Effect of analytical conditions in wavelength dispersive electron microprobe analysis on the measurement of strontium-to-calcium (Sr/Ca) ratios in otoliths of anadromous salmonids

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The use of strontium-to-calcium (Sr/Ca) ratios in otoliths is becoming a standard method to describe life history type and the chronology of migrations between freshwater and seawater habitats in teleosts (e.g. Kalish, 1990; Radtke et al., 1990; Secor, 1992; Rieman et al., 1994; Radtke, 1995; Limburg, 1995; Tzeng et al. 1997; Volk et al., 2000; Zimmerman, 2000; Zimmerman and Reeves, 2000, 2002). This method provides critical information concerning the relationship and ecology of species exhibiting phenotypic variation in migratory behavior (Kalish, 1990; Secor, 1999). Methods and procedures, however, vary among laboratories because a standard method or protocol for measurement of Sr in otoliths does not exist. In this note, we examine the variations in analytical conditions in an effort to increase precision of Sr/Ca measurements. From these findings we argue that precision can be maximized with higher beam current (although there is specimen damage) than previously recommended by Gunn et al. (1992).

Wavelength dispersive electron microprobe analysis (WD-EM) has been used by most researchers, although other methods such as proton-induced *x*-ray emission (PIXE)(Babaluk et al., 1997; Markowitz et al., 2000) have been used. WD-EM remains a common and relatively inexpensive method. The conceptual approach among researchers using WD-EM is similar but the methodological approach or analytical (operating) conditions vary. In a comparison of laboratories using common otoliths, Campana et al. (1997) found among-laboratory variation in mean Sr concentrations that could not be described by otolith variability. Although the laboratories were internally consistent in applying their methods, comparisons between laboratories differed. Campana et al. suggested that the sensitivity of WD-EM to operating conditions might have led to this variation between laboratories.

Development of analytical techniques for measuring Sr/Ca ratios has been reviewed to validate techniques in specific studies (Kalish, 1990; Secor, 1992; Toole and Nielsen, 1992; Limburg, 1995). Gunn et al. (1992) analyzed effects of counting times, beam current, accelerating voltage, and beam diameter on measures of Sr and other elements and they warned that beam powers required for WD-EM were sufficient to cause specimen damage including pitting and chemical change. As a result, Gunn et al. (1992) recommended limiting beam power densities to $< 3 \mu W/\mu m^2$. This recommendation has been followed in most studies using Sr/Ca ratios to reconstruct the chronology of migrations between the freshwater and marine environments (Table 1). Toole and Nielsen (1992), however, concluded that Sr/Ca precision could be increased, with no loss of accuracy, by using analytic conditions that lead to a beam power density of just over $15 \mu W/\mu m^2$ (5-µm beam diameter; accelerating voltage=15 nA; beam current=25 kV). The inherent beam damage was not critical because of the similar behavior of Sr and Ca during progressive beam damage.

In published studies using WD-EM to measure Sr/Ca ratios in otoliths, the operating conditions, including beam power densities, have varied greatly (Table 1). Establishing a microprobe protocol for measurement of Sr/Ca ratios in otoliths involves a balancing act of counting times, beam current, and beam diameter. The selection of optimum conditions is constrained by financial resources, allocation of time for use of instruments, and the required resolution of Sr/Ca ratios for any specific application. Each researcher must weigh the benefits and costs to best answer the question at hand. Generally, these parameters are manipulated to optimize precision and accuracy of analyses in relation to variability within the otolith and implications of the results.

For Sr/Ca ratios to remain an accepted and accurate means of describing migration histories and other life history events, continued analytic and technical refinement and validation are required. We examined the effects of crystal choice, beam diameter, beam current, and beam power densities on Sr/Ca measurements (expressed as atomic ratios) in salmonid otoliths: 1) we measured Sr using both the TAP and PET crystals in regions with high Sr/Ca (>0.003) and low Sr/Ca (<0.001)

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Table 1

Analytic conditions reported by researchers using wavelength-dispersive (WD) electron spectroscopy to measure Sr/Ca ratios in otoliths. Beam power density was calculated for this study and minimum limit of detection is either directly reported from the work cited or from personal communications.

