Age determination procedures for pelagic and benthic species from ICES area in Spanish Institute of Oceanography (IEO)

BIOPEL and BIOBENTON projects

INSTITUTO ESPAÑOL DE OCEANOGRAFÍA 1914

In Memoriam of our colleague 'Manolo Meijide', who passed away in 2015 during the course of this work

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Recommended format for purposes of citation:

Villamor, B., Landa, J., Antolínez, A., Barrado, J., Dueñas, C., Hernández, C., Meijide, M., Navarro, M.R., Riveiro, I. 2015. Age determination procedures for pelagic and benthic species from ICES area in Spanish Institute of Oceanography (IEO). IEO internal Document.

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1 INTRODUCTION

The knowledge of age and growth of fish populations is essential to understand their life cycle (life span, age of recruitment, age of sexual maturity, spawning season, migrations, mortality). In fishery biology it is also important for the study of the demographic structure and its dynamics, which is fundamental to the assessment and management of fish populations subject to fishery exploitation, when age-structured evaluation models are used (Panfili *et al*., 2002).

Over the years, the importance of the study of age and growth in fisheries biology has grown in parallel with the increase in the number of stocks subject to assessment and management (Panfili *et al*., 2002). However, the study of growth remains a complex issue. In various international commissions of fisheries management, especially in ICES (International Council for the Exploration of the Sea), it has been shown the need to review the protocols of age interpretation of many species. Age determination requires a continuous and never-ending process of maintenance of the consistency of the age estimation criterion of the readers of each laboratory and of those of different laboratories (Panfili *et al*., 2002). The ultimate aim of all the work of age interpretation is clear: the establishment of accurate, precise and practical methods, so that they can be routinely used in the age interpretation of all the samples needed for the assessment of exploited species (Panfili *et al*., 2002).

The main aim of this manual is to describe the specific techniques for age determination of commercially important species populations in the ICES area (European Atlantic waters) and where these populations also play an important role in the ecosystem. The manual also provides general information about the morphology, preparation and observation of the calcified structures (CS) (otoliths and illicia), as well as the methods used to measure the accuracy of age estimation. This manual is intended not only to serve as a reference for the IEO age readers, but also to give an idea of the quality of the age estimations to the scientists that used routinely such data. The manual is also intended as a guide for the training of new readers in the preparation and age determination of CS, in addition of the training they received from expert readers. It is also intended as a dynamic guide that can be shifted as new techniques and species populations are evaluated and adopted in the program. It can also serve as a starting point in technical meetings to standardize methodologies and interpretation criteria, as when there are several institutions that share a program of age determination for species included in the manual. Some of the procedures included in this manual are derived exclusively from the IEO experience; others are obtained from the cooperation with other institutions through exchanges of CS and workshops about age interpretation.

2 CALCIFIED STRUCTURES

Within the EU data collection framework (DCF), the biological parameters of 11 species are studied each year (Table 2.1) in the framework of IEO research projects (BIOPEL and BIOBENTON), in Atlantic waters (ICES Subareas VI, VII, VIII and IX) (Figure 2.1).

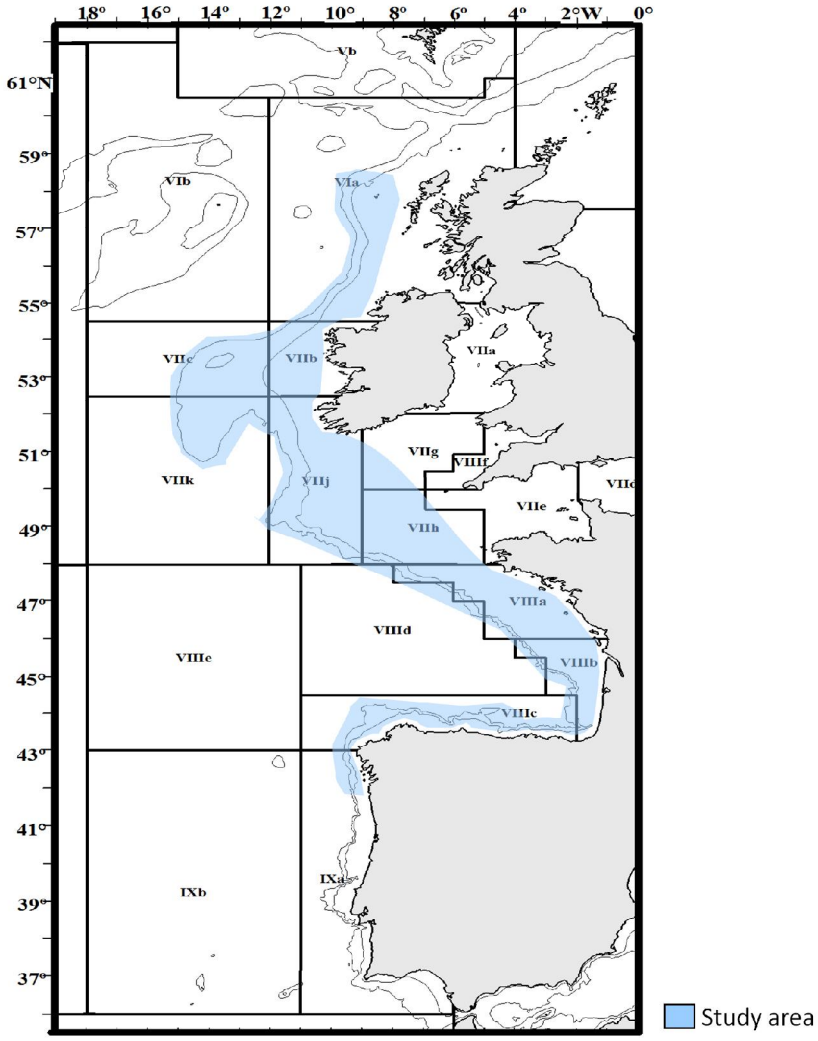


Figure 2.1. ICES Subareas and main sampling areas (approx.).

Table 2.1. Species studied in IEO (BIOPEL and BIOBENTON projects) in ICES areas within the DCF. Type of uses (%) of the age estimation results.

								Aim of study		
Species	Scientific name	Area/stock	Type of structure	Maximum reported age (IEO data base)	Data colletion (period of years)	Number of otoliths (extracted)	Number of otoliths (readings)	Stock assessment/DCF	Environmental studies	Others (validation, corroboration,..)
	Engraulis encrasicolus	VIIIc, VIIIb, VIIIa	Otolith	5	1985-2014	44005	41797	70%	20%	10%
	Engraulis encrasicolus	IXa North	Otolith	5	1995, 2011	2000	2000	100%	0%	0%
	Sardina pilchardus	VIIIc, IXa	Otolith	14	1981-2014	100564	74458	80%	10%	10%
	Scomber scombrus	VIIIc, IXa North	Otolith	18	1982-2014	60000	55000	70%	20%	10%
	Scomber scombrus	VIIIb, VII	Otolith	19	1982-2014	5000	4000	80%	10%	10%
Pelagic species	*Scomber colias*	VIIIc, VIIIb, and IXa North	Otolith	12	2011-2014	7304	1876	80%	0%	20%
	Trachurus trachurus	IXa North	Otolith	30	1982-2014	16000	13600	80%	10%	10%
	Trachurus trachurus	VIIIc, VIIIb	Otolith	30	1982-2014	55000	45000	80%	10%	10%
	Trachurus mediterraneus	VIIIc, VIIIb, and IXa North	Otolith	14	2006-2014	2626	1288	100%	0%	0%
	Micromesistius poutassou	VIIIc, IXa	Otolith	15	1982-2014	50000	35000	95%	0%	5%
						342499	274019	84%	8%	9%
	Lophius piscatorius	VIIb-k, VIIIa,b,d	Illicium	~20	2004-2014	2000	2000	95%	0%	5%
	Lophius piscatorius	VIIIc, IXa	Illicium	~20	1996-2014	12000	12000	95%	0%	5%
Benthic species	*Lophius budegassa*	VIIb-k, VIIIa,b,d	Illicium	-	2004-2014	2000	2000	95%	0%	5%
	Lophius budegassa	VIIIc, IXa	Illicium	-	1996-2014	12000	12000	95%	0%	5%
	Lepidorhombus whiffiagonis	VIIb-k, VIIIa,b,d	Otolith	14	1990-2014	15000	15000	95%	0%	5%
	Lepidorhombus whiffiagonis	VIIIc, IXa	Otolith	14	1990-2014	15000	15000	95%	0%	5%
	Lepidorhombus boscii	VIIIc, IXa	Otolith	12	1990-2014	15000	15000	95%	0%	5%
						73000	73000	95%	0%	5%
Total	**11 species/ 17 stocks**	**VI, VII, VIII, IXa**	**otolith/Illicium**			**415499**	**347019**	**89%**	**4%**	**7%**

2.2 OTOLITHS: MORPHOLOGY, FUNCTION AND STRUCTURES

Fish otoliths, sometimes named ear bones, are CS located in the membranous labyrinth of the inner ear of fishes that play an important role in the hearing, balance and spatial orientation (Popper *et al*., 2005). Otoliths are composed of calcium carbonate, deposited in the form of aragonite (Degens *et al*., 1696; Campana, 1999). All teleost fishes have three pairs of otoliths: asteriscus, lapilli and sagitta. The sagittae are the larger otoliths and are used most often in age determination.

Otoliths vary widely in shape and size depending on the species (Figure 2.2.1 and 2.2.2). Individuals of the same species may have otoliths slightly different in shape. This variation increases with the age. Otoliths of many species grow in one direction until they reach a certain size. Then, the growth axis change, generally to thicken the otolith. This is attributed to the lack of space inside the otic cavity to accommodate further growth in the major axis.

