GREAT LAKES FISHERY COMMISSION

2003 Project Completion Report[1]

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

by:

Edward B. Brothers[2]

[2]EFS Consultants
3 Sunset West
Ithaca, NY 14850

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COMPLETION REPORT TO THE GREAT LAKES FISHERY COMMISSION

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

DATE: **April 30, 2003**

SUBMITTED BY: **Edward B. Brothers, Ph.D.**
EFS Consultants
3 Sunset West
Ithaca, New York 14850
(607) 347-4203

PRINCIPAL INVESTIGATOR: **Edward B. Brothers**

ABSTRACT

Statoliths are small (less than 10 mg) 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 solicited follow-up study for research completed in 1998. The goal of that work was 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. Results demonstrated a statistically significant level of locality-specific elemental associations and ability to correctly classify individuals 60 to 93% of the time. The study clearly demonstrated the existence of site-specific differences in trace element composition and established a procedure to classify samples of unknown origin into the four localities sampled. This research examined newly collected and prepared statolith samples; some prepared samples from earlier studies, and incorporated new instrumentation and several procedures to improve the statistical robustness of the Particle Induced X-ray Emission (PIXE) data. Modification of the mounting and grinding plane, increased acquisition times, smaller spot size and lower beam density improved counting statistics, reduced sample rejection rates, and added data on an additional four elements for a total of ten (Mn, Fe, Cu, Zn, Ba, Rb, Hg, Pb, Ni, and Sr). Efforts emphasizing analysis of statoliths from a St. Marys River locality with known anthropogenic sediment impacts versus samples from a "clean" site demonstrated that statolith composition was consistent with expected environmental trends. Another component of the study was to determine whether elemental composition patterns established in larvae persist into adults. Ammocoetes and adults spawned in the same river were used for this analysis. Adults were generally classified into the appropriate site or at least Michigan rivers, when the alternative was the St Marys system; therefore statolith composition remains relatively constant and diagnostic through the larval/adult phases. Life history transects across statoliths demonstrated that statolith composition is relatively consistent throughout the larval life, however some gradients may be present and require careful attention to preparation and analysis location. Left/right comparisons of concentration within the statoliths of a single individual show that repeatability is reasonable, but some disparity is present and may be due to instrument error and/or within statolith variation and inconsistencies in beam location. Finally, some stream environments in Lake Huron drainages have been demonstrated to be sufficiently different to impact statolith chemistry and produce diagnostic combinations of elements to identify larval origins. Incorporation of isotopic information for abundant elements such as strontium will likely result in significantly improved watershed resolution and classification of adult samples.

INTRODUCTION

Elemental composition of the statoliths of sea lamprey (*Petromyzon marinus*) has been investigated as a means of stock identification required for lamprey control in the Great Lakes. The goal of these investigations 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. Research on lamprey statolith microchemistry is extremely limited and has only been investigated in relation to feasibility studies on lamprey stock identification for Great Lakes populations (Brothers, 1987, 1998; and follow-up letters/reports to the GLFC). These studies employed an ever-improving array of analytical instruments for bulk analysis (more suitable for pooled samples) and beam methods, which allow for characterization of individual statoliths. The abstract for the 1998 project summarizes the most recent status of lamprey statolith information and is the basis for the current work:

> Statoliths are small (less than 10 ?g) 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 were generally below 30 ppm, but were 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.

In order to take advantage of the more advanced analytical power now available, the research reported here utilized an improved Particle Induced X-ray Emission (PIXE) probe to examine the elemental composition of lamprey statoliths. This instrument offers unique sensitivity and minimal complicating interferences for the elements that are likely to be of greatest interest for the stream systems under study. Results from the1997-98 project have already proven the utility of this approach. The Great Lakes Fishery Commission (GLFC) has identified analysis and prediction of the production of transformers in streams as a priority research area. The current research was designed to reinforce and extend the applicability of statolith chemical analysis to lamprey stock delineation in the Great Lakes and specifically addresses two requests in the GLFC solicitation: re-analysis of newly collected St. Marys River samples; and research on known-origin adults.

The operation of the proton probe is based on bombardment of small (circa 10-20 ?m^2) areas of sectioned statoliths with high energy beams, followed by elemental composition determination by analysis of secondarily emitted X-rays (with element specific spectral peaks). Proton probes are capable of detecting many elements to the level of about 1-3 parts per million. As noted above, prior studies have identified several elements 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 known or expected to be elevated in some of the rivers important for the proposed 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.

The CSIRO instrument employed in the current investigation is a new design (Ryan *et. al*, 2001), one of the most sensitive proton probe microanalyzers available, 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). A recent paper also compared accuracy, precision and sensitivity of several techniques as applied to fish otoliths (Campana, et al., 1997). The CSIRO proton probe microanalyzer (also called nuclear microprobe) is described in detail by Ryan, *et al*., 1990 and Ryan *et al*., 2001). Recently completed work identified several possible ways to improve the statistical robustness of the PIXE data. Modification of the mounting and grinding plane, increased acquisition times, smaller spot size and lower beam density were expected to improve counting statistics and reduce sample rejection rates.

The GLFC staff identified the following research activities as most important for continuing the statolith chemistry program:

1. Re-evaluation of larvae from the effluent plume of the St. Marys River. Prior work demonstrated only a weak enhancement of "anthropogenic" metals in the St. Marys animals compared with the other Lake Huron drainage localities. Two factors may have contributed to this result. First, the ammocoetes in the St. Marys sample were unusually small and presumably younger than the larvae in the other samples. Secondly, the larvae may not have been collected in an area impacted by discharges. The effluent plume is restricted to areas of fast current and hugs the shore for a considerable distance. Collections for the current research are located in a region of known sediment impact.

2. Analysis of statoliths of known-origin adults and ammocoetes. Dr. William Swink of the USGS Hammond Bay Biological Station has recaptured tagged adults and resident larvae from the Black Mallard River. Frozen heads of these animals were made available for statolith examination. A small number of additional adult heads from non-Black Mallard River animals were also available and could be included in the sample set but not identified as such; i.e. as "blind" controls. Analysis of the larval and adult statoliths was desired to demonstrate whether elemental patterns in the young lampreys are demonstrable in adults from the same stream origin. If they are not, then statolith-based stock identification may not be a viable approach.

Potential pitfalls to the success of the approach include compositional lability and temporal variability of the stream environment.

Three supporting studies were also desired to assess the robustness of data developed in prior studies and to address intra-individual variation in statolith data from the PIXE probe. The first question could be attacked by re-analysis of existing prepared samples from the localities examined in the 1997-1998 studies. The analysis would employ improved counting conditions and the data could also be combined with the new samples the linear discriminant function testing.

Left/right comparisons for the two statoliths from an individual ammocoete will produce an estimate of internal consistency and allow for assessment of possible instrumental and preparation artifacts. Finally, a transect of analysis points from the statolith origin to the margin can be considered as a "life history scan" to determine whether there is any evidence for ontogenetic changes in composition.

The ultimate goal of the study is to provide the GLFC with data to test the following hypotheses:

1. St. Marys ammocoetes have unique and/or elevated metal concentration patterns in their statoliths which are reflective of elevated metal concentration in river sediments.

2. Adults and larvae with the same stream origin show consistent, unique and identifiable patterns of trace metal concentration in their statoliths and would be classified together when analyzed in a larger data base including samples from other stream localities.

3. Stream environments in Lake Huron drainages are sufficiently different to produce diagnostic combinations of elements in lamprey statoliths

MATERIALS AND METHODS

During the course of the study a total of 144 sets of lamprey statoliths were extracted, embedded, ground and polished. Time did not permit PIXE probe analysis of all of these specimens since the research plan also required repeated runs on some samples for left/right comparisons and transects. A total of 88 successful probe runs for ten elements each were completed representing 60 individual larvae or adults. Fourteen individuals were run twice for the "left/right" comparative analysis. In some cases, element concentrations were below detection limits or otherwise in error (primarily for rubidium). Complete data sets for all ten elements were available for 42 individuals for the linear discriminant analysis. Table 1 summarizes the source, identifying codes, disposition and sizes of all analyzed samples. Many more larvae were received (frozen) than processed. In general, the largest larvae available were chosen for processing.

