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Effects of ethanol preservation on otolith microchemistry

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Solution-based inductively coupled plasma-mass spectrometry was used to examine the effects of exposure time to ethanol (0, 1, 3, 9, 27 and 81 days) and ethanol quality (ACS- *v.* HPLC-grade) on strontium (Sr) and barium (Ba) concentrations in sagittal otoliths of hatchery-raised and wild-caught young-of-the-year walleye *Stizostedion vitreum*. No effect of either attribute on Sr and Ba concentrations were detected, indicating that metabolically inert elements that replace calcium in the calcium carbonate matrix (*e.g.* Sr and Ba) are not influenced by storage in 95% ethanol.

Key words: ICPMS; inductively coupled plasma-mass spectrometry; otolith elemental composition; solution-based ICPMS; *Stizostedion*; stock discrimination.

INTRODUCTION

During the past decade, otolith microchemistry has become a popular tool for fisheries research (Campana, 1999). Many investigations have successfully used otolith microchemistry to discriminate among local spawning populations (Begg *et al.*, 1998; Edmonds *et al.*, 1999; Patterson *et al.*, 1999; Newman *et al.*, 2000), to detect migration events (Secor, 1992; Secor *et al.*, 1995), and to determine natality (Thorrold *et al.*, 1998; Spencer *et al.*, 2000). Because of their widespread application and interest, it is imperative to understand the limitations and caveats associated with otolith microchemical techniques. Although the assumptions regarding the conservative nature of otoliths (resorption and erosion resistance) have been readily addressed (Campana & Neilson, 1985), microchemical alterations (leaching or contamination) caused by storage and handling remain relatively unexamined.

To date, two studies have explored how storage and handling can influence elemental concentrations in otoliths. Milton & Chenery (1998) examined concentrations of Li, Na, Mg, Mn, Co, Sr and Ba in marine fish otoliths and

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found that: 1) freezing fishes increased the concentrations of Na and Mg in the otolith core; 2) Mg concentrations were elevated in otoliths that were left within fishes and then preserved in 70% ethanol, relative to dissected otoliths stored in paper envelopes or in 70% ethanol; 3) Na, Mg, Co and Ba tended to be higher in otoliths that were left within the fish *post mortem*. Proctor & Thresher (1998) also addressed handling and storage effects on the otolith microchemistry of several marine fishes by examining Ca, Sr, Na, K, S and Cl. They found that freezing had the least impact on otolith chemistry (affecting only S), whereas both storage in ethanol and delaying extraction by only 3 h reduced Na at the margin, altered K throughout, and truncated the ultimate range of Cl values. Proctor & Thresher's (1998) general consensus was that immediate removal of otoliths is best, while freezing fishes is the next best alternative.

The conclusions of Proctor & Thresher (1998) and Milton & Chenery (1998), however, need to be heeded with some caution. First, their conclusions were largely based on elements that are metabolically active and may not be tightly bound in the calcium carbonate ($CaCO_3$) matrix. By contrast, both investigations found that Sr and Ba, which are trace elements that substitute for Ca in the $CaCO_3$ matrix, appeared unaffected by handling and storage media, including ethanol preservation. This finding is fortunate, given that the majority of studies that have used otolith microchemical techniques have relied heavily on differences (or changes) in Sr and Ba concentrations (Secor, 1992; Secor *et al.*, 1995; Farrell & Campana, 1996; Edmonds *et al.*, 1999; Bath *et al.*, 2000.) Second, neither Proctor & Thresher (1998) nor Milton & Chenery (1998) examined the influence of handling and storage on otoliths from freshwater species. Given that concentrations of trace elements can be much lower in freshwater systems relative to marine systems (*e.g.* Sr, Ba; Campana, 1999; Thresher, 1999), and correspondingly in fish otoliths (Secor *et al.*, 1992; Bath *et al.*, 2000; Gillanders & Kingsford, 2000), it is important to examine handling and storage effects on freshwater fish otoliths. Finally, neither Milton & Chenery (1998) nor Proctor & Thresher (1998) considered the effect of ethanol purity on otolith elemental composition or incorporated a temporal factor into their analyses. Certainly, the abundance of historic samples preserved in ethanol that could potentially be used in otolith microchemical studies behoves examination of the effects of differences in quality of ethanol, as well as temporal effects of storage.

