GREAT LAKES FISHERY COMMISSION

Project Completion Report[1]

Elemental Composition of the Statoliths of Sea Lamprey (*Petromyzon marinus*)

by:

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November 1998

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COMPLETION REPORT TO THE GREAT LAKES FISHERY COMMISSION

TITLE: Elemental Composition of Statoliths of Sea Lamprey (*Petromyzon marinus*)

DATE: November 10, 1998

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ABSTRACT

Statoliths are small (less than 10 μg)calcareous (apatitic) bodies in the inner ears of lampreys. Elemental composition of the statoliths of sea lamprey (Petromyzon marinus) was investigated as a means of stock identification. Recognition of primary natal streams is required for efficient and cost effective lamprey control in the Great Lakes. The investigation was undertaken as a preliminary study to determine whether there are stream specific elemental "chemoprints" in lamprey statoliths, to identify elements of interest for drainages of Lake Huron, especially the St. Marys River, and to develop techniques necessary to expand the method for routine identification of the "home waters" of adult lampreys. Ammocoetes were collected from four test localities: an "unpolluted" site in the St. Marys River; a "polluted" St. Marys River site downstream of various industrial and municipal inputs; a "pristine" river site on the Pigeon River; and a site on the Rifle River which receives agricultural runoff and municipal inputs. Elemental analysis utilized Particle Induced X-Ray Emission (PIXE) instrumentation. The final data set was based on approximately twenty individuals per site and six elements of interest: strontium, manganese, zinc, copper, lead and mercury. Linear Discriminant Function (LDF) analysis was used to provide a method for predicting which group (locality) a new case is most likely to fall into, or to obtain a small number of useful predictor variables based on statolith elemental composition. Concentrations for five of the elements was generally below 30 ppm, but was in the range of hundreds to thousands of ppm for strontium. The analysis demonstrated a statistically significant level of locality-specific elemental associations and ability to correctly classify individuals 60 to 92% of the time. Variation or "noise" in the data set decreased and classification success increased as analyses were restricted to thicker statolith preparations. The study has clearly demonstrated the existence of site-specific differences in trace elements composition and establishes a procedure to classify samples of unknown origin into the four localities sampled. The generality of the technique to a lake-wide scale is untested. Discussion includes suggestions for refinements in specimen preparation and instrumental operating conditions and the need for further investigation of alternative analytical procedures. A major concern is defining the limits of individual, spatial and temporal variability in statolith composition.

INTRODUCTION

Research on lamprey statolith microchemistry is extremely limited and has only been investigated as part of a feasibility study on lamprey stock identification for Great Lakes populations (Brothers, 1987; also follow-up letters/reports to the GLFC). These items include a discussion of the need for the research as well as the conceptual and practical underpinnings of the approaches taken. A summary of the 1987 project results follows:

> Elemental composition of the statoliths of sea lamprey (*Petromyzon marinus*) was investigated as a means of stock identification required for lamprey control in the Great Lakes. The goal of the investigation was to explore the feasibility of trace element analysis to discriminate the relative proportions of adult lampreys which have spent their larval life in different river systems. The statoliths, although small (maximum diameter - 350 µm; 15 µg per individual), are the only calcified (apatite) structures present throughout larval life and show little additional growth in the adult phase. Six analytical techniques were employed to test for useable trace element chemoprints in the ammocoete lamprey statoliths from five localities (Canada and New York). Comparative materials also examined included various stream and lake waters, N.I.S.T. certified bone samples, and trout otoliths. "Non-destructive" analyses were carried out on individual statoliths (energy dispersive and wavelength dispersive spectrometry of electron microprobe excited x-ray fluorescence, EDS and WDS) or pooled samples from several individuals (x-ray excited fluorescence spectrometry; XRF; and neutron activation analysis, NAA). "Destructive" preparations involving acidic dissolution of pooled samples (2-40 statoliths; 1-20 individuals) were subjected inductively coupled plasma atomic emission or mass spectrometry (ICP-AES and ICP-MS).
>
> The XRF method was capable of detecting Ca and Zn and possibly Fe, but was insufficiently sensitive to other trace elements, particularly in the presence of strong background peaks produced by the sample cups. Even with elimination of this artifact, the instrumentation would not be able to detect the expected concentrations elements in samples of only two statoliths. EDS and WDS analysis demonstrated the presence of Ca, P, Mg, Cl, K, and Na. Detectability of the latter element was questionable with EDS, but positively confirmed with WDS. Differences between localities could not be demonstrated with these methods. Magnesium was found to be distributed in a spatially heterogeneous pattern, possibly associated with presumptive annual zones. NAA revealed the presence of Ca, Cl, Mg, Na, K, and Mn, but the small sample size represented by two statoliths is a significant problem for detecting and quantifying elemental composition by NAA. ICP-AES analyses clearly demonstrated the presence of Ca, P, Mg, Mn, Fe, and Na. Results were reproducible and analysis of "standards" agreed with the expected concentrations for these samples of known composition. There were indications of consistent differences between some

> localities. Modification of the instrumentation to allow for smaller sample volumes would substantially improve sensitivity.
>
> The ICP-MS is generally one to three orders of magnitude more sensitive than the ICP-AES and it also yields data on isotopic fractionation. Due to the low detection limits, contamination artifacts are proportionally more significant and may obscure results for some of the less abundant elements. Quantitative comparisons of ICP-AES and ICP-MS results for the more common constituents (Na, Mg, P, Cr, Fe, Mn, Zn, and Pb) gave rough agreement within about an order of magnitude however some elements yielded inexplicably high values. Although there was only a limited number of samples available for analysis, there was generally good similarity between different samples taken from the same site. Distinctions between sites were either not obvious or possibly influenced of contamination. Site-specific differences in Mn, Fe, Zn, Na, and Cu looked most promising. Future ICP studies should employ axial detectors (for AES) and direct injection nebulizers, laser ablation or electrothermal vaporization to reduce sample dilution.
>
> It is concluded that positive indications in the results support the need for additional analyses, particularly with newer ICP techniques and Particle Induced X-Ray Emission (PIXE). Also required is a greater emphasis on reducing possible contamination.

In the dozen years since the initial project was undertaken, a number of developments have taken place which were expected to significantly enhance the technical feasibility of revisiting the challenge of identifying the home stream of adult lampreys. Although little more has been done on the elemental composition of the statoliths, the chemistry of teleost otoliths has been the subject of many recent studies and several international symposia. An excellent review by Ron Thresher (in press) compiles all available literature and makes critical evaluations of past studies, instrumental advances and conceptual approaches. The current re-investigation of the lamprey problem was stimulated by the availability of a number of new or improved analytical instruments. Bulk analysis methods such as ICP-AES have been improved by a factor of 10 by the incorporation of ultrasonic nebulization and axial configurations and the ICP-MS has become a common instrument. ICP-MS is also now available with laser ablation. Resonance Ionization Spectroscopy (RIS) can use thermal vaporization, ion beam sputtering or laser ablation to atomize sample materials. The most significant improvement is that advances have pushed realistic detection limits to the parts per billion range for the probe or beam methods. These instruments allow for the determination of elemental or even isotopic composition on a fine spatial scale (e.g. 10 μm^2) without the requirement of dissolving the statolith and diluting concentrations. Concurrent with these developments, but still poorly appreciated, is a new understanding of the extreme need to prevent contamination and other alterations of otolith or statolith composition by handling and/or preparation of samples. In order to take advantage of the most advanced analytical power now available, a proton probe (Particle Induced X-Ray Emission; PIXE) has been employed in the present investigation to examine the elemental composition of lamprey statoliths. This instrument offers unique sensitivity and minimal complicating interferences for the elements which are likely to be of greatest interest for the stream systems under study.

The research undertaken here constitutes Stage 1 of a proposed scheme to evaluate the potential for identification of lamprey source streams in Great Lakes drainages. The work was undertaken as a preliminary study to determine whether there are stream specific elemental "chemoprints" in lamprey statoliths.

Two key questions had to be answered before this approach can be used to determine which source streams contribute to the adult lamprey population. These are 1) do stream environments differ enough to produce diagnostic combinations of elements in lamprey statoliths?; and 2) are these diagnostic signatures identifiable in adult populations? The first question is addressed by the current study. If the answer is affirmative, subsequent studies should examine the second question.

