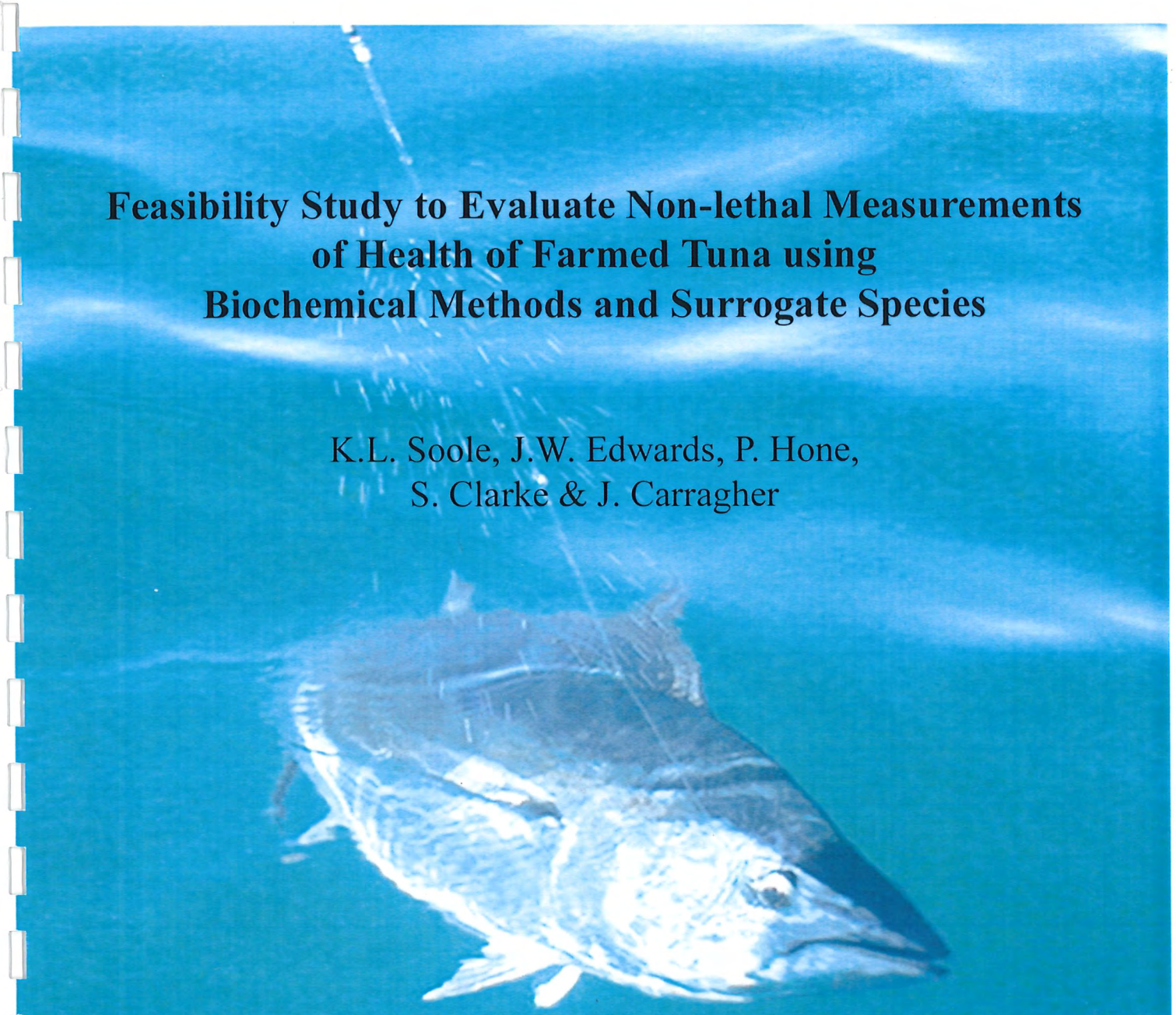
Feasibility Study to Evaluate Non-lethal Measurements of Health of Farmed Tuna using Biochemical Methods and Surrogate Species

K.L. Soole, J.W. Edwards, P. Hone,
S. Clarke & J. Carragher



FLINDERS UNIVERSITY
ADELAIDE, AUSTRALIA

FISHERIES RESEARCH & DEVELOPMENT CORPORATION

Project Nos. 95/082 and 97/307

Feasibility Study to Evaluate Non-lethal Measurements of Health of Farmed Tuna using Biochemical Methods and Surrogate Species

Project Nos. 95/082 and 97/307

K.L. Soole, J.W. Edwards, P. Hone, S. Clarke & J. Carragher

Published by Flinders University, Adelaide Australia

ISBN 0-7258-0779-2

2002

CONTENTS

Cover illustration: Southern bluefin tuna (*Thunnus maccoyii*). Courtesy Trent D'Antignana, Flinders University

95/082 & 97/307	**Feasibility study to evaluate non-lethal measurements of health of farmed tuna using biochemical methods and surrogate species**

PRINCIPAL INVESTIGATOR: Dr. K.L. Soole
ADDRESS: School of Biological Sciences, Flinders University
GPO Box 2100
Adelaide SA 5001
Telephone: 08 8201 2030 Fax: 08 8201 3015

OBJECTIVES:

1. To compare biochemical methods of assessing the effects of pollutant stress in farmed southern bluefin tuna (*Thunnus maccoyii*) with methods currently used. Methods include measurement of adenylates and ATPase in fish tissue samples.
2. To evaluate methods of sampling fish or fish tissues which will reduce handling stress and improve the predictive capacity of samples used to estimate farmed tuna health and stress levels.
3. To evaluate the use of the biochemical responses of other species (e.g. Australian herring - *Arripis georgianus*, blue mussel - *Mytilus edulis*, jack mackerel - *Trachurus declevis*) which cohabit with tuna in farm pens as surrogate indicators of stress experienced by tuna.

NON-TECHNICAL SUMMARY:

OUTCOMES ACHIEVED:

This project has successfully evaluated a variety of biochemical indicators of fish health to be applied to aquaculture of southern bluefin tuna. In addition, the project successfully demonstrated the potential for using common species as surrogate indicators of tuna health, reducing the need for expensive quality assurance assessments in the harvestable resource.

The importance of these approaches to monitoring aquaculture species health is demonstrated in other FRDC research projects, including those on oyster, tuna flesh quality, and prawn bycatch stress measurement. Following the wider dissemination of this report, it is anticipated that industry and fishery managers will be able to use these data to further investigate health and product quality in the aquaculture industry.

Aquaculture of various species is common worldwide. Wild caught southern bluefin tuna are kept in cages at Boston Bay (Port Lincoln, South Australia) until they reach marketable size or market conditions are favourable for sale. Potential water contamination in Boston Bay may affect health and value of tuna. Various indicators have been used to evaluate nutritional conditions, growth rates and health status in fish. These studies generally fail to predict that fish health is compromised at a stage when intervention may prevent further deterioration in health or quality. Biochemical markers of the changes caused by environmental stress are useful as a way of predicting ill health or poor quality in farmed fish. In addition, the use of surrogate species in which the biochemical indicators predict or parallel the responses in tuna reduces the need to use tuna for this testing.

Biochemical methods used in this study include measurement of tissue energy balance (liver and gill adenylate energy charge [AEC], inosine monophosphate load [IMP load] and the ratio of IMP to ATP in tissues), and the measurement of sodium/potassium ATPase (Na^+/K^+-ATPase, an enzyme important in controlling water and salt balance). These were measured in tuna, Australian herring and blue mussels in aquaculture nets at Port Lincoln in 1996 and 1997. Validation of the field studies was performed using aquarium experiments, where Australian herring and blue mussels were exposed to ammonia to simulate conditions of poor flushing of wastes from aquaculture nets.

Gill ATPase in mussel and fish varied widely between monthly samples although this variation was less marked in tuna. There was a general association between ATPase in cohabiting fish and tuna, and also between mussel and tuna. ATPase increased over the month following stocking of tuna nets at the end of 1996 and this increase persisted until a decline between August and November 1997. The initial low level may have resulted from collection and transport stresses from which tuna initially recovered. The later decline may represent an accumulated burden of stress over the 1997 season, or normal physiological and biochemical variation during this time.

AEC in tissues were generally low and displayed considerable variation, especially in gill. Tuna gill AEC dropped to levels associated in the literature with poor health. In tuna liver, values were less variable, but were at levels associated with sub-optimal conditions. This suggests that gill may be more responsive to environmental stress than liver. Correlations between species, although based on few data sampling points, indicate some association between values, suggesting this marker in surrogate species may be useful as a predictor in tuna.

IMPL in tuna were highly correlated with IMPL in cohabiting fish species. This correlation was better in liver samples than in gill. Similarly, ATP/IMP ratios in liver were relatively stable throughout the sampling period and correlations between ATP/IMP in tuna liver and cohabiting fish were stronger than those of gill, suggesting that these two indicators in surrogate species may hold greater potential as predictors of tuna health and quality. There was no correlation between tuna and mussel IMPL or ATP/IMP.

In aquarium studies, mussels with ammonia treatment had enhanced ATPase activity at low ammonia concentrations superimposed by a reduction in activity at higher concentrations. A similar pattern was seen in mussel energy balance. Generally, ammonia did not change AEC in mussel up to 7 days exposure, but there was a slight, significant increase in AEC at 14 days. However, mussel AEC was low in control animals (<0.5) suggesting that these changes may have been an artefact in mussels already stressed by collection and handling. IMPL in mussels fell significantly in all animals, including controls, within 2 hours of commencing ammonia exposure, and levels remained depressed for the 14 day observation period. In Australian herring, ATPase was not consistently nor significantly altered by ammonia treatment. Gill AEC declined after 7 days in all treatment groups (including controls) and declined further at 14 days, ultimately reaching approximately 0.3 – 0.4 compared with commencement values of about 0.6. Liver AEC varied without any apparent pattern. Gill IMPL declined in all groups at 7 days and did not recover by day 14, whereas liver IMPL did not change until day 14 when a statistically insignificant reduction was seen in all groups.

The project found that the biochemical indicators were highly sensitive to sampling and transport procedures resulting in degradation. Sampling strategies have been identified that minimise these effects, ensuring that data collected are representative of events occurring in the field.

The results from this project are not unequivocal, although they suggest that biochemical markers may be useful as indicators of farmed tuna condition. In particular, ATPase, AEC and IMPL in cohabiting fish may closely follow those in tuna, and may provide a basis for a predictive marker of tuna condition and health status. While some of the field data demonstrated poor correlations between markers in mussel and in tuna, others (e.g. mussel AEC vs. tuna liver AEC) were more positive. Pilot experiments using mussels seeded onto tuna farm nets suggested that ATPase and possibly other biomarkers may differ from these measurements in control mussels seeded onto nearby structures. This suggests that biochemical events in mussel may be useful indicators of environmental quality. Further studies based on this feasibility study are strongly recommended.

KEYWORDS: Bluefin tuna, Australian herring, blue mussel, jack mackerel, biomarkers, adenylates, adenylate energy charge, Na^+/K^+-ATPase, IMP load.

Acknowledgements

The research group is extremely grateful to the owners, operators and staff of the tuna farms at Port Lincoln for their kind support in this project. Thanks also to the Tuna Boat Owner's Association for the use of their facilities at Port Lincoln. Aquarium studies at the facilities of SARDI West Beach were supported by SARDI technical staff. SARDI staff also assisted with field operations at port Lincoln. Aquarium studies at the Flinders University were supported by technical staff of the School of Biological Sciences.

Background

Various indicators have been used to evaluate nutritional conditions, growth rates, and health status in a variety of fish and shellfish species. These indicators have then been used to determine improvements and decrements in fish quality or health. However, the majority of studies examining negative impacts on health have concentrated on crude markers that indicate that detrimental effects are already in evidence. They generally fail to predict that fish health is likely to be compromised at an early stage when intervention may prevent further deterioration in health or quality. Biochemical markers of the strain caused to fish by environmental stress are attractive in that these may occur prior to more serious physiological consequences. Intervention at this point may pre-empt the development of frank adverse effects.

Biochemical methods, which have previously been investigated in fish and other marine species, include the measurement of adenylates in various tissues, the estimation of adenylate energy charge (AEC) in these tissues, and the measurement of sodium/potassium ATPase (Na^+/K^+-ATPase). These represent indicators of tissue energy balance and a key enzyme responsible for whole body osmoregulation. The role of inosine monophosphate (IMP, a metabolic precursor of the adenylates) has also been investigated with IMP load (IMPL) and the ratio of ATP to IMP being used to evaluate further the validity of these energy-dependent indicators as biomarkers of stress-response.

Adenylates

These represent the pool of metabolic energy stored in tissues as adenosine nucleotides (adenosine mono-, di-, and tri-phosphates; AMP, ADP and ATP). Depletion of tissue levels of these components or impairment of adenosine nucleotide synthesis will result in a reduction of available energy and will compromise physiological functions. The role of inosine monophosphate (IMP), a metabolic precursor of the adenylates, has also been investigated, with IMP load and the ratio of ATP/IMP being used to evaluate further the validity of these energy-dependent end-points as biomarkers of stress responses in fish.

Adenylate energy charge (AEC)

AEC is indicative of the metabolic energy available to organisms from the adenosine nucleotide pool. This energy is primarily available as ATP, although there is some energy available from ADP. Hence, the AEC is estimated as ([ATP] + 0.5 x [ADP])/([ATP] + [ADP] + [AMP]), and represents a weighted estimate of energy status in tissues. The values for AEC can range from close to 1 in unstressed animals, to 0.4 - 0.5 in animals whose energy status is compromised. Previous work in molluscs (1) and fish (2) have suggested the potential of this measure as a nonspecific indicator of the response to a variety of environmental stresses.

Na^+/K^+-ATPase

This enzyme is the prime mediator of ion transport across epithelial membranes in gills, and is a key enzyme for whole body osmoregulation in aquatic animals (3). Specific activity of this enzyme in gill tissue, the main organ of osmoregulation, has been selected for this project because of its potential as a reliable indicator of metabolic competence in marine organisms. Contaminated environmental conditions have been shown to produce an inhibitory effect on the activity of this enzyme (4,5). The sampling technique is non-lethal, requiring the collection of only a small amount of tissue, permitting the release of animals after sampling. This allows comparative measurements to be made on individual fish on a number of occasions.

IMP load

IMP load (IMPL) is determined by the ratio of the concentration of IMP to that of the adenylates combined (i.e. IMPL = [IMP]/([ATP] + [ADP] + [AMP]) (6) and represents a breakdown product of AMP which allows the maintenance of a high AEC under stress conditions.

ATP/IMP ratio

This is the ratio of the highest energy store to its precursor and represents an overall energy balance marker that varies under the influence of a variety of stressors including handling stress and storage (7).

Each of these assays, or a combination of them, is potentially useful as a method to evaluate the effects of stress in farmed fish. The stresses that are likely to be important in the farming of southern bluefin tuna (*Thunnus maccoyii*) include overcrowding due to high stocking densities, poor flushing of cages and the accumulation of excess nutrients and fish waste products, and environmental contamination such as sewage effluents and urban or rural run-off.