Owing to the potential confounding effects of handling and storage on otolith microchemistry, and hence on its ecological application, it was necessary to re-examine the effects of ethanol storage on otolith microchemistry. This is especially so when considering that removing otoliths immediately after capture or freezing specimens is not always feasible (*e.g.* tropical research). In addition, because most fish specimens (especially larvae) collected in recent years have been preserved in 95% ethanol (as opposed to carcinogenic, otolith-deteriorating formaldehyde solution), this investigation could help determine whether these historical samples could be used in otolith microchemical investigations. Toward this end, solution-based inductively coupled plasma-mass spectrometry (ICPMS) was used to determine how differential storage times in ethanol (0–81 days) and ethanol quality (HPLC- *v.* ACS-grade) influenced elemental concentrations in otoliths of hatchery-raised and wild-caught young-of-the-year (YOY) walleye *Stizostedion vitreum* (Mitchill). A worst-case situation would be that otolith contamination would occur in the ACS-grade treatment

(*i.e.* elements would become trapped in interstitial spaces of otoliths), whereas leaching of trace elements would occur in otoliths stored in trace-metal clean (*i.e.* HPLC-grade) ethanol, and that these effects would magnify with time.

MATERIALS AND METHODS

HATCHERY SPECIMENS

Young-of-the-year walleye ($n = 110$) were obtained frozen from the White Lake Fish Hatchery (Ontario Ministry of Natural Resources, White Lake, Ontario, Canada) in August 2001. All 110 fish were reared in a single enclosure, were exposed to identical environmental conditions, and were members of the same broodstock. On 29 August 2001, all fish were thawed, measured (nearest 1 mm total length, L_T), and randomly assigned to time (0, 1, 3, 9, 27 or 81 days exposure) and ethanol grade [standard laboratory grade (ACS) *v.* high purity (HPLC)] treatments (Table I). Fish were stored individually in 120 ml VWR TraceClean^{TM} high-density polyethylene containers (production number 414 593) and stored with treatments intermixed on shelves.

DISSECTION AND CLEANING PROCEDURES

All instruments used for dissection (*e.g.* slides, probes and vials) were acid-washed, which consisted of: 1) soaking in 13% nitric acid for 24 h, 2) soaking in Milli-Q water (ion exchanged water to reduce trace metals) for 24 h, 3) rinsing three times with Milli-Q water, and 4) drying under a laminar flow fume hood prior to use. All cleaning and processing of both otoliths and glassware occurred in a Class 100 clean room.

For each dissection, the fish was removed from ethanol with Teflon-coated forceps, placed upon an acid-washed glass slide, and the sagittal otoliths were removed using acid-washed glass probes. Once the otoliths were teased away from the saculus, they were rinsed with Milli-Q water and placed in acid-washed 2·5 ml glass vials containing 1 ml of Milli-Q water (to gently remove surface contaminants and rinse any ethanol from the interstitial spaces). Each sagitta was placed in a separate acid-washed glass vial.

Otoliths (mean mass $\pm$ S.E. $= 235{\cdot}1 \pm 3{\cdot}2$ µg, $n = 110$) were further cleaned prior to chemical analysis. After randomly selecting one sagitta per fish, the Milli-Q water was removed with an acid-washed glass pipette. Next, 2·5 ml of Milli-Q water were added, left for 30 s, and then removed. Afterwards, 2·5 ml of trace-metal clean 30% hydrogen peroxide (H_2O_2) were added for 30 s to digest any remaining tissue adhering to the otolith. Three subsequent 30 s rinses with 2·5 ml of Milli-Q water were then conducted to ensure the removal of the H_2O_2. After cleaning, the otoliths were placed into perfluoroalkoxy (PFA) vials, which had been previously acid-washed (as above) and additionally acid refluxed (*i.e. c.* 1 ml 1% HNO_3 was

TABLE I. Sample sizes used to quantify the effects of exposure time (0–81 days) in 95% ethanol and ethanol purity (HPLC- *v.* ACS-grade) on trace elemental concentrations, as determined from solution-based inductively coupled plasma-mass spectrometry of hatchery-raised walleye otoliths. There were not enough walleye for a complete factorial design; thus, the day-0 group served as an isolated control group in the analysis

	Exposure time to ethanol (days)					
Ethanol Grade	0	1	3	9	27	81
None (Control)	10	–	–	–	–	–
ACS	–	10	10	10	10	10
HPLC	–	10	10	10	10	10

added to the PFA vial, heated on calibrated hot plates at 65° C for 1 h, and then rinsed with ultrapure Milli-Q water) twice, to await dissolution.

One drop of 50% HNO_3 was added to each PFA vial to dissolve otoliths. After the reaction had finished, the PFA vials were placed on a hotplate at 65° C to facilitate evaporation of the fluid. When all fluid had evaporated (*c.* 90 min), the residue was re-dissolved in *c.* 2 ml of high purity (non-boiling Teflon-distilled acid diluted with Milli-Q water) 1% HNO_3 (spiked with Be, In and Tl at concentrations of 5, 0·5 and 1 $\mu g\,g^{-1}$, respectively) on a hotplate. Additional spiked 1% HNO_3 was added to the PFA vials to bring the final solution mass to 20·00 g (scale accuracy $\pm$ 0·10 g). The sample solutions were then poured into acid-washed, twice acid-refluxed polypropylene 10 ml auto-sampler vials, where they remained until subsequent analysis. To quantify potential contamination during the cleaning and preparation process, 10 procedural blanks were prepared in the exact same way, from cleaning with Milli-Q water and H_2O_2 to HNO_3 dissolution.

