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1 **Validation of depth-profiling LA-ICP-MS in otolith applications**

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Draft

Abstract

Otolith microchemistry is a widely used technique for elucidating life history patterns in fishes. This typically involves sectioning the otolith and collecting elemental signatures via laser ablation. But this requires time-intensive handling that may influence results. As an alternative to traditional cut/polish/ablate techniques, we tested depth-profiling laser ablation, which offers reduced handling and contamination risk. To validate depth-profiling as a robust method for collecting trace element otolith microchemistry data, we constructed composite otoliths using otolith materials from fishes of different origins (freshwater, seawater). Test ablations were conducted on composite diadromous otoliths at a range of spot sizes and pit depths. We measured tailing and fractionation effects in the following elements; Na, Mg, K, Mn, Zn, Rb, Sr, and Ba. Given appropriate instrument parameters, depth-profiling can accurately collect elemental concentration data both between and within top and bottom layers of an otolith composite across a range of spot sizes and pit depths. Analytical power and lag effects were dependent on spot size, highlighting the importance of optimizing spot size based on sample morphology and instrument parameters.

Key-words: trace elements, otoliths, analytical methods

31 **Introduction**

32 Otolith microchemistry has been a valuable addition to the fisheries scientist's toolbox
33 for elucidating life history patterns and has seen greater use in recent years (Campana
34 1999, Thorrold et al. 2007). Laser ablation – inductively coupled plasma - mass
35 spectrometry (LA-ICP-MS), combining a laser ablation sampling system with an ICP
36 mass spectrometer, is a reliable and increasingly common means of collecting time-
37 resolved elemental concentration and isotopic signatures from otoliths at high spatial,
38 and thereby, temporal resolution (Jones and Chen 2003). A distinct advantage of laser
39 ablation over solution based ICP-MS is the retention of chronologically sequenced
40 chemical signatures within the otolith matrix (Campana 1999, Kingsford and Gillanders
41 2000). Traditional methods for collecting chronologically sequenced otolith
42 microchemistry data using laser ablation sampling include surface profiling spot-
43 transects and raster-transects, whereby a laser is fired repeatedly across a sample
44 surface with a fixed scan rate, after sectioning or grinding and polishing the otolith to
45 expose the plane of interest (Engstedt et al. 2013). However, sectioning otoliths prior to
46 analysis introduces a time-intensive handling and sample preparation step that can lead
47 to contamination (Arslan and Secor 2008), over/under grinding (Ben-Tzvi et al. 2007),
48 and handling loss, especially in smaller otoliths (<300 µm dia).

49
50 Depth-profiling is an alternative method for collecting transect data from otoliths using
51 LA-ICP-MS that does not require grinding, sectioning, or polishing otoliths prior to
52 sample collection. Depth-profiling is when the laser is fired repeatedly at a fixed point
53 on a sample substrate while simultaneously collecting ICP-MS data in a time-resolved
54 manner as the laser ablates through the material (Bleiner et al. 2003). When used on an
55 otolith, this results in an ablation pit beginning on the surface and passing through the

plane of interest, potentially through the whole otolith. In applications where correlating otolith microstructure with microchemistry is not necessary, depth-profiling ablations may offer benefits over more traditional techniques. In addition to reduced handling, the technique may also offer enhanced resolution in comparison with traditional surface-profiling techniques (Griffin et al. 2002). This is because traditional techniques expose a transverse section of the otolith to the laser. When the depth-profiling sampling plane is nominally parallel to the zonation of the otolith, each laser pulse could potentially sample sub-micron intervals, whereas the traditional transverse sample will integrate otolith material in the range of tens of microns. Application of depth-profiling in otolith microchemistry has received more attention in recent years, and results are promising (Macdonald et al. 2008, Crook et al. 2013, Hughes et al. 2014). However, several problems may interfere with its use in collecting accurate whole otolith microchemical profile data. These problems include signal response times influencing the effective temporal resolution of the acquired data through excessive tailing, down-hole effects such as elemental and isotopic fractionation resulting in measured elemental ratios that differ from the true ratios, and laser focusing effects (Fryer et al. 1995, Eggins et al. 1998, Mank and Mason 1999, Woodhead et al. 2004, Jochum et al. 2012). These issues may skew results, particularly when subtle differences in trace elements are being compared, such as in elemental fingerprinting studies.

