Science Series
Technical Report no.143

Improved methods for the preparation and staining of thin sections of fish otoliths for age determination

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Cefas

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1. Introduction

The management of a wide variety of fish stocks is dependent on knowing the age composition and growth rate of individual year classes in the fishery. In some species, age can be determined directly from length frequency distributions (Jones, 1984), but more often it is obtained by counting the annual growth rings on scales, otoliths or other hard structures (Blacker, 1974). In many early studies, age was estimated by counting the annual growth rings in whole otoliths. In many species, though, this can lead to an underestimate of the age, because rings near the edge of the otolith of older fish can be obscured. As a result, a range of techniques has since been developed for improving the reliability of reading annual rings. Christensen (1964) showed that breaking and burning could enhance the contrast between the annual growth rings for a number of flatfish species, and staining has since been found to give similar results (Albrechtsen, 1968; Bouain and Siau, 1988; Richter and McDermott, 1990). In most species, examination of the ring structure can be facilitated using thin sections of otolith (Wiedemann Smith, 1968; Bedford, 1975, 1983). Here, we describe a number of modifications to the original method developed by Bedford (1983) that improve the accuracy, speed and quality of the sectioning processes.

2. Materials and methods

2.1 Preparation of the base

The moulds used for processing otoliths are similar to those described by Bedford (1983). Five pieces of aluminium 12.5 mm (3/8 inch) square are fixed to a base plate 5 mm thick using stainless steel screws, to form two rectangular moulds that take either five or six rows of otoliths (Figure 1).

The top face of each of the three shorter lengths of aluminium making up the sides of the mould has five or six accurately machined slots (0.5 mm wide × 1 mm deep) cut into them to indicate where the otoliths are to be embedded. The accuracy of these side slots is essential for accurate embedding and cutting of the otoliths. The top section has a groove running lengthways which is parallel to the slots in the sides. This is used to align the mould accurately in the jig before the otoliths are mounted. The three side sections and the bottom section are fixed permanently with pins. To remove the blocks from the mould, only the remaining unpinned side needs to be removed. The inside edges of the side sections are sloped to allow easy removal of the resin block. This saves time, maintains the accuracy of the rows, and extends the life of the mould. In order to achieve the high level of precision needed for small otoliths, it is crucial that the mould be manufactured accurately. It must be completely square and the alignment groove must be exactly parallel to the inside edge of the mould and the grooves on the side of the mould.

