Using Hard-Part Microchemistry to Advance Conservation and Management of North American Freshwater Fishes

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ABSTRACT: *Hard-part microchemistry offers a powerful tool for inferring the environmental history and stock assignment of individual fishes. However, despite the applicability of this technique to a wide range of fisheries conservation and management issues, its use has been restricted to only a small fraction of North American species and inland waters. In this article, we provide freshwater fisheries professionals with an accessible review of methods and applications of hard-part microchemistry techniques. Our objectives are to (1) summarize the science of hard-part microchemistry; (2) provide guidelines for designing hard-part microchemistry studies, including sample sizes, laboratory analyses, statistical techniques, and inferential limitations; and (3) identify conservation and management applications where these techniques may be particularly useful. We argue that strategic use of hard-part microchemistry methods (specifically when they are used in concert with other indirect tracer techniques such as stable isotope chemistry and genetics) can advance fish management and conservation across all stages of fish life history.*

Uso de la microquímica de partes duras en pro de la conservación y manejo de peces dulceacuícolas de Norte América

RESUMEN: *la microquímica de partes duras representa una herramienta poderosa para inferir la historia ambiental y la asignación de stocks en peces. No obstante, a pesar de la aplicabilidad de esta técnica a un amplio rango de aspectos de conservación y manejo de pesquerías, su utilización se ha restringido a sólo una pequeña fracción de especies de aguas continentales en Norte América. En este trabajo, se presenta una revisión accesible de los métodos y aplicaciones de técnicas de microquímica de partes duras, dirigida a los profesionales en pesquerías de aguas interiores. Los objetivos son: (1) resumir la ciencia de la microquímica de partes duras, (2) proporcionar guías para el diseño de estudios de microquímica de partes duras, incluyendo tamaños de muestra, análisis de laboratorio, técnicas estadísticas y limitaciones de orden inferencial, y (3) identificar aplicaciones para la conservación y manejo en las cuales estas técnicas puedan ser particularmente útiles. Se argumenta que la utilización estratégica de métodos de microquímica de partes duras (específicamente cuando éstos son usados en conjunto con otras técnicas de rastreo indirecto como química de isótopos estables y genética) puede abonar a la conservación y manejo de los peces durante todos los estadios de vida.*

INTRODUCTION

Successful conservation and management of fishery resources requires understanding fish population structure and dynamics. Fisheries research in rivers and lakes often relies on telemetry and mark–recapture studies to assess individual movements and population processes within and between ecosystems (Pollock et al. 2004). They also rely on population genetics to reconstruct patterns of exchange and isolation among fish populations (Palumbi 2003). More recently, chemical tracers have proven useful for tracking individual fish across longer time periods, thereby complementing other direct and indirect methods (Cunjak et al. 2005; Cooke et al. 2013; Figure 1). This review focuses on hard-part microchemistry and stable isotope techniques; that is, techniques that rely on fine-scale but discrete changes in hard-part (i.e., otolith, statolith, scale, and fin ray) elemental signatures that reflect changes in ambient water chemistry. These techniques have the ability to help fisheries biologists gain insight into lifelong patterns of fish movement and population linkages across chemically heterogeneous landscapes.

Microchemistry analyses of hard parts have proven useful in marine, diadromous, and freshwater fishes, yet in North American freshwaters, they have been applied in relatively few freshwater ecoregions to relatively few taxa (Box 1). These techniques have been used extensively in the Great Lakes, Upper Mississippi, and Colorado freshwater ecoregions but sparsely elsewhere, including the ecoregions with the highest fish richness (i.e., Teays-Old Ohio, Tennessee, and Lower Mississippi ecoregions; Figure 2c), where no studies on wild-produced fish have been conducted. Of the 53 microchemistry studies on North American freshwater fish we surveyed, 31 (60%) have focused on species in the family Percidae (exclusively Yellow Perch *Perca flavescens* and *Sander* spp.) and the family Salmonidae: a small fraction of North American species (Appendix A, Supplemental Material; Figure 2).
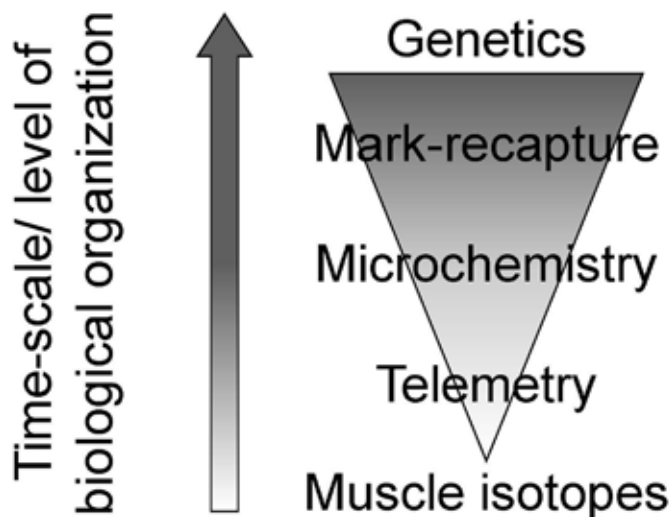
Figure 1. Depiction of techniques for inferring fish life history on population and individual bases ranging from genetics (can infer population-level metrics such as natal homing and stock structure or individual metrics like paternity or genotype) to muscle stable isotope chemistry (infers individual habitat use for last weeks to months). Genetics, mark–recapture, and telemetry techniques all allow for individually based and population-based inferences to be drawn, but inferential power can be maximized by combining multiple techniques because each technique can provide different scales of resolution. For example, though genetics can be useful for inferring natal stock, it provides no insight into individual movements, insight that can be gained through mark–recapture, telemetry, and microchemistry. Investigating a population using multiple techniques in combination can thus increase inferential power.

Our experiences at state and regional fisheries meetings suggest that many freshwater fisheries biologists remain unfamiliar with microchemistry techniques, despite their widespread use in other aquatic systems. This unfamiliarity may stem from lack of access to information; studies utilizing hard-part microchemistry are generally published in specialized journals not commonly subscribed to by government agencies and small academic institutions. In response to this, we have constructed this article with the goal of providing an accessible summary of the methodological details, potential applications, and limitations of hard-part microchemistry approaches for fisheries scientists to facilitate their understanding and broader application in North American freshwaters. Specifically, our objectives in this article are to (1) provide scientific background detailing how and where microchemistry techniques have worked; (2) provide guidelines for designing and implementing hard-part microchemistry studies, including necessary sample sizes, laboratory considerations, data analysis, and key technical caveats; and (3) identify conservation and management applications where these techniques may be particularly useful.

SCIENTIFIC BACKGROUND

Hard-part microchemistry techniques involve a transfer of the elemental chemistry of water to calcified fish structures where they are retained for the life of the fish. The success of these techniques as a tracer of environmental history depends on two key factors: (1) habitats must differ consistently in water chemistry signatures—a function of geologic availability or an-

thropogenic inputs—and (2) these differences correlate to and are preserved in the calcium (Ca)-based molecular matrix of fish hard parts, including otoliths, statoliths, fin rays, and scales (Campana 1999). Fish hard parts typically used for age and growth studies have two qualities that enable them to be used for chemical analysis of environmental history: (1) they grow and lay down new material throughout the fish's life and (2) the material they are made of is not readily resorbed by metabolism of the animal (Campana 1999). If these requirements are met, elemental signatures can be linked to age and growth benchmarks on the hard structure and interpreted with respect to age or life history stages (Figure 3; Box 2). In this way, elemental signatures can be used as natural tags for reconstructing habitat use, migrations, and other fundamental aspects of fish life history that are beyond the spatial or temporal reach of most other approaches (Kalish 1989).

Hard-part microchemistry often focuses on elements that are found in minute amounts in the Ca-based matrix of the hard part to infer environmental history, but the relationship between the elemental abundance of water and that of hard parts is complex (Campana 1999) and genetically determined (Sollner et al. 2003; Limburg and Elfman 2010). For example, Ca is the major constituent by weight in the Ca carbonate ($CaCO_3$) matrix of hard parts, even though the relative weight of Ca in hard parts does not reflect the natural abundance of Ca relative to other elements in water. In fact, all other elements found in hard parts are present at very low concentrations (<1%; Campana 1999). The precise mechanisms by which freshwater fish take up elements from water are complex. In brief, elements are taken up from water as they pass over gills (as opposed to through intestines in marine fish that constantly drink water; Olsson et al. 1998), where they enter blood plasma. In otoliths, elements then cross into the endolymph fluid that surrounds the otolith where crystallization into otolith material occurs (Campana 1999).

