
    - the metagenomic and hydrochemistry approaches outlined in this study are applicable to all types of aquaculture farms, although environmental and microbial baselines will vary between farms, depending on the geographic location and the type of farming undertaken

7. CSIRO has recently developed a new 'fee for service' arrangement for the analysis of molecular microbial diversity and taxonomic information for samples (based on a minimum of ten samples per test). If utilised, this service assumes the farms/hatcheries would obtain their own on-farm water parameters such as temperature, DO and salinity, with CSIRO sending out the water sampling kits (including detailed instructions for use). The fee for service ($250/sample, minimum of 10 samples) results in an extensive, well informed data set and includes:-
    - Sterivex sample kit ($12.50)
    - $30/DNA extraction (using a combination of PowerWater and Spin columns)
    - $15/ARISA fingerprint
    - $110/NGS
    - $80 for sample processing and analyses
    - if required, nutrient analyses could be negotiated separately

Adoption of these techniques and strategies for building on the collected baseline information (including provision of the water sampling kits) now depends on the uptake of ongoing monitoring by the farms. This would need to be undertaken under a fee for service arrangement with CMAR (as outlined above) unless a new externally funded project is established. As discussed previously, a more in-depth longitudinal study (over multiple years) on one, several or all of the Tasmanian farms would provide the local shellfish aquaculture industry with these surveillance and monitoring capabilities however, outside of an externally funded project, the cost of this monitoring needs to be met by the farms. The optimised molecular techniques and protocols developed in this project are currently being deployed by the CMAR team in other project areas and the nutrient analysis techniques (developed in house at CMAR) continue to be utilised by in house capability in a number of research and monitoring projects.

6. Further Development

The extensive data sets collected in this study and our collaborative links with the Tasmanian oyster and abalone farms have provided us with several areas that either require further investigation, future development or platforms from which we can develop new ideas. These include:-

- completion of several experiments – qPCR for all Primary A samples, comparative analysis of the biofilm NGS data, analysis of the Bicheno water storage trials, in-depth analyses of the algal NGS data set (focussing on utility of the eukaryotic primer sets)
- scientific publications from the current study – we envisage two/three publications submitted to the international literature
- ongoing surveillance and monitoring if uptaken by industry; microbial DNA database interrogations if required
- a more in-depth longitudinal study (over multiple years) and multiple locations

- use of ARISA and NGS analyses for product or farm provenance. We are also investigating the utility of microbial communities from fish to provide a fisheries independent tool for location of origin and or provenance testing for fisheries
- if the shellfish industry requires additional quantitative screening and if financially supported, we believe specific qPCR or microarray analyses for important microbial pathogens (as in Shi *et al*., 2012) could be developed. CMAR has the capability and capacity to undertake this research alongside industry partners

In June 2012, we submitted a pre-proposal to the FRDC Aquatic Animal Health Subprogram (SA024, Aquatic Animal Health Subprogram: Standardised monitoring of aquatic pathogens in Australian mollusc aquaculture – Appleyard and Abell). Despite positive support from the Tasmanian Fisheries Research Assessment Board and the Tasmanian shellfish farmers, the pre-proposal through the AAHS was not given a high priority and subsequently not successful. However, during the current project, a public health alert was issued for wild shellfish from the east coast of Tasmania (http://www.dhhs.tas.gov.au/peh/alerts/current_health_alerts/tasmanians_warned_on_eating_wild_shellfish_from_huon_estuary) following the detection of unsafe levels of a paralytic shellfish toxin caused by the algae *Alexandrium tamarense*. In April 2013, a public health alert on oyster contamination (resulting in Novovirus gastrointestinal outbreak) http://www.dhhs.tas.gov.au/peh/alerts/current_health_alerts/barilla_bay_oyster_gastro_outbreak) for oysters harvested near Dunalley was also issued. Neither health alerts were microbial/bacterial in nature and were not related to our four primary shellfish farms (AbTas, Cold Gold and Shellfish Culture) however at Spring Bay Seafoods in Triabunna, the processing of mussels and scallops was affected by the first health alert (our sampling at Triabunna was unaffected as the sampling was from header tanks in their oyster hatchery). We believe that the current research has provided the industry with significant baseline information on microbial communities in the shellfish farms and that the molecular protocols we developed are highly applicable to any new screening studies. Our future metagenomic research will focus on microbial and algal pathogen detection (and assessment of the feasibility of viral detection in the water column).