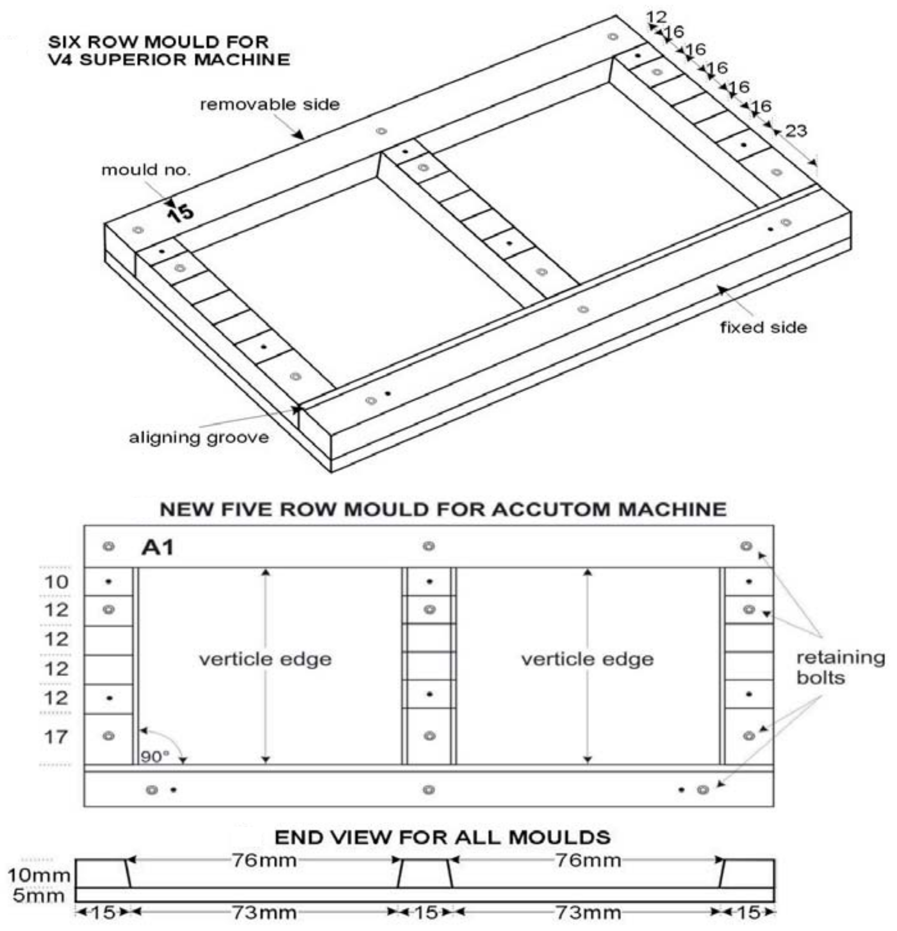

Figure 1. Schematic diagram of the moulds used in otolith processing.

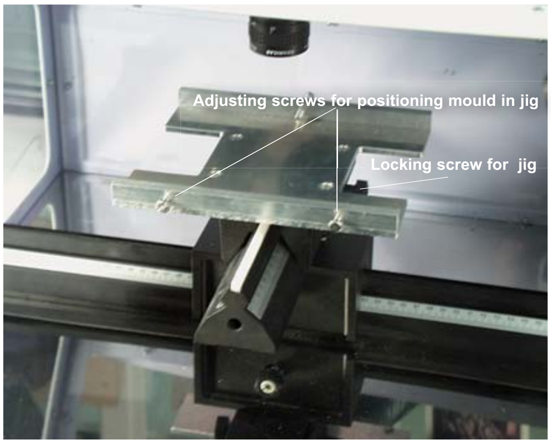

Figure 2. Mounting unit attached to adjustable X-Y jig which allows precise location of the mould under the video camera lens.

Figure 3. View of the mounting box showing the top mounted video camera, extractor vent to remove resin fumes and an alternative X-Y jig.

2.2 Mounting the otoliths

In order to facilitate the accurate and rapid alignment of otoliths the mould is located on an adjustable mounting unit (Figure 2).

This system allows precise transverse and lateral movement of the mould. The mounting unit is enclosed in an open-fronted box constructed from a metal frame with perspex sides. An extractor system fitted to the rear of the box removes the resin fumes to the outside. Otoliths are mounted and aligned in the mould using a video camera and monitor (Figure 3). The monitor has two horizontal lines 2 cm long and spaced 1.5 mm apart drawn onto the centre of the screen for positioning the otoliths. The top line is used for lining up the groove for each row on the side of the mould, and the bottom line for positioning the otoliths. The gap of 1.5 mm between the lines allows for half the thickness of the cutting blade (magnified onto the screen). Once each row is lined up, a locking screw ensures that the lateral movement remains constant as otoliths are placed along the row. Prior to mounting, a thin layer of resin is applied along the row where the otoliths are to be placed. Each otolith is carefully positioned in the resin with its long axis lying at right angles to the line of the cut, resulting in a transverse section.

Once an otolith has been correctly positioned, the block can be moved laterally using the adjustable platform without moving it out of alignment and a second otolith put in place. This process is continued until a line of otoliths extends across the block, each with their centre positioned precisely between the grooves on the mould. Depending on size of the otoliths, up to 25 can be mounted in a single row. Moulds with full and partially completed rows are shown in Figure 4. The use of the video camera and monitor minimizes parallax problems that can occur if an operator tries to align the otoliths by eye, but it is still important that the operator be seated so that his or her eyes are at the same height as the lines on the monitor, or additional parallax errors can be introduced.

