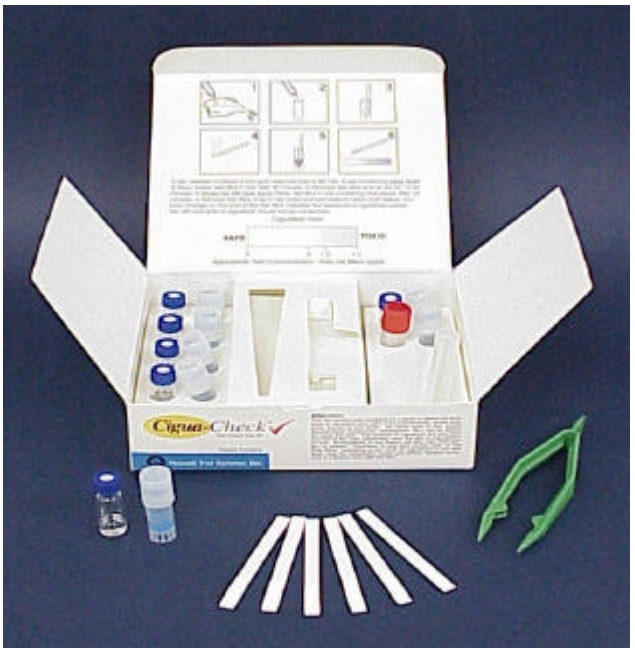
# Evaluating effective quality monitoring methods for the Australian seafood industry

**Sue Poole and Steve Slattery**

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# Evaluating effective quality monitoring methods for the Australian seafood industry

**Sue Poole and Steve Slattery**

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# Non-Technical Summary

**1999/358 Evaluating effective quality monitoring methods for the Australian seafood industry**

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**OBJECTIVES:**

1. Benchmark existing test kits for quality assessment appropriate to fish and prawns
2. Assess the effectiveness of different techniques for measuring quality within fish and prawn processing environments
3. Document measures of fish and prawn quality which can be assessed by test kits within the processing environment
4. Disseminate the information obtained to industry in the form of workshops, printed materials and electronic format

**NON TECHNICAL SUMMARY:**

**OUTCOMES ACHIEVED TO DATE**

A variety of rapid kit methods, both microbiological and chemical, have been evaluated with respect to their accuracy for and application to the Australian seafood industry. The information is now readily available for industry managers to base operational processing decisions on.

A few kits demonstrated value for monitoring total microbial contamination levels within day to day processing line operations. Additionally, two kits were successfully applied within industry for measurement of sulphite and histamine residual levels. However, most kit methods were inappropriate in functional design or provided unreliable results which negate their usefulness for industry application. The information gained is valuable for industry to base business management decisions on. In line with this, summary précis for each rapid test kit evaluated will be available through the Seafood Services Australia website.

Industry response to the sulphite and histamine kits was very positive and several seafood processors have indicated they are using the histamine kit. Additionally, negotiations are occurring with AQIS to have the histamine kit accepted by them as a standard method of testing.

**KEYWORDS: quality measures; test kits; rapid methods; seafood; fish; prawns**

There has always been a need to measure the level of bacteria present within the seafood processing environment. The recent introduction of food safety plan and regulations has strengthened the need for seafood processors to monitor processing procedures. Since the costs of routine sampling and testing by independent analytical laboratories can be excessive, there is an opportunity for seafood processors to monitor microbial levels within processing lines in-house using commercially available test kits. This research evaluated available kits with test samples from the Australian seafood processing chain to determine their appropriateness for use within the seafood industry.

Test kits are commercially available for many microbiological analyses relevant to seafood safety and quality: total bacterial count; coliform count; *Escherichia coli, Salmonella* spp., *Staphylococcus aureus, Listeria monocytogenes.* We evaluated several kits for each of these analyses against samples of fish and prawns, as well as processing surface samples. Kits were also assessed with both pure culture and mixed culture samples.

Of the range of the test kits investigated in this work, the most universally applicable are those that detect total bacterial loads.. The Petrifilm range of kits give an enumeration result and are most applicable for monitoring microbial loads at any point in the processing line. Two other kits, the Environcheck kit and the Hygiene Test kit, provided an indication of total bacterial load by density and were useful for detecting gross contamination. Such kits would be valuable for monitoring and checking efficacy of sanitation procedures within a processing environment. For determing numbers of total coliform organisms present, the Petrifilm Coli Count plate was similarly easy to use and provided reliable estimates of coliforms present. Again, this makes this test kit very acceptable for monitoring hygiene practices within the seafood environment.

For determining specific organisms, rapid test kits are designed to detect a single target and hence provide a quick result for the absence of the organism. Many of these kits are designed to provide an answer in minutes to hours, rather than the days commonly involved with traditional methods. However, since most test kits of this nature have a pre-requirement of growing an enrichment culture from the sample taken they are inappropriate for in-factory industry use. The industry exceptions are those seafood premises that have a separate laboratory facility.

The Petrifilm *E. coli* count plate is one specific organism kit that is different. This kit detected both coliforms and *E. coli* in a one step method. This is accomplished by differentiation of the colony types by colour and gas production. While the concept is ideal for industry application, evaluations of the kit demonstrated that it is very difficult to discern between colony types.

For *Listeria*, there is a simple swab kit available that was considered to have great potential for industry application since it was purported to detect *Listeria* species without the hurdle of an enrichment step first. Despite extensive experimentation with the Transwab kit, too many 'false-positive' results were attained for industry to be able to use the kit with confidence.

The Hanna sulphite test kit was found to be accurate and reliable, although some degree of training of operators is necessary prior to use by industry. Similarly, the Alert histamine kit was simple to use and provided accurate results. Several seafood companies have indicated they will purchase this kit and application has been made to AQIS to have the kit accepted as a standard method.

The CiguaCheck kit for detecting ciguatoxin was found to be completely unreliable. False positive results were prevalent when using the kit, with 82% of randomly purchased fish samples demonstrating a positive result for ciguatoxin. The CiguaCheck kit is availble to the

general public at retail level in many Chemists along the Queensland Coast and hence it is important to extend the kit evaluation findings as widely as possible.

The information gained through this project work provides a sound base for industry to make operational business decisions on. With a few exceptions, the rapid kit methods evaluated lack sufficient sensitivity and specificity for direct testing of samples and their application to the seafood industry is very limited. This will perhaps change as further technological advances occur and are commercialised.

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- David Williams for expert assistance with the chemical analyte kits
- John Nagle and Louise Oliver for perseverance and dedication to trials with the CiguaCheck kit

The project team gratefully acknowledge the many industry participants who willingly expressed opinions and provided comment on practical application issues with respect to various kits. Many also readily volunteered time and effort in trialling commercial kits onsite at their premises and of special mention for helpful discussion and time are:

- Latitude Seafoods
- MG Kailis
- Sam's Seafoods
- Midwest Seafoods
- De Brett's Seafoods
- Cardinal Seafoods

The project team would like to extend particular thanks to those kit supply companies who gave freely of their time and effort to obtain and provide much additional information with respect to specific kits and methods.

The authors would also like to extend grateful appreciation to several Environmental Health Units within Queensland Health for their kind assistance in providing fish samples associated with ciguatera fish poisoning outbreaks.

# 1. Background

There has always been a need to measure the level of bacteria present within the seafood processing environment, from both quality and food safety standpoints. However recently, the impetus for processors to understand the critical points of potential microbial contamination along their processing lines has increased strongly due to the introduction of regulations requiring seafood businesses to have food safety plans in place. The regulatory food authority, Food Safety Australia New Zealand, is to introduce a Seafood Standard which will be incorporated under the Food Standards Code. Such regulation will require that the seafood industry meet specific standards for food handling, storage, processing premises and equipment and industry will therfore need cost effective ways of testing within the processing environment to comply with these standards.

The drive towards implementation of food safety plans at all levels within the seafood industry is supported strongly by many initiatives of Seafood Services Australia including The Seafood Industry's Strategic Plan for Achieving Seafood Excellence; a Risk Assessment of the Australian Seafood Industry; development of a seafood standard through the Seafood Industry Network; and a series of 'how to' choosers to assist industry develop, implement and manage food safety plans for their individual business.

Many seafood operators within the industry are choosing to follow the hazard analysis critical control point (HACCP) system for ensuring the safety of their product and to meet food code accreditation standards. HACCP is a fairly comprehensive plan and demands a thorough knowledge of significant micro-organism contamination and growth potential, as well as a strict monitoring of processing procedures. Hence, implementation of a HACCP system requires a large amount of microbiological testing in the initial stages to assess the effectiveness of sanitation programs, establish the quality of in-coming seafood product, ensure re-contamination of product does not occur and to assure an excellent quality end-product.

Microbiological testing is seldom considered effective for monitoring critical control points during processing due to the time delay inherently involved with traditional culturing methods (NACMCF, 1992) and due to the difficulties of establishing a sampling routine to detect organisms that occur with low numbers and frequency (Kvenberg and Schwalm, 2000). Hence, this indicates the need for rapid testing within quality control programs. The evaluation of the application of rapid test methods to such monitoring was discussed by Stier (1993) who concluded that while the rapid methods are not appropriate for on-line monitoring, they were useful for establishing a quality program, for risk assessment and for assessing cleaning efficacy. Kvenberg and Schwalm (2000) also emphasised the usefulness of rapid test methods for checking the effectiveness of sanitation programs.

The impetus and development of rapid test techniques and kits has come largely from the clinical field of microbiology to automate and fast-track medical diagnosis. Hence, the characteristics of a micro-organism used as the basis of the developed test are relevant to those organisms isolated from clinical specimens and may not necessarily be the same as characteristics present in marine, food or environmental isolates (see review by Austin, 1988). Several researchers have conducted comparative evaluation studies on the developed kits for the medical laboratory application (Holbrook *et al*, 1989; Geiss, 1990; Bouvet and Jean, 1992; O'Hara *et al*, 1992; Banffer *et al*,1993; Hansen and Freney, 1993; Kerr *et al*, 1993; O'Hara *et al*, 1993; Orden *et al*, 1993; Kitch *et al*,1994; Manafi and Willinger, 1994;) and these have also included work with food isolates (Adams and Hope, 1989; Curiale *et al*, 1990; Wieneke, 1991; Bille *et al*, 1992; Fung *et al*, 1984; Fung,1994 and 1995; Patel, 1994). However, it is noted that environmental isolates live under different

selective pressures than clinical ones and hence are more likely to give unusual biochemical reactions that would make them difficult to identify with medical diagnostic bacterial kits (Palmieri *et al*, 1988; Walch *et al*, 1993,) Additionally, it is widely recognised that bacteria within environmental samples can be viable but often non-culturable and this aberration is particularly true within aquatic environments.

Since rapid testing methods have become more widely used, there have been a great number of investigations evaluating various test kits to determine whether their use is appropriate within the food industry. The focus was initially on the dairy industry but has extended to other foods commodities, including meat. As an increasing number of rapid kit methods became commercially available, there was a corresponding number of comparative studies carried out, assessing the efficacy of different kits for different food commodities and applications (for example: Ginn *et al*, 1984; Smith *et al*, 1985; Ingham and Moody, 1990; Chain and Fung, 1991; Cormier *et al*, 1993; Beuchat *et al*, 1998).

Slabyj and Bolduc (1987) were some of the first researchers to look at the applicability of the kits to seafood processing operations and they focussed on the total count of bacteria present at in-line points assessed by several different test methods. Others have studied contamination by specific bacterial species within processing factories (Miettinen, 2001). The applicability of rapid tests for detecting specific pathogens with respect to fish and seafood products has been reviewed by Pearson and Dutson (1994).

A large multi-nation resesarch endeavour, The European Community Fair Programme, has focussed on describing the parameters which can be used to determine the freshness of fish and ways to measure these. Information forthcoming outlines the fast and objective analytical methods which are useful to evaluate fish freshness, but does not provide any information on any commercial test kits that are available to industry.

While some of the rapid test kits available commercially appear to have potential for application to the seafood processing industry, given the inconclusive and sometimes contrary findings of the above researchers, it was not possible to assume that similar kits would function equivalently to findings reported in the literature. Hence, the focus of the research reported here was to evaluate commercially available kits with samples from the Australian seafood processing chain to determine their appropriateness for use.

## 2. Need

The need for effective control and monitoring of safety is obvious following the crisis of confidence caused by the recent viral outbreak which sent shockwaves throughout the seafood industry. Test kits allow industry to control the quality assurance process rather than just follow the recommendations of consultants. They also allow industry to decide the type of processing required and the end use of the products.

The new statutory requirements for food safety plans and the increasing adoption of formal quality management systems, necessitate the development of quality measures that are relevant and quantifiable. For industry to implement quality assurance programmes, there is an essential need for tools by which to monitor the systems. Rapid test kits provide these tools, but are they appropriate and effective for the Australian seafood processing environment? This knowledge is crucial to successful adoption of programme initiatives.

AFFS-Food Technology conducts courses specifically for the seafood industry, on developing HACCP-based food safety plans and it is noted that a frequently raised issue from industry is "What testing procedures do I use to confirm practices have been carried out correctly?" and concern centres round “It’s going to cost me a fortune!”. Hence the industry does have an immediate need for appropriate and effective means for monitoring their handling and processing practices. The need for the research work in this project is further evidenced by feedback from a number of companies who have stated that they found some of the kits promoted by suppliers were not reliable. Others, such as M.G. Kailis, currently use rapid test kits but do not have the facilities to evaluate their reliability.

The one aspect that underlines the success of a quality assurance system is the accuracy and reliability of the test methods being used. Currently there is no pertinent information available directly applicable to the Australian seafood processing industry, to assist in decisions for monitoring their processing practices.

# 3. Objectives

1. Benchmark existing test kits for quality assessment for Australian Seafood species.
2. Assess the effectiveness of different techniques for measuring quality in the Australian processing environment.
3. Document standards for seafood quality in the processing environment which can be assessed by test kit.
4. Disseminate the information obtained to industry in the form of workshops, printed materials and electronic format.

# 4. METHODS

## 4.1 Selection of test kits for use

A comprehensive and exhaustive survey for all commercially manufactured test kits and methods was undertaken and tabulated. The search included:

- Manufacturing companies
- Laboratory supply companies
- Internet searches
- Published literature review
- Technical bulletins
- Food safety regulatory bodies, such as Food and Drug Administration (FDA), United States Department of Agriculture (USDA), Internation Commission for Microbiological Safety of Foods (ICMSF), Food Safety Australia New Zealand (FSANZ)
- Direct communication with microbiology laboratories

From discussions amongst seafood processors, scientists and project team members, a set of criteria were developed against which to rate the various kits (Table 4.1). The test kits for investigation in this work were selected on the basis of meeting the proposed criteria.

**Table 4.1. Criteria for kit selection.**

| Criterion | Scale anchors |
|---|---|
| Operator skill level required | None (1)<br>to<br>Specialised training (5) |
| Clarity of manufacturer's instructions | Simple and clear (1)<br>to<br>Ambiguous, interpretation needed (5) |
| Amount of other equipment required for kit use | None (1)<br>to<br>Specialised instrumentation needed (5) |
| Time to achieve test result | Very rapid (minutes) (1)<br>to<br>Same as for standard method (5) |
| Kit availability in Australia | Readily procurable (next day supply) (1)<br>to<br>Protracted lead time for supply (5) |
| Cost per test | Cheap (< $2 / test) (1)<br>to<br>expensive (5) |

All test kits were rated against these criteria on a 1 to 5 scale to provide an overall ranking. The ranking was used as a guide only for kit selection.

## 4.2 Seafood samples

For microbiological analyses, samples were purchased from different retail outlets ($n$ = 5) as fresh chilled product, most frequently as fillets. Some samples were procured as frozen

product. Samples were also obtained from individual operations of 2 different processing companies.

Fish species:
chilled – mullet, tailor, whiting, sea perch, mackerel, reef fish, tuna
frozen – hoki, barramundi
Prawns:
wild-caught – green and cooked

Processing samples:
stainless cutting surfaces
rinse waters

Selected seafood products were also stored chilled (2$^{o}$C) and sampled periodically over storage time to provide test samples with increased microbial populations.

## 4.3 Microbiological sampling methods

Samples were acquired by either a swabbing technique or by excision of flesh as appropriate. Most frequently sampling was performed in duplicate at each sampling time. Where this was not possible, a single sample was taken. Samples were held on ice for transport to the laboratory and all samples were assayed within 60min of being taken.

### 4.3.1 Swabbing technique

A sterile template, 10 cm$^2$, was used to designate an area for sampling. Swabbing was done with sterile cottonwool applicator swabs (Biolab transwab). The swab was used dry when sampling a wet surface, but pre-moistened in sterile 0.1% peptone diluent for dry surface sampling. After sufficient swabbing of the sample area, the swab was asceptically transferred to a sterile bottle containing 10ml of 0.1% peptone, the swab tip broken off and the bottle cap replaced. Each surface was swabbed in two adjacent areas for experimental replication.

For those test kits which contained a swab as the sampling technique, sampled sites included: bench tops; cutting boards and surfaces; knives; drains; rinse waters. Sampling was undertaken at 2 different seafood processors on multiple separate occasions, as well as the seafood processing room at the research centre whenever seafood product was being processed.

### 4.3.2 Excision technique

A sample of at least 10g was removed from the fish fillet or prawn using a sterile scalpel and tweezers. The excised sample was placed in a sterile plastic bag (Whirlpool). In the laboratory, the flesh sample was weighed and 0.1% peptone diluent added to achieve a 1:10 dilution. The total was then transferred to a sterile stomacher bag (Sarstedt) and macerated by one of two techniques (below).

### 4.3.4 Sample maceration

Samples of fish and prawn flesh were macerated in one of two ways to release any micro-organisms present in the sample and distribute them evenly throughout the liquid diluent medium.

1. Stomaching (homogenised) – the stomacher bag containing the I:10 diluted sample was placed in a Stomacher (Seward) and macerated by the paddle feet for 60 sec.

2. Hand massaging – the stomacher bag containing the 1:10 diluted sample was carefully sealed and placed on a flat surface. The flesh sample was then massaged thoroughly, either by hand movement or by use of a rolling pin (or similar) for 60 sec.

### 4.4 Microbial 'spiking' techniques

When required reference cultures of specific micro-organisms were grown overnight, under appropriate media/temperature conditions, to provide a fresh log-phase culture. This was then serially diluted and plated out to enumerate the number of organisms present. Appropriate dilutions were used to inoculate fish, prawn and surface samples. The micro-organisms were inoculated onto the flesh by a dip method, with surfaces inoculated by direct application of a known aliquot of culture to a set surface area.

For some experiments, a mixed seafood flora population was developed. This was created from the natural flora present on fish samples, isolated on to solid agar plates and then the agar surface rinsed with sterile nutrient broth. The resultant broth was grown overnight at 30$^{o}$C and plated to enumerate the numbers of bacteria present. This mixed culture was then diluted as required to provide a specific number of organisms. The mixed culture was 'spiked' with a specific bacterial species (prepared as above) to known concentration levels.

### 4.5 Microbiological enumeration methods

The conventional (standard) enumeration methods, along with the relevant commercially available test kits trialed are listed in Table 4.2. For all samples tested, bacterial counts attained were the average of a minimum of duplicate plates or kits.

**Table 4.2. The methods used to enumerate bacterial numbers present in seafood.**

| Bacterial species detected | Standard method | Test kit |
|---|---|---|
| Total aerobic bacteria: | | |
| Colony growth | AS 1766.3.5 | Environcheck TVC (Merck) [a]<br>Petrifilm (3M) |
| Protein presence | n/a [b] | Hygiene test (AMS) |
| Coliforms | AS 1766.2.3 | Petrifilm EC (3M)<br>Environcheck C (Merck) |
| *Escherichia coli* | AS 1766.2.3 | Petrifilm EC (3M)<br>Environcheck C (Merck) |
| *Salmonella* | AS 1766.2.5 | VIP *Salmonella* (Biocontrol)<br>*Salmonella* 1-2 Test (BioControl) |
| *Staphylococcus aureus* | AS 1766.2.4 | Petrifilm S.aureus (3M)<br>Dimanco BP Slides (Dimanco) |
| *Listeria* | AS 1766.2.16.1 | VIP Listeria (Biocontrol)<br>Listeria Rapid Test (Oxoid)<br>Isolation Transwab (AMS) |

[a] manufacturing company
[b] not applicable

### 4.6 Kit testing replication

Numbers of separate seafood samples used for individual tests were > 20. Any single sample was tested in duplicate, both for conventional method detection and for any particular kit used. The number of replicates for any specific test kit varied according to likelihood of potential application to the industry. Hence, evaluations of kits that detected specific foodborne pathogens and were based on immunoassays (or ELISA assays) and which required a culture enrichment step prior to use of the kit were limited. However, kits with no pre-enrichment required were evaluated with many different samples. All such kits were assessed in at least five separate situations and with samples containing microbial loads from low (<10cfu/g) ranging to high (>$10^6$cfu/g). Where storage trials of fish or prawn product were carried out, such trials were done as triplicate replications on separate occasions. It was also ensured that different kits from separate manufacturing batches were tested.

### 4.7 Data analysis

Results of microbiological testing were calculated as $\log_{10}$ colony forming units per gram or square centimetre (cfu/g or $cm^2$). Where relevant the kit result was compared directly to interpretive sheets provided by the kit manufacturer.

Where statistical analysis was pertinent, calculations were carried out within the Microsoft Excel program or by using the statistical package, Statistix 1.0.

## 4.8 Specific microbiological methods

## 4.8.1 Total aerobic bacteria

#### 4.8.1.1. Conventional method

A homogenised sample is prepared as above and appropriate dilutions made in 0.1% peptone water. One ml aliquots of the dilutions are plated in duplicate onto plate count agar medium and incubated at 30°C for 48-72h. All colonies that grow are counted.

#### 4.8.1.2. Petrifilm AC kit

All of these kits are based on the same design, with specific media changes according to bacterial species designed for. These kits are suitable for testing liquid and surface samples.

*Basic kit design:* The petrifilm kit consists of 2 plastic films hinged together on one side. The lower film is polyethylene coated paper contains a layer of solid dry nutrients in a rehydratable gelling agent and is printed with a $1cm^2$ grid to facilitate counting of colonies. The upper transparent film is polypropylene coated with indicator dye and gelling agents.

- inoculate with 1 ml of homogenised sample applied to the centre of the bottom film
- for surface samples, gently press the lower film against the surface
- gently cover with the top film and apply pressure (a specially designed plastic spreader is included with the kit).
- wait 1 minute for the gelling agents to solidify
- incubate at 30° C for 24h
- count all red colonies

#### 4.8.1.3 Enironcheck TVC kit

These kits are suitable for testing liquid and surface samples.
*Basic kit design*: consists of a plastic slide coated on both sides with solid agar medium. The slide is attached to the cap of a sterile plastic tube with a flexible joint.

- remove the cap and slide
- for testing surfaces, gently but firmly press the agar slide on the surface. Repeat with the second side of the slide.
- for liquid samples, dip the slide in the sample for 5-10 sec
- replace the slide into the tube and close tightly
- incubate at 30$^{o}$C for 48h
- compare the colony density on the slide with the pictorial density sheet ( included with the kit)

#### 4.8.1.4. Hygiene test

Kits are suitable for liquid or surface testing.
*Basic kit design*: consists of a plastic tube containing solid agar medium incorporating a red dye. A cottonwool tipped swab is attached to the tube cap.

- remove cap and swab
- for surface sampling, swab a known area (10 cm$^2$). If the area is dry moisten the swab with the sterile liquid provided in the kit
- for liquid sampling, dip the swab in the sample for 3-5 sec
- transfer swab to kit tube
- incubate 30$^{o}$C for >14h up 24h
- record colour change of medium, from red to yellow, at regular hourly intervals
- note time of the first sign of colour change and read 'Interpretation of Results' sheet provided with kit. The time taken to change colour is standardised against the equivalent number of bacteria present.

### 4.8.2 Coliforms

A control inoculum of *E. coli* and a blank control were always included in kit trials.

#### 4.8.2.1. Conventional method

A homogenised sample is prepared as above and appropriate dilutions made in 0.1% peptone water.
*Coliforms*: One ml aliquots of the dilutions are plated in duplicate onto violet red bile agar(VRBA) and incubated at 30$^{o}$C for 48h. Positive colonies show as deep red.
*E.coli*: One ml aliquots of sample dilutions are plated onto Eosin Methylene Blue agar and incubated at 44.5$^{O}$C for 24 h.

#### 4.8.2.2. Petrifilm EC kit

These kits are suitable for testing liquid and surface samples.
*Basic kit design:* The petrifilm kit consists of 2 plastic films hinged together on one side. The lower film is polyethylene coated paper contains a layer of solid dry nutrients in a rehydratable gelling agent and is printed with a 1cm$^2$ grid to facilitate counting of colonies. The upper transparent film is polypropylene coated with indicator dye and gelling agents. This kit relies on glucuronidase production for the detection of *E.coli*.

