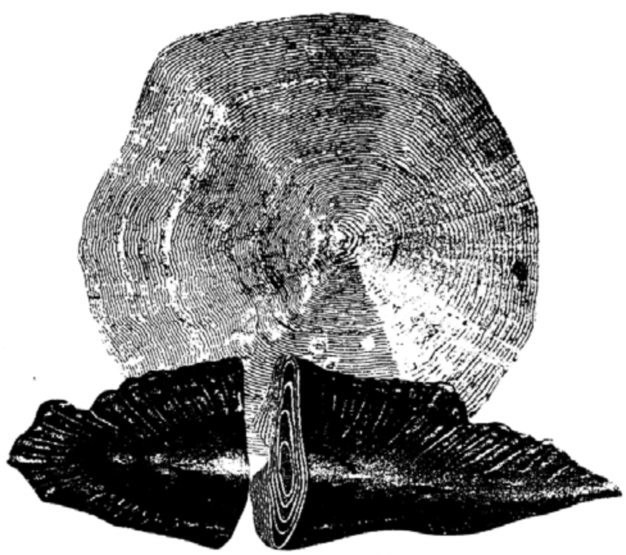
Manual of Standard Methods for the Chippewas of Nawash Calcified Structure Analysis Facility



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Annulus

a) A mark that is subjectively located on or in a calcified structure; is associated with the distal edge of a concentric ring in the form of a check on the scale or a translucent zone in other calcified structures, ***is usually apparent along the entire structure for the first four or five years (prior to maturation) of growth after which the check only appears in the anterior zones of scales and the dorsal lobe of otoliths. To be considered annual the check must be located in both the anterior and anterio-lateral zones of the scale.*** It is considered to separate the check or zone associated with the principal annual cessation or reduction in growth from the tissue deposited when growth resumes or increases. ***During the first three or four years of growth prior to maturation, two annuli may demarcate one calender year after which point, at the onset of reproduction each annulus demarcates one calendar year.*** (CNCSAF)

b) Any zone that forms once a year; frequently an annulus is identified as the zone that forms during or close to a period of no growth or slowing down of growth (Chilton and Beamish 1982)

c) A mark that is subjectively located on or in a calcified structure; is associated with the distal edge of a concentric ring in the form of a check on the scale or a translucent zone in other calcified structures; is found along the entire structure; and is considered to separate the check or zone associated with the principal annual cessation or reduction in growth from the tissue deposited when growth resumes or increases. Two successive annuli are usually considered to demarcate one calendar year of calcified tissue growth (Casselman 1983)

Checks
Marks or growth zones or parts of growth zones that do not form annually. A break in the configurations of the circuli on scales (Casselman and Scott 2000).

Circulus (scale)
Numerous relieved striations, or raised mineralized platelike striations on the surface of a scale which are concentric, or nearly so with the margin of the scale. They mark successive stages in the growth of the scale (Van Oosten 1929; Allen 1985).

Collum
Interruption in the sulcus acoustics, which marks the location of the nucleus (Penttila & Dery 1988).

Crossing over (scale)
This occurs during a period of decreasing growth. When growth first begins to slow the outermost circuli may not extend completely to the posterolateral ridge. As growth slows further, the circuli become increasingly shorter until there my be growth only in the anterior. On the resumption of growth, a full circulus (the annulus) is formed which encloses this zone of progressively shorter circuli (Allen 1985).

Edge
Outer periphery of the age structure, associated with most recent growth (Casselman & Scott 2000).

Edge condition
Categories that quantitatively and qualitatively describe conditions on the edge of the structure in terms of the seasonal growth cycle and zone formation. Makes it possible to relate annulus and zone formation. The summer/winter or opaque/ hyaline deposition occurring on the outer edge of the age structure representing the most recent growth (Casselman & Scott 2000).

Final annulus
The last clearly discernable annulus as one approaches the margin of the structure (CNCSAF).

First annulus
a) scale — The first notable irregularity in regular concentric circuli when examining the scale from the focus toward the margin of the scale. Characterised by broken circuli, crossovers or a detectable difference in circuli spacing (CNCSAF).

b) otolith — The distinct translucent band that is closest to the nucleus

Fragmented and irregular circuli (scale)
Indicate an interruption in the uniform growth of the circuli. When severe and accompanied by a change in spacing of hyaline zone, they are a good indicator of annulus location (Allen 1985).

Hyaline (scale)
A space devoid of circuli. Often associated with crossovers, these zones appear to form during periods of growth cessation (Allen 1985).

Margin
The outer periphery of the structure (Ambrose 1983).

Nucleus (otolith)
Origin of the ageing structure (Chilton & Beamish 1982).

Opaque zone
a) Inhibits the passage of light (Casselman & Scott 2000).

b) Zone that inhibits the passage of light and forms part of the annual growth pattern and is usually the period of faster growth; this zone is often thought of as the summer growth; the zone appears dark under transmitted light and clear under reflected light; in younger fish it is wider than the translucent zone but in very old fish it remains about the same width as the translucent zone (Chilton & Beamish 1982).

Origin (also: focus)
The hypothetical or real point of first growth of the whole or prepared intact structure, where the interpreter chooses to start as a reference point for measurement or enumeration (Casselman & Scott 2000).

Potential annulus
A check that is suspected to be annual but is not readily discernable; appears when contrast is manipulated due to clear differences in circuli spacing and has few breaks or crossovers, comparison among a number of scales from an individual will allow one to discern if potential annuli are actual annual checks (Casselman 1983).

Regenerated (scale)
Refers to a scale that was formed to replace one which was lost or damaged (Ambrose 1983).

Resorption
Removal of calcified material by osteoclast cells in the scale (Simkiss 1974).

Radius
Linear distance from the origin to a specific point, usually the edge (Casselman & Scott 2000).

Reflected light
Light that is shone onto the surface of an object from above or the side (Chilton & Beamish 1982).

Rostrum
Anterior most projection of the sagitta (Secor et. al. 1995).

Sagitta (pl. Sagittae)
Largest of the three pairs of otoliths located in the sacculus of the inner ear of a fish (Penttila & Dery 1988).

Sulcus acoustics
A groove along the medial surface of the sagitta (Secor et.al. 1995).

Transmitted light
Light that is passed through the object from beneath (Chilton & Beamish 1982).

Translucent zone
a) Allows the passage of light (Casselman & Scott 2000).

b) The zone that forms during or close to a period of no growth or slowing down of growth; this zone is often thought of as the winter zone and is frequently identified as the annulus; the zone appears clear under transmitted light and dark under reflected light; in younger fish it is narrower than the opaque zone but in very old fish it remains about the same width as the opaque zone (Chilton & Beamish 1982).

Year class
Fish hatched in a given year (Casselman & Scott 2000).

1. Introduction

1.1. Calcified structure analysis

Lake whitefish is the most important commercial fish species in Southern Georgian Bay and the eastern main basin of Lake Huron, the traditional fishing grounds of the Saugeen Ojibway First Nation. As part of responsible management of the fishery, the Chippewas of Nawash First Nation Fisheries Assessment Program studies the biology, the life cycle and the distribution of the lake whitefish. Accurate age estimation is essential to population assessment and is the foundation upon which valid and conservative fisheries management practices are built. Age-based estimates of growth and mortality are the key to population modeling and ultimately to the setting of safe harvest levels for both recreational and commercial fisheries. The process of age estimation involves the interpretation and counting of growth zones or growth checks which appear in the hard parts of fishes, not unlike those in trees. These growth checks are commonly referred to as annuli and are formed during alternate periods of faster and slower growth and reflect various environmental or internal influences (Tesch 1971).

1.2 Chippewas of Nawash Calcified Structure Analysis Facility (CNCSAF)

To study the age and growth of lake whitefish, the Chippewas of Nawash began developing their Calcified Structure Analysis Facility (CNCSAF) in 2001. The Fisheries Assessment Program has been using scales of lake whitefish to estimate age structure and year class strength since 1999. In 2001 the program began collecting otoliths of lake whitefish and in 2003 both scales and otoliths are used for routine age and growth analyses.

Over the years an increasing number of protocols for age and growth studies were developed on the basis of adaptive management with the following mandate, goals and objectives in mind.

Mandate: Rigorous scientific study of fish calcified structures for the purpose of quantifying and validating fish age and for analyzing calcified structure growth patterns as they relate to somatic (body) growth.

Goal: Provide Chief and Council (managers of the fishery) with accurate age estimates that have been thoroughly validated and based on the best available techniques.

Objectives:

- to increase managers confidence in age estimates.

- to apply the principles of adaptive management, specifically alternative hypotheses and the testing of predictions generated from these hypotheses to the field of fish age estimation.

- to validate age estimation techniques and procedures.
- to minimize sampling error and biases.
- to develop the tools necessary for advances in the field of fish age estimation, particularly digital image analysis.
- to develop known-age, or agreed-age digital reference collections for Lake Huron bloater chub and lake whitefish.
- to develop and adhere to strict principles of quality control.
- to maintain scientific rigor and transparency of our work through web-based open forum and peer review.

This manual includes all the protocols that have been developed for the analysis of calcified structures. It can be used by people who want to learn about processing calcified structures for age estimation of fish and experienced people who want to use it as a reference guide. Standardizing methods for processing calcified structures for age estimation is important to achieve consistency. To standardize the procedures for processing calcified structures it is necessary to follow the instructions in this manual closely. However as new and better methods develop the contents of this manual will be subject to change.

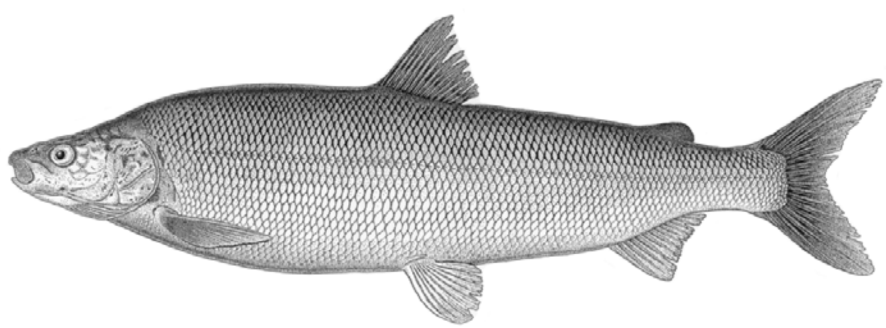


Figure 1.1. lake whitefish (*Coregonus clupeaformis*)
drawing by Paul Vecsei

2. Sampling and sample size

Establishing appropriate statistical sampling rates is a critical exercise which is aimed at ensuring a sample is representative of the population (part of the entire population) being sampled without wastefully over-sampling. The determination of spatio-temporal statistical sampling rates acts to economize the Assessment Program and lends statistical rigour to subsequent analyses. CNCSAF uses sampling rates for Lake Huron / Georgian Bay that are based on the work of Bruce Morrison (2000) (Table 2.1). Specific descriptions of biological sampling procedures are given in Muir and Crawford (2001).

Table 2.1 Seasonal sampling rates for the Chippewas of Nawash Commercial Fisheries Assessment Program.

Species	Season	Sampling rate*	# Fish / Box
Lake whitefish	winter	1 in 10 (10%)	3
	spring	1 in 20 (5%)	2
	summer	1 in 20 (5%)	2
	fall	1 in 50 (2%)	1
Bloater chub	year-round	1 in 50 (2%)	6
Yellow perch	year-round	1 in 50 (2%)	6
All other species	year round	1 in 20 (5%)	2

*Seasonal sampling rates determined for lake whitefish were based on the Fisheries Statistician's analysis of variation in the 1998 biological sample data for lake whitefish (Morrison, 2000).

As depicted in Figure 2.1, there are four levels of subsampling involved in the age estimation process. These can be described as follows;

a. Subsample 1: The first level of sampling is the commercial harvest which is non-random or targeted in both time and space. This sample is characterized by the type of gear employed and the skill of the fishermen responsible for its harvest.

b. Subsample 2: Based on the afformentioned sampling rates, a random sub-sample of individuals is drawn from each commercial landing, and biological samples such as total and fork length (mm), weight (Kg), and scales and/or otoliths are collected from each individual specimen.

c. Subsample 3: From subsample 2, 100 random individuals per region, per season are drawn using random number generation. These individuals represent the first sample of individuals that will be analysed for age.

d. Subsample 4: Of the scales collected from a given individual in the field, three are then selected for analysis. Otoliths are collected from a fraction of the individuals that compose subsample 2. All otoliths collected in subsample 2 are used in the analysis procedures described below.

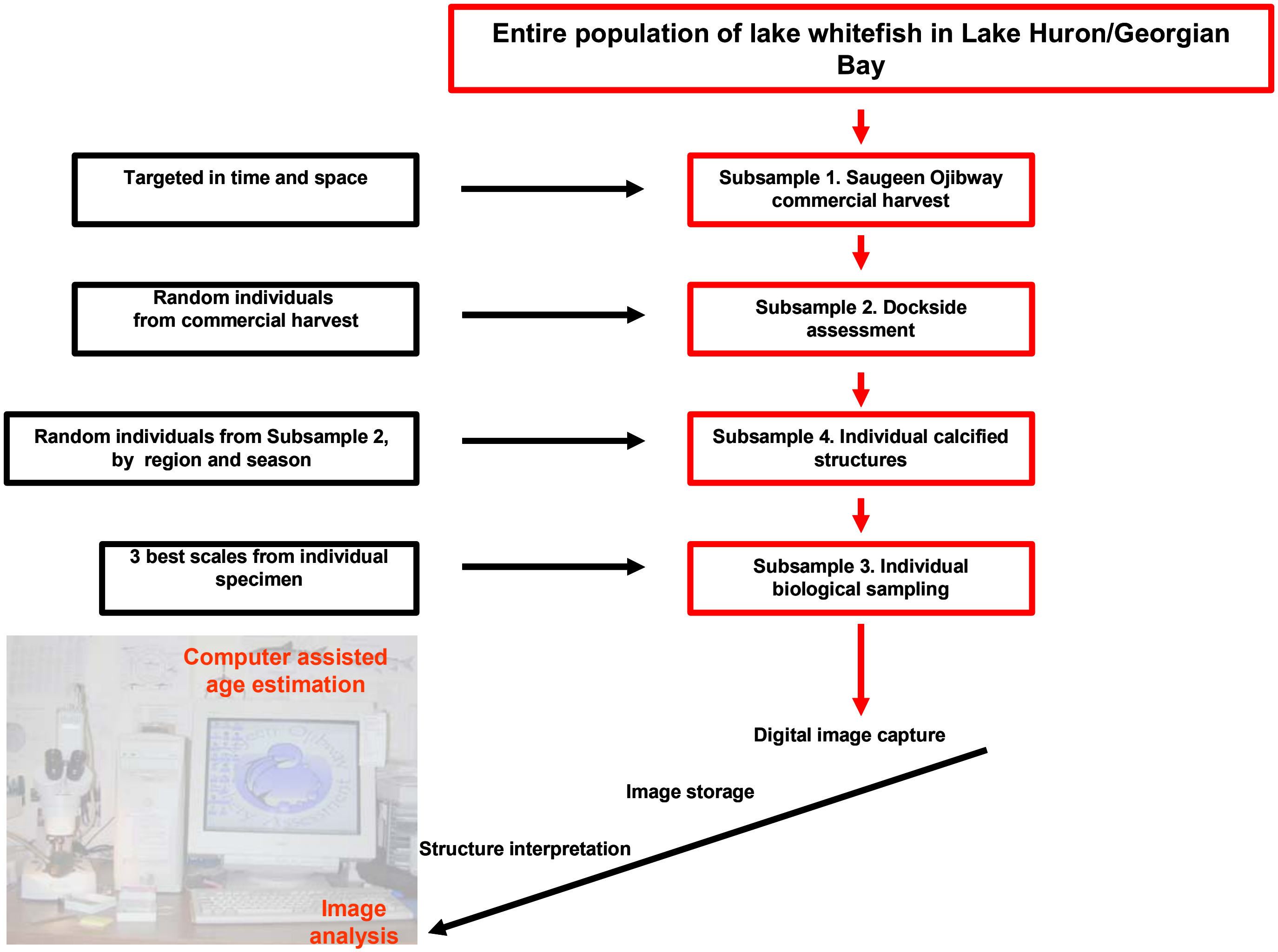


Figure 2.1 Subsampling associated with the Chippewas of Nawash Commercial Fisheries Assessment Program

3. Structure Preparation

3.1 Introduction

The various structures traditionally used for age estimation of lake whitefish, like scales, otoliths, statoliths etc. need some kind of preparation to make the growth bands that are used for age estimation apparent. This chapter details the steps necessary to prepare the structure for age estimation.