Otoliths from roundfish such as cod (*Gadus morhua*), haddock (*Melanogrammus aeglefinus*), whiting (*Merlangius merlangus*) and saithe (*Pollachus virens*) are mounted with the convex side uppermost, so that the processor can

Figure 4. Otoliths of cod (left) and plaice (right) being added to moulds. The top right mould shows a completed mould after the top layer of black resin has been added over the otoliths.

see the mark of the *sulcus acousticus* on the otolith. A small mark with a pencil is usually made along the sulcus to make the central ridge clearer when viewed on the monitor screen. The ridge is aligned along the line of cut, so ensuring that all sections pass through the centre of the nucleus. Roundfish otoliths are also positioned with the anterior end uppermost, so that the section is taken towards the posterior end of the otolith.

Otoliths of flatfish such as plaice (*Pleuronectes platessa*) and sole (*Solea solea*) are placed concave side uppermost. This allows the structure of the first annual ring to be seen on all except the thickest otoliths. A drop of water placed onto the otolith facilitates observation of the nucleus of the thicker otoliths more clearly. If both otoliths are available, the asymmetrical otolith is used and positioned in the mould with the anterior end uppermost.

When all the rows of otoliths have been positioned and the thin line of resin holding them has set, the mould is placed in a fume cupboard onto a flat platform fitted with adjustable feet and a spirit level to ensure that it is level, before filling with resin. Polyester resin pre-mixed with a black pigment is mixed with the hardener and poured into the mould until it is full. Tapping and tilting the mould gently while pouring the resin ensures that no air bubbles are trapped under the otoliths. Two mixes of resin are used: one containing a waxy ingredient ("tack-free"), and one without. The resin mix used for the base coat and for positioning the otoliths does not contain the tack-free ingredient. This helps the two layers of resin bond. The mix for filling the moulds after the otoliths have been positioned contains the tack-free ingredient. This leaves a waxy finish to the surface of the mould, which makes it easier to handle and facilitates scribing the guidelines for cutting. When completely cured, a fine line is drawn across the resin surface between each pair of slots on the sides of the mould, to indicate the centre of the otolith row. A special scribing tool is used for this (Figure 5). The accuracy of the cutting depends on these lines, and they are checked carefully for alignment. The blocks are then removed from the moulds and are ready for sectioning.

2.3 Cutting

The otoliths are cut using either converted surface grinders fitted with an electroplated cutting blade (V4 "Superior", made by Beacon Machine Tools) or using a variable-speed precision cutting machine (Struers Accutom 50) fitted with a metal-bonded diamond blade of 125 mm diameter. The Beacon is faster to use, but the metal-bonded blade on the Accutom produces a slightly smoother finish which is preferred for staining. A key feature of both machines is the digital micrometer controls that allow precise cuts to a resolution of 0.01 mm. In order to take a thin slice, the blade is lined up with the first line on the block and a first cut is taken. The block is then moved and a second cut taken to produce a slice of the required thickness (Figure 6). For most sections, the minimum cut thickness needs to be >0.3 mm to maintain sufficient strength in the slice and to prevent the otolith from breaking. For gadoids and other species, for which the sections are subsequently mounted onto glass slides, the sections are cut 0.45–0.5 mm thick. Sections that are not mounted onto slides are cut 0.5–0.6 mm thick to produce a slightly stronger section. The two methods between them provide the ability to cut a wide range of otolith types and to vary the thickness of the cut to optimize age-reading requirements.

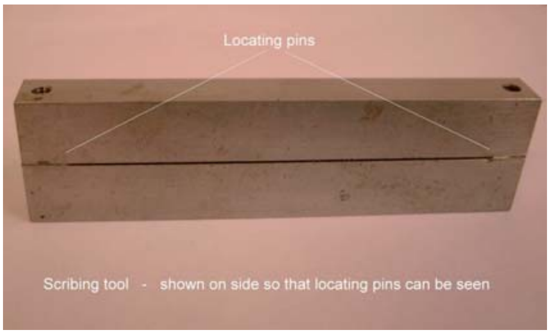

Figure 5. Side view of scribing tool showing the locating pins which fit into the machined slots on the side of the moulds.

Figure 6. Cutting thin sections from the resin block using the Beacon cutter.

