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Fisheries Long Term Monitoring Program Sampling Protocol

Determination of the Ages of Fish at Northern Fisheries Centre, Cairns: (2000-2003)

November 2005

Queensland Government
Department of Primary Industries and Fisheries

Fisheries
Long Term
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Acronyms

DPI&F	Department of Primary Industries and Fisheries
IAPE	Index of average percent error
ID	Identification
MSDS	Material Safety Data Sheet
NFC	Northern Fisheries Centre, Cairns
SMF	Sub-capular meshwork fibre

Background information

Rationale

Age determination has become an important part of analysing fish populations for stock assessment purposes. Until recently, stock assessments by the Department of Primary Industries & Fisheries' (DPI&F) Queensland have relied on catch and effort data and tracking modal progressions of fish lengths through time. Due to the recent availability of data, the Department has enhanced its stock assessments to include age structured information in its fishery models.

Fish ages are determined from otoliths taken from fish specimens provided by fishery independent and fishery sources. Details on specimen collection, removal and storage of otoliths are given in the two field monitoring protocols for the species (DPI&F 2005a, 2005b).

The purpose of these protocols are to provide a practical guide to the procedures used to determine the age of barramundi and Spanish mackerel from otoliths. Step by step methods and techniques involved with preparing the otolith samples and interpreting the marks on them are described. Standardising the ageing methods used since 1999 at the Northern Fisheries Centre (NFC) Ageing Laboratory provides consistent population age structure data inputs for stock assessment of barramundi and Spanish mackerel.

Objective of the fish age determination activities

To provide annual age composition data in a format that can be used in stock assessment analysis of Spanish mackerel and barramundi in Queensland waters.

Otolith structure

Otoliths consist of calcium carbonate crystals in the form of aragonite, radiating from the focus (nucleus) in three dimensions through a protein matrix of otolin (Williams and Bedford 1974). Otoliths are part of a fish's inner ear, which senses movement, momentum, spatial orientation and sound. The fish's inner ear is similar to that of other vertebrates, having three semicircular canals and three otolithic organs; the utricle, lagena, and saccule (Popper and Lu 2000). Each otolithic organ contains a different type of otolith, the lapillus, asteriscus and sagittae respectively. The sagittal otoliths are typically the largest otoliths in a fish, and thus used most frequently for age determination.

Otolith's increase in size by the continuous deposition of material on the otoliths outer surface. Seasonal variation in the composition and rate of deposition of material due to climate, diet or the demands of reproduction and/or growth, cause the formation of alternating zones, with different optical properties (Beckman and Wilson 1995). Under reflected light (source shone directly onto the surface of the otolith), the opaque zones appear as white or light-coloured rings and the translucent zones as dark rings or zones (Figure 1). The formation of successive translucent and opaque zones is considered annual in barramundi (McDougall 2004) and Spanish mackerel (McPherson 1992). The opaque zone is referred to as the annual increment.

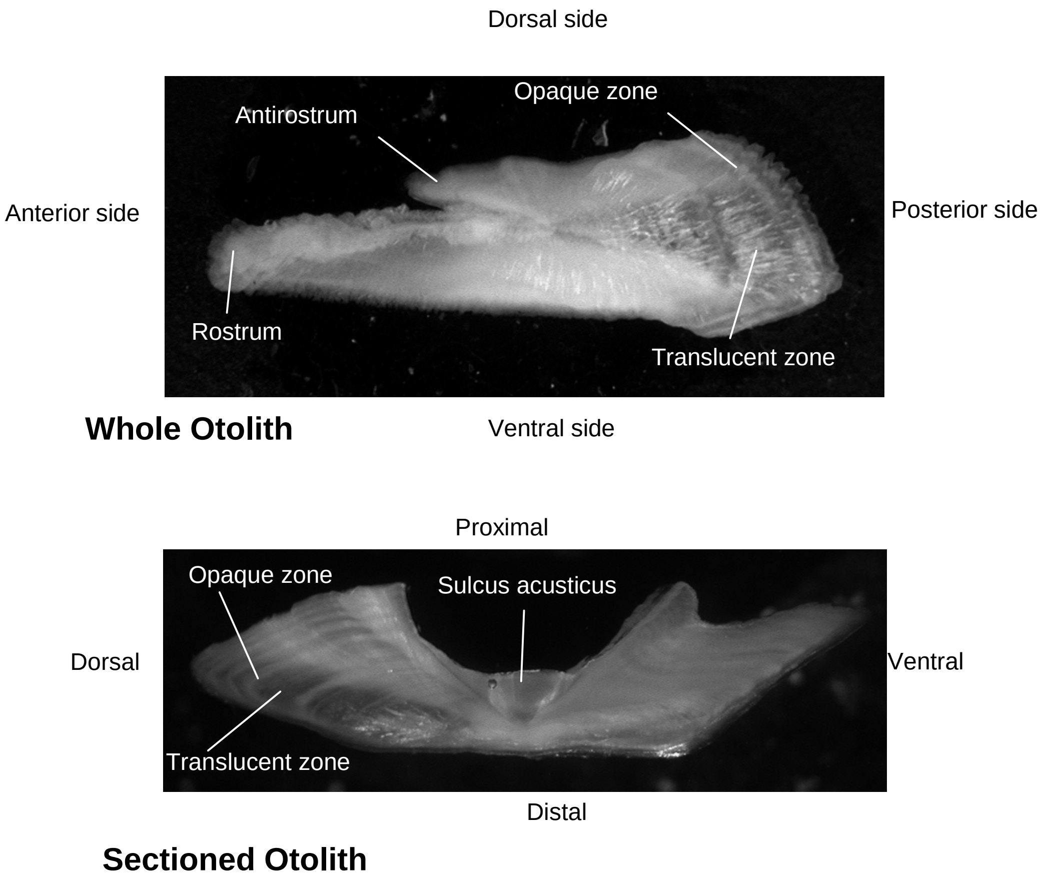

Figure 1. The orientation and internal structures of a whole sagittal otolith and a transverse thin otolith section from Spanish mackerel viewed under reflected light.

Developing an age determination protocol for a fish species

Before ages can be determined for any fish species using otoliths, the internal structures must be viewed and the interpretation procedures documented. This is accomplished at NFC using the following steps:

- Initially, otoliths covering the full range of fish sizes are selected for examination, starting with those from smaller specimens.

- Only those otoliths in which alternating opaque and translucent zones are present should be considered. The zones are then examined to determine their frequency of formation, in order to define their structure and to establish how they are to be interpreted.

- Each otolith is read three times, without knowledge of ancillary fish information.

- After an age is assigned, checks using ancillary information such as sex, length, otolith weight and morphometric measurements are conducted to detect possible outliers in the age estimates.

Laboratory procedures

Otolith Registration

Otoliths received at the NFC are catalogued into a Specimen Register. The NFC Specimen Register records the date received, species, collection location, storage location and the name of the person who registered the specimen. Once registered, the vials containing otoliths are packed into a polystyrene rack (Figure 2) and permanently stored in a cool, dry area. The specimen register is also used to track otolith samples sent to other fish ageing facilities. An example of the NFC Specimen Register is given in Data sheets (see Pg 26), and a blank form is included as Appendix A.

Figure 2. Plastic otolith vials stored safely in a polystyrene rack

Otolith weight

Otolith weight and age are related in many fish species (Milton *et al.* 1994, Cappo *et al.* 2000, McDougall 2004) and the relationship is used to assess potential errors in the age estimates and to examine patterns in otolith growth. An undamaged sagittal otolith is chosen at random from the pair taken from each fish, and is weighed using a GR-200 electrotonic balance (+/- 0.001 g). The GR-200 balance has a serial interface, allowing data to be transmitted directly into an Microsoft® Excel spreadsheet. Detailed instructions for weighing otoliths are given in Appendix B.

Sectioning otoliths

Otolith moulds

Customised, reusable moulds designed to accommodate a range of otolith sizes are made to embed large numbers of otoliths (Figure 3). The silicon rubber moulds are reusable, low cost, and have a high level of tear resistance and outstanding release properties. The large mould accommodates otoliths 15 x 23 mm in size and the small mould holds otoliths 9 x 23 mm. Instructions on making the silicon rubber moulds are given in Appendix C.

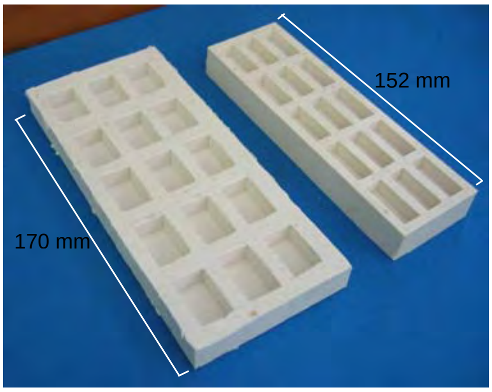

Figure 3. Reusable silicon rubber moulds for embedding the otoliths in resin.

Embedding otoliths

Mounting the whole otoliths in resin reduces the amount of chipping or cracking during sectioning, and provides a stable structure for the wafering saw chuck to grip. One randomly selected otolith from each fish is embedded in a Crystic® polyester resin in a two-stage pour within a fume cupboard. Detailed methods on embedding Spanish mackerel and barramundi otoliths are given in Appendix D.

Sectioning otoliths

Transverse sections of the otolith are cut (across the width) using a low-speed diamond blade wafering saw (Figure 4). Two 0.4 mm serial sections are cut per otolith, the first section incorporating the focus and a second adjacent to the focus. Failure to section through the focus can lead to an underestimate of age by at least one year. Sectioning the otolith on a slight angle may reveal considerable differences in the spacing and appearance of the annual structures. To avoid these problems otoliths are sectioned according to the procedures in Appendix E.

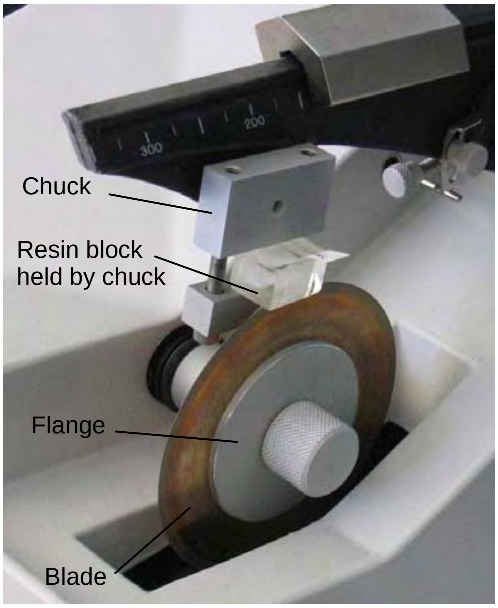

Figure 4. Close up view of an otolith block held in the chuck of a low-speed diamond blade wafering saw.

Mounting the sections

Both otolith sections are positioned on a microscope slide with the sulcus acusticus groove at the top of the slide and the dorsal plane facing the label (Figure 5). The sections are protected with a coverslip and labelled before viewing. Detailed instructions on mounting the sectioned otoliths are given in Appendix F.

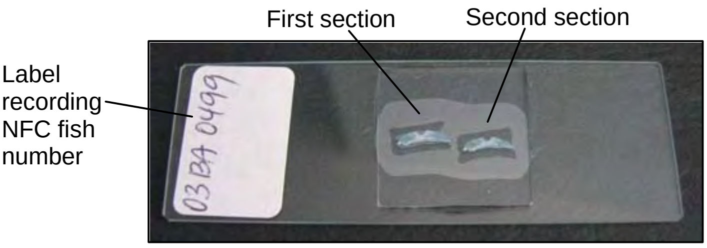

Figure 5. The two otolith sections from fish number (03BA0499) are positioned on the microscope slide. The section closer to the slide label contains the focus and the second section is cut adjacent to the focus.

Quality Assurance

Quality assurance is an essential component of age determination for providing reliable age composition data for stock assessments. Monitoring precision and maintaining consistency throughout the long term data series increases the integrity of the stock assessments. One of the main sources of error is the potential for a change to occur in interpreting the otolith structure over the long term (Campana 2001). This bias error can directly affect stock assessment results and influence estimations of population dynamics and hence management decisions. The quality control program for ageing otoliths at NFC is as follows:

- Reading protocols for interpreting either whole or sectioned otoliths for each species are established to standardise the age determination method (see Pg 11, 16 and 20).
- Otolith readers are trained and their skill maintained by reading the reference collection, of previously aged material, to check consistency (see Pg 8).
- Standard measures used to detect bias and measure precision of age estimates include: an index of average percent error (IAPE), scatter plots along with regression ANOVAs, and interpretation of scatter plots for outliers. In undertaking such analysis, it is recognised that fish growth is highly variable and outliers do not necessarily indicate errors (see Pg 8).
- Each otolith is assigned a readability score from 0 (not rated) to 5 (excellent) (Appendix J). The rankings from 1 to 5 are a subjective ranking of how easy the individual reader found the structure to interpret. Otoliths with a readability index of zero or one (very low) are excluded from subsequent analysis.

Reading the otoliths

The following are steps taken to read a new sample of otoliths

- Otolith readers interpret the reference collection.
- Bias and precision assessment of each readers interpretations of the reference collection are calculated.
- Two readers interpret the new sample of otoliths. A primary reader performs two complete interpretations and an independent reader performs one complete interpretation followed by a 25% randomly sampled re-read.
- Bias and precision assessments of intra-reader and inter-reader are calculate on the new sample.

