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FORENSIC APPLICATIONS OF OTOLITH MICROCHEMISTRY FOR TRACKING SOURCES OF ILLEGALLY STOCKED WHIRLING DISEASE POSITIVE TROUT

Submitted to:
Whirling Disease Initiative, Montana Water Center

Submitted by:
Dr. Brett M. Johnson, Professor
Department of Fish, Wildlife and Conservation Biology, Colorado State University, 1474 Campus Delivery, Fort Collins, CO 80523-1474
Phone: (970) 491-5002; FAX: (970) 491-5091; Email: brett@cnr.colostate.edu

Daniel Gibson-Reinemer, Graduate Research Assistant
Department of Fish, Wildlife and Conservation Biology, Colorado State University, 1474 Campus Delivery, Fort Collins, CO 80523-1474
Phone: (970) 491-2749; FAX: (970) 491-5091; Email: dangr34@hotmail.com

Patrick J. Martinez, Aquatic Researcher
Aquatic Research Section, Colorado Division of Wildlife, 711 Independent Drive
Grand Junction, CO 81505
Phone: (970) 255-6141; FAX: (970) 255-6111; Email: pat.martinez@state.co.us

Dr. Dana Winkelman, Professor and Leader
Colorado Cooperative Fish and Wildlife Research Unit, 1474 Campus Delivery,
Colorado State University, Fort Collins, CO 80523-1474
Phone: (970) 491-1414; FAX: (970) 491-1413; Email: dlw@cnr.colostate.edu

Dr. Gregory Whitledge, Assistant Professor
Dept. of Zoology, Southern Illinois Univ., Carbondale, IL 62901-6501
Phone: (618) 453-7761; Fax: (618) 453-6095; E-mail: gwhit@siu.edu

Abstract

We used naturally occurring chemical markers to trace the environmental history of hatchery trout. Analysis of water and otolith chemistry at hatcheries revealed a high degree of temporal stability, coupled with high variation among hatcheries relative to variation within hatcheries. Proportional relationships between water and otolith chemistry for Sr:Ca, Ba:Ca, and $^{87}Sr/^{86}Sr$ allowed us to use these three quantities as environmental markers in otoliths to classify trout to their hatchery of origin. Multivariate models used to discriminate among hatcheries performed best when all three markers were used, achieving an average accuracy of up to 96% for a group of five hatcheries. Using only Sr:Ca and Ba:Ca, we were able to identify the hatchery of origin with average accuracy rates which varied from 59% using a group of 11 hatcheries to 90% when groups of only two hatcheries were considered. In a rigorous test of the forensic capabilities of otolith chemistry, multivariate models classified a blind sample of at-large fish stocked from hatcheries with 79% accuracy. Our results indicate the most effective use of otolith chemistry in a forensic context will require collaboration with investigators using traditional methods of inquiry to reduce the number of hatcheries classified with otolith markers. We advocate an eclectic approach to source identification using elemental and isotopic markers as a powerful new source of information that can be used to strengthen cases based on multiple lines of evidence.

Introduction

The maintenance of viable, self-sustaining wild trout fisheries is jeopardized by the spread of whirling disease. Illegal stocking of whirling disease positive trout is thought to be an important mode for introducing the disease into uninfected drainages throughout the mountain west and Pacific Northwest. However, it has been virtually impossible to identify where a fish originated from once it is released. Thus, it has been extremely difficult for managers and law enforcement personnel to determine the sources of such illegally stocked fish and prosecute individuals suspected of these violations. The development of new technologies that identify sources would be an

invaluable law enforcement tool as well as a potent deterrent to discourage future violations of this nature (Glenn Smith, CDOW Criminal Investigator, personal communication).

Microchemical and stable isotope analysis of otoliths is emerging as an extremely useful method for tracking origins and movement patterns, or provenance, of fishes (Gao and Beamish 1999; Hobson 1999; Kennedy et al. 2000, 2002; Weber et al. 2002; Wells et al. 2003). Otoliths ("ear stones", calcified structures of the inner ear used in balance and hearing, Bond (1996)) have three properties that suit them to this kind of analysis:

1) Chemical constituents in water are passively absorbed by fish and deposited in their otoliths. Some elements and their isotopes are deposited in the otoliths in proportion to the environmental concentration, making them excellent natural tracers (Campana and Thorrold 2001; Outridge et al. 2002).

2) Otoliths are physiologically inert, so once material is deposited it remains in the otolith for the life of the fish. This is not true for most other parts or tissues in a fish, which may be catabolized or otherwise lost or transformed.

3) Otoliths grow incrementally, even when the fish itself ceases to grow, in a highly consistent manner. Thus, chemical information is deposited chronologically.

Because water chemistry varies from place to place due to variations in lithology, watershed characteristics, and land use and water use, otoliths of fishes from different localities differ in their chemical composition. Further, fish that have moved among locations of differing water chemistry carry a record of where and when they've inhabited the various locations. Thus, otolith microchemisty offers considerable promise as a means to track the origins of illegally stocked trout. Testing the utility of the technique for this application was the focus of this research project.

Most of the research on otolith chemistry has been conducted with marine or diadromous fishes (Campana 2005). However, freshwater systems have the potential to display greater variation in key trace elements than the ocean (Campana et al. 1999), allowing researchers to track environmental histories of fishes originating in geochemically distinct areas. The chemical signatures in different freshwater environments have proven to be useful tools for classifying fish to their location of origin in areas as diverse as the Great Lakes (Ludsin et al. 2006), Arkansas (Bickford and Hannigan, 2005) and Yellowstone National Park (Munro et al. 2005). Encouragingly, freshwater systems have markers such as strontium (Sr) isotope ratios which are not useful in marine environments but can be highly effective environmental tracers in freshwater (Kennedy et al. 2002).

While otolith chemistry shows promise in freshwater systems, critical areas of research need to be examined for it to become a valuable tool in forensic investigations. The use of trace element signatures in otoliths to classify fish to locations in the Mountain West has been accomplished in Wyoming (Munro et al. 2005) and Idaho (Wells et al. 2003), but neither study examined otoliths from more than three locations and both covered relatively small spatial scales. We anticipate investigations of illicit stocking may involve more than three hatcheries and occur over broad spatial scales. The classification accuracy of statistical models in such cases is a major factor in determining how informative otolith chemistry will be. Additionally, no literature to date has examined the variation in groundwater chemical signatures in the Mountain West. The spread of whirling disease in wild rivers in the region has led a number of hatcheries in Colorado to use groundwater sources to avoid contamination. Thus, examining the variation in otolith chemistry among groundwater-fed hatcheries is a vital step in determining the effectiveness of the technique for identifying sources of illicitly stocked trout.

Our investigation was designed to fill in the gaps in the literature and to create a template for forensic use of otolith chemistry. Prior studies have laid a substantial foundation regarding the use of otolith chemistry, but the literature to date has not fully investigated factors relevant to forensic applications of hatchery-reared fish in the Mountain West. We expand upon the current state of the science with an investigation

which is novel in that we: examine variations in surface- and groundwater-fed hatcheries; analyze variation in water and otolith chemistry over hundreds of miles; use multivariate models to classify a number of locations unprecedented in freshwater studies; and subject our data to a rigorous test simulating conditions which may exist in a forensic case.

Materials and Methods

We sampled water and fish from 17 CDOW trout hatcheries, one federal hatchery, and two private hatcheries in Colorado, and one Wyoming Game and Fish (WGF) hatchery during this study. The project originally intended to sample a range of private facilities, but only two vendors agreed to participate in our study. To conserve funds for other objectives and to make the best use of very limited instrument time, we selected a subset of 16 CDOW hatcheries to use for water chemistry analyses and 11 CDOW hatcheries and one WGF hatchery to use for chemical analyses of otoliths (Table 1). The hatcheries spanned a wide geographic and geologic range (Figure 1). The maximum distance between pairs of hatcheries in Colorado was approximately 275 miles (Durango and Watson) and the minimum distance between pairs of hatcheries was less than a mile (Bellvue and Watson).

We collected water from each hatchery in Colorado once per year. To maximize our ability to examine temporal variation we collected samples in a different season each year: summer in 2004, late winter in 2005, and fall in 2006, following the methods of Shiller (2003). Because hatchery water supplies are usually well-mixed to insure that gases are at atmospheric equilibrium, and analytical cost and precision are very high, we collected a single sample per location in 2004 and 2005. In 2006 we collected 3 to 6 samples per location to verify our assumption about precision. We also collected 18 samples of hatchery feed consisting of six size categories representing two major feed manufacturers from several CDOW, one private and one federal hatchery (Table 2) to determine barium (Ba), strontium (Sr) and Sr isotope signatures ($^{87}Sr/^{86}Sr$). Water chemistry and feed analyses were provided by the Center for Trace Analysis at the University of Southern Mississippi using a Finnigan MAT Element 2 high-resolution inductively coupled plasma mass spectrometer. Elemental concentrations were

normalized to calcium concentration because these ratios govern the biological uptake of elements in otoliths (Campana 1999). The replicate samples collected in 2006 were used to approximate sampling and analytical variance in previous years. Because variance tended to increase with element:Ca ratios, we fit a linear regression to the relationship and used that function to calculate estimates of error terms for water chemistry in 2004 and 2005. Strontium:Ca, Ba:Ca, and $^{87}Sr/^{86}Sr$ were analyzed in a multivariate analysis of variance (MANOVA) to test for significant differences among locations, pooling data across years within a hatchery.

Approximately 10 rainbow trout (*Oncorhynchus mykiss*) or hybrids (*O. mykiss x O. clarki*) were collected from each hatchery in summer 2004 and late winter 2005 (Table 3). In fall 2005, we collected ten rainbow trout from the Tillett Springs Fish Hatchery in north central Wyoming. (Hereafter, fish collected directly from hatcheries are referred to as "known origin fish"). At four hatcheries, fish were transferred as fingerlings from one hatchery to another prior to collection (Table 4). In all other cases, known origin fish had resided at the location from which they were collected since hatching. We also collected a sample of 23 rainbow trout from Button Rock Reservoir (BRR) on July 11, 2006; these fish had been stocked from the Bellvue hatchery as sub-catchables (~3-5" TL).

To test the ability of otolith chemistry to identify the provenance of unknown origin fish, we analyzed a blind sample of rainbow trout collected from the wild in 2004 by CDOW Researcher Kevin Thompson (Table 5). These samples were collected in areas where CDOW had stocked rainbow trout and natural reproduction was considered to be unlikely. Therefore, we were confident that all samples obtained in this manner had originated in state hatcheries. (Hereafter, we refer to this sample as "unknown origin fish.") We received randomly numbered fish and a list of 8 hatcheries from which they could have come; only four of those were the true sources. The 8 potential hatcheries of origin were among the 11 from which we chose to analyze otoliths.

Sagittal otoliths were prepared as polished thin sections (Figure 2) following the methods of (Whitledge et al. In Press). Right otoliths were embedded in epoxy and cut transversely using a low speed saw with a diamond blade. Cut otolith sections were

sanded and polished down to the plane of the otolith core. Polished thin sections were mounted on glass slides and cleaned with ultrapure water. We used laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) to collect data on the elemental abundance of 24 elements in transects which were ablated along the axis of growth from the otolith core to the edge. We were thus able to look for changes in the chemical composition of the otolith over time and to separate distinct portions of the otolith corresponding to different environmental signatures.

Otolith elemental analysis was provided by Alan Koenig at the USGS Mineral Resources Laboratory in Lakewood, CO, with a Perkin Elmer ELAN6000 ICP-MS coupled to a CETAC Technologies LSX-500 laser system. External calibration of the system was conducted using a prototype USGS calcium carbonate reference material MACS-1 (Steve Wilson, USGS, personal communication). This reference material is a near matrix match for the aragonite in the otoliths. To control for the amount of otolith ablated, elemental data were standardized relative to Ca. After standardization to Ca, stable portions of transects were integrated to produce a mean concentration as in Longerich et al. (1996) and reported as ppm. In cases where there was a change in the chemical composition within an otolith, stable regions of each zone were integrated to produce an average value while omitting the transition zones. The average values of stable portions were used in multivariate analyses to characterize hatcheries.