TABLE 1- Sample summary; * used for left/right comparisons; ** used for transects

HEADS I.D.	Tag number	Parasitic cohort	Tag Location	Spawning year	Recapture location	Recapture date	Length (mm)	Weight (g)	Sex
B3-02**	2001-21	2000	Black Mallard R.	2001	Ocqueoc R.	6-May-01	418	163.7	M
B4-26	2001-54	2000	Black Mallard R.	2001	Cheboygan R.	15-May-01	462	245.4	F
B4-27	2001-90	2000	Black Mallard R.	2001	Little Thessalon R.	28-May-01	444	219.8	M
B4-28	2001-57	2000	Black Mallard R.	2001	Greene Cr.	18 to 20 May-01	462	226.5	F
B4-29	2001-19	2000	Black Mallard R.	2001	Cheboygan R.	4-May-01	472	201.1	M
B4-30	2001-28	2000	Black Mallard R.	2001	East AuGres R.	2-May-01	399	159.2	M
B4-31	2001-51	2000	Black Mallard R.	2001	Cheboygan R.	11-May-01	461	222.8	F
B4-32	2001-31	2000	Black Mallard R.	2001	Ocqueoc R.	9-May-01	399	142.8	F
B4-33	2001-85	2000	Black Mallard R.	2001	Cheboygan R.	27-May-01	480	231.9	F
B4-34	2001-22	2000	Black Mallard R.	2001	Ocqueoc R.	6-May-01	481	287.8	M
B4-35	2001-48	2000	Black Mallard R.	2001	Cheboygan R.	11-May-01	442	183.4	F
B4-36	2001-15	2000	Black Mallard R.	2001	Tittabawasee R.	3-May-01	478	273.1	M
B4-37	2001-37	2000	Black Mallard R.	2001	Little Thessalon R.	7-May-01	500	261.5	F
B4-38	2001-12	2000	Black Mallard R.	2001	Ocqueoc R.	3-May-01	453	217.9	M
B4-39	2001-89	2000	Black Mallard R.	2001	Cheboygan R.	29-May-01	479	226.9	F
B4-40	2001-49	2000	Black Mallard R.	2001	Cheboygan R.	11-May-01	493	272.5	F
B4-44	2001-55	2000	Black Mallard R.	2001	Cheboygan R.	15-May-01	429	165.1	F
B4-45	2001-87	2000	Black Mallard R.	2001	Cheboygan R.	29-May-01	448	183.1	F

Table 2 continued

BM LARVAE				
I.D.	**Location**	**Year collected**	**Length (mm)**	
A4-1	Black Mallard R.	2000	148.1	
A4-2*	Black Mallard R.	2000	144.6	
A4-3	Black Mallard R.	2000	141.2	
A4-4*	Black Mallard R.	2000	139.3	
A4-5	Black Mallard R.	2000	133.2	
A4-6*	Black Mallard R.	2000	128.0	
A4-7	Black Mallard R.	2000	132.8	
A4-8*	Black Mallard R.	2000	135.5	
A4-9	Black Mallard R.	2000	128.1	
A4-10	Black Mallard R.	2000	143.2	
A4-11	Black Mallard R.	2000	99.8	
A4-12*	Black Mallard R.	2000	128.9	
A4-14*	Black Mallard R.	2000	135.5	
A4-15	Black Mallard R.	2000	140.2	
A4-16*	Black Mallard R.	2000	131.8	
A4-17*	Black Mallard R.	2000	126.6	
			133.6	mean
ST. MARYS "POLLUTED"				
I.D.	**Location**	**Date**	**Length (mm)**	
A2-24*	Hospital Branch, Sta. 11	11-Jul-01	103.2	
A2-25*	Hospital Branch, Sta. 11	11-Jul-01	81.0	
A2-26*	Hospital Branch, Sta. 11	11-Jul-01	82.8	
A2-27	Hospital Branch, Sta. 11	11-Jul-01	85.9	
A2-28	Hospital Branch, Sta. 11	11-Jul-01	73.3	
A2-29	Hospital Branch, Sta. 11	11-Jul-01	77.5	
A2-30*	Hospital Branch, Sta. 11	11-Jul-01	111.1	
A3-33	Hospital Branch, Sta. 11	11-Jul-01	82.5	
A3-34	Hospital Branch, Sta. 11	11-Jul-01	94.5	
			88.0	mean
ST. MARYS "CLEAN"				
I.D.	**Location**	**Date**	**Length (mm)**	
A1-01	Rapids	14-Aug-01	108.0	
A1-02	Rapids	14-Aug-01	108.3	
A1-03*	Rapids	14-Aug-01	104.8	
A1-04*	Rapids	14-Aug-01	98.0	
A1-17	St. Marys GB	14-Aug-01	111.1	
A1-18	St. Marys GB	14-Aug-01	97.9	
A1-22	St. Marys GB	14-Aug-01	99.3	
A1-23	St. Marys GB	14-Aug-01	101.4	
A1-24**	St. Marys GB	14-Aug-01	107.9	
A1-25	St. Marys GB	14-Aug-01	98.9	
			103.6	mean
Misc. Samples				
I.D.	**Location**	**Date**	**Length (mm)**	
R13	Rifle River	1996-1997	161	
R14	Rifle River	1996-1997	130	
R29	Rifle River	1996-1997	115	
P31	Pigeon River	1996-1997	137	
P36	Pigeon River	1996-1997	121	
P49	Pigeon River	1996-1997	136	
P51	Pigeon River	1996-1997	127	
			132.4	Mean

Localities for the collections are shown on Figure 1.

Figure 1 – St. Marys River locations

Figure 2 – Michigan collection sites

Of the 167 heads collected and frozen by Dr. Swink, 91 were sent and of these, nearly half (44) were processed. Eighteen samples were then selected randomly for probe analysis with the expectation that most would be from Black Mallard spawned lampreys and a few would be

from other rivers. Approximately one in 7 of the heads sent were from non-tagged and/or non-black Mallard lampreys, although these were not identified until after the study was completed. Seven preparations from the earlier study (three from the Rifle river and four from the Pigeon River) were re-analyzed as part of the current research.

The two St. Marys collection localities were selected to represent different levels of anthropogenic inputs. Some historical data on elemental composition was available for this and the other rivers and reviewed in the prior study. Unfortunately, spatial and temporal coverage was too incomplete to test correlations between statolith analyses and environmental conditions for the sites. In general terms, metal concentrations in the St Marys River averaged from 1 to several hundred ppm, and levels in the sediment are undoubtedly higher. There was a weak trend for higher concentrations downstream, e.g. for iron.

The statolith preparation procedure used for all samples was as follows:

1. Thawing of frozen ammocoetes and heads 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 and associated membranous labyrinth with stainless steel forceps and teasing away of any adherent tissue (performed in 95% ethanol water followed by a de-ionized water rinse)
4. Air dry and place statoliths in dry gelatin capsule
5. Mount statolith pairs (ventral face down) in Spurr's medium (up to 30 per mount); cure for 24 hr at 70?C;
6. Grind by hand on wet (water) aluminum oxide paper (800 grit); grinding was minimal and removed less than 20 ?m from the ventral face of the statoliths.
7. Polish with 3?m and 1?m diamond paste with brief water rinses.
8. Coat finished preparation by carbon evaporation.

PIXE analyses were run under operating conditions that were modified based on prior experience with statoliths. The instrument used (CSIRO-GEMOC, Heavy Ion Analytical Facility, CSIRO Exploration and Mining, North Ryde, NSW, Australia) is a new generation version proton probe with detection capabilities 4 to 5 times more sensitive than the earlier Si-Li instrument. The conditions for the PIXE analysis were:

1. Up to 6 mounts including 30 samples per mount 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 9 micro coulombs. The time to reach this point is dependent upon the current applied and beam diameter. A 50 ?m rastered square beam was ramped up slowly from less than one to a maximum of 20 NA. Beam diameter was set at a constant for all samples.

3. The analysis package calculates a theoretical sample thickness based on the expected calcium count rate. 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 1.5 to 2 ppm for most elements. A few elements such as barium had MDLs up to 20 ppm. The calculation of MDL was based on three standard deviations, which is a conservative criterion.

5. Uncertainty could be as low as less than 1% for abundant elements (e.g. strontium), but could be as high as 25% for low concentration elements near the detection limits (e.g. lead).

6. For transects, a series of nine or ten samples (spots) was arrayed from the center of the statolith to the edge or margin. Each spot was 10?m square and they were taken at 20?m intervals.