Otolith Reference Collection

The NFC fish ageing laboratory uses reference collections to train new staff and maintain experienced readers skills. Maintaining reader skills improves consistency and reduces errors in determining fish age. Each species aged at the laboratory has a reference collection of otoliths. Otoliths within the collections are from agreed or known aged fish. The collection is referred to each time the otolith reader starts aging a species after a lengthy interval (2 months or more).

Depending on the species the reference collection consists of either whole or sectioned otoliths. Digital images of the otoliths in the collection are stored on file to maintain the longevity of the collection. The reference collection for a species includes up to twenty representative otoliths in each age class. The collection represents otoliths with all readabilities (greater than 1), all collection locations and both sexes. If changes in monitoring methods occur the collection will be expanded as required to include otoliths from other collection times and locations

The otolith reader estimates the age of 100 randomly selected otoliths or images from the reference collection of that species and preparation type (whole/sectioned). These otoliths are interpreted using the standardised ageing protocols (see Pg 11, 16 and 20). The otoliths are read without any additional information on the fish or otolith.

The skills maintenance readings of the reference collection are recorded using a Fish Age Data Sheet (see Pg 27). After all 100 otoliths have been read, the reader will check the reference collection database for the corresponding agreed age values for the fish. The results will be evaluated using age bias plots and IAPE analysis.

Bias and precision

Measurements of bias and precision are used to evaluate the results of the otolith reader's performance during reference collection skills maintenance exercise and in examining new material. The calculations will determine whether the reader has achieved an acceptable level.

An IAPE value is calculated according to Beamish and Fournier (1981) is used to measure the reproducibility of repeated readings of the otoliths (i.e. precision). The precision measure does not take into account the accuracy of the otolith readings but provides a measure for gauging the relative ease of determining the age estimates and to test for intra-reader variability Campana (2001). The IAPE is calculated by:

$$IAPE = 100 / N \sum_{j=1}^{N} \left[1/R \sum_{i=1}^{N} \frac{\left| X_{ij} - X_j \right|}{X_j} \right]$$

Where *N* is the number of fish aged, *R* is the number of times the fish is aged, X_{ij} is the *i*th determination for the *j*th fish, and X_j is the average estimated age of the *j*th fish.

The IAPE values are bootstrapped by a method described by Efron and Tibshirani (1993) which will provide confident intervals to compare the primary readers and independent readers IAPE values.

There is no *a priori* value of precision that can be designated as a target level for aging studies, since precision is highly influenced by the species and the nature of the structure, and not just the age reader (Campana 2001). Generally, an IAPE of less than 5% is considered acceptable for repeated readings (Morison *et al*. 1998b). If the IAPE is >5%, then the otolith reader must study the protocols for interpreting the species and train using the reference collection until the reader achieves an acceptable result.

Age bias plots and regression analysis are used to detect bias errors within the reader's age estimates. The slope and the intercept for the age plot are tested for significance against a line with a slope of one and intercept of zero. The slope and intercept are considered significantly different when the slope and intercept are outside the 95% confident intervals. An acceptable result is achieved when the slope and the intercept is within the 95% confident intervals. If bias is detected between the two readings then the reader must study the protocols for interpreting the species and train using the reference collection until the reader achieves an acceptable result.

Interpreting a new sample

When the reader's skills have been tested for interpreting the species, the otolith reader performs readings of each otolith in the new sample. The first interpretation uses an Optimas® image analysis system (see Pg 9), while the second reading uses only a microscope. These two readings are separated by at least two weeks. To maximize objectivity reference numbers identify otoliths so no ancillary data is known to the reader. The two readings are compared using the techniques outline in Bias and precision (see Pg 8). The second reading is documented using the Fish Age Data Sheet (see Pg 27).

Image analysis system

At NFC an Optimas® image analysis system with a series of customised programs (macros) provides the necessary image analysis and data capturing facilities for otolith reading (Figure 6). Otolith images are captured via a Pulnix® black and white digital camera coupled to a Zeiss Stemi 2000-C® stereo dissecting microscope. The program functions are accomplished via menus, windows and mouse operations. Programs perform different functions such as image capture, calibration of magnifications, age estimation, measurements of otolith dimensions, and image overlays. Directories and subdirectories store and manage large volumes of otolith images and reader data.

Figure 6. Northern Fisheries Centre staff member examining an otolith section with the Optimas® image analysis package.

The many built-in Optimas® image enhancement functions including automatic measurement of grey-level intensity, histogram spreading and filter functions help restore the level of resolution lost by the computer screen. Active manipulation of lighting direction, focal plane, or specimen orientation can also increase visibility of increments, but these options are not available with a captured image.

The program ANNULI4 written by Christopher Donohoe (National Marine Fisheries Service, Santa Cruz laboratory, California) has been customised at the NFC laboratory to assist in ageing barramundi and Spanish mackerel otoliths. The program renamed BarraAnnuli measures and records the following otolith data:

- Otolith radius
- Distance of each annual increment from the focus
- Proportional distance for each annual increment (the ratio of the annual increment distance to the radius distance)
- Captured otolith image with overlaying fish number, microscope magnification and marked positions of annual increments.

Each otolith is read at least once using this Optimas® program and the data transferred into an Excel® spreadsheet (Appendix H). A step-by-step guide for using the BarraAnnuli program is given in Appendix I.

Protocol for age determination of Spanish mackerel from whole otoliths

Initially both left and right otoliths are examined (Appendix B Figure 2), as readability often varies between the pair. Whole otoliths are placed distal or concave side up and immersed in baby oil for reading under magnification. The oil improves the refractive index and light penetration into the otolith, enhancing its readability. Spanish mackerel whole otoliths are viewed using a Zeiss Stemi 2000-C® stereomicroscope at x125 objective and illuminated with polarised, reflected white light on a dark stage.

Age interpretations of whole Spanish mackerel otoliths are based on the method of McPherson (1992). Morphometric measurements and annual increments are read along the posterior plane of the whole otolith. The outer edge of each opaque zone or start of the adjacent translucent zone is considered to be formed annually (McPherson 1992) and is marked in the Optimas® macro with a letter “A”. Age estimation involves counting the number of annual increments from the focus to the posterior margin (Figure 7).

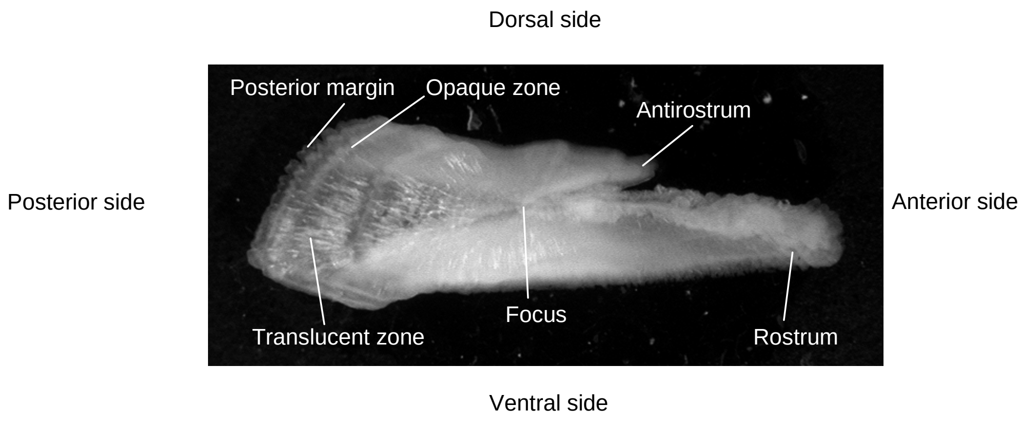

Figure 7. Distal view of a whole sagittal otolith from a Spanish mackerel illustrating the orientations and distinguishing features. The otolith is viewed with reflected light on a dark stage.

Once read, the whole otolith is washed in alcohol, dried and returned to its labelled vial. The vial is left open (for up to 24 hours) ensuring the otolith is completely dry before storage.

Margin interpretation

The otoliths from each region (see DPI&F 2005a) are collected at the same time every year. Therefore, it is assumed the zone represented on the outer margin of the otoliths, collected at this time, will be consistent from year to year. In Spanish mackerel from the Queensland north east coast, the formation of the opaque zone is from June to August (McPherson 1992). The otoliths are collected in November, hence it is assumed the margin should be translucent. Care must be taken when interpreting the margin because opaque areas may be artefacts introduced by the refractive indices of the various otolith materials and the light source. If the outer margin is opaque then the formation of the annual increment is considered to be incomplete (i.e. formed later than usual) (Figure 8). When this occurs the outer annual increment is included in the age estimate. The ageing technique assigns the fish into age cohorts for later use in stock assessment analysis.

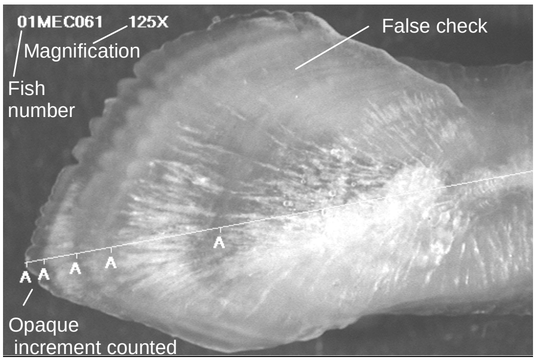

Figure 8. Posterior plane of a whole otolith from five-year-old Spanish mackerel number 01MEC061. The white line is the ageing axis with each annual increment marked with the letter “A”.

First annual increment

The first translucent zone is very large and is characterised by the radial striae (crazing) within the translucent zone (Figure 9). The opaque zone in Figure 9 is very distinct and easily interpreted, however in some otoliths this opaque zone may appear faint or indistinct.

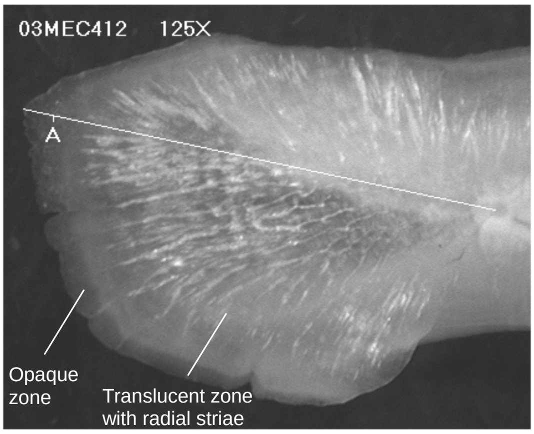

Figure 9. View of the whole otolith of one-year-old Spanish mackerel number 03MEC412. The white line is the ageing axis with the annual increment marked with the letter “A”.

Second annual increment

As with the first year of growth, the second year is characterised by a distinct translucent zone. Otolith from fish number 03MEC124 in Figure 10 has two obvious translucent zones with radial striae. The radial striae pattern within the translucent zones is often used to identify the opaque zones. However in this otolith, the opaque zones are easily identified. In some whole otoliths there is a faint false check between the first and second annual increment. The otolith in Figure 8 has a faint false check. A false check is more pronounced in the sectioned otoliths (Figure 20). The false checks are not counted. Other otolith characteristics may be used to help define the position of the first and second annual increments, for example the stepped shape of the dorsal edge posterior to the antirostrum in fish number 03MEC096 (Figure 10).

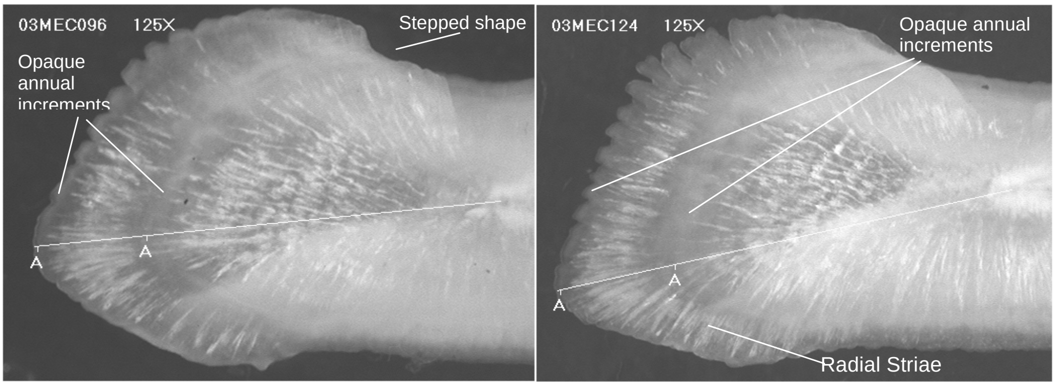

Figure 10. Whole otoliths from two-year-old Spanish mackerel. Fish number 03MEC124 (left) has two very distinct opaque zones. 03MEC096 (right) has a distinct stepping shape indicating the position of the first opaque zone. The white line is the ageing axis with each annual increment marked with the letter “A”.

Subsequent annual increments

After the second or third annual increment, the intervals between the translucent zones is generally smaller and much more uniform. This is well illustrated in the five-year-old fish in Figure 11.