Although usually composed of aragonite, sagittal otoliths in salmonids may also contain portions of vaterite, an alternate crystal form of calcium carbonate. Vateritic portions of otoliths have a different chemical composition from that of aragonite (Gauldie 1996; Melancon et al. 2005) and tend to occur with greater frequency in hatchery-reared fish than in wild fish (Zhang et al. 1995; Bowen et al. 1999). We frequently encountered vateritic portions of otoliths in our transect analyses and could identify them easily based on the characteristically low levels of Sr and high levels of Mg (Gauldie 1996; Melancon et al. 2005). The vateritic portions were excluded from our analyses because they do not reflect the environment in the same fashion as aragonite.

Following analysis of elemental abundance, the $^{87}Sr/^{86}Sr$ ratio was analyzed in a subset of otoliths by Dr. Jon Woodhead at the University of Melbourne. Otoliths were cleaned to remove debris from the first ablation and subjected to a second ablation

along a transect parallel to that of the first ablation line using a Nu Plasma multicollector inductively coupled plasma mass spectrometer. Time resolved scans of the $^{87}Sr/^{86}Sr$ were processed by Alan Koenig and integrated over stable portions. Fish displaying changes in $^{87}Sr/^{86}Sr$ over the transect were identified from the time resolved $^{87}Sr/^{86}Sr$ ratios and average $^{87}Sr/^{86}Sr$ ratios were calculated for each region of the transect.

Discriminant function analysis (DFA) is a statistical method commonly used in otolith studies to evaluate the extent to which distinct groups of fish have unique chemical signatures and to identify group membership of specimens of unknown origin (Wells et al. 2003, White and Ruttenberg 2006). Strontium and Ba were the only elements which displayed a proportional relationship between otolith and water chemistry and were the only elements used in multivariate models to classify known and unknown origin fish. Isotope data were incorporated into models with Sr and Ba for a smaller set of data. Both Sr and Ba were log transformed to meet the assumption of homogeneity of variance (Levene's test for homogeneity of variance $p=0.216$ and $p=0.586$ for Sr and Ba, respectively). We used a cross-validated, leave-one-out approach to classify otoliths of known origin fish (see Wells et al. 2003). There was no significant year effect for Sr or Ba (ANOVA type 3 test of fixed effects, $p=0.177$ and $p=0.158$ for Sr and Ba, respectively; Figure 3, so we pooled data from both years within a location.

As the number of groups classified decreases, the accuracy of the models may be expected to increase. To evaluate the increase in accuracy when number of groups classified decreases, we performed additional analyses using subsets of two to ten hatcheries from the pool of eleven known origin fish. Ten hatcheries were randomly selected for each group size and analyzed in a DFA using Sr and Ba. On average, random chance will classify fish correctly with a percentage inversely proportional to the number of locations being classified and the performance of DFA models should be compared to the accuracy expected due to random chance alone (White and Ruttenberg 2007).

To classify fish of unknown origin, we created a DFA model using the set of eight suspected hatchery sources of the fish. This model was used to classify each of the unknown origin fish to the most likely hatchery of origin. A separate DFA model was

constructed for the subset of otoliths for which both elemental abundance and isotope data were collected.

Results and Discussion

The near lack of private fish grower participation in our study had no negative impact on our ability to test the utility of otolith chemistry for tracking provenance of illicitly stocked trout. In retrospect, it was fortuitous that we used only government hatcheries because they keep meticulous records of fish movements among locations and have no incentive to withhold or provide misleading information regarding the provenance of trout or their rearing practices. The range of geological and water chemistry variation exhibited by the hatcheries included in our study provided an excellent basis for evaluation of the technique. However, while the chemical signatures we acquired form the foundation of a source database, signatures from private vendors will be required in any future forensic application of otolith microchemistry.

Given the prohibitive costs associated with sampling water chemistry frequently, we chose to stratify by season and collect water data over several years rather than several times within a year. Because year was confounded with season in our sampling design, and seasonal variation may actually exceed annual variation (John Stednick, CSU Department of Forest, Rangeland and Watershed Stewarship, personal communication) formal statistical tests of a year effect would be somewhat inappropriate. Despite the inability to partition sampling variance, the variation of water Sr:Ca and Ba:Ca ratios among hatcheries was large relative to variation within hatcheries over time (Figure 4). A similar pattern emerged in $^{87}Sr/^{86}Sr$ ratio (Figure 5) among hatchery water sources. Among hatcheries, the multivariate chemical signature based on Sr:Ca, Ba:Ca, and $^{87}Sr/^{86}Sr$ ratio was highly significant (Pillai's trace, $p<0.0001$). Based on the patterns in water chemistry among hatcheries and the significance of the MANOVA test, our evidence indicates that water chemistry remained stable at a location over years relative to the differences among locations. This conclusion is consistent with our findings from chemical analyses of otoliths. We had only three years with which to examine interannual stability of hatchery water signatures. However, a prolonged drought was temporarily alleviated in 2005 with near

normal runoff in many river basins in the state. This important interannual hydrologic variation did not appear to obscure differences in chemical signatures among the hatcheries.

The significant difference among hatchery water sources is exciting because of the proportional relationship between water and otolith chemistry in freshwater environments. Strontium:Ca ratios in otoliths of hatchery-resident trout varied in proportion to the ratios in the hatchery water sources (Figure 6). Barium:Ca ratios in hatchery-resident trout otoliths tended to display greater within-site variation but also increased with increasing Ba:Ca ratios in water sources (Figure 6). Both Sr:Ca and Ba:Ca display positive relationships between water and otoliths, as expected based on other freshwater otolith studies (Wells et al. 2003; De Vries et al. 2005). No other element we examined showed a discernable relationship between water and otoliths. This finding is also consistent with other freshwater studies which have not yet demonstrated conclusive evidence linking water and otolith concentrations of other elements (as opposed to isotopes).

Our DFA models described the chemical composition or multivariate signature of the otoliths from each hatchery. Chemical composition of individual otoliths can be compared to the models and the otolith will be assigned to the hatchery to which it is most similar. In a verification test of the DFA model using only Sr and Ba, otoliths from the known origin fish from 11 hatcheries were assigned to their hatchery of origin with 59% accuracy (Table 6). While perhaps sounding unimpressive, given the relatively large number of locations which were classified with only two elements, the results are noteworthy. Limitations to the ability to classify fish on the basis of otolith signatures are set by the variation in water chemistry signatures among locations and the variation within otoliths from each location. In this case, the locations displayed a wide range of otolith and water signatures, suggesting that the most effective way to increase the accuracy of classification with Sr and Ba alone is to reduce the number of locations classified. This is demonstrated in the simulation where we decreased the number of hatcheries classified and average accuracy increased considerably beyond what was achieved in a model with eleven hatcheries and was considerably higher than would be expected due to chance alone (Figure 7).

We also performed a validation test of our DFA models using unknown origin fish. This classification of unknown origin fish was a very rigorous challenge of the capabilities of otolith chemistry. The model based on eight potential sources included four "dummy" locations. Further, the unknown samples were otoliths from fish stocked in 2003, while the known origin otoliths on which the model was based were collected in 2004 and 2005. Therefore, we simulated a situation where otolith data were used to identify origins of fish stocked in previous years. Despite these obstacles, the model displayed an overall success rate of 59% (Table 7). This level of success is a testament to the stability of otolith signatures within a location over time as well as the stability of otolith signatures in hatchery fish that have been at large for long periods of time. When only the four true source hatcheries were included in a DFA model, the average accuracy increased to 79%, again, highlighting the improvement of model performance with smaller pools of candidate hatcheries.

Although Sr and Ba were the only elements that proved to be reliable markers, strontium isotopes in otoliths were correlated with strontium isotopes in water (Figure 8. We observed a departure from the expected 1:1 relationship between otolith and water, however, which is consistent with other studies of hatchery fish. Otoliths of wild diadromous fish tend to reflect the unadulterated isotopic ratio of the ambient water (Kennedy et al. 2002; Woodhead et al. 2005), but the influence of marine-derived feed appears to exert an influence on $^{87}Sr/^{86}Sr$ ratios in hatchery-reared salmonids (Ingram and Weber 1999; Kennedy et al. 2002). Seawater has a globally constant $^{87}Sr/^{86}Sr$ value of 0.709172 (Hodell et al. 1990), while freshwater systems have a range of values above and below seawater levels (Graustein 1989). Hatchery-reared fish inhabiting waters with $^{87}Sr/^{86}Sr$ ratios below seawater had otolith $^{87}Sr/^{86}Sr$ ratios higher than that of the ambient water, while hatchery-reared fish inhabiting water with $^{87}Sr/^{86}Sr$ ratios exceeding those of seawater had $^{87}Sr/^{86}Sr$ ratios in their otoliths lower than the ambient water. The marine-derived feed appears to "pull" the otolith $^{87}Sr/^{86}Sr$ ratios towards the seawater average without obscuring the ambient water values. Thus, the $^{87}Sr/^{86}Sr$ ratio appears to be a valuable environmental marker for hatchery fish. Model accuracy improved substantially with the addition of $^{87}Sr/^{86}Sr$ ratios. For the subset of five hatcheries for which both elemental abundance and isotopic ratios were collected,

average accuracy rose from 63% using only Sr and Ba to 96% with the addition of isotope data (Table 8).

Transects of otoliths from fish known to have moved between locations indicate $^{87}Sr/^{86}Sr$ ratios are more sensitive to movements than elemental abundances. In the seven instances where we collected otoliths of fish that were moved from one hatchery to another but resided at each location for more than one month, we observed unequivocal shifts in elemental abundance in only one (TSP to TFH; Table 9). Many of the unknown origin fish we collected also failed to show differences between the core and edge portion, although we know they had moved. However, shifts in $^{87}Sr/^{86}Sr$ ratio were clearly evident in the only two groups of fish for which $^{87}Sr/^{86}Sr$ data are available.

Twenty otoliths from BRR were analyzed for elemental abundance, and a subset of five was analyzed for $^{87}Sr/^{86}Sr$. In 11 of 19 elemental transects, we were able to observe distinct core and edge signatures corresponding to the material deposited at Bellvue Hatchery and BRR, respectively. Transects of $^{87}Sr/^{86}Sr$ were more revealing, distinguishing the core and edge signatures in all five otoliths analyzed; transects of elemental abundance for these same five otoliths only revealed core and edge signatures in two cases. Thus, we believe $^{87}Sr/^{86}Sr$ analysis was the most effective means to discern movement between locations in our study area, as elemental abundance transects often failed to detect movements which are known to have occurred.

Failure to detect movement is likely when source and destination locations have similar water chemistry. However, as more chemical markers are examined in the otolith it becomes increasingly improbable that source and destination water signatures will match in every chemical constituent and a “tattletale” marker will emerge. There are other promising markers being examined in the field of otolith microchemistry that will improve the ability to detect movements of hatchery fish. In situ analyses of sulfur isotopes ($^{34}S/^{32}S$) have been used to reveal source, movements and diet of stocked vs. wild salmon (Weber et al. 2002), and should be evaluated in future research on hatchery trout. Deuterium ($^{2}H/^{1}H$ ratio, δD) in water was recently shown to be highly correlated with otolith δD, proved instrumental for distinguishing pond from river resident fish (Whitledge et al. 2006; Whitledge et al. In press), and we observed large variations

in water δD among hatcheries (Figure 9). Analysis of δD in otoliths is currently restricted to bulk analysis methods which are not well suited to detecting changes within an otolith. With advances in instrument technology (e.g., Weber et al. 2002), it may become possible to examine δD in discrete portions of otoliths. Given the variation we observed in our samples, δD could become a valuable new marker to further identify the origins and movements of hatchery reared trout.

The chemical composition of hatchery feed did not appear to be a significant factor in classifying fish to their hatchery of origin using otolith chemistry. Although some debate exists in the literature, evidence from experimental studies shows feed provides only a minor amount of the Sr and Ba deposited on otoliths (Farrell and Campana 1996; Walther and Thorrold 2006). Further, we observed variations in the chemical composition of hatchery feed of different size pellets (Table 2). If feed was a major determinant of otolith chemistry, we would have seen changes in the otolith chemistry in the line transects as the fish moved from one size of feed to the next. We did not see such changes in transects, and coupled with the existing literature on the subject, we feel confident in assigning a minimal role to feed in elemental abundance of otoliths, in our study. As our otolith:water $^{87}Sr/^{86}Sr$ ratios showed, feed (mean $^{87}Sr/^{86}Sr$ = 0.7074) exerted a predictable "pull" on $^{87}Sr/^{86}Sr$ ratios toward the global seawater average. Although the Sr isotope ratio of hatchery fish otoliths is impacted by feed chemistry, it remains a very useful environmental tracer.

