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Netherlands Institute for Fisheries Research

Development of a prototype Otolith Line for routine age reading of demersal species (OTOLIN) (Final report)

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rivo-dlo

REPORTC027/97
April 1997

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RIVO-DLO FINAL report

Number: C027/97

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Commissioned by: European Commission
DG XIV-Directorate General Fisheries
Unit XIV-C-1
Rue de la Loi 200
BATIMENT J II 99 6/27
B-1049 BRUSSELS, Belgium

Project number: 75511-12

Contract number: 94/116

Approved by: Dr. H.J.L. Heessen
Deputy Head of the Department

Signature:

Date: April 1997

Number of copies: 6
Number of pages: 17
Number of tables: -
Number of figures: 12
Number of annexes: -

"this report does not necessarily reflect the views of the European Commission and in no way anticipates any future opinion of the Commission.

This study has been carried out with the financial assistance of the European Commission".

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Summary

This EU funded study is aimed at the development of an otolith processing line at RIVO-DLO, including a survey of operational systems existing in other laboratories, the development of appropriate equipment and the development of working procedures, which assist in routine age reading of demersal fish species. In the procedure developed, otoliths are embedded in polyester and sectioned through the nucleus with a high precision saw. The rings in the sectioned otoliths are made visible under a microscope or binocular using reflecting or transmitted (polarised) light and interpreted for age determination. The developed technique at RIVO-DLO is a modified system from the one which is in use by Fishery Laboratory Lowestoft. The system has been tested with cod, whiting and plaice otoliths and is implemented for routine age determination for cod and whiting since the beginning of 1997.

Non specialist summary

An otolith is a small calcareous ear stone in bony fish. It mainly consist of calcium carbonate. Each bony fish has two otoliths. These ear stones grow when the fish get older and larger. In the otoliths rings are visible which correspond with changes in growth. In temperate zones the major rings in the otolith correspond with years (like the rings in the stem of a tree). Fishery scientists use these ring patterns for age determination of the fish. Information on the age composition of fish catches is important in studies on the dynamics of fish populations and in growth studies. This report describes the development of a routine method for processing otoliths by RIVO-DLO, which prepares them for age determination. By this method, otolith samples are embedded in black polyester resin. With a high precision diamond saw, sections are cut from the polyester through the nucleus of the otolith. Under a microscope the ring structure of the cut otoliths, is made visible for age determination.

1. Background

Age reading is one of the essential routine activities in biological fishery research. The age of individual specimens of the majority of fish species is determined by counting the annual growth rings in the otoliths. Before this can be done reliably and efficiently, the otolith may need to undergo some kind of preparation. Also different methods of preparation are in use for different species. For some species, the otolith is broken and burnt (sole, turbot, brill, dab, flounder), others are only broken (cod, whiting, haddock) or are read as whole (plaice). However, the choice for using one method over another has often been based on practicalities and not on optimisation criteria.

The procedures applied historically have a number of severe draw backs. These include:

- 1) The process of breaking and burning leads to variable results and may destroy the otoliths for other types of research than just counting rings;
- 2) Age reading is still based on a subjective interpretation of the pattern of rings and the procedures applied do not allow automation of the process if not rigidly standardised. Even cross checks with other readers may be difficult, because routine

methods applied by different readers are often not comparable. Age reading has the character of an art, that can only be performed by experts, and not of a scientific method that can be teached and learned;

- 3) Different methods of preparation may introduce systematic differences. For instance a comparison of the age determination of plaice and dab using the "break and burn" method instead of entire otoliths indicates an underestimate of the age by the latter, in particular for older animals;
- 4) The preparation process is often laborious;
- 5) Revision of the age reading of previously collected data sets, when new insights become available, is virtually impossible because the present procedure is very laborious.

2. Objectives

The ultimate objective of this study is the *implementation of a standardised age reading* procedure based on the development, within the framework of an EU funded contract (reference 94/116), of an otolith processing line at RIVO-DLO with a view of making the collected otolith material permanently available for different types of analysis. This line should include a facility for collecting information for image analysis.

It is envisaged that the otolith line will lead to an improvement in speed, consistency and accuracy of the age reading process, but also will facilitate the implementation of automatic age reading procedures as recently developed at RIVO-DLO and partly financed by the EU (Welleman & Storbeck, 1995, FAR MA 1.45). This project only deals with setting up the procedures, installation of a prototype of the line and developing appropriate logistics. It does not include the routine processing of otoliths. After the prototype has been successfully completed, routine processing should be gradually introduced for all species.

3. Methods and material

The work consisted of two phases:

- 1. Definition of the appropriate methodical aspects.
- 2. Implementation (construction) of a prototype otolith line.

3.1 Definition of the appropriate methodical aspects

This phase can be divided in two sub-phases. During the first sub-phase a general orientation and evaluation was carried out of methods of age reading and otolith preparation applied in fishery research institutes abroad. The evaluation was followed by some experiments in which various sectioning methods and configurations have been tested.

3.1.1 Orientation

After making inquiries in various laboratories in Europe about the procedures they apply to handle otoliths it became clear that, at present, the most advanced system has been developed by CEFAS (Lowestoft). In the Lowestoft procedure samples of otoliths are embedded in (coloured) polyester resin positioned in rows in specially prepared moulds. The embedded otoliths are cut with a high speed diamond grinding disc. A section through the centre of the otoliths is taken and kept for age reading. In some occasions further processing, such as polishing or colouring, of this section is required .

A team of two RIVO technicians has visited Lowestoft in October 1995 and was informed in detail about the procedures applied, the material used and the problems experienced. Also a demonstration of the otolith preparation was given which was taped on video. The "Lowestoft method" is described in Bedford, 1983. About 90% of the otoliths processed in Lowestoft by the sectioning technique were roundfish otoliths. The other 10% were sole otoliths, which were stained afterwards with Neutral red to make the ring structure in the otolith visible. The traditional technique of burning sole otoliths could not be applied because it makes the otolith too crisp for further treatment. Each year 25 000 otoliths are prepared, using a labour capacity of about 1.5 man year.

In Lowestoft technicians have subsequently begun to cut plane otoliths routinely and these are embedded in clear resin and read without staining.