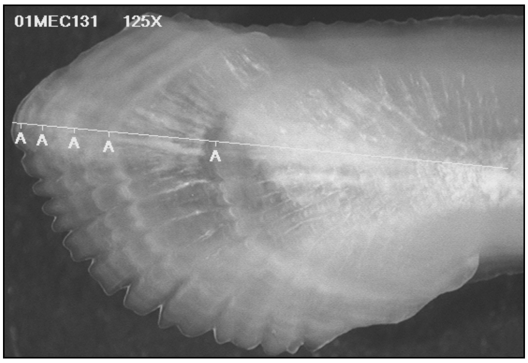

Figure 11. Otolith from a five-year-old Spanish mackerel (fish number 01MEC131) with well defined alternating translucent and opaque zones. The white line is the ageing axis with each annual increment marked with the letter “A”.

Difficulties in interpretation

While opaque zones are usually well defined and are easily recognised in Spanish mackerel otoliths (e.g. Figure 11), the zones may appear thin or translucent in some otoliths (Figure 12). McPherson (1992) found that exposing the otolith to water affected the reading of internal structure by permanently clearing the opaque zones. The lack of contrast between the translucent and opaque zones makes interpretation of the internal otolith structures difficult. Such otoliths are assigned a low level of readability. It is important when extracting the otoliths from the fish's head, to ensure they are removed, cleaned and dried immediately to reduce the likelihood of water clearing the opaque zones.

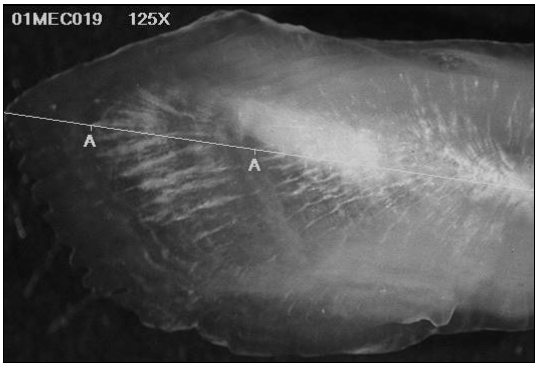

Figure 12. A Spanish mackerel otolith that has been exposed to water and not dried properly before storage.

Figure 13 shows a five-year-old Spanish mackerel with a faint opaque area around the focus within the first annual increment. This zone has formed early in the life of the fish. This mark or "juvenile check" is not considered annual and is not counted in age determination.

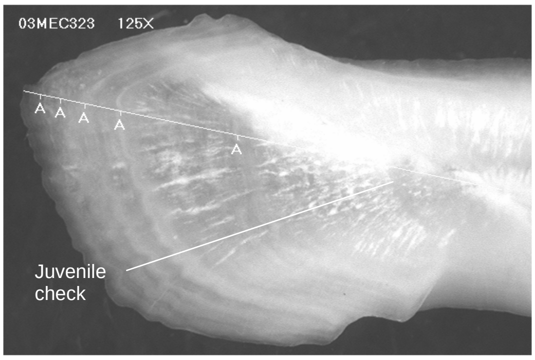

Figure 13. A five-year-old Spanish mackerel (fish number 03MEC323) with a possible juvenile check near the focus. The white line is the ageing axis with each annual increment marked with the letter "A".

In otoliths of Spanish mackerel older than eight years, the contrast between translucent and opaque zones tends to deteriorate toward the outer margin. The whole otolith in Figure 14 could not be aged confidently and was assigned a low score on the readability index. This otolith would need to be sectioned to achieve a more reliable age.

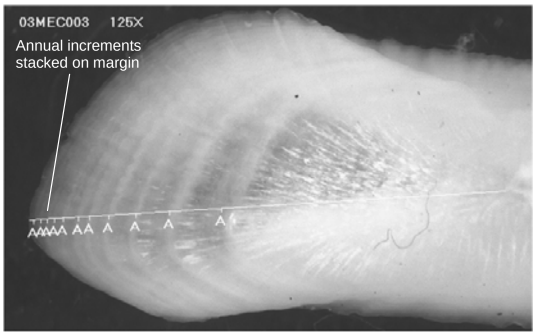

Figure 14. The translucent and opaque zones in this Spanish mackerel (fish number 03MEC003), tentatively aged at eleven years, are crowded on the margin of the otolith making interpretation difficult. The white line is the ageing axis with each annual increment marked with the letter "A".

Protocol for age determination of Spanish mackerel from sectioned otoliths

The main age determination method employed for Spanish mackerel is the reading of whole otoliths. This method was chosen due to time constraints, and the abundance of relatively young fish in the sample (1 to 7 yr olds). Some otoliths have been read both whole and sectioned; further analysis will be conducted to determine the usefulness of sectioned otoliths.

The Spanish mackerel otolith reader conducts a blind reading of 100 sectioned otoliths from the reference collection (see Pg 8). Sectioned Spanish mackerel otoliths are viewed at x40magnification with a Zeiss Stemi 2000-C® stereomicroscope and illuminated using polarised reflected white light on a dark stage (Figure 15).

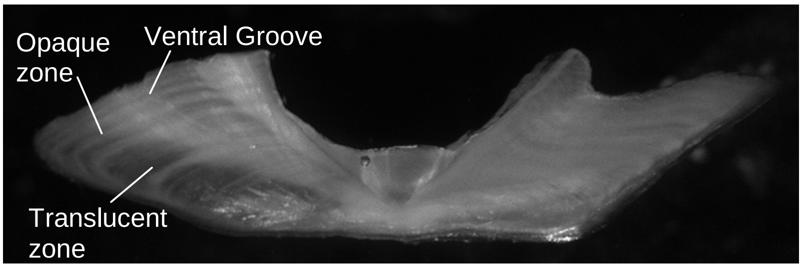

Figure 15. Ventral view of a transverse section of a Spanish mackerel otolith under reflected light on a dark stage.

The alternating opaque and dark zones are most visible on the ventral plane of the sectioned otolith. Within the internal structure of the otolith, a ventral groove can be seen from approximately the first annual increment to the external margin. This feature changes the direction of the opaque zones (Figure 15).

Age determination in sectioned otoliths involves counting the number of annual increments from the focus out to the margin on the ventral plane. The outer edge of each opaque zone or start of the adjacent translucent zone is considered to be formed annually and is marked in the Optimas® macros with the letter "A" (Figure 16). As for whole otoliths from Spanish mackerel it is assumed the margin should be translucent. If the outer margin is opaque the annual increment is considered to be incomplete and is then included in the age determination (Figure 16).

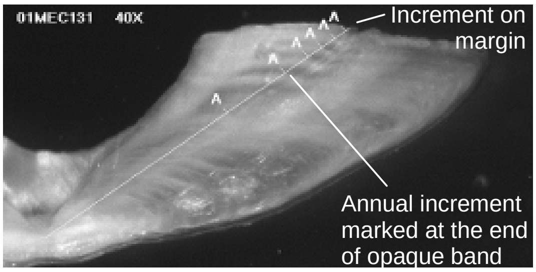

Figure 16. Ventral plane view of a sectioned six-year-old Spanish mackerel otolith (fish number 01MEC131). The white line is the ageing axis with each annual increment marked with the letter "A".

First Annual Increment

The first translucent zone is very large and is characterised by the radial striae within the translucent zone (Figure 17). In some cases the ventral groove on the ventral plane may indicate the location of the first annual increment (Figure 18).

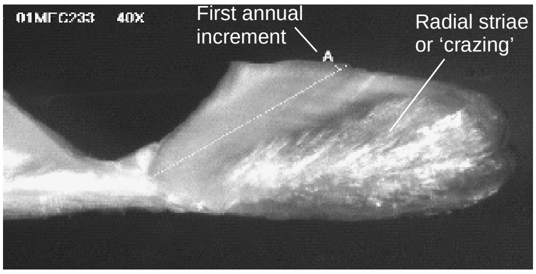

Figure 17. Spanish mackerel sectioned otolith (01MEC233) where the radial striae has been used to identifying the first annual increment. The white line is the ageing axis with each annual increment marked with the letter "A".

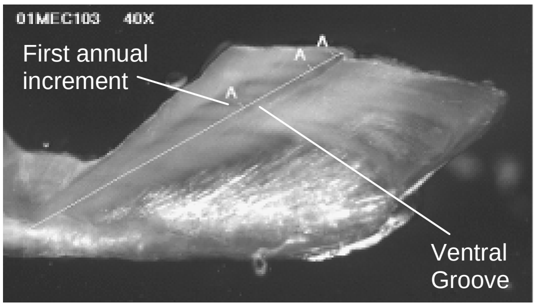

Figure 18. This Spanish mackerel otolith (01MEC103) is a three-year-old fish where the ventral groove assisted the identification of the first increment. The white line is the ageing axis with each annual increment marked with the letter "A".

Second annual increment

The second annual increment may also have 'radial striae within the translucent zone (Figure 19). The rate of otolith growth during this period, is rapid but not as great as in the first annual increment. After the second increment, the distances between the annual increments are closer together.

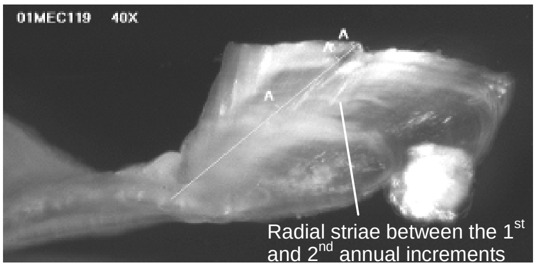

Figure 19. View of a sectioned three-year-old Spanish mackerel (fish number 01MEC119) with radial striae in the translucent zone. The white line is the ageing axis with each annual increment marked with the letter "A".

Between the first and second annual increment there may be a number of faint zones which are false checks (Figure 20). These are also observed in the whole otoliths and are considered to be false because the zonation is not continuous. In some sectioned otoliths, the sulcus acusticus has an inflexion point at approximately the second annual increment (Figure 20). This may be used to indicate the position of the increment.

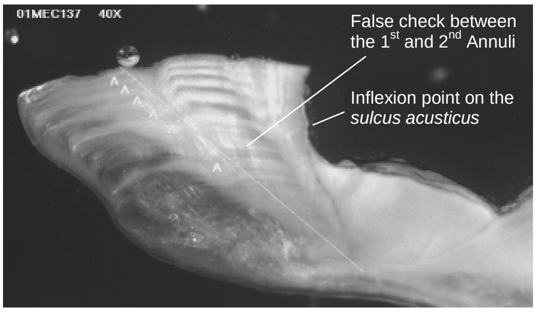

Figure 20. Sectioned Spanish mackerel otolith (fish number 01MEC137) displaying a false check between the first and second annual increment. The white line is the ageing axis with each annual increment marked with the letter "A".

Subsequent annual increments

The annual increments following the third annual increment appear closer together and are more uniformly spaced (Figure 21).

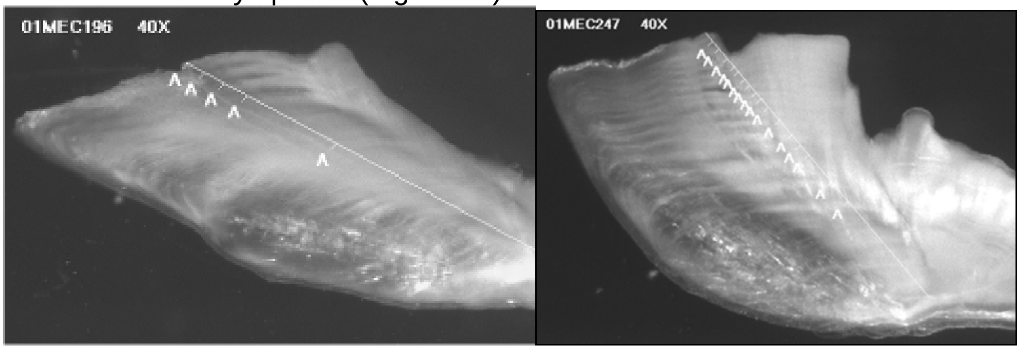

Figure 21. Spanish mackerel sectioned otoliths. Note the older individual (01MEC247) shows decreasing distance between annual increments towards the margin of the otolith. The white line is the ageing axis with each annual increment marked with the letter "A".

Difficulties in interpretation

On occasion, a lack of contrast between the opaque and translucent zones can cause difficultly in interpreting the internal structures especially in the first two years of growth (Figure 22). The contrast may be improved by turning the slide over on the microscope stage and viewing this surface of the section. Irregular growths are often evident in the otolith structure (Figure 23). Normally these did not cause difficulties in assigning age.

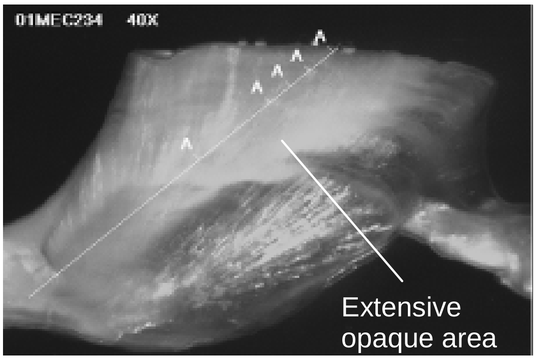

Figure 22. A Spanish mackerel otolith (fish number 01MEC234) with an extensive opaque area. This otolith was given a low readability index. The white line is the ageing axis with each annual increment marked with the letter "A".