Conclusions and Recommendations

In this section we offer several conclusions and recommendations to fishery managers, biologists and law enforcement officers interested in adopting otolith microchemistry to help combat illegal fish introductions. We provide 1) our conclusions regarding the technique's utility and promise, 2) practical considerations and potential pitfalls that may arise when the method moves from the scientific realm to a management and perhaps legal arena, and 3) some recommendations to facilitate the adoption of otolith chemistry as another tool in the fishery manager's toolbox.

Utility for Management

We are confident that otolith chemistry will be useful for tracking origins and movements of illegally stocked trout. We found that a combination of three naturally-occurring chemical markers varied enough among hatcheries to allow us to identify the hatchery of origin of groups of fish with up to 96% accuracy. Although we were not always able to detect movement of fish among hatcheries, the core of the otolith always provided a reliable chemical signature of the location where the fish was first reared. If this is the extent to which otolith microchemistry is informative in some cases, it will provide investigators with information unattainable through any other techniques and could serve as the linchpin in a criminal case. Chemical signatures of hatcheries were stable across several years: interannual variation in water chemistry measurements was insignificant in comparison to variation among hatcheries, and multivariate models developed from fish sampled in 2004 and 2005 were able to classify blind samples of fish captured in previous years. Overall, we conclude that otolith chemistry does indeed have considerable potential as a fishery management tool and that it will be useful for tracking down sources of illegally stocked fish in Colorado. Based on our own findings and on a growing literature (Brenkman et al. (2007); Clarke et al. (2007); Courtemanche et al. (2006); Downs et al. (2006); Ludsin et al. 2006; Munro et al. (2005); Wells et al. (2003); Kennedy et al. (2002)) we believe that otolith chemistry will work for tracking provenance of trout and other salmonids virtually anywhere these fishes are found. The ability of the technique to discriminate fish from two different locations is limited only by the variation in geochemistry.

Otolith chemistry can provide powerful insights into the provenance of stocked fish that are not attainable by other means. To make a comparison to criminal forensics, the technique cannot provide the one-in-a-million accuracy of DNA fingerprinting, but it is capable of providing far greater resolution than that from blood type. The great advantage of otolith chemistry is that it can reveal the locations a fish has inhabited throughout its lifetime. The markers we have used – Sr, Ba, and $^{87}Sr/^{86}Sr$, as well as potential markers like δD – yield reliable information about the environment a fish has inhabited. This cannot be achieved using methods like DNA analysis, as offspring of the same broodstock may go to several different locations.

Further, otoliths are permanent structures – a sort of biological passport capable of indelibly recording the locations a fish has inhabited. These "passports" are present in every trout and allow investigators to look into the residence history of any individual.

Like DNA analysis, otolith chemistry is most useful when it can be compared to that of "suspects". In cases where reference samples are unavailable, chemical analysis of the otolith can be used to develop a "composite sketch" of the suspect source. Thus, even if the suspect is not in the lineup, circumstantial or other evidence can be used to exonerate innocent look-alikes and the sketch can be used to continue searching for more likely suspects based on insights the otolith lends into the water chemistry of the source location and its surrounding geology. Thus, otolith chemistry can be a valuable investigative tool that can direct officers toward the most fruitful lines of inquiry.

Pitfalls and Practicalities

Typically, the chemical signature of otoliths from a source location is described by a multivariate model (we used DFA, other approaches are available). When trying to determine the source of a fish one can use the model to classify the unknown fish to the source in the model that it most resembles. If the true source is not present in the model then the model cannot classify correctly. This scenario is analogous to a police lineup involving a group of suspects that does not include the actual criminal and forcing an eyewitness to pick the suspect who most closely resembles the criminal. Investigators need to be aware of such situations and work diligently to ensure they do not miss any potential suspects. As noted above, investigators must interpret DFA results within the context of other lines of evidence. A lesser risk is associated with considering too many suspect sources. Our Monte Carlo simulation showed that classification accuracy decreases as the number of suspects classified increases. When too many suspect sources are considered, the accuracy of multivariate models will suffer and they may become unreliable.

Another problem with the multivariate models approach is that these models may not be able to discriminate very similar sources. Thus, otolith chemistry and associated statistics can distinguish sources to a finite degree determined by the natural range of differences in water geochemistry from place to place. While the accuracy of

multivariate models based on otolith chemistry is ultimately dependent on the environment, the discriminatory power of models improves as more markers are added, because even sites near to each other are bound to differ in some chemical component. Unfortunately, most markers require extremely sophisticated instruments to measure and interpreting the resulting data requires input from scientific experts. As the number of markers examined increases, the cost of the analysis and the time needed to analyze data increases as well. Currently, it would take three instruments to analyze elemental abundance (Sr and Ba), δD and $^{87}Sr/^{86}Sr$.

There are a handful of excellent laboratories around the world that are doing otolith chemistry analyses on a contract basis (these labs can be readily identified from recently published articles). Costs, sample preparation requirements and turnaround time undoubtedly vary. However, we found that analytical labs often experience high demand and sample turnaround time may not coincide with agency deadlines. We chose to collect, prepare, and in the case of the USGS LA-ICP-MS Lab, partially analyze our own samples. To assist agencies or others considering adopting otolith chemistry as a tool, we have provided an outline of basic procedures (Appendix 1) and estimated costs for each aspect of the process (Appendix 2). Depending on arrangements worked out with the laboratory that will be doing the analytical work, actual costs required to prepare your own samples may be much less. If there are labs that offer complete analysis services then it may be possible to submit whole otoliths and avoid the trouble and expense of gearing up to section and polish otoliths prior to sending them in for analysis. This may be a cost effective option for entities not planning to do much otolith work over the long term.

We discovered that vaterite formation can be a significant problem in otoliths of hatchery trout. Vaterite completely obscured the environmental signature in the otolith in almost 10% of our samples and thus those were unusable. However, we rarely found that both otoliths of the same fish were entirely vateritic, so in most cases at least one aragonitic otolith should be present in each fish collected. Over 25% of the otoliths we collected had vaterite deposits towards the edges of the otoliths. As a result, the core aragonite signature (the signature of the hatchery of origin) was preserved but edge portions were unusable. Therefore, vaterite formation in otoliths is most problematic for

tracking movements, less so for determining the first hatchery in which a fish resided. When vaterite formation begins prior to the movement of fish, otolith chemistry can still yield insight regarding the first environment the fish has inhabited but cannot document any subsequent movements. Future work should inflate sample size estimates by about one third to account for the presence of vaterite in hatchery fish.

As fishery managers and wildlife officers well know, fish stocked by private vendors can take a circuitous route to their final destination; this is part of the impetus for our study. Otolith chemistry is not a silver bullet that will give perfect knowledge of these movements. Several practical and natural constraints must be taken into consideration. In some circumstances movements will go undetected from an examination of otolith chemistry alone, and there were instances in our study where analysis of otolith transects did not reveal movements of fish between locations that were known to have occurred. Refinements in technology may help a little, but in general, otolith chemistry will have a hard time identifying movements of fish under the following circumstances:

1. source and destination waters possess very similar water chemistry,
2. fish are moved between locations with similar water chemistry before they arrive at their final destination, or
3. fish are moved from a location before a discrete chemical signature of that location can be imparted to the otolith.

Consider four hypothetical stocking scenarios and how they may be perceived from an examination of otolith chemistry (Figure 10). Under ideal circumstances, fish are raised at a single source and then stocked at their final destination, and a clear chemical signature of the source hatchery is discerned from the otoliths (Figure 10A). The fish captured from Button Rock Reservoir (BRR) are an example of such a case. Alternatively, fish may be reared at one location for a period of time, transferred to and reared again at another location exhibiting different water chemistry before being stocked at a final destination that also possessed a unique chemical signature (Figure 10B). When the water chemistry of a transient location and the final destination are

similar (Figure 10C) or chemistry is similar among multiple transient locations (Figure 10D) then it becomes much harder to piece together a complete picture of the movement history of the fish. Our data from known hatchery movements (Table 5) are insightful here. In one instance, the elemental markers Sr:Ca and Ba:Ca revealed the movement between hatcheries (TFH-TSP). In the other six cases, Sr:Ca and Ba:Ca did not reveal movement between locations. However, of the six cases where elemental abundance proved uninformative, $^{87}Sr/^{86}Sr$ data were available for two and the movement between hatcheries was detected in both cases. Thus, when fish are moved between hatcheries, our data suggest that multiple types of markers may be required to detect such movements.

Because it may take up to 30 days of residence in a location for a detectable chemical signature to be imparted to the otolith (Forrester 2005), movements at shorter intervals may not be discernible from otoliths (see Kennedy et al. 2002 for an example of transition periods between distinct environments). However, in each case, because trout are generally not moved at a small size post-hatching, the region near the otolith core will provide a reliable chemical signature of the hatchery where the fish originated. That information could become valuable when used in conjunction with other lines of evidence, as we propose below.

Recommendations for Implementation

Otolith chemistry can play a valuable role in identifying the origins and movements of stocked fish. It is ideally suited to fill in gaps left by traditional investigative methods. Like nearly all advances in technology, otolith chemistry is not a panacea, but rather a tool that is highly effective if used appropriately. Critical steps at the outset of an investigation create the conditions necessary for otolith chemistry to be most informative. Before the source of illicitly stocked fish can be identified, evidence in the form of otoliths from fish reared at each suspect source should be obtained so that they may be compared to those of the stocked fish. It is essential to be rigorous and thorough in assembling this reference archive of otolith signatures; this is a foundation on which the rest of the investigation may be built.

Sample size is an important consideration because chemical composition of otoliths varies among fish at the same location, and otolith chemistry is relatively expensive work (however, otoliths are easy to store and one does not have to analyze everything that is collected). Our data can serve as an appropriate guide for statistical power calculations in future studies. At a minimum, we recommend that a sample size of at least 13 fish per site (allowing for vaterite losses) be analyzed. Since this may not be possible in all cases, we expect that our database of otolith signatures may become valuable in situations where investigators are constrained by the number of illicitly stocked fish they have obtained. We also caution that otolith chemistry works best for classifying groups of fish rather than individuals. Even for locations in our study which displayed high overall accuracy rates, individual misclassifications occurred. Thus, we would have less confidence in assigning origins to an individual fish than to a group of fish. Note that otolith chemistry may still offer some useful information in a worst case scenario, where only a few or a single illegally stocked fish is available, and there are no suspects to compare to. In that situation the chemical composition of the illegal fish can be thoroughly described and inferences about source water chemistry and therefore local geology may emerge, thus narrowing the geographic scope of the investigation.

Realistically, we do not believe otolith chemistry is at the stage of being an “off the shelf” technology that agencies can turn to for unambiguous answers from contract labs. As was the case with molecular genetics analysis in the early years, there is considerable potential for misinterpretation and inappropriate conclusions when lab analysts unfamiliar with the local context and agency clients untrained in the intricacies of the methodology collide. Without a scientist intermediary to help ask the appropriate questions, gather the appropriate samples and help interpret the data with the agency clients, the most sophisticated technology can be worse than useless.

Otolith chemistry is a tool that is ready to be applied to some real world problems that agencies are struggling with, foremost among them is illegal stocking. But, we recommend that agencies enlist the assistance of scientific experts from the very beginning of any efforts to use the tool, particularly in a forensic application. In addition to the valid insights an expert brings, other beneficial aspects include quality assurance/quality control of the samples and data, statistical rigor, and maximum

scrutiny of potential markers. The Mountain West could prove to be fertile ground for new markers to be applied to otolith chemistry studies. Novel markers may arise in areas where unique geology or human impacts (e.g., mining or other industrial uses) have occurred. In order for these markers to be useful, care must be taken to identify a priori which new markers may occur in the study area through consultation with geologists, watershed scientists, and ecotoxicologists. In some cases, different instruments or laboratories may be necessary to evaluate new otolith markers due to the sensitivities of the instruments and the chemical properties of the marker. Furthermore, instrumental precision may not be simultaneously maximized for all elements, necessitating careful selection of the suite of elements analyzed prior to analysis. But if new markers can be identified it will become easier to identify where an illegally stocked fish originated, or at least it will be easier to eliminate locations where it could not have originated.

