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**OTOLITH MICROSTRUCTURE PREPARATION,  
ANALYSIS, AND INTERPRETATION:  
PROCEDURES FOR A POTENTIAL HABITAT  
ASSESSMENT METHODOLOGY**

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## **KEY WORDS AND PHRASES**

otolith microstructure analysis, otolith collection and preparation, juvenile Pacific salmon, habitat assessment, daily growth and residence, created and restored estuarine habitats, comparative studies



## INTRODUCTION

The loss and degradation of coastal habitats have placed numerous fish species in jeopardy (Thayer 1992). As considerable amounts of coastal habitat have been lost or degraded in the Pacific Northwest, efforts to restore or replace such areas and their associated ecological functions are becoming more common (Rylko and Storm 1991). However, the success of such efforts in replacing lost functions is difficult to assess without a more complete understanding of the ecological functions associated with estuarine habitats. The use of presence or absence of an indicator or target species, such as a commercially important migratory fish species, is inconclusive. The presence of a species in a habitat provides no information on habitat quality in terms of foraging opportunities, growth, and subsequent fitness of individuals, and does not provide adequate information with which to evaluate habitat. Recent studies on the foraging, growth, and residence of juvenile chinook (*Oncorhynchus tshawytscha*) and coho (*O. kisutch*) salmon rearing in natural and created estuarine habitats in southwestern Washington explored potential methods of habitat assessment (Simenstad et al. 1992, 1993; Miller 1993; Miller and Simenstad 1994). In these studies, we examined diet composition, residence, and otolith microstructure analysis of individual juveniles to evaluate rearing habitat within specific natural and created habitats.

Information on growth, residence, and foraging behavior of juvenile salmon allows for a quantitative assessment of habitat quality and associated fish and wildlife support in natural and created habitats. Residence in estuaries may be an important life-history stage for juvenile Pacific salmon, providing rearing areas with extensive foraging opportunities, refuge from predation, and time for physiological adaptation to increasing salinities (Reimers 1973; Healey 1980; Congleton et al 1981; Levy and Northcote 1982; Iwata and Komatsu 1984; Levings et al. 1986, 1989; Fisher and Percy 1990; Shreffler et al. 1990, 1992; Miller 1993). Rapid growth rates may be a benefit of estuarine residence and contribute to enhanced ocean survival (Parker 1962, 1968; Peterman 1978; Neilson et al. 1985; Tschaplinski 1987; Shreffler et al. 1990). From an applied perspective, short-term growth as an estimator of habitat quality offers a means to effectively measure incremental impacts (i.e., growth deficits or enhancements due to a given type of habitat alteration). Differential growth demonstrated on sufficiently small temporal and spatial scales may discern effects apart from those fish may experience as a result of other, longer-term alterations. Potentially, this approach could be used to estimate specific impacts of a perturbation or stressor (e.g., a dredging event) on an organism against a background of other physical and water quality alterations occurring within the watershed.

Methods appropriate for estimating habitat quality for juvenile fishes are particularly useful, given the perceived sensitivity at early life history stages of fishes and emphasis on potential population level effects. Fish otolith microstructure analysis is one such promising tool for gaining information on short-term growth of juvenile fish in restored, created, and natural systems. Furthermore, using such a tool, researchers can evaluate salmon habitat quality quantitatively and experimentally. In this context, otolith microstructure has been examined fairly extensively in some teleost fishes (Secor et al. 1992, for review). There are a number of benefits to using otoliths in aging and growth studies over other hard body parts, such as scales. Otoliths demonstrate annular and, in younger individuals, daily patterns and, unlike scales, do not appear to be resorbed during stressful periods (Campana 1983). A reference ("check") mark can be artificially induced within

the otolith microstructure by exposing the fish to various stresses (Brothers 1990, Volk et al. 1990). Additionally, daily otolith growth and somatic growth have been found to be positively correlated in several species (Marshall and Parker 1982; Wilson and Larkin 1982; Volk et al. 1984; Neilson and Geen 1985; Campana 1990, 1992; Mugiya and Oka 1991). Although the existence of correlations between fish size and otolith size establishes a basis for growth determinations, little study has been directed toward the use of otolith microstructure analyses as a comparative tool to evaluate relative growth among habitats such as naturally occurring and created or restored habitats.

Since Pannella (1971) discovered daily increments on the otoliths of teleost fishes, considerable debate has been focused on the accuracy and precision of otolith microstructure analysis in determining daily growth rates of younger fishes (Campana 1990, 1992 for reviews). Although strong correlations between somatic growth and otolith growth have been observed, continuous otolith growth during periods of reduced somatic growth or starvation suggests an uncoupling between otolith and somatic growth (Bradford and Geen 1987, 1992; Mosegaard et al. 1988; Maillet and Checkley 1990; Molony and Choat 1990). Such uncouplings occurred when experimental groups of fish experienced significantly different environments with regard to daily ration or water temperature.

Although uncoupling of otolith and somatic growth can inhibit accurate back-calculations of growth and length at age, relative differences or trends in growth may be detectable within the otolith microstructure. Significant differences in growth among habitats or treatments have been detected with microstructure analysis in both field (Neilson et al. 1985) and laboratory settings (Marshall and Parker 1982, Wilson and Larkin 1982, Volk et al. 1984, Neilson and Geen 1985, Bradford and Geen 1992) settings. Neilson et al. (1985) examined juvenile chinook growth in the Sixes River and estuary, Oregon, using otolith microstructure analysis. Although slower rates of growth were determined using otolith increment widths than with actual fish lengths, significant growth differences were still detectable between years and among habitats. Bradford and Geen (1992) found significant differences in otolith increment widths among hatchery-reared chinook sub-yearlings fed variable rations. Bradford and Geen (1992) also found increment widths underestimated overall somatic growth but demonstrated relative differences in growth between treatments. Such research suggests that otolith microstructure analysis may facilitate detection of relative differences in somatic growth rate.

This report describes field methods and otolith preparation, analysis, and interpretation of experiment using otolith microstructure analysis to test relative differences in juvenile salmon growth potential both in various habitats (e.g., natural and altered) and in experimental treatments. Although we developed the procedures described herein in conjunction with studies of juvenile salmonids, the theory and methodology associated with this type of study may be applicable to various juvenile fishes as well as to related questions, such as residence times and ages.

## FIELD STUDIES

Quantitative indices of foraging and subsequent growth of individual fish in specific habitats, whether created, restored, or natural, provide an assessment of habitat quality. Synoptic determination of relative or absolute growth in natural as well as altered habitats enables researchers to evaluate observed growth in altered environments against an unaltered reference habitat. The main components of these studies are the capture, tagging, re-release, and maintenance of fishes in specific habitats of interest. Placing an internal mark on the teleost otoliths through various means, such as exposure to temperature stress, provides a permanent reference marker for future otolith interpretation. Such a tool is invaluable for quantifying short-term residence and growth: internal tags provide reference points for the experiment, information on intra- and inter-individual variability, and a means for determining site-specific residence times.

