Workshop on Methods of Analysis of Heavy Metals in Marine Tissue

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Contents

1. INTRODUCTION

The oceans have always been an important part of Australian environment but in recent years, particularly following the declaration of a 200 mile Australian Fishing Zone in November 1979, they are receiving even greater attention because of their potential as a high quality protein food source. Heavy metals are notable pollutants which can enter the marine food chain, accumulate in biota and be subsequently consumed as food by man or used to feed animals. Whilst it was once thought that heavy metals of terrestrial and anthropogenic orgin remained fixed in estuarine sediments it is now clear that they may be released by various processes of remobilisation and move up the marine food chain.[1] These processes of biological up-take also occur through soluble, collodial and particulate forms of heavy metal compounds existing throughout the aqueous media.

Fundamental to the establishment of data on heavy metals in marine biota is the need for laboratories to be able to measure heavy metal concentrations accurately and precisely. Heavy metals are often present at trace levels in environmental materials. It is generally recognised that many problems exist with the application of different methods for heavy metal determinations at lower concentrations and the only sure way to obtain reliable and comparative results is by carefully adopting appropriate procedures of standardisation. Egan[2] has discussed the role of the collaborative analytical study as the means of

standardising analytical methods. It is important to establish correct analytical data not only to ensure that edible marine biota do not contain excessive levels of toxic heavy metals but also to establish whether there is an increasing trend in levels of land-sourced heavy metal concentrations being introduced into coastal zones as a result of man's activities. Thus it is also a matter of urgent importance to establish present-day baseline levels in seawater, sediments and marine biota such a pelagic fish, crustacea and molluscs.

In recent years an increasing number of environmental test samples of have been circulated to Australian laboratories to examine their capability to undertake trace heavy metal analysis. These have included the determination of lead in blood, acid digest, saline and water[3] and a range of metals in a bovine liver sample.[4] Other published Australian studies have compared the performance of laboratories in the determination of trace metals in river water[5] and mercury in fish[6,7]. The latter is an area where significant improvements in analytical results have been achieved, and reference to this will be made later.

Most of the inter-laboratory surveys described above have shown disagreement in results between laboratories. In part this has arisen because many laboratories have not fully understood the pitfalls of trace analysis and because standardised methods for application of newer instrumental techniques have not been adequately discussed among laboratories. In this respect methods for analysis of Australian marine environmental samples have not been exempt.

The Department of Science and the Environment has been conducting a series of Workshops on Methods of Analysis of Marine Pollutants to assist in the development of accurate, reliable and practicable techniques for measuring various trace substances in the marine environment. Although one objective of this workshop series has been to prepare reference techniques for measuring selected types of marine environmental pollutants at trace levels, it has first been necessary to identify where problem areas exist and if possible, to rectify them. This approach has been recently introduced overseas by groups such as ICES[8]. Once problem areas are clearly delineated, it should then be possible to document more appropriate techniques for use as reference methods which can be used to validate those at present applied in different forms around Australia.

This study deals with an evaluation of the performance of Australian laboratories in the determination of the heavy metals copper, zinc, lead and cadmium in various marine tissues. Arsenic, selenium and mercury were also included but to a lesser extent. The feature of this study which differs from most other Australian inter-laboratory surveys is that this series of studies has included workshops and method discussions between small groups of participating analytical chemists as an integral part of the overall survey of laboratories. Basic to biological and other studies essential to the process of development of regulation and control measures to limit heavy metal concentrations in marine tissue is the need to employ analytical techniques with appropriate reliability, accuracy and

detection limits. It is also essential that regulatory authorities be aware of the scope of application and confidence limits of these techniques in framing future regulations.

2. Inter-laboratory survey

Homogenized samples of various freeze-dried marine tissue (shark, flathead, mussel and crayfish) were prepared by the Victorian Ministry for Conservation Fisheries and Wildlife Laboratories at the Arthur Rylah Institute for Environmental Research, Melbourne. Sets of five marine tissue samples were sent to some thirty Commonwealth, State, Research and Industrial laboratories undertaking measurement of heavy metals in marine environmental materials. A further sample ("low" mussel) was sent to twelve of the laboratories known to be routinely engaged in determining heavy metals in marine tissue.