3.2 Otoliths

Collecting and processing otoliths for age estimation is more labor intensive and time consuming then using scales. However many studies suggest that otoliths give a better representation of age than scales (Secor et. al. 1995; Chilton and Beamish 1982). The reason for this is that unlike scales, growth in otoliths does not slow down in older fish (Casselman 1990), and annuli on the edge of otoliths are less crowded and easier to interpret than those on scales. Because of their unique growth process, otoliths are not subject to resorption and remodeling as are scales. Another advantage of otoliths is that they can be used for other research besides age and growth studies. For example hypotheses regarding stock identification, migration patterns and reproduction schedules, can be addressed using otolith meristics and morphometrics.

CNCSAF uses the sagittal otolith for estimating age of lake whitefish. The sagittal otoliths are the largest of the three pairs of otoliths usually found in bony fish. For convenience the sagittal otoliths will be referred to as otolith for the remainder of this report.

CNCSAF collects and stores both otoliths from each individual, but for routine age and growth assessment we use only one otolith (the right side otolith). Processing one, rather than two otoliths per fish, reduces processing time and the cost per fish. The left side otolith is saved for future research such as age verification, growth estimates, other methods for age estimation practices, or alternatively, for other types of research altogether. The remainder of this section describes the procedures for cleaning, embedding, sectioning and mounting of the otolith for age estimation. In section 3.2.5 a side note is made about taking measurements of the otoliths because if the otoliths are embedded it is too late to take the these measurements. Appendix 3 summarizes structure preparation procedures used by other labs.

3.2.1 Specifications for the saw and blades

CNCSAF uses the Raytech AL-P10S lapidary saw which is equipped with a 1/3 HP motor, that runs at 1725 rpm (Figure 3.1). The power feed moves the carriage at a rate of 25 cm per hour. We installed a 100 mm diameter diamond impregnated blade with a thickness of 0.23 mm (blue blazer 4 x 006 x 009). This is the smallest size blade that can be installed on this saw. The blade sticks only 7 mm out from the surface of the saw table. The blade is run through a sump that is filled with water for cooling. <u>Modifications;</u> To use the 100 mm blades we had to lower the surface where the resin block with the otoliths is placed. We did this by cutting an area from the carriage, and placing the resin block directly on the saw table. We replaced the heavy duty clamp that was standard on this saw with a finer one. The clamp is attached to the cross-feeder. Turning the handle of the cross-feeder a full turn moves the clamp 1.4 mm towards the blade. On the handle of the cross feeder we attached a dial that divides a full turn into 8 equal sections. The amount the dial had to be turned to achieve the right section thickness was determined by trial and error. When we use 0.23 mm blades the sections produced are 0.2 - 0.3 mm when we turn the handle 3/8th of a full turn.

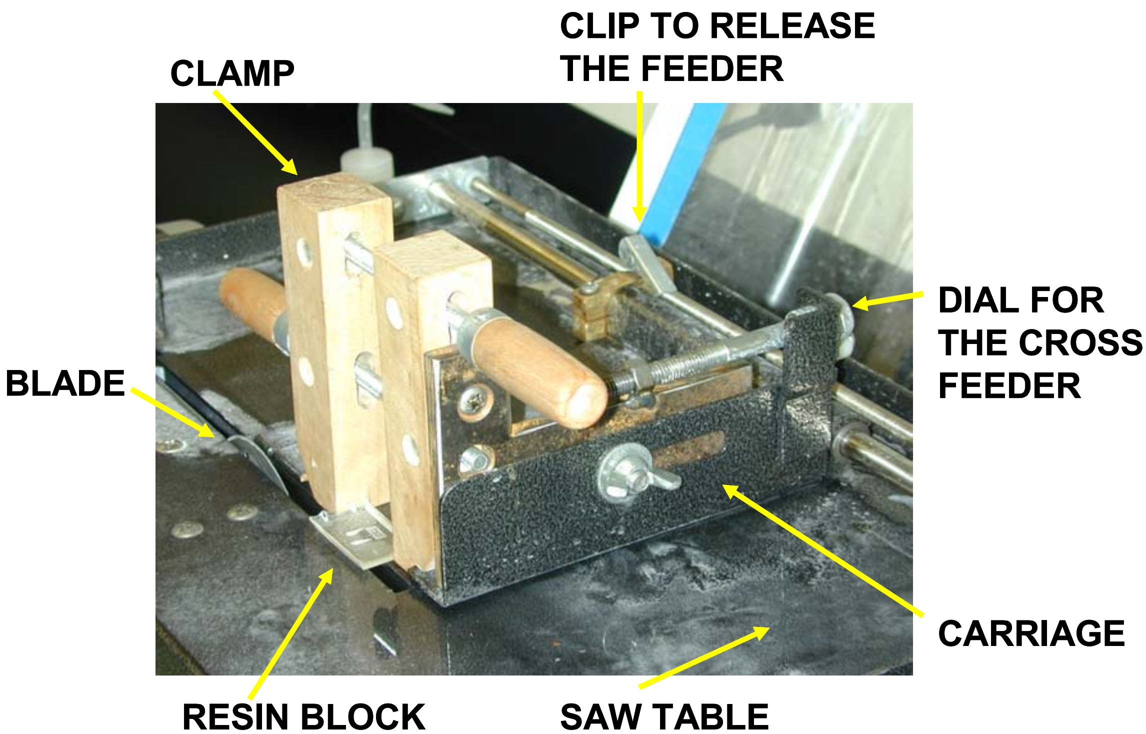


Figure 3.1 CNCSAF modified lapidary saw for sectioning calcified structures.

Saw

- suitable for blades with a diameter of 10 to 12.5 cm (4' - 5')
- a motorized or gravity feed or another mechanism move the sample across the blade
- a clamp that can securely hold the sample
- a dial to position the sample with precision.

Blade

- diamond impregnated
- diameter between 10 and 12.5 cm (4 - 5")

- thickness not less than 0.2 (i.e. the blade is too unstable)
- thickness not more than 0.3 mm (i.e. the blade removes to much otolith material)

3.2.2 Tool checklist

Cleaning and storage of otoliths
- soft brush
- small plastic tubes (Eppendorf)
- manila coin envelopes
- paper towel
- plastic otolith tray with lid (100 squares each 1cm x 1cm)

Embedding otoliths
- resin with measuring cups or syringes
- stirring cups
- stirring sticks
- mold
- paper tags and pencil
- fine grit waterproof sandpaper
- microscopy slides (3" x 1")

Sectioning otoliths
- saw (i.e. Raytech AL-P10S)
- blades (i.e. Blue blazer 4 x 006 x 009)

Measuring otoliths (optional)
- caliper (accurate to 0.001 mm)
- digital scale (accurate to 0.001 grams)

3.2.3 Sample cleaning and storage

When the otoliths are collected from the fish they should be wiped with a paper tissue to remove excessive blood and tissue. The otoliths are temporarily stored in plastic Eppendorf tubes. The tubes are kept in manila coin envelopes with the date, species, length, weight, and fisherman recorded on it. In the lab the otoliths are removed from the plastic tube and checked again for remains of blood and tissue and cleaned if necessary with water, a soft brush, and paper tissue. The otoliths are stored dry in trays with 100 square cubes, each cube containing the otolith pair form an individual sample. A unique individual code is documented and assigned to the otoliths.

3.2.4 Otolith preparation

A) Baking

Baking serves to enhance visibility of the annuli of lake whitefish otolith, since the greater concentrations of organic material in the translucent zones turn brown and contrast with the white opaque zones. The best results are achieved when the otoliths are baked for 20 minutes at 175 ˚C. If the weight of the otoliths is measured it is necessary to bake them to reduce the moisture content to an equal level within all the otoliths. Recommended baking time is 60 ˚C for 24 hours (Beamish 1979). The weight is recorded to the nearest tenth of a milligram (see also section 3.1.5).

B) Embedding

Molds for embedding otoliths are normally made out of silicone rubber or aluminum. Silicone rubber molds can be ordered in several sizes and kits for making these molds are also available. Aluminum molds usually come in two parts to facilitate the removal of the resin blocks. The dimensions of the mold depends on the size of the otoliths and the number of otoliths that one wishes to embedded in the resin blocks. To cut the resin block with a diamond impregnated saw we recommend a height of the resin block of at least 7 mm. A raised grid on the inside of the mold facilitates the placement of the otoliths.

Otoliths can be embedded in either epoxy resin or fiberglass resin. The following properties are desirable in a resin for embedding otoliths: high viscosity, low shrinkage, slow curing, clear, good penetrating qualities but not so much that the resin damages the molds, and non-toxic. Both epoxy resin and fiberglass are hazardous materials that should be handled with care. Read the material safety data sheets (MSDS) of the material you are working with to reduce the chance of accidents and to be prepared if one does happen.

√ *Safety first when working with resin*
- work in a well ventilated room
- protect your hands with gloves
- protect your skin with protective clothing
- protect your eyes with safety goggles

√ *Prepare the bottom layer of resin*
- apply a release agent to the surface of four Pelco flexible locator molds
- prepare 30 ml of System Three Clear Coat Epoxy resin by mixing 20ml of resin with 10 ml of hardener for two minutes
- pour a layer 3 mm thick into each of the molds
- let the resin cure for 48 hours before removing the layer from the mold

√ *Glue the otoliths to the bottom layer*
- with pencil write the unique individual code on the embedding tag that is fixed between the two layers (Figure 3.2)
- glue the tags in the center of the bottom layer, using all purpose contact cement
- place the bottom layer under a dissecting microscope and use transmitted light

- with tweezers place the right side otolith in a drop of fast curing super glue next to the corresponding harvest number. The otoliths are placed with the distal edge upward and the rostrum pointed towards the tags in the center. Ideally the nuclei of all six otoliths are in one line approximately 5 mm from the edge of the resin block (Figure 3.2, 3.4, 3.5). Let the glue cure for a few minutes before covering the otoliths with epoxy resin.

✓ *Cover the otoliths with a layer of resin*

- Prepare 30 ml of System Three Clear Coat Epoxy resin by mixing 20ml of resin with 10 ml of hardener for two minutes
- Cover the otoliths with 4 mm of epoxy resin, the block should not be more than 7 mm thick
- Let the resin cure for 48 hours before sectioning the block

C) Sectioning

The otoliths of mature lake whitefish are thick and growth patterns cannot be distinguished from the surface. Growth patterns can be observed in cross sections of the otolith by sectioning them. Growth patterns are clearly distinguishable in sections of 0.2-0.3 mm in lake whitefish. These growth patterns are most apparent in the areas of slower growth, next to the sulcal groove. Observing growth patterns is easier in transversal sections of lake whitefish otoliths than in the frontal sections. Making sagittal sections is impractical and not likely to give better results than transverse or even frontal sections. Sections are made through, or as close as possible to the nucleus. Only sections that include the nucleus give a complete picture of the growth pattern of the otolith.

Figure 3.2 Twelve right side otoliths of lake whitefish embedded in resin (System Three Clear Coat epoxy). The nucleus is placed as close as possible to the grid line using a dissecting microscope and transparent light. N.B. The paper is a marker which allows for correct identification of the sections after the resin strip has been separated from the block.

Figure 3.3 Two resin strips (System Three Clear Coat epoxy) each containing six sections of lake whitefish otoliths and a paper tag with the corresponding individual codes fixed between two microscopy slides (3" x 1") with System Three Clear Coat epoxy resin.

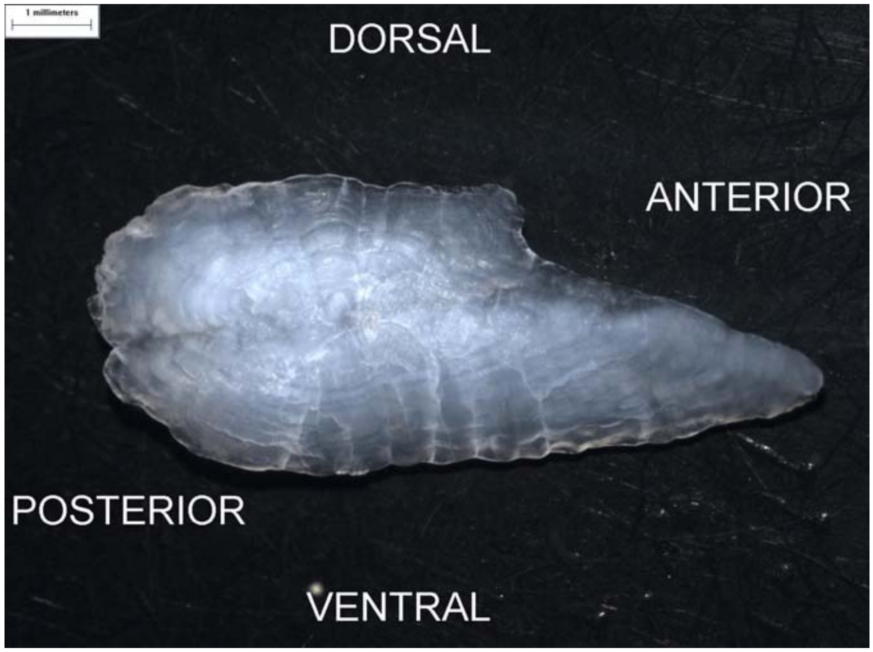


Figure 3.4 Image of the distal (exterior) right side otolith of a lake whitefish from Lake Huron

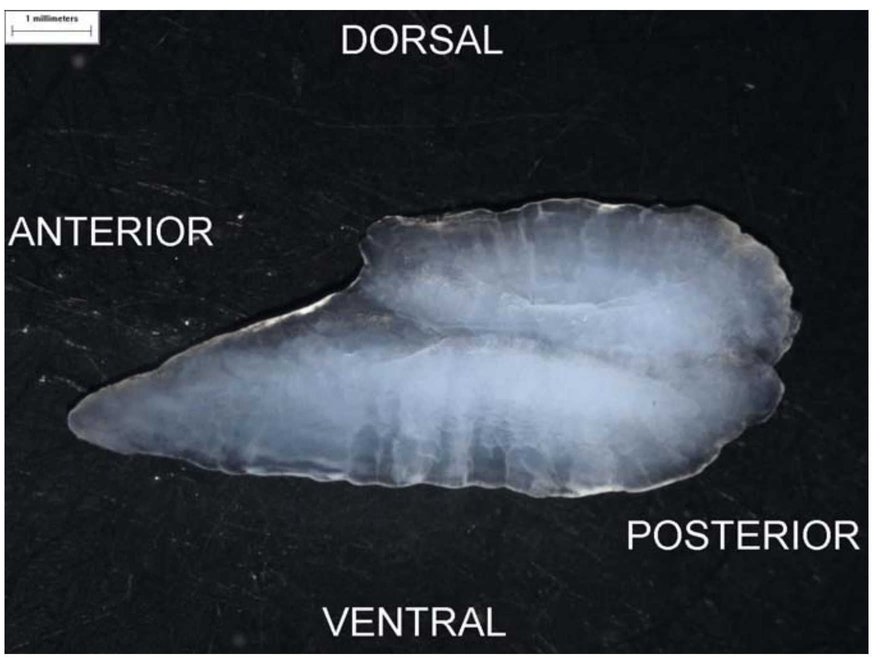


Figure 3.5 Image of the proximal (internal) side of the right hand side otolith of a lake whitefish from Lake Huron. Notice the sulcal groove in the antero-posterior axis.

