

Overseas Travel Report

Workshop on Physical and Biochemical Methods for Analysis for Fish as Food and Subsidiary Activities



AUSTRALIAN
SEAFOOD
COOPERATIVE
RESEARCH CENTRE

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2009/755 Seafood CRC Research Travel Grant: Tom Madigan “PhD workshop on physical and biochemical methods for analysis for fish as food and subsidiary activities”

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OBJECTIVES OF RESEARCH TRAVEL GRANT

1. Attend the PhD workshop on biochemical analysis techniques at the Technical University of Denmark.
2. Evaluate which techniques are applicable to oysters.
3. Familiarisation with the Cells Alive freezing method.
4. Familiarisation with Campden BRI.
5. Establish what techniques these institutes have developed for analysis of quality attributes of oysters.

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Contents

NON TECHNICAL SUMMARY	3
Outcomes Achieved To Date	3
Outputs Developed as Result of Travel Grant	3
BACKGROUND AND NEED	4
RESULTS	4
Workshop on physical and biochemical methods for analysis for fish as food.....	4
Subsidiary Visits	5
OUTCOMES	8
RECOMMENDATIONS.....	9
ACKNOWLEDGEMENTS.....	9
APPENDIX 1: ITINERARY	10
APPENDIX 2: DETAILED INFORMATION ON TRAINING EXERCISES	11

Non Technical Summary

The author attended a PhD training course which provided an introduction to a wide suite of biochemical methods that can be used to assess post-mortem changes in fish. The information gathered will be useful across a range of projects in the Australian Seafood CRC (AS CRC). A number of subsidiary visits were also undertaken to build international collaboration. The author visited the Grimsby Institute and was given an introduction to the Cells Alive System (CAS) which is a novel freezing technique believed to be superior to standard fast freezing systems. The author also visited Campden BRI and discussed their high pressure processing work and with the Centre for Environment, Fisheries & Aquaculture Science (CEFAS) who are playing an important role in the development of European methods for the detection of marine *vibrios*. CEFAS has kindly offered an extensive bank of *Vibrio* strains for use in future AS CRC work. The author has identified and discussed the potential for collaborative links with each of the institutes that were part of the subsidiary visits.

Outcomes Achieved To Date

- The basis for developing biochemical markers for oyster quality will be a direct benefit to the successful completion of the author's PhD. This capacity will also be useful across a wide range of industry projects.
- A professional network has been built with specialists and future specialists from around the world in the area of biochemical analysis of fish muscle post harvest.
- Links with international institutions have been established which can be used to trial the CAS technology for oysters.
- Up-to-date methods have been obtained for the analysis of marine vibrios that will be of direct benefit to the upcoming AS CRC *Vibrio* work in prawns.
- Collaborative research opportunities have been identified with Campden BRI and CEFAS.
- Awareness of the Australian Seafood CRC in international research organisations has been increased.

Outputs Developed as Result of Travel Grant

- A PhD training course on biochemical analysis of fish muscle has been successfully completed.
- The PhD experimental design has been influenced by the findings of this training course.
- The offer of a bank of marine *Vibrio* strains will be an important resource in underpinning future AS CRC projects and assisting industry in future market access negotiations.
- There is potential for revision of the PhD focus to include the Cells Alive technology.

Background and Need

The aim of the PhD course is to provide insight into state of the art laboratory techniques that are recommended for analysis of fish muscle quality associated attributes. This course has been developed specifically as a training course for PhD students by the recognised leaders in seafood quality analysis. Attendance at this workshop has assisted in developing capability to undertake the physical and biochemical techniques that will be undertaken in Seafood CRC projects. This capacity will be extremely beneficial for analysis of quality attributes of seafood for the Australian seafood industry. In particular, it will assist seafood processors to assess the shelf life of potential new product lines.

Results

The training workshop and all meetings were successfully attended. An itinerary for the travel is provided in Appendix 1.