STUDY CONSIDERATIONS

Selecting an Appropriate Study Area

For microchemistry techniques to be useful, it is critical that water chemistry differs at the spatial and temporal scale of interest. Given the fundamental role of water chemistry, it is wise to analyze water samples prior to sampling fish even when the spatial scale is large. Diverse geological makeup, dominant hydrological flow paths (e.g., groundwater versus runoff), habitat (floodplain versus channel in rivers), and water residence time can all contribute to differences in water chemistry. Similarly, anthropogenic signals such as lead (Pb) from mining (Friedrich and Halden 2008, 2010) or nitrogen-stable isotopes (^{15}N) from land-cover differences (Vandermyde and Whitledge 2008) can be present in ambient water chemistry and may serve as a marker in fish hard parts. Water samples for metals (collected by submerging 500-mL polyethylene bottles and filtering water into new 500-mL polyethylene bottles containing 3 mL of trace metal–grade nitric acid in water; see Eaton and Franson 2005) can be sent to a laboratory and characterized using mass

Box 1. Geographic distribution of hard-part microchemistry studies relative to distribution of fish biodiversity.

Microchemical analyses may hold great promise in areas of high fish biodiversity. For example, the geology, soils, land use, and water sources that determine trace element concentrations in freshwaters are often heterogeneous in understudied North American ecoregions (particularly the Teays–Old Ohio), as indicated by designations of MLRAs (Figure 2c). Habitat needs throughout the life history of endemic fishes of special concern in these regions are poorly understood (Warren et al. 2000), and hard-part microchemistry methods could be especially useful for small-bodied fish such as darters and cyprinids that are too small for most mark–recapture and telemetry methods for determining movements. One important note here is that ecoregions where studies are absent may indicate that studies have been attempted but water chemistry differences did not exist at a spatial or temporal scale to answer the question of interest. Because studies with inconclusive findings are generally not published, it is of primary importance that researchers verify that water chemistry differences exist at the spatial scale of interest exist prior to undertaking a hard-part microchemistry study.
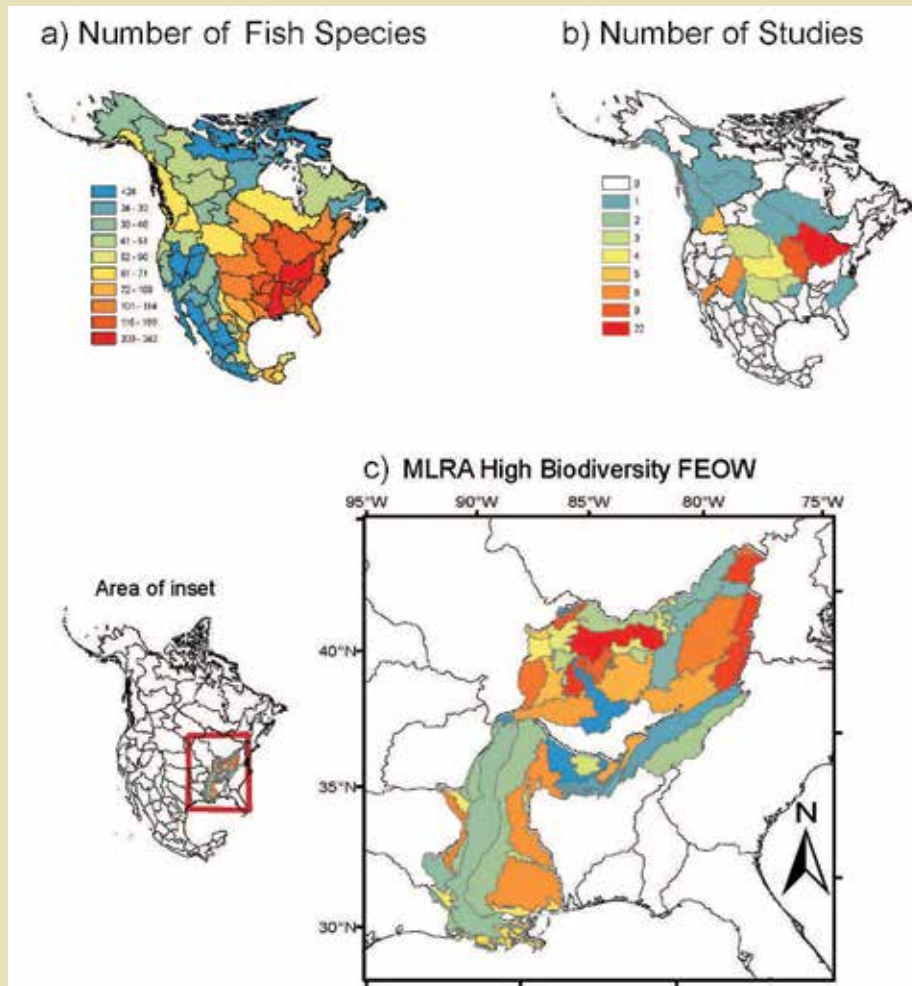

Figure 2. Maps of the freshwater ecoregions of North America (Abell et al. 2008) showing the per ecoregion (a) total number of freshwater fish species, (b) number of studies listed in Appendix A using hard-part microchemistry, and (c) Natural Resource Conservation Service major land resource areas (MLRA) in freshwater ecoregions of the world (FEOW) of highest freshwater fish richness where different colors within each ecoregion represent distinct MLRAs. The MLRA spatial data available from NRCS, http://soils.usda.gov/survey/geography/mlra.

spectrometry for ~$60 per sample. This is a practice that we recommend as pilot work to establish whether spatial differences in trace element concentrations exist in the focal area. An initial impression of potential water chemistry differences can be gained from examining basic geologic maps (Figure 4a) or the Natural Resource Conservation Service's Major Land use Resource Areas (MLRA; Figure 2c); areas grouped together based on characteristics such as geology and land use that may give an initial indication of water chemistry differences prior to running water analyses. Spatial heterogeneity of geological bedrock types may also be a good indicator of water chemistry differences (Newton et al. 1987). However, if spatial heterogeneity is too high or too low (i.e., over very small or very large areas) it may be difficult to determine movement of fishes

among different habitats based solely on hard-part microchemistry (Figure 2c, Figure 4; see also Munro 2004; Dufour et al. 2005; Pangle et al. 2010; Oele 2013).

