

Comparison of accuracy, precision, and sensitivity in elemental assays of fish otoliths using the electron microprobe, proton-induced X-ray emission, and laser ablation inductively coupled plasma mass spectrometry

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Abstract: The elemental composition of fish otoliths is of considerable interest to those who wish to reconstruct temperature, migration, or environmental histories of individual fish based on assays of the otolith growth sequence. However, reported differences in otolith elemental composition among studies may be due in part to performance differences among four of the most popular instruments for targeted elemental analysis: wavelength-dispersive electron microprobe (WD-EM), energy-dispersive electron microprobe (ED-EM), proton-induced X-ray emission (PIXE), and laser ablation inductively coupled plasma mass spectrometry (LA-ICPMS). To rigorously compare the sensitivity, accuracy, and precision of these four analytical tools, the *International Otolith Composition Experiment* distributed blind-labelled real and artificial otoliths of known but varied elemental composition to eight laboratories for assay of 10 selected elements. No one instrument type was sensitive to each element, nor was any one instrument preferred for use in all assays. In general however, abundant elements such as Na and K could only be measured accurately with an electron microprobe, while the trace elements required PIXE or LA-ICPMS. Strontium could be measured with considerable accuracy and precision by WD-EM, PIXE, and LA-ICPMS. The presence of significant, and occasionally large, differences among laboratories suggests that comparisons among published studies should be made cautiously and only after appropriate calibration.

R  sum   : La composition   l  mentaire des otolithes de poisson est d'importance consid  rable pour ceux qui d  sirent reconstruire les ant  c  dents de poissons individuels du point de vue de la temp  rature, de la migration ou de l'environnement    partir d'essais r  alis  s sur la s  quence de croissance des otolithes. Toutefois, les diff  rences signal  es dans les   tudes en ce qui a trait    la composition   l  mentaire des otolithes peuvent   tre attribuables en partie    des diff  rences de rendement dans quatre des appareils les plus utilis  s pour l'analyse d'  l  ments cibles : la microsonde   lectronique    dispersion de longueur d'onde (WD-EM), la microsonde   lectronique    dispersion d'  nergie (ED-EM), l'  mission X induite par proton (PIXE) et l'ablation par laser et spectrom  trie de masse avec plasma induit par haute fr  quence (LA-ICPMS). Pour comparer rigoureusement la sensibilit  , la justesse et la fid  lit   de ces quatre outils d'analyse, l'*International Otolith Composition Experiment* a distribu   des otolithes r  els ou artificiels   tiquet  s    l'insu de compositions   l  mentaires connues mais vari  es   

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huit laboratoires pour qu'ils exécutent le dosage de 10 éléments choisis. Aucun type d'instrument n'a été sensible à chacun des éléments et aucun instrument particulier n'a été préféré pour tous les essais. Toutefois, en général, les éléments abondants comme le Na et K ne pouvaient être mesurés de manière juste qu'avec une microsonde électronique, tandis que les éléments traces nécessitaient la PIXE ou la LA-ICPMS. Le strontium pouvait être mesuré avec une justesse et une fidélité considérables par WD-EM, PIXE et LA-ICPMS. La présence de différences statistiquement significatives, et à l'occasion importantes, entre les laboratoires indique que les comparaisons entre les études publiées devraient être faites de manière prudente et seulement après un étalonnage approprié.

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Introduction

Recent years have seen a rapid growth in interest in the elemental composition of fish otoliths because of its potential for reconstructing the environmental history of individual fish and for distinguishing among populations (Panfili and Troadeuc 1993; Secor et al. 1995a). Otolith elemental assays have been used to infer migration pathways (Secor et al. 1995b; Thorrold et al. 1997), differentiate among fish stocks (Edmonds et al. 1989; Thresher et al. 1994; Severin et al. 1995), reconstruct temperature history (Patterson et al. 1993; Townsend et al. 1995), validate age interpretations through radiochemical dating (Campana et al. 1990; Kalish 1995), and detect anadromy (Kalish 1990; Halden et al. 1995), as well as to detect chemical marks applied through mass marking (Ennevor and Beames 1993). In many instances, the assays target daily or annual growth increments in the otolith, adding an age or time component to the interpretation. The success of the above applications is based in large part on two key properties of the otolith: (i) unlike bone, the otolith is metabolically inert (therefore, newly deposited material is neither resorbed nor reworked after deposition; Campana and Neilson 1985), and (ii) the otolith grows throughout the lifetime of the fish. Because trace element uptake onto the otolith reflects the physical and chemical environment (Fowler et al. 1995; Secor et al. 1995b), albeit with significant physiological regulation (Kalish 1989; Farrell and Campana 1996), the potential exists for the otolith to store a complete age-structured record of exposure history to the environment.

The relative purity of the composition of the otolith has significant implications for its analysis and interpretation. With more than 99% of the otolith being composed of calcium carbonate in an organic matrix (Degens et al. 1969), and with many of the more abundant minor elements being subject to strong physiological regulation (Kalish 1989; Thresher et al. 1994), it is primarily the trace elements (concentrations <0.1%) that are most likely to serve as useful markers of the environment. Yet many of the studies to date report a range of trace element concentrations in the otolith that exceeds that expected of the environment. For example, concentrations of Fe in the otolith have been reported as ranging from 2 to 400 ppm (Sie and Thresher 1992; Arai et al. 1994), while Sr apparently ranges from 80 to 3600 ppm (Gauldie et al. 1986; Secor et al. 1995b). Such diverse estimates of otolith elemental composition may indeed be real, reflecting species effects, physiological effects, anadromy, heterogeneity in the environment, or any combination thereof. However, an alternative explanation is that the observed range also reflects analytical problems associated with the diverse array of sophisticated instrumentation that has been used to make these measurements. Among the more visible candidates for otolith elemental

analysis are the energy-dispersive electron microprobe (ED-EM), wavelength-dispersive electron microprobe (WD-EM), proton-induced X-ray emission (PIXE), proton-induced gamma ray emission, synchrotron X-ray emission, resonance ionization spectroscopy, and laser ablation inductively coupled plasma mass spectrometry (LA-ICPMS). While there are known theoretical differences in performance among the various instruments, the differences in performance characteristics when applied to otoliths remain unknown, and it is these potential differences among instruments that could be contributing to the uncertainty concerning the true elemental composition of otoliths.

