Completion of an analysis of stock structure of orange roughy, based on otolith chemical composition

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and C. J. Mackinnon

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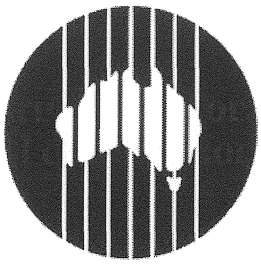

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1. Non-technical Summary

Stock structure of the deepwater fish, orange roughy (*Hoplostethus atlanticus*), remains very much uncertain, despite a variety of techniques being applied to the problem. Resolution of local stock structure of this species would have a direct affect on current management plans for the species and on resolving current uncertainties between Australia and New Zealand on managing 'straddling stocks' of roughy in the Tasman Sea. More broadly, any technique that works on roughy might prove equally applicable to other deep sea fishes, such as the oreo dories.

In 1992/93, we conducted a small pilot study to determine whether regional differences in the composition of roughy otoliths might be a useful stock marker. The approach is based on the hypothesis that slight differences in environmental conditions affect deposition rates of the elements that constitute otoliths, and that these changes are permanent. the pilot study, which was based on samples from five sites in the Australian EEZ, indicated very large differences among sites in concentrations of several elements.

The current study was undertaken to repeat these analyses, to expand coverage to other sites in the Australian EEZ and the Tasman sea, and to assess the reliability of the data, and conclusions drawn. Data reliability was evaluated by incorporating into the study two 'reality checks' : (1) an experimental study on the effects of specimen handling on apparent otolith composition, and (2) a comparison between repeat samples taken at the same sites several years apart.

The 'reality checks' indicate that most of the elements measured in orange roughy otoliths (as well as those in the otoliths of most other fishes) are highly sensitive to the effects of handling or occur at such low concentrations that noise-induced artifacts are common. The key elements that distinguished among sites in the pilot study are all highly sensitive to affects of specimen handling, which renders their use in stock structure analysis highly dubious. The issue is not that they are incorrect or inaccurate, but rather their reliability is uncertain and may be difficult to determine. The sensitivity of otolith composition to handling-induced artifacts has a fundamental impact on the field, and should be factored into any study on otolith composition.

Restricting the expanded analysis to only the few elements that appeared to be reliable nonetheless provided a degree of site separation among roughy. Differences tended to be slight, and of a statistical nature (as opposed to non-overlapping concentrations of different elements among sites). Overall, the results suggest five 'groups' of sites:

(1) the North Atlantic, which differed consistently from all southern hemisphere sites sampled;
(2) the Lord Howe Rise, which differed in several elements from all other sites in both the Australian EEZ and NZ EEZ;
(3) New Zealand and Australian sites broadly differ in two elements; and
(4) within the Australian EEZ, NSW, the GAB and WA samples constitute a group different from a St. Helens/ Maatsuyker/Cascade Plateau group.

An unreplicated sample from WA suggests it may also be distinguishable from the other Australian mainland sites, on the basis of very high Hg levels. This needs to be verified.

We conclude that otolith chemical analysis does provide indications of a structuring of the Australian and Tasman Sea roughy stocks, but that with the exception of the Lord Howe Rise fish, differences are slight and the evidence for stock separations relatively weak. This conclusion is perhaps not surprising, in the light of deep oceanic environments that are likely to be relatively similar over broad geographic areas.

2. Background

Accurate information on stock structure is one the perceived requirements for effective fisheries management. For this reason, a variety of techniques have been employed to assess stock structure, ranging from spatial analysis of demographic parameters to tagging, genetics and, most recently, analysis of otolith composition. Although the basic concepts underlying use of otolith composition as a stock marker were developed over twenty years ago (Thresher, et al., 1997), the discipline has progressed substantially in the last ten years as new and more powerful statistical and analytical tools have become available (see Campana, et al., 1997).

In the early 1980s, apparently large stocks of a deepwater trachichthyid, *Hoplostethus atlanticus* (orange roughy), were discovered off the southern coasts of Australia. A substantial fishery for the species already existed off New Zealand, and there was a rapid increase in effort directed at the Australian stocks. Evidence of subsequent declines in catches in some areas prompted development of interim management plans and scientific efforts to assess stock sizes, productivity and structure. Due to the great depth inhabited by the species (typically deeper than 600 m), conventional tagging was deemed impractical, and evaluation of stock structure was based in indirect methods. Results of this work have not been definitive, however, either because of apparently limited resolving power (e.g., meristics, genetics) or limited sampling effort (parasite studies, otolith chemistry), or both. From a management perspective, the sources of uncertainty arise from, on the one hand, genetic and meristic studies that suggest that the Australian population is largely unstructured and, on the other hand, parasite and whole otolith work that suggest adults are relatively sedentary during non-spawning periods (but say nothing about distributions during reproductive periods or the extent of reproductive independence among 'stocks'). Nor do these results fit well with industry catches of running ripe fish in at least four areas (eastern Tasmania, Maatsuyker/southern Tasmania, Western Australia and New South Wales), which has been taken to imply four 'discrete' stocks.

Two previous studies have assessed stock structure in orange roughy based on otolith composition. Edmonds et al. (1991) analysed element concentrations in whole otoliths of fish from South Australia and two sites off Tasmania. They reported significant differences among all three sites based on, primarily, sodium and magnesium concentrations, and concluded that this implied relatively little movement among adults, as the bulk of the elemental signature derived from otolith mass deposited following the planktonic stage. To obtain data on the otolith primordia, which is deposited shortly after the fish hatches and might permit mapping planktonic mixing, Thresher and Proctor (1995) used probe micro-analysis to measure elemental concentrations independently at the primordia and otolith margins. Sample sizes were small in this pilot study, but generally confirmed significant site-specific differences in the concentrations of several elements and implied a number of stocks in the Australian region.

The issue of stock structure in Australian-New Zealand orange roughy took on a greater urgency when managers were confronted with the issue of 'straddling stocks', fisheries based on grounds that were outside the EEZs of the two countries. A number of such straddling stocks have been fished, the two most prominent of which are those at the Northwest Challenger Plateau and at the Lord Howe Rise. Policies for managing these high-seas fisheries are not yet well developed, and considerable discussion arose as to 'whose roughy' these stocks were most closely affiliated with. For this reason, a priority has been put on assessing the affinities of these fishes.

The current study extends the preliminary data reported on by Thresher & Proctor (1995), under work funded by the Fisheries Resources Research Fund (Project Number 1992/93 #18). Specifically, that study, which was designed primarily to evaluate feasibility of the approach, found highly significant differences between otoliths of fish collected off Western Australia, NSW, the Great Australian Bight and, pooled, eastern and southern zone Tasmanian

specimens. Fish from the GAB and two Tasmanian sites were virtually identical at the centres of the otoliths, suggesting they came from the same, or environmentally similar spawning areas. At the margin of the otoliths, however, all three sites could be readily separated, suggesting the fish move only slightly after settling to the bottom as larvae. The relationship between eastern and southern zone fish is not entirely clear-cut. Fish caught on the St. Helen's Hill during the spawning season cover the range of variability seen in both the eastern and southern zone during non-spawning periods, which is consistent with fish from both areas migrating to the hill to spawn. However, spawning season fish caught in the southern zone are very different from all other fish examined.

In the conclusions of that project, we suggested three possible explanations for the results: 1) they were sampling artifacts, due to the very limited sample sizes, 2) they indicate three, and possibly four stocks of orange roughy in Australian waters, and 3) with particular regard to the relationship between Eastern and Southern Zone fish, the southern zone spawning-type fish could be present there year-round, but constitute a small percentage of the non-spawning population or constitute a wholly undiscovered stock, which migrates to spawning areas in the south from somewhere else and then returns to this unknown area each summer. The results of the pilot study appeared to justify further effort using this technique, but were not sufficient to distinguish among these hypotheses.

3. Need

Nearly all orange roughy stocks within Australian waters are acknowledged to be fully exploited. Since the start of the fishery, considerable effort was put into determining the geographic structure of the stock(s). Unfortunately, as noted above, results of these studies have not been definitive. This uncertainty still needs to be resolved, for two reasons. First, determining whether or not the "eastern" and "southern" populations of orange roughy are the same fish will immediately affect TAC's. If the two nominal stocks are independent, the combined sustainable annual yield for the two increases by about 700 tonnes, adding approximately $2.8 million to the landed value of the catch. Currently the fishery is managed based on a 'best guess' assumption that there is about a 50% mixing of the two 'stocks'. Secondly, the current TAC's are based on the estimated rates of self-regeneration of relatively isolated stocks of an exceptionally long lived and slowly reproducing species. The global distribution of the species, however, implies at least low level genetic exchange over very broad scales, and we cannot discount the possibility that the Australian fishes constitute only one part of a slowly migratory, broadly distributed stock. If so, then the dynamics of stock recovery are determined primarily by the diffusion rates of individuals among population units, and recovery is likely to be much quicker (and consequent TAC's much higher) than that predicted based on local self-regeneration.

The proposed work, therefore, filled four specific needs in addressing the general issue of orange roughy stock structure:

- additional material from each sample site would be examined, which would test logical and statistical validity of the pilot project;
- it would provide more thorough coverage of the Australian and near-Australian stocks, by including samples from a number of hills in the southern zone, as well as the Lord Howe Rise and Cascade Plateau;
- if results of the pilot project were verified, it would develop a strong enough information base to justify a technical publication and an adequate base for management decisions;
- it would provide a basis for determining whether the technique might be useful for other deep-water stocks, such as oreo dories, for which stock structure information is sparse.

4. *Objective*

To improve management of the Australian orange roughy fishery, by determining the relationship between southern and eastern zone orange roughy stocks.

5. *Methods*

All fish for the analysis were caught on commercial vessels. Sample locations and details are provided in Figure 1 and Table 1. Because of the effects of post-mortem handling on otolith composition (see Detailed Results, B), we used only otoliths extracted from fresh or whole frozen fish, except for the sample from Western Australia. For this area, the only samples we could get had been stored in chilled seawater

Procedures for embedding, sectioning and preparing otoliths for analysis follow Gunn et al (1992). *H. atlanticus* sagittae were fixed to the base of an 8 mm embedding capsule with a small droplet of fast setting epoxy resin. The capsule was filled with Araldite D™ resin and placed in an oven at 40°C to aid curing. The embedded otolith parts were sectioned using a diamond saw (Struers Accutom), and the sections fixed to glass rounds with Araldite D™. Coarse grinding to the required plane was performed with 600 and 1200 grade silicon carbide wet/dry paper on a lapping machine. Subsequent fine grinding was performed by hand using 2400 grade wet/dry paper. Sections were polished using 6 µm then 3 µm diamond paste, followed by aluminium oxide powder (Linde B, 0.5 µm). Sections were then ground to expose the primordial core. The completed sections were ultrasonically cleaned and stored in a dessicator. Prior to microanalysis, specimens were coated with a 250 - 300 Å layer of carbon using a sputter coater.

Two probe microanalysers were used in this study. The concentrations of the minor elements (Na, Sr, K, S, Cl, and Ca) were measured using wavelength dispersive (WD) electron probe microanalysis and trace elements (i.e., those present at nominal concentrations less than 50-100 parts per million [ppm]) were analysed using a proton probe microanalyser.

WD microanalysis was done on a JEOL 8900R electron probe fitted with 5 wavelength dispersive detectors. The procedures are detailed in Gunn et al (1992). Data for the current study were acquired using beam defocused to a diameter of 50 µm, with a 15 kV accelerating voltage and a beam current of 50 nA (beam power density of 0.38 µW μm^{-2}). Minimum detection limits and confidence intervals for the concentration estimates are based on equations provided by Ancey et al. (1978) and are tested empirically by Thresher, et al. (1994).

Proton probe microanalysis was done using the micro-PIXE beam line at the CSIRO Heavy Ion Analytical Facility. Procedures and methodology follow Sie and Thresher (1992). In brief, data were acquired at a 50 µm beam diameter, using a 30 nA current accumulated for a total charge of 6 µC for each point. X-rays were detected using an EDS (Si(Li)) detector. A 100 µm Al filter was used to attenuate Ca K lines, which would otherwise 'swamp' the lower end of the spectrum, resulting in high minimum detection limits for heavy elements. Weight fractions and minimum detection limits were calculated using the methods of Ryan et al. (1990), and normalised to 40% calcium.

Two WD-EPMA scans were done on each specimen - one on the primordium itself, and a second about 200 µm from the primordium along the main growth axis. Data for the primordium represents material deposited during late embryonic and early larval stages, whereas the outer point corresponds with deposition during juvenile stages of development. Proton probe microanalyses were done on the primordium, and overlaid the previously obtained WD points. The concentrations of elements measured by the proton probe are unaffected by prior analysis using an electron probe (Thresher & Proctor, unpublished data); this is consistent with the greater depth of X-ray emission induced by protons than by

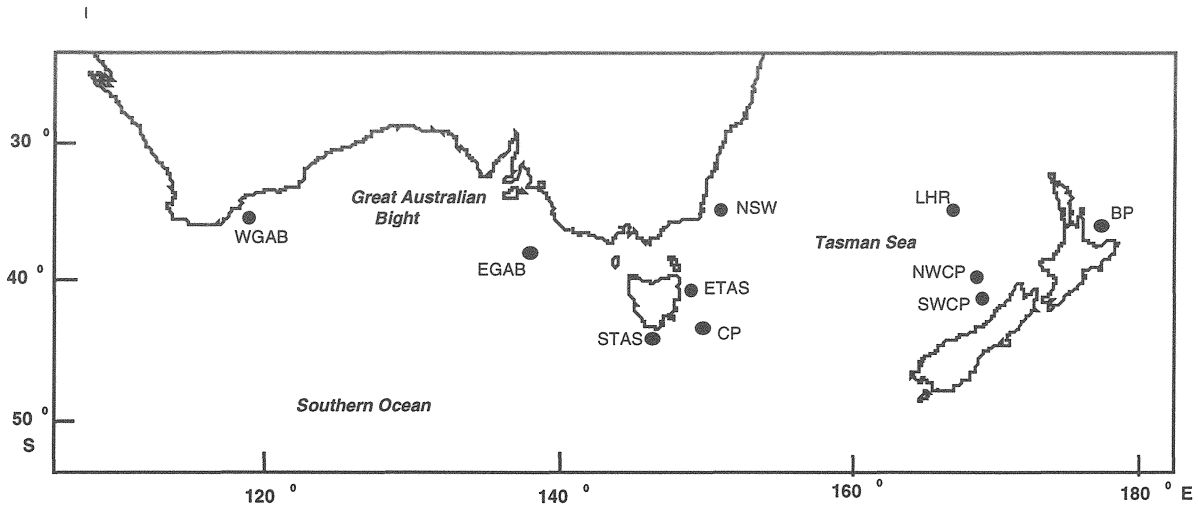


Figure 1. Locations of sites from which orange roughy were sampled for this study. Key to site abbreviations is provided in Table 1.

Table 1. *Hoplostethus atlanticus.* Capture details of individuals analysed in this study (n number of fish; SL standard length). Otoliths from 20 fish were analysed for each site.

Site	Code	Position		Date Caught	Size Range (SL cm)
Western Great Australian Bight	WGAB	35° 25'S	118° 20'E	11 July 1996	31 - 45
Eastern Great Australian Bight	EGAB	37°14' S	138°23' E	10 August 1996	35 - 42
Southern zone Tasmania	STAS	44°12' S	146°11' E	25 July 1996	27 - 41
Eastern zone Tasmania	ETAS	44°14' S	148°46' E	18 July 1996	33 - 43
Cascade Plateau	CP	43°53' S	150°25' E	13 October 1996	38 - 48
New South Wales	NSW	35° 42'S	150° 39' E	28 July 1996	22 - 30
Lord Howe Rise	LHR	35°39' S	165°11' E	25 October 1996	25 - 37
Northwest Challenger Plateau	NWCP	37°18' S	167°16' E	20 June 1996	28 - 40
Southwest Challenger Plateau	SWCP	40°09' S	168°17' E	22 June 1997	31 - 38
Bay of Plenty	BP	36°24' S	176°57' E	2 August 1997	35 - 39

electrons. Sr values from the proton probe are similar in overall patterning to those collected at the 200μm point taken with the electron microprobe, which suggests that the proton probe data is an integration of the entire early life history of the specimen out to its early juvenile stage.

6. *Detailed Results and Discussion*

A. Summary of Results of Pilot Project

The results of the previous, pilot study, are presented in some detail, as they bear on the subsequent decisions as to what elements were examined for evidence of stock structure in orange roughy.

The otoliths used in the pilot study were obtained from fish caught in 6 areas (Table 2). Five sites were off: two samples each from of the 'eastern zone' and the 'southern zone' fisheries off Tasmania, and single samples each from nominal stocks in the Great Australian Bight (GAB), off Western Australia (WA), and off New South Wales (NSW). The sixth sample was from the North Atlantic Ocean, which were examined in part as an out-group for the Australian analysis and to examine the possibility of larger geographic scale differences in otolith composition among conspecific individuals. Where two samples were obtained from a site, one was of running ripe fish taken in the spawning season (June - early September), while the other sample was of fish caught out of season (January - February). As Edmonds (1991) had demonstrated that the otoliths of male and female *H. atlanticus* may differ slightly in chemical composition, we attempted to confine the analyses to female fish only (the limited availability of 'eastern zone' Tasmania non-spawning samples meant that a third of these fish were males). For similar reasons, where possible, we limited analysis to fish within the size range of 30 to 45 cm, in order to minimise effects of possible variation in otolith composition among year-classes. The only fish we were able to obtain from the North Atlantic Ocean were significantly larger than the Australian samples.

As fish were caught on commercial vessels, post capture treatment varied between samples (Table 2). Most fish were in brine-tank storage for varying lengths of time (max. 5 days) after capture, prior to landing in port and subsequent otolith removal; however, some samples from commercial vessels were stored on ice for this period. Only otoliths of the Northern Atlantic Ocean sample were removed from fish immediately following capture. In all cases, otoliths were cleaned, dried and stored in paper envelopes following removal from fish.

Table 2. Catch details of individuals analysed in the pilot study.