The species of interest, characteristics of the study sites, and particular research questions dictate the appropriate sampling design. Whether a paired-site comparison or a broader design that incorporates a variety of sites, all designs need to include adequate sample sizes and replication to ensure statistical power sufficient to detect significant differences in otolith growth or pattern. If information on individual foraging and residence is collected, sampling times and methods must be considered to ensure valid comparisons. Collecting environmental data, such as water temperature, dissolved oxygen, salinity, and depth, provides additional information with which to evaluate respective habitats and to interpret observed differences in growth. In areas influenced by tidal regimes, low tide refugia for experimental fish must be available and may limit the amount of time available for experimentation. If wild fish are to be used, their natural migratory times and patterns need to be incorporated in the study design and sampling methodology. Although they may not be entirely analogous to wild fish, hatchery fish may provide more flexibility in the timing and design of the study, and may offer the only means to guarantee an adequate number of samples of the same size and species of fish. In the case of juvenile salmon, predators and competitors within the habitats being evaluated must also be considered prior to release of experimental fish. If the habitats of interest differ in these respects, fish growth may also be influenced by those factors.

### OTOLITH MARKING AND TAGGING

Many characteristics of otoliths make them useful for field studies. Otoliths are bipartite structures that predominantly comprise protein and calcium carbonate, which commonly occurs in the structural form of aragonite. Calcium carbonate deposition in the otolith is thought to be regulated by circadian rhythm and therefore results in daily deposition of increments (Mugiya 1987; Appendix A). However, periods of stress, such as abrupt changes in temperature, interrupt calcium deposition and cause a check, or stress mark, to form within the otolith microstructure. Such a mark provides an internal tag that can be beneficial for experimental purposes. For example, the stress mark provides a temporal benchmark that can be used to determine daily otolith growth. Information on otolith growth can then be collected from fish residing in different habitats.

A variety of thermal manipulations, including both heating and chilling, have been shown to produce a distinct mark within the otolith structure (Brothers 1990, Volk et al. 1990). A legible check mark will form within the otolith microstructure of fish exposed to water 5°C below ambient temperatures for ~12 h (Simenstad et al. 1992, 1993; Miller 1993). Methods for holding and thermally manipulating fish range from a standardized hatchery operation with high-volume cooling units connected to the hatchery water system (Volk et al. 1990) to a system of portable holding tanks set up at a field station (Simenstad et al. 1992, Miller 1993). In the latter situation, we used large blocks of ice to lower and maintain water temperatures at treatment levels. In the case of on-site maintenance of fish, oxygenation of the water may be necessary depending on the densities of fish being held and the length of confinement.

Although one of the primary advantages of otolith marking is the lack of a change in the fishes' visibility and vulnerability to predation, we highly recommend some method of externally tagging fish to facilitate recapture efforts. External tags facilitate field recapture of marked fish and allow for comparison with external marks of somatic growth. Ideally, morphometric data would be gathered on individual fish prior to otolith marking and re-release to the experimental habitats as well as after recapture to provide as much information as possible. External tags vary from relatively simple and cost-effective techniques, such as variable patterns of fin-clipping, to more elaborate and expensive methods, such as coded-wire tags (see Wydoski and Emery 1983, McFarlane et al. 1990 for reviews). Unfortunately, tagging small fishes (30.0–50.0 mm in fork length [FL]) is expensive and may result in excessive mortalities owing to stress.

## MARK-RECAPTURE EXPERIMENTS

Methods for capturing and restraining experimental fish throughout the experimental growth period must be sufficient to ensure adequate numbers of recaptures for microstructure analysis. However, the method must allow fish to move freely within experimental areas. Recapture rates will vary depending on site characteristics, such as water levels, water velocities, predator communities, and tidal regimes. In the Pacific Northwest, recapture rates between 8% and 15% were attained for coho sub-yearlings in an area characterized by a semi-diurnal tidal regime (Miller 1993). Until average rates of recapture are established for specific areas and methods, large numbers of fish must be used initially to ensure an adequate number of recaptures. The number of fish necessary for valid microstructure analysis will vary depending on the number of habitats being considered and the level of precision desired, as well as the experience of the otolith technician. Researchers should also realize that otoliths may be lost and broken until some level of expertise is gained, and that early preparations may not be adequate to clearly see daily increments; we highly recommend extensive practice using non-essential otoliths.

## FISH PRESERVATION

Appropriate methods of fish preservation are necessary for successful otolith analysis. Otolith dissolution will occur in all samples, and precautions must be taken to minimize its effects. Otoliths preserve well in a 95% ethanol solution. Dissolution is a more significant problem in samples preserved in formalin and ethanol solutions <80%, which can be too acidic (Butler 1992).

Furthermore, ethanol initially used for fish preservation should be replaced within 24 h because waters in fish flesh dilute the preservative (Butler 1992). Some specimen shrinkage will occur regardless of the preservative used. Literature rates for shrinkage can be consulted, but directly determining shrinkage factors associated with individual studies will increase the precision of the estimation measurement of the fish-size/otolith-size relationship and subsequent growth estimates specific to your studies.

## OTOLITH PREPARATION

In this section, we discuss the four preparatory stages for otolith microstructure analysis:

1. otolith removal (from fishes 30–70 mm FL),
2. proper cleaning and handling of the otolith,
3. embedding the otolith for polishing and grinding, and
4. grinding and polishing the otolith.

### OTOLITH REMOVAL

Teleost fishes have three pairs of otoliths: the sagittae, lapilli, and asterisci (Fig. 1). The sagitta is the largest of the otoliths and most commonly used for microstructure analysis. Otolith analyses often focus on the left sagittal otolith, which has a significantly larger total length than right otoliths in some salmonids (Neilson and Geen 1982). Although the left otolith has been found to have a greater total length, no significant differences in increment number or width have been detected between right and left otoliths of chinook salmon (Neilson and Geen 1982). However, we recommend that both the right and left sagittae should be removed from the fish and examined to determine if any systematic differences are present. If either the right or left otolith is not to be used, it can be embedded or archived dry in case the other sagitta is lost or destroyed.