Although otolith removal occurred on different days (*i.e.* 0, 1, 3, 9, 27 and 81 days after preservation), all 110 otoliths were cleaned and dissolved on the same day. Thus, because otoliths were stored after dissection and cleaning in vials containing Milli-Q water, exposure time to ethanol was inversely related to exposure time to Milli-Q water ($P < 0{\cdot}0001$).

WILD-CAUGHT WALLEYE

To ensure that differential exposure time to Milli-Q water did not confound the interpretation of ethanol effects, a second set of otolith microchemical investigations was conducted using wild-caught YOY walleye ($n = 30$), collected in Lake Erie's west basin by bottom trawling during June 1996 by the Ohio Department of Natural Resources, Division of Wildlife. These fish were immediately frozen after capture. Both sagittae were removed from thawed walleye and immediately placed in 1 ml of 30% H_2O_2 for 30 s to remove additional tissue. Otoliths were rinsed with Milli-Q water and placed individually in acid-washed 2·5 ml glass vials. The vials were left uncapped in a laminar flow fume hood for 24 h to dry. Afterwards, individual fish were randomly assigned to one of three treatments. Specifically, one otolith from every fish was stored dry (in its vial), whereas the second otolith was stored: 1) dry (control treatment), 2) in 1 ml Milli-Q water (water treatment) or 3) in 1 ml ACS-grade 95% ethanol (ethanol treatment). All otoliths were stored for 50 days before further cleaning and analysis by solution-based inductively coupled plasma-mass spectrometry (ICPMS). In addition to determining whether storage in Milli-Q water influenced otolith chemical composition, this comparison provided an independent test of the effect of ethanol on otolith microchemistry.

After 50 days, fluids were removed from the water and ethanol treatment vials using new acid-washed glass pipettes. To ensure removal of all tissue from the otolith surface, otoliths were cleaned as follows: 1) 1 ml Ultrapure Milli-Q water was added to each vial and then removed, 2) 1 ml 6% ACS-grade NaOCl was added to the vials, which were then sonicated for 2·5 min and the bleach removed, 3) 1 ml Ultrapure Milli-Q water was added to the vials, which were then sonicated for an additional 2·5 min and the water removed and 4) the otoliths were rinsed two additional times with 1 ml Ultrapure Milli-Q water. Otoliths were removed from vials using unique acid-washed pipettes and deposited in acid-washed, twice-refluxed PFA vials, where they remained until dissolution. Dissolution occurred in the exact same manner as for hatchery-reared walleye, except that wild-caught walleye otoliths were re-dissolved in 4 ml of the spiked 1% HNO_3 solution (instead of 20 ml).

ANALYTICAL PROCEDURES

All samples and procedural blanks were analysed by a Thermo Elemental PQ3 ICPMS coupled to a CETAC 500 auto-sampler. Calibration was accomplished using multi-element standards (made with the same spiked 1% HNO_3 solution) and corrected for matrix and temporal drift using the internal standards Be, In and Tl. Data were acquired in a peak jumping mode and corrected for instrumental background using the 1% spiked

HNO_3 solution. In addition, total processing blanks were analysed for all test procedures and subtracted from the analytical data prior to calculating otolith elemental concentrations. Detection limits for each element were calculated as three standard deviations of the total processing blanks (Table II).

Element selection criteria were comparable to other otolith microchemistry investigations (Thresher, 1999; Rooker *et al*., 2001). Elements were required to have relative standard deviation (RSD) values of $\leq 10{\cdot}5$ (Gillanders & Kingsford, 1996) in the laboratory standards (Table II). Three laboratory standards were created by dissolving 10 adult walleye otoliths from each of the east, west and central basins of Lake Erie (one standard per basin) in separate 1% HNO_3 solutions. In addition, to be included in the analyses, individual elements had to be quantified as three times greater than the S.D. of analytical blanks plus the mean of the blanks, in at least 90% of the samples (Table II). Rubidium, strontium and barium met the RSD criteria but only Sr and Ba subsequently met the detection limit limitations; therefore, Sr and Ba were the only elements compared.

DATA ANALYSIS

Initially, one-way ANOVAs were used to detect differences in L_T among treatments for both the hatchery-raised and wild-caught walleye. For all analyses, significance levels were set at $\alpha = 0{\cdot}05$.