The objective of the *International Otolith Composition Experiment* (IOCE) was to rigorously compare among four of the most frequently used and (or) promising analytical tools for assaying otolith elemental composition: ED-EM, WD-EM, PIXE, and LA-ICPMS. Using a factorial design, we tested for differences across equipment type and elemental concentrations with respect to the features most likely to influence otolith interpretation: (i) limit of detection for each element; (ii) accuracy; (iii) precision; (iv) linearity of response across various concentration levels; and (v) beam or crater size. All assays were based on replicate blind-labelled otolith material of known composition, using either real otoliths or artificial otoliths spiked with varying concentrations of 10 key elements.

Materials and methods

Distribution of identical homogeneous otoliths of known composition to all participants in this experiment would have insured that any observed assay differences were in fact due to instrument effects, rather than sample heterogeneity. Unfortunately, natural otoliths are neither identical nor homogeneous. Therefore we adopted a two-pronged approach: (i) finely ground otolith powder, spiked with varying concentrations of trace elements, was fused into artificial otoliths (beads), thereby insuring homogeneity and a suitable range of concentrations; and (ii) otoliths from juvenile fish of identical age, reared under constant conditions in the laboratory from hatch, were used to insure that interpretation of the bead assays was not unduly influenced by the unnatural consistency of the bead matrix.

Preparation of artificial otoliths

The component matrix of the artificial otoliths was otolith powder obtained from the sagittal otoliths of 100 adult Atlantic croaker (*Micropogonias undulatus*) captured from a single site. The otoliths were cleansed and decontaminated (Fowler et al. 1995), powdered in an agate mortar, sieved to micron-sized particles through an acid-washed, 5- μ m nylon mesh, and then assayed for concentrations of 10 different elements at two independent laboratories. The assay results were then used as the basis for spiking the otolith powder with varying concentrations of MgO, Na₂CO₃, K₂CO₃, Fe₂O₃, NiO, CuO, ZnO, SrCO₃, BaCO₃, and PbCO₃.

Four otolith bead types were prepared, with each containing differing combinations of spike levels for each of the 10 elements, ranging from none to 10-fold depending on the natural concentration of the element. Lead was enhanced up to 100-fold, because of its very low natural concentration ($<1 \mu\text{g}\cdot\text{g}^{-1}$) in croaker otoliths. Therefore, each of the four bead types contained all of the natural otolith constituents, as well as enhanced concentrations for 1–10 elements. Spike levels were randomized across bead types so that, in any given type, one element could be at natural levels; a second element could be enhanced 2-fold; a third, 7.5-fold; etc.

The spiked otolith powder was mixed 1:1 with a pure lithium tetraborate flux, and then divided into equal aliquots. Each aliquot was individually fused into glasslike beads several millimetres in diameter on an electrically heated tungsten filament inside a sealed, nitrogen-filled cell. Fused beads were stored unmounted in acid-washed polyethylene vials in preparation for distribution to IOCE participants. At no time were the otolith beads handled with anything other than acid-washed polyethylene. The beads were subsequently allocated randomly among laboratories, with 10 of each type retained for baseline assay.

While visual examination during the fusion process suggested that the beads were identical and homogeneous in composition, the presence of occasional bubbles and coloured artifacts indicated that complete homogeneity within a given bead was not always obtained. Therefore, participants in the study were instructed to avoid any obvious artifacts during their assays. Replicate beads may also have differed slightly (but significantly) in otolith powder and spike content, as evidenced by consistent reports of differences in elemental concentration among replicates. Differences in otolith powder content among replicates would disappear when standardized to Ca during the assays. However, small differences in the amount of a spike within a given replicate were unavoidable because of the trace quantities involved and undoubtedly contributed to inter-replicate differences. For these reasons, fine-scale comparisons among the various assay results are not justified.

The process used to produce the beads may also have reduced the sensitivity and detection limit of several instruments, which has to be considered when comparisons of the bead assays among instruments are made. Significant quantities of tungsten (>1000 ppm) from the filament were apparently incorporated into the beads during fusion, thus reducing the sensitivity of instruments using energy-dispersive spectrometers (PIXE, ED-EM) to Ni, Fe, and Cu. In addition, the Li present in the lithium tetraborate flux increased the background noise level for all elements detected by PIXE, substantially increasing the limit of detection (LOD) for elements at the low-resolution end of the spectrum, particularly Ba.

Natural otoliths

Sagittal otoliths were removed with acid-washed glass probes from juvenile Atlantic croaker reared in the laboratory from hatch as part of another experiment (Fowler et al. 1995). All fish were reared for 71 days under identical conditions of temperature (25°C), salinity (26‰), and food supply, and all were 30–47 mm in standard length at the time of sampling. Otoliths were subsequently cleansed and decontaminated as was described earlier, then stored unmounted in acid-washed polyethylene vials until distribution. At no time were the otoliths touched by metallic instruments such as forceps. All otolith sectioning and polishing was carried out in the laboratories of the IOCE participants.

Baseline assays of natural and artificial otoliths

To determine the actual elemental concentrations of the natural otoliths and each of the four otolith bead types, random samples of each were submitted to two independent laboratories for dissolution and assay. One laboratory used inductively coupled plasma atomic emission spectrometry (ICP-AES) and atomic absorption spectroscopy (AAS, both graphite furnace and flame), while the other used

ICPMS. Since the two sets of assay results were generally within 20% of each other, the mean value was adopted as the baseline value. The relative concentrations of these baseline values were also consistent with the known additions of each element, with the exception of Cu and Na (for which the baseline assays are considered reliable). Assays for the absolute concentration of Fe were not consistent between the laboratories, although relative values were. As ICPMS is often not well suited to assays of Fe, the Fe values derived from the AAS assays were adopted as baseline concentrations.

Study design

The IOC experiment was factorial in design, with two independent laboratories nested within each of the four instrument types. No one laboratory was allowed to contribute more than one set of assay results (e.g., one instrument per laboratory), and all had extensive prior experience with the instrument selected for their use. Each laboratory was sent two replicate beads of each of the four blind-labelled bead types, and each was asked to conduct five replicate 11-element assays within each of the eight beads. The elements targeted for assay were Mg, Na, Ca, K, Fe, Ni, Cu, Zn, Sr, Ba, and Pb.