Prior to otolith removal, individual fish should be weighed ( $\pm 1.0$  mg) and measured ( $\pm 0.5$  mm). Extreme care should be taken in performing the following procedures. To remove otoliths in fishes of 30–70 mm FL, we recommend the “open-the-hatch” method as described by Secor et al. (1992). Although Secor et al. (1992) recommend this method for otoliths  $>300$   $\mu\text{m}$ , we also found it to be very efficient for otoliths of 100–300  $\mu\text{m}$ . The “open-the-hatch” method requires a dorso-ventral cut through the top of the head that exposes the brain. After the brain is carefully removed, the semi-circular canals that house the otoliths will be visible (Fig. 1). In most cases, a dissecting microscope will be necessary for otolith removal. After locating the semi-circular canals lateral to the brain cavity, look for the lapillus and sagittae, which are floating within the canals and usually covered by a thin layer of tissue, and remove them. If the otolith, especially the sagittae is embedded, tease away the tissue to avoid breakage, especially along the anterior end. Once a technician is familiar with the location of the otoliths, amendment of the removal technique may increase speed and efficiency. Consult Secor et al. (1992) for a review of various removal methods.

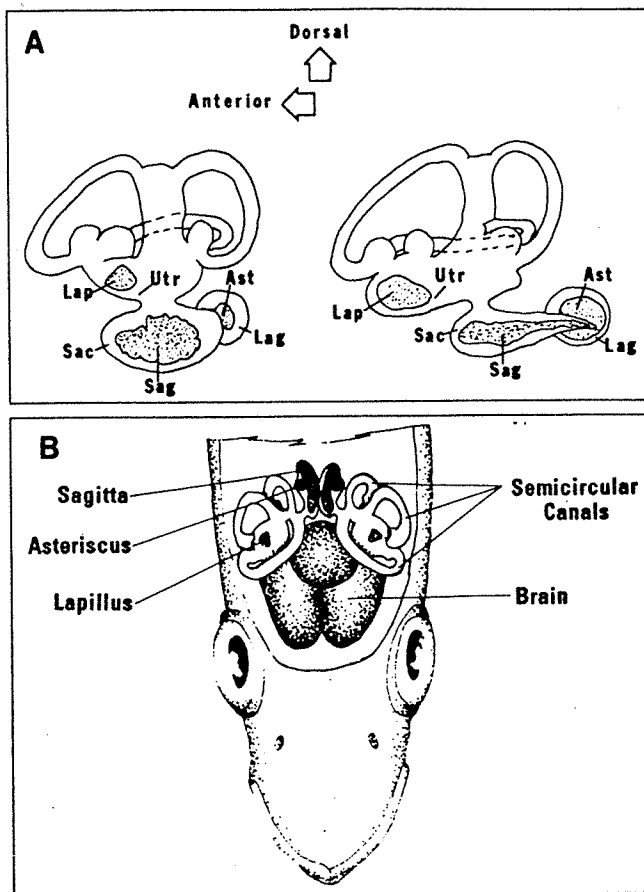


Figure 1. Anatomy of vestibular apparatus. (A) Otoliths within the labyrinth systems of representative teleost and ostariophysan (cyprinoid) fishes. (B) Dorsal view of vestibular apparatus as it sits in a typical teleost. Top of head is cut away. Ast = asteriscus; Lag = lagenar vestibule; Lap = lapillus; Sac = saccular vestibule; Sag = sagittae; sc = semi-circular canals; utr=utricle (Secor et al. 1991)

The occurrence of non-aragonitic otoliths has been reported and can prevent accurate analysis (Mugiya 1972, Dean 1992, Miller 1993). When the calcium carbonate crystallizes in the form of vaterite instead of aragonite, the translucent character of the otolith and lack of clear increments will inhibit interpretation; the organic matrix may be absent or reduced in these cases (Dean 1992). The causes of vaterite formation in some teleost fishes is not fully understood, but when these formations occur, otoliths usually must be removed from analysis. Miller (1993) observed a 15% occurrence of vaterite in experimental juvenile coho otoliths from the Chehalis River, Washington.

Some practice will be necessary before efficient otolith removal is achieved. The lapilli and asterisci can be saved, but both are fairly small and more difficult to remove. If one additional pair were to be collected for all samples, the lapilli would be easier to handle than the asterisci. As mentioned,

otolith dissolution occurs over time regardless of the preservative used. Therefore, samples should be fixed as soon after collection as possible.

## CLEANING AND HANDLING

Remnant tissue on the otolith surface can inhibit otolith analysis and interpretation. Therefore, any excess material (e.g., otolithic membrane) on the dissected otolith must be removed prior to embedding. Otoliths can be cleaned manually by teasing excess tissue off the otolith surface or by soaking the otolith in a diluted (10%) bleach solution for several minutes to 1 h depending on the amount of tissue and otolith size (Secor et al. 1992). If soaked in bleach, otoliths should be rinsed in ethanol or water afterward to prevent crystal formation on the otolith surface. Otolith handling should occur as little as possible to avoid specimen damage or loss; in particular, special care should be taken not to crush or chip the otolith.

## EMBEDDING THE OTOLITH

The quality of the embedded sample and the embedding medium used will largely dictate the clarity of the fixed sample and future grinding success. A variety of embedding mediums are available, such as household epoxy resin, Spurr, Euparal, LR white, and Epon. The method presented here, involving a household epoxy resin made by the Titan Corporation (Appendix B), was found to work well by researchers examining chinook and coho otoliths from individuals <100 cm FL. However, for a more complete discussion of alternative methods, see Secor et al. (1992).

One of the most accessible and easiest to use embedding mediums is thermoplastic casting resin and an associated catalyst that allows hardening. Such products are available at most local hardware stores. Although a well-ventilated lab area is necessary when using most embedding mediums, casting resins do not require additional heat to complete the hardening process. Samples will harden at room temperature within 12–24 h depending on sample thickness.

The ratio of resin to catalyst that results in clear samples of the proper hardness depends on the brand of resin, otolith size, and preferred sample block size. In many cases, six drops of catalyst to one ounce of resin are sufficient. The use of too much hardener results in brittle samples that crack easily while too little hardener creates soft samples that are difficult to grind and often have a cloudy or opaque appearance. When mixing the resin and catalyst, care must be taken not to stir the medium too briskly or air bubbles will be worked into the liquid, which can obscure the otolith after hardening.

The choice of mold used to embed samples also affects future ease of handling fixed specimens. Rounded or slightly oval plastic molds ~1.0 cm in diameter are ideal for otoliths 100–300  $\mu\text{m}$  in total length. A variety of molds are available through Ted Pella, Inc. as well as various other biological supply companies (Appendix B). A word of caution when using molds with numbers carved into the base of the mold for sample identification: the grinding procedure will be inhibited by samples fixed in these molds because an adequate seal can not be formed against the microscope slide. In the absence of adequate molds, the bottoms of unwaxed paper cups work well. In this

case, the shallow circular base of unwaxed cups will hold several otoliths. Samples can then be cut into individual blocks after hardening.