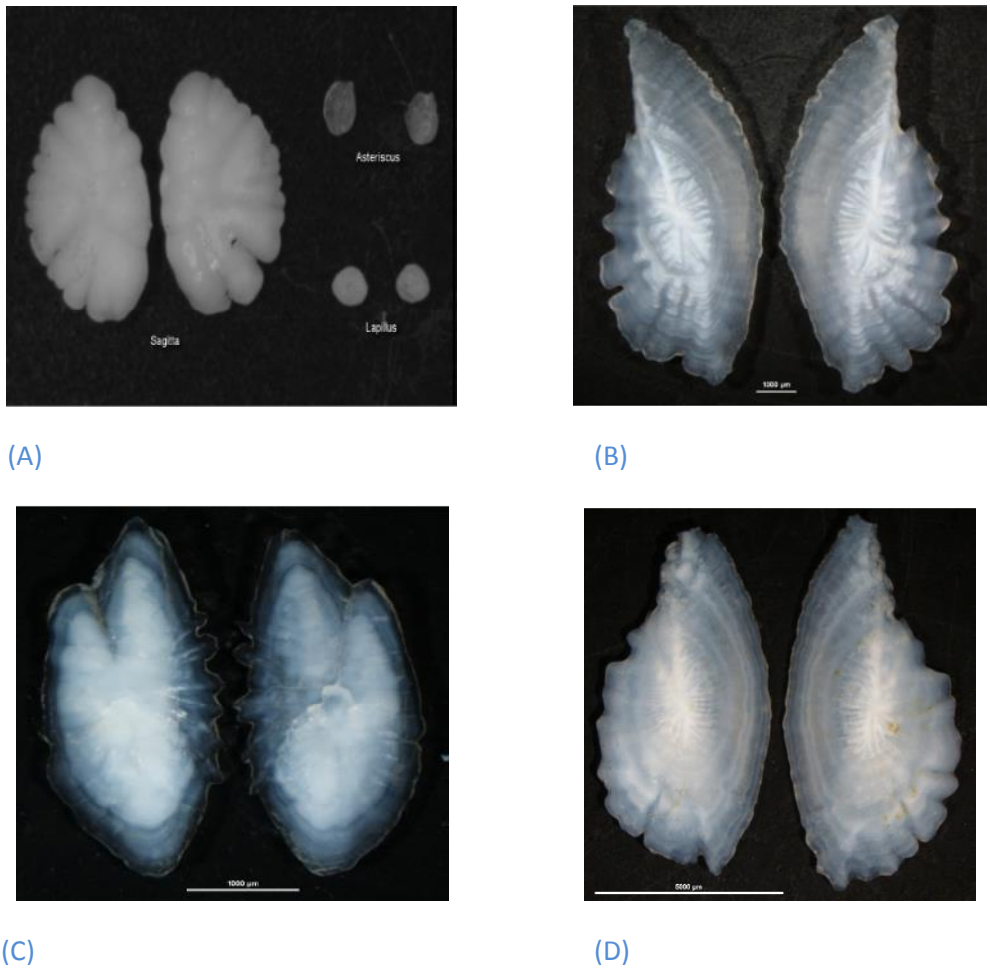


Figure 2.2.1. Otolith types: (A) Three pairs of otoliths of *Lophius budegassa;* otoliths sagitta of (B) *Trachurus trachurus,* (C) *Engraulis encrasicolus* and (D) *Trachurus mediterraneus.*

Figure 2.2.2. Some otolith types of sampled species in EO-Santander. From left to right: *Engraulis encrasicolus, Sardina pilchardus, Scomber scombrus, Scomber colias, Trachurus trachurus, Lepidorhombus whiffiagonis, Lophius budegassa* and *Micromesistius poutassou.*

The names of surfaces, areas and characteristics of a typical otolith are represented in the Figure 2.2.3.

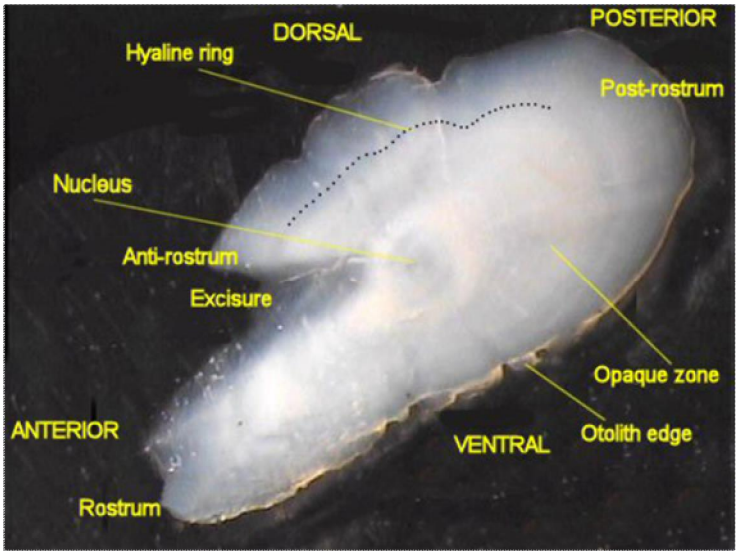


Figure 2.2.3. Surfaces, areas and characteristics of a sardine otolith (ICES, 2005a).

2.3 ILLICIA: MORPHOLOGY, FUNCTION AND STRUCTURES

The fin spines (hard or soft) and the vertebrae (whole or in sections) are the most commonly CS used for age determination, after otoliths and scales. In a similar way as with the otoliths, the differences in the transparency of bone layers deposited during the seasons are interpreted: opaque and translucent areas during periods of rapid and slow growth, respectively (Panfili *et al*., 2002).

In some species with a large size or a high price (eg. tuna or anglerfish) spiny fin rays are easier to obtain, prepare and estimate their age than otoliths. In the case of both European anglerfish species, the first dorsal ray (illicium) is usually used for age estimation (Figure 2.3.1). In the first international workshop of age estimation for both anglerfish species (Anon, 1991), the *illicium* was considered as the better skeletal structure for age estimation, because better results were obtained using them than using otoliths.

Figure 2.3.1 Illicium (first dorsal ray) of anglerfish.

3 SAMPLING PROGRAM

For monitoring fish populations, biological samplings are performed throughout the year. These samplings are carried out once a month or quarter, depending on the stock and species. Samples usually come from the fish market (fish landed from the commercial fleet). Also, biological samplings are performed during research surveys.

3.1 BIOLOGICAL SAMPLINGS ON RESEARCH SURVEYS

The CS (otoliths or illicia) are taken during the annual IEO research surveys carried out in Northeast Atlantic: acoustic surveys (PELACUS) and bottom trawl surveys (DEMERSALES, PORCUPINE), and from triennial ichthyoplankton surveys (CAREVA, JUREVA, SAREVA).

Some years, depending on the sampling program or research project, the CS are also taken by observers on board of commercial vessels.

3.2 BIOLOGICAL SAMPLINGS FROM COMMERCIAL FLEET

Biological samplings of fish landed from the commercial fleet are performed in IEO centers of Santander, Vigo and A Coruña. The fish samples are bought in different fish markets of the Cantabrian coast and Galicia, mainly Santander, Santoña, Avilés, A Coruña, Sada and Vigo. The biological sampling is performed by 2 or 3 people, one or two of them doing the sampling (measuring the fish length, weighting and sexing the specimens, extracting the otoliths, etc), and other person recording all the information on a biological sampling form and collecting the CS in paper envelops or microtubes (Figure. 3.2.1).

In some species, the extracted otoliths are collected in plastic plates, and this extraction is postponed until the end of the sampling. During the sampling, the specimens are placed over a worktop next to where the sampling is being done, in the same order as the one in which they are being sampled. To facilitate the identification of the specimens, the orientation of the fishes is reversed each time that ten specimens have been sampled.

In both anglerfish species, a part of the biological sampling can also be performed in the fish market, measuring and recording only the length and taking the illicia in paper envelops.

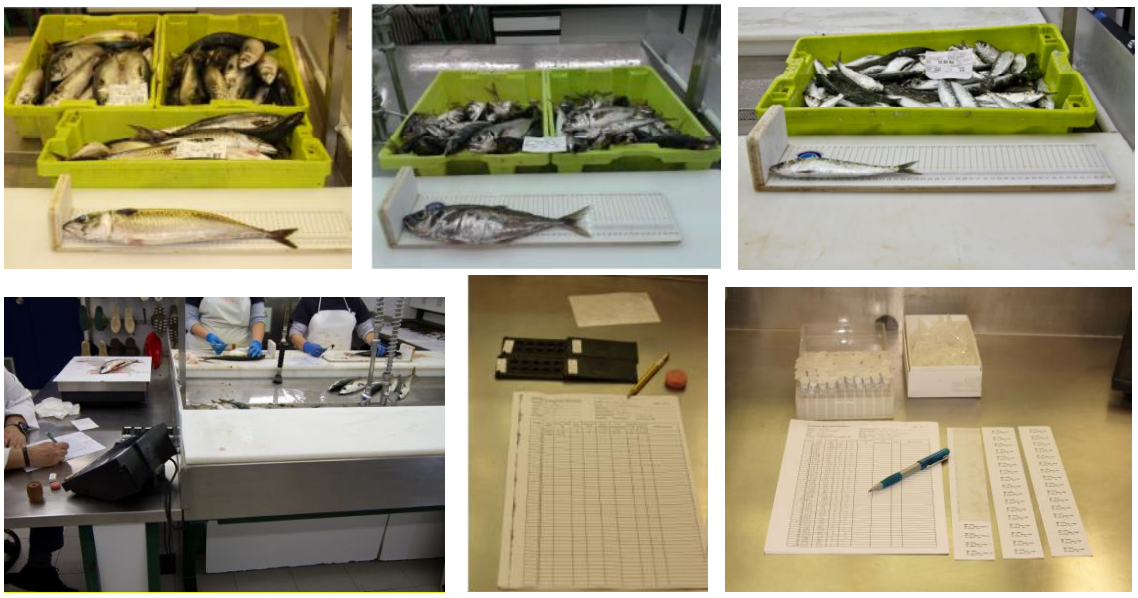


Figure 3.2.1. Performing biological samplings at the laboratory.

4 STORAGE OF CALCIFIED STRUCTURES

Three types of storage for CS are used in IEO: plastic plates with cover, paper envelops and microtubes.

4.1 PLASTIC PLATES WITH COVER

Plastic plates with cover (Figure. 4.1.1) are used to store otoliths of mackerel, chub mackerel, anchovy and sardine. They are black plastic plates (10 x 4.5 cm), with ten circular depressions or wells of 11 mm of diameter and 2.5 mm of depth, located in two rows of five wells each and with a space in the head where it is placed an adhesive label (12 x 30 mm).

At laboratory, about 100 specimens of the aforementioned species are sampled in each biological sampling. On surveys, 40 specimens are sampled in each haul. In both cases, the otoliths of ten specimens are stored in each plate. The wells of each plate are numbered from 1 to 10 and the corresponding specimen numbers are recorded in the label on it (eg, in the plate corresponding to specimens 21-30, the well number 1 corresponds to specimen 21; the well number 2 corresponds to specimen 22 and so on).

Figure 4.1.1. Otolith plates, covers and labels.

Each biological sampling starts in the specimen number 1, using a new plate, even when in the previous sampling there were some empty wells in the last plate.

Each plate must carry two labels with the same information, one of them on the plate head and the other on the cover, so the otoliths can be quickly identified even with the cover on.

The information of the labels at laboratory and surveys samplings includes (Figure. 4.1.2):

- Species
- Fish mark of origin / Survey name
- Biological sampling date / Haul number
- Code of the specimens whose otoliths are stored in the plate (1-10, 11-20, etc.)

Figure 4.1.2 Otoliths plate and cover from a laboratory sampling (left) and a survey (right).