Each laboratory was also sent three of the replicate laboratory-reared otoliths and asked to polish them through the midplane as per their preferred technique. Five assay locations were indicated on each otolith: three assays between the core and postrostrum (core, midway, and 100 μm in from the postrostral tip) and two assays between the core and the ventral surface (midway and 100 μm in from the edge). Assays were requested of each of the 11 elements listed above, although the option to quantify additional elements was presented.

All IOCE participants were urged to use instrumental operating conditions that they would have considered routine for the assay of numerous samples. Variability due to spot size was minimized by targeting a spot diameter of 10 μm (to the extent possible).

Data analysis

Most elemental data were submitted in terms of absolute elemental concentrations ($\mu\text{g}\cdot\text{g}^{-1}$), although one laboratory submitted relative (counts per second) data. While standardization to otolith Ca is a commonly accepted means of reducing intersample variance (based on the premise that Ca concentration in the otolith is relatively invariant), submitted data were not previously standardized to Ca, as per the study design. Data were subsequently standardized to Ca after a preliminary analysis indicated that the coefficient of variation (CV) declined in most samples after standardization to the mean sample Ca concentration:

$$\text{Element}'_{ij} = \text{Element}_{ij} \frac{\text{Ca}_i}{\text{Ca}_{ij}}$$

where $\text{Element}'_{ij}$ is the elemental concentration in assay j of treatment i after standardization to Ca, and Ca_i is the mean Ca concentration across j assays of treatment i . Therefore, all analyses that follow are based on data standardized to Ca, with the exception of the PIXE data, for which Ca measurements were not made.

To convert the one set of relative concentration LA-ICPMS data (LA-2) to absolute concentrations, the element:calcium count ratio for a given element was assumed to be equivalent to the ratio of the absolute concentrations. The element:calcium ratios were then converted to absolute concentrations by assuming a 20% calcium concentration in the artificial otolith beads, as would be the case given an exact 1:1 mixture of otolith powder and flux (see above). Because these calculations do not take into account interbead variations in calcium content, the resulting concentration estimates are almost certainly less accurate than those based on the use of standards. In the case of the LA-2 otolith assays, estimates of absolute concentration were made through standardization to the bead assay results.

Statistical analysis of the otolith beads was carried out with a three-way nested ANOVA for each bead type and element, with five replicate measurements nested within two replicate beads nested

Table 1. Summary of equipment, operating conditions, and standards used by researchers participating in this experiment.

Identifier	Equipment	Model	Operating conditions	Otolith preparation	Data preparation	Standards
ED-1	ED-EM	EDAX DX-4 EDS equipped with a UTW detector	25 kV and 15–20% dead time on the detector. Counting time of 300 s. $10 \times 10 \mu\text{m}$ beam. Working distance of 10 mm	Polished with $0.25\text{-}\mu\text{m}$ Al_2O_3 , then carbon coated	ZAF algorithm	1 mineral standard for each element
ED-2	ED-EM	JEOL 840A electron probe microanalyzer using a NORAN Be-window EDS detector	15 kV accelerating voltage @ 3nA cup current and 25–30% dead time on the detector. Counting time of 60 s. $10 \times 10 \mu\text{m}$ rastered beam. Working distance of 37 mm from pole piece.	Polished with $0.25\text{-}\mu\text{m}$ Al_2O_3 , then carbon coated	ZAF algorithm and Heinrich's mass absorption coefficients. Peak shapes reduced using top hat filters. Matrix correction based on presumed carbon content.	1 mineral standard for each element
WD-1	WD-EM	Cameca Camebex electron microprobe	25 kV @ 5 nA. $10\text{-}\mu\text{m}$ spot size. 20–200 s counting time.	Polished with $0.3\text{-}\mu\text{m}$ Al_2O_3 , then $0.25\text{-}\mu\text{m}$ diamond paste, then carbon coated	ZAF algorithm	1 mineral standard for each element
WD-2	WD-EM	Cameca SX-50 electron microprobe	15 kV @ 4 nA. $9 \mu\text{m}$ spot size. 20–120 s counting time.	Embedded in petropoxy, then polished with $0.05\text{-}\mu\text{m}$ Al_2O_3	PAP algorithm	1 mineral standard for each element
PIXE-1	PIXE	Custom-designed proton microprobe	3 MeV proton beam @ 4 nA for a total of $2.5 \mu\text{C}$ (400 s). Spot size of $6\text{--}15 \mu\text{m}$	Polished with $0.3\text{-}\mu\text{m}$ Al_2O_3 and carbon coated	Background removed with top hat filter method. GUPIX software. Matrix corrections based on fundamental parameters calculation.	USGS basalt glass and NIST steel SRM to determine instrumental constant.
PIXE-2	PIXE	Custom-designed proton microprobe	3 MeV proton beam @ $30\text{--}40$ nA (beads) or $10\text{--}15$ nA (otolith) for a total of $6 \mu\text{C}$. Spot size of $30 \mu\text{m}$ (otoliths) or $100 \mu\text{m}$ (beads).	Polished with Al_2O_3 , then diamond paste to $0.5 \mu\text{m}$, then carbon coated	Otoliths normalized to 40% Ca based on an assumed aragonite matrix.	
LA-1	LA-ICPMS	Custom-designed laser ablation microsampling system coupled to VG PQII+"s" quadrupole ICPMS	Laser wavelength of 266 nm, defocused to $200\text{-}\mu\text{m}$ spot size and 0.2 mJ per shot @ 10 Hz. Data acquired using 1 point/peak, 8.5 ms dwell time for 20 masses. 40 s ablation time.	Polished with $3\text{-}\mu\text{m}$ Al_2O_3 , then sonified.	Multiple isotopes measured for some elements.	NIST 612 glass. Internal Ca in sample (measured with ED-EM).
LA-2	LA-ICPMS	Custom-designed laser ablation microsampling system coupled to a VG Elemental PQ2+ quadrupole ICPMS	Laser wavelength of 355 nm from Nd-YAG laser in Q-switched mode @ 2 Hz and 500-V flashlamp. $10 \mu\text{m}$ beam size. Data acquired in scan mode, 320 ms dwell time for 30 s and 20 masses.	Polished with $3\text{-}\mu\text{m}$ Al_2O_3 , then sonified.	Did not convert counts to absolute elemental concentration. Multiple isotopes measured for some elements.	Internal Ca in sample.