Workshop on physical and biochemical methods for analysis for fish as food

This PhD training workshop was convened by the Technical Institute of Denmark. The Institute has long been regarded as being at the forefront of biochemical research in quantifying changes in fish flesh, post-mortem. The workshop was attended by students from Australia, Denmark, Faroe Islands, Iceland, Malaysia, Norway and Spain. It provided a basic introduction into some of the techniques that the Institute has found most useful for the analysis of post-mortem changes in fish. Students were divided into groups and conducted the following techniques:

- Analysis of water pools using **Nuclear Magnetic Resonance**
- Determination of bound and unfrozen water in fish fillets combined with an estimation of the freezing point using **Differential Scanning Calorimetry**
- Estimation of formaldehyde using the **Nash Test**
- Estimation of activity of the trimethylamine-*N*-oxide aldolase (TMAOase) by **Enzymatic Assay**
- Determination of volatile amines (TMA, DMA), TMAO and cations by **Capillary Electrophoresis**
- Estimation of lipid oxidation using **Dynamic Head Space Gas Chromatography-Mass Spectrometry**
- Estimation of protein oxidation using **Western Blotting**
- **Proteome** analysis of fish muscle.

Further details on these techniques are provided in Appendix 2. The author can be contacted for complete methods not included in this report.

Relevance to Oysters

Several of these techniques may be useful in identifying quality attributes of oysters. In particular, analysis for the presence of trimethylamine will assist in determining whether the spoilage is due to bacteria. Furthermore, the detection of TMAOase in either Pacific or Sydney Rock Oysters would be a noteworthy (publishable) finding and the presence of this lysase may play an important role in oxidation of product during frozen storage. Differential Scanning Calorimetry and Western Blotting may be useful techniques for assessing the denaturation or oxidation of proteins, to demonstrate maintained freshness of product. The use of GCMS to assess lipid oxidation may be an important technique for assessing the effectiveness of different freezing techniques.

To date, the convenors work relating to oysters has only centred on food safety issues.

Subsidiary Visits

Visit to the Grimsby Institute, United Kingdom (23/11/09)

A site visit to inspect the Cells Alive System (CAS) was arranged where the author met with Mike Dillon (Head of School), Martin Croft (Refrigeration and Technical Site Manager) and William Davies (Fish Technologist). This research institute is ideally located at the hub of seafood processing in the United Kingdom. It is estimated that 80% of all seafood processed in the UK is processed in Grimsby. The Grimsby Institute delivers world class food manufacturing training across the UK and Ireland and also delivers a MSc in Productivity and Innovation Development. Within the Humber Institute (Grimsby Institute's specialist seafood unit), they possess capacity (staff and equipment) that is at the forefront of freezing technology.

The CAS was developed in Japan by ABI Co Ltd. It utilises magnetic field generating equipment that generates a weak magnetic field in standard fast freezing equipment. This technology apparently limits the formation of ice crystal within products and maintains a product that is very close to the original fresh state of the food. Images provided by the manufacturer indicate that the system maintains the cellular structure to a higher degree than a standard freezing unit and also minimises drip loss. These units can be used either for initial freezing or for long-term storage and the degree of the magnetic field can be adjusted from 0 to 100%. The Grimsby CAS units are pictured in Figures 1 and 2.



Figure 1: The Cells Alive Systems at the Grimsby Institute.



Figure 2: Interior of a fast freezing Cells Alive unit. Red arrows indicate the electro magnets that encircle the freezing trays.

Initial trials of the CAS at the Grimsby Institute have been qualitative, with no extensive validation having been undertaken. The Institute provided the author with oysters that had been CAS frozen at a variety of settings and then CAS stored. These oysters appeared to retain whiter colour and reduced drip loss in comparison to the oysters stored via a standard freezing mode. However, there was only one oyster from each treatment and the prior physical condition of original oysters was variable. The Grimsby Institute has indicated a desire to undertake joint work with the Australian Seafood CRC to validate the use of the system for oysters. Undertaking work with this Institute would allow the CRC to be co-investigators on a novel project that would be of significant scientific interest world-wide, as there is little (possibly no) published data that validates the effectiveness of this technology.

Whilst the Grimsby Institute has some of the latest technologies in freezing, MAP equipment and extensive product development kitchens, they have little laboratory equipment available and there is no dedicated sensory laboratory. However, there is significant laboratory space available and the Institute is hopeful of developing capacity in this area. Currently, a local scientific company is used for microbial and biochemical analysis. Consequently, any planned future work planned by the CRC should incorporate the availability of equipment into their experimental design. It is recommended that the AS CRC should evaluate the effectiveness of this technology to provide robust data prior to investment by the Australian seafood industry.