- inoculate with 1 ml of homogenised sample applied to the centre of the bottom film
- for surface samples, gently press the lower film against the surface
- gently cover with the top film and apply pressure (a specially designed plastic spreader is included with the kit).
- wait 1 minute for the gelling agents to solidify

- incubate at 30° C for 24-48h
- count all red colonies associated with gas bubbles
- *E. coli* will show as blue colonies with associated gas bubble

#### 4.8.2.3. Environcheck C kit

These kits are suitable for direct surface contact testing or for liquid samples.
*Basic kit design*: consists of a plastic slide coated on both sides with solid agar medium. The slide is attached to the cap of a sterile plastic tube with a flexible joint and contains Chromocult® coliform agar on one side of the slide.

- remove the cap and slide
- for testing surfaces, gently but firmly press the agar slide on the surface. Repeat with the second side of the slide.
- for liquid samples, dip the slide in the sample for 5-10 sec
- replace the slide into the tube and close tightly
- incubate at 30°C for 48h
- colonies show as deep red colour
- compare the colony density on the slide with the pictorial density sheet ( included with the kit)

## 4.8.3 Salmonella

#### 4.8.3.1 Conventional method

A homogenised sample is prepared as above and appropriate dilutions made in 0.1% peptone water. One ml aliquots of the dilutions are plated in duplicate onto Xylose lysine desoxycholate (XLD) and Bismuth sulphite (BS) medium and incubated at 37°C for 24h. Positive colonies show as red with a black centre on XLD and black with an black iridescence surrounding the colony in the agar.

#### 4.8.3.2 Colony isolation and enrichment

Single colonies were picked off the XLD or BS plates and isolated into Tryptose soya broth, with incubation at 37°C for 18h. Enrichment broths were prepared in Rapport medium with incubation at 37°C for 18h. One ml samples of this broth was used for the test kits.

#### 4.8.3.3 Visual Immuno Precipitate Assay (VIP) Salm*onella*

*The kit is designed to confirm the presence of* Salmonella and so requires use of an enriched culture sample.
*Basic kit design:* this is a self-contained immunoprecipitate assay, consisting of a small plastic plate with a well for application of liquid sample and windows for reading the result. After sample added, flow begins across reagent interface.

- inoculate selective medium for overnight enrichment of sample (!)
- add 0.1ml of enriched culture to sample well on kit
- wait while sample flows across kit (2-4min)
- note if blue line appears in first kit window
- observe blue line formation in second window – this is the kit test verification window confirming test completion

#### 4.8.3.4 Salmonella 1-2 Test BioControl

The kit is designed to confirm the presence of Salmonella and so requires use of an enriched culture sample.

*Basic kit design:* this is a plastic kit with two chambers, the first of which contains a peptone-based non-selective motility medium. The second chamber is the result reading window, where a positive result will show as a defined immunocaptured band of cells.

- inoculate selective medium for overnight enrichment of sample (!)
- position 1-2 test unit with black cap facing upwards
- remove cap
- remove chamber plug with sterile forceps
- add 1.5ml enriched (tetrathionate broth) sample to sample well
- reposition unit with white cap upwards and remove cap
- snip off tip under cap and discard
- add one drop of reagent #2 and replace white cap
- incubate with white cap upwards at 35°C for 14-30h
- observe chamber under strong light (preferably fluorescent) while rotating the test kit back and forth
- a positive result shows as a band in the upper half of the chamber
- is a presumptive result only

## 4.8.4 Staphylococcus aureus

### 4.8.4.1 Conventional method

A homogenised sample is prepared as above and appropriate dilutions made in 0.1% peptone water. One ml aliquots of the dilutions are plated in duplicate onto Baird-Parker medium and incubated at 30°C for 48h. Positive colonies will appear as black colonies with an associated halo surrounding them.

### 4.8.4.2 Colony isolation and confirmation

Typical black colonies were picked off the Baird-Parker plates and freshly grown in tryptose soya broth at 37°C for 18h. Individual colonies were inoculated into freshly prepared rabbit serum for the confirmation of coagulase positive *S. aureus*.

### 4.8.4.3 Petrifilm S. aureus kit

These kits are suitable for testing liquid and surface samples.
*Basic kit design:* The petrifilm kit consists of 2 plastic films hinged together on one side. The lower film is polyethylene coated paper contains a layer of solid dry nutrients in a rehydratable gelling agent and is printed with a $1cm^2$ grid to facilitate counting of colonies. The upper transparent film is polypropylene coated with indicator dye and gelling agents, incorporating thermostable nuclease reactive disc.

- inoculate with 1 ml of homogenised sample applied to the centre of the bottom film
- for surface samples, gently press the lower film against the surface
- gently cover with the top film and apply pressure (a specially designed plastic spreader is included with the kit).
- wait 1 minute for the gelling agents to solidify
- incubate at 30° C for 24-48h
- count all red or blue colonies with a pink zone associated

### 4.8.4.4 Dimanco BP Slides

These are solid agar surface contact slides, suitable for liquid or surface samples.
*Basic kit design*: consists of a plastic slide coated on both sides with solid Baird-Parker agar medium. The slide is attached to the cap of a sterile plastic tube with a flexible joint.

- remove slide from vial

- for testing surfaces, gently but firmly press the agar slide on the surface. Repeat with the second side of the slide.
- for liquid samples, dip the slide in the sample for 5-10 sec
- replace the slide into the tube and close tightly
- incubate at 30°C for 48h
- count colonies and compare with the growth pattern chart provided with the kit

## 4.8.5 *Listeria*

### 4.8.5.1 Conventional method

A homogenised sample is prepared as above and 1ml transferred into half Fraser enrichment broth, incubated at 37°C for 19h. After growth, aliquots were transferred into full Fraser for further enrichment and grown for 18h at 37°C. This broth culture was plated out on both PalCam and Oxford solid media to enumerate *Listeria* present and used as inoculum for the test kits.

### 4.8.5.2 Colony isolation and confirmation

Individual colonies were isolated onto PalCam or Oxford agars and onto Nutrient agar, grown at 37°C for 18h. Isolates were then Gram stained and motility tests at both 25°C and 37°C done. An API Listeria identification kit was used for identification of the particular isolates.

To identify bacterial species present within the Transwabs, the swab was removed and either directly spread onto a nutrient agar plate or transferred to diluent from which plates were spread. Colonies of unknown speciation were Gram stained, tested for oxidase reaction and motility. Isolates were then submitted to appropriate API identification kits, most commonly 20E and 20NE.

### 4.8.5.3 Visual Immuno Precipitate Assay (VIP) *Listeria*

The kit is designed to confirm the presence of *Listeria* and so requires use of an enriched culture sample.

*Basic kit design:* this is a self-contained immunoprecipitate assay, consisting of a small plastic plate with a well for application of liquid sample and windows for reading the result. After sample added, flow begins across reagent interface.

- inoculate selective medium for overnight enrichment of sample (!)
- inactivate 1ml of enriched sample at 100°C for 5min, cool
- add 0.1ml of inactivated culture sample to inoculum well on kit
- wait while sample flows across kit (<10min)
- note if dark line appears in first kit window – this is the kit test verification window
- observe a distinct line formation in second window within 10min – this is a positive result

### 4.8.5.4 Listeria Rapid Test

The kit is designed to confirm the presence of *Listeria* species and hence requires use of 2 selected enrichment techniques prior to sample testing.

*Basic kit design*: The Clearview listeria kit contains specific monoclonal antibbodie to flagella antigens common to *Listeria* spp. The kit consists of a plastic unit with an inoculum well and two reading windows. The sample moves through the chambers of the kit by capillary action.

- inoculate selective medium for overnight enrichment of sample (!)
- inactivate 2ml of enriched sample at 80°C for 20min, cool

- add 1.35ml aliquot to the sample well of the kit
- wait for flow through of sample (<20min)
- note if blue line appears in first kit window
- observe blue line formation in second window – this is the kit test verification window confirming test completion
- this test determines the presence of *Listeria* spp. only, it does not differentiate *L. monocytogenes*.

#### 4.8.5.5 Listeria Isolation Transwab

These kits are designed as a presumptive test for *Listeria* species and are based on an enhanced esculin medium.

*Basic kit design***:** consists of a plastic tube containing solid esculin-based agar medium. A cottonwool tipped swab is attached to the tube cap.

- remove cap and swab
- for surface sampling, swab a known area (10 $cm^2$
- for liquid sampling, dip the swab in the sample for 3-5 sec
- remove cap from medium tube and discard
- transfer swab to kit tube with cap fully in place
- incubate 37$^oC$ for up to 48h
- record colour change of medium from light brown to very dark brown or black, commencing at the butt
- any colour change is significant

Specific (additional) experimentation with these kits is specified in the results discussion section.

## 4.9 Specific chemical analyte methodology

### 4.9.1 Sulphite

A comparison of appropriate kits were tested against a range of different sulphite concentrations. The standard sulphite solutions were prepared using AR grade sodium metabisulphite dissolved in distilled water. A comparison of those kits that were suitable for testing dip solutions was undertaken against a range of different sulphite concentrations. These comparative trials were replicated with different kits at separate times. Because of the limited number of tests that were able to be carried out for some brands of kit, several batches had to be purchased.

The kits were also tested using a standard prawn dip. A 1% sodium metabisulphite dip (20L) was used to treat a 4kg batch of prawns for 30 seconds and a 3kg batch for 90 seconds. The kits listed above were used to test the dip before and after the treatment of prawns. The dip samples were then diluted 1:20 to reduce the sulphite concentration to a level suitable for the kits. Dip solutions were prepared fresh before dipping using commercially available sodium metabisulphite dissolved in tap water.

The prawns themselves were tested using the Merckoquant Sulphite Test Strips, the Alert and Boehringer Mannheim Sulphite Test kits.

All solutions and prawn flesh samples were also evaluated using the standard AOAC and NATA accepted Monier-Williams distillation method. All samples were tested in duplicate by each test kit.

#### 4.9.1.1 Standard Monier-Williams method (AOAC, 1985)

- involves complicated distillation and titration:
- place sample in reaction flask with water and acid
- reflux for 1h 45min
- titrate the collected solution to change colour from pink to yellow with a base solution
- calculate sulphite value

#### 4.9.1.2 Alert sulphite kit

This kit is designed for measuring sulphite levels on the surface of prawns. It can test 2 ranges of sulphite concentration and is based on a starch iodine indicator.

- thaw and peel frozen prawns
- add 1 drop of Activator solution (orange cap) onto unpigmented prawn flesh
- add 1 drop of Dye to same area on prawn.
- start timer
- after 1 minute note colour of area treated.
- no change in colour, no sulphite
- blue has turned to violet sulphite >10<100ppm
- no colour remains sulphite >100ppm

#### 4.9.1.3 Boehringer Mannheim kit

Sulphite is oxidised by sulphite oxidase to sulphate in the presence of oxygen:

$$SO_3^{--} + O_2 + H_2O \longrightarrow SO_4^{--} + H_2O_2$$

The hydrogen peroxide formed in this reaction is reduced by the enzyme NADH-peroxidase in the presence of reduced nicotinamide-adenine dinucleotide (NADH).

$$H_2O_2 + NADH + H^+ \longrightarrow 2H_2O + NAD^+$$

The amount of NADH oxidised in reaction 2 is equivalent to the amount of sulphite or to the amount of aldehyde chemically bound sulphite. NADH is determined by means of its light absorbance at 334, 340 or 365 nm.

Sample preparation:

*for testing prawn dip*

- centrifuge turbid dip solutions
- for removal of L-ascorbic acid (if at all necessary when >100mg L-ascorbic acid/L sample) Prawns can be treated with ascorbate but not usually in combination with meta.
- adjust 2mL to pH 5-6 with sodium hydroxide (2mol/L; measuring the volume of the added NaOH and take into account in the calculation)
- add 20 Units ascorbate oxidase (approx. 0.1 mg/mL)
- mix and incubate for 10min.
- adjust the sample to pH 7.5-8.0 with sodium hydroxide (2mol/L; measuring the volume of the added NaOH and take into account in the calculation)
- add 0.1g polyvinylpolypyrrolidone (PVPP) (only necessary for coloured extracts), stir for 1min and filter.
- add 0.1mL of the largely decolourised sample for assay.

*For testing prawn flesh*

- homogenise 100g of prawns for 30s.
- weigh approximately 5g of homogenate into 50mL volumetric flask and add 40mL redistilled water
- close the volumetric flask and incubate at 60°C for 5 min
- Shake from time to time
- cool to room temperature
- fill up to mark with redistilled water, mix and filter
- use the clear solution diluted if necessary

*Testing method*

- use contents of bottle 1 undiluted
- dissolve one tablet of bottle 2 with one mL solution of bottle 1 in a beaker or in a centrifuge tube for each assay (blank and samples) depending on the number of determinations. Use forceps for taking the tablets out of bottle 2. This results in reaction mixture 2
- use contents of bottle 3 undiluted
- use contents of bottle 4 undiluted

#### 4.9.1.4 Hanna Instruments Sulphite kit

The kit works from the basis that sulphites react with iodine under acidic conditions with starch indicator.

- dilute dip (1kg meta/100L) sample 1 in 100 using distilled water only
- transfer 5mL to a 20 mL test vessel
- add 4 drops Reagent 1
- add 4 drops Reagent 2 – mix carefully using swirling motion
- add 2 drops Reagent 3 – mix carefully using swirling motion
- add 1 drop Reagent 4 – mix carefully using swirling motion
- fill syringe fully with Reagent 5
- add Reagent 5 from syringe a drop at a time with constant mixing of the test solution, until solution turns blue
- read millilitres of solution applied and multiply by 200 to obtain mg/L $Na_2SO_3$.
- to obtain the level of sulphur dioxide multiply the reading by 101.64.

#### 4.9.1.5 Merkoquant sulphite test strips

The reaction zone of the test strip, which is impregnated with sodium nitroprusside, potassium hexacyanoferrate (II) and zinc sulphate, turns pink to brick-red depending on the concentration of sulphite ions present.

- dip the test strip into the solution to be tested for 1 second such that the reaction zone is properly wetted
- remove the test strip, shake off excess liquid and after 30 seconds compare the reaction zone with the colour scale

#### 4.9.1.6 Palintest sulphite (Tablet)

The test works by reacting sulphites with iodine under acidic conditions with starch indicator.

- dilute dip (1kg meta/100L) sample 1 in 100 using distilled water only.
- place 50mL of sample in container and seal with a cap.
- add 2 tablets of Sulphite No. 1 – mix carefully using swirling motion.
- add 1 tablet of Sulphite No. 2 HR – mix carefully using swirling motion.
- continue to add Sulphite No. 2 HR tablets until blue colour develops
- calculate concentration from number of Sulphite No. 2 HR tablets used from formula: Sodium sulphite (mg/L $Na_2SO_3$)= (N x 40) – 20.
- to obtain the level of sulphur dioxide multiply the calculation by 0.5082.

#### 4.9.1.7 Palintest sulphite (photometer)

Sulphites react with an indicator dye under buffered conditions.

- place 10mL of sample in glass test tube and seal with a cap.
- add 1 tablet of Sulphitest No. 1.
- crush and mix carefully using swirling motion.
- add 1 tablet of Sulphitest No. 2.
- crush and mix carefully using swirling motion.
- cap the tube and stand for 2 minutes.
- take the test reading using a Photometer at 570nm.
- sulphite concentration can be obtained by comparing % transmittance measurement from supplied chart (mg/L $Na_2SO_3$) Sulphite concentration (mg/L $SO_3$) = (mg/L $Na_2SO_3$) x 0.63

#### 4.9.1.8 Titrets sulphite kit

The test method employs an iodide-iodate titrant in an acid solution and a starch indicator.

- dilute dip (1kg meta/100L) sample 1 in 100 using distilled water only.
- fill sample cup with 20 mL of sample.
- add 5 drops of A-9600 Neutralizer solution.
- stir briefly and wait 30 seconds.
- push a valve assembly onto the Titret ampoule tip until it fits snugly (the valve assembly should reach the reference line on the neck of the ampoule)
- gently snap the tip of the ampoule at the score mark.
- lift the control bar and insert the Titret assembly into the Titrettor.
- hold the Titrettor with the sample pipe in the sample and press the control bar firmly, but briefly, to pull in a small amount of sample. (Never press the control bar unless the sample pipe is immersed in the liquid)
- the contents will turn a deep blue colour. Wait 30 seconds.
- with the sample pipe in the sample, press the control bar again briefly to allow another small amount of sample to be drawn into the ampoule.
- after each addition, rock the entire assembly to mix the contents of the ampoule. Watch for a colour change from blue to colourless.
- repeat steps 9 and 10 until a permanent colour change occurs.
- when the colour of the liquid in the ampoule changes to colourless, remove the ampoule from the Titrettor. Hold the ampoule in a vertical position and read the scale opposite the liquid as mg/L of sulphite ion.
- to obtain the level of sulphur dioxide multiply the reading by 0.8.

### 4.9.2 Histamine

There is only one kit available in Australia, the Alert Histamine screening kit. The kit is intended for the qualitative analysis of histamine in scombroid species of fish, such as tuna, tailor and mahi-mahi. The test kit is designed for use by quality control personnel and others familiar with histamine analysis in fish.

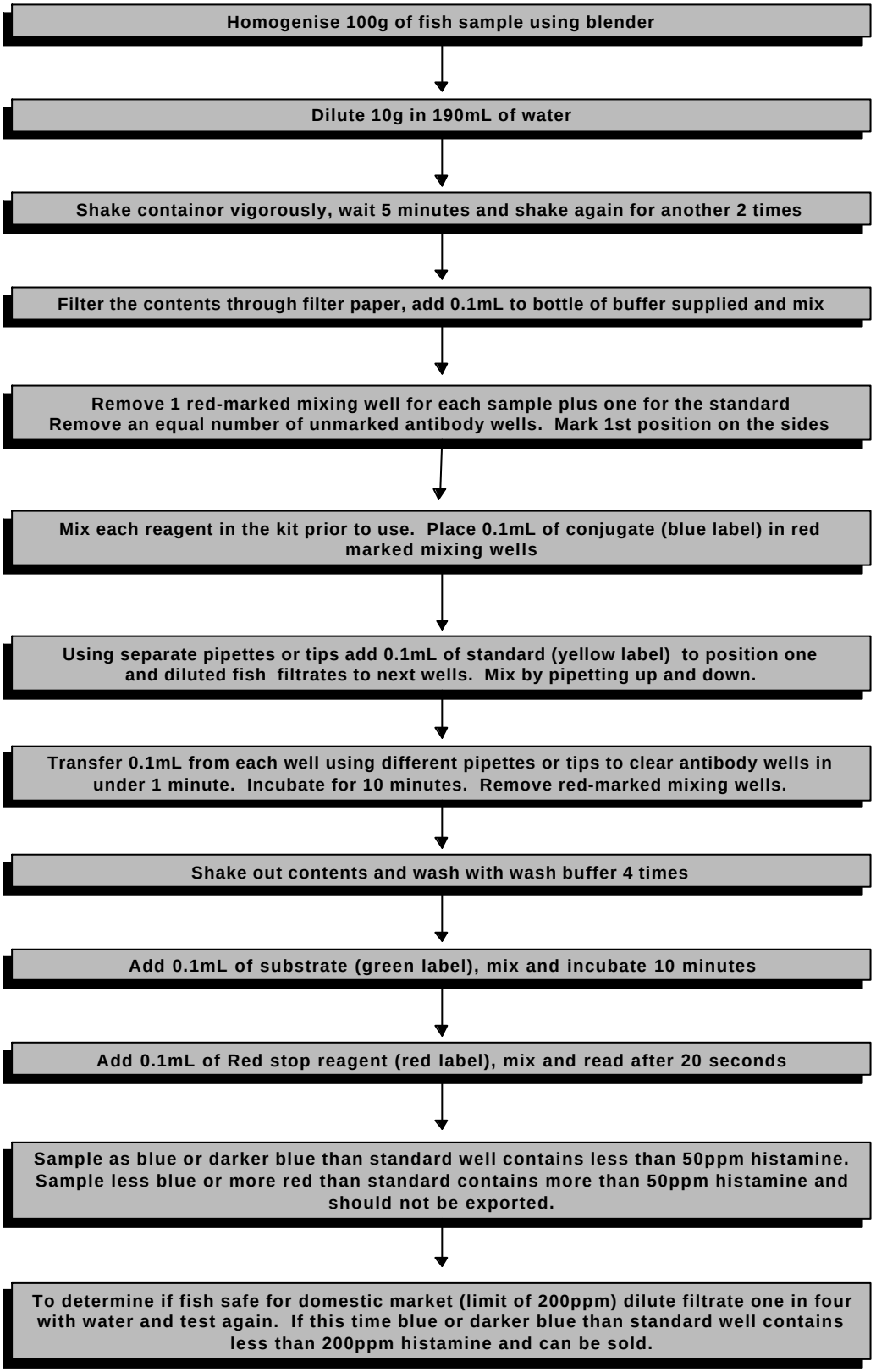
The kit is a competitive direct enzyme-linked immunosorbant assay (CD-ELISA). Histamine is extracted from the sample as a water-based solution. It is filtered and diluted into a buffer solution supplied. The histamine in the buffered solution and a control (a solution of known histamine concentration) compete with enzyme-labelled histamine (conjugate) for the antibody binding sites in the wells. A blank containing no histamine is also tested during the

run. After a wash, substrate is added, which reacts with the bound enzyme conjugate to produce a blue colour. The colour of the sample is visually compared to the colour of the control. If the sample has more blue than the control, it contains less histamine than the control. If the sample contains less blue colour (or more red) than the control, it contains more histamine than the control.

If a result is required in mg/kg histamine then the wells are placed in a Microwell Plate Reader. Several concentrations of control will also be required to produce an integration curve based on the log/log linear relationship of the Optical Density at 650nm. The plate reader should be blanked on air. Most plate readers have programs that integrate the control results and report the test result for samples as a concentration. The flow chart showing the steps involved to test a fish sample.
Two whole albacore tuna were obtained from industry. One fish was refrigerated overnight (Fish A) while the other (Fish B) was temperature abused by being left in the sun for 4 hours and then refrigerated overnight. Removing the loins and excising the darker coloured muscle produced two flesh samples. The samples were then blended to a fine paste. The connective tissue was removed as the samples were blended. Subsamples from each fish were then spiked with enough histamine in solution to produce concentrations that were 0, 10, 20, 40, 60 and 100 mg/kg higher than the initial concentration. Unfortunately there was nothing added to stabilise the histamine so these concentrations dropped during the blending, freezing and thawing process the samples were exposed to.

These subsamples then divided into four batches and frozen. One batch of subsamples were sent to the Australian Government Analytical Laboratory for histamine analysis using Capillary Electrophoresis. The method used was adapted from Mopper and Sciacchitano (1994). At the time of the tests this was the only laboratory routinely testing for histamine. Three individual kits were used to test each batch of spiked subsamples. The Alert histamine test kit has been designed levels above or below 50mg/kg.

# Alert Histamine Test Procedure



## 4.10 Ciguatoxin analyses

A commercially manufactured kit, CiguaCheck, from OceanIT was evaluated. It was envisaged that testing with the kit would be run in parallel with an established ciguatoxin analysis method, however this was not possible. Fish samples used were different from those mentioned above in this section for other kit work and hence are specified in the results section.

Each CiguaCheck kit contains test vials for 5 separate tests, a positive and negative control. The test kits were stored at $4^0$C in a chiller room.

- use a biopsy punch to remove a small rice-grain-sized sample of flesh from the fish underneath the skin
- take care not to touch the sample with your fingers
- put the fish sample into the bottom of the vial containing the clear liquid
- remove a Test Stick from the long tube and place it, paper-covered end down, into the vial containing the clear liquid and the fish sample for 20 minutes
- clean tweezers with water and return to holding slot in kit
- remove the Test Stick and allow it to thoroughly air dry for 15 minutes
- do not touch white paper-covered end of the stick with hands or fingers
- gently shake vial with blue liquid to mix solution
- open and place the dry Test Stick (paper-covered side down) into the vial containing the blue liquid solution
- wait for 10 minutes, remove the Test Stick and gently rinse in tap water
- compare the colour on the end of the Test Stick to the colour strip provided on the inside box lid.
- any colour change on the Test Stick indicates that the fish contains ciguatera poison and should not be eaten.
- the darker the colour the more poison the fish contains

# 5. RESULTS AND DISCUSSION

Traditional methods for detecting the presence of micro-organisms rely on lengthy culturing techniques of growing the organisms in or on culture media with several days incubation before the colonies are countable. To determine the presence of specific bacteria, the incubation phase is often followed by isolation and further biochemical identification. The full method can be expensive in time, labour and media used. Recent advances in technology and knowledge of organism characteristics have allowed the development of 'short-cut' methods to determine the presence of some organisms. These new methods are often referred to as 'rapid methods'. However, this is a rather subjective term as not all rapid methods are quicker to achieve a result than the traditional methods as many often still involve a similar incubation period for the bacteria to grow.

Rapid methods, such as test kits, are generally used as screening techniques. Negative results, implying the absence of the organism being tested for, are usually accepted as is but positive results from a rapid test method often require confirmation using standard official methods (Feng, 1996; FDA, 2001). For strict adherence to Australian Quarantine Inspection Service (AQIS) export requirements and to import regulations in overseas countries, it should be noted that many of the currently available test kit methods have not been validated and hence are not recognised, nor officially approved, for testing for the specific micro-organism.

