AN AUDIT OF METHODS & PROCEDURES FOR LIPOPHILIC DSP TOXIN ANALYSIS USED BY LABORATORIES AT CEFAS, FRS AND DARD, WHICH UNDERTAKE THE STATUTORY MONITORING OF SHELLFISH TOXINS IN THE UK.

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TABLE OF CONTENTS

ANNEX A – CEFAS
ANNEX B – FRS
ANNEX C – DARD
ANNEX D – COMMUNICATIONS FROM OTHER INTERESTED PARTIES

GLOSSARY

CEFAS - Centre for the Environment, Fisheries and Aquaculture Science, Barrack Road, WEYMOUTH, Dorset, DT4 8UB.

FRS - Fisheries Research Services Marine Laboratory, 375 Victoria Road, ABERDEEN AB11 9DB. FRS also includes the Marine Laboratory satellite laboratories and animal handling facilities at the Rowett Research Institute (RRI).

DARD - Chemical Surveillance Department, Veterinary Science Division, Department of Agriculture and Rural Development, Stoney Road, Stormont, BELFAST BT4 3SD.

1. EXECUTIVE SUMMARY

The Food Standards Agency commissioned an independent audit of the three laboratories involved in the UK statutory biotoxin monitoring programme, as part of its programme of work to investigate the atypical responses in the Diarrhetic Shellfish Poisoning (DSP) mouse bioassay (MBA) observed and reported for some cockle and mussel samples by CEFAS and DARD.

This audit* reviewed the application of the routine DSP Standard Operating Procedure (SOP), from receipt of shellfish samples to recording of symptoms in mice and test results in each laboratory. It involved visiting each laboratory to observe the application of the DSP assay method used, comparing these procedures to those described in the agreed interim SOP, recording disparities between the SOP and the procedures as implemented, commenting on the possible effects of any disparities or any other procedure observed, especially with respect to the atypical response to the DSP MBA.

The term atypical response is used in this report to describe the response in the MBA which did not follow the usual pattern of clinical signs for positive DSP results in that the mice died very quickly (within minutes) and more traumatically than is the case with normal DSP toxin. The atypical response consisted of more violent and rapid rear leg and body movements, and agonal breathing after collapse.

The audit found that:

1. No evidence emerged from this audit to support the view that the atypical response is due to the presence of ether in the Tween extract (but this is being separately investigated by the FSA).

2. If the evaporation has been carried out correctly, ether and/or acetone should not be present in significant amounts and it should not be necessary to leave the extract over-night to allow further evaporation of ether.

3. While each laboratory operated a different protocol for the routine DSP assay, all were in accord with the basic methodology outlined by Yasumoto (1984)[1]. No evidence emerged from this audit that obviously supports an argument that the cause of the atypical DSP response is a methodological or procedural artefact. However, if the atypical response is in fact due to a new toxin, what appear to be slight differences in methodology may well have a profound effect on what is present in the final extract and thus injected into the mouse. Under these circumstances, it would be sensible to ensure that all three laboratories operate identical protocols for the DSP assay.

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* which reflects observations made at a single point in time.

[1] Yasumoto, T., Murata, M., Oshima, Y., Matsumoto, G.L., Clardy, J. (1984). Diarrhetic shellfish poisoning. In: Seafood Toxins (Am. Chem. Soc. Symp. Ser.) ed: Ragelis, E.P. 207-214.

4. The procedures used for routine DSP assays in all three laboratories differ to varying degrees from the method described in the SOP. All three laboratories need to address this and ensure that the SOPs in place accurately describe the procedures used in the laboratory, and ensure that SOPs in place are accurately followed. All laboratories must ensure that procedures are regularly audited to maintain compliance.

5. There are different approaches to the determination of positive/negative results by each laboratory. The end-point of the assay, irrespective of differences in analytical procedure prior to that point, has to be standardised. CEFAS require 2/3 or 1/2 mice (depending on amount of shellfish material analysed) to present symptoms within the 5 hour period for a sample to be declared positive. FRS need to observe only symptoms in 1/2 mice - they observe mice closely and kill any that suffer distress, often well before the 5 hour period of observation has ended. DARD observe for 24 hours with death as the end point. When the laboratory audit mussel homogenate was injected into mice at CEFAS the symptoms observed were considered “mild” and as such the result was reported as NEGATIVE, but the same symptoms were observed at FRS and DARD where it was reported as POSITIVE. This is clearly not acceptable. It is strongly recommended that descriptions of symptoms of typical DSP and atypical responses to the DSP MBA are agreed between all three laboratories and clearly tabulated.

6. There is a need to establish the cause of the atypical response and further research is recommended. A possible route would be a comparative LC-MS analysis of extracts that produced negative responses, typical DSP and atypical DSP responses to the MBA. This may indicate a possible cause, but until this research is complete and the cause established, changes in the methodology/procedures used for routine DSP assay should be avoided as the effect of such changes will be unknown, thus possibly exacerbating the problem.

7. There appeared to be no satisfactory internal quality assurance (QA) for the shellfish monitoring protocols in place at any of the three laboratories visited. While the difficulties, described to me, of setting up an effective procedure are recognised, it is felt that they can, at least partially, be overcome and some form of internal QA **MUST** be instituted in each laboratory.

8. The staff of the UK-National Reference Laboratory (NRL) for biotoxins (UK-NRL) are not independent of FRS and, in effect because of their funding arrangements, serve two masters. It is recommended that if possible steps should be taken to establish more clearly the independence of UK-NRL and at the same time consider the role of this laboratory. I suggest that the remit of the UK-NRL should include *inter alia* responsibility for:

    - QA of statutory monitoring laboratories.
    - Liaison with the CRL.

- Monitoring performance of all UK statutory monitoring laboratories.
- Providing an independent objective advice to the FSA and statutory monitoring laboratories, regarding methodology and procedures.
- Undertaking independent research to improve methods with the intention of providing alternative assay system to the present MBA (e.g. Liquid Chromatography – Mass Spectrometry (LC-MS).

9. The UK-NRL should seek to set up at least a UK wide external QA scheme, which in co-operation with the Community Reference Laboratory (CRL) could be extended to the whole of the EU.

10. Telephonic/oral transmission of results should be avoided as it may lead to errors. There should be a clearly described procedure in all laboratories for the approval of results by a named certifying scientist, which would require scrutiny of all the data, including quality control (QC) results, before they are released from the laboratory.

11. CEFAS laboratory has no prior notice of the numbers of samples that are sent for analysis and 20 samples could, with present staffing numbers, be close to overload. Large numbers of samples in a batch increases the possibility of mis-labelling and overload could cause errors in applying SOPs. If numbers of samples in batches exceed those which can be handled easily in one day, overnight storage is required.

2. THE ASSIGNMENT

When commissioning the work the Agency sought to appoint an independent consultant with experience in laboratory procedures rather than an expert in shellfish biotoxin analysis. I was approached to undertake the assignment in the capacity of an independent analytical biochemist with more than 40 years experience.

2.1 Terms of Reference

To undertake on behalf of the Food Standards Agency an independent review of the application of the interim SOP for the DSP test (as agreed on the 2 June 2003) as used by the statutory monitoring laboratories in the UK.

2.2 The Task

To visit the three statutory monitoring laboratories (CEFAS Weymouth, FRS Aberdeen, DARD Belfast) and review the application of the procedures/methods in the DSP interim SOP, from receipt of shellfish samples to recording of symptoms in mice and test results in each laboratory.

The review was to involve:

- Discussing DSP testing arrangements with key personnel involved in the procedure to find out what is done and why, to check compliance with interim SOP documented procedures and to check compliance with QA procedures.

- Observing the sample preparation, extraction, testing by MBA, identification of symptoms and recording of results.

- Writing a final report of the review findings after visiting all laboratories, which would include recommending a course of action to address the Agency's concerns about the application of the test method in the three laboratories

It is understood that the FSA has commissioned CSL to carry out work to develop an optimised and validated SOP for use by the UK statutory monitoring laboratories

2.3 The Brief

To visit each laboratory to review the application of the interim SOP which was introduced during the week commencing 2 June 2003 by each of the three statutory monitoring laboratories. Although FRS introduced the agreed interim SOP on the date stated, it ceased to use it after one week's operation and reverted to its original methodology. I am advised by FRS that this was jointly agreed with FSA and the Home Office.

2.4 Approach

I am not an expert in shellfish testing and accept that there may be information that could be relevant to the assignment, of which I am not aware. I was therefore prepared to consider evidence supplied by any party, which could be substantiated with published work. I have also agreed with the FSA that should any new and relevant evidence based data come to light once this report is published, I would be prepared to review it and draw it to the attention of the FSA for consideration and action.

My audit visits involved observing the application of the DSP assay method routinely used in each laboratory, at the time of my visit, and comparing these procedures to those described in the SOP in use and to the agreed interim SOP. I recorded disparities between the routine SOP and the procedures as implemented, commented on the possible effects of the disparities and other procedures observed, especially with respect to the atypical responses that have been reported by CEFAS and DARD.

I considered drawing up a check list prior to my visit but decided that such a list would be counter-productive in that it might concentrate my attention on preconceived areas.

In preparation for the audit visits, I requested that each laboratory be asked to assay a sample of mussel homogenate which had been prepared for the

purposes of this audit. This sample is referred to throughout this report as the 'laboratory audit mussel homogenate'. This sample would allow a comparison of the end points of the MBA between the three monitoring laboratories and allow major problems in analytical methodology to be identified. The sample would therefore act as an external QC to determine whether the routine procedures followed in each laboratory were equivalent in terms of typical DSP analysis. It specifically does not, however, address the question of the atypical response in the DSP MBA.

Stored mussel samples, previously shown to be negative for typical DSP toxins by MBA, were homogenised on 4 July 2003. A portion of the homogenate was then sent in cold boxes with freezer packs by courier to the participating laboratories. The sample had been stored frozen at -20°C by each laboratory until use. QC samples would normally be analysed blind at the participating laboratory and also at the same time by each laboratory. However, due to practicalities, the laboratory audit sample was identified as such to the analysts. It was analysed while I was visiting each laboratory and was stored for varying amounts of time prior to my visits. I understand that some time after my final visits, a 4g sub-sample was analysed for a suite of biotoxin standards by LC-MS[2]. None were detected.

2.5 Observation of DSP assay

It was made clear to me by FRS, both before and during the visit, that the laboratory audit mussel homogenate could be processed using the interim SOP and that this would be done by a senior member of staff especially for me. FRS however made it clear that the extract would not be injected into mice. Audits such as these are concerned with routine assays. Observations of procedures, carried out especially for the purpose of an audit are not acceptable. In addition, this assay would have had no MBA end point. I considered that observation of an artificial application using a single sample of the interim SOP at FRS would have been incompatible with my remit. I therefore decided that it would be more productive to observe the analytical procedures used in the routine SOP at present in operation, as carried out by the staff, who usually perform the assays. These procedures are common to both SOPs.

2.6 Audit visits to shellfish monitoring laboratories

8-10 July 2003 - CEFAS

5-7 August 2003 - FRS which also includes the Marine Laboratory satellite laboratories and animal handling facilities at the Rowett Research Institute (RRI).

1-2 September 2003 - DARD

[2] okadaic acid (OA), dinophysistoxin-1 (DTX1), dinophysistoxin-2 (DTX-2), pectenotoxin-2 (PTX-2), azaspiracid-1 (AZA-1) azaspiracid-2 (AZA-2) azaspiracid-3 (AZA-3) azaspiracid-4 (AZA-4), yessotoxin (YTX), homo-yessotoxin (45-OH YTX), and gymnodimine.

Over a period of 2-3 days in each laboratory, I observed the whole assay protocol from receipt of samples to the final observation of the mice post injection with the extracts. All staff at CEFAS, DARD and FRS were very helpful and there were no problems in obtaining information or observing procedures during the DSP assay. My detailed comments on the SOPs are given in the Annexes. Laboratories were given the opportunity to read this report in draft to ensure that the detailed observations recorded in the Annexes were correct. Agreed inaccuracies were remedied before publication of the Final Report.

3. BACKGROUND

The statutory monitoring laboratories that carry out the testing on behalf of the Agency are CEFAS for England and Wales, DARD for Northern Ireland and FRS for Scotland. Although the NRL for shellfish biotoxins is based at FRS, it provides a distinct service which is separate to the shellfish monitoring functions carried out by FRS.

During 2001, at an UK-NRL network meeting, it was noted that variations in the DSP extraction procedures were used by the monitoring laboratories. At that time, CEFAS and FRS were using variations of the Yasumoto (1984) method and DARD was using a variation of the Yasumoto (1978) method. By mid March 2001 DARD was also using the Yasumoto 1984 method. Towards the end of 2002, the FSA became fully aware of discrepancies in the numbers and volumes of solvent extractions employed by each UK biotoxin monitoring laboratories for the detection of DSP in shellfish. The work initiated by the Agency to help standardise testing procedures across the UK, took account of the findings of work undertaken by the UK-NRL network prior to February 2002.

In February 2003, a meeting of the UK NRL network on DSP was held, at which each of the existing protocols for the DSP test used by the laboratories were discussed by representatives from DARD, CEFAS, FRS, RRI, BIOSS (Biomathematics and Statistics Scotland), the UK-NRL for biotoxins and the FSA. As part of the work to standardise testing procedures across the UK, interim DSP testing arrangements were developed in consultation with the three laboratories involved in statutory marine biotoxin testing. Agreement was reached on the different stages of the test method, but not the precise details of how each stage of the test method was to be carried out. Each laboratory was asked to use this information to adapt their existing SOPs and produce an “interim SOP”.

At the time it was appreciated that to implement the interim SOP would require each laboratory to make changes to their current operating procedures for the DSP test. The Agency agreed to review the interim SOP arrangements once the method had been introduced to see whether the changes had caused any material difference to the extract or the test results.

When the interim method was implemented at the beginning of June 2003, it was found that the FRS experience of using new procedure differed to the other two laboratories. Following implementation at FRS, 18 samples were tested, these being 15 mussel, 2 scallop and 1 oyster. Three of the samples produced extreme reactions in mice. FRS staff also recorded a strong smell of ether and it was this observation that led FRS, the Home Office and a veterinary surgeon from the Rowett Institute to conclude that the results were most likely to have been caused by ether.

Until issues associated with the extraction stage at FRS had been resolved, it was jointly decided (FRS, FSA and HO) that the interim SOP should be suspended at FRS. The decision was risk based and took a number of factors

into consideration including the circumstances surrounding the application of the testing method at FRS in the week commencing 2 June 2003. The interim SOP remained in place at the other two statutory testing laboratories, where it was introduced without any difficulties and where it is giving comparable results to those seen previously.

4. DSP METHODOLOGY

The testing arrangements operate within a clear legal framework. However this report does not comment on or describe the testing requirements laid down by legislation. This is a matter for the FSA.

The DSP assay system used in all three laboratories is based on that described by Yasumoto in 1984. It is a very simple analytical process involving preparing a homogenate of flesh from the shellfish, extracting the DSP toxin with acetone, evaporating off the acetone leaving an aqueous extract, from which the hydrophobic DSP is extracted with diethyl ether. The ether extract is washed with water and finally evaporated to dryness. The final extract is suspended in Tween and injected intraperitoneally into mice. The assay is positive or negative depending on the symptoms/death of the mice after injection.

This is a very simple assay, the specificity of which depends upon the final mouse bioassay.

5. CEFAS VISIT REPORT

5.1 UKAS accreditation

The laboratory is accredited by UKAS for DSP, PSP and ASP assays. I am not entirely happy about the frequency or methodology of UKAS inspections, which do not appear to be sufficient to maintain an awareness of the importance of proper QA systems. Tight external scrutiny is an important part of the maintenance of proper analytical standards. I suggest that UKAS be asked to provide FSA with a schedule of visits they have made and intend to make to CEFAS (and the other laboratories, where relevant), both announced and unannounced and what brief is given to their inspectors. It would I think be valuable if UKAS could use the same staff for all three accredited laboratories.

5.2 Standard Operating Procedures (SOPs) for routine DSP assay (See Annex A for detailed comments)

5.2.1 Agreed interim SOP

The interim SOP is not a stand-alone document. All labs have accommodated changes to their existing SOPs to implement the revisions recommended at the UK-NRL network meeting in February 2003. The interim SOP therefore consists of the appropriate collection of different SOPs operated, of course, in the correct sequence:

MFS Microbiology S0Ps:

SOP 07 (issue 4 – dated 15 May 2003) RECEIPT & CONTROL OF SHELLFISH SAMPLES

SOP 10 (issue 6 – dated 30 June 2003) OPENING AND HOMOGENISING SHELLFISH FOR ASP, PSP AND DSP EXTRACTION

SOP 12 (issue 8 – dated 8 May 2003) DSP EXTRACTION FROM SHELLFISH

SOP 13 (labelled as issue 4 but is probably issue 3 – dated 28 April 2003) MOUSE BIOASSAY

General SOPs

In addition there are up to 19 other general SOPs relating to topics such as balances, pipettes etc. I have looked at all of these but only a few were relevant and these were examined more carefully:

SOP 09 deals with in-house calibration and testing and refers at paragraph 12 to checks on syringes for biotoxin analysis. SOP 16 deals with QC of SOP's themselves and details genesis and up-dating etc.

5.3 Comments on SOP as a result of observations

I observed the whole process covered by SOPs 07, 10, 12. Many of the procedures described in SOP 12 were not in fact carried out as written. All such deviations have been recorded (Annex A).

I observed all stages of the DSP test from receipt to the preparation of extracts for injection. There were 3 samples for DSP assay plus one from the day before [mussels & Pacific oysters (BTX/2003/454 and/455), mussels (BTX/2003/458) plus a cockle sample (BTX/2003/448) from the batch of 20 the day before, which had been refrigerated (4^{o}C) overnight and shucked before I had arrived. The batch therefore contained five samples including the laboratory audit mussel homogenate.

CEFAS laboratory has no prior notice of the numbers of samples which are sent for analysis and 20 samples would seem to be close to overload. In any event, large numbers of samples in a batch increases the possibility of mis-labelling and overload could cause errors in applying SOPs.

I note that neither in this batch (nor in the batch started the day before - n=18) were any QC samples included.

SOP 07 requires details of samples received to be recorded by the receiving laboratory. For Biotoxin analysis, this is described in Paragraph 9 (page 5/9). The BTX/2003/ number is recorded sequentially on the left hand side of the page of the workbook.

SOPs 10 and 12 deal with the shucking and extraction for DSP. Further information has to be recorded in the workbook identified at para 8.2 of SOP.

Since 2001 it is understood that 1 in 50 samples giving a negative response to the MBA and all DSP positive samples (including atypical responses to DSP MBA) are routinely analysed by LC-MS for certain DSP toxins for which certified reference standard solutions are available (OA, PTX-2, YTX, AZA-1, Gymnodimine, desmethyl spirolide C). For samples showing atypical responses to the DSP MBA, none of the above toxins have thus far been identified.

Microbiology SOPs 07, 10 and 12, are not followed as described therein or at least they were not followed by the operators on the day of my inspection.

There are a few general points, which should be mentioned here:

1. SOPs 12 & 13 appear to have been produced by a single person who was the author, reviewer and the issue authoriser on both the SOPs. This is undesirable as in practical terms it may mean that the SOP has only been seen by one person. General SOP 16 deals with the QC of SOPs and paragraphs 6.1. 6.2 and 7.1 clearly suggest (rightly) that the author, the reviser and the authoriser should be different individuals. If this procedure is followed the SOP will have been seen by at least three personnel and

thus errors both in content and syntax are more likely to have been spotted and remedied.

2. The laboratory keeps a record of the reagents it receives, which are allocated a laboratory batch number, which can be seen recorded in the Workbook (SOP 12, para. 8.2). What however is not recorded is the batch or lot number of the manufacturer, although it is possible that such data is recorded on the invoice or delivery note. This is a potential source of lack of traceability, should the manufacturer withdraw or report problems with a particular batch. This should be dealt with in the General SOP 14 which deals with media (produced in house, prepared from external sources and externally supplied material).

The following day I observed SOP 13, the injection and subsequent observation (these observations are required at 30 minute intervals over a period of 5 hour). I only made observations every hour. On the previous evening I had been shown a rather poor quality video recording of the atypical mouse response.