Fig. 1. Comparison of mean elemental assay results with actual concentration for each of four artificial otoliths (beads) and four instrument types (two laboratories for each). If the results for a given instrument type differed significantly among laboratories, the latter are plotted separately. Only those laboratories that detected significant differences among bead concentrations are presented. Error bars represent 95% confidence intervals. The solid line represents the 1:1 line.

Table 2. Baseline elemental analysis of artificial otoliths (beads) and the otolith powder used to prepare them.

Sample and analysis	Elemental concentration ($\mu\text{g}\cdot\text{g}^{-1}$)										
	Na	Mg	K	Ca	Fe*	Ni	Cu	Zn	Sr	Ba	Pb
Otolith powder used in beads											
AAS (GF and flame)	1180	35	1400	—	3.0	<1.0	<2.0	10.0	1 830	6.8	<2.0
ICP-MS	1470	28	1640	—	—	3.2	1.1	10.7	1 890	7.0	<0.05
Bead 1											
GF-AAS	650	429	—	—	130	38.4	3.9	25.2	9 930	7.1	<0.10
ICP-MS	617	222	286	234 000	453	32.3	6.2	20.0	10 500	4.6	0.13
Bead 2											
GF-AAS	5250	111	2790	—	65.6	13.1	24.0	73.6	607	62.8	<0.20
ICP-MS	4290	106	5110	217 000	231	8.4	43.1	163	1 150	106	<0.03
Bead 3											
GF-AAS	4800	82	2640	—	18.2	8.3	11.6	20.9	5 300	15.3	1.30
ICP-MS	4710	53	2900	215 000	119	4.4	19.4	13.0	5 980	16.5	1.56
Bead 4											
GF-AAS	2620	44	1560	—	125	35.8	12.5	6.2	11 400	37.7	<0.10
ICP-MS	3010	18	728	212 000	192	38.1	14.1	5.0	12 300	46.5	0.15

Note: Beads were prepared by spiking powder from adult croaker otoliths with known concentrations of various elements, and then fusing in a 1:1 mixture with lithium tetraborate.

*The absolute values of the Fe assays in the beads appear somewhat higher than expected based on spiking levels, although the relative concentrations are correct; Fe assays based on GF-AAS are probably more accurate than assays based on ICPMS.

within two laboratories nested within four instrument types. Since this is a simple hierarchical design, each factor was tested over the mean square error of the factor below it in the nested design. To simplify the interpretation, each of the four bead types was analyzed separately (a significant bead effect is known to be present a priori).

Inaccuracies in the analysis of Sr in the otolith beads by the WD electron microprobes quickly became apparent, presumably because of unsuspected effects of the lithium tetraborate flux on the X-ray background. Although the uncorrected Sr data are presented as measured, corrected Sr concentrations in the beads for the two WD-EM instruments were later calculated by subtracting the mean real-otolith Sr background from the peak counts measured in the assays of the beads. The resulting net counts were converted to corrected absolute concentrations on the basis of the regression between the original PAP-corrected Sr concentrations and the original net counts. Note however that application of background measurements from one matrix for use in another matrix is not normally a recommended practice and was applied in this case only, because few alternatives were available.

To quantify the relationships between observed and actual concentrations, measures of accuracy (deviation of observed from actual values), precision (reproduceability of measurements), and correlation were calculated. Accuracy calculations were based on the mean within-bead concentrations (to insure independence) and were computed as the sum of squared deviations of the observed and actual concentrations for a given element and laboratory. Precision was calculated as the mean within-bead CV for a given element and laboratory. The correlation coefficient between observed and actual mean within-bead concentrations was also calculated for each element and laboratory as a measure of linearity. However, this statistic was not useful as it was found to be unduly leveraged by the highest concentration in elements with an uneven distribution across their range.

Results

Allowing for the specific requirements of each instrument

type, there remained considerable diversity among laboratories in the operational protocols applied to the assay of the IOCE samples (Table 1). The differences in hardware associated with any one equipment type were relatively minor; however, operating conditions such as currents, counting times, laser wavelengths, and dwell times often differed. The implications of these operating differences were not immediately apparent, although those laboratories that used large spot sizes (e.g., LA-1, PIXE-2) would presumably see reduced variance associated with any sample heterogeneity. Most laboratories used external standards to calibrate their readings, and given the option, most would have reduced variance by standardizing to the Ca concentration in the sample. The degree of otolith preparation was relatively similar among instrument types, with the exception of LA-ICPMS, which required less exacting sample preparation. None of the instruments resulted in much more than shallow beam-width pitting of the otolith samples, with the exception of LA-ICPMS, which produced shallow craters several times larger in diameter than the beam size.

As was expected based on otolith assays of other species, the elemental composition of the otolith powder used to prepare the artificial otoliths was dominated by Ca, Sr, Na, and K (>0.1% each), with the remaining elements each accounting for less than 40 parts per million (ppm) (Table 2). Trace element concentrations in the beads were, by design, more variable than in the powder but reflected the same gross variations. Note that the elemental concentrations in the beads relative to Ca are approximately double those presented in Table 2, because of dilution by the lithium tetraborate flux.

No one instrument type could detect each element in the otolith beads under a single instrument setup (Fig. 1). The detection capability for a given element varied most among