The experimental design for the exploration of stream chemoprints involved examination of approximately 20 pairs of statoliths from large ammocoetes collected at four Lake Huron drainage sites. The collection localities were selected to include at least two streams expected to differ markedly in water chemistry due to anthropogenic pollution. The St. Marys River region includes sites which have received significant levels of contaminated effluents from steel plants and paper mills. This large area has not been treated with lampricides, has abundant ammocoete habitat, and is adjacent to Lake Huron sites characterized by high rates of lamprey scars on lake trout (Eshenroder *et al.*, 1995). Ammocoete collections in the region can easily target larvae exposed to mill polluted water and sediments. For comparison, samples were also to be obtained from upstream sites or other nearby areas not washed by contaminated waters. Samples from two such areas, "contaminated" and "clean" would constitute the main test case for the proton probe analyses. Two additional collection sites, representing a geographically separated and geochemically distinct watersheds, will help to determine the sensitivity of the technique to natural variation in the lake basin.

It was hoped that the results of Stage 1 would determine whether there is scope for naturally occurring elemental signatures diagnostic of drainages and/or whether the only distinguishable signatures are likely to be from unusual (in most cases, artificially distorted) drainages. The results would also indicate which elements are useful in stream diagnosis, their concentrations in statoliths and, hence, the analytical instrument best suited for cost-effective continuation of the project.

METHODS and MATERIALS

Statoliths were examined using two probe microanalysis techniques: electron probe and proton probe microanalysis. Both are based on bombardment of small (circa 10-20 µm^2) areas of sectioned statoliths with high energy beams, followed by elemental composition determination by analysis of secondarily emitted X-rays (the energy spectra of which is element specific). The two techniques differ, as suggested by their names, in the nature of the beams used to excite the secondary X-rays. Electron probes are relatively common and inexpensive. But are generally limited in sensitivity to about 100 parts per million (.01 wt %), due to X-ray "noise" induced by deceleration of primary electrons in the target material. Proton deceleration is much less pronounced, however, such that proton probes are capable of detecting many elements to the level of about 1-3 parts per million. A pilot study (1987) identified several elements of interest in lamprey statoliths in the greater than 10 part per million range, suggesting they would be well resolved by proton probe microanalysis. Because of the nature of the background spectrum (the "brehmstrahlung"), proton probe microanalyzers are particularly useful for analysis of trace metals, such as those associated with pollution (Fe, Zn, Pb, Cu, Cr, Hg are expected to be enriched in some of the rivers important for the study). Proton bombardment is also far less destructive than most other beam analyzers, such as the electron probe and laser ablation-ICPMS, permitting replicate analysis of the same point, if required, to verify elemental composition.

Electron probe studies were very limited and only served as a coarse screening procedure to identify any existing "gross" differences in statolith composition. Proton probe microanalysis of lamprey statoliths was carried out by Drs. R. E. Thresher and S. Sie, at the Australian CSIRO Heavy Ion Analytical Facility. Dr. Thresher is a senior scientist in the CSIRO Division of Fisheries. He has worked on the use of elemental analysis on stock identification of fishes for more than seven years, and has published a number of major papers on the subject. Dr. Sie is Head of the Heavy Ion Analytical Facility, and is an internationally respected theoretical physicist who designed and built the CSIRO proton microprobe. This instrument is one of the most sensitive proton probe microanalyzers in the world, and the only one whose operating conditions are well documented when used to assess elemental composition of calcified structures of fishes. To date, analytical accuracy and precision have been robustly determined for only two micro-analytic techniques for fish calcified structures - the Electron probe (by Gunn, et al., 1992) and the proton probe, by Sie and Thresher (1992). The CSIRO proton probe microanalyzer is described in detail by Ryan, et al. (1990).

Live lamprey ammocoetes, collected in Michigan and Canadian waters, were obtained with the assistance of Roger Bergstedt of the USGS, Biological Resources Division, Lake Huron Biological Station, Millersburg, MI. Five sets of samples were received. Pertinent data are summarized in Table 1 and collection localities for the four test localities [P, R, SMR(U), SMR(D)] are plotted in Figs. 1-3.

Collection Locality	I.D.	Number Processed	Number Analyzed	Description
Platte and White Rivers, MI		32(16+16)		Held at USGS laboratory for several months before receipt. Used for testing of preparation and analysis procedures
Pigeon River, MI	P	50	17	"pristine" river site
Rifle River, MI	R	49	19	river in agricultural region
St. Marys River, ON (up river site)	SM(U)	51	18	little or no industrial inputs;
St. Marys River, ON (down river site)	SM(D)	49	19	locality receives industrial and municipal effluents

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Table 1 - Ammocoete samples and locality descriptions.

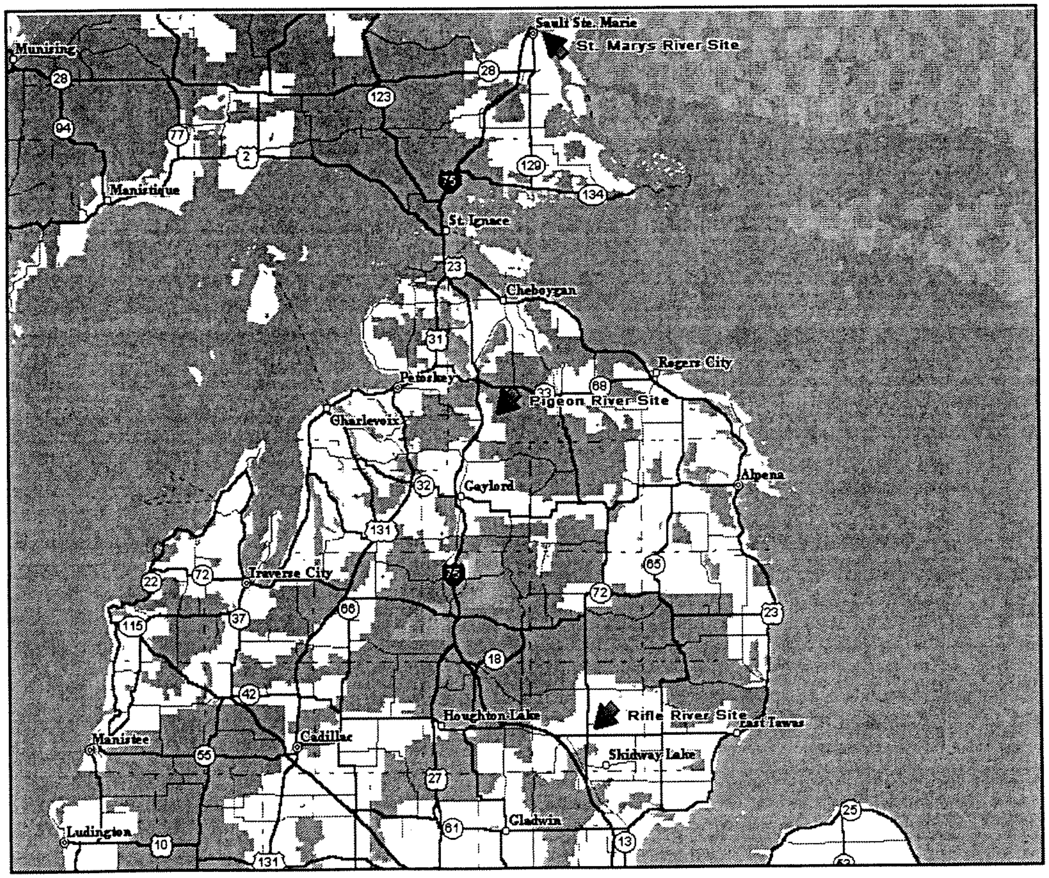

Figure 1 - Collection sites in Michigan, USA and Ontario, Canada.

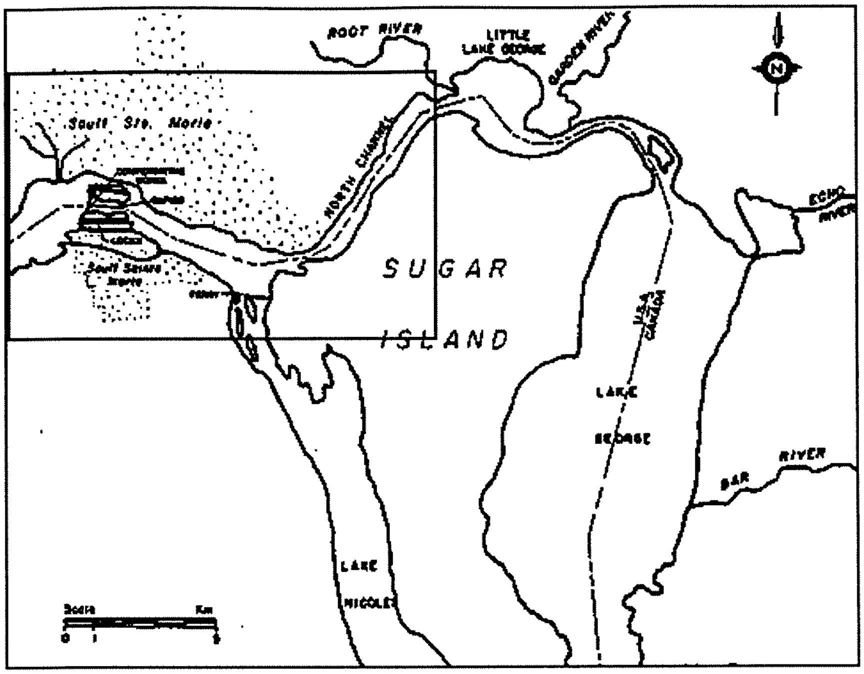

Figure 2 - General location of Canadian sites; SMR(U) and SMR(D); boxed area enlarged in Fig. 3.