Once the otoliths are fixed on resin (see section 7.1), the plates are stored inside cardboard boxes (19.5 x 4.5 x 10.5 cm) with dividers (Figure 4.1.3). Each box with a divider can store 12 plates in a vertical position. All the plates from a biological sampling are stored in the same cardboard box (Figure 4.1.4). Sometimes storing plates from successive samplings is possible, but only when there is enough space in the box for all the plates of the same sampling, otherwise, a new box should be used, even if there is still space in the previous one. The same is performed for the storage of otoliths plates from surveys. The cardboard boxes are filled with plates from successive hauls, whenever the plates of the same sampling are stored in the same box (Figure 4.1.4).

Figure 4.1.3. Cardboard box of otolith plates: dismantled (left) and mounted (right).

The cardboard boxes are labeled with the following information (Figure 4.1.4):

- Species
- Laboratory /Survey name
- Biological sampling date/ Haul number

Figure 4.1.4. Cardboard box with otoliths plates from a laboratory sampling (left) and a survey (right).

Anchovy otoliths are not fixed in resin, but are kept loose in their plates. These are stored with their cover on and with adhesive tape tightening the center of the plate. The plates are placed inside a cardboard box in a horizontal position to avoid the displacement of the otoliths inside the plate (Figure 4.1.5).

Figure 4.1.5. Storage of plates with loose otoliths of anchovy.

The cardboard boxes with otoliths plates of mackerel and chub mackerel are stored in a drawer of a sideboard placed in the microscope laboratory (Figure 4.1.6). They are stored in their corresponding drawer by species, year and origin (laboratory or survey) and are kept this way while they are being used for age estimation, pictures, growth increment measures, etc (usually over a year).

Figure 4.1.6. Sideboard for the temporal storage of otolith plates.

When they are no longer needed, the cardboard boxes with the otoliths plates are placed orderly inside bigger cardboard boxes. These new boxes are labeled with the appropriate information (species, year, date of the sampling, survey, etc.). A code is also assigned to each box, which consists in a number (the following to the last box) plus a letter (a different letter by species: J for horse mackerel, A for anchovy, C for mackerel, and E for chub mackerel, which are the initials of each species name in Spanish). These boxes are permanently stored in a store of the center (C.O. Santander) (Figure 4.1.7). The same is performed with the envelopes and microtubes of horse mackerel otoliths.

Figure 4.1.7. Final storage of otoliths plates: cardboard boxes (left) and boxes in the store (right).

The otolith plates of sardine, once the resin is dry, are packed and sent to C.O. Vigo (see below).

4.2 ENVELOPES

Otoliths of horse mackerel, Mediterranean horse mackerel, blue whiting, megrim and four-spot megrim have been stored inside square cardboard envelopes (4.5 x 4.5 cm) until the first quarter of 2012. Nowadays, this type of envelopes is still used to store otoliths of megrim and four-spot megrim, but the otoliths of the other species are stored in microtubes. The illicia of black anglerfish and white anglerfish are also stored in rectangular paper envelopes (8.9 x 8.7 cm) (Figure 4.2.1).

The following data are printed on the front of the envelopes:

- Code
- Species
- Fish length
- Sex
- Date / Haul number
- Survey name
- Sexual maturity (anglerfish envelopes)
- Area (anglerfish envelopes)

These data are filled by pencil during the sampling. The IEO logo is printed on the envelope back.

The envelopes with otoliths inside are organized in groups of ten using a rubber band (of 6 cm) that holds them together. Special care must be taken to not over tighten the envelopes with the rubber band to prevent damage to the otoliths. A single round of rubber band is recommended, grouping the envelopes by their top, where the otoliths are not located. The envelopes are stored in cardboard boxes (19.5 x 4.5 x 10.5 cm).

Each illicium of black anglerfish or white anglerfish is placed inside the envelope with its base first, and the top part of each illicium that stand out of the envelope is cut. The illicium skin is no retired (Figure 4.2.1).

The envelopes with the illicia inside are stored in cardboard boxes, properly labeled. The illicia that are yet not processed remain stored in their envelopes. The boxes with the illicia envelopes are kept in the store of the center. The envelopes whose illicia have been processed are eliminated.

Figure 4.2.1. Envelopes and boxes used to the storage of otoliths (above) and illicia (below).

4.3 MICROTUBES

The use of plastic microtubes of 1.5 ml has been progressively introduced since 2012 (Figure 4.3.1) to store otoliths of horse mackerel, Mediterranean horse mackerel, blue whiting, black anglerfish and white anglerfish.

An adhesive label (35.6 x 16.9 mm) is placed on each microtube. The gaps in the labels are filled by pencil during the sampling. The following information is printed on the labels used in the biological samplings performed at laboratory and surveys:

- **Sp** (species, already printed)
- **Laboratory** (already printed for lab samplings) / **Survey** (name of the survey, already printed for survey samplings)
- **Date** (year already printed for lab sampling) / **Haul** (for survey samplings)
- **Code**
- **Area**
- **Length**

During the biological sampling, the microtubes are placed in a plastic square box (140 x 153 x 53 cm) to avoid splashes and spots. After the sampling, the microtubes are moved to a square cardboard box (150 x 150 x 55 cm) where they will be stored. This box has 10 rows x 10 columns, with a total of 100 wells, so each box can contain the otoliths of the 100 specimens from a laboratory sampling (Figure 4.3.1).

On a survey, a maximum of 40 specimens is sampled in each haul. When the sampling is performed, the microtubes are moved from the temporary plastic box to the cardboard box, which is filled with the microtubes from some hauls until the box is completed.

When the otoliths are no longer needed, all the cardboard boxes with the microtubes are placed inside bigger cardboard boxes for a permanent storage, in the same way as the otolith plates (see section 4.1).

Figure 4.3.1. Final storage of otoliths microtubes boxes (left) and boxes in the store (right).

5 BIOLOGICAL PARAMETERS

When a fish is being sampled with the aim of estimate its age, the record of the biological parameters (length, weight, etc.) and the sampling information (date, fishing harbour, etc.) are necessary. All this information is entered in the IEO Database Manager, SIRENO (In Spanish: Seguimiento Informático de los Recursos Naturales Oceánicos), whose display set for data consists in two sections (Figure. 5.1).

A head that contains the common fields to all species:

- Sampling date
- Fishing harbour
- Origin
- Institution
- Vessel name
- Fishing gear
- Species
- Category
- Total weight
- Sample weight
- Total number of specimens
- Number of specimens of the sample
- Person in charge of the sampling
- Person in charge of the age estimation
- Person in charge of the sampling form
- Sample origin
- Landing date
- Age estimation criteria
- Maturity criteria
- Comments

There is also a main section where the sampling biological data of each specimen are entered. The relation between the different fields of this section varies depending on each species particularities, being the most common ones:

- Sampling type
- Code
- Length
- Weight
- Sex
- Sexual maturity

- Age
- Otolith
- Gonad weight
- Gutted weight
- Liver weight
- Fatness
- Otolith edge
- Age estimation quality

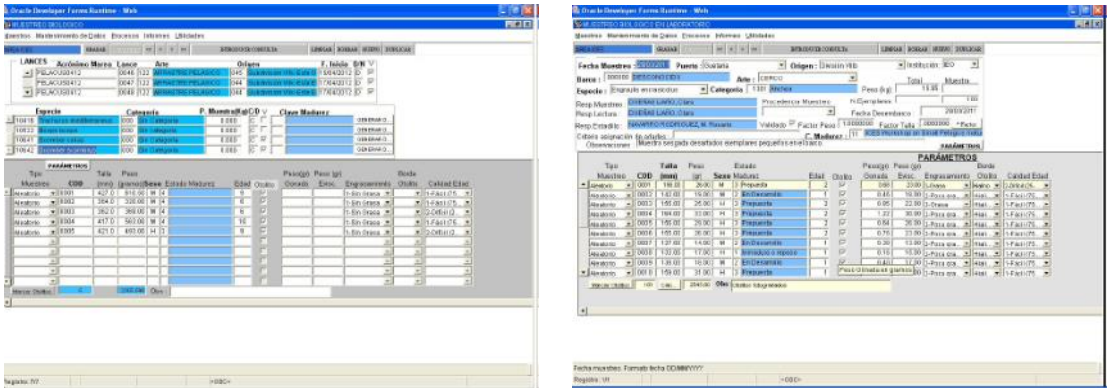


Figure 5.1. Display set for the introduction of biological sampling data from surveys (left) and fish market (right) in SIRENO.

6 DELIVERY OF CALCIFIED STRUCTURES

Some of the CS are extracted in an IEO laboratory and then sent to another one, where they are processed and analyzed. Thus, CS of some stocks of sardine, blue whiting, Mediterranean horse mackerel, megrim and four spot megrim obtained in Santander and Coruña laboratories are currently sent to Vigo laboratory; CS of some stocks of mackerel, chub mackerel, horse mackerel, anchovy, white anglerfish and black anglerfish obtained in Coruña and Vigo laboratories are currently sent to Santander laboratory.

The guidelines to follow for this process are:

- Time of delivery: after each sampling, the CS should be sent as soon as possible to the destination laboratory by internal courier. Thus, those otoliths that are stored loose, without being fixed, should be sent the day after the sampling if it is possible; the otoliths that have to be fixed with resin are sent once the resin is dry. It is recommended to fix the otoliths with resin one or two days after the sampling, so they can be sent to the destination laboratory within two weeks.
- Contact person: in each laboratory, a person is responsible of the biological sampling of each species. This person sends the CS to the person in charge of the processing and age estimation of that species in other lab.
- Biological parameters: the biological sampling data are entered into the S.I.R.E.N.O. Access application by the person in charge of the biological samplings of each species in each laboratory. This must be done as soon as possible after each sampling. Subsequently, the data have to be checked with the data of the sampling form and validated.
- Packing: CS must be carefully packed to avoid their breakage. Usually, the envelopes containing CS, the otoliths plates or the microtubes are introduced in a cardboard box which, in turn, is stuffed into a padded envelope.

7. PREPARATION OF CALCIFIED PIECES USED IN AGE ESTIMATION

7.1 WHOLE OTOLITHS MOUNTED ON RESIN

This procedure is used in IEO (C.O. Santander) for otoliths of mackerel, chub mackerel, sardine and anchovy, although the anchovy otoliths are being observed for age estimation immersed in water since 2009.

Once both sagittal otoliths have been extracted from the fish in a laboratory sampling, the organic residues are very carefully removed from the otoliths to prevent breakage. Then they are rinsed with distilled water and are put aligned into their plates, inside their corresponding well (see section 4.1). A small drop of distilled water is put in the centre of every well to make easier that the otoliths stay inside it (Figure 7.1.1).