Participating laboratories were requested to analyse each sample by their own routine methods for zinc, copper, cadmium, lead, arsenic, selenium, mercury and any other metals as convenient. It was anticipated that not all laboratories taking part in the survey would be equipped to analyse all the metals listed and if this were the case, results of a limited selection of metals would be acceptable.

Laboratories having supplies of known standardized sample reference materials such as USNBS Orchard Leaves 9SRM 1571 or "Bovine Liver" 9SRM 1577 were also requested to include analysis of such materials for subsequent statistical interpretation of the general result.

Participants were asked to report results in note form on the Methods Report pro-forma supplied and include operational details of the analytical methods used. They were also asked to include other information on aspects of technique which they may have found from experience to influence accuracy and reproducibility such as sample loss, contamination and solution stabilities. Participating laboratories were identified according to a given laboratory code number and advised that other participants would not be informed of their identities.

Specific instructions for sample preparation were provided to participants. The powdered samples had 80% of their original weight removed as moisture and had to be re-constituted strictly as indicated in order to provide wet macerated tissue equivalent to normal field samples. This step was intended to avoid any undue severity in the early stages of the reaction caused by contact of finely divided dry organic material with strong digestion reagents. For every 1.00 g of wet tissue required for each analysis, 0.80 mL distilled water was added to 0.20 g freeze-dried tissue weighed accurately into the required digestion vessel. This was allowed to stand for at least sixty minutes (preferably overnight) with occasional agitation.

3. Inter-operator study

The inter-operator study was undertaken to examine sources of variation in some methods used for the determination of heavy metals in marine tissue under controlled conditions and

to undertake a comparison with the inter-laboratory survey results.

Sixteen participants from various Australian laboratories (selected as experienced) attended the inter-operator workshop in Hobart, March 1979. The facilities of the Chemistry Department, University of Tasmania and the Tasmanian Department of the Environment were used for the 3-day workshop.

Certain limitations were imposed on the experimental work attempted during the inter-operator study. These were:

- the number of participants;
- the available time; and
- the available apparatus and facilities.

As a result it was necessary to design the experiments with restrictions placed on:

- the number of methods employed;
- the number of samples analysed;
- the number of heavy metals determined; and
- the extent of apparatus preparation by the participants.

The inter-operator study was restricted to the determination of copper and zinc by flame atomic absorption spectrometry (AAS) in two of the marine tissue samples (shark (No. 1) and high mussel (No. 5)) using two different acid digestion procedures. Choice of acid digestion procedures was made as follows:

- digestion with concentrated nitric acid was a procedure used routinely by the host laboratories. Apparatus, particularly pre-cleaned glassware (racks of test tubes with 10 ml calibration marks) was readily available.

- digestion with tri-acid mixture (nitric/perchloric/sulphuric acids) as an example of a digestion method giving total oxidation of organic matter.

It was recognised, that digestion with di-acid mixture (nitric/perchloric acids) is more widely applied in Australian laboratories than tri-acid mixtures. However at an earlier workshop in Perth November 1978, which considered arsenic and selenium, it was pointed out that better recovery of As (organically bound) was achieved with a tri-acid mixture.[9]

Methods involving ashing and fusing oxidation procedures, Teflon lined bombs and closed refluxing digestion apparatus were not applicable because of the limited availability of apparatus. Flame AAS was the only feasible instrumental

technique to use. The two acid digestion procedures chosen were representative of a variety currently in use. The resulting digestion solutions were either in 10% nitric acid or 10% sulphuric acid.

not there

The experimental design of the inter-operator study is shown in Figure 1. Participants were allocated numbers at random by drawing lots. Each participant was required to undertake analysis of samples as specified under one of four groups. The prime comparisons were between:

- digestion;
  - concentrated nitric acid or
  - tri-acid mixture.
- Standard solutions;
  - use of own-prepared or
  - issued standard solutions.

Various control solutions made up in appropriate final acid solutions were also analysed by the four groups:

- a control standard solution whose concentration was unknown to the participant; and

- bulk digestion solutions of the two marine tissue samples and blank solutions both diluted 1:10 in respective final acids.

The control solutions were prepared by an independant participant (not one of the sixteen) who also prepared the issued standard solutions.