As our analyses showed, the multivariate models classified fish to their source location (hatchery) more accurately when there were fewer candidate locations and when there were more classifying variables (markers). We found that a small number of markers (e.g., Sr, Ba) could not distinguish otoliths from locations with similar water chemistry but adding another piece of information about the locations ($^{87}Sr/^{86}Sr$) allowed the model to eliminate some locations because their chemical signatures no longer overlapped. While it may not always be possible to definitively identify a source with otolith chemistry alone, otolith chemistry can assist investigators by narrowing their search in a process of elimination in which various independent lines of evidence serve to filter out possible sources until the most likely source emerges. In this “Eclectic Approach to Source Identification” (Figure 11), evidence from otolith chemistry complements that derived from classical detective work and more traditional forms of stock identification (e.g., genetics, Feyrer et al. 2007). We believe the Eclectic Approach will help make the results more clear to those unfamiliar with otolith chemistry and increase the confidence in the outcome. Just as criminal cases are bolstered when DNA evidence is used along with more traditional types of evidence, so too will investigations of illicit stocking become stronger when otolith chemistry is used with other lines of evidence.

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Table 1. Codes, names, and locations of hatcheries sampled during 2004-2006. Configuration of each facility's water supply is also given. All hatcheries except TFH were operated by the Colorado Division of Wildlife; TFH was operated by Wyoming Game and Fish Department. Hatchery codes in bold text indicate that otoliths of fish from the hatchery were analyzed for elemental abundance.

			Water supply		
Code	Hatchery name	UTM	Type	Source	n
BLV	Bellvue	13T 485700 4497678	Ground	Well	3
CCL	Chalk Cliffs	13S 401752 4289786	Surface	Chalk Creek	3
CRU	Crystal River	13S 310143 4361016	Ground	Spring, well	3
DUR	Durango	13S 245031 4129967	Ground	Springs	3
FRH	Fish Research Hatchery	13T 485700 4497678	Ground	Wells	2
FRO	Finger Rock	13T 337021 4441493	Ground	Springs	2
GSU	Glenwood Springs (hatch house)	13S 296419 4383375	Ground	Spring	3
GSU	Glenwood Springs (raceway)	13S 296419 4383375	Surface	Mitchell Creek	3
MOH	Mt. Ouray	13S 409394 4268124	Ground	Spring	3
MSH	Mt. Shavano	13S 411108 4266683	Ground	Spring	3
MVU	Monte Vista	13S 406628 4154264	Ground	Wells	3
PIK	Pitkin	13S 366560 4273141	Ground	Springs	3
PRU	Poudre Rearing Unit	13T 439979 4505679	Surface	Cache la Poudre River	3
RIF	Rifle Falls	13S 268465 4397368	Ground	Springs	3
ROJ	Roaring Judy	13S 338886 4286770	Ground	Spring, well	3
SLS	San Luis Valley	13S 412821 4122781	Ground	Well (irrigation)	3
TFH	Tillet Springs Rearing Unit	12T 732695 4979547	Ground	Springs	0
WAT	Watson	13T 485700 4497678	Surface	Cache la Poudre River	3

Table 2. Barium, strontium and strontium isotope signatures of trout feed sampled from several CDOW hatcheries, one private and one federal hatchery in 2004 and 2005.

Hatchery/date sampled	Feed manufacturer	Feed size	Ba:Ca (nmol /µmol)	Sr:Ca (nmol/µmol)	$^{87}Sr/^{86}Sr$
CDOW					
07/19/04	Rangen	#0	0.046	1.727	0.7070
07/20/04	Rangen	#1	0.109	0.801	0.7120
07/19/04	Rangen	#2	0.095	0.654	0.7110
07/20/04	Rangen	#3	0.169	1.019	0.7080
07/19/04	Rangen	#4	0.151	0.802	0.7080
07/19/04	Rangen	3/32"	0.223	0.820	0.7060
07/19/04	Rangen	1/8"	0.234	1.539	0.7070
08/20/04	Rangen	1/8"	0.131	0.957	0.7080
03/16/05	Rangen	1/8"	0.188	0.986	0.7060
05/04/04	Rangen	3/16"	0.167	1.220	0.7060
07/19/04	Rangen	3/16"	0.227	0.972	0.7050
		Mean	0.158	1.045	0.7076
Private					
07/21/04	Nelson	#0	0.020	1.687	0.7040
07/21/04	Nelson	#0	0.095	0.602	0.7090
07/21/04	Nelson	#2	0.060	1.214	0.7090
		Mean	0.058	1.168	0.7073
Federal					
03/18/05	Nelson	#1	0.062	1.628	0.7110
03/19/05	Nelson	#2	0.066	1.675	0.7040
03/20/05	Nelson	#4	0.115	0.619	0.7070
03/21/05	Nelson	3/32"	0.075	0.679	0.7070
		Mean	0.080	1.150	0.7073
		Grand mean	0.099	1.121	0.7074

Table 3. Collection site, year, species, and total length of trout collected directly from hatcheries and used for chemical analyses of otoliths. Mean total length (TL, mm) is reported with standard deviation in parentheses. Dashes denote location/year combinations when no fish were collected.

	2004			2005		
Hatchery	Date sampled	Mean TL	n	Date sampled	Mean TL	n
BLV	09/03/04	116 (15)[1]	10	04/08/05	56 (4)	10
CCL	07/2104	294 (13)	10	03/15/05	251 (13)	10
				03/17/05	288 (39)	10
CRU	07/19/04	311 (19)	10	03/17/05	70 (8)	10
DUR	07/20/04	276 (20)	10	03/14/05	244 (18)	10
GSU	07/19/04	221 (14)	10	03/17/05	121 (14)	10
MSH	07/21/04	139 (15)[1]	10	03/15/05	154 (19)	10
PRU	09/10/04	231 (27)[1]	10	04/08/05	230 (20)	10
RIF	07/20/04	283 (24)	10	03/17/05	230 (22)	10
ROJ	09/09/04	230 (27)	11	03/16/05	236 (34)	11
SLS	07/21/04	223 (12)	10	03/15/05	208 (17)	10
TFH	--	--	--	10/24/05	303 (24)	10
WAT	09/03/04	276 (23)	10	04/08/05	236 (16)	10

[1]rainbow x cutthroat hybrid

Table 4. Samples of fish that were known a priori to have resided at multiple hatcheries. We collected the fish from the destination hatchery at the specified size at collection on the date of collection shown (n = 10 in each case).

Hatchery of origin	Size at transfer (mm)	Date of transfer	Destination hatchery	Size at collection (mm)	Date collected
BLV	140	March 2004	WAT	276	September 2004
BLV	127	August 2004	WAT	236	April 2005
BLV	127	August 2003	PRU	231	September 2004
BLV	191	August 2004	PRU	230	April 2005
MOH	76	November 2003	SLS	223	July 2004
MSH	76	September 2004	SLS	208	March 2005
TSP	64	June 2004	TFH	303	October 2005

Table 5. Hatchery of origin, site, date, and mean length (TL, mm) of at-large fish collected by CDOW researcher Kevin Thompson and provided to CSU as blind samples ("unknown origin fish") for use in testing DFA classification models. Mean length is shown with SD in parentheses; mean length of fish collected in December was determined from fish grouped into size classes. Fish originating from DUR and RIF were known to be of the 2003 year class; other fish were of unknown age.

Hatchery of origin	Collection site	Date collected	Mean TL	n
ROJ	ROJ channel	12/01/04	253 (--)	45
DUR	ROJ ponds	11/12/04-11/30/04	301 (41)	27
RIF	ROJ ponds	11/12/04-11/30/04	304 (23)	18
ROJ	ROJ ponds	11/12/04	282 (19)	11
MSH/RIF	Spring Creek	09/08/04	252 (18)	28

Table 6. Classification accuracy of 11 CDOW hatcheries using a discriminant function analysis with only Sr and Ba as classifiers. Accuracy is the percentage of otoliths from each location that were classified to the correct hatchery of origin by the discriminant function; n is the number of otoliths analyzed from each location. Bold numbers along the diagonal also indicate the percentage of otoliths from each hatchery that were correctly classified to their hatchery of origin. Some rows do not sum to exactly 100 due to rounding error. Average accuracy among locations was 59%.

			Location/accuracy (percent)										
Location	Accuracy (percent)	n	BLV	CCL	CRU	DUR	GSU	MSH	PRU	RIF	ROJ	SLS	WAT
BLV	70	17	**70**	0	10	0	0	0	0	0	0	0	20
CCL	76	17	0	**76**	18	0	0	0	0	6	0	0	0
CRU	39	28	0	21	**39**	7	0	0	0	11	0	0	21
DUR	84	19	0	0	0	**84**	0	5	0	5	0	5	0
GSU	67	18	5	0	0	0	**67**	0	5	0	4	0	0
MSH	58	19	0	0	0	11	0	**58**	0	0	0	32	0
PRU	40	20	10	5	5	0	0	0	**40**	0	25	0	15
RIF	72	18	0	0	17	11	0	0	0	**72**	0	0	0
ROJ	83	18	11	0	0	0	6	0	0	0	**83**	0	0
SLS	29	14	0	0	0	36	0	36	0	0	0	**29**	0
WAT	29	14	0	7	14	0	0	0	50	0	0	0	**29**

Table 7. Classification accuracy (percent) of DFA models for a blind sample of CDOW hatchery-reared fish captured at large after stocking. The 8 location model includes the four true sources as well as four hatcheries which were not sources of the fish, while the 4 location model uses only the four hatcheries from which the fish were stocked. The row "MSH/RIF" includes fish that were captured from locations that had been stocked by Mount Shavano and Rifle hatcheries. Otoliths from this group that were classified as MSH or RIF in a DFA model were classified as accurate, although we cannot provide further resolution for those samples.

Hatchery of origin	n	8 location model accuracy	4 location model accuracy
ROJ	57	84	96
DUR	27	64	73
RIF	18	53	73
MSH/RIF	28	36	68
Average accuracy		59	79

Table 8. Percentage of otoliths classified to each of 5 hatcheries in a DFA model using Sr:Ca, Ba:Ca, and $^{87}Sr/^{86}Sr$ and a DFA with only Sr:Ca and Ba:Ca (in parentheses). Average accuracy was 96% for the model including $^{87}Sr/^{86}Sr$ and 63% for the model without $^{87}Sr/^{86}Sr$.

Hatchery	n	CCL	CRU	MSH	PRU	SLS
CCL	5	100 (60)	0 (40)	0 (0)	0 (0)	0 (0)
CRU	5	0 (60)	80 (20)	20 (0)	0 (0)	0 (20)
MSH	4	0 (0)	0 (0)	100 (75)	0 (0)	0 (25)
PRU	3	0 (0)	0 (0)	0 (0)	100 (100)	0 (0)
SLS	5	0 (0)	0 (0)	0 (40)	0 (0)	100 (60)

Table 9. Fish originating in public hatcheries and moved to a different location and the ability of otolith chemistry to detect such movements. Strontium abundance was analyzed for fish from all hatcheries, but fish from only three hatcheries were analyzed for $^{87}Sr/^{86}Sr$ (n = number of otoliths analyzed for each marker, SD in parentheses). The first row (BLV to BRR) represents fish collected in Button Rock Reservoir, CO, and the last row shows fish collected from Tillet Fish Hatchery (TFH) in Wyoming (see Table 4).

Hatchery of origin	Collection site (year)	Mean Sr (ppm)			Mean $^{87}Sr/^{86}Sr$		
		n	Core	Edge	n	Core	Edge
BLV	BRR (2006)	19	Change between core and edge in 11 of 19		5	0.7112 (0.0004)	0.7345 (0.0006)
BLV	PRU (2004)	10	No changes detected		0	Not analyzed	
BLV	PRU (2005)	10	No changes detected		3	0.7112 (0.0002)	0.7170 (0.0027)
BLV	WAT (2004)	4	No changes detected		0	Not analyzed	
BLV	WAT (2005)	10	No changes detected		0	Not analyzed	
MOH	SLS (2004)	4	No changes detected		0	Not analyzed	
MSH	SLS (2005)	10	No changes detected		5	0.7105 (0.0011)	0.7085 (0.0005)
TSP	TFH (2005)	10	412 (95)	860 (41)	0	Not analyzed	

Figure 1. Geologic map of Colorado showing approximate locations of 16 CDOW trout hatcheries sampled during 2004-2006. The 11 hatcheries that were used for developing the DFA models are shown in green.

