NPAFC---
DOC. 396
Rev. ____

# DISCRIMINATION OF MULTI-COUNTRY THERMAL MARK CODES BY AUGMENTATION OF CODING SCHEMES OR MARKING MECHANISMS

by

Kristen M. Munk

Alaska Department of Fish and Game
CWT & Otolith Processing Lab
P.O.Box 25526
Juneau, Alaska, 99802
United States

submitted to the

North Pacific Anadromous Fish Commission

by

United States of America

March 1999

THIS PAPER MAY BE CITED IN THE FOLLOWING MANNER:
Munk, K.M.1999. Discrimination of multi-country thermal mark codes by augmentation of coding schemes or marking mechanisms. (NPAFC Doc. 396). Alaska Department of Fish and Game - CWT & Otolith Processing Lab, Box 25526, Juneau, Alaska, 99802, 14p.

# DISCRIMINATION OF MULTI-COUNTRY THERMAL MARK CODES BY AUGMENTATION OF CODING SCHEMES OR MARKING MECHANISMS

Kristen M. Munk

For the past 4 years the research community has enjoyed the luxury afforded by release location-specific thermal mark codes identified in samples of salmon otoliths collected in the ultimate mixed-stock population, the North Pacific. Because of increasing interest by the international research community in examining oceanic salmon populations, and the soaring interest in thermal marking at hatcheries in Russia, Japan, Canada, and the United States, coordinating and ensuring thermal mark quality across this vast marking network becomes imperative to the integrity of this valuable and necessary research. However, this "golden-child" of fish mass-marking -- thermal marking of otoliths-- cannot support this interest. Simply stated, not enough feasibly applied or easily discernible, discrete thermal mark codes are available to accommodate all thermal marking interests at hatcheries which contribute to the North Pacific's mixed-stock salmon population.

In addition to past and present high-seas thermal mark recovery (tmr) efforts, this technology has performed extremely well for localized tmr programs which look at modest complexes of thermal mark codes in samples collected from near-shore interception fisheries, to rivers-of-origin or otherwise. These programs are relatively few, with centralized coordination of thermal mark coding handled within each country or state with few exceptions. While little to no coordination has occurred between these locations, an awareness of the other's marks is necessary and attempted. Otolith samples from the North Pacific have begun to highlight the inadequacy of this after-the-fact awareness. Some duplication of mark codes has unknowingly occurred within and between Alaska, British Columbia, and Russia. These duplications generally are not a problem because secondary pattern characters, and "mark characterization" measurements and observations of the thermal mark in relation to the otolith core provide comfortable classification. However, other times these duplicated thermal marks are a challenge for even the experienced observer.

The strengths of thermal marking have been proven. But the points which make it incapable of singularly satisfying international marking interests need to be addressed. It is impossible to adequately discuss these details in this brief document, however the two prominent points are presented:

1) Coordination may be difficult. Thermal marking is dynamic throughout the marking process which makes true coordination difficult in the event of a system failure during marking operations at one hatchery. The overseeing mark coordinator will need to quickly communicate a mishap to other coordinators, and mediate the compromised code with the other countries in mind. Indeed, even without mishaps, achieving equity in thermal mark assignment is extremely challenging (though some hatcheries in Alaska where temperature differential is achieved with zero monetary cost have agreed to assignment of the more complex marks).

2) The total number of thermal mark codes is limited. The 'marking range' within the otolith is limited and bisected by the hatch event (Figure 1), during which marking should not be conducted. While more complex mark codes increase the total number possible, this will create additional costs (financial, logistical) to the hatcheries. Costs will increase for tmr programs as well: complex codes slow turnaround of data by taking longer to decode; Level 2 classification error may increase; and, more highly trained personnel will be required, etc.