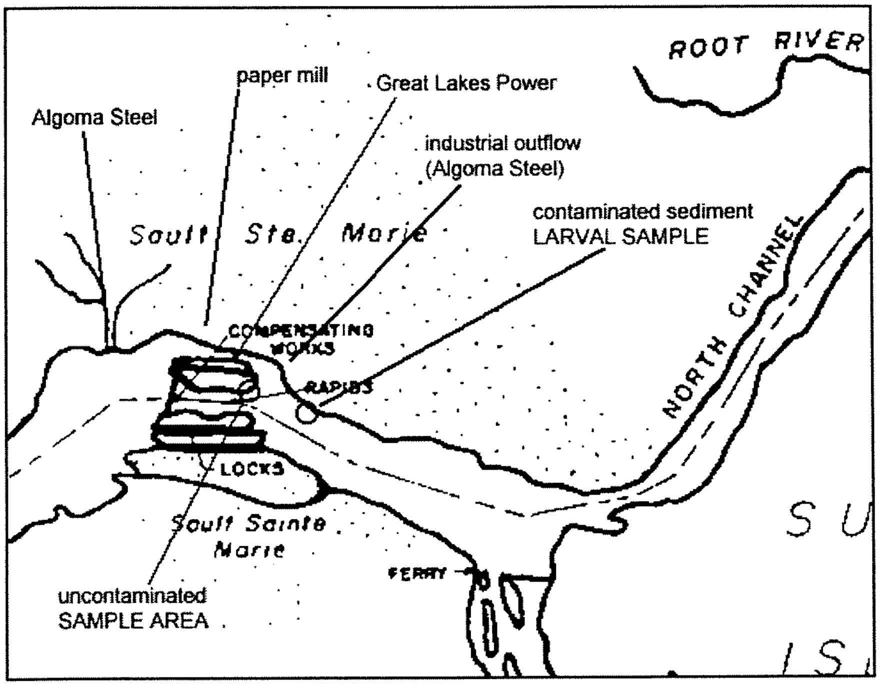

Figure 3 - Detail of area around Sault St. Marie, Ontario, Canada.

These localities were selected to represent four distinct aquatic environments (water and sediment characteristics), with different levels and types of anthropogenic inputs. Some historical data on elemental composition was available and reviewed. Unfortunately its spatial and temporal coverage was too incomplete to test correlations between statolith analyses and environmental conditions for the sites. Mean levels of some elements are summarized in Figs. 4 and 5. In general terms, metal concentrations averaged from 1 to 100 ppm, and levels in the sediment tended to be slightly higher than the water levels (as measured for the Rifle River).

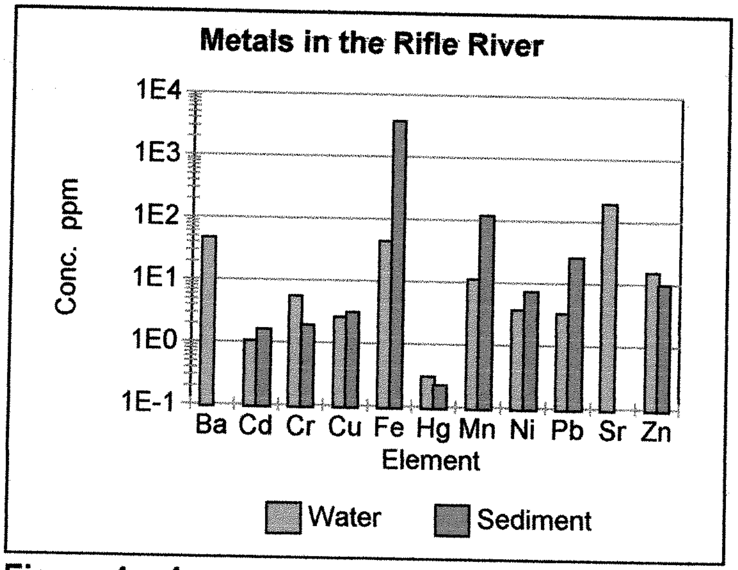

Figure 4 - Average metal concentration in Rifle River analyses.

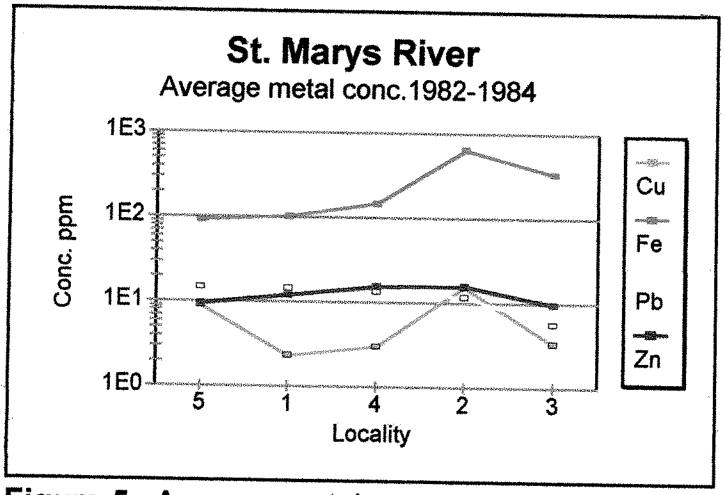

Figure 5 - Average metal concentration in St. Marys R. analyses. Localities ordered upstream to down (L to R).

Size distributions of collected ammocoetes for the four test sites varied, with the downstream St. Marys River location having distinctly smaller individuals (Fig. 6). The largest individuals available were selected for statolith extraction and analysis. Length distributions of the 73 individuals used in the final statistical analysis are shown in Fig. 7

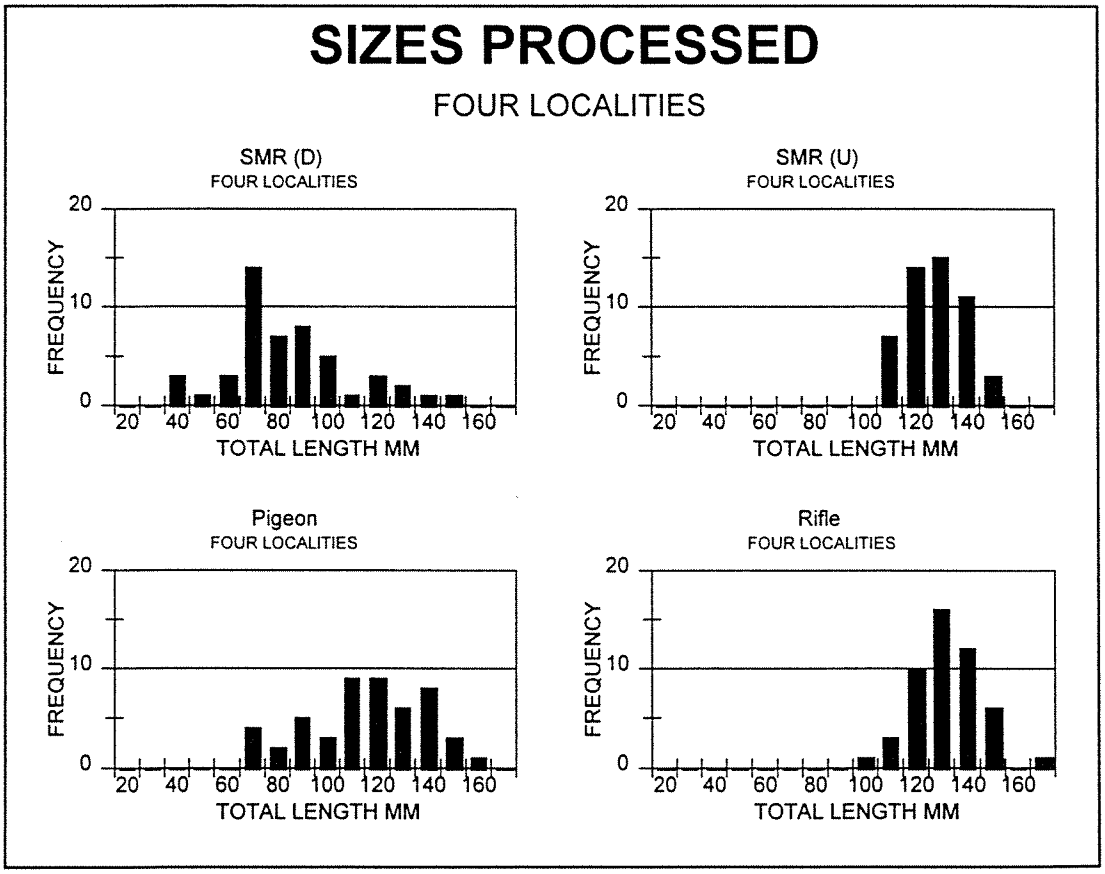

Figure 6 - Size distribution of sampled ammocoetes.