In some cases, such as where the target species is rare or valuable, or there are other reasons preventing sufficient numbers to be collected for analysis, the health of alternative species may be investigated as surrogate indicators of the health status of ecosystems or specific ecosystem components. The authors have previously carried out a study of biochemical and cytogenetic effects measured in whiting and mullet in South Australian gulf waters, and found that these indices were associated with environmental stresses such as industrial pollution, urban and agricultural run-off, and sewage treatment effluents (2). Hence the examination of these biochemical indicators in species cohabiting with tuna in farming cages (such as Australian herring, *Arripis georgianus*, jack mackerel, *Trachuris declevis* or blue mussel, *Mytilus edulis*) may provide an indirect method of assessing tuna health.

Need

Reduced quota levels and the need to value add the tuna caught has seen the development of methods for farming tuna in Boston Bay at Port Lincoln. Fish caught from the wild are transferred to holding pens in Boston Bay where they are maintained until they reach a size suitable for sale or until market conditions are favourable for sale. The water quality in Boston Bay is affected by a variety of potential contamination sources, including treated sewage emissions from Port Lincoln, urban and agricultural run-off, and in the areas of the holding pens by nutrients released from the

fish feeds and fish excreta. These factors may affect the health of growing tuna, causing them to be more susceptible to infection, and reduce the quality of their flesh hence reducing their market potential.

In 1993, when the market value of Port Lincoln farmed tuna was over 30 million dollars, 2.5% of the farmed animals were lost due to disease mortality. In 1994/5 up to 10% of animals in some nets died. Assuming an average loss of 5% this represent a potential loss of over 5 million dollars (of an estimated market value of 50 million dollars per annum) over this period. In addition, there is a perception amongst consumers that farmed fish are of poorer quality than wild caught tuna. This difference in quality may be due to stress-related factors associated with their confinement.

Present methods of assessing fish health result in high mortality of fish which are caught and returned to the pens, or require sampled fish to be killed, further reducing the yield of the farming process. Non-lethal and less stressful methods of assessing tuna health and environmental quality need to be developed to allow fish to be harvested before any deterioration occurs. This may be achieved by either improving capture and restraint procedures and sampling techniques to reduce mortality in the sampled individuals, or by use of alternative surrogate species in which the selected indicators predict or parallel the responses in the target species. This project is an experimental approach to the development of indicators that may in future be used in the evaluation of alternative farm management protocols.

The approach described in this project also complements other research protocols, such as investigations of disease or nutrition in farmed species, and may provide a method of assessing stress in fish associated with disease or nutritional status.

Objectives

1. To compare biochemical methods of assessing the effects of pollutant stress in farmed southern bluefin tuna (*Thunnus maccoyii*) with methods currently used. Methods include measurement of adenylates and ATPase in fish tissue samples.
2. To evaluate methods of sampling fish or fish tissues which will reduce handling stress and improve the predictive capacity of samples used to estimate farmed tuna health and stress levels.
3. To evaluate the use of the biochemical responses of other species (e.g. Australian herring - *Arripis georgianus*, blue mussel - *Mytilus edulis*, jack mackerel - *Trachurus declevis*) cohabiting with tuna in farm pens as surrogate indicators of stress experienced by tuna.

Methods

Phase 1 - field studies

During 1996, tissue adenylates, AEC and Na/K-ATPase levels were measured in gill and livers of cohabiting fish species (Australian herring, mackerel) and gill of blue mussels from tuna nets. Groups of 10 - 20 fish and mussels were intended to be collected from tuna nets at intervals of 4 - 6 weeks, coinciding with tuna harvesting or sampling operations. At times, operational factors superseded collection of research samples. Gill and liver samples were also obtained from harvested or sampled tuna. During the 1997 season, samples were collected from tuna, mussels, and on a few occasions from cohabiting fish species.

Collection methods

Cohabiting fish were caught by hand line, rapidly brought into the boat and killed immediately by a blow to the head. Gill and liver samples were excised immediately and frozen using liquid nitrogen-cooled tongs. Tissues were placed into aluminium foil packets, labelled and stored in liquid nitrogen. Mussels were sampled from nets by hand, were opened *in situ*, and mantle frozen, labelled and stored as for fish samples. Tuna were rapidly brought aboard the boat, were pithed with a spike in the head, and gill and liver immediately excised, frozen, labelled and stored as before. All samples were transported by road under liquid nitrogen to Flinders University for biochemical analysis. Samples were stored at -80°C until analysis.

Phase 2 - aquarium studies

During 1996, Australian herring and blue mussel were collected from control sites remote from aquaculture sites at Boston Bay. They were transported to the West Beach laboratories of SARDI where they were transferred to indoor holding tanks. Fish and shellfish were kept in 300 -600 L tanks with a constant supply of sand-filtered seawater (5 L/min). Animals were fed daily with 4 mm formulated pellets (Australian herring) or mixed algal culture (mussel). Groups of 5 fish were transferred to 40 L tanks and groups of 10 mussels were transferred to 20 L tanks and exposed to various environmental conditions (elevated ammonia, reduced oxygen) for 1, 7, or 14 days. After animals were fed their daily allowance, half of the water in the experimental tanks was replaced with freshly treated seawater. After their exposure to experimental conditions, herring were netted and mussels obtained by hand. Tissue samples were collected as in phase 1 and were transported to Flinders University for analysis.

In 1997, aquarium studies of ammonia exposure in Australian herring were repeated. Exposures were made at 0, 1 and 5 mg/l with exposure periods of 2 hour, 7 days and 14 days.

Analytical methods

Tissue adenylates

Tissue samples were pulverised, homogenised and centrifuged with all tissue handling steps carried out as expediently as possible and with all tissues maintained in liquid nitrogen to prevent decay of adenosine nucleotides. Adenylates were extracted, buffered and measured by HPLC using the method of Stocchi et al (1987) (8)

AEC

This was estimated from the tissue concentrations of adenosine nucleotides measured by HPLC as described above. AEC was determined according to the relationship

$$AEC = ([ATP] + 0.5\,[ADP]) / ([ATP] + [ADP] + [AMP])$$

described by Haya and Waiwood (1983) (5).

ATPase

ATPase in extracted tissues was measured by the colorimetric assay of Tucker and Matte (1980) (9), in the presence and absence of ouabain, a specific inhibitor of Na^+/K^+-ATPase. The results were expressed relative to protein concentrations measured in tissue extracts using the method of Bradford (1976) (8).

IMP load and ATP/IMP

IMPL and ATP/IMP were determined in tissues following quantitation of adenylates and IMP by HPLC as described above. IMPL was determined according to the relationship

$$IMPL = [IMP] / ([ATP] + [ADP] + [AMP])$$

described by Caldwell and Hinshaw (1994) (6). ATP/IMP was the molar ratio of these materials in tissues (10).

Statistical analysis

Data from sample groups were analysed using GraphPad Prism 2.01 for Windows 3.1 (GraphPad Software Inc, San Diego CA). Data were tested for normality and were analysed using analysis of variance followed by Tukey's post hoc test to determine differences between groups. Correlation analysis used a simple linear model (Pearson r), where r^2 represents the proportion of variation in the Y variable explained by changes in the independent variable (X axis). For example, an r^2 of 0.81 in a correlation of gill AEC in herring with gill AEC in tuna indicates that 81% of the variation in the herring data is explained by variations in the tuna data.

Results and discussion

General

Project 95/082 was to have commenced in July 1995 and original milestone dates were based on this. Funding from FRDC was delayed until November 1995 and changes were made to the milestone dates as a result. With this delay, tuna nets were empty at the start of this project and Phase 1 was not commenced. This time instead was spent on assay development and validation, logistic details of sampling, freight, and analysis were worked out, and additional planning and coordination included visits by the research group to Port Lincoln to meet with TBOA staff and examine facilities. The projected date for conclusion of sampling and analysis was shifted to 30 March 1997. Further funding was granted by FRDC (97/307) to continue the project for a further season, and to finalise aquarium studies being performed as part of ongoing BSc honours projects at the School of Biological Sciences, Flinders University.

Major achievements

- The assay for ATP, ADP and AMP was to have been by a colorimetric technique, requiring a separate assay for each of the adenylates measured. We have developed a high performance liquid chromatography assay for simultaneous automated measurement of these adenylates. This assay also permits the simultaneous measurement of inosine monophosphate (IMP), which is an important precurser for ATP, ADP and AMP synthesis and maintenance.

- Establishment of an assay for ATPase and its stability, and reliability. While this assay has proven reliable we hope in future to modify it so we can use the same HPLC technique as that used for measuring ATP, ADP and AMP. This will streamline analyses in further studies.
- Analysis of tissues from tuna, cohabiting fish species and mussels from the 1996 and 1997 tuna farming seasons. These have been correlated and seasonal changes have been noted in the biomarkers examined.
- Changes in IMPL, AEC and ATPase in tuna were associated in the 1997 season with capture and transport stress. Further changes in these markers in tuna, mussel and cohabiting fish species may have been related to environmental stresses or to seasonal physiological changes.
- Useful correlations were observed between IMPL, AEC and ATPase in tuna and cohabiting fish species. The approach to monitoring farmed tuna stress responses using data collected from surrogate species, cohabiting in tuna nets, appears promising and may provide the basis for predictive markers of stress response in farmed tuna.
- Biochemical markers in mussel may be useful as indicators of environmental conditions in nets of farmed tuna.

Progress against milestones

(i)Phase 1 (Originally 1/10/95, revised to 1/12/95 with agreement of FRDC)

Phase 1 measurements were incorporated in the design as part of Phase 2 studies, and were measured during the course of the project.

(ii)Phase 2 (Originally 1/3/96, revised to 1/5/96 with agreement of FRDC)

It was planned that Phase 2 would begin in April 1996, as harvesting operations from tuna nets began. Sampling was to be on a 6 weekly cycle. In April 1996 the tuna aquaculture industry at Port Lincoln suffered a major setback when a severe storm caused adverse conditions in Boston Bay which resulted in the death of 70% of tuna stocks. We had to redefine the sampling plan in the light of reduced fish stocks and a delayed harvesting program. Sampling resumed in June 1996 with samples being collected on a 4 weekly cycle. This sampling regime was continued until the end of the 1996 season. (November/December 1996). Further funding (97/307) permitted sampling from tuna nets to continue until November 1997.

(iii)Phase 3 (Originally 1/11/96, revised to 1/1/97 with agreement of FRDC)

Mussel aquarium studies began in April 1996, examining the influence of environmental factors on biomarkers. Factors examined were temperature, salinity, nitrate levels and reproductive status of mussels.

Aquarium studies of cohabiting fish species and their response to these environmental factors are have been completed, but the inconclusive nature of the results led us to believe that these studies should be repeated in 1997. These studies were performed by BSc Honours students at the School of Biological Sciences, Flinders University in 1997 and 1998.

Scientific findings

1. Gill ATPase - This is shown in figures 1a and 1b with data for samples from tuna, mussel and cohabiting fish species represented. These show wide monthly fluctuations in ATPase, although this variation was less marked in tuna. Statistically significant elevations in tuna ATPase were seen only in June 1996 and May 1997. These may represent seasonal fluctuations in ATPase activity. It is unclear whether this was a consequence of the earlier storm and its associated high mortality or to normal seasonal physiological events. Seasonal variation of ATPase in cohabiting fish species was most marked, with significant elevations in winter 1996. The seasonality of this response in Australian herring and mackerel could not be confirmed in 1997 due to reduced sampling activity. ATPase levels in cohabiting fish species were greater than those in tuna in winter 1996. ATPase activity, measured here in tuna, Australian herring and mackerel, was similar in magnitude to levels measured in wild fish (yellow eye mullet) in South Australian waters (2).

   Correlations between ATPase in cohabiting fish and tuna, and in mussel and tuna were generally poor, both in 1996 and 1997 seasons and in the combined 1996/1997 seasons (figures 1c, 1d, 1e and 1f). the outlying value (June 1996) represents samples collected soon after the severe storm of 1996. This data point may be unrepresentative, and its omission substantially improves the correlation between data (e.g. fig 1e. Pearson r changes from 0.231 to 0.582). One explanation for the unrepresentativeness of this point may be that the tuna, captive in the nets at the time of the storm, were more severely affected than the cohabiting fish species (that were able to leave the nets to seek shelter) and the mussels (able to close and survive under anaerobic conditions for substantial periods). Alternatively, June 1996 samples were the first to be collected in this project and sample quality and viability of results may be suspect. Notwithstanding these concerns, it appears that the predictive capacity of ATPase in cohabiting species with respect to ATPase in tuna is reasonable, but requires further investigation to be developed as a useful field marker.

2. AEC in gill (figures 2a and 2b) - This was quite low in gill of some species and displayed quite a lot of variation. In tuna, this dropped to levels associated in the literature with poor health. In tuna liver (figures 2c and 2d), values were less variable, approximating 0.6 - 0.75; levels associated with sub-optimal condition. AEC in liver and gill of cohabiting species were also generally low (between 0.25 and 0.60) with occasional mean values around 0.75. These values were considerably below those reported by Hamann et al (2) in wild fish from South Australian waters exposed to urban and industrial outflows. This parameter is most sensitive to tissue collection and storage conditions. Although the liver tissue collection in particular required dissection of the tuna, the sample was still rapidly obtained after tuna were removed from nets (i.e. within 45 sec). Correlations between species (figures 2e to 2h) indicate some association between values, suggesting this marker may be useful as a biomarker in tuna. The correlations may have been skewed by the extreme conditions during the period when tuna mortality was high, although samples were not available from that time for comparison. Correlations were generally improved by the omission of the data from June 1996.

3. Variations in IMPL are shown in figures 3a, 3b, 3c and 3d, and the correlations between IMPL in tuna and in cohabiting fish and shellfish are shown in figures 3e, 3f, 3g and 3h. IMPL and AEC are not independent. It appeared that as tuna gill AEC varied in monthly samples, IMPL varied in the opposite sense. That is, the changes in IMPL were mirror

images of the changes in AEC. This may be a consequence of the role of IMP as a regulator of AEC by the deamination of AMP (12). Although depression of AMP in favour of IMP occurs in crustacea only at high levels of stress (11), this reduction of the adenylate pool occurs in exercising vertebrates as a means to remove inhibition of enzymes by AMP (12, 13). Changes in IMPL in tuna were also highly correlated with IMPL in cohabiting fish species. This correlation was better in liver samples than in gill, especially when the outlying values from June 1996 were censored (e.g. Pearson r for liver IMPL in tuna and cohabiting fish changed from 0.895 with June 1996 data to 0.935 without). There was no correlation between tuna IMPL and mussel IMPL.

4. ATP/IMP ratios (figures 4a to 4d) and their correlations between species (figures 4e to 4h) are shown. ATP/IMP ratios in liver were relatively stable throughout the sampling period, excluding samples from tuna in June 1996, and from Australian herring in August 1996. Gill sample data were more variable during the same period. Correlations between ATP/IMP in gill of tuna and cohabiting fish were weak (Pearson r = 0.547) whereas those in liver were strong following censoring of June 1996 outlying data (Pearson r = 0.963). Mussel ATP/IMP data did not correlate with those in either gill or liver of tuna.

5. Aquarium studies were performed to investigate the effect of ammonia on these biochemical markers in Australian herring and mussel. Ammonia treatment resulted in sporadic changes in ATPase in mussel, most of which were not statistically significant. The main observation (figs 5, 5a), reproduced in other studies, was an enhanced ATPase activity at low ammonia concentrations (1 mg/l) superimposed by a reduction in activity at higher concentrations (5 mg/l). Generally, ammonia did not change AEC in mussel up to 7 days exposure (figs 6, 6a), but there was a slight, significant increase in AEC at 14 days. However, mussel AEC was low in control animals (<0.5) suggesting that these changes may have been an artefact in mussels already stressed by collection and handling. IMPL in mussels (figs 7, 7a) fell significantly in all animals, including controls, within 2 hours of commencing ammonia exposure, and levels remained depressed for the 14 day observation period.