Site	Code	Position	Date Caught	Sample size	Size Range (F.L. cm)
East Coast Tasmania	ET	Lat.44°14'S Long.148°45'	21 June 92	40	31 - 41
East Coast Tasmania (off season)	EO	Lat.41°37'S Long.148°39'	14 Feb 88 8 - 23 Feb 89	24	33 - 43
Southern zone Tasmania	ST	Lat.44°15'S Long.147°5'E	1 July 93	24	30 - 46
Southern zone Tasmania (off season)	SO	Lat.44°15'S Long.147°5'E	28 Jan 92	20	34 - 41
Great Australian Bight	GA	Lat.35°36'S Long.133°46'E	2 - 9 Aug 90	26	33 - 42
Western Australia	WA	Lat.33°00'S Long.114°00'E	1 - 7 June 94	12	36 - 44
North Atlantic	NA	Lat.61°31'N Long.11°27'W	22 Feb 94	10	48 - 57

Procedures for embedding, sectioning and preparing otoliths for analysis were reported above. Statistical procedures in general followed Sokal and Rohlf (1981), after which groupings of sites and specimens were tested and quantified by linear discriminant function analysis (LDFA), using the SYSTAT package.

In the pilot project, seventeen elements were detected in *H. atlanticus* otoliths, six by EPMA and eleven by micro-PIXE, in concentrations similar to those we've observed in other species (e.g., Sie and Thresher 1992); sodium (Na), strontium (Sr), potassium (K), sulphur (S) and chlorine (Cl) occurred in mean concentrations of 100 to 5000 ppm; and a variety of 'trace elements' occur at concentrations less than 10 ppm. By comparison with the other species we've examined, trace element concentrations in otoliths of orange roughy were relatively high. Copper (Cu), for example, was detected in all specimens examined, and manganese (Mn), iron (Fe), zinc (Zn) and mercury (Hg) were detected in a large fraction of the samples.

At the primordium, five of the six macro- and microconstituents measured differed significantly ($p<0.01$) among samples, the exception being S (Fig. 2). The pattern of site-specific differences was similar for four elements (Na, K, Cl and Ca), with concentrations at two sites (WA and NSW) higher than those at the other sites. For Sr, mean concentrations were higher among specimens collected in the North Atlantic than for any of the Australian specimens, though overlap among sites was broad. Of the eleven trace elements detected at the primordium, four (Zn, Br, Hg and Se) differed significantly among samples (Fig. 3). However, post-hoc analysis (Scheffe's test) indicated that most differences could be attributed to one sample, that from the North Atlantic. By comparison with the Australian samples analyzed, individuals from the North Atlantic had significantly higher concentrations of Zn and Pb and lower concentrations of Br and Se. Re-analysis excluding the North Atlantic sample indicates the measured concentrations of only one trace element, Se, differed significantly among Australian samples (ANOVA $F_{4,66} = 3.04$, $P<0.05$). Post-hoc analysis of the reduced data set indicated no major separation among sites, however; the largest pair-wise difference was between samples from the Great Australian Bight and non-spawning fish from eastern Tasmania, which differed at the $P< 0.1$ level.

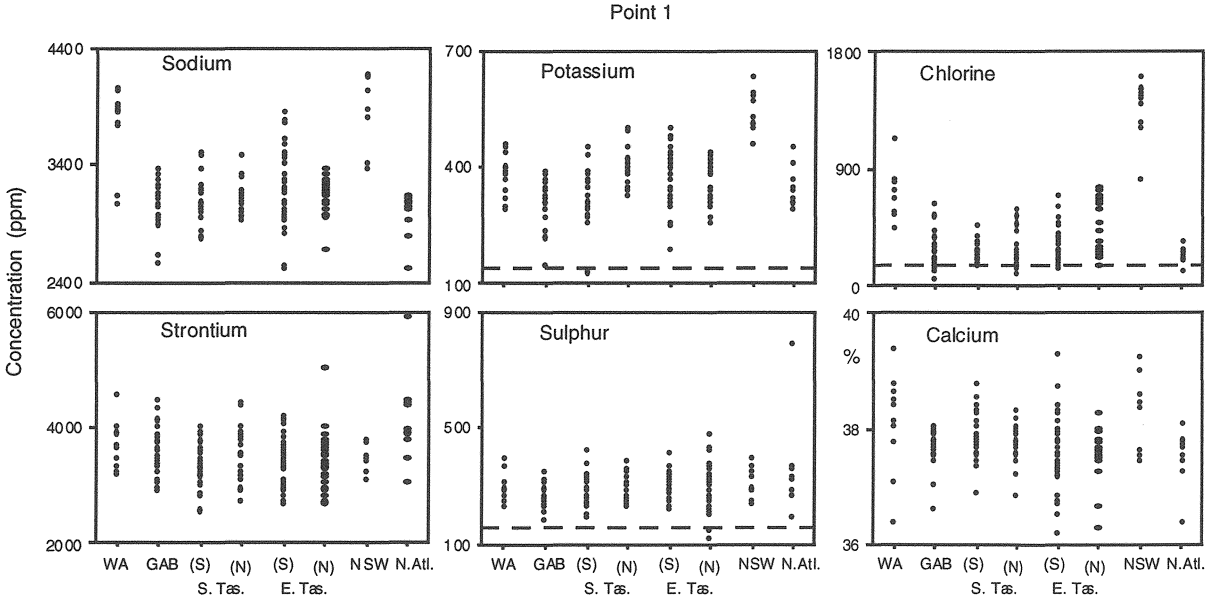


Figure 2. Distribution of concentrations of elements, measured by EPMA at point 1 (adjacent to primordial core), across sites. Horizontal dashed lines indicate minimum detection limits. Differences among samples were significant (by ANOVA, $p<<0.01$) for all elements except Sulphur. (S) = spawning season, (N) = non-spawning season.

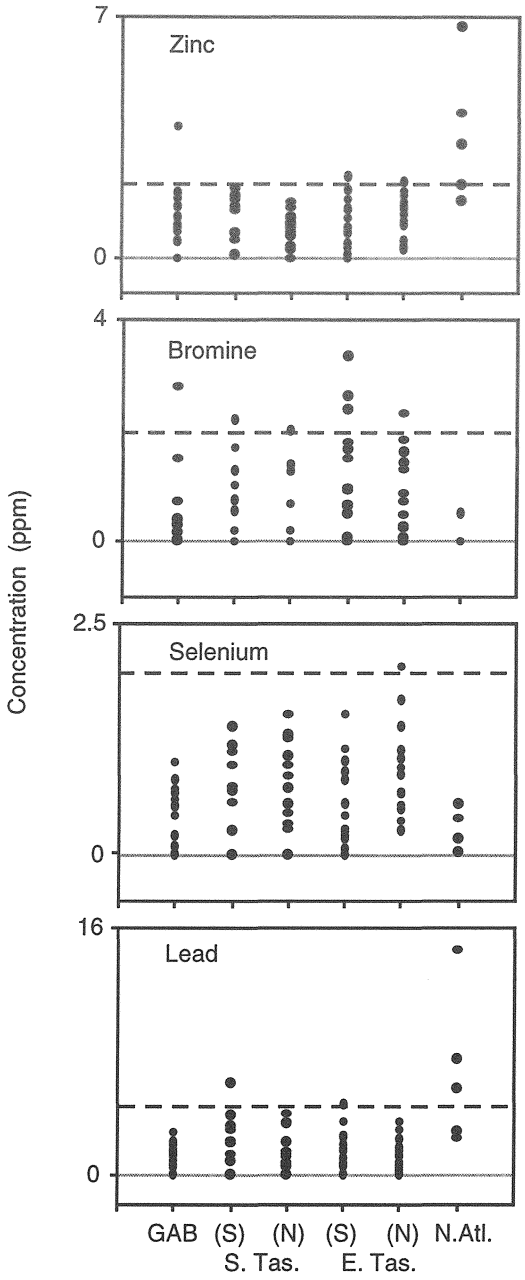


Figure 3. Distribution of concentrations of elements, measured by micro-PIXE at point 1 (adjacent to primordial core), that differed significantly ($p<0.01$) across sites. Horizontal dashed lines indicate minimum detection limits. (S) = spawning season, (N) = non-spawning season.

Consequently, site discrimination and groupings of sites, using linear discriminant function analysis (LDFA), was based entirely on the 5 microconstituents and Ca. This analysis indicated a highly significant separation among samples (sites and seasons) (Wilks' lambda = 0.12, $F_{42, 716} = 9.96$, $p << 0.001$) (Fig. 4), based on four significant ($P<0.01$) discriminant factors. Post-hoc analysis of the factor loadings indicated that factors 1 and 2 distinguish among samples from WA, NSW and all other sites, pooled; factor 3 further separates the North Atlantic sample from all remaining Australian samples; and factor 4 does not clearly distinguish any sub-set of samples. Plots of samples in discriminant factor space clearly indicated the separation of the WA and NSW samples from the remaining samples, less clearly show a separation for the North Atlantic sample, and indicated broad overlap among the remaining Australian samples.

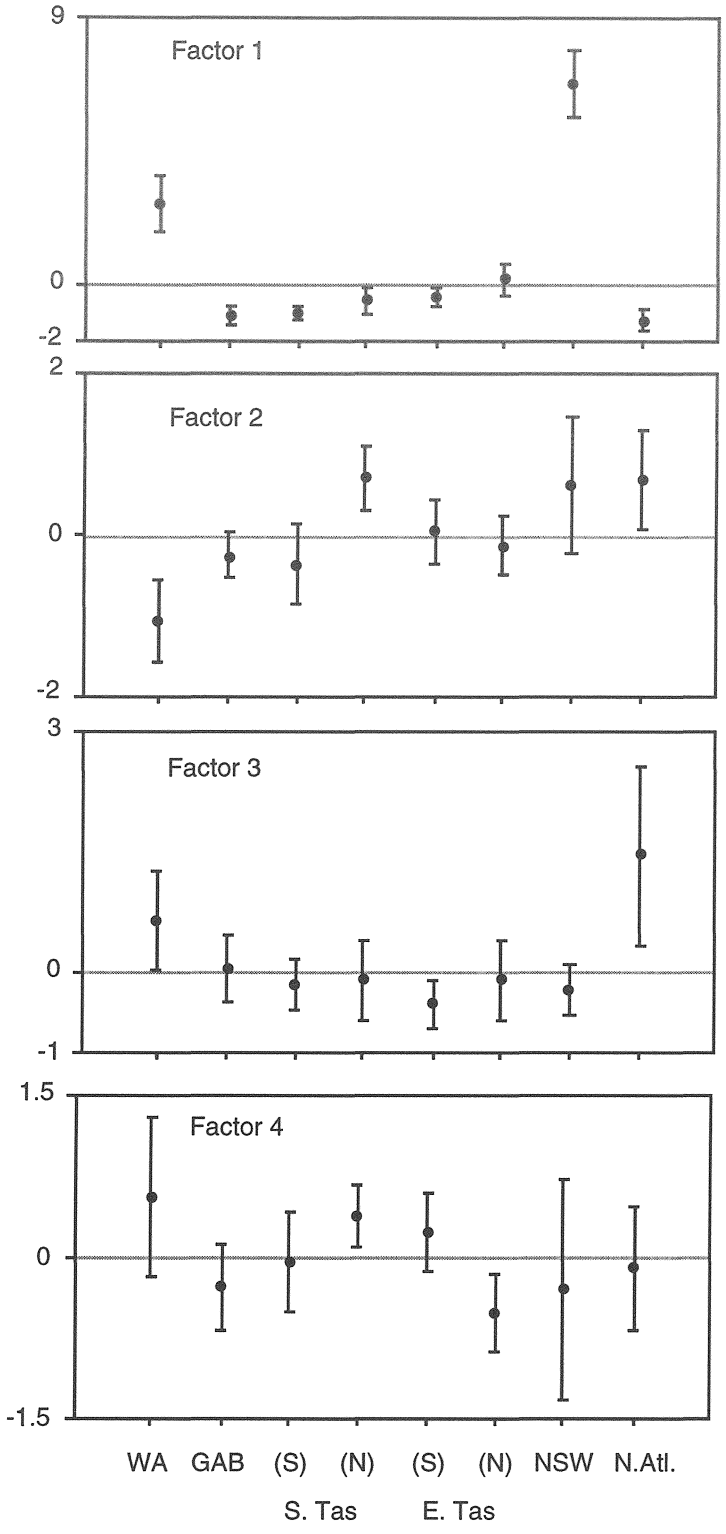


Figure 4. Means of linear discriminant factors, from LDFA of element concentrations measured by EPMA at Point 1 (adjacent to the primordial core), across sites. All factors shown are significant at $p<0.01$. Error bars are 95% confidence intervals. (S) = spawning season, (N) = non-spawning season.

Variation in composition at the two other points examined, 200μm from the primordium and adjacent to the otolith margin, was generally similar to that reported from the primordium. In common with Point 1, concentrations of Na, Cl and K differed significantly among samples (by ANOVA, significant at $p << 0.01$), and the pattern of site-specific differences for these elements was similar, i.e. concentrations for WA and NSW were higher than at other sites The largest differences were for Na, Cl, and K, in descending order of magnitude. LDFA of the point 2 and margin compositions, based on concentrations of Ca and the microconstituents only, reveals the highly significant differences among samples (sites and seasons); Wilks' lambda = 0.09, $F_{42,707} = 11.22$, $p << 0.001$), with four significant ($p << 0.001$) discriminant factors. Margin data discriminated among samples collected in the GAB and off Tasmania. Separation of the GAB sample occurred primarily in factor 3; post-hoc analysis indicated differences between the Tasmanian and GAB samples are significant at $p<0.01$. The element contributing most to this separation was Sr, which are consistently lower at the margin for GAB than Tasmanian specimens.

Detailed analysis of the spawning and non-spawning samples from the eastern and southern zones off Tasmania also suggested significant ($p<0.001$) differences among these samples at the margin. The principal elements generating the site separation are Na and Cl. Distribution of the specimens from the four samples in along Na and Cl axes indicates four points (Fig. 5). First, mean Na values for spawning season fish in both areas are higher than those for fish caught in the non-spawning season. Second, Cl values for southern zone specimens collected in the non-spawning season are lower than those of eastern zone fish at the same time of year. Third, chlorine values for spawning season fish caught in the eastern zone (St. Helens Hill) overlap almost exactly the combined values for southern and eastern zone fish caught during the non-spawning season. And fourth, Cl values for spawning season fish in the southern zone are higher than those of any other Tasmanian sample, including non-spawning, southern zone fish.

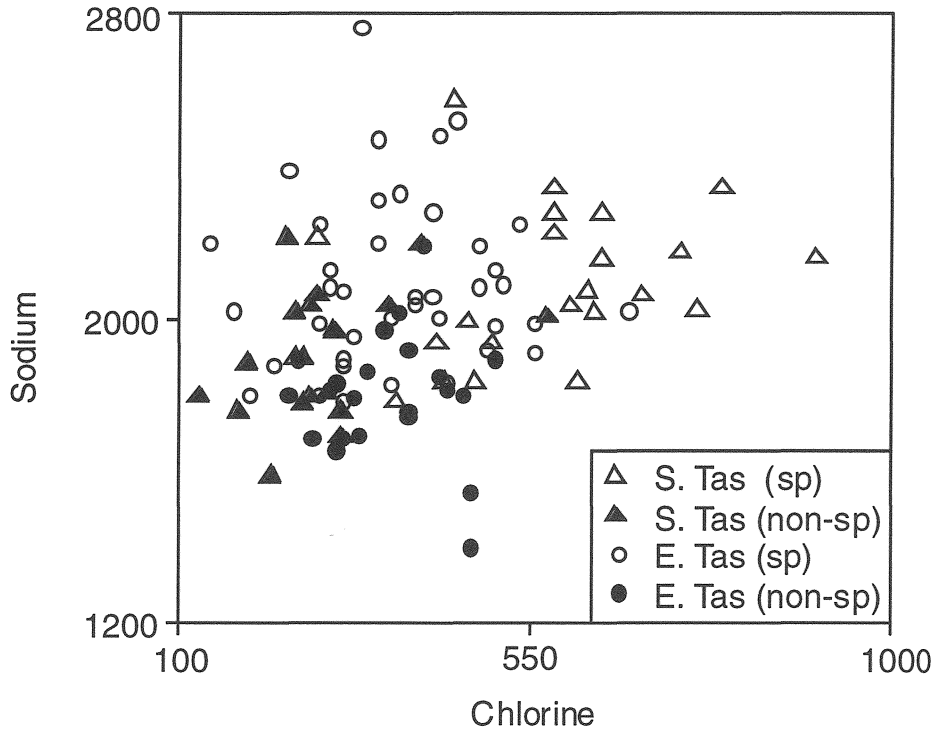


Figure 5. Relationship between Na and Cl concentrations, measured by EPMA at the otolith margin of Tasmanian samples, split by zone and season.

The principal conclusions from the work summarised above was that, first, overall element concentrations in orange roughy were not substantially different from those of other species we've examined, second, that within the Australian context, trace elements contribute nothing to discriminating among samples and third, that differences among Australian samples in micro-constituent concentrations were substantial. Linear discriminant function analysis applied to these differences distinguished among three or four groupings of sites, depending upon the position in the otolith analysed. At the primordium and immediately adjacent to it (Pts 1 and 2 in the analyses), the samples from Western Australia and NSW are readily separated from those taken in the Great Australian Bight (GAB) and off Tasmania; close to the otolith margin, differences also emerge between the GAB and Tasmania.

Regional differences at and near the primordium were based on, primarily, three elements - sodium, chlorine and potassium. All three elements loaded heavily onto discriminant factor 1, which separated the NSW and WA samples from other Australian samples (as well as each other); factor 2 was primarily loaded onto by potassium, and further separates the NSW and WA samples from one another. Separation among sites at point 2, about 200 microns from the primordium, was similar to that at the primordium itself and involved the same elements. Indeed, the same basic pattern of sample discrimination also occurs at the margin, with the addition that site-specific differences in strontium become significant, principally distinguishing between the GAB and Tasmanian samples.