7. Planned Outcomes

Our research addressed the following Seafood CRC public benefit output:-

1.3 - Removal or reduction of key production constraints in selected aquaculture systems

We achieved this by undertaking strategic metagenomic technologies for abalone and oyster culture and used this molecular information to inform baseline (and if uptaken by industry, long term) strategies for these industries. The outcomes of this research includes:-

- standardising sampling and the analysis pipelines for the generation and comparative analysis of data (*abiotic*, nutrient and microbial community composition) from abalone and oyster aquaculture sites
    - the water sampling was shown to be suitable for incorporation into routine water quality assessment protocols
    - three different DNA extraction techniques, molecular (ARISA, NGS, qPCR) screening protocols and nutrient analysis (for ammonium, nitrate, phosphate and silicate) protocols were optimised
    - detailed sample spreadsheets and enduring databases of farm specific information (including *abiotic*, nutrient and molecular information) informed from 14 months of monitoring
    - demonstrating the utility of NGS for algae identification

- developing an open access Database of DNA sequence data describing the microbial community structure and associated chemical data

    - this is still in a draft form, however it will be available for use following the project's CMAR completion date of June 2013
    - historical datasets can now be screened for the presence of 'newly described' microbial pathogens if they arise

- establishing cost effective microbial monitoring with archival capacity
    - during the current sampling, archival samples were taken and these are maintained at -80℃

- improving husbandry efficiencies through identification of important water chemistry factors and tools that enable the prediction of microbial presence
    - protocols for on-farm monitoring of important water parameters, including salinity and dissolved oxygen were developed in consultation with farm staff
    - an easy to use field sampling kit was produced
    - baseline inventories of microbial species associated with abalone and oyster hatcheries/grow-out areas in Tasmania were recorded
    - automated ARISA monitoring enables rapid and detailed screening/evaluation of farm and environmental samples
    - ARISA profiles and NGS species counts were shown to be farm specific
    - univariate and multivariate models for microbial diversity and water covariates were established

- detecting the presence of microbial species of interest, including those considered pathogens in abalone and oyster culture
    - over 650 000 microbial DNA sequences detected, over 2 240 bacteria taxa identified
    - DNA from pathogenic bacteria including *Vibrios* and *Pseudomonas* detected

8. Conclusion

To our knowledge, this is the first Australian study using state-of-the-art molecular techniques (including NGS) to identify the diversity (and community structure) of microbial species found in the water columns at both oyster and abalone farms. The wealth of data generated from multiple molecular approaches was coupled with the first longitudinal evidence of *abiotic* and nutrient information from across multiple locations in Tasmania. Regular screening and testing of eight water quality parameters such as salinity, temperature, dissolved oxygen, ammonium and silicate afforded the project with information that was not previously recorded on any of the farms. Our research served to demonstrate the type of analyses that can be undertaken on NGS datasets such as that acquired during this and future studies, rather than an exhaustive catalogue of trends in all microbial species across all farms.

During the project, 235 samples were tested for community fingerprints using ARISA; 152 samples were screened for bacterial identification using NGS; 35 of these same samples were screened for Eukaryota and Plantae identification using NGS; and a small subset (n = 27) were tested for bacterial abundance using qPCR. We detected DNA from over 2 240 bacterial species and over 100 algal/Eukaryota taxa. This is highly important as the presence of a diverse microbial community in the grow-out tanks, water tanks and source water that is used on farm is important for maintaining healthy water quality though ecosystem activities such as nitrification and ammonification. While we observed differences in both diversity and microbial composition among the five shellfish farms, we did not observe any mass mortalities or sub-optimal conditions for spawning, setting or grow-out. Given the practices that each of the farms undertake as part of their on-farm husbandry activities (e.g., regular clearing of faeces/food from tanks, use of flow through systems, storing water at increased temperatures and carbon filtration), this ability of the farms to maintain a balance between innocuous, beneficial and pathogenic bacteria (i.e., the overall marine microbial community) in their aquatic hatcheries and grow-out sites is highly important.

We present here substantial evidence to show that metagenomic analyses were effective in resolving taxonomic identity, community profile changes and assessment of microbial abundance from water samples without the need for cultivation of microbes within the laboratory. We believe these molecular techniques and water quality protocols are innovative, proactive measures towards improving production efficiencies for shellfish aquaculture. Importantly for the wider aquaculture industry, approaches deployed in this study can be used to screen any sourced water – be that from aquaculture farms, grow-out areas, aquarium tanks or blue water sites. These approaches are microbial specific, not aquaculture type specific, hence we could just as easily deploy them to test filtered water samples from salmon and tuna pens, prawn ponds or scallop hatcheries.