3.1.2 RIVO tests

It was decided to aim for a stepwise implementation of a system similar to the one used in Lowestoft. However, the surface quality of the otolith sections obtained by the Lowestoft procedure was not considered to be smooth enough to use these sections for image analyses in the future. Therefore, tests were carried out at the end of 1995, using a high precision diamond grinding machine, the CUTO1, purchased for this purpose.

The sections made with the CUTO1 with a fine cutting wheel were of good quality. However the machine was too slow to be applied for routine purposes. In order to improve the capacity of the machine, tests were carried using various cutting wheels. The capacity of the grinding machine improved, but again the quality of the sections was not considered good enough for future image analyses. In particular, the cutting surface of the sections was rather course and would require further polishing to make the ring structures better visible. Therefore, enquiries were made on the market about available high precision saws with a better performance.

3.2 Construction of a prototype otolith line

This section gives a short technical description of the equipment purchased and developed to section otoliths and the procedures developed for otolith sectioning using this equipment.

3.2.1. Material

3.2.1.1. high precision grinding machine

After a thorough orientation, a LABCUT 230 grinding machine was purchased. This commercial machine is manufactured by D.R. Bennet LTD, 105A Barkby Road, Leicester LE5 7LG UK. The LABCUT 230 is adapted with a coolant recirculating system (water + anti-oxidant) and a hydrocheck cutting disc pressure regulator. The cutting area is protected by a polyester cover with perspex windows and the cutting disc is only operative when the cover is closed. The work speed is 3600 rpm.
The grinding disc used for sectioning in the LABCUT 230 is an EXTEC diamond wavering blade. The grinding disc is mounted to the machine (see Figure 1) with flanges and secured by a nut. The specifications of the disc are: high concentration; size 154 mm in diameter; 12.5 mm bore; 0.53 mm. thick; reorder number. XL 12215.

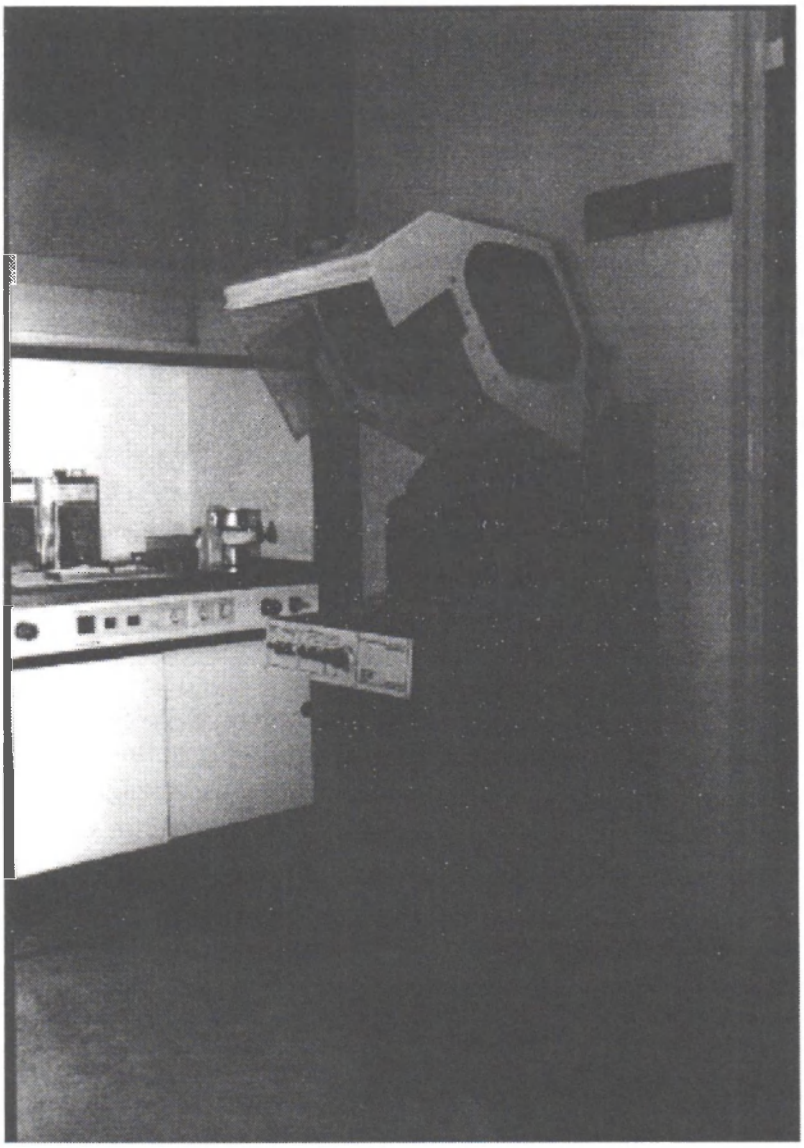


Figure 1. High precision grinding machine.

3.2.1.2. sectioning table

The polyester blocks with embedded otoliths are mounted under the high precision cutting disc in a sectioning table which fixes the position of the block by a jig (see Figures 2 and 3). This table and jig, developed by RIVO-DLO provide a very simple working routine and a constant thickness of the sections of 0.49 mm. The firm basing of the polyester block on the sectioning table avoids vibrations. This results in a very smooth surface of the sections.

3.2.1.3 moulds

It is necessary that the moulds, which are used to prepare the polyester blocks with otoliths, can be disassembled in order to release the polyester blocks. The moulds represent a modification of those used in Lowestoft. These modifications were necessary because of the different sectioning table.

In each mould two polyester blocks with otoliths can be prepared. The moulds are made of a base plate 180x170x6 mm, two longitudinal strips 180x12x12 mm, three lateral strips 146x12x12 mm. The left and middle lateral strips measure on the bottom 10 mm, as they have a bevel right side of 10^{o}. Across the top of the lateral strips 6 precisely spaced slots are cut. These are parallel to each other and to the longitudinal strips. Each slot is 0.4 mm wide and cut to a depth of 1 mm. The spacing of the slots is 12 mm from the longitudinal strip on top, the next five slots are equally spaced at 16 mm intervals. The material used for the base and strips is aluminium, the screws to fix the strips are made of stainless steel. A total of 10 identical moulds have been constructed. The components of the moulds are exchangeable between all moulds and assembly of each part is only possible in one position.

3.2.1.4 X-Y table with video display

An X-Y table with a fixed video camera above it has been constructed to position the otoliths in a prepared mould. This table allows to change the position of the mould on the X-Y table in lateral and longitudinal direction.