The same distilled water, prepared reagents, volumetric flasks, pipettes, burettes, balances and spatulas (for dispensing marine tissue) were used throughout where applicable. Details of procedures specified to be used by participants and the operating parameters for the flame AAS are given in Appendix 1. Glassware for digestions was supplied pre-cleaned and used as received. This glassware was used routinely in the host laboratory for trace metal analyses.

The high mussel sample contained a relatively large zinc concentration. For this reason participants were asked to dilute their mussel digest solutions and blanks 1:10. Participants using issued standards were also asked to measure the zinc concentration by means of the flame AAS burner rotation method. A set of issued high range standards for zinc was provided. These were also prepared by the independent participant.

4. Treatment of Data

4.1 Inter-Laboratory Survey

All results were placed on computer file according to laboratory number, marine tissue sample, heavy metal, sample digestion procedure and method of analyte measurement. Other

details were also filed for later use. The full data set comprised aggregations of subsets based on the particular sample and metal combinations. Each sub-set was further sub-divided into those observations falling either within or outside two standard deviations (o (x)) from the mean (x) where:

$$\bar{x} = \frac{1}{n} \sum_{i=1}^{n} x_i \qquad (1)$$

and

$$2\,o(\bar{x}) = \frac{2}{n(n-1)} \sum_{i=1}^{n} (x_i - \bar{x})^2 \qquad (2)$$

Subsequently data treatment was undertaken according to whether sub-sets referred to (i) "all results" or (ii) "results within two standard deviations of the mean". This approach was adopted so that the influence of outliers on the mean of any particular sub-set could be compared.

One-way analysis of variance (ANOVA) was conducted on each sub-set of all heavy metals. These sub-sets were further classified according to (i) and (ii). The source of variation was broken into two components. These were:

- within laboratory (or residual variation) (intra-laboratory)
- between laboratory variation (inter-laboratory).

The variance ratio (F-test) tested the significance of the between laboratory component.

Further one-way ANOVA were conducted on sub-sets for copper, zinc, cadmium and lead. The sub-sets were also classified according to (i) and (ii). Practically all laboratories used an acid digestion procedure and an AAS method of measurement for these four metals. Only one laboratory employed anodic stripping voltammetry (ASV) and only a few laboratories used ashing procedures.

Therefore it was possible to examine sources of variation as follows:

- acid digestion procedure
  - nitric only
  - nitric/perchloric
  - nitric/perchloric/ sulphuric
- AAS method of measurement
  - direct flame AAS
  - solvent extraction/ flame AAS
  - graphite furnace AAS

and undertake to establish real areas for laboratory improvement.

With all ANOVA the level of significance was taken as 5% (i.e. $P < 0.05$). The assumptions made in the analysis of variance were that treatment and environmental effects were

additive and that experimental errors were independently distributed in the normal distribution with a common variance[10]. At worst, failure of this assumption would affect the significant level and sensitivity of the F-tests, hence the true significance probability would be larger than the apparent one (i.e. too many significant results obtained). However in the majority of experiments these disturbances were not sufficiently great to invalidate the technique. Consequently significant levels must be considered as approximate rather than exact[10].

4.2 Inter-Operator Study

The various groups of zinc and copper results as well as calibrated data were also examined by ANOVA to establish if any significant relationships existed.

The experimental design was a 2 x 2 x 2 random effects model with some missing values (zinc in high mussel) and two replications in each cell. The random effect in this treatment due to operator variation was taken into account to avoid individual differences contributing to observation for that particular cell.

An alternative semi-quantitative treatment of calibration data was also undertaken to examine the degree of scatter of each participant's calibration lines. The line of best fit for each calibration data set was computer calculated using linear regression. The degree of scatter of each calibration line was expressed as the percent relative standard

deviation (%RSD) of the slope (slope variance with respect to mean slope).

5. Results and Discussion

5.1 Inter-Laboratory Survey

Results reported on a wet weight basis are given in Table 1. Examination of various sub-set pairs revealed a general improvement in %RSD when outliers were rejected. Significantly, mercury data gave the lowest %RSD's. A possible reason was that each laboratory used a similar analytical method of measurement. All laboratories measured mercury concentration by cold vapour generation/AAS. One of the earlier marine pollution workshops in this series[11] considered methodology problems in detail and a draft reference method of analysis for mercury in fish had since been fairly widely circulated. The % RSD's for marine biota (Table 1) also reflected the improved performances generated by groups participating in these laboratory exercises.