Statistical evaluation for the final data set (60 individuals divided into six localities or groups and based on results for up to ten 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 localities or groups. Differing levels of resolution were tested; e.g. individual stream localities, St. Marys River versus Michigan Rivers. Of particular interest was testing the statistical separation of ammocoetes from the “polluted” St. Marys locality from animals collected in the “clean” upstream locality.

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

3. Establish procedures for classifying individuals into groups (i.e. assign a lamprey from an unknown locality into a probable locality group). In particular, would adult with a known origin (Black Mallard River) be correctly grouped with ammocoetes from the same river?

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 ten elements of interest (Mn, Fe, Ni, Zn, Rb, Sr, Cu, Ba, Hg, and Pb) revealed some distinctive composition patterns when all individuals (N=60) from the six groups were compared (Figures 3A and 3B). Analysis of variance determined that although there were some significant differences in mean element concentrations between the groups (for Mn, Fe,

Zn, Rb, and Sr), complete utilization of all of the data by multi-element analysis via discriminant function would be required to separate groups on the basis of statolith chemistry.

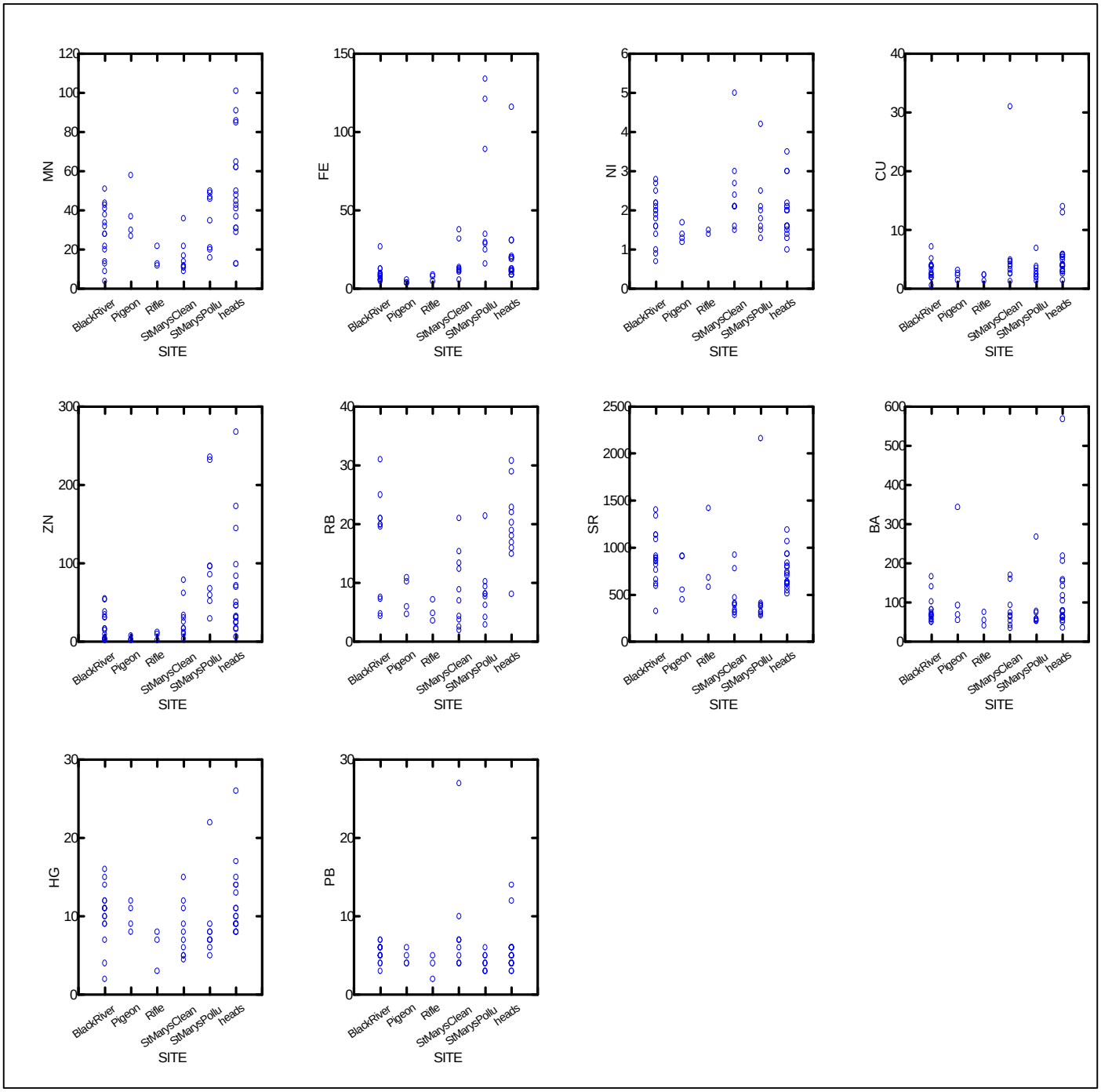


Figure 3A – Individual values of element concentration (ppm) for all specimens used in the LDF analysis.

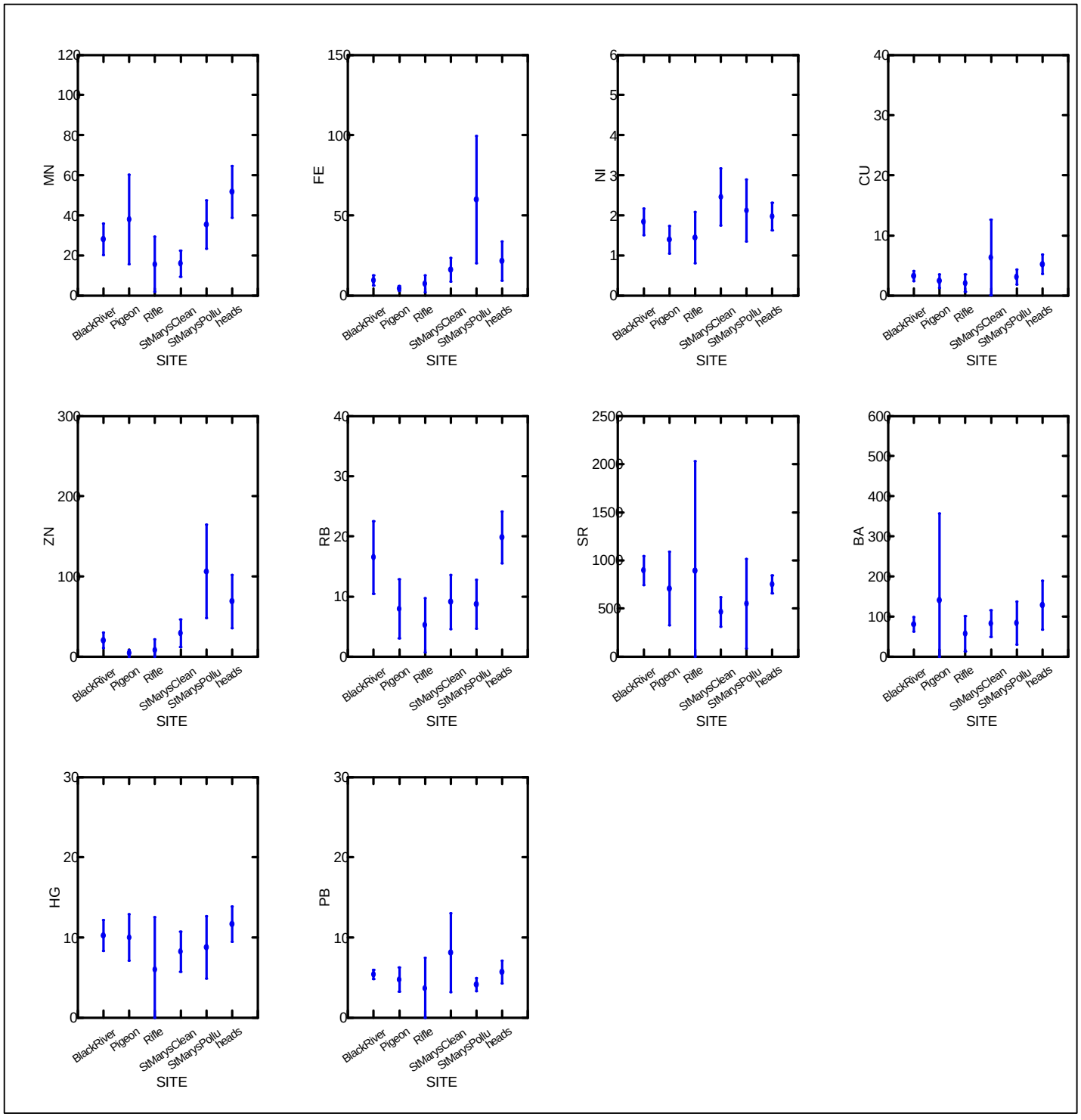


Figure 3B – Means and 95% confidence limits for element concentrations (ppm) for all specimens used in the LDF analysis

Table 2 presents summary statistics for all combined elemental concentrations (ppm). Note that due to either missing values or concentrations below detection limits, a total of only 42 individuals had a complete complement of values for all ten elements. Most of the missing values were for rubidium (12 of 60).