2.4 Mounting on slides

Otoliths of most species can be read directly from thin sections, although a better image can be obtained by embedding the sections in clear resin. Covering with a glass cover slip further protects the surface from scratching and dust. As each resin strip is removed from the cutting blade, it is placed onto a standard glass slide 76 × 51 mm in size, which has been labelled with the relevant details of the otoliths. A thin layer of UV-stabilized clear resin is poured over the strips and a glass cover slip carefully placed on the surface of the resin, taking care not to trap air bubbles under the glass. Each slide can take up to four strips and, when set, the resin forms a permanent mount which remains transparent to light and does not discolour with age (Figure 7). When the cover slips are positioned, a purpose-built, level mounting platform is used. This consists of a perspex tray containing 16 raised blocks that are slightly smaller than the glass slides. Slides are placed on the blocks and any excess resin drips into the tray, which has been lightly waxed so that dried resin can be removed easily.

Figure 7. Examples of completed sections embedded in clear resin on glass slides

2.5 Staining

The use of histological stains together with sectioning increases the contrast between the translucent and opaque zones in a number of fish species (Richter and McDermott, 1990). The method has been adapted and used successfully to stain material from sole, turbot (*Scopthalmus maximus*), brill (*Scopthalmus rhombus*), lemon sole (*Microstomus kitt*), dab (*Limanda limanda*), gurnard (*Triglidae* spp.), flounder (*Platichthys flesus*), yellowtail flounder (*Limanda ferruginea*), thickback sole (*Microchirus variegatus*), sand sole (*Pegusa lascaris*), sollenette (*Buglossidium luteum*) and sea bass (*Dicentrarchus labrax*).

The resin strips containing the sectioned otoliths are removed from the cutter, and a graphite pencil is used to label the sample information directly onto the black resin surface. Each strip is immersed in 100 ml of a solution of neutral red stain acidified with 0.5% ethanoic (glacial acetic) acid and 1 g of

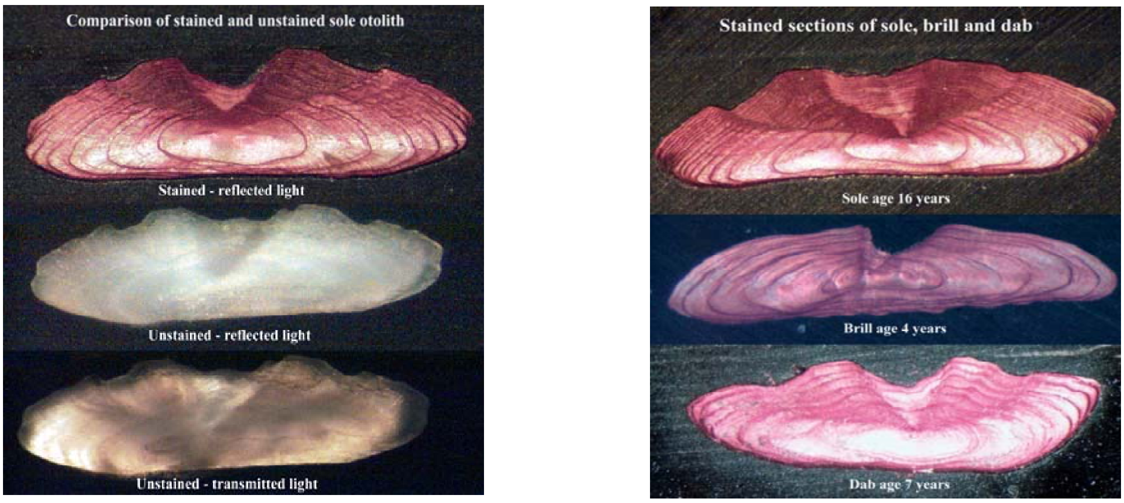

Figure 8. Left panel: Comparison between a sole otolith section stained to show annual rings and unstained sections under reflected and transmitted light. Right panel: Stained sections of sole, brill and dab otoliths.

sodium chloride for 30 minutes. The effect of the acidification is to cause mild de-calcification of the surface of the otolith, with the result that the protein bands in the annuli are slightly exposed and take up the stain more readily. Figure 8 shows a comparison between stained and unstained otoliths of sole. Following staining, the strips are rinsed in tap water to remove excess stain, dried rapidly in a current of air and stored dry in plastic wallets until required. The slides are mainly examined dry, but water is brushed onto the surface of some otoliths to aid interpretation of the ring structure.