Characterization of hard-part microchemistry of resident (nonmigratory) fishes provides a convenient reference point for interpreting microchemical differences within or between more mobile species. For instance, to determine whether strontium (Sr) could be a useful tracer of migratory fish movements in the Wisconsin and Mississippi rivers, we mapped Sr concentrations from these systems reported in Garbarino et al. (1995) and compared them with otolith Sr:Ca ratios between resident Smallmouth Bass (*Micropterus dolomieu*), a species that has been widely shown as nonmigratory (Lyons 2011), from each
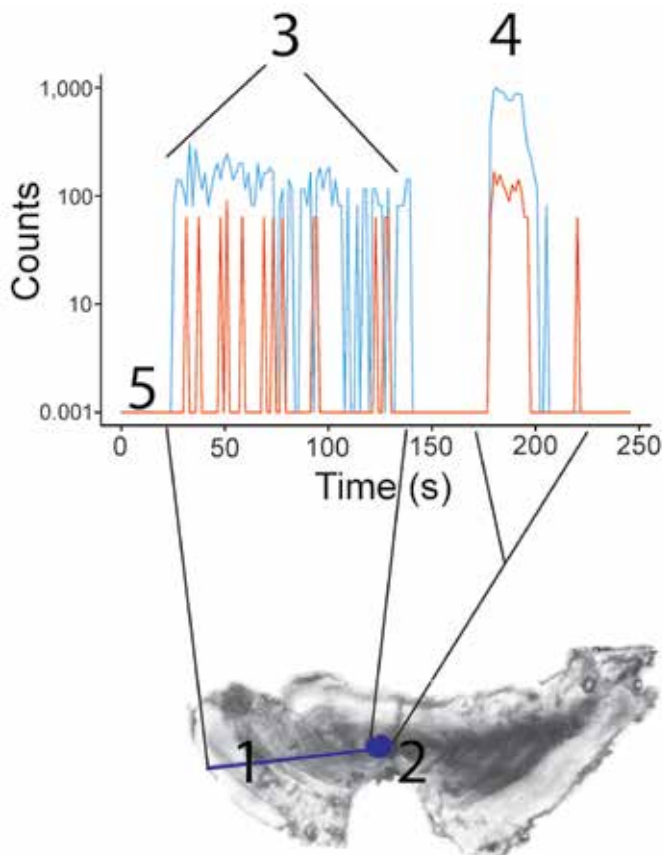
Figure 3. Western Sand Darter (Ammocrypta clara) otolith showing (1) laser transect on otolith, (2) laser spot on otolith, (3) two element transect output, (4) two element laser spot output, and (5) two element background concentrations. This output graph depicts uncorrected, raw count per second data (i.e., number of times the spectrometer encounters each element per second) from the LA-ICP-MS on the y-axis and the number of seconds since data recording began on the x-axis. In order to quantify the background concentrations of elements in the inert carrier gas and in the spectrometer chamber, the ICP-MS is allowed to collect data for ~30 s before the LA system is turned on. As a result, raw counts from the first ~30 s of the output are relatively low and sharply increase when the laser is turned on.
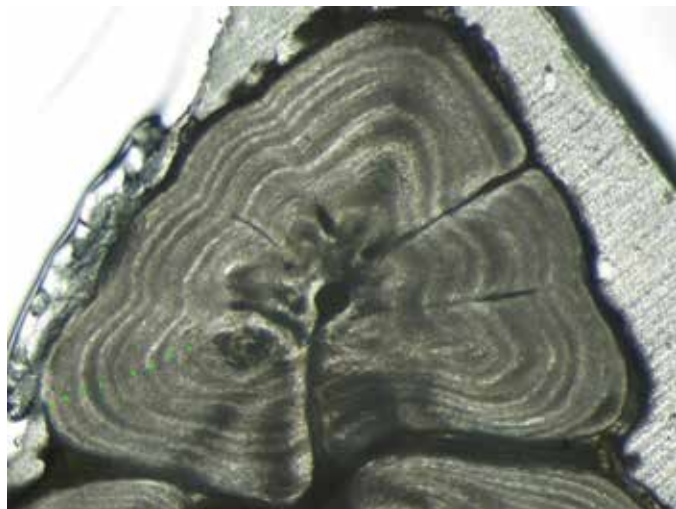
Shovelnose Sturgeon (*Scaphirhynchus platorunchus*) pectoral fin ray. Photo credit: Dan Walchak and Paul Kanehl, Wisconsin Department of Natural Resources.

river. Even with modest sample sizes (N = 18 Mississippi River, N = 20 Wisconsin River), these two rivers proved to have distinct Sr signatures (Figure 4). These differences set the stage for interpreting ontogenetic changes in Sr:Ca of migratory fish species in each river.

Choice of Instruments and Elements to Be Analyzed

Among the most important study considerations is deciding what elements will be analyzed because the target elements dictate laboratory needs and preparation methods (see Campana et al. 1997). The question being addressed and the geochemical setting for a study should guide these choices. To some degree, the elements selected for quantification may depend on proximity, availability, and cost of instrumentation (which routinely exceeds $1,000 per day). Ideally, consideration should be given to likely predictors of water chemistry differences that may be caused by differences in geology or hydrologic connectivity.

Looking to literature on marine and diadromous fishes for guidance on element selection may not be especially useful due to large chemical differences between fresh- and saltwater and differences in metabolic functions of fish between systems. For example, Sr has been central to the work on diadromous fishes because saltwater has much higher Sr concentrations than freshwater, leading to a steep increase in hard-part Sr concentrations when a fish transitions between freshwater and saltwater. In freshwater, Sr is also commonly informative because, like barium (Ba), it replaces Ca in the $CaCO_3$ matrix, is stable over time (Hedges et al. 2004), and also has high spatial variability relative to other elements. On the other hand, manganese (Mn) proved useful in 96% of the studies we surveyed involving marine fishes and 100% involving diadromous fishes but has been much less useful in freshwater systems (Figure 5). The reduced utility of Mn in freshwater systems may be metabolic in nature; Gibson-Reinemer et al. (2009) found no relation between concentrations of water and otolith Mn in Rainbow Trout (*Oncorhynchus mykiss*) in addition to no relation between zinc (Zn), water, and otolith concentrations. Furthermore, the biogeochemistry of Mn in fishes is complex and appears to change with fish growth (Limburg et al. in press); we therefore recommend that caution be used if hard-part Mn is quantified.

Techniques exist to quantify a wide variety of elements and their isotopes. Elements should be ideally selected based on three criteria: (1) the element is incorporated into the hard parts in proportion to its concentration in the water (tested by fitting a regression line between water samples and fish collected at the same site; see Gibson-Reinemer et al. 2009; or through laboratory exposure of fish to varied elemental concentrations; see Collingsworth et al. 2010; Phelps et al. 2012); (2) elements should show spatial variability but temporal stability (at least over the spatial and temporal scales of the study); and (3) isotopes should have high relative abundance within the hard part or the portion of the hard part corresponding to the life history stage of interest such that concentration of these isotopes are routinely above the detection limits of the instrument used. Ele-

Box 2. Hard-part microchemistry analysis using laser ablation–inductively coupled plasma–mass spectrometry.

LA-ICP-MS is the most commonly used and preferred method of microchemistry quantification where possible (Ludsin et al. 2006). This technique is versatile because it can quantify a wide variety of elements, particularly heavy metals, including Ba and Sr, commonly used in movement studies (Appendix A). The LA-ICP-MS uses a laser to ablate a portion of the fish hard part (either a transect [Figure 3 part 1] or spot [Figure 3 part 2]). The ablated material is then carried via argon (Ar) or other inert carrier gas to a plasma torch inside the machine that ionizes the sample. The atomic mass of each ion is then recorded by a mass spectrometer based on mass to charge ratios and is quantified as the counts of each element recorded by the ICP-MS each second (counts per second; Figures 3 parts 3 and 4). These chemical signatures can then be linked to temporal landmarks on the hard structure (e.g., annuli). Other ICP-MS-based techniques also exist for quantifying heavy metals, the most commonly used of which is solution-based ICP-MS (SO-ICP-MS). This technique relies on the dissolution of the entire otolith in acid and is generally used when larval and young of year fishes are of interest (Ludsin et al. 2006; but see Schaffler and Winkelman [2008] for use on juvenile fish).

Determining whether to sample a spot or transect will depend on research objectives. Studies determining natal origin or homing or stock assignment, for instance, may use the laser to sample spots (Figures 3.2, 4) because these studies are interested in discrete points of life, generally the core (natal signature) and edge (recent signature). On the other hand, using the laser to sample a transect (Figures 3.1, 3.3) to give a more continuous depiction of environmental history may be better suited for studies of larval dispersal and migration history. We recommend the use of transect-based data collection for studies inferring movement from LA-ICP-MS data.

Transect data can present unique problems for analysis because calculations must be performed to interpret the data with respect to physical landmarks on the hard structure such as annuli. If the research objective requires understanding chemical signatures at specific ages, for example, annuli must first be enumerated and identified on the hard structure using standard age and growth techniques (Quist et al. 2012). However, before identifying chemical signatures by fish age, a link must be created between the time (in seconds since laser ablation was started) that a chemical signature was recorded by the mass spectrometer and the placement of each annulus on a hard part. We use this equation, which is based on the Fraser-Lee back-calculated length-at-age method (Quist et al. 2012):

$$T_{Li} = \left[\frac{A_i * T_{ttl}}{L_{ttl}} \right] + T_c, \qquad (1)$$

where T_{Li} is the time in seconds (from LA-ICP-MS output) that laser transect crosses annulus i, A_i is the length of the hard structure from core to annulus i, L_{ttl} is the total length of the laser transect on the fin ray, T_{ttl} is the total time (s) elapsed over the laser transect, and T_c is the time the laser crossed the hard-part core.