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10. Appendix 1 - IP

There are no patents arising from the research.

11. Appendix 2 Staff

Staff engaged on the project:

Principal Investigator
Dr Sharon Appleyard CSIRO Marine and Atmospheric Research

Co-Investigator
Dr Guy Abell CSIRO Marine and Atmospheric Research

Research staff
Ms Ros Watson CSIRO Marine and Atmospheric Research

12. Appendix 3 - other

Farm Questionnaire

As part of our ongoing discussions and keeping in mind that we are aiming to develop a pipeline for analyses that are relevant and suitable for farm use, we would appreciate you taking a couple of minutes to consider the following questions. Your answers to these questions will help us to tailor the sampling in the future.

1. Do you believe that ongoing water sampling is necessary for your farm's success

   Yes / No

2. Do you believe that ongoing sampling and collecting baseline water parameter information will benefit your farm

   Yes / No

3. How often do you think water sampling should be undertaken on your farm

   Never

   Once a month

   Once a season (i.e. summer, autumn, winter, spring)

   Only when husbandry and or culture issues arise

   Regularly during spawning and hatchery activities

4. With respect to ongoing water sampling, please rank the following in order of importance to your farm's culture activities; sampling

   _____microbes (bacteria and archaea)

   _____algae

   _____viruses

   _____hydrochemistry parameters (ammonium, nitrate, silicate, phosphate)

   _____water temperature

   _____water pH

   _____salinity, conductivity

   _____dissolved oxygen

   _____chlorophyll –a

   _____pigment analyses

   _____other – please explain _____

5. If you or your farm staff were undertaking the ongoing water sampling, how much time per month could be set aside for this task

   _____1 hour per month

   _____2 hours per month

_____4 hours per month

_____8 hours per month

_____>8 hours per month

6. Please indicate which of the following is already on farm and could be used for water sampling

_____Room temperature storage (i.e. bench top, cupboard)

_____Fridge

_____-20℃ freezer

_____Ultra low freezer (-80℃)

_____Liquid nitrogen dewar and regular access to liquid Nitrogen

_____Mains power

_____Handheld pump (with either vacuum or pressure facilities)

_____Portable peristaltic pump

_____Portable vacuum pump or vacuum line

_____Workbench, lab area

_____Temperature & pH meter

_____Multi-probe meter (temp, pH, salinity, conductivity)

_____Dissolved oxygen meter

_____Measuring cylinders, 2L water carboys

_____Plastic ware - pipettes, tips

_____Plastic ware - nutrient analyses tubes (volume?), 2ml screw cap tubes, 50ml Falcon tubes, 60ml syringes

7. If samples needed to be stored short term on farm (e.g. up to 3 months), would your farm have the appropriate storage space

Yes / No

8. If samples needed to be sent to a laboratory for analyses, do you have access to reliable and fast courier service or postage

Yes / No

9. How quickly would you prefer results to be made available to your farm

_____Within a week of samples arriving in the testing laboratory

_____Within a month of samples arriving in the testing laboratory

_____Within 3 months of samples arriving in the testing laboratory

_____Once every 6 months

_____Once a year

10. If your farm was part of a wider collaborative project looking at the spatial and temporal variability of sampling, would you be open to

    _____Data from your farm being uniquely identifiable

    _____Data from your farm being treated confidentially but still part of the wider project (e.g. Farm A, Farm B, Farm C)

    _____Data from your farm not be used in any collective down stream analyses apart from those directly applicable to your farm

11. What type of data report would be the most informative and useful for your farm

    _____Highly detailed with analysis methodology implicitly explained

    _____Brief and concise with a focus on results and implications for husbandry rather than method based

12. If in the future, water sampling was integrated into your farm's regular husbandry activities, would you be prepared to pay for diagnostic analyses

    Yes / No

Water sampling for microbial communities and nutrients using Sterivex™ filter units

Sharon Appleyard
Update for Farms
29 Jun 2012

Introduction

Based on our recent questionnaire and with an aim of establishing autonomous water sampling methods for the shellfish microbial project, presented here is a method for sampling using Sterivex™ filter units.