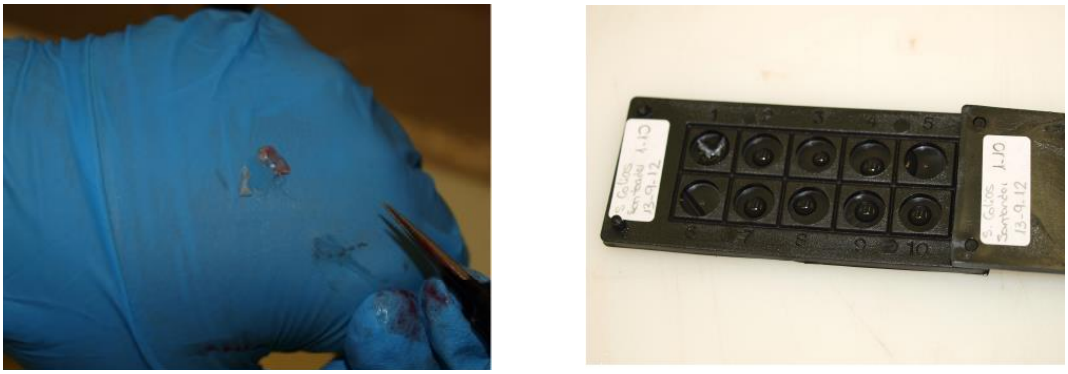


Figure 7.1.1. Cleaning an otolith after its extraction (left) and plate with water drops inside the wells (right).

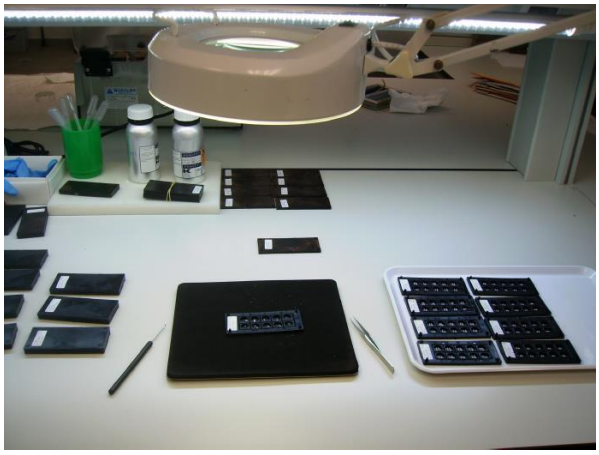
After the sampling, the otoliths are left to dry under the extractor hood for 24 hours (without covering with the plate lid) (Figure 7.1.2).

If the otoliths come from a survey, they are stored loose inside their plates with their lid on until they are brought to the laboratory. To prevent their displacement inside their plate (due to the movement of the ship and while the otoliths are being transported to the laboratory) the plates are kept closed with their lids on, with adhesive tape tightening the central part of the plate. The plates from each sampling are gathered together with an elastic band and put inside a cardboard box. The plates of several hauls of the same species are stored in the same box (Figure 7.1.3).

Figure 7.1.2. Otoliths left to dry under the extractor hood.

Figure 7.1.3. Plate with adhesive tape in the central part (left) and storage of otoliths plates in surveys (right).

Each box is labelled with the sampling information (species, survey name). The plates and the boxes must be kept in a horizontal position all the time. In turn, when a cardboard box is full, it is stored in a higher plastic or metal box.



At the laboratory, the otoliths coming from both, surveys and fish market, are placed in the centre of their wells with the assistance of fine tweezers and a punch, leaving a small space between both otoliths to facilitate their further age estimation and the image capture of both otoliths together (Figure 7.1.4).

Figure 7.1.4. Location of the otoliths before being mounted on resin.

Sometimes the otoliths have organic residuals that make difficult the age estimation. In this case, these residuals must be very carefully removed to prevent the breakage of the otoliths.

Once the otoliths are cleaned and placed correctly in the wells, they are covered with transparent resin (Eukitt). This process must be done using the proper protective equipment (mask, glasses, latex gloves) under the extractor hood. The resin is poured over the plate using a Pasteur pipette, filling first the grooves which delimit the wells. After that, each well is filled up until the resin overflow it (Figure 7.1.5). It is very important to use enough resin (it must overflow the wells), because when it is dry, the remaining space will be occupied by the resin. If only a small amount of resin is poured, the light of the microscope will produce uncomfortable glints and therefore, the age estimation and image capture will be difficult.

Figure 7.1.5. Protective equipment (left) and otoliths being fixed with Eukitt.

While the resin is being poured, the otoliths can be displaced inside their well. Some air bubbles can also be trapped under and around the otoliths, but using a fine punch, the otoliths can be repositioned and the bubbles expelled.

After that, the resin of the plates are left to dry under the extractor hood during 5-7 days (Figure 7.1.6).

Figure 7.1.6 Otoliths drying under the extractor hood after being mounted on Eukitt.

When the resin is dry, the lids are placed over their corresponding plates and all the plates are stored in a cardboard box with dividers, which keep the plates in a vertical position. The box is labelled with the corresponding sampling data (species, laboratory, date) (Figure 7.1.7) (see section 4.1). For the age estimation, the plates will be placed under the binocular microscope with reflected light.

Figure 7.1.7. Storage of otoliths mounted on resin once the resin is dry.

7.2 WHOLE OTOLITHS IMMERSED IN A FLUID

This procedure is used in IEO (C.O. Santander and C.O. Vigo) for otoliths of anchovy, horse mackerel, Mediterranean horse mackerel, megrim and four spot megrim (Figure 7.2.1).

In the case of the anchovy otoliths, these are left to dry in their plates under the extractor hood for 24 hours after sampling. Once dry, the plates are kept closed with their lids on, with adhesive tape tightening the central part of the plate. The plates from a sampling are gathered with an elastic band

and put inside a cardboard box. Plates and boxes must be kept all the time in a horizontal position. The box is labelled with the corresponding sampling data (species, laboratory, date) (see section 4.1). When the anchovy otoliths are going to be observed for estimating the age , the wells of the plates are filled with water using a 1 ml Pasteur pipette. The otoliths are oriented and located with the sulcus downwards using fine tweezers and a punch, and are observed directly under the binocular microscope.

After the age estimation, the water is removed from the wells using the pipette, being careful of not to absorb the otoliths at the same time. Again, the plates are kept closed with their lids on, with adhesive tape tightening the central part of the plate and put inside their cardboard box.

In the case of horse mackerel and Mediterranean horse mackerel, for the age estimation, the otoliths of specimens with a length less than 27 cm are placed inside a small container filled with a mixture of alcohol and glycerine and are directly

A similar procedure is performed for otoliths of megrim and four spot megrim, but in this case, the otoliths are immersed in just water. After the age estimation, the otoliths are left to dry over a piece of blotting paper, and, once dry, they are returned to their respective envelopes.

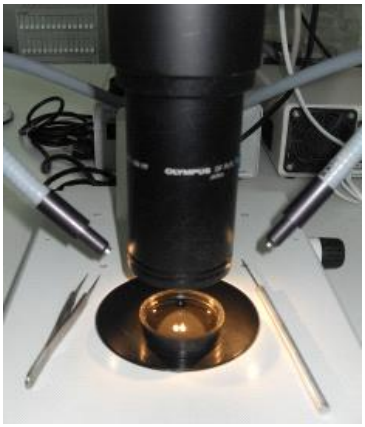


Figure 7.2.1. Otoliths directly observed under the binocular microscope

7.3 BURNING OF OTOLITHS

This technique was used in IEO (C.O. Santander) for the age estimation of horse mackerel otoliths, although it is not currently in use. It allowed the identification of annuli on otoliths of specimens of great length.

Whole otoliths are required to use this technique. The otolith is placed onto a semi-soft surface (like play-dough), and the centre of the otolith nucleus is pressed with a small blade knife. The impact generated when the otolith is sectioned in half, transversely to the sulcus, is absorbed by the surface where the otolith is placed.

The otolith section which includes the post-rostrum is placed between the fingertips and is wetted with water before being polished with a piece of very fine sandpaper, until the largest flat severed surface is smooth out. Afterwards, these otolith sections are placed inside a well of a metallic plate (Figure 7.3.1).

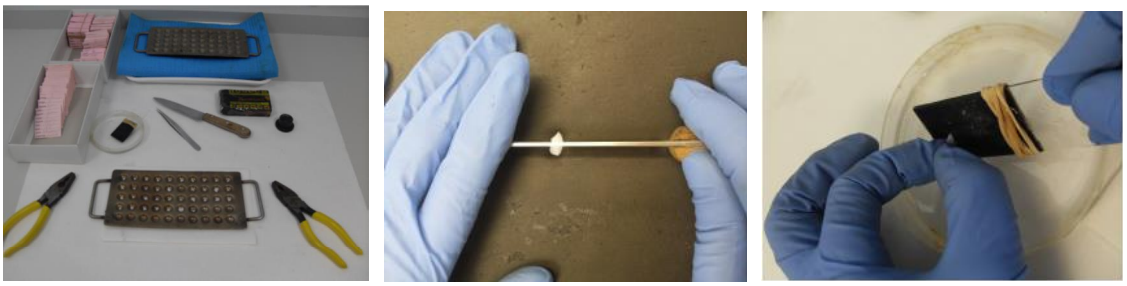


Figure 7.3.1. Mounting equipment (left), otolith being sectioned in half (centre) and otolith section being polished in water (right).

Each polished otolith section of the other specimens belonging to the same sampling is placed in each well of the metallic plate, in the same numerical order as in the sampling. The plate is then placed over a heat source. When the plate is being manipulated, it must be taken by the handles, using a pair of pliers for each handle, to be insulated from the heat.

As the otolith sections are heated, they gradually change from their original white colour to different shades of brown colour (Figure 7.3.2).

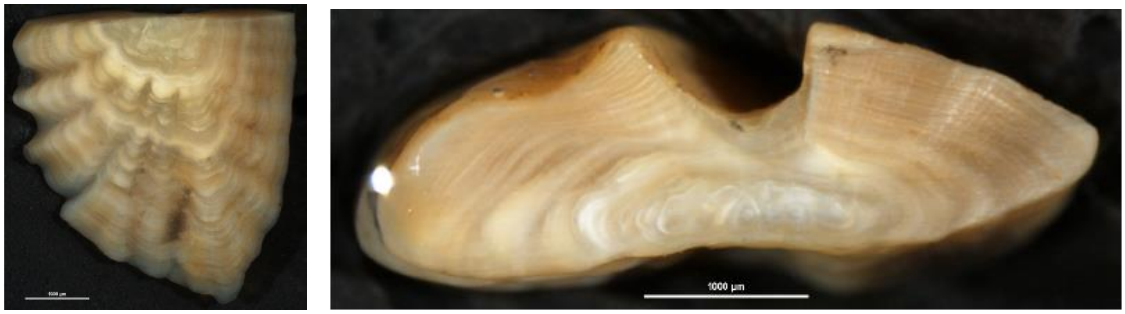


Figure 7.3.2. Horse mackerel otoliths after the burning process.