Prior to data analysis, quantification of hard-part microchemistry requires postprocessing: calibration of data to known standard reference materials, correction for instrument drift, and subtraction of background concentrations of elements. These data processing tasks can be time-consuming, particularly when many elements are measured along a long sample transect, as is often the case in LA-ICP-MS studies. Postprocessing of LA-ICP-MS can be accomplished using free graphical user interface software like the Analysis Management System (Mutchler et al. 2008; www.geochem.geos.vt.edu/fluids/laicpms/ams.shtml) or the free download Fathom Toolbox for Matlab (Jones 2001; available at: www.marine.usf.edu/user/djones/matlab/matlab.html, although Matlab is not freeware). These software packages, as well as some proprietary alternatives, reduce the time required for the postprocessing step by accomplishing background and machine-drift correction of data, conversion of raw elemental counts into parts per million concentrations, and integration of elemental signals over a specified time period that corresponds to an age or life history stage into one step. These software packages require designation of an internal standard—that is, an element that is relatively invariant in concentration throughout the hard part—to quantify the relative abundance of the other elements. Generally, Ca is used as the internal standard due to its high concentration in hard parts relative to other elements. Software will require specification of concentrations of the internal standards either in parts per million or by weight. For these inputs, Ca makes up approximately 38% of the weight of the otolith weight (Campana 1999) and 23% of the weight of fin rays and scales (Clarke et al. 2007).

ments used in studies of freshwater fish that have been shown to display these traits include (but are not limited to) Sr, Ba, deuterium (^{2}H—a stable isotope of hydrogen), ^{18}O (stable isotope of oxygen), and ^{15}N (but not for small fish; see Vandermyde and Whitledge 2008), although it is not commonly used (Figure 5). Other elements may be locally useful, such as magnesium (Mg), which has been used in a number of studies of freshwa-

ter fish (Figure 5), but researchers should ensure relationships between water and hard-part elemental signatures prior to their use because some elements may also be under physiological control. Utility of elements may also depend on the hard part examined. For example, commonly used trace metals such as Ba and pollutant heavy metals such as mercury (Hg), copper (Cu), Pb, or nickel (Ni) did not accumulate equally in both statoliths
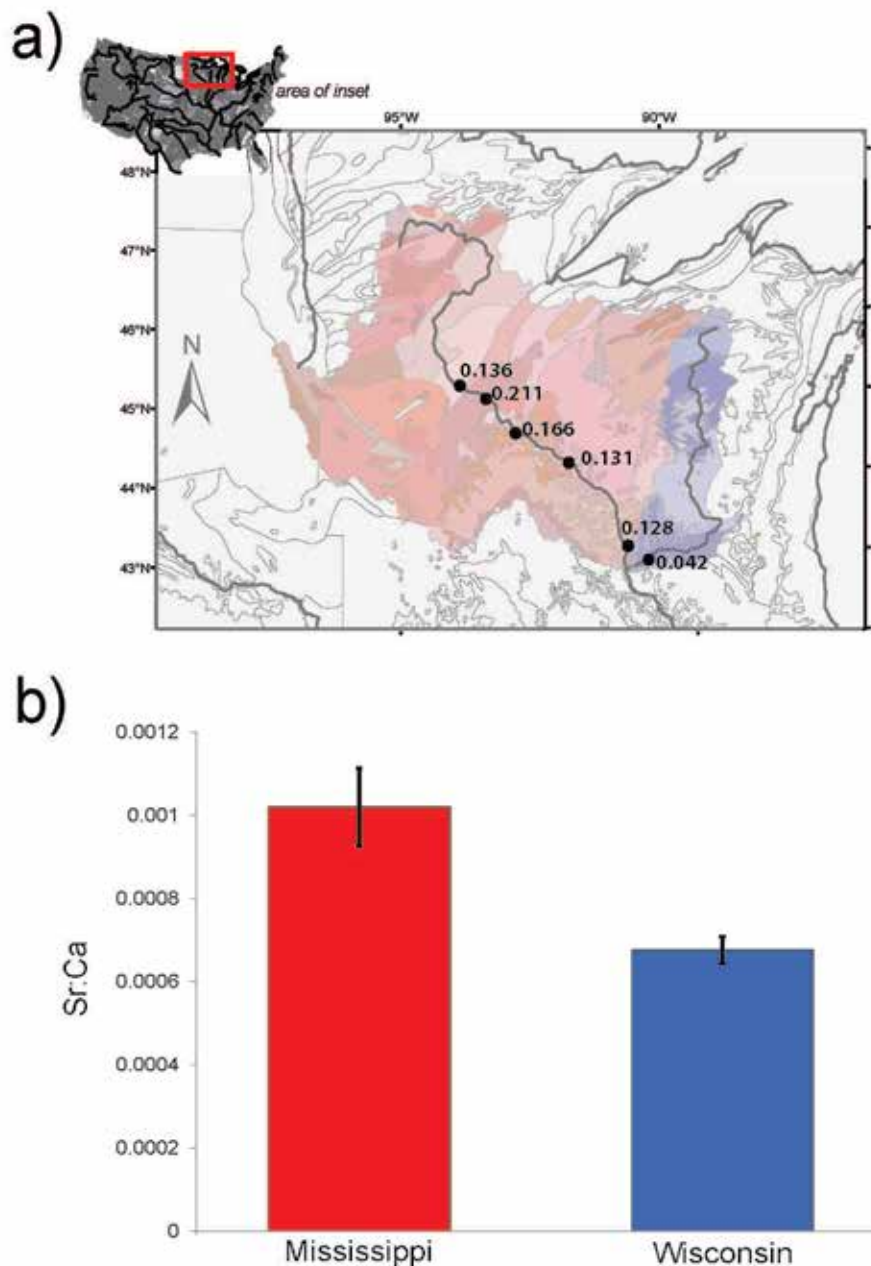
Figure 4. (a) Map of geological units in the Mississippi River Basin (reds) upstream of the Wisconsin River Basin (blues). Shades and tints of colors in each watershed represent a different geological unit based on bedrock and surficial geology. Values on map indicate Sr values from water samples taken in the Mississippi and Wisconsin rivers reported from the October 1991 sample in Garbarino et al. (1995). Spatial data available from United States Geological Survey, http://mrdata.usgs.gov/geology/us. (b) Bar charts of otolith Sr values from Smallmouth Bass (*Micropterus salmoides*) collected in the Mississippi River (N = 18) near the downstream-most point (red bar) and Wisconsin River (N = 20) approximately 80 km upstream of the Sr sampling point. Error bars represent standard error. Otoliths were analyzed using a CETAC Technologies LSX-213 LA system (CETAC Technologies, Omaha, NE) coupled to a Perkin-Elmer ELAN DRC II ICP-MS (Perkin-Elmer, Waltham, MA) located at the University of Massachusetts, Boston. In this procedure, the laser ablated a transect across otoliths extending from the core to edge (beam diameter: 25 µm; speed: 15 µm*s⁻¹, pulse rate: 20 Hz; energy level: 70%; nebulizer gas: 94%). United States Geological Survey solid standard MACS-3 was analyzed every 15 samples to correct for instrument drift. Each sample and standard run was preceded by a ~30 s gas blank measurement to enable calculation of background concentrations. We then used Analysis Management System (AMS) data reduction software (Mutchler et al. 2008) for integrating output across the entire otolith, background subtraction, machine drift correction, and conversion of LA-ICP-MS output from counts per second to µg*g⁻¹.

(Brothers and Thresher 2004). In addition, though statolith iron (Fe), Cu, Pb, Mg, and Sr were found to be stable through metamorphosis from larvae to adult, rubidium (Rb) concentrations change after metamorphosis (Lochet et al. 2013). Considering the large number of freshwater studies that have found Sr to be useful for habitat discrimination coupled with its stability among hard parts, we recommend that this element should always be quantified in studies examining heavy metals. However, the utility of other elements will depend on the study area and hard structure examined.