When the otolith is ready to be embedded, it is placed sulcus acousticus (the groove in the proximal surface of the otolith) side down on the mold before being covered with resin (Fig. 2). Immediately after gently pouring the resin, use a thin metal forceps or wooden dowel to tap the surface of the otolith to prevent air bubbles from being trapped under the otolith, which can obscure the otolith and subsequently prevent accurate interpretation of the microstructure. A thin resin block ( $\leq 2.0$  cm) is usually adequate for grinding and polishing. As the otolith grows, its longitudinal plane curves and complicates the grinding procedure. This requires samples to be fixed in larger resin blocks and sectioned in order to ensure that grinding occurs along a consistent plane. However, the longitudinal planes of otoliths  $< 300 \mu\text{m}$  are still level.

## OTOLITH GRINDING AND POLISHING

The grinding and polishing process is the most detailed and time-consuming stage of otolith preparation and is very important for accurate microstructure interpretation. The goal of grinding and polishing is to remove material obscuring the otolith core in a consistent plane and, ideally, clearly expose daily increments in a standardized region of the otolith (Fig. 3). A clear preparation of the otolith from its core to its outer edge is often difficult to attain. As research goals vary, the aim of polishing may be modified: When recent growth within specific habitats is of interest, a clear preparation of the otolith edge would be the primary concern (Fig. 4); when age at hatch is desired, the primary aim would be a clear preparation of the otolith core area.

A variety of instruments and methods can be used for otolith preparation. The use of light or scanning electron microscopy (SEM) will dictate whether a grinding or etching process is employed. Haake et al. (1982) present an extensive discussion of SEM preparations for larval fishes. The preparation of otoliths (specifically salmonid otoliths between 100 and 300  $\mu\text{m}$  in total length) for light microscopy is presented in the following discussion. Depending on otolith size and amount of embedding material used, the sample will be either affixed directly onto a microscope slide or sectioned prior to being affixed to a slide. The curvature and distortion of the otoliths  $< 300 \mu\text{m}$  are usually minimal and permit samples to be attached directly to the slide and ground and polished along the sagittal plane.

Various mounting methods and materials are available for securely affixing samples onto a microscope slide. A thermoplastic glue (CrystalBond®) is often recommended (Secor et al. 1992). However, the authors had greater success with a removable adhesion wax sold by Struers®. The adhesion wax does not form as strong a bond, melts at lower temperatures, and is generally easier to work with than CrystalBond®. For the initial grinding process, microscope slides are heated to  $\sim 40\text{--}60^\circ\text{C}$  before the adhesive is liberally applied to the slide. At that time, the block (otolith section) is held in place on the slide, sulcus side up, until the adhesive cools and hardens. The use of a removable adhesive allows samples to be re-positioned for handling ease as well as flipped in order to grind both sides of the otoliths.

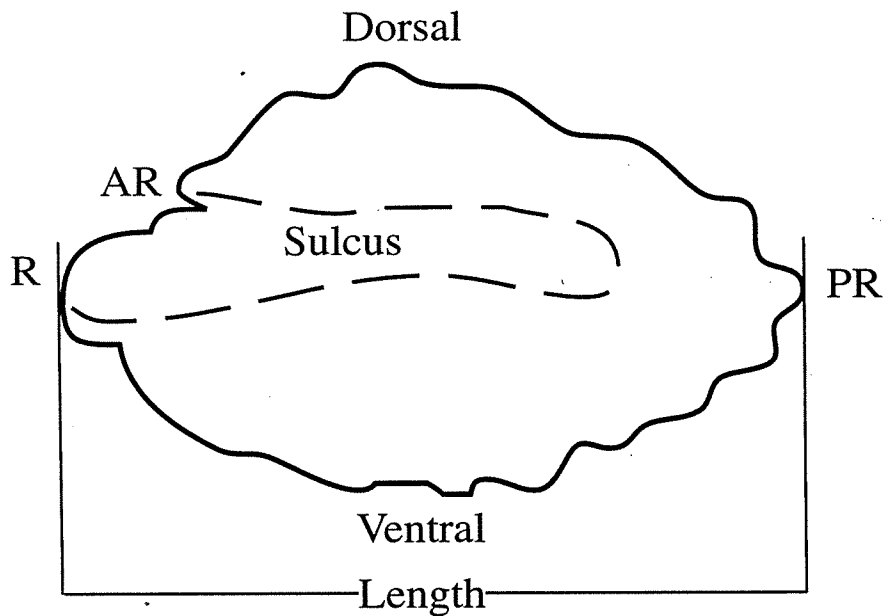


Figure 2. Medial view of right sagitta of typical teleost. In all sagittae, the sulcus faces medially and the rostrum defines the anterior margin of the sagitta. Recognition of the sulcus and rostrum will allow determination of right or left sagitta. AR = antirostrum, PR = postrostrum, R = rostrum. Adapted from Stevenson and Campana (1992).

Although some workers choose to polish samples manually, the use of an automated polishing wheel can increase preparation efficiency, enable initial preparations of large numbers of otoliths and, with experience, enhance precision. When preparing samples for interpretation of daily structure with light microscopy, technicians should grind both sides of the otolith. A variety of devices are available, including a dual wheel system developed by Struers® called Rotopol-V (Appendix B). The Rotopol-V has variable rotation speeds ranging from 60 to 600 rpm. Although an automated specimen holder plate is available for the machine, it usually cannot be used without sacrificing the precision and accuracy necessary for interpretation of daily increment microstructure. However, in cases where determining the absence of a crude check mark or distinct pattern on the otolith is the desired endpoint, an automated specimen holder may be of value in increasing processing speed.

After the block is secured to the slide, the initial grinding process can begin. As the sulcus side of the otolith, which will be ground initially, has less material obscuring the core than the distal side, the core will be reached relatively quickly. Therefore, using a finer paper (800–1200 grit) at moderate speeds (~180–350 rpm) can prevent grinding through the core at first. When one attains familiarity with the otolith microstructure of the species examined, a coarser paper (500 grit) and faster rotations can be used to rapidly approach the core. The initial signs of the core are the dark, rounded primordia surrounding the core. When the primordia are visible, we urge caution until the polisher has gained experience with the time required to reach the core without grinding through it. Once the core is penetrated and the primordia are gone, the sample is no longer usable. A com