Table 2 – Descriptive statistics for pooled samples

ELEMENT	N	Range	Minimum	Maximum	Mean	Std. Deviation
Mn	57	97	4	101	34.75	22.03
Fe	58	130	4	134	20.91	27.54
Ni	57	4.3	0.7	5	1.98	0.76
Cu	60	30.4	0.6	31	4.21	4.21
Zn	60	266	2	268	47.57	58.02
Rb	48	29	2	31	12.85	8.09
Sr	60	1886	277	2163	715.55	350.22
Ba	59	533	35	568	98.88	85.17
Hg	60	24	2	26	9.89	4.16
Pb	60	25	2	27	5.60	3.45
Valid N (listwise)	42					

A matrix of linear correlation coefficients (Pearson's) indicates only weak associations when elemental pairs (outliers not excluded) are considered on at a time; however several are significant at the .05 level (Table 3).

Table 3 – Correlation matrix for paired element relationships

Matrix of Correlation Coefficients		Mn	Fe	Ni	Cu	Zn	Rb	Sr	Ba	Hg	Pb
Mn	Pearson Correlation	1									
	Sig. (2-tailed)	.									
	N	57									
Fe	Pearson Correlation	**0.303**	1								
	Sig. (2-tailed)	0.022.									
	N	57	58								
Ni	Pearson Correlation	0.180	0.207	1							
	Sig. (2-tailed)	0.193	0.130.								
	N	54	55	57							
Cu	Pearson Correlation	0.220	0.084	**0.627**	1						
	Sig. (2-tailed)	0.099	0.531	0.000.							
	N	57	58	57	60						
Zn	Pearson Correlation	**0.516**	**0.783**	0.098	0.077	1					
	Sig. (2-tailed)	0.000	0.000	0.466	0.559.						
	N	57	58	57	60	60					
Rb	Pearson Correlation	**0.572**	-0.201	0.054	0.132	0.213	1				
	Sig. (2-tailed)	0.000	0.176	0.725	0.373	0.146.					
	N	46	47	45	48	48	48				
Sr	Pearson Correlation	-0.053	**-0.320**	-0.139	-0.131	-0.242	0.119	1			
	Sig. (2-tailed)	0.694	0.014	0.301	0.318	0.063	0.422.				
	N	57	58	57	60	60	48	60			
Ba	Pearson Correlation	0.225	-0.068	0.129	0.144	-0.052	0.113	**0.410**	1		
	Sig. (2-tailed)	0.096	0.617	0.344	0.277	0.697	0.448	0.001.			
	N	56	57	56	59	59	47	59	59		
Hg	Pearson Correlation	**0.432**	-0.026	**0.410**	0.226	0.056	**0.513**	0.114	**0.337**	1	
	Sig. (2-tailed)	0.001	0.849	0.002	0.082	0.672	0.000	0.386	0.009.		
	N	57	58	57	60	60	48	60	59	60	
Pb	Pearson Correlation	-0.032	0.003	**0.542**	**0.818**	-0.089	0.046	-0.077	0.193	0.231	1
	Sig. (2-tailed)	0.811	0.981	0.000	0.000	0.499	0.754	0.557	0.142	0.076.	
	N	57	58	57	60	60	48	60	59	60	60
BOLD	Correlation is significant at the 0.05 level or better (2-tailed).										

LDF analysis was first performed on the full data set (60 individuals; 6 sites/groups). Under these conditions the head samples were considered as an independent "locality." As noted above, complete elemental were available for 42 individuals and this is the number of valid cases in the data set. Wilks lambda values in Table 4 show that Mn, Fe, Zn, Rb, Sr, and Ni are the most important variables to the discriminant function; the same elements displaying some locality specific differences in means (see above).

Table 4 – Correlation matrix for paired element relationships

	Wilks' Lambda	F	df1	df2	Sig.
Mn	.553	5.814	5	36	.000
Fe	.515	6.788	5	36	.000
Ni	.819	1.596	5	36	.186
Cu	.879	.988	5	36	.439
Zn	.517	6.730	5	36	.000
Rb	.636	4.127	5	36	.005
Sr	.567	5.503	5	36	.001
Ba	.897	.827	5	36	.539
Hg	.921	.615	5	36	.689
Pb	.824	1.535	5	36	.203

Of the five calculated discriminant functions, the first two are statistically significant, and the remaining three are incrementally less important. Iron, zinc and strontium values were most highly correlated to function 1; manganese, lead and nickel to function 2; rubidium to function 3; barium to function 4; and mercury and copper to function 5. A plot of the first two functions and the group centroids is shown in Figure 4.

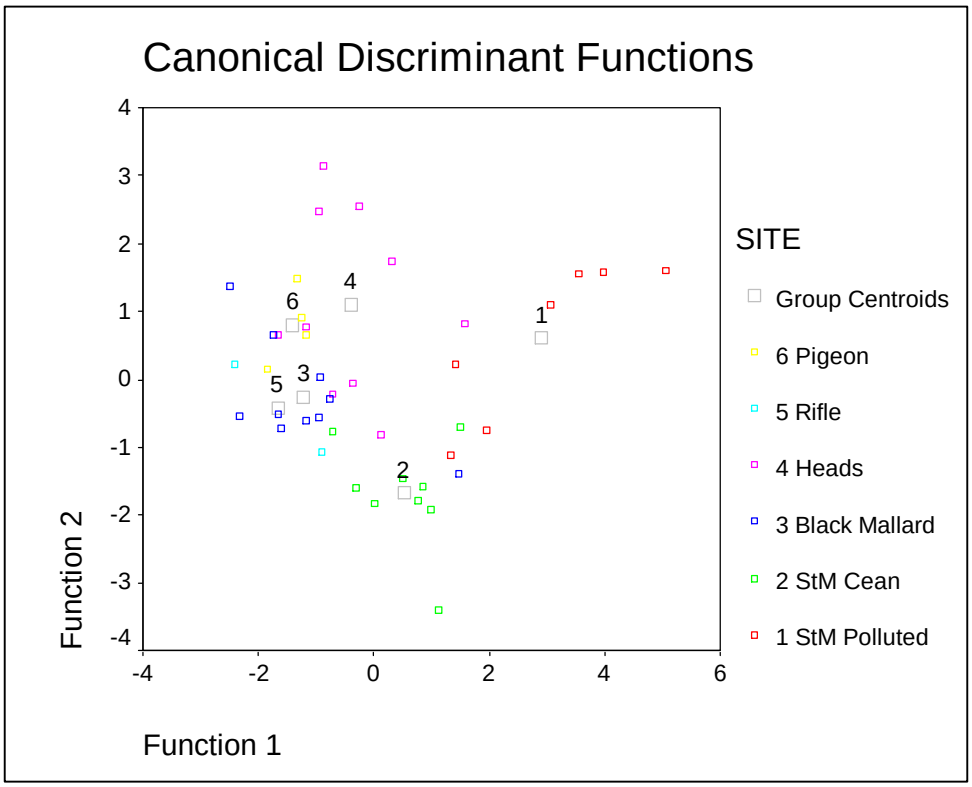


Figure 4 – Discriminant function 2 plotted against function 1

There is good separation of the centroids for the St. Marys River fish (sites 1 and 2), while 3, 4, 5, and 6 are more clustered in the upper left portion of the plot. Another way to visualize the separation and relative importance of all of the functions is seen in the pair wise comparisons of Figure 5. Function 1 best separates the St. Marys "polluted" samples and the addition of function 2 helps to separate all St. Marys fish from Michigan fish. A summary table (Table 5) shows the rates of correct and incorrect classification for this data set. The overall total of correct classification was 71.4%. If the twelve misclassifications are examined on a case-by-case basis, two are within the St. Marys system, seven are within the Michigan rivers and heads, and only three are misclassifications between St. Marys and Michigan samples. Therefore, only 25% of the "errors" in classification (3 out of the 42 fish analyzed) are between major drainage systems.