HATCHERY-REARED WALLEYE

The resulting blank-subtracted data were $\log_{10}$-transformed to achieve normality, and analysed for time and ethanol-grade effects using a two-factor MANOVA (homogeneity of variances tested by Box M, $P = 0{\cdot}08$) with an isolated, day-0, control group. Stock discrimination applications of otolith microchemistry typically have used linear discriminant function analysis (DFA) to quantify stock-specific elemental signatures and to assign unknown individuals to specific stocks (Gillanders & Kingsford, 1996; Thorrold *et al*., 1998; De Portual *et al*., 2000). Thus, this technique was used to help identify if treatment (*i.e*. stock) differences existed. An absence of distinctive clustering among samples from the same treatment on resultant plots of DFA roots and an inability to accurately assign samples to treatment groups would indicate an absence of confounding storage effects.

WILD-CAUGHT WALLEYE

Data were $\log_{10}$-transformed to achieve normality and analysed for differences within the otolith pairs and among treatments using analysis of covariance (ANCOVA), where the covariate was fish L_T and the main effect was treatment (control *v*. water *v*. ethanol). The first step was to look for a significant interaction term for each element. If none was detected (*i.e*. slopes did not differ), the interaction term was removed from the model and secondary analysis explored whether a treatment effect existed (*i.e*. intercept differences). *Post hoc* comparisons were conducted using least-square means (Bonferroni adjusted) to test for treatment effects.

RESULTS

No differences in walleye (hatchery-raised) L_T were evident among ethanol-time treatments (one-way ANOVA, d.f. = 109 and 10, $P = 0{\cdot}99$). All mean L_T ranged from 38 ± 3 to 40 ± 4 mm ($\pm$, accuracy of the calipers) across treatments. A MANOVA with an isolated control group (day-0 walleye) detected no effect of ethanol grade ($P = 0{\cdot}54$) or exposure time ($P = 0{\cdot}22$) on concentrations of Sr and Ba calculated from analysis of Sr^{86}, Sr^{88}, Ba^{137} or Ba^{138} isotopes (Fig. 1). A DFA verified that there were no differences among the treatment groups

TABLE II. Relative standard deviation (RSD) and limit of detection (LOD, ppm) values for elemental concentrations within laboratory standards created by dissolving and combining 10 sagittal otoliths from adult walleye collected in the east (EB), central (CB) and west (WB) basins of Lake Erie. RSD values <10·5 (bold) were required for all three standards in each experiment (hatchery-raised or wild-caught walleye) for an element to be of analytical use. In addition, for an element to be of analytical value, >90% of the samples must have had concentrations above the limit of detection (LOD = 3 × S.D. of blanks + blank mean) (bold). Data are shown for at least one isotope of each element monitored. Only Sr and Ba met both criteria. Elemental concentrations were quantified using solution-based inductively coupled plasma-mass spectrometry

	Hatchery-raised					Wild-caught				
	EB RSD	CB RSD	WB RSD	LOD	% >LOD	EB RSD	CB RSD	WB RSD	LOD	% >LOD
Li^{7}	64·1	167·3	67·1	0·9442094	**9**	**8·2**	151·2	120·1	0·5182106	**100**
Na^{23}	23·7	14·2	12·7	2910·6214	**99**	11·9	**9·8**	19·7	11754·179	**100**
Mg^{25}	51·9	24·8	17·4	86·726108	**95**	12·5	30·2	29·2	19·010812	**100**
K^{39}	65·8	**4·6**	79	−7·707	26	104·2	290·5	95·8	−71·730	58
Ca^{43}	**0**	**0**	**0**	1629·173	**100**	**0**	**0**	**0**	20·439	**100**
Ca^{44}	**7·4**	**7·7**	**9·3**	2349·820	**100**	**3**	**2**	**5·3**	105·008	**100**
V^{51}	79·4	145·7	85·9	0·047	34	42·8	13·2	30·9	0·062	23
Cr^{53}	43·2	46·6	53·3	4·075	17	38·8	22·5	71·8	1·376	2
Fe^{54}	46·3	35·2	46	165·892	36	36·7	51·5	141·6	61·240	12
Mn^{55}	50·4	27·1	276·5	2·742	55	**7**	47·5	49·6	1·982	88