✓ *Making a thin section through the nucleus of the otolith*

- place the block with the otoliths in the clamp
- line up the block parallel to the blade
- tighten the clamp
- move the feeder carriage back until the block is in front of the blade
- adjust the cross feeder so the first cut will close to the nuclei
- turn on the power of the blade and the automatic feeder
- when the first cut is complete stop the saw and return the carriage to the start position
- turn the cross feeder 3/8th of a turn (if you are using the Raytech 4” Blue Blazer) to position the block for the next section.
- turn on the power to the blade and the automatic feeder
- turn off the power of the automatic feeder and the blade just <u>before</u> the block is completely cut
- remove the strip of resin with the otolith sections by cutting it off with a sharp pair of scissors.
- make another strip in the same way (and more if required)

D) Mounting otolith sections

The resin strips containing the otolith sections are labeled with unique individual codes and fixed between two micro slides with epoxy resin. Mounting the resin strips between two slides reduces glare when the sections are observed through a microscope. Fixing the sections in resin makes them more transparent and as a result the clarity of the annuli increases. This method results in better image quality and the sections are better protected for long term storage. We prefer using this method over others such as; fixing the sections to one slide with transparent acrylic paint or fixing the sections to one slide using Crystal Bond.

Sanding the sections lightly before mounting them makes the surface very smooth and is recommended. Although staining with glycerin or cedar oil does seem to make the sections more transparent, we found the annuli can be read just as well without this treatment.

✓ *Sanding the sections*

- rub the sections for one minute over 1500 grit Silicon Carbide waterproof sand-paper wetted with water
- rinse the sections with water
- dry the sections for two hours before mounting them on slides

✓ *Fixing the sections between two slides with resin*

- wear gloves and goggles for protection!
- prepare 15 ml of System Three Clear Coat Epoxy resin by mixing 10ml of resin with 5 ml of hardener for two minutes
- write the individual code on a micro slide tag with pencil

- place the sections with the proximal edge above on the micro slide
- place the tag on the slide, above the sections (Figure 3.3)
- pour four or five drops of resin on the sections and the tag
- place another micro slide on the sections and gently press, to remove all the air from underneath the micro slide
- wipe excess resin from the slides carefully and place the slides horizontally on an elevation to prevent the slides from getting glued to the table
- let the resin cure for at least 24 hours.

3.2.5 Side Note; Taking measurements of the otoliths

The dimensions and the weight of the otoliths contain important information about the lake whitefish. It is useful to measure these characteristics before the otolith is embedded and this information is lost. Otoliths are suitable to identify and discriminate among stocks of fish because their structure is less variable and their growth is less extreme than that of scales and is less influenced by short-term environmental conditions (Casselman 1978).

Length and height are measured from an image of the distal (exterior) side of the otolith. These measurements can be taken from a calibrated digital image. Image capture relies on a zoom stereo microscope with 1.2 X magnification and reflected light from an angle. To reduce reflection, the otolith is placed in a black tray and submerged in water. Thickness of the otolith is measured at the thickest point with a caliper. A good reference for shape analysis of otoliths is Casselman (1981).

If the weight of the otoliths is measured it is necessary to bake them to reduce the moisture content to an equal level within all the otoliths. Recommended baking time is 60 $^{\circ}$C. for 24 hours (Beamish 1979). The weight is recorded to the nearest tenth of a milligram.

3.3 Scales

The purpose of this section is to detail the tools and the procedures for preparing, and analysing lake whitefish scales for the purpose of age estimation (Figure 3.5). Age estimation for lake whitefish in Lake Huron began with the work of John Van Oosten. Van Oosten (1923) investigated periodicity of lake whitefish scales which he raised in the New York Aquarium. Based on the finding that scales from these artificially reared whitefish "*showed the same number of 'annuli' as that of the number of winters in the fish's life*", Van Oosten claimed that accurate ages could be estimated from the lake whitefish scale.

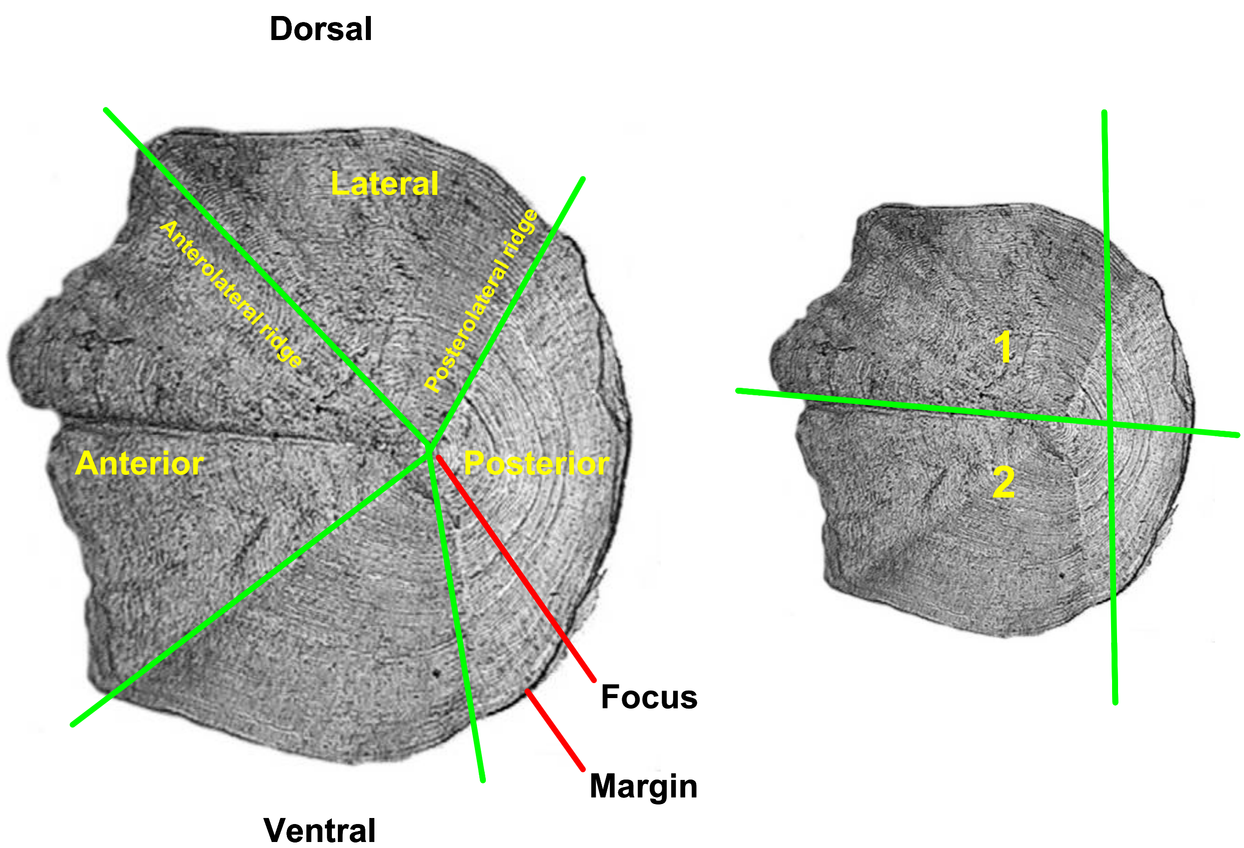


Figure 3.5 Lake whitefish scale features and division of scale into quadrants for image capture

3.3.1 Tool checklist

- 3 X 1 inch non-frosted glass microscope slides
- toothbrushes
- paper towel
- bibulous drying paper
- tweezers
- Scotch tape
- acetone and soap
- Petri dishes
- water

3.3.2 Scale storage

At the time of collection all scale samples are stored between paper in manilla coin envelopes. The envelopes are clearly labeled with a unique identification number and the date, species and effort number from the associated harvest.

3.3.3 Scale preparation

A) Cleaning

✓ *Clean the scales*

- when selecting the scales hold them up to the light to ensure that they are not regenerated (Figure 3.6)
- ensure that all three scales selected are of approximately equal shape and size
- place 3 scales form an individual in a petri dish
- add a mild detergent solution and let soak for approximately one minute
- using tweezers take one scale at a time out of the water, and scrub both surfaces vigorously using the toothbrush
- ensure that all mucus, dried blood, epithelium and any pigment is removed
- dry scale thoroughly using bibulous drying paper, or other lint-free towel

B) Mounting

✓ *Mount the scales*

- place scale on glass slide with the medio-lateral (shiny) side facing down and the anterior of the scale facing to the left (Figure 3.7)
- once all three scales have been cleaned and positioned, place a second clean, dry slide overtop of the scales
- use Scotch tape to seal the scales securely between the two slides

✓ *Label the sample*

- the individual sample number, the year, the region and the effort numbers are recorded on the tape at one end of the slide

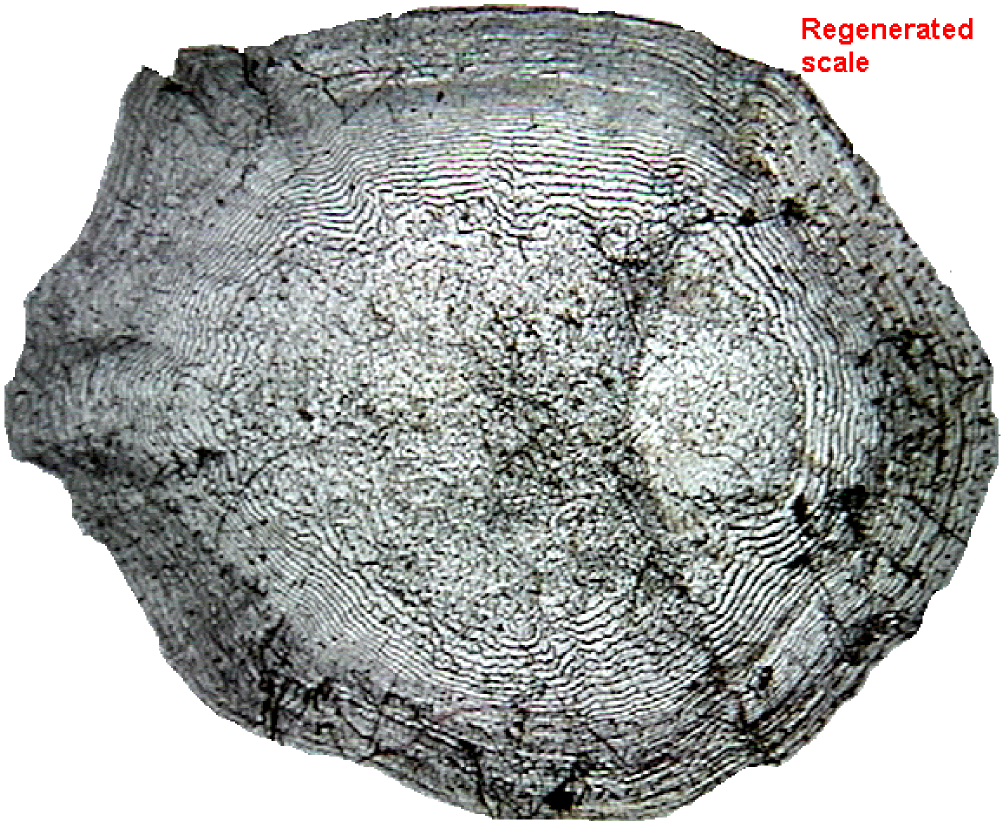


Figure 3.6 A regenerated scale of a lake whitefish from Lake Huron. An easy rule to identify scales which are regenerated is by holding them up to the light. If all ridges extending from the margin converge to a common point at the nucleus of the scale then the scale is not regenerative. If the ridges do not meet at the center of the scale, then it is likely regenerative.

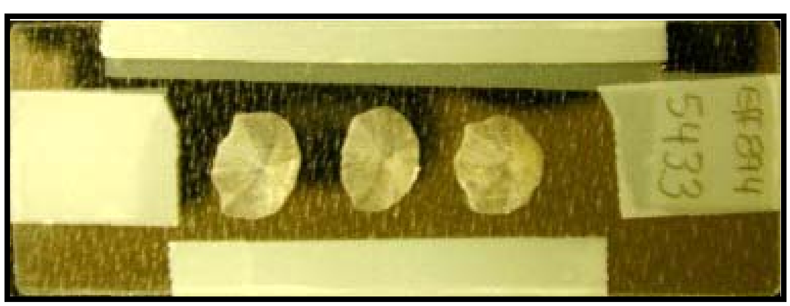


Figure 3.7 Three scales of lake whitefish between microscopy slides (3" x 1")

4. Image Capture

4.1 Introduction

Images are acquired using a colour digital camera attached to a microscope and captured using an Integral Technologies MV Lite Image capture board. The Image capture phase of the age estimation process relies on image analysis software. CNCSAF uses Image Pro Express software to enhance scale features, quantify and tag image features, to analyse image tagging data, and ultimately assign an age estimate in years to each individual. Once captured, images are enhanced, and stored on the hard-drive (C:/) as *.jpeg* files. Once all images for a given project are captured, they are then transferred to CD's for permanent long-term storage.

4.1.1 Image capture equipment specifications

- Digital camera (i.e. Hitachi KP-D50U CCD Color Camera, Roper Scientific CoolSNAP-Pro *cf* Color).
- Image capture board (i.e. Integral Technologies MV Lite)
- System microscope with 2X or 4X magnification (otoliths only) (i.e. Fisher Scientific Micro-master Microscope or Olympus BX41) **OTOLITHS ONLY**
- dissection microscope with a 0.4 X magnification (i.e. MITUTOYO, Olympus SZ40) **SCALES ONLY**
- Image Analysis Software (i.e. Image Pro Express 4.0)
- Personal computer with Pentium a 4 processor, 20GB available storage capacity, Microsoft windows 2000 operating system, a A CD-ROM Drive for reading and writing is recommended.
- 17 " Color monitor is minimum standard (i.e. Dell Optiplex GX260, Dell M992 Color monitor).

4.1.2 General image capture procedures

√ *Preview the image*
- start Image Pro Express
- secure a sample on the stage of the microscope
- left-click on the drop-down *Acquire menu* and select the *Setup Acquire* option
- left-click the *Start Preview* button in the FlashBus/32 window
- adjust the microscope so that the image is focussed
- adjust the position of the sample until the desired area is in view (see Appendix 2)
- adjust the light intensity and filters

√ *Capture the image*
- left-click the *Stop Preview* button
- left-click on the *Snap* button

√ *Storing the image*

- left-click the drop-down *File* menu and select the *Save-as* option
- in the *Save File As* window select *JPEG* in the *Save as type* box
- give the image a *File name* according to the conventions in Figures 4.1 and 4.2
- save the image to the appropriate folder in the *fish ageing database*

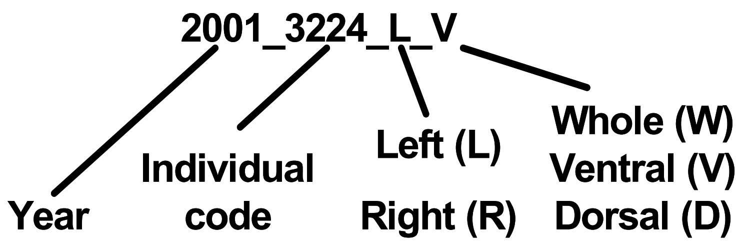


Figure 4.1 Otolith image file storage convention

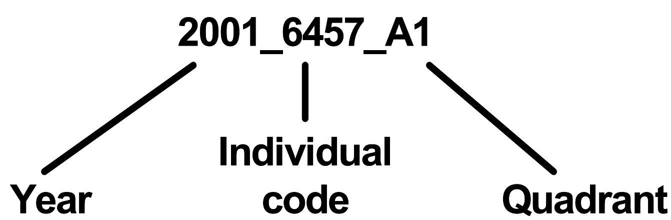


Figure 4.2. Scale image file storage convention

4.2 Otolith image capture protocols

If the left and the right otolith are sectioned, images of both otoliths should be captured, and saved with code L for left and R for right. If more than one section of the same otolith is available, one section should be selected based on the quality of the section i.e. clarity of the annuli, transparency of the section, sharpness of the bands.
Three images are taken of each otolith. If more sections are available only the "best" section should be captured and saved in a file.