B. Effects of Specimen Handling and Otolith Preparation on the Apparent Concentration of Elements in Fish Otoliths

An assumption implicit in the pilot project, as well as virtually all otolith composition-based studies to date, is that analyses accurately measure the 'real' composition of an otolith. This assumption has two components: that the analytical instruments measure composition accurately and without bias, and that the otoliths, when analysed, retain the composition they had in the live fish.

Instrumental accuracy has been tested, in part, by documenting the effects of operating conditions on precision and sensitivity (Toole and Nielsen 1992; Gunn et al. 1992; Sie and Thresher 1992), and, in part, by comparing the performance of a variety of analytical tools using real and artificial otoliths of 'known' composition (Campana et al. 1997). The assumption that an otolith's composition does not change significantly between when in the fish and when analysed has not been tested, though it is fundamental to the science. In fact, there are a number of reasons to doubt the assumption is valid. It is well known, for example, that otoliths dry out when removed from fish and readily take up fluids (e.g. ethanol and immersion oil) when re-immersed, implying movement of at least fluids into and out of otoliths. Gauldie et al. (in press) quantify this process, and note that fluid migration has considerable potential for moving even large molecules through the protein-aragonite matrix that constitute otoliths (Gauldie and Coote in press). Otolith chemical analysis is also known to be prone to a variety of artifacts due to contamination, particularly as levels of nominal detectability approach the part per million level (E. Brothers unpublished data; Sie and Thresher 1992). Because of these issues, participants at a 1992 workshop on otolith composition urged considerable caution in interpreting the results of compositional studies (see Thresher et al. 1997).

Two additional observations led us to question the assumption that otoliths, when analysed using standard techniques, retain their *in situ* composition. First, when comparing left and right otoliths from individual fish we often found differences that greatly exceeded instrumental error (see Thresher et al. 1994). These differences indicate either asymmetric deposition of elements in otolith pairs or a post-mortem alteration somehow induced by differences in the way the two otoliths were handled, prepared and analysed. Second, our early, unpublished attempts to analyse the composition of otoliths removed from ethanol-preserved fish larvae resulted in apparent compositions that not only did not match any of the

nominal source populations, but which did not match any known fish. This suggested that how a fish was preserved had a large effect on the composition of its otoliths.

These observations led us to undertake a series of experiments involving a number of Australian species to determine (1) which, if any, of the elements we routinely measure in otoliths using the electron probe microanalyser are susceptible to modification by post-mortem treatment, (2) what effect, if any, the standard procedures used to obtain and prepare otoliths for analysis have on their composition, (3) the procedures that distort otolith composition the least, and (4) the magnitude of these artifacts relative to apparent ontogenetic and stock differences in fish. With specific regard to the pilot study for orange roughy, we sought to determine the reliability of apparent differences among putative stocks, given what we knew were differences among samples in the way in which they were handled following capture.

The general methodology used to test for an impact of a particular handling technique was to remove one otolith from a freshly killed fish (the 'control' otolith), then preserve or otherwise handle the fish prior to removing the second otolith in the pair (the 'treatment' otolith). Both otoliths were then prepared for microanalysis at the same time using techniques as nearly identical as possible, and their composition compared. This approach assumes that within the limits of our analytical sensitivity the left and right otoliths in an individual fish are naturally identical in composition. We randomly assigned left and right otoliths as controls or treatments.

Two analytical instruments were used in this study: energy dispersive (ED) and wavelength dispersive (WD) electron probe microanalysers. The latter is 5-10 times more sensitive than the former, but less readily available. Procedures, in general, follow closely those described above.

Experiments and Results

Experiment #1. Effects of delayed extraction and preservation on the composition of otoliths of juvenile jackass morwong, *Nemadactylus macropterus*.

The otoliths of *N. macropterus* have been examined in detail by Thresher et al. (1994). For the current study, we used juveniles (7 - 11 cm FL) caught on December 7, 1993 using baited hand-lines, off Cygnet Jetty, south east Tasmania. Between capture and experimental treatments the next day, the fish were held together in a 200 litre aquarium, at room temperature (24 °C).

For the experiments, fish were individually killed by immersion in a concentrated solution of rotenone in seawater. Death occurred in one to two minutes. Immediately following death, each fish was rinsed in seawater and treated using one of the protocols listed in Table 3. Fish were assigned randomly to the treatments. Otoliths were removed through small 'windows' (about 5 x 5 mm) cut in the dorsal musculature posterior to the eyes. In the control experiment, both otoliths were removed immediately after death. In the experimental treatments, only one otolith was removed immediately and the plug of flesh excised to allow its removal was pinned back in place with dorsal spines, so as to minimise the movement of fluids into and out of the brain-case prior to removing the second otolith. Following removal, otoliths were cleaned using forceps and a soft tooth brush, then dried overnight in an oven at 35 °C, and stored in a moisture-free cabinet until prepared for analysis using our standard procedures. Their composition was measured using the WD microprobe (15 kV, 25 nA, 20 µm beam diameter) at two positions in each otolith: at a point immediately adjacent to the primordium and at a point adjacent to the posterior margin. Life history scans (sequential analyses equally spaced along the growth axis primordium to posterior margin, 15 kV, 25 nA, 14µm beam diameter, 25 µm steps) were also done on a few specimens to obtain finer scale information on apparent effects of handling.

Table 3. Experiment # 1. *Nemadactylus macropterus*. "Effects of delayed extraction and preservation" experiment — Treatment of otoliths following death. Left and right sagittae in each fish were randomly assigned as either Otolith - Side 1 or Otolith - Side 2.

Treatment	Otolith - Side 1	Otolith - Side 2	No. of fish
A	Extracted immediately following death	Extracted immediately following death	12
B	Extracted immediately following death	Extracted after fish had been lying on its side (the side containing otolith) for 3 hours at room temp. (24˚C)	12
C	Extracted immediately following death	Extracted after fish had been in a brined* and iced seawater slurry for 48 hrs	12
D	Extracted immediately following death	Extracted after fish had been frozen at -20 ˚C for 60 days	12
E	Extracted immediately following death	Extracted after fish had been preserved in 100% ethanol for 60 days	12

- 2 kg rock sea-salt added to 10 litre seawater + ice, and kept in the 4 ˚C coldroom.

Results

Differences between pairs of otoliths in the control set were within the range of expected measurement error (Fig. 6). The slope of the regression between otolith pairs did not differ significantly from 1.0 for any of the six elements measured.

In contrast, all treatments B - E affected the concentrations of at least one element. Freezing had the least effect, resulting only in concentrations of S higher than in the controls (Fig. 6). Preservation in ethanol depressed Na levels at the otolith margins, elevated K and S levels at both the primordium and margin and a dramatically reduced range of variation in Cl concentrations; differences among specimens at the margin were virtually erased, with Cl values throughout the otoliths converging on about 900 ppm. Delaying otolith extraction by three hours had similar effects: Cl concentrations converged on a value about 400 ppm, and high levels of Na near the margin were reduced. S concentrations were apparently unaffected, however, whereas concentrations of K were overall lower than in the control otoliths. Delayed extraction also affected Ca, which was slightly elevated in the treatment otoliths. Reflecting this, delayed extraction also lowered the mean and range of Sr/Ca ratios. Finally, preserving fish in a chilled hyper-saline solution markedly increased Cl concentrations, up to nearly 7000 ppm in one individual, while also increasing Na and S concentrations and depressing K values.

Measured concentrations of K, S and Cl were elevated all along the growth axis of otoliths removed from frozen and ethanol-preserved samples. Apparent ontogenetic variability for these elements in preserved samples bore little or no resemblance to that measured in fresh fish.

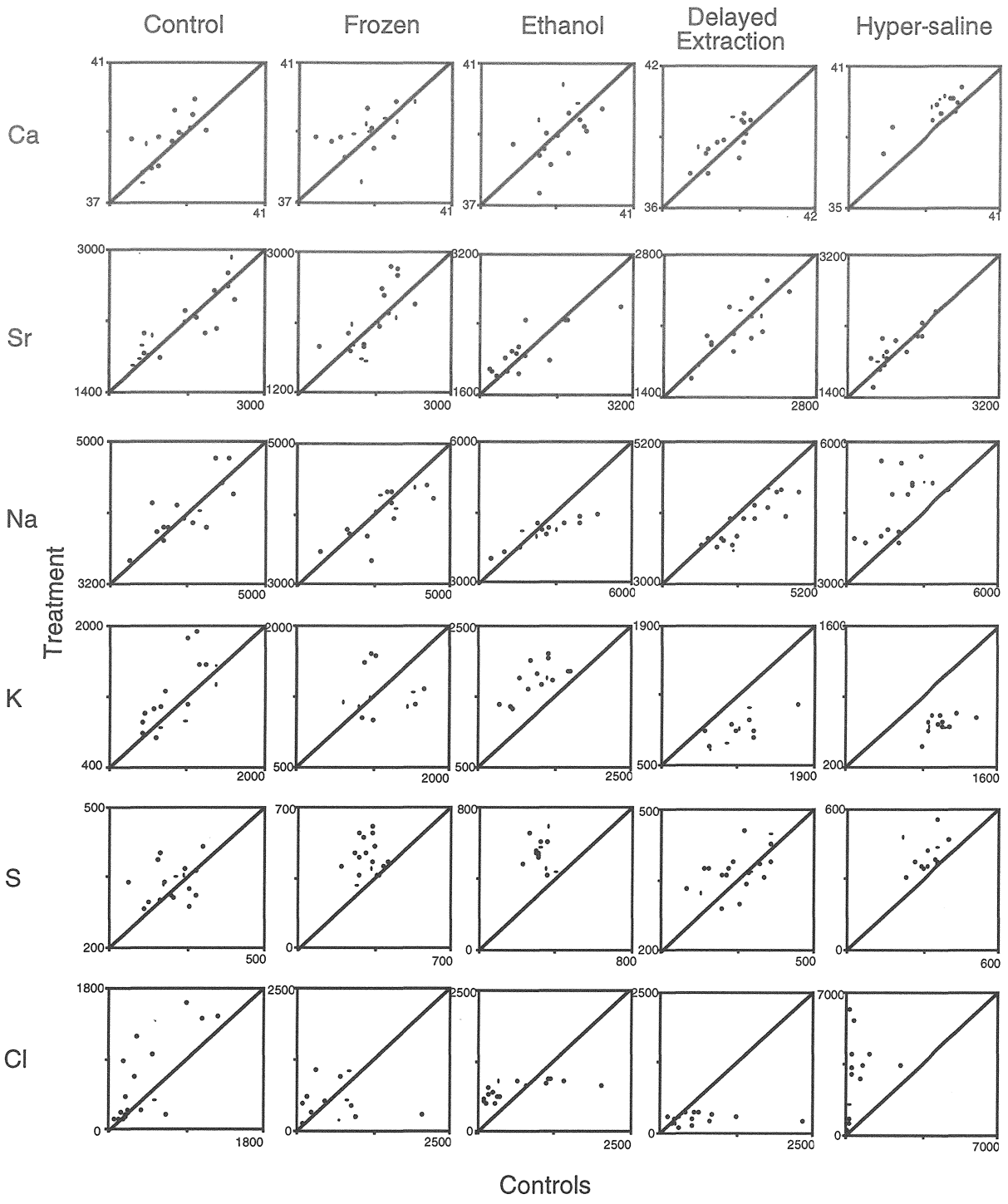


Figure 6. Effects of different post-mortem handling techniques on apparent concentrations of the minor elements in otoliths

Experiment #2. Effects of storage in chilled seawater on the composition of otoliths of orange roughy, *Hoplostethus atlanticus*.

A standard procedure for storage of commercial catches at sea is to refrigerate whole fish in chilled (-1 to 0 °C) sea water. To test the effect of this practice on otolith composition, we removed one otolith from each of twenty-five adult orange roughy (35-40 cm SL) immediately after they came on board from a trawl off south western Australia in June 1994. As in Experiment #1, the flesh excised to remove each otolith was pinned back into place, after which the fish were put with the rest of the catch in the refrigerated tanks. The second otolith was removed in port, 57 hours later. Following extraction, the otoliths were brushed clean and stored dry until preparation for analysis.

Otolith margins was analysed using the ED microprobe (20 kV, 14 x 12 µm raster). Replicate analyses were done on some margins (adjacent to each other along the margin, but with no overlap of 'burn-scars'), in which case mean concentrations were calculated for each individual. The primordia of five pairs of otoliths were analysed using the WD microprobe (15 kV, 50 nA, 50 µm beam diameter).

Results

As expected with the ED system, measurement errors are large for the margin analyses. Nonetheless, concentrations of Na and Cl decreased significantly following storage in chilled seawater (Fig. 7). Sr and S appear to be unaffected, while there is evidence of increased variability in K values, though most of the scatter is within calculated measurement errors.

Sample sizes for analyses at the primordium are small, but there is no obvious effect on this region of the otolith.

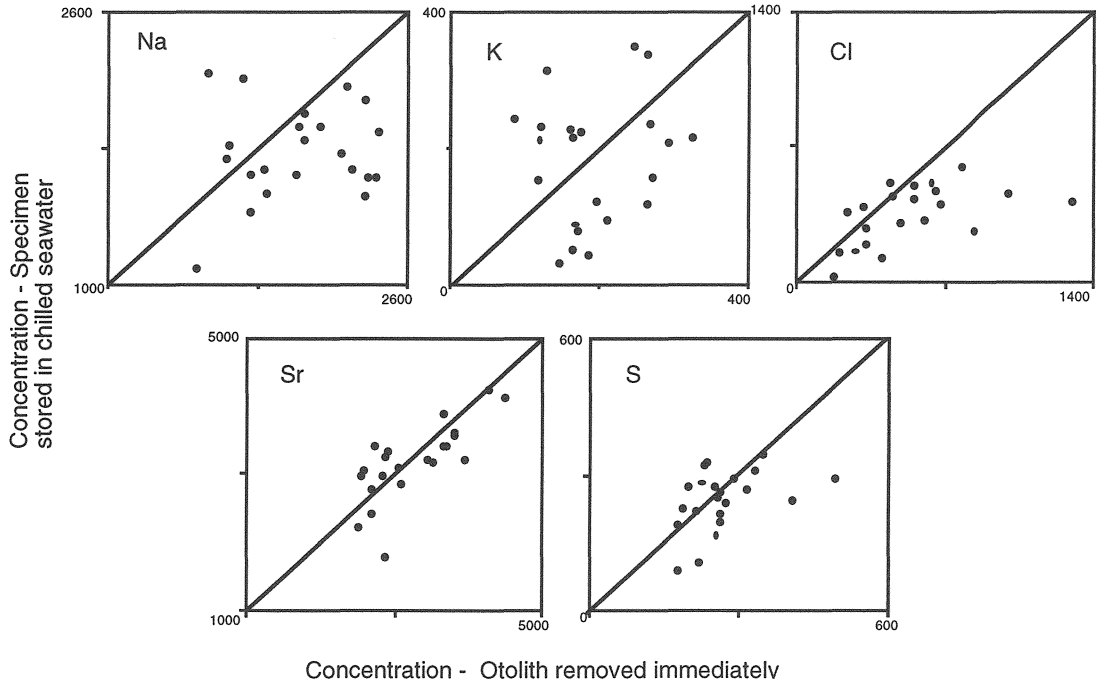


Figure 7. Effects of storing orange roughy otoliths in chilled seawater on apparent concentrations of the minor elements in their otoliths.

Experiment #3. Effects of delayed extraction and preservation in ethanol on the composition of otoliths of juvenile sole, *Rhombosolea tapirina*

To assess the effect of the procedures typically used to collect, process and preserve larvae on otolith composition, we experimented with short-term (30 min) delayed extraction (approximating the time a larva might be dead in a plankton net) and preservation in ethanol (which is typically used for larval samples) for three weeks. As larval fish were not available to us, we used instead newly settled (65 days post-hatching) greenback flounder, *Rhombosolea tapirina*, reared in captivity. Although post-settlement, the size of the fish (8 to 13 mm SL) and their otoliths was similar to larvae. Experimental protocols paralleled Experiment #1. Otoliths were analysed using the WD microprobe (15 kV, 25 nA, 14 μm beam diameter).

Results

Delaying otolith extraction by thirty minutes had only a slight effect on the composition of the flounder otoliths (Fig. 8). Of the six elements measured, only Sr showed a consistent deviation from the expected 1:1 relationship between control and treatment otoliths, with all but one of the values higher in the treatment than in the corresponding control. The difference between control and treatment is highly significant, with an intercept value for the regression about 200 ppm higher than expected if the treatment had no effect. Sr/Ca ratios are correspondingly also elevated in the treatments. For the other five elements, there was a very wide scatter of points about the predicted regression line, but no systematic loss or increase in apparent concentrations.

Preservation of the small juveniles for three weeks in ethanol had a dramatic effect on apparent Cl concentrations, which declined overall (paired t-test, $p<0.001$) and converged on a common value of about 200 ppm (Fig. 8). Ca concentrations generally declined as well.