Co^{59}	16·4	15·5	35·9	−0·004	5	22·8	**6·1**	15·6	0·068	**100**
Ni^{62}	110·8	227·7	260·7	1·010	57	42·6	109·5	558·4	−0·088	19
Cu^{63}	63·7	27·7	25·1	21·956	41	**3·2**	94·1	114·7	0·938	58
Zn^{68}	43·6	**6·8**	117·2	130·858	2	22·2	78·3	119·1	33·821	38
Rb^{85}	**8·3**	**7·9**	**7·2**	0·200	75	**3·6**	13·2	69·8	0·006	85
Sr^{86}	**1·6**	**7·1**	**1·7**	2·270	**100**	**0·4**	**1**	**2·6**	0·142	**100**
Sr^{88}	**1**	**6·8**	**1·7**	2·259	**100**	**0·6**	**0·9**	**2·6**	0·027	**100**
Y^{89}	11·5	28·3	27·1	0·012	**100**	17·4	16·3	46·6	0·001	**100**
Cd^{114}	44·8	55	68·1	0·324	61	78·9	77·4	138·3	0·032	62
Cs^{133}	44·9	151·8	154·6	0·007	68	81	81·6	38·8	0·003	46
Ba^{137}	**3·6**	**7·1**	**3·6**	2·676	**100**	**3·1**	**6·3**	10·9	0·022	**100**
Ba^{138}	**4**	**6·9**	**2·4**	2·733	**100**	**3·3**	**6·8**	**9·5**	0·041	**100**
Ce^{140}	52·4	40	30·9	0·060	45	**8·1**	50·5	65·3	0·004	**96**
Pb^{206}	51·2	124·8	83·3	0·766	57	25·6	143	148·6	0·203	44
Bi^{209}	53	**8**	890·1	0·031	27	**9**	**3·5**	205·2	0·005	31

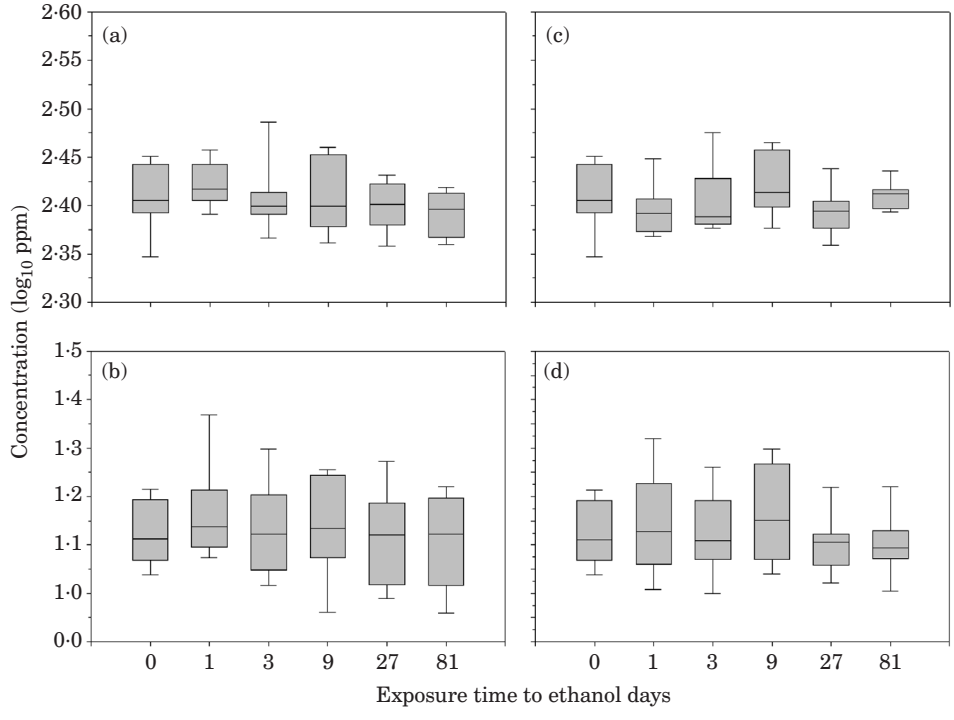

FIG. 1. Mean elemental concentrations ±1·96 SE (▒) and ranges (I) of (a), (c) Sr and (b), (d) Ba in walleye otoliths stored for various times within (a), (b) HPLC- or (c), (d) ACS-grade 95% ethanol ($n = 10$ per day × treatment). No differences were detected among exposure-time and ethanol-grade treatments ($\alpha = 0{\cdot}05$) . Elemental concentrations were quantified by detection of ^{88}Sr and ^{138}Ba using solution-based inductively coupled plasma-mass spectrometry.

($P = 0{\cdot}903$). Because none of the DFA roots were useful in discriminating among treatments, there was no distinct clustering of treatments on the DFA plot and accurate classification of samples to treatment groups was not possible (classification accuracy ranged 0–50%; Table III).

No differences in walleye (wild-caught) L_T were evident among treatments (one-way ANOVA, d.f. = 2 and 28, $P = 0{\cdot}956$). The overall ANCOVA model was significant for all isotopes (all $P < 0{\cdot}05$), owing to a strong correlation between otolith pairs (all $r > 0{\cdot}69$). Except for a significant interaction between treatment and the control otolith concentrations for Sr^{88} calculations ($P = 0{\cdot}002$), which was caused by an extreme deviation in the water treatment from both the control and ethanol treatments, none of the ANCOVAs for any of the isotopes (of either Ba or Sr) showed significant interaction effects (all $P \geq 0{\cdot}13$). No intercept differences were detected for either Ba isotope (all $P \geq 0{\cdot}28$; Fig. 2). Intercept differences, however, were found between the control and water treatments for Sr^{88} ($P = 0{\cdot}047$; Fig. 2) and between the ethanol and water treatments for Sr^{88} ($P = 0{\cdot}049$; Table IV). In both of these cases, otolith elemental concentrations were higher in the water treatment. In no cases were differences evident between ethanol and control otoliths.