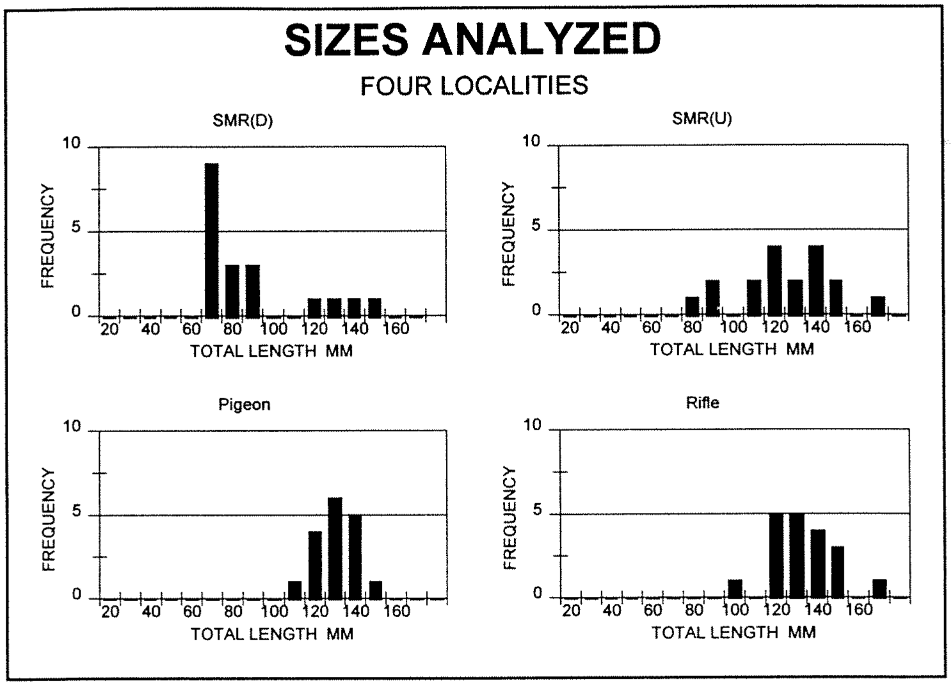

Figure 7 - Size distribution of ammocoetes in the final analysis.

Initial tests on the White and Platte River samples were designed to determine the best procedures for extracting, cleaning, orienting and mounting, grinding and polishing, and then acquiring and finally analyzing X-ray energy spectra with the PIXE instrumentation. The following alternative protocols were applied to a total of 48 different samples:

Handling prior to dissection - A) 5 minutes in liquid nitrogen
B) 24 hours in 95% ethanol at approx. -10°C
C) Freezing (dry) for 24 hours
*D) 5 minutes in 95% ethanol and held on ice for less than 30 minutes

Mounting Medium - A) Araldite 502
B) Spurr's low viscosity medium
*C) 50:50 Araldite and Spurr's

Orientation in mount - A)Ventral face up
*B) Ventral face down

Evaluation of the PIXE results demonstrated little evidence for artifacts produced by handling and preparation. The low concentrations of the metals being analyzed made it difficult to detect significant differences between the treatment groups. The analyses did not include other elements which have been shown to be particularly susceptible to handling artifacts (e.g. Cl, Na, Fe). In our final results, we also eliminated two samples which had extraordinarily high zinc values and were assumed to be contaminated. Initial screening included the following elements: Ca, Cr, Mn, Fe, Ni, Cu, Zn, Br, Sr, Rb, Mo, Pb, Hg, Cd, Ga, Se, Co, Ba, Ti, and U. A final set of six elements was chosen on the basis of several criteria: low likelihood of contamination or artifact; biological or environmental interest; consistent detection above calculated minimum limit and demonstration of significant differences between localities. These elements were strontium, zinc, manganese, copper, lead and mercury. Larger sample sizes and enhanced spectral acquisition (see below) may allow for the inclusion of additional elements.

The procedure used for all subsequent samples incorporated alternatives D, C and B (marked * above). The complete protocol was as follows:

1. Immersion of live ammocoetes in 95% ethanol for 5 minutes followed by holding on ice for not more than 30 minutes
2. Frontal section to expose and open the otic capsules
3. Removal of statoliths with stainless steel forceps and teasing away of any adherent tissue (performed in de-ionized water; less than 1 minute)
4. Air dry and place in dry gelatin capsule
5. Mount statoliths (ventral face down) in a 50:50 mixture of Araldite 502 and Spurr's medium; cure for 24 hr at 70°C
6. Grind by hand on wet (water) aluminum oxide paper (800 grit); polish with 3μm and 1μm diamond paste with brief water rinses

PIXE analyses were run under a series of conditions which was based on successful results with small otoliths of teleost fishes. Otoliths are primarily composed of calcium carbonate as aragonite (sagittae). Lamprey statoliths are apatite or calcium phosphate, however both calcified materials have relatively low concentrations of elements other than those making up the apatite or aragonite, and any organic matrix. The conditions for the PIXE analysis were:

1. Up to 20 mounts or samples were loaded into the chamber at one time.

2. Dwell time for each sample was approximately 10 to 15 minutes. The exact time was determined by a cutoff at a total accumulated charge of 6 micro coulombs. The time to reach this point is dependent upon the current applied and beam diameter. Beam diameter was set at a constant for all samples and the current was ramped upwards until a optimal count rate was reached.

3. The analysis package calculates a theoretical sample thickness based on the expected calcium count rate. This value is express as "mass" in micrograms, however it

is not equivalent to actual statolith mass. It is simply a value to express how good the counting statistics are and is a function of sample thickness. Low "mass" is an indication of the sample being too thin; masses of 10 μg represent "infinite" thickness with respect to beam penetration and x-ray generation. Mass corrected data were used for the subsequent statistical analyses.

4. A minimum detection limit (MDL) was calculated for each element in each sample. These were somewhat higher (e.g. 4-6 ppm) than the usual values (1-2 ppm) due to the limiting size and thickness of the statoliths. The calculation of MDL was based on three standard deviations which is a conservative criterion.

Statistical evaluation for the final data set (73 individuals divided into four localities or groups and based on results for six elements) utilized Linear Discriminant Function analysis (LDF). When applied to multiple groups, the technique provides a method for predicting which group a new case is most likely to fall into. Linear combinations of independent variables (element concentrations) are used as the basis for group (locality) classifications. Comparison of groups proceeds by the calculation of individual scores by summing the products of weighting factors by element concentrations. Averaging the scores derives a group centroid; one centroid results for each group. Comparing centroids shows how far apart the groups are along the dimension (location) being tested. The objectives for the LDF analysis were:

1. Determine if there were statistically significant differences among the four localities

2. Establish procedures for classifying individuals into groups (i.e. assign a lamprey from an unknown locality into a probable locality group)

3. Determine which elements account for most of the differences between the localities

Discriminant functions can be evaluated for statistical significance and ranked on the basis of their relative importance in distinguishing the groups. A calculated value, Wilks Lambda, is used for statistical testing at a particular significance level (95% in the present study). This statistic varies between zero and one; zero indicates perfect separation (no overlap) and a value of one denotes complete overlap.

RESULTS

Analyses for the six elements of interest (Sr, Mn, Zn, Cu, Pb, Hg) revealed some distinctive patterns when all individuals (N=73) from the three sites were compared (Fig 8). Table 2 presents summary statistics for these data.

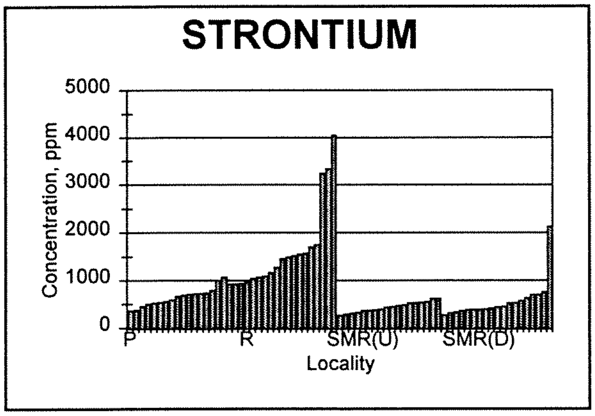

Figure 8A - Strontium concentrations.