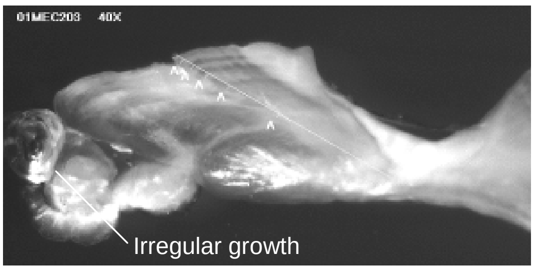

Figure 23. View of a Spanish mackerel otolith section (fish number 01MEC203) with an irregular growth. The white line is the ageing axis with each annual increment marked with the letter "A".

Barramundi ageing protocol for sectioned otoliths

Initial examination has indicated that whole barramundi otoliths are very difficult to interpret especially in older individuals and fish from the Gulf of Carpentaria. Sectioning the barramundi otoliths improved the resolution of the internal structures examined. Comparison of the results of the two ageing techniques indicated whole otoliths are unsuitable for determining the ages of the older fish, as the aged whole otoliths underestimated the age of the samples. To remain consistence within the reading method, all barramundi otoliths are interpreted as sectioned otoliths.

The barramundi otolith reader conducts a blind reading of 100 sectioned barramundi otoliths from the reference collection (see Pg 8). Sectioned barramundi otoliths are viewed using a Zeiss Stemi 2000-C® stereomicroscope at x125 objective and illuminated using polarised reflected white light.

The alternating opaque and translucent zones are most visible on the ventral plane of the sectioned otolith (Figure 24). In the ventral plane, the opaque annual zones are clearly visible along the ageing axis near the sulcus acusticus (Figure 24). The outer edge of each opaque zone or start of the adjacent translucent zone is considered to be formed annually and is marked by Optimas® in the image analysis macro with the letter “A”.

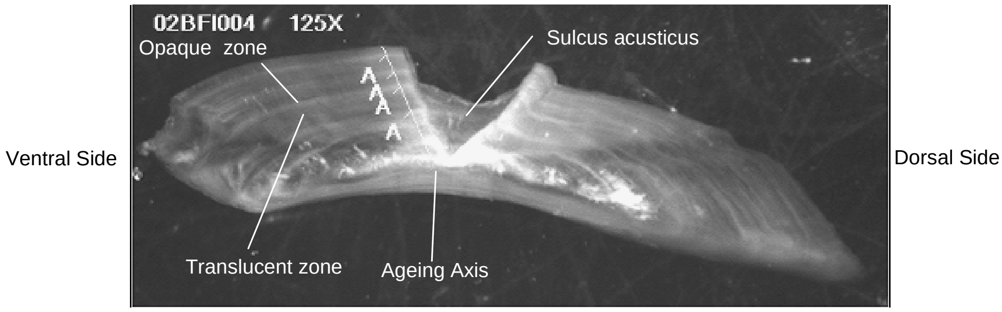

Figure 24. Transverse section of a barramundi otolith (fish number 02BFI004) under reflected light on a dark stage. The white line is the ageing axis with each annual increment marked with the letter “A”.

In some sectioned barramundi otoliths, annual increments can be seen in the sulcus acusticus (Figure 25). These zones may give an indication of the position of annual increments in otoliths that are difficult to interpret. The final age estimate for the sectioned otolith is performed along the ventral ageing axis.

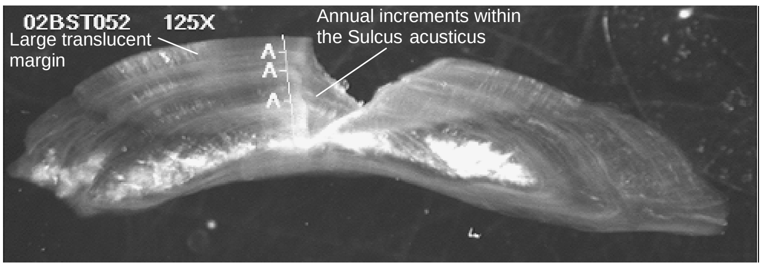

Figure 25. Sectioned barramundi otolith (fish number 02BST052) with annual increments visible in the sulcus acusticus. The white line is the ageing axis with each annual increment marked with the letter “A”.

The dorsal plane of the sectioned barramundi otolith often contains areas that are difficult to interpret, as well as numerous false checks or double banding (Figure 26). This is possibly caused by spawning. To maintain consistency in sectioned otolith interpretations, this plane is only referred to as a guide.

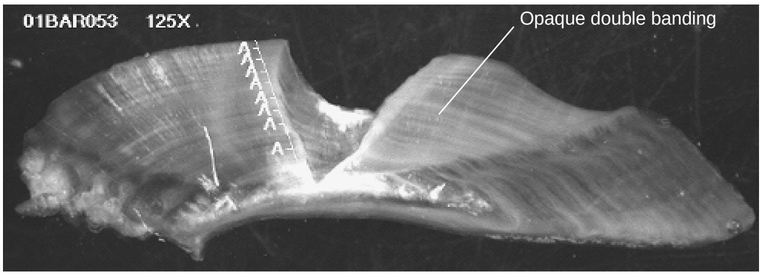

Figure 26. Sectioned barramundi otolith (fish number 01BAR053) with double banding on the ventral plane. The white line is the ageing axis with each annual increment marked with the letter "A".

Margin interpretation

The fish from which otoliths are taken are collected at the same time of year in each river system (see: DPI&F 2005b). Therefore, it is assumed the zone represented on the outer margin of the otolith will be consistent from year to year for each individual river. During these collection times the outer margin on the otolith's ventral plane is usually a large translucent zone, and is assumed the formation of the next opaque zone has not yet commenced (Figure 27).

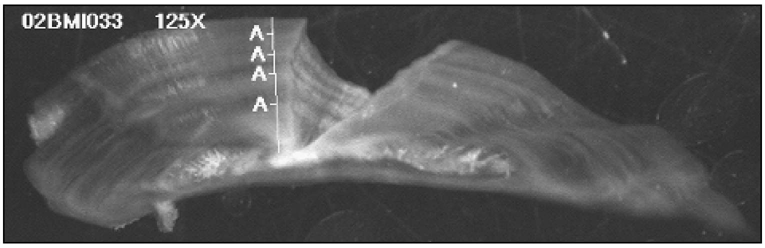

Figure 27. Sectioned barramundi otolith (fish number 02BMI033) with a large translucent zone on the outer margin. The white line is the ageing axis with each annual increment marked with the letter "A".

In a small percentage barramundi of otoliths, the opaque zone is evident on the margin. Where this occurs the estimated age of the fish is n -1, where n equals the number of opaque zones (Figure 28). This technique ensures all fish are assigned into the consistent age cohort.

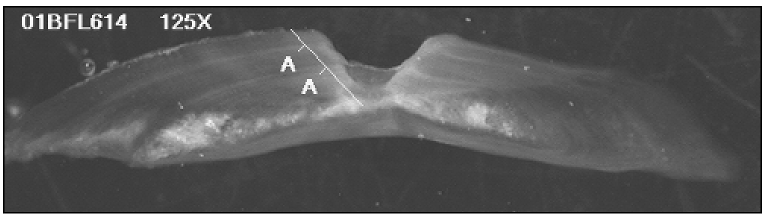

Figure 28. Sectioned barramundi otolith (fish number 01BFL614) with an opaque zone on the outer margin. This opaque zone was not included in the final age estimate. The white line is the ageing axis with each annual increment marked with the letter "A".

First annual increment

The position of the first annual increment in barramundi can vary considerably from region to region, among the sectioned otoliths. The variation may be due to the date of birth as barramundi has an extended spawning period from October to February. The first increment is characterised by a high degree of radial striae around the focus (Figure 29). In some sectioned otoliths there is a juvenile mark consisting of a dense opaque core area formed close to the centre of the otolith. This increment is not considered annual and is not counted in age determination.

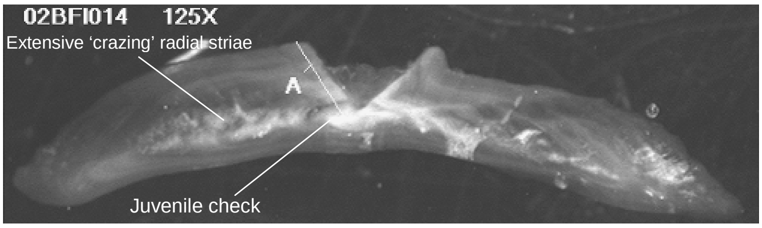

Figure 29. Sagittal otolith section of a one-year-old barramundi (fish number 02BFI014) with a juvenile mark around the focus. The white line is the ageing axis with each annual increment marked with the letter "A".

A sub-capular meshwork fibre (SMF) zone may be used to identify the first annual check (Morison *et al*. 1998a). This fibre is a dark line radiating from the focus to the ventral margin of the otolith. The fibre changes direction when it intercepts the first annual check (Figure 30). To date a small number of SMFs have been observed in the sectioned otoliths.

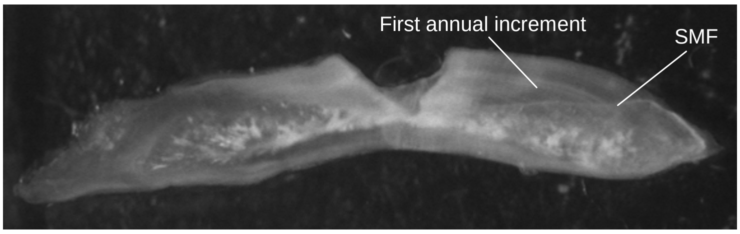

Figure 30. Sagittal otolith section of a one-year-old barramundi with a sub-capular meshwork fibre (SMF) zone indicating the position of the first annual increment.

Second annual increment

As with the first year of growth, the second increment on the sectioned otolith has a very wide translucent zone. The large translucent zones are indicative of the rapid growth rate in barramundi during this life history stage (Figure 31). In some otoliths, both the first and second translucent zone may contain radial striae.

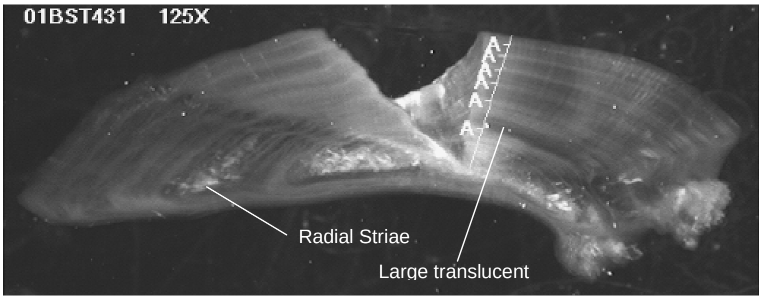

Figure 31. Sectioned barramundi otolith (fish number 01BST431) with relatively large first and second translucent zones. The white line is the ageing axis with each annual increment marked with the letter "A".

Subsequent annual increments

The first two annual increments in the sectioned barramundi otoliths are usually the most difficult zones to interpret. Beyond the second increment, the intervals between the translucent zones are generally more uniform (Figure 32). In older individuals, the annual increments decease in width as growth slows and become crowded on the otolith's outer margin.

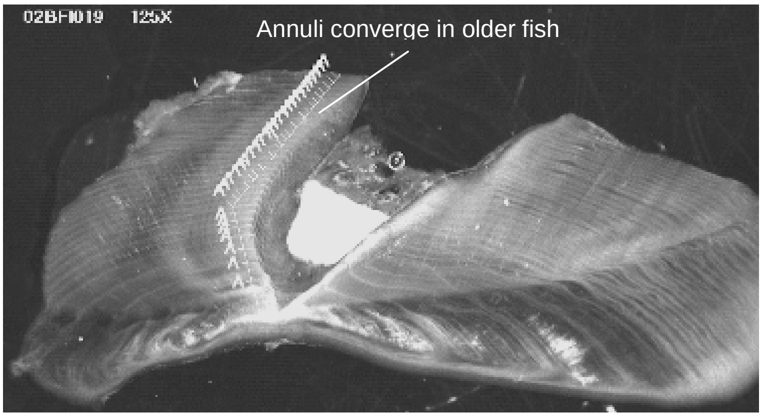

Figure 32. Sagittal otolith sectioned from an old barramundi (fish number 02BFI019). Note the reducing interval between the annual increments in this specimen. The white line is the ageing axis with each annual increment marked with the letter "A".

Difficulties in interpretation

If the sectioned otolith can not be marked along the ventral axis in the Optimas® macro then an alternative axis is applied. This action may be required in interpreting the otolith of older fish and in those individuals with broad areas of uninterpretable opaque material (Figure 33).

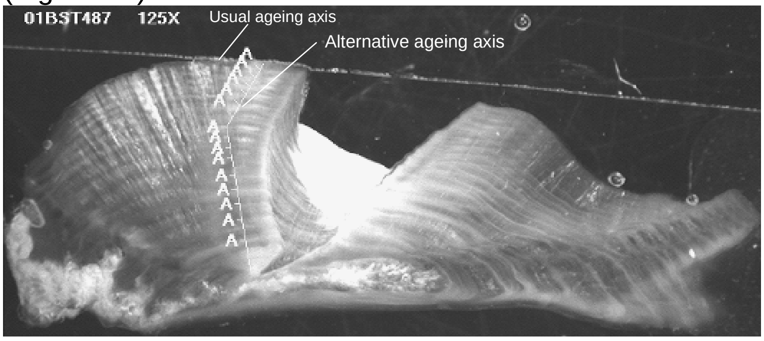

Figure 33. Alternative ageing axis used in fish number 01BST487. The white line is the ageing axis with each annual increment marked with the letter "A".