Visit to Campden BRI (24/11/09)

Campden BRI is an institute that provides a research platform for the United Kingdom food industry. Individual businesses can become a member to the company and gain access to scientific literature, reports, a brief consultancy for troubleshooting, updates on technology and changes to legislation, discounts on their wide variety of training courses and also access to the research platform. They undertake a wide variety of research projects at their Chipping Campden location. They provide a “one-stop-shop” for industry where in the one location they have the capacity for food processing, product development, sensory, microbiological and biochemical analysis, imaging equipment, training facilities and bread and grain research.

A site visit to Campden BRI was undertaken to discuss their high pressure work as part of a Seafish funded project. During this visit, the author met with Craig Leadley (Food Managing Technologist).

Discussions with Craig Leadley regarding their recent high pressure processing project were beneficial as it confirmed the need to identify appropriate methods for quantifying post-mortem changes in oysters (the author’s current area of work). Craig indicated that the sensory method used in their study was subjective and to date, they have not undertaken any other work relating to quality analysis of oysters.

Craig stated that Campden BRI would be very interested in collaborative work with the AS CRC. He feels that useful collaborations begin at the scientist to scientist level and if this proves beneficial, it provides an excellent platform for a formalised agreement. He suggested that there may be an opportunity to work together to assess a new technology by Sequid, which is based on dielectric spectroscopy. Reportedly, this equipment can measure values that are correlated with storage time and with the Quality Index Method (QIM) based freshness values. In the author’s opinion there is significant scope for the AS CRC to collaborate with Campden BRI in a variety of future projects and this should be further explored by the AS CRC. If the AS CRC intends to investigate the effectiveness of the CAS, a collaborative project between AS CRC, Grimsby and Campden BRI would be ideal, as Campden BRI can provide the analytical capability for the project.

Visit to CEFAS Weymouth Laboratory (26-27/11/09)

Unlike Australia, the United Kingdom has had only one confirmed case of illness associated with marine vibrios. However, like Australia they are aware of the increasing international interest in these pathogenic species. Consequently, CEFAS implements an extensive research program in this area. A site visit to CEFAS was undertaken to discuss their current work in marine vibrios. The author met with Rachel Rangdale and Craig Baker-Austin to discuss and observe their *Vibrio* methodologies.

Part of their current work is in the development of improved extraction methods that allows previously qualitative PCR methods to become quantitative via the use of a Proteinase K digestion step and use of DNA preparation kit. This is a particularly useful process as it can then be used with any combination of real-time methods. Furthermore, it will allow a quantitative result within 3-5 hours. Whereas the US FDA method currently being trialled in Australia takes 24-30 hours and only provides a semi-quantitative result. It is likely that this method will become the accredited European standard as it provides a fast result and also provides information on the potential for pathogenesis from the species identified.

As part of the laboratory visit, both conventional and real-time PCR analysis were undertaken to gain an understanding of the equipment used within their laboratories. Real-time PCR

results are presented in Figure 3. These results were targeting a newly discovered gene of *Vibrio vulnificus* that allows differentiation between clinical and environmental strains. Figure 3 depicts positive amplification in the clinical strain, indicating that this isolate likely has the potential for pathogenesis in humans. The author was previously unaware of this method and was very impressed with its usefulness. Particularly, as there have been several cases of fatalities in Australia associated with this organism.