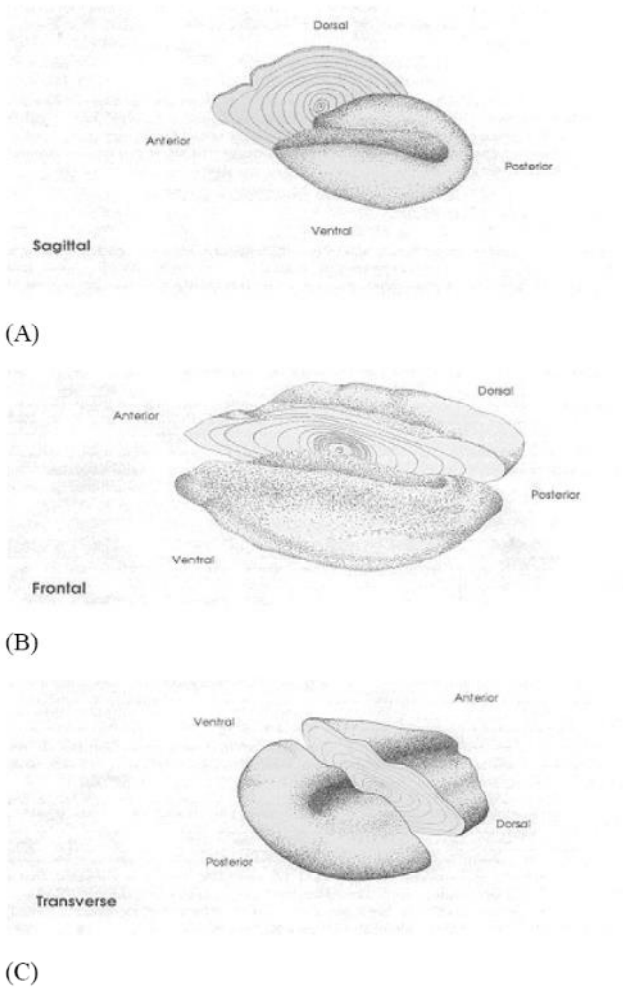
Afterwards, the metallic plate with the otoliths is left to cool on a wet dishcloth. When everything is cool, each burned otolith is placed inside its corresponding envelope.

The burned calcified structures are observed directly using a binocular microscope. Each processed section is stuck by its post-rostrum side in a piece of black play-dough, leaving a flat surface upside so it can be well illuminated (Figure 7.3.3).

Figure 7.3.3. Observation of otoliths after the burning process.

7.4 SECTIONS OF CALCIFIED STRUCTURES

The aim of the process of cutting and sanding CS is to obtain a fine section of such pieces. The otoliths of many fish species are extremely opaque or too thick, so the growth increments cannot be easily identifiable by a binocular microscope. The observation of fine sections of these CS improves the readability of the growth increments (Bedford, 1975; 1983). Therefore the sectioning is necessary in the age estimation studies of these species.



The CS sections can be obtained in different planes (Figure 7.4.1) by sectioning or sanding. All sections must go through the otolith nucleus to avoid the growth increments loss, and thus, to avoid a possible age underestimation.

The section orientation is important because some otoliths grow asymmetrically. For sagitta otoliths with internal or sulcal priority growth, transversal or oblique sections are recommended. For otoliths with longitudinal priority growth, transversal sections can contain many growth discontinuities and therefore, frontal sections are recommended.

In the first study stages of a species, several otoliths sections of different length is necessary to obtain for determining the possible best section plane (Morales-Nin, 1992).

In both anglerfish species, transverse illicia sections are used for the age estimation.

Figure 7.4.1 Types of sagitta otoliths sections (Secor, *et al*., 1992).

7.4.1 SECTIONING

This process is performed in IEO (C.O. Santander) for black anglerfish and white anglerfish (illicia), and also for large (> 26 cm) horse mackerel and Mediterranean horse mackerel (otoliths).

7.4.1.1 Material

The material used for the mounting of CS (otoliths and illicia) in plates is the following (Figure 7.4.1.1.1):

- Polyester resin (Estratil A-250 TY pre-accelerated)
- Universal black colouring (Standard for all kind of paintings)
- Catalyst / hardener (Butanox M-60)
- Vaseline
- Acetone (solvent)
- Paper cup or another kind of container for the mixture
- Metal bar
- Metal plates (mould)
- Tweezers
- Punch
- Scissors
- Rule paper (to place the illicia)
- Sellotape
- Labels (12 x 30 mm)
- Spaghetti
- Microscope slides
- Slicing machine (Benetec OTO-LABCUT 230F). It is essential a machine with a cutting speed of 2000 rpm or higher, a diamond sectioning blade and a cooling system.
- Mounting station for CS processing (Benetec OTO-922). It consists of: mounting jig with X-Y positioning, video camera and monitor with fixing jig, extractor hood.

Figure 7.4.1.1.1. Expendable equipment needed to prepare CS sections

Preparation of the moulds. A metal plate (mould) is greased with vaseline (to remove the resin block from its mould when it is solid). Two spaghetti are placed at an angle of 45º (to indicate the number or position of each cut). The mould code is written on the edge of the mould using a permanent maker (Figure 7.4.1.1.2).

Figure 7.4.1.1.2. Metal plates (moulds) ready.

The resin: preparation of the fixing medium. A jet of previously stirred black colouring is poured into a paper cup (the jet comes out when the bottle is pressed softly only once). Then, 100 g of polyester resin is added and all is well mixed using a metal bar, avoiding the bubbles formation. Finally, 1 ml of catalyst/hardener is added to accelerate the reaction and to harden the resin faster, and all is mixed again (Figure 7.4.1.1.3). The variation in the amount of catalyst to add is no so important since the mixing is already pre-accelerated and the reaction will depend mainly on environmental factors (as temperature or humidity) in the lab.

Afterwards, the mixture is poured inside the mould placed on the mounting jig and under the extractor hood, until approximately half its capacity. The mould is placed on a holder, which is adapted to the video camera, and it is left to dry until the mixture is solid enough to place the otoliths there without sinking. The mixture must to be spongy but not sticky. This usually takes 30-60 minutes, depending on

factors such as the temperature or the resin viscosity. However, it must be checked with a punch every 10-15 minutes (Figure 7.4.1.1.3).

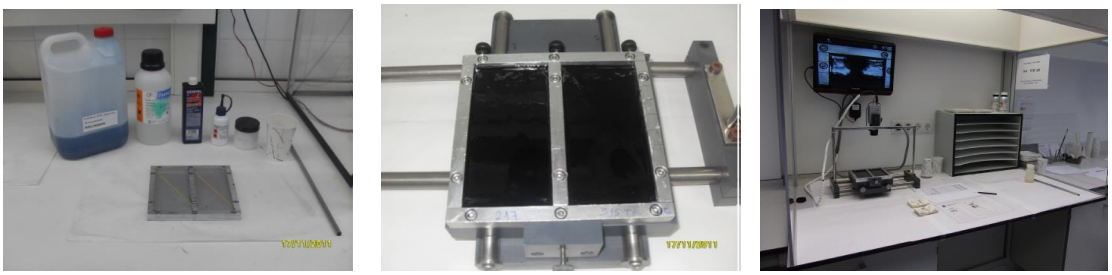


Figure 7.4.1.1.3. Preparation of the fixing medium (left), mounting jig with X-Y positioning (centre), and video camera and monitor with fixing jig (right).

Preparation of the slicing machine First of all, it must be check that the oil level of the slicing machine oil tank reaches the mark (Figure 7.4.1.1.4). If the oil level does not reach the mark, enough quantity of oil must be prepared to cover this level. The mixture is a dilution to 1/60 of oil in water (for preparing 1 litre: 16.66 ml of oil are mixed with 983.3 ml of water).

Figure 7.4.1.1.4. Slicing machine "Benetec OTO-LABCUT 230F"

7.4.1.2 Obtaining fine sections of otoliths/illicia

7.4.1.2.1 Placing the otoliths/illicia on the plates

Illicia preparation: The illicia for sectioning are selected and located by their code. The spare parts of each illicium, both the base and the tip, are removed just leaving the main part of the illicium to be cut (~2.5 cm of illicium). It is important to be careful with the skin of the illicia and it is recommended not to remove it from the illicium or take it away but without touching the skeletal part, so the external growth increments do not disappear.

Each illicium is placed on a laminated sheet marked with groups of lines (Figure 7.4.1.2.1.1). 10 illicia are placed in each line, separated in two groups of 5 (1a group and 1b group). Thereby, up to 50 illicia can be placed by mould.

The illicia are stuck with sellotape to avoid their movement (Figure 7.4.1.2.1.1), so they are placed in the cutting area. This cutting area is placed to a certain distance of the top of the illicia, and this distance depends on the fish length. So, for illicia of small anglerfish (e.g. ~25 cm), the cutting area will be located

to ~1 mm from the illicium top; and for larger fish (e.g. ~100 cm), it will be placed to a higher distance (~5 mm from the illicium top) (Figure 7.4.1.2.1.1).

The cutting area is marked over each illicium with a marker.

Figure 7.4.1.2.1.1. Preparation of the illicia before being mounted on resin.

Otoliths preparation: Otoliths of horse mackerel and Mediterranean horse mackerel are collected in 1.5 ml microtubes, labelled and stored in cardboard boxes (see section 4.3). From each sampling, those otoliths belonging to specimens with a length >26 cm are separated to be sectioned. Following the same numeration order than in the sampling, one of the otoliths is taken from its microtube and placed it in its corresponding plate well. It is important to take always the same otolith (right or left) for sectioning (in IEO Santander the right otolith is chosen whenever it is possible).

These otoliths are placed in a 50-well plastic plate with a lid (14 x 8 cm), with 10 columns and 6 lines of which it is used only the first five lines. These are named using the first five letters (A, B, C, D and E). At the same time, a file is created and printed with the biological data of each otolith and their position in the plate, which is the same position they will occupy in the resin plate.

Once the 50 wells are completed with their corresponding otoliths, a horizontal line is marked with a pencil over each otolith and through its nucleus (Figure 7.4.1.2.1.2).