TABLE III. Linear discriminant function analysis classification table of hatchery-reared walleye otoliths to 95% ethanol treatment groups [ethanol-grade (HPLC *v.* ACS) × exposure time (0, 1, 3, 9, 27 or 81 days)] using concentrations of Sr and Ba, as determined from solution-based inductively coupled plasma-mass spectrometry by detection of ^{88}Sr and ^{138}Ba. Control, treatment in which otoliths were not exposed to ethanol. Per cent correct, the percentage of samples classified correctly. Other columns indicate treatments to which samples were misclassified

Treatment	Per cent correct	Control	ACS-1	ACS-3	ACS-9	ACS-27	ACS-81	HPLC-1	HPLC-3	HPLC-9	HPLC-27	HPLC-81
Control	0	0	1	0	1	0	3	2	0	1	0	2
ACS-1	30	0	3	0	1	1	0	1	0	0	0	4
ACS-3	0	0	2	0	0	0	3	2	0	0	0	3
ACS-9	20	0	2	0	2	0	2	2	0	0	0	2
ACS-27	0	1	1	0	0	0	1	1	0	0	2	4
ACS-81	50	0	0	0	0	0	5	2	0	1	0	2
HPLC-1	20	0	2	0	1	0	5	2	0	0	0	0
HPLC-3	0	0	2	0	0	0	2	2	0	0	0	4
HPLC-9	0	0	3	0	0	0	3	2	0	0	0	2
HPLC-27	0	0	4	0	0	0	3	1	0	0	0	2
HPLC-81	30	0	2	0	0	0	2	2	1	0	0	3
Total	**13·64**	**1**	**22**	**0**	**5**	**1**	**29**	**19**	**1**	**2**	**2**	**28**

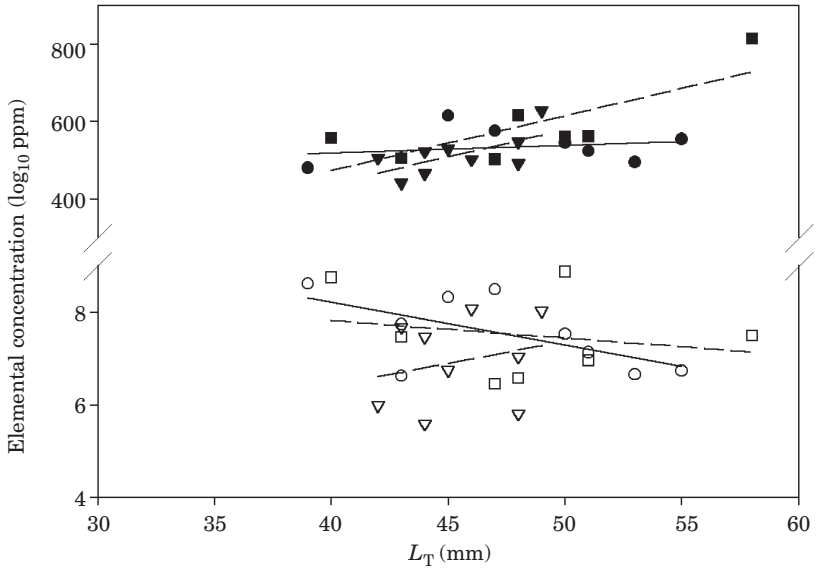

FIG. 2. Linear regressions of concentrations of Sr and Ba within walleye otoliths calculated by analysing ^{88}Sr or ^{138}Ba, which were either stored dry (C) ($n=9$), in water (W) ($n=8$) or in ethanol (E) ($n=9$) (●, C Sr^{88}; ▼, E Sr^{88}; ■, W Sr^{88}; ○, C Ba^{138}; ▽, E Ba^{138}; □, W Ba^{138}) for 50 days post-dissection prior to elemental analysis. Total length was not measured for one of the water treatment individuals. Elemental concentrations were quantified using solution-based inductively coupled plasma-mass spectrometry.