The positioning is done with the help of a video camera, projecting the magnified otoliths on a monitor. A line is fitted in the lens of the camera for positioning the otoliths.Having the line in the camera has the advantage that the projection of the line on the screen of the monitor is free of parallax .

3.2.1.5 levelling table

A transportable small levelling table has been constructed,. A level surface is desirable, when preparing the layers of polyester in the moulds in order to have a smooth surfaces with constant thickness. The levelling table is also used for gluing the polyester sections with otoliths on a glass microscope slide.

3.2.1.6. Resins

Resin for the fist layer: A casting resin, resulting in a slightly sticky surface. The resin is stained with 5 % black dye and 0.8% hardener is added.

Positioning "glue": As mounting medium for the otoliths some casting resin, stained with 5 % black dye (hardener 0.6% instead of 0.8%) is used.

Embedding layer: Laminate resin with paraffin is used for the top layer, resulting in a non sticky surface. The resin is stained with 5 % black dye and 1% hardener is added.

"Slide glue": As mounting medium for the section on a glass microscope slide some transparent casting resin, (hardener 0.6% instead of 0.8%) is used.

3.2.2. Methods

When preparing the polyester sections with embedded otoliths the following procedure is applied

3.2.2.1. preparing the moulds

When the mould is firmly assembled, the inner surface is slightly smeared with releasing grease. This makes it possible to remove the polyester block afterwards. The mould is placed on the levelling table and a first 3 mm thick layer of prepared resin for the first layer is poured in each half of the mould. A casting resin is used for this layer, resulting in a slightly sticky surface. When the resin is hardened the positioning of the otoliths can start. This stage can be prepared well in advance for further processing.

3.2.2.2. Positioning the otoliths

It is important to make sure that a sample number is well attached on the mould. When the mould is placed on the X-Y table underneath the camera we make sure that slots of the first row are in one line. Make sure that the line on the video screen goes through the centre of the slots, indicated on the longitudinal strip of the mould, by adjusting the X-Y table.

The positioning of the otoliths starts from left to right (Figure 4), The bevel right side of the left and middle lateral strip makes it always possible to see on what side is started. To mount the otoliths on the first polyester layer positioning "glue" is used. The otoliths are positioned with the centre of the nucleus on this line. In the case of very large otoliths, it may be necessary to break of the ends of the otolith to avoid overlap with the other slots. When all otoliths of the sample have been put in position, the samples are stored for at least 4 hours.

3.2.2.3. Finishing the moulds

In the next step the mould is placed on the level table again and is filled with a laminate resin containing paraffin (embedding layer). This has the advantage of a non sticky surface. When ready, the samples are stored for one night.

Lines are drawn on the polyester blocks with a special bar. The position of the lines is indicated by the slot marks on the longitudinal strip of the mould. These lines indicate the position of the centre of the nucleus of the otolith in the resin. The use of the bar ensures a parallax free line.

3.2.2.4. Sectioning the otoliths

By disassembling the moulds, the polyester block with otoliths are taken out and placed in the jig of the sectioning table under the cutting wheel of the grinding machine (see figure 2). The grinding disc is placed on the edge of the first line. The first cut is along the centre of the nucleus (Figure 5). The next cut, producing the actual section, is positioned by a brass matchblock. Each cut only takes a few seconds. All following sections taken from the polyester block are adjusted by this matchblock.

3.2.2.5. Final treatment of the otolith sections

All sections of the same sample are collected and glued on a glass microscope slide (50 * 76 mm) using "slide glue". This is also done on the levelling table. The sections might be covered by a thin cover glass but this is not necessary. A label, containing information of the sample (species, year, sample number) is attached. After hardening of the glue the "product" is ready and age determination can start.

4. Results

The result of the project is the establishment of an otolith processing line at RIVO-DLO as an instrument used for routine age determination of flatfish and roundfish otoliths. Sectioning experiments using the equipment and procedure described above have been carried out with cod, whiting and plaice otoliths in order to test the performance with regard to estimate the production capacity and the quality of the images of the otolith structure.

Preparing the moulds with a first layer of resin can be done well in advance and takes about 10 minutes per mould. Positioning of the otoliths is the most time consuming part of the whole routine. Positioning of a market sample cod (60 otoliths) takes 30 minutes, whiting (50 otoliths) 20 minutes, and plaice (60 otoliths) 20 minutes. Preparing of the positioning "glue" is included. The time necessary for sectioning of a block with embedded otoliths depends on the number of otolith rows in the block. One slide takes about 30 seconds sawing time. Adjusting, grinding etc. takes all together less than 10 min.

The actual preparation time of a standard otolith market sample, using the sectioning method is less than one hour including all the processing stages. The estimated additional time required for administration and storage is 0.5 hour.

The sections prepared with the system developed have a very smooth surface and a constant thickness of 0.49 mm and are considered suitable for age reading. The method applied, however, allows the production of even thinner sections if required. With very little modifications to the sectioning table sections of 0.39 mm were made.

The images of the otoliths can be inspected with direct reflected light (Figure 6) or with transmitted light (Figure 7) using a binocular microscope. In both cases the contrast between the annual rings in the otoliths is improved by wetting them with water or microscope conversion fluid. In general the best results were obtained using transmitted light. A promising result was achieved by viewing sectioned plaice otoliths with transmitted polarised light (Figure 8). Using polarised light has an advantage over extra time consuming techniques such as staining. The annual rings in these otoliths were very distinct and bright. Our most experienced readers were enthusiastic, because the annual rings were clearer compared to the old method, using transmitted light though the whole otolith. This finding is similar with those of the Lowestoft technicians, who have read the otoliths with both reflected and transmitted light.

With regard to roundfish otoliths, the new method makes less difference compared to the method traditionally used at RIVO-DLO. Using the old method, roundfish otoliths were broken and read using reflected light. Using the new method the "readability" of the cod otoliths has improved because the otoliths are sectioned now through the nucleus and the sections have a smooth surface (Figure 9). The "readability" with reflected and transmitted light (Figure 10) is about the same. Whiting appears to be more difficult to read compared to cod. The whiting otoliths also showed improved images, both with reflected (Figure 11) and transmitted light (Figure 12). Using transmitted polarised light did not improve the readability.