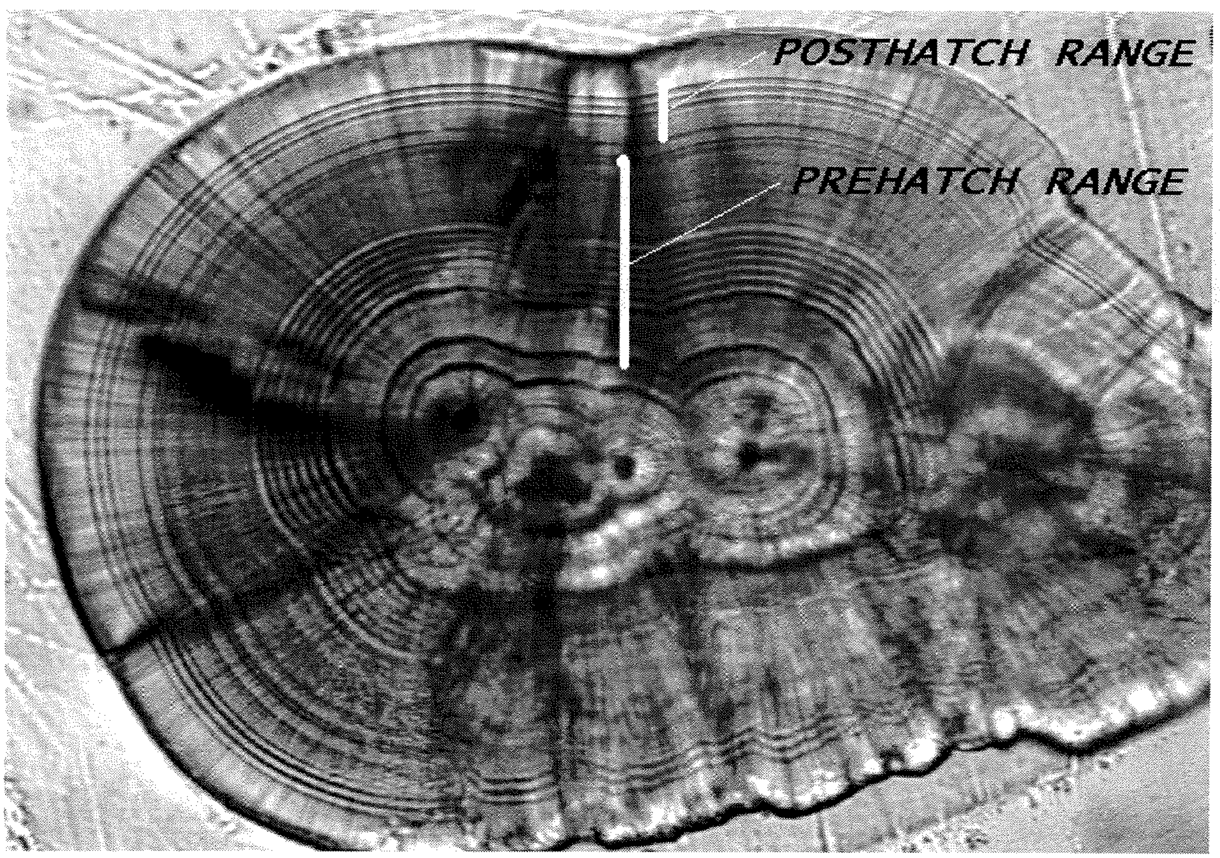


Figure 1. Prehatch and posthatch marking range in otolith, with thermal mark coding visible in each (width of marking range is modified by development, and is not consistent).

The effect these two concerns would have on a coordinated international marking program are profound. Even assuming excellent networking of all individuals will not mitigate the lack of marking-range, or 'real-estate', within the otolith.

Therefore, augmentation to the thermal mark code technique to enable a greater number of discrete, release location-specific codes is warranted. Interim treatment of the problem may be dealt with through careful and "quasi-centralized" coordination/direction of thermal mark coding, and perhaps a trial of country-specific thermal mark coding (see Recommendation at end of this document). Ultimate resolution of the problem will undoubtedly involve other "mass-marking" techniques. These other techniques include either applied or natural discriminators and are herein generally discussed. Consideration of each of these is given relative to the baseline thermal mark recovery process, aquacultural concerns, mark application, etc.