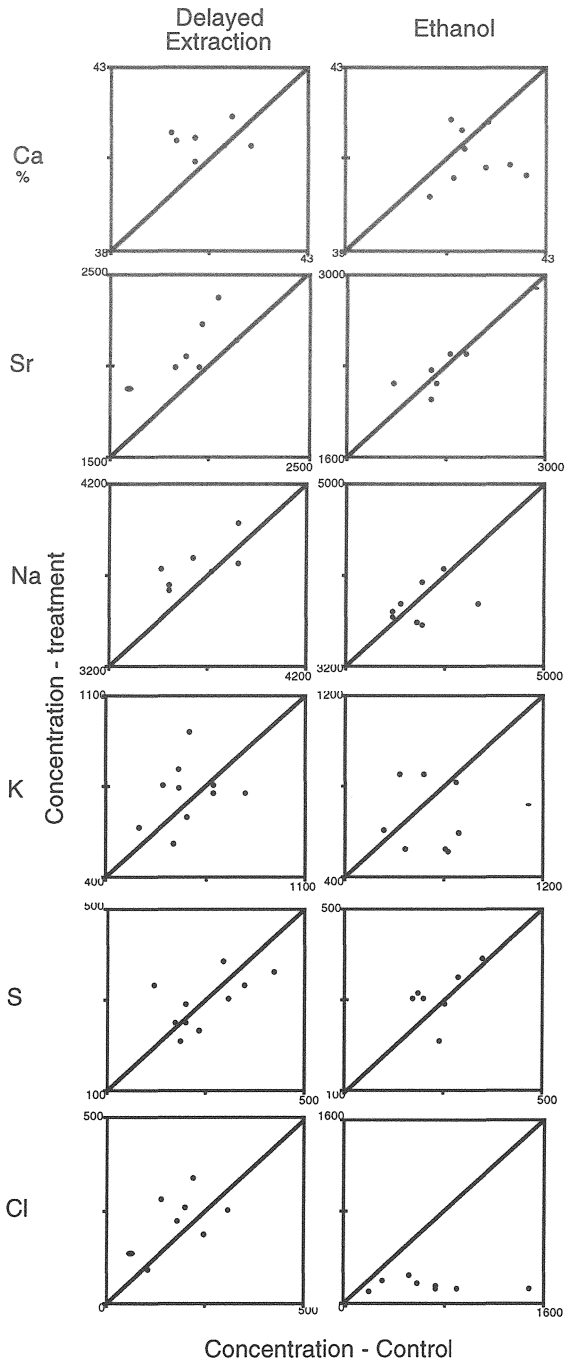


Figure 8. Effects of delayed extraction and ethanol preservation on apparent concentrations of the minor elements in otoliths of larval flounder, *Rhombosolea tapirina*.

Experiment #4: Effects of immersion in freshwater on the composition of otoliths of adult jackass morwong, *Nemadactylus macropterus*

Thresher et al. (1994) reported large, inexplicable differences in the composition of otolith pairs removed from adult *N. macropterus*. A review of the treatment of these otoliths indicated that in all cases one member of each pair had been 'aged' by the Australian Central Aging Facility (CAF) prior to microanalysis. Analysis of four additional pairs of normal and

'aged' otoliths confirmed large and consistent differences, particularly with regard to K and Cl.

The CAF 'aged' the otoliths whole by visual enumeration of annuli, but immersed them in tap water during the viewing period ("one to four minutes") to improve the clarity of the growth bands. To test whether immersion in freshwater could account for the observed differences in composition, we conducted two experiments. In the first, we prepared one otolith from a frozen adult morwong as normal, but immersed the second otolith in Hobart, Tasmania tap water for 20 mins and then dried it at 40° C for 3 hours prior to processing. The second trial was similar, but involved otoliths from freshly killed adult morwong and distilled water. Life history scans were done on all otoliths, using the WD microprobe (15 kV, 25 nA, 14 μm beam diameter, 25 μm steps).

Results

The experiment in which one otolith was immersed in tap water produced data similar to the 'aged' otoliths. By comparison with its control, the tap-water immersed specimen had very low concentrations of K and Cl, slightly depleted S levels and slightly elevated Na concentrations (Fig. 9). Apparent ontogenetic patterning also differed substantially between the normal and tap-water immersed specimens.

In contrast, immersing fresh otoliths in distilled water had only a slight effect on most elements. For both pairs of otoliths, mean concentrations and gross ontogenetic patterns are similar for Sr, S, Ca and Cl. The Cl data may not be very informative, however, as by chance both of the controls had consistently low Cl concentrations anyway. Na and K are also generally similar between controls and treatments, though in both pairs K is depleted in the outer third of the immersed otoliths.

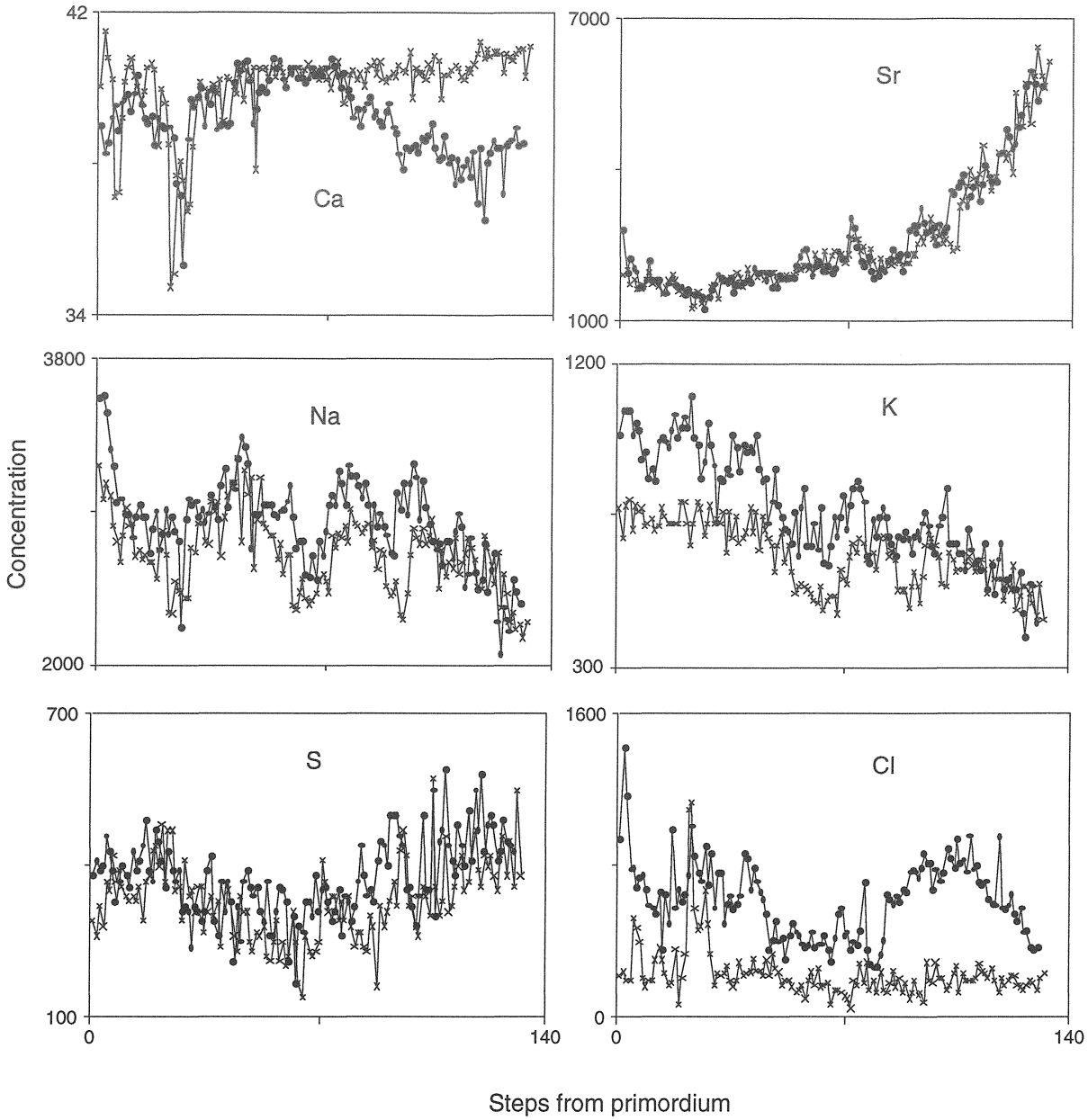


Figure 9. Effects of immersion in tap water (crosses) on the apparent concentration (ppm, except Ca which is % wt fraction) of elements along the growth axis of a jackass morwong otolith. Filled circles are the controls.

Experiment #5. Effects of surface preparation on apparent composition of otoliths of adult jackass morwong, *Nemadactylus macropterus*

The standard procedure to prepare an otolith for probe microanalysis is to section it to expose internal growth structures. Sectioning is typically done using a geological cutting saw, during which water is dripped onto the specimen to cool it (preventing heat fracturing) as it is cut. Following cutting, otoliths are also usually ground and polished using sand papers and abrasives, also usually wet. Finally, at different points in the process, the otoliths and sections are typically cleaned in an ultrasonic bath, to remove residual abrasives, again in water or a solvent such as ethanol.

Given the apparent effects of water immersion on otolith composition, we tested whether preparing the surface of an otolith for analysis affected its apparent composition. Specifically,

we compared the composition of a prepared surface with that from an 'unprocessed' fractured surface. Otoliths from frozen, adult *N. macropterus* were fractured dorso-ventrally through the primordium and the composition of suitable fracture plains, close to the otolith margin, were assessed using the ED electron probe (20 kV, 14 x 12 µm raster). Three analyses were done on each of five otoliths, at the probe positions approximately 30 µm apart (surfaces permitting). The otolith fragments were then embedded in resin and prepared using our standard procedures to expose sections adjacent to and encompassing the same daily growth increments as the fracture points analysed.

If surface preparation did not affect apparent composition, the regression plots of the paired analyses should show a moderately wide scatter (due to inherent imprecision of the ED probe microanalyser), but also consistently lower values for the fractured surface (due to effects of surface topography on X-ray absorption and scattering - see Gunn et al. 1992). That is, the data points should predominantly lie above and to the left of the 1:1 regression line on a plot with values for the fractured surface as the abscissas and values for the processed surface as the ordinates.

Results

Suitability of the fractured surfaces for probe analysis was evaluated by examining the apparent Ca concentrations; Ca is particularly sensitive to the effects of surface topography on apparent concentrations (Gunn et al. 1992). For one of the fifteen fracture surfaces analysed, measured Ca was less than 30% (as compared with an expected value of about 38%) and the point was discarded. For the other surfaces, Ca ranged from 35.6 to 38.4% (Fig. 10).

Of the six elements measured, only Ca and Sr plotted as if surface preparation had no impact on apparent composition (Fig. 10). Measured concentrations of all four other elements are higher on the fracture surfaces than on the prepared surfaces. For S, K and Cl, the means and variances differ significantly between the two surface types ($p<0.01$ in all cases); the means do not quite differ in Na (paired $t = 1.83$, $p<0.1$), but given a null hypothesis that apparent concentrations would be higher on processed surfaces, the tendency towards a higher mean on fracture surfaces is probably meaningful. Ratioing the other elements to Ca has no effect on the results.

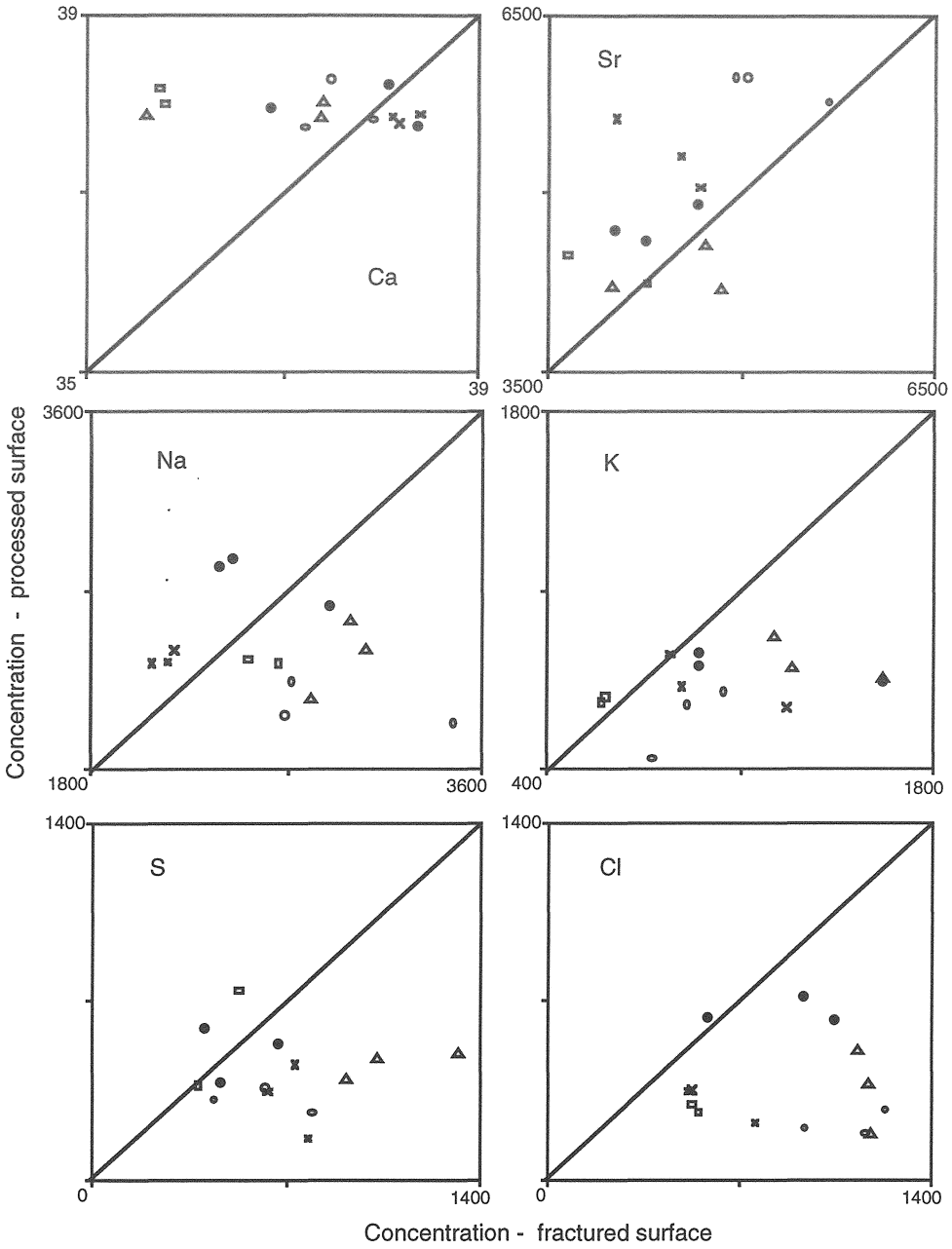


Figure 10. Effects of surface preparation on concentrations (ppm, except Ca which is % wt fraction) of six elements in otoliths of jackass morwong (different symbols indicate multiple measurements in different otoliths).

Discussion and Conclusions

The assumption that otoliths, when analysed, necessarily or even typically retain the composition they had in the live fish is clearly not valid. Every element we measured was affected by at least one treatment, and a range of very commonly used procedures, such as washing otoliths in water or collecting them from frozen fish, significantly alters the composition of an otolith from what it was pre-handling. The ubiquity of some of the factors that alter otolith composition is particularly concerning. In the light of what appear to be massive effects of cutting and polishing on four of the six elements examined, for example, we have to conclude that electron probe-based studies of otoliths that involve these elements are of dubious validity. This includes the pilot study on orange roughy stock structure, where all of the differences among sites, to a very large extent, derive from analysis of Na, K and Cl. All three elements are highly sensitive to the effects of post-mortem handling.

Not all such studies are necessarily invalid, however. Although all of the elements we measured were affected by one or more of the experimental procedures, the elements clearly fell into two groups based on their sensitivity. Sr and Ca (and Sr/Ca ratios) were unaffected by most of the procedures we tried. Delayed extraction had sporadic effects on these elements, suggesting it would be prudent in the future to base results on otoliths removed immediately from fresh or freshly frozen specimens, but otherwise we conclude that these elements, at least, are relatively robust to the types of specimen handling and sample preparation typically reported in the literature.

Na, K, Cl and S were much more sensitive to the effects of handling. Overall, the pattern of responses was consistent among experiments, indicating a lability that cannot easily be ascribed to small sample sizes or to unusual conditions. Beyond this conclusion, however, interpreting the data is not straight-forward. For example, that cutting and polishing an otolith alters the composition of the surface being prepared implies that the data we collected to test the effects of preservation techniques incorporate both the effects of surface preparation and the effects of preservation per se. Our use of control and experimental otoliths in each test allows for some partitioning of the two effects, but how the two interact is not clear, and may well vary from experiment to experiment. As an example, the decline in Cl levels in otoliths of *Hoplostethus atlanticus* stored in chilled seawater after capture may be (1) the result of the preservation technique itself, (2) due to the 57 hour delay in extracting the otolith (since delayed extraction had a similar effect on Cl), (3) because Cl is more easily washed out of a preserved surface than a fresh one, or (4) some combination of any or all of the above. Our experiments are not adequate to discriminate among these hypotheses. We also suspect, but do not have sufficient data yet to test, that sensitivity to, for example, preservation in ethanol differs among species, perhaps as a function of absolute elemental concentrations.

The sensitivity of Na, K and Cl to ethanol, water and brine suggests that the artifacts are due to the movement into and out of otoliths of ions that are only loosely bound in the otolith matrix. The effects of delayed extraction could derive from the same mechanism, as ions move in response to the failure of active mechanisms that maintain the ionic disequilibrium between endolymph and blood (Kalish 1989, 1991, Radtke et al. 1996). An easy movement of water-soluble ions into and out of otoliths is consistent with the earlier noted movement of fluids in and out of otoliths (see Gauldie, in press), the staining of structures inside otoliths by externally applied dyes (Albrechtsen 1968, Gauldie 1990) and recent studies that measure directly the movement of molecules into otoliths (Gauldie and Coote in press). Gauldie and his colleagues (West et al. 1994) have argued, not only that otoliths are more permeable than generally suspected, but also that this permeability significantly complicates radionuclide-based age determination (for a counter argument, see Fenton et al. 1991).

This lability suggests that measuring real ontogenetic variation in otoliths is going to be difficult for many elements due to what may be unavoidable post-mortem artifacts. Several almost universal factors that could influence composition seem likely, such as the potential redistribution of soluble elements in otoliths as they dry, redistribution and leaching during cleaning and surface preparation and stress and death-induced changes in endolymph composition (Kalish 1991) that presumably could propagate in a wave-like fashion through

an otolith. Even an observation that apparent ontogenetic variation of an element is consistent within or among species is not compelling evidence that the variability is 'real', as the otoliths could well have been collected and prepared for analysis in much the same way, producing similar artifacts.