## 5.1 Selection of kits

A list of commercially available kits was compiled by sourcing information from:

- manufacturing companies
- laboratory supply companies
- internet searches
- published literature review
- technical bulletins
- food safety regulatory bodies, such as FDA, USDA, ICMSF, ANZFA
- direct communication with microbiology laboratories

A huge number of test kits are available commercially for both specific micro-organisms and for determining the total number of micro-organisms. However, despite the large number apparently existing, closer inspection notes that most kits are based on same or similar underlying principles of test method.

Rapid methods can be grouped according to the assay principle on which the test is based. The main groups are:

- Modified traditional methods – based on conventional agar with additional indicators, inhibitors, selective chemicals
- Miniaturised biochemical kits – based on same selective media but usually carried out in micro-well plates
- ATP based – measures total ATP present, dead or living, costly initial outlay
- Antibody-based kits – based on single micro-organism target, form antigen –antibody complex, very sensitive
- DNA based – requires sophisticated technique and instrumentation

Such a large number of test options can be confusing and rather overwhelming to a user, but it is worth repeating that although there are many individual kits are available, most work on very similar principles and hence the number is realistically reduced. The test kits focussed on in this work are those that seemed likely to be appropriate for use within a seafood processing operation, based on selection criteria (Table 5.1). Evaluations of rapid methods show that some perform better with some foods than others and this is attributed to interference by different food components.

**Table 5.1. Criteria used for determining suitability of test kits.**

| Criterion | Scale anchors |
|---|---|
| Operator skill level required | None (1)<br>to<br>Specialised training (5) |
| Clarity of manufacturer's instructions | Simple and clear (1)<br>to<br>Ambiguous, interpretation needed (5) |
| Amount of other equipment required for kit use | None (1)<br>to<br>Specialised instrumentation needed (5) |
| Time to achieve test result | Very rapid (minutes) (1)<br>to<br>Same as for standard method (5) |
| Kit availability in Australia | Readily procurable (next day supply) (1)<br>to<br>Protracted lead time for supply (5) |
| Cost per test | Cheap (< $2 / test) (1)<br>to<br>expensive (5) |

The range of test kits can be readily categorised into groups according to the principles of design and operation of the test. After gathering detailed information about each test kit, the kits were ranked against the selection criteria. It was considered that several groups were automatically knocked out with respect to practicality for the seafood industry due to either complication of method (skilled technical expertise or expensive instrumentation required for operation) or expense of unit (initial outlay and per test). This reduced the options to three groups, any kit of which seemed to have potential for direct use within industry.

# 6. INDUSTRY APPLICATION

One of the aspects universal for use of any rapid kit method within industry is the need for prior sample preparation. Aspects for industry operations directly related to this step were investigated to ensure that methods are as practical as possible. The aspects included here were driven by industry comments and feedback received in direct discussions with various seafood processing stakeholders.

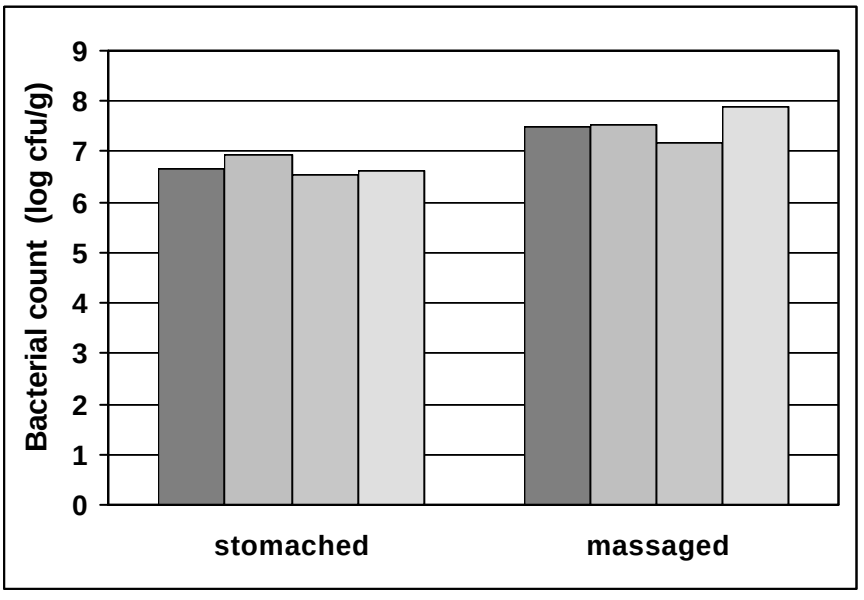
## 6.1 Sample preparation

For samples of seafood product, the usual microbiological laboratory practice to distribute the microbial load to be determined evenly throughout the sample is by homogenising the sample in a Stomacher machine or by using specific homogenising instrument (Waring Blendor or Bamix). While homogenisers are readily available to industry (for purchase), there remains an issue of ascepsis between samples requiring that instruments used need to be sterilised before use. This imposes restrictions for industry and applicability of kit use.

Alternative methods for distributing the microbial load within a sample were investigated. A simple massaging of sample with sterile diluent in a sterile plastic bag by use of a rolling pin (or similar) or finger massaging for a set time was standardised. There is ready availability of sterile bags and diluent for industry to acquire.

Comparing the two sample preparation methods, results showed that very similar counts were achieved (Figure 6.1) and value differences were within acceptable standard error for microbiological enumerations

**Figure 6.1. Bacterial counts attained from stomaching and massaging different fish species.**

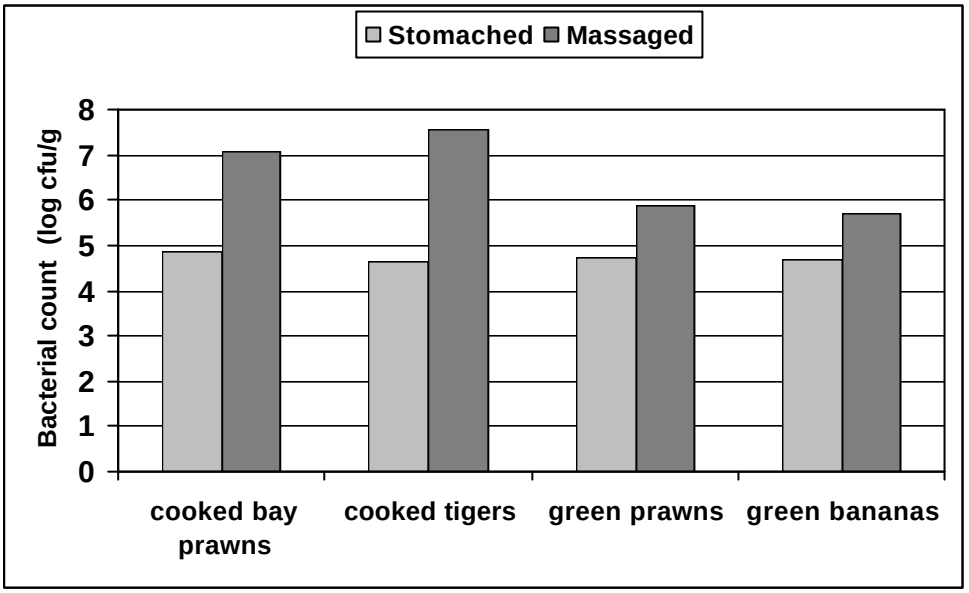



These findings are in agreement with Cormier *et al* (1993) who found good correlation between homogenisation and massaging methods. However, Slabyj and Bolduc (1987) found that massaged samples always provided a lower enumeration of bacterial load (34-77% recovery rates) than homogenised samples, although these workers selected the

massage method for further research due to practical considerations within a processing plant.

In some instances, especially with samples of cooked prawns, massaging often gave higher counts compared to that of stomaching (Table 6.1). This phenomenon can be attributed to the fact that the greater proportion of the total bacterial load carried on a prawn will be borne on the shell or surface of the prawn. Hence, including full cross-sections of the prawn in the sample as occurs with the standard method for stomached samples, slightly reduces the bacterial load according to weight to surface area ratio of the sample taken.

**Table 6.1. Bacterial counts attained from stomaching and massaging different prawns.**




The results from these trials demonstrate that specialised blending equipment is not required to achieve accurate results when using rapid kits within a factory situation.

### 6.2 Swab methods for sampling

The availability and use of different swab types were investigated, as this method of sampling is applicable universally, even for very small operations and is particularly appropriate for processing surface sampling. Several types are available, varying on swab tip material component. Many are adaptable in use by incorporating appropriate/specific liquid medium into the sterile swab holder tube.

Comparative trials were not undertaken within this project as there are many published accounts and reviews discussing the efficacy of swab sampling compared to excision methods (Favero *et al*, 1968; Lee and Fung, 1986; Dorsa *et al,* 1996; Gill, *et al*, 2001).

### 6.3 Sample dilution

Dilutions of the primary prepared sample are usually required for seafood product samples , as well as for samples that are suspected to contain high loads of bacteria. Experiments were conducted with a range of reduced volumes of sample delivered to or onto the kit.

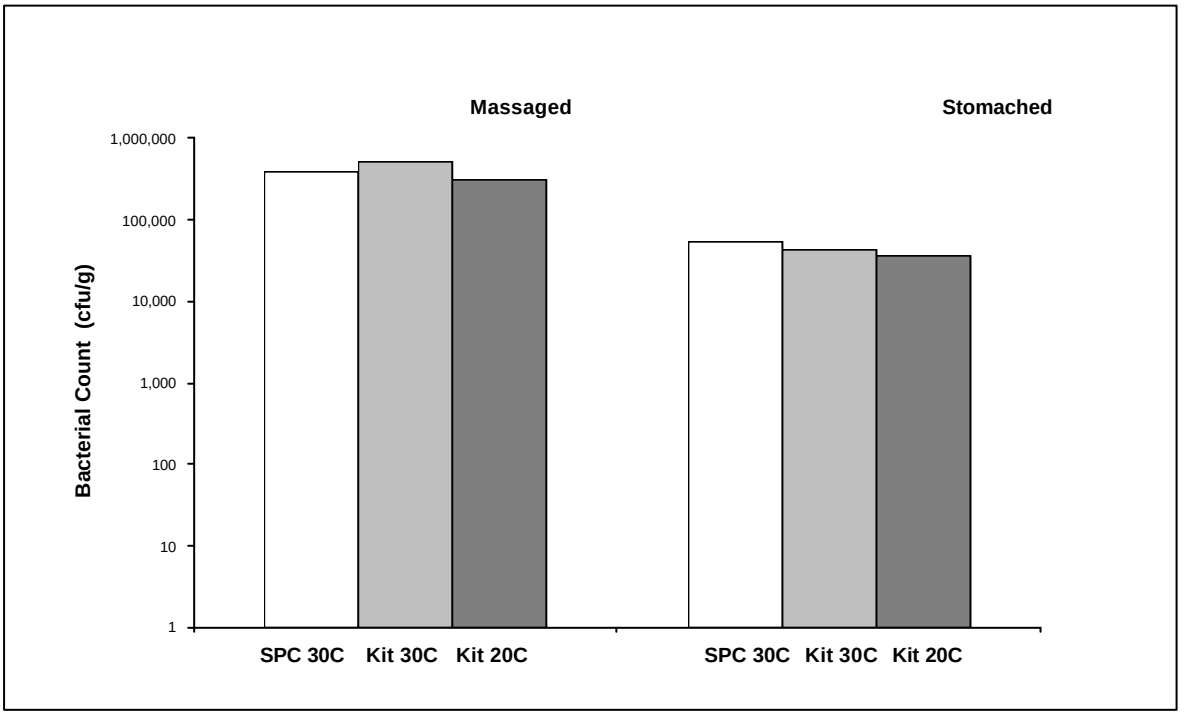
Results indicated that a volume of <1ml does not disperse on the solid medium well enough to make colony growth countable, hence is inappropriate.
Simple sterile equipment is needed to make dilutions and this is readily purchased at little cost.

### 6.4 Kit incubation temperature

All the rapid kits specify definite time/temperature conditions for incubation of the kit to allow colony growth to occur. For kits detecting total bacterial loads, the conditions are frequently 30°C for 24-48h. As it is unlikely that small to medium size seafood factories will have a constant temperature incubator available for use, investigations were carried out to determine the critical of incubation temperature. Various kits were incubated at the temperature recommended by the manufacturer and duplicate kits prepared with the same samples and incubated at 20°C, a temperature chosen as likely to represent an average ambient for many seafood premises.

Importantly, it was found that for most kits, similar counts are obtained from incubation of inoculated sample at 20°C as at 30°C for the same incubation period (Figure 6.2).

**Figure 6.2. Bacterial counts attained with kits incubated at different temperatures.**




This finding has great significance as it negates the need by seafood companies which have no lab facilities, to invest in incubation equipment (the smallest appropriate incubators are c.$500)

# 7. Total aerobic bacteria

The total number of bacteria present in samples from the processing environment or on seafood itself is frequently used as an excellent indicator of hygiene practices within that environment (Mayer and Ward, 1991). It can also be used as a microbial index to assess the potential storage life of seafood products (Jay, 1992).

Conventional culturing methods are hampered by expensive materials and equipment, skilled technique training required and often a very long time interval of incubation for growth before results are known (Swanson *et al*, 1992; Jett *et al*, 1997). Hence the need for a rapid technique which preferably avoids these hurdles.

## 7.1 Selection of total aerobic bacteria kits

There are a large number of test kits available commercially for the determination of total bacterial load. They can be readily categorised into groups according to the principles of design and operation of the test. After gathering detailed information about each test kit, the kits were ranked against the selection criteria.

Several kits were obtained from each grouping according to availability in Australia (and/or ease of procurement) and investigated further. It was found that all kits within any one grouping were practically identical, the only difference being the manufacturing company and the country of origin. After screening several kits within each group, three separate types were selected as representative of each test kit principle. The selected kits were the most readily available in Australia were: Petrifilm Aerobic Count Plate, Environcheck, and Hygiene Test .

The Petrifilm Aerobic Count Plate (PetrifilmAC) and Environcheck (EnvCh) are both kits based on pre-prepared solid media, with the difference being incorporation of a dye in PetrifilmAC and the physical form of the kits. The Hygiene Test (HygT) is based on an incorporated dye within the test vial liquid medium which changes colour in response to microbial growth.

## 7.2 Petrifilm AC

Among the various methods developed for determining total aerobic count, Petrifilm in particular has gained wide acceptance as an alternative to conventional plating methods (Mizuochi and Kodaka, 2000). For various food commodities, researchers have found excellent correlation between bacterial numbers attained with petrifilm plates and conventional agar plating methods. The Petrifilm AC test method has received validation as an alternative method to standard plate count methods from the Association of Official Analytical Chemists (AOAC) and the French Standards Association (AFNOR), although the latter organisation has not validated it for seafood.

*Kit ingredients*: self-contained test unit, including a spreader for even distribution of sample on the film. Doesn't contain sample preparation equipment – need sterile sample bags, sterile diluent, sterile 1ml transfer pipette and incubation temperature of 30$^{o}$C.
*Clarity of instructions*: very simple and clear, including reading the result

The quantitative comparison of microbial loads on various seafood samples, comparing Petrifilm AC with conventional culturing methods using standard plate count agar was found to be exceptionally good (Table 7.1).

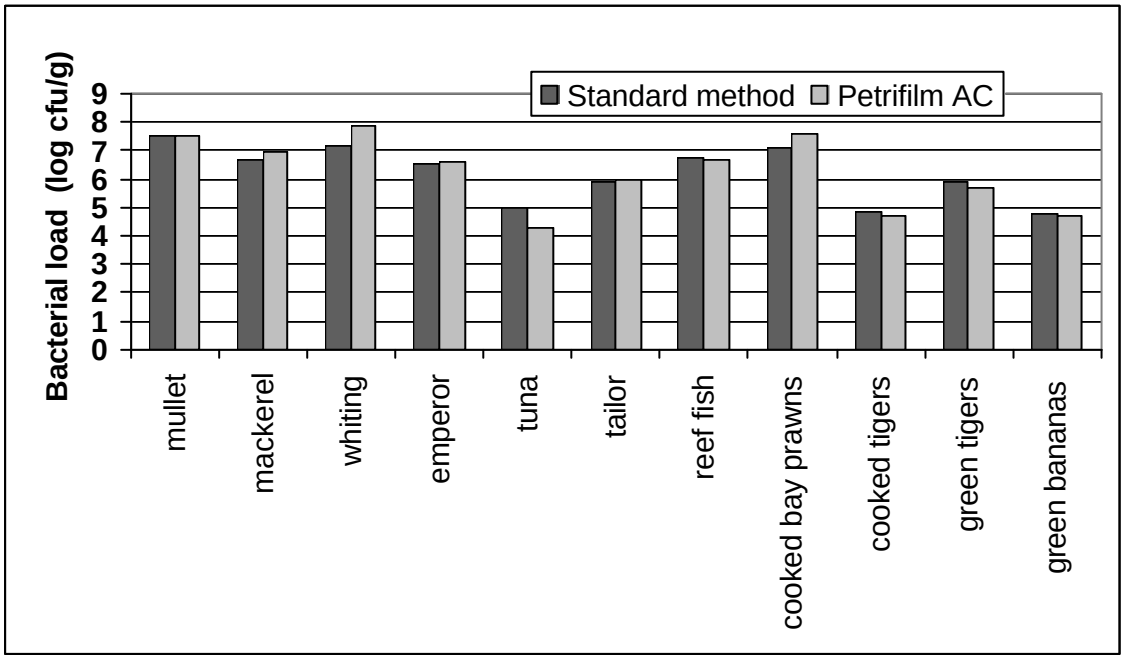
**Table 7.1. Correlations between total aerobic counts achieved with Petrifilm AC and standard method.**

| Sample type | Correlation coefficient |
|---|---|
| All samples | 0.98 |
| Fish | 0.96 |
| Prawns | 0.99 |
| Surfaces | 0.98 |
| Rinse waters | 0.98 |

This high degree of correlation agrees with other researchers findings in comparative studies with Petrifilm and traditional agar plate methods (Ginn *et al*, 1986; Smith *et al*, 1985; M$^{c}$Goldrick *et al*, 1986; Bishop and Juan, 1988; AFNOR,1989). Investigations demonstrating the quantitative comparisons between conventional plating methods and Petrifilm AC from fish and seafood samples have reported variable results. Fung and co-workers (1987) reported a correlation coefficient of 0.99 when analysing seafood samples and Mizuochi and Kodaka (2000) a similar coefficient of 0.96, stating that 10% of the Petrifilm AC results were higher than TVC and 7% lower. However, Slabyj and Bolduc (1987) claimed to only recover 8% of the microbial population on white hake fillets with Petrifilm AC compared to that detected with standard plate count methods.

Many of the samples tested in this investigation were from the retail level and therefore could be expected to demonstrate a microflora contaminated with terrestrial bacteria as opposed to micro-organisms directly from marine environments. This fact could perhaps affect the results and cause the high correlation achieved. However, in a detailed study using samples from throughout the processing line, Abgrall and Cleret (1990) found no need to incorporate salt into the recovery media as there was no statistical difference in the counts obtained. This is providential as the Petrifilm AC media-gel used contains very little salt. There was good comparison of bacterial numbers between both methods irrespective of sample type (Figure 7.1).

**Figure 7.1. Comparison of bacterial numbers from Petrifilm AC and standard methods with fish species.**



For retail purchased samples, the total bacterial load was generally medium ($10^5$ – $10^6$) and again, as most samples tested fell within this range, this may be directly related to the high correlation achieved with the testing methods. To determine whether this was the case, fish samples were obtained immediately post-filleting and held chill-stored ($2^oC$) for a length of time. From the samples tested at intervals throughout the storage period we attained microbial loads ranging from $10^3$ to $10^8$ cfu/g and correlations remained high at 0.997 for loads >$10^5$/g and 0.967 for loads less than this. However, there were some functional issues with the PetrifilmAC kits when levels of bacteria were high, if the sample was not diluted (see discussion below, under operational observations). Other researchers, Slabyj and Bolduc (1987), described results that indicated PetrifilmAC performed less well compared to the standard method with increasing seafood sample age (longer storage time).

It is encouraging to note the good correlation between PetrifilmAC and the standard method counts obtained occurred irrespective of species and sample type. Different species and variant handling practices, including extended chilled storage, will result in different microbial population composition (Shewan, 1961). Such differences in bacterial species present on the samples does not seem to have a significant effect on the enumeration of the population by either method. Other researchers have cited the population compositional difference to explain variances in quantitation of microbial loads (Abgrall and Cleret, 1990). They identified that *Photobacterium phosphoreum* constituted 70-99% of the microflora present on the whole fish tested and that this bacterial species exhibited enhanced growth on PetrifilmAC media compared to standard plate count media. As there is little difference between the two media, except for a dye incorporated into the Petrifilm plate, Abgrall and Cleret suggested that it was the physical micro-environment of the Petrifilm test kit that favoured the growth of *P.phosphoreum* and proposed this as an explanation for disparity in counts obtained between the two methods. We did occasionally observe higher counts on the Petrifilm kit than the TVC agar but did not isolate and identify the colonies from those plates.

**Kit operational observations:**

Multiple kits were used at any sampling time for replication and results obtained from were consistently reproducible. This pertained to kits from different manufacturing batches also.

The kits are straight-forward and basically easy to use, although sample dilution for samples suspected of having high microbial levels is desirable. For samples with high levels of bacteria that were not diluted, as would be beneficial for industry use, the incubated Petrifilm plate turned an even deep pink with no discernable colonies visible. This sort of result should be recorded as ‘too numerous to count’ (TNTC), which is a void enumeration. However, when the bacterial load is at a lower level than this even though still TNTC, it was found that an estimation can be made by counting the colonies (red dots) in one grid square and multiplying by the number of grids (20). The estimated counts obtained this way were in close agreement with those obtained on the TVC plates. For practical purposes within a seafood factory, a Petrifilm plate that resulted in a TNTC would still be useful as it decidedly indicates the bacterial levels are too high for acceptance. This is fitting for almost all samples likely to be taken within a processing line. The only occasion it may not be pertinent is for assessing end-product quality, where an actual count is required.

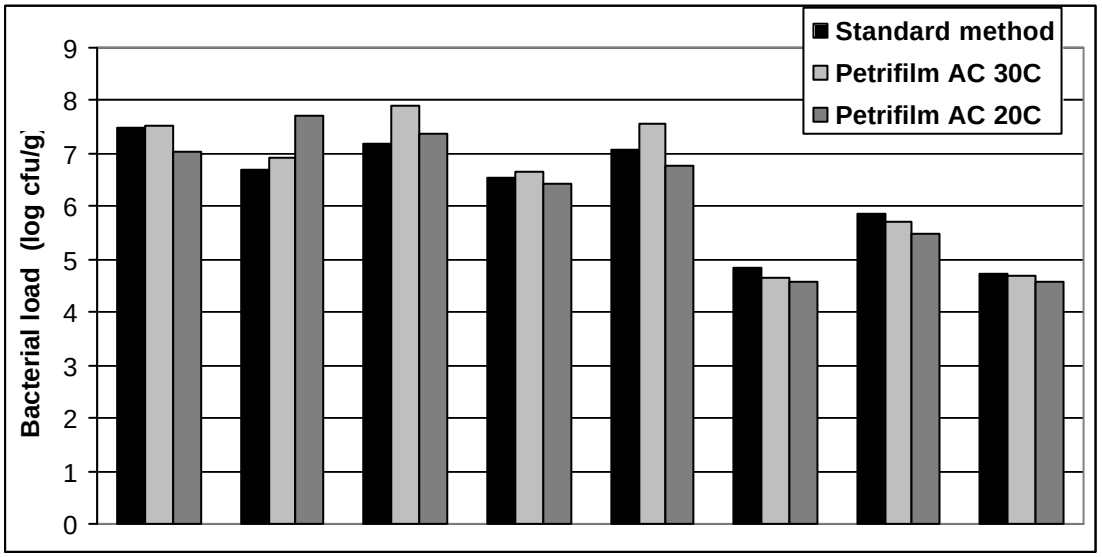
The Petrifilm kit is adaptable to different sample types. For example, ‘wet’ or fluid samples can be added directly (1ml) to the kit; a dry surface sample, a cutting board prior to use for example can be taken with a wet swab method which can be transferred to the Petrifilm or the film can be rehydrated with 1ml of sterile water and, after solidification of the medium (ca. 1 min) can be used in direct contact with the surface required to be tested.

The bacteria show as red dots after incubation and all are counted regardless of shape or size. A practical observation with respect to seafood samples and reading the PetrifilmAC plates is that of particulate matter, shreds of fish or prawn flesh, present within the sample. Even if small, it was noted that it can interfere with separating colonies for counting. We found avoiding counting those grids where this occurs and multiplying the rest by the appropriate factor, precluded this issue from negating a result. Similarly, due care needs to be taken when overlaying the top film of the kit after adding the 1ml sample so as to avoid formation of any air bubbles which can confuse after incubation of the Petrifilm. Should it be required, the kit allows for 'picking' a single colony for isolation and identification by a microbiology laboratory (the seafood processor could simply send the entire Petrifilm plate to the laboratory).