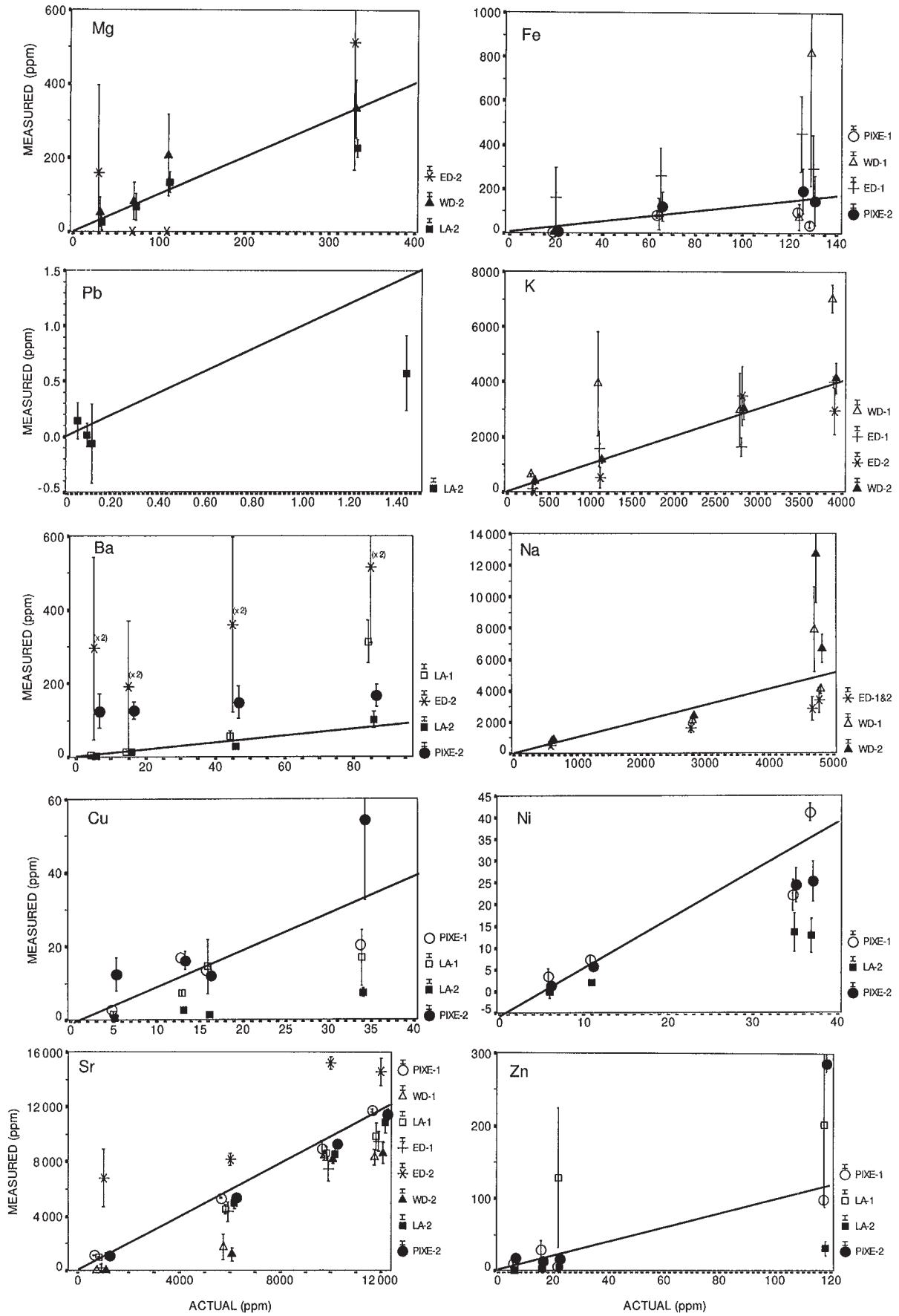


Table 3. Limits of detection (ppm by weight) by instrument type and laboratory for each element assayed in juvenile croaker otoliths.

Instrument and laboratory	Limit of detection (ppm)									
	Na	Mg	K	Fe	Ni	Cu	Zn	Sr	Ba	Pb
ED-EM										
ED-1	21 300	17 200	3400	1100	1100	1300	—	12 500	2900	21 000
ED-2	1 300	1 600	780	1900	700	890	1200	910	1600	120
WD-EM										
WD-1	260	—	70	40	—	—	—	175	—	—
WD-2	230	250	330	—	—	—	—	480	—	—
PIXE										
PIXE-1	—	—	—	5	2.6	2.2	0.5	1.0	40	6
PIXE-2	—	—	—	2	0.4	0.4	0.3	0.5	20	3
LA-ICPMS										
LA-1	—	—	—	—	0.4	0.06	0.2	2	0.04	0.02
LA-2	—	0.09	—	—	0.1	0.04	0.1	0.7	0.04	0.02

Note: Because there is no generally accepted method for estimating elemental limits of detection for ED-EM, the limits for ED-EM must be viewed in a relative sense only.

instruments but also between laboratories using the same instrument. Indeed, the only element that could be measured in all laboratories was Sr. Abundant elements such as Na and K were only reported by the ED and WD electron microprobes, while trace elements such as Ni, Pb, Cu, and Zn required either PIXE or LA-ICPMS. On average, where concentration differences among bead types were detected, the relative difference among bead types was accurately estimated. However, absolute concentration was estimated accurately with less frequency. It is important, though, to avoid interpreting the actual (baseline) composition of the beads in Fig. 1 too strictly; given the variability apparent in the baseline assays of several elements (Table 2), the actual composition of some of the beads may differ slightly from that indicated in Fig. 1. For instance, the distribution of assay estimates around the two highest Sr concentration values suggest that the baseline values overestimated actual concentrations by 10–15%. However, errors of this magnitude do not change any of the overall patterns or conclusions concerning the relative accuracy of each instrument, nor do they explain the surprisingly inaccurate measurements of bead Sr by WD-EM.

Analyses of variance of the otolith bead assay results were consistent with the general patterns apparent in Fig. 1. Replicate otolith beads differed significantly within most concentrations and laboratories, suggesting that, while bead composition may have been internally homogeneous, significant differences existed among beads within any given batch. For elements such as Mg, Cu, Ni, Na, and K, instrument and laboratory effects were generally nonsignificant across most bead concentrations. Significant instrument effects were apparent in the assays of Ba, Fe, and Pb. However, in the other elements, most of the remaining variance could be attributed to laboratory effects nested within instruments, rather than to the instruments themselves. For example, much of the variance in the Sr assays was due to the large and significant difference between the two laboratories using ED-EM. When the analysis was repeated without the ED-EM assays, the instrument effect became significant and the laboratory effect less so, with a posteriori contrasts pointing to the WD-EM assays as the most significant source of variance. There were no consistent differences between the PIXE and LA-ICPMS assays across all bead concentrations.

While the assays of the otolith beads were useful in determining assay accuracy over a range of elemental concentrations, the bead matrix was too dissimilar to that of real otoliths to be useful in determining LOD (minimum detection limit, defined as 3 SD of the blank) for many of the elements. Limits of detection based on the assays of the laboratory-reared otoliths indicated that most elements were detectable by ED-EM only at concentrations exceeding 1000 ppm, although the actual ED-EM LOD values varied substantially depending on the method used to calculate them (Table 3). WD-EM was the more sensitive of the two electron microprobes, requiring concentrations of more than 100 ppm (Table 3). Detection limits for PIXE and LA-ICPMS were much lower again, at around the ppm level for most elements. Sub-ppm LODs for LA-ICPMS may or may not be realistic, since they can be calculated only on the basis of argon gas blanks without an attendant laser pulse. For all instruments, LODs in the otoliths were lower than those in the otolith beads, although the patterns were generally consistent between the two.