The issue is pseudo-replication; consistency does not imply accuracy, with regard to either ontogenetic or putative geographic differences. Differences in handling could profoundly affect apparent composition, generating consistent differences between samples wholly unrelated to real differences, if any, among specimens in the wild. It is not difficult to imagine scenarios in which fish from different habitats or sites are collected and preserved in different ways (e.g. speared versus netted, brought to the surface live in short duration trawls versus dead for several hours in long ones, held in a catch bucket for a hour before being brought back to the laboratory versus frozen immediately), any of which could generate internally consistent, but spurious 'differences' between samples. Obtaining samples from several sources minimises the potential for pseudo-replication, but does not avoid it altogether, particularly where samples are drawn from a fishery. Fishers converge on a common 'best' practice for catching and preserving fish, which may well be uniform among the fleet and may differ substantially depending on the depth and topography of the fished grounds and their distance from ports of landing. Without direct evidence that a particular element in a particular species/situation is not sensitive to post-handling artifacts, it is difficult to falsify an hypothesis that apparent differences are artifacts due to pseudo-replication, as there could always be some subtle or unknown difference between the way otoliths are handled from two habitats or sites that could account for perceived differences in composition.

Testing this, and other hypotheses about otolith composition, is not simple. The apparent porosity of otoliths and the relative instability of some of the elements in them suggests that obtaining data on 'real' composition is much more difficult than has been generally assumed, again with the apparent noteworthy exception of Sr. Contamination (e.g. addition of elements not naturally present in the otolith), deletion and depletion of elements that are present and the re-distribution of elements in otoliths and on prepared surfaces can occur at any of numerous steps in the process. Our results suggest several obvious approaches to minimising these artifacts, though they cannot be ruled out entirely. These approaches include: always use otoliths removed immediately from freshly killed specimens; catch and handle specimens from different sites as similarly as possible prior to otolith extraction; minimise immersion in water; and avoid long periods of ultrasonic cleaning. As well, it might be better to wash otoliths in diluted seawater that is isotonic with endolymph than in freshwater, as it might reduce exchange rates into and out of otoliths.

Three alternative approaches might also be useful. First, where samples are known to have been caught, preserved or generally handled differently, artifacts could be tested for statistically and either factored out or demonstrated to be slight relative to apparent site- or habitat-specific differences in composition. This type of retrospective analysis may often require a sampling design more complex than is typical in most otolith studies. Second, experiments can be done to demonstrate that handling-induced artifacts are not significant for a particular species, situation and suite of elements. Third, labile elements can be ignored or leached from samples prior to their analysis. Washing otoliths in distilled water for 24 hours prior to analysis might eliminate the labile component of its composition, and perhaps provide a substrate for analysis that is unaffected by handling-induced artifacts.

The extent to which these problems affect trace elements (present at concentrations less than 100 ppm) is not known. Milton and Chenery (ms.) report mixed effects of specimen preservation (without considering confounding effects, such as alteration due to specimen preparation) on the composition of clupeoid otoliths, as measured using laser ablation ICP-MS. Most elements nominally detected were affected only slightly, though otoliths removed from frozen fish consistently tended to differ from otoliths of freshly killed samples. On first principles, the lability of some trace elements should be predictable from their chemical similarity to the elements we tested. Hence, given that Ca and Sr were minimally affected in our experiments, Mg and Ba are also likely to be relatively insensitive to effects of handling. Conversely, F and Br are likely to be as unstable as Cl, Se probably responds to the same

factors as affects S, and Li and Rb are likely to be similar to Na and K. Overall, we suggest it would be prudent to test for post-mortem artifacts on trace metal concentrations before undue reliance is placed on these elements as indicators of population structure.

C. Expanded and Revised Analysis of Stock Structure of Australian Orange Roughy, Based on Otolith Composition

In combination, the previous two sections indicate that the results of the pilot study, while perhaps accurate, are unreliable. The sensitivity of the key elements involved in the initial stock separation - Na, K and Cl - to virtually all forms of post-mortem handling suggests that even slight differences between sites in the way specimens were handled, the otoliths extracted or even in the way they were cleaned and prepared for analysis could have substantially distorted the results. The distortion could either be in the form of generating apparent differences among sites that are not real (in the sense of not reflecting *in vivo* otolith composition) or obscuring differences that would otherwise be present. Given the implications of management decisions based on evidence of stock structuring in the fisheries, it would seem unwise to base those decisions on information derived from elements whose reliability is uncertain.

Hence, the up-dated and expanded evaluation of stock structure of orange roughy involves three modifications to the pilot project:

1. increased sample sizes and sources for all sites, including replicating the original samples/sites;
2. increased coverage, to include more sites in and immediately adjacent to the Australian EEZ; and
3. re-analysis based only on elements demonstrated or likely to be insensitive to the effects of post-mortem handling and specimen preparation.

All fish for the analysis were caught on commercial vessels. Sample locations and details are provided in Figure 1 and Table 1. Even though we examined only those elements we think are largely unaffected by post-mortem processing, we nonetheless only used otoliths extracted from fresh or whole frozen fish, wherever possible. The exception was the single sample we were able to obtain from Western Australia, which had been stored in chilled seawater.

As in the prior work, we detected seventeen elements in *H. atlanticus* otoliths, six using the electron microprobe and eleven using the proton microprobe. Two trace elements detected occasionally, cobalt and molybdenum, are almost certainly contaminants (see Sie and Thresher, 1992) and were ignored in subsequent analyses; for the same reason, we also excluded the occasional very high reading for iron and nickel.

Life history scans of juvenile and adult roughy indicate high levels of variability within and among specimens (Fig. 11). Among the Australian specimens, only strontium shows evidence of a consistent ontogenetic patterning, with all specimens exhibiting a single or double peak in Sr concentrations within 1000 μm of the primordium, followed by an irregular decline to the exterior of the otolith. Sulphur shows little or no overt patterning in any specimen, whereas sodium, potassium and chlorine differ in pattern from specimen to specimen, but tend to co-vary within specimens. Concentrations of chlorine correlate with those of sodium in all four juveniles we've examined to date (correlation coefficient ranging from 0.54 to 0.93, $p < 0.001$ in all cases); otherwise correlations among elements within specimens are inconsistent among specimens. Ontogenetic variability is also high in the trace elements. However, in most instances, detection is just above the respective minimum detection limits (which differ among elements), and there is little or no indication of long term ontogenetic trends in concentration.

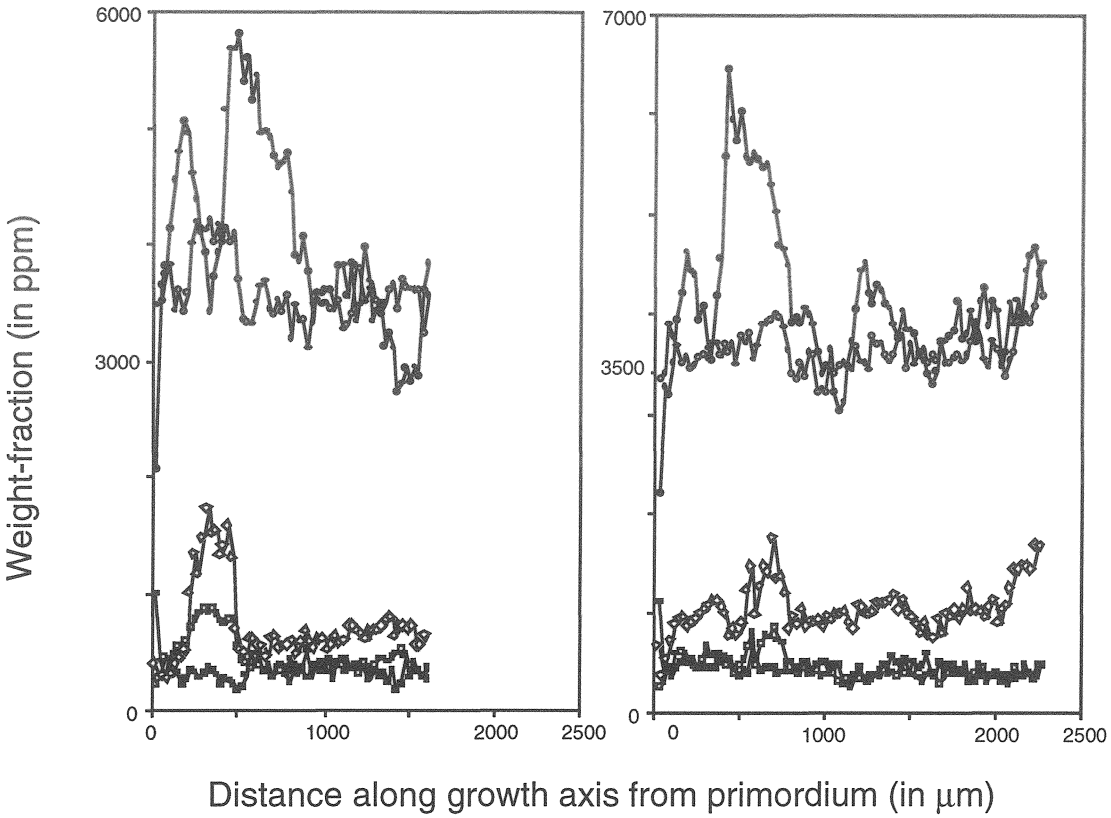


Figure 11. Life history scans of juvenile (<10 cm.) orange roughy, indicating variability across otoliths in concentrations of the minor elements.

As discussed above, four of the five micro-constituents routinely measured in otoliths provide unreliable information regarding stock structure. These elements are sodium, potassium, chlorine and sulfur. Also as noted earlier, several elements measured using the proton probe are also of dubious validity - in particular iron and nickel - due to conspicuous effects of stainless steel contaminants on measured concentrations. Consequently, of the elements we measure using the two analytical instruments, only five (Sr, Zn, Cu, Hg and Pb) routinely occurred at high enough concentrations and appeared to be free of artifacts, to justify their use in analysis of stock structure. We also excluded Ca for the analysis, on the basis that it can be expected to occur at concentrations about 38% (by weight) and deviations from that are likely to be statistical artifacts, rather than real differences.

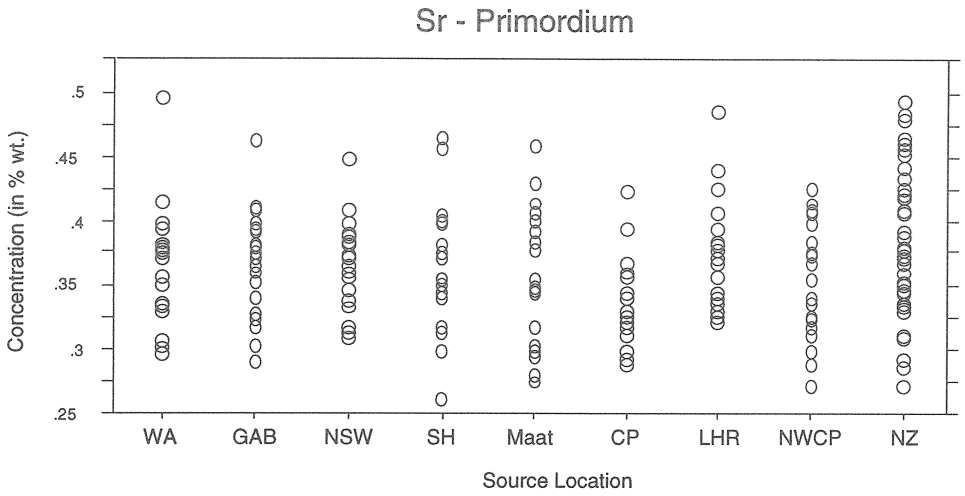
Analysis of New Samples: Primordium

For an analysis of the Australian stocks, we pooled samples from two sites in the NZ EEZ (South Challenger Plateau and Bay of Plenty) to constitute a NZ out-group. Obtaining samples from almost all sites proved difficult, largely due to uncertainties of targeting by individual vessels and to recent changes in fishing effort. Reflecting the latter, we had particular difficulties getting samples from the Southern Zone and from the Lord Howe Rise. In both instances, only winter (spawning season) samples were available, so analysis was restricted to only that time of year.

Samples were examined in two groups, both consisting of a random mix of individuals from all sites. The target sample size of twenty individuals was met for all but a few sites on the electron probe. However, because of beam time limitations and limited availability of samples from some sites, we were able to only probe about 15 specimens per site on the proton microprobe.

For the analysis, Sr data are given for both the primordium and for the position 200 μm from the primordium, both obtained using the electron probe. Data for the remaining elements, which were collected using the proton microprobe, are an integration across that distance, due to the deeper analytic depth of the beam.

Basic results are given in Figs 12a-f. Each depicts a) a scatterplot of individual values from each of the sites (or sites pooled, in the case of the NZ EEZ), b) summary output of a one factor ANOVA of concentration against site, and c) pair-wise comparisons among sites, based on a post-hoc analysis of the ANOVA (a Fisher's PLSD test), to identify the significant outlying sites, if any.



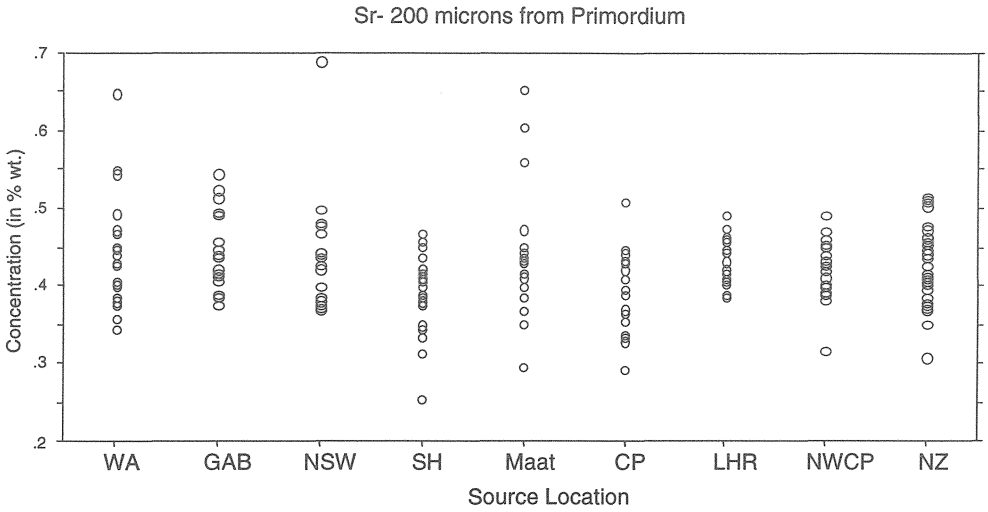
ANOVA Table for Sr - Primordium

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Source Location	8	.036	.004	1.986	.0502
Residual	187	.420	.002		

Fisher's PLSD for Sr - Primordium
Effect: Source Location
Significance Level: 5 %

	Mean Diff.	Crit. Diff	P-Value	
WA, GAB	-2.000E-4	.030	.9894	
WA, NSW	-.001	.030	.9522	
WA, SH	.004	.030	.7976	
WA, Maat	.013	.030	.3744	
WA, CP	.026	.030	.0947	
WA, LHR	-.010	.030	.5346	
WA, NWCP	.010	.030	.5077	
WA, NZ	-.020	.026	.1238	
GAB, NSW	-.001	.030	.9628	
GAB, SH	.004	.030	.7874	
GAB, Maat	.014	.030	.3673	
GAB, CP	.026	.030	.0921	
GAB, LHR	-.009	.030	.5431	
GAB, NWCP	.010	.030	.4992	
GAB, NZ	-.020	.026	.1275	
NSW, SH	.005	.030	.7517	
NSW, Maat	.014	.030	.3431	
NSW, CP	.026	.030	.0837	
NSW, LHR	-.009	.030	.5736	
NSW, NWCP	.011	.030	.4702	
NSW, NZ	-.019	.026	.1413	
SH, Maat	.010	.030	.5271	
SH, CP	.022	.030	.1555	
SH, LHR	-.013	.030	.3843	
SH, NWCP	.006	.030	.6846	
SH, NZ	-.024	.026	.0672	
Maat, CP	.012	.030	.4243	
Maat, LHR	-.023	.030	.1382	
Maat, NWCP	-.003	.030	.8208	
Maat, NZ	-.034	.026	.0110	S
CP, LHR	-.035	.031	.0256	S
CP, NWCP	-.016	.030	.3068	
CP, NZ	-.046	.026	.0007	S
LHR, NWCP	.020	.030	.2063	
LHR, NZ	-.011	.027	.4347	
NWCP, NZ	-.030	.026	.0220	S

Figure 12a. ANOVA of Sr - Primordium by Source Location



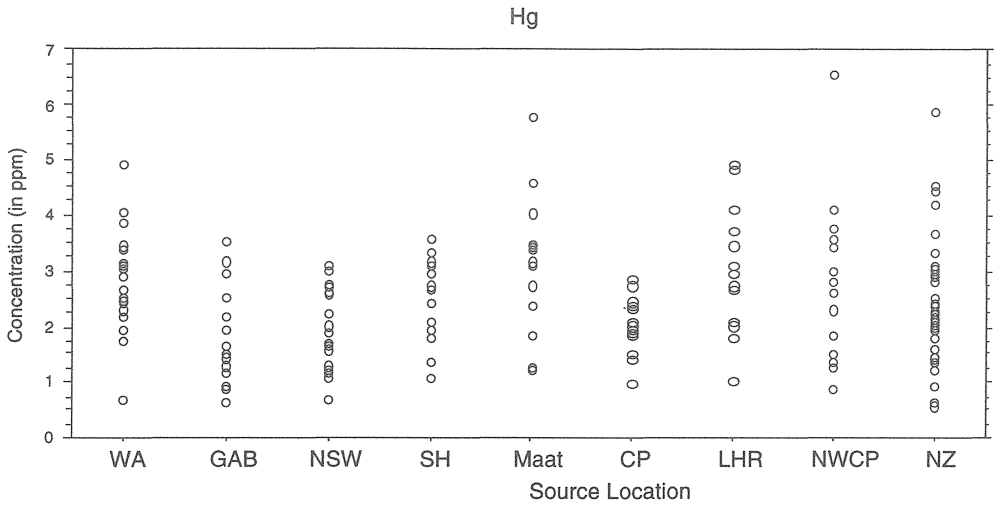
ANOVA Table for Sr - 200 microns

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Source Location	8	.072	.009	2.821	.0056
Residual	187	.600	.003		