3 Assessing the accuracy of sectioning

The main difficulty in sectioning small otoliths is the possibility that the cut has not been taken through the centre of the nucleus. This problem is reduced by positioning the otoliths concave side down and with the wider (anterior) end uppermost, so that progressive cuts are made toward the posterior (pointed) end. The sulcus mark is pencilled in so that it can be seen easily, and the otoliths are positioned so that the blade passes exactly through the "V" of the sulcus. Otoliths are positioned vertically (see Figure 4) to maintain a precise transverse section through the otolith. The effect of missing the centre could be to miss the first annual ring, resulting in misinterpretation of the age. The techniques described above are designed to minimize this happening. In particular, precision-made moulds and the use of the video camera positioned in each mounting cabinet ensures that each otolith is mounted precisely along the line of cut. Precision is also maintained by lining each resin block directly under the centre of the saw blade.

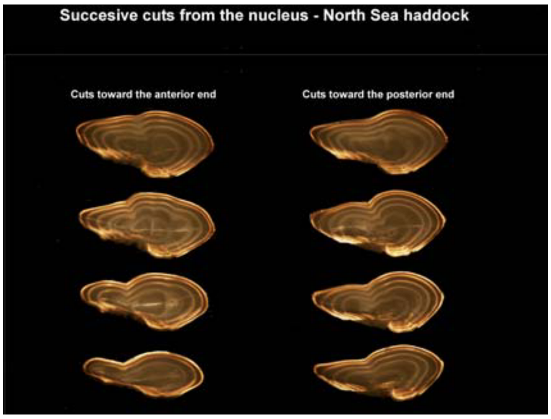

Figure 9. The effect of progressively cutting away from the nucleus is illustrated with North Sea haddock.

After cutting, all otoliths are checked for an unusual nucleus shape that would indicate cutting off-centre. The effect of cutting progressively off-centre, for a species such as haddock, is shown in Figure 9. The top otoliths in the figure are the first cut through the centre. As the cut moves away from the nucleus, the central opaque and hyaline rings become increasingly smaller, until the first annual ring is lost altogether. The first ring often assumes a characteristic figure-of-eight shape in the centre when the cut is not through the nucleus. In the case of haddock and other gadoids, if cuts are taken posteriorly, up to four off-centre cuts are possible before the central rings are lost, compared with only two if the cuts are made anteriorly.

Flatfish otoliths are placed with the sulcus side against the resin. The nucleus can be seen more clearly that way up, and they are positioned so that the bottom line on the monitor passes directly through the nucleus. The asymmetrical otolith should be positioned with the anterior part uppermost. If the symmetrical otolith is used, it can be positioned either way up as long as a transverse section is taken.

4. Comparison between ages determined from whole, burnt and sectioned plaice otoliths

In order to ensure that sectioned otoliths provide an effective means of ageing otoliths, a comparison was made between ages determined for 113 North Sea plaice that were aged using three widely used techniques. The otoliths were initially aged whole in a black glass dish using reflected light, then one otolith of each pair was broken and burnt while the second was sectioned and mounted on a slide in clear resin. All otoliths were read independently by four experienced readers. The ages are pooled and the results are shown in Table 1 and Figure 10. The percentage agreement was high for all three methods. The sectioned otoliths had the closest agreement across all ages, whereas the whole otoliths appeared to be underestimating the ages of fish older than 8 years.

Table 1. Comparison between the percentage agreement, bias and precision estimated as the coefficient of variation (CV) between four experienced readers using three different methods.