The assay results of the laboratory-reared otoliths were in many respects similar to those of the beads: elements that were measured accurately in the beads by a given instrument were also measured accurately in the otoliths (Table 4). On average, the ED and WD electron microprobes performed well in measurements of the more abundant elements Na and Ca (much less so in the case of K by ED), while PIXE and (or) LA-ICPMS were required for accurate measurements of the trace elements Mg, Fe, Ni, Cu, Zn, Ba, and Pb. With the exception of one of the ED instruments, all laboratories appeared to provide accurate measurements of Sr in the otoliths. While the potential for local variations in Sr concentration across the otolith reduces our ability to compare assay accuracies too precisely, it is worth noting that many of the Sr estimates differed significantly among laboratories (Table 4). Analysis of variance with laboratory as a main effect indicated that there were no significant differences in elemental concentration associated with assay location on the otolith ($p > 0.05$).

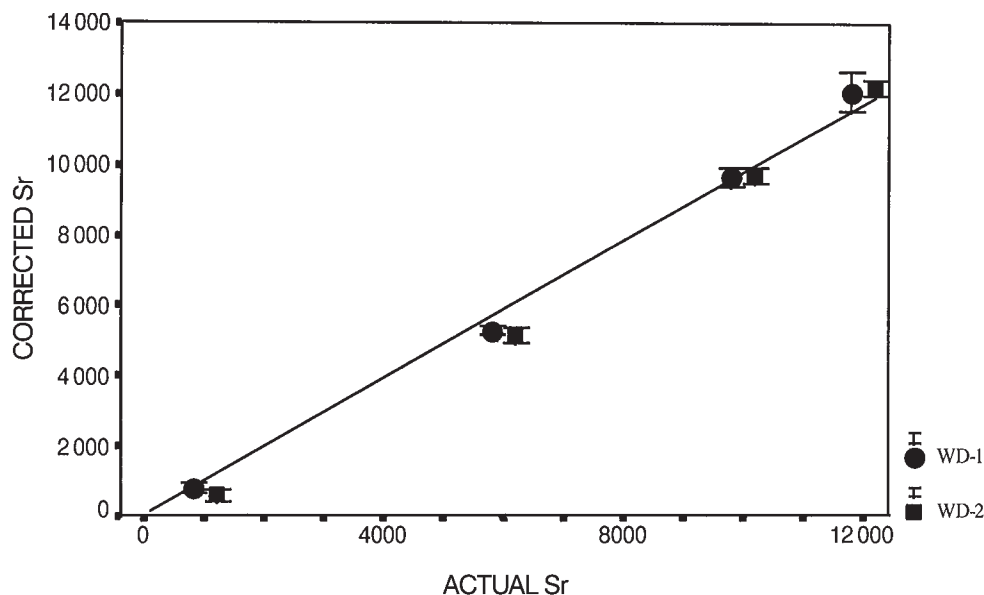
In light of the accuracy of the Sr measurements in the otoliths as measured by WD-EM, the inaccuracy noted in the Sr assays of the beads by the same instruments was puzzling. Further investigation uncovered anomalously high background spectra in the beads, resulting in distorted calculations

Table 4. Elemental analysis of juvenile croaker otoliths reared under constant environmental conditions from the time of hatch.

Instrument and laboratory	Elemental concentration ($\mu\text{g}\cdot\text{g}^{-1}$)										
	Na	Mg	K	Ca	Fe	Ni	Cu	Zn	Sr	Ba	Pb
ED-EM											
ED-1	1300±431	—	120±35	366 000±2400	340±94	490±83	0±95	—	1720±228	0±113	—
ED-2	500±99	—	100±37	385 400±700	0±35	600±122	—	—	0±94	300±75	—
WD-EM											
WD-1	3038±147	—	1691±180	388 000±1000	—	—	—	—	2258±88	—	—
WD-2	3790±220	80±20	1150±67	400 000±1200	—	—	—	—	1650±102	—	—
PIXE											
PIXE-1	—	—	—	—	0±0.7	1.0±0.2	1.0±0.3	1.0±0.3	1870±77	—	—
PIXE-2	—	—	—	—	2.1±1.5	1.3±0.2	2.9±0.1	1.9±0.1	2280±93	18±4	1.5±0.2
LA-ICPMS											
LA-1	—	—	—	—	—	0.8±0.2	—	0.6±0.2	1494±71	2.5±0.3	—
LA-2	—	15±2	—	—	—	—	—	3.2±7.8	2336±193	3.7±1.0	—
Baseline											
ICP-OES	—	<50	1350	364 500	<10	<125	<5	<250	1600	<5	<25
ICP-MS	—	<50	—	370 000	<10	<1	<2.5	<10	1800	4.2	0.25
ICP-MS	—	71	—	364 600	—	5.1	2.5	31	1955	5.7	1.2
ICP-MS	2480	30	1385	326 000	125*	6.7	1.2	11	1550	3.1	0.5

Note: The baseline elemental composition was determined in four independent assays of whole dissolved otoliths. Values are median \pm SE for five probe observations on each of three otoliths by each of eight laboratories. Elements measured by only a single laboratory are not reported.

*Assays of Fe by ICP-MS are probably not reliable.