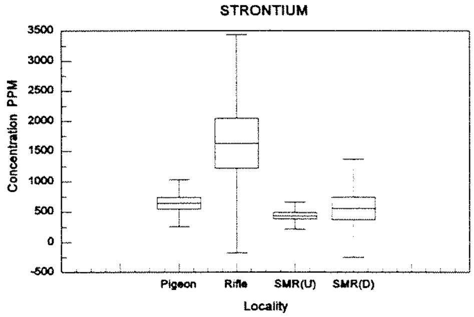

Figure 8B - Sr; Mean, 2 Std Dev, and 2 Std Err by locality.

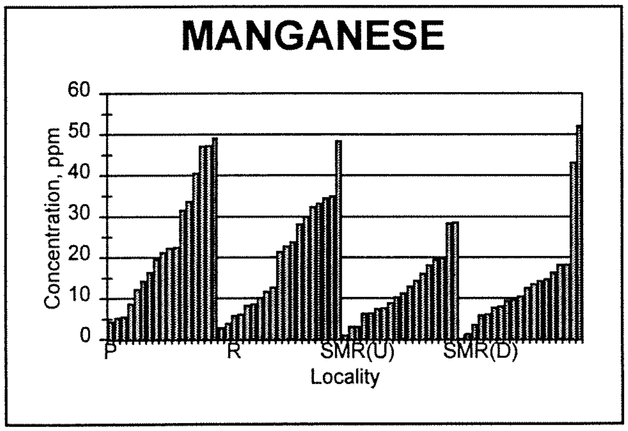

Figure 8C - Manganese concentrations

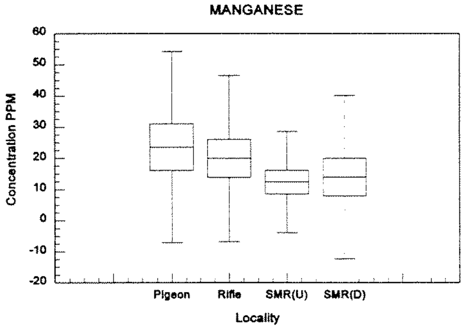

Figure 8D - Mn; Mean, 2 Std Dev, and 2 Std Err by locality.

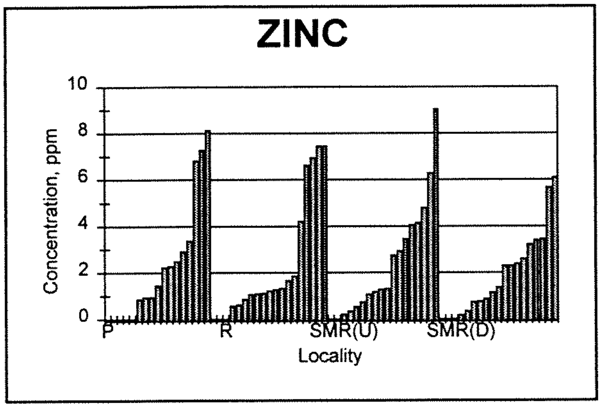

Figure 8E - Zinc concentrations.

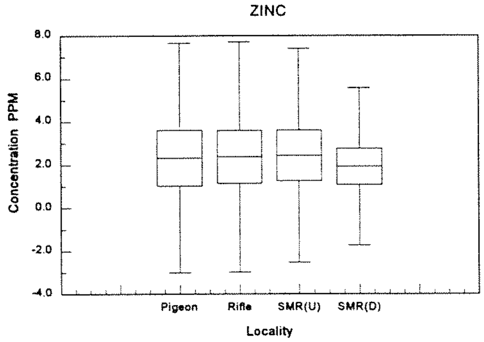

Figure 8F - Zn; Mean, 2 Std Dev, and 2 Std Err by locality.

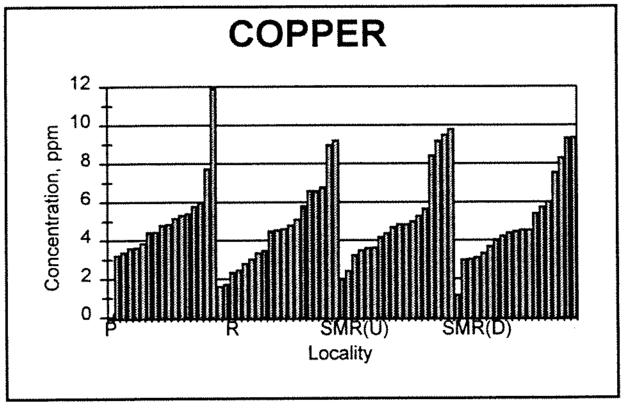

Figure 8G - Copper concentrations

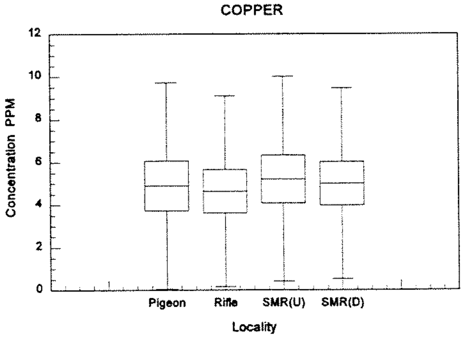

Figure 8H - Cu; Mean, 2 Std Dev, and 2 Std Err by locality.

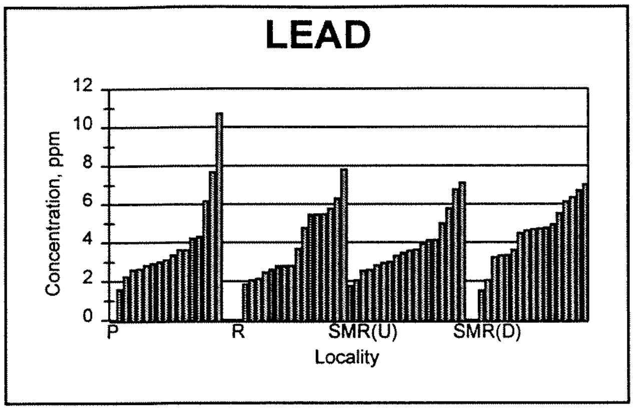

Figure 8I - Lead concentrations

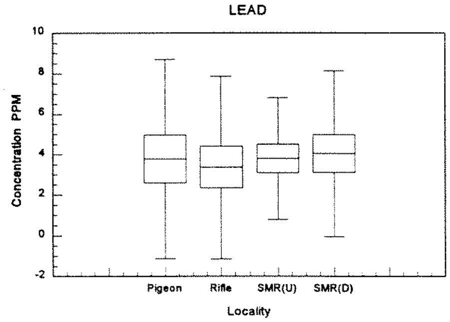

Figure 8J - Pb; Mean, 2 Std Dev, and 2 Std Err by locality.

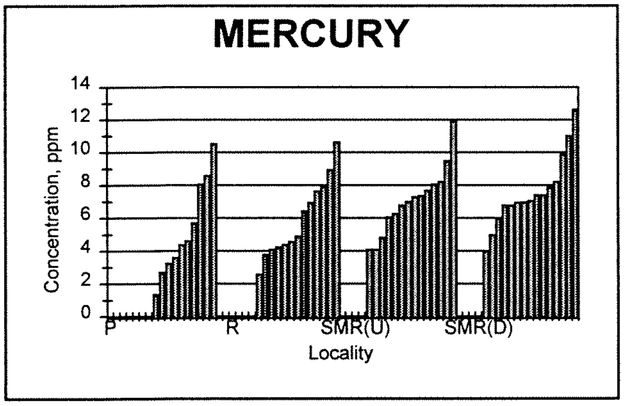

Figure 8K - Mercury concentrations

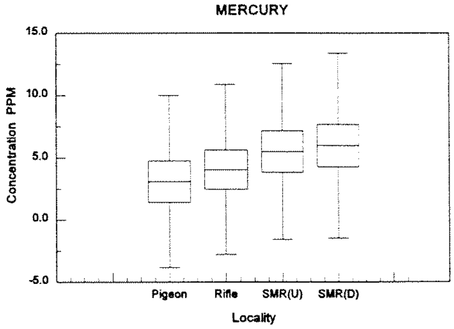

Figure 8L - Hg; Mean, 2 Std Dev, and 2 Std Err by locality.