## THERMAL MARK TECHNOLOGY CONSIDERATIONS

A brief discussion of logistics of this technology, apologetically from the sole vantage point of the Alaska Department of Fish and Game, is worthwhile when considering difficulties in international thermal mark coordination or mark assignment, application of complementary marking, or the need to preserve integrity of existing programs. Thermal mass-marking was identified by the US-Canada Treaty's Transboundary River Project technical subcommittee to identify enhanced stocks of sockeye in both countries in an effort to monitor a harvest-sharing agreement, as well as addressing other commercial fisheries management concerns within southeast Alaska. For this to succeed, the managers needed prompt turnaround of accurate data (today, from time of sampling fish from the fishery to release of data on 100 otoliths is typically 8 hours). It was this consideration along with constraints at the hatchery, discussed below, which drove both the development of minimum requirements for marking within the RBr code structure (Munk, in prep) and also the otolith processing techniques in use at the CWT & Otolith Processing Lab in Juneau (Munk, in prep).

The unit of a thermal mark is called a thermal ring (or thermal increment). Produced by one thermal cycle and with an approximate differential of four degrees centigrade, it is composed of one relatively cool event of generally 24 hours duration, and one relatively warm event of 24-48 hours duration (the higher end duration is required if the ambient incubation temperature is low, which is often the case in Alaska). Shorter duration for cool and warm events has been used, but generally only for a series of thermal rings which contrast with more pronounced rings using longer duration. This cycle is repeated a minimum of four times for a one thermal-band mark. All combinations generally begin from this point (two or more thermal bands have fewer rings per band, but the overall number of cycles will be greater). "Four times 48 hours equals 192 hours equals 8 days…" so for the most-fortunate hatchery, it takes only 8 days to induce their hatchery code. Thermal marking systems at production hatcheries in Alaska generally can only simultaneously mark a small proportion (perhaps 35 million embryos) of the inventory. This amount may require six iterations of the marking schedule (one hatchery in Alaska has a yearly inventory of >200 million pink salmon), therefore 48 days are now expended to the marking effort, *for the least cumbersome thermal mark*!

While hatcheries try to economize time and energy by weaving iterations together -- for example, while one row of incubation units receive heated water, the other row receives cold – the logistics of staying within the marking window for each row makes this challenging. The "marking window" is defined as the period of time from which the otolith is developed enough to be thermal marked, to just short of hatching. And again, the post-hatch marking range begins shortly after hatch, through the mid-alevin stage (late-alevin marking is generally discouraged). If a hatchery misses the marking window, their mark, and perhaps that of others, is compromised.

In Alaska, for the 1998 brood year there were 5 discrete basemarks identifying hatcheries marking pink salmon (plus 3 more identifying treatment groups within a hatchery). For sockeye, there were 14 discrete mark codes, 4 for chinook, and 2 for coho. Over 7 discrete codes were applied for chum salmon, and coordinated with an additional 3 in Japan.

Aside from physiological and cultural considerations above, physical system limitations also have great impact on marking. Most thermal marking systems in use today have been custom built for thermal marking. They are sized with all hatchery parameters in mind. Other systems are simply opportunistic piecing to existing infrastructure. Fuel flow problems, power outages, and sensor difficulties have contributed to compromised marks, known as "glitch patterns", in the past. Other glitched marks arise from the "human element"…. Effects of these are minimized through immediate contact with the thermal mark coordinator. This means the mark coordinator is "on call" throughout the duration of thermal marking, generally late August through March.

While much emphasis seems to be placed on the limitations described above, it is done so with the concept of international coordination in mind; on a local level, the thermal mark programs are managed around the above described limitations to the point where none are perceived.