On Petrifilm plates from some fish samples, particularly those that had been stored chilled for an extended time, we observed that the solid gel medium had liquefied in spots. It is suggested this could be caused by proteolytic micro-organisms present in the sample added on to the plate as enzymes from this type of bacteria could readily attack the components solidifying the Petrifilm medium. This is supported by noting that the phenomenon rarely occurred with samples of fresh fish or prawns and only evidenced in older stored product. The incidence was a not common however and again we found the solution was to simply not count those grids affected.

Of practical consideration for seafood industry use of the Petrifilm kits is the manufacturer's recommendation that incubation be carried out at 30°C. This would necessitate the seafood processor procuring an incubator capable of accurately holding this temperature. Experiments were carried out comparing the total counts achieved after incubation at 30°C and at 20°C. The latter temperature was chosen as it was considered close to ambient in an office environment. The comparative counts are shown in Figure 7.2 are from Petrifilms incubated for the same length of time.

**Figure 7.2. Bacterial counts attained on Petrifilm incubated at 20°C and 30°C.**




***Benefits***

- easy to operate, use and read
- minimal additional equipment required and is readily available (sterile graduated 1ml sample deliverer, sterile diluent or physiological saline)

- kit adaptable to different sample types, wet or dry
- minimal storage space require for kits compared to standard method requirements
- kit has a long shelf life because of the dehydrated medium
- inexpensive per unit test

***Limitations***

- addition of inoculum to the film needs practice and care
- a degree of ascepsis is needed
- particulate matter in some samples can interfere with counting
- major drawback – results not obtained rapidly, still require 2-3 days incubation

***Summation:*** simple, practical and useful in everyday processing operations and for verification of sanitation programs within the factory.

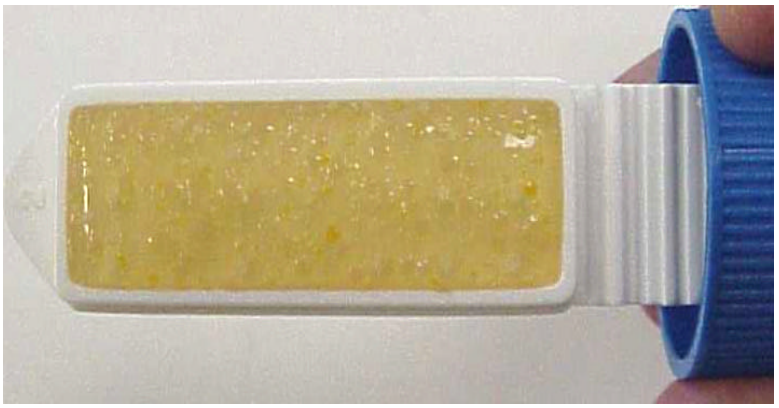
## 7.3 Environcheck

The Environcheck kit is a solid media dip slide that can be used for liquid or direct contact surface samples. Bacterial colony growth is compared to a density chart to give an indication of total bacterial load, but not an actual enumeration of bacteria present.

*Kit ingredients:* the kit is a complete unit with no extra equipment required
*Clarity of instructions:* straightforward and clear

Reading the slides after incubation is a little subjective (Plate 7.1). These slides are not designed to allow individual colonies to be counted, but rather the overall density of colony growth is matched to a visual comparison sheet provided with the kit. By its nature, this provides equivalent log ranges of density and hence results are "in the order of $10^x$ cfu/ml".

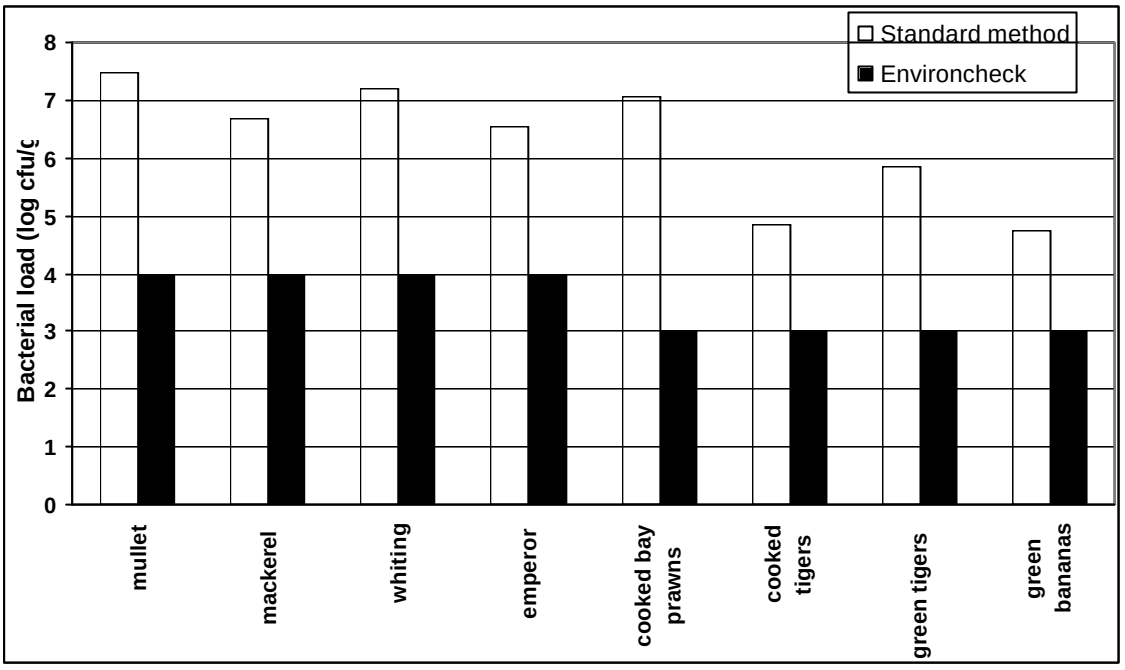
**Plate 7.1. Environcheck slide after incubation.**



Even given this limitation however, results obtained with the kit were frequently 2 or 3 logs lower than those obtained with standard plating methods. Statistical analysis was not done with these samples as an actual count is not attained, hence the data points for cases cannot be achieved. Results using the Environcheck kit are presented in Figure 7.3.

The Environcheck kit was found suitable for rinse water samples and wet surfaces, but not effective at all when used in direct contact with dry surfaces.

**Figure 7.3. Comparison of bacterial numbers from Environcheck kit and conventional plating method.**




***Benefits:***

- very easy to use
- inexpensive per test

***Limitations:***

- density of colonies interpretation too subjective
- bacterial loads measured as too low compared to standard method counts

***Summation:***

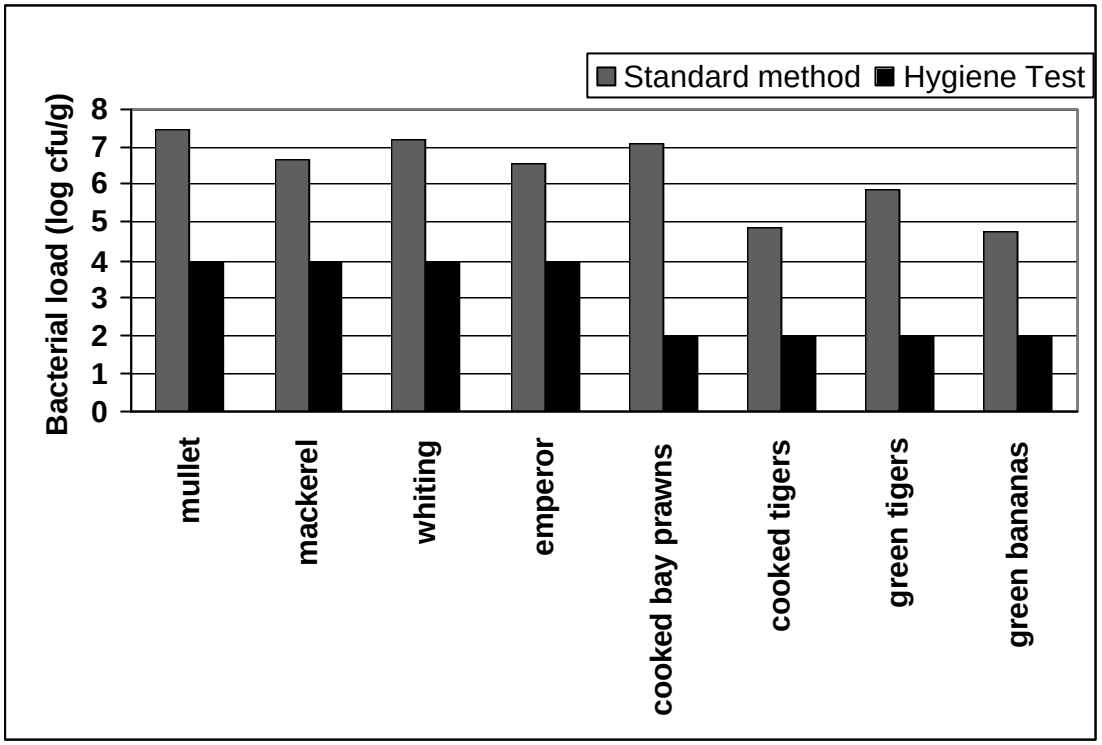
Most suitable for water quality assessment and liquid homogenate samples. It can also be used for assessing fillet flesh or surfaces by direct contact. However, microbial load measured with this kit is always a good deal lower than the actual load.

## 7.4 Hygiene Test

This test kit does not enumerate bacterial numbers in samples, but rather gives an indication of the load within fairly large ranges. The test is based on an colour change from red to yellow with time. It is clearly noted in the marketing information supplied that the time lapse prior to yellow colour development needs to be correlated to bacterial load for individual operations. Then, it can be used to monitor contamination levels at critical control points along the processing line.

*Kit ingredients*: the test kit is complete in itself, except for incubation at greater than ambient
*Clarity of instructions*: very simple and clear
The Hygiene Test was applied mostly to seafood processing line samples, although some fish and prawns were swabbed with the kit swab. Results from fish samples are presented in Figure 7.4.

**Figure 7.4. Comparison of bacterial numbers from Environcheck kit and conventional plating method.**




Attempts were made to develop a standard guideline graph to show the relationship between time for colour change and bacterial load, incubating the Hygiene Test at 30°C and 20°C. However, this was not highly successful. For any one trial, a reasonably straight line was obtained for the top end of the graph where bacterial load was high ($10^4$ – $10^8$ cfu/ml). At these bacterial numbers, the colour change occurred within 12 hours. However, when bacterial load was <$10^4$ cfu/ml as determined by standard plating method, the Hygiene Test tubes frequently did not show colour change at all or at best, sometimes went partially yellow only after a prolonged period (>30h). Similar findings occurred from many trials undertaken, indicating that it was not a function of the microflora composition on any one sample. Such results negated the ability to produce a straight line graph of correlation.

Where bacterial loads were medium to high (>$10^4$cfu/ml), total bacteria present determined by the kit compared reasonably with total bacteria enumerated by standard plate methods. It is important to note that the time lapse should be recorded at the first sign of colour change occurring (Plate 7.2).

**Plate 7.2. Colour changes in inoculated Hygiene Test kits**

**First colour change** **Complete change**


Investigations were carried out comparing incubation of the Hygiene Test tubes at temperatures of 20°C and 30°C. Colour change was observed at either temperature, with the time lapse to colour change merely extended for those kit tubes at 20°C. The prolonged time lapse appeared consistent at all bacterial load levels. These findings are of practical consideration for industry use of the kit, as there is no need to acquire specialised incubation chambers.

The Hygiene Test kit reaction is based on total protein present and therefore any flesh residual present in the sample will affect the reaction time. This was evidenced during early investigations when very rapid colour changes occurred in samples taken from knives and cutting boards. The result implied a very high number of bacteria present, but the counts obtained from standard plating methods were low ($10^3/cm^2$). This anomaly was further demonstrated when fish fillets were sampled with the conclusion that this kit is not appropriate for fish product. However, for assessing cleaning/sanitation practices within a factory environment a rapid colour change is pertinent as a high protein content remains indicative of inadequate cleaning of surfaces.

The lack of correlation at low level bacterial loads restricts the usefulness of this kit for monitoring sanitation procedures within a processing operation. Discussion with the company supplying the kit did not reveal any further useful information in this area and no reference was found on use of the Hygiene Test in the literature. However, this kit measures gross contamination and can detect such within hours as opposed to days with the standard method. For the specific purpose of verifying sanitation programs within processing operations, the Hygiene Test kit is useful for detecting contamination. A prerequisite for this use is that the colour change time needs to be correlated to normal processing bacterial levels for an individual factory i.e. in-house standards developed. Once this is done, the kit can be readily used as a simple method to trigger alarm bells with respect to bacterial levels present in environmental samples from the processing line or post-cleaning. The lit provides a rapid check rather than an enumeration of microbial load.

***Benefits:***

- inexpensive per test
- very straightforward to use with minimal operator skill required
- provides a result rapidly (hours)
- excellent for monitoring against established standard levels

***Limitations:***

- protein-based reaction, therefore flesh protein can affect result
- low levels of bacteria are not readily detected
- does not give an actual count of bacterial present

***Summation:***

The Hygiene Test kit is a colour-change based test using swab sampling. It is very easy to use and inexpensive per test. Detection of bacterial load is optimal $>10^5$ $cfu/cm^2$. Where fewer bacteria are present, the time/colour change relationship becomes unreliable. However, this test kit could be a very useful tool for monitoring sanitation practices within a seafood operation.

# 8. Coliforms and *Escherichia coli*

Faecal coliforms and *E. coli* are recognised as useful marker organisms for indicating sanitary quality and environmental contamination, hence there has been a concentration of effort in developing rapid techniques for detecting these bacteria. The research work included an early focus on aquatic systems and seafood due to the importance of pathogen presence with respect to shellfish consumption (Qadri *et al*, 1974; Andrews *et al*, 1975; Havelaar and During, 1988)

## 8.1 Selection of kits

There are several test kits available for the determination of coliforms and *Escherichia coli*. Detailed information about each test kit was gained and the ranked against the selection criteria. The kits can be divided into two categories:

1. enumeration or estimation of coliforms/ *E.coli* directly from samples
2. confirmation of *E. coli* strains

The second category is not of primary usefulness for industry application but more appropriate to microbiological laboratory use, to hasten result attainment.

Within the first category, all the kits are based on direct contact between the culture media of the kit and the test sample. The kits simply contain variations on standard solid media as a growth supporting technique, only differing in whether the kit would detect just coliforms or coliforms and *E. coli*. After screening several kits, only the Petrifilm kits and Environcheck C were chosen as representative of the kit types and most likely to be useful for the seafood industry. These two kits were also readily available in Australia.

## 8.2 Petrifilm *E. coli* count plate

The Petrifilm kits available provided a range of options:

- coliform count plate – determines total coliforms present only
- rapid coliform count plate – detects coliform contamination at levels >1000/g
- high sensitivity coliform count plate - measures coliforms, accommodating up to 1g of sample (avoids sample dilution requirements)
- *Enterobacteriaceae* count plate – determines all members of the *Enterorbacteriaceae* family, including coliforms and non-coliforms
- *E.coli* /coliform count plate – enumerates both organisms in one test kit

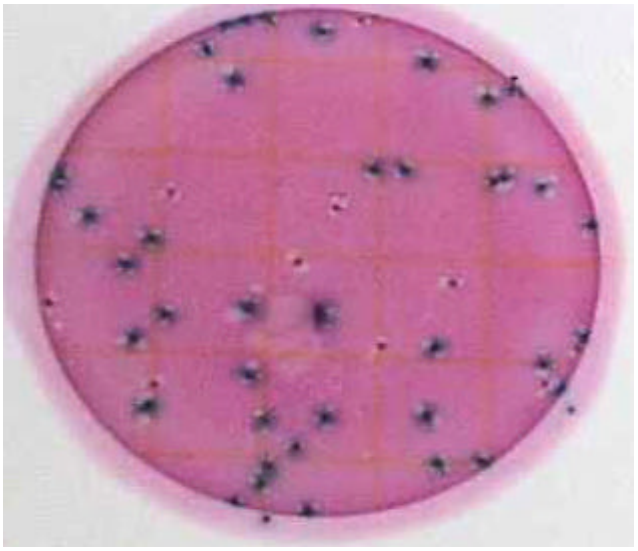
The *E. coli* count plate kit was chosen as the likely useful option for seafood industry use and preferable over a coliform count only. Within the industry, it is not common that coliforms would be at the levels detected by the rapid coliform count and for the high sensitivity test kit, flesh particulate matter interferes (even from direct sampling of processing surfaces. The *Enterobacteriaceae* count kit has too broad an application for seafood industry use.

The Petrifilm *E. coli* count kit has similar physical structure as other Petrifilm kits with a grid based bottom agar film containing nutrients, selective agents and indicator dyes similar to those used in standard method violet red bile agar.

*Kit ingredients*: a self-contained test unit, including a spreader for even distribution of sample on the gel-film. Strict incubation temperatures are required.
*Clarity of instructions*: very simple and clear, good positive-result descriptions

Coliforms show as red colonies with associated gas bubbles trapped by the top film of the kit. The parameter that differentiates *Escherichia coli* from coliforms is the production of a glucuronidase enzyme by most strains of *E. coli* and when this reacts with the indicator in the gel, causes a blue precipitate to form around the colonies. Most *E. coli* also produce gas during growth (Plate 8.1).

**Plate 8.1. Coliform and *E. coli* growth on Petrifilm plate kit.**



Petrifilm kits frequently gave higher detection levels of both coliforms and *E. coli* compared to the count obtained from the standard method. It appeared these kits were giving a large number of false positive reactions, where the colony growth showed as 'positive' according to kit instruction description. Coliform and non-coliform colonies also show as red on this kit gel but the latter are not associated with gas bubbles. Interpretation of which colonies have gas bubbles is often quite subjective, even after much practise. The plate films were always difficult to read when high numbers of organisms are present.

In order to familiarise the operator with colony type formation, many colonies were isolated off the film and subjected to further biochemical testing. Particularly confusing were the colonies associated with blue precipitate (*E. coli*) as with high numbers of bacteria present – not necessarily *E.coli* – the background agar gel turned from a light pink to a deep pink/purple colour. This made reading of the blue colonies extremely hard and mis-reading of the films was evidenced by only about 20% confirmed as *E. coli* with further isolation and tests. All subsequent trials included an *E.coli* organism control run simultaneously with the seafood samples.

Results with fish samples indicate reasonable comparability between coliforms present as determined by the kits and enumeration by standard methods. It is important to emphasise that the correlation between the methods was good when coliform load in the sample is low, which is reassuring as this would be the most likely scenario within the industry samples.

When bacterial load in the sample was high, the gel plate looked as though no colonies were present and the natural assumption is that the sample was 'clean'. Closer inspection the film showed that the colony number was 'too numerous to count' and hence the colonies were extremely small and the gel a uniform deep red colour. This result with samples containing high counts needs to be emphasized for industry application of the kits. .

These findings are in contrast with those of Blackburn *et al* (1995) who reported that standard method violet red bile agar generally gave higher counts compared to those obtained with Petrifilm coliform count.

For coliform colonies and typical *E.coli* colonies to be differentiated and countable it was found that the number of colonies enumerated on Petrifilm plate kit needs to be <100 organisms per plate (which is equivalent to per ml). With this number, both *E.coli* and coliforms are readily discernible and can be removed easily for further identification if required. For industry use of this *E. coli* kit, it is therefore recommended that the user run several serial dilutions in order that the organism capture range falls within 1-100 per plate.

These kits were only trialed with samples from the retail level since such samples are more likely contain coliform organisms than most of those straight from the sea. For the many samples tested it was found that coliform numbers were at low levels, if present at all. This finding is, of course, good but not very useful for assessing the worth of the kits for detecting the organisms. Therefore further experiments involved samples of fish and prawn flesh that had been 'spiked' with low levels (10 and 100 organisms/g) of *E.coli*. Recovery of the organisms was not high (<30%) with either the standard VRB agar method nor the Petrifilm EC plate. With such low recovery levels, the result are inconclusive, but it appeared that the Petrifilm was not as effective in detecting *E. coli.* A limitation with the Petrifilm EC is that it relies on the *E.coli* producing the enzyme glucoronidase, which some strains of *E.coli* don't have. Hence there is a possibility of incorrect low counts being obtained and this may contributed to the poor recovery.

Consideration was given to inoculating samples with higher numbers of organisms, however whether this improved recovery or not is irrelevant to industry application of this kit as permissible levels of faecal contamination are low (<230cfu/100g sampled) and hence it is low level presence that is needed to be detected.

**Kit operational observations**

Sample dilution can be important with solid medium kits although, frequently, fewer dilutions are needed as coliforms and *E.coli* are not usually present in high numbers. In instances where bacterial numbers were high (>2.5 $x10^2$cfu/g) the kits were completely uncountable, while the standard method agar plates were still able to be estimated. This is a disadvantage with solid medium kits and necessitates a prior estimate of likely contamination level. However, again it should be mentioned that, for most samples, coliforms and *E. coli* will be present at low levels (<100 cfu/g) or even absent.

Where numbers of coliforms and *E. coli* present were low, interference due to the presence of large numbers of bacteria present that are not coliforms appeared to reduce the sensitivity in both the Petrifilm EC plates.

In an industry use environment, where likely organism numbers are unknown, it would be useful to develop a set of pre-determined visual templates. These could involve a series of fixed photographs and give the kit user a comparative template. In this situation, the interpretation would be an approximation only of the actual count present in the sample, but would prevent null test results.

Particulate matter from the fish/prawn sample was obvious on the plate kit surface but is not mistaken for colony growth. Therefore the nature of the sample does not interfere with kit accuracy and that makes this kits suitable for seafood flesh samples. Furthermore, there was good correlation achieved between the kit result and that of the standard method

whatever seafood sample was tested: oily fish flesh, lean flesh or prawn flesh. Therefore, our findings showed the kit is suitable for all seafood sample types.

A surprising finding was that the Petrifilm EC kit appears to function equally as well at 20$^{o}$C and within the same time period as colony formation the recommended 44.5$^{o}$C . This finding is significant for industry use of the kit as it negates the need for controlled temperature incubators and implies that kit plates can be simply left in the office area overnight (18-24h) and read next day.

***Benefits***

- Petrifilm EC is suitable for all seafood sample types
- Easy to use
- inexpensive
- Both coliforms and *E. coli* can be differentiated in kit

***Limitations***

- Film plates can be difficult to read
- Interference from high levels of bacteria present

***Summation:***

The kit is simple to use but needs a degree of familiarisation for reading accurately. Within industry application, the kit would be beneficial in detecting serious contamination along the processing line.

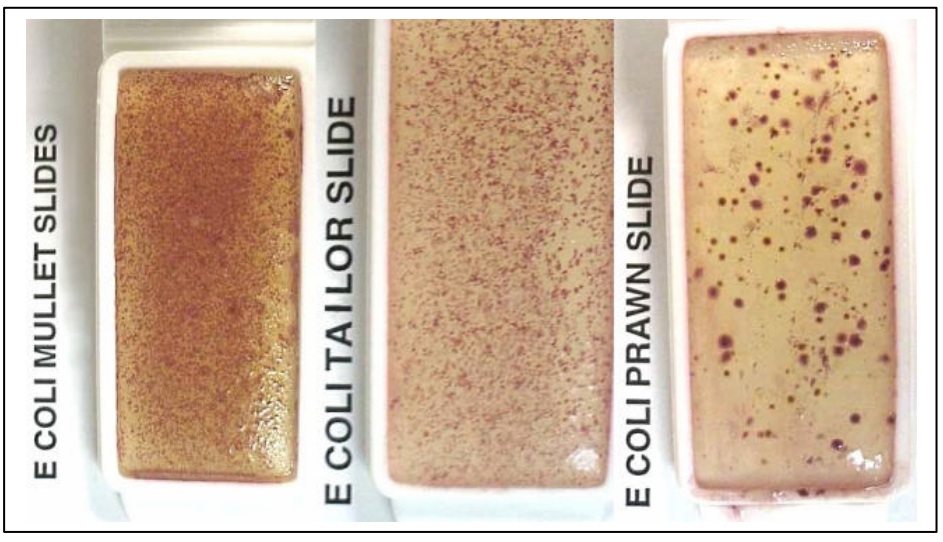
### 8.3 Environcheck coli slide

The Environcheck kit is a solid media dip slide that can be used for liquid or direct contact surface samples. Bacterial colony growth is compared to a density chart to give an indication of total bacterial load, but not an actual enumeration of bacteria present.