The % RSD's for all metals (Table 1) in low mussel (sample 4) were generally lower than other metal/tissue combinations apart from mercury and high mussel (sample 5) lead and cadmium. In the latter case there were factors of 20 and 200 in analyte concentration for lead and cadmium respectively compared with other marine tissue samples. The results for low mussel (sample 4) revealed that the experienced laboratories produced "better" data but this should be viewed in the light of comments below.

(INSERT TABLE 1)

Results of ANOVA applied to sources of variation "between laboratories vs. within laboratories" are given in Table 2. These results showed that a significant difference existed in laboratory error and that there was a real variation from laboratory to laboratory in all but a few notable cases such as selenium and lead in low mussel (sample 4) and selenium in high mussel (sample 5). In considering results within 2 S.D's of mean, selenium in high mussel improved dramatically with the rejection of outliers ($P < 0.001$ —— $P > 0.05$).

Closer examination of "procedures" and "methods" reported by the respective laboratories revealed that the use of similar "digestion procedures" and "methods of analyte measurement" were common features of the above three cases. By comparison most laboratories employed a variety of digestion procedures for other metal/tissue combinations. This may explain why data sets for mercury (all samples), arsenic (all samples) and selenium (samples 1-3, 6) gave significant differences according to the ANOVA treatment (Table 2) even though comparable methods of analyte measurement were used for each of these elements (mercury - cold vapour generation/AAS, arsenic-hydride generation/AAS, selenium-hydride generation/AAS and spectrofluorimetry). The real variation from laboratory to laboratory did not appear to be a direct function of analyte concentrations compared with analytical technique sensitivities.

The ANOVA treatment summarized in Table 3 gives further support to the comments above. There was insufficient data to

compare the combined effects of "digestion procedure" and "AAS method". It should also be noted that classifications of "digestion procedure" and AAS method" [section 4.1) were arbitrary because few laboratories used exactly the same combinations of acid volumes, digestion times, final volumes, AAS instruments and operating conditions. Nevertheless certain general features emerged from the ANOVA treatment (table 3) as specified.

Under the designation "digestion procedure" certain cases (zinc in low mussel, cadmium and lead in high mussel) gave improvement ($P < 0.05$ —— $P > 0.05$) upon rejection of outliers. However in others (copper in low mussel and lead in lobster) the reverse effect applied ($P > 0.05$ —— $P < 0.05$).

Similarly under the designation "AAS method" improvement upon rejection of outliers (copper in lobster, zinc in low flathead and cadmium in shark) was observed ($P < 0.05$ —— $P > 0.05$) together with the reverse effect in other cases (copper in high flathead and low mussel, cadmium in high flathead and high mussel) ($P < 0.05$ —— $P > 0.05$).

An explanation of the "reverse effect" described above was revealed by the data summarized in Table 4. Comparison of grand means and sub-group means (extracted from ANOVA treatment Table 3) for "digestion procedure" and 'AAS method" (results within 2 S.D.'s of the mean only showing $P > 0.05$) revealed why significant differences arose. The most glaring example was for lead in low flathead. By comparison no result was given by the

ANOVA treatment of lead in low mussel (Table 3.A) because practically all laboratories used the same "digestion procedure" (also supported by the ANOVA treatment given in Table 2).

Obviously a lack of uniformity in the techniques employed for analysis has caused the differences shown in Table 4. However the determination of copper and lead by AAS are both less sensitive than for zinc and cadmium. In general a real problem appears to exist with the determination of sub-parts per million concentrations (wet wt) of copper and lead in marine tissue. These are in fact the normal levels. Another possible problem area appears to exist with respect to the type of marine tissue, particularly for mussel and flathead.

In terms of practical significance the observed differences for many of the results given in Table 4 may be of little consequence. In this respect the confidence intervals are more informative than the significance tests.