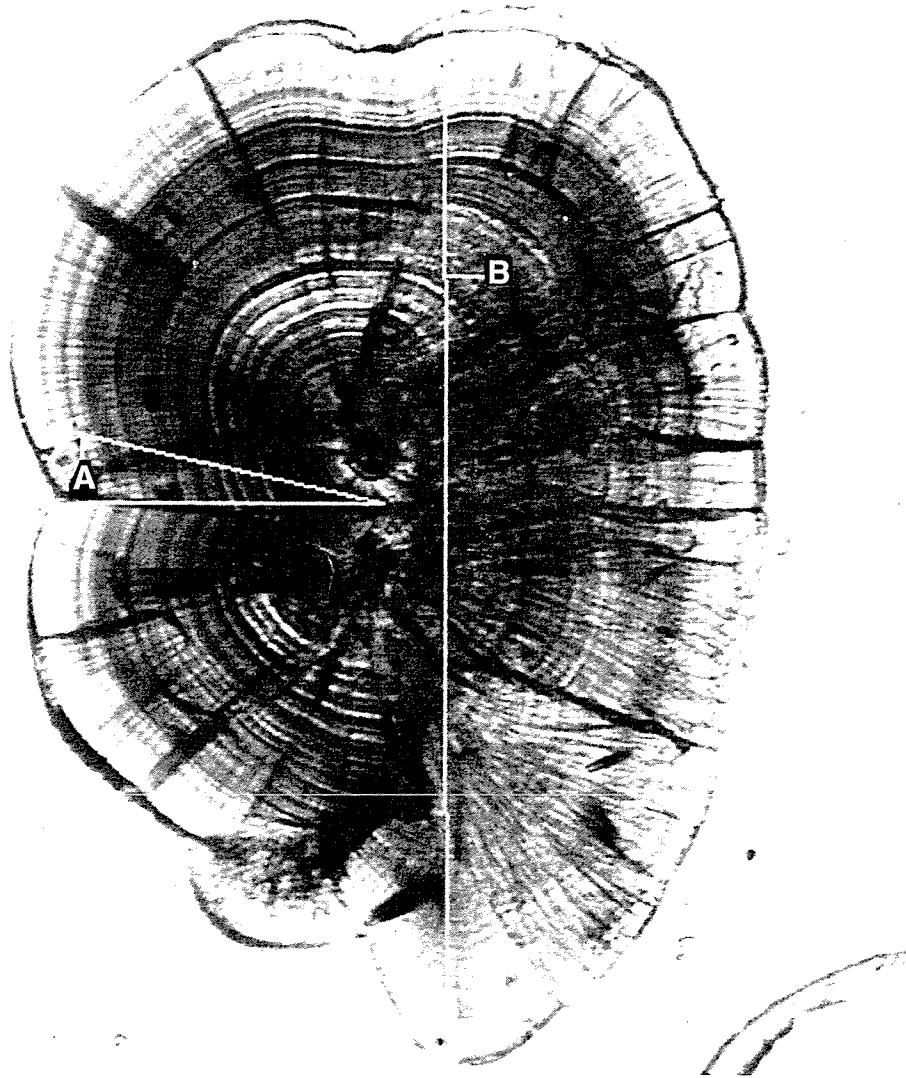


Figure 3. Photograph depicting measurement transects on the sagittal otolith of a sub-yearling coho salmon from the Chehalis River, Washington: (A) delineates otolith section used for daily increment width analyses; (B) represents transect used for total otolith length measurements.

pound microscope is necessary to frequently check the grinding progress through the otolith. We advise initially erring on the conservative side and flipping the specimen when the core and primordia are just visible. However, the goal should be to remove as much material from the core area as possible in order to enhance future interpretation. Therefore, when the primordia are visible, using fine-grit paper or an alumina slurry ( $0.5\text{--}3.0\ \mu\text{m}$ ) at rapid speeds (400–600 rpm) allows more material to be removed from the otolith at a conservative rate. When the primordia and

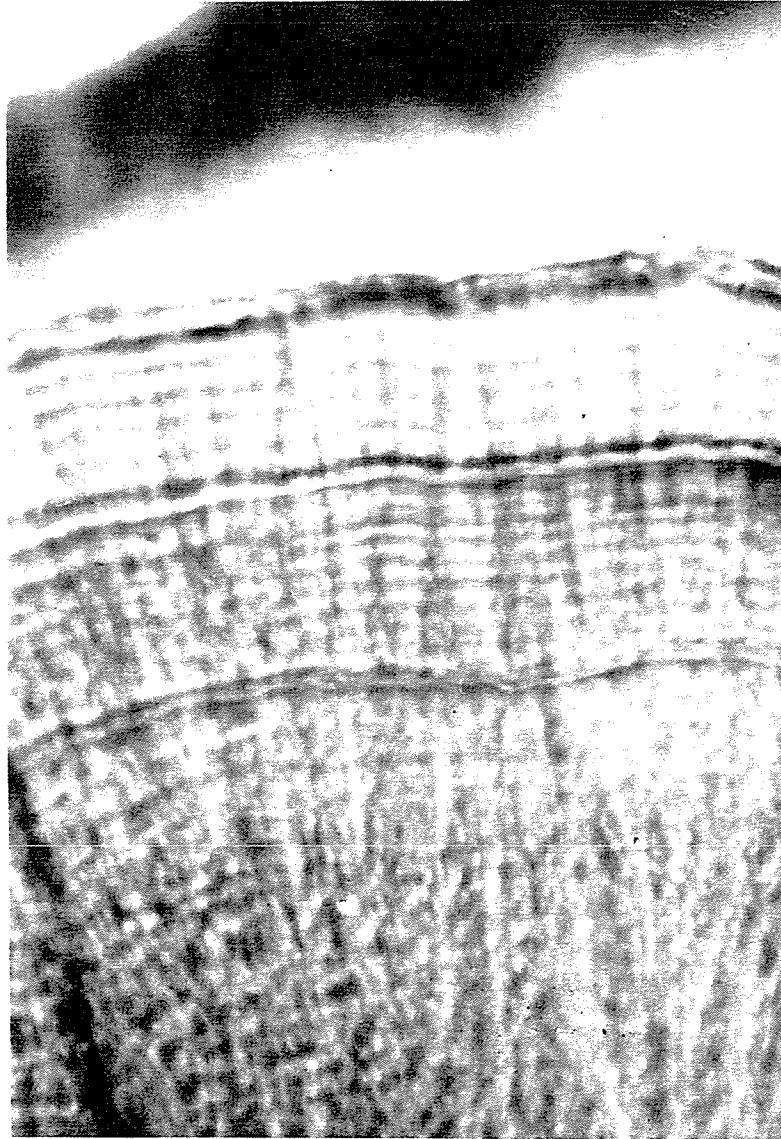


Figure 4. Photograph of region (A) from Figure 3 depicting outer section of the sagittal otolith of a sub-yearling coho salmon from the Chehalis River, Washington. A temperature-induced stress mark and five increments representing daily growth are evident at the outer edge of the otolith.

core are clearly visible, a final polish with an aluminasilicate slurry can enhance the final preparation.