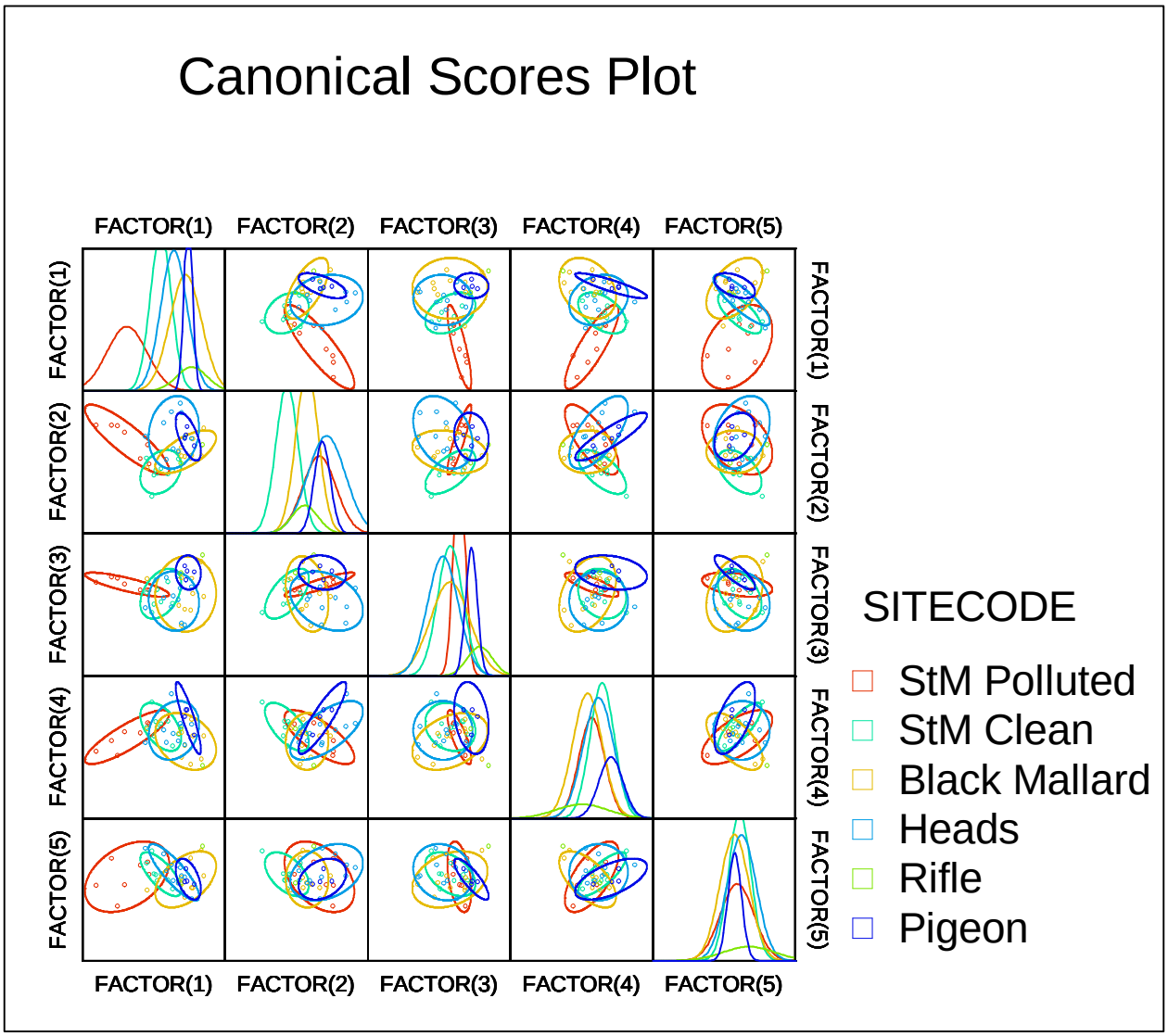


Figure 5 – Pair wise comparisons discriminant function distributions with individual points and bounding ellipses

Table 5 – Summary for LDF using all ten elements and samples divided into 6 groups (as in Fig. 4)

Classification Results

			Predicted Group Membership						Total
		SITE	1	2	3	4	5	6	
Original	Count	1	5	2	0	0	0	0	7
		2	0	7	2	0	0	0	9
		3	0	1	8	0	1	0	10
		4	0	1	3	6	0	0	10
		5	0	0	1	0	1	0	2
		6	0	0	1	0	0	3	4
	%	1	**71.4**	28.6	0	0	0	0	100
		2	0	**77.8**	22.2	0	0	0	100
		3	0	10	**80**	0	10	0	100
		4	0	10	30	**60**	0	0	100
		5	0	0	50	0	**50**	0	100
		6	0	0	25	0	0	**75**	100

71.4% of original grouped cases correctly classified.

The relatively poor separation among sites when all six sites are treated as separate reflects the very large overlap among statoliths from the Rifle, Pigeon, Black Mallard and heads, i.e., fish from the same drainage systems. To test specific hypotheses (i.e., are statoliths from the “polluted” St. Marys different from the “clean St. Marys?; are St. Mary’s fish different from Michigan fish?) a series of LDF analyses were performed on data sets with localities/groups variously combined or excluded; some elements excluded (e.g. rubidium; to increase valid cases); and some with the addition of data from previous studies (Brothers, 1998) where six elements were analyzed with different instrumentation. The last examples are clearly of only limited values since direct comparison of re-analyzed samples from the Pigeon and Rifle Rivers (7 larvae), show some correlation, but a slope significantly different from 1 for all elements except strontium (Fig. 6). Some of the variation in the “old” values for mercury, copper and zinc appears to be highly suspect and due to either contamination, or more likely, instrument limitations.