I observed an atypical response in the MBA from a sample extracted from cockles. Two mice were used as the final extract was only 3ml. One showed rapid onset of convulsions and died after 9 minutes. The other appeared less affected and after about one hour seemed to recover well. According to SOP 13 (dated 28 April 2003) POSITIVE results (and thus subsequent bed closure) are only recorded if death (1/2 or 2/3, depending on numbers used) supervenes within the 5h period. However, in an email from CEFAS subsequent to my visit, I was informed that these criteria had been changed from death to clinical signs of typical DSP within the same 5 hours on 16 June 2003, in accordance with the recommendation from the February 2003 UK NRL Network meeting. Two mice are used only when the weight of the homogenate is insufficient to provide 25g of original homogenate per ml of Tween for injection. This is not made entirely clear in the SOP. The presence of an atypical response to the MBA without death is NOT reported as POSITIVE.

The laboratory audit mussel homogenate (BTX/2003/Professor M sample) had also been extracted and injected into 3 mice. At 1.5-2hours observation, there appeared to be signs of typical DSP poisoning in all three mice. Mice were lethargic and cold with difficulty moving back legs. These symptoms persisted up to 5 hours after injection but none of the mice died within the 5h period. This response would be reported by CEFAS as negative, as the symptoms observed were not judged to be sufficiently pronounced (i.e. mice did not lose grip reflex).

I was provided with a copy of the e-mail reporting results and a list of the results as sent to local authorities.

5.4 Quality assurance

The QA Manager, whom I did not meet, is based at Lowestoft. The Manager visits several times a year to audit the implementation of the SOPs. UKAS also monitor performance. For internal QA negative controls are included in the batch but there is no positive control. In fact (*vide supra*) there were no controls in either of the batches examined when I was there. There is clearly no formalised external QA though it is clear that all the UK testing laboratories and others within the EU are in contact with each other and the capability for informal transfer of samples between laboratories exists and such transfer is taking place from time to time.

There are clearly two aspects to QA. Firstly each laboratory must have proper internal QA, which must be agreed and monitored by the QA Manager. The institution and maintenance of the internal QA system should involve UKAS as well as CEFAS staff. Each section should appoint its own QA manager, who is responsible for ensuring that proper QC samples are run with each batch and for maintaining records of performance, which should be displayed where all staff can see results. Every SOP should include details of QC samples and where and how they are to be processed and there must be clear indications of what results are expected and what action is to be taken if the QC samples are outside the agreed parameters. In an ideal world, QC samples should be introduced in such a way that the analytical staff are not aware they are any different to normal samples. In the case of the DSP assays, this would be very difficult.

The second consideration with regard to QA is the institution of external QA, to which all UK laboratories should belong. As the requirement for shellfish toxins is mandatory in EU countries, it would seem sensible to explore the possibility of setting up a European external QA scheme. There are difficulties with storage and transport but these are not insurmountable.

I have seen a document on QA, which I have been told comes from CEFAS, which consists of 9 numbered paragraphs. I have read this carefully but see nothing in it to make me wish to alter my remarks above. My comments may be regarded as a counsel of perfection, which cannot be achieved in practice but this is not necessarily a view with which I would agree).

5.5 Conclusions

There are a number of comments about analytical procedures at CEFAS:

- The SOPs specifically relating to DSP analyses at CEFAS leave a great deal to be desired. They must be re-written so as to present the procedures in a clear unambiguous way, removing extraneous matters which are not immediately relevant.

- I advise that the SOPs should initially be written by those who carry out the procedures at the bench and subsequently revised by more senior personnel. The most senior person in the group should issue the agreed

SOPs only after all group members have read them and agreed that they are accurate.

CEFAS has since advised FSA that SOPs are prepared by experienced and senior analysts with a thorough working knowledge of the methods; bench-tested by the operators, and information fed back from the 'bench' to inform the SOP revision, approval and issuing process. It has been explained that additional background information may be included in the SOPs to assist the technician's understanding of the methodology.

- Some sort of internal QA must be instituted as soon as possible. At a minimum a negative sample must be run with every batch and also attempts should be made, preferably in agreement with other laboratories, to devise a positive sample for the DSP assay. It might be possible to use the negative sample to which an agreed concentration(s) of okadaic acid or other DSP had been added. All laboratories in the UK should agree on some sort of external QA scheme, which should be accredited by a suitable body. In addition, discussions should be started to organise a European QA scheme as soon as possible. SOPs should include information about QC samples and what action to take if the required result is not obtained. Performance data for both internal and external QA should be displayed in the laboratories in graphical form so that all staff can see how they are performing.

- I am surprised that the deviations from the SOPs have not already been spotted by the CEFAS Quality Manager but also by UKAS. Both UKAS and CEFAS should be asked to tighten up their surveillance procedures and both organisations should deal with the questions of QA.

- I am concerned that steps should be taken to prevent the presence of fluid (?water) in the final extract. If present, this will dilute the Tween used for re-constitution. Investigations might centre on filtration of the ether extract, or use of some drying agent.

- CEFAS laboratory has no prior notice of the numbers of samples which are sent for analysis and 20 samples would seem to be close to overload. Large numbers of samples in a batch increases the possibility of mis-labelling and overload could cause errors in applying SOPs.

- In my opinion it is desirable to have a single SOP, which fully documents the procedures to be carried out. For the SOP in effect to have to be assembled by the proper and sequential combination of a collection of other SOPs is not in my view satisfactory.

6. FRS VISIT REPORT

6.1 UKAS accreditation

Both the original and interim methods, described in the two different sets of SOPs, are UKAS accredited to ISO17025. UKAS visit annually but can visit without warning whenever they wish, although they have not done so.

6.2 Standard Operating Procedures (SOPs) for routine DSP assay. (See Annex B for detailed comments)

6.2.1 SOP for routine DSP assay

There are two SOPs for DSP assay:

A. Agreed 'interim SOP'. This is the SOP which all labs agreed to use from June 2003. This consists of SOPs 2217 (issue 5.00), SOP 2247 (issue 9.00) and M2290 (issue 8.00).

B. Actual SOP used during my inspection: This consists of SOP 2215 (issue 6.00), SOP 2245 (issue 10.00) and M2300 (issue 9.00).

Although my remit was to audit the performance of the interim SOP, FRS was not operating this procedure when I visited them and had in fact not done so since June 2003. FRS had received the laboratory audit mussel homogenate and was prepared to process it by the 'interim SOP' but not inject it into mice. This process would have been carried out specially for me by the most experienced analyst, and would therefore not be representative of the routine assay carried out at FRS.

I took the view that artificially to process it in the way suggested was pointless and unless the interim SOP was used routinely, it was a waste of time to use it for a single sample, particularly if no mice were to be injected. The purpose of an audit of this kind is to observe procedures which are being routinely carried out by technical staff, to see if something untoward was being done in the course of the routine work. Audits such as these are designed in effect to examine the implementation of a SOP at the bench level by technical staff and NOT to have a procedure demonstrated by a highly trained staff member going through it just for the purpose of the audit. This frustrates the whole purpose of the audit, which is to identify unrecorded procedures or short-cuts which diverge from those described in the SOP.

All the procedures used in the FRS SOP which are being used routinely are identical to those in the written interim SOP. The differences between the SOP operated at the moment and the interim SOP are highlighted in Table 1 (see page 20). It can clearly be seen that there are only four places where the two SOPs diverge and these relate to weight of homogenate, volume/number of acetone extracts, number of ether extractions and when the Tween suspension is made up. There are NO DIFERENCES in procedures used in the two SOPs which are identical. I have in the course of the audit of the

routine FRS SOP seen all the necessary procedures, which would be used in the interim SOP.

It might of course have transpired that the four changes mentioned above had the effect of radically altering the response of mice injected with the final extract, which is of course the reason why FRS ceased to use the interim SOP. However FRS was not prepared to carry out this important end-point determination. I would therefore have had no means of knowing whether the procedure I had witnessed was the same as that carried out in June.

I decided therefore that it would be more productive to observe the analytical procedures used in the routine SOP at present in operation, as carried out by the junior staff who usually perform the assays, because these procedures are common to both SOPs. I came to the conclusion that it might be useful to get the laboratory audit mussel homogenate processed through the FRS routine method, simply as a QC sample, to see if FRS got the same response as CEFAS and DARD. There was clearly little value in using the laboratory audit mussel homogenate as a QC for the interim SOP, as there was no question of injecting the extract into mice, if processed using this SOP and as such I would not be able to observe the test end point. The Home Office Inspector was consulted by FRS as to whether the extract prepared using the standard SOP could be injected into mice and was initially against it. Further arguments were put to him, that the quality of results are as important or even more important than the results themselves and that there must be consistency between laboratories. FRS pointed out that the laboratory audit mussel homogenate had been frozen since receipt and that this might affect the toxin. In fact it was finally agreed by the HO inspector, after further consultation with FSA, that the laboratory audit homogenate extract could be injected into mice.

I am advised by FRS that when the interim SOP was used in June 2003, abnormal responses were seen in 3 of 18 samples which were attributed to ether. After reverting to the original methodology, which is what is used today, no responses of this type are observed.

6.3 Comments on SOP as a result of observations

I noted that the process whereby these SOPs were produced was not apparent from the SOPs themselves and that I felt that such documents should indicate who the author(s) were, who had revised/checked the document and who had issued it and all three (at a minimum) should be different persons.

Staff informed me that the bench workers were asked to draft these SOPs, before being looked at by more experienced staff who revised the text if necessary. The original authors then looked at the revised text, made any corrections and returned them to more experienced staff**,** who then authorised the final version. All such SOPs are signed off by the QA manager. I pointed out that this process is not apparent from the SOPs themselves, though I am assured that the process is fully documented. There is a SOP [SOP0055

relating to how such SOPs are written] but it does not address the question of authorship nor the process through which the SOP goes before being issued/authorised.

There are a number of other SOPs, most of which, while important, deal with routine aspects of the process (i.e. SOP 2230: Operation of the rotary evaporator). Some perhaps are more important:

> SOP 2210: Receipt of samples for Shellfish toxin testing at Rowett Lab. There is also a new version in draft, which includes the use of the new LIMS system.
> SOP 2213: Care of animals used in bioassay.
> SOP 2222: Progress of bioassay samples for shellfish toxin testing through the LIMS system [in draft].

FRS have in place an extremely comprehensive and fully documented technical/quality system. This perhaps arises because it is centralised and adequately staffed.

On 6.8.03, I had a brief discussion with staff about the differences between the standard SOP, which they are operating today, and the so-called interim SOP. The two methods consist of four major specific SOPs each – these consist of an Introductory SOP, which summarises the procedures and refers the reader to specific SOPs [M2300 for operational method and M2290 for the 'interim agreed SOP'], and three other SOPs dealing with reception and processing to the homogenate stage [2215 & 2217], with preparation of extract for mouse bioassay [2245 & 2247] and finally the bioassay itself [2255] which appears to be common to all methods.

The differences between the two methods are presented in Table 1.

Table 1.

Process	Agreed Interim	Actual Method
	[2217, p.9.5]	[2215, p.9.6]
Homogenate weight Used:	100g	75g
Acetone extraction:	[2247, p.9.1/9.4] TWICE (2 X 100ML) Ultra-Turrax T25 (13,500RPM)	[2245, p.9.1] ONCE (225ML) Ultra-Turrax T50 (6,400RPM)
Ether extraction	x 3	x2
TWEEN suspension	[2247, p.9.13] Immediate & store At 4^{o}C	[2245, p. 9.11] Stand extract overnight. Suspend in TWEEN following day.

It seems that agreement to the production of a common method to be applied by all three testing laboratories extended to some but not all aspects relating to the mouse bioassay protocol because some of the issues needed resolving with the Home Office. It was therefore agreed that each laboratory should stick with its existing SOP, although weights of mice used would be standardised.

During my visit I observed testing of two mussel samples from the monitoring programme and the laboratory audit mussel homogenate. All three responses were described as POSITIVE for DSP. None of the animals died but sufficient symptoms of typical DSP were observed and the animals were stunned and necks dislocated before the 5 hour observation period ended.

Although not part of the SOP, FRS are running a parallel study, taking the surface temperature of the mice (back of head), as one of the symptoms of DSP is body temperature depression. Results observed by me were:

Mouse	Time of death After start (hrs)	Temperature (^{o}C) of each animal
Not affected	NA	32/34
Sample 5659	4.75h	27/28
Sample 5665	4.25h	27/26
Laboratory audit homogenate	2.5h	25/25

6.4 Quality Assurance

I had a discussion with staff about the need to institute both internal and external QC. I suggested that at the very least three QC samples/batch were essential [negative control and a negative control plus okadaic acid standard added at a concentration of 110-120% of the EU threshold and at 5x the threshold]. I pointed out that Okadaic acid was, for example, available in pure form from Sigma-Aldrich. Staff indicated that spiking samples with pure standards did not always work. I did not regard this as a reason for not using such a system. I indicated that it was essential that some sort of internal QA was instituted and also said that I felt that after 19 years (i.e. since 1984) some sort of external QA system should have been instituted. Ring trials have been tried (organised by UK-NRL and CRL in Vigo) but apparently there has been no feed back to FRS on performance (although I note that there has been a report from the CRL in Vigo about the ring trial carried out in 2000 and a report from UK-NRL in April 2002).

I indicated that I felt that a member of the monitoring group staff should be appointed to monitor the internal QA performance and results should be displayed in the laboratory so that all staff were aware of how they performed.

Following my visit FRS informed FSA that it has an extensive QA system which encompasses the staff involved in the DSP bioassay. This QA system is said to incorporate staff records, competencies, permitted areas of work etc. FRS has also subsequently advised that within the relevant Group, it has a UKAS Deputy Quality Manager who is ultimately responsible for signing-off the data. There is, however, no structured or organised internal or external QC (this being specific to the method), athough, the UK-NRL and the CRL have organised ring-trials. FRS has participated in these trials.

6.5 Conclusions

- I am concerned that the staff of the UK-NRL are not independent of FRS and, in effect because of their funding arrangements, serve two masters. I recommend that FSA consider carefully whether it is possible to provide funds to support all UK-NRL staff, albeit still based at FRS. I think it is important to provide the UK-NRL with the necessary funding to ensure that the advice it gives is, and is universally recognised as, independent.

- There were a number of small deviations from the SOPs, which were in the main not significant. I have noted these and recommend that the SOPs are revised to take account of actual practice. I noted that the process whereby these SOPs were produced was not apparent from the SOPs themselves and that I felt that such documents should indicate who the author(s) were, who had revise/checked the document and who had issued it and all three (at a minimum) should be different persons. I note that there is no record of batch numbers of reagents used and thus in the event of some problem with a particular reagent, there is no traceability. NOTE – Following my visit, FRS commented that it operates an electronic tracking system called “Workbench Professional – Document Control

Module" which tracks the history of a document from creation (author, date and time) through edits to approval and also tracks revisions to approved documents, but this was not mentioned to me during my visit and I have not seen it.

- FRS have no specific internal or external QA for the DSP assay which they use. This really is not acceptable. Whatever the difficulties which exist or are alleged to exist, attempts must be made to deal with this deficiency.

7. DARD VISIT REPORT

7.1 Accreditation

The methods used are accredited to Good Laboratory Practice (GLP) (EEC Directive 88/320) and the Laboratory is inspected by Department of Health who are responsible for the UK GLP Compliance Programme, monitoring laboratories every two years. DARD were last inspected on 25th September 2001. I am informed that DARD had started the process to obtain UKAS accreditation but it has been delayed because of the atypical response problem.

7.2 Standard Operating Procedures (SOPs) for routine DSP assay (See Annex C for detailed comments)

7.2.1 SOP for routine DSP assay

I was provided with two SOPs for DSP assay at DARD:

Agreed 'interim SOP'. This is the SOP which all labs agreed to use from June 2003. Hand written heading 'updated SOP' - formally described as SOP RES 212 V5. This was the SOP in operation during my inspection.

I have also been supplied with the SOP used prior to the introduction of the agreed interim SOP – SOP RES 212 V3.

I was also supplied by DARD with a third SOP (SOP RES1 V6), which describes in generic terms the reception, logging and storage of samples. The part of this SOP dealing with shellfish poisons is at paragraphs 5.0 & 6.0 (pages 7 & 8).

DARD had received the laboratory audit mussel homogenate and it was processed for me on 1st/2nd September. It had been frozen for several weeks before being used.

7.3 Comments on SOPs as a result of observations

There appears to be no written procedure for the production of SOPs but the two SOPs supplied to me by DARD are signed by three independent people and thus appear to have been produced by an acceptable procedure. NOTE since my visit, I understand that FSA have been supplied by FRS with document (QAU1 v8), which provides guidelines on writing a SOP and laboratory SOP responsibility. I have not seen this document

Laboratory audit mussel homogenate – all three mice were examined at approx. 2h after injection (examined at 4.30pm, having been injected at 2.30pm approx). Typical DSP symptoms very similar to those seen at FRS and CEFAS. No observation at 5h but at 0815h the next day, all three mice were found dead. Reported as POSITIVE result.

During my visit I also saw Cockle extracts collected as part of the monitoring programme being tested. All gave immediate mild atypical symptoms which wore off after 15-20 minutes. Mice were lethargic and hyperventilating. At 2 hours observation all mice were normal and the result was subsequently recorded as negative. It was not the atypical response I had seen at CEFAS.

7.4 Quality Assurance

I had the same discussion with staff at DARD as I had at CEFAS and FRS about the need to institute both internal and external QC: I suggested that at the very least three QC samples/batch was essential [negative control and a negative control plus okadaic acid standard added at a concentration of 110-120% of the EU threshold and at 5x the threshold]. I pointed out that okadaic acid (OA) was, for example, available in pure form from Sigma-Aldrich. Staff at FRS had indicated that spiking samples with pure standards did not always work. I did not regard this as a reason for not using such a system. I indicated that it was essential that some sort of internal QA was instituted and also said that I felt that after 19 years (i.e. since 1984) some sort of external QA system should have been instituted. DARD did not take part in the UK NRL ring trial because their Home Office licence at that time did not permit mice to be used for this purpose. I find this unacceptable, as such minimal controls are necessary, if proper evaluation of results are to be carried out. I recommend that the Home Office be approached about this matter.

I indicated again that I felt that a member of the monitoring group staff should be appointed to monitor the internal QA performance and results should be displayed in the laboratory so that all staff were aware of laboratory performance.

7.5 Conclusions

- There were at DARD a number of small deviations from the SOPs, which were in the main not significant. I have noted these and recommend that the SOPs are revised to take account of actual practice.

- DARD has insufficient internal or external QA. This really is not acceptable. I was told that difficulties exist, whatever these are, attempts must be made to deal with this deficiency.

8. DISCUSSION

There are a number of points of general and specific concern, which are detailed in the body of this report. These deal primarily with quality assurance, consistency of SOPs and some minor procedural points.

Since a large part of this assignment relates to the SOPs operated by the laboratories, the SOPs used by the laboratories audited in this report have been included in the relevant Annexes to the report.

8.1 Comparison of methodology

Required changes had been made to some existing SOPs, which were combined in order to accommodate the agreed changes for the interim SOP. It soon became apparent during my observation in all laboratories, that there were distinct differences between the assay as actually carried out in the laboratory and that described in the SOPs. Such differences are not unusual and most often represent short cuts introduced without an appreciation of whether such changes are important. While such changes are or may appear to be trivial, as we do not know the cause of the atypical response, it is not clear at this stage whether these differences are significant.

The disparities between numbers of mice used at each laboratory as the end point of bioassay, clinical signs or death, and variations in observation period (between 5 and 24 hours) has led to discrepancies in the way results are reported. While what appeared to me to be identical mice symptoms for the laboratory audit sample were observed at each of the laboratories, the results of the assay were interpreted and reported differently. The symptoms observed by CEFAS were not regarded as sufficiently significant to report a typical DSP result, therefore CEFAS reported this as NEGATIVE. FRS culled the mice after 2.5 hours, reporting the result as POSITIVE. All three mice at DARD died within the 24 hours observation period and the result was POSITIVE.

The audit sample was prepared from 6 mussel samples that had all been shown to be negative in the MBA and had subsequently been stored in a freezer for up to 6 months

I am not entirely clear whether all the laboratories are in agreement as to what constitutes an atypical response. I observed the atypical response in mice during my visit to CEFAS and saw a video showing it, which was produced by CEFAS and showed that the atypical response seen does not always lead to death of all or any of the mice, which may subsequently recover. If the mice survive the 24 hour period, DARD would regard such a reaction as a negative response, whereas FRS would almost certainly cull the mice after 2-3 hours. While FRS had made a video of the abnormal responses observed during their short implementation of the agreed interim SOP, I did not see it due to technical difficulties and so cannot compare it to the atypical response observed at CEFAS. During my visit to DARD I observed injection of cockle extracts into mice, producing what DARD described as mild atypical DSP

symptoms, which were apparent within 2 minutes of injection. At 2 hours observation all mice were normal and the result was subsequently recorded as negative.