   In Australian herring, ATPase was not consistently nor significantly altered by ammonia treatment (figs 8, 8a). Gill AEC (figs 9, 9a) declined after 7 days in all treatment groups (including controls) and declined further at 14 days, ultimately reaching approximately 0.3 – 0.4, compared with commencement values of about 0.6. Liver AEC (figs 10, 10a) varied without any apparent pattern, although there was an initial, concentration-dependent decline in liver AEC in all groups at 2 hours. Gill IMPL (figs 11, 11a) declined in all groups at 7 days and did not recover by day 14, whereas liver IMPL (figs 12, 12a) did not change until day 14 when a statistically insignificant reduction was seen in all groups. These data support the field data observations that biochemical markers in gill may be more responsive to environmental stresses than the same markers in liver. The data are also consistent with the biochemical model in which IMP mediates the attenuation of changes in AEC.

6. Other studies were conducted by BSc Honours students at the School of Biological Sciences, Flinders University, that were complementary to the investigations performed in this project. Westcott (1996) (14) showed that mussels seeded onto tuna farming nets had significantly lower ATPase than mussels of the same age and size seeded onto a marker buoy over 100 metres from the tuna nets. This work also showed that in the mussels seeded onto the tuna nets, those attached to the inner ring of the net had lower ATPase than those attached on the outer side.

Baker (1998) (15) determined the conditions under which various tissue samples could be taken from fish (Australian herring) exposed to an acute stressor (capture from the wild by hook and line) such that tissue AEC and IMPL values were not compromised (figs 13, 13a). By sampling groups of fish at various times up to 30 minutes after capture it was determined that:

liver AEC was significantly decreased (by up to 50%) after only
10 minutes of confinement (no shorter time period tested)
5 minutes of anaesthesia using benzocaine solution
10 minutes after fish were killed immediately following capture

liver IMPL was
not significantly affected by confinement stress or anaesthetic (although there were trends for both to increase with time)
was significantly increased at 30 minutes post-mortem

gill AEC and IMPL were not affected by 30 minutes of confinement stress

The conclusions taken from this study were that for Australian herring:

capture and handling stresses need to be minimised to avoid possible confounding effects on AEC
IMPL is not as labile as AEC and thus there is a greater window available to take samples for analysis of in situ levels
there are marked differences between tissues in responsiveness to stressors.

7. The sampling strategies adopted in this project were sound, but were limited by the sometimes conflicting demands made on personnel. In particular, samples were collected by a field officer at Port Lincoln, whose major duties were directed at other projects involving measurements and samples taken from farmed tuna. This dilution of effort, while unintended, may have compromised the quality of the data collected for this project. Secondly, sample quality may have been degraded during transport to the laboratories at Flinders University. Although samples were freighted in liquid nitrogen, in some cases samples arrived at Flinders University after being spilled or after liquid nitrogen had evaporated. Any further studies of this type would now avoid these difficulties as Flinders University personnel are now stationed at Port Lincoln and these staff are able to coordinate and perform field sampling procedures. In addition, development of extensive laboratory facilities at the Flinders University Marine Science Centre means that HPLC and enzymatic analyses of samples are able to be performed locally. Hence samples can be processed promptly, minimising the potential for degradation of sample components.
8. The results from this project appear equivocal, although they suggest that the biochemical markers may be useful as indicators of farmed tuna condition. In particular, ATPase, AEC and IMPL in cohabiting fish may closely follow those in tuna, and may provide a basis for a predictive marker of tuna condition and health status.

   Specifically;

- Mussel ATPase data are as good as gill ATPase in cohabiting fish species, and these are reasonably correlated with this marker in tuna.
- Correlations between mussel AEC, IMPL and ATP/IMP and the corresponding marker in tuna are poor.
- Correlations between AEC, IMPL and ATP/IMP in cohabiting fish species and the corresponding marker in tuna indicate that these markers have promise as surrogate indicators of tuna condition. This is particularly true of liver-derived measurements.
- Biochemical measurements in gill appear to be more responsive to external stresses than those in liver, and these may provide markers of short-term changes in environmental conditions.
- Of the available biochemical measures, IMPL appears to be the most indicative of stress-response in tuna.

List of figures

List of figures (continued)

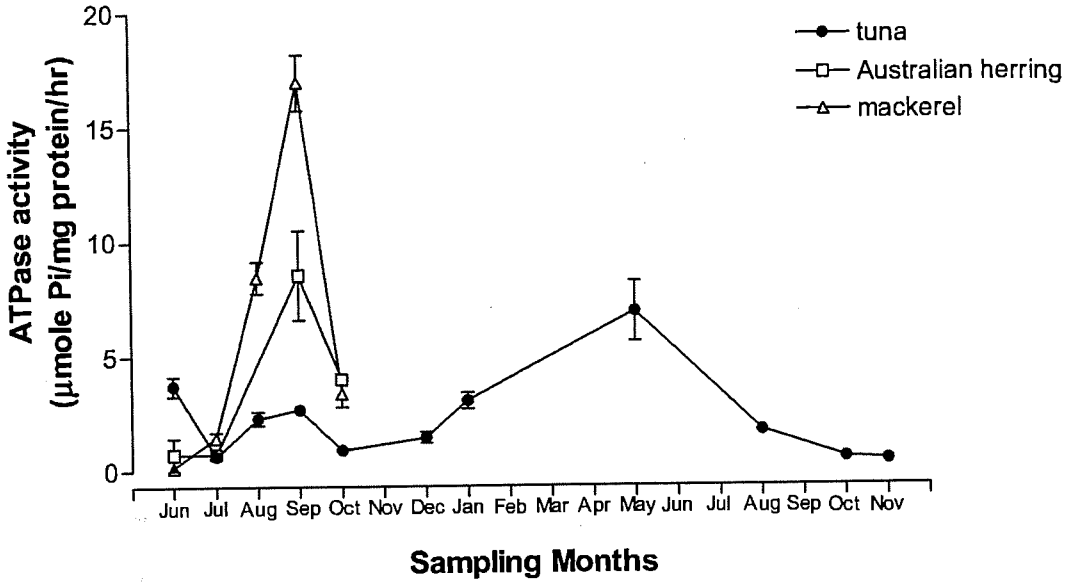


Figure 1a. ATPase in gill of tuna and cohabiting fish species during 1996/1997 tuna farming season

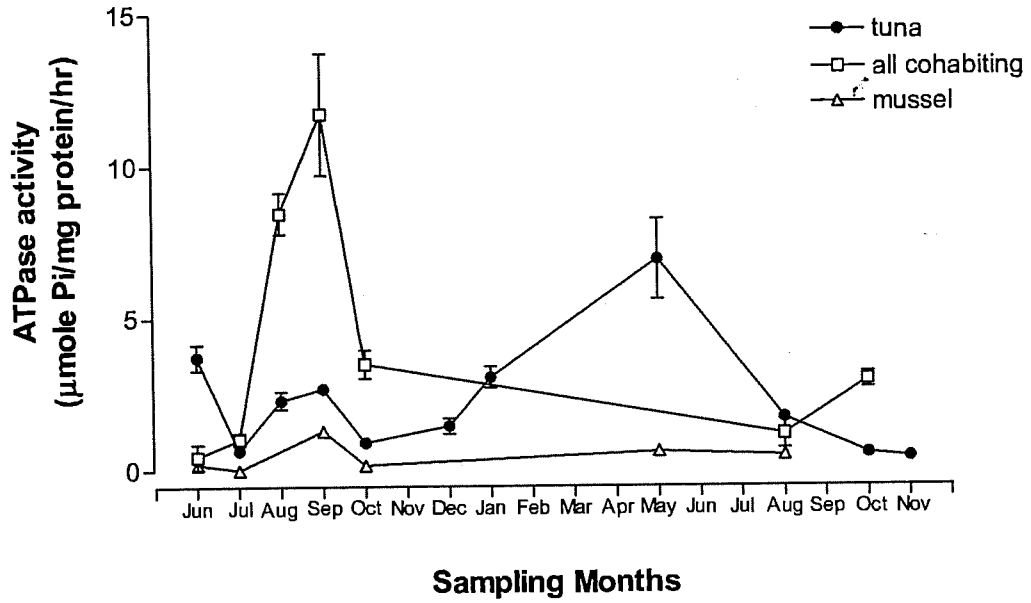


Figure 1b. ATPase in gill of tuna and cohabiting fish species, and mussel during 1996/1997 tuna farming season

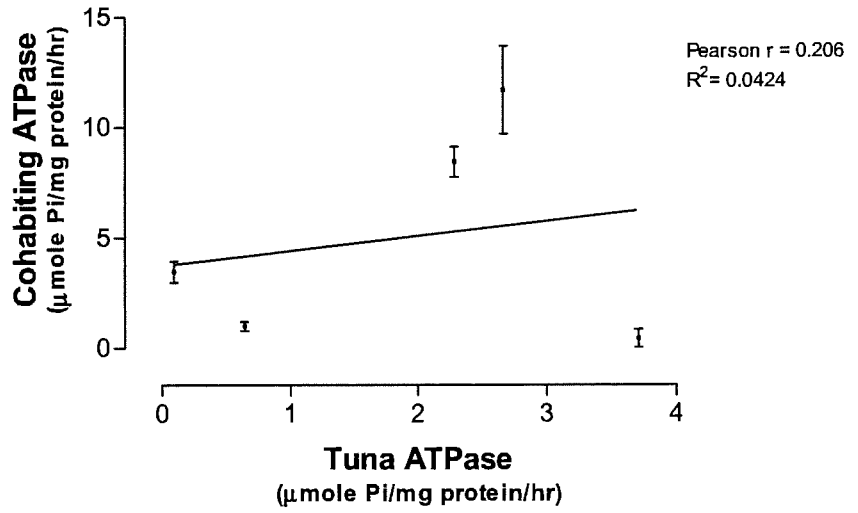


Figure 1c. Correlation between ATPase in gill of tuna and cohabiting fish species, during 1996 tuna farming season (June 1996 value arrowed)

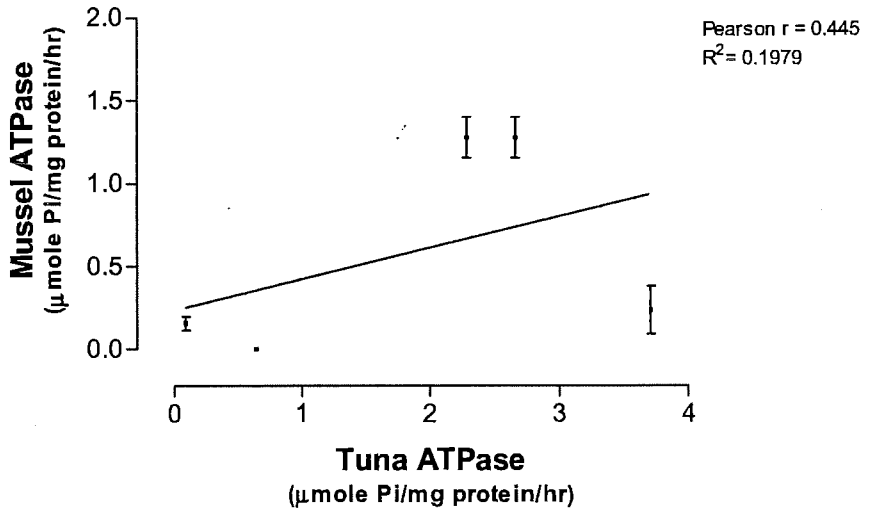


Figure 1d. Correlation between ATPase in mussel and in gill of tuna during 1996 tuna farming season (June 1996 value arrowed)

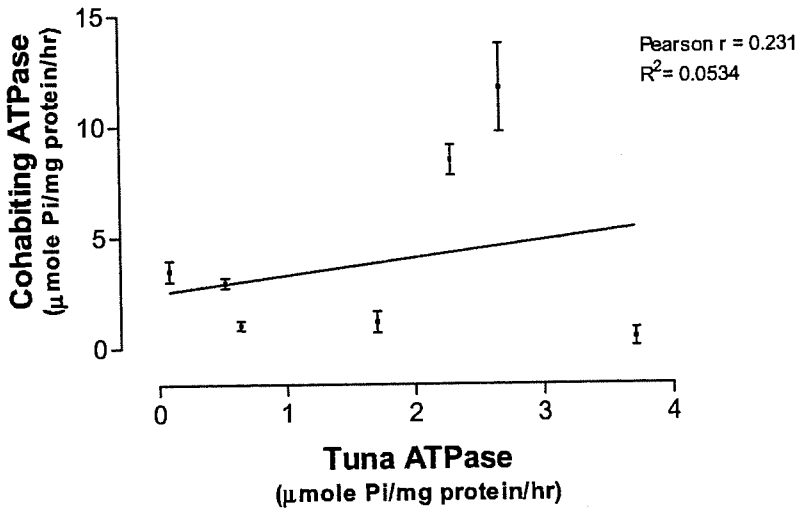


Figure 1e. Correlation between ATPase in gill of tuna and cohabiting fish species, during 1996/1997 tuna farming season (June 1996 value arrowed)

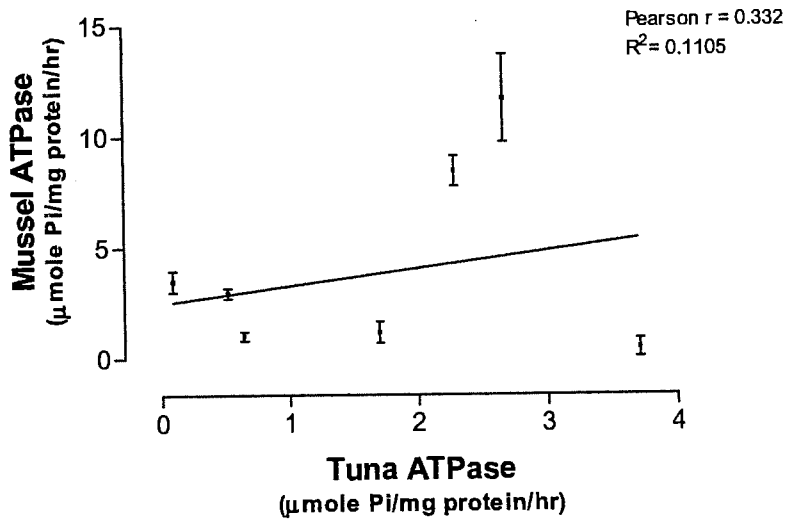


Figure 1f. Correlation between ATPase in gill of tuna and in mussel during 1996/1997 tuna farming season (June 1996 value arrowed)