5.2 Inter-Operator Study

Results for the inter-operator study are summarized in Figure 2 (dry weight basis, note that a factor of 5 converts wet weight results to dry weight results). The control standard solutions measured by each participant were of unknown concentration to the participant but were in fact 0.60 mgL^{-1} in both copper and zinc. The purpose of the control standard solution was to establish the subsequent validity comparison of digestion data by establishing whether significant differences

were obtained from measurements of a common standard. The ANOVA treatment on copper and zinc control standard solution data (Table 5) revealed no significant difference between various data sets tested. This important finding provided the basis of further statistical comparisons.

The ANOVA treatments for zinc in shark, copper in shark and copper in mussel data are summarized in Table 6. All respective data corresponded to direct aspiration of digestion solutions.

No significant differences were found for both zinc in shark and copper in shark respectively. In the case of copper in mussel a significant difference was found between solution types (10% nitric acid and 10% sulphuric acid), but not between other data sets and interactions. A possible reason is discussed later.

The ANOVA treatment for zinc in mussel data was undertaken in four categories (Table 7). Comparison between diluted (1:10) and un-diluted (burner rotation) solutions was required and some data sets were not complete (bulk digest solutions for participants 1-8 and some results showing contamination were rejected). A significant difference was only found in one case (set 2, between participants). This may have been related to cross-contamination of the wash solution (distilled water) with mussel digest solutions containing relatively high zinc concentrations. This did not occur with copper and was overcome when recognised during the inter-operator

study by changing the wash solution at regular intervals. No significant difference was found between techniques of burner rotation or dilution 1:10 for high zinc levels (Table 7).

The concentrated nitric acid digestion did not give complete destruction of the fatty matter in isolated marine tissue samples leading to occasional nebulizer capillary blockage by particulate material. This was overcome by frequently cleaning the nebulizer with a wire. Mussel sample solutions (tri-acid digestion) occasionally gave a white precipitate upon cooling and dilution with distilled water. This precipitate (probably calcium sulphate) could also have caused nebulizer blockage but this was not observed with shark tissue digests. Both these sources of nebulizer blockage may be the reason for the observed significant difference in copper in mussel data (Table 6).

The results for the semi-quantitative examination of participants' calibration data are shown schematically in Figure 3. Three calibration lines showed a relatively high degree of scatter. When these were excluded, the average %RSD of respective calibration line slopes were observed to be approximately the same (1-2%). Visual examination of Figure 3 showed that the calibration lines scatter for 10% sulphuric acid solutions tended to be greater. This may have arisen because these participants were the last to make flame AAS readings and some build-up of residue in the nebulizers may have occurred. The use of %RSD of the slope of a calibration line was a valuable parameter for comparison of individual calibration techniques.

Solutions from zinc in mussel digests were diluted 1:10 so that their relatively high zinc concentrations (approximately 1.4 absorbance units) could be read using normal range zinc standards (0-1.0 μg L^{-1}). The diluted digestion solutions gave readings of about 0.3 absorbance units compared with about 0.2 absorbance units for burner rotation with high range zinc standard (0-10.0 μg L^{-1}). Shark digest solutions of much lower analyte concentrations were also diluted 1:10 in certain cases.

The %RSD's of all absorbance readings (digestion and control standard solutions) were also calculated and examined for any trends. One trend observed was the loss of precision in reading absorbance following dilution at the lower ends of the calibration lines. Various examples displayed schematically in Figure 4 show this effect and indicate that measurement of analyte concentration at very low absorbance levels should be avoided. This is in agreement with the reported[12] deterioration in precision observed when absorbance values decrease below 0.050 and particularly below 0.010.

5.3 Comparison of inter-laboratory survey and inter-operator study

It was originally not intended to prepare quantities of marine tissue samples for more than one series of analytical studies. Long-term concentration variation of components had not been established over a sufficiently long period at the time of this inter-operator study to allow confident re-use of the

samples as reference materials. Comparison between means (dry wt. basis) from the inter-laboratory survey and group means from the inter-operator study group were made using Student's t-test. Significant differences were observed in some cases indicating that unqualified comparisons of corresponding sample metal means may not be valid. Preservation problems have been reported in other studies involving preparation of shark powder/paste materials[13].