As a result of the new procedure, otoliths samples can be stored in an orderly system and are easily accessible for re-examination.

5. Deviations and modifications from the original work plan

It was originally envisaged to mount the otoliths in a non-toxic resin substrate, which hardens when exposed to blue light. This is, however, only possible with transparent resin. In our case staining of the resin is required. In those cases the traditional toxic resin has still to be used. A good air extraction system is required to keep the toxic fumes below the standard set for health regulations.

In the original project it was intended to develop the new otolith processing routine for plaice otoliths. In our communication with Lowestoft technicians is became clear that it is possible that, when using stained resin, this may mask the last (youngest) growth ring in the plaice otolith. This problem may be avoided by using clear resin. Therefore it was decided to postpone the use the new method for plaice otoliths and to focus on roundfish (cod and whiting) otoliths first. However, the results so far indicate that it will be possible to use the same system also for other species. It was considered desirable to do comparison experiments between the old en new method first, before implementing the new method for plaice.

6. Conclusions

Under this contract study a otolith processing system has been developed at RIVO-DLO. At this stage the system is ready to produce slides of sectioned otoliths which allow age reading by experts using a binocular microscope and in the future by image analysis. This line is operational since 1-1-97. From 1997 onwards, roundfish (cod and whiting) otoliths are being processed with the new method, because there appear to be no further complications. Personnel will be trained for these activities. An archiving system for the otolith slides, allowing for easy retrieval of individual samples, has still to be developed.

7. Follow up

Future developments at RIVO-DLO relating to the otolith line fall outside the scope of the contract.

It is the intention to apply the sectioning technique to plaice otoliths from 1998 onwards. In 1997, plaice otolith comparison experiments have been planned to investigate the consistency between age readings between the old and the new method.

Further tests will be carried out with otoliths of other commercial and non-commercial species as well, to find out whether the method is suitable for routine application. Also difficult types of otoliths - for instance horse mackerel and sole - will be tested.

It is envisaged that in the near future a semi-automatic transport system through a computer vision system will be developed allowing images to be taken of each otolith with standard orientation and for routine computer image analyses (Welleman & Storbeck, 1995).

8. References

Bedford, B.C. 1983. A method for preparing sections of large number of otoliths embedded in black polyester resin. J. Cons. int. Explor. Mer, 41: 4-12

Welleman, H.C., and F. Storbeck, 1995. Automatic Ageing of Plaice (*Pleuronectes platessa* L.) Otoliths by means of Image Analysis. *In* Recent developments in fish otolith research / David H. Secor, John M. Dean, Steven E. Campana, editors : Anne B. Miller, associate editor. 12 p. cm.-(Belle W. Baruch library in marine science ; no. 19)

9. Figures

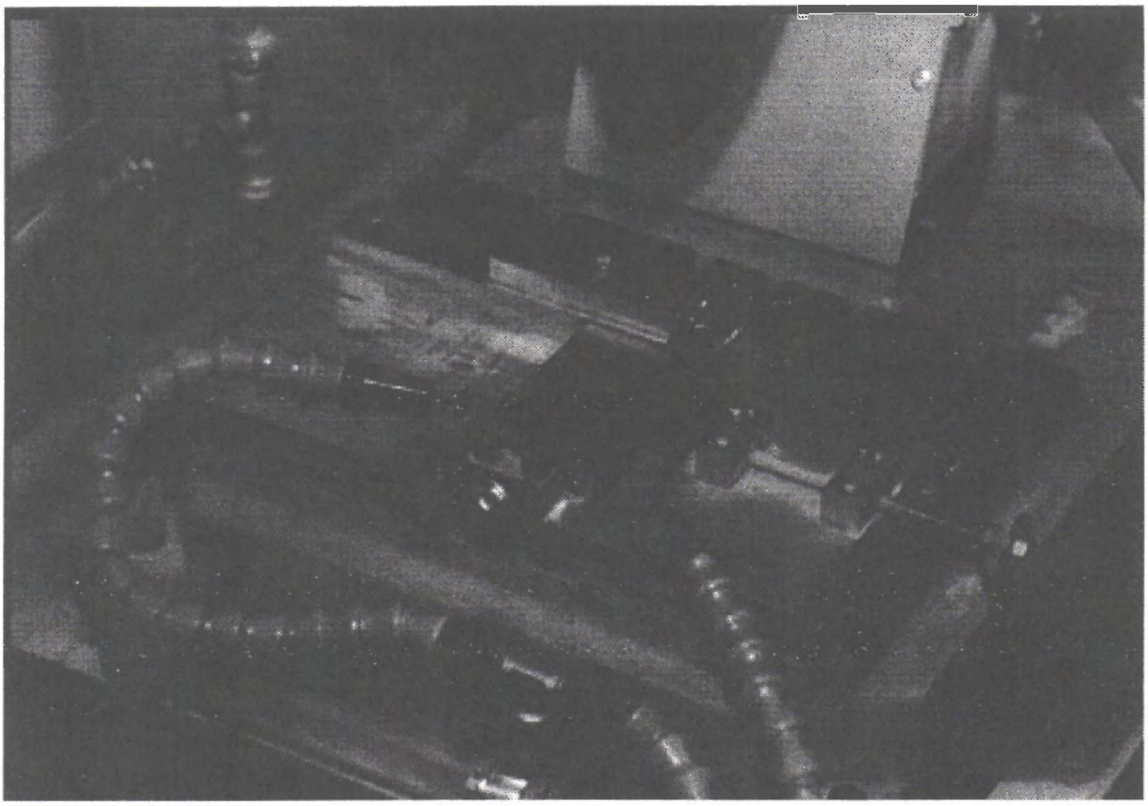
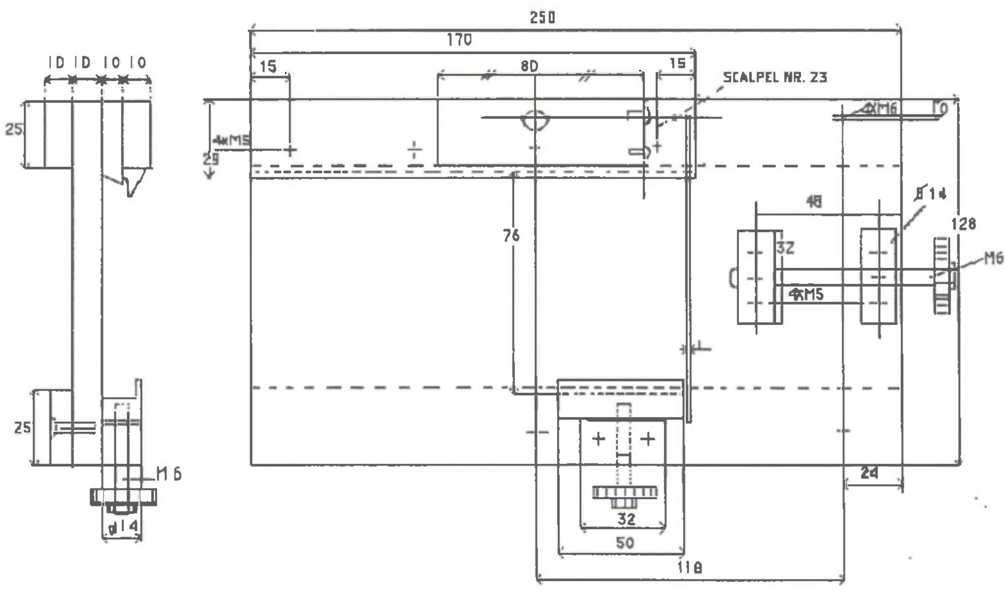