Source	Beam diameter (µm)	Accelerating voltage (kV)	Beam current (nA)	Beam power density (µW/µm^2)	Minimum limit of detection (Sr ppm)
Brown and Severin (1999)	6	15	20	10.61	—
Campana et al. (1997) WD-1	10	25	5	1.59	175
Campana et al. (1997) WD-2	9	15	4	4.25	480
Kafemann et al. (2000)	5×8	15	10	3.75	490
Kalish (1990)	10×10	15	10	1.5	—
Kawakami et al (1998)	1	15	50	954	—
Limburg (1995)	20	20	25	1.59	290
Radtke (1995)	5	15	10	7.63	—
Rieman et al. (1994)	5	15	50	38.14	—
Secor (1992)	5×5	25	20	20.00	580
Thresher et al. (1994)	14	15	25	2.44	311
Toole et al. (1993)	5	15	20	15.27	—
Volk et al. (2000)	10	15	15	2.86	237
Zimmerman and Reeves (2000)	7	15	50	19.50	43

levels; 2) we then compared the results of repeated Sr/Ca measurements collected at the same spots using various beam diameters, while holding accelerating voltage and beam current constant to determine the effect of beam damage on Sr/Ca measurements; and 3) we compared the results of repeated Sr/Ca measurements collected at the same spots using various beam currents, while holding accelerating voltage and beam diameter constant. We argue that increased precision of Sr measurements afforded by higher beam current (and hence, higher beam power densities) is preferable for studies where only measurements of Sr/Ca ratios are required.

Materials and methods

Otolith preparation

Sagittal otoliths from an adult sockeye salmon (*Oncorhynchus nerka*) collected in the Deschutes River, Oregon, and a juvenile chinook salmon (*O. tshawytscha*) collected in the Umatilla River, Oregon, were used to represent high (>0.003) Sr/Ca and low (<0.001) Sr/Ca ratios, respectively (Zimmerman, unpubl. data). High Sr/Ca ratios characterized the saltwater growth region in the sockeye salmon otolith and low Sr/Ca ratios characterized the freshwater growth region of the chinook salmon otolith. Each otolith was mounted sulcus side down with thermo-setting plastic resin on a microscope cover slip attached at one end with super-glue to a standard microscope slide. The otolith was then ground with 1200-grit sandpaper in the sagittal plane to the level of the nucleus. The mounting medium was heated and the otolith turned sulcus side-up. The otolith was then ground with 1200-grit and 2000-grit sandpaper in the sagittal plane to the level of the primordia and polished with 0.05-µm alumina paste. The cover slip was then cut with a scribe and mounted with other prepared otoliths (those used in other studies) on a petrographic slide for microprobe analysis. The slide containing several otoliths was rinsed with deionized water, air dried, and carbon coated (400 Å). Elemental analysis was conducted with a Cameca SX-50 wavelength dispersive microprobe. Strontiantite ($SrCO_3$, USNM R10065) and calcite ($CaCO_3$, USNM 136321) were used as standards for Sr and Ca, respectively. Standards were calibrated with a 30-µm-diameter beam and 10-s counts resulting in minimal effects of beam damage.

Effect of spectrometer (crystal) choice

To evaluate differences in diffracting crystals, we conducted a series of tests where Sr was measured by using both the PET and TAP crystals. A 15 kV, 50 nA beam was used for these comparisons. With a 7-µm-diameter beam, Sr was measured by using the TAP crystal (Sr Lα) and Ca was measured by using the PET crystal (Ca Kα). Two transects of 10 points each were sampled so that the points on adjacent transects covered the same temporal location on the otolith. Sr and Ca were analyzed simultaneously; counting times for the Sr and Ca peaks were 40 s, and background counts were 40 s. A second set of transects covering the same temporal locations in the otolith was sampled, but Sr was measured with the PET crystal (Sr Lα). Because our microprobe has only one PET crystal, simultaneous measurement of elements was not possible. Transects were conducted on both high and low Sr/Ca regions. Sr/Ca ratios were calculated from normalized mole fractions of Sr and Ca. Limit of detection (3σ; Potts, 1987) was calculated for all points in both high and low Sr/Ca regions.