The first of these potential problems is tailing. Tailing is when a signal wanes at an interface point instead of returning instantaneously to a new value due, for example, to the mixing of 'low' and 'high' concentration layers for a perceptible time interval (Bleiner et al. 2005). However, modern low volume ablation chambers have signal response times on the order of tens or hundreds of milliseconds, in comparison with

tens of seconds for older style high volume ablation chambers (Gurevich and Hergenröder 2007). Recent reductions in LA-ICP-MS instrument response times provide an opportunity to explore depth-profiling as a technique for sampling otoliths. But other issues may still interfere with the accurate collection of data. Laser focus and wavelength have been found to affect elemental fractionation and ablation pit morphology during depth-profiling ablation (Mank and Mason 1999, Hathorne et al. 2008). Elemental fractionation is a process by which elements are enriched or depleted in relation to their respective concentrations in the substrate, resulting in anomalous measured concentrations relative to the actual elemental concentration (Fryer et al. 1995). Departure from “top hat” pit morphology, whereby the cross section of the ablation pit possesses parallel sides and a perpendicular and flat pit bottom, occurs through poor laser focus during ablation. This can result in non-target ablation (i.e. ablation of the pit walls) and reduced resolution (Eggins et al. 1998). Both of these effects may worsen during depth-profiling as the ablation front proceeds further down-hole.

Woodhead et al. (2008) recommend characterizing downhole elemental fractionation using matrix matched standards, and the construction of layered composites of known elemental composition and depth to assess the magnitude of tailing effects prior to using depth-profiling in LA-ICP-MS analysis. Following their recommendation, we examined down-hole elemental fractionation at a range of spot sizes (50-90 μm), depths (100-200 μm) and masses (^{23}Na , ^{24}Mg , ^{39}K , ^{55}Mn , ^{64}Zn , ^{85}Rb , ^{88}Sr , and ^{138}Ba) in a matrix-matched calcium carbonate standard. Matrix effects, involving a different ablation response for materials of differing major element composition (e.g. carbonate versus silicate substrates), can influence accuracy during laser ablation, and matrix-matched standards result in greater accuracy (Jochum et al. 2012). When assessing the migratory

life history of diadromous fishes using depth-profiling, sampling the marine larval region requires ablating through the freshwater adult region. To assess top layer influence and tailing on bottom layer values, we constructed composite otoliths with boundaries of known depths with materials taken from non-migratory populations of freshwater and marine fishes. Because otolith trace element concentrations are often different between freshwater and marine systems (Elsdon and Gillanders 2005), we wanted to simulate the sampling of bottom layers through a top layer with different elemental concentrations. We then characterized the effects of spot size and layer depth on the resolution of elemental concentrations through top layers of opposing freshwater and marine origins.

Methods

Otolith Collection and Preparation

An artificial composite otolith simulating the concentration of trace elements found in a diadromous fish was constructed from otoliths of freshwater and marine origin.

Composite otoliths were constructed by gluing polished layers of freshwater and marine otolith material to each other. Freshwater otoliths were obtained from a population of land-locked non-migratory brown trout (*Salmo trutta*), and saltwater otoliths were obtained from gurnard (*Chelidonichthys kumu*).

The sagittal otoliths were removed from each fish, cleaned of tissue with a synthetic bristle brush, rinsed with high-purity Milli-Q (Millipore Ltd, U.S.A.) water, and stored in a laminar flow hood in 0.5 ml polypropylene microtubes to dry for at least 24 hours.

Prior to mounting, otoliths were fractured along the sulcus using a scalpel. The largest fragment was retained and mounted proximal face up in thermoplastic glue

(Crystalbond, Aremco Products Inc., New York). Otolith fragments were polished until the majority of the surface was flat and level using a graded series of either wet lapping films or lapping powders in order to present a good mating surface when bonding the layers. Otoliths were then sonicated for 5 minutes in Milli-Q water and allowed to air dry. The thickness of samples forming the base (lower layer) were measured and recorded using a micrometre screw gauge. Fresh and saltwater otolith materials forming the top (upper layer) were heated and freed from the thermoplastic glue, with care being taken not to contact or contaminate the polished otolith surface with glue or instruments.

Composite otoliths were constructed by adhering the polished freed otoliths to polished base otoliths of contrasting origin (freshwater *S. trutta* otoliths and saltwater *C. kumu* otoliths, hereafter referred to as “fresh” and “salt” respectively) using cyanoacrylate, and then repeatedly polishing and measuring sample thickness until a value equal to the base plus the target top layer thickness (60, 80 or 100 μm) was obtained. Cyanoacrylate was used because it is a rapid-setting, low-viscosity glue that resulted in good adhesion. Polishing resulted in samples with a veneer of otolith materials of contrasting origins (fresh/salt) with 60, 80, and 100 μm top layer thicknesses. Three composite otoliths with fresh/salt 60 μm , salt/fresh 80 μm , and fresh/salt 100 μm , were prepared for testing.