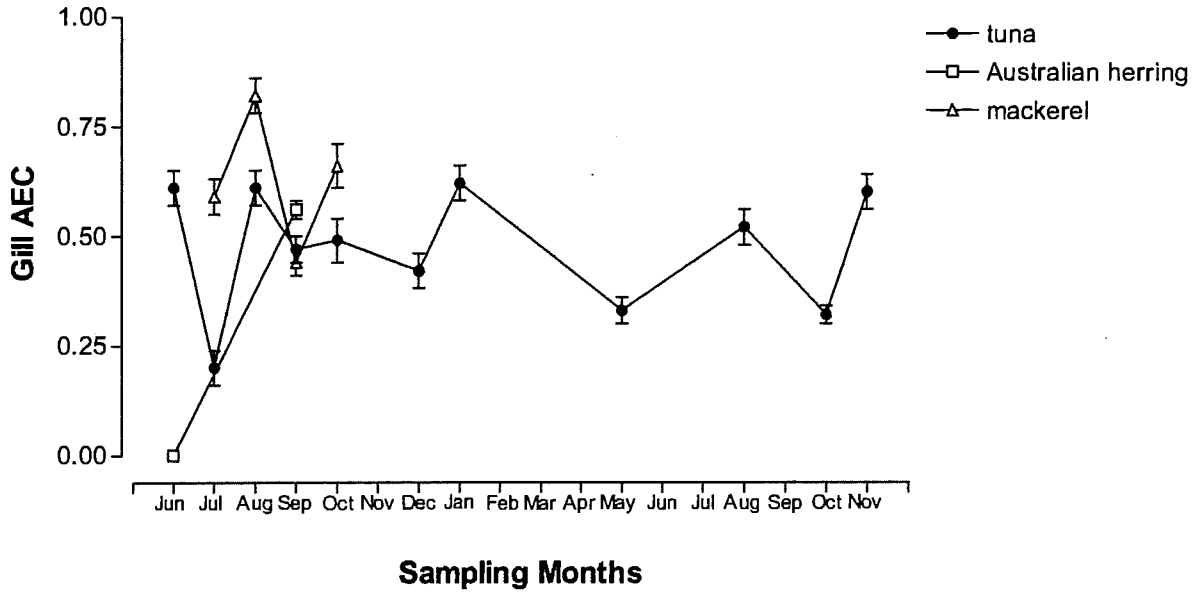


Figure 2a. Adenylate energy charge in gill of tuna and cohabiting fish species during 1996/1997 tuna farming season

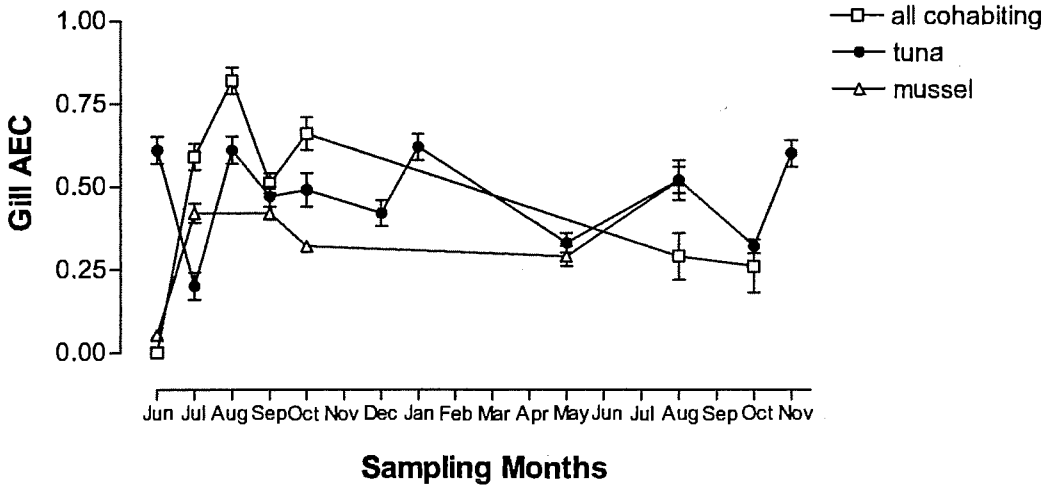


Figure 2b. Adenylate energy charge in gill of tuna and cohabiting fish species and in mussel during 1996/1997 tuna farming season

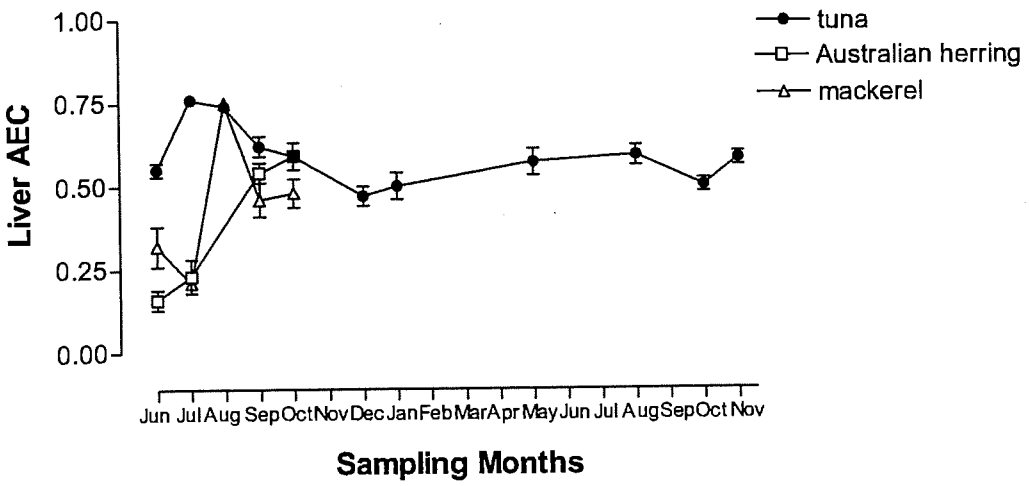


Figure 2c. Adenylate energy charge in liver of tuna and cohabiting fish species during 1996/1997 tuna farming season

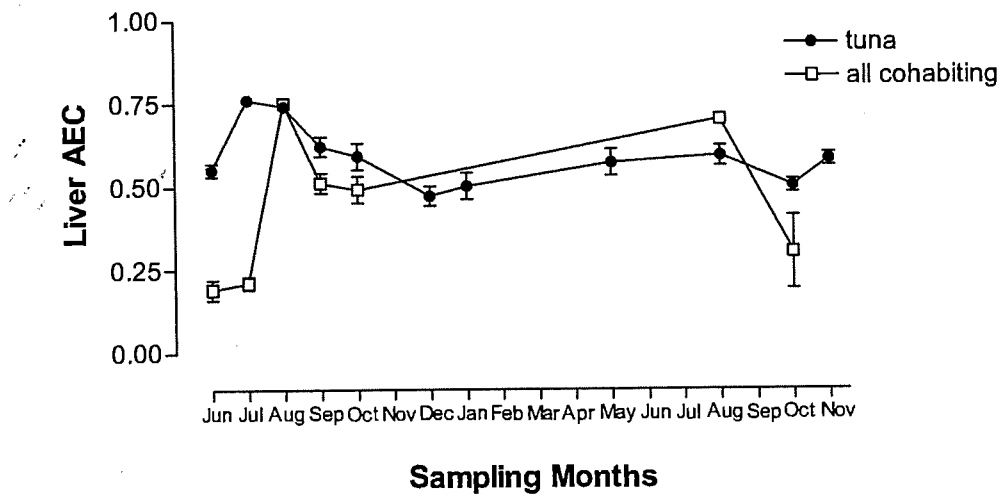


Figure 2d. Adenylate energy charge in gill of tuna and cohabiting fish species during 1996/1997 tuna farming season

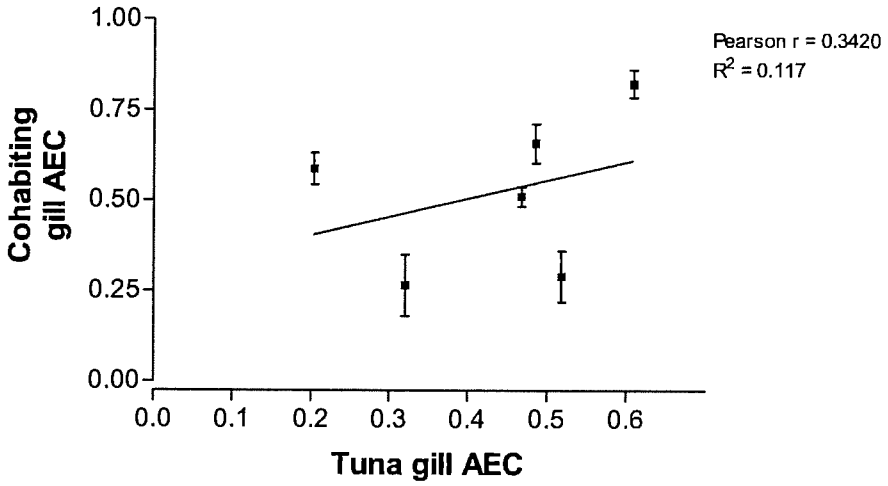


Figure 2e. Correlation between adenylate energy charge in gill of tuna and cohabiting fish species during 1996/1997 tuna farming season

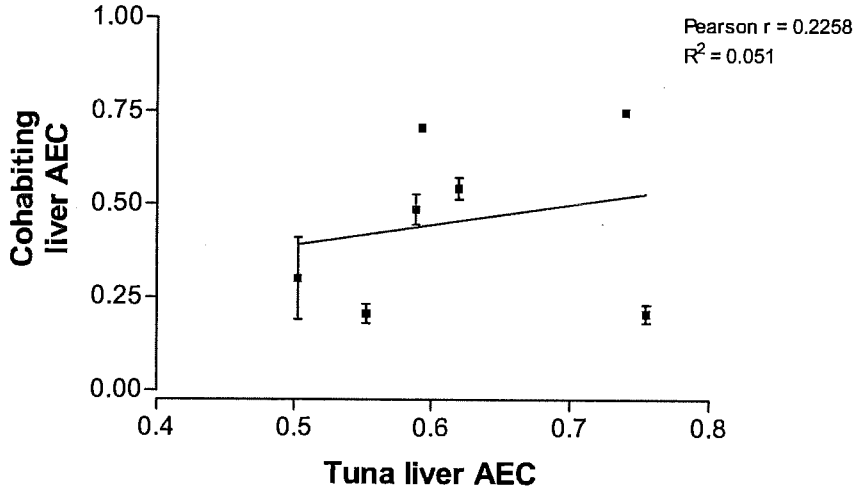


Figure 2f. Correlation between adenylate energy charge in liver of tuna and in cohabiting fish species during 1996/1997 tuna farming season

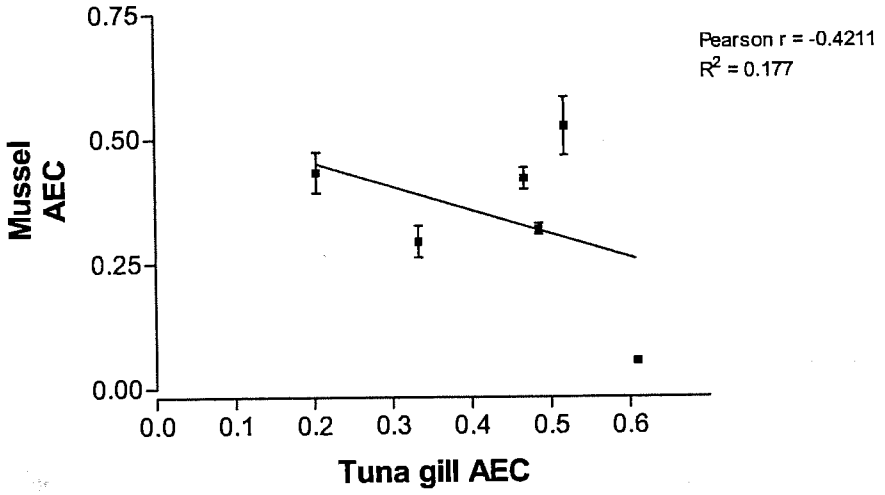


Figure 2g. Correlation between adenylate energy charge in gill of tuna and in mussel during 1996/1997 tuna farming season

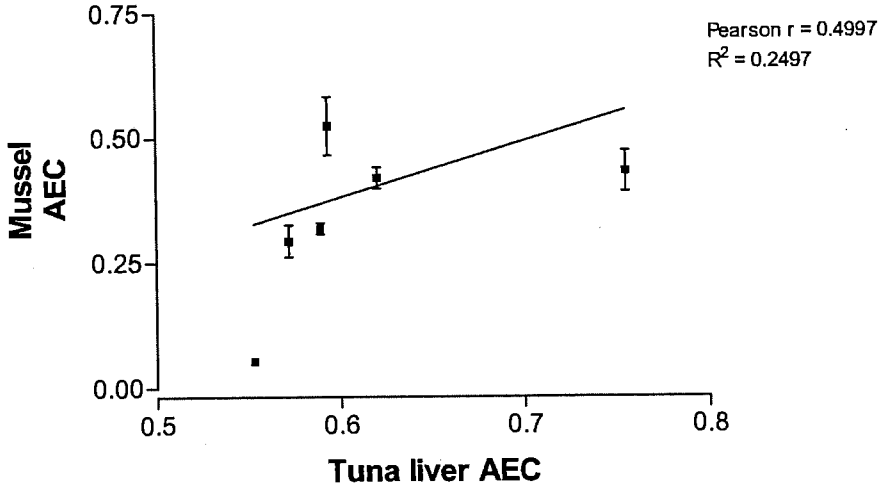


Figure 2h. Correlation between adenylate energy charge in liver of tuna and in mussel during 1996/1997 tuna farming season

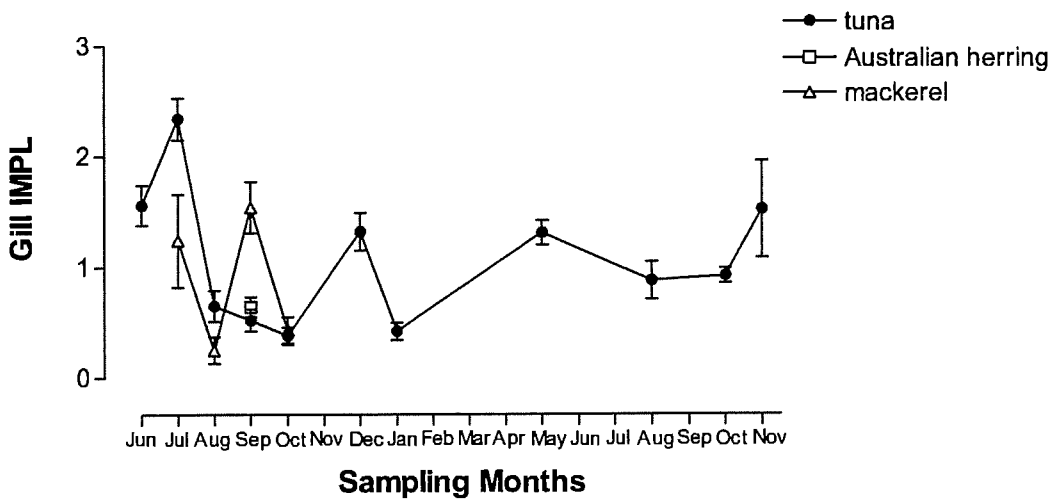


Figure 3a. IMP load in gill of tuna and cohabiting fish species during 1996/1997 tuna farming season