Effect of beam diameter and beam current

We conducted five repeat measurements at each of five points within the high and low Sr/Ca regions using a 1-, 7-, 15-, 20-, and 25-µm-diameter beam at 15 kV and 50 nA. This resulted in beam power densities of 961, 19.5, 4.2, 2.39, and 1.52µW/µm^2, respectively. We conducted five repeated measurements at each of four locations within the high and low Sr/Ca regions using beam currents of 5, 10, 20, and 30 nA with a 10-m-diameter beam and accelerating voltage of 15 kV, with resulting beam power densities of 1.0, 1.9, 3.8, and 5.7µW/µm^2, respectively. The coefficient of variation (CV) of Sr/Ca ratios was calculated as the $SD_{Sr/Ca}$ × Mean Sr/Ca^{-1} for each beam power density. If beam damage affects precision and accuracy of Sr/Ca ratios, subsequent measurements at the same spot should be increasingly divergent from the first and such divergence should be evident in high coefficients of variation. The limit of detection was used as a measure of precision. Limit of detection (3σ; Potts, 1987) for Sr was calculated for the first measurement taken at each beam power density in each region.

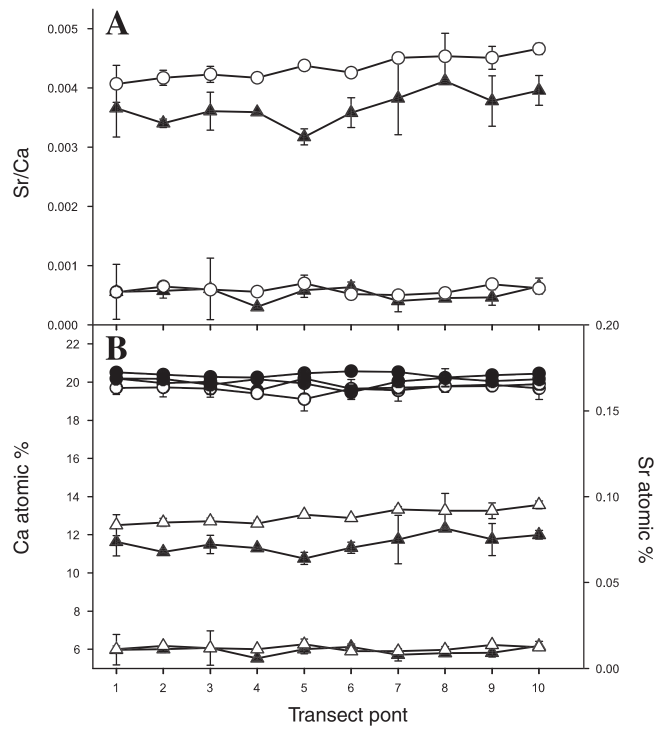

Figure 1

Transects of (**A**) Sr/Ca atomic ratio and (**B**) Ca (circles) and Sr (triangles) as atomic percentage when Sr is measured on the PET crystal (solid symbols) and on the TAP crystal (open symbols). Error bars represent 95% confidence intervals.

Results

Effect of spectrometer (crystal) choice

Spectrometer (crystal) choice for the measurement of Sr had an apparent systematic effect on Sr/Ca ratios at high Sr/Ca levels but no effect at low Sr/Ca levels (Fig. 1A). The mean Sr/Ca level in the high Sr/Ca region was significantly lower (~15%) when Sr was measured on the PET crystal (t=7.189; P<0.001; df=38). Crystal choice had an effect on measurement of both Ca and Sr in the high Sr/Ca region. Ca did not differ significantly between the high and low Sr/Ca regions (P>0.05) but was approximately 2% lower when Sr was measured with the PET crystal (Fig. 1B). This difference was attributable to beam damage, which occurred as Sr was measured. The mean Ca was 197,400 ppm when Sr was measured on the PET crystal and 202,100 ppm when Sr was measured on the TAP crystal.