Otoliths with irregular growths are usually rare within the barramundi samples collected (Figure 34). Where possible, these otoliths are aged. The sectioned barramundi otolith in Figure 34 was aged but assigned a low readability index (see Appendix J).

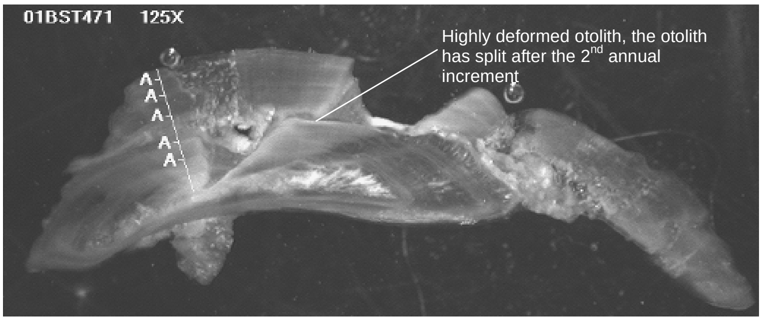

Figure 34. Sectioned barramundi otolith, fish number 01BST471, with irregular growth. The otolith has split and rotated between the second and third annual increments. The white line is the ageing axis with each annual increment marked with the letter "A".

Barramundi otoliths that are difficult to interpret with little contrast between the alternating opaque and translucent zones are assigned a low readability index. Such otoliths require the examination of the sulcus acusticus, ventral and dorsal planes of the sectioned otolith in determining the age (Figure 35).

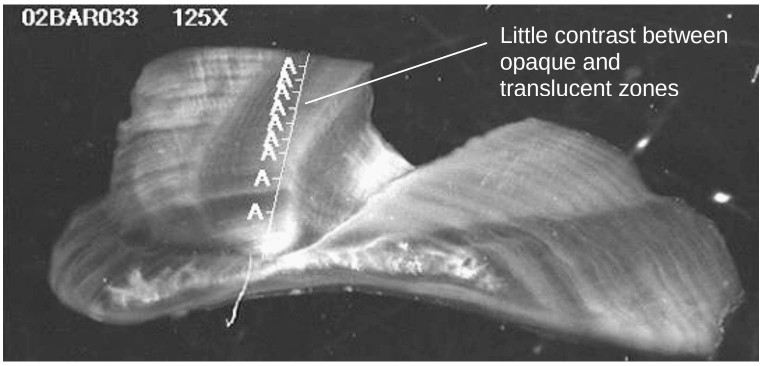

Figure 35. Sectioned barramundi otolith (fish number 02BAR033) with little contrast between the opaque and translucent zones. The white line is the ageing axis with each annual increment marked with the letter "A".

Training and safety

Staff of the NFC fish ageing laboratory, must be trained in the various techniques and methods outlined above. The otolith readers need experience in interpreting both whole and sectioned otoliths. When working in the NFC laboratory, all staff must comply with the NFC Laboratory Safety Manual (http://ournet/Business/centres/North/Northern+Fisheries/default.htm) which outlines the procedures and protocols that ensure all employees work in a safe manner. Technical staff using chemicals, for example polyester resin to embed otoliths must read and understand the relevant Material Safety Data Sheet (MSDS).

Data sheets

Specimen Register

On arrival at NFC all otolith specimens are documented on the Northern Fisheries Centre Specimen Register (Figure 36). Blank forms are included as Appendix A.

Queensland Government
Department of Primary Industries and Fisheries

Northern Fisheries Centre Specimen Register

Date	Species	Tissue Type	Collection Location	Staff	Storage Area
12/02/03	*L. calc.*	*otolith*	*Fitzroy*	*DBR*	*Cupboard 1 shelf 4*
14/02/03	*S. comm..*	*otlolith*	*Townsville*	*DBR*	*Cupboard 3 shelf 1*

Figure 36. completed example of the Northern Fisheries Centre Specimen Register

Specimen Register field descriptions

Date: Date the specimens are received by NFC dd:mm:yy
Species: Scientific name of the species (may be abbreviated)
Tissue type: Sample type received
Options:
Otolith
Gonad
Other
Collection Location: Site the fish was collected from
Staff: Personnel registering the samples, abbreviate as 3 initials of the person's name
Storage Area: NFC location where the samples are stored – list cupboard and shelf number

Fish Age Data Sheet

Reading of all otoliths is documented in a Fish Age Data Sheet (Figure 37.). Blank forms are included as Appendix G.

Queensland Government
Department of Primary Industries and Fisheries

Northern Fisheries Centre
Fish Age Data Sheet

Page ……*4*….. of …*4*…
Verify ……*WLH*……..
Checked

Date Read: …*23/02/01*……… Year Fish Caught …*2001*……. Reader: ………*WLH*……….	Read Number (circle): A B C	Read type (circle): Whole / Sectioned

Fish Number	Age	Readability	Comments
01MEC133	*5*	*3*	*Difficult 1st increment*
01MEC069	*2*	*4*	*Good otolith*

Readability Index: 0 – not rated; 1 – very low; 2 – low; 3 – medium; 4 – high; 5 – very high

Figure 37. A completed example of the Northern Fisheries Centre Ageing Laboratory Fish Age Data Sheet

Fish Age Data Sheet field descriptions

Page ___ of ___: Page number and total number of pages, recorded to ensure that data are not misplaced

Verify: Name of person who verified the data, by checking that all fields are legible and completed
Name abbreviated as 3 initials
Fill in after verification complete

Checked: Name of person who checked the data entered in LTMP database against the data sheet
Name abbreviated as 3 initials
Fill in after data checking is complete

Date Read: Date the reading is made
dd:mm:yy

Year Fish Caught: Year the sample was collected
yyyy
If training maintenance read of reference collection enter REFERENCE

Reader: Name of otolith reader
Name abbreviated as 3 initials

Read Number: Number of times the otoliths have been read
Circle the number
Options:
- 1 – 1st read
- 2 – 2nd read
- 3 – 3rd read

Read Type: Type of otolith preparation being read
Options:
- Whole
- Sectioned

Fish Number: Individual identification number of the fish

YYFSS###], where

YY= year

F = species

B = barramundi

M = mackerel

SS = Site fish caught

= Individual number

Age: Reader’s estimation of the fish’s age

Readability: The ease with which the otolith was read (Appendix J)

Readability Index Options:

0 – not rated

1 – very low

2 – low

3 – medium

4 – high

5 – very high

Comments: Any comments relevant to the reading of the otolith

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Appendix A - NFC Specimen Register

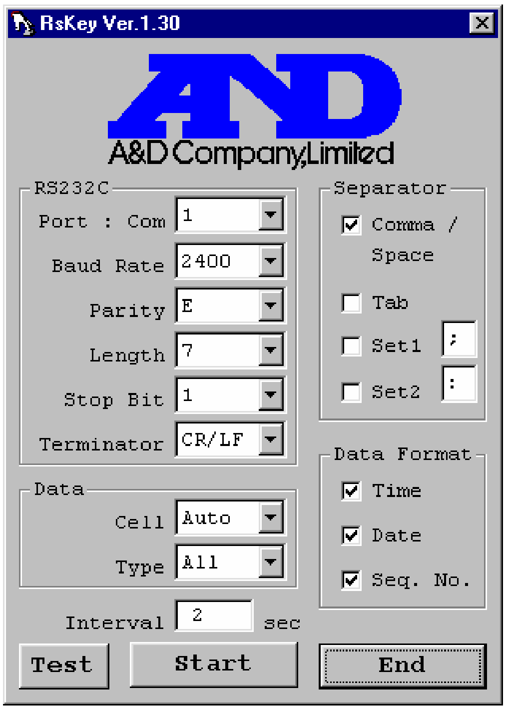
Queensland Government
Department of Primary Industries and Fisheries

Northern Fisheries Centre Specimen Register

Date	Species	Tissue Type	Collection Location	Staff	Storage Area

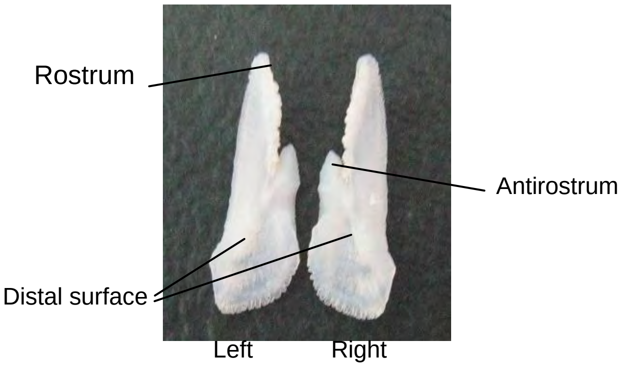
Appendix B - Operating the GR-200 Balance

1. Use **Shortcut** on the Windows Desktop called **Shortcut to RsKey,** or from the **Start menu** choose Programs/Windows Explorer /C/Program Files/WinCT/RsKey (application)
2. The settings for RsKey are as shown in Appendix B Figure 1. Check these settings before weighing the otoliths



Appendix B Figure 1 Settings for RsKey for the GR-200 Balance

3. Open the Excel® file Monitoring/Otolith/Otolith Weight/<year>/<species>/…. Otoliths are entered in excel workbook under Site sheet.
4. With the Excel® file open, press Start in the RsKey Window. RSKey minimises to taskbar and the red dot flashes while running.
5. Sort otoliths into numerical order and place in foam block
6. **Turn the balance on and allow to warm up** for at least an hour before use. Check that the balance is level. Calibrate balance – press CAL (see balance manual, section 7, page 18-20)
7. Place the otoliths in order of fish number to streamline the weighing and data entry process.
   - Remove both otoliths from the vial and randomly select one (Page 4)
   - If the selected otolith is broken or chipped use the other
   - Only whole otoliths are weighed
8. Enter Left or Right in the **Side** field, to record the side of the fish the otolith came from. (Appendix B Figure 2)



Appendix B Figure 2 Spanish mackerel left and right hand side whole sagittal otoliths sulcus acusticus facing down (i.e. concave side up).

9. Caution - This program has problems with the Num and Caps Lock, turning them off each time the Print button is pressed on the balance.
10. Place the curser in the ID field, wait for the weight reading to stabilise (O symbol on display), then **Press Print on the GR-200 balance.** An example of results is seen in the table below:

ID&Header	Weight (g)	Side	Mode	Time	Date	Seq. No
ST	0.4856	Left	g	11:57:23	07/09/2001	2
ST	0.4852	Right	g	11:57:28	07/09/2001	3
ST+000.2797			g	18:01:46	09/28/2001	67

If the weight has not stabilised, the result will be similar to the last entry in the table. Delete it, wait for the balance to stabilise, ensure curser is in the ID field, and press Print again.

11. Complete otolith ID data in upper case.
12. Weigh the next otolith by placing the curser in the next ID field, and pressing Print on the GR-200 balance.
13. To end the weighing session - **Save** the file and press **End** in the RsKey Window.

Cautions for weighing operation and to maintain accuracy:

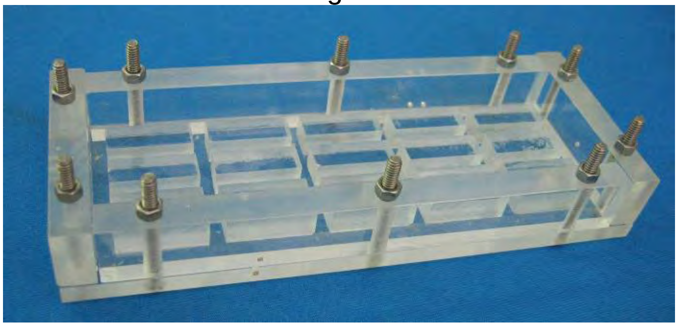
- Press re-zero key before each otolith is weighed on the pan.
- Flashing '**<**' on display indicates instability. Do not weigh until stability is achieved.
- Close the balance door between samples.
- Use forceps to handle the otoliths.
- Shorten the operation time as much as possible. The weight reading will gradually increase if the otolith is left on the weighing pan for a longer than 10 minutes (Remove otolith, run calibration, re-weigh).
- Calibrate the balance daily before use and every 2hrs during the course of weighing the otoliths.

Appendix C - Methods for Making Otolith Mould

- Read and understand the MSDS for Silastic 3481® resin and Silastic 81® curing agent before using the material
- The silicon rubber moulds are made within a fume cupboard
- The two-part mix for the silicon rubber is 100 parts Silastic 3481® resin to 5 parts Silastic 81® curing agent.

Materials list

- Silastic 3481® (a local supplier of this is The Fibreglass Centre – phone (07) 3208 4850)
- Silastic 81® curing agent (a local supplier of this is The Fibreglass Centre – phone (07) 3208 4850)
- Latex gloves
- Lab Coat
- Fume cupboard
- Clear Perspex® die (Appendix C Figure 1).
- One stirring rod
- Weighting trays
- Syringes 60 ml and 1 ml
- Artist paint brush
- Tissues and detergent



Appendix C Figure 1 Clear Perspex® die used for making otolith moulds.