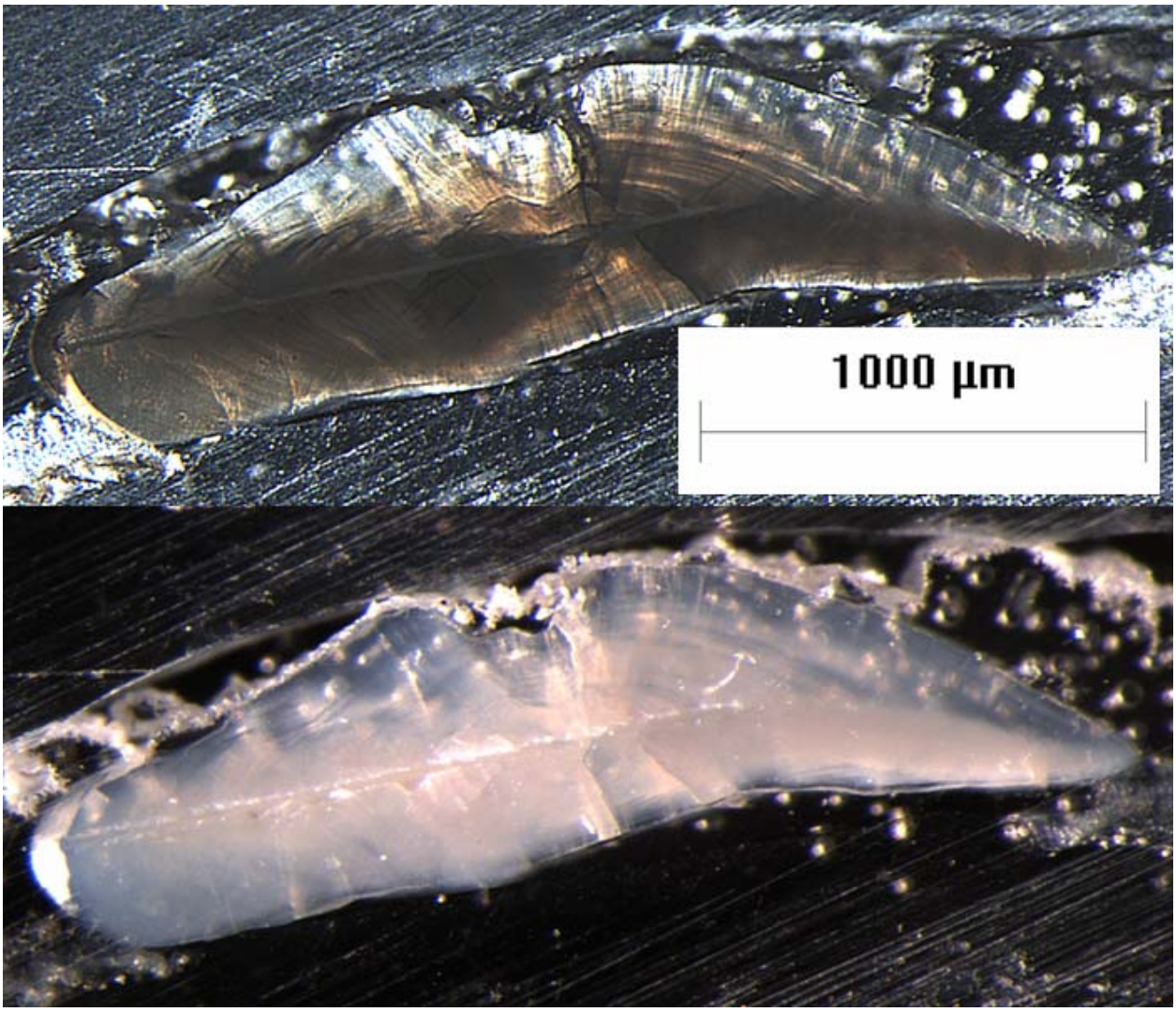

Figure 2. Polished thin section of an otolith extracted from a rainbow trout collected from the Crystal River Hatchery on March 17, 2005, viewed under transmitted light (upper panel) and reflected light (lower panel). A furrow ablated by the LA-ICP-MS laser can be seen running longitudinally from the left side of the otolith to a point about 250 µm to the right of the otolith's core.

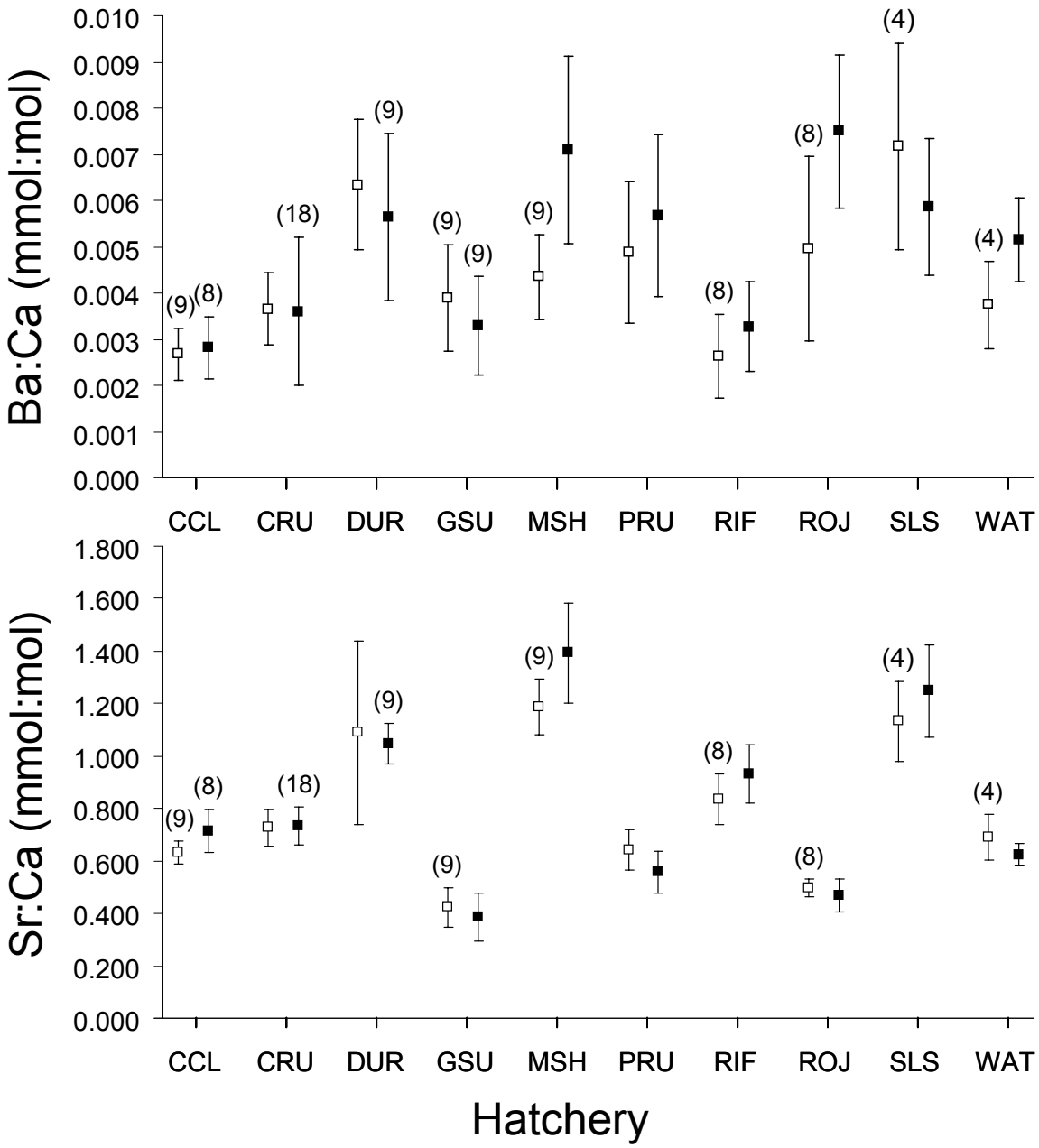

Figure 3. Mean Barium (Ba) and strontium (Sr) concentrations (± SD) in otolith samples from 10 CDOW trout hatcheries sampled in 2004 (□) and 2005 (■). Data from BLV were not used because of physical and chemical abnormalities in otoliths collected in 2005. Sample size was 10 fish unless shown.

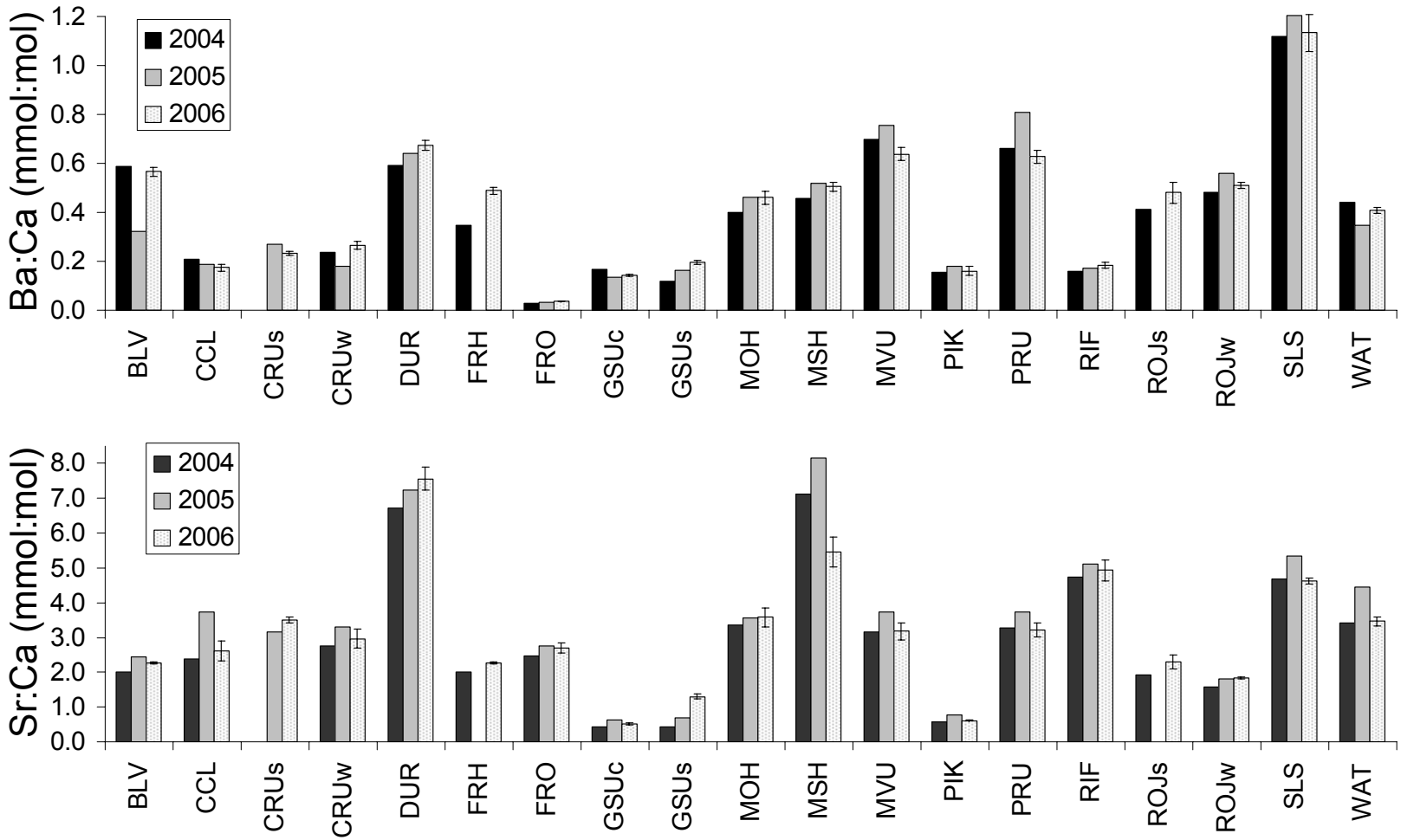

Figure 4. Strontium (Sr) and barium (Ba) concentrations (normalized to calcium) in water samples collected at 16 CDOW trout hatcheries during 2004, 2005, and 2006. Multiple water sources were sampled at CRU, GSU, and ROJ; subscripts "s", "w", and "c" denote spring, well, and creek samples, respectively. All other hatcheries had only one water supply type. Replicate samples were only collected in 2006; bars represent the mean of three to six samples per location collected on a single day, plus or minus one standard deviation. No data were available for some site/years.