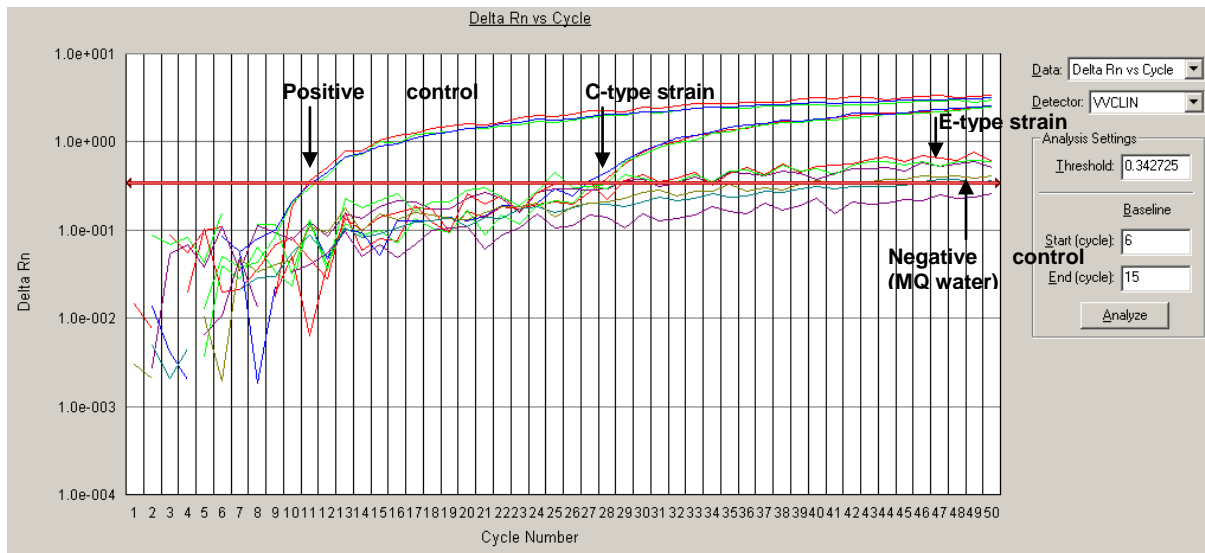


Figure 3: Results of realtime PCR analysis assessing two strains of *Vibrio vulnificus*.

CEFAS has offered to send an extensive bank of *Vibrio* strains (30-50) to the SARDI laboratories in Australia in exchange for some Australian strains. This bank of strains will be invaluable in the development of Australia specific methods and will assist in the development of a reference capability for the detection of these bacteria in Australia. CEFAS has also kindly provided electronic copies of their methodologies (including PCR primer and probe sequences), which will be extremely useful in determining the correct methodologies for use in Australia. This visit builds upon the real time PCR capability developed as part of a current AS CRC project (2007/719) and importantly lays a foundation for a potential AS CRC project in prawns, which is currently under consideration by the Research Advisory Committee to utilise the latest technologies and position Australia amongst the world leaders in the analysis of marine *Vibrio* spp. Increased international interest may also result in detentions of other marine species such as finfish, crustacea and molluscs (e.g. abalone). Consequently, the outcomes from this section of the travel are pertinent to all industry sectors of the AS CRC.

Outcomes

- The basis for developing biochemical markers for oyster quality will be a direct benefit to the successful completion of the author's PhD. This capacity will also be useful across a wide range of industry projects.

- Information has been gathered on the latest biochemical techniques for the analysis of quality changes post mortem. These techniques have relevance for all sectors of the AS CRC.
- A professional network has been built with specialists and future specialists from around the world in the area of biochemical analysis of fish muscle post harvest.
- Links with international institutions have been established which can be used to trial the CAS technology for oysters.
- Up-to-date methods have been obtained for the analysis of marine vibrios which will be of direct benefit to the upcoming AS CRC *Vibrio* work in prawns.
- Collaborative research opportunities have been identified with Campden BRI and CEFAS.
- Awareness of the Australian Seafood CRC in international research organisations has been increased.

Recommendations

- AS CRC to consider funding a collaborative trial with the Grimsby Institute to validate the benefits of the CAS. The author will investigate whether funding can be obtained via a collaborative research grant through the European Union.
- AS CRC should consider undertaking collaborative work with Campden BRI to investigate the usefulness of the Sequid system in Australia. AS CRC could consider taking out an associate institute membership to Campden BRI. This would allow access to information updates, publications, events, newsletters, food law alerts (for the EU) and a new technologies bulletin.
- Incorporate the CEFAS developed methods into the upcoming AS CRC *Vibrio* work in prawns. Use this work to develop further collaborations with CEFAS. In early 2010, SARDI and CEFAS will undergo an exchange of bacterial strains.
- A summary report of the travel should be submitted for inclusion into Seafood Stories.

Acknowledgements

The author would like to gratefully thank the staff from the Grimsby Institute, Campden BRI and CEFAS for their kind hospitality and willingness to take time from their busy schedules to convey what types of work they undertake at their respective institutes. Lastly, the author would like to thank the Australian Seafood CRC, Marine Innovation South Australia and the South Australian Research and Development Institute for funding this study tour.