*Kit ingredients:* the kit is a complete unit with no extra equipment required
*Clarity of instructions:* straightforward and clear

Reading the slides after incubation is a little subjective. These slides are not designed to allow individual colonies to be counted, but rather the overall density of colony growth is matched to a visual comparison sheet provided with the kit. By its nature, this provides equivalent log ranges of density and hence results are "in the order of 10$^{x}$ cfu/ml" (Plate 8.2)

**Plate 8.2. Visual representations of coliforms on Envirnoncheck slides.**



The Environcheck kit was found suitable for rinse water samples and wet surfaces, but not very effective when used in direct contact with dry surfaces. However, within these limitations, the slide is a simple way to determine the faecal contamination level within a processing line as any coliforms demonstrated should trigger alarm bells.

***Benefits:***

- very easy to use
- inexpensive per test
- useful as a contamination indicator for processing lines

***Limitations:***

- density of colonies interpretation too subjective
- bacterial loads measured as too low compared to standard method counts

***Summation:***

Most suitable for water quality assessment and liquid homogenate samples. It can also be used for assessing fillet flesh or surfaces by direct contact. However, microbial load estimated from colony density with this kit is always a good deal lower than the load determined by standard method plating techniques.

# 9. *SALMONELLA*

Salmonella is a foodborne pathogen, but present as a contaminant on seafood. It is carried by birds and animals and hence its presence within the seafood processing environment is indicative of poor sanitation practices or contamination from seafood workers. The organism can cause serious gastro-enteritis like illness and therefore regulatory authorities set limits on its presence in food products, including many seafoods. The common permissible level for most importation of seafood is absent in 25 grams of product, that is a zero tolerance.

## 9.1 Selection of kits

A wide search of commercially available kits evidenced that there was no kit manufactured that would detect the presence of Salmonella species directly from food samples. Of the kits available, all were designed to hasten the screening of foods after an enrichment step. In a microbiology laboratory situation this is of considerable benefit as use of such kits negate the need for further lengthy incubation techniques after primary isolation of the organism. However, within a seafood processing environment it is untenable to undertake an enrichment step for the selection of Salmonella, since the potential for subsequent contamination of the seafood premises with a more or less pure culture of the organisms is high. Hence, rapid kits of this type are unsuitable for most seafood industry use.

Notwithstanding the above qualification, a brief study of two antigenic-based kits was undertaken. These kits will only be appropriate for large sized seafood businesses that have separate attached laboratory facilities.

## 9.2 Visual Immuno Precipitate Assay (VIP) for *Salmonella*

The kit unit is a self-contained immunoprecipitate assay. It is based on an antibody-antigen binding method incorporating a dye indicator. It is specific for *Salmonella* and detects both motile and non-motile forms. The VIP kit detects the presence or absence of Salmonella but is not quantitative.

The kit worked extremely well with pure cultures of several laboratory strains (previously isolated from different foods). Obvious blue lines were obtained in the reading window within 1-2 mins of culture inoculum application. The kit produces an accurate result in 25h of which 24h is required for the enrichment step. This equates to about 48h less than standard method procedures. For the limited number of strains examined (5) no false-positive or false-negative results were attained. The control and blank inoculum samples illustrated that the kit demonstrated completely accurate results.

In trials where mixed seafood microflora populations were ‘spiked’ with very low levels of *Salmonella* (10-20/ml), results demonstrated high levels of validity and repeatability with all seafood products tested. This indicated that there was no residual interference from bacterial species of the commensal flora. This is unsurprising as the enrichment method is very selective for *Salmonella,* however, it was worth checking.

Retail samples of both prawns and fish species were obtained and enriched for *Salmonella*, with inoculums applied to the kit. No *Salmonella* was detected in any sample.

***Benefits:***

- The actual kit is very easy to use and produces accurate results

- The kit itself is relatively inexpensive

***Limitations:***

- Before the kit can be used an enrichment step is required on the sample- this entails specialised media, technique and incubation temperature (42$^{o}$C)
- Different methods apply for raw food samples
- Use is restricted to laboratories

***Summation:***

The kit is marketed as a "*convenient and accurate method for screening Salmonella in food and environmental samples*". However, this is only true given the limitations of an enrichment step being undertaken. The kit provides very accurate results and is reliable but the enrichment step precludes its appropriateness within most seafood factories.

### 19.3 *Salmonella* 1-2 Test

The *Salmonella* 1-2 test from Biocontrol is a rapid qualitative method for the detection of motile Salmonella. Motility is a characteristic of Salmonella species commonly used as the basis of differentiating the species from other enteric micro-organisms (Holbrook et al, 1989). It is recommended for use with processed food products and environmental samples. The 1-2 Test has Association Of Official Analytical Chemists International approval as an official method for use with all food testing.

This kit is also based on an antigen-antibody binding reaction, but unlike the VIP test there is no dye indicator incorporated. Visualisation is by *Salmonella* being immobilised in a motility medium, which results in development of a well-defined band of cells (the immunoband). Hence, a positive 1-2 test shows an immunoband in the upper half of the motility chamber gel. The immuno-band is a white band and is U-shaped following the meniscus outline. It is a 3-dimensional effect through out the gel and can be seen when rotating the 1-2 tester unit against a strong light source. Presence of immunobands indicate presumptive motile *Salmonella*. The kit comes with additional reagents and these dictate a very short shelf-life of around 6 weeks.

This kit has a simpler sample preparation and enrichment process than the VIP assay and a corresponding reduction in required chemicals, media and specific incubation temperatures. The test operation is relatively straightforward, but some degree of laboratory technique knowledge is required. The results or immunobands were difficult to read without practice, hence would required skilled personnel. It was found essential to run a control *Salmonella* organism with each kit test to assist in reading the immunoband by comparison. The test takes a between 14-30h with incubation at a specific temperature of 35$^{o}$C.

The same samples used for the VIP kit were used to trial the *Salmonella* 1-2 test. The results were again accurate for all test carried out, though reading results with some strains was doubtful. It is worth noting that tests performed with kits a little outside their expiry date still gave accurate results with pure cultures of *Salmonella* strains.

***Benefits:***

- the kit was relatively easy to use
- results attained quicker than standard conventional methods
- less preparation required than the VIP kits

***Limitations:***

- requires an enrichment step
- short storage life of kits
- a degree of skill and practice required to read kits
- comparison with a *Salmonella* control test considered essential

***Summation:***

The kit was very accurate once skill in reading the immunoband was gained, but considered inappropriate for general industry use.

# 10. Staphylococcus aureus

The presence of *Staphylococcus aureus* is used as an indicator of hygiene of food processing environments. The organism is primarily from human origin and actually carried by about 10% of the population in their nasal passages. Hence, its presence on seafoods indicates poor personal hygiene practices within the seafood operations.

## 10.1Selection of kits

All kits available are similar in principle and based on solid agar medium methods. Two different kinds were selected for testing on the main basis that they were readily procurable within Australia and inexpensive.

## 10.2 Rapid *S. aureus* Count Plate (Petrifilm S. aureus)

The kit has similar physical structure as other Petrifilm kits with a grid based bottom agar film and incorporating a thermostable nuclease disc as the differentiating parameter for *S. aureus*. The kit combines the enzymic metabolism characteristics specific to *S. aureus* that standard method Baird-Parker media relies on. Differentiation by these features are usually separate steps requiring incubation periods and individual colony isolation within standard methods. Hence, the combination of selective isolation and confirmation of *S. aureus* within a 1-step kit is of rapid advantage.

Several types of retail seafood samples were tested including both fish and prawn samples. A reference strain of *Staphylococcus aureus* and a blank were used as controls in all trials.

The kit was found to be easy to use, although a degree of care is needed in placing the thermonuclease disc on the growth gel film. This step then requires a 1-4h incubation at 62$^{o}$C and the temperature is critical. This factor is going to cause some difficulty and extra expenditure for use of the Petrifilm kit at the industry level. However, the kit can be used to the first stage only which provides enumeration of *S. aureus*.

Overall, results obtained with the Petrifilm S. aureus kit were similar to the counts from conventional Baird-Parker agar plates. The two methods were not subjected to statistical analysis to determine the correlation coefficient between them as sample numbers (*n*) were too low at 9. The counts of positive colonies from both methods were almost exactly the same, however, for all samples and this is not unexpected as both are based on Baird-Parker agar.

Interpreting the positive *S. aureus* colonies on the Petrifilm plate (Plate 10.1) needs consideration of:

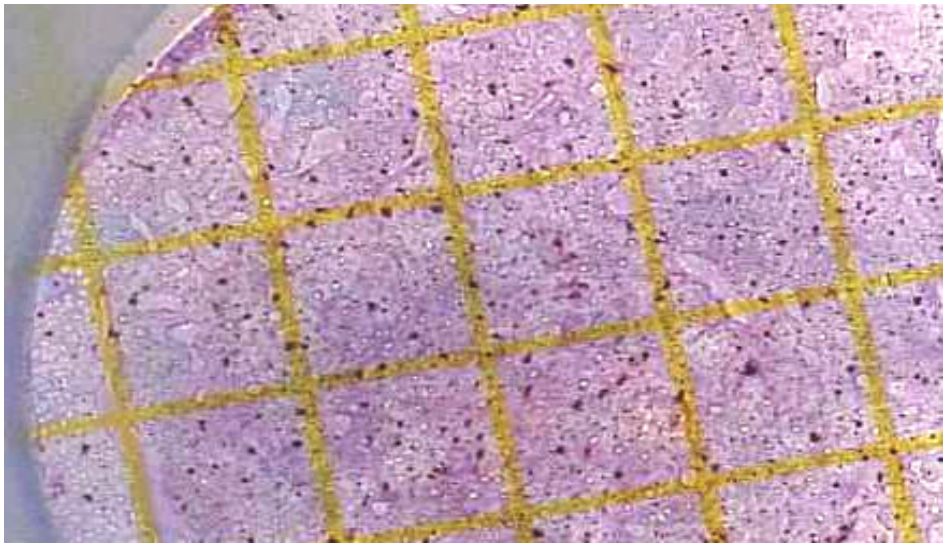
1. high levels of total organisms present in the sample can cause over-crowding on the film and thereby make colour interpretation of the *S. aureus* colonies difficult
2. colour interpretation is also harder when high counts of *S. aureus* are present

Additionally, the blue, red, pink colours of differentiation can make reading the plates difficult for those people with colour blindness (one of the researchers involved in these tests is colour-blind and hence a useful participant for these types of kits).

Where kit plates were overcrowded it was found useful to count the pink-zoned colonies in just one region of the background grid and multiply by the grid squares (each is 1cm$^2$). Ideally, the kit works most effectively with low counts of bacterial in the sample (< 100/g) and due the need for observing clear zones surrounding colonies for the confirmation of *S.*

*aureus*, the ideal number is around 20 per film plate. At these low levels, the Petrifilm kit repeatedly provided results exactly the same as the standard method for coagulase positive

**Plate 10.1. Petrifilm plate showing positive *S. aureus colonies.***



staphylococci. Above this range, human interpretation error and product sensitivity seemed to combine to produce difficulties involving pink zone over lap and inability to discern between red colonies and pink zones.

Several comments are worth noting for successful kit use within industry:

- dispersion of sample aliquot must be even for enumeration ease and success
- the correct dilution range of between 1-100 organisms is ideal
- pink colonies can be difficult to discern at >100 colonies/g
- even *S. aureus* controls tests can be misinterpreted at >100 colonies
- for the thermonuclease disc, temperature control at 62$^0$C, times and circulating air are critical for reducing false positives.
- a degree of skill/practise is required in removing the thermonuclease insert with out damaging the readable colonies present on the gel film
- given the above, blue and red colonies with pink zones can be discerned successfully and in agreement with levels attained by the standard method.

***Benefits:***

- inexpensive and easy to use, with some care
- no extra equipment needed for screening for *Staphylococcus*

***Limitations:***

- dilution of sample may be needed
- practise of reading the colours of colonies needed
- confirmation of coagulase *Staphylococcus aureus* not always possible

***Summation:***

The Petrifilm *S. aureus* kit is relatively easy to use and provides accurate results compared to standard methods for enumerating organisms. If thermostable nuclease confirmation is required for the organisms, this can be achieved but more care and greater expense is involved. Overall, the kit is a useful tool for screening seafood samples and or factory environments for *S. aureus*.

## 10.3 Dimanco BP Slides

The Dimanco BP Agar slide consists of a slide or paddle covered on both sides with Baird-Parker agar within a sterile plastic vial. It has a total surface area of 25cm$^2$. The selective BP agar contains chemical components that the *S. aureus* bacteria metabolise to result in classic black colony growth. These colonies are then counted and calculated for the surface area sampled.

The same samples as were tested for the Petrifilm kit were used in testing this kit too. Results from all samples showed that there was not good correlation between the number of colonies enumerated by the kit and that number detected by the standard method. Though convenient, the major concern with the Dimanco BP slides was that there was at least 1 log fewer colonies detected by the slides as compared to standard method counts. This greatly reduces the kit's effectiveness as an enumerating tool. When counts of *S. aureus* were <30/g as detected by the standard method, the BP slides showed no growth at all.

A second concern with this kit was the time to attain visible growth on the slide: growth generally did not occur until after the 24-48h recommended in the instructions. It was only after 72h that colony growth could be observed. This makes it a longer method than the standard method. Even when the slide was dipped in a reference culture of *S. aureus*, growth of colonies was not visible until around 60h. It was expected that the pure culture would be readily detected on the BP slide as there was no other possible interference to growth of the organism.

However, the BP slides did show much greater accuracy than this when used in direct contact with the surface of fish fillets. Therefore, the suggestion that this kit method is most effective for direct contact samples. However, the BP slide did not demonstrate accuracy in detection of *S. aureus* when sampling prawns, with little or no flat contact surface. In these cases, the operator would have to homogenise the sample in liquid and dip the slide into that, but in so doing enumeration accuracy appears to reduce. The slide was not very effective when the organism is within a liquid matrix, where recovery of *S. aureus* was low. This finding restricts the potential application uses of this kit to solid surface testing.

This test kit does not confirm differentiated coagulase positive *S. aureus*.

***Benefits:***

- extremely convenient and easy to use
- colonies are easy to count
- inexpensive per test
- very effective for processing surface and environment sampling

***Limitations:***

- some seafood samples may need homogenisation with liquid to provide an appropriate contact between slide and sample - this reduces the kit accuracy by at least 1 log/g.
- incubation time required by the kit is greater than the standard method, hence results are delayed rather than rapid.

***Summation:***

The Dimanco BP slides are very convenient and inexpensive, but their application use should be restricted to direct surface contact sampling. If required to be used with diluted homogenised sample, the slide should be in contact with the sample dilution for a prolonged contact period, followed by a thorough drain and dry. The slides should also be incubated for greater than the 48 hours recommended.

# 11. *Listeria*

*Listeria* spp. and particularly *L. monocytogenes,* is recognised as a major concern in seafood products (Hartemink and Georgsson, 1991; Dillon and Patel, 1992; Embarek, 1994; Johansson *et al*, 1999). It is especially of concern with smoked seafood products (Rorvik *et al*, 1995) as vacuum packaging, cold storage and high salt content may favour growth of pathogenic *L. monocytogenes* over competing microbial flora.

*Listeria* has also been found prevalent in the seafood processing environment (Miettinen *et al*, 2001). Currently very little is known about the survival of *Listeria* in processing environments (Bremer and Osborne, 2001) and hence it would be of enormous advantage for seafood industry businesses to be able to monitor for *Listeria* within their day to day operations.

## 11.1 Kit selection

Of the several kits available commercially, none were designed to detect the presence of *Listeria* species directly from food samples. Similar to other pathogen detecting kits, they are to assist to accelerate the screening of foods following an enrichment step. In a microbiology laboratory situation this is of considerable benefit as use of such kits negate the need for further lengthy incubation techniques after primary isolation of the organism. However, within a seafood processing environment it is unreasonable to undertake an enrichment step for the selection of *Listeria*, as the potential for subsequent contamination of the seafood premises with a grown culture of the organisms is high.

However, similar to work with *Salmonella* spp., two rapid kits were assessed for their ability to confirm the presence of *Listeria* spp. They give qualitative, not quantitative, results by detecting the presence of *L.monocytogenes* and related species. The two kits are similar in design basis and hence considered together here.

## 11.2 Oxoid Listeria Rapid Test *Listeria* Visual Immunoprecipitate Assay (VIP*) Listeria*

Both kits establish the presence or absence of *L.monocytogenes*. They are based on incorporation of an antibody attached on the kit surface which then binds specifically to *L.momocytogenes* antigen when present. A dye is included and reacts when antigen is bound, hence giving a visual colour band. Due to the antibody-antibody complex reaction of the kits, they are very specific for the organisms and have a high level of sensitivity. Both kits claimed detection levels as low as 5-7 cfu/g of *Listeria* organisms.

Both kits were similar in method required for use and come with reasonably clear instruction on methods for enrichment. The VIP kit uses modified Fraser Broth as the enrichment medium similar to the standard medium used and worked well giving clear and unambiguous kit readings. The Oxoid kit provided a number of pre-made additives that were not available with the VIP kit. In a standard laboratory environment this is not so important as ingredients would be readily available, however in an industry scenario the total support approach would be beneficial for the user. The heat shock step to release specific antigens from the organisms grown in the enrichment broth was simpler with the VIP kit as the temperature used was 100$^{o}$C (i.e. boiling water temperature). The same with the Oxoid kit required 80$^{o}$C and hence a specialised waterbath capable of holding this temperature constant is required.

Trials using either kit with samples from fish resulted in a high number of apparent false positives attained when compared to detection of *Listeria* presence in the same samples by standard method techniques. The standard method produced a negative result for *Listeria* presence, while the kit results indicated they were present. These findings warranted much further investigation, as testing which gives in false-positive results would have an enormous economic effect on the seafood processor and operations. The high proportion of false-positives obtained was considered to be serious weakness of the kit system. In trials using 'spiked' fish flesh samples it was found that the rapid confirmation kits are only sensitive to enrichment cultures containing $>10^3$-$10^4$ cfu/g.

Interestingly, in trials with stored samples, both kits and the standard method gave positive results. From all the results attained, it is suggested that it is possible the confirmation kits are detecting *Listeria* presence earlier, or at a lower level, than the standard method does. Further investigation in this area is probably warranted and may provide a direct detection system for monitoring for *Listeria* presence.

***Benefits:***

- use of the kit (after enrichment of sample) is very simple and easy
- identification of the presence of *Listeria* is extremely quick - the Oxoid kit took 20sec for a positive result and only 2 min for a negative

***Limitations:***

- the kit cannot be used on a straight sample
- for any sample type an enrichment step is required
- many apparent false-positive results were attained

***Summation:***

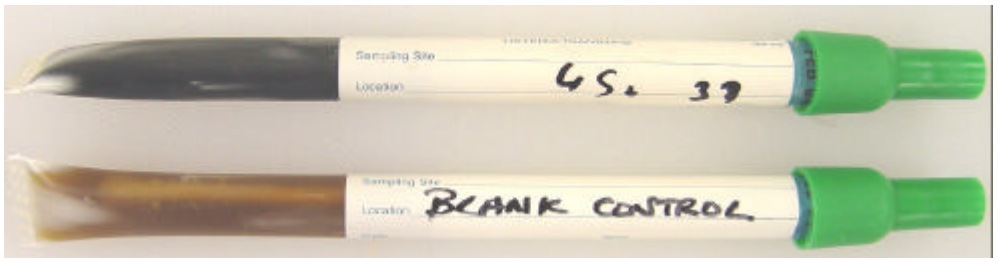
The kits give extremely rapid results (~20sec) and confirm *Listeria* presence readily. More work needs to be carried out regarding the seemingly high number of false-positive results attained. The kits are useful within the laboratory however, use of the kit is not appropriate for seafood processors unless they have a separate analytical lab on site. The stumbling block for seafood industry use of these kits for monitoring purposes is that should *Listeria* be present in the original sampled material, the kit procedure dictates the growth of a concentrated culture of *Listeria*.

### 11.3 Listeria Isolation Transwab

During the extensive search for all commercially available kits, the researchers discovered a swab kit designed for sampling for further *Listeria* identification. This kit is simply a "swab and incubate system". A swab is supplied which is used to sample the required test material (seafood, benchtop, drain etc). After sampling the surface, the swab is placed into a gel medium contianed inside the swab tube. This medium changes colour from brown to black in the presence of *Listeria* species and provides a preliminary detection of the organism. The *Listeria* isolation Transwab is designed to be used alongside traditional selective methods for detection of *Listeria* species

The kit medium relies on the characteristic of *Listeria* species to be able to hydrolyse a chemical compound esculin, which not many enteric bacteria are capable of doing. Esculin is incorporated into the gel medium gel, the hydrolysis of which results in a distinctive black precipitate. This is readily visible and causes the colour change in the medium (Plate 11.1).

**Plate 11.1. Colour change reaction in the Transwab kit.**




The swabs worked beautifully with control strains of *Listeria,* when the swab was dipped in diluted overnight broth cultures. Colour change was obvious and rapid 4-6h when incubated at 37$^{o}$C. These results were highly encouraging.

However when tested with seafood samples and/or mixed populations of microflora species, practically all swab tubes turned black. By the comparative standard plating methods, only a very low percentage of samples showed positive for *Listeria* spp. The swab kit appeared to be giving an extraordinary large number of false-positive results. Endeavouring to find out why, it was noted that of the 36 micro-organism species that the swab had been tested against by the manufacturer company, few were organisms that exhibit esculin hydrolysis for example: *Lactobacillus, Streptococcus, Leuconostoc* or *Pediococcus*. These genera of bacteria are quite likely to be present on processed seafoods and in land-based prcoessing environments, hence could contribute to the colour change reaction.

An intensive effort was allotted to further investigations with the Transwab kit due to its potential for application within the processing environment. If there was some definable conditions that could be applied to the use of this kit which allowed the differentiation of *Listeria* spp from other commensal flora species, it would be excellent in practical terms.

Initially, studies focussed on defining the reaction of the gel media in the kit with pure cultures of strains of *Listeria monocytogenes.* Standard method plating was always run in parallel with swab trials to determine the exact number of organisms being inoculated into the swabs.

It was noted that the while the average volume absorbed by the Transwab for inoculation of the gel medium was often less than the standard 0.1ml (some were found to be as low was 0.076ml), this was considered not to affect low dilution inoculations by an amount to be of concern. The Transwabs were quite variable in tip bud size which was the likely reason for slightly different volumes of sample being absorbed.

### 11.3.1 Time for colour change in Transwab

Studies were carried out with several strains of *Listeria monocytogenes,* grown overnight as broth cultures. Trial results indicated that the Transwab reacted accurately even when few *Listeria* were present in the sample. (Table 11.1).

**Table 11.1. Colour change of Transwab kit with *Listeria monocytogenes* (#18)**

| Concentration of *Listeria* in broth culture sampled (cfu/ml) | Time for colour change (h) |
|---|---|
| **2000** | **< 17** |
| **200** | **< 19** |
| **20** | **< 24** |
| **2** | **< 41** |

As these trials were conducted with pure cultures, the question arose as to the colour change reaction times when few *Listeria* are present in a mixed microflora population. Investigations were conducted with a mixed population of bacteria, of known proportions, originally isolated from seafood product and inoculated with a range of concentrations of different strains of *Listeria*. The mixed microflora population was grown to concentration of around $10^8$/ml and comprised of 9–10 different bacterial species selected for the characteristic that they did not hydrolyse esculin and cause a colour change in the media. Results (Table 11.2) show that *Listeria* species grow well and hydrolyse esculin even at low numbers in a mixed population.

**Table 11.2. Colour change time when *Listeria* present as part of mixed flora.**

| **Culture concentration (cfu/ml)** | ***L.monocytogenes* #31** | ***L.monocytogenes* #18** | ***L.innocua*** |
|---|---|---|---|
| 2400-2800 | <16 ♣ | < 17 | < 22 |
| 240-280 | < 20 | < 19 | < 22 |
| 28-24 | < 21 | < 21 | < 22 |
| 3 | < 36 | > 48 | < 22 |

♣ hours

These investigations demonstrate that the Transwab kit is very capable of detecting the presence of *Listeria* from seafood samples against a mixed flora background. This fits with the information from the manufacturing company of the Transwab kit who state that the gel medium within the kit contains inhibitors which stop the growth of non-*Listeria* species. They further claim that only *Listeria* species will grow within the medium to produce a black precipitate due to esculin hydrolysis. However, it is suggested by some researchers that *Listeria* are particularly difficult to select for and it is reported that even Oxford listeria selective agar can support dense bacterial growth which is not equated to *Listeria* spp. (Neamatallah *et al*, 2003) and hence this could mask the growth of *Listeria* species. Hitchins and Duvall (2000) suggest that growth of *Listeria* and *L. monocytogenes* in particular may be hindered by the presence of other organisms.

Additional fish samples were investigated using the Transwab and again a high number of samples tested returned positive reactions from the Transwabs. For example, total bacterial loads of ~$10^5$ – $10^7$ on fish flesh ( sweetlip, nile perch, snapper, mullet and prawns

purchased from seafood retail outlets) yielded positive Transwab reactions within 16 - 24h and yet no *Listeria* were recovered from the samples by the standard method procedures. In fact, for all samples of fish purchased only two, a rainbow trout and a mullet, demonstrated *L. monocytogenes* and *L.welshimeri*, respectively.