Fisher's PLSD for Sr - 200 microns
Effect: Source Location
Significance Level: 5 %

	Mean Diff.	Crit. Diff	P-Value	
WA, GAB	-5.000E-5	.035	.9978	
WA, NSW	.003	.035	.8650	
WA, SH	.051	.035	.0049	S
WA, Maat	.001	.035	.9711	
WA, CP	.054	.036	.0033	S
WA, LHR	.010	.036	.5879	
WA, NWCP	.022	.035	.2118	
WA, NZ	.012	.031	.4394	
GAB, NSW	.003	.035	.8628	
GAB, SH	.051	.035	.0049	S
GAB, Maat	.001	.035	.9689	
GAB, CP	.054	.036	.0032	S
GAB, LHR	.010	.036	.5860	
GAB, NWCP	.023	.035	.2108	
GAB, NZ	.012	.031	.4375	
NSW, SH	.048	.035	.0081	S
NSW, Maat	-.002	.035	.8936	
NSW, CP	.051	.036	.0054	S
NSW, LHR	.007	.036	.7065	
NSW, NWCP	.019	.035	.2804	
NSW, NZ	.009	.031	.5632	
SH, Maat	-.050	.035	.0055	S
SH, CP	.003	.036	.8645	
SH, LHR	-.041	.036	.0271	S
SH, NWCP	-.029	.035	.1128	
SH, NZ	-.039	.031	.0134	S
Maat, CP	.053	.036	.0036	S
Maat, LHR	.009	.036	.6124	
Maat, NWCP	.022	.035	.2253	
Maat, NZ	.011	.031	.4644	
CP, LHR	-.044	.037	.0190	S
CP, NWCP	-.032	.036	.0829	
CP, NZ	-.042	.031	.0087	S
LHR, NWCP	.012	.036	.4995	
LHR, NZ	.002	.032	.8976	
NWCP, NZ	-.010	.031	.5064	

Figure 12b. ANOVA of Sr at 200µm by Source Location



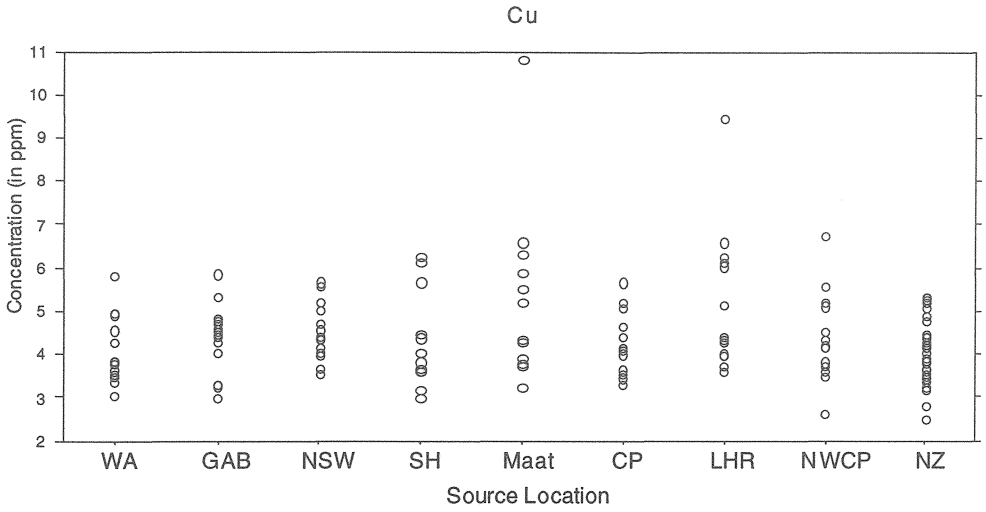
ANOVA Table for Hg (ppm)

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Source Location	8	24.841	3.105	2.890	.0051
Residual	150	161.183	1.075		

Fisher's PLSD for Hg (ppm)
Effect: Source Location
Significance Level: 5 %

	Mean Diff.	Crit. Diff	P-Value	
WA, GAB	.936	.716	.0108	S
WA, NSW	.875	.693	.0137	S
WA, SH	.352	.746	.3525	
WA, Maat	-.301	.730	.4164	
WA, CP	.795	.730	.0329	S
WA, LHR	-.080	.716	.8251	
WA, NWCP	.042	.730	.9100	
WA, NZ	.365	.584	.2182	
GAB, NSW	-.061	.726	.8680	
GAB, SH	-.584	.776	.1392	
GAB, Maat	-1.237	.761	.0016	S
GAB, CP	-.140	.761	.7161	
GAB, LHR	-1.016	.748	.0081	S
GAB, NWCP	-.894	.761	.0217	S
GAB, NZ	-.571	.622	.0720	
NSW, SH	-.523	.755	.1731	
NSW, Maat	-1.176	.739	.0020	S
NSW, CP	-.079	.739	.8325	
NSW, LHR	-.955	.726	.0102	S
NSW, NWCP	-.833	.739	.0275	S
NSW, NZ	-.509	.595	.0929	
SH, Maat	-.653	.789	.1041	
SH, CP	.444	.789	.2684	
SH, LHR	-.432	.776	.2731	
SH, NWCP	-.310	.789	.4386	
SH, NZ	.013	.656	.9680	
Maat, CP	1.096	.774	.0058	S
Maat, LHR	.221	.761	.5674	
Maat, NWCP	.343	.774	.3829	
Maat, NZ	.666	.638	.0408	S
CP, LHR	-.876	.761	.0244	S
CP, NWCP	-.754	.774	.0563	
CP, NZ	-.430	.638	.1849	
LHR, NWCP	.122	.761	.7518	
LHR, NZ	.445	.622	.1593	
NWCP, NZ	.323	.638	.3183	

Figure 12c. ANOVA of Hg by Source Location



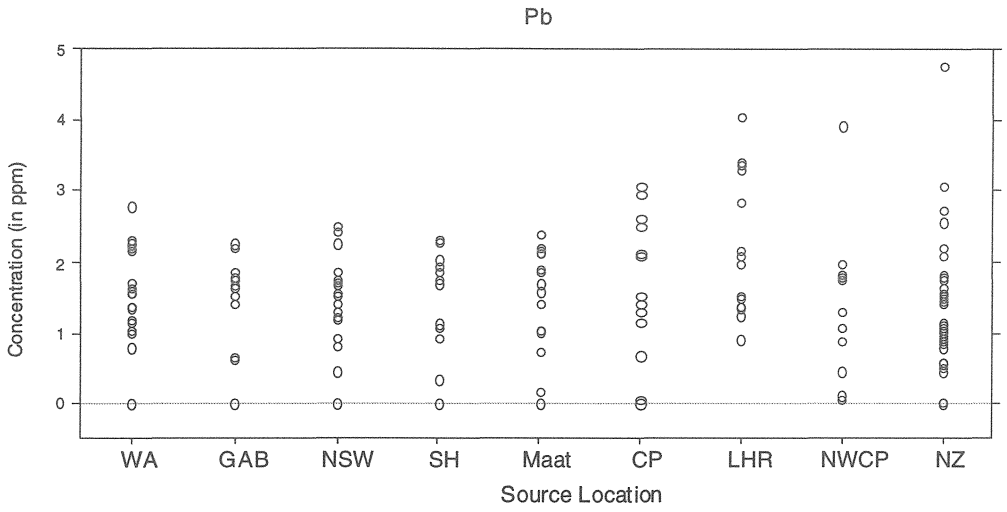
ANOVA Table for Cu (ppm)

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Source Location	8	18.471	2.309	2.042	.0454
Residual	144	162.789	1.130		

Fisher's PLSD for Cu (ppm)
Effect: Source Location
Significance Level: 5 %

	Mean Diff.	Crit. Diff	P-Value	
WA, GAB	-.185	.767	.6338	
WA, NSW	-.330	.755	.3890	
WA, SH	-.029	.796	.9431	
WA, Maat	-.861	.781	.0309	S
WA, CP	-.016	.796	.9689	
WA, LHR	-.834	.767	.0334	S
WA, NWCP	-.096	.781	.8079	
WA, NZ	.144	.641	.6584	
GAB, NSW	-.145	.755	.7052	
GAB, SH	.157	.796	.6982	
GAB, Maat	-.676	.781	.0893	
GAB, CP	.170	.796	.6744	
GAB, LHR	-.649	.767	.0969	
GAB, NWCP	.089	.781	.8219	
GAB, NZ	.329	.641	.3119	
NSW, SH	.301	.785	.4491	
NSW, Maat	-.531	.769	.1744	
NSW, CP	.314	.785	.4297	
NSW, LHR	-.504	.755	.1894	
NSW, NWCP	.234	.769	.5487	
NSW, NZ	.474	.626	.1370	
SH, Maat	-.832	.809	.0439	S
SH, CP	.013	.824	.9750	
SH, LHR	-.805	.796	.0475	S
SH, NWCP	-.067	.809	.8695	
SH, NZ	.172	.675	.6144	
Maat, CP	.845	.809	.0408	S
Maat, LHR	.027	.781	.9451	
Maat, NWCP	.765	.794	.0590	
Maat, NZ	1.005	.657	.0030	S
CP, LHR	-.818	.796	.0441	S
CP, NWCP	-.080	.809	.8445	
CP, NZ	.159	.675	.6415	
LHR, NWCP	.738	.781	.0639	
LHR, NZ	.978	.641	.0030	S
NWCP, NZ	.240	.657	.4717	

Figure 12d. ANOVA of Cu by Source Location



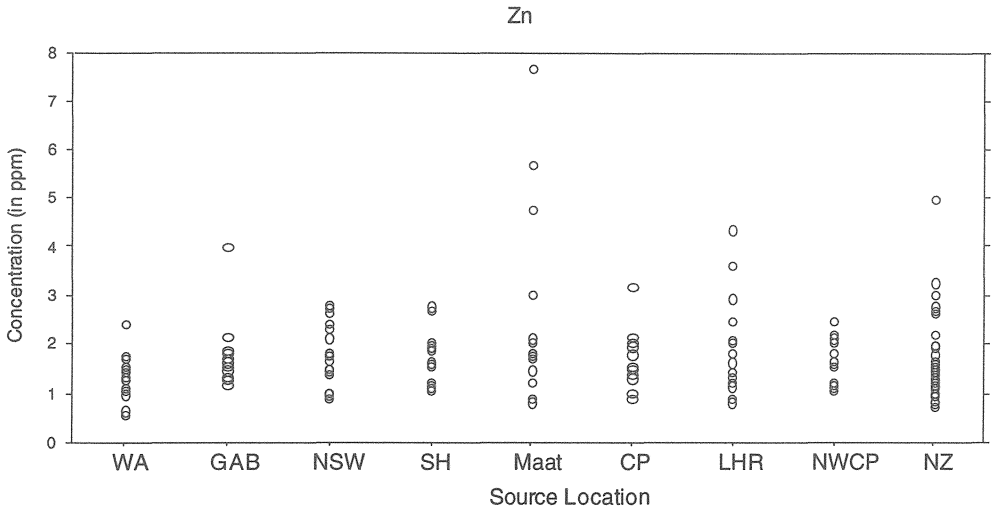
ANOVA Table for Pb (ppm)

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Source Location	8	10.697	1.337	1.923	.0604
Residual	151	104.988	.695		

Fisher's PLSD for Pb (ppm)
Effect: Source Location
Significance Level: 5 %

	Mean Diff.	Crit. Diff	P-Value	
WA, GAB	.124	.576	.6723	
WA, NSW	.050	.549	.8581	
WA, SH	.038	.600	.9000	
WA, Maat	.063	.587	.8329	
WA, CP	-.137	.587	.6446	
WA, LHR	-.781	.576	.0082	S
WA, NWCP	.176	.587	.5553	
WA, NZ	.117	.469	.6218	
GAB, NSW	-.074	.576	.8005	
GAB, SH	-.085	.624	.7874	
GAB, Maat	-.061	.612	.8448	
GAB, CP	-.261	.612	.4011	
GAB, LHR	-.905	.602	.0034	S
GAB, NWCP	.052	.612	.8668	
GAB, NZ	-.006	.501	.9808	
NSW, SH	-.012	.600	.9696	
NSW, Maat	.013	.587	.9651	
NSW, CP	-.187	.587	.5298	
NSW, LHR	-.831	.576	.0050	S
NSW, NWCP	.126	.587	.6725	
NSW, NZ	.068	.469	.7762	
SH, Maat	.025	.635	.9391	
SH, CP	-.176	.635	.5855	
SH, LHR	-.819	.624	.0104	S
SH, NWCP	.137	.635	.6693	
SH, NZ	.079	.528	.7670	
Maat, CP	-.200	.623	.5264	
Maat, LHR	-.844	.612	.0072	S
Maat, NWCP	.113	.623	.7208	
Maat, NZ	.055	.513	.8336	
CP, LHR	-.644	.612	.0394	S
CP, NWCP	.313	.623	.3222	
CP, NZ	.255	.513	.3283	
LHR, NWCP	.957	.612	.0024	S
LHR, NZ	.899	.501	.0005	S
NWCP, NZ	-.058	.513	.8231	

Figure 12e. ANOVA of Pb by Source Location



ANOVA Table for Zn (ppm)

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Source Location	8	14.121	1.765	2.091	.0403
Residual	144	121.570	.844		

Fisher's PLSD for Zn (ppm)
Effect: Source Location
Significance Level: 5 %

	Mean Diff.	Crit. Diff	P-Value	
WA, GAB	-.351	.653	.2901	
WA, NSW	-.458	.642	.1612	
WA, SH	-.365	.678	.2888	
WA, Maat	-1.249	.665	.0003	S
WA, CP	-.288	.678	.4021	
WA, LHR	-.612	.665	.0708	
WA, NWCP	-.228	.665	.4993	
WA, NZ	-.332	.541	.2267	
GAB, NSW	-.107	.653	.7467	
GAB, SH	-.015	.688	.9666	
GAB, Maat	-.899	.675	.0094	S
GAB, CP	.062	.688	.8582	
GAB, LHR	-.261	.675	.4451	
GAB, NWCP	.123	.675	.7195	
GAB, NZ	.018	.554	.9484	
NSW, SH	.092	.678	.7884	
NSW, Maat	-.792	.665	.0199	S
NSW, CP	.169	.678	.6227	
NSW, LHR	-.155	.665	.6465	
NSW, NWCP	.230	.665	.4956	
NSW, NZ	.125	.541	.6486	
SH, Maat	-.884	.700	.0136	S
SH, CP	.077	.712	.8313	
SH, LHR	-.247	.700	.4867	
SH, NWCP	.137	.700	.6983	
SH, NZ	.033	.584	.9118	
Maat, CP	.961	.700	.0074	S
Maat, LHR	.637	.686	.0686	
Maat, NWCP	1.021	.686	.0038	S
Maat, NZ	.917	.568	.0017	S
CP, LHR	-.324	.700	.3618	
CP, NWCP	.061	.700	.8644	
CP, NZ	-.044	.584	.8813	
LHR, NWCP	.384	.686	.2703	
LHR, NZ	.280	.568	.3320	
NWCP, NZ	-.105	.568	.7160	

Figure 12f. ANOVA of Zn by Source Location

Overlap in measured concentrations among sites is extensive for all elements. Nonetheless, for two of the six parameters measured (Sr - 200 μm and Hg), differences among sites are highly significant ($p<0.01$). For all four of the other parameters measured, differences among sites all hover around the $P=0.05$ level.

Two sites are uniquely chanracterised by high levels of individual elements. The sample from the Lord Howe Rise contains Pb at levels significantly higher than any other site examined (which in turn were all similar, with Pb levels at or near the minimum detection limit for the element). Similarly, the distribution of Zn measurements among fish from Maatsuyker was significantly higher than those from all other sites. In this case, however, the modes overlap substantially and the differences are driven entirely by a few outlying individuals from Maatsuyker.

The results of evaluating the pattern of pair-wise comparisons are summarised in Fig. 13. The nine source locations aggregate into apparent six groups, based on consistently significant differences among adjacent sites. No element differs significantly between the samples from the eastern GAB and NSW, between St. Helens and the Cascade Plateau, nor between the NW Challenger Plateau sample and those from the NZ sites. Otherwise each source location can be uniquely characterised by one or more elements. The sample from Western Australia differs from its nearest neighbors on the basis of a elevated Hg concentrations; Maatsuyker differs from its nearest neighbors in concentrations of four elements (noting that differences in Zn are suspect); whereas the Lord Howe Rise sample has uniquely high Pb levels and separates from its nearest neighbors as well in Cu concentrations.