Laser ablation–inductively coupled plasma–mass spectrometry (LA-ICP-MS; Box 2) is by far the most common technique currently used to assay hard-part microchemistry in freshwaters. This technique has been used in North American freshwaters for addressing a wide variety of research objectives (Appendix A) and is also among the most precise techniques available (Campana et al. 1997). LA-ICP-MS is used to quantify elements with high atomic weights (e.g., heavy metals), but other instruments are needed to quantify lighter elements that can be useful in investigations of fish habitat use such as hydrogen (H), carbon (C), nitrogen (N), oxygen (O), and their stable isotopes (^{2}H, ^{13}C, ^{15}N, ^{18}O). Use of lighter elements can provide different perspectives on fish environmental history and have been used to provide records of ambient water temperature (using ^{18}O; Dufour et al. 2005; Weidel et al. 2007), river of origin (e.g., using ^{13}C; Limburg et al. 2013), habitat type (e.g., river vs. floodplain lake using ^{2}H, ^{13}C, ^{18}O; Zigler and Whitledge 2010, 2011), and watershed land cover (using ^{15}N; Vandermyde and Whitledge 2008). Quantifying these elements requires use of high-resolution–inductively coupled plasma–mass spectrometry (HR-ICP-MS), high temperature conversion elemental analyzer–isotope ratio–mass spectrometry (TC/EA-IR-MS), or ion microprobe (secondary ion mass spectrometry). Like LA-ICP-MS, techniques used for quantifying low-atomic-weight elements can be linked to temporal landmarks on the hard structure because sample material is collected using a high-precision drill—a micromill—that can sample specific portions of life history, similar to using a laser to vaporize a spot (Box 2; Figure 3). Several other techniques
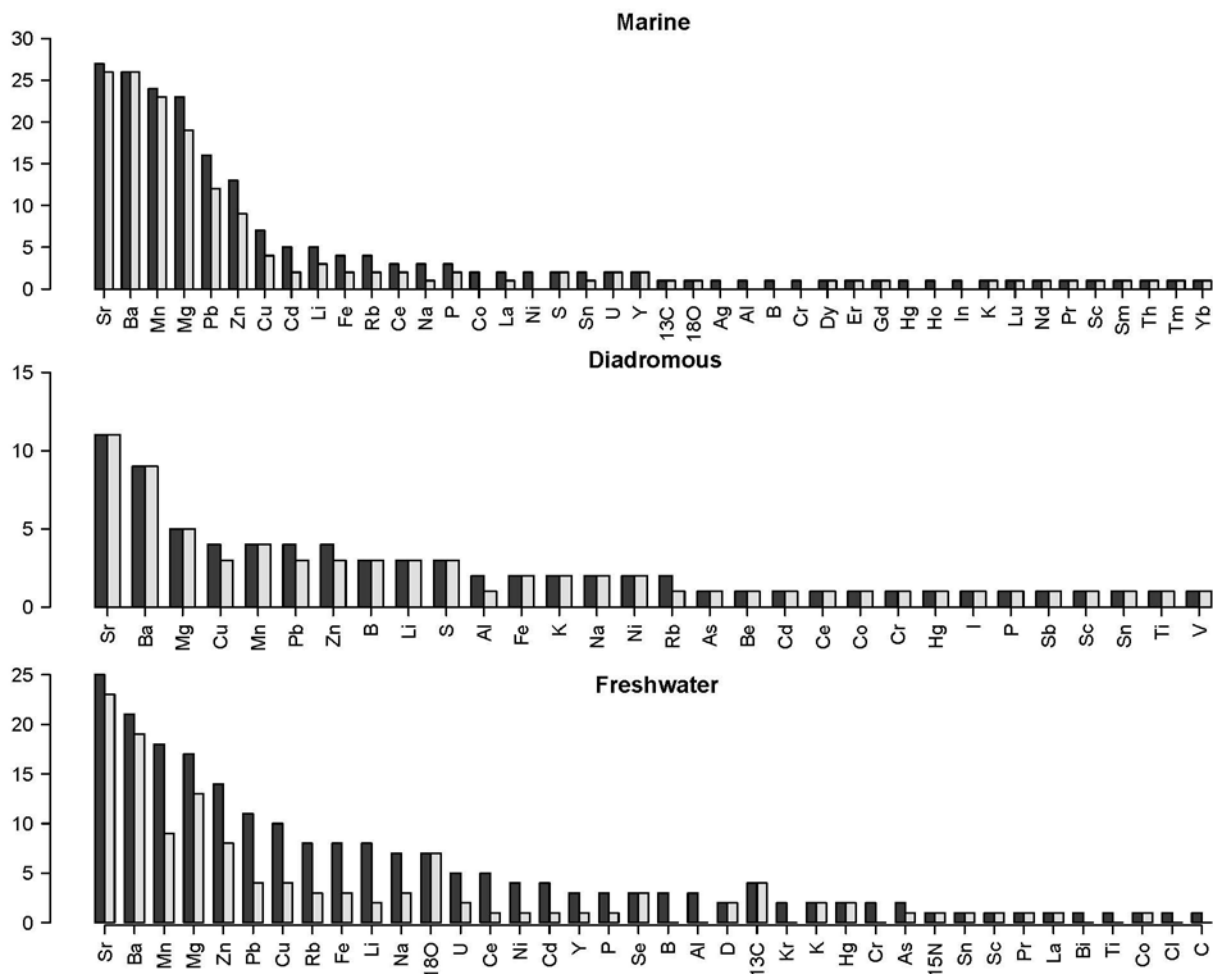
Figure 5. Number of published studies of North American obligate freshwater fish, and selected anadromous and marine fish from around the world (literature cited for this figure in Supplemental Material), that measured a particular element or isotope (dark bar) in a hard part (otolith, spine, fin ray, scale) in a fish hard part and used it in statistical analyses (light bar). For studies examining more than one element, each element is counted separately (i.e., one study can be counted for multiple elements).

are also available for quantifying hard-part microchemistry but are much less common. Examples of applications of these techniques and the elements they quantify are provided in Appendix A.

Hard-Part Selection and Preparation

Hard parts used in microchemistry analyses are typically the same ones used for age and growth studies for a given species. Otoliths, especially the large sagittal otoliths, have been the most commonly used. Lapilli otoliths have also been used in methodological tests (see Brazner et al. 2004) but are seldom studied. Fin rays and scales are generally thought to be comparable to otoliths and do not require lethal sampling, making them appealing alternatives for microchemistry studies (Clarke et al. 2007). Pectoral fin rays have been shown to provide a similarly permanent record of habitat use despite being composed of apatite (Ca phosphate mineral found in tooth enamel and bone material) rather than carbonate (Wells et al. 2003; Clarke et al. 2007; K. T. Smith and Whitledge 2010). However, the lumen of pectoral and fin rays can degrade, resulting in an incomplete record of early life history, especially in older fish (Davis-Foust et al. 2009). Similarly, scales are also associated

with incomplete environmental histories because they are often regrown following damage. Regrown scales will only contain information since scale regrowth (Wells et al. 2003; Clarke et al. 2007). Additionally, certain elements may not provide equally good records across all hard parts; for example, Pb concentrations in fish scales have been shown to be uncorrelated with that of otoliths from the same fish (Muhlfeld et al. 2005), although scale concentrations of Sr, cadmium (Cd), and Ba are correlated with water chemistry (Wells et al. 2003). Statoliths of lamprey (Brothers and Thresher 2004; Hand et al. 2008; Lochet et al. 2013), the only calcified structure for these fishes, are also thought to be comparable to otoliths but require killing fish and may lose some elemental information after larval metamorphosis (Howe et al. 2013; Lochet et al. 2013).