1) whole section (2x magnification)	Code **W**
2) ventral side, with a large part of the nucleus (4x magnification)	Code **V**
3) dorsal side, with a large part of the nucleus (4x magnification)	Code **D**

The images of otoliths are taken with transparent light. The sections of lake whitefish otolith always appear dark in the center and bright at the edge when viewed with a transparent light source. Adjust the light until the edge of the bands at the edge of the otolith are just visible. The center should not be too dark.

4.3 Scale image capture protocols

For our purpose scales are arbitrarily divided into quadrants based on existing scale features (Figure 3.5). This is done for the following reasons: 1) Image Pro Express does not permit the capture of a high resolution image of the entire scale, 2) the posterior of the scale does not contain useful information with which to estimate lake whitefish age in Lake Huron, and 3) because we are only interested in the anterior of the scale, quadrants permit within-fish replication. The image of a quadrant is taken in a way that the entire focus and first two annuli are visible. In summary, we use three scales from each individual fish, and are capturing images of the two anterior quadrants from each scale. We therefore, end up with six images for each individual fish.

5. Structure Interpretation

5.1. Introduction

This chapter details the general conventions used for structure interpretation by CNCSAF. At CNCSAF each species in each water body is treated uniquely when it comes to interpretation. Distinction is also made between the type of structure that is being analysed to estimate age. This is done because, evidence suggests that age estimates are strongly influenced by growth (Casselman 1983) which in turn is influenced by the environment. For these reasons, the definitions and criteria for scale feature identification detailed below have been developed. The sections of the definitions which differ from conventional definitions appear in italics.

5.1.1 Image presentation

In many conventional age estimation methodologies the analyst is made aware of the date of capture, the identity of the individual and known information on the growth of the individual or of the population from which that individual was used. This information is factored into the analysis making some conventional methodologies inherently biassed in their design. CNCSAF has taken a different approach. CNCSAF uses a blind design in which scale quadrants are randomly displayed and analyzed with no knowledge of the biological or effort data associated with it's harvest.

During analysis, 25 random, unknown quadrant images are opened at once using the Image Pro software. The analysis process involves further image enhancement or manipulation and tagging of annuli. Tagging data are then exported to a Microsoft Excel spreadsheet where mean, median and mode, variance and statistical confidence are automatically calculated, and analyst tagging confidence and comments are manually assigned to each image. A confidence ranking system is an integral part of the CNCSAF age estimation process. It is a form of quality control that provides a powerful filter with defined thresholds to eliminate information that is not of superior quality. The specific procedures for analysis are detailed in Appendix 2.

5.1.2 Allocation of fish birthdays

According to the literature, it is believed that growth ceases or slows in the winter and as such an annual check is laid down on scales separating the period when growth resumes in the spring. Growth continues throughout the summer until water temperatures begin to cool in the fall at which point it slows prior to the onset of spawning to complete the cycle. As it has never been validated, the timing of annulus formation in lake whitefish in Lake Huron is uncertain. CNCSAF are currently addressing hypotheses regarding the timing of annulus formation, but based on the literature we have adopted the convention of arbitrarily assigning a birthday for all fish on January 01 (Hile 1936).

Birthday decision rule: Individual fish are corrected for season of capture post-analysis, taking into account the conventional January 01 birthday. The decision rule states that: if an individual was captured between January 01 and May 01(the onset of growth in the spring), an extra calendar year is automatically added to the estimated age of that individual.

5.1.3 Edge interpretation

The edge of the structures is characterized as either new growth present or new growth absent beyond the final annulus. The reason for this distinction is that if one considers the general birthday rule, it assumes that at the time of capture an annuli has not been laid down on a structure for the past years growth. By coding the amount of new growth, and tying this to the birthday decision rule, potential errors in counting annuli more than once are minimized.

5.2 Interpretation of Lake Huron lake whitefish otoliths for age estimation

In temperate-zone waters, fish exhibit seasonal growth patterns which are believed to be indicative of age. The seasonal growth patterns appear as dark and light bands on the cross section of the otoliths. In most fish, when transmitted light is used, the summer growth zone appears opaque or dark, and the winter growth zone appears as a hyaline or translucent zone and is called the annulus. The former represents a zone of active growth. When using reflected light the “opposite” is observed, the annulus is dark and the growth zone is light. The initial few years, as seen on an calcified structures are represented by large wide growth zones. As fish get older and growth slows down, the annual growth zone laid down is much smaller and the opaque and translucent zones may be equal in width. (Chilton and Beamish 1982). Marks or growth zones or parts of growth zones that do not form annually. Are called checks. Rapidly growing otoliths sometimes produce wide growth zones which often contain these checks. However, by examining an area of slower growth it is usually possible to eliminate the problem of having to identify checks (Chilton and Beamish 1982).

5.2.1 The Lake Huron lake whitefish otolith

When viewed with transparent light the sections of lake whitefish appear very dark around the nucleus and bright towards the edge. The material in the center appears to be much denser than that of at the edges.
Definition: This annulus is considered to be any zone that forms once a year; frequently an annulus is identified as the zone that forms during or close to a period of no growth or slowing down of growth.

As in most otoliths of fish from temperate waters, the annuli of lake whitefish are translucent and they appear bright when the sections are viewed with transmitted light (see Figure 5.1). The annuli are associated with slower winter growth. The wider and darker bands that are associated with the summer growth often contain many checks. The checks in the faster growth areas of lake whitefish otoliths can easily be distinguished from the areas of slower growth.

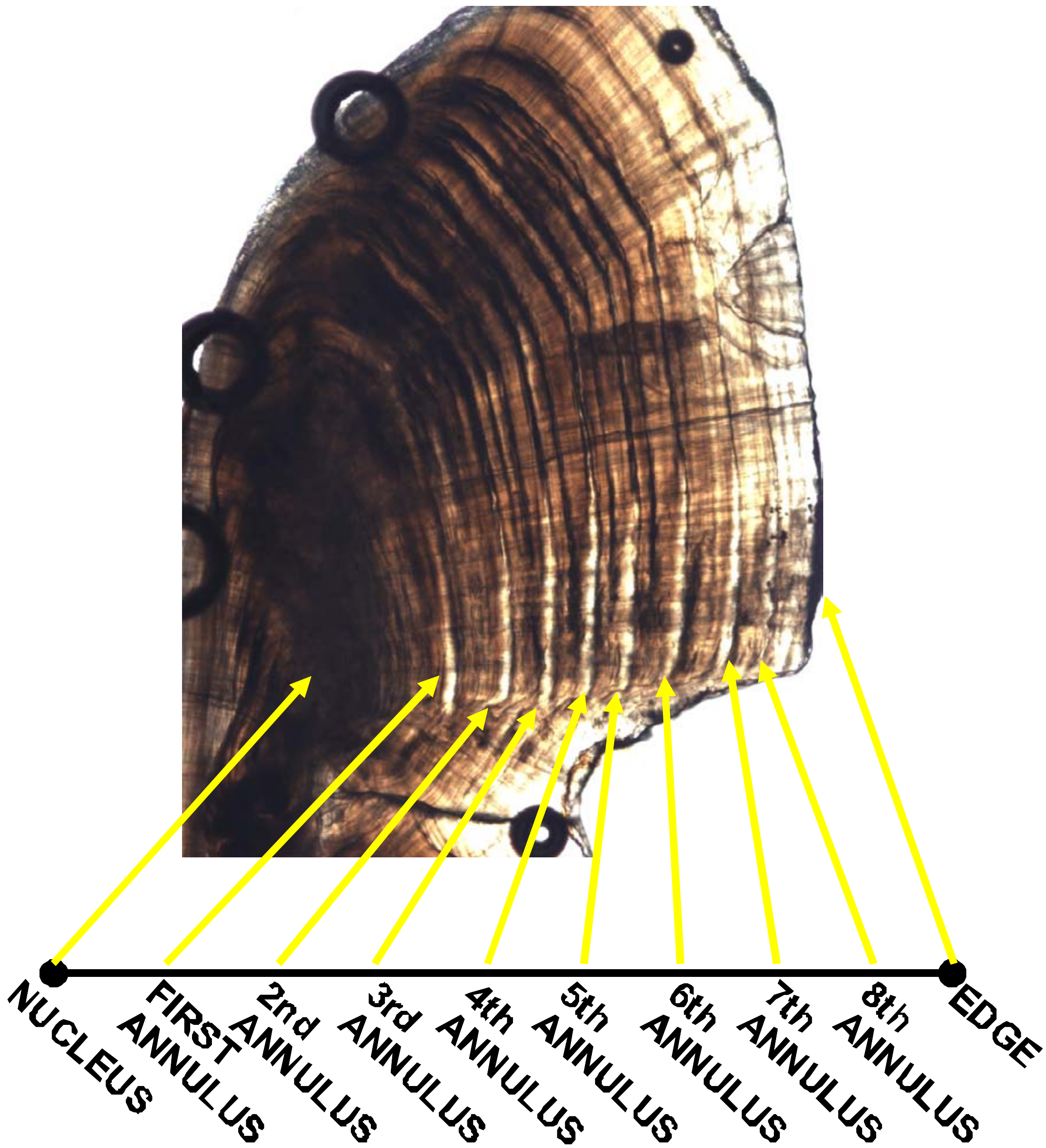


Figure 5.1. Ventral lobe of a transverse section through the nucleus of the otolith of a lake whitefish from Lake Huron.

5.2.2 The First annulus (Figure 5.1)

Definition: The distinct translucent band that is closest to the nucleus.
Since the core of the otolith of lake whitefish is very dense, the area around the nucleus can appear too dark. This can make the first annulus hard to distinguish.

5.2.3 Interpretation of the otolith edge

Definition: The edge represents the portion of the otolith between the final annulus and the otolith margin.

✓ *Features*

- the amount of growth on the edge will vary depending on the time of capture
- a great deal of subjectivity is typically inherent to interpreting of the edge of otoliths

✓ *Rules*

- if the final annuli is right on the edge, then the edge code must reflect no new growth
- if there is some growth beyond the final annulus then the edge code must reflect the amount

5.3 Interpretation of scales for age estimation

5.3.1 The annulus on the scale of the lake whitefish from Lake Huron (Figure 5.2)

Definition: A mark that is subjectively located on or in a calcified structure; is associated with the distal edge of a concentric ring in the form of a check on the scale or a translucent zone in other calcified structures; *is usually apparent along the entire structure for the first four or five years (prior to maturation) of growth after which the check only appears in the anterior zones of scales*. *To be considered annual the check must be located in both the anterior and anterio-lateral zones of the scale.* It is considered to separate the check or zone associated with the principal annual cessation or reduction in growth from the tissue deposited when growth resumes or increases. *During the first three or four years of growth prior to maturation, two annuli may demarcate one calender year after which point, at the onset of reproduction each annulus demarcates one calendar year*.

✓ *Criteria for identification*

- the annulus must be located in both the anterior and anterio-lateral zones of the scale (Figure 5.2)
- change in circuli spacing (Figure 5.3)
- crossing over of circuli (Figure 5.3)
- fragmented or irregular circuli (Figure 5.3)
- hyaline regions (Figure 5.4)

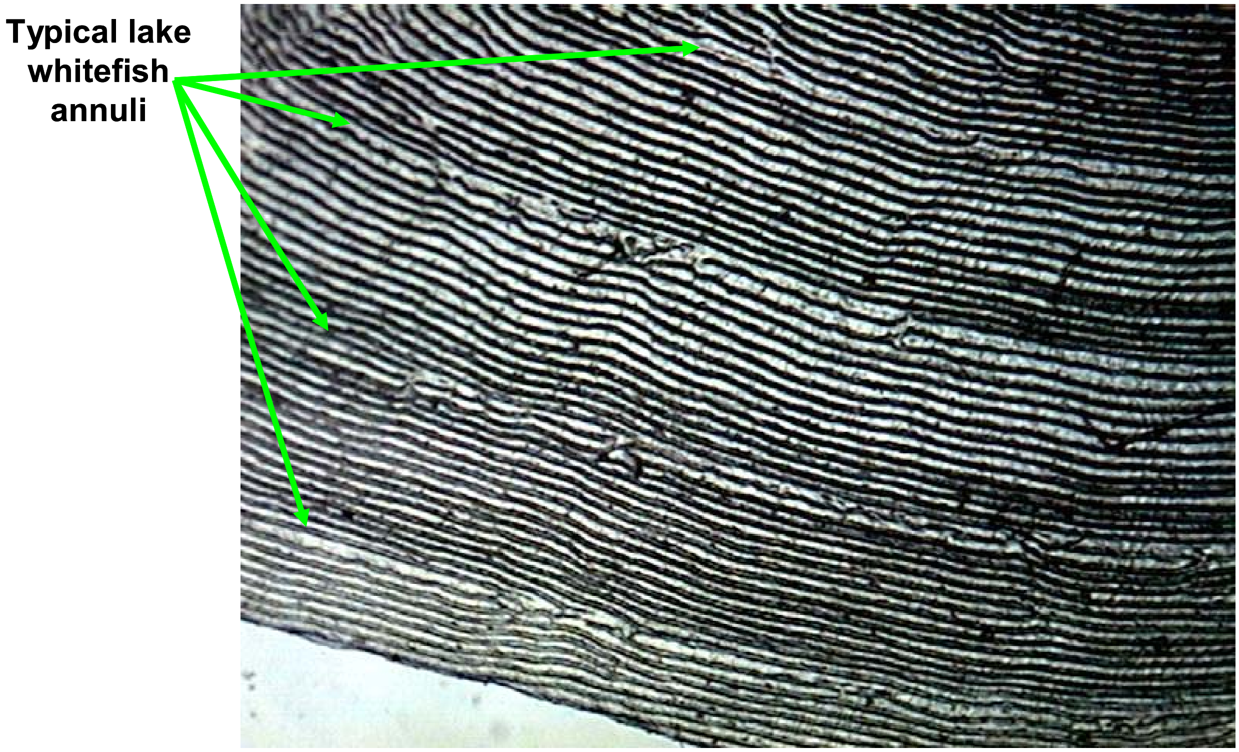


Figure 5.2 Typical annuli of lake whitefish in Lake Huron. Annuli are not always this clear and easily distinguished.