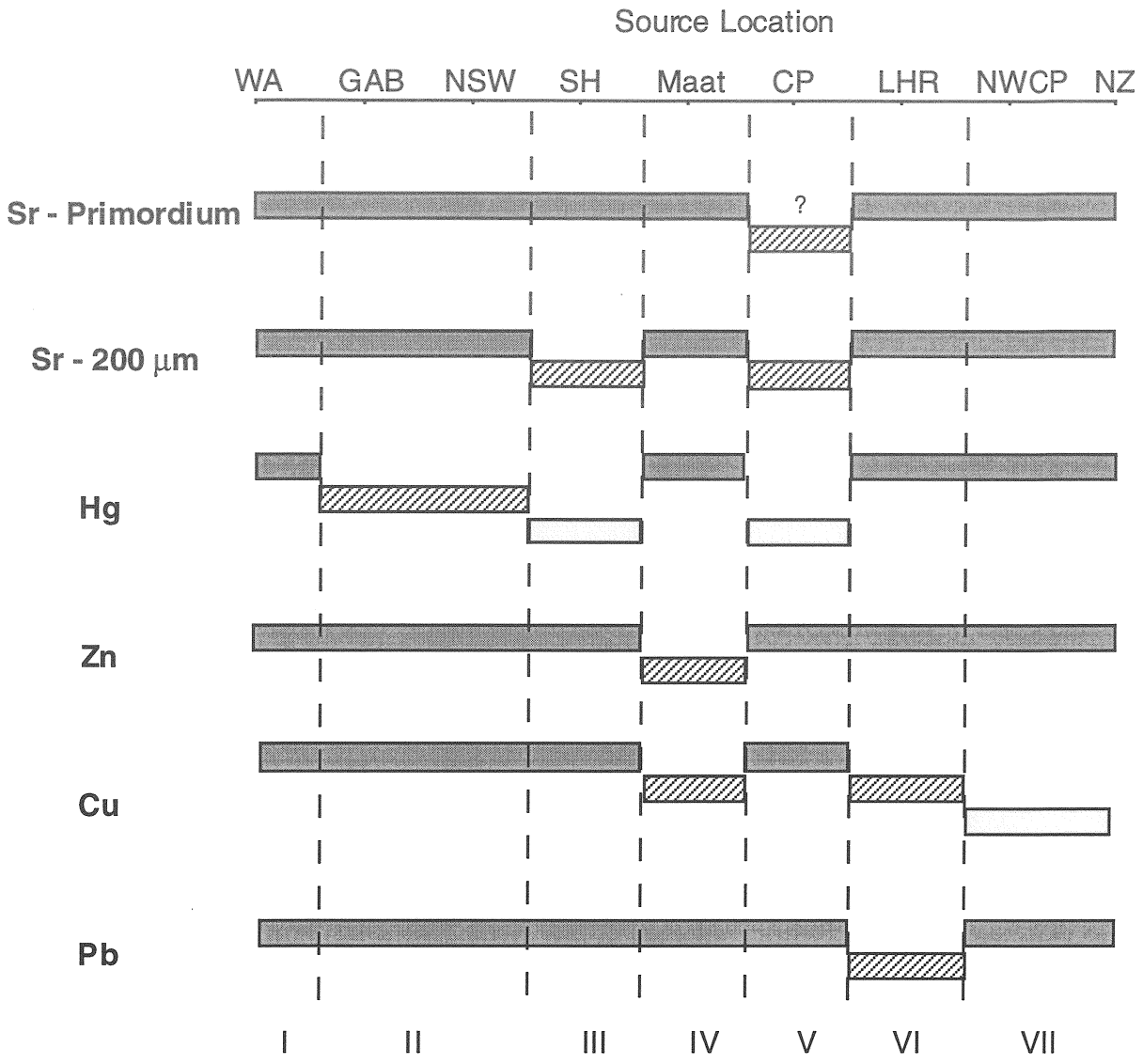


Figure 13. Apparent separaion between source locations, based on pair-wise comparsions of adjacent sites.

Analysis of New Samples: Margin

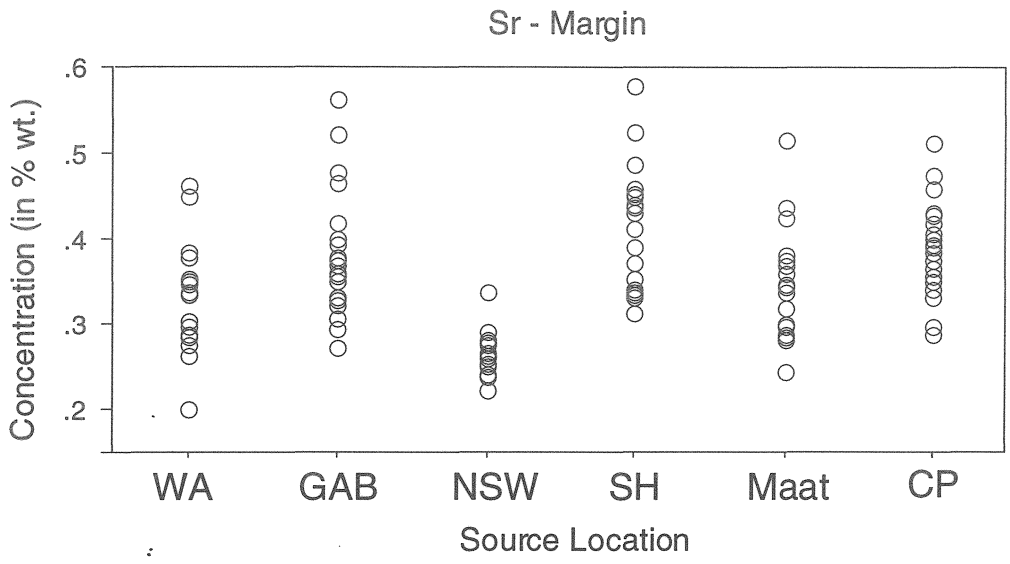
Margin scans of otolith composition were undertaken on a sub-set of the total samples, with a particular focus on potentially resolving stock issues between coastal Australian stocks. Because of time constraints, analysis include one sample each from the western GAG, the eastern GAB, NSW, eastern Tasmania (Saint Helens), southern Tasmania (Maatsuyker) and the Cascade Plateau. All samples were collected in winter, each from a single trawl in each region. The number of fish in each sample prevented the ability to confine the analysis to one sex as was previously attempted. as above, in order to minimise the effects of possible variation in otolith composition among year classes on geographic patterns it was attempted to limit the analysis to fish within the size range of 30 to 45 cm.

As the samples included fish caught on commercial vessels without an observer, post capture treatment varied between samples. This was kept to a minimum by only storing fish that could not be sampled immediately after capture on ice. No fish from brine-tank storage were used. In all cases otoliths were cleaned, dried and stored in paper envelopes following removal from fish.

The electron probe scan was done on the posterior margin of each otolith. Conditions and procedures for the analysis follow those described above.

Because of the sensitivity of other elements measured using the electron microprobe to post-mortem artifacts, only the strontium data was analysed for purposes of stock delineation.

The main results are shown in Figure 14. Strontium concentrations at the otolith margins strongly separate specimens from all adjacent sites, i.e., the specimens from WA differ significantly ($p<0.005$) from those from the eastern GAB, which differs from NSW fish ($p< 0.001$), and so forth. The only near-neighbor sites that did not differ were specimens from the Cascade Plateau and eastern Tasmania.



ANOVA Table for Sr - Margin

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Site	5	.288	.058	14.600	<.0001
Residual	114	.450	.004		

Fisher's PLSD for Sr - Margin
Effect: Site
Significance Level: 5 %

	Mean Diff.	Crit. Diff	P-Value	
CP, GAB	.003	.039	.8704	
CP, Maat	.052	.039	.0098	S
CP, NSW	.128	.039	<.0001	S
CP, SH	-.015	.039	.4657	
CP, WA	.065	.039	.0015	S
GAB, Maat	.049	.039	.0153	S
GAB, NSW	.125	.039	<.0001	S
GAB, SH	-.018	.039	.3724	
GAB, WA	.062	.039	.0024	S
Maat, NSW	.076	.039	.0002	S
Maat, SH	-.067	.039	.0011	S
Maat, WA	.013	.039	.5258	
NSW, SH	-.143	.039	<.0001	S
NSW, WA	-.063	.039	.0019	S
SH, WA	.079	.039	.0001	S

Figure 14. ANOVA of Sr at the margin by Source Location

D. Comparison with Results of the Pilot Study

The results above could derive from 1) real differences among stocks, 2) artifacts due to small sample sizes, or 3) artifacts due to handling, etc. To begin to assess the reality of the apparent differences among sites, we compared the results of the 'new' analysis with data from approximately the same sites collected as part of the pilot program. In practice this apparently simple comparison was limited by the few sites sampled in the original project and by difficulties in matching closely site and seasons of collection. Because of the limited goals of the pilot project, proton microprobe data were only collected for three sites and electron probe data for only five sites. Because of large changes in fishing effort among sites in the intervening years, it was impossible for us to replicate the WA sample, which in the pilot project was obtained from a ridge SW of Bunbury and in the second was collected off Albany. As well, the NSW samples, although both collected in the same general area, were taken during the winter spawning period in the pilot project, but in the summer subsequently. To the extent that seasonal variations in environmental conditions are incorporated into the growing otolith margin, margin analyses of winter and summer samples could well be expected to differ, even if both were drawn from the same stock; providing the two samples are from the same stock, differences in season collected should not affect analyses at or near the otolith primordium

The basic comparisons are shown in Figs. 15 and 16. For most elements, there are high levels of variability within sites, among sites and between the two samples. Visual inspection of the data suggests a degree of consistency for Cu, for Sr at the primordium and for Sr at 200 µm from the primordium. For the three other trace metals, in particular, the lack of consistency suggests that apparent differences among the three sites sampled are artifacts due to small sample sizes, rather than consistent differences among stocks.

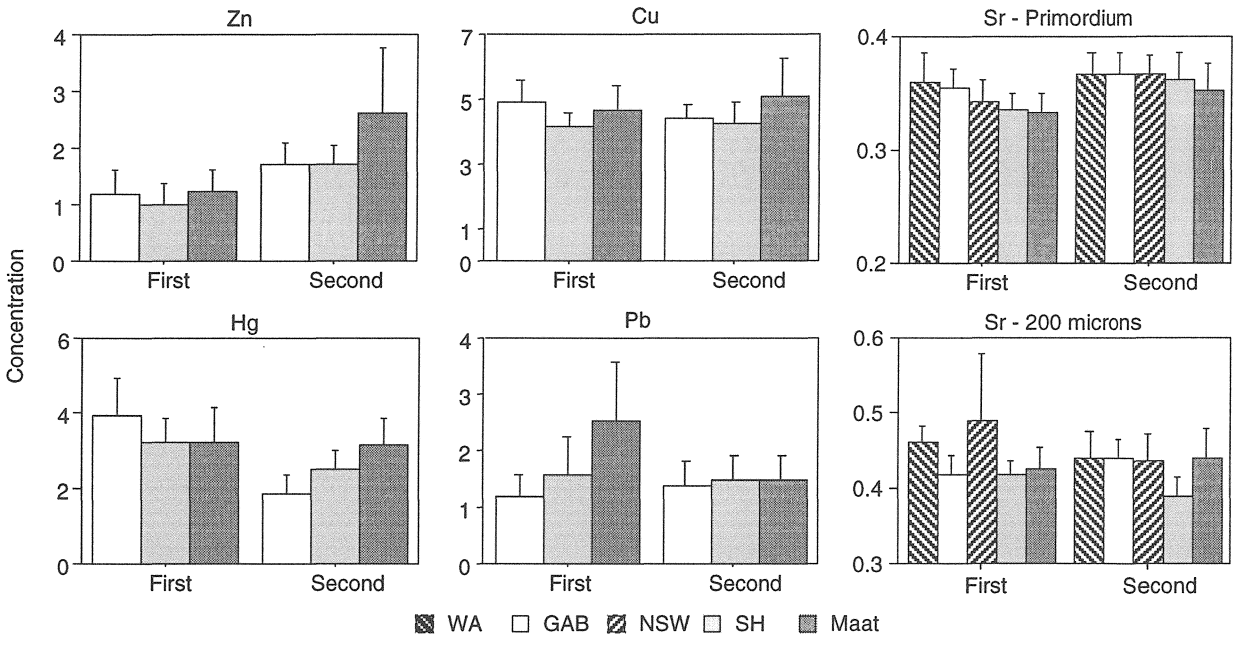


Figure 15. Comparison of apparent differences among sites run in the pilot study and in the re-analysis

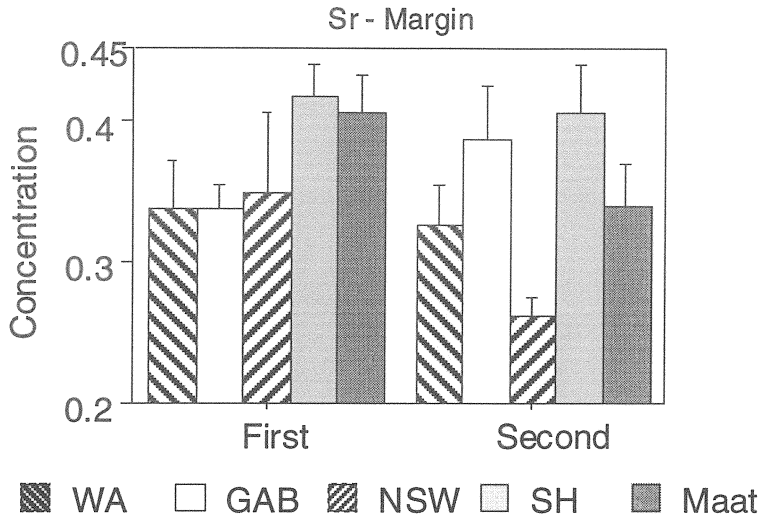


Fig. 16.´ Comparsion of apparent differences among sites run in the pilot study and in the re-analysis, for Sr at the margin

For five of the seven parameters examined, the interactive term of a two-factor (source location X sample) ANOVA is not significant; however, differences among sites are also not significant for all but two (Sr - 200 μm and Pb, at $p<0.005$ and $p<0.05$, respectively). The results for Sr - 200 μm are unaltered by removing the questionable WA comparison from the analysis. The parameters for which the interactive term is significant (i.e., there is a significant change in ranking of sites between samples) are Sr - Margin and Hg, at $p < 0.001$ and $p< 0.05$, respectively. The interactive term remains significant for the former even when NSW and WA are removed from the analysis.

E. Synthesis and Discussion

The expanded analysis of orange roughy stock structure incorporated two elements of reality checking: first, we evaluated the effects of post-mortem processing on measured concentrations of apparently key elements, and second, we compared apparent site differences as determined in the pilot project with those from roughly the same areas in the re-analysis. These two reality checks set the limits of what can reliably be inferred from applying analysis of otolith composition to questions of stock structure in roughy.

Overall, the results suggest that of the fifteen elements we can measure in roughy otoliths, about half cannot be relied upon to provide useful information on stock structure. Four of the six elements measured using an electron microprobe are potentially distorted during the process of preparing an otolith for analysis, and the comparison of site differences among samples suggest that a further four trace elements inconsistently differ among sites. For these reasons, the very large and highly significant differences we found in the pilot study between NSW and GAB roughy, or between spawning and non-spawning fish in the southern zone, have to be discounted, not because they are necessarily wrong but because the elements that drove those differences are too susceptible to post-mortem modification to be relied upon.

The analysis of orange roughy stock structure using all but the dubious micro-constituents, shown in Figure 13, indicates most sites to have regionally diagnostic 'fingerprints', implying localised roughy stocks. In particular, we were unable to distinguish, on the basis of elemental signatures 'encoded' during early larval stages (i.e., spawning areas), only between fish from the GAB and NSW and between the NW Challenger Plateau and two sites in the NZ EEZ. Otherwise each site appeared to support its own spawning population of fish, though the distinction between fish from St. Helens and the Cascade Plateau is not strong. The separation of the nine sites into six 'populations' was based on two criteria: significant differences between adjacent sites in one or more elements, and restricting pooling to adjacent sites only. The latter is based on the hypothesis that even though fish from, e.g., Western Australia are similar in elemental composition to those from, e.g., Maatsuyker, the fact that both differ from all intervening areas suggest that the similarity is fortuitous rather than evidence of some very complex pattern of larval drift and stock movement.

With the noteworthy exception of the Lord Howe Rise, which had diagnostically and regionally distinct high levels of Pb (being virtually the only site at which lead was routinely measured above the nominal minimum detection limit of the element), differences among most sites are one of distributions within a range of values that were generally similar among sites. Hence, it is not possible even with the full set of elements to unambiguously 'fingerprint' a fish as being from a particular spot, but rather to state that the population of fishes, as a whole, from Cascade Plateau, for example, differ significantly from the sample, as a whole, from Maatsuyker, and therefore most likely represent a different population. Used in this way, otolith composition is similar in practical use to meristics or population genetics, and is subject to the same problems of sampling adequacy.

Eliminating from the site comparisons the dubious trace elements changes the site separation picture only slightly (Figure 17), even though it is now based on only four of the parameters initially measured, and only three of the fifteen elements we started with. As compared with six discernible assemblages of similar fishes spanning the nine sites, the reduced analysis still suggests six assemblages. The only separation lost is that between WA and the GAB, which was based on exceptionally high levels of Hg at the former. This difference could still be real (it certainly looks real), but we have been unable to obtain another sample from the site to verify it.