In the high Sr/Ca region, mean Sr was significantly lower when Sr was measured on the PET crystal (t=11.58; P<0.001; df=38) (Fig. 1B). The mean (±SD) was 7270 ±4 ppm when Sr was measured with the PET crystal and 8870 ±3 ppm when Sr was measured with the TAP crystal. This difference is also reflected in the higher minimum limit of determination for Sr for PET in both the high Sr/Ca (695 ppm) and low Sr/Ca regions (126 ppm). Using the TAP crystal to measure Sr, we found that the minimum limit of determination for Sr was 103 ppm and 65 ppm in the high Sr/Ca and low Sr/Ca regions, respectively. To achieve similar counting statistics for Sr with the PET crystal, count times would need to be increased to 200 seconds on both the peak and background.

Effect of beam diameter and beam current

In repeated measurements with different beam diameters (same beam current) at the same locations, Sr/Ca ratios did not vary greatly with the exception of measurements made with the 1-µm beam (Fig. 2A). The CV of the Sr/Ca ratios for the 1-m beam was high and led to significant variation of Sr/Ca ratios in subsequent measurements at the same point (Table 2). The Sr/Ca ratio was least variable for the 7-µm beam in the high Sr/Ca region (Table 2). Limit of detection (3σ) for Sr ranged from 80 ppm to 172 ppm in the low Sr/Ca region and from 299 ppm to 315 ppm in the high Sr/Ca region under the various beam diameters (Fig. 3A).

Beam current had a significant effect on variation of Sr/Ca and limit of detection for Sr. The greatest variation in Sr/Ca ratios was observed at beam currents 5nA and 10 nA (Fig. 2B). The CV of Sr/Ca ratios was negatively related to beam current (r=–0.93; P>0.05) (Table 3). The CV of Sr was high in all treatments, ranging from 0.23 to 1.17 in the high Sr/Ca region and from 0.11 to 0.46 in the low Sr/Ca region. The CV of Ca was 0.04 for all beam configurations. The limit of detection of Sr as measured at the first sample

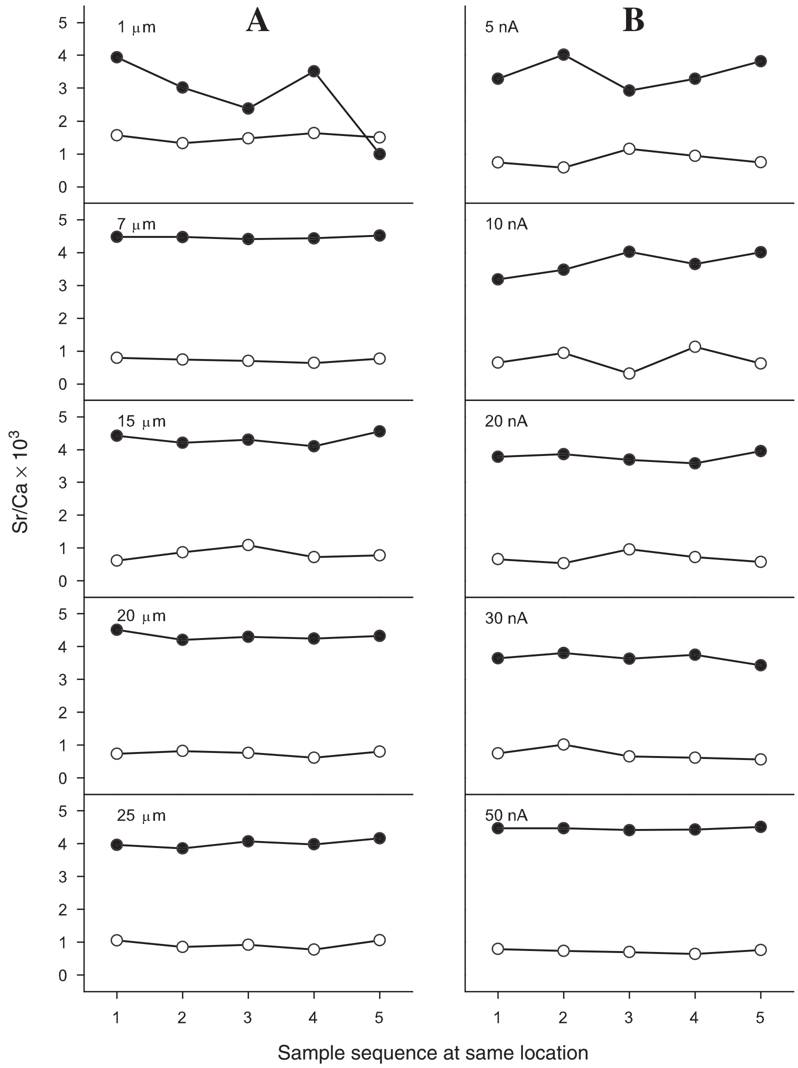

Figure 2

Repeat measures of atomic Sr/Ca with (**A**) varying beam diameters, common beam current (50 nA), and (**B**) varying beam currents, common beam diameter (10 μm). Open circles represent low Sr/Ca regions and solid circles represent high Sr/Ca regions.