14. Clean the surface of the die with detergent and wipe dry.
15. Initially pour 20 ml of silicon rubber and 1 ml of curing agent into the weighing tray. Stir until thoroughly mixed (not cross-contaminate the containers). When stirring ensure the stirring rod is not removed from the substance, this will prevent air bubbles being formed in the silicon rubber.
16. Using the artist brush, paint a thin layer (1-2 mm) of the initial pour over the surface of the die. Assist any entrapped air to escape by gently tapping mould on the bench.
17. The final pour is 100 ml of silicon rubber base and 5 ml of curing agent. Stir until thoroughly mixed. When pouring the silicon rubber into the die make sure no air bubbles are entrapped.
18. Leave the mould to cure for 24 hours in the fume cupboard. The final mechanical properties of the mould are reached within 7 days.
19. After 7 days ease mould away from the die using your hands, the mould can then be used.

Appendix D - Embedding Otoliths in Synthetic Resin

- Read and understand the MSDS for the clear casting resin and polyester resin catalyst.
- Ensure that the fume cupboard is operational (Appendix F Figure 1)

Materials list

- Fume cupboard
- Latex gloves
- Lab coat
- Otolith labels displaying fish numbers
- Syringe - large (60 ml)
- Syringe – 1 ml with needle18G x 1 1/2 inch
- Clear Casting Resin® (1 L tin) Resin – Crystic Polyester, DG 3 Flammable (distributed by Dadson (07) 4053 4000)
- Polyester Resin Catalyst MEKP® (20 ml) DG 5.2 Organic peroxide (distributed by Dadson (07) 4053 4000)
- Large and small silicon rubber otolith moulds
- Stirring rods
- Jewellers Forceps – very fine point or Micro dissecting Fine tip forceps (12cm) from ProsciTech® "Inox Tweezers No. 4 Biology"
- Weighing trays
- 3 ml disposable plastic bulb pipettes (Transfer Pipettes, disposable, Biolab® #BER225)
- Tissues and Scissors

1. Remove any old resin from the otolith mould by peeling it off
2. Prepare the otolith labels and print on waterproof paper.
3. Ensure otoliths are in numerical fish number order in polystyrene rack (Figure 2). Sort into large otoliths for large moulds, and small otoliths for small moulds, in separate racks.
4. Prepare resin using large (60 ml) syringe to measure the resin and small (1 ml) syringe to measure the catalyst (Re-use syringes - keep separate). Proportions are 1.5% by weight of catalyst to resin (use Appendix D Table 1 as a guide). For a one person operation an initial pour of 30 ml resin/0.45 ml catalyst is a workable amount. This quantity is sufficient for filling two small moulds or one large mould. Use weighing tray and a stirring rod to mix thoroughly. Avoid creating air bubbles by not removing the stirring rod from the solution until mixing is complete.

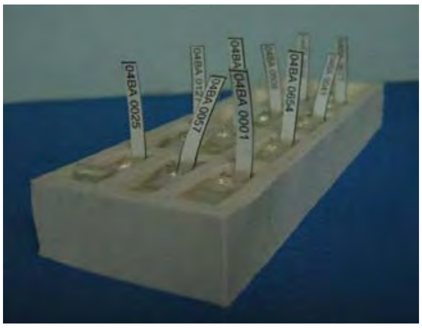
Appendix D Table 1. Required amounts of resin and catalyst for embedding

No. of Mould	Initial Pour		Final Pour	
Small Moulds	Resin (ml)	Catalyst (ml)	Resin (ml)	Catalyst (ml)
1	15	0.23	24	0.36
2	30	0.45	48	0.72
3	45	0.68	72	1.08
4	60	0.90	96	1.44
5	75	1.13	120	1.8
Large Moulds	Resin (ml)	Catalyst (ml)	Resin (ml)	Catalyst (ml)
1	30	0.45	45	0.68
2	50	0.75	90	1.36
3	75	1.13	135	2.04

5. Half fill the mould (approx 1.0 ml for the small mould and 1.5 ml for the large mould) using a 3 ml disposable plastic bulb pipette (Appendix D Figure 1). (Fill the bulb pipette and allow air bubbles to rise to top).

Appendix D Figure 1 Sagittal otolith from a Spanish mackerel placed in a mould half-filled with resin.

6. The resin will remain workable for up to 20-30 min, add otolith label after 15-20 min just prior to setting (Appendix D Figure 2).



Appendix D Figure 2 Otoliths embedded in resin with fish identification labels.

7. Once this resin has set, the otoliths can be positioned in the mould and more resin added.
   a. Make up more resin as above (48 ml resin/0.72 ml catalyst is a workable amount for one person).
   b. Dispense another 1-1.5 ml resin into each of 3-6 moulds.
   c. One otolith from the fish is randomly selected. Carefully place an otolith into the labelled mould using forceps. Slide the otolith in with the concave surface facing upwards. Placing the posterior end of the otolith in first helps to avoid air bubbles becoming caught in the sulcus.
   d. Positioning of otoliths:

      Barramundi - rostrum away from label
      Spanish mackerel - rostrum closest to label

Label

Rostrum

Spanish mackerel

Rostrum

Label

Barramundi

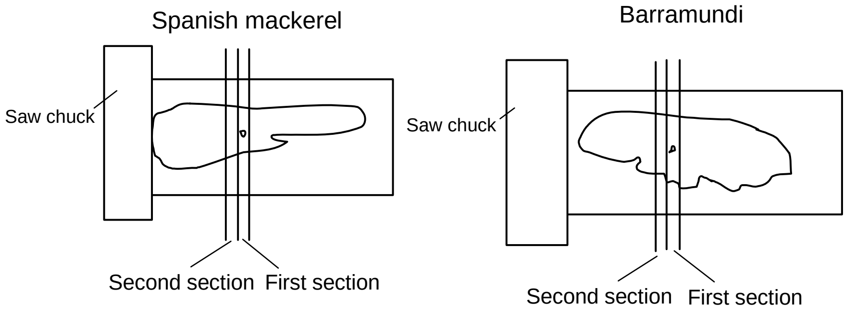
Appendix D Figure 3 Position of labels for embedded Spanish mackerel and barramundi otoliths

8. Repeat step 7 until all are completed.
9. Allow to cure for 24 hours in fume cabinet before removing from mould.
10. Ease the embedded otoliths from the mould, loosen by bending mould, pressing gently from behind and pull out by number tag.

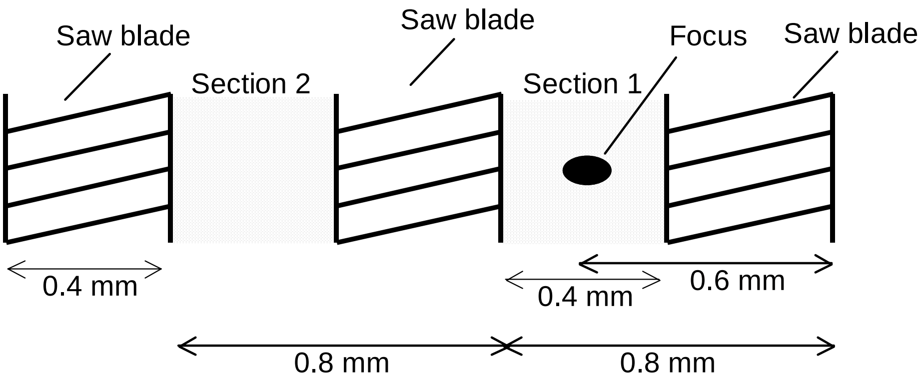
Production Time: Using this method you can pour approximately 7-10 moulds per 7 hour day or 105-150 otoliths can be embedded per day.

Appendix E - Sectioning otoliths

Cut two 0.4 mm transverse sections per otolith, Appendix E Figure 1 and Appendix E Figure 2 are diagrammatic representations of the process.



Appendix E Figure 1 Orientation of Spanish mackerel and barramundi otoliths held in the wafering saw chuck

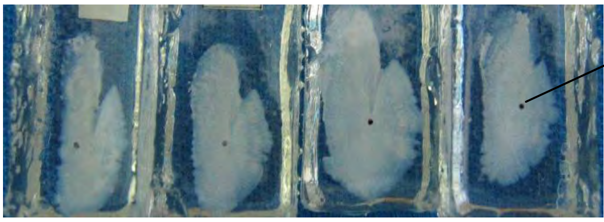


Appendix E Figure 2 Diagrammatic representation of cutting the two otolith transverse sections.

Materials list:

- Buehler® Isomet or Struers® Saw
- Dishwashing detergent
- Microscope slides - glass
- Permanent fine felt tip marker pen (e.g. Sanford® "Sharpie" extra fine point permanent marker #35000)
- Microscope slide labels
- Fingernail clippers
- Saw Allen key - 4mm CRV
- Stereomicroscope with transmitted light base (Wild® MZ3 or Nikon® SMZ10)
- Dial callipers, Stainless steel, 150mm, 0.05mm divisions. Mitutoyo® #505-633-50
- acid-free oil (e.g.WD-40® lubricant or similar)

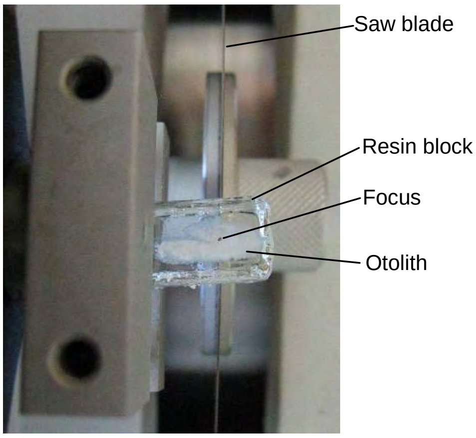
1. Mark focus of the otolith with a fine-tipped permanent pen, using a stereomicroscope and transmitted light source (Appendix E Figure 3).



Appendix E Figure 3 Embedded barramundi otoliths with the focus marked

2. Label slide with the fish number using permanent marker pen
3. Saw Settings and Using the Saw
   - Read saw instructions paying particular attention to safety, use and care
   - IMPORTANT: check that the saw blade will cut through the specimen block without the flange hitting the specimen holder.
   - When resuming cutting after a break of over a month, practise first on blank blocks and check section thickness with callipers.
   - If one of the sections cut is too thick (greater than 0.45 mm) or thin (less than 0.35 mm) then cut a third section, by repeating Step 12. Sections between 0.35 mm and 0.45 mm are acceptable.
   - If the section is thicker at one end and thinner at the other, discard the section, Remove the saw blade, wipe the blade and flange clean and turn the blade around. Ensure the blade is firmly clamped in flange to prevent excess wobble of blade as this will break of the blade. Cut a replacement section by repeating step 12.
   - Correct speed setting is 2 (rpm x 100)
   - Water reservoir. To tap water add ~3 ml detergent to ¾ fill reservoir (lubricant for saw blade)
   - Always check block number against slide label, especially when both saws are in use
   - Store saw blade wheel in Perspex® case when not in use
   - Dress saw blade with dressing stick and lubricate saw blade flanges with acid-free oil at end of each day's cutting
   - Before adjusting saw micrometer screw always align arm of chuck as low as possible without the blade touching.
   - The thickness of wheel blade is approx 0.4 mm (width cut away)
   - The counter weight setting for the Buehler Isomet saw is 229 grams and the specimen holder arm weight is 149 grams.
   - The counter weight setting for the Struers saw is fixed and the specimen holder arm setting is 150 units.

4. Securing resin block in chuck
   - Place the block in the bone saw chuck, flat surface on base and fish ID label facing up and out. (Appendix E Figure 4).
     Barramundi – through widest part of dorsal edge with the rostrum closest to chuck.
     Spanish mackerel – align ventral edge otolith at right angles to chuck with the rostrum furthest from chuck.
   - Orientate block to obtain true cross section (Appendix E Figure 1).
   - Trim resin if necessary with nail clippers so the block is held squarely and firmly in the chuck.



Appendix E Figure 4. Focus of otolith in resin block aligned to saw blade.

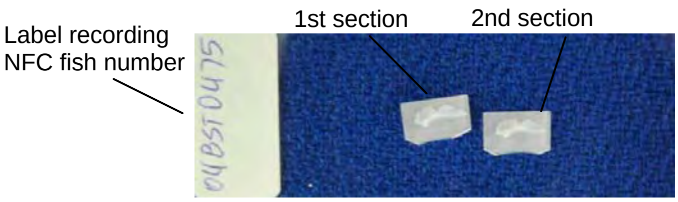
5. First cut - Line up the saw wheel with the black dot marking the focus (Appendix E Figure 4).
6. Tighten with Allen key (do not over-tighten as the block may snap off)
7. Lower the chuck until the resin block is above the top of diamond wheel without touching.
   - Note the number on the dial of the saw micrometer screw, paying particular attention to the line position (Appendix E Figure 5)
   - Always adjust the micrometer screw with the chuck aligned at the same height
   - Line position is dependent on the height of chuck arm above diamond wheel

Appendix E Figure 5. Saw micrometer screw adjusted to cut the otolith.