Otolith trace elements analysis

LA-ICP-MS was used to conduct depth-profiling transects of the composite diadromous otoliths. We used an Agilent 7500cs ICP-MS coupled to a Resonetics (now ASI) RESolution M-50 laser ablation system located in the Centre for Trace Element Analysis

at the University of Otago, Dunedin, New Zealand. The ablation system was powered by a Coherent 193 nm ArF excimer laser. Otolith mounts were placed in the M-50 (Laurin Tech) sampling cell and visually located via a 400X magnification video imaging system. Each sample was ablated repeatedly in a vertical transect from the distal surface to the proximal surface using a range of circular spot sizes. These diameters were 50 μm , 75 μm , 90 μm , and 130 μm . The laser was operated with a 5Hz. repetition rate. Average on-sample fluence for all ablations was 2.19 J/cm². Ablation occurred in an atmosphere of pure helium to minimize re-condensation of ablated materials and potential elemental fractionation artefacts (Eggins et al. 1998). Laser software-controlled gas flows of helium and nitrogen and ICP-MS-controlled argon carrier gas were tuned to maximise signal intensities on ¹³⁸Ba and ⁸⁸Sr while minimising oxides and mass fractionation by monitoring the NIST SRM 612 certified reference calibration glass (National Institute of Standards and Technology, U.S.A.). Each ablation of the composite otoliths was carried out for ~400 seconds to ensure that both layers of the composites were sampled. Prior to all ablations, a 60-90 second background ion count was measured. To reduce lag time associated with washout, no signal smoothing manifold was employed (Woodhead et al. 2008).

Table 1. Parameters of the LA-ICP-MS instrument.

Composite diadromous otoliths were analysed for the following elements; Na, Mg, K, Mn, Zn, Rb, Sr, and Ba, with Ca as the normalizing element to correct for reduced ablation yield downhole. Sr and Ba were chosen because of their strong correlation with freshwater and marine residency (Elsdon and Gillanders 2004). Sodium, Mg, K, Zn and Rb were also monitored; although not as strongly associated with environmental

conditions (Elsdon et al. 2008) these elements can still be informative when developing elemental signatures for populations (Thresher 1999). Importantly, the selected elements, with the exception of Zn, are lithophilic, and therefore much less prone to elemental fractionation effects (Jochum et al. 2012). The NIST SRM 610 and 612 calibration glasses were analysed every 5 ablations, using identical spot sizes to those employed for the composite otolith ablations, but with 40 second ablation periods. SRM 612 was selected as the calibration standard, and SRM 610 was used as a reference standard. USGS MACS-3 calcium carbonate standard (United States Geological Survey, U.S.A.) was used to assess the effects of down-hole elemental fractionation in a carbonate matrix. Working reference values for MACS-3 were taken from Chen et al. (2011). Working values for NIST SRM 610 and 612 glasses were taken from Jochum et al. (2011).

In order to provide values to compare with results from the laser ablation, approximately half of each otolith used to construct the composite laminates was retained for dissolution and analysis by solution nebulisation ICP-MS. Otolith materials were rinsed in 0.1% v/v ultrapure HNO₃, then de-ionized water (>18.2 MOhm-cm at 25C), and then dried. The cleaned material was weighed and then dissolved in ultrapure HNO₃ in a PFA vessel (SavilleTM). The resulting solution was quantitatively transferred to a larger vessel and diluted gravimetrically to give a final acid concentration of 2%v/v. An aliquot was removed, diluted as required and had a cocktail of reference elements added. The elemental concentrations were measured by solution-nebulisation ICP-MS using NIST traceable multi-element concentration standards. Blank samples were processed identically but no correction was required. The instrument was operated following manufacturer's recommendations for robust conditions. Different solutions

for the calibration of calcium and strontium were used to eliminate any possible bias due to doubly-charged strontium affecting the calcium calibration. Concentration results were converted to mole ratios.

Laser ablation data collection

To characterize the effects of ablation spot size on the resolution of transitions between zones, we recorded the time required to ablate completely through the top layer and into the bottom layer in the composite diadromous otoliths (Fresh/ Salt 60 μm , Salt/ Fresh 80 μm , Fresh/ Salt 100 μm). Freshwater otoliths have high Ba/Ca and low Sr/Ca ratios relative to marine otoliths and abrupt changes in Sr/Ca and Ba/Ca ratios were used to detect transitions between freshwater and marine layers. Transition times between layers were then defined by the onset and cessation of rapid changes in Sr/Ca and Ba/Ca readings. The laser on time in seconds to reach the transition at the three top layer thicknesses tested (60, 80, 100 μm) was also used to estimate a general ablation rate through the otolith materials ($n = 58$) used in our study. To assess the additive influence of top layer concentrations on bottom layer values, we collected data from periods representing top and bottom layers within an ablation trace and compared the values (Fig. 1). We compared mean elemental concentrations in two ways. First, we bracketed layer transitions, as characterized by rapid changes in the concentration of Sr/Ca, with 25-second sample periods. This allowed us to compare the measured concentrations immediately before and after a transition (Fig. 1). Second we chose 25 second intervals from the top and bottom layers at fixed time periods that were outside the transition zone for all ablations of the composite otoliths. This resulted in top layer periods being collected from 5 seconds to 30 seconds 'laser-on' time. Due to differences in the depth of the top layer across the different composite otoliths, bottom layer

periods for the Fresh/Salt 60 μm and Salt/Fresh 80 μm were collected from 145 seconds to 170 seconds laser on time. Bottom layer periods for the Fresh/Salt 100 μm were collected from 195 to 220 seconds laser-on time. This ensured that bottom layer periods did not overlap layer transitions, allowing for a comparison of concentrations outside the influence of the putative transition period (Fig. 1).