for each beam-current configuration ranged from 99 ppm to 290 ppm in the low Sr/Ca region and from 312 ppm to 844 ppm in the high Sr/Ca region (Fig. 3B).

Discussion

In our experiment, for measuring Sr/Ca ratios in otoliths with WD-EM analysis, the TAP crystal was the best choice for the measurement of Sr because it provided the advantage of higher count rates and higher resolution of Sr. Use of the PET crystal to measure Sr has not been reported in the literature. In fact, crystal choice is frequently not reported, making it difficult to know whether TAP or PET crystals have been used to measure Sr. Personal communication with several researchers confirmed that the TAP crystal is commonly used to measure Sr and has been cited as the crystal used (Kalish, 1990; Thresher et al., 1994). Given the different results possible with the use of different crystals to measure Sr, reporting the crystals used should be included in papers reporting Sr/Ca ratios measured on WD-EMs. Note that even though measurements of Sr with the TAP crystal appeared to be the best method for otoliths, the TAP crystal should not be used to measure Sr in materials containing Si because of analytical interference from Si.

Variation of beam diameter has very little effect on the limit of detection of Sr, even at extremely high beam current densities. Rather, variation in beam current had

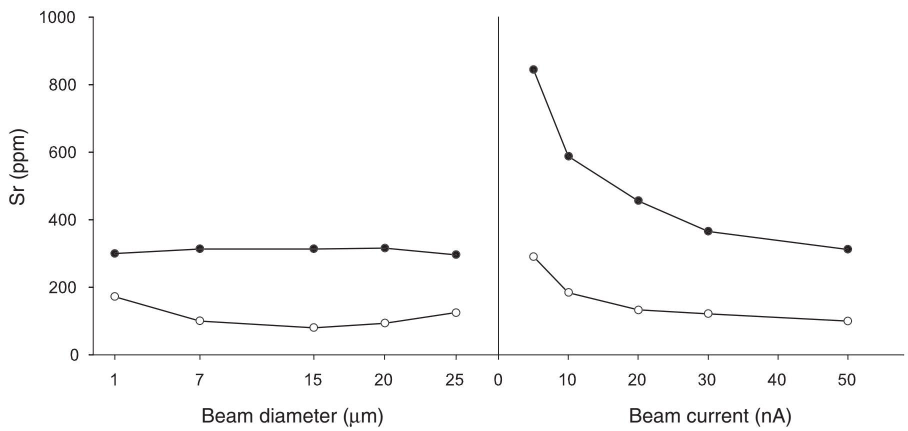

Figure 3

Detection limit of Sr with (**A**) varying beam diameter and (**B**) varying beam current. Open circles represent low Sr/Ca regions and solid circles represent high Sr/Ca regions.

Table 2

Coefficient of variation of Sr/Ca ratios and Sr (in parentheses) in high and low Sr/Ca regions of otoliths.

Beam diameter (µm)	CV Sr/Ca and Sr in low region	CV Sr/Ca and Sr in high region
1	0.076 (0.02)	0.416 (0.41)
7	0.082 (0.07)	0.009 (0.04)
15	0.218 (0.23)	0.041 (0.05)
20	0.107 (0.03)	0.028 (0.02)
25	0.133 (0.13)	0.030 (0.04)

Table 3

Coefficient of variation of Sr/Ca ratios and Sr (in parentheses) in high and low Sr/Ca regions of otoliths under varying beam current.