Further effort was focussed on establishing whether the growth conditions for those bacteria that were causing the false-positives could be manipulated so as to minimise their effect on colour change in the media. It had already been shown that *Listeria*, if present, would cause a definite color blackening of the media in the Transwab and hence, the hypothesis was: can the 'false-positive-causing' organisms be diluted sufficiently to reduce or delay their effect on the media colour.

Investigations were conducted where, subsequent to showing a positive black reaction, the primary Transwab was removed from the tube and transferred to a fresh Transwab gel medium. Additionally, the positive swab was subjected to decimal dilutions and plated onto standard method PalCam and Oxford agars. Results illustrated that all the 'second' swabs also turned black and within 15h. It was concluded that a simple dilution factor of the positive-causing bacteria was not effective in reducing their ability to cause a medium colour change.

Last attempts concentrated on the time/temperature conditions stipulated with the Transwab kit. It was considered possible that while *Listeria* species are known to grow favourably at 20$^{o}$C, those bacterial species causing false-positive reactions may not do so as readily at lower temperatures. Preliminary experiments checked that *Listeria* strains would still hydrolyze esculin at lower temperatures (30$^{o}$C, 25$^{o}$C and 20$^{o}$C) and this was shown to be correct but with a corresponding extended period for the reaction to occur (Table 11.3).

**Table 11.3. Transwabs inoculated with *Listeria* strains, incubated at different temperatures.**

| ***Listeria* culture** | **37$^{o}$C** | **30$^{o}$C** | **25$^{o}$C** | **20$^{o}$C** |
|---|---|---|---|---|
| ***L.monocytogenes*** | 8-22 h | 8-22 h | 33-48 h | 48-72 h |
| ***L.monocytogenes* in a mixed culture*** | 8-22 h | 8-22 h | 33-48 h | 48-72 h |
| ***L.innocua*** | 8-22 h | 8-22 h | 33-48 h | 48-72 h |
| ***L.innocua* in a mixed culture*** | 8-22 h | 8-22 h | 33-48 h | 48-72 h |

* composed of commensal flora of seafoods without bacterial species that cause positive colour changes (concentration: 1.2x10$^{8}$ cfu/ml)

Although the exact time for colour change was not able to be observed, these results provided confirmation that *Listeria* species will still hydrolyze esculin at temperatures lower than 37$^{o}$C.

A series of experiments was undertaken combining *Listeria* (strain 31) at different inoculum levels and a range of mixed bacterial populations, with an without the presence of bacterial

strains that had proven to cause blackening of the Transwab medium. All swabs were incubated at either 37°C or 25°C (Table 11.4).

**Table 11.4. Time for reaction of Transwab medium inoculated with different levels of *Listeria* and different mixed populations of bacteria.**

| Background culture type | *Listeria* **170** cfu/ml | | *Listeria* **17** cfu/ml | | *Listeria* **2** cfu/ml | | *Listeria* **0** | |
|---|---|---|---|---|---|---|---|---|
| | **37°C** | **25°C** | **37°C** | **25°C** | **37°C** | **25°C** | **37°C** | **25°C** |
| None | 20-21* | 28-40 | 25-26 | 28-40 | >68 | >68 | >68 | >68 |
| Mixed - $10^{8}$ φ | 23-24 | 28-40 | 24-25 | 28-40 | 24-25 | 40-42 | >68 | >68 |
| Mixed - $10^{5}$ | 25-26 | 28-40 | 28-40 | 40-42 | 40-42 | >68 | >68 | >68 |
| FB 9 α - $10^{9}$ | 25-26 | 28-40 | 25-26 | 28-40 | >68 | >68 | >68 | >68 |
| FB 9 α - $10^{6}$ | 25-26 | 28-40 | 28-40 | 40-42 | >68 | 40-42 | >68 | >68 |
| S 5 α - $10^{8}$ | <16 | 28-40 | <16 | 28-40 | <16 | 48-68 | <16 | 48-68 |
| S 5 α - $10^{6}$ | 25-26 | 28-40 | 25-26 | 48-63 | >68 | >68 | >68 | >68 |

* hours to black
φ cfu/ml
α FB9 and S5: isolates capable of causing media colour change to black

It is seen that when *Listeria* was present at >2cfu/ml, the Transwab always returned a positive result whatever the background microflora. This occurred at both incubation temperatures, with a delayed reaction time at the lower temperature. Where *Listeria* levels were very low (2cfu/ml) a positive reaction was not always observed. One anomalous result occurred with low level *Listeria* within the FB 9 population (at $10^{6}$cfu/ml), where a positive reaction was noted more rapidly at 25°C than at 37°C. It is suggested that this could be a result of < 2 Listeria cells being transferred to the Transwab. This was not confirmed by carrying isolation work.

It is of note that when *Listeria* was absent, the only positive results obtained were from microflora populations which contained the S5 isolate and this isolate caused a rapid change of the gel medium to black illustrated by the reaction times. The FB9 isolate which previously had consistently demonstrated the ability to hydrolyse to esculin within the Transwab kit and caused typical 'listeria-like' reactions on PalCam and Oxford agar plates, did not cause a similar positive reaction within these experiments. Similar variable reactions had been observed with other isolates which gave a 'false-positive' blackening reaction at primary inoculation and subsequently did not consistently produce the effect.

These investigations revealed that there is no simple trend indicating that *Listeria* spp. react and cause blackening of the Transwab gel medium more rapidly than other bacterial species at low incubation temperature. This was disappointing and warrants further investigation as experimentation in this area was restricted by research project constraints.

Work on the identification of various isolates causing a black reaction in the Transwab medium revealed a range of bacterial types. Several were identified as Gram +ve cocci, tentatively suggested to be *Micrococcus* spp. after further biochemical characteristics were dertermined. Others were Gram –ve short rods: some showing a yellow pigmentation of colonies and suggested to be of the *Flavobacterium* group; non-pigmented colonies were identified as *Pseudomonas vesicularis*. All these bacterial species are commonly found in the commensal flora within aquatic envirnoments. In fact, Bremer and Osborne (2001) reported that the number of *L. monocytogenes* cells attaching to stainless steel surfaces increased when in the presence of a mixed microflora containing *Flavobacterium* spp. Therefore, as these organisms are capable of turning the Transwab medium black, it negates the applicability of this kit for use within the seafood industry environment.

***Benefits:***

- extremely simple and easy kit to use
- could be applied for monitoring processing environments with the qualification of requiring further laboratory confirmation of positive results

***Limitations:***

- not reliable with microflora from seafood, especially when present in high numbers
- too many false-positives attained

***Summation:***

The Transwab is an excellently simple kit concept which potentially removes the need for an enrichment culture grown within the factory environment, but too inaccurate to be useful for industry. The kit did work superbly with tested *Listeria* species, even when the were present in very low numbers and when against a high level background flora. However, for some commensal floras from fish and the seafood environments, the presence of specific bacteria capable of hydrolysing esculin causes false-positive results from the Transwab.

The lack of a functional and industry-use appropriate kit for determining presence of *Listeria* within factories is a disappointing outcome of the project work. Such a rapid kit would be of enormous use and benefit to industry. However, it is deemed unwise to suggest industry use of the transwab kit currently available.

# 12. Sulphite

Metabisulphite is commonly used on prawns as a retardant to the development of melanosis or black-spot. However, there are restrictions as the allowable residual levels permitted in end product. In Australia for domestic raw product, a residual sulphite concentration of 30 mg/kg (or ppm) is permitted, while an export level is 100mg/kg. As these levels are subject to strict control by AQIS (among other regulatory bodies) it is useful for industry to be able to test the concentration of sulphite residual in dipping tanks and prawns.

## 12.1 Test kits assessed

There are several kits available commercially (Table 12.1).

**Table 12.1. Commercial test kits for measuring sulphite.**

| Test kit name | Sample type and range |
|---|---|
| Alert sulphite test kit | Solutions and flesh surface<br>>10 ppm but <100 ppm [1]<br>>100 ppm |
| Boehringer Mannheim sulphite test kit | Solutions and flesh<br><0.3g/L |
| Hanna Instruments sulphite test kit | Solutions<br>0 to 20 ppm $Na_2SO_3$ or 0 to 10 ppm $SO_2$<br>0 to 200 ppm $Na_2SO_3$ or 0 to 100 ppm $SO_2$ |
| Merckoquant sulhpite test strips | Solutions and flesh surface<br><400 ppm |
| Palintest sulphite (Tablet) | Solutions<br>0 to 500 ppm $Na_2SO_3$ [2] or 0 to 250 ppm $SO_2$ [3] |
| Palintest sulphite (Photometer) | Solutions<br>0 to 500 ppm $Na_2SO_3$ or 0 to 250 ppm $SO_2$ |
| Titrets sulphite test kit | Solutions<br>10 to 100 mg/L $SO_3$ (ppm) or 8 to 80 ppm $SO_2$<br>50 to 500 mg/L $SO_3$ (ppm) or 40 to 400 ppm $SO_2$ |

[1] ppm = mg/L
[2] $Na_2SO_3$ = sodium metabisulphite
[3] $SO_2$ = sulphur dioxide

All 7 sulphite test kits were evaluated with either dipping solutions or prawns flesh as appropriate.

## 12.2 Sulphite kits for testing dip solutions

A comparison of those kits that were suitable for testing prawn dip solutions was carried against a range of different sulphite concentrations. This comparison has been replicated in a second evaluation trial. Because of the limited number of tests able to be carried out by some brands of kit, several individual kits had to be purchased.

None of the kits were suitable for direct testing of prawn dip sulphite concentration. A dilution of 1 in 20 was required to reduce the sulphite concentration to within the effective ranges of the individual kits. This action requires some accuracy on behalf of the tester. Some kits were ineffective and the components were exhausted before satisfactory data could be obtained. Testing of a second batch of these kits produced results indicating that the previous kits had been faulty. Chemical kits do have a finite shelf life and it is likely to have expired while in the hands of the supplier. This situation has ramifications for use of

some chemical kits by the seafood industry, as it is unlikely that regular application of standard curves would be possible during routine operation. It would also make that particular kit much more expensive to use.

The results produced by each kit were compared statistically with the known sulphite concentration of the standard sodium metabisulphite solutions tested. The results obtained from the trials were analysed using Pearson's correlation coefficients. These reflect the extent of the linear relationship between the standard and the test kit result. These are presented in Table 12.2 below to show how close the result was to the true value. The correlation coefficient and probability from this analysis is present in the following table.

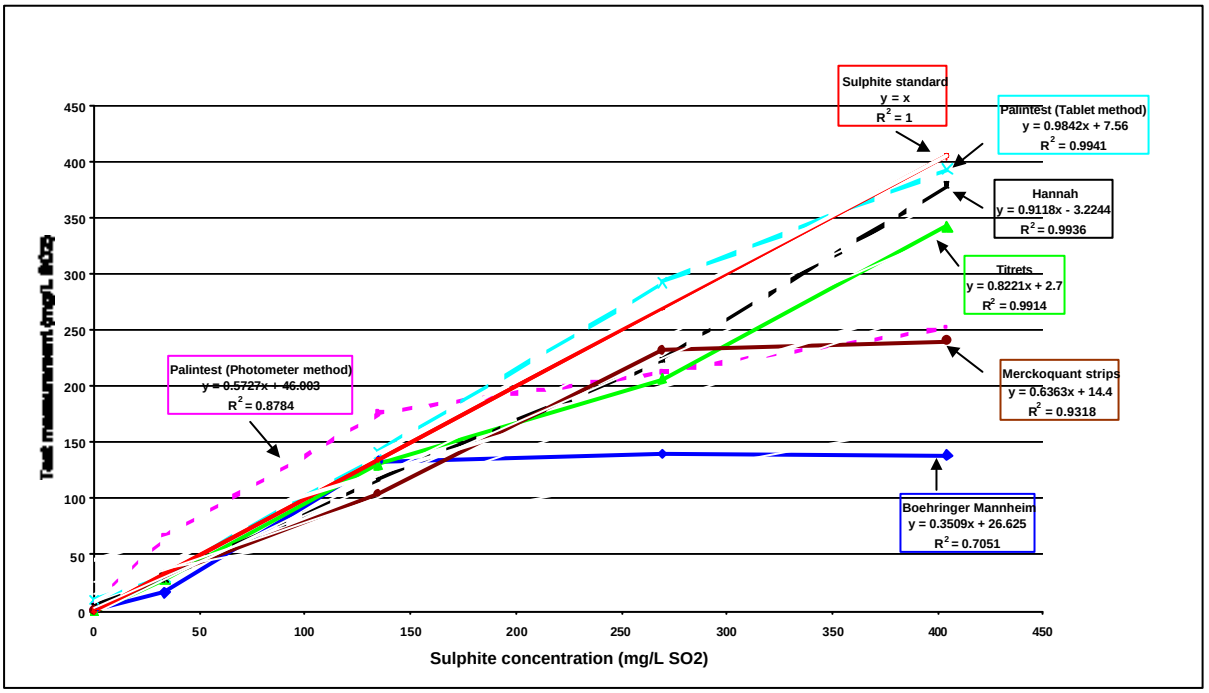
**Table 12.2. Comparison of sulphite kit result against a sodium metabisulphite standard curve.**

| Kit | Correlation coefficient ($R^2$) | P-Value |
|---|---|---|
| Boehringer Mannheim Sulphite Test Kit | 0.5643 | 0.1451 |
| Palintest Sulphite Test Kit Photometer method | 0.9047 | 0.002 |
| Titrets Sulphite Test Kit Titration method | 0.9904 | 0 |
| Palintest Sulphite Test (Tablet method) Kit | 0.9935 | 0 |
| Hannah Instruments Sulphite Test Kit | 0.9884 | 0 |
| Merckoquant Sulphite test strips | 0.8774 | 0.0042 |

Results that correlate highly have a coefficient close to 1 and a probability close to zero. With the exception of the Boehringer Mannheim Sulphite Test Kit, the kits recorded sulphite concentrations that correlated well with the standards used.

These relationships can also be displayed in graph form. A plot of $SO_2$ curves produced by comparing the sulphite value obtained by the kit against the concentration of the known standard can be seen in the following figure, Figure12.1.

**Figure 12.1 Sulphite standard curve, linear correlation and equation for various sulphite test kits.**



Statistical analysis of the data allowed the identification of differences between individual kits of the same brand. The differences found support the earlier conclusion that deterioration of kits can occur during storage. This led to a lower accuracy for that particular brand of kit.

The accuracy of a kit can be determined by a number of values displayed in the above plot. The first value to be considered is the linear nature of the curve that can be expressed as the linear correlation ($R^2$ value). As the curve for the standard solutions has an $R^2$ value of one, only those kits, which produce $R^2$ values approaching one, can be considered to produce accurate results.

The second value of note is the slope of the curve (the factor associated with **x** in the linear equation that describes the line). Again this value should approach one to be considered accurate. For the test to be able to produce a reading close to zero concentration, the cofactor in the equation following the slope term should be close to zero. The larger the value the less sensitive the test is at low concentrations.

For this investigation the kits that produce results that are the most accurate are the Palintest Sulphite Test (Tablet method) Kit and the Hannah Instruments Sulphite Test Kit. The Titrets Sulphite Test kit was the third closest to the standard.

While the Palintest Sulphite Test (Tablet method) Kit was the easiest to use and the most accurate, the sensitivity of the kit was not as effective as the Hannah Instruments Sulphite Test Kit. Due to the use of tablets to supply the reactive agents, the Palintest Sulphite Test Kit cannot give a zero measurement as shown by the curve cofactor of 7.56. This also causes the results of the kit to increase by 40 mg/L increments of $Na_2SO_3$. The Hannah Instruments Sulphite Test Kit does start at a zero measurement and increases by 2 mg/L $Na_2SO_3$ increments. The standard curve for this kit had a negative cofactor but it is smaller than that describing the Palintest Sulphite Test (Tablet method) curve. This aspect is probably only of interest if extreme accuracy was required. Either kit would be suitable for measuring prawn dip concentrations. A dilution factor of 20 times the original solution should be applied so that the kits can produce a result without excessive use of the test components. At this time they both cost the same per test and appear to have good shelf life.

The results of residual sulphite levels in prawns tested using a standard prawn dip are given in Table 12.3.

**Table 12.3. Sulphite measurement (mg/L) of a sodium metabisulphite dip using available test kits.**

| Kit | Dip before prawns applied | Dip after prawns applied |
|---|---|---|
| Monier-Williams Distillation Method | 57 | 52 |
| Boehringer Mannhein Sulphite Test Kit | 7.8 | 10.9 |
| Palintest Sulphite Test (Photometer method) Kit | 228.5* | 164.3* |
| Palintest Sulphite Test (Tablet method) Kit | 71 | 71 |
| Titrets Sulphite Test Kit (10-100ppm $SO_2$) | 13 | 12.5 |
| Titrets Sulphite Test Kit (50-500ppm $SO_2$) | 150 | 148 |
| Hannah Instruments Sulphite Test Kit | 57.9 | 59.4 |
| Merckoquant Sulphite test strips | >80<180 | >80<180 |

* Test resulted in high readings for a blank.

The Hannah Instruments Sulphite Test Kit produced measurements closest to the sulphite residue as determined by the Monier-Williams method. The Palintest Sulphite Test (Tablet method) Kit was the next kit close to the real residue level. While unable to give a definitive numerical measurement, the Merckoquant Sulphite test strips were third in order of accuracy. This result is consistent with the performance of the kits against the standard curve.

### 12.3 Sulphite kits for testing prawn flesh

When a prawn is dipped for a short time in a chemical solution the majority of residue remains at the surface. The testing of only the surface of the prawn does not lead to an effective evaluation of the sulphite for the whole of the flesh. Legislation relating to sulphite residues refers to the edible portion and only by extracting the sulphites from the whole of the flesh will a result be obtained that will be comparable to the standard method known as the Monier-Williams method. To determine whether prawn pieces or a homogenised sample were most appropriate for testing with the Alert Sulphite Test Kit a homogenate was made from 25 individual prawns. This and whole prawns from the same treatment were tested (Table 12.4).

**Table 12.4. Sulphite measurement of prawns commercially treated with a sodium metabisulphite dip using the Alert kit.**

| Kit | Type of Sample | Sulphite residue (mg/L $SO_2$) |
|---|---|---|
| Monier-Williams | Individual prawns | <10 |
| Alert Sulphite Test Kit | Individual prawns | 5 at <10, 5 between 10 and 100 |
| Alert Sulphite Test Kit | Homogenised sample | 2 at <10, 1 between 10 and100 |

The result for this kit makes it difficult to identify which method is best for testing. There was difficulty in reading the Alert result, as often there was no violet colour development just the fading of the blue. The Alert Sulphite Test Kit can only give two measurements, greater than 10 but less than 100 mg/L sulphite or greater than 100 mg/L sulphite. This does cover the sulphite concentration of 30 mg/L sulphite that is pertinent to legislation about domestic production of prawns. The sensitivity of the Merckoquant Sulphite Test Strips is also low with large increments between each reference concentration.

Both kits also were difficult to read when testing the surface of prawns. The Merckoquant Sulphite Test Strips not only picked up sulphite but also protein from the surface of the prawn. This was much darker than the surface of the strip and masked the reading that could be made. This could lead to much higher measurements being recorded than were actually displayed by the strip. Because the Alert Sulphite Test Kit is composed of solutions, these readily roll off the surface of the prawn possibly even washing away some of the sulphite residue. This made it difficult to interpret and could lead to lower measurements of sulphite residue being recorded than was actually present.

The researchers were unable to obtain effective sulphite measurements from prawns using the Boehringer Mannheim Sulphite Test Kit. While the chemical supplier promotes the kit as suitable for prawns, a method of application for prawns was not present in instructions accompanying the kit. One method that was recommended as suitable for testing samples with similar viscosity to prawn flesh homogenate, such as jam, appeared to be suitable. This method recommended heating a homogenised sample in a 60°C water bath for 5 minutes

with regular shaking. Treated prawn flesh was then evaluated using the three kits promoted as suitable for evaluating solid material.

The Boehringer Mannheim Sulphite Test Kit did produce inconsistent measurements for prawn flesh (Table 12.5).

**Table 12.5. Sulphite measurement (mg/L) of prawns exposed to a sodium metabisulphite dip using available test kits.**

| Kit | Material tested | Prawns dipped for 30 seconds | Prawns dipped for 90 seconds |
|---|---|---|---|
| Monier-Williams Distillation Method | homogenate | 16 | 33 |
| Boehringer Mannheim Kit | homogenate | 22.3 | 0.3 |
| Alert Sulphite Kit | homogenate | <10 | <10 |
| Merckoquant Sulphite test strips | homogenate | 10 | 1 at 80<br>1 at>80<180 |
| Alert Sulphite Test Kit | individual prawns | 6 at>80<180<br>4 at 180 | 7 at >10<100<br>3 at >100 |
| Merckoquant Sulphite test strips | individual prawns | 6 at>80<180<br>4 at 180 | 2 at 80<br>4 at>80<180<br>3 at 180<br>1 at >180<400 |

As the homogenate led to lower measurements than the Monier-Williams method, indicating that some of the sulphite was being bound to the matrix, individual prawns were utilised for the final trial. A range of prawn treatments resulted in sulphite residues between <10 and 160 mg/L $SO_2$ as measured by Monier-Williams were tested by the three kits. The Merckoquant Sulphite Test strips and the Alert Sulphite Test Kit were used on 10 individual prawns from each treatment while the Monier-Williams method and the Boehringer Mannheim Sulphite Test Kit were applied in duplicate to prawn pieces derived from 10 individuals. The number of correct measurements with respect to the Monier-Williams method that was recorded by each kit is presented in Table 12.6.

**Table 12.6. The number of correct flesh sulphite measurements identified by three test kits.**

| Kit | % of correct measurements | % of incorrect measurements |
|---|---|---|
| Merckoquant Sulphite test strips | 16.9 | 83.1 |
| Boehringer Mannheim Sulphite Test Kit | 11 | 89 |
| Alert Sulphite Test Kit | 53.9 | 46.1 |

The Alert Sulphite Test Kit produced the most number of correct measurements. Unfortunately it produces just as many incorrect measurements. The results show that none of these kits are reliable to test for the residue in prawn flesh, either whole or homogenised.

### 12.4 Summary of sulphite kits

Table 12.7 reviews the kits tested that were suitable for solutions.

## Table 12.7. General Comparison of Sulphite solution Test Kits.

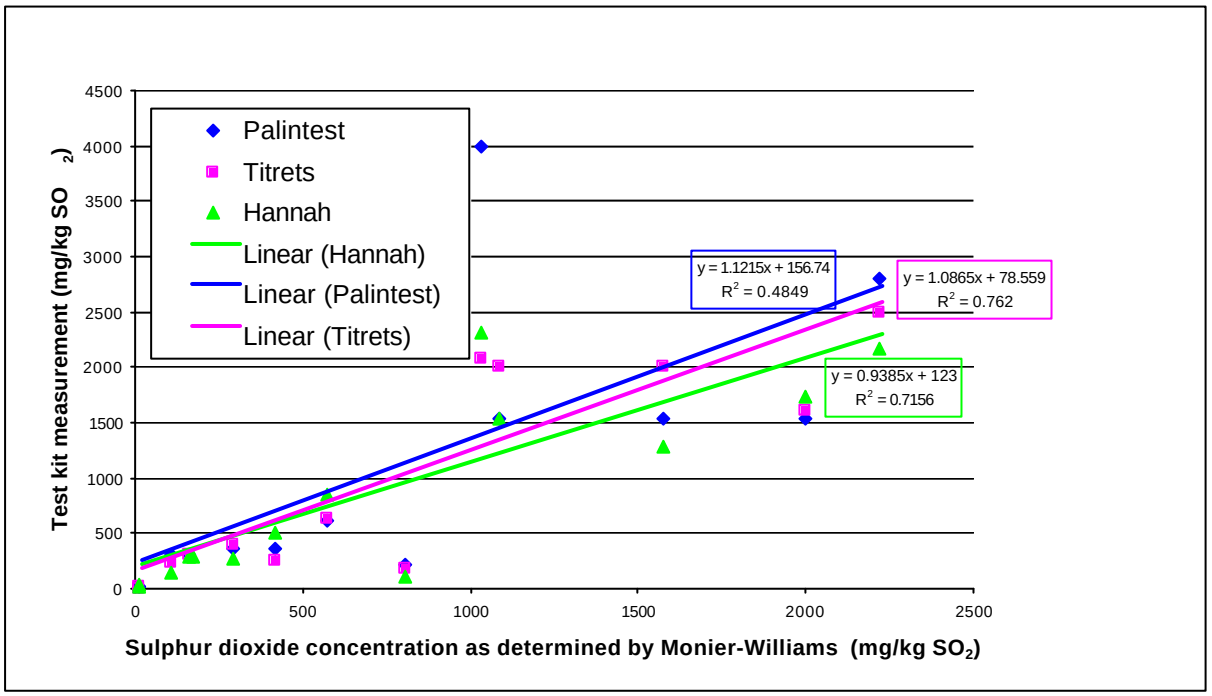
| | Hannah Instruments | Titrets Titration method | Palintest Tablet method | Palintest Photometer method | Merckoquant test strips | Boehringer Mannheim |
|---|---|---|---|---|---|---|
| **Supplied reagents** | All equipment and reagents supplied in plastic case | Everything supplied | Reagents supplied only | Reagents supplied only | Everything supplied | Reagents supplied only |
| **To test prawn dip (1kg sulphite/100L)** | Dilute 1:100 | Dilute 1:100 | Dilute 1:100 | Dilute 1:100 | Dilute 1:20 | Dilute 1:100 |
| **To test holding tank (50g sulphite/100L)** | Direct | Direct | Direct | Direct | Direct | Direct |
| **Addition of reagents** | 5 different reagents | 1 addition only then titrate | 2 different types of tablet | 2 different tablets | None | 5 different reagents |
| **Testing time** | 1 minute | Approx. 3 minutes | 1 minute | Approx. 4 minutes | 30 seconds | Approx. 40 minutes |
| **Method of test and reading of results** | Visual colour change stops titration, read delivery pipette | Visual colour change stops titration, read reaction tube volume | Visual colour change to stop addition of tablets | Photometer measures transmittance | Colour of strip referenced to chart supplied | Spectrophotometer measures absorbance |
| **Calculating results** | Simple multiplication of reading | Simple multiplication of reading | Simple multiplication of reading | Direct reference to chart provided | Direct result | Requires involved calculation |
| **Accuracy** | Accurate result produced | Accurate result produced | Accurate result produced | Accurate result sometimes produced | Limited accuracy due to stepped reference colours | Accurate results obtained under rigid operating conditions |
| **Stability of reagents** | Room stable | Room stable | Room stable | Room stable | Refrigerate | Refrigerate |
| **Average cost** | 71c / test | $8.80 / test | $1 / test | $1.36 / test | 60c / test | $10 / test |
| **Comments** | Easy to follow directions and use | Several steps to method, can over-titrate easily | Easy to follow directions and use | Needs some equipment, old stock can be defective | Easy to use, strips deteriorate with temperature | Needs expensive equipment, training, old stock can be defective |

## 12.5 Commercial trials of sulphite test kits

The three most accurate kits have been tested by industry in Western Australia, South Australia and Queensland. Individuals from four different companies responsible for preparing and testing sulphite treatments appraised the Palintest Sulphite Test (Tablet method) Kit, Titrets Sulphite Test Kit (10-100ppm $SO_2$), Titrets Sulphite Test Kit (50-500ppm $SO_2$) and Hannah Instruments Sulphite Test Kit.