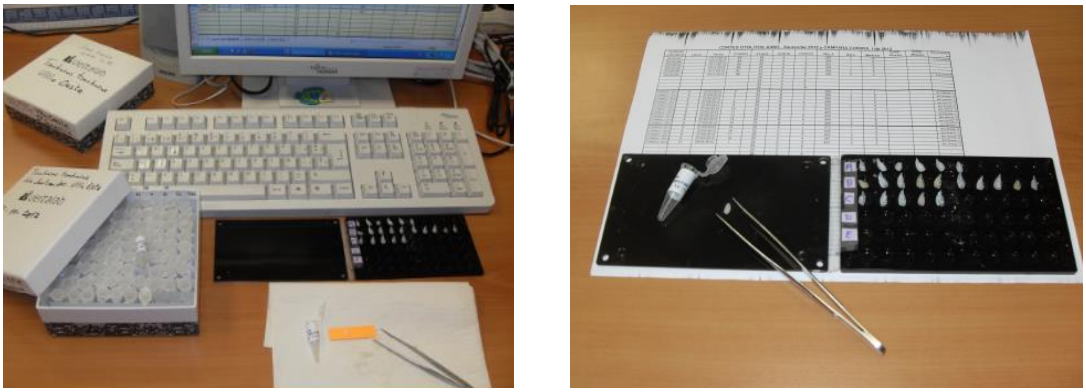


Figure 7.4.1.2.1.2. Preparation of otoliths before being mounted on resin.

Placing the otoliths/illicia on the plates: This process is performed using the mounting station for CS processing: on the mounting jig with X-Y positioning, using the video camera and monitor with fixing jig, and under an extractor hood.

With the video camera on, the side marks of the mould are aligned with the mark showed in the monitor. A metal sheet is placed over the mould so that the hand can be rested on it without staining it with the resin and making the process easier by this way.

The otoliths are placed, using tweezers, one by one following the line of the monitor, so that the painted mark over the otoliths/illicia coincides with the monitor line. 10 otoliths/illicia are placed per row, with a total of 50 otoliths/illicia by plate (Figure 7.4.1.2.1.3)

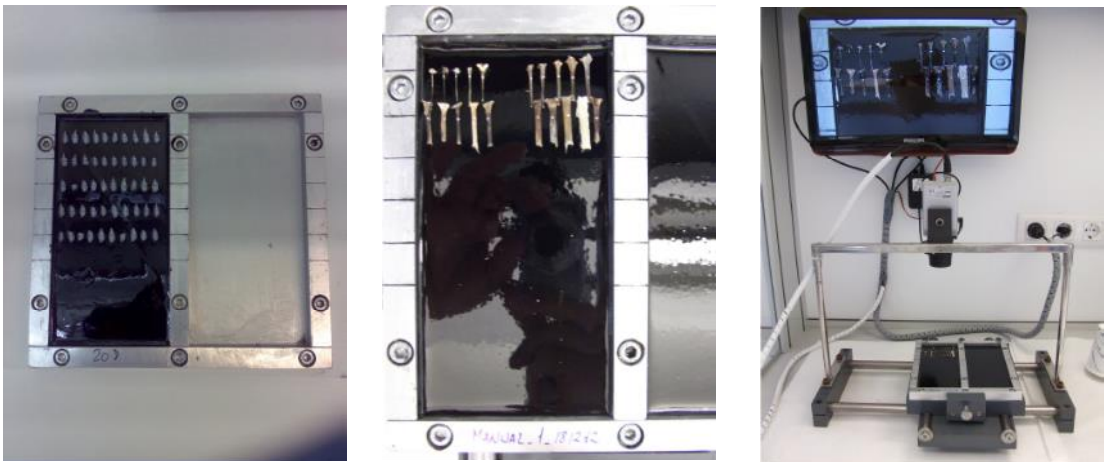


Figure 7.4.1.2.1.3. Placing the otoliths (left)/illicia (centre) on the resin; mounting jig, video camera and monitor with fixing jig (right).

When all the otoliths/illicia are placed on a mould, the process continues in the same way with the next one. Other mixture of polyester resin is prepared in the same way as above and very carefully poured over the otoliths to prevent their displacement. In the case of the illicia, the resin is poured over them in a zigzag way. Enough quantity of mixture has to be added to cover all the mould capacity. It is left to dry until is completely hard (~ 2 days).

Marking of the cutting lines on the resin blocks: Once the resin is dry, the cutting line is marked on it using a guide wire and a blade (or metallic cutter). The wire guide must coincide with the mould marks (Figure 7.4.1.2.1.4). The resin block code (the same code as the mould) is marked with a punch over the resin, at the bottom of each plate.

Figure 7.4.1.2.1.4. Marking of the cutting lines.

Removing the polyester blocks from their moulds: An allen wrench is used to remove the resin blocks from their moulds. The central column that separates the two moulds is unscrewed and both blocks are released using a sharp knock. The columns of both sides can be also removed if it is necessary (Figure 7.4.1.2.1.5). When the blocks are released, the mould is cleaned with acetone.

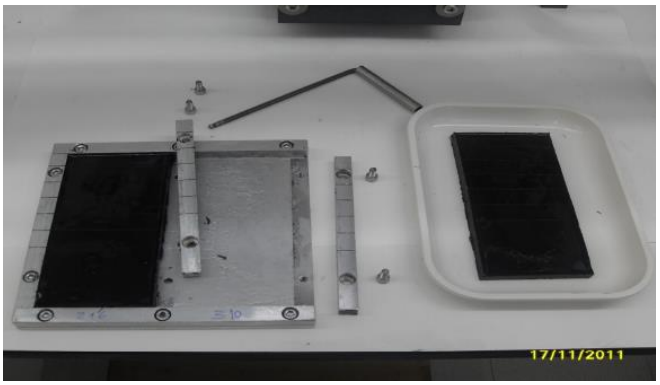


Figure 7.4.1.2.1.5 Removing of the polyester blocks.

7.4.1.2.2 Sectioning.

Switching on the slicing machine: The slicing machine (Benetec OTO-LABCUT 230F) is turned on (by rotating the red button on the back side to a vertical position). A control panel with a switch for the light, an ON/OFF button for the power cutting head (blade) and an ON/OFF button for the coolant (water jet) is located on the front side of the slicing machine. The light switch is turned ON. Then the power cutting head and the coolant buttons are also turned ON to wet the base, where the resin block is placed. It is necessary to turn the power cutting head and coolant buttons OFF before opening the machine cover (Figure 7.4.1.2.2.1).

Figure 7.4.1.2.2.1. Control panel of the slicing machine.

Placing the polyester blocks in the slicing machine: The metal pointer (or slicing guide) is lifted and the resin block is placed matching the pointer with the second mark of the block. The pointer is lowered only to align it with the block mark. Immediately after, it is lifted again so that it does not touch the block as it is very fragile and can be easily twisted. The nut is tightened until the block is immobilized (Figure 7.4.1.2.2.2).

Figure 7.4.1.2.2.2 Immobilizing of the polyester block inside the slicing machine.

Making of the sections: When the machine cover is closed the cutting head and coolant buttons are turned ON. Two jets of water are sent from a pair of small hoses and both are met over the cutting blade area. Both hoses must be kept always out of the cutting area. The lever is lowered with a light pressure until the cut is completely done. Then, the lever is lifted and the cutting head and coolant buttons are turned OFF.

The cover is opened and the first section is removed. The nut is loosened and a metal guide-pattern is put in the place of the first cut. The resin block is moved towards the metal guide and re-adjusted with the nut (Figure 7.4.1.2.2.3).

Figure 7.4.1.2.2.3 Re-adjusting of the polyester block after the first cut.

The cover is closed again and the cutting head and coolant buttons are turned ON. The lever is lowered with a light pressure once more. When the cut is completely done, the cutting head and coolant buttons are turned OFF before lifting the lever. The cut will be on the blade. The cut and the metal guide are removed, and the resin block is placed once more with the pointer over the second mark of the block. The nut is tightened until the block is immobilized. All the process is repeated until obtaining 5 cuts by block.

As they are being extracted, the resin sections are cleaned using absorbent paper. The pieces of each block are joined together using sellotape. This way, the block will be available in a right order if any section is needed to be repeated.

Each resin section is marked with a permanent marker. At least 3 positions of CS are marked to facilitate their further identification under the microscope. The positions 1, 5 and 9 are usually marked with a letter associated to the section. The block code, year and species are also marked in at least 2 of the 5 cuts.

Once all the sections have been done, the slicing machine is switched OFF by rotating the red button on the back side to a horizontal position. The slicing machine is cleaned and left with the cover open, if it is possible.

7.4.1.2.3 Fixing of the otoliths/illicia sections.

It is recommended to clean the new slides with acetone, so the sections can stick to them easily. Under an extractor hood, the resin sections are soaked inside a bottle of Eukitt (a transparent and fast-drying fixing medium) using tweezers. Once they are impregnated with Eukitt, the sections are placed on a slide. Five sections are usually placed on each slide. The slides are labeled with the resin block code, year and species (Figure 7.4.1.2.3.1). The slides are left to dry under the extractor hood for ~2 days.

Figure 7.4.1.2.3.1. Fixing of otoliths (centre) and illicia (right) sections.

During the process, the data of each resin block must be available, so it can be checked in case it is necessary (Figure 7.4.1.2.3.2).

Figure 7.4.1.2.3.2. Slide of otoliths sections with their corresponding data.

7.4.1.2.4 Storage of otoliths/illicia sections.

When they are dry, the slides with the CS sections are stored in cardboard boxes with dividers (otoliths) or specific boxes for storing microscope slides (illicia). The boxes are properly labeled (Figure 7.4.1.2.4.1).

Figure 7.4.1.2.4.1. Storage of slides with otoliths (left) and illicia (right) sections.

7.4.1.2.5 Maintenance and cleaning of the material

For a correct maintenance, the slicing machine must be cleaned after each use and the levels of oil must be replenished to be ready for the next time.

The resin residue remaining on the instruments used in the process are eliminated with acetone.

7.4.2 SANDING AND POLISHING

This preparation method is used to analyze the otolith microstructure and to study the daily growth of juveniles of anchovy, sardine, mackerel, white anglerfish and black anglerfish.

7.4.2.1 Material

- Adhesive: Crystalbond (Mounting wax 40-8150). It is a thermoplastic adhesive (a solid substance melted by heat). Using this adhesive, the otoliths are stuck and unstuck to the microscope slide as often as necessary during the sanding process.

- Heater: hotplate SBS-Cpc 135 D.

- Glass slides.

- Sanding pieces: teflon clamp tight to the slide.

- Polisher-devastating (devastator): polisher with one plate (Buehler meta serv 2000 model), with a diameter of 8" and speed of 50-500 rpm, allowing to obtain different thickness.

7.4.2.2 Process to obtain fine otolith sections

7.4.2.2.1 Otolith selection

The *sagitta* is the otolith most frequently used, although the *lapilli* is also used in some studies. It is recommended to use always the same kind of otolith (eg *sagitta* or *lapilli*) in the same daily growth study of a species, due to the difference in the initial growth among the three pairs of otoliths of each species.

When an otolith is crystalline, or when it is damaged while being manipulated, the other otolith of the pair can be used, as both otoliths have the same growth rate.