5.3.2 First Annulus

Definition: The first notable irregularity in regular concentric circuli when examining the scale from the focus toward the margin of the scale. Characterized by broken circuli, crossovers or a detectable difference in circuli spacing**.**

✓ *Criteria for identification*

- typically close to the focus
- may be demarcated by a shift in the actual orientation of the focus in relation to the rest of the scale
- may appear as a darkened zone separating it from subsequent growth

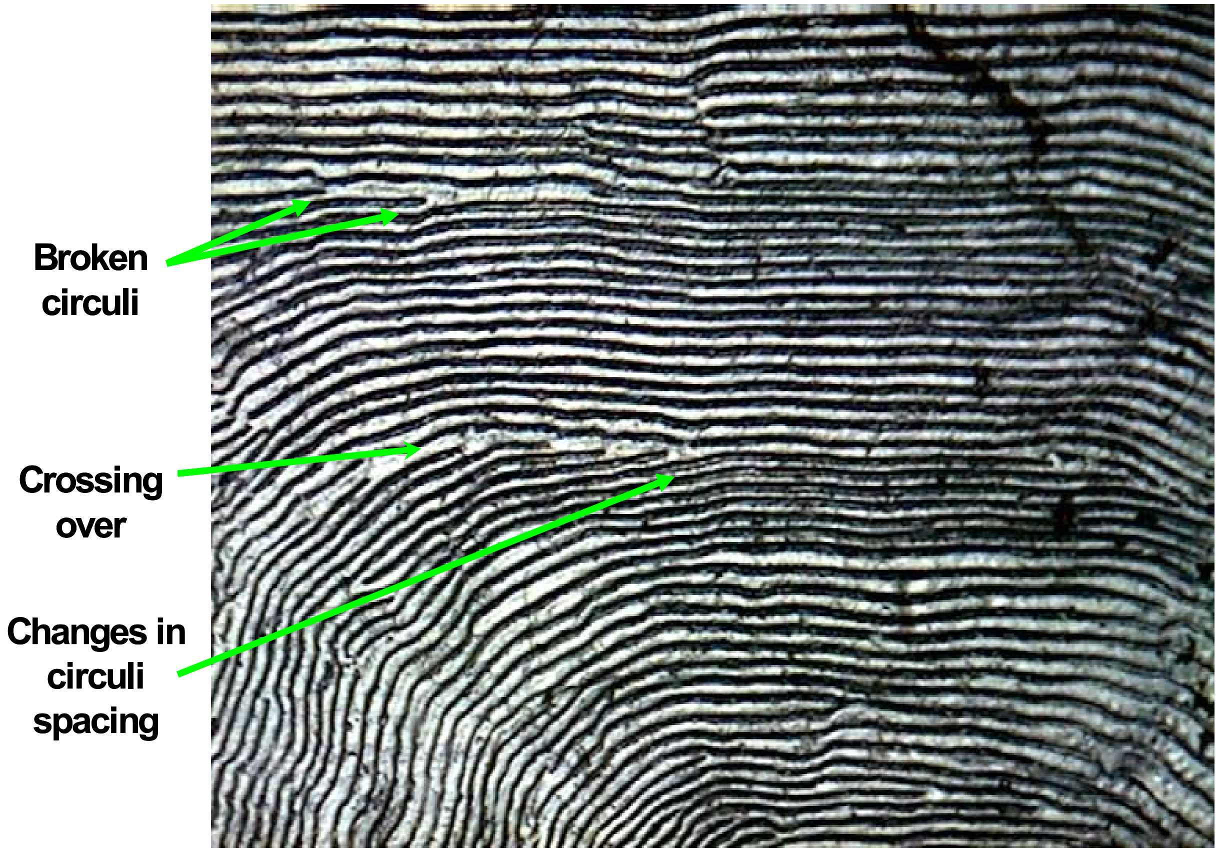


Figure 5.3 Lake whitefish scale showing common features associated with the detection of annuli

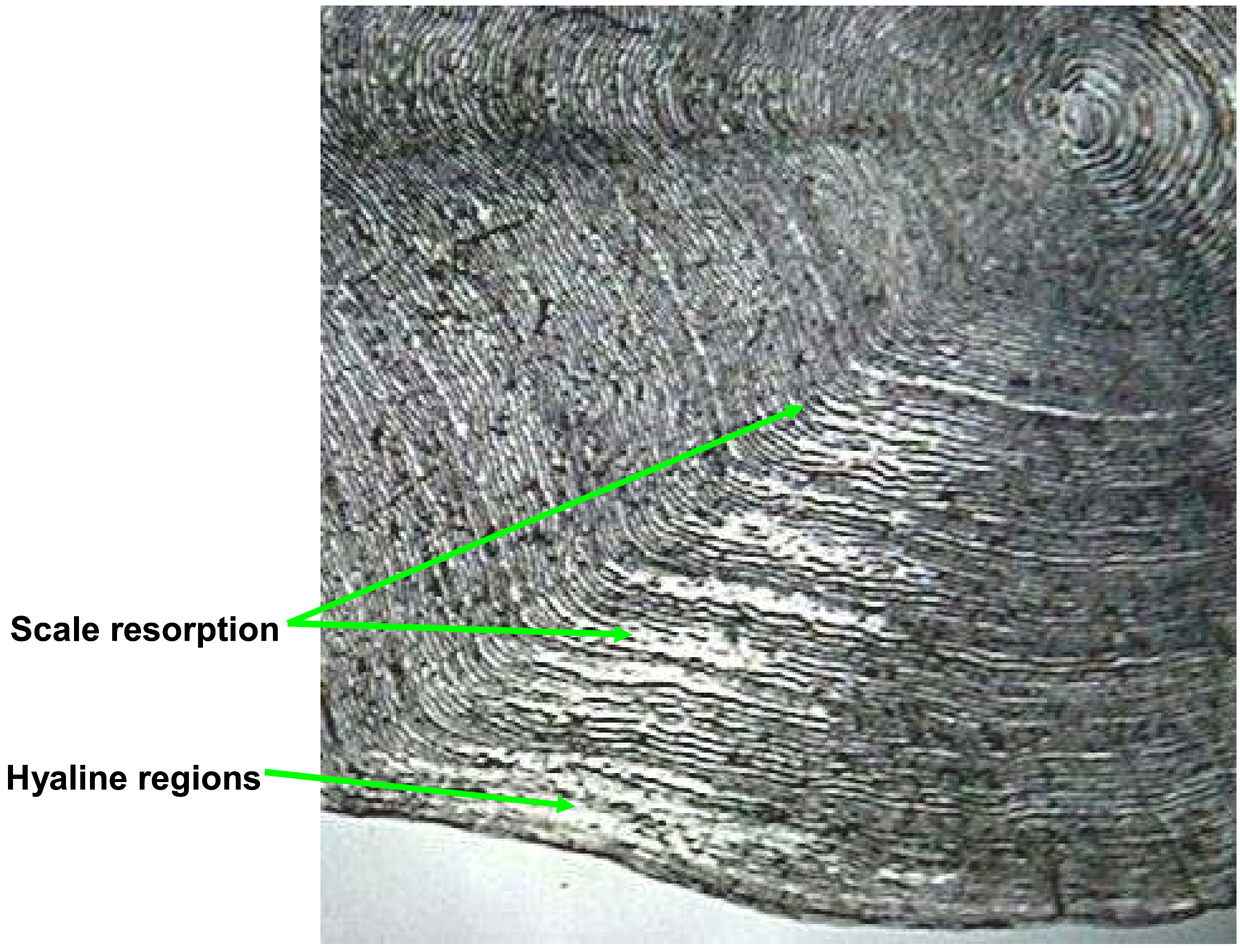


Figure 5.4 Lake whitefish scale showing some resorption and the associated hyaline zones

5.3.3 The final annulus

Definition: This represents the last clearly discernable annuli as one approaches the margin of the scale. It is important to be sure that what is deemed the final annuli, has indeed fully formed around the anterior and the anterio-lateral of the scale.

✓ *Criteria for identification*

- is extremely difficult to identify if it is located close to the edge, or if edge is crowded or eroded
- criteria for identification are the same as those for the annuli
- often a band of hyaline material if final annulus is near edge

5.3.4 Potential annuli

Definition: A check that is suspected to be annual but is not readily discernable throughout both the anterior and anterio-lateral sections of the scale; appears when contrast is manipulated due to clear differences in circuli spacing but has few breaks or crossovers. By identifying this feature on scales uncertainty is admitted. This uncertainty is addressed in Appendix 2.

√ *Criteria for identification*

- often takes the form of an annulus, but is only present in a portion of the quadrant
- little to no hyaline and little crossing over
- may be changes in circuli spacing

5.3.5 Interpretation of the scale edge

Definition: The edge represents the portion of the scale between the final annulus and the scale margin.

√ *Features*

- the amount of growth on the edge will vary depending on the time of capture
- a great deal of subjectivity is typically inherent on interpreting the edge of scales

√ *Rules*

- if the final annuli is right on the edge, then the edge code must reflect no new growth
- if there is some growth beyond the final annuli then the edge code must reflect the amount and the regions of the scale in which it is present

LITERATURE

Allan, R.J. 1985. Analysis of scale age and growth for lake whitefish *(Coregonus clupeaformis)* from Lake Simcoe. Report 1985-5. Ontario Ministry of Natural Resources. 154 p.

Ambrose, J. (Jr.) 1983. Age Determination. Pp. 301-324 *in* L.A. Nielsen and D.L. Johnson (Eds). Fisheries Techniques. American Fisheries Society. Bethesda, Maryland.

Anas, R.E., and S. Murai, 1969. Use of scale characters and a discriminant function for classifying sockeye salmon (*Oncorhynchus nerka*) by continent of origin. Int. North Pac. Fish. Comm. Bull. 26: 157-192.

Beamish, R.J. 1979. New information on the longevity of pacific ocean perch (*Sebastes alutus*). J. Fish. Res. Bd. Can. 36: 1395-1400.

Casselman, J.M. 1978. Calcified tissue and body growth of northern pike, *Esox lucius Linnaeus*. Ph.D. thesis. University of Toronto. Toronto, ON. 782 p.

Casselman, J.M., J.J. Collins, E.J. Crossman, P.E. Ihssen, G.R. Spangler, 1981. Lake whitefish (*Coregonus clupeaformis*) stocks of the Ontario waters of Lake Huron. Can. J. Fish. Aquat. Sci. 38: 1772-1789.

Casselman, J.M. 1983. Age and growth assessment of fish from their calcified structures - techniques and tools. Pp. 1-18 *in* E.D. Prince and L.M. Pulos, (Eds). Proceedings of the international workshop on age determination of oceanic pelagic fishes: tunas, billfishes, and sharks. NOAA (National Oceanic and Atmospheric Administration) Technical Report 8, Washington, D.C.

Casselman, J.M. 1990. Growth and relative size of calcified structures of fish. Trans. Amer. Fish. Soc. 119: 673-688.

Casselman, J.M., and K.A. Scott. 2000. A general procedures manual for CSAGES-Calcified Structure Age-Growth Data Extraction Software (Version 5.2). Special Publication of the Glenora Fisheries Station, Ontario Ministry of Natural Resources. Picton, ON. 96 p.

Chilton, D.E. and R.J. Beamish. 1982. Age determination methods for fishes studied by the groundfish program at the Pacific Biological Station. Can. Spec. Publ. Fish. Aquat. Sci. 60: 107 p.

Hile, R. 936. Age and growth of the cisco, *Leucichthys artedi* (LeSueur), in the lakes of the northeastern highlands, Wisconsin. U.S. Bur. Fish., Bull. 48: 211-317.

Irie, T. 1960. The growth of the fish otolith. J. Fac. Fish. Anim. Husb. Hiroshima Univ. 3: 203-229.

King, M. 1995. Fisheries biology: assessment and management. Fishing News Books. London.

Morrison, B.J. 2000. Sampling lake whitefish from the Chippewas of Nawash First Nation Commercial Fishery. University of Guelph. 74 p.

Muir, A. and S.S. Crawford. 2001. Standard methods for the Chippewas of Nawash Fisheries Assessment Program. Technical report prepared for Chippewas of Nawash Chief and Council. 60 p.

Neilson, J.D., and G.H. Geen. 1982. Otoliths of chinook salmon *(Oncorhynchus tshawytscha*): daily growth increments and factors influencing their production. Can. J. Fish. Aquat. Sci. 39: 1340-1347.

Penttila, J. & L.M. Dery. 1988. Age determination Methods for Northwest Atlantic Species. National Marine Fisheries Service, Woods Hole, MA. Northeast Fisheries Center.

Secor, D.H., J.M. Dean, and E.H. Laban. 1992. Otolith removal and preparation for microstructural examination. Can. Spec. Pub. Fish. Aquat. Sci. 117: 19-57.

Secor, D.H., J.M. Dean, and S.E. Campana. 1995. Recent developments in fish otolith research. Belle W. Baruch Institute for Marine Biology and Coastal Research. University of South Carolina Press. 19: 735 p.

Simkiss, K. 1974. Calcium Metabolism of Fish in Relation to Ageing. Pp 1-12 in T.B. Bagenal, (ed.). Ageing of Fish. Unwin Brothers Limited, London

Tesch, F.W. 1971. Age and Growth. Pp. 93-123 *in* W.E. Ricker (Ed). Methods for assessment of fish production in fresh waters. Blackwell Scientific Publications, London. 348 p.

Van Oosten, J. 1923. The whitefishes (*Coregonus clupeaformis*): a study of the scales of whitefishes of known ages. Zoologica 2: 381-412.

Van Oosten, J. 1929. Life history of the lake herring (*Leucichthys artedi* LeSeur) of Lake Huron as revealed by its scales, with a critique of the scale method. U.S. Bureau of Fisheries Bulletin. 44(1053): 265-428.

APPENDIX 1 List of materials

ITEM	PRICE	CONTACT
Raytech AL-P10 SAW	$1000	www.dadsrockshop.com P.O.Box, 10649, Ft.Mohave, AZ 86427 USA 1-800-844-3237
Raytech Blue Blazer 4"	$15	www.dadsrockshop.com
Waterproof Sandpaper, Silicon Carbide 1500 Grit	$0.99	Canadian Tire (General Hardware Store)
Pelco Deep Trough Flexible Locator Mold (Blue)	$8.00	Pelco International P.O. Box 492477, Redding, CA 96049-2477, USA Telephone: 530 243 2200;1 800 2373526 Canada / www.pelcoint.com
System 3 Clear Coat Resin	$65/pint	Kitchener Fiberglass Products Ltd. 319 Bridge St. East, Kitchener, On (519) 744 3681/ www.systemthree.com
Wooden Stirring Sticks	$1.71 / 200	Ultra Food and Drug Market, Guelph, On
Gloves, Vinyl	$12.49 / 100	Canadian Tire (General Hardware Store)
Le Page, Accu-flo Super Glue automatic pen	$3.19	Canadian Tire (General Hardware Store)
Safety Glasses	$4.99	Canadian Tire (General Hardware Store)
Mastercraft All purpose cement	$2.00	Canadian Tire (General Hardware Store)
Dow Corning 1101 Emulsion, Release Agent	$10.00/ pound	County Services, 500 Maltby rd, East, Guelph, On (519) 826 6769
“Rite in the rain” All-weather Copier Paper 8.5 x 11 inch	$40.19/ 100 sheets	Halltech Environmental Inc. 129 Watson Rd S. , Guelph, On, www.htex.com
Microscopy Slides (3" x 1")	$13.06/ 72	Boreal Laboratories ltd. phone: 1-800-387-9393 399 web: www.boreal.com
Bibulous Drying Paper	$ 3.00/50	Boreal Laboratories ltd. (General Hardware Store)

APPENDIX 2 CNCSAF Image Analysis Ageing procedures

1. Lines

1.1 Creating lines

Measurement lines will act as references for image tagging. They are not essential to analysis and need not be saved however, they will prove to be a useful reference tool.

Line Feature

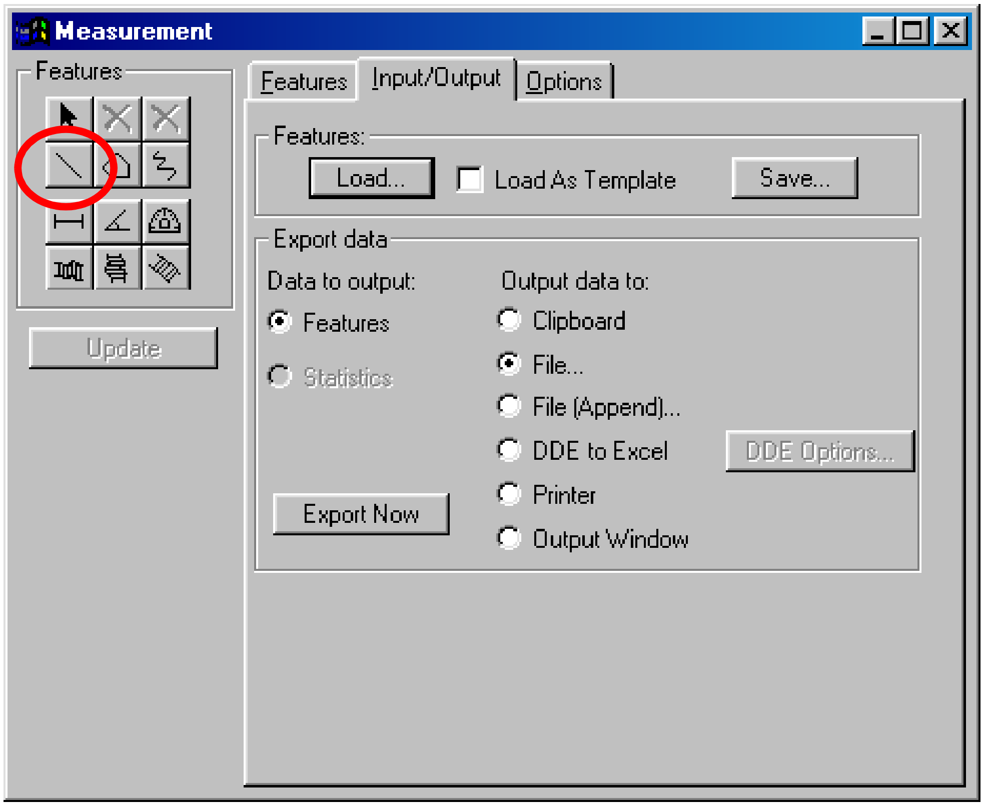


Figure A2-1. Image Pro Express 4.0 Measurement window

a. Left-click on the pull-down menu entitled *Measure* and select the *Measurement* option. This will bring up the *Measurement window* (Figure A2-1).
b. Select the *Line feature* from the tools provided in the left hand corner of this window.
c. Minimize the *Measurement window*.
d. Select the image that you are working in by clicking on its title-bar.
e. Place the cursor on the image and hold down the left button on the mouse to draw the line. To complete the line simply release the left button on the mouse.
f. Maximize the *Measurement window* and unselect the line tool.