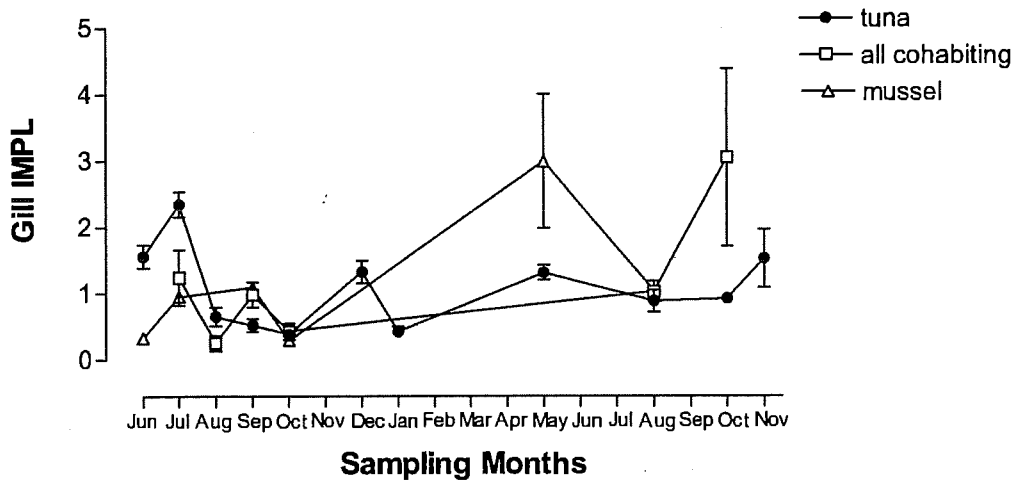


Figure 3b. IMP load in gill of tuna and cohabiting fish species and in mussel during 1996/1997 tuna farming season

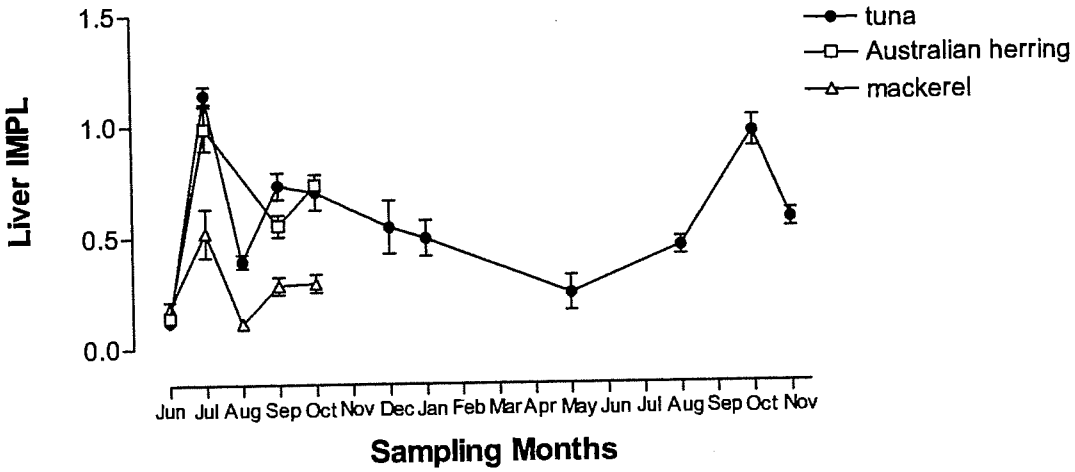


Figure 3c. IMP load in liver of tuna and cohabiting fish species during 1996/1997 tuna farming season

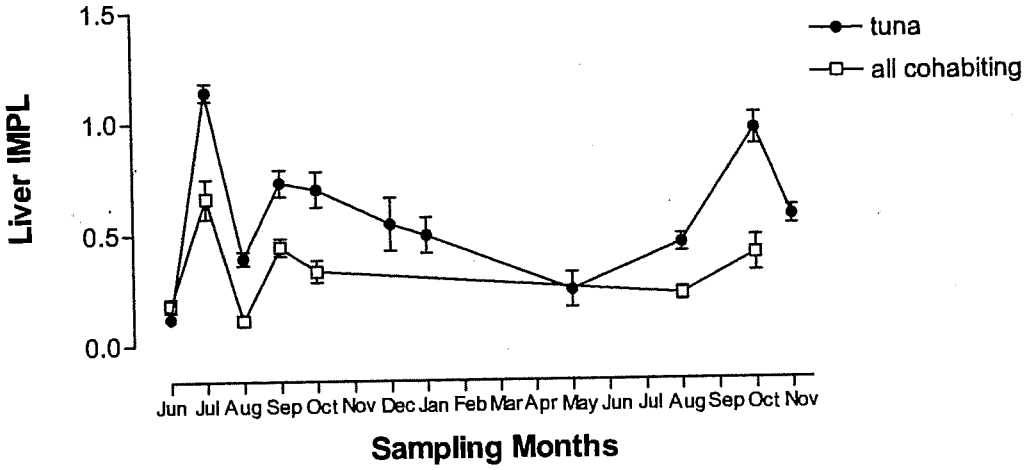


Figure 3d. IMP load in liver of tuna and cohabiting fish species during 1996/1997 tuna farming season

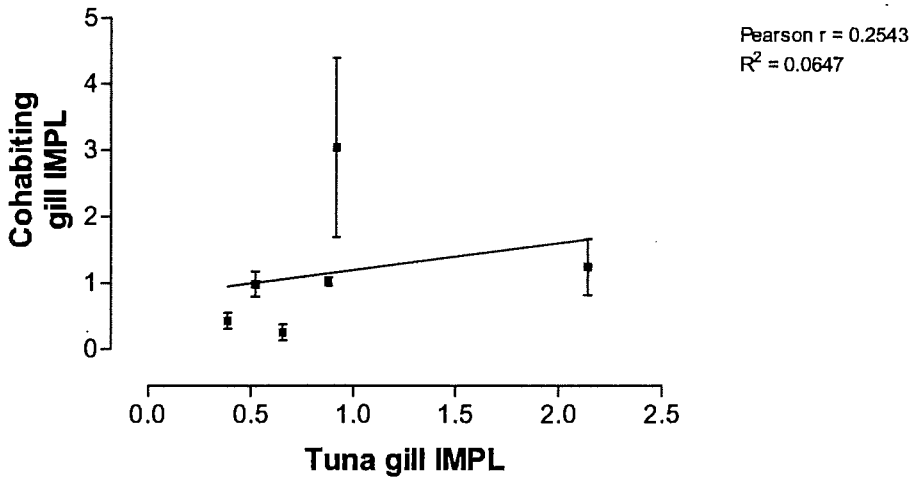


Figure 3e. Correlation between IMP load in gill of tuna and cohabiting fish species during 1996/1997 tuna farming season

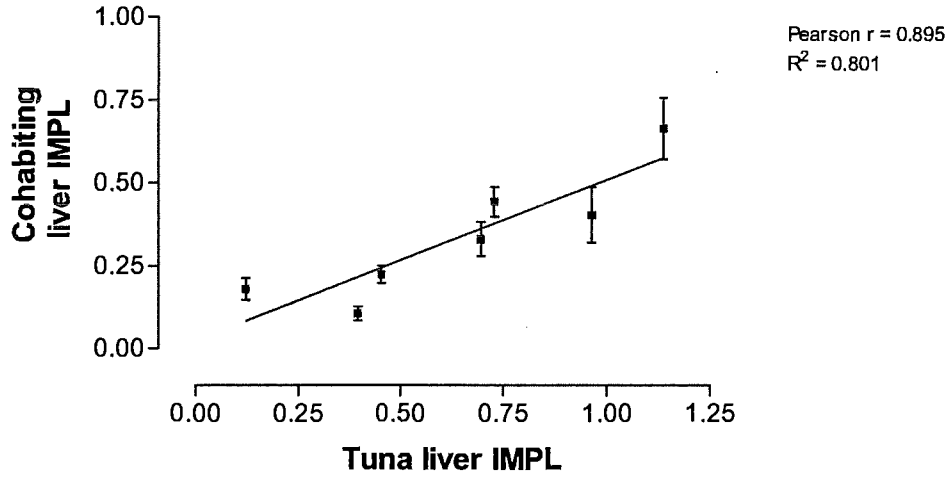


Figure 3f. Correlation between IMP load in liver of tuna and cohabiting fish species during 1996/1997 tuna farming season (June 1996 value arrowed)

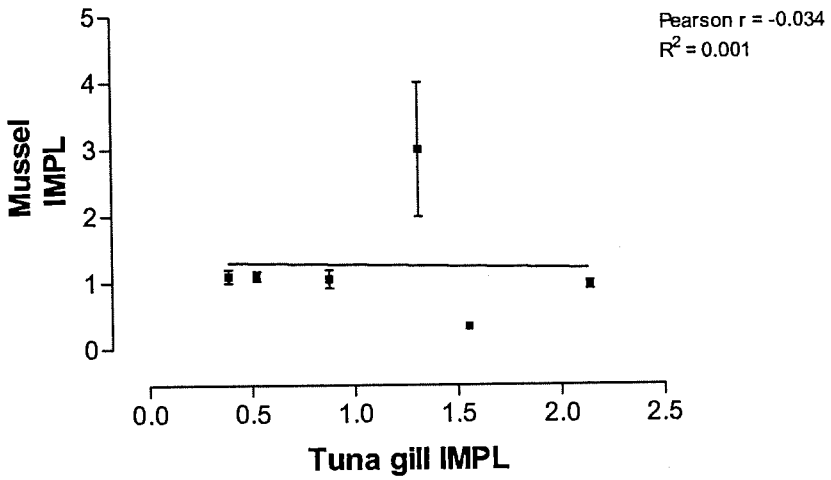


Figure 3g. Correlation between IMP load in gill of tuna and in mussel during 1996/1997 tuna farming season

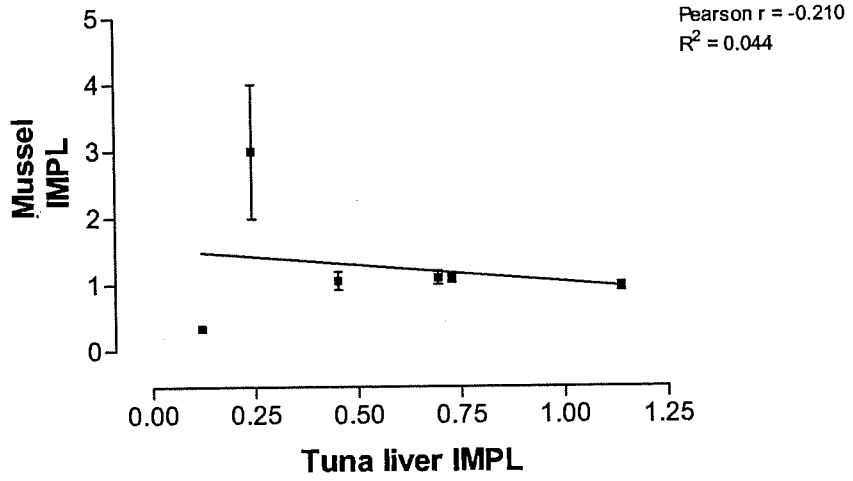


Figure 3h. Correlation between IMP load in liver of tuna and in mussel during 1996/1997 tuna farming season

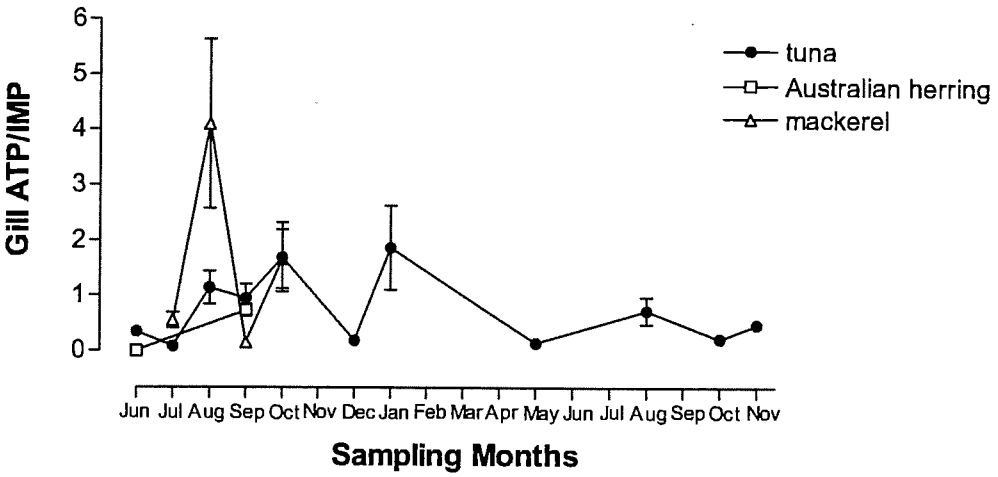


Figure 4a. ATP/IMP ratios in gill of tuna and cohabiting fish species during 1996/1997 tuna farming season

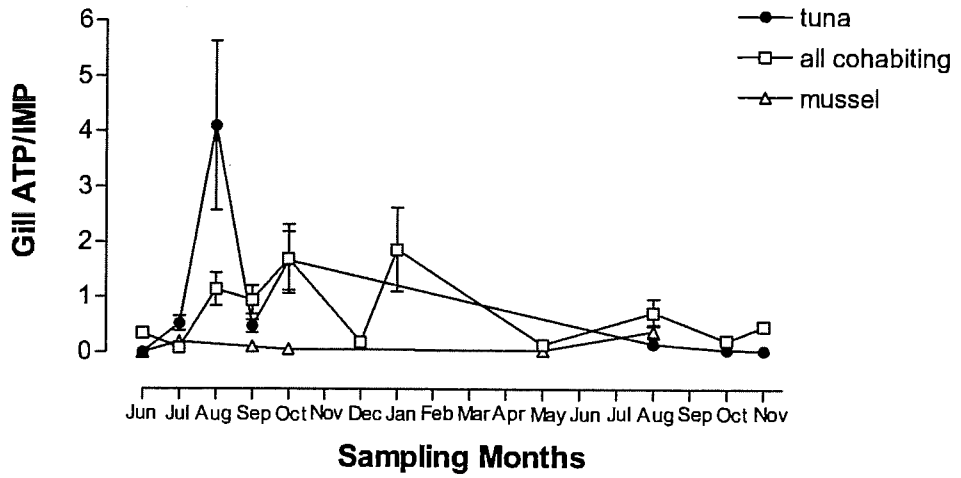


Figure 4b. ATP/IMP ratios in gill of tuna and cohabiting fish species and in mussel during 1996/1997 tuna farming season

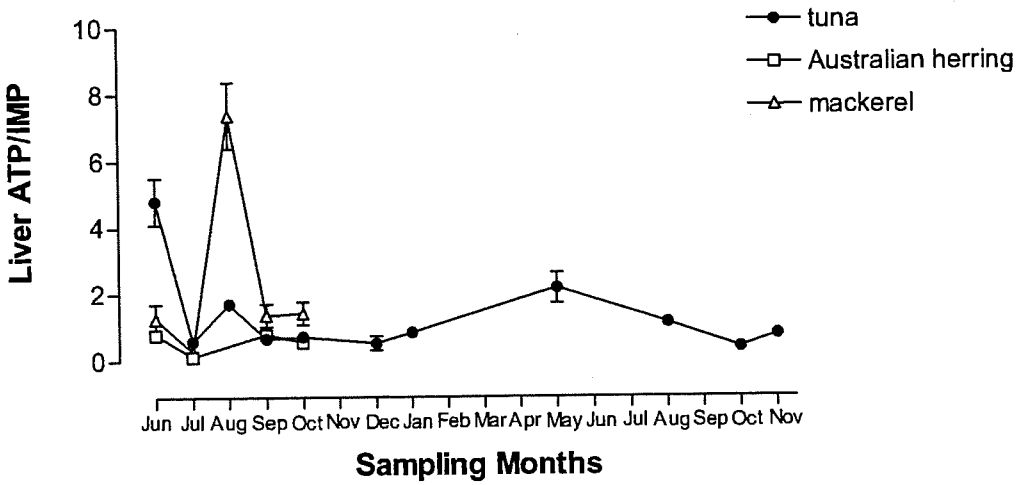


Figure 4c. ATP/IMP ratios in liver of tuna and cohabiting fish species during 1996/1997 tuna farming season