Archived samples can be used in microchemistry analyses provided that hard parts were both immediately removed from the fish and stored dry, or whole fish samples can be preserved by freezing or storing in ethanol shortly after death (Proctor and Thresher 1998; Milton and Chenery 1998; Hedges et al. 2004). Large fish are commonly frozen—a preservation method that has similar results to hard parts that are immediately removed
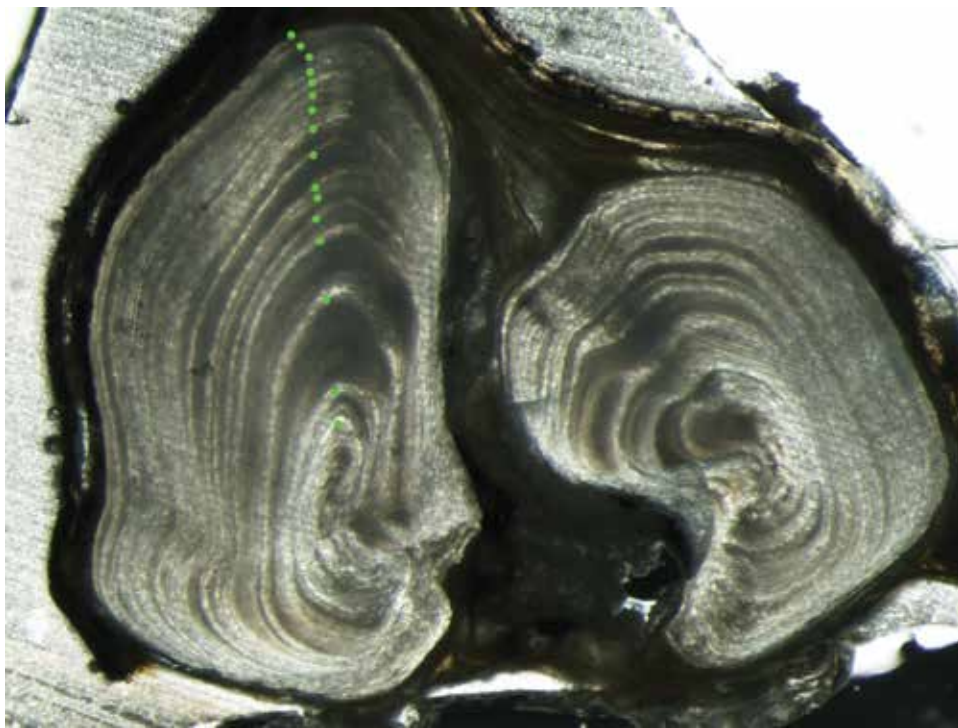
Blue Sucker (*Cycleptus elongatus*) pectoral fin ray. Photo credit: Dan Walchak and Paul Kanehl, Wisconsin Department of Natural Resources.

removed with a metal blade because they must be sectioned prior to microchemical analyses. Preparation of samples after this point will depend on the analytical method chosen.

For LA-ICP-MS analysis (Box 2), whole or sectioned otoliths or statoliths are mounted on a petrographic glass slide with thermoplastic resin and polished. Polishing is accomplished with a lapping wheel using a series of alumina or diamond slurries or by hand using wet sandpaper (see Brothers and Thresher [2004] and Hand et al. [2008] for detailed statolith methods and Hamann and Kennedy [2012] for detailed otolith methods). Analysis of fin rays requires structures to be dried, mounted in epoxy, and sectioned using a low-speed saw before being mounted on glass petrographic slides, whereas scales

from the fish and analyzed (Proctor and Thresher 1998), but small fish are frequently preserved in ethanol. In these cases, storage of fish in different grades of 95% ethanol (e.g., high-performance liquid chromatoraphy vs. American Chemical Society grade; Hand et al. 2008) or 70% ethanol (Milton and Chenery 1998) have all been shown to provide similar results to freezing samples. However, though elements that replace otolith Ca such as Sr, Mn, Ba, Mg, and Zn are not thought to be affected by storage of fish in ethanol, elements involved in biological regulation like sodium (Na), potassium (K), sulfur (S), chlorine (Cl; Proctor and Thresher 1998; Milton and Chenery 1998; Hedges et al. 2004), and ^{18}O (Storm-Suke et al. 2007) may be altered by this preservative. Unfortunately, samples preserved with formalin cannot be used because the acidic properties of the preservation medium degrade otoliths (Morales-Nin 1992). Ideally, these preservation options could enable analysis of historical samples that could be valuable in reconstructing behavior and habitat use by fishes prior to shifts in management or restoration activities.

Careful handling of hard parts for analysis is essential for avoiding contamination, but the initial steps of sample preparation are similar to those used for analysis of age and growth. Secor et al. (1991) provided an excellent guide for preparing otoliths for microstructural analysis that can be a useful initial guide to microchemistry preparation. For statloiths, otoliths, and scales, sample preparation begins with removing hard parts using nonmetallic forceps (or metal forceps wrapped in Teflon tape to avoid contamination with metals) and then carefully cleaning structures of other biologic material. Hard structures are then stored dry or frozen inside clean glass, plastic, or paper containers, typically washed with trace metal–grade nitric acid, and dried before samples are placed in them. Fin rays may be

do not require sectioning after mounting. Fin rays and scales generally require little or no polishing to expose clean annuli. For all of these structures, it is important to triple rinse and even sonicate samples that have been sectioned and polished to remove residue from the saw blade and/or polishing step. Full preparation details are provided by Phelps et al. (2012) for fin rays and Clarke et al. (2007) for scales. In contrast, preparing samples for solution-based inductively coupled plasma–mass spectrometry (SO-ICP-MS) analysis involves dissolving entire otoliths in an acid solution for direct injection into the ICP-MS and there are no sectioning or polishing steps required.

The SO-ICP-MS method has been shown to provide results similar to the LA-ICP-MS, but the LA-ICP-MS has slightly higher precision and is the recommended method where available (Ludsin et al. 2006). Samples being analyzed by secondary ion mass spectrometry have a similar preparation, but it is of the utmost importance that the hard part surface is thoroughly polished to a very flat surface because ions can become trapped in even the tiniest pits in the surface. Once polished, the sample is rinsed of all impurities with ultrapure water and coated in a gold thin film prior to analysis (Weidel et al. 2007).

Sample Sizes of Fishes and Study Site Selection

As in other aspects of fishery science, sample sizes are a primary determinant of inferential power from microchemical analyses, and the minimum number of fish needed to reach robust conclusions will depend on the research question and chemical variation among study areas (Appendix A). Published studies of North American freshwater fishes have used sample sizes as small as two (Weidel et al. 2007) and as many as 138 fish per site (Whitledge et al. 2007), with a median of 16 (Ap-

pendix A, Supplemental Material). With respect to research objectives, studies seeking to conduct stock assessments or examine natal sites like Bronte et al. (1996; 26 fish per site) or Brazner et al. (2004; 34 fish per site) may require more fish to detect rare immigrants. Studies interested in lifetime environmental history may require fewer fish because they extract a large amount of temporal information from each fish (e.g., Weidel et al. 2007; *n* = 2).

The sample size per site appropriate for any given study depends on the variance in elemental composition within versus between sites and the number of distinct chemical histories present within each site (Hayden et al. 2013; Limburg et al. 2013). For instance, otolith microchemistry patterns can emerge with as few as five replicate individuals per site when locations have fixed chemical differences (Friedrich and

Walleye (*Sander vitreus*) otolith. Photo credit: Connie Isermann, Fisheries Analysis Center, University of Wisconsin-Stevens Point.

Halden 2010), but within-site temporal variation can sometimes swamp the variance between sites in highly seasonal environments. Moreover, microhabitat differences and short-term movements of fishes may enhance the variability in elemental signatures even among a resident population of fish. When movement between sites occurs, then larger sample sizes are required to robustly separate resident from immigrant microchemical profiles. In certain situations, investigators have limited control over within habitat signature variability. For instance, habitat signatures are frequently characterized using larval or young of year fish (i.e., Reichert et al. 2010; Oele 2013) that may drift to other locations. It is important to collect individuals prior to these movements in order to accurately characterize (and minimize variability) within-habitat signatures. Once within- and between-habitat variability is known, we also recommend using a power analysis constructed with variances of hard-part chemistries of resident fish or a small sample of the species of interest to determine the appropriate sample size for each system.