Statistic			Element			
	Sr	Zn	Mn	Cu	Pb	Hg
Standard Error						
Mean	828.37	2.27	17.32	4.94	3.75	4.68
Standard Error	82.07	0.28	1.55	0.27	0.24	0.43
Median	569.00	1.27	13.40	4.54	3.45	4.81
Standard Deviation	701.23	2.38	13.24	2.28	2.06	3.64
Variance	491728.32	5.68	175.20	5.21	4.25	13.27
Kurtosis	8.56	0.49	0.25	0.63	0.87	-1.07
Skewness	2.72	1.22	1.03	0.80	0.49	0.04
Range	3793	9.03	52.1	11.9	10.7	12.6
Minimum	257	0	0	0	0	0
Maximum	4050	9.03	52.1	11.9	10.7	12.6
Count	73	73	73	73	73	73
Confidence Level(0.95)	160.86	0.55	3.04	0.52	0.47	0.84

Table 2 - Summary statistics for all elements.

A matrix of correlation coefficients for the elements indicates only weak associations when element pairs are considered one at a time (Table 3).

	Matrix of Correlation Coefficients					
	Sr	Zn	Mn	Cu	Pb	Hg
Sr	1					
Zn	0.129493	1				
Mn	0.127483	0.225797	1			
Cu	0.19749	0.245029	0.22489	1		
Pb	-0.12193	0.061912	-0.23234	-0.05047	1	
Hg	-0.10431	-0.14495	-0.2292	-0.23396	0.175725	1

Table 3 - Correlations between elements.

Pairwise comparison of sample means for the elements at each locality revealed some significant differences for Sr, Hg and Mn. Non-overlap of the 95% confidence interval of the means (approx. two standard errors of the mean) in Figs 8 can be viewed as graphical representation of which sites were distinctly different or whether trends in metal concentrations were in evidence. Scattergram plots of elements (in two or three dimensions) show only the most obvious locality distinctions (Fig.9).

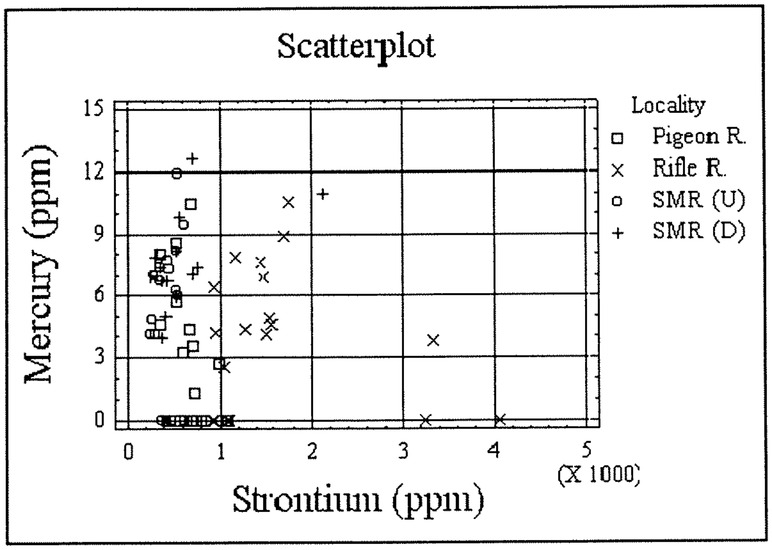

Figure 9A - Strontium x Mercury.

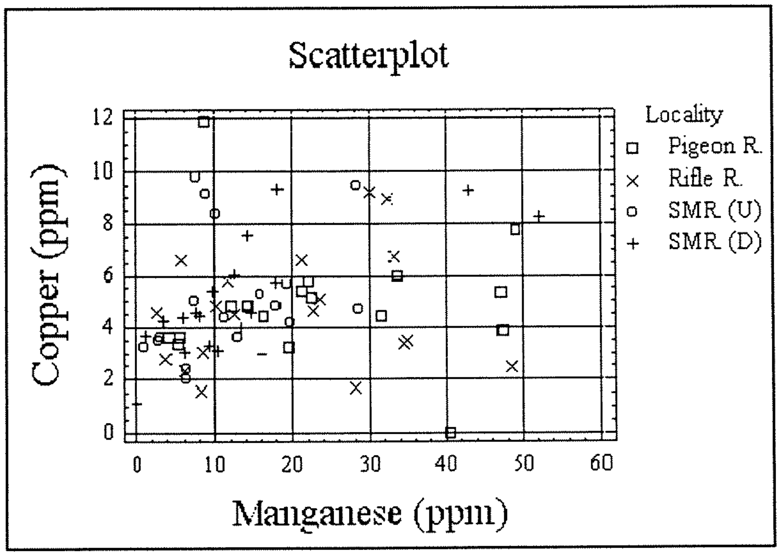

Figure 9B - Manganese x Copper.

For example, in Fig. 10 the Rifle River site separates out on the strontium axis and the Pigeon River samples are separated on the manganese axis. This plot includes the three elements showing the greatest degree of site difference. As seen in the data presented earlier and in multi-element scattergram plots, copper, zinc and lead showed less difference between sites when considered one element at a time or in combination with each other. In order to utilize all available information from the PIXE analysis, the

LDF procedure considers the contributions from the latter elements as well since it calculates a discriminant function based on a combination of the six elements measured. Mercury was the only element which demonstrated a possible cline in concentration with the downstream St. Marys River site having the highest average level. Comparison of the upstream and downstream St. Marys River sites did not reveal anticipated higher metal concentrations below the pollution inputs. The smaller size (and younger age) of the ammocoetes at the downstream site may have been a contributing factor however.

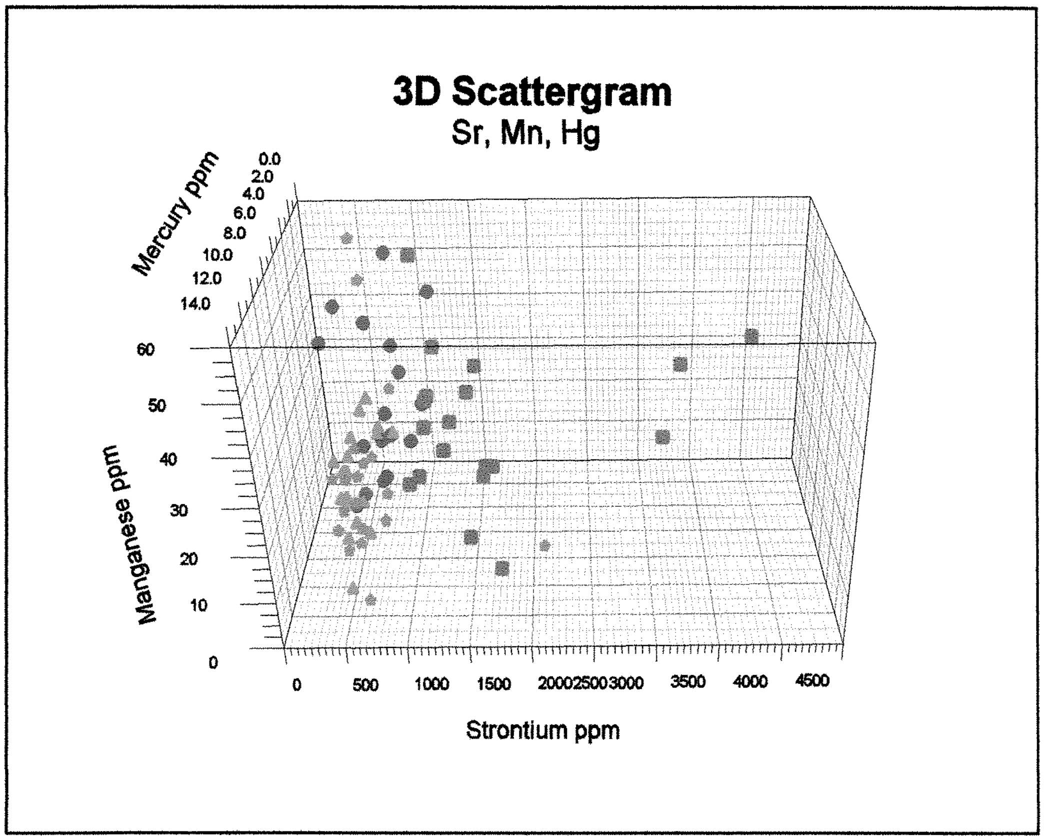

Figure 10 - Strontium x Manganese x Mercury.

● Pigeon R.

■ Rifle R.