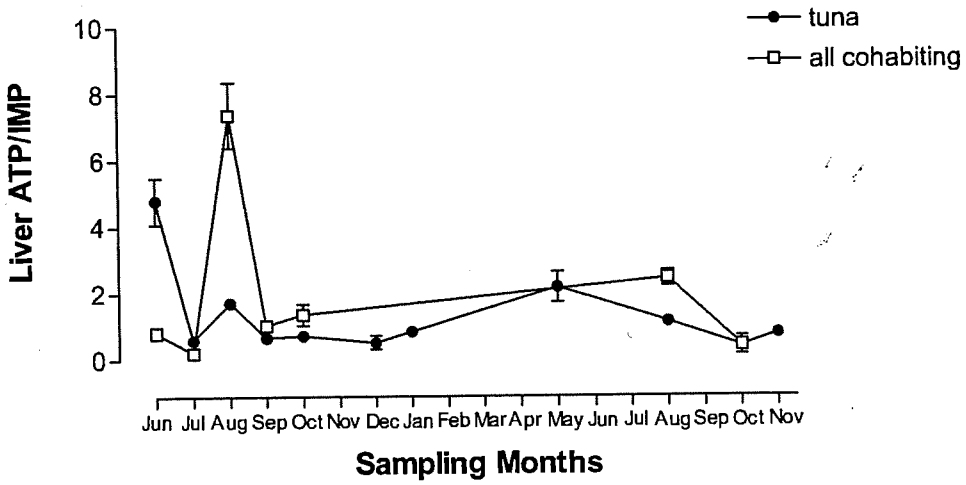


Figure 4d. ATP/IMP ratios in liver of tuna and cohabiting fish species during 1996/1997 tuna farming season

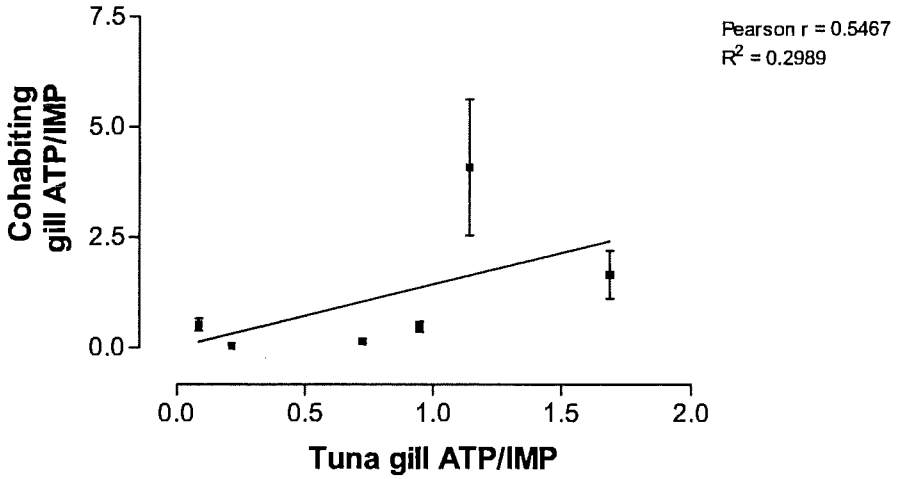


Figure 4e. Correlation between ATP/IMP ratio in gill of tuna and cohabiting fish species during 1996/1997 tuna farming season

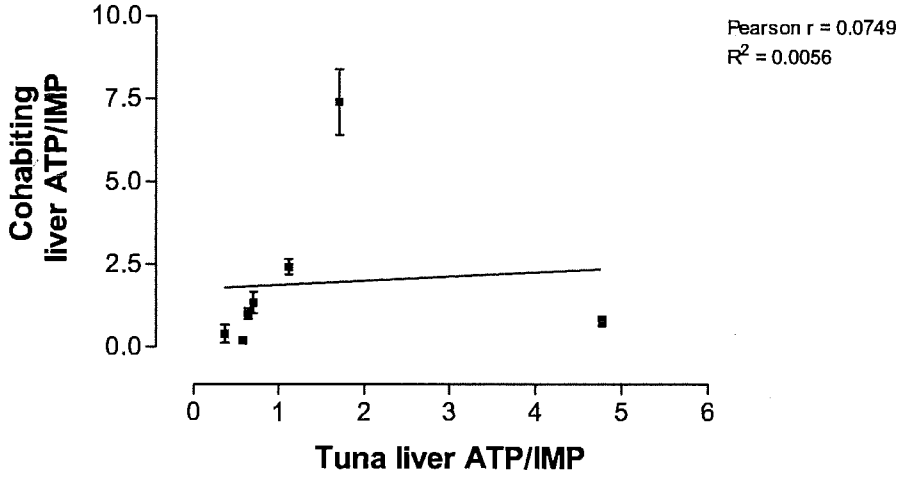


Figure 4f. Correlation between ATP/IMP ratio in liver of tuna and cohabiting fish species during 1996/1997 tuna farming season (June 1996 value arrowed)

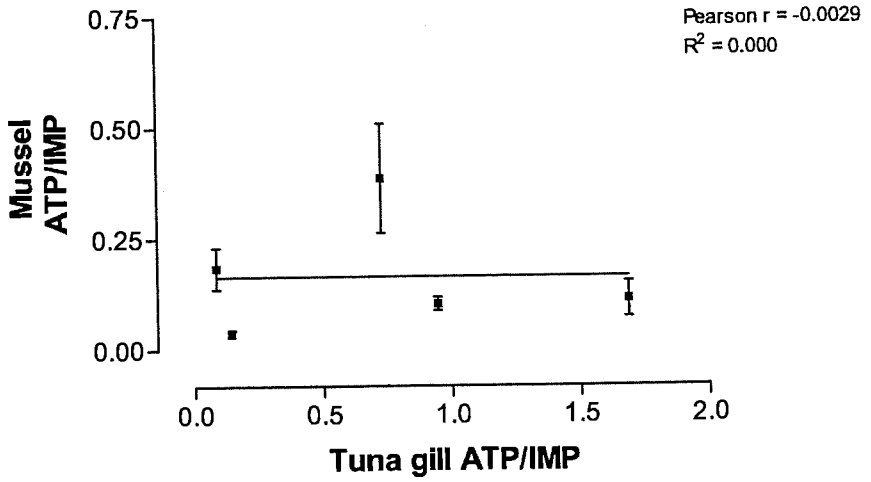


Figure 4g. Correlation between ATP/IMP ratio in gill of tuna and in mussel during 1996/1997 tuna farming season

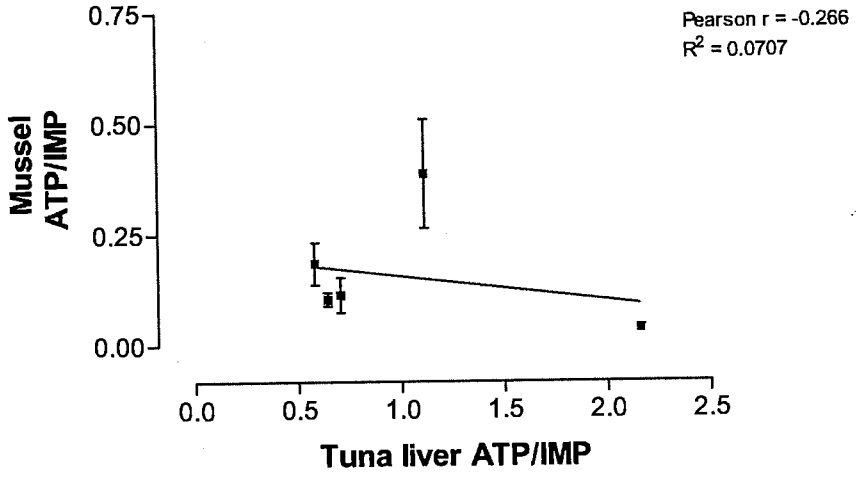


Figure 4h. Correlation between ATP/IMP ratio in liver of tuna and in mussel during 1996/1997 tuna farming season

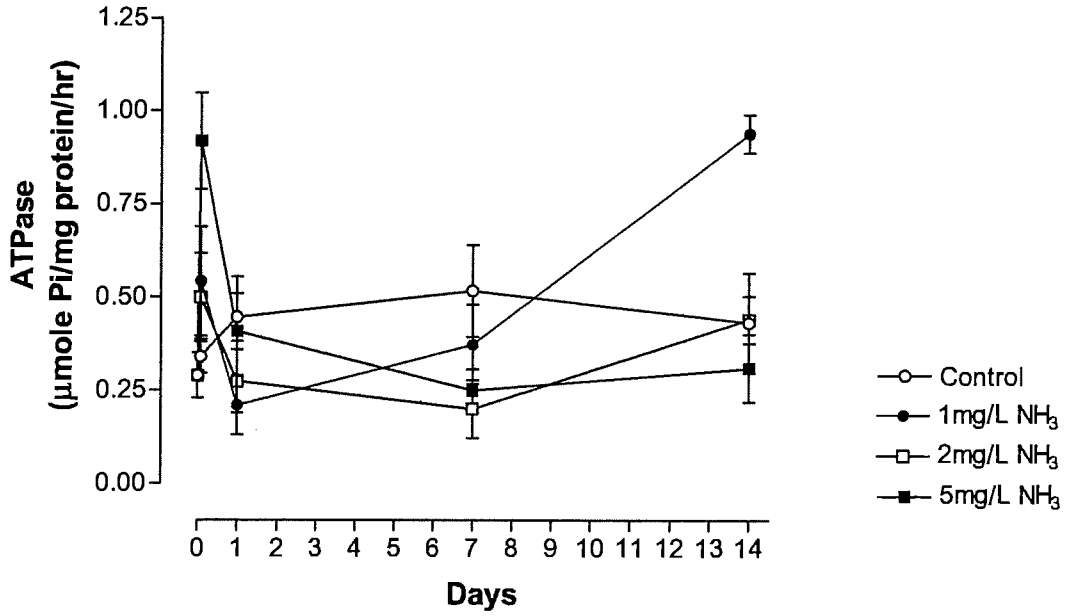


Figure 5. ATPase in mussel exposed in aquaria to ammonia over 14 days

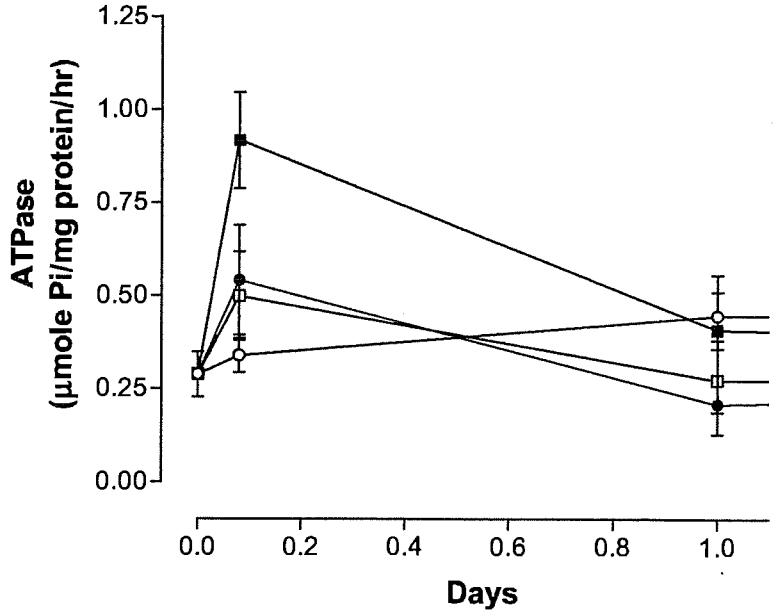


Figure 5a ATPase in mussel exposed in aquaria to ammonia over 14 days (enlargement of plot to day 1)

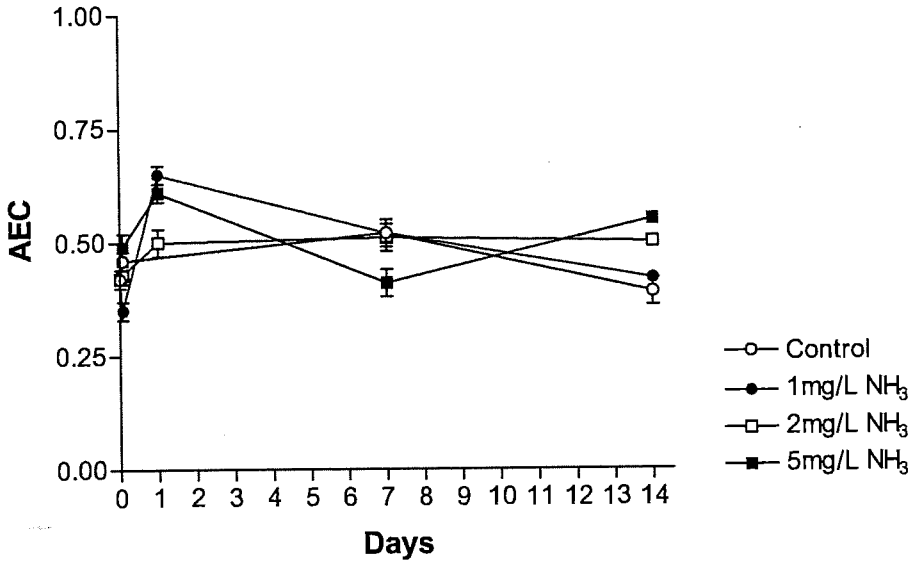


Figure 6 AEC in mussel exposed in aquaria to ammonia over 14 days

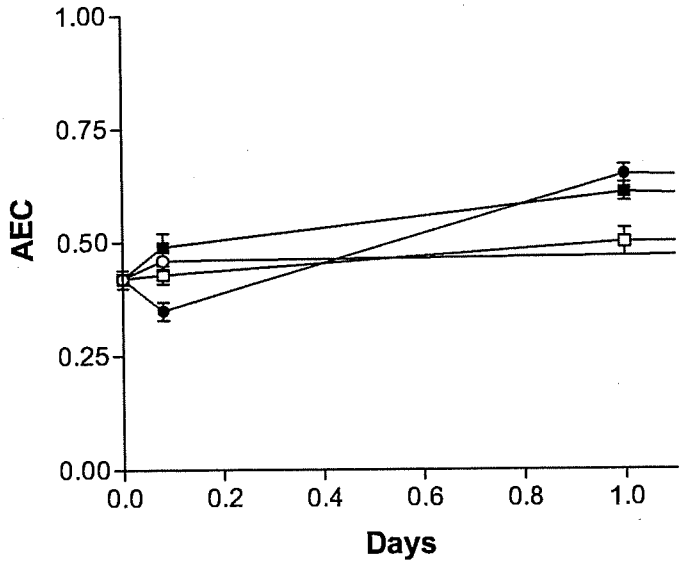


Figure 6a. AEC in mussel exposed in aquaria to ammonia over 14 days (enlargement of plot to day 1)