A second key aspect of study design is the number and spatial separation of sites being compared. Microchemical comparisons are most fruitful at the interface between water bodies with fundamental differences in chemistry because spatial distance and chemical differences are decoupled at geochemical borders. This often occurs near anthropogenic inputs or features (Palace et al. 2007; Murphy et al. 2012; Freidrich and Halden 2010) at confluence points in river networks (Freidrich and Halden 2008; Humston et al. 2010; Phelps et al. 2012) or where tributaries enter lakes (Hand et al. 2008; Schaffler and Winkelman 2008; Reichert et al. 2010). Longitudinal sampling along river channels, or comparisons within a large lake, is less
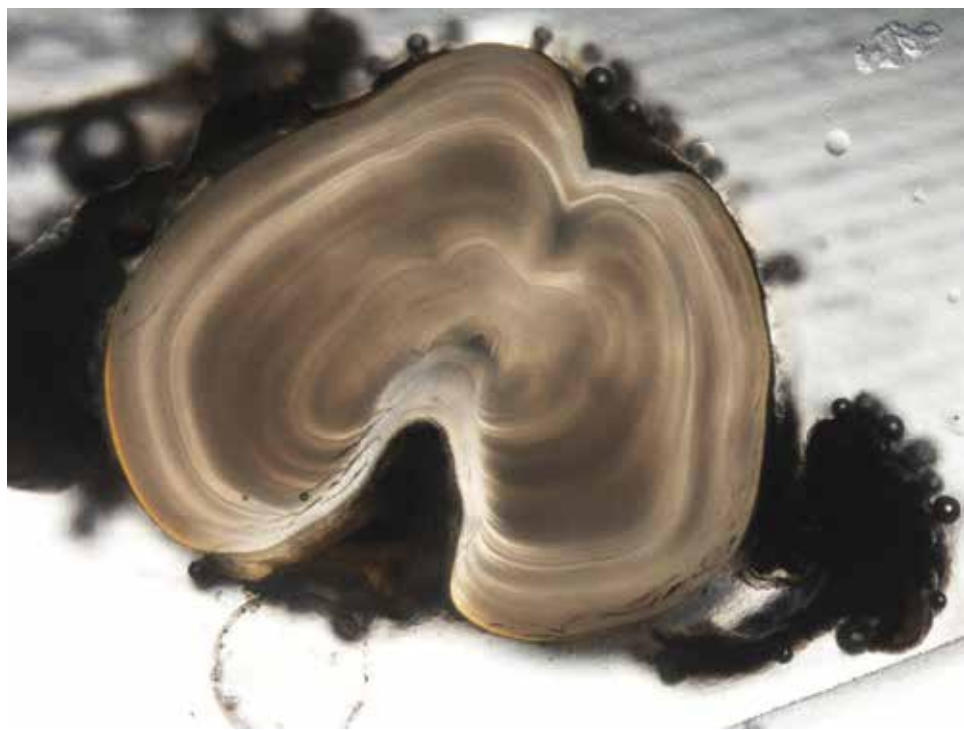
likely to show distinct chemistries (Dufour et al. 2005; Oele 2013). We recommend using pilot analysis of water samples or nonmigratory fish species to identify sharp natural or anthropogenic chemical boundaries. Comparing a few sites on each side of such boundaries provides maximum inferential power for a given total sample size by focusing on replicate individuals from each site rather than a large number of sites. Regardless of the target number of sites and individuals to be analyzed, we also recommend collecting additional individuals as well as extra sites at the same time as resources allow. These collections provide backup options if unexpected patterns emerge, and they avoid complications from comparing samples collected at different times (and thus potentially different microchemical regimes).

Data Analysis

After deriving multi-element signatures from each sample, most studies use statistical assignment tests to determine source population, natal area, or environmental history. Linear discriminant function analysis (LDFA) is the most commonly used assignment technique for grouping individuals with similar microchemistry to elucidate which sites are distinctive (21 of 53 studies; Appendix A, Supplemental Material). Various types of regression analyses (analysis of variance, analysis of covariance, multivariate analysis of variance) are also widely used to test for differences among sites (Appendix A, Supplemental Material), but they lack the predictive modeling and validation steps of LDFA and its nonlinear counterpart, quadratic discriminant function analysis (QDFA). All of these statistics require that microchemical data meet normality assumptions, so log-transformation is common. LDFA and QDFA can use a

Walleye otolith. Photo credit: Connie Isermann, Fisheries Analysis Center, University of Wisconsin-Stevens Point.

ing a high signal-to-noise ratio and stable chemical differences among populations. Bayesian techniques have only been used in one study of obligate freshwater fishes of North America (Pflugeisen and Calder 2013), but use of these approaches will doubtless increase as Bayesian statistical methods become more common. Nonetheless, direct comparisons indicate that if there is a training data set, LDFA can provide results similar to those of computationally intensive approaches like Bayesian mixture models (Munch and Clarke 2008) when sample sizes are >30 or to artificial neural networks (machine learning) and random forests (resampling) when less than four elements are being used for discrimination (Mercier et al. 2011). A new technique, k-sample nearest-neighbor discriminant analysis, can assign individuals of unknown origin to groups and is also robust to deviations from normality, although its performance has not yet been compared against other statistical methods (Gao et al. 2013). Though these statistical approaches may seem daunting, they are increasingly accessible through

training data set that usually involves young-of-year of focal species to characterize putative source populations—a step that can be used to present *a priori* characterizations of elemental signatures of source populations—although use of the training data set restricts assignment of fish of unknown origin to these source populations (e.g., a migrant from an uncharacterized source location will still be assigned to a characterized population). LDFA is the most commonly used technique, so we will restrict our discussion to comparisons between it and alternative methods of statistical assignment.

Researchers using otolith microchemistry in marine systems have addressed statistical limitations by employing assignment methods developed for population genetics (Cornuet et al. 1999) such as Bayesian, machine learning, or resampling techniques. These techniques are generally robust to deviations from multivariate normality and may also be able to assign individuals to unsampled sources. For example, Bayesian mixture models can allow for assignment of individuals to sampled or unsampled source populations (Standish et al. 2008; Neubauer et al. 2010; Pflugeisen and Calder 2013). In fact, the latest statistical models can estimate the likely number of unsampled source populations (Neubauer 2012; Hogan et al. 2014), an assessment that is not possible with LDFA, QDFA, or regression-type analyses. As with any discrimination technique, successful application of Bayesian methods is predicated on the data hav-

no-cost statistical packages (e.g., R package; R Development Core Team 2014) that can execute computationally intensive analyses using a standard personal computer. We recommend use of Bayesian techniques in concert with LDFA, QDFA, or k-sampled discriminant analysis to ensure robustness to violations of multivariate normality and other statistical assumptions about otolith microchemistry data.

Technique Limitations

Hard-part microchemistry techniques also have a number of limitations that are important to consider prior to beginning a study, some of which are limitations for all studies and some that are particular to studies of adult fishes. For instance, all studies using hard-part microchemistry require distinct water chemistry among habitats for discrimination. If these differences do not exist at the scale of interest, then hard-part microchemistry techniques will not be of use, even at relatively large spatial scales (see Munro 2004; Dufour et al. 2005; Pangle et al. 2010; Oele 2013). Unfortunately, it may be difficult to ascertain where hard-part microchemistry may not be of use from the published literature because studies with negative results are seldom published (see Munro 2004; but see Howe et al. 2013). Temporal stability of water chemistry signatures is also an important limitation that all studies must consider. For instance, larval Yellow Perch showed unique Sr concentrations among

tributaries of Lake Erie, yet inter-annual variability was sufficiently high that fish collected in one year could not be used to assign fish collected in a different year (Pangle et al. 2010). As a result, Pangle et al. (2010) recommended building a multiyear database of larval Yellow Perch signatures from several tributaries to help overcome this limitation. All studies must also consider analytical limitations, namely, that laboratory and statistical analyses required to conduct a hard-part microchemistry study have steep learning curves. Though we have made an attempt in this article to reduce this barrier, it is no replacement for a colleague or mentor who can help guide new investigators through this process. Additionally, equipment needed for laboratory analysis is common at large universities (often in found geology laboratories) but not elsewhere. As a result, access to

Young-of-year Northern Pike (*Esox lucius*) otolith. Photo credit: Dan Oele, University of Wisconsin-Madison.

both analytical expertise and instrumentation may be a limitation for fisheries biologists at government agencies and smaller academic institutions. Thus, in addition to using this and other papers to guide study design and inferences, we strongly recommend that new investigators seek advice or collaboration from colleagues with direct experience in chemical analyses of hard parts.