8.2 Accreditation and Compliance

A QA manager's responsibility is for accreditation, compliance and document control. I am surprised that the SOP deficiencies at all labs (to varying degrees) have apparently not been noted by the internal QA manager and/or during UKAS/GLP compliance visits and dealt with.

8.3 Quality Control and Quality Assurance

There was no internal quality assurance in place for this assay at any of the three laboratories visited. There is really no valid justification for carrying out an assay such as the DSP procedure without some internal and external QA in force. FSA take action as a result of positive/negative results reported by these laboratories and it is essential that the validity of the results in terms of at the very least, within- and between laboratory precision can be clearly demonstrated.

QA is not only a matter for each individual laboratory but is also a collective responsibility, both nationally and internationally. Internal QA systems are important but external QA surveillance has equal and added value, particularly in demonstrating between laboratory consistency. FRS felt that adoption of QC samples was not possible at the present time, due to the nature of Home Office licences.

8.4 The atypical response to the DSP MBA

Having now carefully reviewed the analytical procedures carried out in all three laboratories, it is clear to me that there is no obvious methodological explanation for the atypical response observed primarily when analysing cockles. The fact that this response is observed mainly in cockles but not from every sample (I have been given for the incidence in cockles of approx. 40%), does not suggest a general methodological problem.

The extracts prepared during the visits, which were obtained after the ether had been evaporated were significantly different between CEFAS and the other laboratories for all the samples. The CEFAS extracts were often very dirty and contained liquid whereas the FRS and DARD residues (that I saw) were usually dry with little or no liquid (i.e. no water present). This suggests the possibility that CEFAS are getting water carry-over, perhaps because of the way that CEFAS carries out the extraction (slightly more vigorously shaken than FRS and DARD, who use a gentle swirling action). If water comes through to this final residue, it may carry over water-soluble products and/or make it more difficult to evaporate the ether dissolved in it. Both FRS and CEFAS empty their extracts out through the separating funnel tap, which means that the ether layer is inevitably contaminated with water as the ether is the top layer and the lower water layer has already passed through the tap

and the stem of the separating funnel. Consideration should be given to pouring off the ether layer through the top of the funnel, as is done at DARD, and in addition collecting it through a filter paper (there are filter papers available which are designed to remove water) or use of anhydrous sodium sulphate or other such drying agent.

I am not convinced that there is any firm evidence from this audit to support the view that the atypical response is due to the presence of ether in the Tween extract but clearly this is an issue which should be, and I understand is being, investigated. I am not in possession of all the evidence, but have seen a letter from DARD to FSA sent in November 2002, describing*, inter alia*, ether injection experiments. While these DARD results can be criticised, they do seem to suggest that the ether concentration needed to produce symptoms comparable to the fully fledged atypical response at CEFAS is in the region of 100 l/ml, nearly 10x higher than the concentration at which ether could easily be detected by smell. In none of the extracts which I have observed during my visits to the three laboratories have any smelt of ether. Removal of ether by rotary evaporation is the last process before the extract is suspended in Tween for injection into mice. If the evaporation has been carried out correctly, ether and/or acetone should not be present in significant amounts and it should not be necessary to leave the extract over-night to allow further evaporation of ether. The rotary evaporation procedure used during the routine method I observed at FRS, is identical to that used in the interim SOP and is almost identical to the procedure used at CEFAS and DARD.

9. MAIN FINDINGS & RECOMMENDATIONS

1. No evidence emerged from this audit to support the view that the atypical response is due to the presence of ether in the Tween extract (but this is being separately investigated by the FSA).

2. If the evaporation has been carried out correctly, ether and/or acetone should not be present in significant amounts and it should not be necessary to leave the extract over-night to allow further evaporation of ether.

3. While each laboratory operated a different protocol for the routine DSP assay, all were in accord with the basic methodology outlined by Yasumoto (1984)[3]. No evidence emerged from this audit that obviously supports an argument that the cause of the atypical DSP response is a methodological or procedural artefact. However, if the atypical response is in fact due to a new toxin, what appear to be slight differences in methodology may well have a profound effect on what is present in the final extract and thus injected into the mouse. Under these circumstances,

[3] Yasumoto, T., Murata, M., Oshima, Y., Matsumoto, G.L., Clardy, J. (1984). Diarrhetic shellfish poisoning. In: Seafood Toxins (Am. Chem. Soc. Symp. Ser.) ed: Ragelis, E.P. 207-214.

it would be sensible to ensure that all three laboratories operate identical protocols for the DSP assay.

4. The procedures used for routine DSP assays in all three laboratories differ to varying degrees from the method described in the SOP. All three laboratories need to address this and ensure that the SOPs in place accurately describe the procedures used in the laboratory, and ensure that SOPs in place are accurately followed. All laboratories must ensure that procedures are regularly audited to maintain compliance.

5. There are different approaches to the determination of positive/negative results by each laboratory. The end-point of the assay, irrespective of differences in analytical procedure prior to that point, has to be standardised. CEFAS require 2/3 or 1/2 mice (depending on amount of shellfish material analysed) to present symptoms within the 5 hour period for a sample to be declared positive. FRS need to observe only symptoms in 1/2 mice - they observe mice closely and kill any that suffer distress, often well before the 5 hour period of observation has ended. DARD observe for 24 hours with death as the end point When the laboratory audit mussel homogenate was injected into mice at CEFAS the symptoms observed were considered “mild” and as such the result was reported as NEGATIVE, but the same symptoms were observed at FRS and DARD where it was reported as POSITIVE. This is clearly not acceptable. It is strongly recommended that descriptions of symptoms of typical DSP and atypical responses to the DSP MBA are agreed between all three laboratories and clearly tabulated.

6. There is a need to establish the cause of the atypical response and further research is recommended. A possible route would be a comparative LC-MS analysis of extracts that produced negative responses, typical DSP and atypical DSP responses to the MBA. This may indicate a possible cause, but until this research is complete and the cause established, changes in the methodology/procedures used for routine DSP assay should be avoided as the effect of such changes will be unknown, thus possibly exacerbating the problem.

7. There appeared to be no satisfactory internal quality assurance (QA) for the shellfish monitoring protocols in place at any of the three laboratories visited. While the difficulties, described to me, of setting up an effective procedure are recognised, it is felt that they can, at least partially, be overcome and some form of internal QA **MUST** be instituted in each laboratory.

8. The staff of the UK-National Reference Laboratory (NRL) for biotoxins (UK-NRL) are not independent of FRS and, in effect because of their funding arrangements, serve two masters. It is recommended that if possible steps should be taken to establish more clearly the independence of UK-NRL and at the same time consider the role of this laboratory. I suggest that the remit of the UK-NRL should include *inter alia* responsibility for:

- QA of statutory monitoring laboratories.
- Liaison with the CRL.
- Monitoring performance of all UK statutory monitoring laboratories.
- Providing independent objective advice to the FSA and statutory monitoring laboratories, regarding methodology and procedures.
- Undertaking independent research to improve methods with intention of providing alternative assay system to present MBA (e.g. Liquid Chromatography – Mass Spectrometry (LC-MS).

9. The UK-NRL should seek to set up at least a UK wide external QA scheme, which in co-operation with the Community Reference Laboratory (CRL) could be extended to the whole of the EU.

10. Telephonic/oral transmission of results should be avoided as it may lead to errors. There should be a clearly described procedure in all laboratories for the approval of results by a named certifying scientist, which would require scrutiny of all the data, including quality control (QC) results, before they are released from the laboratory.

11. CEFAS laboratory has no prior notice of the numbers of samples that are sent for analysis and 20 samples could, with present staffing numbers, be close to overload. Large numbers of samples in a batch increases the possibility of mis-labelling and overload could cause errors in applying SOPs. If numbers of samples in batches exceed those which can be handled easily in one day, overnight storage is required.

ANNEX A - CEFAS

Observations on implementation of and recommendations for improvement of the interim SOP

1. MFS Microbiology SOP No; 07 (issue 4 15.5.03)

The relevant part of this SOP relating to DSP assay is at para. 9

Para 9.1.

It would be sensible to re-name the workbook referred to in this paragraph as 'Sample receipt book'.

Para. 9.3.

This is NOT carried out at this point and in fact is delayed until after para. 8.7 of SOP No; 10.

Para 9.7.

For routine DSP assay, homogenates of whole shellfish flesh are always used. References to homogenates of digestive glands should be removed from the body of the SOP and put as an Appendix, indicating clearly in what circumstances these alternative homogenates are to be used. Similarly only 5 hour observations are used for routine DSP assay. Incorporating such alternatives at this point in the SOP without any indication of reasons why the alternative approach might be used, is potentially confusing.

Para. 9.9.

The containers used were, where suitable, the containers in which the samples were packed on arrival, which were mostly plastic lunch boxes. This paragraph should read

'If samples not in suitable containers, transfer into alternative container. Label all containers with appropriate sample number (see para. 9.1)'.

2. MFS Microbiology SOP No; 10 (issue 6 - 30.6.03)

Para 8.1.

While this paragraph is correct and was acted upon throughout, the operator indicated that on occasions it was best to use only one pair of gloves when handling cockles, as the use of two pairs of gloves made it difficult to handle the small shells.

Para 8.2.

The shellfish samples are rinsed under a tap before shucking. This is achieved by filling the box containing the shellfish with running water and washing the shellfish in the box under the running water. In exceptional circumstances (if the shellfish are very contaminated with silt or mud) the shucked shellfish are rinsed in fresh water in a sieve.

If samples arrive in plastic bags, they would either be rinsed in the bags in the sink or decanted into plastic boxes and then rinsed.

These instructions should be incorporated into the SOP as well as some indication of how long the washing takes. The use of the words ‘running’ and ‘fresh’ should be more carefully defined in the SOP. I understand the term ‘running’ as meaning mains water direct from the tap and ‘fresh’ as distilled water.

Para. 8.3.

The first two sentences were NOT carried out at and in fact the container used was the container in which the shellfish had arrived (see para 9.9, SOP 07).

Para 8.4.

Beakers were not used. Instead a pre-weighed weighing boat was used. These boats have all been shown to weigh 5g. In fact this procedure was not carried out at this point.

Para. 8.5.

References to digestive gland dissection, which is not used in routine DSP assays should be removed at this point.

Para. 8.6.

Flesh from each animal is in fact placed on a sieve not in a beaker and liquor allowed to drain. When sufficient flesh has been collected, it is tipped from the sieve onto the pre-weighed and labelled weighing boat. Sieve is rinsed with tap water in between each sample.

Similar procedure is adopted for other shellfish (i.e. absorbent tissue is not used as described). For opening oysters, there is a special board to assist.

Para. 8.7.

Dissection of the digestive gland is not done routinely during DSP assay and thus should not be in this part of the SOP.

NB It is after this paragraph (8) that Para 9.3 of SOP 07 is carried out (i.e. Recording sample number of each lot of flesh into the Marine Biotoxins: shellfish extraction of DSP analysis workbook.

Para. 9

There are two sizes of Waring blender. Usually the smaller one is used after being labelled with the appropriate sample number but a larger one is used for mussels if excessively large amount of flesh were to be used. This is a potentially confusing point as the degree of homogenisation depends upon speed of blender (revs/min), time and frequency of blending but also the size of the blender in comparison to the weight of flesh. I recommend that only ONE blender size be used with an agreed weight of tissue.

Footnote comment at end of para. 9.2 refers to this Waring blender point.

Para 9.2

References to digestive gland should be removed. In fact 100g of flesh is placed into screw capped plastic centrifuge pots.

NB two asterisk footnote actually refers to comments above para. 9.1

3. MFS Microbiology SOP: 12 (issue 8 dated 8.5.03)

Para. 4

The lamina flow cabinet in which ether extracts stand and are subsequently evaporated is inadequate and allows too much ether vapour to escape into the environment. Since there are a large number of electrical devices in the laboratory, which could cause a spark and thus an explosion, this practice should cease and a proper fume cupboard with safety glass should be obtained. All ether extractions, storage and evaporation should be carried out therein. The UltraTurrax should not be used in this fume cupboard and all other electrical contacts should also be removed.

Para. 6.

This needs to be modified in view of existing practice. Items listed at paras. 6.5, 6.7, 6.9 and 6.15 are apparently not used.

6.3 are plastic screw capped centrifuge pots.

6.4.A filter funnel is used. These are unspecified but in fact plastic (?teflon) ones were used.

6.11. A separating funnel (not column) is used.

6.20 These are plastic TC tubes (12 x 75mm) with snap on top.
Para. 7.

The procedure used here should include note of manufacturer's lot or batch number for each reagent. Traceability of each batch back to the manufacturer should be possible.

Para 8.2

It is undesirable if weights of homogenate of <100g are used. If they are then the volume of acetone used for the extraction should be reduced pro-rata and this should be recorded here. Description of use of shellfish digestive gland homogenate should be put in separate paragraph or even separate SOP as it is not done routinely and interpolation at this point is likely to cause confusion.

The first two sentences of this have already been done and data recorded in paras. 9.2.1-9.2.3 of SOP 10. Reference to reducing Tween volume is not appropriate at this point.

Para. 8.3.

Turrax probe was cleaned with acetone before starting. Check that probe is properly secure before starting. Samples were blended for 40 seconds, not 30 seconds.

Last sentence should be deleted as it seems not to be correct.

Para. 8.4

250ml glass screw capped flasks are not used - replace with '1 litre round bottom flask'.

In fact the Whatman filter paper is 113V and they are pre-folded 32cm diameter. Filter papers should be smaller (11cm) as at the moment they are too big for the funnels, which are plastic (?Teflon).

I was told that the large filter papers had been ordered in error and for the sake of economy, it was felt should be used until they were finished.

Para. 8.5

Insert at end of first sentence '… 30 seconds (section 8.3). Repeat…'

Para. 8.6.

Solid is scraped out using a large plastic spatula.

Para. 8.7.

First sentence should be re-written as, 'Add acetone extract through the same filter paper as previously used in para. 8.4 into the 1 litre round bottom flask.'

Apparatus used for evaporation:

Water bath (Buchi B-490) set at 45^{o}C

Buchi Rotavapor R-200 + Buchi vacuum controller V-800

Para. 8.8.

Initial vacuum setting for Buchi vacuum controller was around 430millibar, which is held for around 15-20 min until bubbling stops. At this point continuous pumping is started and pressure drops to 245/250millibar and the pumping ceases at around 130millibar. In the evaporations I watched this second continuous pumping stage took about 10 mins.

Para. 8.9.

Volume of final extract is about 50-60mL.

Para. 8.10.

In line 1, it should be a 'glass separating funnel'. This is used below and elsewhere in the SOP.

Para. 8.11

Shaking is not defined anywhere (and indeed is difficult so to do) but in the examples I watched, the operator shook from side to side for around 10-20 seconds. I asked how they knew how long to shake and they indicated timing using a watch but my timing showed an occasional time span of 20 seconds. [This applies also in paras. 8.12 & 8.13]

In line 3, again it should be 'separating funnel'.
In line 4, the bottom layer is collected into the original round bottom flask, 'to which the appropriate volume of ether has already been placed'.

Para. 8.12.

Line 1 should be deleted. Paragraph should start 'Add this mixture (para. 8.11) to the…'
Line 5 should be modified to read, 'into the round bottom flask, into which the ether for extraction has already been added, and…'

Para. 8.13

In line 1 it should be 'Pour the mixture of ether and the bottom layer into the separating funnel and…'

[NB The ether layer is uppermost and the procedure used allows the lower aqueous layer to be run off through the tap, after which the ether layer is also run off through the tap. I am not sure this is desirable as it is inevitable that

this will allow through some aqueous layer as well. It might be sensible to pour the ether layer out through the top of the filter funnel and possibly also to filter to remove any water.]
Para. 8.14

The use of a measuring cylinder as described is not what is done . In fact it should read (having deleted first 10 words), 'Add 5±1ml of distilled water to the combined ether extracts and shake the separating funnel for 10 seconds. Allow to settle…' Again shaking is not defined but I observed a similar pattern to that described above (para. 8.11).

Para. 8.15.

Should read 'Repeat section 8.13 using another 5±1ml aliquot of…'
Evaporation uses the same rotary evaporator system as already described in para. 8.7. Set on continuous pumping and when it reaches around 110 millibar, evaporation is considered to be finished. This usually takes around 10 -15 minutes.

Para. 8.16

Should read, 'Re-suspend the extract in the round bottom flask in 2.5ml of 1% Tween 60. Gently swirl the liquid in the flask while immersed in the ultrasonic water bath until suspension is judged to be complete (see GENERAL SOP 15 : Ultrasonic Bath'). After re-suspension, remove liquid with a Gilson 1ml pipette and place in a plastic TC? tube and make up to appropriate volume (dependent upon original weight of tissue used – 1ml of Tween per 25g homogenate – i.e. 4ml for 100g and 3ml for 75g)'.

[NB The final extracts after ether is evaporated often contain liquid (possibly water), which will thus reduce the percentage of Tween in the final solution for injection. Suspension is often a very subjective process as the extracts vary widely from liquid to layers of solid. The latter are often difficult to re-dissolve. These extracts need cleaning up in some way].

The lids are snap on and parafilm is unnecessary unless storage or transport to another laboratory are required.

Para. 8.17

Not done at this time. This paragraph should be removed from this SOP and placed into the mouse bioassay SOP.

Para 8.18

Should be re-numbered para. 8.17. Storage is in plastic TC tubes NOT in universal vials

4. Mouse bioassay (10.07.03) MFS Microbiology SOP 13

[NB Although this SOP is recorded as being Issue 4 and dated 28.4.03 examination of history of procedure on second page, indicates that it should in fact be issue 3. I note that this SOP has remained essentially unaltered since 7.12.01]. DSP assay is specifically referred to in SOP at Para.11.

Para. 1. Introduction

Summarises what a positive result is for DSP as death of 2 out of 3 mice within a 5 hour period after injection. The 24 hour period mentioned here refers to research samples and should be removed from this SOP or to a different part of the SOP, clearly distinguished from that part applying to the routine DSP assay.

Para 4.2 The SOP states that a positive result is when 2/3 mice die within 5 hours. The SOP does not indicate what the positive result is if 2 mice are used for the assay.

I subsequently discovered that CEFAS had, in June 2003, changed the end point in mice from death to symptoms within 5 hours. This of course begs the question 'How severe do the symptoms have to be?' This is not defined anywhere and should be.

Para. 6.3

This should include 23-gauge needles which are used for sucking up extracts which cannot be got into the syringe using 25-gauge needles (*vide infra*).

Para. 8

During my observations, female Charles River CD1 mice (Margate, Kent) were used. It was explained that females were less aggressive and had a slower growth rate than males.

If this is the case, the SOP should restrict the use to CD1 female mice and not allow a choice.

Para.11.1

In fact, prior to the assembly of mice described in para. 11.1.1, the syringes for injection were prepared by sucking up 1ml into each syringe. It was indicated that CEFAS injected a 23 gauge needle, but 25 gauge needles

might be used if there was difficulty in getting extract into the syringe, although 23 gauge needles would still be used for injection.

I am not sure of the logic behind this, as, if 25 gauge needles were used as described, there would presumably be equal difficulty in getting the extract out through a 23 gauge needle.
[This emphasises the need to clean up these extracts before injection].

The filled syringes are arranged on the bench each set facing a different way, with the labelled extract in a universal container behind.

This procedure is in fact paragraph 8.17 of SOP 12 (issue 8) and thus should be moved to this SOP and removed from SOP 12. As stated the syringes were not themselves labelled nor were the batch numbers of the syringes recorded anywhere while I was observing. While the procedure adopted seemed reasonable for the small number of extracts being analysed, I wondered how effective it is for larger numbers. If the arrangements of syringes on the bench were to be disturbed in some way, it would not be possible to identify which extract was in which syringe.

The SOP should specify the needle sizes used in the way described above and should take account of the procedure as actually carried out. The extracts were in plastic TC tubes, which were shaken before the extract was removed. Although this SOP indicates that three mice were to be injected (except in certain circumstances described in paragraph 11.1.1), in one extract only two syringes were loaded. This was because only 3ml of extract had been prepared [see para. 8.16 of SOP 12].

Para. 11.1.1

The SOP indicates weight range 16-21g should be used. During observation, the range had been reduced to 19-21g inclusive. These were placed into a single cage ready for injection and the tail of one of the three mice was marked. Extract number and weight were recorded in the MB Results of DSP Mouse Test 2003 v 1.04 workbook.