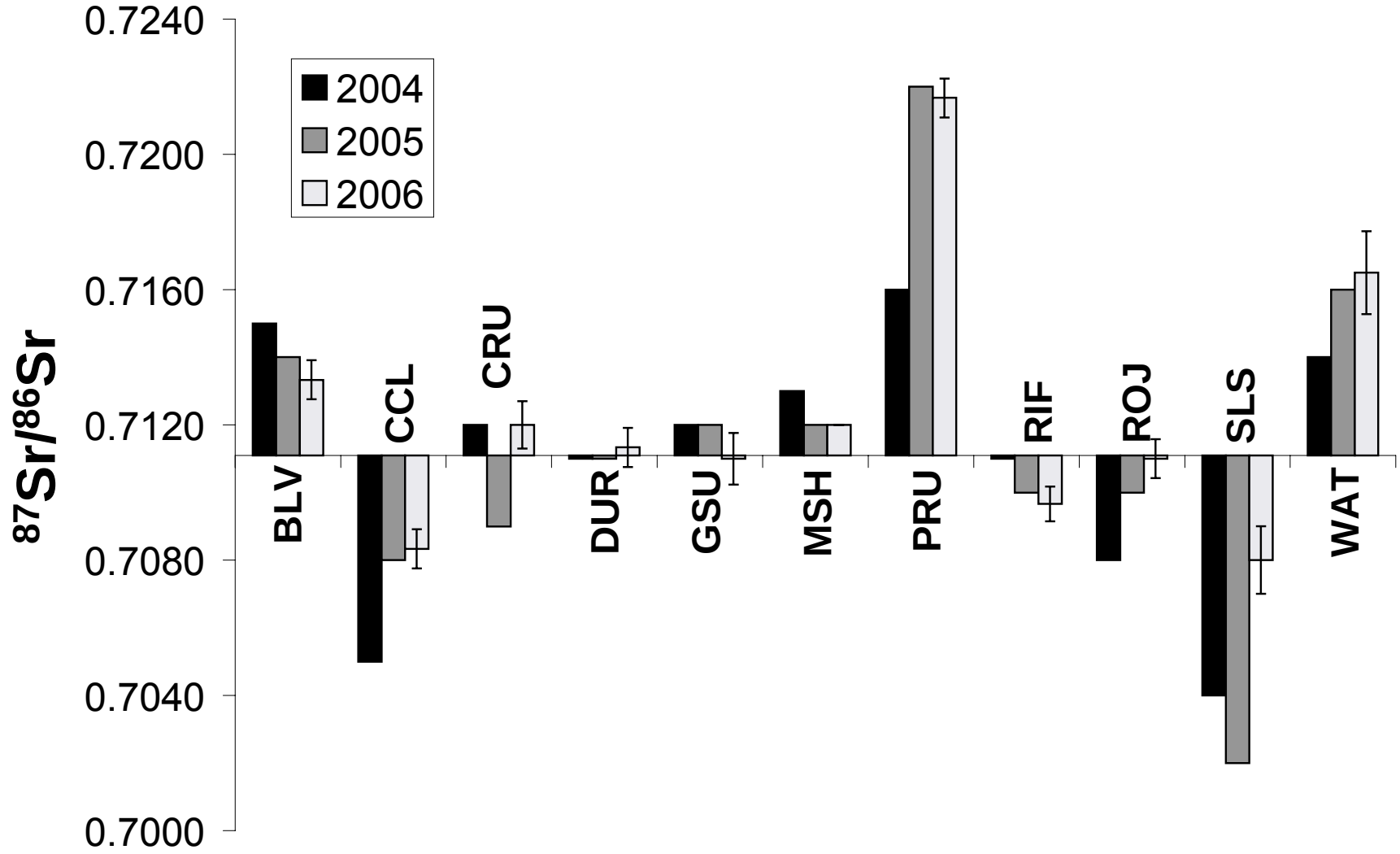

Figure 5. Strontium isotope ratio of water samples collected from 11 CDOW hatcheries plotted as difference from the global freshwater mean (0.711; Graustein 1988). Replicate samples were only collected in 2006; bars represent the mean of three to six samples per location collected on a single day, plus or minus one standard deviation.

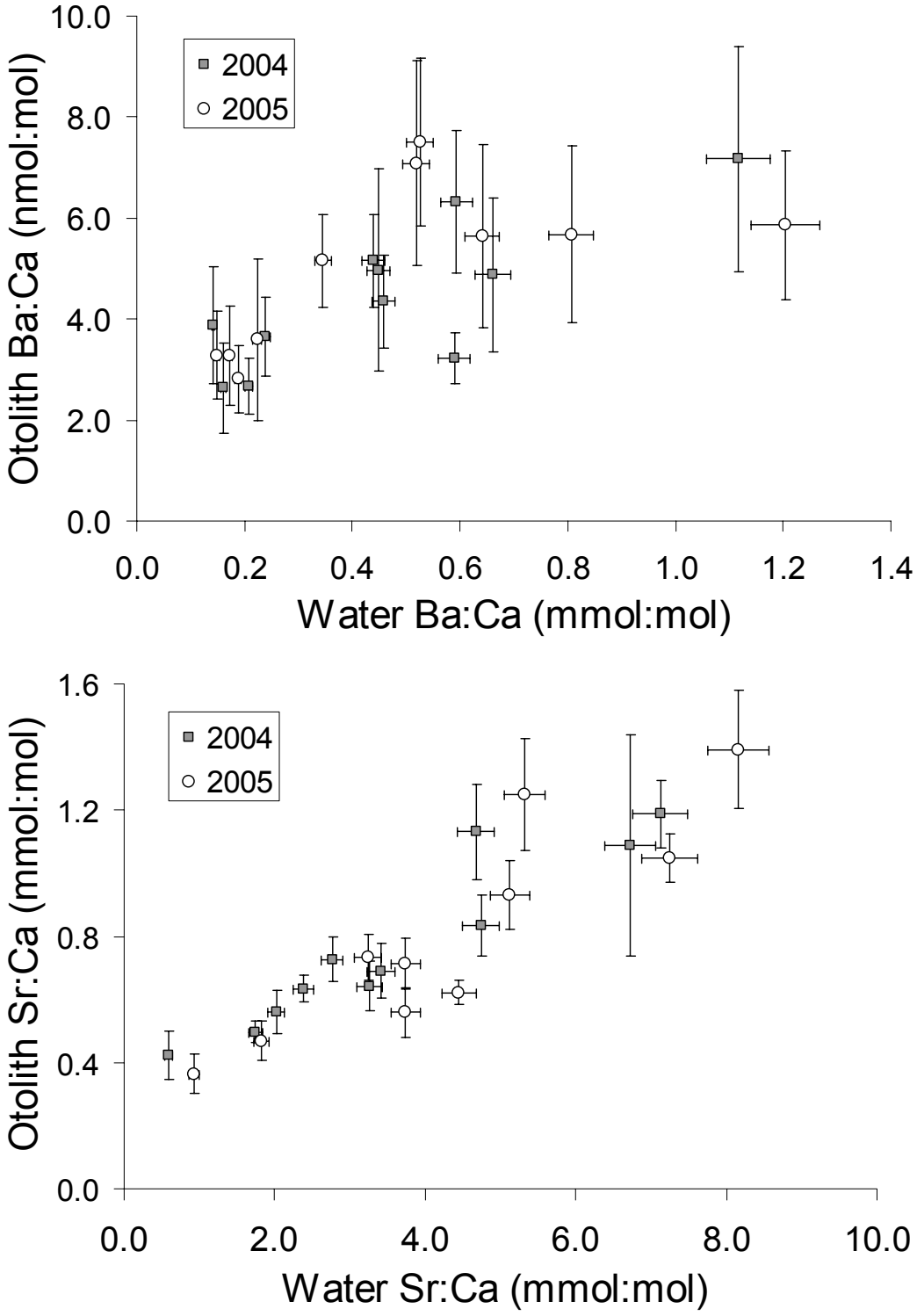

Figure 6. Mean barium (Ba) and strontium (Sr) concentrations (± SD) in otoliths and in water samples at 11 CDOW trout hatcheries sampled in 2004 and 2005.

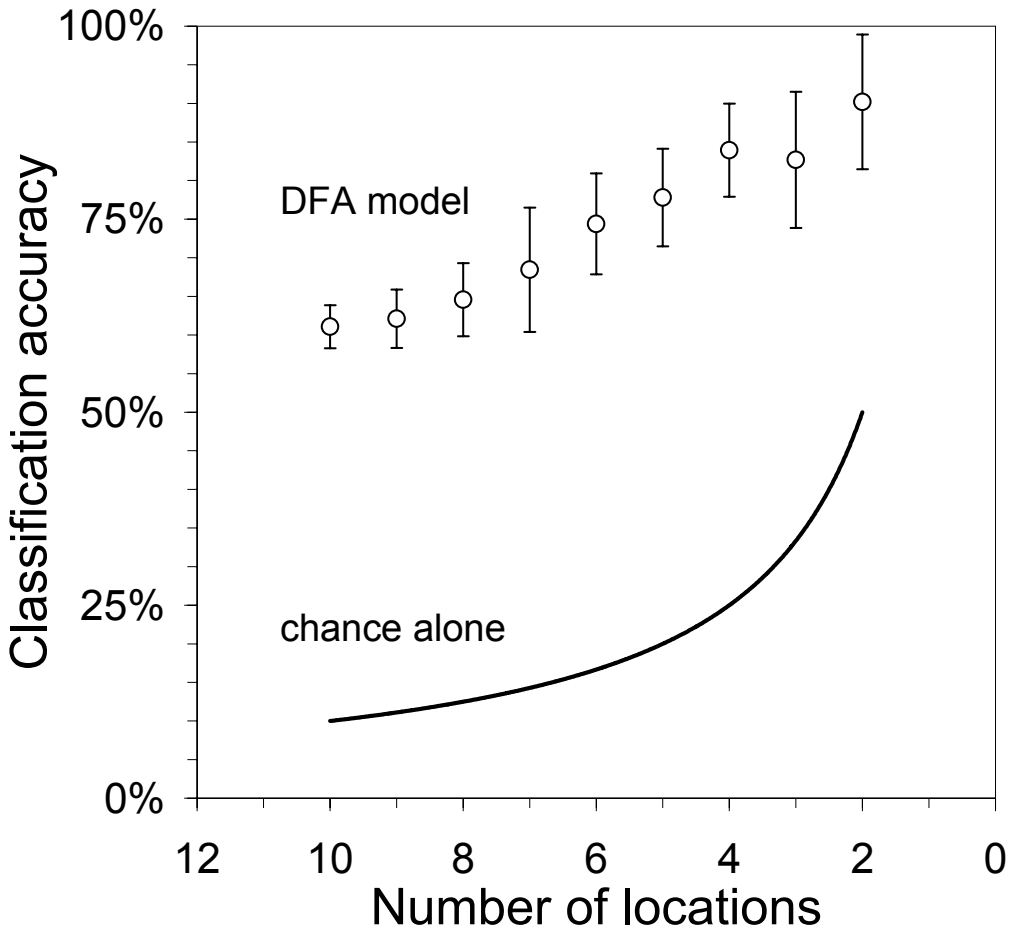

Figure 7. Results of Monte Carlo simulation showing effect of group size on classification accuracy when sets of 10 to 2 hatcheries were randomly selected from the pool of 11 study hatcheries. Circles represent the average accuracy (plus or minus 1 SD) of models based on 10 analyses per group size (all 11 combinations of 10 hatcheries were used for the group size of 10). The solid line represents the expected accuracy of models due to chance alone.