The application of these kits has been evaluated both on board vessels and in the processing factory (Figure 12.2). Samples of the test solutions were returned to the laboratory for evaluation by the Monier-Williams method. The accuracy of these kits was consistent with the previous trials conducted in the laboratory. The following graph shows the results from these trials.

**Figure 12.2. Commercial evaluation of sulphite test kits**




The linear correlations show that the Titrets sulphite test kit was the most accurate. The Hannah Test kit followed this closely. The Palintest kit performed badly. This is hard to explain as a fresh batch of kits was obtained for these trials.

The industry evaluations of the three best performing kits found that the Hannah test kit was the most preferred of the three kits. The selection of a particular kit appeared to depend on familiarity of the individual with chemical test methods. The more complex methods were selected more often as the preferred kit. The difficulty of titrating using the Titrets resulted in this kit being given a lower rank than the Hannah.

Most of these industry evaluations were performed in the presence of one of the researchers. This helped the industry participant with information about individual kits and actions required to obtain valid results. When the evaluation was conducted by industry without the researcher present the results obtained from the kits were unreliable. It is obvious that even the three best performing kits were not able to give precise results if the

operator was inexperienced in diluting test samples accurately. This outcome indicates that even if a company decides to use a particular sulphite test kit there should be some initial training of the staff responsible and regular backup testing of samples by an independent laboratory.

## 12.6 Brief evaluation opinion of each test kit:

### 12.6.1 Alert sulphite kit

*Benefits:*

- Measuring prawn flesh not solutions.
- Quick result (1 minute)
- Range important to industry

*Limitations:*

- The drops diffuse over the surface of the prawn making it difficult to be sure of the result.
- The dye pools in the grooves between segments. Colour changes were more noticeable in areas where the solutions were thin.
- The method tests only the surface of the prawn. The result produced will always be higher than the sulphite residue present for the whole of the prawn. Food standards for sulphite residues refer to the edible portion.
- When a homogenised sample was tested the viscous nature of the sample resulted in a negative result even when a high residue was present.

### 12.6.2 Boehringer Mannheim sulphite kit

*Benenfits:*

- This kit was inaccurate and therefore has no benefits for industry

*Limitations:*

- Needs to be refrigerated below 4°C.
- Specialised equipment needed such as spectrophotometer and homogenisers.
- Accurate results obtained only under rigid operating conditions.
- Test concentration of sulphite must be between 0.03 and 0.3 g/L.
- Solid or semisolid samples should be crushed or homogenised and extracted with or dissolved in water and filtered.
- Turbid solutions need to be filtered or centrifuged.
- Solutions need to be pH adjusted. Acid and weakly coloured samples need to be adjusted to pH 7.5-8 by adding sodium or potassium hydroxide solution.
- Strongly coloured samples must be treated with polyvinylpolypyrrolidone (PVPP).
- Does not appear to work on prawn flesh. Inconclusive results were obtained for dip solutions.

### 12.6.3 Hanna Instruments Sulphite test kit

*Benefits:*

- All equipment supplied in sturdy plastic case.
- Easy to follow directions and to use.
- Precise control of titration possible.
- Accurate result produced.
- No pH adjustment required.

*Limitations:*

- Dilution of at least 1:100 is required to test normal prawn dips.
- Designed for sodium sulphite, calculations needed for other compounds.
- Choice of test vessel important for obtaining correct result.
- Plastic test vessels fragile.

- Cannot use tap water for dilutions

### 12.6.4 Merkoquant sulphite test strips

*Benefits:*

- No equipment required for testing if pH of test solution lies between pH 6 and pH 12.

*Limitations:*

- Needs to be refrigerated below 4°C.
- Dilution of at least 1:100 is required to test normal prawn dips.
- Stated as suitable for dip solutions and prawn flesh, but no procedure for the latter is supplied.
- The method tests only the surface of the prawn. The result produced will always be higher than the sulphite residue present for the whole of the prawn. Food standards for sulphite residues refer to the edible portion.
- pH of test solution should lie between pH 6 and pH 12. Solutions below pH6 should be adjusted to a pH value of at least 7-10 with sodium acetate or sodium hydroxide solution. All prawn dips will have a lower pH than 6.
- A slight change of colour between levels makes it difficult to determine concentration of solutions other than specific levels on reference chart.

### 12.6.5 Palintest (Tablet)

*Benefits:*

- Easy to use and calculate result.
- Quick result (1 minute)
- Large range which is more important to industry

*Limitations:*

- Dilution of at least 1:100 is required to test normal prawn dips.
- No sample container.
- Requires a lot of the reagents to test high concentration dips.
- No zero measurement possible.
- Sensitivity poor because due to 40 mg/L $Na_2SO_3$ increments.

### 12.6.6 Palintest (Photometer)

*Benefits:*

- Quick result (2 minutes standing then reading with Photometer)
- Large range of residues important to industry can be tested.

*Limitations:*

- Specialised equipment required. Battery operated photometers are available.
- Dilution of at least 1:100 using distilled water required for testing normal prawn dips.
- No sample container provided.
- Use distilled water for any dilutions. Gives low results in the presence of tannic acid or tannin treated waters and no result if nitrites, ferrous ions or sulphide present.

### 12.6.7 Titrets

*Benefits:*

- All materials supplied.
- Accurate test procedure supplied.
- Larger range which is more important to industry
- Separate kits required for testing dips or holding tanks.

*Limitations:*

- Test procedure not very easy to follow - must be read several times to understand how to use equipment.
- Dilution of at least 1:100 is required to test normal prawn dips.
- Must be stored in dark place.

- Difficult to control titration resulting in less accuracy

# 13. Histamine

There was difficulty in obtaining more than one histamine test kit. Even chemical supply companies listed as being agents for particular kits were no longer supplying any histamine test kits. In the second year of the project while searching for information on histamine test kits, a review paper written by researchers from Davis University was obtained which compared six different histamine test kits (Rogers and Staruszkiewicz, 2000). As much time had been lost waiting for deliver of further histamine test kits and a review had already been published it was decided to rely of this data rather than to repeat similar work.

When it became clear that only one kit would be obtained during the term of the project, the researchers decided that as the review article had identified the effectiveness of this particular kit that it should be evaluated on its own as it is the only one available to Australian processors.

## 13.1 Alert Histamine Screening Test kit

13.1.1 *Qualitative results:*
Table 13.1 gives qualitative results obtained for the laboratory evaluation of the kit.

**Table 13.1. Histamine test kit qualitative performance.**

| **Treatment** | **A** | | | | | | **B** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Spiked concentration | 0 | 10 | 20 | 40 | 60 | 100 | 0 | 10 | 20 | 40 | 60 | 100 |
| Results by capillary electrophoresis (mg/kg)* | 0 | 0 | 7 | 21 | 33 | 63 | 0 | 0 | 14 | 29 | 28 | 61 |
| Results from Kit 1 | –[α] | – | – | ± | ± | + | – | – | – | ± | ± | + |
| Results from Kit 2 | – | – | ± | ± | ± | + | – | – | ± | ± | ± | + |
| Results from Kit 3 | – | – | ± | ± | + | + | – | – | ± | ± | + | + |

* As determined by AGAL
[α] – = 0mg/kg, ± = <50mg/kg, + = >50mg/kg

The blue of the blank was quite different to the pink colour of samples that contained some histamine. It was quite easy to identify samples that contained histamine levels less than 50mg/kg. The amount of colour strength due to the histamine concentration provided such sensitivity to the test that three levels of result could be determined. The visual evaluation of the test shows that the kit is quite accurate but there were several incorrect determinations that may be due to error of the two individuals evaluating the colours. There were two samples tested by Kit 1 that were incorrect but this would not have had an impact on the shipment of a fish as the level of histamine was much lower than the 50 mg/kg action level set by FDA. There were two samples tested by Kit 3 that gave incorrect results that would be of more interest to processors. These samples produced colours that were interpreted as being equal to or higher than the 50 mg/kg action level set by FDA. These false positives are of greater concern but at least there was an indication that some histamine was present even if the levels were not above 50 mg/kg.

13.1.2 *Quantitative Results:*
The advantage of this kit is that the final colour of the test cell can be evaluated in a more empirical way based on the optical density (O.D.). When a number of standards are included along with the samples being tested, a formula based on the log/log linear relationship of the concentration with the O.D. can be derived. The concentration of histamine in the test sample can then be calculated. This application does require the use of

expensive equipment such as a plate reader but it does have the advantage of removing false positives and negatives. Figures 13.1 and 13.2 show how accurate the kits are when compared to the standard method of capillary electrophoresis.

**Figure 13.1. Standard curve for histamine concentration as determined using the Alert Histamine Kits.**

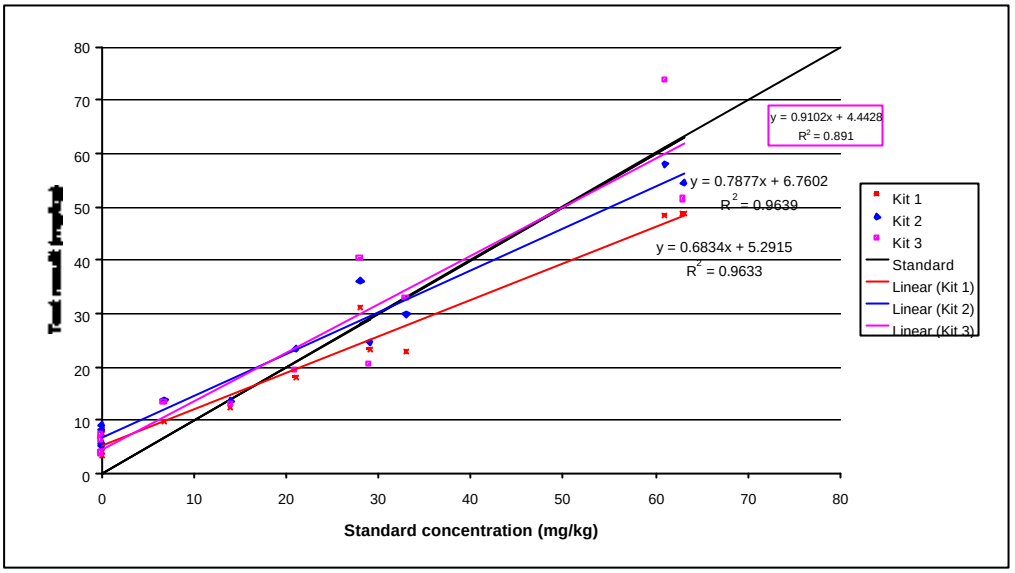
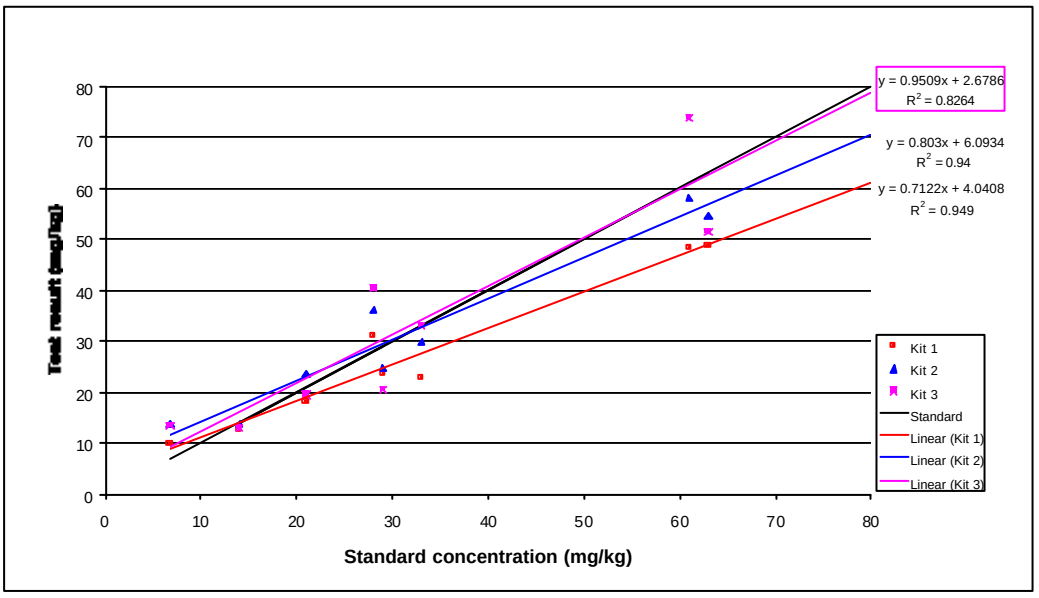



Figure 13.1 shows that the concentrations obtained by the Alert Histamine Test Kits were very similar to those obtained by the standard method. The $R^2$ values show that the results from each kit were quite linear and therefore consistent over the range of concentrations tested. The gradients (0.68 or higher) of the lines were close to that of the standard curve. The intercept values were also small.

Because the capillary electrophoresis method has little accuracy when the histamine level is below 10mg/kg, samples containing such low amounts are usually reported as <10 mg/kg. The standard curves have been redrawn in Figure 13.2 using only data greater than this level.

**Figure 13.2. Standard curve for histamine concentration >10mg/kg as determined using the Alert Histamine Kits**.



The figures show that the kits produced results that are consistent with the standard method. There were no significant differences between the kit results and the standard method. The accuracy improves when data of <10 mg/kg are removed with higher $R^2$ values and gradients closer to 1.

### 13.1.3 Industry evaluation

The test kit was demonstrated to or trailed by representatives from 11 different companies or government bodies. A questionnaire was given to each participant after the kit was demonstrated to complete. A brief analysis of industries opinions on the test kit follows. At this time there is only one laboratory testing fish for histamine but not all participants forward samples for testing. Several companies said that they had tried or had thought of obtaining kits in the past but had not obtained any. After the demonstration most industry representatives indicated they would purchase this kit. Industry found that the kits provided results in a form that was suitable for their needs. Non-industry wrote that they would recommend the kit to their contacts. Most were happy to send samples to laboratories that used the kits with only one stating to keep the testing in house. All participants stated that they benefited from the demonstrations with the education extending further than just the histamine test kit into general processing.

***Benefits***

- The kit can be used in the processing area with very little sample preparation and a minimum of equipment.
- A result can be obtained on fish being packed for export before they leave the factory.
- It can provide both qualitative and quantitative results (depending upon equipment available)

***Limitations***

- Some colour vision impaired individuals may have difficulty in determining test results for borderline histamine concentrations.
- As small samples of 10g may not be representative, several pieces from different parts of the fish should be pooled as a composite sample.
- Repeating the test for samples with histamine concentrations close to the 50mg/kg test control (also the action level set by FDA) may be advisable.
- Samples that appear to be above the 50mg/kg level should be diluted 1 in 4 before further testing to determine whether the level is above the domestic 200mg/kg limit.

***Summation***

The kit is quite accurate and reliable. The cost per test is much cheaper than sending off samples to the laboratory. It does not need much in the way of expensive equipment or much scientific training for the kit to be used in a factory situation. For the cost of a hand held blender or food processor, some plastic beakers, filter paper and filter funnels and a pipette that dispenses 100µL volumes a factory QA Manager can have histamine results within an hour of sampling fish.

Care should be taken in selecting staff approved to use the kit so that you avoid individuals with difficulty in determining colour. This aspect can be negated with the use of a plate reader. Most plate readers also have integration programs so that the results can be expressed directly as mg/kg histamine. Using the kit in this way will reduce the number of fish that can be tested, as three standards must be included with each run. This kit makes it easier to test more fish more often. This should encourage processors to test their production more frequently, thus ensuring a safer and better

quality product for consumers. The kit could be incorporated into QA systems currently in use by processors.

**Adoption by AQIS**

Under commercial conditions, staff from 11 factories or government bodies have evaluated the kit so far. The kit has been demonstrated at a WA Pelagic Longline workshop held at Challenger TAFE in May. The individuals who used the kit expressed a desire to use the kits in their routine operation but some have expressed the reservation that they would want AQIS to approve of the use of the kit first. This has led the project staff to approach AQIS management to review the data compiled on the kit during this project. The following points were the basis of the proposal submitted.

Proposal for adoption of the Alert Histamine Test Kit for routine histamine testing by the seafood industry in Australia and acceptance of kit by AQIS.

- The Centre for Food Technology has been evaluating a histamine test kit as part of the FRDC Project No. 99/358 "Evaluating effective quality monitoring methods for the Australian seafood industry".
- There is just one histamine rapid analysis kit available domestically to the Australian seafood industry. It is the Alert® for Histamine ELISA Screening test (distributed by the Neogen Corporation).
- The kit has been well received by the seafood industry during factory trials and state that it would be an asset to their QA or HACCP programs but have expressed a reluctance to use a test kit that AQIS has not approved.
- AQIS has stated that they require histamine to be analysed by "the standard method" and defer to Standards Australia.
- There is no standard method for histamine analysis recommended by Standards Australia.
- The ANZFA regulations do not specify a preferred method.
- The US FDA has approved a Fluorometric (also the only method accepted by Codex) and an ELISA method for histamine and the performance of these test methods have been endorsed by the AOAC under the "Performance Tested Methods Program".
- The FDA approved ELISA test kit is the Histamarine kit produced by Immunotech (a Coulter Company).
- Rogers and Staruszkiewicz (J. of Aquatic Food Product Technology Vol. 9 (2) 2000 p. 5-17) state the Alert kit has several practical advantages over the Histamarine and other ELISA kits because it is more accurate at low and high levels of histamine, easier to extract samples, it contains all of the reagents and plastic ware required to do the analysis, all the volumes are the same requiring one pipette only, the difference in intensity of the colour development is visually easier to distinguish, and the results can be read visually (qualitatively) or with a plate reader (quantitatively).
- At this time the only laboratory we are aware of that commercially tests for histamine is AGAL which uses the Capillary electrophoresis method. This method is less accurate than the Alert test kit at low concentrations of histamine with reports stating these results as <10mg/kg.

- The Centre for Food Technology tested histamine spiked samples using the Alert Histamine Test kit and has had replicate samples tested at AGAL by the Capillary electrophoresis method. Evaluation of the results obtained show linear constants ($R^2$ values) for both methods above 0.93 and no significant differences between the Alert kit results and the Capillary electrophoresis method.

The response was that AQIS only accepts histamine results that have been obtained used the “Standard Method”. Standards Australia has stated that they do not have a standard method for histamine testing. This has led to an application by the Centre for Food Technology in conjunction with Safefood NSW for the Alert® for Histamine ELISA Screening test to be a New Standards Australia International Publication. At this time the application is still under review.

# 14. Ciguatoxin

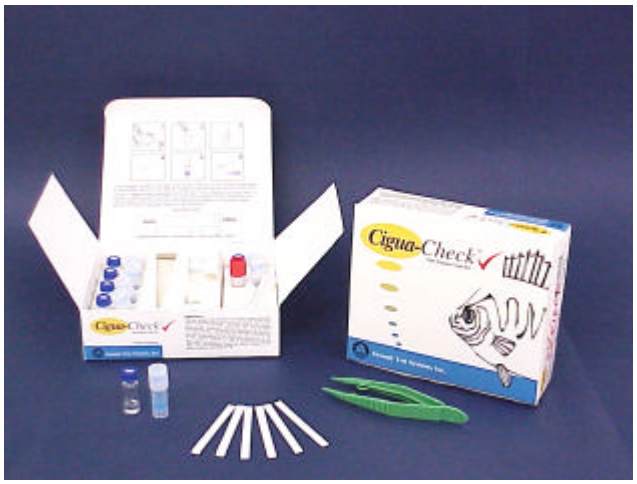
Ciguatera fish poisoning has been identified as the greatest seafood hazard in fish taken from tropical waters. The risk management of this hazard is currently the most difficult issue facing the Queensland and Australian seafood industry. Recently, there has been a significant increase in reported ciguatera fish poisoning incidents in both Queensland and interstate. There has also been a high incidence of cases within our biggest export markets (Hong Kong) which were traced to fish originating from Queensland waters. The connection to Queensland fish has a strong negative effect on Australian tropical seafood exports in general. Additionally, interstate markets are reacting and hence seafood industry revenue within tropical fisheries is strongly affected.

The established method for determining ciguatoxin levels in fish flesh is complex, labour intensive and involves expensive instrumentation. The method requires a lengthy and exacting extraction process to separate the toxin from the lipid matrix in which it is bound. The collected fraction sample is then subject to multiple steps of gas chromatography and mass spectroscopy. The entire procedure is protracted and demands a high level of expertise from the operator. Hence, while very sensitive and accurate, this is not a method that can be applied routinely to fish samples for the screening for ciguatoxin.

A few alternative methods have been tried for detecting ciguatoxin and most have proven to be ineffective. The mouse bioassay has been found reliable, as has a bioassay involving chickens or cats, but it is rare (in fact, as the author knows, impossible) to get approval for methods involving animals from the Animal Welfare Research Ethical Committees. A method using a monolayer of mouse nerve cells, the neuroblastoma assay, has shown to be reliably accurate for detecting saxitoxin in molluscs and it has potential to be used for ciguatoxin. Other methods investigated include a brevetoxin assay which is a competitive-binding-based assay and immunobead tests. However, these methods have not been validated.

A simple and cheap test for detection of ciguatoxin presence in fish is potentially addressed by the availability of the Cigua-Check test kit. CiguaCheck is marketed and promoted as a simple, easy field test kit for detecting ciguatoxin. The kit (Plate 14.1) was developed by Oceanit Test Systems, Inc. in Hawaii as a rapid test for ciguatera and hence it was assessed within this research work.

Plate 14.1. CiguaCheck kit.

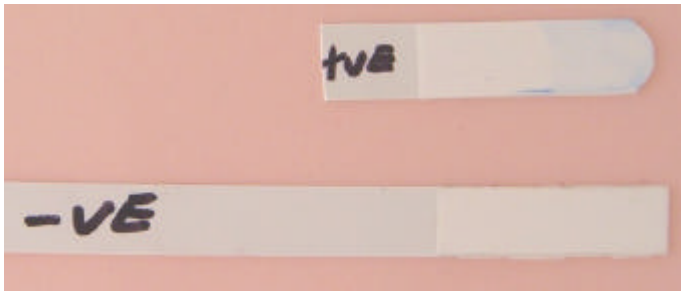


Collaborative efforts with the National Research Centre for Environmental Toxicology attempted to set up the brevetoxin assay to run in parallel with the CiguaCheck kit testing. However, this was not successful due to a series of complications and difficulties. Therefore, unfortunately the CiguaCheck kit testing was carried out with no reference possible to validate the results. All samples used for the kit tests have been retained, however and will be tested by an alternative method as soon as available.