1.2 Saving lines

Unfortunately Image Pro Express does not allow the user to burn lines or features to the image. Features therefore, must be stored as separate files and then reloaded onto the image as required.

a. Maximize the *Measurement window* and select the *Input/Output tab*; in the *export data frame*, ensure that *Output data to: file* is selected then left-click the *save* button.
b. The lines should be saved with the same filename as that of the active image but the file extension will be **.msr*. The **.msr* file will be saved to the folder entitled *lines* within the subdirectory containing the individual that is being analysed.

1.3 Loading existing lines into an image

a. Left-click on the pull-down menu entitled *Measure* and select the *Measurement* option. This will bring up the *Measurement window* (figure A2-1).
b. Select the *Input/Output* tab.
c. In the feature frame left-click the load button and select the appropriate **.msr* file from the directory. The file should be within the folder for that individual and the filename should correspond to the name on the title bar of the image that is being analysed.

2. Manual Tags

Tags are used to identify features and are essential tools for analysis of calcified structures. The tagging data is automatically recorded in the Manual Points Count table and when tagging is complete, is exported to a working Microsoft Excel file for analysis. Unfortunately Image Pro does not let the user burn the tags into the image so they must be stored as separate files and reloaded onto the image.

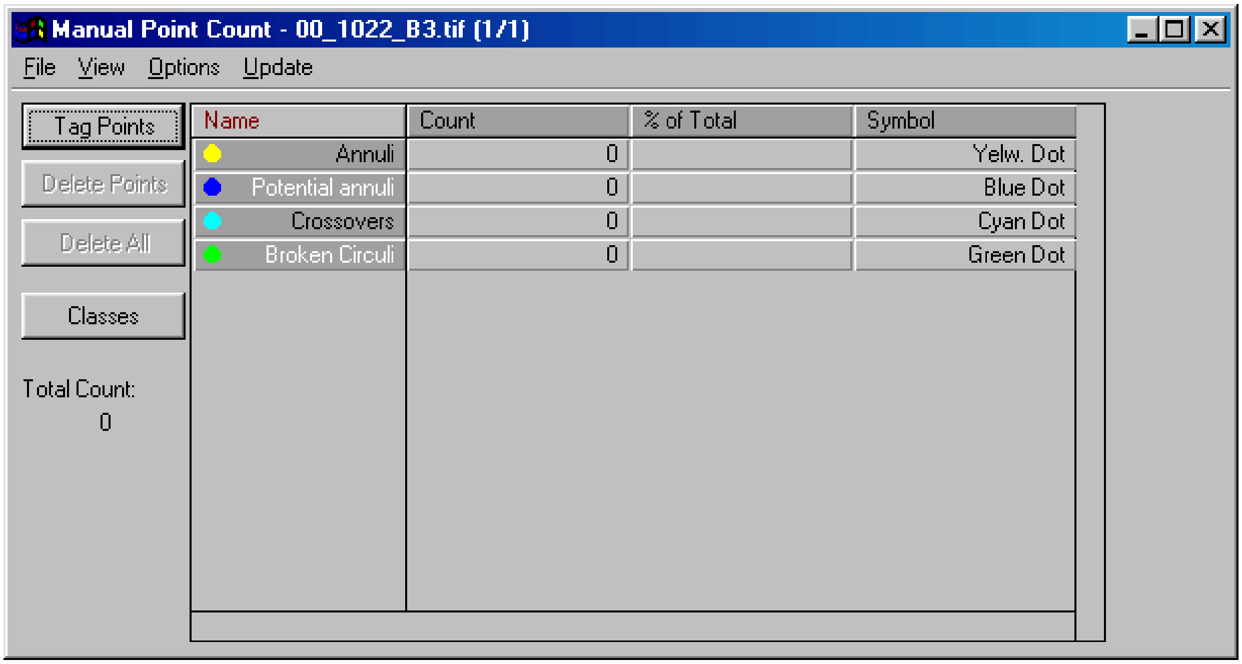


Figure A2-2. Image Pro Express 4.0 Manual Point Count Window

2.1 Creating manual tags

a. Left-click on the pull-down menu entitled *Measure* and select the *Manual Tag* option. This will bring up the *Manual Point Count window* (Figure A2-2).
b. Left-click on the pull-down *File menu* and select the *Load Settings* option.
c. In the *Fish Ageing* directory double-left-click on the file entitled *Tag_settings.tag* and it will automatically load the tag settings that are defined in Table A2-1.
e. Once the tag settings have been loaded, left-click on the *Tag Points* button on the left-hand side of the *Manual Point Count window* and this will bring up the *Add New Points window*.
f. Use the drop down list to select the *Tag Class* appropriate for the feature you intend to tag.
g. Refer to Table A2-1. and begin by visually identifying the first annulus and subsequently identify annuli, potential annuli, and the final annulus and give them appropriate tags by positioning the cursor over the image feature and left-clicking. All tags will be automatically tallied and recorded in the *Manual Point Count table* (Figure A2-2).
h. Once tagging is complete, click the OK button on the Tag Class window and this will return you to the Manual Point Count window.

Table A2-1. Manual tag classes, symbols and their definitions that are employed by CNCSAF to characterize Lake Huron lake whitefish calcified structures.

Class	Symbol	Definition
Annual check	● yellow ☞	**1. Conventional:** A mark that is subjectively located on or in a calcified structure; is associated with the distal edge of a concentric ring in the form of a check on the scale or a translucent zone in other calcified structures; is found along the entire structure; and is considered to separate the check or zone associated with the principal annual cessation or reduction in growth from the tissue deposited when growth resumes or increases. Two successive annuli are usually considered to demarcate one calendar year of calcified tissue growth (Casselman 1983) **2. Anterior check definition:** A mark that is subjectively located on or in a calcified structure; is associated with the distal edge of a concentric ring in the form of a check on the scale or a translucent zone in other calcified structures, ***is usually apparent along the entire structure for the first four or five years (prior to maturation) of growth after which the check only appears in the anterior zones of scales and the dorsal lobe of otoliths. To be considered annual the check must be located in both the anterior and anterio-lateral zones of the scale.*** It is considered to separate the check or zone associated with the principal annual cessation or reduction in growth from the tissue deposited when growth resumes or increases. ***During the first three or four years of growth prior to maturation, two annuli may demarcate one calender year after which point, at the onset of reproduction each annulus demarcates one calendar year.***
Potential Annulus	● blue	A check that is suspected to be annual but is not readily discernable, appears when contrast is manipulated due to clear differences in circuli spacing and has few breaks or crossovers, comparison among a number of scales from an individual will allow one to discern if potential annuli are actual annual checks. This tag is no used to characterize the edge annuli. CNCSAF attempts to quantify the edge rather than put an uncertainty on it.

Class	Symbol	Definition
Final Annulus	● green	This tag marks the last clearly discernable annulus as one approaches the margin of the structure. Any features beyond the Final Annulus are characterized in Edge comment column of the working Microsoft Excel Data file.
First Annulus	● red	The first notable irregularity in regular concentric circuli when examining the scale from the focus toward the margin of the scale. Characterised by broken circuli, crossovers or a detectable difference in circuli spacing.

2.2 Saving manual tags

a. Image tags are saved by left-clicking on the pull-down *File menu* and selecting the *Save Points* option.

b. In the *Save Points window* choose to save the tags in the sub-folder entitled tags within the folder for the individual being analysed and name the file with the filename indicated in the title bar for the active image but with the file extension *.tag

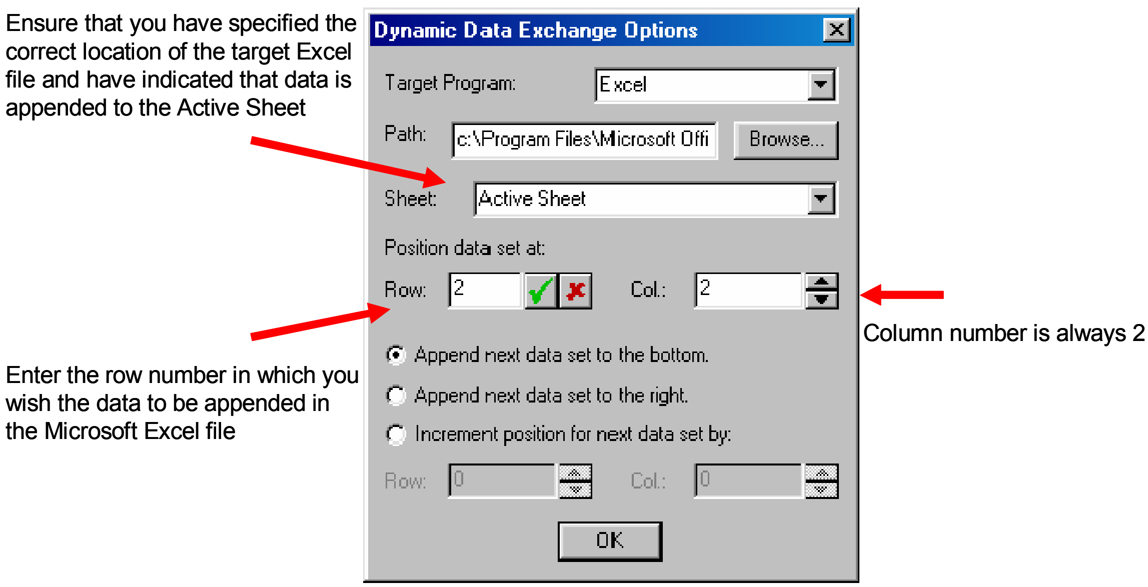


Figure A2-3. Image Pro Express 4.0 Dynamic Data Exchange Options window

2.3 Saving manual tag data to the MS Excel file

a. If you are working on year 2000 samples, you would use the Excel file entitled *2000_AgeData*

b. Leave this Excel file open while working within Image Pro because once you append data from the *Manual Point Counts table* to the Excel file you will need to add supplemental information.

c. In Image Pro Express, left-click on the pull-down *File menu* and scroll down to *Save Options*. When this option is highlighted a sub-list appears containing two elements *Top Line* and *Left Column*. Uncheck *Top Line*.

e. To save the data in the *Manual Point Count table* to Excel, left-click on the pull-down *File menu* and left-click *DDE Options* (Figure A2-3)

f. Within the *Dynamic Data Exchange Options window* ensure that the settings are the same as in Figure A2-3. However, when the Excel file already contains records then you must select which *Row* you want the data to be appended, but the *Column option* will always be *2*. Once you have configured the *DDE options* you will not need to reconfigure them again for that working session.

g. Left-click *OK*

h. Left-click the pull-down *File menu* and select the *DDE To Excel* option. The data will automatically be appended to the active Excel file for that year, if you have selected the correct options.

2.4 Loading existing tags into an image

a. Within the active image file Left-click on the pull-down menu entitled *Measure* and select the *Manual tag option*. This will bring up the *Manual Point Count window* (Figure A2-4).

b. From the pull down *File menu* select the *Load Points option*.

c. Within the *Fish Ageing* directory double-left-click on the subdirectory within which you are working and select the tags folder.

d. Select the **.tag* file for the individual that you are working on and the existing tags will be loaded into the active image. Always ensure that the tags that you load are for that individual by cross- referencing the filenames.

3. The Excel Age Data File

A Microsoft Excel file has been created to store ageing data for each year. The filename convention that is used is as follows: year_AgeData (eg. 2000_AgeData). This file serves a number of functions; 1) it receives tagging and measurement data from Image Pro through *DDE (Dynamic Data Exchange)*, 2) It allows you to input other data such as comments and various data to characterize the image, and
3) has a semi-automated spreadsheet for data analysis. All three of these functions will be described in the following sections.

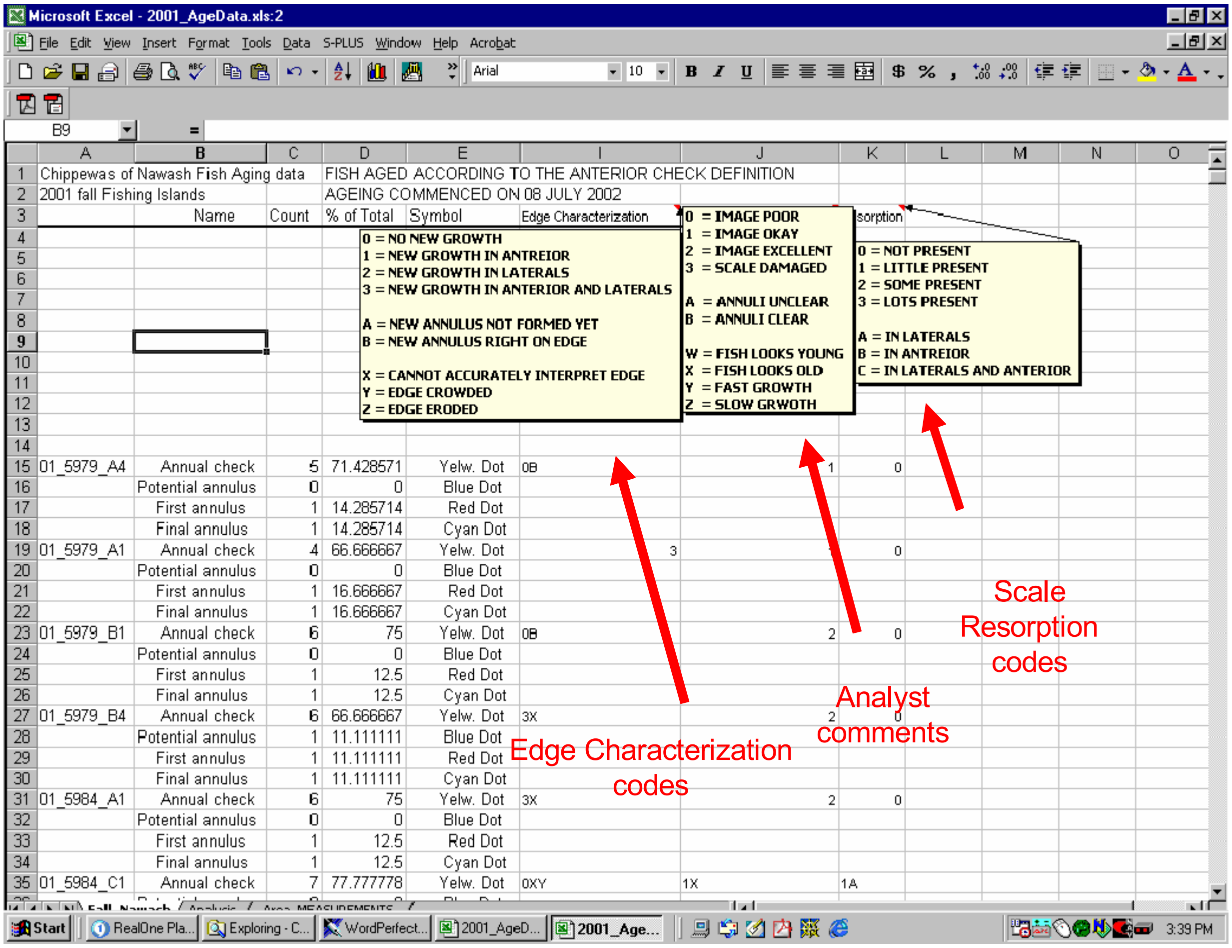


Figure A2-4. The tagging *Data worksheet*

4. The data worksheet

As detailed in section 2.3, all of the tagging data gets exported to Microsoft Excel through the *DDE* function. This data goes into the *Data worksheet*. Once the tagging data from an individual has been sent to the *Data worksheet*, the analyst enters an *Edge Characterization Code*, *Comment* and a code that quantifies the amount and location of *Resorption* (Figure A2-4). The codes and comments are alpha numeric so that the data can be summarized or queried by the researcher.