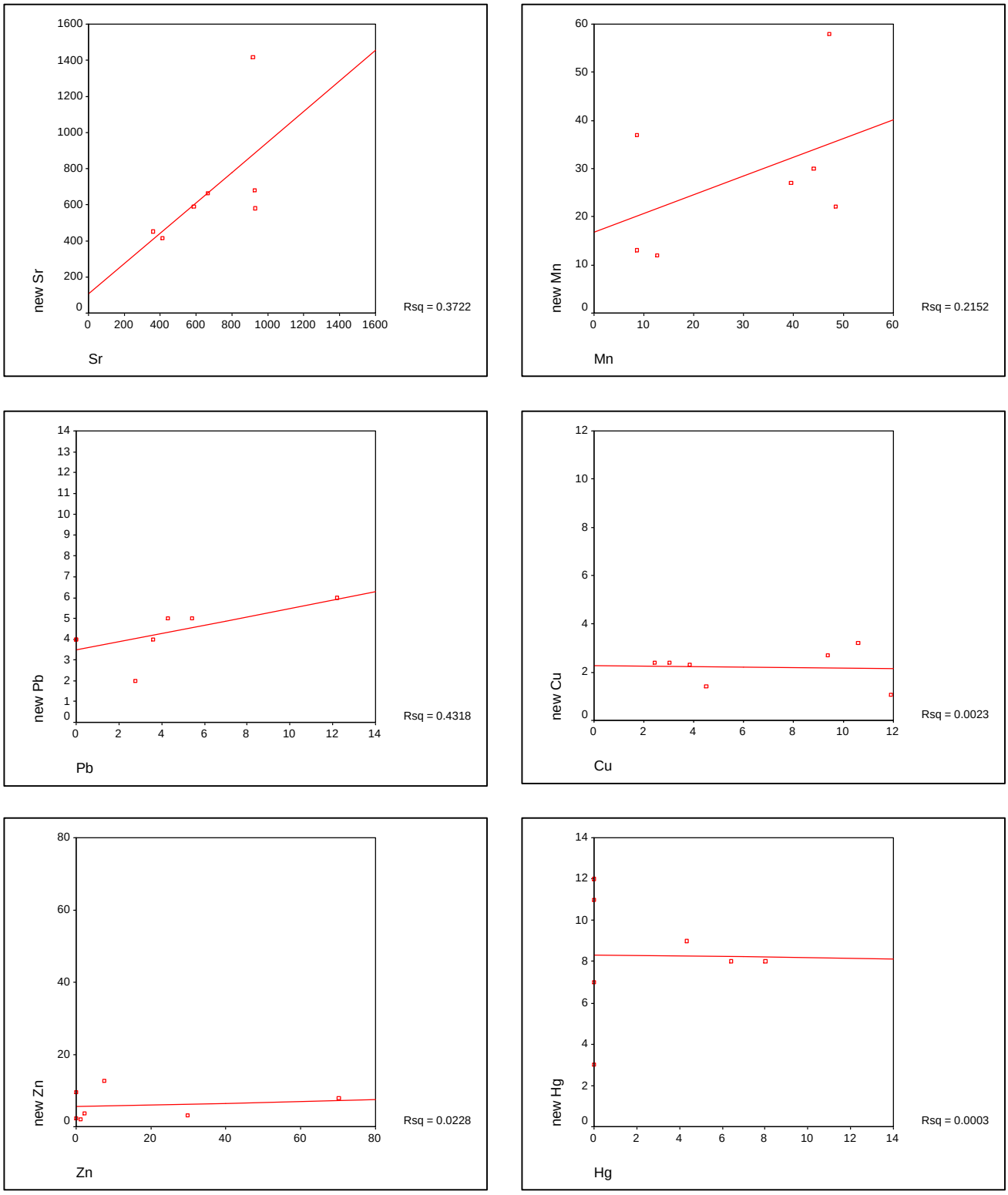


Figure 6 – Plots of 1998 data (x-axis) versus new analyses for the same samples (y-axis). Units are ppm.

The conditions (number of elements, groups, data set etc.) and summarized results (as percent correctly classified) for thirteen different LDF analyses are shown in Table 6. Two values for correct classifications are given:"OCC" and "CVCC". The latter is a based on a highly conservative method in which each case in the analysis is classified by the functions derived from all cases other than that case. After the data collection and initial analyses were concluded, it was learned via information from Dr. Swink that none of the eighteen randomly chosen head samples were from lampreys spawned in rivers other than the Black Mallard. For this reason some subsequent analyses combined Black Mallard ammocoetes and the heads into one group. The following conclusions may be drawn from this table:

1. St. Marys ammocoetes could be distinguished from those from Michigan sources at greater than 90% accuracy (analysis G) (except when the "old" data are included in the analysis, when the success rate drops to 87%; analysis M).

2. Ammocoetes from the "clean" and "polluted" parts of the St. Marys could be separated at greater than 93% accuracy (analysis J)

3. Inclusion of rubidium can significantly improve classification success. It is the most important element in discriminant function 3 (analyses A and B).

4. Inclusion of the heads in the data sets results in even greater percentages of correct classification (analysis G versus I and A versus H). This may be due to the inclusion of several outliers in the head samples.

5. Addition of the "old" data degraded classification success unless the analysis was limited to only the St. Marys versus Michigan rivers (analyses K, L and M).

In conclusion to this section, the analyses have demonstrated the ability to use trace element concentrations to correctly classify or identify ammocoetes from the St. Marys River versus all other localities tested (Michigan, Lower Peninsula). Strontium levels are the most important variable affecting this discrimination. However one St. Marys individual showed an anomalously high strontium value. Secondly, St. Marys upstream ("clean") ammocoetes can be separated from downstream individuals ("polluted") on the basis of inclusion of a second function, which is weighted by iron, and zinc levels. Distinguishing of larvae from stream systems within the Michigan Lower Peninsula was not possible for the relatively small number of samples available. The Black Mallard River is also in fairly close proximity to the Pigeon and they probably share very similar watershed characteristics. Head samples were generally correctly classified as Michigan fish, however some were classified as Rifle River or Pigeon River fish. Results from a few heads were anomalous and distinct outliers and may represent contamination artifacts or instrument error.

Table 6 - Comparison of LDF runs; OCC=percent of original grouped cases correctly classified; CVCC=percent of cross-validated grouped cases correctly classified

Analysis	Sample size	Valid	OCC	CVCC	Elements	Groups	Description
A	60	42	71.40	45.20	10	6	all elements; all groups
B	60	53	56.60	39.60	9	6	exclude Rb; all groups
C	60	42	69.00	54.80	10	5	all elements; heads and Black Mallard as one group
D	60	53	67.90	49.10	9	5	exclude Rb; heads and Black Mallard as one group
E	60	42	76.20	64.30	10	3	all elements; St. Marys, Michigan and heads
F	60	53	77.40	66.00	9	3	exclude Rb; St. Marys, Michigan and heads
G	60	42	90.50	85.70	10	2	all elements; St. Marys versus Michigan (including heads)
H	60	32	84.40	62.50	10	5	all elements; heads excluded from data set
I	60	32	93.80	84.40	10	2	all elements; St. Marys versus Michigan; heads excluded from data set
J	60	16	93.80	62.50	10	2	all elements; only two St. Marys groups
K	133	130	57.70	46.90	6	6	add old data; Rb, Ba, Fe, Ni excluded; all groups
L	133	130	58.50	54.60	6	5	add old data; Rb, Ba, Fe, Ni excluded; heads and Black Mallard as one group
M	133	130	87.60	86.70	6	2	add old data; Rb, Ba, Fe, Ni excluded; St. Marys versus Michigan

Transects or “life history scans” were performed to assess the presence of spatial variability within a statolith. Although the section plane and orientation of transects were not optimal for the purpose, such data may be expected to yield information about temporal variation in deposition rates of different elements. Inspection of the raw data suggested the presence of contamination at the statolith margin and therefore the last point in the transects were not included in further analysis. Data for the two fish analyzed were relatively consistent in that they showed similar patterns of change across the statolith (Fig. 7). Most elements exhibited either a downward trend towards the margin or no significant pattern. The results suggest that it may be important to carefully orient the location of the proton beam to reduce the variability of results. In the present study, the single spectrum acquired for each fish was from a relatively large area (50 ?m square) and always located near the center or origin of the statolith.