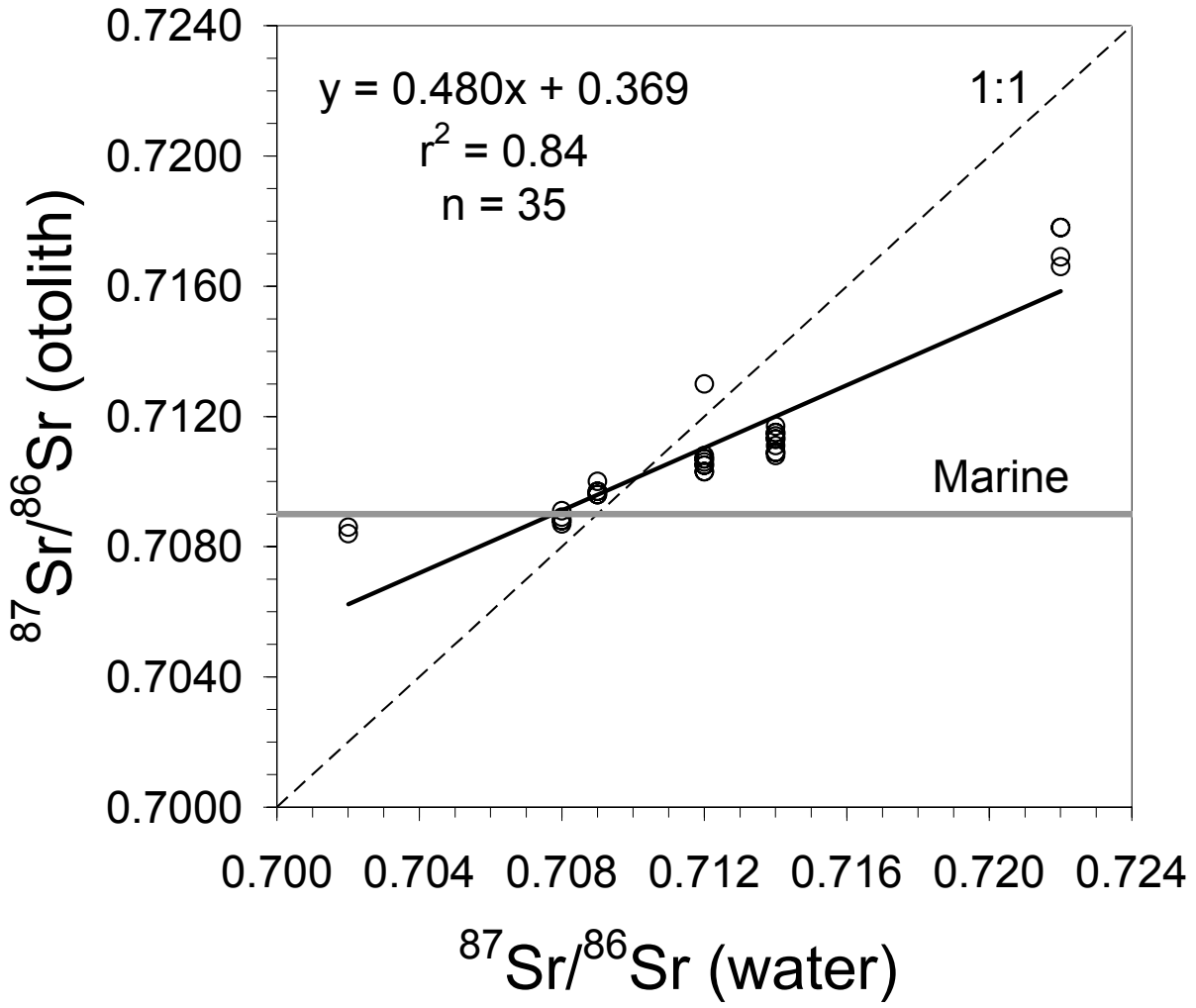

Figure 8. Strontium isotope ratios in hatchery reared trout as a function of the isotope ratio in the water at each hatchery. The 1:1 line represents the slope that would be expected in wild fish (Kennedy et al. 2002; Ingram and Weber 1999). The solid black line represents the slope of the relationship between otolith and water chemistry in our samples, indicating a strong "pull" of marine derived hatchery feed. The horizontal "Marine" bar indicates the global seawater value of $^{87}Sr/^{86}Sr$.

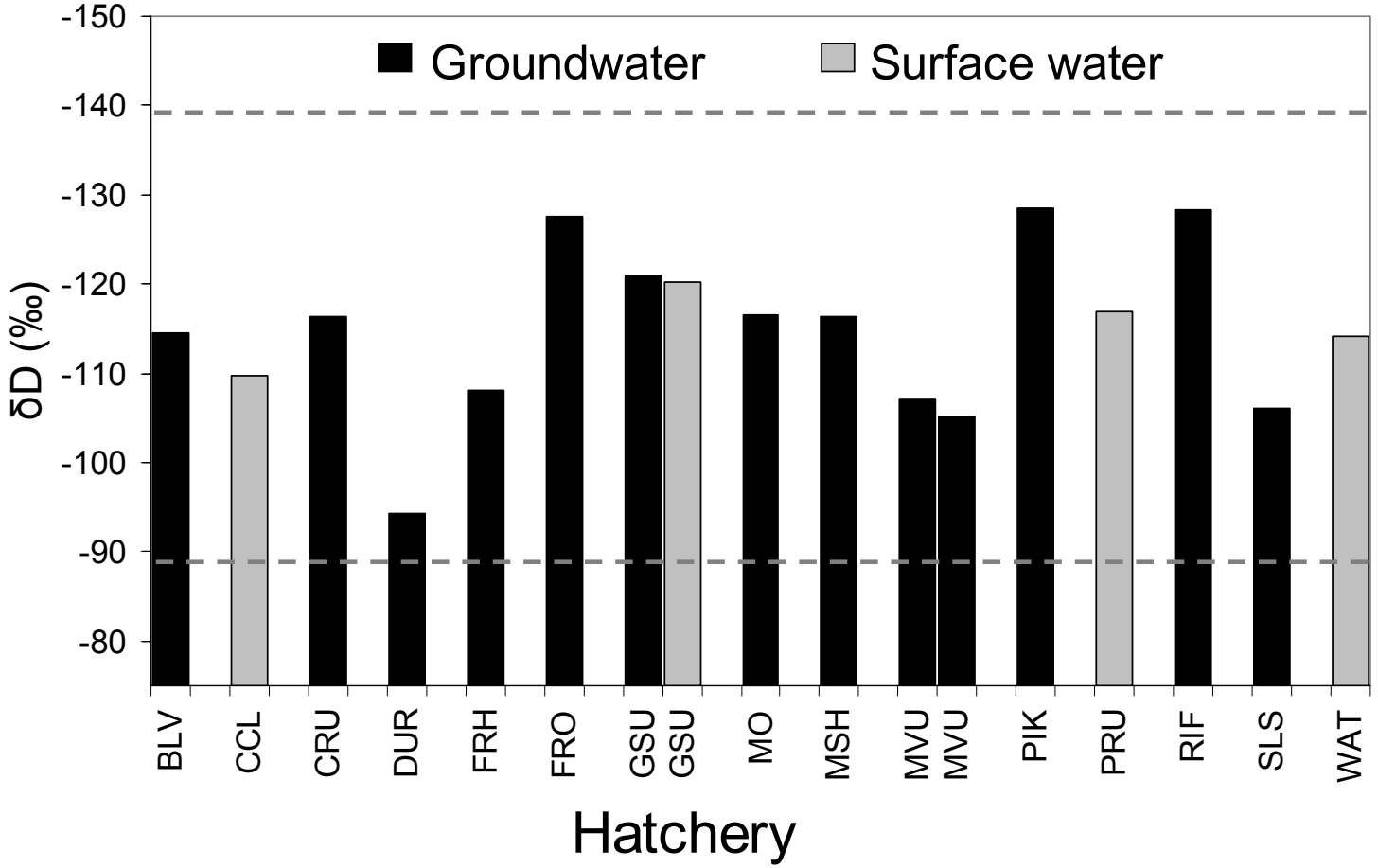

Figure 9. Deuterium signature (δD) of water samples taken from 15 trout hatcheries during July 2004. Three facilities had exclusively surface water supplies (CCL, PRU, WAT), all others were supplied by groundwater sources or a mix of surface and groundwater. Dashed lines show the maximum and minimum δD reported for Colorado surface waters in Coplen and Kendall (2000). Two measurements at MVU represent samples from a shallow (18 m) well and a deep (760 m) well.

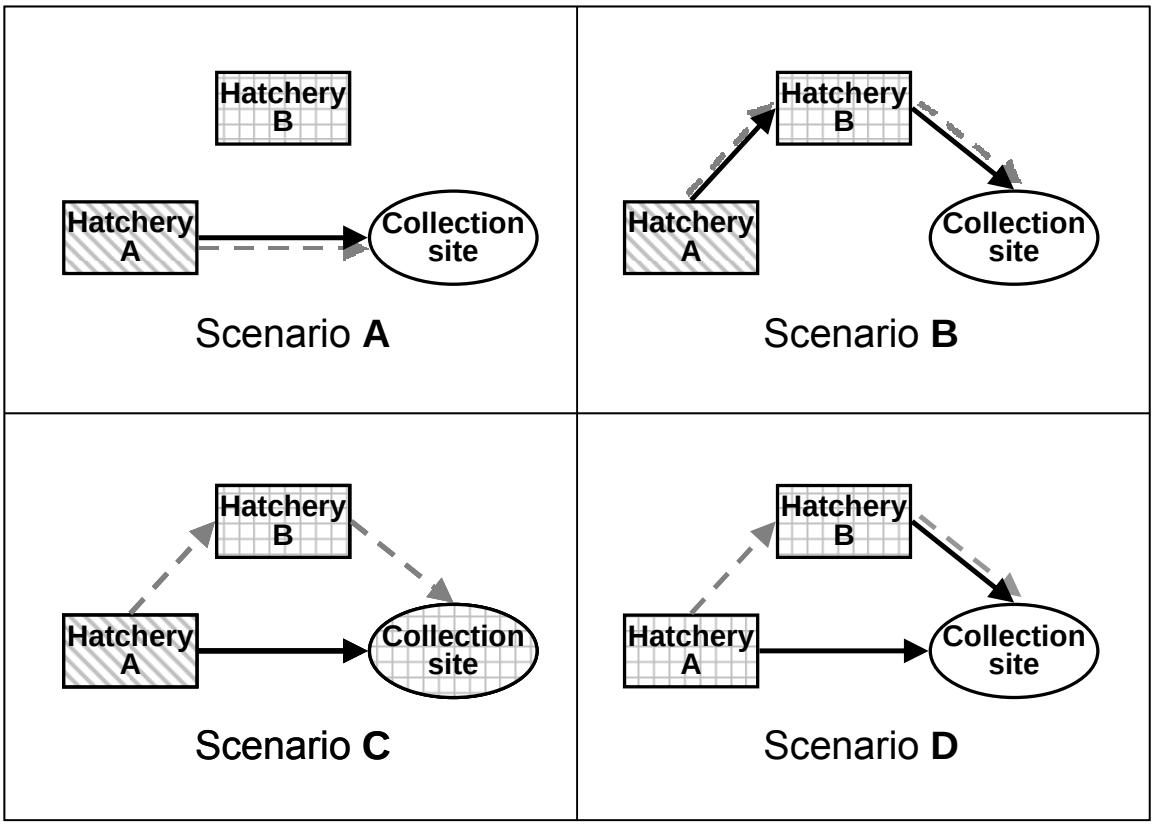

Figure 10. Four hypothetical stocking scenarios and how they are perceived by examination of otoliths of the stocked fish. In each panel, arrows represent direction of fish movement (solid lines = perceived, dashed lines = actual), Hatchery A is where the fish were hatched and reared to some size before being stocked at their final destination (Scenario A) or being moved to Hatchery B (Scenario B, C, D) and subsequently being stocked at their final destination. Cross-hatching represents water chemistry; in Scenarios A and B water chemistry differs among the three locations, but there are only two unique chemistries in Scenarios C and D. In Scenario C, water chemistry of Hatchery A differs from that of Hatchery B and the Collection site, which share the same water chemistry; thus, fish transferred from Hatchery A to B before being stocked at the final destination appear to have been stocked directly from Hatchery A, based on otolith chemistry. This outcome could also arise if fish are moved from Hatchery A to Hatchery B for a short time before being stocked at the Collection site, regardless of the distinctiveness of Hatchery B's water chemistry. In Scenario D, fish may be moved between hatcheries with similar water or not prior to stocking, neither movement nor the exact source are discernible from otolith chemistry.