## CONTEMPORARY MASS-MARKING METHODS IN APPLICATION TO "COUNTRY-CODE"

There are several mass-marking techniques in use today each with their own pros and cons as applied to the question of country-code identifier. None provide the tremendous advantage of thermal marking, that is 100% mass-marking at extremely low cost with subsequent ease and accuracy in recovery of thermal marks. However, to overcome the hurdle which thermal marking cannot, proper application of one or several of these methods to the thermal marking process will accomplish the discrete identification required. The challenge of course, is finding the complementary method which most efficiently achieves the goal without disproportionately burdening those who have the least to gain.

### Applied Fluorescent Chemical-coding

Several chemical compounds have been used with mixed success in mass-marking applications. Fluorescing compounds such as alizarin complexone, oxytetracycline, fluoriscene, and calcein have been applied in multiple coding sequences to produce marks which are permanent throughout the life cycle of the fish (Tsukumoto, 1985; Beckman and Schultz, 1996; Brooks et al, 1994; Hendricks et al, 1991; Monaghan, 1993). Embryos, alevins, or juvenile fish may be exposed to the chemical via immersion or injection. Successful mark application is sensitive to water condition: the parameters of temperature, pH, time, and concentration must be carefully controlled. Typical duration of immersion treatment which produces one viable "chemical ring" is 24 hours. Longer or shorter duration may be possible, or required. If ambient incubation temperature is low, temperature of the water must be increased for adequate uptake of some of the chemicals (which could be done simultaneous with thermal marking).

Recovery of the complementary chemical mark in relation to an identified thermal mark code requires greater attention to detail in the various steps. Handling/dissection of an extracted otolith may require modification because some fluorescing compounds like OTC are "light labile", that is, they begin to decay upon exposure to light (otoliths from mature fish may afford some protection during handling). Preparation of otoliths may also require modification because fluophors require a non-fluorescing fixative. Half-sectioning the otolith is similar to tmr but will now need two distinct stages entailing both additional labor and equipment.

During conventional production tmr, proper evaluation of the microstructure requires a grinding wheel, stereomicroscope, and a transmitted-light compound microscope. Recovery of fluorescing chemical codes requires an "epi-fluorescent tube" attachment to the compound microscope which enables UV-filter excitation of the chemical (in other words, recovery of the chemical mark is done within the tmr lab). Once the otolith is sectioned to expose the core and identification of a thermal mark is made (with brightfield illumination), the ultraviolet light is switched on to determine presence or absence of a fluorescing mark (above mentioned compounds fluoresce with a different color, suggesting yet another mark complement). A technician with substantial expertise would be required to process these samples and would have to do so expeditiously and with certainty. A second-reading by another experienced technician much later may not be a viable option due to possible degradation of the chemical.

Despite the possibilities and reported successes, use of these chemicals in application and mark recovery is not without problems. Because tetracycline compounds are light-labile, special consideration in handling the "raw" or half-sectioned otolith must be made to preserve the integrity of the chemical code during its active life of consideration. Use of these chemicals are still highly regulated in some countries. In the United States for example, the Food and Drug Administration requires permitting for any chemical introduced into animals meant for consumption (for fish marking, this would require an INAD exemption, for *investigational* use of chemical only). Other countries may not have this government regulation, but instead, societal constraints which discourage the use of chemicals that give the appearance of "tainted" fish.

## Applied Elemental Coding – Strontium Chloride and "Rare Earths"

Fish entrain strontium in otoliths and tissues through natural processes, reflecting availability in the environment. Ambient strontium levels are significantly higher in saltwater than freshwater. For mass-marking, strontium chloride is introduced into incubation water (Behrens-Yamada et al, 1979). As a calcium-analog, it is taken up into the otolith in notable excess to natural background levels of strontium. Complex codes are possible with multiple exposures (Schroder et al, 1995).