The results were expressed as %RSD about the grand mean for respective sets (Table 8). The magnitude of the %RSD's follow the order:

> Inter-laboratory survey (all results): greater than Inter-laboratory survey (results within 2 S.D.'s of mean): greater than Inter-operator study (own digest): greater than Inter-operator study (bulk digest).

This comparison is supported by the ANOVA data given in Table 2. There was also a perceptible difference between the %RSD for inter-operator bulk digest and own digest results. These differences were indicative of errors arising from handling and preparation of samples and revealed an important area for further examination. Some of the increase in %RSD for inter-operator own digest results may have been introduced by lack of familiarity with apparatus and methods. Nevertheless the %RSD's in this case were lower than for the overall inter-laboratory survey results.

The comments on the digestion of high mussel tissue with concentrated nitric acid and tri-acid mixture (section 5.2) seem to be reflected in the inter-laboratory survey results for the same tissue (Table 4). This points to differences in digestion efficiency with respect to tissue type.

The deterioration of precision at low absorbance values (Figure 4) is closely linked with the low sensitivity of copper and lead determinations by AAS (section 5.1) particularly direct flame AAS. Increasing the sensitivity through the use of concentrating techniques (e.g. solvent extraction, ion exchange) or directly by using graphite furnace AAS or ASV should be strongly preferred rather than direct flame when the lower limits of detection of direct flame AAS measurement are approached. Techniques such as scale expansion simply amplify the signal instability rather than reduce the background noise levels, not necessarily improving the precision of results.

6. Conclusions

Examination of data from the inter-laboratory survey showed a wide spread of results for heavy metals in different marine tissue samples. This indicates that careful attention should be directed to improving aspects of sampling, analysis and intercalibration. Regulatory and control authorities need to take into account realistic confidence limits when interpreting measurement results from different laboratories. There is an obvious need for greater uniformity of techniques.

The examples of low mussel (sample 4) and mercury (all examples) (Table 1 and section 5.1) indicated that experience and the use of uniform techniques contributed significantly towards reporting comparable results. Nevertheless it was the use of common digestion procedures and methods of measurement which ultimately yielded the most acceptable results (lead in low mussel compound with lead in low flathead). It would be important also to understand the extent of the sources for, and ways to eliminate, laboratory contamination[14].

Lack of appreciation of these factors can lead to erroneous conclusions, an example of which has been recently described.[15] More recent examples of intercomparison studies of heavy metals in tissue were reported by Stoeppler and Nurnberg.[16]

Very few laboratories reported the routine use of certified standard reference materials (SRM's). This may reflect the lack of suitable marine tissue SRM's. Some of these, however, have recently become available from the NBS in Washington[17] (SRM 1566 Oyster Tissue) and the IAEA[18] in Monaco (MA-A-1 Dried Copepoda and MA-A-2 Homogenized Fish Flesh).

More thorough examination of factors influencing sample stability should be undertaken to develop specifications for future supplies of Australian marine tissue reference materials. The need for such materials was endorsed by several participants in the workshop discussions who had experienced calibration problems due to differences in the effectiveness of digestion techniques applied to marine tissue compared with non-

marine tissue standard materials, in which the metals are likely to be present as different chemical species.

A more thorough examination of standardization procedures is warranted and a future exercise is proposed which will involve circulation of a series of "standard solutions" as inter-calibration samples. A similar exercise conducted by ICES[8] revealed that certain laboratories were deficient even in applied calibration techniques.

The inter-operator study demonstrated ways to examine factors influencing methods of determination of heavy metals and interactions between the methods. The purpose of the study was not to decide preferences for methods but rather to indicate where problem areas were likely to exist. Both digestion methods examined revealed deficiencies.

Participants in the inter-operator workshop discussions expressed a general preference for nitric/perchloric di-acid digestion mix rather than the tri-acid digestion mix, although some metals (e.g. arsenic) required the increased severity of tri-acid mix to ensure adequate recoveries from marine tissue samples. Concentrated nitric acid is the preferred acid for graphite furnace AAS whereas ASV requires destruction of all organic matter. Thus choice of acid digestion system will most likely be determined by the instrumental technique employed.