Figure 1.

Tailing is an important aspect of top layer effects that needs to be characterized. To characterize tailing, we collected data in five 15-second intervals, beginning at the inflection point for ^{88}Sr during the layer transition. This resulted in a series of time intervals beginning at the mid-point of the layer transition and covering the 75 seconds post transition (Fig. 1, Stepped Period). Then we calculated the mean and standard deviation of each period. We tabulated the number of 15-second periods that were greater than and did not overlap the distribution of the fifth period. This gave an approximate measure of the time duration associated with tailing.

Elements that had higher concentrations in the top layer than in the bottom layer were considered informative for testing tailing and top level effects. For two composite otoliths (Fresh/Salt 60 μm , Fresh/Salt 100 μm), this resulted in the following elements being used to test for top layer effects; Na, Mg, K, Mn, Zn, Rb and Ba, monitored as the following respective isotopes; ^{23}Na , ^{24}Mg , ^{39}K , ^{55}Mn , ^{64}Zn , ^{85}Rb , and ^{138}Ba . For the remaining sample matrix (Salt/Fresh 80 μm), this resulted in ^{88}Sr being used.

We characterized elemental fractionation during long (200 second) depth-profiling ablations of the USGS MACS-3 calcium carbonate standard at three spot sizes (50, 75, 90 μ m). Elemental fractionation factors, defined as the degree of elemental fractionation, were calculated as the ratio of total ion counts determined in the second half of the measurement to ion counts measured in the first half of the measurement and normalized to ^{43}Ca (Fryer et al. 1995). We calculated fractionation factors for two time periods, 0-100 seconds and 0-200 seconds of laser-on time. This was done to compare elemental fractionation factors with previously reported values (Jochum et al. 2012) (100 seconds) and to observe elemental fractionation effects at pit depths of 140 μ m which are required for otolith analysis (200 seconds).

Data processing was done using Lolite version 2.5, which is free software for deconvoluting time-resolved laser ablation data (Paton et al. 2011). Background counts were subtracted and data reduction took place under the Trace_Elements and Trace_Elements_IS data reduction schemes, using ^{43}Ca as the internal standard. NIST SRM 612 was used as the calibration standard, with NIST SRM 610 and USGS MACS-3 as reference standards. Measurement precision, calculated as % relative standard deviation (%RSD) was based on NIST SRM 610 (n = 6) and MACS-3 (n = 6). Respective %RSD for NIST 610 and MACS-3 was as follows; Na/Ca 1.0/6.8, Mg/Ca 1.0/5.4, K/Ca 2.1/na, Mn/Ca 2.2/5.3, Zn/Ca 1.2/7.6, Rb/Ca 1.3/na, Sr/Ca 2.1/7.1, Ba/Ca 1.9/8.4.

Statistical Analysis

To describe the relationship between transition time and spot size, we employed a linear regression model. Transition time in seconds was set as the response variable

and spot size in microns was the predictor variable. The *lm* function in the base package of the R statistical computing environment v. 3.1.1 was used (R Core Team 2014).

To test the effects of layer depth on transition times, we used a generalized linear mixed effects model with nested random effects. Transition time (in seconds) was the response variable and top layer thickness (Fresh/ Salt 60 μm , Salt/ Fresh 80 μm , Fresh/ Salt 100 μm) was the predictor variable. Spot size nested within otolith was set as the random effect in order to control for differences in transition time between spot sizes, and to control for potential matrix effects associated with preparation of the composite otoliths. The *lme* function in the *nlme* package (Pinheiro et al. 2014) in the R statistical computing environment v. 3.1.1 was used (R Core Team 2014).

To assess the influence of top layer elemental concentrations on bottom layer concentrations, a generalized linear mixed-effects model with nested random effects was used. We employed the same model on both the bracketed and fixed time periods. The centred mean elemental concentration of the bottom layer was the response variable and the centred mean elemental concentration of the top layer was the predictor. Spot size nested within otolith was set as the random effect to control for potential non-independence of ablation values within a specific otolith preparation. The *lme* function in the *nlme* package (Pinheiro et al. 2014) in the R statistical computing environment v. 3.1.1 was used (R Core Team 2014).

To assess the effect of spot size on tailing time, a generalized linear mixed-effects model was used. Tailing time, as represented by the number of 15-second periods with a greater non-overlapping distribution than the fifth period was the response variable.

Spot size in microns was the predictor variable, with element nested within otolith as the random effect. The *lme* function in the *nlme* package (Pinheiro et al. 2014) in the R statistical computing environment v. 3.1.1 was used (R Core Team 2014).