8. Adjust thickness by advancing the micrometer screw +0.6 mm (increasing numbers) - count up 6 divisions (0.6 mm). Refer to Appendix E Table 1 for settings for the Struers Saw & Buehler Isomet Saw.
9. Lift block holder until above wheel blade and press start. Lower the chuck slowly onto the moving blade
10. Cut block into 2 parts. Appendix E Figure 1 and Appendix E Figure 2 show diagrammatic representations of the two sections.
11. First section
   - Adjust thickness by retarding the micrometer screw by -0.8 mm (decreasing numbers) i.e. count 8 divisions (0.8 mm) (Appendix E Table 1)
   - Cut and check thickness with callipers – should be 0.4 mm (+/- 0.05 mm)
   - Trim the corners of the section with nail clippers to remove the slag. Rinse section in tap water, leave wet and place carefully on a microscope slide with sulcus acusticus facing uppermost.
   - The otolith section containing the focus (the first section) is positioned closest to the microscope slide label (Appendix E Figure 6)

12. Second section

- Cut a second section is cut in the same method as for the first section, by retarding the micrometer by a further –0.8 mm (8 divisions) (Appendix E Table 1)
- Cut, check and trim as per first section
- Second otolith section is positioned farthest from the slide label (Appendix E Figure 6).
- If the micrometer adjustments have been made correctly, the finishing dial number on the micrometer will be the same as the starting dial position (Appendix E Table 1)



Appendix E Figure 6. Position of the otolith sections on the microscope slide for barramundi fish number 04BST0475.

Production Time: One saw will cut approximately eight sections per hour, utilising both saws will allow approximately thirteen sections to be cut per hour

Appendix E Table 1. Buehler Isomet and Struers saw micrometer screw settings.

Action	Screw Adjustment	Buehler's Isomet ® Saw					Struers ® Saw									
Start	initial reading	0	10	20	30	40	0	10	20	30	40	50	60	70	80	90
First cut	advance 6 divisions (+0.6 mm)	40	0	10	20	30	60	70	80	90	0	10	20	30	40	50
Second Cut (to obtain first section)	retard 8 divisions (-0.8 mm)	20	30	40	0	10	80	90	0	10	20	30	40	50	60	70
Third Cut (to obtain second section)	retard 8 divisions (-0.8 mm)	0	10	20	30	40	0	10	20	30	40	50	60	70	80	90

Note: micrometer dial 1 unit = 0.01mm 1 division = 10 units = 0.1mm

Buehler Isomet® micrometer numbered 0 to 49 then becomes 0 again

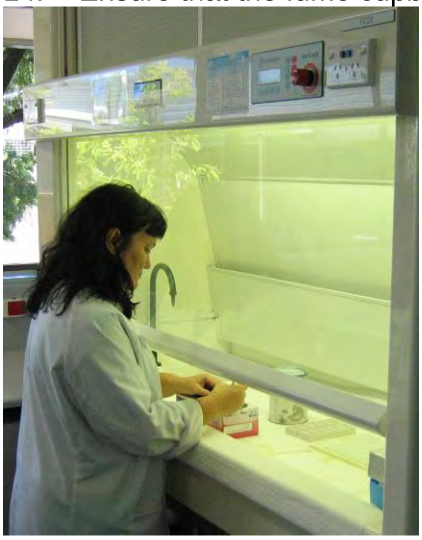
Stuers® Saw micrometer numbered 0 to 99 then becomes 0 again

Appendix F - Mounting Otoliths

Materials List

- Fume cupboard
- Latex gloves
- Lab coat
- Syringe - large (60 ml)
- Syringe – 1 ml with 21G needle
- Clear Casting Resin (1 L tin) (distributed by Dadson (07) 4053 4000)
- Resin – Crystic Polyester, DG 3 Flammable (distributed by Dadson (07) 4053 4000)
- Polyester Resin Catalyst MEKP (20 ml) DG 5.2 Organic peroxide
- Stirring rods
- Jewellers Forceps – very fine point
- Weighing trays
- 3 ml disposable plastic bulb pipettes
- Microscope coverslips – glass 22 x 22 mm and 22 x 32 mm

13. Read and understand the MSDS for the clear casting resin and polyester resin catalyst.
14. Ensure that the fume cupboard is operational (Appendix F Figure 1)

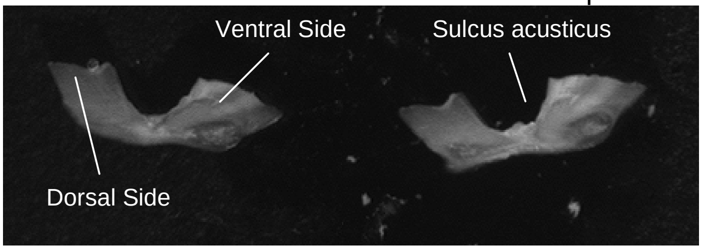


Appendix F Figure 1. Fume cupboard used when mounting the otolith sections in clear resin

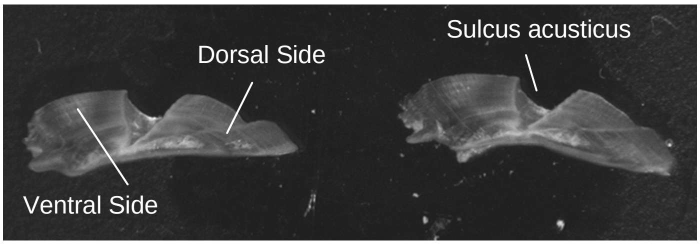
15. Prepare resin
    - 3 ml clear casting resin + 0.045 ml polyester resin catalyst. Use 3 ml graduated plastic transfer pipette - Reuse one pipette for resin only, keep separate from mixed resin.
    - Mix carefully in weighing tray using stirring rod. Prevent air bubbles forming by non removing the stirrer from the solution.
    - Squeeze the pipette bulb before placing in the solution. Fill the pipette with the resin mix.
    - 3 ml quantity will do approximately
      10 small sections on slides (coverslip 22 x 22 mm) or
      6 large sections (coverslip 22 x 32 mm)

16. Remove otolith sections from the microscope slide. Check that the otolith section has been trimmed with nail clippers so that section sits flat on slide.
    - Place the two sections on black cardboard; be careful to maintain the order of the sections - first section (focus) and second section (Appendix F Figure 2).

- Check each section is orientated correctly and ready to be positioned onto the slide. Orientate the sulcus acusticus upwards.



Section 1 **Spanish mackerel** Section 2



Section 1 **Barramundi** Section 2

Appendix F Figure 2. Position and orientation of first and second otolith sections on microscope slide

17. Clean the microscope slide with a damp tissue and dry
18. Place a small amount (1 drop) of resin onto the slide and carefully position the otolith sections
19. Cover the section otolith with more resin (1 drop) avoiding air bubbles where possible
20. Carefully lower coverslip into place. Push down gently on coverslip over section, on one edge first, to displace air bubbles.
21. Sit the microscope slide horizontally and allow the resin to spread evenly to the cover slip edges, the resin sets in 20-30 minutes.

Production Time: One-person can mount twenty otolith slides per hour.

Appendix G - NFC Fish Age data sheet

Page ……….. of ………..

Verify ……………………..

Checked ………………....

Queensland Government
Department of Primary Industries and Fisheries

Northern Fisheries Centre

Fish Age Data Sheet

Date Read: …………………… Year Fish Caught ………………… Reader: ……………………………	Read Number (circle): A B C	Read type (circle): Whole / Sectioned

Fish Number	Age	Readability	Comments

Readability Index: 0 – not rated; 1 – very low; 2 – low; 3 – medium; 4 – high; 5 – very high

Appendix H - Optimas® measurement recording

Optimas® outputs data into a Excel® spreadsheet called "Counts" and "distance". The fields appearing in the output are:

Counts output

Fish number: This is the number that the fish specimen was allocated. This field is called [Id Label] in Optimas®.

Side: This refers to the position in the fish's head from which the otolith is removed

L - Left side
R - right side
U - unknown.

Otolith type: This will always be the Sagittal (S). This field is recorded automatically by Optimas® but can be removed for importing into the central database.

Replicate: This is a character to signify the reading number.
A - first reading,
B - second reading,
C - third reading etc

Otolith radius: Radius of the otolith from the focus to the antirostral.

Total number of annuli: The total number of annual increments counted in the otolith.

Readability Index: The confidence that the reader has that he or she has counted the total number of annuli (annual increments) in the otolith (Appendix J).

Readers initials: The three initials of the person who read the otolith.

Date/time: The date and time the reading was made.
Recorded as day month date time(24 hour hh:mm:ss) year
e.g. Tue Nov 05 15:49:52 2002

Comments: Any comments relating to the otolith.

Distance Output

Fish number: This is the number that the fish specimen was allocated. This field is called [Specimen ID] in Optimas®.

Replicate: This is a character to signify the reading number.
A - first reading,
B - second reading,
C - third reading etc.

Annulus number: The number of the annulus starting from 1 in the centre to n at the outer edge.

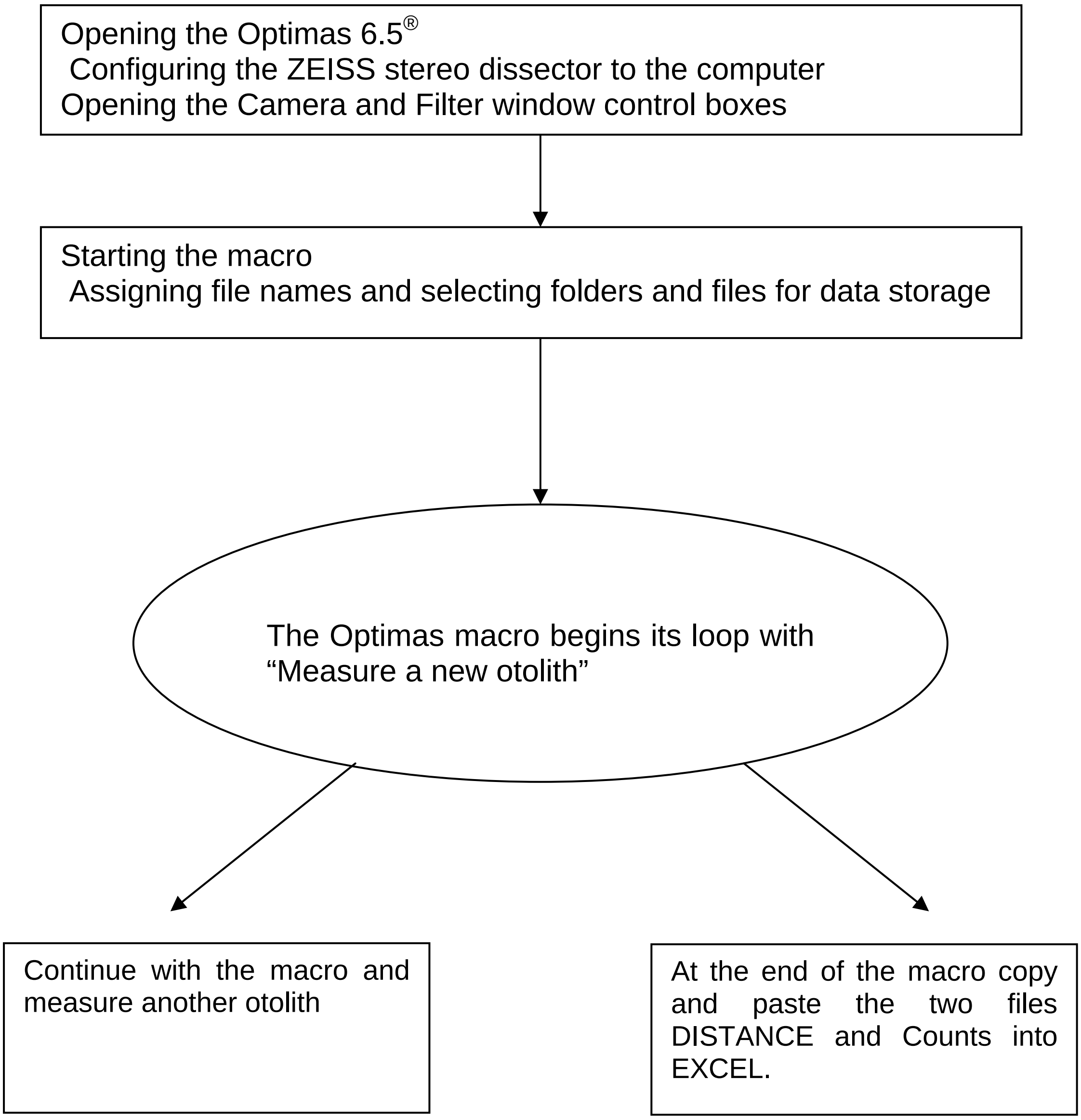
Annulus distance: The distance between the annulus and the focus in millimetres.

Proportional distance: The annulus distance divided by the radius of the otolith (as measured from the focus to the antirostral).

Appendix I - Optimas® program macro BarraAnnuli

This program macro is used to measure the radial distance from the focus to each annual increment of the otolith. The output is in two flies Distance and Counts. The Distance file records the radial distances from the focus to the annual increment. Counts records the description of the otolith, estimated age of the otolith, quality assurance of the estimate and comments. The macro saves an image of the otolith with the specimen label in the upper left corner. A flow diagram of the main steps is included as Appendix I Figure 1 and each of these steps are detailed below.