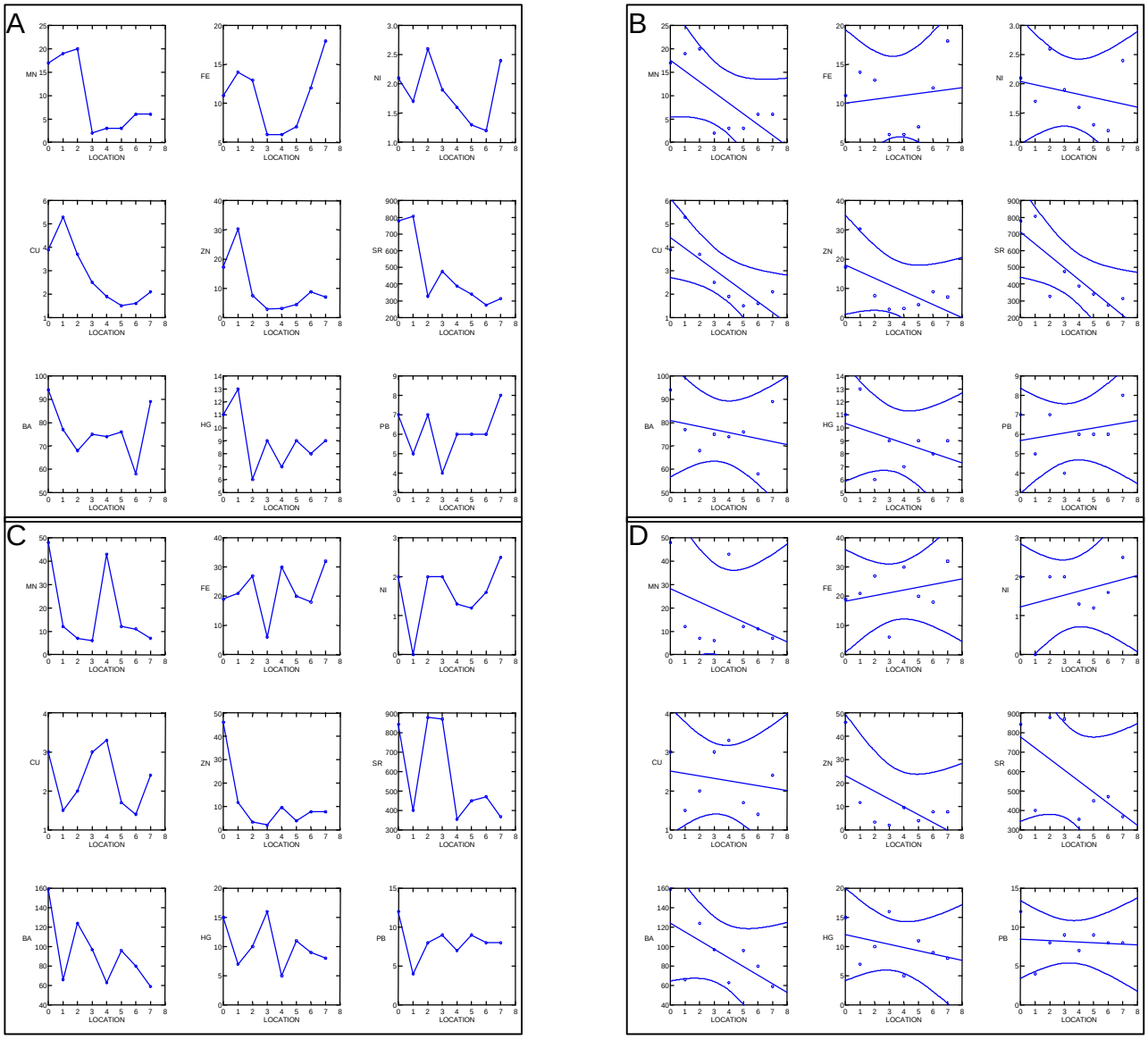
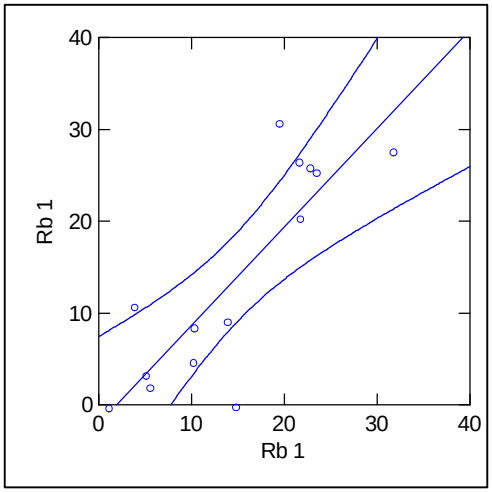
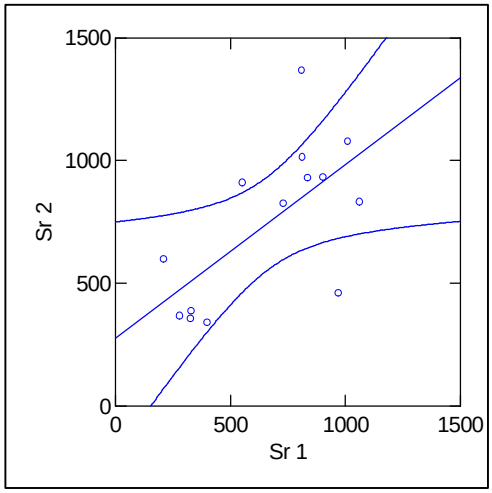
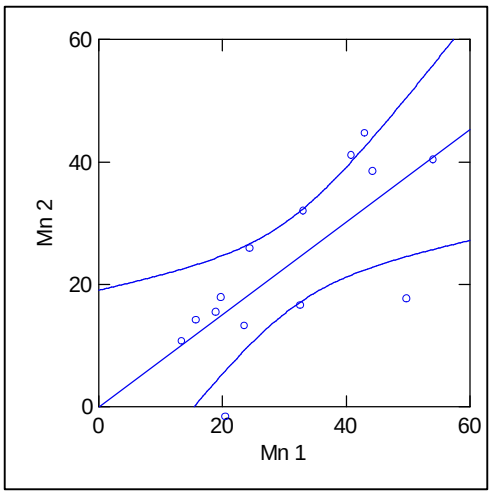


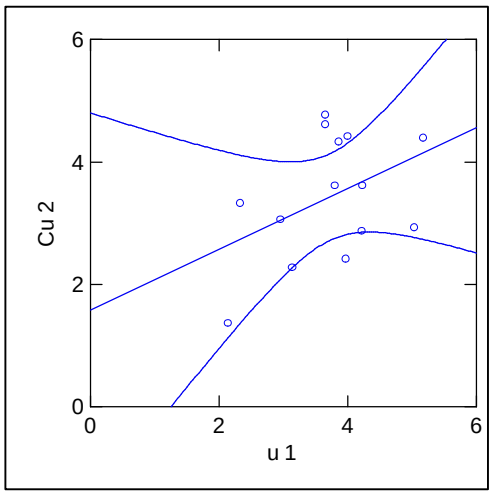
Figure 7 – Element concentration (ppm) versus statolith position ("0" at the center, "8" near the edge) for two lampreys: St. Marys larva A1-24 (A and B) and Black Mallard adult B3-04 (C and D). The graphs on the right show linear regression trends and 95% confidence envelopes.

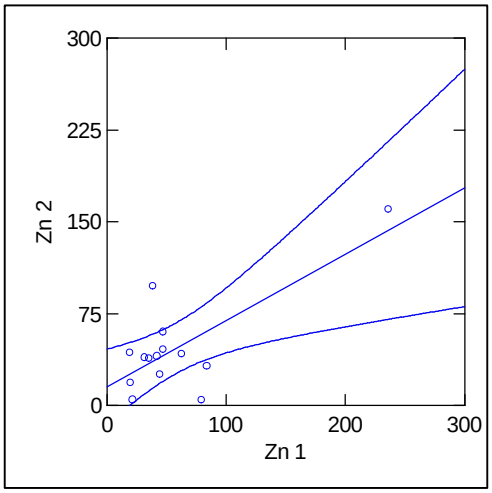
The final supporting study involved the fourteen "left/right" comparisons of data obtained from the two statoliths from a single individual. Summary plots with fitted linear regression lines and 95% confidence limits for the relationship are shown in Figure 8. In general, the values show good consistency within an individual with a slope of 1 (perfect agreement between statoliths) included with the calculated limits. In some cases a poorer fit was heavily influenced by a single outlying value. Manganese, strontium, and rubidium showed the best left/right correlations.