Figure 2. Sectioning table with polyester block with embedded otoliths on it. The photograph also shows the jig used to position the block on the table and the two outlets of the coolings system.



Fligure 3. Technical lay out of the sectioning table.

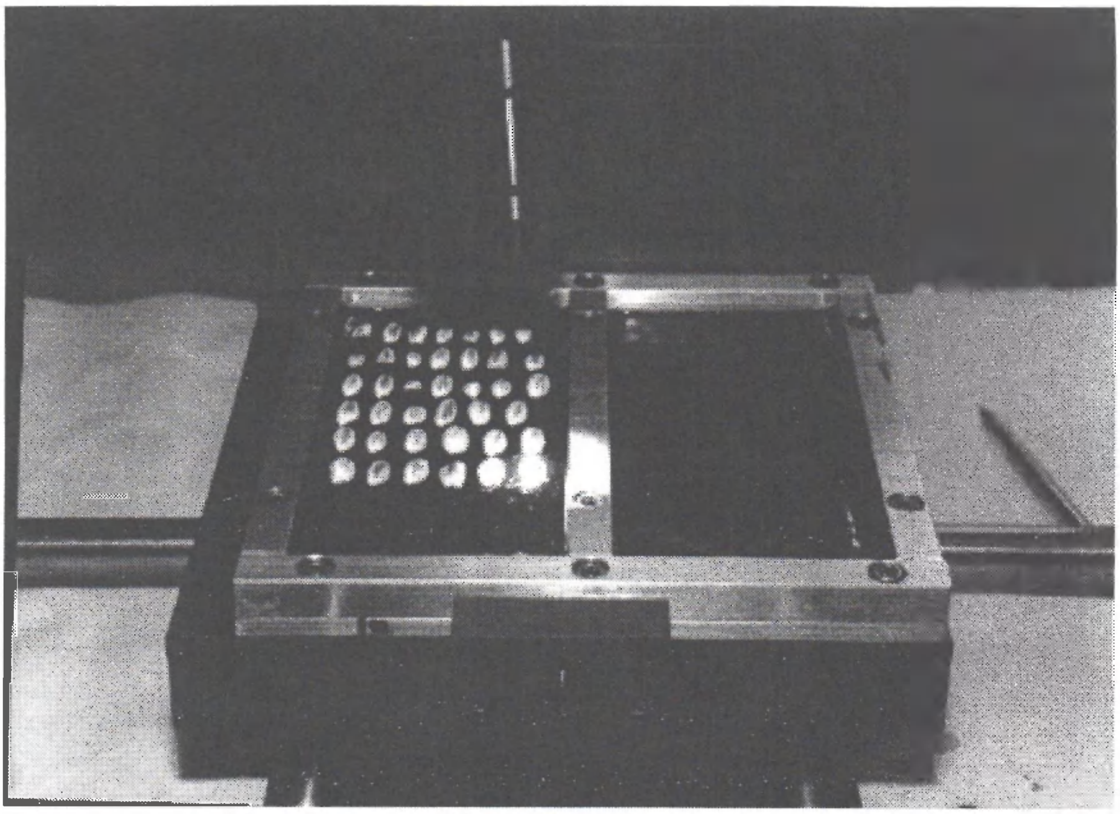


Figure 4. Mould with positioned otoliths on X-Y table.

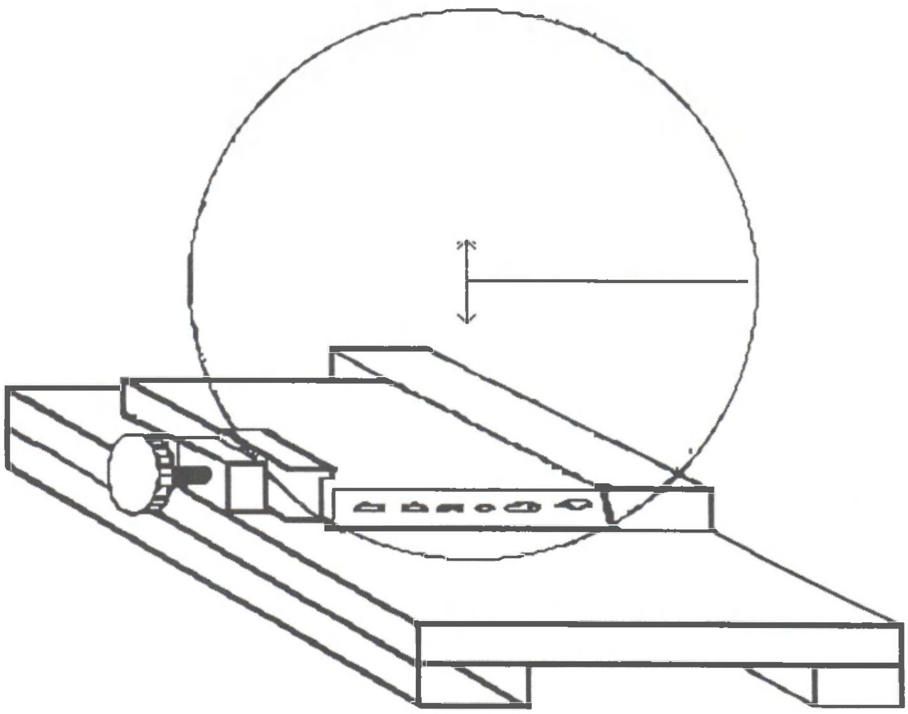


Figure 5. Drawing of polyester block in sectioning table after first cut.