Flow Diagram for the Optimas® Macro BarraAnnuli



Appendix I Figure 1 Flow Diagram for the Optimas® Macro BarraAnnuli

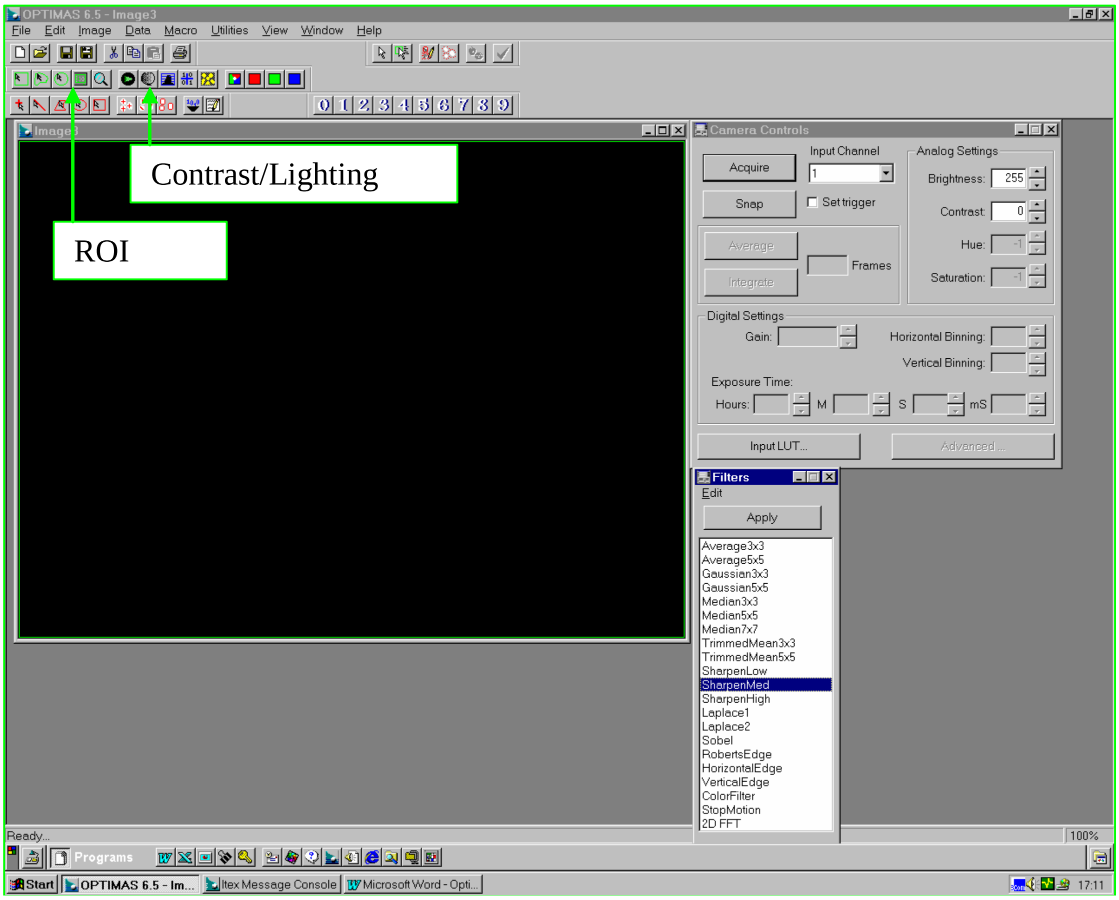
1. Opening and Initialising the Program
    - Open the program Optimas 6.5®
    - Configure the ZEISS® stereo dissector to the computer.
    - Load the configuration file by clicking on the File menu and selecting **OPEN CONFIGURATION**
    - The system will be looking in a Configuration file, change this to **Annuli** then select **OTCAL**
    - Opening the Camera and Filters
    - From the **Image** menu select **Acquire Camera**
    - Again from the **Image** menu select **Filters**
    - Opening the macro
    - From the **Macro** menu select **Open Macro**
    - Select **Optimas6.5\Annuli** look in all files and open the macro **BarraAnnuli**.
    - Once opened move the macro to the bottom of the screen for ease of operation

2. Starting the macro.

Assigning file names and selecting folders and files for data storage

- Run the Macro
  From the menu list in the Macro window choose **RUN!**
- Enter ROOT Name for 2 data files (5 letter maximum) e.g. 1231a.
  This will create two data files that the will be exported into Excel®. This does not work at this stage and so the files are called **DELME**. At the end of each ageing session these files should be deleted.
- Select Folder
  This selects the folder where the data is to be stored
  For e.g. The 2000 Barramundi data goes in
  e:\otolith\OTOLITH MEASURE\Barra\Whole_2000
- Select Source
  This allows choice from captured images and loaded images from disk
  Choose captured images
- Attention
  Save images to disk?
  Choose **YES**
- Select Folder
  Choose for e.g. e:\otolith\OTOLITH IMAGE\Barra\2000c
- Enter your initials
  Enter your **three initials** in Capitals

3. BarraAnnuli begins its loop Figure 4 shows a diagrammatic representation of the macro.
    - Attention
      Measure a new otolith?
      This is the beginning of the loop in the macro
      Choose **Yes**
    - Attention
      Press OK and Position the otolith under microscope
      Adjust contrast and focus
      Then click the Freeze icon (Red Box icon)
      Use the **contrast/lighting button** (Appendix I Figure 2) to alter the light levels and grey-scale by moving the mouse on the screen
      From the filters window select either **MEDIUMSHARPEN OR LIGHTSHARPEN**, then press APPLY. Ignore the Freeze icon as you are already within this option.
      Adjust the Region of Interest by pressing the **ROI icon button** (Appendix I Figure 2).



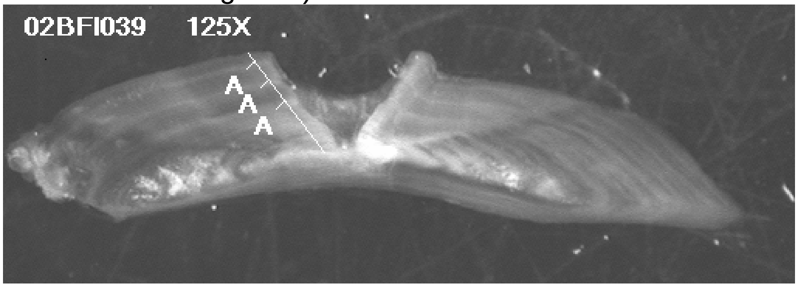
Appendix I Figure 2 Location of shortcut buttons within the Optimas® macro

- Enter ID label
  - The previous otolith was:
  - Enter the full label in capitals and numerals
  - e.g. The year 2000 Staaten River barramundi fish number 12. **00BST012**
- Enter the Letter for this replicate
  - e.g. **A,B,C**,….
  - This enters the otolith reading number
  - Enter this in capitals
- Which otolith?
  - Asteriscus
  - **Sagittae**
  - Lapillus
  - Right
  - Left
  - **Unknown**
  - A
  - B
  - This gives a brief description of the otolith
  - Choose Sagittae and Unknown
- Attention
  - The current calibration file is Default_Frame28cmWide
  - Do you want to change it?
  - Set the calibration to coincide with what the ZEISS® magnification is
  - For e.g. ZEISS_10 then click **SWITCH**
  - Note: This window has a time limit so be quick
- Attention

Draw line from core to edge

Choose OK, then left click on the focus and drag the line to the edge of the otolith and right click. The line should follow the same direction as the rostrum (Appendix I Figure 3)

- Attention
  - Correct Placement?
  - Choose Yes or No
- Attention
  - Press OK and Edit Flags
  - Add flags by depressing A and clicking Left mouse Button
  - Delete flags by SHIFT and clicking LMB
  - Click Right Mouse Button to Exit editing
  - The flags are marked on each check after the opaque zone (Appendix I Figure 3).



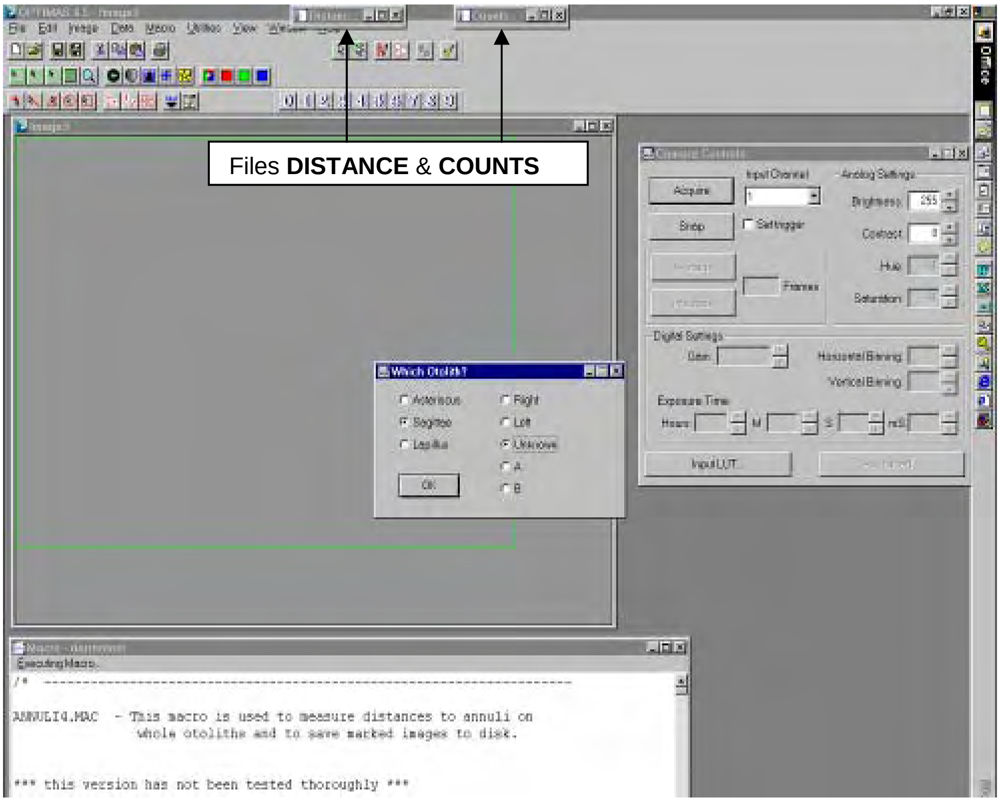
Appendix I Figure 3 Marking a line for the ageing axis and flagging the annual increments with the letter "A".

- Attention
  - Save this data?
  - Choose **Yes**
- Select confidence level (Appendix J)
  - VERY HIGH – all increments distinct
  - HIGH – most areas distinct
  - MEDIUM – some areas difficult
  - LOW – some guessing
  - VERY LOW – structural abnormality
  - NOT RATED
  - Choose the one which best fits your age estimate
- Enter comments (do not use commas)
  - Comment on the outer margin of the otolith
  - For e.g. translucent or opaque
- Attention
  - Draw box around otolith
  - Leave space in upper Left corner for label
  - Do this by left clicking in the left corner of the image and dragging the box open
- Attention
  - Correct region?
  - Choose Yes

4. BarraAnnuli ends loop

- Attention
  - Measure a new otolith?
  - This is the end of the loop
  - To end the macro choose NO to measure another otolith choose YES this will take you back to number 13 where you are asked to adjust focus and light levels.

- At the end of each ageing session the files DISTANCE and COUNTS need to be copied into Excel®. **Remember** to go back and delete the two DELME files in

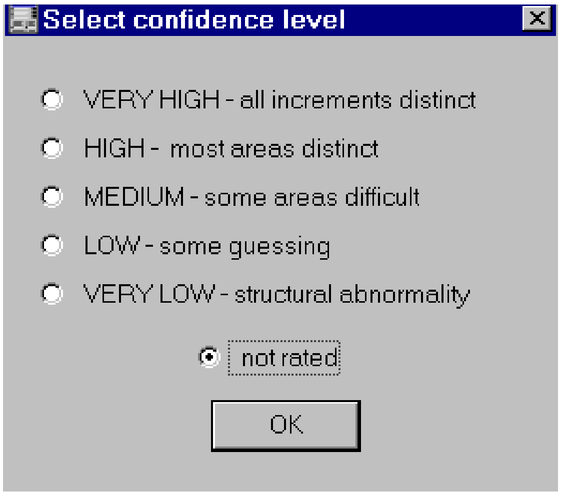


e:\otolith\otolithmeasure\Barra (Appendix I Figure 4).

Appendix I Figure 4 Locating the files for each ageing session on the screen display using Optimas®

Appendix J - Readability Index

The following criteria are used to score the readability of the otolith. This is recorded as a confidence level in Optimas® program (Appendix J Figure 1).



Appendix J Figure 1 Optimas® confidence level menu which rates the readability of the otolith

5) VERY HIGH

- All annual increments distinct
- Very confident estimate

4) HIGH

- Most areas distinct
- Annual increments are interpretable

3) MEDIUM

- Some areas confusing
- Annuli difficult to define in some areas
- Reasonable level of confidence of estimate
- In some regions along the ageing axis, annual increments may/must be estimated

2) LOW

- Very difficult to read
- Zones difficult to interpretable
- Could include minor calcium abnormalities
- Otolith condition translucent, opaque, chalky

1) VERY LOW

- Otolith with structural abnormalities
- Calcium abnormality
- Otolith data only used for radius analysis

0) NOT RATED

- Empty jar
- No label, or two labels the same
- Two otoliths from different fish in the same jar
- Broken, shattered otolith
- Otolith discarded from analysis