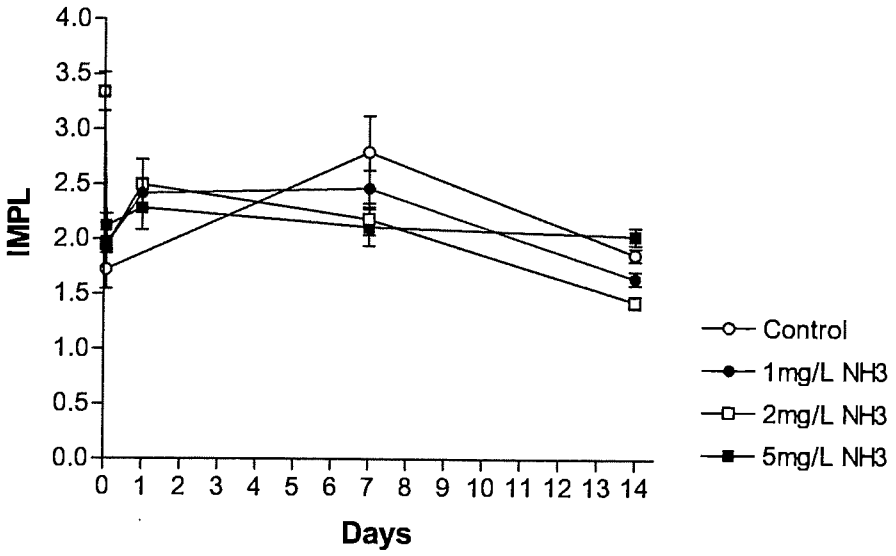


Figure 7. IMPL in mussel exposed in aquaria to ammonia over 14 days

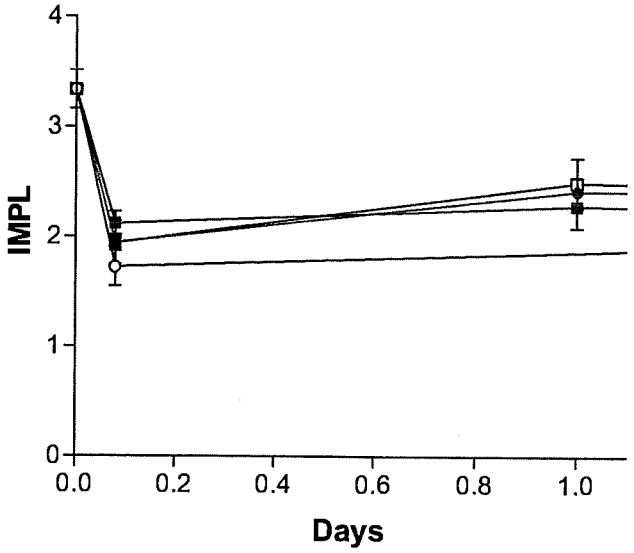


Figure 7a. IMPL in mussel exposed in aquaria to ammonia over 14 days (enlargement of plot to day 1)

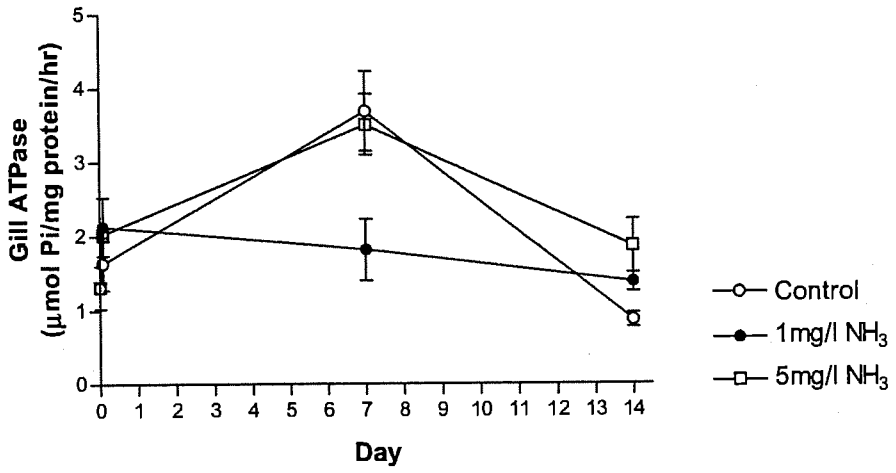


Figure 8. Gill ATPase in Australian herring exposed in aquaria to ammonia over 14 days

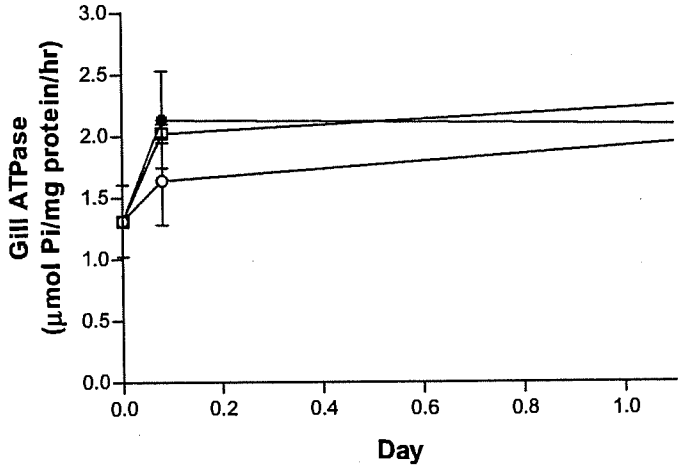


Figure 8a. Gill ATPase in Australian herring exposed in aquaria to ammonia over 14 days (enlargement of plot to day 1)

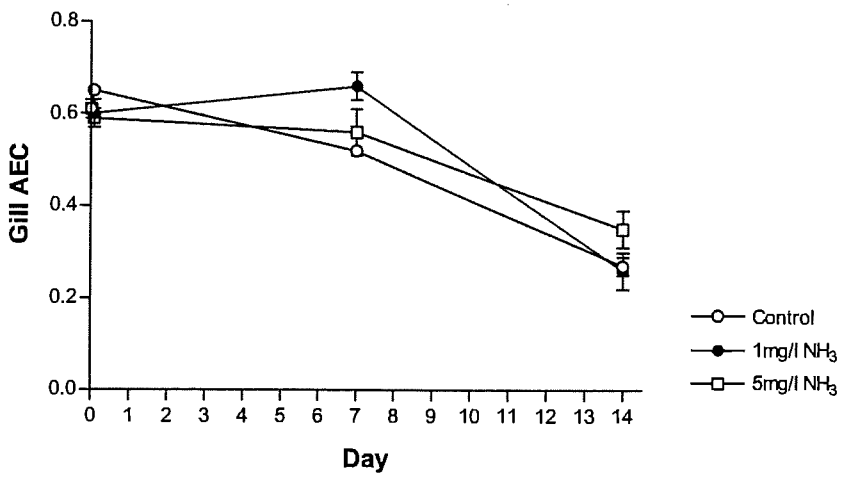


Figure 9. Gill AEC in Australian herring exposed in aquaria to ammonia over 14 days

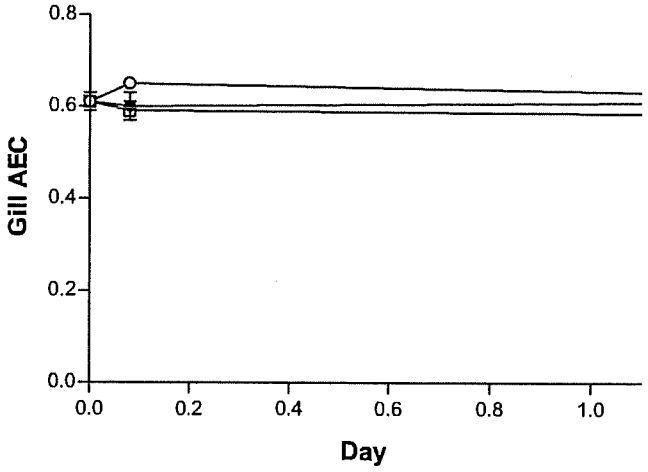


Figure 9a. Gill AEC in Australian herring exposed in aquaria to ammonia over 14 days (enlargement of plot to day 1)

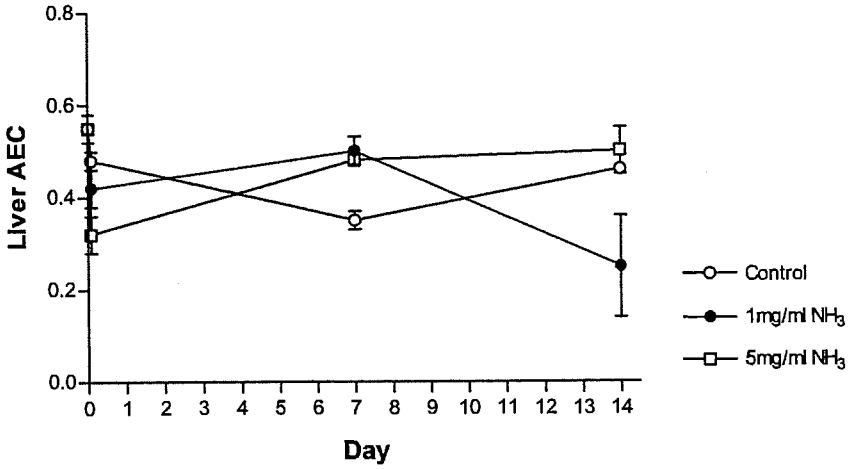


Figure 10. Liver AEC in Australian herring exposed in aquaria to ammonia over 14 days

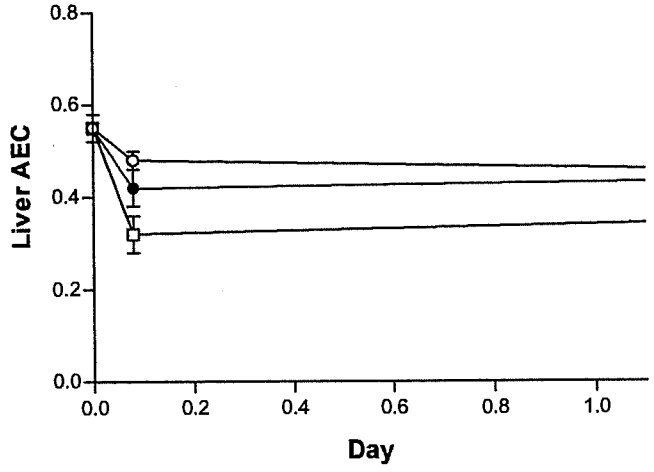


Figure 10a. Liver AEC in Australian herring exposed in aquaria to ammonia over 14 days (enlargement of plot to day 1)

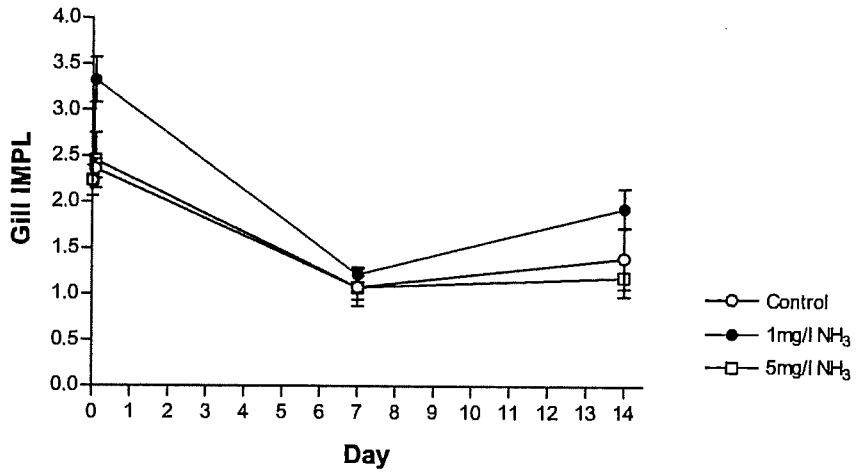


Figure 11. Gill IMPL in Australian herring exposed in aquaria to ammonia over 14 days

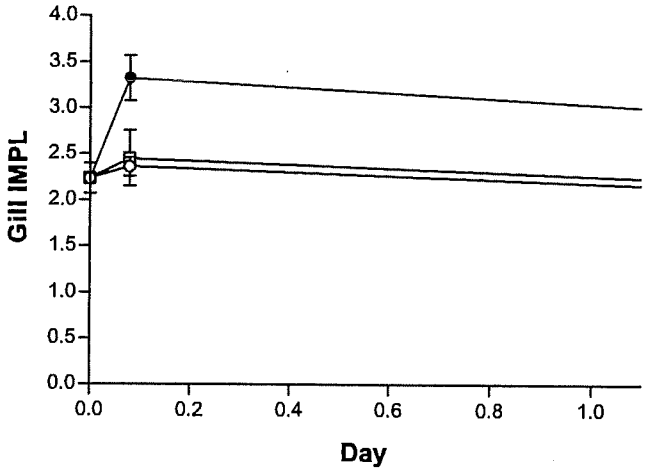


Figure 11a. Gill IMPL in Australian herring exposed in aquaria to ammonia over 14 days

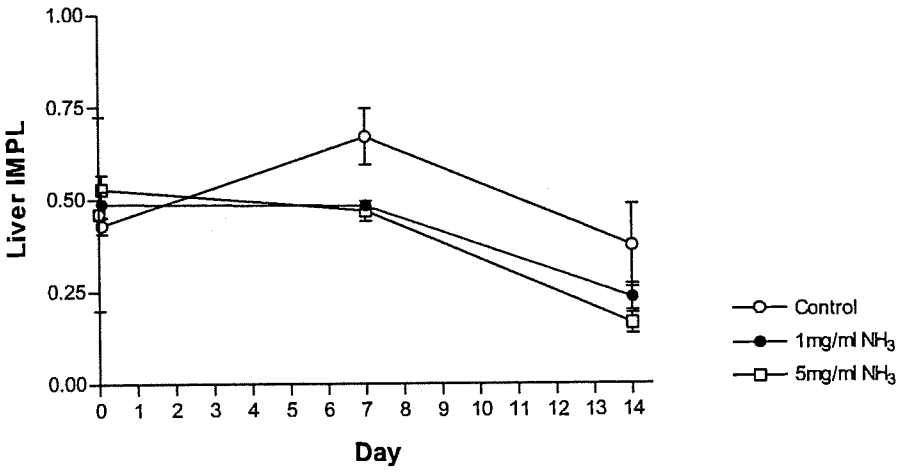


Figure 12. Liver IMPL in Australian herring exposed in aquaria to ammonia over 14 days

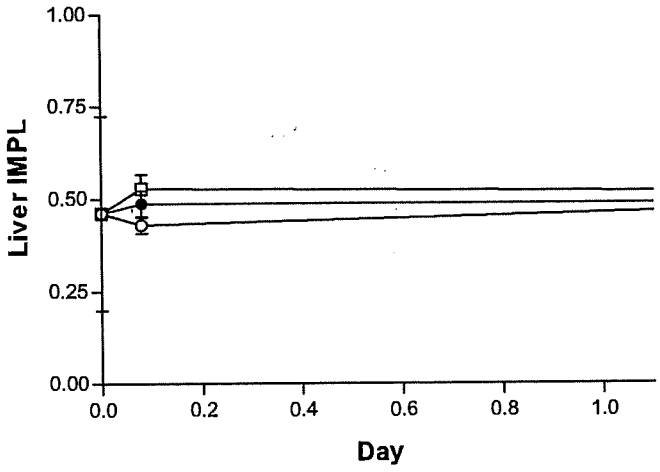


Figure 12a. Liver IMPL in Australian herring exposed in aquaria to ammonia over 14 days (enlargement of plot to day 1)