When the sulcus side is sufficiently ground and polished, heat the slide just enough to remove the section. Re-affix the section with the polished side now facing the slide. A strong, durable medium, such as Duro® Quick Gel™ no-run super glue, works well at this point. Any brand or type of super glue that is thick and viscous, not runny, will achieve an effective seal. At this stage,

allow the super glue to thoroughly dry (12+ h) to prevent any slippage or leakage during grinding. After the glue is dry, applying clear nail polish along the edges of the block will further seal the sample. If the section is not adequately affixed to the slide, water will invade the sample as it thins during polishing and can ruin the otolith.

After all adhesives have thoroughly dried, return to the grinding wheel. As mentioned, because of the embedding method, a greater amount of excess material surrounds this side of the core.

Therefore, depending on the thickness of the block, grinding with 500 grit paper at higher speeds (400–600 rpm) will hasten preparation time. When the core becomes barely visible, return to the 800–1200 grit paper for finer grinding. Again, when the core and primordia are visible, move to the fine alumina slurries to refine the preparation. When daily rings are clearly distinguishable, a fine polish can again be used to finish the sample preparation.

## **MICROSTRUCTURE ANALYSIS**

Recent advances in fish otolith microstructure interpretation have opened the way for improved and expanded analyses. In addition to age analyses, information on the timing of life-history events, mortality rates, stock differentiation, environmental conditions during the life of the fish, and improved methods for growth estimations have resulted from otolith microstructure analysis (Jones 1992). However, such analyses require validation of certain assumptions, such as daily increment deposition, without specific verification or testing of these assumptions. Caution must be exercised to avoid making erroneous conclusions from the data. Although daily increment deposition has been seen in larval and juvenile stages of numerous species, a good deal of subjectivity exists in microstructure analysis. Fluctuations in environmental parameters, such as temperature and daily ration, can cause changes in individual metabolic rates and result in the deposition of sub-daily rings and other types of “noise” within the microstructure that inhibit accurate and consistent interpretation. Such inconsistencies, found even among experts, have resulted in formal attempts to standardize methods of interpretation (Stevenson and Campana 1992).

For both age and growth estimates, several steps can be taken to minimize subjectivity, improve accuracy and precision, and standardize data collection techniques. As increment formation varies within the otolith microstructure, it is important to use a standard measurement radius in order to ensure comparable information. One of the first steps in this process is to select a counting axis (Fig. 3). This axis should be a region along the otolith growth axis with the most consistent increment clarity. Whether the analysis includes the entire life of the fish or only a recent growing period, the axis that consistently contains the most complete increment sequence should be chosen and used for all samples. Initially, numerous samples may need to be examined before determining which region consistently has the greatest clarity.

## LIGHT MICROSCOPY AND IMAGE ANALYSIS

Light microscopy is the most commonly used method for interpretation of otolith microstructure. The following discussion is focused on optimum image resolution and analysis with light microscopy. The basic requirements for microstructure analysis include the following:

- a compound light microscope;
- binocular eyepieces with video or another camera to capture the image, or a photo eyepiece and associated image analysis computer software;
- a moveable specimen stage;
- a substage condenser; and
- an aperture diaphragm.

Magnification ability will vary depending on the objectives, eyepieces, and focal length of the equipment used. It has been suggested that the greatest number and most significant interpretation errors are associated with light microscopy (Dean 1992). Although a magnification of at least 1000X is recommended for accurate microstructure examination of otolith increments widths between 1.0 and 2.0  $\mu\text{m}$ , image resolution can vary greatly (Campana 1992). The greatest resolution will be attained with the lens objective with the greatest numerical aperture (NA) (Table 1). Because of air along the light path, the NA is limited to 1.0 unless an oil immersion is used. The constraints of the equipment need to be acknowledged and addressed in order to ensure accurate microstructure interpretation.

A computer image analysis system, although not absolutely necessary, provides the ability to manipulate, enhance, and save individual images as computer graphics files and to automatically quantify increment information. Image analysis systems allow manipulations of an image by converting the projected image into an array of numbers. The image is divided into pixels that each represent a position on the image; the pixel value corresponds to the gray level, or light intensity, of the image within that area. This allows any mathematical computation that could be done with an array of numbers to be done with the projected image. A number of computer image analysis programs are available (e.g., Optimas<sup>®</sup> of Optimas Corporation (formally Bioscan), SigmaScan<sup>™</sup> by Jandel Scientific, and Optical Pattern Recognition System<sup>™</sup> (OPRS) by BioSonics). Optimas<sup>®</sup>, which will be discussed here, offers a variety of enhancement techniques (i.e., mathematical, digital processes that enhance or characterize the basic shape of objects in the image). The most commonly used tools for enhancing otolith increments are filters. A filter can remove noise and enhance features. For example, Optimas<sup>®</sup>' high-pass filters sharpen image contrast and enhance the edges of a selected object, which can greatly increase otolith increment clarity. An "Output LUT" software routine allows for alteration of the gray values in an image and thus can also improve image clarity by removing noise.

Optimas<sup>®</sup> will run on any Microsoft<sup>®</sup> Windows<sup>™</sup> compatible DOS computer. The Optimas Corporation recommends at least an Intel 80386-based system. The program requires Windows version 3.0 or later and PC- or MS-DOS<sup>®</sup> version 3.1 or later. Optimas<sup>®</sup> is a powerful program that is applied extensively in the medical sciences. Depending on the version used (i.e., version 4.0 and above), data, such as increment number, can be sent directly to a spread sheet, such as Microsoft<sup>®</sup> Excel. A wide variety of measurements are possible with the data collection box (e.g., the length of

Table 1. Limiting characteristics of a compound microscope using each of the major objective lens types. All numbers assume a perfectly aligned and optimized optical system. Adapted from Eastman Kodak Co. (1980) and Campana (1992). NA = numerical aperture.

	Typical NA of objective	Overall NA of microscope	Resolution under green light ( $\mu\text{m}$ )	Maximum useful magnification	Depth of field under green light ( $\mu\text{m}$ )
Achromat					
10X	0.25	0.25	1.10	250X	8.52
45X (dry)	0.65	0.65	0.42	650X	0.99
100X (oil) <sup>a</sup>	1.25	1.00	0.27	1,000X	0.30
100X (oil) <sup>b</sup>	1.25	1.25	0.22	1,250X	0.30
Aprochromat					
10X	0.32	0.32	0.86	320X	5.83
45X (dry)	0.95	0.95	0.29	1,000X	0.19
100X (oil) <sup>a</sup>	1.40	1.00	0.27	1,000X	0.16
100X (oil) <sup>b</sup>	1.40	1.40	0.20	1,400X	0.16

<sup>a</sup>Using immersion oil between specimen and objective, but not between condenser and slide.