Recovery of the strontium code is separate from the thermal mark recovery process. Once the microstructure is evaluated for a thermal mark, during a separate session the otolith tmr preparation (or the other otolith) is polished to substantial finish. A spatial analysis of the otolith surface using an electron microprobe is generally used in profiling the strontium signals. The strontium mark can also be viewed optically after completing one step toward final preparation for the electron-microprobe. This step produces a "backscatter image" which reveals the strontium events. If strontium were induced simultaneous to thermal marking, this backscatter image may be confounded by the thermal mark pattern, both of which will show under reflected light.

Some "rare earth" elements such as dysprosium and others in the lanthanide series have also recently been applied to mass-marking of fish (Ennevor and Beames, 1993). The mechanism for marking fish and recovery of the code is similar to that of strontium chloride.

Elemental and strontium chloride marking are not difficult, and studies suggest no deleterious impact to fish survival. Two disadvantages of strontium chloride or elemental marking complementary to thermal marking are the high cost and turnaround time of processing samples (SrCl more-so than rare-earth elements???). The preparation of the otolith for microprobe analysis takes significant additional time, and expensive mass-spectrometry equipment is required.

<u>Natural Coding- "Rare Earths"</u>

Rare-earth elements (lanthanide series) are naturally present in solution, in bodies of water inhabited by fish. Otoliths (and other bone) are 'typed' with these elemental signatures through natural physiological incorporation. Mass-spectrometry (and other spectrometric methods) is used to reveal these profiles, which have been proposed to identify continent of origin or some subregion of that continent[1]. At times spectrochemical analysis of data suggest greater geographic resolution, for example discrimination of sockeye stocks from Naknek vs Illiamna Lake within Bristol Bay[2].

Recovery of the suite of elements within the otolith is through any spectrometric method, however one conventional method is "inductively coupled plasma mass spectrometry" (ICP-MS). Either chemical dissolution or laser-ablation (i.e. vaporizing) of the otolith core prior to spectrometric analysis has been successfully used. The laser-ablation technique provides a broader analysis which for our application is preferable. The proposed mechanism for semi-tandem recovery to reveal this type of "country code" is similar to that described for applied-coding above. Spectrometric data are then analysed for country classification of the thermal marked otolith. This implies too that otolith reference samples from fish reared in a region have been collected, to which data are compared.

## DISCUSSION OF COMPLEMENTARY CODING TOOLS

The complementary marking scenario where natural vs applied codes may be preferred because it would obviate the need for mark application time and cost. It assumes that natural signals provide sufficient discrimination to identify country-of-origin; it must be remembered that this complementary mark need not discriminate to level of hatchery or release location --- the thermal mark will do this once a gross geographical region has been indicated. Continuing technological advancements will undoubtedly make future examination of naturally occurring rare-earth profiles a fish identification tool of choice, next to thermal marking.

Otolith processing costs are comparable between the natural vs applied elemental marking, and cheaper than recovery of a strontium code. A current estimate for examining otoliths using laser ablation ICP-MS is US$75.00, dropping to US$60.00 for greater than 300 specimens. Cost using isotopic dilution ICP-MS is US$100.00 per otolith. Estimates for processing otoliths to recover a Sr

[1] Brown, Robert, of Elemental Research, Inc., Vancouver, BC. 1989 April 5, Seminar at ADFG Headquarters, Juneau, Alaska. "Application of Inductively Coupled Plasma Mass Spectrometry (ICP-MS) to Mass Marking of Hatchery Stocks and to Fish Stock Identification in General."

[2] Straty, Richard R., of NOAA-National Marine Fisheries Service's Auke Bay Laboratory. 1972. "Summary of the Spectrochemical Analysis of the Otoliths (Ear Bones) of Juvenile Sockeye Salmon."

mark are well in excess of US$100.00. A range of costs should be considered however, including cost of chemicals, application, specimen processing, mark discrimination success, and additional otolith handling constraints which may impact conventional tmr practices.