Results

Table 2. LA-ICP-MS and solution ICP-MS concentration data for the four otoliths used to construct the composite diadromous otoliths. Otoliths were calibrated against NIST 612 for the laser ablation analysis. Laser spot sizes used were 50, 75, and 90 μm . Mean values were calculated from the means of 5 ablations at each spot size from each otolith ($n = 15$). Periods were from 5 seconds after onset of ablation to 35 seconds after onset of ablation, resulting in 30 seconds of ablation time used to calculate each mean. Freshwater otoliths had higher trace element concentrations than saltwater otoliths for all elements except Sr. Elemental concentrations that were below limits of detection for solution analysis are marked no detection (*nd*).

Table 3. LA-ICP-MS concentration data normalized to ^{43}Ca for NIST glasses and USGS calcium carbonate reference materials. NIST 610 and USGS MACS-3 were calibrated against NIST 612. NIST 612 calibrated against NIST 610. Reference values (RV) for NIST glasses from Jochum et al. (2011). Reference values for USGS-MACS3 from Chen et al. (2011). Laser spot sizes used were 50, 75, and 90 μm . Mean values were calculated from 2 ablations at each spot size from each standard ($n = 6$). Periods were from 5 seconds after onset of ablation to 35 seconds after onset of ablation, resulting in 30 seconds of ablation time used to calculate each mean.

Results from both the LA-ICP-MS and solution ICP-MS demonstrated significant differences in elemental concentrations between otolith materials from the two different sources (fresh and salt), with the freshwater material possessing higher concentrations of all elements except Sr (Table 2). Concentrations obtained from the solution ICP-MS were used as comparison values when assessing LA-ICP-MS data. We observed orderly and predictable ablation profiles at all spot sizes and layer thicknesses when ablating the composite diadromous otoliths and standards (Table 3). Transitions between top and bottom layers of freshwater and marine origin were readily apparent from rapid changes in concentration of all measured elements. Transitions were most easily characterized by rapid and simultaneous opposing changes in the concentrations of Ba/Ca and Sr/Ca (Fig. 2).

Figure 2.

Otolith materials of a specific origin (fresh, salt) were used to construct the composite otoliths without regard to orientation within the original structure of the donor otolith. Because of this, within a layer, there was no specific orientation of traditional zones of interest (i.e. edge-core, interior-exterior, larval-adult). This resulted in unpredictable variation in trace elements both within and between ablations on otolith materials of a specific origin. The variation in trace element concentrations observed within otolith materials was more pronounced in the freshwater otoliths. In addition, this variation was greater in some elements than others, specifically Mn and Zn, and to a lesser extent Ba and Mg. Serendipitously, this variation within materials provided an additional test of whether elevated concentrations in the top layer affected observed values for the bottom layer within individual depth-profiling ablations.

Spot Size Effects on Resolution

Spot size had a significant positive effect on transition times between layers of the composite diadromous otoliths, irrespective of depth (Fig. 2). Concentrations were generally stable in otolith materials of either freshwater or saltwater origin, making layer transitions obvious. As spot size increased, transition time increased. From the results for the linear model, for every 10 μm increase in spot size, there was a mean 3.5 ± 0.2 second increase in transition time (r-squared: 0.78, t value: 12.9, p value: $2\text{e-}16$) (Fig. 3). We also tested whether layer thickness had any significant effect on transition times. The results of a general linear mixed effects model indicated that layer thickness at the depths we tested (60, 80, 100 μm) had no significant effect on transition time (t value: 0.82, p value: 0.56).

Figure 3.

Top Layer Effects

We tested the influence of top layer concentrations on bottom layer concentrations by sampling at specific periods along individual ablation traces (Bracketed and Fixed Periods, Fig. 1). The results of a generalized linear mixed effects model indicated that for the bracketed periods, top layer concentrations had significant effects on bottom layer concentrations for both Sr (t value: 2.04, p value: 0.065) and Zn (t value: 3.44, p value: < 0.01). For the other elements, there was no significant influence of top layer concentrations on bottom layer concentrations. The results of the mixed effects model on the fixed period indicated that there was no relationship between top layer concentrations and bottom layer concentrations for any of the elements tested. This

indicated that the observed influence of top layers on bottom layers in some elements (Sr, Zn) was limited to the time period immediately following the transition, but that influence did not propagate through the entire trace.

Tailing

When individual traces were examined, some elements displayed noticeable tailing during ablation. This was observed as an initially rapid decrease in concentration at the layer transition, followed by a period of equilibration where the concentration slowly decreased to the mean concentration of the bottom layer. This resulted in traces with extended tails on waning signals for these elements (Fig. 4).

Figure 4.