Para.11.1.2

The mice were injected intraperitoneally (although I cannot comment on the technique employed as I have not done this for more than 20 years, itt certainly seemed to be appropriate). I am however surprised that such dirty extracts are permitted by the Home Office to be injected into mice. In addition, it is extraordinary that 1 ml can be injected into a 20g mouse without some sort of symptom being expected. The time of injection of the second mouse was recorded in the same workbook as described above. In order to avoid injecting the same mouse twice, the first mouse to be injected is an unmarked one, leaving the marked mouse in the cage. Once injected the second unmarked mouse is removed from the cage before the first mouse is replaced. The final mouse for injection is thus the marked mouse. The extract inside the universal container is placed on the cage in order to identify the

extract injected into the mice in that cage. This procedure should be described in the SOP.

Para 11.1.3.

Timing started from the injection of the SECOND mouse. The SOP should be adjusted to record this.

Para 11.1.3-5

Records were completed as described. A photocopy of the relevant pages from the Mouse Observations 2003 v 1.03 is provided).

Para. 11.1.6

I did not see this workbook or any such recording taking place.

SOP 07 :RECEIPT AND CONTROL OF SHELLFISH SAMPLES (ISSUE 4)

Production Summary

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HISTORY OF PROCEDURE

Issue	Date Issued	Changes
1	**26/01/01**	
2	**02/05/01**	
3	**10/07/01**	**Removal of points 9.2 and 9.3**
4	**15/05/03**	**Change in distribution, reference to MICROBIOLOGY SOP 01 and temperature alterations**
5		

1 INTRODUCTION

It is important that all samples submitted for microbiological testing are uniquely identified and their details recorded from the moment of receipt until final reporting of results.

2 SCOPE

This SOP describes the procedures for receipt of samples, both those submitted by external organisations and research samples generated internally. Samples covered in this SOP are those submitted for *E. coli,* male-specific RNA bacteriophage, *Salmonella* spp. or biotoxin testing and describes the procedure for reporting of results.

3 TRAINING

This procedure may only be carried out by staff who have received appropriate training. Training records must be completed accordingly.

4 *SAFETY PRECAUTIONS*

Before performing this procedure staff should have read and understood the following risk assessments:

4.1 *Wey/Gen 2. -20°C and 4°C walk in fridges and freezers.*

5 REFERENCES / ASSOCIATED DOCUMENTS

N/A

PROCEDURE

6 Equipment

6.1 Plastic bags

6.2 Tags

6.3 Marker pens

6.4 Refrigerator 2-6°C

7 *RECEIPT OF EXTERNAL SAMPLES FOR MICROBIOLOGICAL ANALYSIS*

7.1 Immediately on receipt in the laboratory of external samples the temperature in which the shellfish are being kept must be taken. Place a calibrated thermometer or probe into the packaging as close

to the centre of the shellfish as possible and read the temperature. Record the temperature on the **Sample Preparation Worksheet.**

7.2 Information concerning external samples must be written down in the **shellfish hygiene sample book.**

7.3 Record the consecutive sample number in the book and allocate this to the sample. This is the CEFAS sample reference number.

7.4 Record the date of sample collection and date of receipt of sample in the book.

7.5 Record the location / address / source of the sample in the book in the 'description' column and record the species.

7.6 Indicate which analysis is required by drawing a line through the results field of those analyses not required. Choice of analysis will generally be known from previous testing of samples from the same source or from the analysis requested by the sender. Where any doubt exists advice should be sought from a senior member of staff.

7.7 File paperwork supplied by the sender of external samples in the submission file.

7.8 Inspect the sample ensuring shellfish are not dead and are in a suitable condition for testing and that the minimum number of shellfish required for analysis are present. (Refer to section 9.3 of MICROBIOLOGY SOP 01)

7.9 If shellfish are not suitable for testing they can not be tested and the sender must be informed as soon as possible. A senior member of staff should also be informed immediately if this occurs. Make a record of the fact that the sender has been informed in the comments section of the shellfish hygiene sample book.

7.10 If the minimum number of shellfish required is not present the sample may still be tested but a note of this must be made in the shellfish hygiene sample book and on the final report form. The decision as to whether to test samples with insufficient numbers of shellfish should be made following consultation with a senior member of staff.

7.11 Transfer samples into containers labeled with the appropriate CEFAS sample reference number.

7.12 Either analyse the samples on the day of receipt or store at 2-6ºC until the following day. Samples for bacteriophage analysis may be frozen at <-15ºC for testing at a later date.

8 *RECEIPT OF INTERNAL SAMPLES FOR MICROBIOLOGICAL ANALYSIS*

8.1 On arrival in the microbiology laboratory of internal samples i.e. those from laboratory based trials, the temperature in which the shellfish are being kept must be taken. Place a calibrated thermometer or probe into the container as close to the shellfish as possible and read the temperature. Record the temperature on the **Sample Preparation Worksheet.**

8.2 Information concerning internal samples must be written down in the **shellfish hygiene sample book.**

8.3 Record the consecutive sample number in the book and allocate this to the sample. This is the CEFAS sample reference number.

8.4 Record the date of receipt in the microbiology laboratory of the sample in the book.

8.5 Record the any other relevant information relating to the sample in the book in the 'description' column and record the species.

8.6 Indicate which analysis is required by drawing a line through the results field of those analyses not required.

8.7 Inspect the sample ensuring shellfish are not dead and are in a suitable condition for testing and that the minimum number of shellfish required for analysis are present.

8.8 Transfer samples into containers labeled with the appropriate CEFAS sample reference number.

8.9 Either analyse the samples on the day of receipt or store at 2-6ºC until the following day. Samples for bacteriophage analysis may be frozen at <-15ºC for testing at a later date.

9 *RECEIPT OF EXTERNAL SAMPLES FOR BIOTOXIN ANALYSIS*

9.1 A sample request form must be completed in-house for all biotoxin samples requested, using information provided by the sender of the samples. The submission form is in the workbook titled 'Marine Biotoxins; sample request forms.'

9.2 Complete the rest of the sample request form as far as possible at this stage and with the type of analysis requested.

9.3 Transfer the sample number from the sample request form into the extraction workbook for the relevant analysis, i.e. into the workbook 'Marine Biotoxins; shellfish extraction for DSP toxins' or the workbook 'Marine Biotoxins; shellfish extraction for PSP toxins.'

9.4 Inspect the sample ensuring shellfish are not dead and are in a suitable conditon for testing. Also ensure that the species match the information provided by the sender and that the minimum number of shellfish required for analysis are present. Record on the sample request form whether or not the sample is suitable for testing.

9.5 If shellfish are not suitable for testing, samples can not be tested and the sender must be informed as soon as possible. Record on the sample request form when the sender has been informed that the samples are not suitable for testing. A senior member of staff should also be informed immediately if this occurs.

9.6 If the minimum number of shellfish required is not present the sample may still be tested but a record of this must be made on the sample request form in the relevant section and also on the final report form in the comments section. The decision as to whether to test samples with insufficient numbers of shellfish should be made following consultation with a senior member of staff.

9.7 Complete the 'analysis information' section on the sample request form with the type of analysis and whether DSP analysis is for the digestive gland or the whole shellfish (and either a 5 hour or a 24 hour result).

9.8 The date extracted, date analysed and date reported must be completed as the sample proceeds through the analytical process.

9.9 Transfer samples into containers labeled with the appropriate sample number.

9.10 Either analyse the samples on the day or store the sample between 2-6ºC until the following day.

10 RECORDING RESULTS FOR MICROBIOLOGICAL ANALYSIS

10.1 Results that have been recorded on the worksheets for the different analyses must be transferred into the shellfish hygiene sample book.

10.2 The analyst transferring the results must initial the original worksheet in the relevant section once the results have been transferred. To ensure no transcription errors occur during this process, a second analyst must check the shellfish hygiene sample book against the original worksheets. This analyst must initial the shellfish hygiene sample book in the relevant column.

11 RECORDING RESULTS FOR BIOTOXIN ANALYSIS

Results for biotoxin analysis are recorded in the biotoxin workbooks.

12 REPORTING RESULTS

12.1 Reporting results for internal samples

Results for internal samples do not need to be reported on a report form and it is the responsibility of the member of staff who submitted the sample to obtain the result either directly from the workbooks or from a relevant member of staff.

12.2 Reporting results for microbiology samples.

12.2.1 When all the results for an external sample have been entered into the shellfish hygiene sample book a report sheet titled 'Results of microbiological examination of shellfish hygiene samples' can be completed. Any comments regarding the sample which have been entered into the comments section of the shellfish hygiene sample book should be transferred to the comments section of the report sheet.

12.2.2 If any delay in either obtaining or reporting results occurs, a senior member of staff must be informed as soon as possible to decide whether it is necessary to inform the sender.

12.2.3 Once a report sheet has been completed it must be given to the microbiology technical manager for scrutiny and signing. The names of the technical managers who have the authority to sign a report sheet are printed on the report.

12.2.4 Reports must be signed by the microbiology technical manager and then sent either by fax (if required) or by post. A copy of the signed report sheet must be taken and filed in the report sheet file.

12.2.5 The technical manager, or another member of staff nominated by the technical manager, may give results to customers by telephone if required urgently. A subsequent confirmation report sheet must still be sent. A note must be made in the shellfish hygiene sample book that the result has been given by telephone.

12.3 Reporting results for biotoxin samples.

12.3.1 Results that have been recorded in the workbooks can, if necessary, be transferred onto the report form 'Results of algal biotoxin examination of shellfish hygiene samples. Any comments pertinent to the sample should be written into the comments section of this form.

12.3.2 If any delay in either obtaining or reporting results occurs, a senior member of staff must be informed as soon as possible to decide whether it is necessary to inform the sender.

12.3.3 Once a report sheet has been completed it must be given to the biotoxin technical manager for scrutiny and signing. The names of the technical managers who have the authority to sign a report sheet are printed on the report.

12.3.4 Reports must be signed by the biotoxin technical manager and then sent either by fax (if required) or by post. A copy of the signed report sheet must be taken and filed in the report sheet file.

12.3.5 The technical manager, or another member of staff nominated by the technical manager, may give results to customers by telephone if required urgently. A subsequent confirmation report sheet must still be sent. A note must be made in the workbook that the result has been given by telephone.

13 REVIEW

This procedure will be reviewed as a minimum on the timescales given in the review programme for SOPs. A record of the review will be filed. Any amendment arising from such review or from operating requirements will result in the issue of the entire procedure as a new 'issue'.

14 RECORDS

This procedure, its review sheets and its subsequent revisions constitute records in themselves and each master copy will be retained in a file as arranged by the Quality Manager.

14.1 Sample Preparation Worksheet

14.2 Shellfish Hygiene Sample Book

14.3 'Marine Biotoxins; sample request form'

14.4 'Marine Biotoxins; shellfish extraction for DSP toxins'

14.5 'Marine Biotoxins; shellfish extraction for PSP toxins'

14.6 Results of microbiological examination of shellfish hygiene samples

14.7 Results of algal biotoxin examination of shellfish hygiene samples

SOP 10: OPENING AND HOMOGENISING SHELLFISH FOR ASP, PSP AND DSP EXTRACTION (ISSUE 6 – UPDATED SINCE VISIT)

Production Summary

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HISTORY OF PROCEDURE

Issue	**Date Issued**	**Changes**
1	**23/01/01**	
2	**02/05/01**	
3	**08/11/01**	**Highlighted**
4	**03/02/03**	**Title alteration**

		Distribution list M Gubbins added **5.2.4 addition SOP16** **9.3 section on ASP added**
5	**31/03/2003**	**9.3 DSP replaced ASP**
6	**30/6/2003**	**9.2.1 replace "approximately 100g" with 100±1g"**
7	**21/07/2003**	**Add section 6.12 "plastic sieve"** **8.6 remove "absorbant paper" replace with "plastic sieve"** **History of Procedure numbering corrected. Previous numbering missed out issue 4.**

Note: The SOP has been updated to reflect audit observations.

1 INTRODUCTION

Bivalve molluscan shellfish are prepared for paralytic shellfish poisoning (PSP) and diarrhoeic shellfish poisoning (DSP) analysis by opening and homogenisation in blenders. Standardisation of this step is critical in achieving reproducible results in these assays.

2 SCOPE

This SOP describes the procedure for opening and homogenising bivalve molluscan shellfish for use in PSP and DSP analysis. Shellfish which are specifically covered in this SOP are oysters (*O. edulis, C. gigas*), mussels (*M. edulis*), cockles (*C. edule*) and clams (*M. mercenaria, T. semidecussatus and T. decussatus*) although other bivalve shellfish may be dealt with in a similar fashion.

3 TRAINING

This procedure may only be carried out by staff who have received appropriate training and training records must be completed accordingly.

4 SAFETY PRECAUTIONS

Risks of cuts and minor physical injury exist when performing this procedure and measures to reduce these risks are described in the risk assessment Wey/Shell 3 Opening and homogenising shellfish. Homogenisation of shellfish should be performed in a laminar flow cabinet to reduce the risk of infection from aerosols. As well as being fully trained in performing this procedure staff should have read and understood the following risk assessments.

4.1 *Wey/Gen 2. -20°C and 4°C walk in fridges.*

4.2 Wey/Shell 3. Opening and homogenising shellfish.

5 REFERENCES/ASSOCIATED DOCUMENTS

5.1 *REFERENCES*

5.1.1 AOAC (1990) Paralytic Shellfish Poison. Biological method. Final action. In: Hellrich, K. (eds), Official Methods of Analysis. 15th Edition, pp. 881-882, sec 959.08. Association of Official Analytical Chemists, Arlington, Virginia, USA.

5.1.2 Yasumoto, T.; Murata, M.; Oshima, Y.; Matsumoto, K.; Clardy, J. (1984). Diarrhetic Shellfish Poisoning. In: Ragelia, E.P. (ed.), Seafood Toxins, ACS Symposium Series, 262, pp. 207-214, American Chemical Society, Washington, D.C.

5.2 ASSOCIATED SOPS

5.2.1 MICROBIOLOGY SOP 07 'Receipt and control of shellfish samples.'

5.2.2 MICROBIOLOGY SOP 11 'Extraction of shellfish for PSP extraction.'

5.2.3 MICROBIOLOGY SOP 12 'Extraction of shellfish for DSP extraction.'

5.2.4 MICROBIOLOGY SOP 16 'Extraction and clean up of shellfish for ASP.'

5.2.5 GENERAL SOP 05 'Balances.'

PROCEDURE

6 Equipment

6.1 Balance

6.2 Forceps

6.3 Freezer <-18°C

6.4 Laminar air flow cabinet (Class ll)

6.5 Latex gloves

6.6 Refrigerator at 5±3°C

6.7 Safety gloves

6.8 Scissors

6.9 Shucking knife

6.10 Sterile glassware

6.11 Waring blender and jars

6.12 Plastic sieve

7 MEDIA AND REAGENTS

N/A

8 SAMPLE PREPARATION

For recording the receipt of a biotoxin sample see MICROBIOLOGY SOP 07 'Receipt and control of shellfish samples.' Once the sample has been given a CEFAS reference number proceed with the sample preparation as follows.

8.1 To protect hands wear a latex glove over the top of a protective glove throughout the sample preparation steps.

8.2 Discard any dead or gaping shellfish and wash remaining shellfish under running water.

8.3 Select the appropriate number of shellfish depending on the species (Appendix 1). If there are insufficient shellfish use those that are available. Place the selected shellfish into a clean container labelled with the appropriate CEFAS reference number. Place any remaining shellfish and a tag labelled with the CEFAS reference number with a permanent marker pen into plastic bags. Label the bag externally with the CEFAS reference number using a permanent marker pen and freeze at less than -18°C.

8.4 Preweigh and label with the CEFAS reference number an appropriate sized beaker depending on the expected weight of the shellfish flesh.

8.5 For some biotoxin extractions all the shellfish flesh is required for use. However, some extractions require only the digestive gland which has to be dissected out. (For the dissection of the digestive gland see section 8.7). For the amount of shellfish flesh required see section 9.

8.6 Open all selected shellfish as described below with a shucking knife. Place flesh into the beaker. When opening shellfish ensure that the hand holding the shellfish is protected with a heavy duty safety glove to prevent cuts and attempt to remove the shellfish flesh as a whole.

Oysters and Clams

Insert the knife between the two shells towards the hinge end of the animal. Push the knife further into the animal and prise open the upper shell, allowing any liquor to drain to waste. Push the blade through the animal and sever the muscle attachments by sliding across the animal. Remove the upper shell and scrape the contents of the lower shell into the Plastic sieve.

After a minimum of 5 minutes draining, weigh the required amount of shellfish tissue into the tared beaker as described in section 9.

Mussels and Cockles

Insert the knife in between the shells of the animal and separate the shells with a twisting motion of the knife. Allow the liquor to drain to waste. Cut the muscle between the shells and scrape the contents into the plastic sieve.

After a minimum of 5 minutes draining, weigh the required amount of shellfish tissue into the tared beaker as described in section 9.

Dissection of the Digestive Gland

8.7 Remove meat from shell as described above and place on absorbent paper. Orientate meat so that the digestive gland is ventral (see appendix 2). Using small sharp scissors and forceps remove overlying mantle then turn meat over, remove the foot and cut the retractor muscles. Finally clean the digestive gland of residue meat.

9 HOMOGENISATION

Sufficient shellfish meat for both the PSP and DSP extractions should be placed in a blender and homogenised at high speed for approximately 1 minute (4 bursts of 15 s with at least 5 s between bursts). When PSP and DSP extractions are to be carried out on a sample 150g of shellfish meat should be homogenised and 50g taken for PSP extraction. When only PSP is to be extracted from the sample then 100g of shellfish meat should be homogenised. If digestive gland is being extracted for DSP testing then blending should take place separately to the PSP shellfish meat.

9.1 Homogenate for PSP extraction

9.1.1 Weigh approximately 50g of homogenised shellfish flesh into a beaker, the homogenate is now ready for PSP extraction.

9.1.2 If less than these weights of material are available it will be necessary to reduce proportionally the volume of HCL in the PSP extraction step, (MICROBIOLOGY SOP 11).

9.1.3 Record the weight of homogenised shellfish flesh in the workbook 'Marine Biotoxins; shellfish extraction for PSP toxins'.

9.2 Homogenate for DSP extraction

9.2.1 Weigh100±1g for the whole shellfish flesh homogenate or 20g for digestive gland homogenate into a 200ml blender jar** and label the jar with the sample number. The homogenate is now ready for DSP extraction.

9.2.2 If less than these weights of material are available it will be necessary to reduce proportionally the volume of Tween used in the DSP extraction step, (MICROBIOLOGY SOP 12).

9.2.3 Record the weight in the workbook 'Marine Biotoxins; shellfish extraction for DSP toxins' in the either the section

for 'weight of whole shellfish (g)' or 'weight digestive gland (g).'

**If shellfish are particularly small or only small numbers are available it may be necessary to use a smaller blender to achieve a consistent homogenate.

9.3 Homogenate for ASP extraction

9.3.1 For each sample, weigh 4±0.02g of the shellfish homogenate into an appropriately labelled 50ml centrifuge tube.

9.3.2 Record the weight in the workbook: ‘Marine Biotoxins; shellfish extractions for ASP toxins’.

10 REVIEW

This procedure will be reviewed as a minimum on the timescales given in the review programme for SOPs. A record of the review will be filed. Any amendment arising from such review or from operating requirements will result in the issue of the entire procedure as a new 'issue'.

11 RECORDS

This procedure, its review sheets and its subsequent revisions constitute records in themselves and each master copy will be retained in a file as arranged by the Quality Manager. Records will be retained for a minimum of 6 years.

11.1 'Marine Biotoxins; sample request form'

11.2 'Marine Biotoxins; shellfish extraction for DSP toxins'

11.3 'Marine Biotoxins; shellfish extraction for PSP toxins'

Appendix 1

Oysters	*Ostrea edulis*	6-15 (10)*
	Crassostrea gigas	6-15 (10)
Mussels	*Mytilus edulis*	10-30 (15)
Cockles	*Cerastoderma spp*	20-50 (30)
Hard shell Clams	*Mercenaria mercenaria*	6-15 (10)

Manila Clams	*Tapes semidecussatus*	20-50 (30)
Razor Clams	*Ensis spp.*	10-20 (10)
Pallourdes	*Tapes decussatus*	20-50 (30)

*Figures in brackets represent minimum sample sizes for classification and end-product testing for *E. coli* for statutory purposes.

Appendix 2
Dissection of the digestive gland.