Beam current (nA)	CV Sr/Ca and Sr in low region	CV Sr/Ca and Sr in high region
5	0.354 (0.94)	0.099 (0.11)
10	0.429 (1.11)	0.098 (0.41)
20	0.239 (1.12)	0.039 (0.45)
30	0.248 (0.23)	0.039 (0.46)
50	0.082 (1.17)	0.009 (0.11)

significant effects on the limit of detection of Sr. As a result, higher beam currents (>20 nA) were appropriate for measuring Sr/Ca ratios in spite of beam damage observed at higher beam power densities. Beam diameters between 7 and 10m provide the best temporal resolution (i.e. covering fewer daily increments). The lower CV of Sr/Ca ratios observed with the 7-m beam diameter was likely due to the lower temporal variation afforded to smaller beam diameters (compared to larger beam diameters) and lower error related to specimen damage (compared to the 1-m beam). The lower CV of Sr/Ca ratios at the 7-µm beam diameter suggested that in spite of beam damage, the Sr/Ca ratio was not dramatically affected by beam damage. However, the increase in CV for the smaller diameters suggested that there are limits to usable beam densities.

Greater precision of Sr/Ca measurements is critical to understanding life history of some species (Markowitz et al., 2000) or in situations where differences in environmental Sr/Ca raios are less than those observed between ocean water and freshwaters (Rieman et al., 1994; Volk et al., 2000). Volk et al. (2000) found that timing of freshwater entry and length of freshwater residence by summer steelhead (*O. mykiss*) and spring chinook salmon had effects on otolith core or primordia Sr/Ca levels. Summer steelhead and spring chinook enter freshwater and stay for up to several months before spawning. Volk et al. (2000) suggested that significant egg development during this extended prespawning freshwater residence led to a dilution of the Sr signature in these anadromous fish. Zimmerman and Reeves (2000, 2002) were able to distinguish between resident rainbow trout and summer steelhead in the Deschutes River, Oregon, by comparing the Sr/Ca ratios in primordia and the first summer of juvenile growth (freshwater growth region). In essence the freshwater growth region acts as a proxy for the freshwater environment and significantly higher Sr/Ca ratios in the primordia suggest an anadromous maternal origin. The greater precision of Sr measures afforded by higher beam currents may be important in distinguishing differences in seasonal ecotypes, such as summer steelhead and spring chinook salmon, or in distinuishing estuary habitats from freshwater and ocean environments.

These results are applicable only to otolith calcium carbonate in the mineral form of aragonite. Like Brown and Severin (1999), we have found that crystalline structure affects the distribution of Sr. Vateritic regions should be avoided when measuring Sr/Ca ratios in otoliths. In vateritic portions of otoliths from chinook salmon and steelhead, Sr is often below our minimum detection limit of 43 ppm, yet the concentration of Ca does not differ from that found in aragonitic otolith regions (Zimmerman, unpubl. data).

Studies of fish migration between marine and freshwater environments are based on the general difference between Sr in marine and freshwater environments. Sr concentrations in seawater are generally an order of magnitude greater than in freshwaters (Bagenal et al., 1973; Kalish, 1990). Sr is substituted for Ca in the calcium carbonate matrix of the otolith at levels that correspond to those in the environment (Kalish, 1989; Farrell and Campana, 1996). Given this relationship, it has become a convention to report Sr as a fraction of Ca (Secor and Rooker, 2000). However, Secor and Rooker (2000) pointed out that Ca is relatively invariant in aragonitic otoliths and rarely varies more than 5% within an individual fish. At 8074 points sampled in the primordia, freshwater growth regions, and saltwater growth regions of several species of salmonids the Sr/Ca ratio was entirely driven by differences in Sr (Zimmerman, unpubl. data). At these 8074 points, Sr was highly correlated with the Sr/Ca ratio (r^2=99.45%) and Ca was not correlated with the Sr/Ca ratio (r^2<0.01%). Given this relationship, increasing precision of Sr is desirable to increase precision of the Sr/Ca ratio.

Our results suggest that tests of hypotheses related to Sr/Ca ratios can be conducted at higher beam power densities than suggested by Gunn et al. (1992). High beam power densities resulting from higher beam current and beam diameter of 7 to 10-µm provide greater precision (spatial on the otolith and temporal in the life of the fish) of Sr. This is not true for studies of stock discrimination, such as those described by Thresher (1999), that rely on absolute values of multiple elements, including Sr.

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