Characterization of the number of periods with positive non-overlapping distributions greater than the fifth period in the stepped periods indicated a similar trend to the bracketed analysis. Zinc and Sr, the two elements implicated as having influence on bottom layer concentrations in the bracketed comparisons, had the longest average tail-out times, with a mean tail-out time of 51 ± 6 and 32 ± 7.5 seconds respectively. The other elements examined (Ba, Na, Mg, K, Mn, Rb) had more abbreviated transitions periods, averaging 30 seconds or less (Table 4). The results of a linear mixed model of the effect of spot size on tailing time indicated a small but statistically significant interaction. For every 10 μm increase in spot size, there was a 1 ± 0.3 second increase in tail-out time (t value: 2.68, p value: 0.01).

Table 4. Mean Tailing period (in seconds) for tested elements. The tailing time was calculated by multiplying the mean number of positive periods by the seconds per period (15).

Elemental Fractionation

Elemental fractionation factors for 100 second ablations (estimated pit depth is 70 μm) are presented here to compare the results of this study with similar work on elemental fractionation effects in biogenic carbonates (Jochum et al. 2012). Pit depths were calculated using a mean ablation rate of 0.71 μm per second of laser on time at the given ablation parameters, and assumed that ablation rates in the MACS-3 carbonate standard were roughly equivalent. Elemental fractionation factors for the 200-second ablations (estimated pit depth is 140 μm) are also presented to investigate the viability of obtaining accurate elemental concentration results for whole otoliths using aspect ratios greater than 1:1 (75 μm , pit depth 140 μm , 2:1 aspect ratio).

Our results were similar to the elemental fractionation factors presented by Jochum et al. (2012) for 100 second ablations of MACS-3 using a 193 nm excimer laser. For the lithophile elements, average fractionation factor across the three spot sizes tested was 0.999 ± 0.022 , as compared with their reported values of 1.031 ± 0.004 . For the single chalcophile element in our analysis, Zn, the average elemental fractionation factor was 0.930 ± 0.035 , while Jochum et al. (2012) reported a value of 1.03 ± 0.02 . In the 200-second ablations, we observed elevated fractionation values. The lithophile elements exhibited a mean fractionation factor of 1.029 ± 0.036 , and the chalcophile Zn had a factor of 0.985 ± 0.050 . The slightly elevated fractionation factor for lithophiles in the 200-second period was driven by the 50 μm spot (Fig. 5: Spot Size 50 μm , 140 μm

depth). These were the only ablations with fractionation factors approaching 10% (mean value: 1.078 ± 0.025). These were also the only ablations with aspect ratios greater than 2:1, which may have contributed to the elevated fractionation factor we observed at this spot size (Woodhead et al. 2004).

Lithophile elements (Na, Mg, Ca, Sr, Ba) had elemental fractionation factors approaching 1 in both the 100-second and 200-second periods. This indicated little or no fractionation, with the exception of the 50 μm 200-second ablation (Fig. 5). For the single chalcophile element Zn, there was significant fractionation at some spot sizes and depths (fractionation factor at or greater than $\pm 10\%$), but no consistent trend.

Figure 5. Effects of spot size on fractionation factors in the MACS-3 calcium carbonate standard. Two depths, representing 100-seconds and 200-seconds of laser-on time are presented. Estimated laser depth at that laser-on time is listed. Three spot sizes were tested: 50, 75, and 90 μm

Discussion

We investigated the reliability of depth-profiling as a technique for sampling trace element concentrations in otoliths. Our results indicate that the technique is robust, and that it can be used to reliably collect these data. There are however important factors to consider when deploying depth-profiling, particularly when attempting to compare small differences in elemental composition within zones representing specific phases of a fish's life history (eg. larval/juvenile/adult). These factors include tailing effects, elemental fractionation, and spot size effects on resolution. Top layer contamination is strongly associated with tailing effects when transitioning from high to low elemental

concentrations during an ablation trace (Mason and Mank 2001). Establishing time periods associated with transitions between layers, defined as the duration of the tailing period, will be critical for the reliable assessment of elemental concentrations in different zones of an otolith (eg: larval vs. adult). Furthermore, elemental fractionation has been implicated as worsening during long downhole ablations (ablation of a sample for periods greater than 20 seconds) (Woodhead et al. 2004) and needs to be rigorously assessed prior to using depth-profiling in an otolith application where long ablation times are needed. Finally, laser spot size has a large effect on resolution of layer transitions and tailing when going from high to low trace element concentrations.

Instrument performance is likely to have deterred the use of depth-profiling in previous investigations of otolith microchemistry. Signal response times and laser effects have a strong influence on time resolved signal resolution during ablation (Gurevich and Hergenröder 2007). Prolonged washout associated with older high volume ablation chambers would have made interpretation of depth-profiling data difficult due to excessive tailing when going from high to low concentrations (Mason and Mank 2001). Our work, using a modern low-volume ablation chamber, showed that elemental concentrations in the upper layers of the composite otoliths had an influence on concentrations in the bottom layers during depth-profiling ablations, but that influence was restricted to the zone immediately after the transition. This tailing effect decreased over time as the ablation front progressed through the transition. For the lithophile elements tested (Na, Mg, K, Mn, Rb, Sr, Ba), the waning signal was generally 30 seconds or less. For the one chachophile element (Zn), the waning signal was detectable up to 60 seconds after the transition. The large difference in tailing time between Zn and the

lithophiles raises the possibility that Zn's prolonged tailing time may be driven by elemental fractionation processes.