▲ SMR(U)

✱ SMR(D)

Stepwise elimination of small "mass" statoliths from the analysis (e.g. only including those 1 μg or greater; 2 μg or greater, etc.) resulted in increasing levels of significance when comparing mean metal concentrations between sites. The same progressive approach to the LDF analysis (see below) also demonstrated increasing levels of discriminant function significance and decreasing levels of mis-classification as the apparent size of statoliths increased.

Examination of the covariance and correlation relationships between elemental concentrations within groups (localities; Table 4) illustrates that there are weak relationships (for covariance, these are unscaled values; the sign is more important than the magnitude for comparisons). For example, strontium is positively related to zinc and manganese, but negatively related to lead and mercury.

Results for the LDF analysis on the full sample set (N=73) are presented in Table 5 along with results for a reduced sample set of statoliths calculated to have a "mass" of 4 μg or greater. These data demonstrate that the total data set shows a single significant derived function (P-value <0.05) and the smaller data set has two significant functions.

Plots of the derived functions (and centroids showing average trends) illustrate the degree of separation between localities. Separation of sites (as the mean or centroid) along a given axis shows which function is responsible for most of the discrimination. Examination of the loadings for the functions then shows which elements are the most influential for that function. The 4 μg and up sample group (Figs. 11-13) showed good

	Within-Group Covariance Matrix					
	Sr	**Zn**	**Mn**	**Cu**	**Pb**	**Hg**
Sr	266989.00					
Zn	197.23	5.89				
Mn	179.52	7.17	161.63			
Cu	428.45	1.39	7.79	5.39		
Pb	-71.67	0.35	-6.06	-0.29	4.37	
Hg	-28.84	-1.19	-6.34	-2.18	1.21	12.47
	Within-Group Correlation Matrix					
	Sr	**Zn**	**Mn**	**Cu**	**Pb**	**Hg**
Sr	1					
Zn	0.16	1.00				
Mn	0.03	0.23	1.00			
Cu	0.36	0.25	0.26	1.00		
Pb	-0.07	0.07	-0.23	-0.06	1.00	
Hg	-0.02	-0.14	-0.14	-0.27	0.16	1.00

Table 4 - Within-group covariance and correlation matrices.

All Samples (N=73)				
Functions Derived	**Wilks Lambda**	**Chi-Square**	**DF**	**P-Value**
1	0.358	68.799	18	0.0000
2	0.830	12.486	10	0.2539
3	0.984	1.109	4	0.8928
Samples 4 ug or greater (N=42)				
Functions Derived	**Wilks Lambda**	**Chi-Square**	**DF**	**P-Value**
1	0.176	62.567	18	0.0000
2	0.565	20.529	10	0.0246
3	0.888	4.293	4	0.3678

Table 5 - Summary for LDF analyses

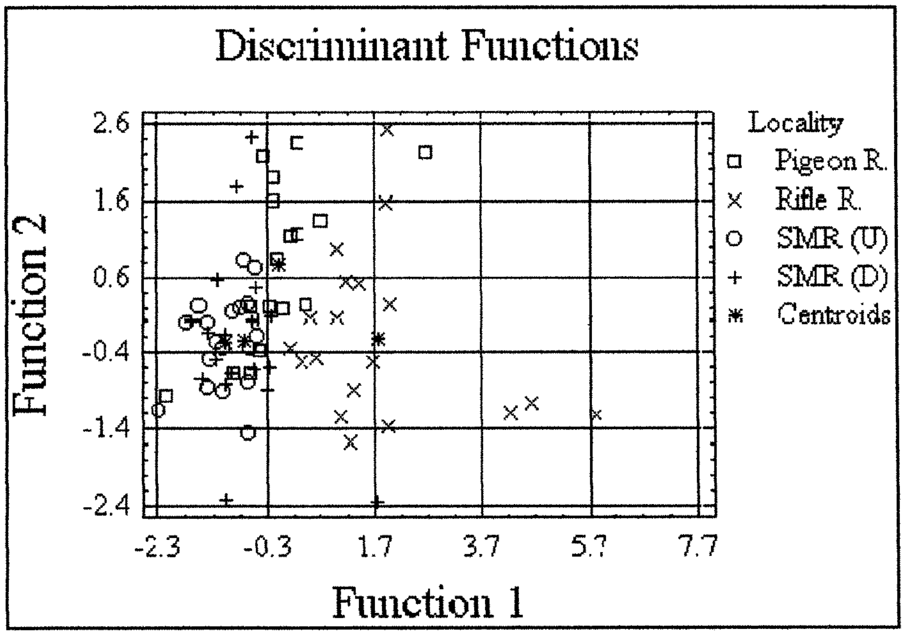

Figure 11 - Function 1 x Function 2; samples with statoliths of 4µg or larger

separation and still included over 40 individuals. More inclusive groupings showed similar separations, but with a somewhat higher degree of scatter. Graphing Function 1 versus Function 2 shows separation of the Rifle R. and Pigeon R. samples. Axes for Functions 1 And 3 break out the Rifle R. and SMR(U). Axes for Functions 2 and 3 also separate the Pigeon R.

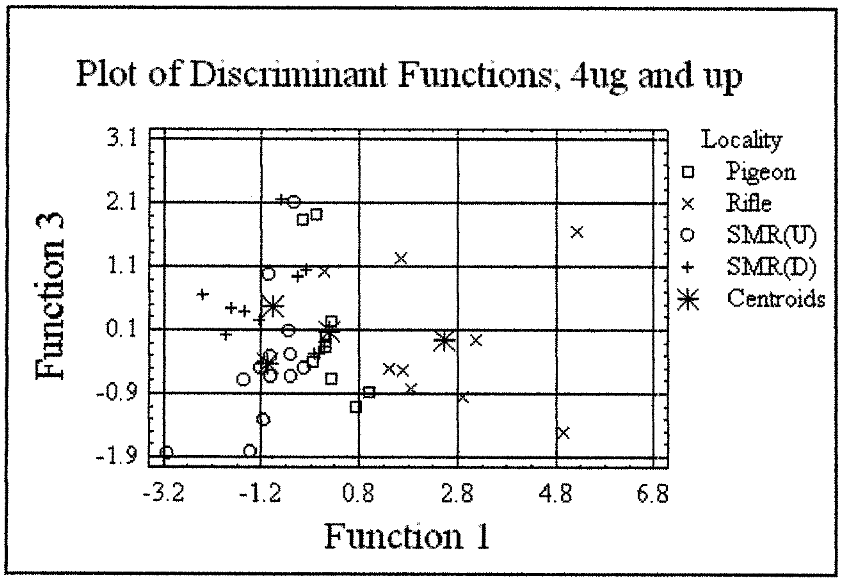

Figure 12 - Function 1 x Function 3; samples with statoliths of 4µg or larger

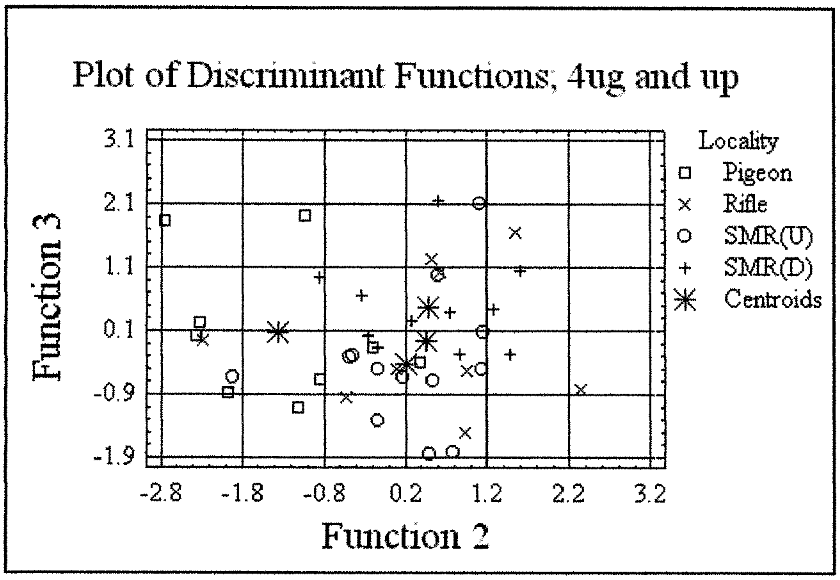

Figure 13 - Function 2 x Function 3; samples with statoliths of 4µg or larger

Comparison of the LDF analyses by statolith "size" (Table 6) illustrates decreasing values for Wilks Lambda. Careful examination of the relative magnitude of the standardized discriminant function coefficients also allows for identification of the most important elements being used to discriminate amongst the localities. The last column in the table shows the percentage of samples correctly classified by the analysis. This value increases to over 90% when samples are limited to statoliths of larger mass or thickness. The probable effect of limiting analysis to larger statoliths is the reduction of measurement noise. Graphical representations of these trends are presented in Figs.14 and 15.