TABLE IV. *Post hoc* comparison of treatment means (least-square means, Bonferroni-corrected; *P* values are presented) from ANCOVAs conducted on wild-caught walleye otolith pairs (total length was the covariate). For the comparisons, one sagittal otolith was stored dry and the second was either stored dry (C) ($n=9$), in Milli-Q water (W) ($n=8$) or in ACS-grade 95% ethanol (E) ($n=9$) for 50 days. Significant *P* values ($\alpha=0{\cdot}05$) are in bold (in both cases ^{88}Sr was higher in the water treatment). One fish from the Milli-Q water treatment was excluded from the analysis because its otolith was broken

Comparison	^{137}Ba	^{138}Ba	^{86}Sr	^{88}Sr
C *v.* E	0·1877	0·1332	1·0000	1·0000
C *v.* W	1·0000	1·0000	0·4637	**0·0470**
E *v* .W	0·0604	0·0516	0·1742	**0·0487**

DISCUSSION

Previous studies have suggested that otolith handling and storage procedures can influence otolith elemental composition (Milton & Chenery, 1998; Proctor & Thresher, 1998,). Most notably, they found that elements not tightly bound in the $CaCO_3$ matrix (*e.g.* Na, K, Mg and S) are prone to alteration, whereas those that are tightly bound (Sr, Ba) are not. The present results support this

latter conclusion in that no effect of ethanol preservation or ethanol quality was found on Sr and Ba concentrations in walleye otoliths.

Initial speculation was that ethanol purity might affect otolith elemental concentrations. Both ACS- and HPLC-grade ethanol are initially prepared to the standards of the American Chemical Society. Additionally, HPLC-grade ethanol is sub-micron filtered and contamination is reduced by storage in cleaned polytetrafluoroethylene-lined bottles and packing with inert gas. Specifically, owing to a lack of trace metals in HPLC-grade ethanol, it was suspected that leaching of elements might occur. Conversely, contamination, *via* trapping of trace metals in interstitial spaces of otoliths, potentially could have occurred in the ACS-grade treatment. It was found, however, that concentrations of Sr and Ba in walleye sagittae were unaffected by storage in either ACS- or HPLC-grade ethanol, and that persistent exposure to ethanol (up to 81 days) did not change these results. Although it is possible that ethanol effects might occur after 81 days of exposure, this is unlikely. Certainly, this finding will be of value to researchers that seek to use historical samples, preserved in ethanol, for ecological applications (*e.g.* stock discrimination, tracking dispersal patterns and ontogenetic changes).

By contrast to ethanol, storage in Milli-Q water caused enhanced Sr concentrations relative to other treatments. This was probably the result of Milli-Q water being undersaturated in calcium carbonate relative to the otolith. In turn, slightly acidic water (measured pH of Milli-Q water varies from 7·03 for freshly prepared water to 6·06 for stored water) could dissolve significant amounts of small otoliths with time. Unfortunately, why Sr concentrations would increase cannot be explained. In fact, if Milli-Q water was dissolving otolith edges more than the core (due to greater exposure of edges to water), a reduction in otolith Sr in the water treatment would be expected, given that 1) the outer edges (*i.e.* outer growth bands) were probably formed during warm summer months and 2) otolith Sr and temperature are typically positively related. Certainly, a detailed investigation of Sr concentrations across juvenile walleye otoliths (from core to edge) might help to understand this result. Regardless, the consistency in elemental concentrations between otoliths from the dry and ethanol treatments (wild-caught walleye) indicates that ACS-grade ethanol was not dissolving the surfaces of the otoliths, while the occurrence of dissolution in the water treatment (apparent from the elevated concentrations of Sr) provides confidence in the stability of ethanol storage by comparison. The consistency of elemental concentrations between dry and ACS-grade ethanol stored otoliths, considered with the stability of elemental concentrations between otoliths stored in ACS- and HPLC-grade ethanol, bolsters the conclusion that storage in ethanol, whether ACS- or HPLC-grade, has no effect on concentrations of Ca replacing elements within otoliths stored for up to 81 days.

It should be cautioned, however, that these results only pertain to elements that bind tightly into the $CaCO_3$ matrix. These would include elements such as Sr, Ba, Mn and Rb, whose ionic radius and ionic charge match that of a free Ca^{2+} ion (Campana, 1999; Thresher, 1999). Thus, other elements that do not readily replace Ca in the $CaCO_3$ matrix and that are physiologically regulated (*e.g.* K, S, Mg and Na), may indeed be influenced by storage, such as in ethanol (Milton & Chenery, 1998; Proctor & Thresher, 1998).

In addition to supporting the use of historical specimens, these findings suggest that small fishes or larvae, which typically are stored in ethanol because they cannot be easily frozen or dissected upon collection, may be used for otolith microchemical analysis. This is important because larval fishes may provide a key for many stock discrimination applications. This is especially so in systems where individuals are transported from their natal site early in life. For example, in Lake Erie, although walleye spawn in geographically distinct locations in the western basin (*e.g.* adjoining tributaries and open-lake reefs; Goodyear *et al.*, 1982, Roseman *et al.*, 1996, Mion *et al.*, 1998), mixing of stocks typically occurs during the larval stage in offshore waters of the western basin, because of water movement caused by river discharge and longshore currents. Thus, in this case, because larvae from different local spawning populations typically do not remain at natal sites, or reside in distinct nursery areas through the juvenile stage, the larval otolith provides the only means to characterize site-specific signatures in a natal spawning area. Only after the development of a library of site-specific signatures can unknown origin fish be typed back to their natal area (using laser-ablation inductively coupled plasma-mass spectrometry, LA-ICPMS).