7.4.2.2.2 Otolith sticking on the slides

A clean slide is heated over the hotplate to a temperature between 85ºC - 100ºC (range in which the crystal bond is softened until it is transformed in liquid). The thermoplastic adhesive is applied over the slide (Figure 7.4.2.2.2.1). The slide is retired from the heat and the otolith is placed over the adhesive, ensuring that it is fixed in the right side. The sanding plane must be in parallel with the slide surface. The slide is labeled with the corresponding data.

Afterwards, the slide is adjusted to the sanding piece (created in IEO C.O. Santander) (Figure 7.4.2.2.2.1).

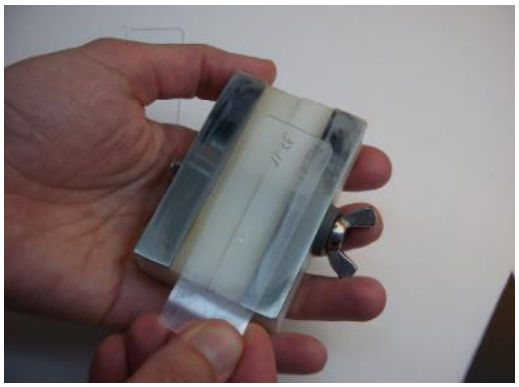


Figure 7.4.2.2.2.1 Sticking of the otoliths on the slide (left) and adjusting of the slide to the sanding piece (right).

7.4.2.2.3 Sanding and polishing

To obtain fine otolith sections firstly the otolith is sanded until the desired plane is obtained. Subsequently, the otolith is polished to eliminate their surface irregularities (Figure 7.4.2.2.3.1).

Figure 7.4.2.2.3.1 Sanding of the otolith

In the first stage, a grinding diamond disk (0.5 µm) is used to a variable speed. Later, the sections are polished with a polishing cloth lubricated in an aluminum oxide solution (0.05 µm) until the otolith nucleus and the growth micro-increments can be observed.

This process must be carefully performed. The polishing plane is checked continuously under the microscope, so the nucleus is not exceeded and the otolith edges are not eroded.

7.4.2.2.4 Sections storage

The polished sections (stuck to the slides) are stored in specific boxes for storing microscope slides (Figure 7.4.2.2.4.1). The boxes are labeled with the corresponding information. The storage of the otolith sections on slides facilitates their handling in the microscope observation process.

Figure7.4.2.2.4.1 Storage box of slides with polished otolith sections.

7.5 SUMMARY OF THE PREPARATION METHODS BY SPECIES

Table 7.5 shows a summary of the methods of CS preparation by species used in IEO for age estimation.

Tabla 7.5. Methods of CS preparation by species used in IEO for age estimation

Scientific name	CS	Annual age				Daily age
		Whole, immersed in liquid	Whole, mounted in resin	Burned	Sections mounted on black polyester resin	Sanded and polished
Engraulis encrasicolus	Otolith	X	X			X
Sardina pilchardus	Otolith		X			X
Scomber scombrus	Otolith		X			X
Scomber colias	Otolith		X			
Trachurus trachurus	Otolith	X		X	X	
Trachurus mediterraneus	Otolith	X			X	
Micromesistius poutassou	Otolith	X				
Lophius piscatorius	Illicium				X	X*
Lophius budegassa	Illicium				X	X*
Lepidorhombus whiffiagonis	Otolith	X				
Lepidorhombus boscii	Otolith	X				

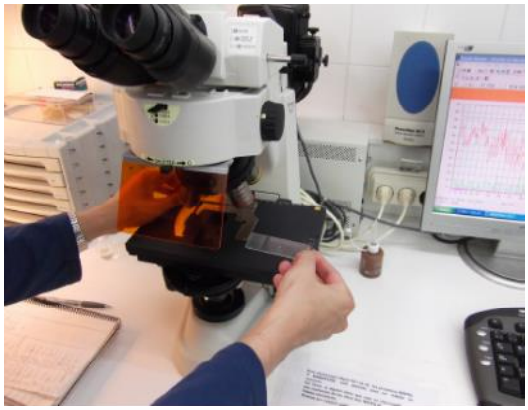
*Otolith

8 OBSERVATION TECHNIQUES

8.1 MICROSCOPE

A microscope (Nikon Eclipse 50i) with 5 objectives (from 4x to 100x) is used in Santander laboratory for the age estimation in CS (Figure 8.1.1). It includes unique phase contrast lens, for the study of small structures, and epi-fluorescence, that allows fluorescent microscopy analysis.

A digital camera (Sony DF-W SX910) is attached to the microscope. The analogical output signal is digitized by an acquisition card connected to a computer, which carries adapted an images analysis system (TNPC 4.1, VISILOG 6.4) that integrates the image capture, the data administration and the analysis.



The microscope is used for the age estimation of illicia sections of both species of anglerfish, and otolith sections for daily growth studies of anchovy, sardine, Atlantic mackerel and both species of anglerfish.

Figure 8.1.1. Microscope

8.2 BINOCULAR MICROSCOPE

A binocular microscope (Nikon SMZ1500) with a zoom ratio of 15x that covers a range from 0.75x to 11.25x, is also used in Santander laboratory for the age estimation of CS (Figure 8.2.1). A total magnification range from 3.75x to 540x can be obtained depending on the combination between eyepieces and lens selected, allowing the selection of the best magnification, extending from macro to micro-observation. It has a standard diascopic base C-DSS with a light source and an energy source (transformer) built in its body to dispose of reflected light and an additional external illuminator for projected light.

A digital camera (Nikon DS-5M) is attached to the binocular microscope. Its analog output signal is digitized by an acquisition card connected to a computer, which has a Nikon image analysis system (NIS-Elements Viewer 4.0), that integrates the image capture, the data management and the analysis.

The binocular microscope is used for the age estimation of whole otoliths of anchovy, Atlantic mackerel, chub mackerel, sardine, blue whiting, megrim and four spot megrim and otolith sections of horse mackerel and Mediterranean horse mackerel.

Figure 8.2.1. Binocular microscope

8.3 PROFILE PROJECTOR

A profile projector (Nikon Model 6C) is a measurement optical tool that magnifies the characteristics of a sample surface to allow the measurement on a circular/linear scale (Figure 8.3.1).

The profile projector projects a magnified image of the profile of an area, or the characteristics of a CS, on a screen, normally using diascopic illumination. Dimensions can be directly measured on the screen or can be compared with standard reference to the right magnification. Frequently, the screen has a grille that can be rotated 360 degrees in order to align with the edge shown on the screen. Points, positions, measurements and estimates can also be performed using a simple digital reading deviced.

The profile projector is also used for the age estimation of illicia sections of both species of anglerfish.

Figure 8.3.1. Profile projector

8.4 IMAGE ANALYZER

8.4.1 TNPC. DIGITAL PROCESSING OF CALCIFIED STRUCTURES

TNPC software (developed by IFREMER with the Noesis company) is used to acquire and interpret images of calcified objects, especially otoliths, for age estimation.

This system consists of 3 units (Figure 8.4.1.1):

- An image acquisition unit (camera Sony DFW-SX910).
- A central unit of treatment, storage and visualization: a computer with a large random access memory (min. 2Gb) for digital analysis with two screens (Screen 1: visualization of the CS; Screen 2: biological parameters or image analysis).
- A software unit (TNPC 4.1 software, VISILOG 6.4) for the CS image acquisition and treatment.

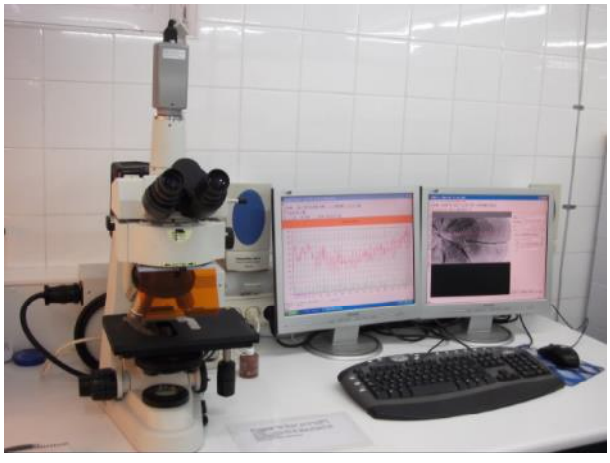


Figure 8.4.1.1. Computer aided system for age and growth estimation

This computer aided system for age and growth estimation is routinely used for acquisition, interpretation and saving of CS images (otolith section for daily growth studies). The CS image

acquisition and interpretation follow the standardised process used of TNPC software (Mahé *et al*., 2006).

- Image acquisition: CS images are directly obtained with the TNPC software (Figure 8.4.1.2). The whole image of the CS (otolith, illicium) observed at the microscope, once processed, is too large to be captured whole. Therefore, it has to be captured in several parts (of each CS) that will be subsequently joined with the macro option, to obtain an image of the whole CS. Previously to the interpretation; each image has to be calibrated. The image format is IM6, which allows the record of all the components related to the image.

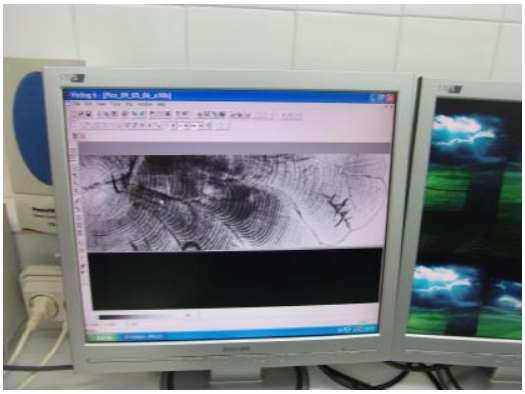


Figure 8.4.1.2. Image acquisition directly with TNPC software.

- Growth structures interpretation: age estimation is performed with aid of radials (Figure 8.4.1.3), which are directly integrated in the image database previously created. Each radial is recorded with 3 files (rad, pro and iid), which allows to measure the distance between the nucleus, the growth increments and the CS edge.