Fractionation is largely platform dependent, and modern long-focus short-wavelength excimer lasers (193 nm ArF) exhibit minimal fractionation and excellent ablation characteristics, especially on biogenic carbonates (Hathorne et al. 2008, Jochum et al. 2012). This makes them particularly well suited for depth-profiling analysis of otoliths. Fractionation is also influenced by lithophile/chalcophile/siderophile classification, with lithophilic elements exhibiting less fractionation. We observed large negative fractionation factors for the one chalcophile element we tested (Zn). The larger negative factors observed for Zn may be the result of mass loading effects as similar observations of depressed fractionation factors for Zn have been made by Kroslakova and Günther (2006) and Jochum et al. (2012). They observed depressed Zn concentrations with increasing spot sizes and concordant signal intensities in carbonate matrixes. We were unable to assess fractionation for the lithophile elements Rb or K due to low concentrations in the MACS-3 standard, but assume that their fractionation behaviour would not be significantly different from other lithophile elements. In our tests, using the 193 nm laser, down-hole fractionation for lithophile elements was minimal, but increased at aspect ratios greater than 2:1. In combination with previous work on down-hole effects during depth-profiling ablations, it is prudent to restrict analysis in real otolith samples to aspect ratios around 2:1 (Eggins et al. 1998, Woodhead et al. 2005). The implication of this is that as target sample depth increases, spot-size should also increase. But as spot-size increases, spot-size-related lag effects will increase transition times between layers, potentially hindering interpretation of ablation traces.

Laser spot size is a critical and often overlooked aspect of laser ablation analysis of otoliths. The laser spot size defines the otolith area sampled, and may integrate signals from multiple zones containing variable elemental concentrations if the borders of the spot overlap those zones (Hoover and Jones 2013). A larger area of integration may blur or obscure changes that would otherwise be evident by employing a smaller spot size. Because of this, choice of spot size will affect both surface- and depth-profiling ablations in a similar manner. In our work, spot size had a large and predictable effect on the resolution of layer transitions during depth-profiling ablations of the composite otoliths. Smaller spot sizes resulted in sharper transitions and reduced tailing times. The sharper transitions associated with smaller spot sizes resulted in tightly resolved ICP-MS traces and well defined concentration changes, even when going from high to low concentration. The degree of responsiveness observed in the smaller spot sizes could allow for fine scale resolution of concentration changes previously collected via more traditional surface ablation techniques (Elsdon and Gillanders 2005). The trade-off is reduced ablation yield at smaller spot sizes and reduced analytical precision, which may limit the ability to reliably characterize less abundant elements. Two possible explanations for the observed relationship between transition time and spot size are postulated. One possibility is that layers in the composite were presented to the laser at a slightly non-perpendicular angle, with larger spot sizes then interacting with angled surfaces as they progressed through the transition of the otolith composites (Fig. 6a). The other possibility is that pit morphology departed from the ideal 'top-hat' form over the course of the ablation and non-target ablation occurred (Eggins et al. 1998) (Fig. 6b). Either or both processes may be occurring. In applications analysing whole otoliths, layers are likely to have a non-perpendicular presentation, and researchers will be faced with similar patterns to those presented here.

Figure 6.

In conclusion, depth-profiling laser ablation is a robust technique for collecting time-resolved trace element signatures from otoliths and is a viable alternative to more common methods of sampling trace element concentrations in otoliths. The technique offers reduced handling and lower contamination risks, which should improve accuracy and reduce costs. But it requires an appropriate instrument platform, including a long-focus short-wavelength laser and modern low-volume ablation chamber. Concordant with the parameters of the instrument being used are element choices. We recommend restricting elemental selection during depth-profiling assays to elements with well-understood fractionation properties, specifically lithophilic elements. Additionally, spot-size-related lag effects on transition times between zones of changing concentration may limit application of the depth-profiling technique to smaller otoliths because of constraints on ablation pit aspect ratio. The application is limited in that it does not allow for simultaneous examination of otolith microstructure during ablation, but it is still critical to match spot size to otolith microstructure. Investigators should select the smallest appropriate spot size, based on target depth, as constrained by a 2:1 depth-ratio. In order to do this, some preliminary knowledge of the microstructure of the otolith is necessary, specifically, which zones (larval, juvenile, adult) are to be sampled and what the depth of the target zone is within the chosen transect. With these caveats in mind, depth-profiling should be particularly well suited to applications where investigators are handling large numbers of small otoliths and assaying life history patterns, such as in studies of diadromous migrations.