<sup>b</sup>Using immersion oil between specimen and objective and between condenser and slide.

a line, distance from an object, area of an object). However, because distance between selected lines is not an option, a separate program must be written. If the researcher desires to measure increment width, using a separate program or macro will greatly enhance the precision and efficiency of data collection. If assistance beyond the scope of user and technical reference material is necessary to accomplish this, the Optimas Corporation may assist in preparing a program for your needs.

Optimas® has both a pull-down menu and a band of icons in the lower left-hand portion of the screen for commonly used commands. Initially, the image must be “acquired,” after which it will be “frozen” and available for enhancement and data collection. Prior to collecting any data, the program must be calibrated with an object of known length to provide an accurate scale for objects in the camera’s field of view. If no calibration has been installed, a default calibration will appear. In order to calibrate Optimas®, refer to the user guide. After Optimas® is calibrated at each magnification that will be used for data collection, a configuration file with the selected calibrations can be saved for future use. Each time data collection is begun, the configuration file must be opened and the appropriate calibration selected. The data collection box provides the tools for marking, or flagging, and measuring a selected image.

## OTOLITH MICROSTRUCTURE INTERPRETATION

Determining age and growth from otolith increment data can provide valuable and interesting information, but it is also under a number of logistical and theoretical constraints. An important consideration in ageing and subsequent growth studies is the validation of daily increment deposition (Geffen 1992). The recognition of some landmark or examination of otoliths from fish of known age is necessary for accurate age and growth interpretations based on increment count data. Daily increment formation has been reported in chinook, sockeye (*O. nerka*), and chum (*O. keta*) salmon (Wilson and Larkin 1980, Neilson and Geen 1982, Volk et al. 1984) and for coho (Miller 1993). However, evidence suggests that environmental conditions (i.e., temperature, photoperiod, or ration) can obscure the diel cycle of increment formation (Neilson and Geen 1982, 1985; Campana 1983). Therefore, validation of daily increment formation under specific study conditions is desirable until the mechanisms regulating increment deposition are more thoroughly understood. The use of a landmark, such as a known hatch check or a stress-induced reference mark, can serve this purpose (Fig. 4; Miller 1993, Miller and Simenstad 1994).

Various methods and discussions on the appropriate uses of otolith increment data in growth studies are common in the literature (Secor et al. 1989, Campana 1990, Francis 1990, Campana and Jones 1992, Secor and Dean 1992). Any growth determination from otolith microstructure has two implicit assumptions: (1) a periodic, or daily, formation of increments occurs, and (2) increment width is proportional to fish growth. The initial assumption can be met through the methods discussed above. The second assumption is usually met with strong correlations between fish growth and otolith growth. A variety of growth models have been used to back-calculate length and predict growth rates, including the traditional regression model, the Fraser-Lee model, and the biological-intercept model (Campana and Jones 1992). All of these models assume the relationship

between otolith and fish growth is linear through time and can therefore lead to erroneous estimates if this assumption is not met. The relationship between otolith growth and fish growth can vary, especially during periods of reduced fish growth (Jones 1992). Such evidence complicates growth back-calculations. Additional information on a greater range of ages and environmental conditions is necessary to verify growth relationships throughout the life of the fish. However, it is important to remember that examining only a short period of growth, such as the rearing habitat studies discussed here, can result in intrinsically curvilinear data appearing linear.

The most commonly used method of estimating growth from otolith increment data has been a simple linear regression model based on the otolith size to fish size relationship (Campana 1990). As a result, such models are subject to bias and error associated with the generation of both the allometric relationship and the resulting regression model. In order to avoid some bias, the researcher must consider the variables involved in model generation. For example, the sampling variability associated with length data used to establish the correlative relationship, the range of ages empirically represented, and the method used to generate the regression model will determine what conclusions can be drawn from a data set and affect the accuracy of those estimates.

An advantage of the biological-intercept model (Francis 1990) is that it incorporates information on when otolith growth and fish growth proportionality begins (i.e., the point in the life history of a species when otolith growth commences). The mean size of individuals at that time is then incorporated into the growth models as the intercept. This method requires information on fish size and otolith size at young ages to determine the intercept value but can improve growth estimates because separate growth curves are determined for individual fish. However, as the three pairs of otoliths do not always form simultaneously, models need to be specific to the otolith pair under consideration (Neilson et al. 1984). Additionally, using fish length as the dependent variable is appropriate in otolith-determined growth models. However, for any model, residuals must be randomly distributed and the variance must be constant across the data range or estimates may be inaccurate, biased at certain ages or sizes, and improperly influenced by outliers.

Methods of model generation may be changed by recent information suggesting otolith increment width is more closely associated than somatic growth with metabolic rate and temperature (Mosegaard et al. 1988, Wright et al. 1990, Wright 1991, Secor and Dean 1992). If metabolic rate and temperature are consistently shown to influence increment width, growth models will need to incorporate such variables and will undoubtedly become more complex in order to accurately predict growth under a range of environmental conditions. Additionally, the reported cases of a time-lag or uncoupling between changes in somatic growth and otolith growth suggest further caution to researchers attempting to draw conclusions at too fine a scale (Bradford and Geen 1987, Mosegaard et al. 1988, Molony and Choat 1990). This evidence, in conjunction with the recognition that slower-growing fish have larger otoliths than faster-growing fish of the same size (Secor and Dean 1989, Campana 1990, Wright et al. 1990), calls for caution when determining growth estimates through microstructure analyses without incorporating important environmental variables and validation experiments. One method for avoiding some of these biases is to examine the growth trajectories of otoliths from individual fish to determine if there are differences in increment formation during a specific time period (Fig. 5). This allows for a direct examination of otolith growth patterns for fishes residing in different environments, regardless of body size. Such