Sampling occurs monthly on each of the four Tasmanian shellfish farms - for early July, farm staff will undertake the sampling (including taking water measurements such as temperature and pH) and then storing filters and water filtrate at -20°C. CSIRO will pick up the frozen samples during the next field visits (scheduled for the end of July 2012).

Sampling points (be they inlet pumps/tanks, grow-out tanks, water storage tanks) are indicated below. Where possible, water quality measurements should be made *in situ* and at each of the sampling sites. Sampling at each point consists of filtered water samples, water measurements and Sterivex filters for microbial samples. Filtered water samples are stored in capped 10ml nutrient analysis tubes; hydrochemistry analyses for ammonium, nitrate, silicate and phosphate on these water samples are then undertaken at CSIRO. DNA is extracted from the Sterivex filters and various molecular analyses are undertaken at CSIRO. Table 1 outlines the sampling that has been undertaken to date. If possible, the same sampling sites (or part thereof) should be sampled for July 2012. To ensure sample integrity and consistency of environmental conditions, please sample within one week of receiving the kits.

Table 18 Sampling points across the four shellfish farms

Farm	Sampling Sites
AbTas	A4, A7, C12, D2, inlet pipe
Cold Gold	A16, C7, D36, E37, inlet pump
Shellfish C – Bicheno	wharf, header tank, 5day, 1day, 0 day, other water storage tanks
Shellfish C - Pipeclay	header tank, beach

Water sampling methods

Sampling kits and equipment provided

- 1 × 1L PP bottle with screw cap lid – this bottle is used for water sampling. Buckets or other wide opening containers can also be used
- Syringe – the same syringe should be used for all sampling points per farm, water can be flushed through the syringe in between sampling points
- Ziplock plastic bags
- 4 × 10ml white screw cap nutrient analyses tubes (tubes for ammonium sampling have a yellow dot, tubes for nitrate, silicate and phosphate have a red dot – all are labelled) + 1 × labelled Sterivex™ filter unit (1 per sampling site) per sampling site. Each filter unit also comes with an inlet and outlet cap which should be attached to the appropriate end after filtration
- Polystyrene tube rack
- waterproof pen, paper and field sampling sheets (pre-labelled). There is a sampling sheet for each set of Sterivex filter unit & nutrient analysis tubes
- sampling instructions

- electronic versions of the sampling instructions and farm specific excel sheets for data entry
- please note – there is no spare sampling kits provided due to cost

At each of the sampling points, the protocol below should be followed. At each sampling point, water measurements, filtered water in nutrient analysis tubes and a Sterivex filter is required. The syringe can be re-used.

Step 1 Water measurements

- identify the sampling point, and using a water meter, take temperature, pH, dissolved oxygen etc (depending on water meter) measurements and record the data on sheets provided

Step 2 Sampling the water

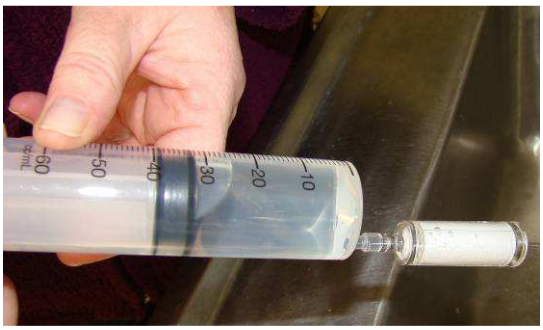
- keeping hands out of the water and flow as much as possible, use a bucket, carboy or the 1L provided Nalgene bottle. Rinse through at least 100ml of water into the sampling container – briefly swirl this water through the container and discard
- then sample 1L of water from the sampling point. In header and water storage tanks, the water is taken from the surface of the tank. For grow-out tanks at Cold Gold, the water is sampled at the surface near the outflow area and at AbTas at the surface behind the circulating arm. Water from the beach and the jetty at PipeClay Lagoon and Bicheno respectively is also taken from the surface. For the inlet water at Cold Gold and AbTas, the 1L Nalgene bottles are used to collect water from the pipe and pump with water measurements taken from the water collected in the Nalgene bottle




Step 3 Filtering for microbial communities and nutrients
Using the provided Sterivex filter unit, filter 500ml of sampled water. When filtering do not touch or contaminate the ends of the Sterivex filter units. 500 ml of water should be filtered through each Sterivex unit using the syringe