SOP 12: DSP EXTRACTION FROM SHELLFISH ISSUE 8 (UPDATED SINCE AUDIT VISIT)

Production Summary

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HISTORY OF PROCEDURE

Issue	Date Issued	Changes
1	**22.01.01**	
2	**10/04/01**	
3	**04/06/01**	**Alterations to 8.6**
4	**23/07/2002**	**Alterations highlighted**
5	**17/09/2002**	**Alterations to distribution list, 8.2,8.5,8.14,8.16**

6	**9/12/02**	**7. addition AnalaR**
7	**31/03/2003**	**8.4 filtration method** **8.18 storage time of extract**
8	**08/05/2003**	**8.4 filtration method**
9	**21/07/2003**	**8.4 replace "250ml screw top flask" with "1L evaporating flask"** **8.7 replace "Pour the prepared sample into…flask" with "Attach the 1L evaporating flask containing the acetone extract to the rotary evaporator"**

Note: The SOP has been updated to reflect audit observations.

1 INTRODUCTION

Bivalve molluscan shellfish are processed for the diarrhoeic shellfish poisoning (DSP) mouse bioassay by lipid extraction using acetone and diethyl ether with volatile extraction by rotary evaporation under vacuum. Standardisation of this step is critical in achieving reproducible results in this assay.

2 SCOPE

This SOP describes the extraction of DSP from shellfish.

3 TRAINING

This procedure may only be carried out by staff who have received appropriate training. Training records must be completed accordingly.

4 SAFETY PRECAUTIONS

Diarrhoeic shellfish poisons are harmful and tumourigenic and should be handled in accordance with the relevant risk assessment (WEY/SHELL 22 and 23).

Before performing this procedure staff should have read and understood the following risk assessments:

4.1	WEY/SHELL 22/23 Extraction of Biotoxins and Handling of mice during the PSP/DSP BIOASSAY	
4.2	WEY/GEN 2	-20°C and 4°C walk in fridges
4.3	WEY/GEN 3	Use of centrifuges
4.4	WEY/GEN 12	Use of circulating heated water baths
4.5	WEY/GEN 14	Use of rotary evaporators
4.6	COSHH 08	DSP, PSP Extraction

5 REFERENCES / ASSOCIATED DOCUMENTS

5.1 REFERENCES

Yasumoto, T.; Murata, M.; Oshima, Y.; Matsumoto, K.; Clardy, J. (1984). Diarrhetic Shellfish Poisoning. In: Ragelia, E.P. (ed.), Seafood Toxins, ACS Symposium Series, 262, pp. 207-214, American Chemical Society, Washington, D.C.

5.2 ASSOCIATED SOPS

5.2.1 MICROBIOLOGY SOP 10 ‘Opening and homogenising shellfish for PSP and DSP extraction.’

5.2.2 MICROBIOLOGY SOP 13 ‘Mouse Bioassay.’

5.2.3 GENERAL SOP 02 'Temperature Monitoring.'

5.2.4 GENERAL SOP 07 'pH meters.'

5.2.5 GENERAL SOP 09 'In-house calibration and testing.'

5.2.6 GENERAL SOP 13 'Rotary Evaporator.'

5.2.7 GENERAL SOP 14 'Media.'

5.2.8 GENERAL SOP 15 'Ultrasonic Bath.'
5.2.9 GENERAL SOP 17 'Centrifuges'

PROCEDURE

6 EQUIPMENT

6.1 Ultra Turrex T25 basic

6.2 Centrifuge

6.3 Centrifuge tubes - 750ml

6.4 Funnel
6.5 Glass beaker

6.6 Glass measuring cylinder

6.7 Glass screw capped flask
6.8 Needles - 25 gauge (Becton Dickinson)

6.9 Pipette
6.10 Rotary evaporator and associated glassware
6.11 Separating column

6.12 Spatula

6.13 Sterile disposable syringes 1ml or 2ml
6.14 Ultrasonic bath

6.15 Universal vial

6.16 Water bath, thermostatically controlled at 45±2°C
6.17 Whatman filters 113, 11cm

6.18 Working thermometer

6.19 250ml Conical flask

6.20 4ml measuring vial

7 MEDIA AND REAGENTS

For the preparation of reagents see GENERAL SOP 14 ‘Media’.

7.1 Acetone AnalaR grade
7.2 Diethyl ether AnalaR grade
7.3 1% Tween 60

8 PROCEDURE FOR DSP EXTRACTION

8.1 For the DSP extraction use the shellfish homogenate in the blender jar as previously prepared according to MICROBIOLOGY SOP 10 ‘Opening and homogenising shellfish for PSP and DSP extraction’

8.2 Weigh 100g ± 1g of whole shelllfish homogenate or 20g± 0.5g of shellfish digestive gland homogenate into a 750ml centrifuge tube. Record the weight in the workbook: ‘Marine Biotoxins; shellfish extractions for DSP toxins’. If the weight of homogenate is less than that specified adjust the final tween volume accordingly. Add 100±2ml acetone to the homogenate in the centrifuge tube, record the batch number and the volume of the acetone in the workbook 'Marine Biotoxins; shellfish extraction for DSP toxins'.

8.3 Blend using an Ultra Turrex T25 basic set at level 1 (11000rpm) for a minimum of 30 seconds. Clean the dispersing element between samples by wiping with a clean paper towel and then blending in clean acetone in a 250ml conical flask for a minimum of 30 seconds, then wipe clean again using a paper towel. The cleaning acetone should be changed every 5th sample. After every 10 samples the dispersing element should be dismantled and cleaned thoroughly. If multiple samples are being processed all blenders, screw capped flasks and round bottomed flasks should be labelled with sample number.

8.4 Centrifuge the acetone homogenate at 3500rpm in the Mistral 3000i centrifuge at 20°C for 10 minutes (See General SOP 17 'Centrifuges'). Once centrifugation is complete pour off the supernantant through a 113, 11cm filter into an appropriately labelled 1 litre evaporating flask.

8.5 Add 100±2ml acetone to the solid homogenate in the centrifuge tube and blend using the Ultra Turrex T25 basic for a minimum of 30 seconds. Repeat section 8.4.

8.6 Discard any solid sample remaining after the second and final acetone extraction into an autoclave bag.

8.7 Attach the 1 litre evaporating flask containing the acetone extract to the rotary evaporator. Evaporate the filtered sample under vacuum using the rotary evaporator with water bath maintained at 45±2°C. (See GENERAL SOP 13 'Rotary Evaporator.') Record the temperature of the water bath on the worksheet 'Temperature records for waterbaths (Room 271)'. Use a working thermometer which has been checked against a UKAS reference thermometer. (See GENERAL SOP 02 'Temperature Monitoring').

8.8 Evaporate the sample until distillation (bubbling) stops, leaving an aqueous suspension.

8.9 Pour the aqueous suspension into a glass measuring cylinder to measure the volume. (Record the volume of the aqueous suspension in the workbook 'Marine Biotoxins: shellfish extraction for DSP toxins').

8.10 Place the suspension in a 250ml glass separating column and add an equal volume, (± 5ml), of diethyl ether. The diethyl ether should first be poured into the original round bottom flask to wash out any residue before being poured into the separating column. Record the batch number of the diethyl ether in the workbook 'Marine Biotoxins; shellfish extraction for DSP toxins.'

8.11 Shake the mixture for about 10 seconds and then allow to settle, (depending on the sample this can be anywhere between 2 and 30 minutes). Using the tap on the base of the separating column remove the bottom layer into the original round bottomed flask. If a thin middle layer appears remove with the bottom layer. Remove the top layer into a conical flask labelled with the correct sample number.

8.12 Into the bottom layer in the round bottomed flask add the same volume of diethyl ether as previously used. Add this mixture to the separating funnel, shake the mixture for about 10 seconds and then allow to settle, (depending on the sample this can be anywhere between 2 and 30 minutes). Remove the bottom layer into the round bottomed flask and pour top layer into the correctly labelled conical flask, combining it with the first top layer.

8.13 Replace bottom layer the separating column and repeat step 8.12 to give a total of 3 diethyl-ether extractions. Discard bottom layer into the waste bottle for acetone and diethyl ether stored in the fume cupboard. Pour the combined top layers in the labelled conical flask into the separating funnel so that all 3 ether top layers are now in the separating funnel.

8.14 Place 10±1ml of distilled water into a measuring cylinder and add 5±1ml of this to the combined extracts and shake the column for 10 seconds and then allow to settle, (depending on the sample this can be anywhere between 2 and 30 minutes). Remove the bottom layer into a 250ml beaker and discard into the waste bottle for acetone and diethyl ether stored in the fume cupboard.

8.15 Repeat section 8.13 using the remaining distilled water. Place the sample in a correctly labelled 250ml round bottomed flask and evaporate to dryness in the rotary evaporator. Continue to maintain the water bath at 45±2°C

8.16 Resuspend the dried extract in the round bottomed flask using the ultrasonic water bath at room temperature. (See GENERAL SOP 15 'Ultrasonic Bath.'). For the resuspension use a 1ml gilson pipette and 2.5ml of 1% Tween 60. After resuspension remove extract using a 1ml gilson pipette and place in a measuring vial. Make solution upto 4ml by adding additional tween (final extract: 1ml of Tween per 5g of digestive gland or 1ml of Tween per 25g of whole shellfish) and seal lid with parafilm. Record the batch number and volume of the 1% Tween 60 in the workbook ‘Marine Biotoxins; shellfish extraction for DSP toxins.’. . If the original weight of homogenate was less than 100g adjust the resuspension tween and final tween volume accordingly.

8.17 Load 1±0.1ml of extract through a needle into three sterile disposable syringes (which have been batch tested according to GENERAL SOP 09). It may be necessary to run the extract through the needle several times to break up any lumps. Record the batch number of the syringe in the workbook 'Marine Biotoxins; shellfish extraction for DSP toxins'. Label the syringes with the sample number and the test, i.e. DSP.

8.18 The extract is now ready for the DSP mouse bioassay (see MICROBIOLOGY SOP 13 ‘Mouse bioassay’). The extract can be stored in universal vials at 5±3°C for a maximum of 5 days and preferably no longer than 48 hours if the sample is not to be used immediately for the mouse bioassay.

9 REVIEW

This procedure will be reviewed as a minimum on the timescales given in the review programme for SOPs. A record of the review will be filed. Any amendment arising form such review or from operating requirements will result in the issue of the entire procedure as a new 'issue'.

10 RECORDS

This procedure, its review sheets and its subsequent revisions constitute records in themselves and each master copy will be retained in a file as arranged by the Quality Manager. Records shall be retained for a minimum of six years.

10.1 'Marine Biotoxins; shellfish extraction for DSP toxins'

SOP 13: MOUSE BIOASSAY (ISSUE 4)

Production Summary

Author	
Reviewed by	
Date	January, 2001
Issue Authorisation	
Position	
Signature	
Date	

Distribution of Copies

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HISTORY OF PROCEDURE

Issue	**Date Issued**	**Changes**
1	**24.01.01**	
2	**9/04/01**	
3	**07/12/01**	
4	**28/04/2003**	**change to distribution list**
5	**22/07/2003**	**History of procedure numbering corrected. Previous numbering omitted revision 2.**

1 INTRODUCTION

The diarrhoeic shellfish poisoning (DSP) and paralytic shellfish poisoning (PSP) toxins from the extracted shellfish are tested separately by mouse bioassay. Death of the mouse indicates the presence of these toxins.

The mouse bioassay for DSP toxicity is considered to be semi-quantifiable, as the prolonged observation time would suggest. It is less reliable as a determinant of acute toxicity than is the corresponding AOAC (American Organisation of Analytical Chemists) mouse bioassay for PSP toxicity. The precise cause of death in rodents by intraperitoneal administration of DSP toxins is not yet known.

PSP toxins are quantified by a bioassay standardised using calibrated saxitoxin reference material and units of measure are recorded as µg Saxitoxin equivalence / 100g shellfish flesh.

DSP toxins are detected by a semi-quantifiable mouse bioassay. DSP mouse bioassay is a positive or negative result with death of 2 out of 3 mice in either a 5 hour (routine samples) or 24 hour (research samples) observation time. Death after 5 or 24 hours indicates the presence of DSP toxins, after 24 hours this corresponds to approximately 3.2µg DTX1 (dinophysistoxin) or 4µg OA (okadaic acid).

2 SCOPE

This SOP describes the mouse bioassay which is the approved EC method for the detection of paralytic shellfish poisoning (PSP) and diarrhoeic shellfish poisoning (DSP) toxins. It consists of an intraperitoneal injection of shellfish extract with the death of the mouse within a certain timespan indicating the presence of toxins.

3 TRAINING

This procedure may only be carried out by staff who have received appropriate training. Training records must be completed accordingly.
Staff responsible for intraperitoneal injection must possess a Home Office Personal Licence for small animal handling.

4 SAFETY PRECAUTIONS

Always use rubber gloves when handling materials which may contain shellfish poison.
When working with animals or in the animal house always follow the Home Office guidelines.
Diarrhoeic shellfish poisons are harmful and tumourigenic and should be handled in accordance with the relevant risk assessment (WEY/SHELL 22 and 23).
Before performing this procedure staff should have read and understood the following risk assessments:

4.1 WEY/SHELL 22 Saxitoxin
4.2 WEY/SHELL 23 Toxin extraction for mouse injection

5 REFERENCES /ASSOCIATED DOCUMENTS

5.1 REFERENCES

5.1.1 AOAC (1990) Paralytic Shellfish Poison. Biological method. Final action. In: Hellrich, K. (eds), Official Methods of Analysis. 15th Edition, pp. 881-882, sec 959.08. Association of Official Analytical Chemists, Arlington, Virginia, USA.

5.1.2 Yasumoto, T.; Murata, M.; Oshima, Y.; Matsumoto, K.; Clardy, J. (1984). Diarrhetic Shellfish Poisoning. In: Ragelia, E.P. (ed.), Seafood Toxins, ACS Symposium Series, 262, pp. 207-214, American Chemical Society, Washington, D.C.

5.1.3 Official Journal of the European Communities. Directive 91/492/EEC, 15 July 1991. 'Laying down the health conditions for the production and the placing on the market of live bivalve mollluscs.'

5.1.4 'Personal Licence : Animal Services Unit Home Office Licensee Manual'. Central Veterinary Laboratory, Weybridge.

5.2 ASSOCIATED SOPS

5.2.1 MICROBIOLOGY SOP 10 ‘Opening and homogenising shellfish for PSP and DSP extraction’.

5.2.2 MICROBIOLOGY SOP 11 ‘PSP extraction from shellfish.’

5.2.3 MICROBIOLOGY SOP 12 ‘DSP extraction from shellfish.’

5.2.4 GENERAL SOP 07 'pH meters'.

5.2.5 GENERAL SOP 09 'In-house calibration and testing'.

5.2.6 GENERAL SOP 14 'Media'

PROCEDURE

6 EQUIPMENT

6.1 Balance
6.2 Calibrated timer
6.3 Needles -25 gauge (Beckton Dickinson)
6.4 pH meter

6.5 Pipettes
6.6 Sterile disposable syringes
6.7 Universal vials
6.8 Volumetric flask

7 MEDIA AND REAGENTS

For preparation of toxins see MICROBIOLOGY SOP 11 ('PSP extraction from Shellfish') and MICROBIOLOGY SOP 12 ('DSP extraction from shellfish.')
For the preparation of reagents see GENERAL SOP 14 'Media.'

7.1 PSP standard solution – saxitoxin dihydrochloride (STXdiHCl-C) certified callibration standard obtained from the National Research Council Canada.
Concentration 25±1µg/ml (STXdiHCl-C) calibration solution.
7.2 0.1M HCL
7.3 0.01M HCL
7.4 PSP toxin extract
7.5 DSP toxin extract
7.6 Acidified distilled water (pH 3.0±0.5)

8 ANIMAL SOURCES

Outbred female mice obtained from :-

8.1 Charles River
Margate
Tel : 01843 823575
Fax :01843 823497
CD-1 outbred mice female

8.2 Harlan UK ltd
Bicester
Tel : 01869 243241
Fax :01869 246759
CD-1 outbred mice female

9 PROCEDURE FOR PSP BIOASSAY

The calculation of the PSP toxicity requires a conversion factor which is obtained from the results of the calibration of PSP bioassay described in section 10 of this SOP.

9.1 Receipt of mice

9.1.1 On receipt of a batch of mice complete the workbook 'Marine Biotoxins; record of mouse strain and supplier.'

9.1.2 Complete the section 'Animal House, Mouse Stock No. M…' with the consecutive number from the previous page.'

9.1.3 Complete the remaining sections, (arrival date, arrival time, received by, batch number, number of mice, sex of mice), and label the mice in the cages accordingly.

9.2 PSP bioassay

9.2.1 Record PSP test, the date, analyst recording, analyst injecting and animal batch number in the workbook 'Marine Biotoxins; results of mouse test.'

9.2.2 Take two test mice and label each one with an identifying mark using a permanent marker pen. Mark the first mouse with a band around the base of the tail and the second mouse with a band around the tip of the tail. Weigh each mouse to the nearest 1g and record the weight and the sample number of each mouse in the workbook ‘Marine Biotoxins; results of mouse test.’ Mice should be within the range 16-21g.

9.2.3 Intraperitoneally inoculate the two test mice with 1±0.1ml PSP extract each. (For preparation of PSP extract see MICROBIOLOGY SOP 11).
(See diagrams in Appendix 1 on correct mouse positioning for injection.)

9.2.4 Start a calibrated timer (see GENERAL SOP 09 ‘In-house calibration and testing’). Note the time of injection of the mouse half way through the injection. On completion of the injection record the injection time. Record this in the workbook ‘Marine Biotoxins; results of mouse test.’

9.2.5 Observe the mice for one hour, viewing closely every 5 minutes, noting any symptoms in the workbook ‘Marine Biotoxins; observations of mouse test.’ Note the time of death as indicated by the last gasping breath. Record the time of death from the calibrated timer in the workbook ‘Marine Biotoxins; results of mouse test.’

9.2.6 Calculate mouse death time as 'time of death' minus 'time of injection' and record in the workbook 'Marine Biotoxins; results of mouse standardisation test.'

NOTE : FOR RESEARCH PURPOSES ONLY (NOT FOR ROUTINE MONITORING SAMPLES)

9.2.7 If the median death time of the two mice is <5 minutes make dilutions of the PSP extract to obtain death times of 5-7 minutes. (See section 9.1.6).

9.2.8 Use distilled water for the dilutions. If large dilutions are necessary (i.e. over 1:1 dilutions), adjust the pH by adding diluted HCl (0.1M or 0.01M) to achieve a pH of between 2 and 4, (never >4.5), (see GENERAL SOP 07 'pH meters').

9.2.9 Repeat sections 9.1.1 to 9.1.5 with the new dilution expected to give a median death time of 5-7 minutes.

9.2.10 Repeat section 9.1.7 as necessary until a median death time of 5-7 minutes has been achieved.

9.2 Calculation of PSP toxicity

9.2.1 Using the mouse death time determine the mouse units (MU) from Sommer's table, (see Appendix 2), and record in the workbook 'Marine Biotoxins; PSP calculations of mouse test.'

9.2.2 The MU require a weight correction factor (WCF) dependent on the weight of the mouse. To obtain the WCF take the weight of the mouse and look up the corresponding mouse units in the table in Appendix 3, ('Correction table for weight of mice'). Record the corresponding mouse units in the workbook 'Marine Biotoxins; PSP calculations of mouse test.'

9.2.3 These mouse units are then multiplied by the original MU taken from Sommer's table to give the corrected mouse units (CMU). Record the calculated CMU in the workbook 'Marine Biotoxins; PSP calculations of mouse test.'

9.2.4 Calculate the average CMU for the two mice and convert the CMU to µg PSP/100g shellfish flesh using the following equation : -

µg poison/100g shellfish flesh = median CMU x CF x dilution factor x 200

Where CF = conversion factor taken from the calibration of PSP bioassay, (see section 10 of this SOP and section 10.4.5 for the actual CF value).

Record the µg PSP/100g shellfish flesh in the workbook 'Marine Biotoxins; PSP calculations of mouse test.'

9.2.5 Record this result in the workbook 'Marine Biotoxins; shellfish extraction for PSP toxins' as STXdiHCl-C equivalent µg/100g shellfish flesh or not detected (ND).

NOTE :

9.2.6 Any value >80 µg PSP/100g shellfish flesh is hazardous and unsafe for human consumption.

10 CALIBRATION OF PSP BIOASSAY

10.1 Preparation of dilutions for PSP bioassay calibration

10.1.1 The calibration must be carried out once per month or if the assay is performed less frequently then the calibration can be carried out with each batch of mice on which the PSP assay is performed.