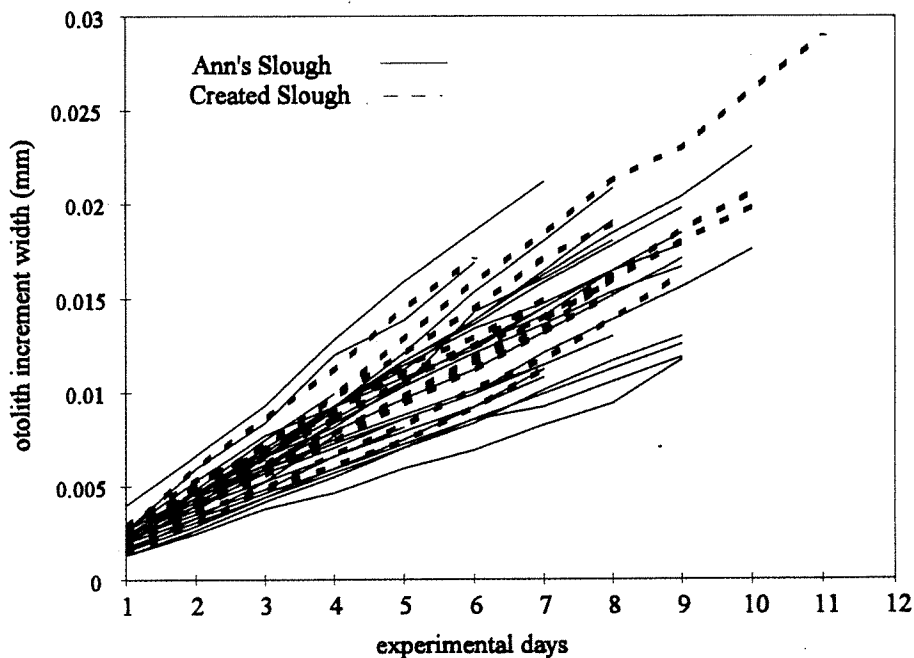


Figure 5. Individual otolith growth curves for coho (*Oncorhynchus kisutch*) salmon from the Chehalis River, Washington, May 1992.

comparisons, in conjunction with environmental, foraging, and morphometric data, provide an individually based analytical tool for comparison of fish habitat quality.

## SUMMARY

Fish otolith microstructure analysis has the potential to provide a tool for habitat assessment. Although caution must be exercised when making growth determinations under many conditions, especially when using experimental manipulations, microstructure information can quantify an individual fish's growth response to prevailing habitat conditions. In conjunction with information on environmental parameters, foraging behavior, and residence times, otolith microstructure can provide a means of evaluating available fish habitat. As more information on factors regulating increment deposition and check formation is attained, microstructure analysis may provide more detailed information specifically on how fish experience their habitats. Whether somatic growth, temperature, metabolic rate or, most probably, a combination of all of these proves to regulate increment deposition, otolith microstructure provides unique information about individuals and populations and, potentially, their environment.

## RECOMMENDATIONS

Although fish otolith microstructure offers great potential for unambiguous assessment of individual fish growth, we need to conduct further laboratory research in order to understand the processes regulating otolith increment deposition and width. Currently, research indicates that environmental variables, most probably through subsequent effects on metabolic rates, are correlated with changes in the number and width of otolith increments. Otolith formation and increment deposition also vary among species. Controlled growth experiments with individuals can provide information needed to adequately assess microstructure patterns observed in naturally variable field settings. Until such processes are understood, confidence in interpretation of otolith increment microstructure under variable conditions will be seriously limited. Therefore, we suggest the following, additional research:

- determine whether a time-lag exists between changes in somatic growth and a subsequent change in otolith microstructure under constant environmental conditions,
- determine the level of precision in growth estimates derived from otolith increment analysis (i.e., otolith growth may demonstrate a negative *trend* in fishes with reduced somatic growth but will not provide a precise measure of somatic growth),
- determine the effect of daily ration on increment formation (i.e., attempt to discern the minimum difference in ration that leads to detectable differential somatic growth under a range of environmental conditions), and
- examine the effects of activity on increment formation (i.e., determine whether different levels of activity are reflected in the otolith microstructure even among fish with similar growth rates).

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## **APPENDICES**



## APPENDIX A. GLOSSARY OF OTOLITH AND MICROSTRUCTURE TERMS

<b>Accretion zone</b>	Component zone of daily increment that is predominantly composed of aragonitic calcium carbonate as demonstrated by Dunkelberger et al. (1980) and Mugiya (1987). Ultrastructural examination has shown elongate crystals in this zone that are perpendicular to the periphery of the otolith. Also termed “incremental zone”.
<b>Antirostrum</b>	Anterior thumb-like” projection of the sagitta. It is located dorsal to the rostrum.
<b>Core</b>	Calcified area occurring within the earliest deposited increment (contained within the first discontinuous zone). Related terms, “nucleus” and “kernal” are ambiguous and not commonly used in microstructure studies (Wilson et al. 1987).
<b>Discontinuous zone</b>	Component zone of daily increment comprised predominantly of organic matrix as demonstrated by Mugiya (1987). Zone preferentially dissolves when weak acids (e.g., low concentration HCl or EDTA) are applied, resulting in narrow grooves being observed in SEM examination.
<b>Growth axes</b>	Axes within the otolith along which proportionally rapid rates of deposition occur. Axes within the microstructure where increment widths are greatest. Otoliths can have more than one growth axis, in which case axes are sometimes referred to as major and minor.
<b>Increment</b>	Bipartite concentric ring composed of alternating zones of predominantly calcium carbonate accretion zone and predominantly organic discontinuous zones. Daily increments are those that have been validated to occur at a daily rate.
<b>Increment width</b>	Linear measure of increment, comprising one <b>accretion zone</b> + one <b>discontinuous zone</b> . Usually measured along major growth axis.
<b>Otolithic membrane</b>	Noncellular membrane that adheres to portions of the otolith.
<b>Macula</b>	Sensory epithelium composed of sensory hair cells and supporting cells. Cilia bundles of the macula serve as mechanoreceptors in hearing. In the saccular vestibule, it is located along the sulcus of the sagitta.
<b>Primordia</b>	Initial deposition sites of organic matrix and calcium carbonate. Usually located in the core, primordia may fuse or remain separate, forming multiple cores.
<b>Postrostrum</b>	Posterior-most projection of the sagitta.
<b>Rostrum</b>	Anterior-most projection of the sagitta.
<b>Sulcus</b>	Sculptured groove along the medial face of the sagitta. Sulcus rests on the <b>macula</b> .
<b>Vestibule</b>	Sac structure which contains the otolith. Composed of epithelial tissue. The <b>lagenar vestibule</b> contains the lapillus, the <b>saccular vestibule</b> contains the sagitta, and the <b>utricle vestibule</b> contains the astericus.

APPENDIX B. ORGANIZATIONS PROVIDING EQUIPMENT NECESSARY FOR  
OTOLITH MICROSTRUCTURE ANALYSIS

:

Ted Pella, Inc. (molds)  
P. O. Box 492477  
Redding, CA 96049-2477  
Telephone: General: 916-243-2200  
U. S. A.: 1-800-237-3526

Struers, Inc. (grinding equipment)  
26100 First St.  
Westlake, OH 44145-1438  
Telephone: 216-871-0071

Buehler (polishing mediums)  
41 Waukegan Rd.  
Lake Bluff, IL 60044

Optimas, Inc. (image analysis software)  
170 W. Dayton, Suite 204  
Edmonds, WA 98020  
Telephone: 206-775-8000