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Example trace with the three sample schemes demonstrated. Bracketed periods were placed immediately adjacent to layer transitions. Fixed periods were located away from layer transitions. Stepped periods characterized tailing during layer transitions.

Figure 1.
581x357mm (72 x 72 DPI)

Four example ablation traces of the composite otoliths showing Sr/Ca in black on the right axis and Ba/Ca in grey on the left axis. These example traces illustrate the increase in transition time between layers as spot size increases.

Figure 2.

685x1365mm (72 x 72 DPI)

Boxplots of transition time in seconds vs. spot size, showing an increase in transition time with increasing spot size.

Figure 3.

177x177mm (150 x 150 DPI)

Example of Zn tailing behaviour within a single time resolved ablation trace. Ba tailing trace included for comparison purposes. During a waning signal, Zn takes approximately 40 seconds to equilibrate to the bottom layer concentration, while Ba takes approximately 30 seconds. Spot size is 50 μm . Zn is indicated by the black line, and Ba by the grey line. Dashed grey box indicates tailing period for the two elements.

Figure 4.

388x357mm (72 x 72 DPI)

Effects of spot size on fractionation factors in the MACS-3 calcium carbonate standard. Two depths, representing 100-seconds and 200-seconds of laser-on time are presented. Estimated laser depth at that laser-on time is listed. Three spot sizes were tested: 50, 75, and 90 µm.

Figure 5.
655x559mm (72 x 72 DPI)

Two diagrams of ablation pit layer interactions. Diagram A illustrates the effects of different spot sizes on transition times, assuming some non-perpendicular presentation of layers. Diagram B illustrates how the same effect could be produced through a departure from the top-hat ablation profile.

Figure 6.

156x199mm (300 x 300 DPI)

LA-ICP-MS operating conditions	
Laser wavelength	193 nm
On sample fluence	$2.19 \pm 0.09 \text{ J/cm}^2$
Laser frequency	5 hz
Ablation duration	400 s
Spot size range	50, 75, 90, 130* μm
Dwell Time	10 ms
Total Cycle Time	121 ms
Elemental masses	23, 24, 39, 43, 55, 64, 85, 88, 138

** 130 μm spot sizes were only used for layer transition timing*

Draft

Element	Freshwater Otolith A			Freshwater Otolith B			Saltwater Otolith A			Saltwater Otolith B		
	Mean	SD	solution	Mean	SD	solution	Mean	SD	solution	Mean	SD	solution
<i>μmol/mol</i>												
Na	3549	190	3410	3146	125	3210	2800	222	2800	2372	292	2890
Mg	28.8	5.3	51.0	17.1	3.6	42.0	12.3	2.0	43.0	11.5	6.8	41.0
K	1226	109	1400	1007	66	1160	500	67	543	493	56	529
Mn	8.2	13.2	4.2	9.9	14.9	2.0	2.3	1.4	<i>nd</i>	3.2	1.4	2.0
Zn	40.4	42.5	34.4	3.5	3.0	23.5	1.0	1.6	<i>nd</i>	0.4	0.1	<i>nd</i>
Rb	0.26	0.04	<i>nd</i>	0.30	0.05	0.37	0.05	0.02	<i>nd</i>	0.13	0.12	<i>nd</i>
Sr	575	24	639	822	55	779	3113	432	3100	2026	185	2460
Ba	14.8	2.6	19.5	2.4	1.0	6.7	1.4	0.4	1.2	0.8	0.2	1.0

Element	NIST SRM 610				NIST SRM 612			
	RV	U	LA-ICP-MS		RV	U	LA-ICP-MS	
			Mean	SD			Mean	SD
<i>μmol/mol</i>								
Na	99415	2225	99736	950	101632	2200	101260	915
Mg	432	29	521	5	68.0	5.1	60.7	0.6
K	464	21	484	10	62.3	2.4	62.6	1.3
Ca	82144	1140	82106	758	85000	700	85088	792
Mn	444	13	464	10	38.7	0.9	40.5	0.9
Zn	460	18	453	5	39.1	1.7	39.3	0.5
Rb	425.7	1	423	6	31.4	0.4	31.6	0.4
Sr	515.5	1	527	11	78.4	0.2	76.7	1.6
Ba	452	9	461	9	39.3	0.9	37.1	0.7

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USGS-MACS3			
RV	SD	LA-ICP-MS	
		Mean	SD
5900	400	6017	407
1880	300	2068	112
1.1	0.8	2.4	1.2
376900	5200	382950	24061
532	43	559	30
111	6	93	7
<i>nr</i>	<i>nr</i>	0.0	0.0
6570	340	7112	503
58.9	4.2	61.7	5.2

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Element	Tailing Period (seconds)
Mn	10.5 ± 9.0
Rb	11.0 ± 7.7
Na	16.0 ± 11.8
Mg	22.0 ± 9.4
K	26.0 ± 9.0
Ba	29.5 ± 6.1
Sr	32.0 ± 7.5
Zn	51.5 ± 6.1

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