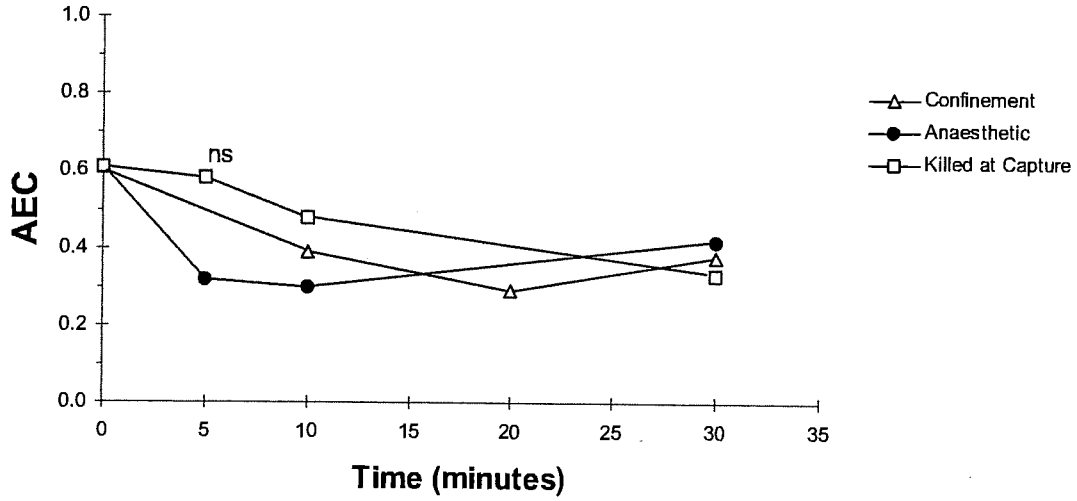


Figure 13. Liver AEC in Australian herring subjected to various treatments immediately after capture from the wild. All points, except that shown by 'ns', were significantly different from the initial value. Error bars have been omitted for clarity

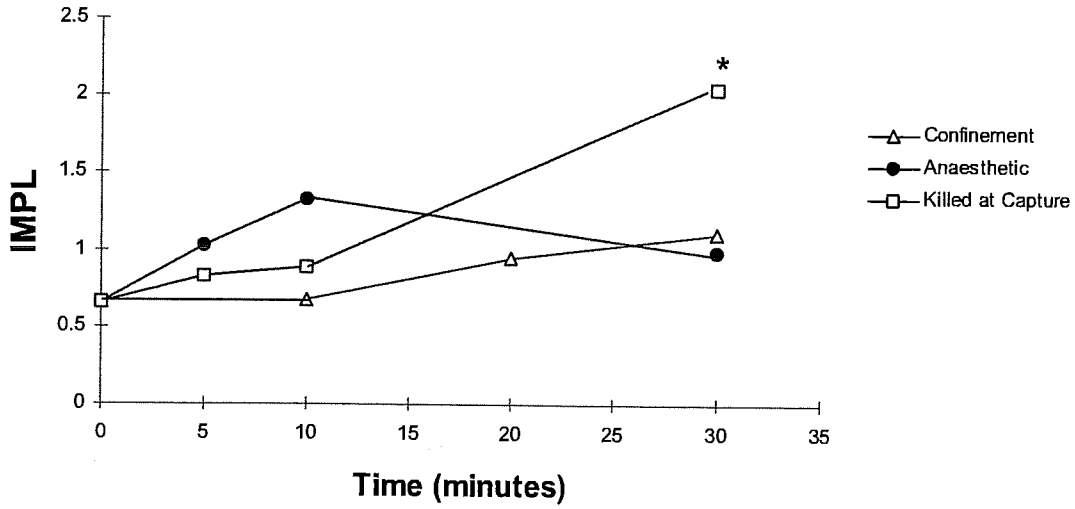


Figure 13a. Liver IMPL in Australian herring subjected to various treatments immediately after capture from the wild. The point accompanied by the asterisk was significantly different from the initial value. Error bars have been omitted for clarity.

Benefits

This was a feasibility study to develop and evaluate techniques that will be applicable to a wide range of aquaculture industries in which the health of farmed species may be periodically assessed. This includes fish and mollusc aquaculture industries in all Australian states.

The biochemical measures described here may allow detrimental effects on farmed species to be detected at an early stage. This will permit remedial steps to be taken to reduce stress or to harvest aquaculture stocks before their condition deteriorates and reduces their market value.

This project also describes an approach in which surrogate species, cohabiting in nets and pens with aquaculture stocks, may be used as indicator organisms to predict detrimental effects in target species. This reduces losses of aquaculture stock in sampling and analysis procedures.

Further development

The variability in the results of this feasibility study were in part a consequence of a variety of factors beyond the control of the investigators. Most notable of these was the high tuna mortality in April 1996 which interrupted sampling programs and may have influenced the parameters under investigation. However, the data appear promising in terms of the establishment of surrogate bioindicator programs to monitor the health of farmed tuna, and possibly other species. Specific further studies should include;

- Further monitoring studies using the field protocols of this feasibility study as a model. They should concentrate on establishing the specific relationships between markers in tuna and in cohabiting species.
- A biochemical or physiological marker of tuna flesh quality must be incorporated into the experimental design to allow the objective analysis of the performance of the biochemical markers as predictors of stress responses in tuna. Current work by Finders University staff at Port Lincoln aims to investigate some of these potential parameters in tuna muscle. Combination of these 2 projects would result in exciting outcomes in this area of research.
- Analysis of biochemical markers at the Flinders University laboratories at Port Lincoln, reducing the potential for sample spoilage in transport to Adelaide.
- Environmental studies of mussel responsiveness should be better coordinated with mussels seeded onto tuna farm nets and control sites for convenient sampling throughout the tuna farming season.

The ultimate benefit of this work will rely upon identifying and quantitating the relationships between biochemical markers in surrogate species cohabiting with farmed tuna and similar markers in the tuna themselves. These biochemical markers in tuna will also need to be correlated with outcome measures more directly applicable to the tuna farming industry. Crude markers, such as market value at the time of harvesting or mortality in nets, are likely to be insensitive to the physiological changes experienced by the tuna at a time when intervention may improve fish quality. Therefore, markers of tuna quality, relating to the commercial and aesthetic status of the fish, must be explored.

Planned outcomes

The development of this approach to monitoring health of farmed tuna may be applied to a wide range of other aquaculture species. In particular, the identification of factors affecting integrity of field samples used for biochemical analysis of environmental stress has resulted in the development of improved sampling and handling protocols that are currently being applied to research projects including those investigating tuna flesh quality, environmental stresses and oyster quality, stress in prawn bycatch and stress responses in farmed mussels and abalone. This report, and associated manuscripts submitted for publication in the scientific literature will provide a valuable resource to investigators in this area of research.

Wider dissemination of the findings of this report, through industry publications and primary industries agencies of State and Commonwealth governments, will also lead to a better understanding by industry and fisheries managers of the usefulness of this approach to monitoring condition and health of the aquaculture species. This may be especially true of the innovative use of surrogate species as indicators of the health of the target species, reducing the need for destructive testing of harvestable resource.

This project has identified specific issues that must be considered in a surrogate monitoring program, including verification of the capacity of the biomarkers in surrogate species to predict health outcomes in the target species. This requires understanding of interindividual and seasonal variations in target species and their influence through appetite, condition factor, stocking density and other factors upon the measured stress indicators. Similar seasonal and individual changes in the surrogate species must also be investigated.

Conclusion

This project aimed to compare biochemical methods of assessing the effects of pollutant stress in farmed southern bluefin tuna with methods currently used. Proposed methods included the measurement of markers of tissue energy balance (tissue adenylates, adenylate energy charge, IMP load and ATP/IMP ratios) and Na^+/K^+-ATPase in samples collected from tuna. It was observed early in the project that the use of capture-sample-release methods for monitoring health markers in tuna was labour intensive, time consuming, and stressful for sampled tuna, with a consequent risk of adverse quality outcomes. Destructive sampling of tuna, where samples were collected from fish that were then processed for market was also seen as undesirable, since sufficient tuna samples were required to gain a representative estimate of stress levels with a corresponding negative impact on yield from the tuna nets. While these sampling procedures may be coordinated with harvesting operations from May/June each season, this coordination is unlikely during the early pre-harvest part of the season.

The second aim was to evaluate methods of sampling fish or fish tissues which will reduce the observed handling stress and improve the predictive capacity of samples used to estimate farmed tuna health and stress levels. This subsidiary aim was partly successful, as the method of tuna tissue sampling was refined to reduce the time tuna were handled prior to pithing and tissue sample collection.

The major aim of the project was to evaluate the use of the biochemical responses of other species (e.g. Australian herring - *Arripis georgianus*, blue mussel - *Mytilus edulis*, jack mackerel - *Trachurus declevis*) which cohabit with tuna in farm pens as surrogate indicators of stress experienced by tuna. These would then provide an inexpensive pool of subject animals, in which

stress biomarkers would predict adverse impacts in tuna. This was achieved by demonstrating that changes in AEC, IMPL and ATPase in cohabiting fish species correlated with these same biomarkers in tuna. Within this part of the project, a HPLC method for simultaneous analysis of adenylates (AMP, ADP, ATP), IMP and their metabolic precursors in fish and shellfish tissues was refined and will provide a useful and inexpensive biochemical marker of stress responses in a range of species.

References

1. Ivanovici, A.M. 1980. The adenylate energy charge in the estuarine mollusc, *Pyrazus ebeninus*. Laboratory studies of responses to salinity and temperature. *Comparative Biochemistry and Physiology* **66A**:43-55.
2. Hamann, M., Boxall, V.A., Edyvane, K.S., Edwards, J.W. and Soole K.L. 1999. Biochemical and cytogenetic biomarkers of pollutant exposure in marine fish (*Aldrichetta forsteri* Valenciennes and *Sillago schomburgkii* Peters) near industrial and Metropolitan centres in South Australia. *Biomarkers* **4**:290-302
3. McCormick, S.D. 1993. Methods for non-lethal gill biopsy and measurement of Na,K-ATPase activity. *Canadian Journal of Fisheries and Aquatic Sciences* **50**:656-658.
4. Caldwell, R.S. 1974. Osmotic and ionic regulation in decapod crustacea exposed to methoxychlor. In '*Pollution and Physiology of Marine Organisms*'. Vernberg *et al.* editors. Academic Press, New York. 1974. 197-223.
5. Haya, K. and Waiwood, B.A. 1983. Adenylate energy charge and ATPase activity: Potential biochemical indicators of sublethal effects caused by pollutants in aquatic animals. In '*Aquatic Toxicology*'. Nraigu, J.O. editor. Wiley and Sons, New York. 1983. 307-333.
6. Caldwell, C.A. and Hinshaw, J.M. 1994. Nucleotides and the adenylate energy charge as indicators of stress in rainbow trout (*Oncorhynchus mykiss*) subjected to a range of dissolved oxygen concentrations. *Comparative Biochemistry and Physiology* **109B**:313-323.
7. Erikson, U., Sigholt, T. and Seland, A. 1997. Handling stress and water quality during live transportation and slaughter of Atlantic salmon (*Salmo salar*). *Aquaculture* **149**:243-252.
8. Stocchi, V., Cucchiarini L., Canestrari, F., Piacentini, M.P. & Fornaini,G. 1987. A very fast ion-pair reversed-phase HPLC method for the separation of the most significant nucleotides and their degradation products in human red blood cells. *Analytical Biochemistry* **167**:181-190
9. Tucker, R.K. & Matte, A. 1980. In vitro effects of cadmium and lead on ATPases in the gill of the rock crab, *Cancer irroratus Bulletin of Environmental and Contamination Toxicology* **24**:847-852.
10. Erikson, U., Beyer, A.R. and Sigholt, T. 1997. Muscle high-energy phosphates and stress affect k-values during ice storage of Atlantic salmon (*Salmo salar*). *Journal Food Science* **62**:43-47.
11. Paterson, B.D. 1993. The rise in inosine monophosphate and l-lactate concentrations in muscle of live penaeid prawns (*Penaeus japonicus*, *Penaeus monodon*) stressed by storage out of water. *Comparative Biochemistry and Physiology* **106B**:395-400.
12. Greenaway, P. 1991. Nitrogenous excretion in aquatic and terrestrial crustaceans. *Mem. Qld. Mus.* **31**:215-227.
13. Krumschnabel, G. and Lackner, R. 1993. Stress responses in rainbow trout *Oncorhynchus mykiss* alevins. *Comparative Biochemistry and Physiology* **104A**:777-783.

14.Westcott, N. 1996. Honours project. School of Biological Sciences, Flinders University, Adelaide South Australia

15.Baker, J. 1998. Honours project. School of Biological Sciences, Flinders University, Adelaide South Australia

Appendix 1. Intellectual Property

The value of intellectual property of this project rests in the strategy and methodology of monitoring aquaculture species' health using a combination of biochemical indicators and surrogate species. The generation of intellectual property and uses for the strategies developed during the project will be assessed in future research.

Appendix 2. Staff engaged in the project

Principal Investigator

Dr. Kathleen Lydia Soole
Senior Lecturer, School of Biological Sciences
Flinders University of South Australia
GPO Box 2100
ADELAIDE SA 5001
email: kathleen.soole@flinders.edu.au

Co-Investigators

Dr. John William Edwards
Senior Lecturer, Department of Environmental Health
School of Medicine
Flinders University of South Australia
GPO Box 2100
ADELAIDE SA 5001
email: john.edwards@flinders.edu.au

Mr. Steve Clarke
Senior Research Officer, SA Research and Development Institute
PO Box 120
HENLEY BEACH SA 5022
email: clarke.steven@pi.sa.gov.au

Dr. Patrick Hone*
Senior Research Officer, SA Research and Development Institute
PO Box 120
HENLEY BEACH SA 5022
* Now at
Fisheries Research and Development Corporation
PO Box 222
DEAKIN WEST ACT 2600
email: phone@frdc.com.au

Dr. John Carragher
Lecturer, School of Biological Sciences
Flinders University of South Australia
GPO Box 2100
ADELAIDE SA 5001
email: john.carragher@flinders.edu.au

Other Participants

Ms. Trish Shields
Research Assistant, School of Biological Sciences
Flinders University of South Australia
GPO Box 2100
ADELAIDE SA 5001
email: patricia.shields@flinders.edu.au

Ms. Kirsten Rough
Tuna Boat Owners Association of Australia
PO Box 1146
PORT LINCOLN SA 5606
email: rough.km@pl.camtech.net.au

Mr. Nigel Westcott
Honours Student, School of Biological Sciences
Flinders University of South Australia
GPO Box 2100
ADELAIDE SA 5001

Mr. Jeremy Baker
Honours Student, School of Biological Sciences
Flinders University of South Australia
GPO Box 2100
ADELAIDE SA 5001

Ms. Jolanta Ciuk
Research Assistant, Department of Environmental Health
School of Medicine
Flinders University of South Australia
GPO Box 2100
ADELAIDE SA 5001