Several limitations to hard-part microchemistry techniques are particular to adult fishes. For example, brief sojourns into habitats with different chemistries will not be detected when residence is too short for sufficient new hard-part accretion to occur. This situation could be more likely to occur during time periods in a fish's life when the accretion rates are very slow; for instance, during spawning migrations when fish are devoting energy to reproduction rather than growth, movements during winter, or those of older fish that are growing very slowly. Difficulties may also exist in ageing and interpreting the hard parts of adult fishes because of uncertainty about how many annuli a fish has, what constitutes an annulus, or where annuli are placed. This limitation muddies how chemical changes recorded in the hard structure align with specific ages or life history events and can be partially overcome by averaging signals that correspond to a particular life history stage across parts of a hard structure for an individual fish.

CONSERVATION AND MANAGEMENT APPLICATIONS

Judicious use of hard-part microchemistry techniques has enormous potential to advance understanding of freshwater fish populations, just as it has for the marine and diadromous species where these methods have been pioneered (see reviews

by Secor et al. 1995; Campana 1999; Secor and Rooker 2000; Campana and Thorrold 2001; Gillanders 2005a and 2005b; Elsdon et al. 2008; Brown and Severin 2009; Chang and Geffen 2012). The potential for hard-part microchemistry to provide insights into lifelong patterns of habitat use by fishes also makes this technique ripe for conservation and management application. In this section, we discuss how hard-part microchemistry techniques are suited to address some widespread conservation and management challenges.

Fisheries Law Enforcement

Hard-part microchemistry can provide a retrospective view of where a fish has been throughout its life and thus may provide evidence of illegal stocking or harvesting to law enforcement authorities. For example, otolith chemistry has been used to identify the source and estimate of date of illegal stocking of Lake Trout (*Salvelinus namaycush*) into Yellowstone Lake (Munro et al. 2005), as well as the source of invasive species in the Upper Colorado River (Whitledge et al. 2007). Microchemistry techniques may be especially powerful for law enforcement when used alongside other methods to identify the source of illegally harvested or imported fish. For instance, genetics can be a powerful tool for identifying the location of fish harvest (Ogden 2008), and coupling genotyping with microchemistry techniques can increase the resolution of stock assignments (Bradbury et al. 2008; Collins et al. 2013). In fact, freely available software now allows joint analysis of genetic and microchemical data (J. S. Smith and Campana 2010).

Merging hard-part microchemistry, genetics, and muscle stable isotopes—a technique that can provide insight into the last several weeks to months of habitat use (Cunjak et al.

Walleye otolith. Photo credit: Connie Isermann, Fisheries Analysis Center, University of Wisconsin-Stevens Point.

locations that can be protected to enhance conservation goals. For example, Yellow Perch declines coupled with their importance to sport fisheries in the Laurentian Great Lakes have prompted multiple studies focused on linking habitats to successful recruitment, such as Reichert et al. (2010), who used otolith microchemistry to show that larval Yellow Perch in Lake Erie tributary plumes have higher survival than those in open water. Similarly, in order to focus Bighead Carp (*Hypopthalmichthys nobilis*) and Silver Carp (*H. molitrix*) eradication efforts in the Upper Mississippi and Illinois rivers, Norman (2013) used otolith microchemistry to determine habitat use of these species throughout their life history. Results of this study indicated that control efforts focused on early life history should target floodplain lakes, whereas those focused on adults should target river channel habitats. In these cases, microchemistry provided insight into habitat use that could not have been derived from genetics (these sites are used by the same stock) or tagging studies (which cannot track larval fish effectively in large ecosystems). Accounting for such complexity of habitat use through the life cycle is essential for improving local management and conservation efforts and can also contribute to prioritizing habitat restoration and threat alleviation at large spatial scales (e.g., Januchowski-Hartley et al. 2013; Pracheil et al. 2013; Martinuzzi et al. 2014).

2005)— to provide several layers of resolution for pinpointing fish harvest location may be especially important in regulation of roe fisheries. Currently, it is difficult to regulate these fisheries because fish are highly mobile, harvest regulations vary from state to state, and commercial value of caviar-bearing species is sharply increasing as global sturgeon stocks collapse (Bettoli et al. 2009; Koch and Quist 2010; Pracheil et al. 2012). Combining multiple techniques may hold promise for enforcement of illegal harvest of fishes such as Lake Sturgeon (*Acipenser fulvescens*) that have genetically structured populations (DeHaan et al. 2006; Forsythe et al. 2011) and demonstrated utility for understanding movement and habitat use with fin-ray microchemistry (K. T. Smith and Whitledge 2011).

Designating and Prioritizing Conservation and Management Efforts

Fish often make use of different habitats in each major phase of their life history, and understanding how these habitats are connected through fish life cycles is essential for successful conservation (Wilcove and Wikelski 2008). Hard-part microchemistry offers a glimpse into the environmental conditions of spawning and nursery sites and provides a description of the environmental conditions that are encountered throughout fish life history. These conditions can be useful in pinpointing specific

Evaluating Recruitment and Stocking Contributions

In a system where fish populations are supplemented through stocking, determining long-term survival and dispersal of stocked fish after their release can be challenging. Hard-part microchemistry has proven effective in determining whether wild-caught adult fish are of hatchery or wild origin. Such studies take advantage of the fact that the chemistry of hatchery waters is often dramatically different from the water chemistry of the stocking location, yielding a distinctive hatchery signature of stocked fish during early life history. For example, Gibson-Reinemer et al. (2009) found that hatchery Rainbow Trout could be assigned back to their hatchery of origin based on otolith microchemistry signatures with a high degree of accuracy. Similarly, Bickford and Hannigan (2005) used elemental signatures from otolith cores to assign hatchery of origin of stocked Walleye (*Sander vitreus*). Hard-part microchemistry can thus facilitate insight into relative mortality and year-class

strength among different hatchery stocks for informing future stocking endeavors, as well as distinguishing the relative contributions of different hatchery stocks from wild-spawned fish.

Identifying per habitat contributions to recruitment of wild-produced fish is an important application of hard-part chemistry techniques for establishing targeted population management and conservation actions. Early studies demonstrating that hard-part microchemistry could be used to identify the river of natal origin examined Sr concentrations in otoliths of anadromous Atlantic Salmon (*Salmo salar*)—a fish that spends its early life history in freshwaters—to estimate contributions of Connecticut River tributaries to recruitment (Kennedy et al. 2000, 2002). More recent studies have used hard-part microchemistry techniques to determine the river of natal origin for obligate freshwater fish including Asian carps (Bighead Carp [*Hypophthalmichthys nobilis*] and Silver Carp [*H. molitrix*]) in the Illinois River (Norman 2013) using otolith Sr and Ba to identify river and otolith ^{13}C and ^{15}N to determine

Walleye scale. Photo credit: Connie Isermann, Fisheries Analysis Center, University of Wisconsin-Stevens Point.

natal habitat (e.g., floodplain, channel). In this case, understanding the source of fish recruitment both in terms of river and habitat within the river using microchemistry is helping to focus Asian carp control efforts in the study system and in other rivers as the range of these fishes expand.

CONCLUSIONS

There is a growing need for large-scale, progressive management of freshwater fisheries (Martin and Pope 2010; Post 2013; Pracheil et al. 2012) as habitat degradation, climate change, and land conversion continue to expand (Vörösmarty et al. 2010; Martinuzzi et al. 2013). Hard-part microchemistry techniques offer a valuable tool for understanding aspects of fish life history and habitat use that have been difficult to resolve using traditional techniques or even the latest genetic tools. In particular, microchemical approaches provide insight into movement patterns during early life history; a stage that has remained enigmatic despite advances in fish biology (Rose 2000).

Although there are questions that can be resolved using microchemical methods alone, they are the most powerful when used in combination with other techniques like mark–recapture, telemetry (Pollock et al. 2004; Cooke et al. 2013), and genetics

(Collins et al. 2013). In that context, microchemistry can fill in information that cannot be gained during early life history, between encounters, when fish move outside of the search area, or within a single genetic stock. In additon, it is important to note that we are not advocating that microchemical methods are devoid of limitations and challenges but rather that the examples presented herein indicate ample opportunity to apply these techniques more widely across North American freshwaters. The resulting insights into fish movements, population dynamics, and life history are necessary for managing resilient freshwater fisheries now and into the future.

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