Method	Number of fish	Percentage agreement	Relative bias	CV(%)
Whole	113	87.6	0.00	5.8
Burnt	113	92.9	–0.04	1.2
Sectioned	113	95.6	0.01	4.4

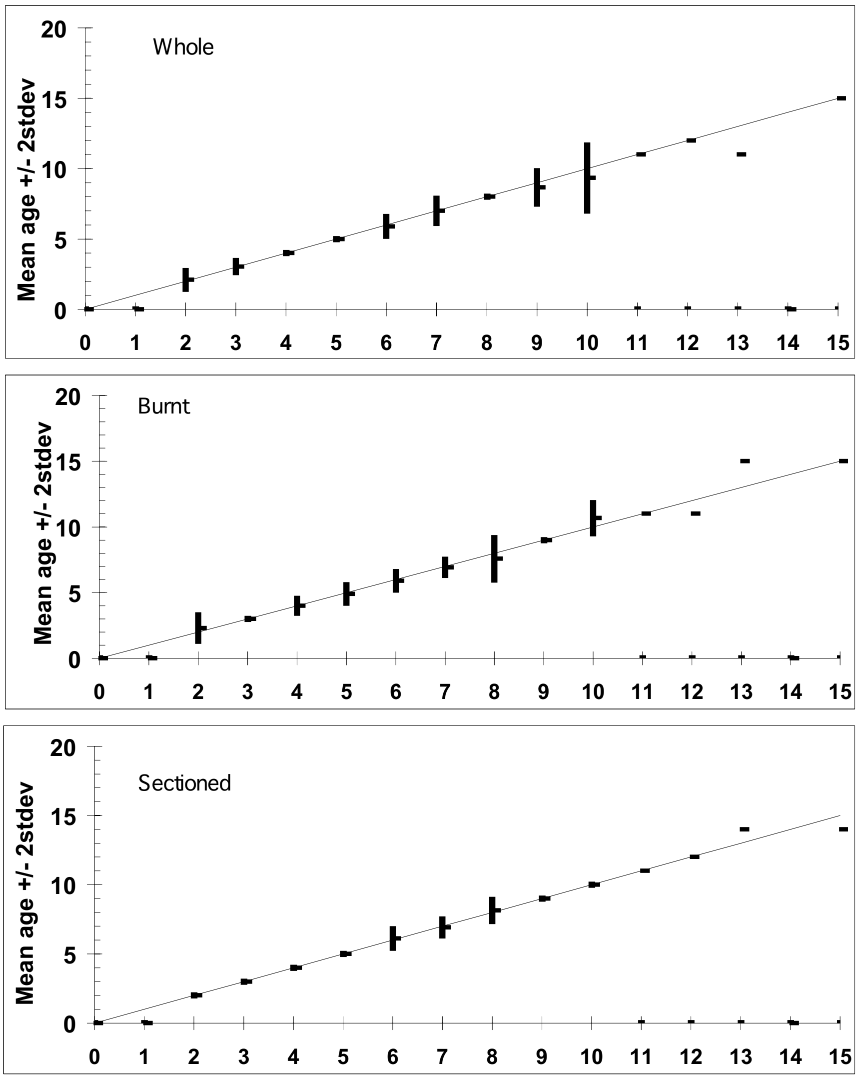

Figure 10. Comparison of percentage agreement for three ageing methods using plaice. Bars are +/- 2 standard deviations. All methods give consistent results up to age 8. Whole otoliths appear to under-age the older fish.

5. Conclusion

The methods described above have been used routinely to section up to 50 000 otoliths annually at Cefas. The main advantages over previously described procedures are the increased speed and precision of the mounting and cutting process.

Thin sections of otoliths mounted in black resin offer a number of advantages over the traditional methods of ageing. The contrast between opaque and translucent zones is greatly improved, and transmitted or reflected light can be utilized to view each otolith. In particular, reflected light allows exact identification of the ring structure on any part of the otolith. Although these sections take a considerable time to prepare, once produced, an experienced reader of otoliths can age many more otoliths than was previously possible, and with more confidence because of the increased clarity of the zones. In flatfish species such as sole, a combination of thin sections together with staining significantly enhances the ring pattern, improving reading speed and precision and providing a permanent record for training or further biological analysis, such as back-calculation of growth. All this would be more difficult and/ or impossible using the traditional burning methods.

The staining techniques have been applied successfully at Cefas to a wide range of species and used in routine age reading with a high degree of precision.

A comparison of ages obtained using sections together with reading the otolith whole or broken and burnt showed that, in plaice, sectioning resulted in no loss of information compared with the other methods and provided significant gains in speed of reading, training and quality control compared with traditional methods.

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