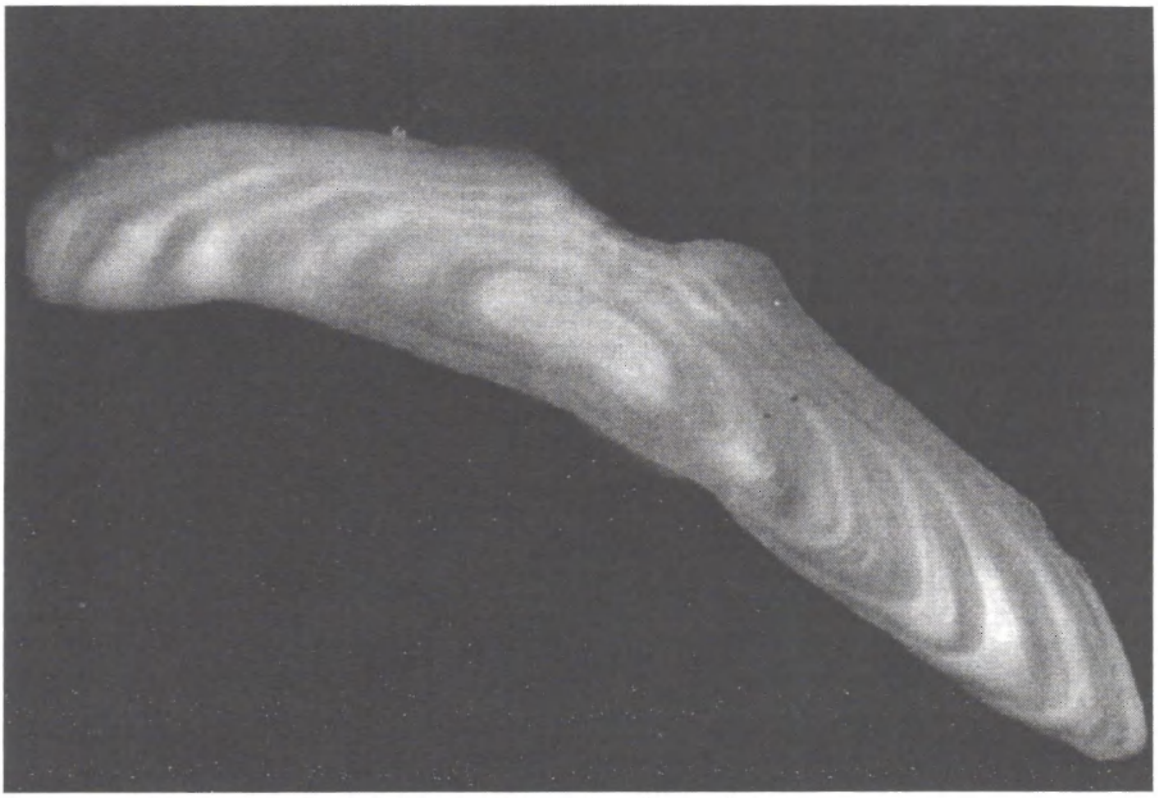


Figure 6. Example of sectioned plaice otolith with reflected light.

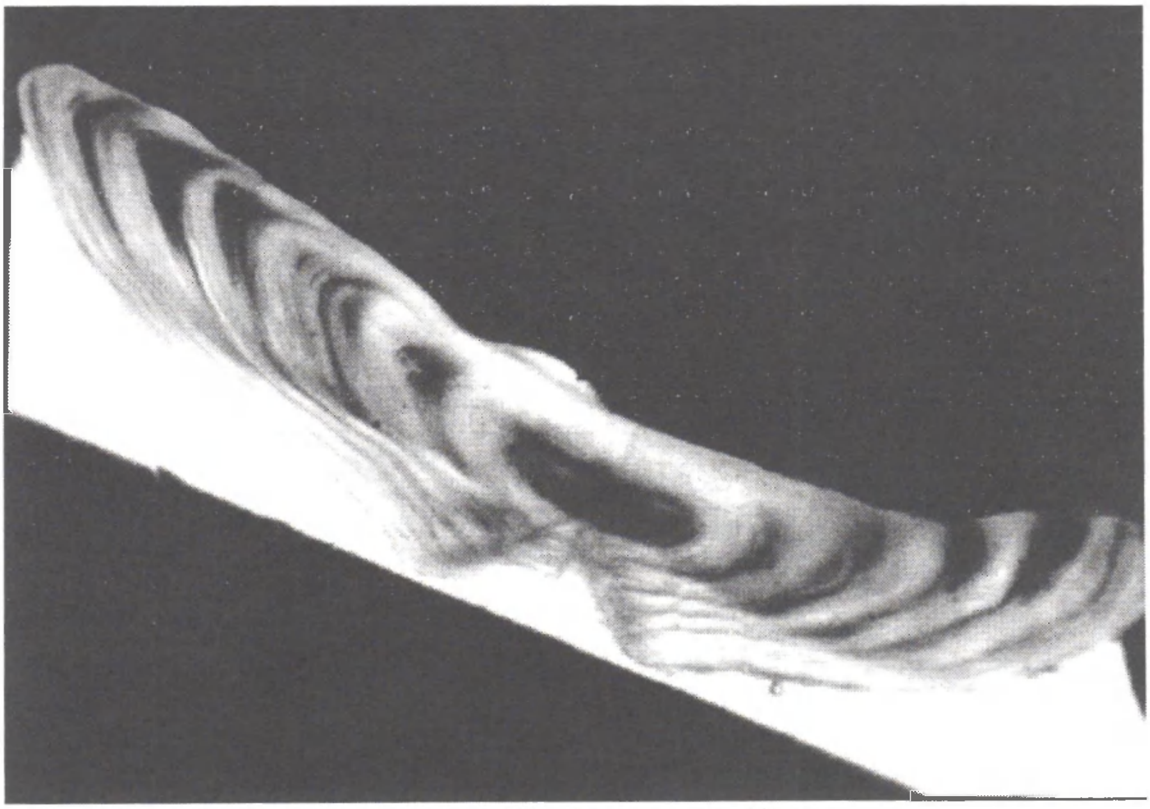


Figure 7. Example of sectioned plaice otolith with transmitted light.