Appendix 1: Itinerary

Thursday, 12 November 2009	Leave Adelaide
Friday, 13 November 2009	Arrive Lyngby, register for workshop and collect course reading materials
Monday, 16 November 2009	Workshop
Tuesday, 17 November 2009	Workshop
Wednesday, 18 November 2009	Workshop
Thursday, 19 November 2009	Workshop
Friday, 20 November 2009	Workshop
Monday, 23 November 2009	Grimsby
Tuesday, 24 November 2009	Campden BRI Grimsby
Wednesday, 25 November 2009	Travel to CEFAS (funded by SARDI)
Thursday, 26 November 2009	CEFAS (funded by SARDI)
Friday, 27 November 2009	CEFAS (funded by SARDI)
Saturday, 28 November 2009	Return to Adelaide

Appendix 2: Detailed information on training exercises

Low Resolution Nuclear Magnetic Resonance and Differential Scanning Calorimetry

Nuclear magnetic resonance (NMR) was used to study the water distribution in frozen cod and angler subjected to a variety of frozen storage parameters (correctly stored and abused). Fish muscle tissue is comprised of approximately 80% water. Consequently, the distribution of water within muscle tissues (e.g. bound to proteins or unbound) can play an important role in the deterioration of product. Therefore, analysis of these “water pools” can be a useful tool. Low Resolution NMR measures the spin of nuclei in all hydrogen containing solutes and solvents following an electromagnetic pulse. Specifically, the method measures the rate of relaxation after the pulse as a function of time. Further multivariate (3-way) analysis is required to manipulate data to assess differences between samples.

Differential scanning calorimetry (DSC) can be used to calculate the freezing temperature of a sample and determine the amount of unfrozen water in a sample. This technique measures the change in enthalpy of a product as it is frozen. At the freezing point, there is a clear change in enthalpy. In addition to the aforementioned points, DSC can also be used to qualitatively examine the denaturation of proteins. However, these changes occur on a markedly lower scale of enthalpy than the changes noted for freezing and also require further analysis of the data to detect differences between samples. The ability to examine denaturation of proteins provides an effective way to differentiate between fresh and deteriorating products.

Capillary Electrophoresis, Nash Test and the TMAOase Enzymatic Assay

Trimethylamine Oxide (TMAO) is present in all marine animals and also in some freshwater species. TMAO can be reduced to trimethylamine by spoilage bacteria, which leads to characteristic “off odours” associated with spoiled seafood. TMAOase is present in abundance in the internal organs of gadoid (cod) species of fish and to a lesser extent in their muscle tissues and there have been unconfirmed reports of this lysase in some shellfish. TMAOase is responsible for the formation of formaldehyde in gadoid fish muscle during frozen storage, which can result in deterioration of fish muscle texture. The activity of this lysase is most active down to -10°C ; however below this temperature the activity is dramatically slowed (not stopped).

Capillary electrophoresis (CE), the Nash Test (NT) and the TMAOase Assay (TA) were used to assess differences in cod and angler (negative control) to explore the relationship of TMAOase in white fish muscle and the formation of formaldehyde in relation to storage temperature.

CE is a useful technique that is essentially a combination of chromatography and electrophoresis. A liquid is passed through a fine capillary by a positive charge and osmotic flow. It can be used to detect both anionic and cationic solutes in a single analysis. Solute can be identified and quantified against a set of standards.

The NT which, was developed in 1953 uses an approximately neutral solution of acetylacetone and ammonium salts together with absorbance at 416nm in comparison to a set of standards to quantify the amount of formaldehyde present in a sample.

The TA is a two step enzymatic assay that incorporates an indicator sample at the end point and the changes during the 20 minute reaction can be used for quantification. The reaction is halted using a pH shift and the samples can then be kept for later analysis.

These techniques are highly suitable for use in AS CRC projects that are examining shelf life of Australian seafoods.

Dynamic Head Space Gas Chromatography-Mass Spectrometry and Western blotting

Increased awareness of the benefits of consuming fatty fish is driving demand for these products. However, during frozen storage lipid oxidation can lead to off tastes, odours and textural changes. There is an increasing consideration that oxidation of proteins may also lead to the formation of volatiles. In this series of experiments, Dynamic Head Space Gas Chromatography-Mass Spectrometry (GCMS) was used to assess the formation of volatile substances against several samples that had been subjected to a range of treatments. The differences between samples can clearly be seen in Figure A2.1, with little formation of volatiles in the VP and A treated samples. Despite the usefulness of the Dynamic Head Space technique to capture volatile compounds, static headspace capture is recommended as it is ideally suited to a large sample throughput.