Side note: Campana, et al (1997) suggested caution in comparing elemental analyses between labs using the range of popular equipment like wavelength dispersive (WD-EM) and energy-dispersive (ED-EM) electron microprobes, proton-induced X-ray emission (PIXE), and ICP-MS. If applying an elemental mark, structuring the code should take this into account and create simple and unambiguous codes which do not challenge resolution of even the most sophisticated recovery equipment.

## CONCLUSION AND RECOMMENDATION

As one might understand from the above, there is no perfect answer to the problem of extending thermal marking to the international arena using a complementary coding mechanism to identify country of origin of similar thermal marks. I believe further thought and work should occur after adoption of basic assumptions and guidelines which the NPAFC and other concerned agencies must develop and therein subsequently operate. I provide the following for consideration:

1) Given that the concern for expanding thermal marking to include a "country of origin" code is motivated by "high-seas" research interests seeking release-location specific identification of thermal marked fish, most if not all additional costs of marking and mark recovery should be borne by those interested parties, or by some mutual agreement with the marking facilities.

2) Accept and support that established thermal mark recovery programs that presently have "local integrity" must not be compromised.

3) Recommend and/or create a North Pacific Thermal Mark Committee (NPTMC).

4) Develop or assist PSMFC in creating "terms of reference" for this proposed NPTMC.

5) Convey strong support to participating agencies for funding a Thermal Mark Coordinator (TMC) position. Deadlines needing to be met by these coordinators require significant dedicated time; impact to programs when deadlines are not met can compromise coordination of marking.

I further recommend that short-term handling of the coding/coordination problem should take the following course:

For 1999 Brood Salmonids (thermal marking to occur fall and winter of 1999/2000)

1) **Attempt coordination amongst countries and states through thermal marking alone**, and experiment with complementary marking schemes*.
2) As part of thermal mark coordination and country coding**, attempt subset of thermal mark code which might aid future transition during mark-congested years** (if not outright meet country-specific thermal mark coding). Recommendations for accomplishing subset-code are in process.
3) **Identify a thermal mark coordinator for each country and or state.** Given the established programs in Washington and Alaska, these states should continue coordinating their own statewide marking programs; California has tentatively agreed to coordinate their few marks *through* Alaska for 1999 brood. Have chairperson responsibilities rotate among TMC's.
4) **All thermal mark coordinators should send an electronic and hardcopy list of hatcheries which will be thermal marking their 1999 brood, to each thermal mark coordinator by July 1, 1999.** This list should also include: species, discrete release sites (if they are identified by one thermal mark code), thermal mark code, temperature shift direction from ambient, thermal mark schedule, graphic depiction of proposed mark (and in relation to hatch), and any anticipated problems or history of problems by each marking facility.
5) **Identify code conflicts on the distributed lists and attempt to resolve by July 31, 1999**. TMC's for each duplicated code should resolve the conflict between themselves, and with consideration of other proposed thermal marks.
6) **Mark Committee Chairperson should report the thermal mark codes to all mark coordinators plus the Pacific States Marine Fish Commission by August 15, 1999**. Prompt completion and distribution of this report is critical, as many facilities begin thermal marking by

late August. This report should discuss code disputes and resolution proceedings. It should re-iterate need to maintain discrete marks during thermal marking system failures, recognizing that immediate intervention in creating discrete codes is required and may not allow concensus of all TMC's.

*1999 Complementary-Code Investigations

1) **Conduct the recovery scenario for naturally occuring elemental profiles from otoliths with undisputed thermal mark codes,** from pre-existing otoliths sampled in 1999 during the Offshore Carrying Capacity project. It is likely that thermal marks from Prince William Sound, Southeast Alaska, and Vancouver Island will occur in these samples. Once a positive thermal mark identification is made, send the companion otolith to a lab capable of ICP-MS or other appropriate mark-recovery device.
2) **Attempt simple chemical/element inducement of marks**, for example, 1$^{st}$ mark group = one single event mid prehatch on the otolith, 2$^{nd}$ mark group = one event mid-alevin, 3$^{rd}$ mark group = one event at start of the thermal mark and 1 event at the end of the thermal mark (i.e. chemical/element rings bound the thermal mark, and induced during "warm" event of thermal cycle ). Follow-up on mark inducement with practical mark recovery operations.