10.1.2 Open two ampoules of STXdiHCl-C, the PSP standard solution. Each ampoule contains 0.2ml of 25µg/ml in 0.003M dihydrochloric acid. Using a labelled, calibrated P200 Gilson pipette, transfer the PSP standard into a 10ml volumetric flask. Use approximately 2ml of distilled water to rinse out the ampoules and place this solution in the 10ml volumetric flask. Record the expiry date of the STXdiHCl-C in the workbook 'Marine Biotoxins; results of mouse standardisation test.'

10.1.3 Make the PSP standard solution in the volumetric flask up to 10ml using acidified distilled water (pH 3.0 ±0.5) to give a 'reference' solution of 1µgSTX/ml. Decant this solution into a universal vial. Record the batch number of the acidified distilled water in the workbook 'Marine Biotoxins; results of mouse standardisation test.'

10.1.4 Prepare dilutions in labelled universal vials by taking the appropriate volume of the reference solution and diluting as follows :-

2ml reference solution and 2ml distilled water to give 0.50 µg STX/ml
2ml reference solution and 3ml distilled water to give 0.40 µg STX/ml
2ml reference solution and 4ml distilled water to give 0.33 µg STX/ml
2ml reference solution and 5ml distilled water to give 0.29 µg STX/ml
1ml reference solution and 3ml distilled water to give 0.25 µg STX/ml

10.2 Determination of median death time for calibration of PSP bioassay.

10.2.1 Take two test mice and label each one with an identifying mark using a permanent marker pen. Mark the first mouse with a band around the base of the tail and the second mouse with a band around the tip of the tail. Weigh each mouse to the nearest 0.5g and record the weight and the sample number of each mouse in the workbook 'Marine Biotoxins; results of mouse standardisation test.' Mice should be within the range 16-21g. Record the standard concentration (µg STX /ml) being used in the same workbook.

10.2.2 To determine the required median death time of 5-7 minutes intraperitoneally inoculate the two test mice with 1±0.1ml each of the median dilution (33 pgSTX/ml). (See diagrams in Appendix 1 on correct mouse positioning for injection.)

10.2.3 Start a calibrated timer (see GENERAL SOP 09 'In-house calibration and testing'). Note the time of injection of the mouse half way through the injection. On completion of the injection record the injection time. Record this in the workbook 'Marine Biotoxins; results of mouse standardisation test.'

10.2.4 Observe the mice closely for ten minutes, noting any symptoms in the workbook 'Marine Biotoxins; observations of mouse test.' Note the time of death as indicated by the last gasping breath. Record the time of death from the calibrated timer in the workbook 'Marine Biotoxins; results of mouse test.'

10.2.5 If the death time is outside the 5-7 minute range then repeat section 10.2.1 to 10.2.5 with a different dilution according to the results obtained.
It may be necessary to carry out further slight dilutions around one of the reference solutions to cause mouse death close to 5-7 minutes.
For example, to reduce the reference solution from 0.33 µg STX/ml to 0.31 or 0.32 µg STX/ml dilute as follows : -

1ml 0.33 µg STX/ml + 50 µg distilled water = 0.31 µg STX/ml
1ml 0.33 µg STX/ml + 25 µg distilled water = 0.32 µg STX/ml

10.2.6 When an intraperitoneal injection, into two test mice, of a 1±0.1ml volume causes a median death time of 5-7 minutes use this dilution to inject three further mice to obtain a minimum of five results at the median death time.

10.3 Bioassay for PSP calibration

10.3.1 Take five test mice and label each one with an identifying mark using a permanent marker pen. Mark the first mouse with a band around the base of the tail and the second mouse with a band around the tip of the tail. The third mouse can be left unmarked. Place three mice per cage and label each cage accordingly to ensure the correct identification of the mice. Weigh and record the weight of the five mice to the nearest 0.5g in the workbook 'Marine Biotoxins; results of mouse standardisation test.' Mice should be within the range 16-21g.

10.3.2 Intraperitoneally inoculate each mouse with 1±0.1ml of the pre-determined dilution, (as prepared in section 10.2). (See diagrams in Appendix 1 on correct mouse positioning for injection.)

10.3.3 Start a calibrated timer (see GENERAL SOP 09 'In-house calibration and testing'). Note the time of injection of the mouse half way through the injection. On completion of the injection record the injection time. Record this in the workbook 'Marine Biotoxins; results of mouse standardisation test.'

10.3.4 Observe the mice closely for ten minutes, noting any symptoms in the workbook 'Marine Biotoxins; observations of mouse test.' Note the time of death as indicated by the last gasping breath. Record the time of death from the calibrated timer in the workbook 'Marine Biotoxins; results of mouse standardisation test.'

10.3.5 Calculate the median death time for the group of ten mice (the five mice used as described in section 10.2 and the five mice used as described in section 10.3).

10.4 Calculation of PSP toxicity for calibration of PSP bioassay

10.4.1 Using the mouse death time determine the mouse units (MU) from Sommer's table (see Appendix 2) and record in the workbook 'Marine Biotoxins; results of mouse standardisation test.'

10.4.2 The MU require a weight correction factor (WCF) dependent on the weight of the mouse. To obtain the WCF take the

weight of the mouse and look up the corresponding mouse units in the table in Appendix 3, ('Correction table for weight of mice'). Record the corresponding mouse units in the workbook 'Marine Biotoxins; results of mouse standardisation test.'

10.4.3 These mouse units are then multiplied by the original MU taken from Sommer's table to give the corrected mouse units (CMU). Record the calculated CMU in the workbook 'Marine Biotoxins; results of mouse standardisation test.'

10.4.4 Divide the concentrations of the reference solution (µgSTX/ml) by the CMU to obtain the conversion factor (CF). This value represents µg poison equivalent to one MU. Record the CF in the workbook 'Marine Biotoxins; results of mouse standardisation test.'

10.4.5 Calculate the average CF from the ten mice and record in the workbook 'Marine Biotoxins; results of mouse standardisation test.' If CF varies by over 20% more than once every 5 calibrations then the possibility of uncontrolled or unrecognised variables in the method must be investigated.

11 PROCEDURE FOR DSP BIOASSAY

11.1 DSP bioassay

11.1.1 Take three test mice and weigh each one before injection and record the weight and the sample number in the workbook 'Marine Biotoxins; results of mouse test.' Mice should be within the range 16-21g. When performing repeat testing for an area which is closed after more than one DSP positive it is possible to reduce the number of mice injected to two. The use of three mice should resume once the area is negative and reopened.

11.1.2 Intraperitoneally inoculate the three test mice with 1±0.1ml DSP extract each. (For preparation of DSP extract see MICROBIOLOGY SOP 12). (See diagrams in Appendix 1 on correct mouse positioning for injection.)

11.1.3 Start a calibrated timer (see GENERAL SOP 09 'In-house calibration and testing'). Note the time of injection in the workbook 'Marine Biotoxins; results of mouse test.'

11.1.4 Throughout the 5 or 24 hour assay time observe the mice every half an hour for poisoning and record any symptoms

in the workbook ‘Marine Biotoxins; observations of mouse test.’

11.1.5 Record the number of mice deaths or survivals in the workbook ‘Marine Biotoxins; results of mouse test’ when the assay time is complete.

11.1.6 Record the results of the bioassay as negative (<2 mice dead) or positive (>2 mice dead) in the workbook ‘Marine Biotoxins; shellfish extraction for DSP toxins.’

11.2 DSP TOXICITY

The mouse bioassay is positive if 2 or 3 out of 3 mice die within 5 hours (routine samples) or 24 hours (research samples) assay time.

12 ANALYTICAL CONFIRMATION

12.1 Procedure

12.1.1 If routine monitoring samples are being processed one out of every 50 homogenates for mouse bioassays for PSP and DSP will be confirmed by HPLC. (For example every 50 samples from sample BTX/2001/1, i.e. BTX/2001/50 and then BTX/2001/100 etc). The sample number must be recorded in the workbook 'Marine Biotoxins; Analytical Confirmation' together with the result of the PSP or DSP analysis.

12.1.2 The sample homogenate (stored in the fridge) must be packaged and sent in a cool box to the Burnham laboratory for HPLC analysis. A record that samples have been sent for HPLC analysis and the name of the analyst sending the samples should be recorded in the workbook 'Marine Biotoxins; Analytical Confirmation.'

12.1.3 When results produced by the Burnham laboratory are returned to CEFAS they must be recorded in the workbook 'Marine Biotoxins; Analytical Confirmation.'

12.2 Analytical confirmation for PSP analysis

12.2.1 If the variation between the mouse bioassay and analytical result is more than 20% of the total toxin value for PSP then the procedure must be inspected for factors causing variability outside of the mouse bioassay confidence limits.

12.2.2 A departure log must be completed to record the action taken.

12.3 Analytical confirmation for DSP analysis

12.3.1 If the analytical results for DSP do not agree with the DSP mouse bioassay, (i.e. more than 30µg/100g±20% does not cause mouse death or less than 20µg/100g±20% does cause mouse death), then the procedure must be inspected for factors causing variability outside of the mouse bioassay confidence limits.

12.3.2 A departure log must be completed to record the action taken.

13 EUTHANASIA AND DISPOSAL OF MICE

Mice which have not died during the bioassay must be dealt with according to the information given in 'Personal Licence: Animal Services Unit Home Office Licensee Manual'. Central Veterinary Laboratory, Weybridge.

14 UNCERTAINTY OF TEST RESULTS

Factors influencing the final results of the PSP and DSP test results include potential variability in mouse batches and mouse injection. Uncertainty can also arise through individual analyst variability in the extraction procedure.

To take account of these uncertainties within the PSP analysis, the average conversion factor is calculated. If the conversion factor varies by greater than 20% it is investigated and recorded on a departure log. In addition one out of every fifty homogenates for the mouse bioassay is confirmed by HPLC. If the variation between the mouse bioassay and analytical result is more than 20% of the total toxin value for PSP then the procedure is investigated for factors causing variability outside of the mouse bioassay confidence limits. All findings are recorded on a departure log.

To take account of these uncertainties within the DSP analysis, if the analytical results, (from HPLC analysis), do not agree with the DSP mouse bioassay, (i.e. more than 30µg/100g±20% does not cause mouse death or less than 20µg/100g±20% does cause mouse death), then the procedure must be inspected for factors causing variability outside of the mouse bioassay confidence limits. All findings are recorded on a departure log.

15 REVIEW

This procedure will be reviewed as a minimum on the timescales given in the review programme for SOPs. A record of the review will

be filed. Any amendment arising from such review or from operating requirements will result in the issue of the entire procedure as a new 'issue'.

16 RECORDS

This procedure, its review sheets and its subsequent revisions constitute records in themselves and each master copy will be retained in a file as arranged by the Quality Manager. Records will be retained for a minimum of six years.

16.1 'Marine Biotoxins; results of mouse test'

16.2 'Marine Biotoxins; observations of mouse test.'

16.3 'Marine Biotoxins; results of mouse standardisation test.'

16.4 'Marine Biotoxins; PSP calculations of mouse test.'

16.5 'Marine Biotoxins; shellfish extraction for PSP toxins.'

16.6 'Marine Biotoxins; shellfish extraction for DSP toxins.'

16.7 'Marine Biotoxins; record of mouse strain and supplier.'

16.8 'Marine Biotoxins; Analytical Confirmation.'

Appendix 1

Photographs showing:
- correct mouse positioning for injection.
- correct mouse positioning for injection.
- pre-innoculation position

Appendix 2

Sommers Table (not reproduced here)

Appendix 3

Correction table for weight of mice (not reproduced here)

Other information supplied by CEFAS

CEFAS provided me with the following documents:

- Copies of all the forms filled in during the analytical process.
- Preliminary investigations into the causative ‘toxin’ responsible for atypical DSP positive mouse bioassay results in the algal biotoxin monitoring programme for England and Wales. July 2002 (Higman et al.)
- BESU SOP BTX 001 (revision 00) SOP for the determination of DSP toxicity by mouse bioassay [ex Prof Yasumoto]
- BESU SOP BTX 001 (revision 01) SOP for the determination of DSP toxicity by mouse bioassay [ex Prof Yasumoto]
- Mouse Assay for detection of Oas, DTXs, PTXs, YTXs and AZAs) Protocol 1 and protocol 2 [ex Prof Yasumoto]
- PP2A assay. [ex Prof Yasumoto]

Annex B - FRS

Observations on implementation of and recommendations for improvement of the routine SOP

1. SOP 2215 (Issue 6.00, dated 16.6.03) Processing of shellfish samples for toxin analysis:

Para. 2.

This paragraph requires two different logging in procedures depending on whether the staff are 'bioassay' (use SOP 2210) or 'chemistry' (use SOP 60). This is undesirable and is not acceptable and a single logging in system should be instituted.

[NB They are running a new LIMS (Laboratory Information & Management System) in parallel with the existing paper one. SOPs are being written for this (LIMS1 and LIMS2 (both Introduction/user guides), 2210 (Receipt of shellfish samples), 2222 (Progress of shellfish samples through the LIMS system) & 2258 (Reporting of shellfish toxin results)].

Para. 4.

2% Trigene, for disinfectant purposes, is specified here, though Mr. Muscle domestic cleaner was used when I was there.

Para. 5.

Measuring cylinders and spray bottle for Trigene preparation were not used.

Para. 6.

Cold running water was used for rinsing here.

Para. 8.

I was given an information leaflet sent by FRS to all those involved in sampling. This describes sample collection protocols and provides names, e-mail addresses and phone numbers of FRS staff who can be contacted for information. This is to be commended.

Once again (see para 2 above) two different kinds of staff use different procedures (or refer to different SOPs). This should cease and a single procedure should be used. The procedure I observed used the Rowett 1 Record sheet (a photocopy is attached).

Para. 9.1.

The samples I observed arrived in plastic bags inside plastic lunch boxes. The unique sample number is written on the luncheon box. These sample numbers are allocated by the Technical Manager's staff to each sample batch on arrival and recorded in Rowett 1. The last number used is recorded on a piece of paper kept in the laboratory, which be be used to checkthe numbers already used on the previous page of the Rowett 1 form. The details of source are on the label attached to each sample. The plastic bags were removed, opened and the contents tipped back into the same box. The rinsing process then followed.

Para. 9.2.

The shellfish were indeed rinsed under running tap water but for varying times by the three operatives. The number of rinses varied between two and seven. This may be acceptable as the amount of rinsing clearly depends on how dirty each sample is.

Para. 9.3.

Routine scallop testing uses whole tissue homogenate. If gonads are to be used, the reason must clearly be stated here or this must be removed.

[NB I have had this explained to me but it needs further explanation in the SOP. All scallop samples arrive at FRS monitoring lab. The Shellfish Research Laboratory collect them and prepare homogenate for ASP screening by HPLC-DA. Then, 120g aliquots of homogenate are returned to the monitoring group for DSP and PSP].

The flesh is placed in pre-weighed weighing boats, which have been labelled with the unique sample number.

Para. 9.4.

Sufficient sample is not defined here and should be defined. Staff inform me that 150g is required. Prioritisation is also not properly defined but FRS indicated that experienced staff would do this. The choice depends upon season of year and likely incidence of DSP or PSP. At this time of year (August) DSP is more likely than PSP and, if tissue weight is less than 150g, DSP is given priority. This should be explained in the SOP. Weights are checked on a balance (see Para. 9.5).

Again two different logging systems are described. This should be resolved. When I observed this process, Rowett 1 was used.

Para. 9.5.

A plastic container was not used when I observed the process. The homogenisation was carried out using a Christison EN 1083 domestic blender. There are two sizes of blender and the smaller one is used if weights are less than 50g. One sample used in the larger container weighed around 100g and I noted considerable difficulty in getting good homogenisation. The 20 second period is timed using a clock.

Para. 9.6

This is unclear and needs tidying up. The title 'chemically prepared samples' is ambiguous and I am not clear why there are two paragraphs here. Para. 9.6.2 describes what I saw. Details were recorded on Rowett 2, which together with Rowett 1 is sent with samples to the Rowett for bioassay. Rowett 4 is filled in at the RRI on receipt.

[NB No animal facilities at FRS for historical reasons. Torry had such facilities but these were transferred to RRI when Torry closed. Lab and animal house are at RRI. Homogenates prepared at FRS and then transferred to RRI for processing].

Para. 13.

Special safety risks are identified in this paragraph. I asked whether the staff involved had read these risk assessments, understood them and signed indicating this. I did not receive a satisfactory response to my question.. I did establish that there were no signatures, which in my view is the proper way to indicate that relevant staff have indeed seen these assessments and are aware of risks and precautions which are necessary.

2. SOP 2245 (Issue 10.00 dated 16.6.03) – Extraction of DSP Toxin from Shellfish tissues for bioassay.

Para. 2.

This should specify the particular kind of Ultra-Turrax used (T50) and the rpm associated with the setting chosen.

Para. 5.

Calibrated balance not used in this SOP. Should be removed from the list.

100ml flasks should be more clearly described. Those used were pear-shaped (Quickfit FD 100/3 24/29). [NB Comment by staff that these shape flasks were much better when suspending in Tween in comparison to standard round bottom flasks]

Conical flask is 100ml.

Ultra-Turrax model should be specified.

Para. 9

Rowett 4 form is filled in here. Should be deleted from previous SOP (2215) at para. 9.6.

Para. 9.1.

The 225ml was dispensed in three 75ml aliquots.

Para. 9.2.

The Ultra-Turrax T50 setting at 3 gives revs of 6400rpm. During the timed 30 seconds, the container is swirled around by the operator.

Para. 9.3.

After rinsing with acetone, the rotor is wiped with a tissue.

Para. 9.4.

After filtration, the filter papers containing the homogenate has the last bit of acetone squeezed out and collected.

Para. 9.5.

The evaporation procedure is described in general SOP 2230, even though it is headed, 'Operation of rotary evaporator for shellfish toxin extraction at Rowett lab.' I think that SOP 2230 should be incorporated into this SOP as it gives details of waterbath (Clifton unstirred waterbath) temperature (48oC) and vacuum settings which accord with my observations. Atmospheric pressure today was 1016 millibar (as recorded on the knf Neuberger vacuum controller. Automatic setting pumps down to 580 millibar (approx.) and falls to approx. 100 millibar when evaporation is complete. It should not be necessary to have to use another SOP to obtain information on this process.

Para. 9.7.

Volume must be recorded in Rowett 4.

The extraction process is really not much more than a mixing, which is carried out by swirling the contents of the flask.

Para. 9.8.

The actual process is that the lower aqueous layer is run off through the tap into the original 500ml flask, to which the ether is then added. The upper layer (ether) is also run off through the tap into the 100ml conical flask.

Para. 9.10.

'mix well' in this paragraph has a different meaning to that in para. 9.7 as here it means vigorous shaking in a separating funnel. I noted three (each lasting around 3-4 seconds) vigorous shakes, opening the tap after each 4 sec shake. Very occasionally emulsions form but there is nothing in the SOP to advise what action is to be taken.

Para. 9.11.

Ether layer run off into 100ml flask through Teflon tap.
SOP 2230 is again quoted here. As with para. 9.5, I recommend that salient details are incorporated into this SOP (2245).

The extract, once dried, is left in the fume cupboard overnight and para 9.12 (suspension in Tween) is carried out on the following day.

NB I noted that the residue after evaporation was dry and unlike CEFAS no liquid was present.

Para. 9.12.

Carried out on following day. Suspension was much easier and more complete at FRS than at CEFAS, perhaps because the extract was dry and no liquid was present. The suspension is transferred into screw cap glass Universal containers, which are labelled with the appropriate sample number. This information should be added to this paragraph.

[NB There is no record made during this process either here or previously of the batch number or any other identifier of the reagents used in relation to particular batches. There is thus a traceability problem if manufacturers should report faulty batches of solvents. It was pointed out that use of internal QA would overcome such difficulties and should identify faulty batches. There are two general SOPs, which I was led to believe deal with this problem – SOPs 1375 and 1400. However, on inspection, they do not].

3. SOP 2255 (Issue 4.00, dated 14.5.03) – Analysis of extract to assess level of DSP toxin by bioassay.

Para. 5.

The mice used during my observation were Female CD1 mice supplied by Harlan (Batch 31 which had arrived on 5th August – NB two days acclimatisation before use).

Para. 6.

Animal house temperature maintained between 19-25^{o}C.

Para. 9.

During my visit paras. 9.1 and 9.2 were carried out in reverse order and cage referred to in para. 9.1 was labelled in the way described after two mice of appropriate weight had been placed in the cage. Mice are weighed and weight printed out automatically. Weights are subsequently recorded in 'injection book' but on occasion of my visit fell within limits defined in para. 9.2.