4.1 Analysing the edge

The *Data worksheet* requires a code to identify the average scale-edge condition among images for a given individual or the average between repeated measures from other structures such as otoliths or fin-rays (Table A2-2, Figure A2-4). The purpose of analysing the edge is to characterize whether or not the final annuli for a given year has been deposited on the structure.

The edge is interpreted at the time of tagging and the analyst enters a code in the *data worksheet*. Here we detail why the edge is interpreted and how it fits into the analysis worksheet described in section 5. Edge analysis is critical because it eliminates confusion over the commonly accepted convention of the fish's birthday. It is assumed that in the Northern Hemisphere, all fish lay down an annulus in the winter, and therefore have a 'birthday' on January 01 (Hile 1936). Under this assumption, one year is typically added to the age estimate if the fish was captured 'after the period of seasonal growth' but before it had laid down an annulus for that 'growing season', regardless of whether it had actually laid down the annulus or not. By employing the edge characterization code, the *Analysis spreadsheet* overrides the conventional 'fish birthday' rule, when correcting fish age. This creates another level of objectivity in the age estimation process. The converse of the latter scenario is that if the fish was captured during the 'period prior to annulus formation' and had in-fact not laid down an annulus for that growing season, then one year would indeed be added to the final *corrected age* estimate by the analyst.

To be clear about this methodology, consider the following scenario. A lake whitefish was fish was captured in the month of December and it was found to have 8 annuli on all of it's scales, but *no new growth* was observed beyond the *final annulus*; the final annulus was right on the edge of the scale margin. According to convention, this individual would have been captured after that years *'growing season'* had ended, but prior to the assumed time of *annulus formation*. Under the conventional methodology, that individual would be estimated to be 9 years old. In contrast, employing the edge characterization methodology described above, the *Analysis spreadsheet* would have found that the fish was indeed captured in December, but had in fact already laid down the annulus for that growth season. In this case the spreadsheet would correctly assign that individual an age of 8 years, whereas according to conventional methodology, that individuals age would have been overestimated by one year.

Table A2-2. CNCSAF age estimation edge characterization codes and definitions

Code	Edge Characterization	Description
0	NO NEW GROWTH	final annulus right at the margin
1	NEW GROWTH IN ANTERIOR	there is a clear annulus near the margin but there is some discernable growth in the anterior but not in the laterals of the scale
2	NEW GROWTH IN LATERALS	there is a clear annulus near the edge but there is some discernable growth in the laterals but not in the anterior of the scale
3	NEW GROWTH IN ANTERIOR AND LATERALS	the final annulus is very close to the margin but there is new growth in both the anterior and the laterals of the scale
4	NEW GROWTH IN VENTRAL LOBE	new growth is observed beyond the final annulus in the otolith
A	NEW ANNULUS NOT FORMED YET	This code is only used when there is significant growth after the final annulus and no indication of an annulus on the margin
B	NEW ANNULUS RIGHT ON Margin	No new growth and a clear annulus is formed right at the margin
X	CANNOT ACCURATELY INTERPRET EDGE	This code is used when the analyst cannot accurately interpret the edge but there is no clear reason why this is the case
Y	EDGE CROWDED	This code is used when there are a number of annulus close to the margin of the structure and there is very little edge beyond what the analyst calls the final annulus.
Z	EDGE ERODED	This code is used if there is visible damage or missing tissue beyond the final annulus

4.2 Analyst comments

Analyst comments are manually added into the *data worksheet* (Table A2-3)
Table A2-3. CNCSAF age estimation comments and descriptions

Code	Comment	Description
0	IMAGE POOR	The image has low resolution, is not properly cleaned or focused
1	IMAGE OKAY	The image itself is not that bad, but the structure is extremely difficult to interpret
2	IMAGE EXCELLENT	The image is great, and the structure is easy to interpret
3	STRUCTURE DAMAGED	The calcified structure is clearly damaged and therefore is difficult to interpret
A	ANNULI UNCLEAR	The annuli are difficult to distinguish
B	ANNULI CLEAR	The annuli are clear and easy to interpret
W	FISH LOOKS YOUNG	The image is incredibly clear; the spacing between annuli is wide; the spacing between circuli is constant and clear
X	FISH LOOKS OLD	The structure is poor; the spacing between annuli is narrow; the spacing between annuli is irregular; the spacing between circuli is highly irregular
Y	FAST GROWTH	The image is incredibly clear; the spacing between annuli is very wide
Z	SLOW GROWTH	The spacing between annuli is narrow

4.3 Quantifying resorption

In order to address hypotheses regarding the growth of calcified structures, the amount and location of any resorption on scales is quantified thorough a system of codes (Table A2-4).
Table A2-4. CNCSAF Scale age estimation resorption codes and definitions

Code	Comment	Description
1	LITTLE PRESENT	resorption is present in less than 1/8 of the image
2	SOME PRESENT	resorption is present in less that 1/4 of the image
3	LOTS PRESENT	resorption is present in greater than 1/4 of the image
A	IN LATERALS	marks the primary location in which the resorption occurs
B	IN ANTERIOR	marks the primary location in which the resorption occurs
C	IN LATERALS AND ANTERIOR	marks the primary location in which the resorption occurs

4.4 Organizing the data

One of the shortfalls with Image Pro Express is that it is limited in it's data handling abilities. For this reason, once *tagging data* is sent to the *Data worksheet*, all subsequent data manipulation is manual. When the analyst is done one session of tagging, all of the data that has been sent to the *Data worksheet*, must be grouped according to Individual code. Data is out of order because, as detailed in the manual, images are presented to the analyst in a random blind fashion. Data organization is a manual process that is accomplished by using the *cut / insert cells* tools.

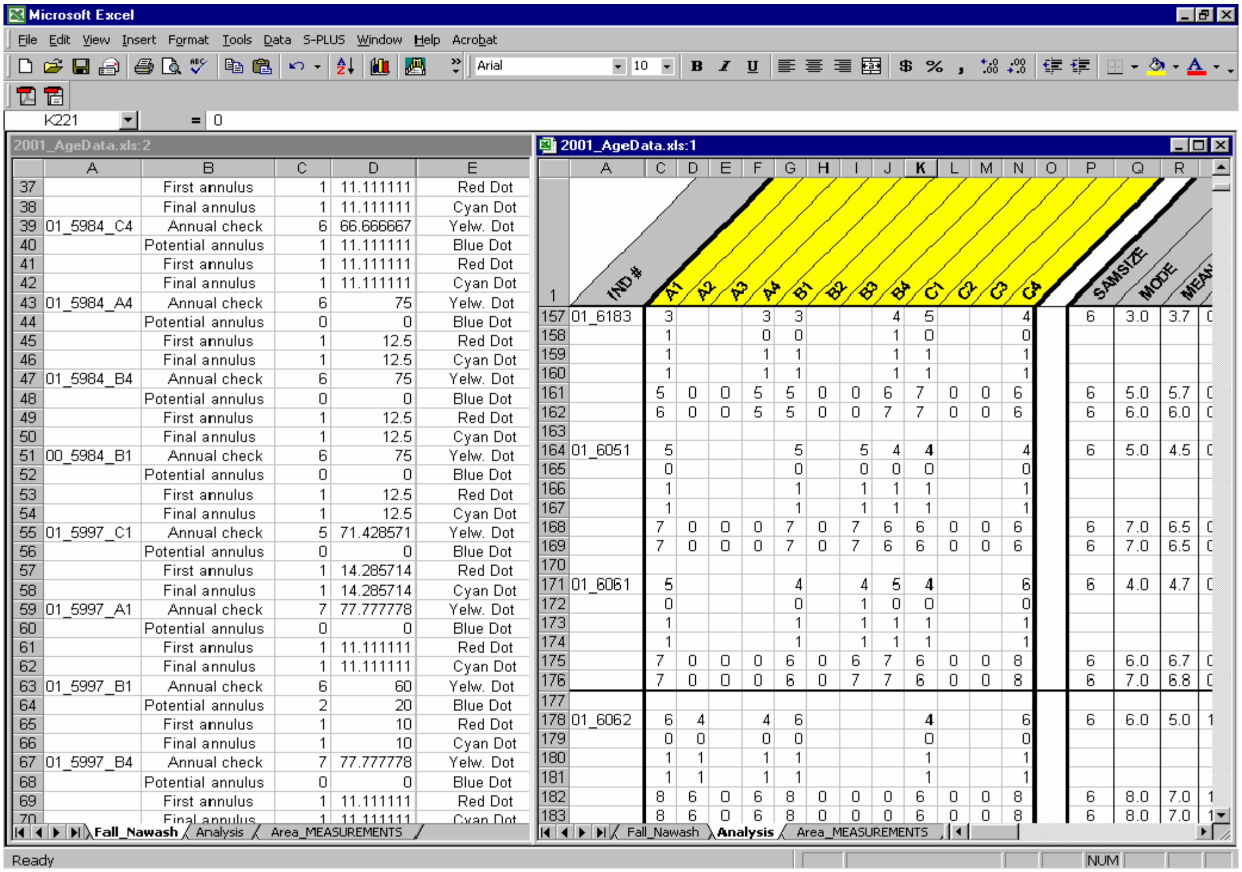
5. The analysis worksheet

This section will detail how to transfer tagging data from the *Data worksheet* into the *Analysis worksheet*, describe the design of the *Analysis worksheet* and the procedures for entering appropriate supporting data, and detail the automated process for estimating age.

5.1 Transferring tagging data from the data worksheet to the analysis worksheet

Once the data has been grouped according to individual number, the analyst transfers the tagging data into the analysis worksheet (Figure A2-5).

a. Left-click on the pull-down menu entitled *Window*, and select *New Window*.
b. Left-click on the pull-down menu entitled *Window*, and select *Arrange*, and then select *Tiled*.
c. In the left-hand window, select the *Data worksheet* and in the right-hand window, select the *Analysis worksheet* (Figure A2-6).
d. In the window displaying the *Analysis worksheet*, type the individual number for the individual that you are working on and the year (e.g. 2003_5979) in the appropriate space.
e. Select the *Data worksheet* and copy the data for that individual into the *Analysis worksheet*.
f. The *edge code* for each individual must be entered into the appropriate spot in the *Analysis worksheet* (Procedures detailed in *4.1*).
g. In the *Analysis spreadsheet*, the edge code is limited to either new growth present, or new growth absent beyond the final annuli. The analyst must therefore decipher the average edge code for that individual from the *Data worksheet.*
h. The *month of capture* must be input into the appropriate column of the *Analysis worksheet*.



The Data worksheet

The Analysis worksheet

Figure A2-5. Transferring tagging data from the data worksheet to the analysis worksheet

5.2 The Analysis worksheet (Figures A2-6 & A2-7)

The *Analysis worksheet* was designed to eliminate potential annuli. Potential annuli are identified as false annuli if they do not appear in the majority of the images, and if this is the case, they are eliminated from subsequent calculations. Conversely, if potential annuli, do appear in the majority of the images they are identified as annuli and are then added into the calculations. This is done by comparing sums of annuli and potential annuli between images. The spreadsheet, therefore, automatically identifies and eliminates false annuli lending an important degree of objectivity to the age estimation process.

5.3 Semi-automated analysis

Sums of annuli and potential annuli are compared among images and mean, mode, variance and statistical confidence are automatically calculated by the spreadsheet based on these comparisons. Table A2-5 details the functions within the *analysis worksheet*.

Table A2-5. CNCSAF age estimation tagging data automated analysis

Automated features	Description	Purpose
Sample size	tallies the number of images analyzed for a given individual	represents the number of images or replicates for a given individual
Mode	the arithmetic mode of tagging data	returns the most frequently occurring value in the array of tagging data from all images or replicates for a given individual
Mean	returns the arithmetic mean of tagging data	returns the mean as a descriptive measure of central tendency in the tagging data from all images or replicates for a given individual
Standard deviation	returns the statistical standard deviation	quantifies the variance in tagging data from all images for a given individual
95% confidence interval	returns the interval for which we are confident that 95% of the data fall into	is a measure of variability in tagging data or repeated measures from the same structure
precision	returns an estimate of precision based on Chi squared statistics	further quantifies the variability in tagging data or repeated measures from the same structure
confidence (see section 5.3.1)	gives a measure of the variability in the tagging data	based on the standard deviation, the spreadsheet assigns a confidence to the data from all images or replicates from a given individual; 0-5 = no confidence (too much variability in tagging data), 10 = high confidence (no variability in tagging data)
age estimate	returns the age estimate based on the least variable set of tagging data	this field considers the confidence of the age estimate based on all images for a given individual; if the confidence in the data is below 5 then the data is considered too variable, and the age of fish cannot be estimated with confidence
round age	rounds the *age estimate* down to the nearest whole number	if the age estimate is based on the mode of the tagging data, then there is no need to round the age down but if it is based on the mean, the age must be rounded down to the nearest whole age
corrected age	corrects the age estimate based on the edge code, the time of capture and the conventional January 01 birthday	this represents the final age estimate for a given individual; it has been arrived at through semi-automated analysis of image tagging data; it is a fairly robust and objective estimate of fish age

Once all of the required data are entered into the spreadsheet, the number of annuli for a given individual is then automatically estimated using the mean and mode, and based on the average edge condition and the month of capture. The age estimate is then automatically corrected and the individual is assigned to a year class.

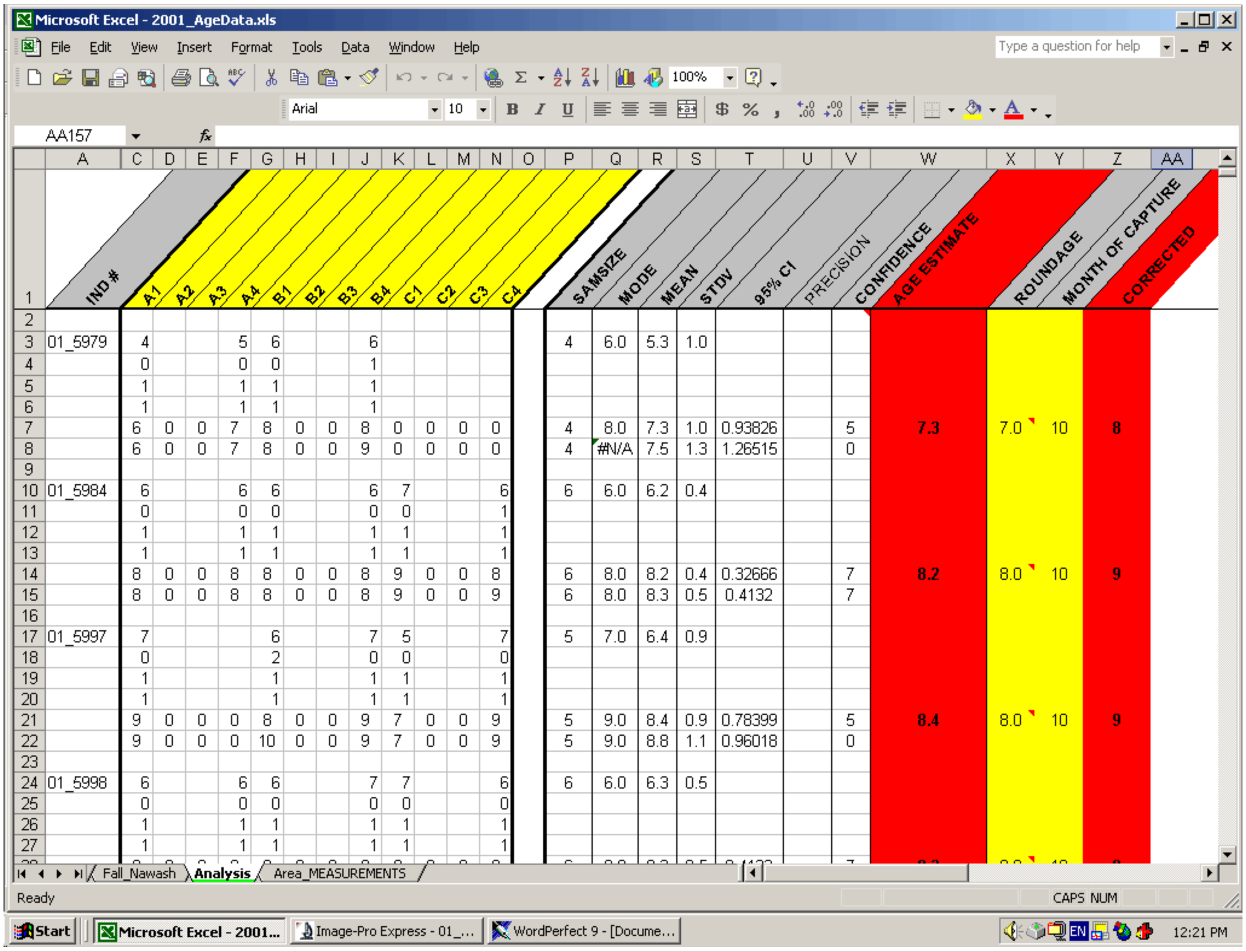
5.3.1 Confidence ranking

One of the most powerful features of the *Analysis worksheet* is the ability to integrate quality management into the age estimation process. The process of confidence ranking involves automatic attribution of a numeric rank which is calculated by the *analysis worksheet* based on the variance associated with the tagging data for a given individual. A *low variance* in tagging data from all images for a given individual or repeated measures from a given structure= *high confidence (0 variance = confidence 10)*. The way the spreadsheet was coded was based on the logical decision rules presented in Table A2-6.