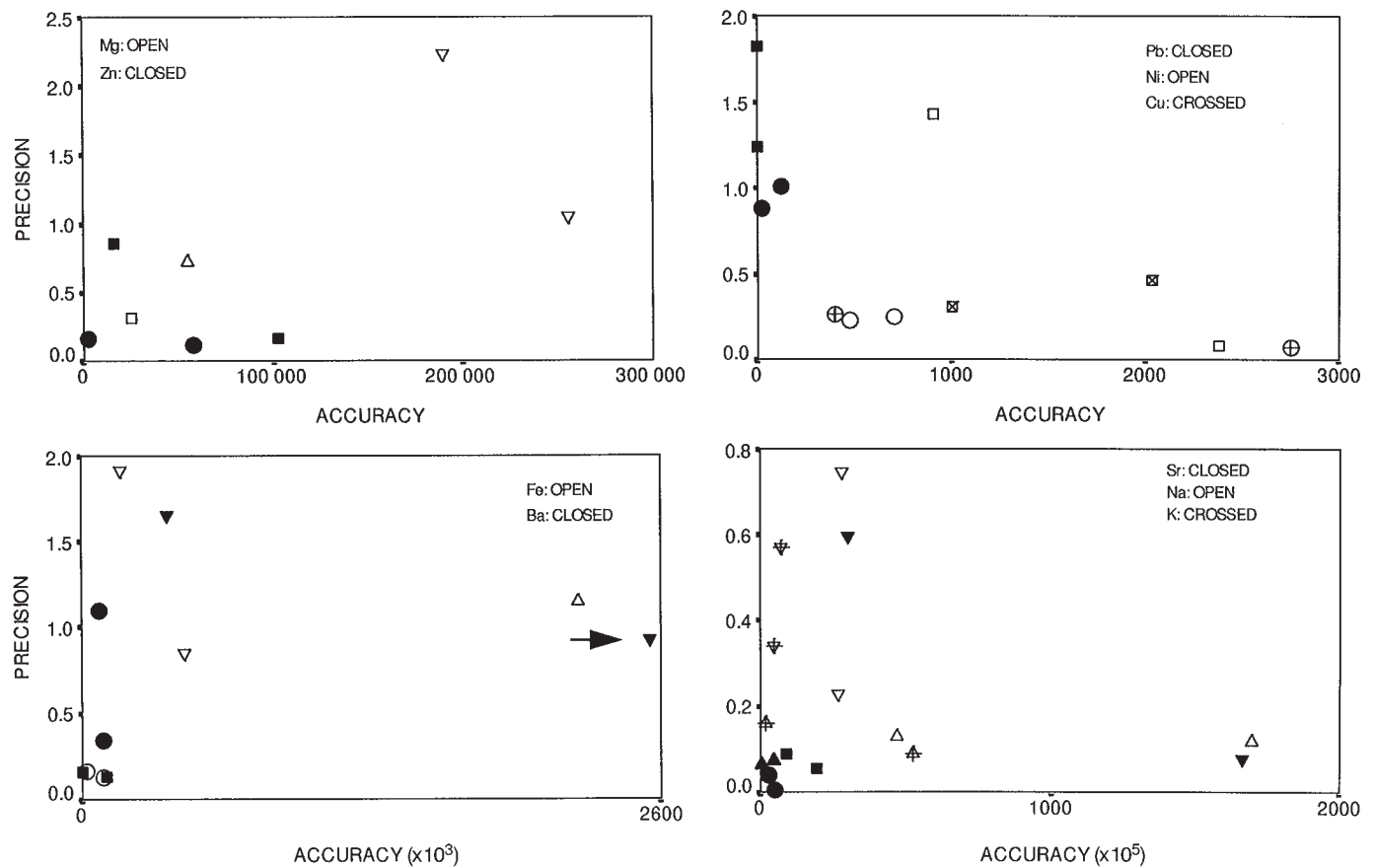
Fig. 2. Comparison of Sr measurements by WD-EM, corrected for background anomalies (see Materials and methods), with actual concentrations in the artificial otoliths (beads). Error bars represent 95% confidence intervals. The solid line represents the 1:1 line.

for Sr concentration. Substitution of background counts measured in real otoliths for those in the beads (see Materials and methods) resulted in corrected Sr concentrations that were much more accurate than originally calculated (Fig. 2). On the basis of these corrected values, it appears that WD-EM is comparable with PIXE and LA-ICPMS in its measurement accuracy and precision of Sr.

Measures of precision varied markedly among both instruments and elements and not necessarily in concert with measures of accuracy (Fig. 3). For example, assays of Ba by LA-ICPMS and Fe by PIXE were both accurate and precise.

However, the most precise assays for Ni and Cu (by LA-ICPMS and PIXE, respectively) were also the least accurate. Conversely, the least precise estimates for K were the most accurate. In general, ED-EM provided the least precise measurement for any given element, while PIXE was most precise. For almost all elements, the precision of the bead assays was better than that of the otolith assays, suggesting that the beads were more uniform in elemental composition. Indeed, the most readily measured elements in the otolith were never measured with CVs of less than 15–20%, suggesting that this may represent the best precision possible in a random sample of natural

Fig. 3. Mean assay accuracy and precision of artificial otoliths (beads) by element and laboratory. Only those laboratories that detected significant differences among bead concentrations are presented. The calculations for Sr by WD-EM have been corrected for background anomalies (see Materials and methods). The arrowed symbol in the bottom left panel is offscale and should be located at $x = 5000$. (□) LA-ICPMS; (○) PIXE; (△) WD-EM; (▽) ED-EM.



otoliths from the same environment. Of course, improved precision is likely if the assays were to be restricted to a specific growth zone within a single otolith.

In addition to the 11 elements selected for assay in this study, several laboratories reported the presence of additional isotopes and elements in the croaker otoliths. Multiple isotopes of many of the elements were measured with LA-ICPMS; however, for the purposes of this study, concentrations of individual isotopes were averaged to produce elemental concentrations. In addition, the elements C, O, Cl, S, and P were reported as being present in significant quantities by one of the WD-EM laboratories, while one of the LA-ICPMS labs reported significant quantities of Cr, Ce, and La. However, the presence of Cr, Ce, and La was unconfirmed by other laboratories and was not reported in conjunction with LODs.

Discussion

Otoliths are intrinsically difficult to assay, most likely because of the relative purity of the aragonite matrix. This difficulty was reflected in the variance around the solution-based estimates of croaker otolith elemental composition, which in principle, should be relatively accurate and precise. On the basis of the assays reported here, croaker otoliths are composed 96.2% by weight of calcium carbonate, with an additional 0.73%

present as nonorganic trace impurities. By differencing, the organic matrix would make up the remaining 3.1%. Other broad-spectrum elemental assays of otoliths have also resulted in trace element concentrations of less than 1% (Edmonds et al. 1992; Sie and Thresher 1992; Proctor et al. 1995; Severin et al. 1995), and protein concentrations of 1–8% (Degens et al. 1969; Asano and Mugiya 1993; Hoff and Fuiman 1993). Thus, the croaker otoliths assayed as part of this study appear to be representative of a broad range of species.