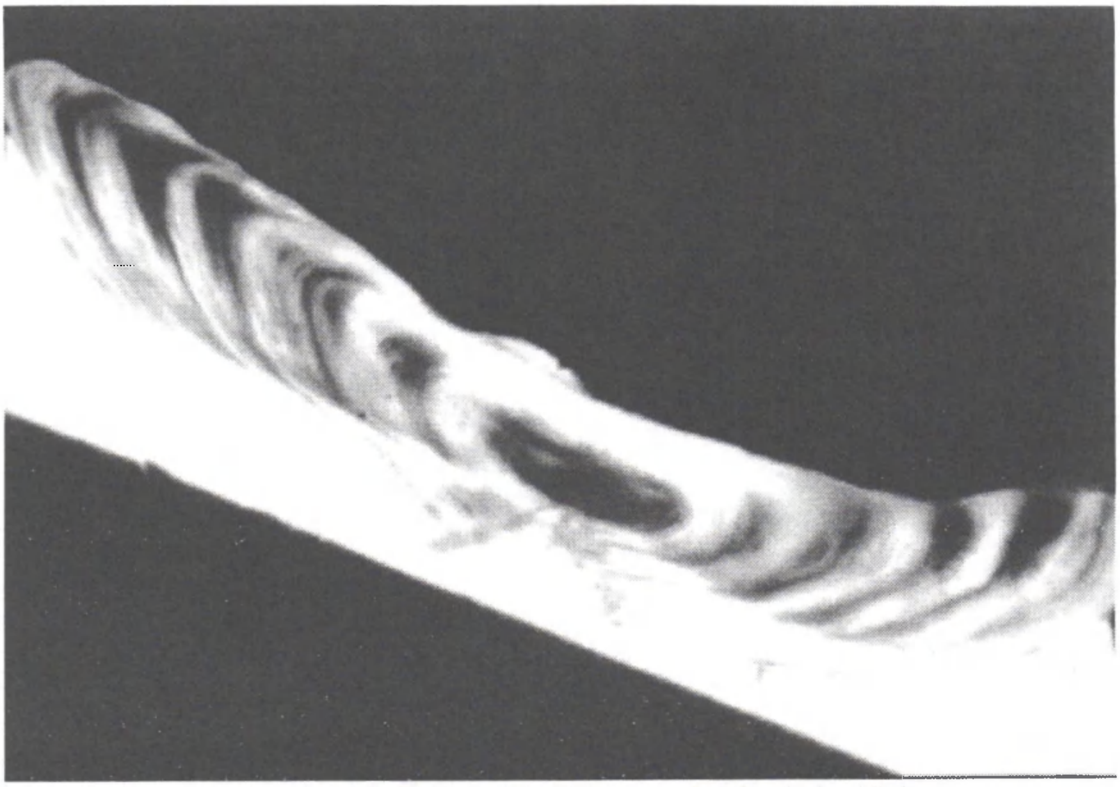


Figure 8. Example of sectioned plaice otolith with transmitted polarised light.

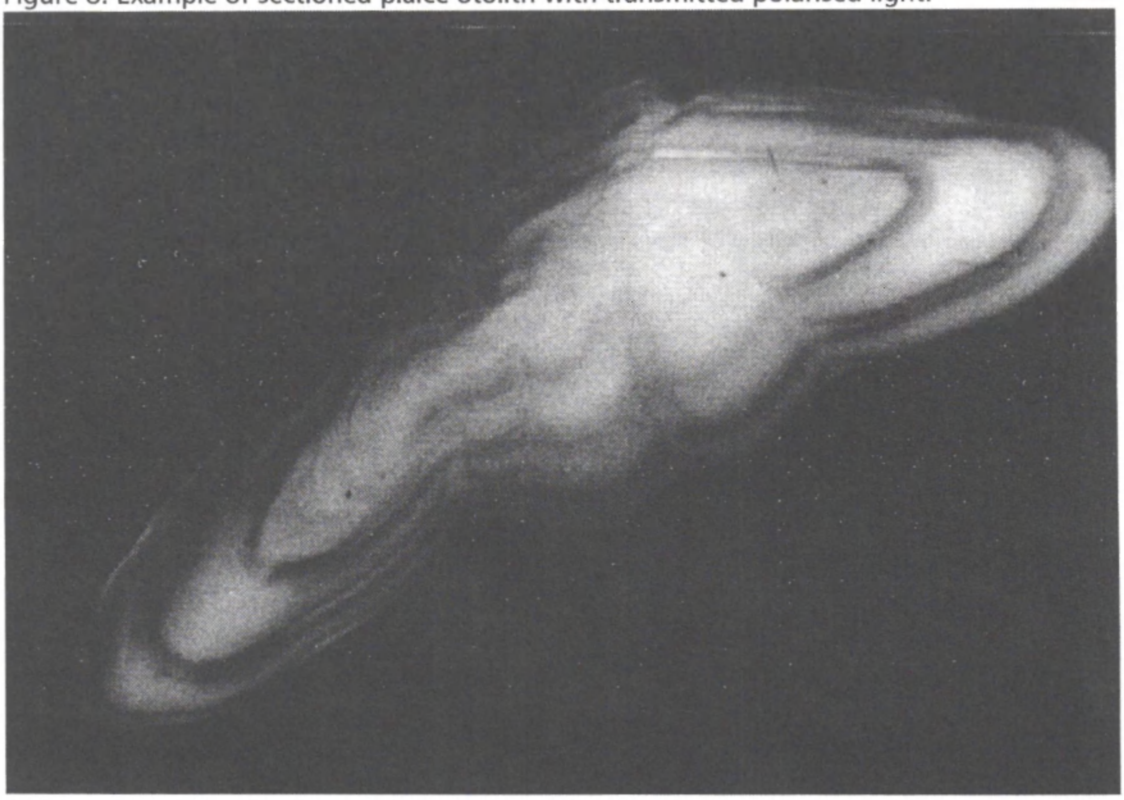


Figure 9. Example of sectioned cod otolith with reflected light.