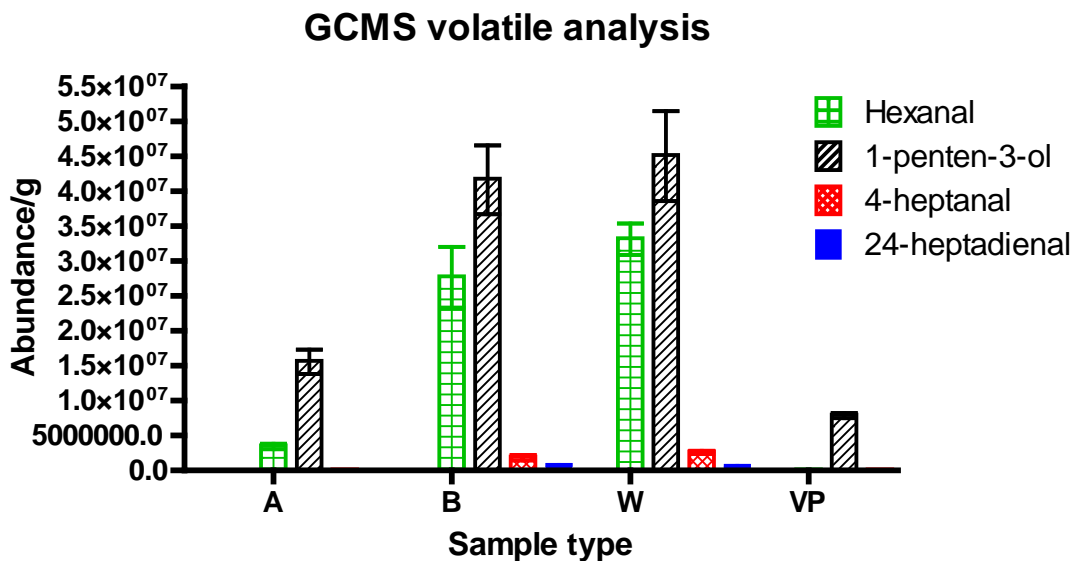


Figure A2.1: Results (abundance per gram of volatile compounds) from the Dynamic Head Space Gas Chromatography-Mass Spectrometry analysis of the four experimental treatments.

Western Blotting (WB) was also used to detect for the presence of oxidised proteins in the sample. In this technique the concentration of protein is determined using a commercially available kit. The concentration is then used to add the same amount of protein to the wells off an SDS PAGE gel in duplicate. This separates the proteins according to the isoelectric point and size if the proteins. Oxidised proteins react with carbonyl groups of the protein. The proteins contained within the gel are then transferred to a membrane and oxidised proteins are detected via the use of an antibody and a chemiluminescent reaction. If oxidised proteins are

detected, they can be cut from the duplicate gel (which is stained with coomassie) and identified by mass spectrometry and sequence analysis. It is worth noting that several groups of students achieved poor results using this technique, which highlights the complexity of the method.

These techniques are highly suitable for use in AS CRC projects that are examining the suitability of Australian seafood products for frozen storage.

Proteome analysis

The proteome is reflective of the phenotype and represents the gene expression of an organism. Proteomics is an extremely useful technique that identifies changes in the proteome that can be used to develop information on a variety of environmental factors. It can be used to identify environmental factors such as stress, chemical contamination, season and feed (including the identification of changes due to replacement of marine based lipids with land based). Proteins are separated according to their isoelectric point and their molecular size. Following a staining procedure, gels are calibrated using specially designed software to align proteins between gels. Gels are then compared using the software to identify changes in the regulation of proteins. As with the WB, the proteins can be identified by mass spectrometry and sequence analysis. In practising this technique, two groups of fish were assessed: stressed and unstressed. Much of this technique was only demonstrated due to the complexity. However, from this experiment several proteins were identified that were clearly different between groups of fish, which indicates that these proteins may play a role in the response of fish to a stress event.

This technique would be highly useful in a range of AS CRC projects, particularly in projects examining the effects of stress during harvesting and the replacement of marine based lipids and proteins in feeds with land based ingredients.