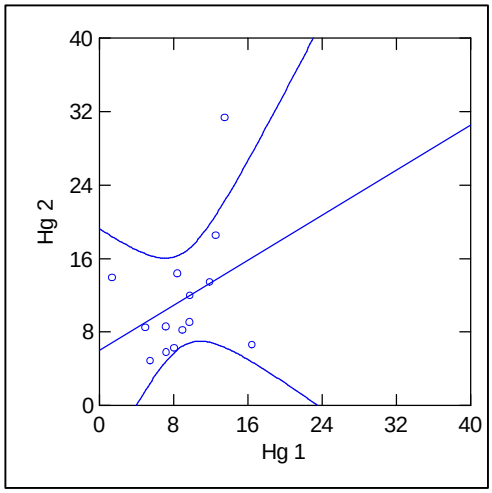

Rb 1
Rb 1
0
10
20
30
40


Sr 2
Sr 1
0
500
1000
1500


Mn 2
Mn 1
0
20
40
60


Cu 2
u 1
0
2
4
6


Zn 2
Zn 1
0
75
150
225
300


Hg 2
Hg 1
0
8
16
24
32
40

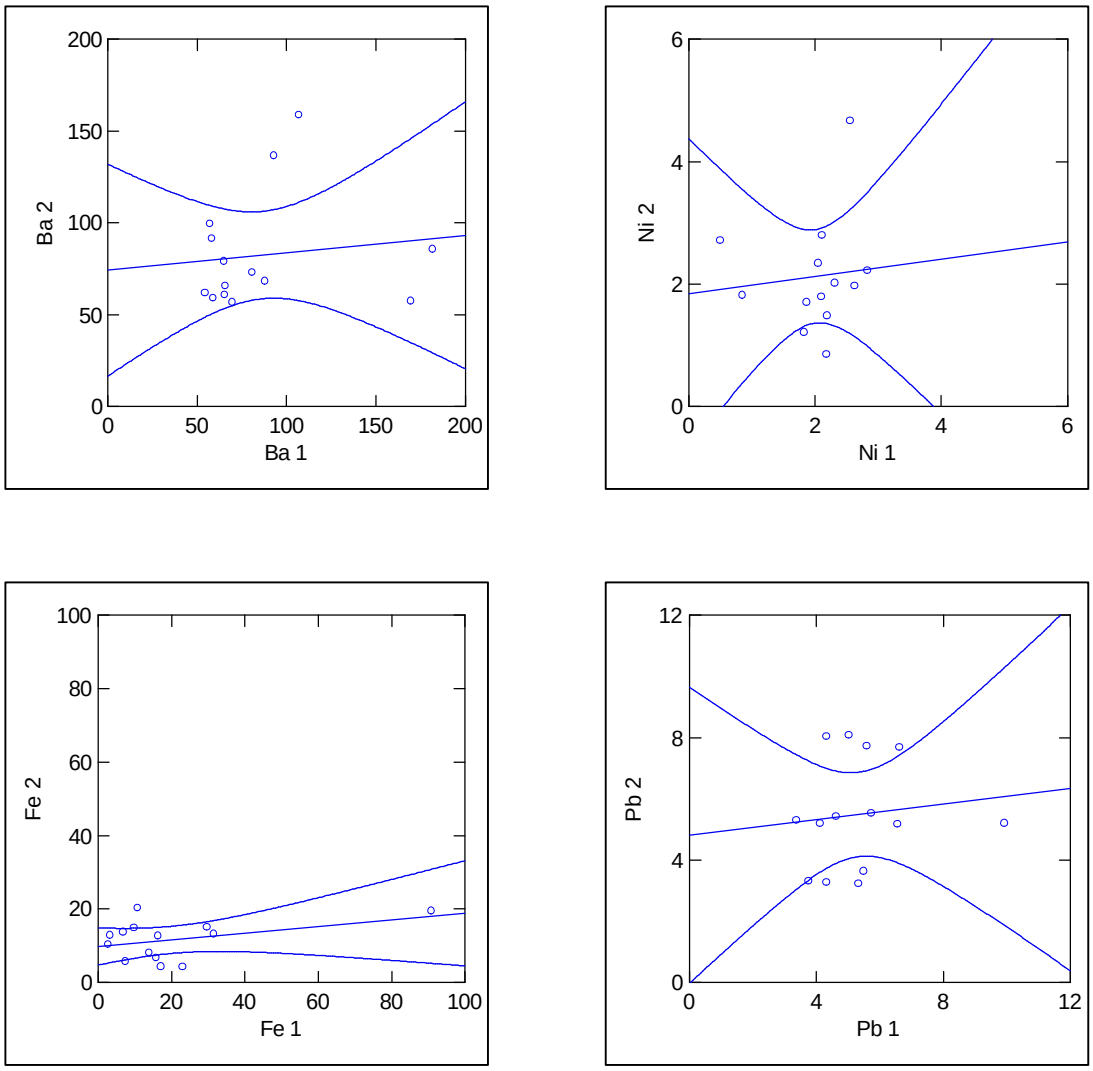


Figure 8 – Element concentrations (ppm) for left/right statolith pairs for 14 individual lampreys. The plotted line is the linear regression estimate surrounded by the 95% confidence envelope. Perfect agreement between both statoliths would produce a diagonal line with a slope of 1 (from the origin to the upper right corner).

DISCUSSION AND SUMMARY

Application of improved handling and preparation methods, instrumentation, and operating conditions during spectrum acquisition has resulted in more robust analyses with improved sensitivity and the inclusion of four elements (Ba, Rb, Cu, and Fe) added to the six used in the earlier studies (Sr, Mn, Zn, Pb, Hg, and Ni). Consistency of the PIXE probe data was tested by comparison of the two statoliths from a singe individual (14 cases). Repeatable measurements were obtained for most elements; especially those found to be most important in the LDF analysis. Elements showing more scatter or randomness to the relationship had outliers and concentrations close to the detection limits that undoubtedly had a negative effect on the outcome. Spatial homogeneity within a single statolith was also tested by multiple sample

points along a transect from the statolith section center to the margin. For the two individuals tested in this manner, some common patterns of distribution were weakly suggested; often a decline in concentration towards the otolith margin. However, scatter along the path was substantial and it is unknown whether it is real or due to measurement artifacts. Overall, there was sufficient evidence for the reliability and precision of the concentration measurements to justify their use in LDF analysis for the six collection groups represented in the investigation. No direct check of accuracy, i.e. an independent test of element concentration was attempted.

A variety of LDF analyses including different data sets (element lists, group combinations and exclusions, and incorporation of data from prior studies) were performed to determine whether samples could be correctly assigned to their stream origin. A second question asked whether adults maintain their larval "signature." The results demonstrated that St. Marys River larvae could be distinguished from Michigan Upper Peninsula drainage samples with a high degree of certainty. Furthermore, upstream ("polluted") St. Marys River larvae could be distinguished from downstream ("clean") larvae.

The key element that distinguished St. Marys fish from those collected in Michigan streams was strontium, which was generally higher in the Michigan fish. This difference is also evident in the "old" data (Brothers, 1998), suggesting that it is consistent and a robust difference between the drainages. Strontium concentrations differ widely among Great Lake drainages, reflecting a diverse geological history, and it is likely the low values for statoliths collected in the St. Marys reflect overall lower Sr concentrations in Lake Superior and its drainages than in the "lower" lake drainages. Irrespective of the underlying factor, Sr differences are easily measured using most analytical techniques, and are among the least affected by specimen handling, which suggest that the differences between the two sites could be easily used to discriminate among fish from each. The elements that separate other sites are also consistent with broad expectations. The elements most influential for the separation of upstream and downstream St. Marys River larvae ("clean" versus "polluted") were those having likely elevated anthropogenic origins (e.g. iron, manganese, lead, and nickel). Rubidium also appears to be a potentially important element in site separations, which is consistent with the high but variable presence of this element in the Great lakes region; Michigan is one of only two states in the US where rubidium is mined commercially.

Unfortunately, random selection of adult heads for analysis inadvertently excluded any individuals known to have a spawning origin from a river other than the Black Mallard. In general, the head data resulted in assignment to Michigan rivers; 90% probability. A finer discrimination was not successful due to the fact that the statolith compositions from the Michigan rivers were relatively similar to one another and there were insufficient sample sizes for other rivers (Rifle and Pigeon). Some skewing of element concentrations and a few notable outliers were seen amongst the heads. The cause of this observation is unknown, however the difference handling necessitated by the large size of the heads may be an additional source of variation. Several papers (Proctor and Thresher, 1998; Milton and Chenery, 1998) have pointed out the importance of handling to otolith trace element analysis and statoliths are markedly smaller in dimension and mass than all otoliths used in these studies.

This study has clearly demonstrated the usefulness of elemental data to identifying river origins for lampreys and suggests it is a potentially powerful tool for distinguishing adults of St.

Marys origin from those derived from Michigan drainages. With regards to the three hypotheses outlined in the introduction, the following may be concluded:

1. Statoliths of downstream St Marys River ammocoetes display elevated metal concentrations consistent with presumed elevated concentrations in river sediments impacted by anthropogenic impacts.

2. Adults and larvae from the same stream origin (Black Mallard River) are relatively consistent in the concentrations of trace metal elements and are classified together when compared to all St. Marys samples. Weak spatial/temporal gradients may also be present in statolith element concentrations even within the larval life. Lampreys from the Black Mallard were not consistently discriminated from samples obtained in other Michigan Upper Peninsula streams. Minor differentiation of larval and adult statolith chemistry remains unexplained and may be attributed to contamination during processing, however this requires further investigation.

3. Some stream environments in Lake Huron drainages have been demonstrated to be sufficiently different to impact statolith chemistry and produce diagnostic combinations of elements to identify larval origins.

Increased geographic resolution may be possible using isotopic information (Kennedy *et al*., 1997; Weber, 2002; Weber *et al*., 2002), but whether the techniques used for isotopes can be applied to specimens as small as lamprey statoliths is not clear.

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