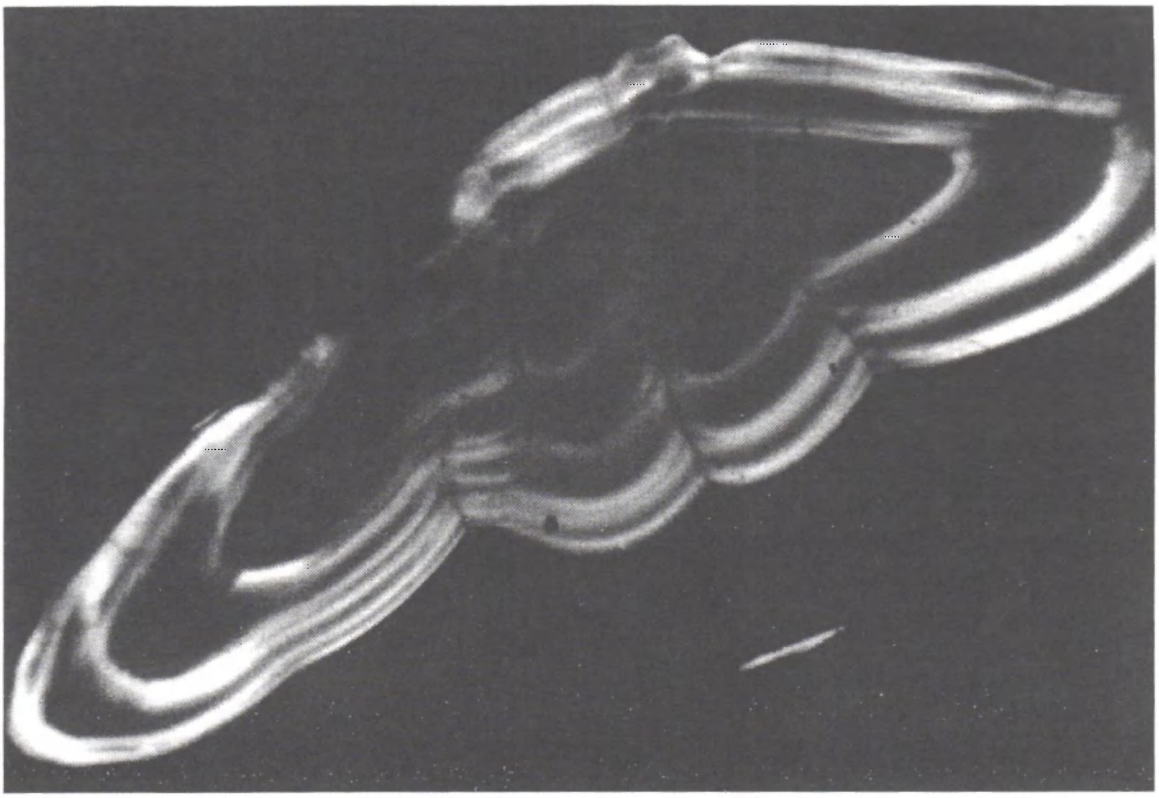


Figure 10. Example of sectioned cod otolith with transmitted light.

Figure 11. Example of sectioned whiting otolith with reflected light.

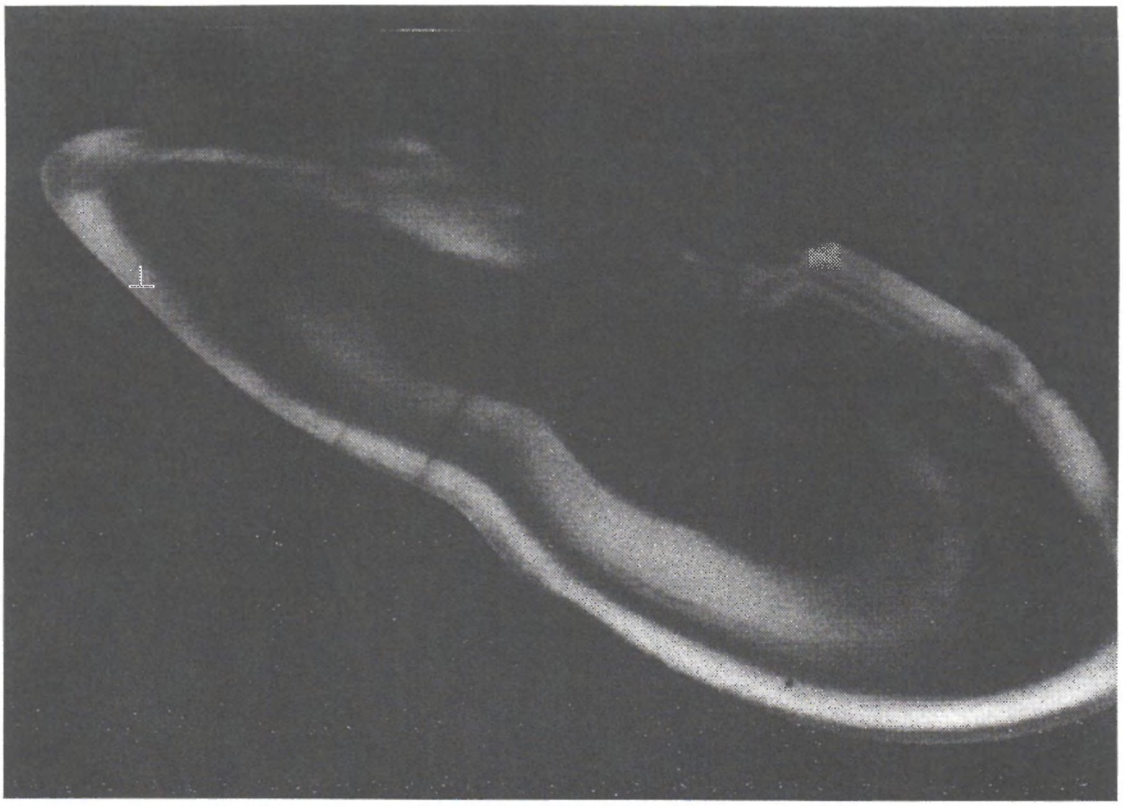


Figure 12. Example of sectioned whiting otolith with transmitted light.