Para. 9.3.

Used 21-gauge needles for injection. Syringes, once filled and air removed, are arranged in rack (12 spaces but gaps between each second syringe, so each rack contains 8 syringes). Syringes are not labelled but are arranged from left to right in ascending order.

Each set of two mice is injected from left to right with appropriate syringe. If the operator is interrupted (as happened during my observation) it might be possible to forget which animals had been injected and if the injection had caused no bleeding, it might be difficult to decide if mice in that cage had been injected. It would sensible to require that cages were moved to the left or put in some area away from the remaining cages containing the un-injected mice.

Para. 9.5

Results of these mouse bioassays were indeed recorded as described in this paragraph. I have a copy of the completed Rowett 6 form for the batch of samples I had observed.

[NB Although not strictly part of my remit – observation restricted to analysis – I did note the process of reporting [described in FRS SOP 2258 – issue 1.00, dated 7.2.03]. So far as I could see this SOP describes the procedures accurately. I was given a copy of the report for the period (1-7th August) which included the batch of samples, whose progress I had been monitoring.

Para. 9.1.4. This deals with giving of a so-called interim report to the client - presumably FSA (Scotland). It is suggested that this is done verbally or otherwise. Telephonic or other oral communication of results is not desirable as it can lead to mistakes in transmission or reception of data. Results should not in my view be communicated except in writing and only to an agreed destination or person. Interim results should be kept within the laboratory and NOT communicated until they have been through the complete process and have been confirmed as valid by the certifying scientist. Electronic reporting should be implemented by FRS.

SOP 2210 : RECEIPT OF SAMPLES FOR SHELLFISH TOXIN TESTING AT ROWETT LAB (ISSUE 3)

1. Introduction and Scope

This procedure covers the receipt and logging in of shellfish tissue samples for algal toxin testing by bioassay staff.

2. Principle of the Method

Samples are received and details are recorded on the Sample Login Sheet (R1).

3. Reference Material

Not applicable

4. Reagents

Not applicable

5. Equipment

log-in sheet(s)
pen

6. Environmental Control

Not applicable

7. Interferences

Not applicable

8. Sampling and Sample Preparation

Shellfish samples are received from shellfish sites or offshore boxes as part of a monitoring programme or from commercial companies.

9. Analytical Procedure

9.1 Take samples to fish house (if they have not already been delivered there by handymen), remove plastic containers from outer cardboard boxes. (See SOP 2205) Discard outer boxes. Samples received from other departments may be wrapped in polythene bags only or in sample pots. In both these instances the sample will already have a number allocated and corresponding paperwork will be attached.

9.2 Record details from container label on log-in sheet (R1) at next available number (sequential number/year as yyyy). Samples from other departments will be recorded using the sample number already allocated to them. This must include site location and/or bed location and date of collection. Other optional information may include grid reference, name of collector, and number of boxes left with collector. If the sample is unsuitable, this is recorded and reason noted in comments box. Samples may be unsuitable for a number of reasons eg they are smelly due to delays in post, shells are not of marketable size, etc

9.3 Open plastic container, check species of shellfish received and record this on log-in sheet. If sample is unsuitable for processing record this and give reason in ‘comments’ box on log-in sheet.

10. Calculation of Results

Not applicable

11. Precision, Bias and Limit of Determination

Not applicable

12. Reports

Not applicable

13. Safety

Wear laboratory coat and disposable gloves.

14. Literature References

Not applicable

SOP 2215 : PROCESSING OF SHELLFISH SAMPLES FOR TOXIN ANALYSIS (ISSUE 6)

1. **Introduction and Scope**

This procedure covers the processing of shellfish tissue samples prior to extraction of Amnesic Shellfish Poisons (ASP) and Diarrhetic Shellfish Poisons (DSP) for chemical analysis. Samples are also processed for DSP and Paralytic Shellfish Poisons (PSP) by bioassay.

2. **Principle of the Method**

Samples are received and logged according to SOP 2210 by bioassay staff or SOP 60 by chemistry staff. The shellfish are shucked and homogenised prior to extraction of toxin.

3. **Reference Material**

Not applicable

4. **Reagents**

2% Trigene solution.
Preparation: Decant 20ml of Trigene into a measuring cylinder and make up to 1 litre with water. The 2% Trigene solution is kept in spray bottles.

5. **Equipment**

disposable gloves
weigh boat
plastic box
kitchen sieve
shucking knife
food processors (domestic)
calibrated balance
calibrated timer
plastic screw capped containers – wide necked and narrow necked
spatula
measuring cylinders
spray bottle

2. **Environmental Control**

The bowl and lid of the food processor, sieve, knife and spatula are washed under hot running tap water between samples. Benches are wiped if there is a spillage and cleaned with detergent at the end of the day. All unused samples and waste are disposed of in yellow waste bins.

7. **Interferences**

Not applicable

8. Sampling and Sample Preparation

Shellfish samples are received from shellfish sites as part of a monitoring programme or from commercial companies. On receipt, samples are logged (SOP 2210 (bioassay staff) or SOP 60 (chemistry staff)) on Record Sheet R1 (SOP 2210) or B137 (SOP 60). Sample information includes a unique

number, sample location (ie grid reference or bed location), sample collection date, sample receipt date, sample species, number of shells used per sample and the analysts initials.
Samples arriving late are stored overnight in the fridge or frozen if stored for a longer period. Results must be available within 5 working days of receipt.

7. Analytical Procedure

9.1 Wearing disposable gloves, remove bags of shellfish from sample container and tip shellfish onto the draining board or into an empty box.

9.2 Rinse shells under tap water to remove external debris

9.3
Scallops
Open shells by cutting adductor muscle with a shucking knife. Rinse the opened scallop under running cold water and remove all the tissue from both shells and transfer to a clean plastic container. If only the gonad is required for analysis, rinse the scallop under cold running water, then remove the gonad by breaking the connecting membranes and transfer the rinsed gonad to a clean plastic container. In a scallop sample sufficient shells are used to give at least 30g of gonad which is analysed separately and all the contents of the remaining shells are used as a whole sample (PSP by bioassay and ASP and DSP by chemical assay).

Mussels and other shellfish.
Open shells by cutting adductor muscle with a shucking knife, remove meat from shell and place in kitchen sieve propped over a clean sample box. The whole animal is used for testing in all species except scallops. (PSP and DSP by bioassay and ASP by chemical assay)

9.4 Continue step 9.3 until all shells are opened or there is sufficient sample for analysis. If there is less tissue suitably experienced staff prioritise tests. The number of shells opened is recorded on the log-in sheet B137 (SOP 60) or R1 (SOP 2215).

9.5 Place sample from sieve into a labelled weighboat and transfer meat from weighboat or plastic container to a food processor and homogenise at the high speed setting for at least 20 seconds.

9.6.1 Chemical prepared samples
Transfer the homogenate, using a clean spatula, to uniquely labelled plastic vials/tubs taking care not to overfill as follows:

Minimum of 4g in a universal tube for each of ASP and DSP chemical assays, 10g to 100g into wide necked screw capped container for DSP and PSP bioassay.

As the samples warm up there may be a build up of gas pressure in the containers causing the sample to explode on opening. Underfilling the container reduces this risk.

9.6.2 Bioassay prepared samples
Weigh empty containers labelled with sample number, re zero balance then transfer aliquots of homogenate to containers as below:

> 75g ± 1g into wide necked screw capped bottle for DSP bioassay
> 10g to 100g into narrow necked screw capped bottle for PSP bioassay
> Minimum of 4g in universal tube for each of ASP and DSP chemical assays

Note weight on outside of containers (except for DSP which is always 75g), on worksheets (R3 – PSP and R4 - DSP) and on weight record sheet (R2).

9.7 Sample homogenates may be frozen or refrigerated until required for chemical analysis or bioassay. Excess homogenate is disposed of as in SOP 60.

10. Calculation of Results
Not applicable

11. Precision, Bias and Limit of Determination
Not applicable

12. Reports
Not applicable

13. Safety
Laboratory coat and disposable gloves are worn at all times. Care is essential when using shucking knives. This procedure is covered by Risk assessments ITA 018, RL1 and RL2.

14. Literature References
Not applicable

SOP 2245: EXTRACTION OF DSP TOXIN FROM SHELLFISH TISSUES FOR BIOASSAY (ISSUE 10)

1. Introduction and Scope

This procedure covers the extraction of toxin from processed shellfish tissue homogenate. The extraction is then analysed to assess the level of DSP toxin

2. Principle of the Method

This process involves homogenised shellfish tissues being blended together with acetone using an Ultra-Turrax ®, filtration of the resulting mixture, evaporation, ether separation, further evaporation and suspension in Tween to yield the extract for analysis

3. Reference Material

Not applicable

4. Reagents

acetone AnalaR
diethyl ether AnalaR
1% Tween 60 (SOP 2220)
distilled water

5. Equipment

disposable gloves
calibrated balance
wide necked screw capped bottles
narrow necked screw capped bottles
113V 100cm fluted filter paper
large plastic funnel
universal tubes
50ml measuring cylinder
disposable pastette
water bath
rotary evaporator
glass flask 100ml and 500ml
conical flask
separating funnel + stopper
cork ring
clamp stand
Ultra-Turrax ®
calibrated dispenser
calibrated timer
sonicator

6. Environmental Control

Not applicable

7. Interferences

Not applicable

8. Sampling and Sample Preparation

Shellfish samples are prepared as per SOP 2215 and an aliquot is received in a wide necked screw capped bottle labelled with sample number.

9. Analytical Procedure

Before starting the extraction, the sample number is recorded on record sheet (R4). Each further stage is also recorded and initialled on this record sheet

9.1 To 75g ± 1g tissue in screw capped bottle add 225ml ± 5ml acetone using dispenser pump on Winchester bottle

9.2 Homogenise using Ultra-Turrax ® at speed setting 3, for at least 30 seconds.

9.3 To clean Ultra-Turrax ® between samples, wipe with tissue to remove excess shellfish tissue from openings, remove stuck tissue with forceps and rinse by operating with acetone only in a spare screw capped bottle at setting 3 for 10-15 seconds

9.4 In a fume hood, using a large plastic funnel and 113V fluted filter paper, filter homogenate into a narrow necked screw capped bottle labelled with sample number

9.5 Pour filtrate into labelled, 500ml, glass, round bottomed flask and evaporate off acetone (SOP 2230). Acetone is completely evaporated when there are no obvious signs of liquid running back into the flask.

9.6 Remove flask from evaporator and continue process in fume cupboard.

9.7 Pour contents of flask into 50ml measuring cylinder and measure volume. Return liquid to flask, add equal volume of ether, mix well, transfer to separating funnel and allow to separate.

9.8 Place same volume of ether as above into original round bottomed flask and add bottom layer from separating funnel. Allow to separate and transfer top layer from separating funnel to labelled conical flask.

9.9 Mix contents of round bottomed flask, transfer to separating funnel and allow to separate again. Discard bottom layer into round bottomed flask and add portion in conical flask to separating funnel.

9.10 Using a wash bottle add at least 5ml distilled water to separating funnel, stopper and mix well. (release pressure in separating funnel by opening tap when funnel is inverted) Leave to separate then discard bottom layer into round bottomed flask. Repeat 9.10. Pour contents of round bottomed flask into Winchester bottle labelled ‘waste chemicals’.

9.11 Run remaining contents of separating funnel into labelled 100ml glass round bottomed flask and evaporate (SOP 2230) until dry + 5-10 mins. Flask may be left overnight in fume cupboard to ensure thoroughly dry.

9.12 Using a disposable pipette add 3ml ± 10% Tween 60 (SOP 2220) to flask and mix with contents of flask. Squirt contents up and down using a pastette until contents are suspended in Tween 60 and sides of flask are clean. Sonicator may be used to help dislodge contents – pour warm water into sonicator and switch on. Immerse flask in water two or three times for at least 20 seconds each time.

10. Calculation of Results

Not applicable

11. Precision, Bias and Limit of Determination

Not applicable

12. Reports

Not applicable

13. Safety

Wear laboratory coat and disposable gloves and safety specs if required. See risk assessment RL2

14. Literature References

Yasumoto *et al*., Diarrhetic Shellfish Poisoning, American Chemical Society, 1984, pp207-214.
Yasumoto *et al.,* Occurrence of a New Type of Shellfish Poisoning in the Tohoku District, *Bull. Japan. Soc .Sci. Fish.,* **44**(11), 1978, pp1249-1255

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SOP 2255 : ANALYSIS OF EXTRACT TO ASSESS LEVEL OF DSP TOXIN BY BIOASSAY (ISSUE 4)

1. Introduction and Scope
This procedure covers the analysis of extract to assess the level of DSP toxin by mouse bioassay

2. Principle of the Method
This process involves the intraperitoneal injection of DSP extract into mice. This is the EC approved method for detection of toxin.

3. Reference Material
Not applicable

4. Reagents
DSP extract as per SOP 2245

5. Equipment
disposable gloves
calibrated balance
cage for mice
mice (CD1, female, Harlan/B&K or equivalent)
2ml syringe
21 gauge needle
marker pen
calibrated timer

6.Environmental Control
Not applicable

7. Interferences
Not applicable

8.Sampling and Sample Preparation
DSP toxin is extracted as per SOP 2245 and extract is received in a universal tube labelled with sample number

9.Analytical Procedure

Note - Only holders of the relevant Home Office personal licence may perform injections

9.1 On cage label write cage number and details of sample number, personal licence number of person performing injections and mean start time of test.

9.2 Take cage of mice from holding room to procedure room in animal house. Check weight of mice, select two per test which are 18g – 22g, colour one with marker pen to distinguish and note weight and colour in injection book. Place mice in separate cage. A separate cage is used for each sample to be analysed.

9.3 Place needles on two syringes and fill to at least 1ml with extract. If analysing more than one sample use rack to hold syringes in the correct order. Expel air bubbles and excess liquid into piece of cotton wool to leave 1ml ± 0.1 in each syringe.

9.4 Remove mouse from cage and hold in position, inject contents of syringe into abdomen of mouse. Repeat for second mouse. Start timer. If analysing more than one sample then timer is started when the first sample is injected as the running time for analysis is 5 hours +/- 15 minutes. All injections must be completed within 15 minutes.

9.5 Observe mice for first 30 minutes and at 20 – 40 minute intervals thereafter throughout the test. If one or both mice die note time elapsed since injection in injection book (this is a positive result). Mice may present signs of DSP intoxication and appear distressed – in this case experienced staff will humanely kill them and this will also be a positive result. If mice survive 5 hours (+/- 15 minutes) then they are humanely killed by a Schedule I method (typically CO_2 and dislocation of neck - SOP 2270). Ref - Guidance on the Operation of The Animals (Scientific Procedures) Act 1986.

9.5 The result is recorded in the injection book and the DSP Test Sheet (R6).

10. Calculation of Results

Not applicable

11. Precision, Bias and Limit of Determination

Refer to M 2300 - Analysis of shellfish for the presence of DSP toxins by bioassay.

12. Reports

Not applicable

13. Safety

Wear laboratory coat and disposable gloves and safety specs if required. See risk assessment RL5

14. Literature References

Yasumoto *et al.*, Diarrhetic Shellfish Poisoning, American Chemical Society, 1984, pp207-214
Yasumoto *et al.,* Occurrence of a New Type of Shellfish Poisoning in the Tohoku District, *Bull. Japan. Soc .Sci. Fish.,* **44**(11), 1978, pp1249-1255
Guidance on the Operation of The Animals (Scientific Procedures) Act 1986.
Project Licence PPL 60/2842

Other information supplied by FRS

- Copies of forms for batch observed by me
- Rowett 1 (logging in sheet)
- Rowett 2 (Analytical schedule and weight record sheet)
- Rowett 4 (DSP extraction sheet (Rowett))
- Rowett 6 (DSP test sheet)
- Example Final Report.
- Document produced at my request with regard to NRL.
- Organogram provided by NRL, indicating NRL staff.
- Draft document (version 3 15 April 2003) 'Remit of the UK National Reference Laboratory for Marine Biotoxins'.
- Milestones, deadlines, target dates and costs' April 2003-March 2004 (draft vers. 2, 13 June 2003) - only first two pages.
- Comparison of SOPs currently in use in UK shellfish monitoring laboratories for the extraction of lipophilic compounds from shellfish and their application in the mouse bioassay' F. Mackintosh and S. Gallacher. August 2003 UK-NRL Report 02/03
- An overview of activities of the UK-National Reference Laboratory for marine biotoxins – a discussion document for future operation of the reference laboratory' S. Gallacher, March 2003-08-05
- Monitoring of Algal toxins in shellfish in Scotland, April 2002 to March 2003. G. Howard
- UKNRL Report (April 2002) 'An inter-laboratory exercise on DSP toxin determination by mouse bioassay (MBA) and LC-MS
- Summary of the UKNRL results from the EU-NRL's intercalibration exercise on DSP determination by mouse bioassay and comments on the EU-CRL report. Susan Gallacher & Lesley Bates (Stobo) – 18 May 2001.
- Report on the EU-NRLs intercalibration exercise on DSP determination, April 2001.
- UK NRL Annual Report April 2002-March 2003.
- Document prepared by FRS for UKAS as part of the case for getting accreditation for the 'agreed NRLUK protocol' in May 2003 [only in part].

ANNEX C. - DARD (NI).

Observations on implementation of and recommendations for improvement of the SOP

1. SOP RES1 V6 (dated 1.6.01) - Reception, logging and storage of samples, the scheduling of tests and the recording of results for the assay of antibiotics in animal feedstuffs and detection of algal toxins in shellfish.

I was not present for reception of samples and the logging in procedure was demonstrated to me only with regard to the laboratory audit mussel homogenate. The cockle samples I observed on 2nd September had arrived while I was observing the homogenate processing. Samples arrived in plastic bags and in case of cockles received on 1.9.03, did not require washing – see below.

Para. 5.1.2.

DARD has a computerised database which allows for recording of receipt and all other necessary information. This system is not unique to shellfish but applies to all assays carried out in the Department. Once the data is entered, the computer generates a unique number for each sample and prints out a work sheet (described as a Daybook sheet – para. 5.1.3). I was given a copy of the completed sheet for the laboratory audit mussel homogenate processing.

2. SOP RES 212 V5 (dated 1.6.03) – Mouse bioassay for the detection of diarrhetretic (*sic*) shellfish poison (okadaic acid and dinophysistoxins), yessotoxins, azaspiracid and pectenotoxins in bivalve molluscs.

Para. 1.0.

This paragraph covers SAFETY and refers to (a) the VSD Safety Code, (b) 'appropriate risk assessments' and (c) COSHH assessments. All staff knew of the VSD Safety Code and claimed to have read it. All were unaware that they could access this Safety Code on the DARD Intranet. None were aware of (b) nor that these were generic assessments, copies of which were available. All had read the COSHH assessments for the processes in which they were involved. I have inspected the COSHH records and all staff have signed the appropriate method. All the staff had made their own assessment of the risk posed to them by the procedure described in the SOP and correctly identified the major hazard as ether.

Para. 4.1

It should be noted here that ether, acetone & Tween are subject to control procedures, where aliquots are evaporated to dryness and injected into mice to ensure no response. This occurs whenever new Tween is made up and

when new lots/batches of ether and/or acetone are started. Data on this are recorded in a file, ‘Calibration Control Log’:

RU 73a: Ether/Acetone & Tween control mouse bioassay results
RU 77a: Tween
RU 77: records control number (also on side of bottles of reagents), start sample number and acetone lot and ether batch numbers.
RU 5: Records animal house balance checks.

This allows for checks on quality of reagents but also allows for traceability. This procedure should be described either in the SOP here or elsewhere - if elsewhere, reference should be made to this process here. The procedure is to be commended.

Solvent control number is recorded on check list.

Para. 4.2

More specific details of the apparatus used here would be appropriate (i.e. para 4.2.5). Apparatus not used should be deleted (i.e. para. 4.2.8).

Para. 5.1.1

Only one batch of cockles arrived while I was at DARD. These were very clean and did not need further washing.

Para. 5.1.3.

Rinsing inside the shellfish ‘if necessary’. This was needed only for scallops when full of sand.

Paras. 5.1.5 and 5.1.6

These are the wrong way round. Procedure described in 5.1.6 came before 5.1.5.

Para. 5.1.7

The blender used was a Christison Laboratory Blender (using the LOW-1 setting).

Para. 5.2.2

Weighed using a top pan balance (Denver XE510).

Para. 5.2.3

Should instruct operator to use safety glasses. Should also at this point require operator to record solvent control number on checklist (described as Batch ID at para. 6 of checklist).