In general, assay precision by each of the instruments was not a good measure of assay accuracy and vice versa. Many assays (with the exception of those by ED-EM) were relatively precise ($CV < 0.5$). Yet accuracy varied widely within this range of precision for any given element. In only one instance (Mg) was the most precise estimate also the most accurate. The absence of reference standards was not the problem here, because virtually all laboratories used mineral reference standards to calibrate their instruments. However, none of the mineral standards matched the aragonite matrix present in the otolith, which may have introduced some bias into the results. Indeed, various participating laboratories noted that the presence of lithium (which was expected) and tungsten (which was unexpected) in the otolith beads degraded their ability to quantify the concentration of other elements. Certified reference materials (CRM) are often developed to insure

accuracy in the assay of specific materials (Quevauviller et al. 1992; Catterick et al. 1995); the development of a CRM for otoliths may be worthwhile.

Inaccuracy in estimating absolute concentration has significant implications for comparisons among published studies: it suggests that apparently significant differences among laboratories in otolith elemental concentration for a given fish species or population may be due to analytical differences among laboratories rather than actual differences in otolith composition. Thus, inferences concerning stock differences based on comparisons among laboratories may or may not be valid. Therefore, if more than one laboratory is to be involved in a project, intercalibration among laboratories would appear to be necessary. On the other hand, the relative concentration of most detectable elements was accurately estimated by the laboratories in this study, even if the absolute concentrations were in error. Such internal consistency would lead to far more robust conclusions than if multiple laboratories were involved. Indeed, absolute differences in otolith composition would seldom be expected to yield conclusions any different than those based on relative differences, with the exception of situations where elemental concentrations are close to detection limits.

While differences in accuracy, precision, or sensitivity among instrument types are to be expected, differences among laboratories using the same instrument are less easy to explain. Nor were interlaboratory differences consistent across elements. For example, only one of the PIXE laboratories reported the relative concentration of Ba in the beads, and only one of the ED-EM laboratories was able to measure the relative concentration of Fe. A similar situation was apparent in comparisons of the otolith assays by the two LA-ICPMS laboratories: the laboratory that was able to accurately measure Mg in the otoliths could not detect Ni, and vice versa. In some cases, laboratory-specific operating conditions were responsible for these differences, such as the better quantification abilities of ED-1 relative to ED-2 associated with a counting time that was five times as long. However, in the majority of cases, a more likely source of the difference is the conversion of detector counts to concentrations, during which matrix corrections, removal of spectral interferences, and other, often laboratory-specific, data preparation steps can combine to influence final values. Whatever the cause, differences among laboratories using similar instrumentation can be a significant source of error (Campana and Moksness 1991) and provides further justification for use of CRMs in calibration.

Is there a single instrument that is to be preferred for use in determining the elemental composition of otoliths? The answer depends upon the hypothesis being tested and, by corollary, the elements being examined. Each of the four instrument types performed well with specific subsets of elements (Fig. 3); in particular, trace elements were best measured with PIXE and LA-ICPMS while measurement of the abundant K and Na ions required use of an electron microprobe. In general, the ED-EM proved to have the highest LOD and to be the least precise of the instrument types, as has been noted by other workers (Gunn et al. 1992; Kalish 1990). Given the LODs noted in this and other studies, a previous report of stock discrimination based on otolith trace element detection using ED-EM (Mulligan et al. 1987) appears to be in error. With respect to Sr, all but the ED-EM instruments were capable of providing accurate and precise measurements. However, there were

significant differences in mean otolith Sr concentrations among laboratories that cannot be fully explained by local otolith variability. This finding has significant implications for those using Sr:Ca ratios to reconstruct temperature or salinity history (Radtke 1989; Townsend et al. 1995; Secor et al. 1995b). In particular, it suggests that inconsistencies in calibrating WD-EM-based Sr:Ca measurements with other analytical techniques (e.g., Townsend et al. 1995) may in fact be due to analytical bias among instruments or laboratories, rather than to real differences in otolith composition. Although other workers have noted the sensitivity of WD-EM measurements to operating conditions (Gunn et al. 1992; Toole and Nielsen 1992), the work described here supports the assumption that measurements by a single instrument are internally consistent but that comparisons among instruments are not necessarily valid.

Given the observed variability among laboratories in assaying the same material, it is reasonable to question the biological significance of published differences in otolith elemental concentrations among species and studies. This is particularly true of Sr assays using WD-EM, which make up the majority of published work. On the basis of the croaker otolith assays (Table 4), the CV for Sr among all laboratories using either WD-EM, PIXE, or LA-ICPMS was 18%; the CV for the WD-EM laboratories alone was 22%. However, the variability in otolith Sr across published studies appears to be considerably larger. Limburg (1995) reported a CV for otolith Sr of 38% across 7 freshwater species, while Secor et al. (1995b) reported CVs of 41% and 34% for 6 freshwater and 23 saltwater species, respectively. While a comparison of CVs across independent studies and species is hardly conclusive, it does suggest that sources of variance due only to instrument and laboratory effects are insufficient to explain more than about half of the observed variance in otolith Sr across independent studies. Given the much lower variances observed within laboratories (as opposed to among laboratories), there is no reason to doubt the validity of the majority of the published studies on Sr. The same conclusion does not necessarily apply to elements such as Pb, Ni, Cu, Fe, and Zn, for which instrumental differences and variability were much larger.

While electron microprobes, PIXE, and LA-ICPMS are among the most popular instruments for targeted assays of otoliths, there are other instruments which conceivably could provide superior assay capabilities for one or more elements. Indeed, this study did not consider the many elements that are present in otoliths at the sub-ppm level (e.g., Cd, Sn, Rb, U), which are probably not detectable in otoliths even with PIXE and LA-ICPMS. Resonance ionization spectroscopy (Arlinghaus et al. 1993), synchrotron monochromatized X-rays (Ishikawa et al. 1991), and laser-induced fluorescence spectroscopy (Coutant 1990) could conceivably fill this gap, as well as secondary ion mass spectrometry, Fourier transform infrared spectroscopy, micro Raman spectroscopy, and others (Jackson et al. 1993; Jambers et al. 1995). In general, bulk and (or) solution-based elemental assays such as isotope dilution ICPMS (Fassett and Paulsen 1989), accelerator mass spectrometry (Rucklidge 1995), and neutron activation analysis (Schmitz et al. 1991) are capable of better accuracy, precision, and (or) sensitivity than are beam-based assay techniques. However, bulk techniques cannot take advantage of the chronological growth sequence recorded in the otolith, and as

such, are better suited to questions of stock discrimination than to questions concerning migration pathways or anadromy that involve ages or dates.

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