- fill the syringe to the 50ml mark

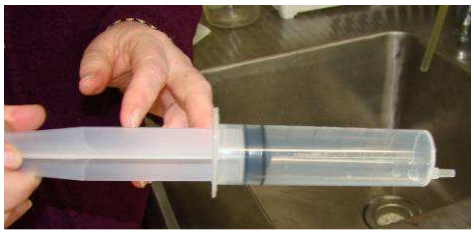
- attach the syringe firmly to the Luer lock end of the filter unit and depress the plunger



- using the water that is being filtered out of the other end of the filter unit, rinse each of the four nutrient analysis tubes by recapping the tubes, swirling the water and inverting the tubes, then empty the tubes (this cleans the tubes and prepares them for the water sample)

- now proceed to re-fill the syringe and push through the rest of the 500ml of water. To do this, carefully remove the filter each time from the syringe and draw up 50ml into the syringe. Then reattach the syringe to the filter and push through the water sample (this will need to be done ten times)
- Use the filtered water that comes out of the other end to carefully fill each of the four nutrient analysis tubes to the marked line. The ten passes of the filled syringe usually takes about 8 minutes to push through the Sterivex unit. If more than 500ml of water is filtered, please mark this on the sheet provided as the estimate of microbial abundance is based on a standard 500ml volume

- the white filter in the Sterivex unit may start to go a slight brownish colour – this is OK, it is just the particulates from the water being captured on the filter
- after filtering, remove as much of the remaining liquid as possible in the Sterivex filter unit by pushing through a syringe volume of air into the Sterivex unit. Then cap the Sterivex unit with the provided inlet and outlet caps – both caps twist on, the larger cap goes on the smallest end of the filter unit



- the capped Sterivex unit should be put back into its small plastic bag. It should then be stored with its' associated water samples together in the larger provided plastic bag at -20°C until CSIRO picks up the samples and takes them back to the labs. If possible, please ensure no freeze/re-thawing of the samples

- all data from the field sheets should be entered into the provided spreadsheets, with paper copies retained for archival records

If there are any questions or problems with the Sterivex sampling, please contact Sharon on 0400876388 (or sharon.appleyard@csiro.au) or Guy on 0424993605 (or guy.abell@csiro.au).

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FOR FURTHER INFORMATION

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Lysosyme:lysis buffer recipe for DNA extraction

Lysis buffer
200mM $NaH_2PO_42H_2O$ (monobasic)
200mM Na_2HPO_4 (dibasic) MW 142 142g/1L =1M 5.68g/200mL = 200mM

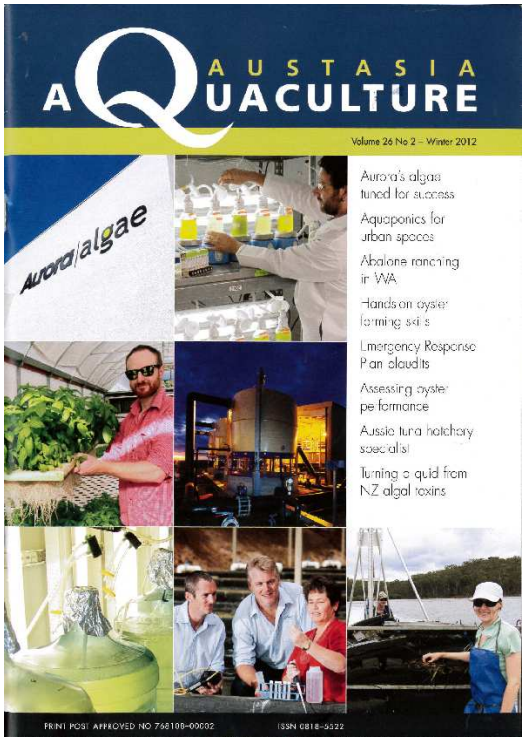
To make up 200mL lysis buffer
39mL 200mM NaH_2PO_4
61mL 200mM Na_2HPO_4
17.54g NaCl
2g CTAB
4g PVP K30
+ ddH20 to make up to 200ml
Adjust to pH 7.0 (using NaOH)

Publications and presentations

- media coverage for the project (Fishing Today April/May 2012 & Austasia Aquaculture Winter 2012)

Fishing Today Volume 25(2), pg 32 and front cover

Austasia Aquaculture 26(2), 30-32 and front cover



- oral presentation at the July 2012 Australian Marine Science Association conference (https://www.amsa.asn.au/conference/amsa-nzmss2012_hobart/index.php)