The very first sample tested with the kit was fish flesh consumed by an elderly man showing strong symptoms of ciguatera fish poisoning and the test result showed a strong blue, indicating the sample was positive for ciguatoxin. This was extremely encouraging, as it seemed the CiguaCheck kit worked and worked well.

A positive result with the CiguaCheck kit is demonstrated by the end of the white test strip turning blue (Plate 14.2).

**Plate 14.2. Positive and negative control strips.**




Many fish were obtained and sampled, with the rapid realisation that all was not simple (Table 14.1). Results were very difficult to read on the strip, making a decision as to a positive or negative result almost arbitrary in some cases.

**Table 14.1. Results from samples fish species purchased.**

| Sample type | Positive | Negative | Total | % positive |
|---|---|---|---|---|
| Coral trout | 5 | 0 | **5** | 100 |
| Cod | 1 | 0 | **1** | 100 |
| Hussar | 1 | 0 | **1** | 100 |
| Parrot fish | 4 | 0 | **4** | 100 |
| Red emperor | 3 | 1 | **4** | 75 |
| Sweetlip | 4 | 0 | **4** | 100 |
| Mackerel | 5 | 5 | **10** | 50 |
| Snapper | 3 | 0 | **3** | 100 |
| | | | | |
| Flake | 2 | 0 | **2** | 100 |
| Mullet | 3 | 0 | **3** | 100 |
| Swordfish | 1 | 0 | **1** | 100 |
| Tuna | 1 | 0 | **1** | 100 |
| Whiting | 1 | 0 | **1** | 100 |
| Atlantic salmon | 1 | 0 | **1** | 100 |
| Sea perch | 1 | 0 | **1** | 100 |
| Scallops | 1 | 0 | **1** | 100 |
| Nile perch | 0 | 1 | **1** | 0 |
| Farmed barramundi | 1 | 0 | **1** | 100 |

It is commonly accepted that levels of 1.0 ng/g within the human system will result in symptoms of ciguatera poisoning appearing (Lehane and Lewis, 2000). The test kit is suggested to be semi-quantitative, measuring ciguatoxin level in the range 0.8 – 1.4 ng/g as determined by depth of blue colour. The researchers in this investigation did not consider that colour development on the test strips was consistent enough to assess a toxin level and regard the kit as qualitative only. It is recognised that, at best, the kit will only detect ciguatoxin presence at levels > 0.9 ng/g.

Table 14.1 shows a very high proportion of positive results among the randomly purchased fish samples, suggesting a large number of fish are ciguatoxic. This deduction is unlikely as, if true, it would dictate that a very high number of consumers are suffering from ciguatera poisoning. At the times of fish purchase for the research, there was no corresponding increase in notified ciguatera fish poisoning cases to the Queensland Health Department.

The fish species listed at the top of Table 14.1 are reef dwellers or taken from near-reef waters and hence can be regarded as more potentially likely to carry ciguatoxin. Therefore positive results attained from these fish are possible, however given that the samples were randomly purchased from retail seafood shops, the proportion showing as positive is unlikely.

The fish listed in the lower half of the table are not usually associated with ciguatera and yet most returned a positive test result with the CiguaCheck kit. A few species were included that would definitely not be ciguatoxin carriers, for example the farmed freshwater barramundi (800g); scallops; sea perch, Atlantic salmon. Yet these too, gave positive blue test strips.

In North Queensland, results of randomly sampled fish illustrate a similarly doubtful picture (Table 14.2).

**Table 14.2. Results of samples from North Queensland.**

| Species | Total | positive | negative |
|---|---|---|---|
| Barramundi *(Lates calcarifer)* | **1** | | 1 |
| Black Blotched Reef Eel *(Gymnothorax favagineus)* | **1** | 1 | |
| Blackspot Tuskfish *(Choerodon schoenleinii)* | **2** | | 2 |
| Blacktip Reef Shark *(Carcharhinus melanopterus)* | **1** | 1 | |
| Bludger Trevally *(Carangoides gymnostethus)* | **1** | 1 | |
| Chinaman fish *(Symphorus nematophorus)* | **3** | 3 | |
| Coral Trout *(Plectropomus spp)* | **24** | 23 | 1 |
| Footballer Trout *(Plectropomus laevis)* | **1** | 1 | |
| Gold Spot Cod *(Epinephelus coioides)* | **1** | 1 | |
| Golden Trevally *(Gnathanodon speciosus)* | **1** | 1 | |
| Grassy Sweetlip *(Lethrinus laticaudis)* | **1** | 1 | |
| Gray Reef Shark *(Carcharhinus amblyrhynhcos)* | **3** | 3 | |

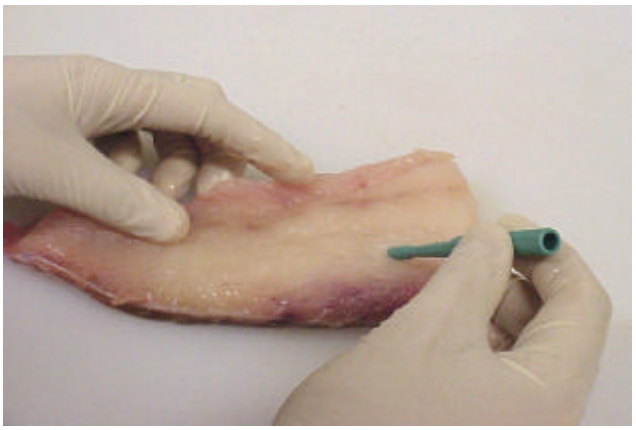
Table 14.2 contd

| Species | Total | positive | negative |
|---|---|---|---|
| Hammerhead Shark (*Sphyrna zygaena*) | **6** | 6 | |
| Hussar *(Lutjanus adetii)* | **1** | 1 | |
| Long Nose Emperor *(Lethrinus rostratus)* | **1** | 1 | |
| Midnight Sea Perch (*Macolor macularis*) | **1** | 1 | |
| Minifin Parrotfish *(Scarus altipinnis)* | **1** | 1 | |
| Parrotfish *(Scarus* spp*)* | **1** | 1 | |
| Red Bass *(Lutjanus bohar)* | **1** | 1 | |
| Red Emperor *(Lutjanus sebae)* | **1** | 1 | |
| Red Spot Sweetlip *(Lethrinus or Plectrorhinchus* spp *?)* | **1** | 1 | |
| Reef Shark *(Carcharhinus* spp) | **2** | 2 | |
| Reticulated Sweetlip *(Lethrinus reticulatus)* | **1** | 1 | |
| Robinson's Bream *(Gymnocranius grandoculis)* | **1** | 1 | |
| Sea Perch *(Psammoperca waigiensis)* | **3** | 3 | |
| Snapper (*Chrysophrys auratus?*) | **1** | | 1 |
| Spangled Emperor *(Lethrinus nebulosus)* | **2** | 2 | |
| Spanish Mackerel (*Scomberomorus commerson)* | **3** | 1 | 2 |
| Spotted Bream *(Gymnocranius* spp) | **1** | 1 | |
| Swordfish (*Xiphias gladius*) | **1** | | 1 |
| Tiger Shark *(Galeocerdo cuvier)* | **6** | 6 | |
| Tille Trevally *(Caranx tille)* | **2** | 2 | |
| Whiting (*Sillago ciliata*) | **1** | | 1 |
| **TOTAL** | **78** | **69** | **9** |

Inconsistent results were recorded on multiple samples taken from the same fish. This was unrelated to manufacturing batch lot of the test kits and all kits were used within their expiry period. In order to avoid any variability in results being due to operator error, a standard operating method for the test was developed. This was to ensure consistency between test kits and operators.

The first factor considered was test sample size. The instruction included in the kit specifies a "rice grain" size of flesh. To make the sample size consistent every time, various sampling devices were tried. The best found was a simple surgical biopsy punch (Plate 14.3) and this was used for all further sampling. A separate biopsy tool was used for each sample to guarantee there was no cross over contamination between samples.

**Plate 14.3. Biopsy tool used for sampling fish flesh.**

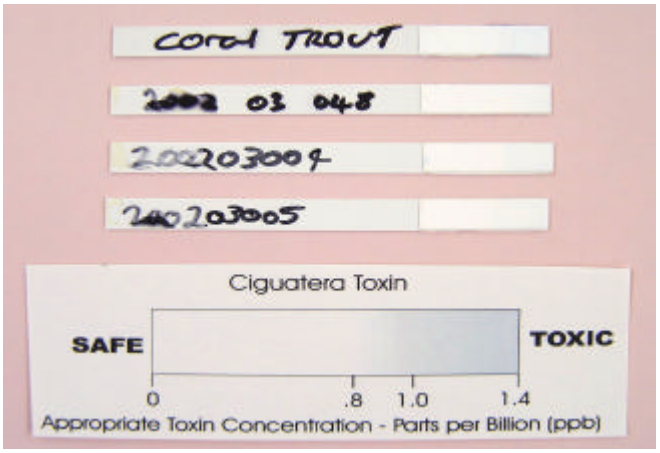


The instructions supplied with the kits are adequate but general user tips and use limitations could be improved. Cautions about substances that interfere with the test result need expanding. A warning about contamination from oil on the hands is the only one present. Not mentioned within the kit instruction sheet but revealed from discussion with the manufacturer (Oceanit), is that blood residues in the fish flesh will also interfere with the test and can cause a false positive result. While gloves can be worn to avoid the hand lipid issue, it is considered unlikely that all traces of blood would be absent from fish flesh and hence, it is possible that this factor is constantly interfering with the result attained.

There are some discrepancies discovered with the printed instructions and those provided from discussions with OceanIT. The instruction sheet recommends the test strip be rinsed with tap water, but Dr Joanne Ebesu (Director of Research, Oceanit Inc.) recommends 0.85% saline. The kit states that a purple colour on the positive control strip indicates a positive reaction, but in reality this colour change is a pale blue. Kit storage conditions are permitted as room temperature, however further correspondence with OceanIT suggests refrigerated storage prolongs the shelf life of the kit and is preferable.

For the test kit itself, the procedural steps were simple and easy, but reading of the strips was often very subjective. The blue indicator dye of the test strip was very faint and hard to visualise as a change in colour (Plate 14.4)

**Plate 14.4. Colour development on test strips.**



Various visual conditions were evaluated to maximise observation of the colour. The test strips were viewed under different light sources (natural light, fluorescent and ultra violet) and using different coloured backgrounds (grey, blue, green, pink). It was found that a light pink background gave the best contrast when reading the test strip, along with strong natural light.

The positive control strips supplied with the CiguaCheck kit were most frequently very faint in blue colour. This concerned the operators, as the test strips were even paler. Of serious concern was the finding that many of the positive control strips did not show any blue colour whatsoever. This, despite following the kit's instructions to the letter. The implication of such non-reaction is that that kit (containing five tests) is mis-functional and not to be used. Records show that only 54% of the positive control strips demonstrated a blue colour change; 16% a doubtful change and 30% no colour change. The negative control strip returned a negative result 100 percent of the time.

Communications with Oceanit did not provide any satisfactory answers on these findings.

We were kindly provided with fish samples that had been implicated in cases of ciguatera fish poisoning. We tested all these, but under the proviso that any result gained was not confirmed and should not be used for any further action. Table 14.3 presents the results from these samples.

**Table 14.3. Results of fish samples implicated in ciguatera outbreaks** *

| Species | Total | Positive | Negative | % Positive |
|---|---|---|---|---|
| Spanish mackerel Brunswick Heads | 64$^{\alpha}$ | 50 | 14 | **78** |
| Spanish mackerel Townsville | 4 | 4 | 0 | **100** |
| Spanish mackerel Caloundra | 3 | 2 | 1 | **66** |
| Spanish mackerel Cooked steaks | 3 | 2 | 1 | **66** |
| Mackerel (200108001) | 3 | 2 | 1 | **66** |
| Mixed Reef Brisbane | 5 | 2 | 3 | **40** |
| Coral trout (200206001) | 1 | 1 | 0 | **100** |
| Red Bass Cairns | 4 | 3 | 1 | **75** |
| Grunter Hervey Bay | 3 | 3 | 0 | **100** |
| Coral trout Sydney NSW | 2 | 1 | 1 | **50** |
| Trevally Hervey Bay | 3 | 3 | 0 | **100** |
| **Total** | **95** | **73** | **22** | **76** |

* as provided from Qld Environmental Health Units
$^{\alpha}$ individual steaks from 4 fish

The high number of mackerel samples involved are not individual fish but include multiple steaks (42) from 4 fish. The high number of positive results attained with these samples

could be expected as the fish were suspected in cases of ciguatera. However, we currently have no way of confirming these results as positive. All samples are being retained for future testing. Of interest, are the grunter samples – these fish were small (500-600g) and yet all three tested as positive with the CiguaCheck kit.

With similar samples provided from north Queensland, the results were comparable (Table 14.4) with 89% returning a positive result with the CiguaCheck kit. Again it was emphasised that these results are not confirmed.

**Table 14.4. Fish implicated in ciguatera poisonings in North Queensland**

| Species | Total | Positive | Negative | Unsure |
|---|---|---|---|---|
| Barracuda (*Sphyraena jello)* | **1** | 1 | | |
| Butterfish (*Parastromateus niger*) | **2** | 2 | | |
| Chinamanfish (*Symphorus nematophorus)* | **1** cooked | 1 | | |
| Cod (*Epinephelus* spp) | **1** | 1 | | |
| Collared Bream (*Gymnocranius audleyi)* | **2** | 1 | 1 | |
| Coral Trout (*Plectropomus maculatus* ) | **16** | 4 | | |
| Coral Trout - Leopard Spot (*Plectropomus leopardus*) | **1** | 1 | | |
| Deep Sea Bream (*Gymnocranius* spp) | **1** | 1 | | |
| Mackerel (*Scomberomorus* spp) | **5** | 5 | | |
| Mangrove Jack (*Lutjanus argenticulatus*) | **1** | 1 | | |
| Mirror Dory (*Zenopsis nebulosus*) | **1** | | | 1 |
| Moray Eel (*Gymnothorax* sp.) | **4** | 4 | | |
| Morwong (*Nemadactylus douglasii*) | **2** | 2 | | |
| Queenfish (*Scomberoides lysan*) | **4** | 2 | 2 | |
| Red Bass (*Lutjanus bohar*) | **1** | 1 | | |
| Spanish Mackerel (*Scomberomorus commersoni*) | **21** | 17 | 1 | 2 |
| Spotted Mackerel (*Scomberomorus munroi*) | **1** | 1 | | |
| Spotted Cod (*Epinephelus* spp.) | **1** | 1 | | |
| Stripey (*Katsuwonus pelamis*) | **2** | 2 | | |
| Yellowtail Kingfish (*Seriola lalande*) | **3** | 3 | | |
| Spotted Cod Liver | **1** | 1 | | |
| Unknown fillets Cooked or raw | **12** | 10 | 2 | |
| TOTAL | **84** | 75 | 6 | 3 |

The Table illustrates some interesting findings:

- of the 16 coral trout (*Plectropomus maculatus*) tested only 4 were positive (25%)
- of the 21 spanish mackerel (*Scomberomorus commersoni*), 17 (81%) were positive

These two species are commonly regarded as of high risk with respect to carrying ciguatera toxin and while the Spanish mackerel indicates that this is correct, results from the coral trout do not.

**Summation**

The Cigua check test kit is easy to use and relatively inexpensive. The kit can be used by any person, as there is no scientific expertise required. The instructions included with the kit are set out with simple clarity, step by step.

Results attained from using the kit are unreliable. There were inconsistencies between test kits and many apparent false-positives. The colour change indicator is often faint and very hard to read, making result decisions rather subjective.

For those samples of fish implicated in ciguatera outbreaks, 82% returned a positive result. For samples of fish randomly purchased, the kits indicated that 87% of all samples were positive for ciguatoxin. This would imply a very high number of consumers were suffering from ciguatera poisoning. At the times of fish purchase for the research, there was no corresponding increase in notified ciguatera fish poisoning cases to the Queensland Health Department. The kits returned positive results from fish samples that were extremely unlikely to be ciguatoxic.

While the results from the CiguaCheck kits could not be validated by an alternative test method, on the data obtained in this work it is concluded the kits do not provide a reliable result to indicate whether a fish is carrying ciguatoxin or not.

# 15. Benefits and Adoption

The sector of the seafood industry to benefit most from the successful application of rapid test kit methods is the processing sector. With the increased requirement to produce food under an established food safety plan, there is a strong need for monitoring of process lines within seafood factories to identify process failures. While testing requirements for regulatory purposes remain the province of independent certified analytical laboratories, in-house monitoring of sanitation practices and hygiene efficacy can become self-assessed.

Many seafood processors within the industry are choosing to follow the HACCP system which has a mandatory requirement for frequent monitoring and verification of procedures. With respect to food safety, microbiological testing is of critical importance but can be excessively expensive to undertake. Reliable test kits providing rapid results allow a big reduction in implementation costs for the seafood processing sector.

Rapid test kits commercially available can be divided into functional groups with respect to industry adoption. Those that involve direct contact between the kit and the sample are the simplest for use and are usually inexpensive per kit, most currently costing $1-2 /test. This compares with a cost of c. $20/sample submitted for a similar test undertaken at an analytical laboratory.

There was strong interest from many individual seafood processors in the findings of this project. However, given the unreliability of most rapid kits assessed there has been little uptake of the methods. For the kits currently available, this is considered a correct and appropriate decision.

A major impediment for industry use to many of the kits that detect specific bacterial species is the enduring requirement for a preliminary enrichment step before the kit can be used. Such a step needed for use renders the kit completely unpractical for seafood processors as, should the bacterial species be present in the sample, enrichment produces an almost pure culture of the organism in high concentration and this is not advisable within the processing factory.

A possibility to assist seafood processors in this area, is a compromise system where the seafood processor would be responsible for the sampling with a simple swab or similar and then that sample be sent for laboratory analysis. Such sterile swabs suitable for this purpose are commercially available (e.g. that manufactured by Oxoid). This style of sampling swab can incorporate any medium desired in the empty tube of the kit into which the swab is placed. Hence it is possible to include a microbial transport medium or a specific micro-organism enrichment broth in the swab kit. The material to be sampled is then swabbed, the swab re-placed into the kit and then the whole unit sent off to a testing laboratory.

This system has the advantage of being less expensive for industry per test due to reduction of sample freight costs. As well it would result in a reduction in laboratory costs per test as testing time is less because there is no sample preparation to perform. Additionally, the total length of time to attain a result is reduced.

Discussions with seafood processors has evinced no interest in undertaking this idea, most citing the complications handling and freighting unfamiliar items: “it is easier to just sample as we know how”.

For chemical analytes, two kits were found to be reliable and suitable for industry use: the Alert histamine test kit and the Hanna Instruments Sulphite test kit.

The Hanna sulphite kit was trialed by four different seafood companies processing prawns in Western Australia, South Australia and Queensland. The feedback was positive on the ease of use of the kit but the results obtained from industry kit use and research result were not always the same. It is suggested that some training of industry personnel is necessary prior to adoption of the kit within processing operations.

The Alert histamine kit was also successful when trailed by representatives from 11 different companies or government bodies. Industry participants commented that they found that the kits provided results in a form that was suitable for their needs and that they would purchase this kit. Non-industry users informed us that they would recommend the kit to their contacts. Several seafood companies have indicated they will purchase this kit and application has been made to AQIS to have the kit accepted as a standard method.

With respect to the CiguaCheck kit, it is currently readily available to everyone from many chemists up and down the Queensland coast. As our research has shown that the kit to be completely unreliable, and while understanding we cannot interfere with private commercial business considerations, we have (and continue to ) expound the findings of our evaluation of the kit within this project work.

Although the findings from this research work are disappointing with respect to successful identification of kits suitable for industry use, it is considered that summary comments for each kit evaluated are worth having available to industry. It is not considered pertinent to go to the initially proposed expense of publishing kit data sheets for industry distribution but it would be relevant to extend them electronically, most appropriately through the Seafood Services Australia website. This would ensure that the information was available for any interested seafoods industry managers

# 16. Further Development

The Listeria Transwab warrants further investigation. Notwithstanding the limitations of its purpose of manufacture, it appears its potential is great and if procedures are able to be refined, its usefulness to industry as a self-monitoring tool would be enormous. It is suggested that a double-stepped procedure using the Transwab first, followed by a specific antigen-antibody test kit should the Transwab gel medium turn black, may be appropriate. It is unsure whether the antibody based kit will pick up target antigens amongst the background proteins of other bacterial species present, but it is worth investigating. These experiments are being developed as follow on work within AFFS-Food Technology.

The unreliability of the CiguaCheck kit and the results obtained with it need to be broadcast widely. Particular extension focue is needed within the Hervey Bay and North Queensland regions, but all stakeholders need to be included: all sectors of the fishing industry; charterboats; general public; retail level; and the tourism sector. It is considered appropriate that this extension is undertaken in conjunction with SSA and Safe Food Queensland under a total risk communication umbrella.

It remains to chase up AQIS re the adoption of the Alert histamine kit as an accepted standard method.

## 17. Planned Outcomes

The major outcome intended by the research project undertaken was to facilitate the adoption and implementation of food safety programs by the Australian seafood industry. The facilitation would be in the form of identifying rapid methods suitable for industry to use to allow for self-monitoring and verifying their day to day operational processes. Successfully categorized kits would provide reduce associated laboratory analysis costs for seafood businesses.

However, with some few exceptions, the findings from the research work have indicated that rapid kit methods are not appropriate for industry use.

Although the findings from this research work are disappointing with respect to industry application, the information is of value for industry to know as there is now a detailed evaluation of those kits commercially available undertaken with samples directly relevant to their industry. Advances in technological achievements are yet required before rapid kits are suitable for general industry use.

## 18. CONCLUSIONS

Rapid test kits are ideal for assessing effectiveness of quality control programs or for screening large numbers of samples. Bacterial numbers within these types of samples are frequently low and hence the test kit can be used without the need for diluting the sample and provide a quick guide to process controls throughout the line.

The most widely used method for monitoring hygiene is swabbing and cultivating the collected bacteria within the sample. Direct contact agar methods are the easiest to carry out for routine monitoring hence the focus in this research was on selecting kits of this type. These kits were found to have limitations the main one being that they still rely on microbial growth to provide a result and this often has the consequence of no timing advantage compared to standard methods.

Of all the test kits investigated in this work, those that detect total bacterial loads are the most universally applicable. The Petrifilm range of kits give an enumeration result and are most applicable for monitoring microbial loads at any point in the processing line. The Environcheck kits and the Hygiene Test kit provide an indication of total bacterial load by density. Therefore they are useful for detecting gross contamination but applicability is restricted to monitoring and checking efficacy of sanitation procedures. The Petrifilm coliform count kit falls into this grouping as well.

For determining specific organisms, rapid test kits are designed to detect a single target and hence provide a quick response for negative results, that is absence of the organism. Many of these type of kits are designed to provide an answer in minutes to hours, rather than the days involved with traditional methods. However, most test kits of this nature have a pre-

requirement of growing an enrichment culture from the sample taken and therefore this step in their use precludes these kits from being appropriate for industry use. The industry exceptions are those seafood premises that have a separate laboratory facility.

An exception is the Petrifilm *E. coli* count plate which detects both coliforms and *E. coli* in the one step kit. This is accomplished by differentiation of the colony types by colour and gas production. While the concept is perfect for industry application, evaluations demonstrated that discernibility between colonies on this kit is very difficult.

For *Listeria*, there is a simple swab kit available that was considered to have great potential for industry application since it was purported to detect *Listeria* species without the hurdle of an enrichment step first. Despite an enormous amount of effort expended with this Transwab kit, it was found that too many 'false-positive' results were attained for industry to be able to use the kit with confidence.

The Hanna sulphite test kit was found to be accurate and reliable, although some degree of training of operators is necessary prior to use by industry. Similarly, the Alert histamine kit was simple to use and provided accurate results. Several seafood companies have indicated they will purchase this kit and application has been made to AQIS to have the kit accepted as a standard method.

The CiguaCheck kit for detecting ciguatoxin was found to be completely unreliable. False positive results were prevalent when using the kit, with 82% of randomly purchased fish samples demonstrating a positive result for ciguatoxin. These findings need to be extended to both industry members and the general public through whatever means available.

Rapid test kits frequently involve complex designs and formats and, combined with the difficulties often inherent in analysing foods, there is a need for caution and care when selecting kits for a specific purpose. They also emphasised the strong requirement for kits to be thoroughly evaluated within a given environment. This was the purpose of this current research work.

The conclusion being that, with a few exceptions, rapid kit methods evaluated still lack sufficient sensitivity and specificity for direct testing of samples and their application to the seafood industry is very limited. This will perhaps change as further technological advances occur and are commercialised.

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# APPENDICES

## Appendix 1. Intellectual Property

There is no intellectual property arising from this project work

## Appendix 2. Staff

Engaged on the project:

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|---|---|
| **Principal Investigator:** | Sue Poole |
| **Co-Investigator:** | Steve Slattery |
| **Technical staff:** | Ross Naidoo<br>Reg Reeves<br>David Williams |
| Contributed to project work: | John Nagle<br>Louise Oliver |