Statolith "size"	N	Wilks Lambda	Sign. Factors	Major Elements	% Correct
All	73	0.358	1	Sr, Mn	60.27
≥2 μg	68	0.311	1	Sr, Mn	58.82
≥3 μg	58	0.255	1	Sr, Mn	68.97
≥4 μg	42	0.176	2	Sr, Mn	76.19
≥5 μg	13	0.002	1	Sr, Cu, Hg	92.31

Table 6 - Trends in LDF results with statolith size.

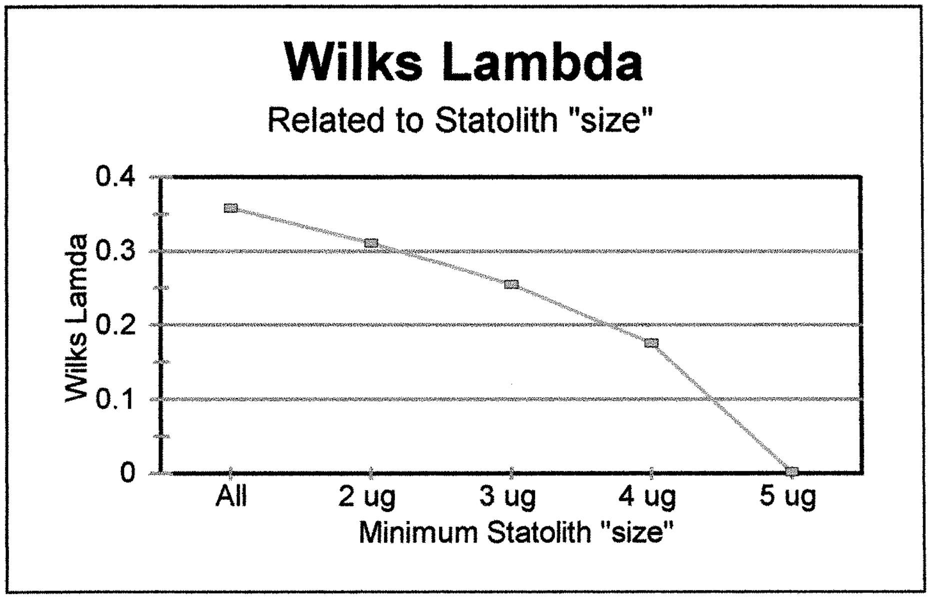

Figure 14 - Wilks Lambda as a function of statolith size. Smaller Lambda values have higher significance level and indicate less overlap between groups (localities).

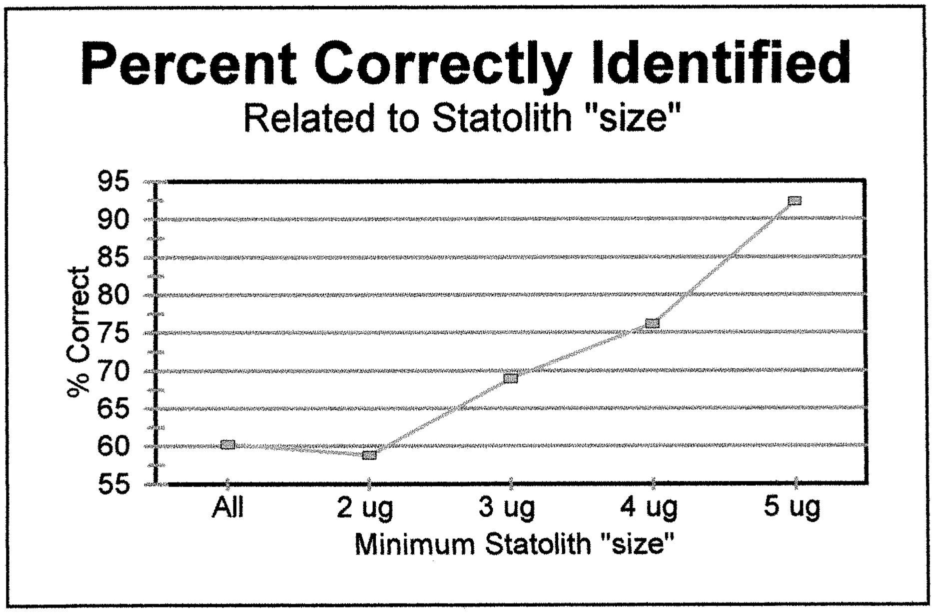

Figure 15 - Percent of individuals correctly classified to their location of origin as a function of statolith size.

DISCUSSION

PIXE analysis of ammocoete statoliths combined with Linear Discriminant Function Analysis for six elements has produced correct classification rates of over 90%. Elements selected in this study were those which are reliably detected with the instrumentation; relatively free from artifact effects; and some are likely related to anthropogenic sources (e.g. heavy metals). Strontium also proved to be of great interest since it is abundant in the statoliths (hundreds to thousands of ppm) and was strongly weighted in the derived functions. Its relative abundance in the Rifle River samples may be related to agricultural runoff and/or geological characteristics of the watershed. Recent studies have demonstrated the usefulness of isotopic fractionation of strontium when analyzing salmon otoliths (Kennedy et al., 1997).

The most significant problem encountered in the project was the effect of small statolith mass on the calculated detection limits of the PIXE analysis. The inherent operational factor is actually sample thickness, a variable which is dependent on animal size and can be only slightly modified by changes in mounting orientation; grinding procedures, beam targeting. More reliable/less noisy spectral traces could also be obtained by acquiring x-rays for longer times; increasing probe current; and increasing beam diameter. There are trade-offs to such procedural modifications they will result in greater damage to the samples and decreased spatial resolution.

The results presented here demonstrate the ability of the PIXE probe to analyze statolith composition with sufficient sensitivity to successfully classify ammocoetes to their home “stream “ or location in a larger river system. Some elements (e.g. mercury) used for the analysis were probably strongly influenced by current anthropogenic inputs and long-time accumulations in the sediment. Increased emphasis on such elements might require additional or alternative analytical techniques which target particular elements or isotopes and also have detection limits far below what is capable with PIXE. Table 7 summarizes average elemental concentrations in the environment (Rifle R.) and in statoliths from the current study and by two other methods employed in the 1987 project. There are some unexplained differences in the values which may be the result of a combination of factors such as true variation in source localities, introduction of contamination artifacts, and typical instrumental bias. It is known that PIXE and laser ablation ICP-MS underestimate nickel and zinc by up to an order of magnitude when compared to bulk analyses by ICP and ICP-MS (Ron Thresher, pers. com.). Accurate results are important, however for the purposes of stock identification, consistency and precision are essential. Exploration of alternative analytical methods which provide adequate sensitivity and precision for identified elements/isotopes of interest should be alternative analytical procedures continued.

There are many levels of variation which must be understood with respect to stock identification program. Due to the limitations of funding, the question of repeatability within the sample set investigated in the current study has not been adequately addressed. Left/right comparisons and multiple analyses of the same statolith are simple tests which should be performed. At a higher level, description of individual variation was barely achievable with the relatively small sample sizes available. The next level, the existence of temporal and spatial variability at a given location, is also of major concern and can only be investigated with longer term studies covering multiple sites within a river system. The compositional differences identified in the current study were slight, even though localities were selected represent large differences in environmental conditions. Unfortunately there were inadequate direct and current environmental measures to correlate to statolith results. Nevertheless, it should be expected that intermediate sites would produce intermediate compositions which would make separation of unknowns more difficult. At this stage of the research, it is imperative that new samples be obtained from the same sites as well as additional sites. In this way a range and pattern of variability can be developed to characterize statolith composition typical of the region as a whole. Samples from adults should also be incorporated into the scheme to see if any identifiable or classifiable compositional patterns can be associated with the stream results. Stage 2 of the project could be titled – Determination of ranges of elemental concentrations in statoliths of adult lampreys, and detailed analysis of diagnostic stream signatures.

	Mn	**Ni**	**Cu**	**Zn**	**Sr**	**Cd**	**Hg**	**Pb**		
Rifle R. (water)	11.4	3.7	2.6	15.3	194.5	1.0	0.3	3.3	Environmental	
Rifle R. (sedimen	120.0	7.5	3.1	9.9		1.6	0.2	26.1	ppm	
Current Study	17.3		4.9	2.3	828.4		4.7	3.7	Statoliths	
									ppm	
ICP-AES	35.0		30.0	100.0		20.0		10.0		
ICP-MS results	24.9	35.3	236.4	176.4	69.3	3.6	52.4	46.0		

Table 7 - Average metal concentrations in statoliths and the environment (Rifle R.).

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