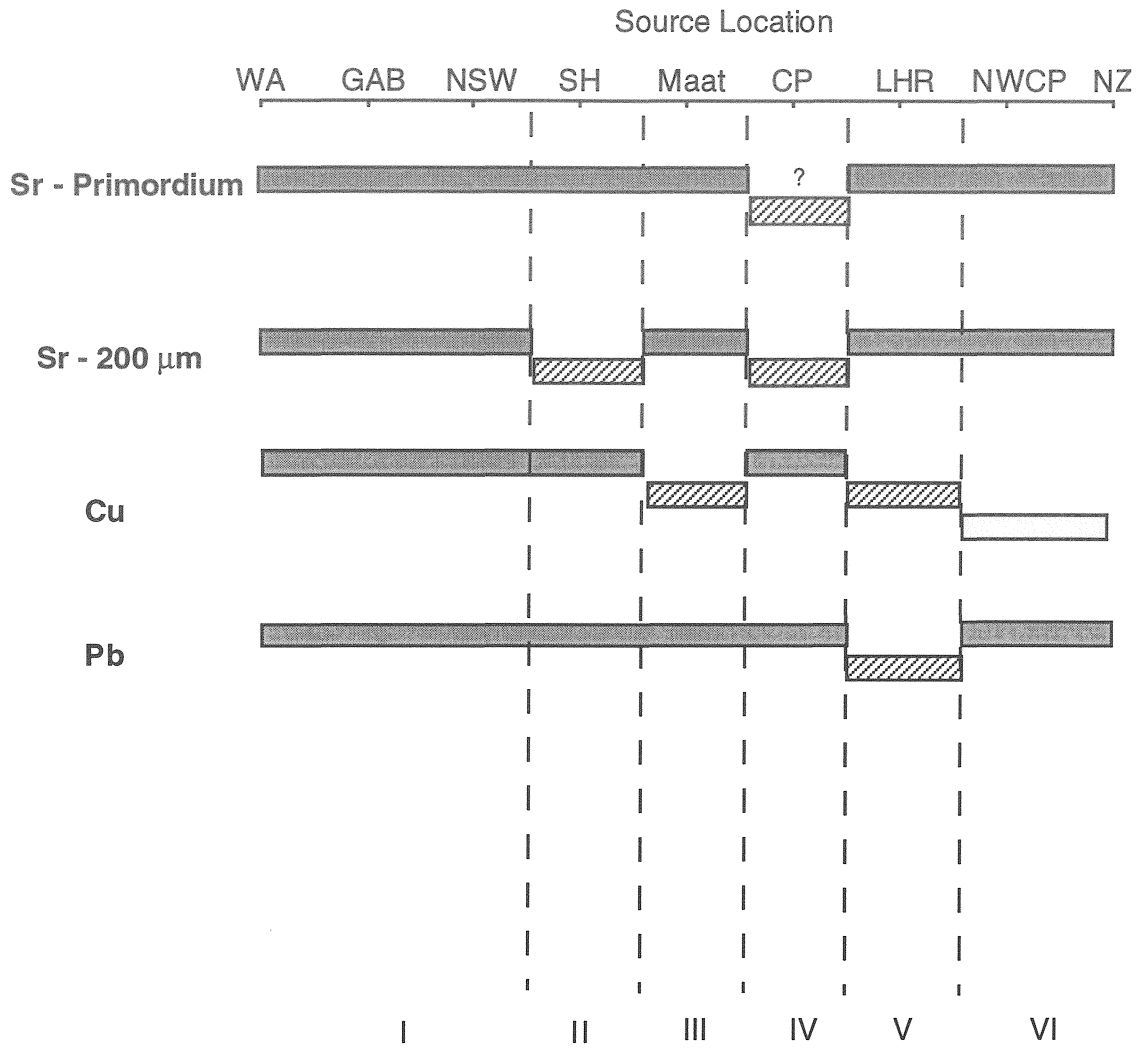


Figure 17. Apparent separation among source locations based on pair-wise comparsions of apparently reliable elements among adjacent sites

Ultimately and unfortunately, however, perusal of the data, the extent of overlap among sites, and the differences between the pilot and recent analyses provide little basis for drawing confident conclusions about spawning stock structure in roughy. The only really 'strong' difference in the data set is that between the Lord Howe Rise fish and all others, on the basis of lead levels that are consistently high. The only other roughy we've seen with such high lead levels are from the North Atlantic. The significant difference between, as groups, a WA/GAB/NSW assemblage and St. Helens/Cascade Plateau fish, is broadly consistent with industry expectations, but is based on only one parameter (Sr at 200 μm), values for which overlap broadly between the two groups. The same is true for the significant site differences that separates Maatsuyker from its near neighbors; copper levels and strontium at 200 μm are both significantly higher at Maatsuyker than either St. Helens or the Cascade Plateau, but the differences are so slight that one is inclined to attribute them to artifacts of small sample sizes.

Taking this argument one step further, if we assume that differences within sites between the pilot study and re-analysis are principally artifacts of small sample sizes, pooling the two data sets for the four parameters deemed reliable provides a statistically more substantial basis for comparison. The expanded analysis, which now includes the North Atlantic sample, indicates all four parameters differ significantly among sites; the weakest separation was for Cu, which differed at the P=0.01 level; differences for Pb and for the two Sr parameters were all significant at $p<<0.001$. The combination of the four data broadly distinguishes among five aggregates of sites. First, all four parameters distinguish between the North Atlantic and southern hemisphere samples. Second, elevated Pb and Sr levels at the primordium (as compared with nearby sites) distinguishes the sample from the Lord Howe Rise from all other southern hemisphere sites. Third, the samples from New Zealand differ or nearly differ significantly from most sites in the Australian EEZ in both Cu levels and concentrations of Sr at the primordium. And fourth, Sr at the primordium also distinguishes between, as groups, WA/GAB/NSW and St. Helens/Maatsuyker/Cascade Plateau, as well as between the NW Challenger Plateau and the NZ EEZ samples.

Conclusions about adult mobility, based on differences at the otolith margin, are also relatively weak, though perhaps for different reasons. The differences at the margin among repeated sites are very significant (at $P<<0.001$) and to an extent consistent; Sr values at the margin are highest for fish from St. Helens (compared with the GAB and Maatsuyker) in both the pilot and recent analyses. The results are broadly consistent with limited movement by adults among these sites. However, the sample-by-site ANOVA also indicates a strong and highly significant interactive term, indicating either that otolith composition at the margin differs depending on when the sample is taken (which is a very plausible hypothesis) or that they are driven by small sample sizes. Unfortunately, the only element for which reliable differences at the margin were measured in this study involve Sr, which of all elements is the one that shows the highest levels of what appear to be age or date related variability. Other work suggests that Sr concentrations vary as functions of water temperature, salinity or both, either or both of which might reasonably be expected to vary substantially and semi-regularly over time, even at the depths roughy occupy. The only practical way of overcoming this problem is to base marginal comparisons on more than a single point for each fish, an analytic approach we used in the absence of expectations about likely site-based differences and in order to keep project costs to a minimum. If Sr differences near the margin are diagnostic among sites, then ideally data from full life history scans, which reflect long-term (multi-year) patterns, should be compared and contrasted among individuals from different sites. We have done such scans for a handfull of roughy, and initial indications are promising (i.e., the three adult fish from Maatsuyker that we have run all showed similar ontogenetic patterns of Sr variability), but expanding such an effort to include substantial numbers of individuals from different sites would be expensive and time consuming. One life history scan, even for a rapidly acquired datum like Sr, takes about 12 hours of beam time. Comparing ontogenetic patterns for, say, twenty individuals each from the GAB, St. Helens and Maatsuyker would require about 12 weeks of beam time. Even if

we developed a sub-sampling regime that was less data intense, the cost of such an exercise in beam time alone would be around $30,000. The statistics of such a comparison would also be difficult, particularly without reliable means of converting positions along a growth axis to fish ages and hence dates, that could be used as a common basis for comparison.

Consequently, we conclude that otolith chemical analysis does provide indications of a structuring of the Australian and Tasman Sea roughy stocks, but that with the exception of the Lord Howe Rise fish, the evidence for separations derived from the currently available techniques among sites is relatively weak. To the level of the technology we currently apply to the problem, analysis of otolith composition does not constitute a 'silver bullet' for solving stock resolution problems in the species. In practice, this conclusion is probably not surprising. By comparison with coastal habitats, where we do find good indications of stock structuring, deep oceanic environments are likely to be relatively similar over broad geographic reasons. Hence, to the extent that otolith composition reflects environmental heterogeneity, its applicability to species like orange roughy might be slight.

There is, of course, always the potential to apply more sensitive, and often more expensive, techniques to the problem. A variety of analytical tools have been used to examine otolith composition, up to and including tuned X-ray radiation, a technique that potentially resolves composition to the few parts per trillion level (as opposed to the 2-3 parts per million level we applied in this project). There would be no shortage of advocates of these more exotic technologies. And it is possible that these techniques, because they target a slightly different mix of elements (no single technique measures equally well all elements) and, perhaps, isotopes could provide stock delineation not evident in the elements we examined. Those techniques would still be subject to the same problems we documented on effects of specimen handling, however, and should be used cautiously.

A good example is the work on orange roughy by Edmonds et al (1991), who applied ICP-MS (inductively coupled plasma-mass spectroscopy) to analysis of whole otoliths. ICP-MS is relatively crude, to the extent that it deals with whole otolith composition rather than deposition at discrete stages in the life history, but has an advantage of apparently high levels of sensitivity (to the tens of parts per trillion range). At this level of sensitivity, Edmonds et al reported highly significant differences among samples of roughy taken at three sites in the Australian EEZ (the GAB and eastern and western Tasmania). However, one of the elements that drove this separation was sodium, which our studies, detailed above, indicates is one of the elements most affected by specimen handling and preparation. On that basis, we would be highly suspicious of the principal conclusions drawn by Edmonds, et al., despite the nominally much higher sensitivity of the analytical technique they used. The main message is that while potentially very promising, the science of analysis of otolith composition is still developing, and major methodological issues still need to be worked out and factored into the science. Ultimately, no one yet fully understands the mechanisms of elemental deposition or the magnitude of the factors that can alter this deposition. Until the field matures and differences among individuals and stocks can be unambiguously replicated using a range to techniques, management decisions based on otolith chemistry as a sole or major input would be unwise.

From a fishing industry perspective, the optimal solution still seems to be that of providing small amounts of funds to encourage development of the technology, because of its potential when applied to species like orange roughy, while also encouraging application of existing otolith technologies as one of a suite of approaches that can provide useful inputs to stock resolution of inshore and coastal species.

Literature Cited

Albrechtsen, K (1968). A dyeing technique for otolith age reading. J. Cons. Coms. Int. Explor. Mer 32: 278-280.

Ancey, M., Bastenaire, F., and Tixier, R. (1978). Applications of statistical methods in microanalysis. In: Microanalysis and scanning electron microscopy. Proceedings of the Summer School at St-Martin -d'Heres, 1978. F.Maurice, , Meny, L., and Tixier, R., (eds.) Les Edition de Physique , Orsay, France. 319-343.

Campana, S.E. et al. (1997) Comparison of accuracy, precision and sensitivity in elemental assays of fish otoliths using the electron microprobe, PIXE and laser ablation ICPMS. Submitted to Can. J. Fish. Aquat. Sci.

Edmonds, J. S., Caputi, N., and Morita, M. (1991). Stock discrimination by trace-element analysis of otoliths of orange roughy (*Hoplostethus atlanticus*), a deep-water marine teleost. Aust. J. Mar. Freshwat. Res. 42: 383-389.

Fenton, G.E., Short, S.A. and Ritz, D.A. (1991). Age determination of orange roughy, *Hoplostethus atlanticus* (Pisces: Trachichthydae) using ^{210}Pb:^{226}Ra disequilibria. Mar. Biol. 109: 197-202.

Gauldie, R.W., Coote, G.E. and Vickridge, I. (1990). The relationship between organic material and check rings in fish otoliths. Comp. Biochem. Physiol. A 97: 461-474.

Gauldie, R.W. and Coote, G.E. (in press). Diffusion of cacodylate (di-methylarsinic acid) ion into the otolith of *Hoplostethus atlanticus*. Submitted to Fisheries Science.

Gauldie, R.W., Thacker, C.E., West, I.F., and Wang, L. (in press). Movement of water in fish otoliths. Submitted to Comp. Biochem. Physiol. A.

Gunn, J. S., Harrowfield, I. R., Proctor, C. H., and Thresher, R. E. (1992). Electron probe microanalysis of fish otoliths - evaluation of techniques for studying age and stock discrimination. J. Exp. Mar. Biol. Ecol. 158: 1-36.

Halden, N. M., Babaluk, J. A., Campbell, J. L., and Teesdale, W. J. (1995). Scanning proton microprobe analysis of strontium in an arctic charr, *Salvelinus alpinus*, otolith: implications for the interpretation of anadromy. Environ. Biol. Fish. 43: 333-339.

Kalish, J. (1992). Formation of a stress-induced chemical check in fish otoliths. J. Exp. Mar. Biol. Ecol. 162: 265-277.

Kalish, J. M. (1989). Otolith microchemistry: Validation of the effects of physiology, age and environment on otolith composition. J. Exp. Mar. Biol. Ecol. 132: 151-178.

Kalish, J. M. (1990). Use of otolith microchemistry to distinguish the progeny of sympatric anadromous and non-anadromous salmonids. Fish. Bull. 88: 657-666.

Kalish, J. M. (1991). Determinants of otolith chemistry: Seasonal variation in the composition of blood plasma, endolymph and otoliths of bearded rock cod *Pseudophycis barbatus*. Mar. Ecol. Prog. Ser. 74: 137-159.

Milton, D.A. and Chenery, S.R. (ms.) An evaluation of the effect of storage method on otolith elemental composition detected by laser-ablation ICPMS.

Mugiya, Y. and Uchimura, Y. (1989). Otolith resorption induced by anaerobic stress in goldfish, *Carassius auratus*. J. Fish Biol. 35: 813-818.

Mulligan, T. J., Martin, F. D., Smucker, R. A., and Wright, D. A. (1987). A method of stock identification based on the elemental composition of striped bass *Morone saxatilis* (Walbaum) otoliths. J. Exp. Mar. Biol. Ecol. 114: 241-248.

Proctor, C. H., Thresher, R. E., Gunn, J. S., Mills, D. J., Harrowfield, I. R., and Sie, S. H. (1995). Stock structure of the southern bluefin tuna *Thunnus maccoyii*: an investigation based on probe microanalysis of otolith composition. Mar. Biol. 122: 511-526.

Radtke, R. L., Townsend, D. W., Folsom, S. D., and Morrison, M. A. (1990). Strontium:Calcium concentration ratios in otoliths of herring larvae as indicators of environmental histories. Environ. Biol. Fish. 27: 51-61.

Radtke, R.L., Showers, W., Moksness, E. and Lenz, P. (1996) Environmental information stored in otoliths: insights from stable isotopes. Mar. Biol. 127:161-170.

Rees, A.J. and Proctor, C.H. (in prep.) Uses of an EDS (energy dispersive spectroscopy) X-ray microanalysis system for microchemical analysis of fish otoliths.

Severin, K. P., Carroll, J., and Norcross, B. L. (1995). Electron microprobe analysis of juvenile walleye pollock, *Theragra chalcogramma*, otoliths from Alaska: a pilot stock separation study. Environ. Biol. Fish. 43: 269-283.

Sie, S. H. and Thresher, R. E. (1992). Micro-PIXE analysis of fish otoliths: methodology and evaluation of first results for stock discrimination. Internat. J. PIXE 2: 357-380.

Thresher, R. E., Proctor, C. H., Gunn, J. S., and Harrowfield, I. R. (1994). An evaluation of electron-probe microanalysis of otoliths for stock delineation and identification of nursery areas in a southern temperate groundfish, *Nemadactylus macropterus* (Cheilodactylidae). Fish. Bull. 92: 817-840.

Thresher, R.E., Mills, D.J., Proctor, C.H., and Ianelli, J. (eds) (in press). Proceedings of the "Skeletal Microchemical Analysis of Marine Fish Stocks" Workshop, Hobart, Australia, 1992. CSIRO Division of Marine Research Report # ?

Toole, C. L. and Nielsen, R. L. (1992). Effects of microprobe precision on hypotheses related to otolith Sr:Ca ratios. Fish. Bull. 90: 421-427.

West, I. F. and Gauldie, R. W. (1994). Determination of fish age using Pb-210-Ra-226 disequilibrium methods. Can. J. Fish. Aquat. Sci. 51: 2333-2340.

7. *Benefits*

This project has potential and real benefits at two levels.

First, it was an attempt to apply a new set of technologies to resolving stock issues in the commercial deepwater fish, orange roughy. As such, the benefits a primarily potential, to the extent that improved stock information could alter management of the species and have flow on effects to resource allocation and harvesting strategies. The one apparently unambiguous result, that the Lord Howe Rise fishery is based on a stock different from either of those fished in the Australian and NZ EEZ's could well affect issues of access to the fishery via input to resolving the Tasman sea straddling stocks issues. Otherwise, however, separation among stocks within the Australian EEZ are too weak to justify modifying existing management structures.

Second, the results of the project provide a clear cautionary note for the unbridled application of otolith chemical analysis to stock issues in Australia in general. The potential of the technique is substantial, but it is currently being applied without regard to fundamental issues of data quality. A quick perusal of the recent literature indicates a number of studies whose principal conclusions are dubious, in the light of our experimental studies indicating the marked sensitivity of many elements to common laboratory usage. There is still a very strong tendency to treat otolith chemical analysis as a 'black box' science: otoliths in, data out, without any real understanding of the issues involved in assuring data quality. Hence the current project has considerable value to the fishing industry as a whole, to the extent that it minimises management errors based on faulty interpretations of the otolith chemical studies now beginning to be used widely across the field.

8. *Intellectual Property*

The intellectual property developed in this project are jointly owned by the CSIRO and the FRDC. It has no apparent commercial value. The support of the FRDC will be acknowledged in any technical publications that arise from the study.

9. *Further Development*

With specific regard to orange roughy, two further developments are recommended.

First, additional samples should be examined from Western Australia and the Lord Howe Rise. Both differed significantly from other sites in having usually high concentrations of trace metals (Hg and Pb, respectively), suggesting separate stocks. However, both are based on only a single sample from each site, and until the results are verified they should be used in management with considerable caution. Nonetheless, the issue of the Lord Howe Rise fishery, in particular, as a Tasman Sea straddling stock, may well justify the effort of verification.

Second, apparently significant differences among sites in Sr concentrations at the otolith margin could well indicate limited mobility among adults. The single point analysis used in this study, an approach taken in the light of the pilot study that showed significant differences found in many elements, are not sufficient when applied to only a single element known to vary substantially as a result of environmental conditions. To make this assessment robust, multiple points should be measured along the growth axis, so that effects of year-to-year and seasonal variation can be estimated and factored out of the comparison. Such an approach is fairly routine, but will require additional effort to be put into the analysis. As a first cut, to assess the extent to which the approach might be useful, a small number of life history scans should be taken for fish from widely separated sites, such as NSW and the GAB, that can be compared with the small amount of data we already have for the southern zone.

With regard to general issues about application of otolith chemical analysis to stock issues, there are also two points worth developing further.

The first is alluded to above. The potential of otolith composition to clarify stock issues is being widely touted, despite limited understanding of the factors that affect composition. Already, erroneous conclusions are creeping into the scientific and fisheries literature, as advocates pursue apparent differences among specimens and species too uncritically. Hence one strong development that should be pursued is to provide cautionary advice to practitioners in the field, alerting them to potential confounding problems. In practical terms, the FRDC should ensure that applicants for funding to do otolith chemical studies are aware of the relevant confounding problems.

Second, nonetheless, there are a suite of emerging technologies that do offer higher resolution and more precise information about otolith composition. Properly designed studies that use these technologies could well help resolve stock issues, even in species like orange roughy. In practice, the ambiguity associated with current approaches to resolving stock structure do not look to be disappearing any time soon, and there remains a need to pursue these emerging technologies, at an appropriate level of support, to assess the extent to which any might assist in the development of effective management regimes.

10. Staff

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