Although concerns about effects associated with larvae dying before or during capture in nets, as well as effects owing to fluid (*i.e.* ethanol) immersion, seem valid (Thresher, 1999), preliminary otolith microchemistry data from Lake Erie walleye (Hedges, 2002) and yellow perch *Perca flavescens* (Mitchill) (S.A. Ludsin & B.J. Fryer, unpubl. data) larvae suggest that these concerns are unwarranted.

In addition to finding differences in elements bound 'loosely' to the $CaCO_3$ matrix, Proctor & Thresher (1998) observed that Ca concentrations were slightly affected by delayed dissection, ethanol preservation and hyper-saline solutions. Since Ca is used by many analytical techniques, and especially with ICPMS, as a standard for comparing and calibrating other elements, artificial alterations in Ca could cause artificial estimates of elemental concentrations in other elements. Stoichiometric considerations require that changes of the Ca concentration (from 40·0% by mass) in aragonite ($CaCO_3$) be accounted for by the substitution of another cation (reduction of Ca concentration) for Ca^{2+}, or by replacing the CO_3^{-2}, by another, lighter anion with the same net negative charge (increase in Ca concentration). All likely cations (except for Ba and Mg which are typically present at levels of *c.* 10–100 ppm in otoliths) and anions were analysed in Proctor & Thresher (1998), and none was of sufficient abundance to account for the calculated variations in Ca. Therefore, the observed variations in Ca among storage media were probably analytical artefacts possibly related to different surface polishing characteristics of the otoliths (to which electron microprobe analyses are sensitive; Potts, 1987) associated with the different treatments, or to the leaching of protein which can make up to 3% by mass of otoliths.

Initially, this study was designed to include Mg, Mn, Rb and Zn in the analysis, other elements which can replace Ca. Detection limits of these elements, however, fell below the quality criteria (RSDs $< 10{\cdot}5$), which were based on three in-house reference materials created from adult walleye collected in the east, central and west basins of Lake Erie. Interestingly, traverses across adult walleye otoliths (transverse sections), using LA-ICPMS, have shown that otoliths from walleye in Lake Erie are depauperate of trace elements outside of the otolith core (except for Sr and Ba), but that concentrations of many trace

elements are high in the core region [*i.e.* cores were characterized by Mn peaks orders of magnitude higher than the surrounding material (B.J. Fryer & K.J. Hedges unpubl. data)]. Thus, it is extremely likely that examination of otolith cores (synonymous with larval otoliths) with LA-ICPMS would have provided additional elements for analytical use. In fact, a comparison of elemental concentrations from yellow perch otolith pairs analysed with LA-ICPMS and solution-based ICPMS (one technique per otolith) has demonstrated that the dilution of core material within the entire otolith and the further dilution of the entire otolith within solution apparently can decrease concentrations of the core-localized elements to analytically challenging levels (unpubl. data). LA-ICPMS conducted on larval walleye otoliths has also allowed Mn, Zn and Mg to be incorporated into the suite of analytically detectable elements (Hedges, 2002). These findings clearly indicate a potential advantage to using LA-ICPMS when dealing with otoliths from larvae and freshwater fishes. Certainly, a follow-up investigation that explores differences at across 'zones' of the otolith (*e.g.* edge *v.* core) is warranted, and may help explain why storage in water causes elevated Sr levels relative to storage in ethanol (or dry).

As for the potential effect of ethanol storage on Mn, Zn and Mg, it was initially speculated that these elements would not be susceptible to significant alteration by ethanol because each is able to easily replace Ca in the otolith matrix (as all are +2 cations known to form their own carbonate minerals and substitute for Ca in $CaCO_3$, in geologic environments). Indeed, Milton & Chenery (1998) found no effect of storage on Mn. Milton & Chenery (1998), however, did find that Mg was altered by both ethanol storage and delayed extraction, although the observed instability was rather small (10–100 ppm), which may be related to some of the Mg being tied to the protein in otoliths rather than all being incorporated in the aragonite lattice. As such, the use of Mn and Zn, and possibly Mg, in microchemical analysis of otoliths preserved in ethanol seems warranted.

Overall, the present study has indicated that 95% ethanol can serve as a good storage medium for fishes to be used in otolith microchemical analyses. Important elements, such as Sr and Ba, demonstrated no 'ill effects', owing to ethanol purity (HPLC- *v.* ACS-grade) or exposure duration (0–81 days). Thus, with an appropriate selection of elements (*i.e.* those that bind tightly into the $CaCO_3$ matrix), immediate otolith extraction, freezing fishes and ethanol preservation are all valid methods of specimen storage.

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