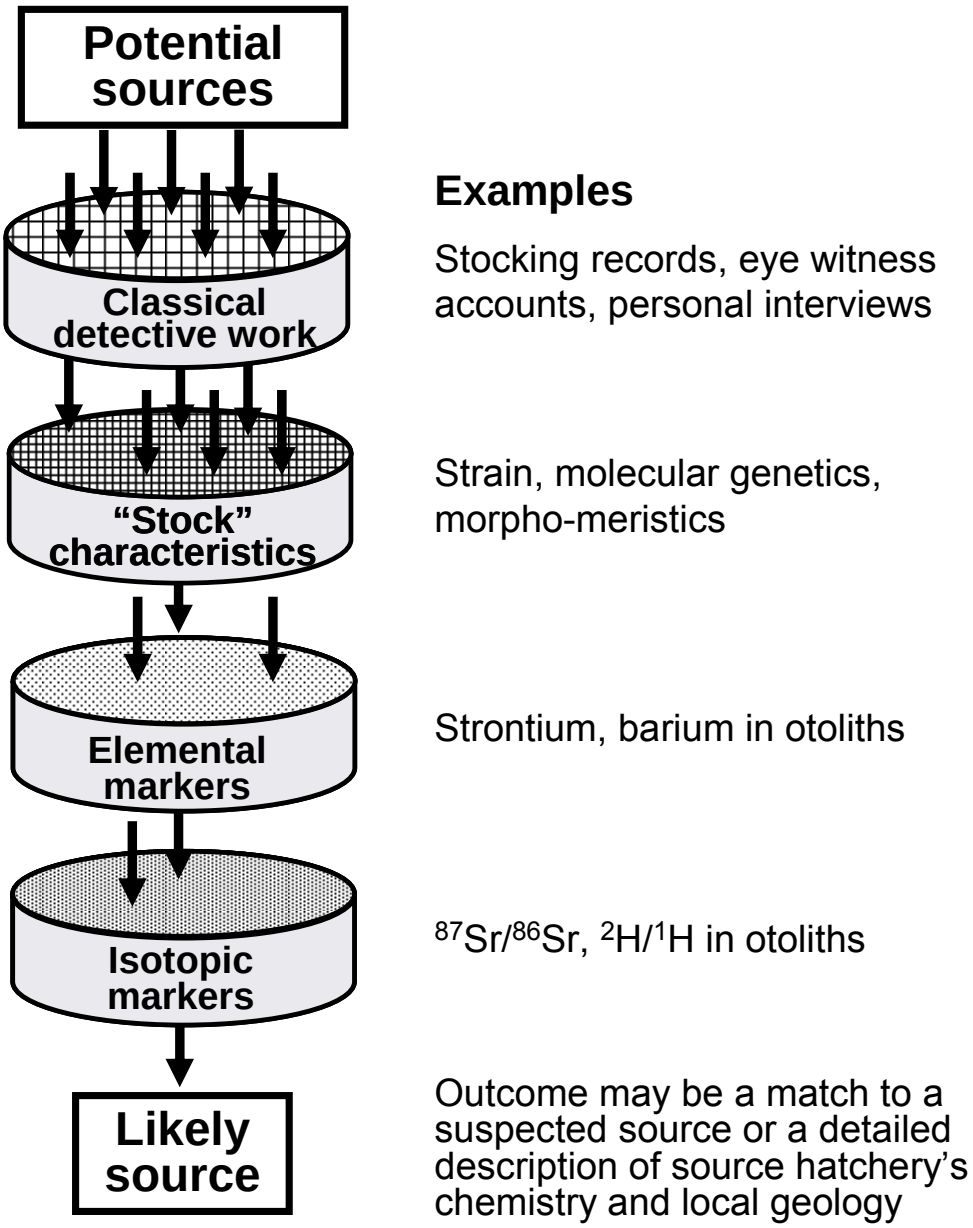

Figure 11. In the "Eclectic Approach to Source Identification" multiple lines of evidence are used to narrow the pool of suspects until the most likely source of an illegal introduction is identified, or until a detailed chemical signature of the source hatchery and its surrounding geology can be constructed from the illegal fish's otoliths. With this approach investigators glean new information from otolith chemistry unattainable by conventional methods while their conventional methods serve to narrow the pool of suspects, thereby enhancing the effectiveness of classification models developed from otolith chemistry.

Appendices

Appendix 1. Procedures

Appendix 2. Cost and labor estimates

Appendix 3. Non-technical project summary

Appendix 4. Photos related to the project.

Appendix 1. Procedures

Table A1.1. Abridged (not complete!) procedures for the collection of samples for determination of origin and movement of illegally stocked fishes. We recommend that both otoliths and tissue samples be taken from fish; this allows for both microchemical analysis and molecular genetic analysis. It is essential that tissue and otolith samples be given the same identifier so data from each can be matched up later.

Otoliths

We assume that otoliths will be subjected to microchemical analysis by LA-ICP-MS. Note that risk of contamination is much greater for solution-based approaches, as opposed to the laser transect methods we used. See Campana et al. (2000) for additional precautions necessary for handling otoliths prior to solution-based analysis.

1. The number of fish to collect can be determined from a power analysis or based on the present study's guideline: 13 fish per location.
2. Handling otoliths with nonmetallic forceps is not critical but is recommended.
3. Record detailed collection information (date, collection site, length, weight, species/strain, etc.)
4. Remove saggital otoliths from fish immediately after capture (or freeze fish until otoliths can be removed). Do not store fish or otoliths in liquid preservative.
5. Remove all tissue adhering to otoliths and rinse with deionized distilled water.
6. Place otolith pair in labeled polyethylene microcentrifuge tube, and store tube in labeled coin envelope.
7. Store coin envelopes in sealed Whirlpak or Ziploc bag until otoliths can be embedded, sectioned, and polished or sent to analytical laboratory.

Tissue samples

We recommend following the protocol for collecting trout tissues for genetic analysis developed in 2007 by Kevin Rogers, Aquatic Wildlife Research Biologist, CDOW (Kevin.Rogers@state.co.us). In a nutshell, this protocol states:

1. Use scissors to remove at least a 1-cm^2 piece of the top of the caudal fin.
2. Store tissue in 15 mL polypropylene, "plug-seal" centrifuge tube with denatured reagent grade ethanol diluted to 80% with distilled water.
3. Do not place anything (e.g., a label) inside the centrifuge tube with the tissue/ethanol or it might compromise the DNA analysis. Rather, write on the outside of the tube with a special purpose laboratory marker.

Table A1.1. Abridged procedures- continued.

Water

We followed the procedure of Shiller (2003; alan.shiller@usm.edu) to collect clean water samples for trace element and isotope analysis. This protocol is best accomplished with two people, a "clean hands" person and a "dirty hands" person. Great care must be taken to avoid sample contamination. The procedure consists of two parts:

1. **Samples collection**. We used Method B. Immerse a pre-cleaned 250 or 500 mL bottle in the water source, rinse a couple times, then immerse and invert under water and cap it.
2. **Sample filtration**. This is quite tedious and time-consuming, and this is usually the stage with the most serious potential for sample contamination.
    a. Given the windy and dusty conditions typical of the mountain west, we strongly recommend filtration be done indoors, if possible. However, filtration should also be done soon after samples are collected. When away from buildings, we did our filtration inside a tent or inside the topper of a pickup truck.
    b. There are several steps to this protocol, resulting in 2 replicate 15 mL samples of filtered water. You will double bag the plastic sample bottles in ziplocks, and keep them cool and in the dark until you can ship them to Dr. Shiller's lab for trace chemistry analysis.

Appendix 2. Cost and labor estimates

Table A2.1. Required supplies, sources, and approximate costs ($US, 2006; laboratories may charge higher rates for commercial or private clients) for sample collection, preparation and analysis associated with the use of otolith microchemistry for forensic applications. Asterisked items are not essential but very useful.

Sample Collection			
Supplies/equipment	Source	Cost	Otoliths per unit
Coin envelopes (2-1/2" X 3-1/2")	Office supply outlets	$20 per 500	One pair
Gloves, other field supplies	various	$100	$100 per additional 100 samples
Microcentrifuge tubes (1.5 mL)	Scientific supply outlets	$20 per 500	One pair
Non metallic forceps	Scientific supply outlets	$10 per each	Thousands
Ultra-clean collection water kits	Center for Trace Analysis, Univ. of Southern Mississippi	$25 per each	N/A
Otolith Preparation			
Supplies/equipment	Source	Cost	Otoliths per unit
Isomet Low Speed Saw	Buehler Ltd.	$4,500	Thousands
Saw blades (Norton)	Grainger Industrial Supply	$100	>75
Other saw supplies (dressing sticks, cutting fluid)	Buehler Ltd.	$100	Dozens
Epoxy mounting kit	Electron Microscopy Sciences	$150	>200
Sandpaper, slides, miscellaneous supplies	Hardware stores	$200	$100 per additional 100 otoliths
Stereomicroscope, camera*, image analysis software*	Scientific supply outlets	≥$10K	Unlimited
Lapidary polishing machine*	Ameritool Inc.	$329	Thousands
Chemical Analysis			
Sample:analytes	Cost per sample	Laboratory used in this study	
Water:elements and $^{87}Sr/^{86}Sr$	$85 (minimum $350)	Center for Trace Analysis, University of Southern Mississippi	
Water:^{2}H	$27	Water and Environmental Research Center, University of Alaska-Fairbanks	
Otolith: elements	$10; $1,200/day	USGS Mineral Resources Laboratory, Lakewood, CO	
Otolith: $^{87}Sr/^{86}Sr$	$65 (min. $1,270)	Isotope & Trace Element Geochemistry Group, University of Melbourne, Australia	

Table A2.2. Labor (person-hours) requirements for various tasks associated with water sample collection and filtration, and otolith preparation for LA-ICP-MS analysis. To allow time for drying, not all steps in the otolith process can be accomplished in the same day.

Sample type/task		Labor (h)
Water		(per sample)
Sample collection, ultra-clean methods		0.1
Filtration, ultra-clean methods		0.4
	Sum	0.5
Otoliths		(per otolith)
Dissection, extraction, cleaning		0.15
Embedding in Epofix		0.1
Sectioning with low speed saw		0.1
Mounting on slides, polishing		0.15
Cleaning (sonication)		0.1
	Sum	0.6

Appendix 3. Non-Technical Project Summary

One of the continued threats to viable trout populations in the Mountain West is the spread of whirling disease via illegal stocking of diseased trout. Attempts to halt such introductions and prosecute violators have been thwarted because it has been virtually impossible to trace the origins of a diseased trout once it has been stocked. Naturally occurring chemical markers in fish tissue have shown promise as a method to track the origins of fish in previous studies. However, research to date had not looked at the potential for these markers to work adequately in hatchery environments over large areas or to distinguish many potential source hatcheries. We evaluated the use of chemical markers in fish otoliths, or "ear stones," to determine the hatchery of origin of stocked trout.

We found that otolith markers could be highly effective markers of the past environmental history of trout. We sampled 11 hatcheries and several populations of stocked trout captured from public waters, simulating conditions that may occur in a forensic case. Our ability to correctly identify the hatchery the fish came from increased with the number of chemical markers used (and hence cost) and when there were fewer "suspect" hatcheries. Otoliths are capable of providing information about the location a fish has inhabited, a feat not achievable with any other technique. The information from otoliths is best used to fill gaps in cases where traditional methods of investigation have been adequately conducted. The result of this research will provide law enforcement with a valuable tool to prosecute those who have illegally stocked trout and serve as a deterrent to future illegal stockings. Thus, we have provided a useful tool to help preserve the biological and economic health of trout fisheries.

Appendix 4. Photos related to the project.

Figure A4.1. Watson hatchery uses surface water from the Cache la Poudre River, visible in bottom left corner. The water is diverted into a Watson Lake (visible to the right of the road on right side of picture) before coming into the raceways. We sampled fish from Watson that had previously resided at Bellevue, less than a mile away. Photo provided by Jim McKissick, CDOW.

Figure A4.2. Rifle Falls Hatchery, with raceways and hatch-house pictured at left, is fed by a mix of 5 springs collected less than a mile from the hatchery. The right photo shows the area where the springs mix prior to entering the hatchery. The springs produce a consistent supply of water at a year-round temperature of 59ºF.

Figure A4.3. Water was collected using clean techniques. In some instances, hatcheries used multiple water sources and samples were collected after they had been thoroughly mixed prior to entering raceways, as shown above (photo: P. J. Martinez).

Figure A4.4. Configuration of the trout culture facilities sampled in this study varied greatly from place to place. Above: Finger Rock hatchery. Right: Mount Shavano hatchery (photo: Jim McKissick, CDOW).