It is in fact an ULTRATURRAX. Type and setting should be specified in SOP (T25 and setting 3). Carried out on bench, whereas it should perhaps be done in a fume cupboard (but NOT when ether is being used!). Ultraturrax cleaned with acetone.

Paras. 5.2.4 and 5.2.5

Combine these two which should read, 'Filter the homogenate/acetone mixture through Whatman 113V filter paper, collecting the acetone fraction into a (500ml pear shaped FD500/4RE) rotary evaporate flask, previously labelled with the appropriate sample number on at least two separate places on the flask.'

Not sure that this is wise and suggest that sticky labels with printed number should be used instead (*vide supra*).

Para. 5.2.6

It should be noted that after the first filtration, the remaining acetone is squeezed out of the filter paper using a metal spatula. The homogenate is then recovered from the filter paper and scraped back into the same screw cap plastic bottle (see para. 4.2.12). After the second acetone extraction, the same filter paper is used for the second filtration.

Para. 5.2.7

No indication of water bath temperature, except by thermometer. No record on the thermometer used as to whether or when it had been calibrated. The specific type of equipment used here should be specified in para. 3.0. Water bath was a Bibby RE200B. Rotary evaporator was a Bibby RE200 rotating at 30rpm (speed control setting 2).

NB. I note here that, although nothing is said about this in the SOP, the operators were applying Dow Corning High Vacuum Grease to the end of the rotary evaporator to prevent the flask from sticking. This is an undesirable practice, even if the grease had been tested for a mouse response, which I understand it hasn't, as the solvents may dissolve some of this grease and carry it through to later stages of the extraction process. Distilled water provides a simple and effective replacement for the grease.

Pumps used are KNF Neuberger LABOPORT. No pressure indication. The vacuum connection was at the bottom end of the condenser rather than the top and thus solvent was being sucked into the pump, which must decrease efficiency of pumping.

This evaporation process took around 20 minutes.

Para. 5.2.8
In the case of the laboratory audit mussel homogenate, the final residue was 63ml. The cockle extracts were however around 40ml. The flask was cooled under running tap water before ether was added.

Para. 5.2.10

In fact the measuring cylinder was not rinsed as described. The total volume of ether to be used (equal to the volume of the aqueous phase measured in para. 5.2.8) is measured in the measuring cylinder, poured into the flask, shaken and then poured into the separating funnel.

Para. 5.2.11

Gentle shaking is in fact a rocking motion repeated 8-10 times.

Para. 5.2.12

With very dark extracts (e.g. the laboratory audit mussel), it is sometimes very difficult to see the interface between the phases. The cockle extract is much lighter and thus easier to distinguish where the interface is. Operators always erred on side of caution and let anything which they suspected of being aqueous run through the tap, even if that lost some of the ether.

Para. 5.2.13

DARD is the first laboratory to appreciate the problem of contamination, if the ether layer is run out through the tap. The ether is decanted out through the top of the funnel.

Para. 5.2.18

In fact de-ionised water was used here NOT distilled water.

Para. 5.2.21

Evaporation took around 10-15 minutes. Not always possible to get it to dryness. The laboratory audit sample extract was very oily (approx. 0.5ml). The cockle extracts were relatively dry. All were sniffed by two operators to test for presence of ether. None was detected by them or by me.

Para. 5.2.22

Ultrasonic bath not used when I was present.

Para. 5.2.23

Small glass screw-cap vial (LIP 5179 2/5). 5ml indicated on label but they used a similar vial filled with 4ml water as a means of assessing 4ml.

Para. 5.3.1

Used top pan balance, regularly calibrated and record kept in RU5 in Calibration Control Log. Weight checked before use using a standard. Laboratory audit mussel homogenate extract was injected together with three other extracts. Copy of RU73a is attached (see Appendix 1). Note that each mouse was in a separate cage.

For laboratory audit homogenate, mice weighing 20.1, 21.1 & 19.5g were used. Range required 18-22g.

Para. 5.3.2

Vials shaken before use. 1ml removed and injected. Each cage labelled with Project licence number, code for DSP (03/2). Numbers of the four personal licences & Departmentally allocated ID, samples A, B & C (labels are written by hand, with one operator calling out the identification number. This is not ideal as errors can occur if number is mis-heard. Suggest use of printed labels.

Veterinary Sciences Division, Stoney Road, Stormont, BELFAST BT4 3SD

TITLE: MOUSE BIOASSAY FOR THE DETECTION OF DIARRHRETIC SHELLFISH POISON (OKADAIC ACID AND DINOPHYSISTOXINS), YESSSOTOXINS, AZASPIRACID AND PECTENOTOXINS IN BIVALVE MOLLUSCS

SOP RES 212 V5

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1.0 **SAFETY**

1.1 All procedures must be carried out in accordance with the VSD Safety Code and appropriate risk assessments.

1.2 All chemicals should be used under the conditions stated in the COSHH assessment

1.3 Personal safety equipment and clothing should be worn at all times as detailed in the appropriate COSHH assessment. A protective glove must be worn when using knives and scalpels.

2.0 INTRODUCTION / SCOPE

Diarrhetic Shellfish Poisoning (DSP) complex (Okadaic Acid (OA) and Dinophysistoxins (DTXs)), Pectenotoxins (PTXs) and Azaspiracids (AZAs) pose a serious hazard to human health when present above certain limits in shellfish. In accordance with Commission Decision 2002/225/EC the maximum levels in edible shellfish are set at 160ìg OA equivalents / kg for OA, DTX and PTX together and 160ìg AZA equivalents / kg for azaspiracid. The regulatory limit for Yessotoxin (YTX) is set at 1000ug / kg. The toxins are produced by the planktonic algae (mainly dinoflagellates), upon which the shellfish feed. DSP is generally observed in people as a mild gastrointestinal disorder, i.e., nausea, vomiting, diarrhea, and abdominal pain accompanied by chills, headache, and fever. Azaspiracid (AZP) also causes gastrointestinal disorder but the original source of this toxin is unknown. The toxins are extracted from shellfish tissue using acetone, followed by an ether clean-up and water wash. The final residue is resuspended in 1% Tween 60. Although the efficiency of diethyl ether in terms of YTX recovery is not as good as the other toxins, due to the high inter-peritoneal toxicity of YTX diethyl ether is good enough to detect the presence below the regulatory limit. Three mice are inoculated intraperitoneally with 1 ml of the extract and the death time recorded. The death of more than one mouse within 24 hours is reported as a positive result.

3.0 EQUIPMENT

3.1 Balance (0-60.000g, 0-600.00g)
3.2 Refrigerator
3.3 Adjustable pipette 1 - 5ml
3.4 Blender (RES 157)
3.5 Ultra-turex homogeniser
3.6 Rotary evaporator
3.7 Fume cupboard
3.8 Ultrasonic bath

4.0 MATERIALS

4.1 CHEMICALS

All chemicals are analar grade unless otherwise stated.

4.1.1 Acetone
4.1.2 Diethyl Ether
4.1.3 Distilled water
4.1.4 Tween 60

4.2 APPARATUS

4.2.1 Glass beakers, 600ml
4.2.2 Sieve
4.2.3 Shellfish knife/scalpel
4.2.4 Plastic bucket
4.2.5 Quick-Fit flasks for Rotary Evaporator
4.2.6 Graduated cylinder 100ml
4.2.7 Syringe 2 ml
4.2.8 Needle 23 G x 1" or 25 G x ”
4.2.9 Timer clocks
4.2.10 Separating funnel 100ml
4.2.11 Conical flasks 100ml
4.2.12 Plastic, 250ml screw-cap bottles

4.3 REAGENTS

4.3.1 1% Tween 60.
1ml (±0.05ml) of Tween 60 made up to 100ml with distilled water.

4.4 MICE

4.4.1 Outbred CD1 female mice weighing between 18g and 22g. If alternative breeds and/or male mice are used note the deviation on the checklist. Do not re-use mice.

4.5 SAMPLES

4.5.1 Shellfish samples may be stored chilled (<4°C) for a maximum of 4 days but preferably for less than 24 hours prior to testing.

5.0 METHOD

5.1 PREPARATION OF SAMPLE

5.1.1 Clean the outside of the shellfish with fresh, cold running water.
5.1.2 Open by cutting the abductor muscles.
5.1.3 Rinse inside, only if necessary, with fresh, cold running water.
5.1.4 Remove the tissue from the shell with an oyster knife or scalpel.
5.1.5 Collect approximately 150g (± 5g) of tissue in a 600ml beaker.
5.1.6 Place the tissue on a sieve and allow to drain.
5.1.7 Blend the tissue in a blender until homogeneous (approx. 1 minute, up to 4 bursts of 15 seconds with a minimum of 5 seconds between bursts).

5.2 EXTRACTION PROCEDURE

5.2.1 Prior to starting the extraction procedure fill in the appropriate checklist. As each stage of the procedure is completed the analyst should initial the relevant section in the checklist. Any deviations from the procedure must also be noted.

5.2.2 Weigh 100g (± 1g) of the homogenate into a 250ml plastic screw-capped bottle (4.2.12). Note the weight of tissue on the checklist.

5.2.3 Add 100ml of acetone and place on the Ultraturex for 30 seconds, (13,500 rpm). Note the volume of acetone on the checklist.

5.2.4 Filter the homogenate through Whatman 113V filter paper.

5.2.5 Transfer or collect the acetone fraction in a rotary evaporator flask.

5.2.6 Repeat steps 5.2.3 to 5.2.5, once.

5.2.7 Combine the acetone fractions and evaporate the acetone using a rotary evaporator at 47 ± 2^{o}C. To prevent excessive foaming, the vacuum may need to be released occasionally during evaporation. Record the temperature of the water bath on the checklist.

5.2.8 Measure the volume of aqueous phase by transferring it to a measuring cylinder. Note the volume on the checklist.

5.2.9 Transfer the aqueous phase to a separating funnel.

5.2.10 Rinse the measuring cylinder and the rotary evaporation flask with a small volume of diethyl ether and transfer the rinse to the separating funnel.

5.2.11 Add further diethyl ether to the separating funnel (the total volume of ether should be approximately equal to the aqueous phase) and mix gently by hand for 10 seconds.

5.2.12 Collect the lower aqueous layer and any emulsion layer.

5.2.13 Decant the ether layer in to a 100ml rotary evaporator flask. To avoid carry over of aqueous phase, do not use the separating funnel tap.

5.2.14 Transfer the aqueous phase to the separating funnel.

5.2.15 Repeat steps 5.2.10 and 5.2.14, twice.

5.2.16 Discard the lower aqueous layer.

5.2.17 Combine all three ether fractions in the separating funnel.

5.2.18 Add approximately 5ml of distilled water from a wash bottle and mix gently by hand for 10 seconds.

5.2.19 Discard the lower aqueous layer.

5.2.20 Repeat steps 5.2.18 and 5.2.19.

5.2.21 Evaporate the ether fraction to dryness using a rotary evaporator at 47 ± 2^{o}C. Record the temperature of the water bath.

5.2.22 Resuspend the residue in approximately 2.5ml of 1% Tween 60 (using an ultrasonic bath sparingly, if necessary).

5.2.23 Transfer the suspension to a 5ml graduated glass screw top vial and make up to 4 ml with 1% Tween 60.

5.2.24 The resuspended extract may be stored at +4°C for up to 5 days, but preferably no longer than 48 hours. Record the storage time.

5.3 MOUSE TEST

5.3.1 Weigh 3 mice and place in separate boxes. Mouse weights must be between 18g and 22g. Record the weights on Form RU 73a

5.3.2 Inoculate each mouse interperitoneally with 1ml of extract (5.2.24) and record the start time. Use the narrowest gauge needle possible (25g). Record the batch number of the syringe and the value of the check weight on form RU 73a.

5.3.3 Observe for the first 15 minutes and then check regularly up to 24 hours post-inoculation.

5.3.4 Make a note of any symptoms observed on RU 73a.
Mice react to the presence of DSP and AZA in different ways. After an inoculation of a positive DSP extract the mouse will show progressive symptoms over several hours. It will become subdued, unresponsive, its extremities will become bluish and it will feel cold to the touch. The mouse will become prostrate and the rear legs may be extended. Some of these effects will become apparent within 30 minutes post inoculation. In the case of AZA, after inoculation the mouse may display disorientation, apparent paralysis of the hind legs, breathing difficulties and a quite violent jumping reaction just prior to death. This will occur with samples containing at least 0.4µg AZA / g tissue.

5.4 DEGREE OF TOXICITY

5.4.1 The test is positive if more than one mouse dies within 24 hours.

5.5 MEASUREMENT OF UNCERTAINTY

Factors influencing the final results of the DSP test include potential variability in mouse batches and in mouse injection. Uncertainty can also arise through individual analyst variability in the extraction procedure.

Veterinary Sciences Division, Stoney Road, Stormont, BELFAST BT4 3SD

TITLE: RECEPTION, LOGGING AND STORAGE OF SAMPLES, THE SCHEDULING OF TESTS AND THE RECORDING OF RESULTS FOR THE ASSAY OF ANTIBIOTICS IN ANIMAL FEEDSTUFFS AND DETECTION OF AGAL TOXINS IN SHELLFISH

SOP RES 1 V.6

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1.0 SAFETY

1.1 All procedures must be carried out in accordance with the VSD Safety Code and the Residue Testing and Physiology Sections Code of Practice on Safety.

1.2 Animal feedstuffs (meals) contain fine particles which may cause irritation by inhalation or ingestion. Some samples contain concentrated levels of antibiotics. Other hazards are not known.

1.3 Personal safety equipment and clothing should be worn at all times.

1.4 For the COSHH assessment refer to "The Preparation of Samples" in Appendix 1 of the SOP for the meal grinder, SOP RES 2.

2.0 INTRODUCTION

The Residues department receives animal feedstuffs for the detection and quantification of antibiotics under the "Medicines (Animal Feeding Stuffs) (Enforcement) Regulations 1985".
The feedingstuffs may be complete feedingstuffs, concentrates or premixes and are collected from mills and farms by DANI inspectors. Divisional Veterinary Offices, other VSD departments and Veterinary Practitioners may also send samples for antibiotic residue analysis.
Shellfish samples are submitted by local Borough Councils on behalf of the Food Standards Agency. The samples are tested for the presence of algal toxins.
All samples arriving in the Residues Department are logged on to the Residues Database and given a unique Residue sample number (see SOP RES 85, Instructions for the use of the Residues Database). Following log-in, samples are processed or transferred to the appropriate section for testing.

3.0 EQUIPMENT

3.1 Disposable gloves

3.2 Dust mask

4.0 FEEDSTUFFS METHOD

4.1 Reception and logging of feedstuffs samples.

4.1.1 Using the mill reference located on the sample label, arrange the samples in numerical order.

4.1.2 Ensure the mill reference is written on the sample bag.

4.1.3 Open the sample bag and remove the documentation.

4.1.4 Check the mill number, additive and additive level on both labels and on the outer ticket of the bag. If they do not correspond, consult the supervisor.

4.1.5 Identify the tests required from the sample label. Unmedicated meals are scheduled for Inhibitory Substances and Zinc Bacitracin screening test.

4.1.6 Enter the sample details on the Residues database, which will allocate each sample a unique Residues sample number.

4.1.7 Record the Residues sample number on the documentation and staple the documentation together.

4.1.8 Generate a daybook sheet(s) from the database.

4.1.9 Generate a sample transfer sheet for those samples requiring tests performed by the Chemical Confirmation Unit.

4.1.10 Record the Residue number and additive details on a label and attach to the appropriate sample bag.

4.1.11 Place 50g of samples being transfered (4.1.9) into a minigrip bag and seal.

4.1.12 Transfer the sample to Biochemistry Chemical Confirmation Unit with the sample transfer form.

4.1.13 Meal samples which require transfer to the Hormones section for further analysis must be accompanied by a transfer form RU14.

4.1.14 Re-seal the original sample bag with masking tape and place in appropriate storage bin.

4.1.15 Sign the database entry record and pass it and all sample documentation to the supervisor.

4.1.16 The supervisor must check that all labels and database entries are correct.

4.1.17 If correct, the supervisor must countersign the daybook sheets.

4.1.18 Authorised personnel may modify incorrect database entries see (RES 85). The daybook sheet must be corrected accordingly and initialled.

4.1.19 The species and type of each sample is determined using the following guide lines.

SPECIES	TYPE
Porcine	**Sow** (Sows, breeding sows and gilts, pig grower to 16 wks/20kg, piglet weaning to 15kg/7 weeks) **Finisher** (Pig finisher, pig growers and fatteners up to 6 mths, pigs 20kg to 60kg)
Bovine	**Calf** **Finisher (**Beef fattening nuts, bull nuts, finishing cattle and store cattle) **Dairy** (Dairy, 16%-19%)
Avian	**Layers** (Hens, Layers) **Starter** (Breeder) **Finisher** (Chicken broilers/growers, finishers and turkey grower/finisher)
Ovine	**Ovine**
Equine	**Equine**

4.1.21 The sample documentation is retained in the Pending box file in a filing cabinet until a result is obtained.

4.2 Storage of meal samples in the residues section.

4.2.1 Meal samples are stored at ambient temperatures in appropriately labelled storage boxes. Medicated and unmedicated samples must be stored separately.

5.0 SHELLFISH METHOD

5.1 Reception and logging of samples.

5.1.1 Locate the documentation for each sample and check the details against any labels on the sample bags. If they do not correspond, consult the supervisor.

5.1.2 Enter the sample details on the Residues database, which will allocate each sample a unique Residues sample number and schedule required tests.

5.1.3 Generate a daybook sheet(s) from the database.

5.1.4 Record the Residue number on a label and attach to the appropriate sample bag.

5.1.5 Sign the database entry record and pass it and all sample documentation to the supervisor for checking.

5.1.6 If correct, the supervisor must countersign the daybook sheets.

5.1.7 Authorised personnel may modify incorrect database entries see (RES 85). The daybook sheet must be corrected accordingly and initialled.

5.1.8 For those samples requiring tests performed by the Chemical Confirmation Unit take a sub-sample and generate a sample transfer sheet from the database.

5.2 **Storage of shellfish samples in the residues section.**

5.2.1 Shellfish samples may be stored overnight in a refrigerator or for longer periods at –20°C .

6.0 **RECORDING OF RESULTS**

6.1 Results are recorded in the appropriate day book and entered on the database as they become available by an officer of at least scientific officer grade.

6.2 The concentration of the analyte is recorded along with the units. In the case of inhibitory substance test, if the sample is positive, the zone size is recorded. For High Voltage Electrophoresis any antibiotics identified are listed.

6.3 For assays which are in-valid (RES SOP 68). "Assay Invalid Interfering Substance Present" is entered into the results column.

6.4 Shellfish bioassay results are recorded in the daybook as positive or negative. Details of mouse deaths may be recorded where appropriate.

6.5 Results from the Chemical Conformation Unit are received on a CCU report form. When a report form is delivered by a member of the CCU to the Residues department, an officer of at least

scientific officer grade must sign the relevant section and record the result in the appropriate day book.

6.6 Final results must be entered on to the Residues database.

7.0 **REPORTING OF RESULTS**

7.1 When the results of all analyses for a sample are available the results may be reported (see SOP RES 66).

8.0 **RAW DATA ARCHIVING**

8.1 The Residue raw data and the report form are archived according to SOP RES 67 after the results have been reported.

Other information provided by DARD

Letter from Dr. John McEvoy (DARD) to Dr Jonathan BACK (FSA HQ in London) dated 27 November 2002.

ANNEX D - COMMUNICATIONS WITH OTHER INTERESTED PARTIES

On 7th July 2003, before I had visited any of the laboratories, I had a telephone conversation with Dr Jim Cocckrill of IRF International Ltd. I received subsequently an e-mail and a letter (both dated 9th July 2003). I indicated to Dr. Cockrill that I was trying to approach the audit with an open mind and that I did not really want to listen, at that stage, to possible explanations for the atypical response. I indicated to him that I hoped to be able to meet him before finalising this report.

However, I managed to speak again with Dr. Cockrill on 11 September and although we arranged to speak again on the 15 September we did not manage to make contact with each other. During those conversations I explained my remit and that I could not comment on issues, but that I was prepared to listen to him and consider any issues he wanted to raise, but that I was not prepared to release any part of the report until published.

At the request of FSA, I spoke to David Anderson at the Home Office on 26 September 2003. I briefly outlined the assignment remit and advised that during my audit of DSP testing practices at CEFAS, DARD and FRS I had identified two major areas of concern - lack of controls and non-consistent end-points. I indicated that when he had read the Report, I'd be happy to talk with him again.