Table A2-6. Chippewas of Nawash Age Estimate Confidence Ranking Scale

Rank	Description	Outcome
<5	tagging data too variable, range between annual tags > 3 years between quadrants or replicates; >½ images poor quality; annuli not easily discernable	Cannot estimate age reliably
5	tagging data variable, range between annual tags 3 years between quadrants or replicates; >4 images poor quality; annuli difficult to discern	Age estimated based on the mode of annuli and potential annuli
6	tagging data variable, range between annual tags 2 years between quadrants or replicates; >2 images poor quality	Age estimated based on the mode of annuli and potential annuli
7	some variation in tagging data but minimal when taking into account potential annuli; some images poor quality	Age estimated with good confidence
8	some variation in tagging data; >70% of the images have the same number of annual tags; image quality good	age confidently estimated based on mode of annual tags
9	little variation in tagging data, >80% of the images have the same number of annual tags; image quality excellent	age confidently estimated based on mode of annual tags
10	no variation in tagging data; all images are excellent quality	age estimated with 100% confidence

As an example, consider the following scenario; if a group of images for a given individual has a confidence ranking below 5, the spreadsheet will automatically display the message *"Cannot age"*, and that individual is discarded from the analysis.


Microsoft Excel - 2001_AgeData.xls

	IND #	A1	A2	A3	A4	B1	B2	B3	B4	C1	C2	C3	C4		SAMSIZE	MODE	MEAN	STDV	95% CI	PRECISION	CONFIDENCE	AGE ESTIMATE	ROUNDAGE	MONTH OF CAPTURE	CORRECTED
2																									
3	01_5979	4			5	6			6						4	6.0	5.3	1.0							
4		0			0	0			1																
5		1			1	1			1																
6		1			1	1			1																
7		6	0	0	7	8	0	0	8	0	0	0	0		4	8.0	7.3	1.0	0.93826		5	7.3	7.0	10	8
8		6	0	0	7	8	0	0	9	0	0	0	0		4	#N/A	7.5	1.3	1.26515		0				
9																									
10	01_5984	6			6	6			6	7			6		6	6.0	6.2	0.4							
11		0			0	0			0	0			1												
12		1			1	1			1	1			1												
13		1			1	1			1	1			1												
14		8	0	0	8	8	0	0	8	9	0	0	8		6	8.0	8.2	0.4	0.32666		7	8.2	8.0	10	9
15		8	0	0	8	8	0	0	8	9	0	0	9		6	8.0	8.3	0.5	0.4132		7				
16																									
17	01_5997	7				6			7	5			7		5	7.0	6.4	0.9							
18		0				2			0	0			0												
19		1				1			1	1			1												
20		1				1			1	1			1												
21		9	0	0	0	8	0	0	9	7	0	0	9		5	9.0	8.4	0.9	0.78399		5	8.4	8.0	10	9
22		9	0	0	0	10	0	0	9	7	0	0	9		5	9.0	8.8	1.1	0.96018		0				
23																									
24	01_5998	6			6	6			7	7			6		6	6.0	6.3	0.5							
25		0			0	0			0	0			0												
26		1			1	1			1	1			1												
27		1			1	1			1	1			1												

Figure A2-6. The analysis worksheet

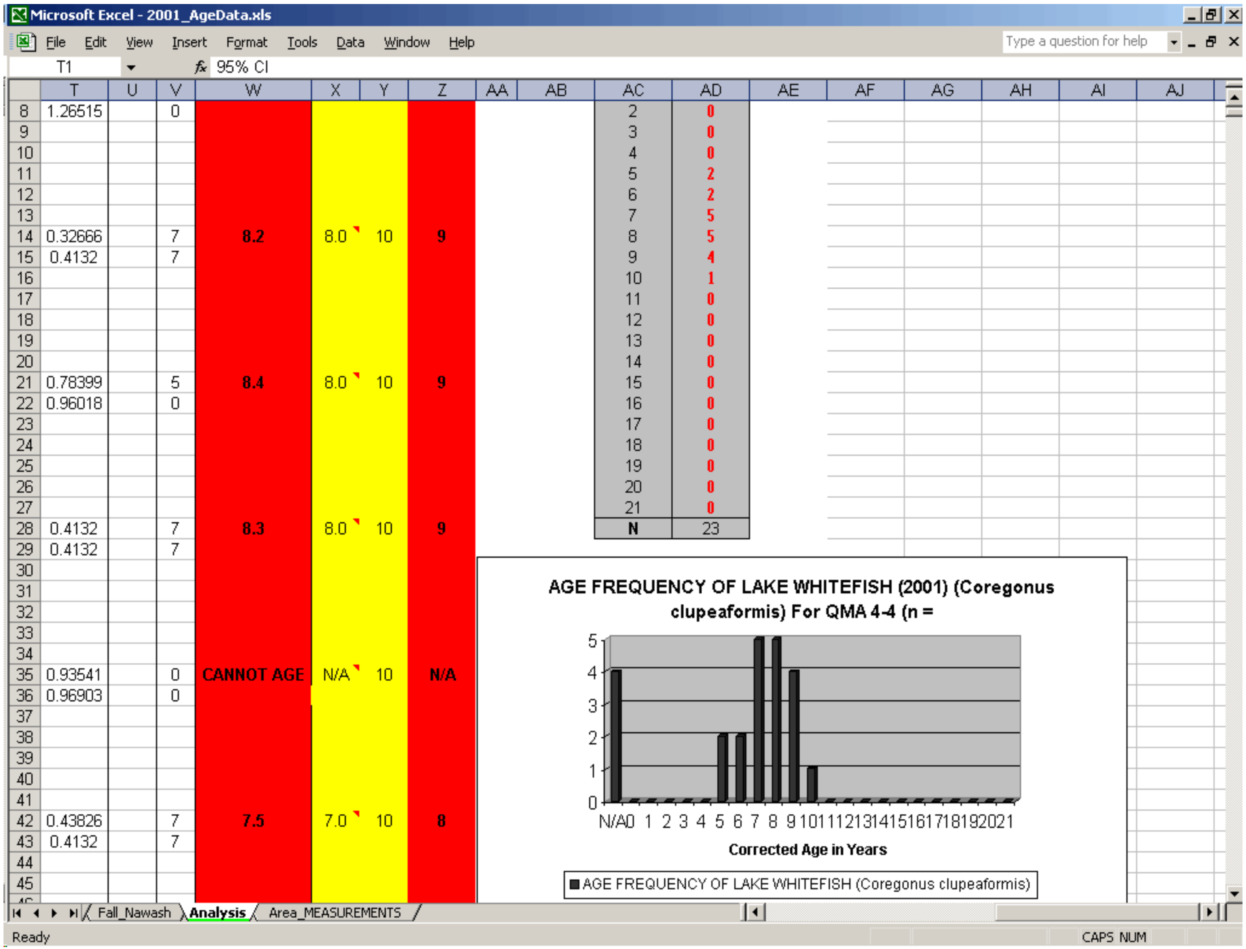


Figure A2-7. The analysis worksheet

APPENDIX 3 Otolith processing methods used by other labs

Many thousands of otoliths are processed for ageing every year, nevertheless practical information about embedding and sectioning otoliths is hard to come by in the literature. This is a summary of the procedures that we received from various laboratories around the world. A list of all the sources and the contact information is printed at the bottom of this document.

- Procedures

The otoliths of lake whitefish from the commercial harvest are relatively large (1 cm), therefor only the procedures that are macroscopical in nature are included in this summary. Because the Chippewas of Nawash calcified structure analysis facility (CNCSAF) uses the sectioning method to prepare otoliths for ageing other methods like grinding are not included in this summary. The species studied by the various labs mentioned in this summary include gadoids (cod, haddock, etc), whitefish (*Coregonus lavaretus*), and Baltic herring (*Clupea harengus*). The smallest otoliths that are processed by macroscopic procedures in this way are 2-3 mm in length by the DFO -Marine Fish Division (DFO-MFD) and by the Finnish Game and Fisheries Research Institute (FGFRI) 2-3mm Baltic Herring otoliths. For all these species transverse sectioning was used.

- Heat treatment of the otoliths

Heat treatment to improve the clarity of zones was reported by two laboratories. The DFO-Marine Fish Division bakes cod (*Gadus morhua*) otoliths at 150 ºC for a few minutes prior to sectioning. NIWA reported baking otoliths of Patagonian toothfish (*D. eleginoides*) in an oven at 275ºC for 12 minutes, until amber colored.

- Embedding

All the laboratories use a variation of the following procedure to embed the otoliths: 1) A bottom layer is prepared out of resin; 2) The otoliths are fixed on this layer; 3) The otoliths are covered with a layer of resin.

- Resin for embedding

All laboratories used either polyester resin (also called fiberglass resin) or epoxy resin for embedding the otoliths. The Center for Quantitative Fisheries Ecology (CQFE) uses “a quick drying epoxy-resin”. The Central Ageing Facility (CAF) does not recommend the use of epoxy resins if rubber molds are used because the hardness of the epoxy seems to attach to the resin and the blocks are very hard to remove from the silicon molds without tearing them. They suggest using clear casting Resin F61209 Polyester resin in styrene 46% W/V MAX. This product is not as hard as the epoxy and is easier to section. The FGFRI uses black resin to improve contrast at the edge of the otolith, The DFO-MFD added black pigment to the polyester resin fro the same reason.

- Molds for embedding

The molds, that are used to hold the resin are made of either Silicone rubber or Aluminum. The aluminum molds are made out of two pieces that can easily be taken apart to remove resin blocks. To facilitate the release of the block from the mold, some laboratories use a release agent like Teflon.

- Saw

The saws used for otolith sectioning by the laboratories are: Buehler Isomet low speed saw The Northeast Fisheries Science Center (NEFSC), Struers Accumulator-50 (FGFRI), and the Extec Labcut 1010 by the Netherlands Institute for Fisheries Research (RIVO). (see table A3-1)

TABLE A3-1 Prices of saws and blades used for sectioning otoliths

SAW	PRICE	BLADE 100 mm/0.23 mm
Raytech AL-P10S	US $800	US $16 (4” Blue Blazer)
Extec Labcut 1010	US $4.000	US $220
Struers accutom 50	US $15.000	US $151 (100CA)
Buehler isomet low speed	US $4.000	US $250

- Blades

All laboratories used diamond impregnated blades cooled by water or mixture of soap and water for sectioning. Sizes of the blades varied between 7.6 cm to 15 cm. Most laboratories used a single blade for cutting slices. One laboratory uses two blades that are separated by thin plastic or metal spacers to produce a thin section with one cut. All other labs used a single blade that was moved after each cut (Table A3-1).

- Sectioning

All laboratories made sections of 0.3 mm and 0.55 mm thickness. Most laboratories used a single blade making several multiple passes to section the otolith. Some laboratories use double blades and make a single pass to section the otolith.

- Staining

Staining the sections depends on the species and the preference of the researcher. The Finnish Game and Fisheries Research Institute used Neutral Red to stain the sections of whitefish (*Coregonus lavaretus*), The DFO - Marine Fish Division occasionally tried some staining using various solutions (alizarin, weak HCl, etc) that differentially stain or etch the calcium carbonate matrix. Although most labs have tried staining with some material the results do not match the effort required and most laboratories do not practice on a regular basis.

- Mounting

The only source that mentioned how the sections were mounted was the DFO - Marine Fish Division. They glue the sections on a microscopy slide with a transparent acrylic spray.

- Contact List

Chippewas of Nawash Calcified Structure Analysis Facility (CNCSAF)
RR # 5, Wiarton, On
N0H 2T0, Canada
(519) 534 1689 fax (519) 534 21 30
email: assess@bmts.ca

The Northeast Fisheries Science Center (NEFSC),
166 Water Street, Woods Hole, MA 02543-1026
www.nefsc.noaa.gov
Department of Fisheries and Oceans Canada (DFO - MFD)
Marine Fish Division, Gulf of Maine Section
531 Brandy Cove Rd, St. Andrews, NB E5B 2L9,
Joseph J. Hunt, huntjj@mar.dfo-mpo.gc.ca

Center for Quantitative Fisheries Ecology (CQFE)
Old Dominion University,
Arthur C. Clarke (wpersons@odu.edu)
http://www.odu.edu/sci/cqfe/age&growth/Aging_Fsh/structures.htm

Finnish Game and Fisheries Research Institute (FGFRI)
Turku Game and Fisheries Research / RKTL,
Turun riistan- ja, kalantutkimus, Itäinen Pitkäkatu 3, FIN-20520 Turku, Finland
Jari Kämäräinen (jari.kamarainen@rktl.fi)
Jari Raitaniemi (jari.raitaniemi@rktl.fi)

Central Ageing Facility (CAF)
Marine and Freshwater Resources Institute,
Queenscliff, Victoria, Australia 3223

Netherlands Institute for Fisheries Research (RIVO)
Postbox 68 1970 AB IJmuiden, The Netherlands
Telephone: +31 255 56 46 46, : +31 255 56 46 44
Internet: www.rivo.wag-ur.nl
Dr. Loes Bolle l.j.bolle@rivo.dlo.nl

TABLE A3-2 Dimensions and the type of material the molds are made of by some laboratories and the type of resin used for embedding the otoliths.

	Mold Dimensions			Mold Material	Resin used for embedding
	Width mm	Length mm	Depth mm		
CAF	65	70	12	Silicone	Polyester resin
FGFRI	4	76	10	Aluminum	?
DFO - MFD	?	?	?	Aluminum	Black Polyester resin
CCAMLR	?	?	?	?	Epoxy resin
CNCSAF	35	60	11	Silicone	Epoxy resin

TABLE A3-3 Otolith size, specifications for the blade

	Otolith size mm	Section thickness µm	Blade Diameter mm	Blade Thickness µm
CAF	?	300 – 400	?	250
FGFRI	?	400	150	400
DFO M	0.5 -1.5	?	150	?
NEFSC	?	150 - 300	76	147 (006)
RIVO	?	550	154	530
CNCSAF	0.7-1.3	300 - 400	102	196 (008)