Right from the outset, synergistic effect of thermal plus other applied marking should be examined. Several of the bone-seeking chemicals and elements have been shown to increase uptake during elevated temperatures. Introduce chemical/element simultaneously with the warm event of the thermal cycle and evaluate mutual benefit: increased uptake of chemical/element, possible "whitening" of the warm zone in the otolith microstructure (personal observations, unpublished work). One seemingly obvious mechanism may be that increase in uptake is proportional to increase in fish metabolism. Another possibility is that the solute increases in availability for uptake due to greater dissolution with increasing temperature (though concentration presumably is controlled at the outset).

For 2000 Brood Salmonids (thermal marking to occur fall and winter of 2000/2001)

Upon outmigration of the 1999 brood fish, and well before mark proposal deadlines identified above, the NPTMC should meet and discuss recent thermal marking operations and difficulties. They should evaluate the outcome of investigations into analysis of naturally occuring element profiles and applied chemical or elemental marking. Reorganization of the "marking cooperatives" (state or regional hatcheries coming under one TMC) should take any expansion of marking programs into account. If the level of programs/request for number of thermal mark codes has exceeded possibility, additional and complementary coding should be rigorously applied. If the complementary code of choice is "examination of natural elemental profiles", it should be accompanied by a statement of commitment by the initiating agency.

## REFERENCES

Beckman, D.W. and R.G. Schultz. 1996. A simple method for marking fish otoliths with alizarin compounds. Trans. Amer. Fish. Soc. 125:146-149

Behrens-Yamada,S., T.J.Mulligan, and S.J. Fairchild.1979. Strontium marking of hatchery reared coho salmon, Oncorhynchus kisutch, Walbaum. J.Fish.Bilo. 14:267-275

Brooks, R.C., R.C.Heidinger, and C.C. Kohler.1994. Mass-marking otoliths of larval and juvenile walleyes by immersion in oxytetracycline, calcein, or calcein blue. N.A. J.of Fish.Mngmt. 14:143-150.

Campana, S.E. (and 19 coauthors).1997. Comparison of accuracy, precision, and sensitivity in elemental assays of fish otoliths using the electron microprobe, proton-induced X-ray emission, and laser ablation inductively coupled plasma mass spectrometer. Can.J.Fish.Aquat. Sci. 54:2068-2079.

Ennevor, B.C., and Beames, R.M. 1993. Use of lanthanide elements to mass mark juvenile salmonids. Can.J.Fish.Aquat.Sci.50:1039-1044.

Hendricks, M.L., T.R. Bender,Jr., V.A. Mudrak. 1991. Multiple marking of American shad otoliths with tetracycline antibiotics. NA J. Fish Mngmt. 11:212-219.

Monaghan, J.P.Jr. 1993. Comparison of calcein and tetracycline as chemical markers in summer flounder. Trans. Amer. Fish Soc. 122:298-301

Munk, K.M. in prep. Thermal Marking Manual: A guideline to the induction of thermal marks in otoliths for the purpos of mass-marking hatchery stocks. ADFG special publication.

Munk, K.M. in prep. Production otolith processing to reveal core microstructure for recovery of thermal mark codes. ADFG special publication.

Schroder, S.L., E.C.Volk, C.M.Knudsen, and J.J. Grimm. 1995. Marking salmon fry with strontium chloride solutions. Can.J.Fish.Aquat.Sci. 52:1141-1149

Tsukamoto, K. 1985. Mass-marking of ayu eggs and larvae by tetracycline-tagging of otoliths. Bull.Jap.Soc.of Sci.Fish 51(6),903-911.