The workshop provided a unique opportunity for informal discussion and exchange of experience between analytical chemists

engaged in routine determination of heavy metals in marine tissue. Many other valuable comments and observations on techniques for sampling and analysis were made during the course of the workshop, which will be taken into account in a later more detailed report of these surveys and experiments. The department of Science and the Environment intends to publish this in the series of Marine Environment Reports in due course.

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Appendix 1

Inter-operator study - Outline of methods

A. Digestion with concentrated nitric acid (two samples)

1. Weigh 0.20 to 0.25 g sample into test tube. Tubes will be supplied pre-cleaned in racks. Prepare samples in duplicate.

2. Moisten with approx. 0.8 mL distilled water.

3. Add 2.0 mL. conc. HNO_3 from stock supplied in burette. Also prepare two reagent blanks.

4. Place test tubes in water bath on hotplate. Bring water to boil as directed over approx three quarters of an hour.

   Caution: Initial heating may result in frothing and continual attention is required. Frothing may be controlled by gently tapping tube.

5. Continue heating until clear. This may require about 4 hours.

6. On completion of digest, cool test tube and make up to 10 mL calibration mark with distilled water. Mix contents briefly using eccentric mixer.

7. If applicable prepare a 1:10 dilution of each mussel digestion solution. Transfer 1.0 mL to another test tube and make up to the 10 ml calibration mark with stock 10% HNO_3. Mix contents briefly using eccentric mixer.

B. Digestion with tri-acid mixture HNO_3 : $HClO_4$: H_2SO_4 - 3:1:1 (Two samples)

1. Weigh 0.20 to 0.25 g sample into supplied 100 mL Erlenmeyer flask. Prepare samples in duplicate.

2. Moisten with approx. 0.8 mL distilled water.

3. Add 5.0 mL tri-acid mixture from stock supplied in burette. Also prepare two reagent blanks.

4. Place flasks on hot plate and heat as directed until gently refluxing. Use watchglass on top of flask to control reflux at this stage.

   Caution: Regulate any undue frothing by gently swirling. Reaction must be carefully watched to avoid charring. Proceed for three quarters of an hour and distill off HNO_3 carefully until $HClO_4$ fumes observed, taking due precautions to avoid carbonisation as the last of the HNO_3 is removed.

5. Heat further until fumes H_2SO_4 observed. (approx 20min.).

6. Cool, carefully add water (caution - conc. acid!) and transfer to calibrated test tube.

7. Allow contents to cool thoroughly. Add distilled water and boil gently for a few minutes. Re-cool and transfer solution to test tube making up to 10 mL calibration mark with additional distilled water. Mix contents briefly using eccentric mixer.

8. If applicable prepare a 1:10 dilution of each mussel digestion solution. Transfer 1.0 mL to another test tube and make up to the 10 mL calibration mark with stock 10% H_2SO_4. Mix contents briefly using eccentric mixer.

C. Standard solutions to be prepared

1. Use stock solution containing 10 mg L^{-1} of both zinc and copper and selecting appropriate acid mixture (10% HNO_3 or 10% H_2SO_4).

2. Prepare 100 mL of each of the following:-

0.25 mg L^{-1}	using	2.5 mL	of	10ppm	stock	from	burette	in 100mL
0.50 mg L^{-1}	.."	5.0 mL	"	"	"	"	"	" 100mL
0.75 mg L^{-1}	"	7.5 mL	"	"	"	"	"	" 100mL
1.00 mg L^{-1}	"	10.0 mL	"	"	"	"	"	" 100mL

3. Retain a portion of the acid as 0.0 mg L^{-1} standard.

4. Transfer solutions to alternative storage vessels if advised (i.e. shortage of volumetric glassware etc).

D. Flame AAS operating conditions

Instruments Two Pye-Unicam SP1950 atomic absorption spectrophotometers with automatic deuterium lamp background correction

Flame	Air/acetylene, lean Air flow rate - fixed Acetylene flow rate - slight adjustments allowed by participants if required during setting-up.	
Resonance line	Copper	324.7 nm
	Zinc	213.7 nm
Hallow Cathode lamp Current	Copper	2 mA
	Zinc	5 mA
Other aspects (i)	Burner alignment and height adjustment carried out by participant to maximise response.	
(ii)	Four absorbance readings per solution.	