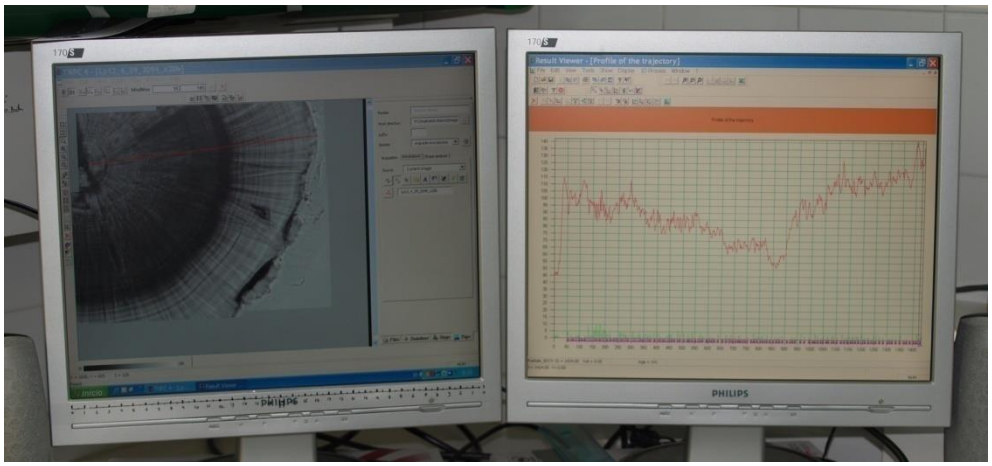


Figure 8.4.1.3: Interpretation of a CS using the TNPC software (growth increments identified by marked lines, Mahé *et al*, 2006).

8.4.2 NIS-ELEMENTS VIEWER 4.0

The NIS-Elements software (developed by Nikon) facilitates image acquisition, objects measurement and counting, databases and reports creation (for whole otoliths, otolith sections and illicia sections). The functionality core includes manual measurements of length and areas, automated counting of objects

using thresholds, feature restrictions and data export, as well as advanced macros constructor for a more complex customized programming (Figure 8.4.2.1).

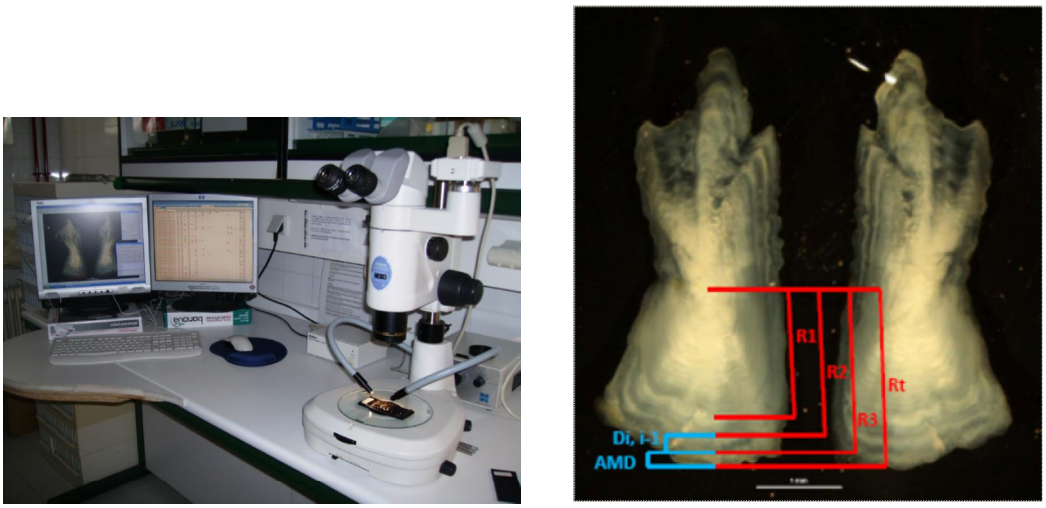


Figure 8.4.2.1. Image capture and biometric measurements of CS using the NIS-Elements Viewer 4.0 program, with a binocular microscope and an attached camera.

9 AGE ESTIMATION

9.1 CALCIFIED STRUCTURES IDENTIFICATION

The calcified material is settled in concentric layers around the CS nucleus; a process that continues even after the fish has stopped growing in length (Campana and Thorrold, 2001). This material consists of alternate layers (opaque and translucent/hyaline) that differ in density and optical properties, and their appearance depends on the microscope and light source used to observe them. When they are observed in a dark background with reflected light, the translucent growth areas are seen as dark areas whereas the opaque growth areas are seen as light areas. The opposite occurs when they are observed with transmitted light.

In general, a year of a CS growth consists in an opaque growth area and a translucent growth area (Figure 9.1.1). In temperate water fishes, the opaque growth area is typically wider and goes with quick growth periods, whereas the translucent growth area is typically tighter and goes with slow growth periods. Therefore, the opaque area is sometimes known as the “summer area” and the translucent area as “winter area”. However, these names can be a bit confusing as the growth of the translucent area sometimes occurs in spring or at the beginning of summer. The exact moment when the growth areas are settled depends on a series of factors such as the fish age, temperature, geographic area and species.

Age readers often use the name “ring” to describe an annual growth area (typically the translucent growth area). However, the word “annual mark” or “annulus” is more appropriate, since as Panfili *et al*. (2002) pointed out.

The assumption of the annual growth area deposit is fundamental for age estimation, as it provides a time mark that corresponds to CS growth and fish age. This way, the name “annual mark” or “annulus” refers to any growth area settled annually, the name “ring” can be used, although less frequently, to

refer to concentric growth areas that can be or not be deposited over an annual base. False rings or "checks" are irregular translucent growth areas that sometimes appear in the CS (Figure 9.1.1). Checks are no annual marks and, often, they are generated as a result of the physiologic or environmental stress that the fish has experienced along its life. Checks can correspond with events of the fish life history, like colonization, migration, sex maturity or spawning (Penttila and Dery, 1988). Checks can be usually differentiated from real annual marks by their irregular width, their relatively weak appearance and their lack of continuity along the CS (Figure 9.1.1). Double rings are a special check type, where two or more translucent growth areas, closely spaced, are settled in one year alone.

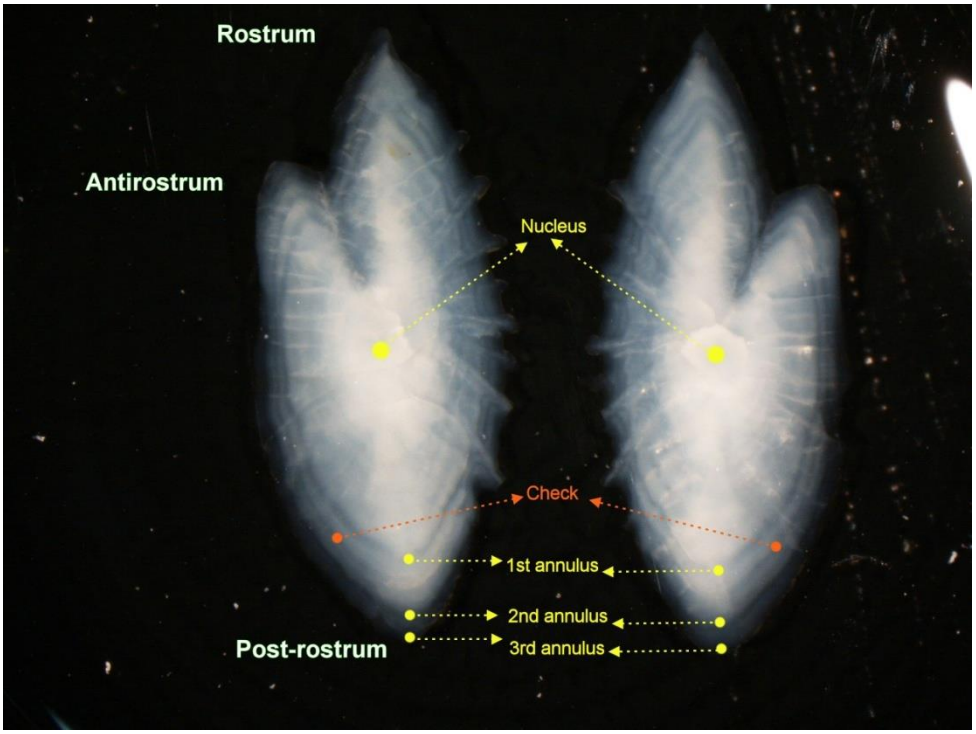


Figure 9.1.1. 3-years-old anchovy otolith showing growth areas and the terminology used referring to the otolith structures.

The number of checks observed in the CS varies depending on the species and the geographic area and can increase the difficulty in the growth pattern interpretation. To be familiar with the general growth pattern of a species through the experience helps age readers to differentiate between checks and annuli. IEO readers usually count the growth translucent areas from the nucleus to the edge for age estimation. It is necessary to identify the checks to avoid their inclusion in the final age estimation. Age readers also have to interpret the opaque/hyaline growth of the CS edge. The identification of the first annual mark is a critical step in age estimation. In several species, checks are frequently observed within the first years of life, frequently related with diet or environment changes, so information about the life history of the species can be useful to guide in age estimation. The knowledge of the spawning period can provide useful information to the age readers about the expected growth until the first annulus formation. I.e., species that spawn early in the year have more time to settle the opaque material around the nucleus, which results in a wider first annulus than the species that spawn later.

Morphology helps age readers to identify the CS reading axes. A reading axe is a track from the nucleus to the CS edge along which an age reader counts annual marks. Depending on the species, some axes are more suitable for age estimation than others. Illicia sections are rounded in shape, but otoliths are not spherical, so the new calcified material is not settled in the same proportion between the axes; some axes growth faster than others, which can influence in the type of CS preparation method for age

estimation. When possible, the most advisable should be to compare the age estimations from different axes within the same CS to obtain consistent annual marks recounts.

9.2 GROWTH STRUCTURES INTERPRETATION

The assignation of the 1st January as the birth date was agreed by international convention for many stocks/species in Northeast Atlantic waters, with independence of the real spawning date. This international convention of birth date allows to determinate the birth year and to assign each fish with the right cohort, which is an integral part of an efficient assessment.

The birth date convention can affect the age estimation when the opaque growth is observed at the edge of a CS. Age readers have to assign the right year to the growth area observed at the edge. In other words, age readers have to decide if the opaque growth observed at the edge was generated along the year in which the fish was captured or during the previous year. The catch date is a vital piece of information that allows the age readers to make this decision. I.e., a fish captured in August will usually have an opaque growth at the edge, and so the age estimation is equivalent to the number of translucent growth areas observed in the CS. However, a fish captured in January with the same amount of opaque growth at the edge (but in which the translucent growth area that mark the end of the previous year is not jet settled), the opaque growth at the edge of its CS is attributed probably to the previous year growth cycle. The estimated age is one year more than the number of translucent growth areas observed, as 1st January is already happened. In the same case but for a fish captured in December, it would be assigned an age one year younger.

Therefore, once all CS growth marks are justified, the following information is needed to assign the age to an individual:

- Catch date
- Birth date
- Growth mark considered
- Nature of the CS edge

The general criteria used to the age estimation of the species here described are:

- Birth date is considered to be 1st January.
- These species are assumed to form each year a translucent and an opaque area.
- Interpretation of the age: An overall age assignation criteria is described in Table 9.2.1. The fish will have the same number of years as the number (n) of complete hyaline bands (H) observed, but with the following considerations, according to the year semester in which the fish was caught and the CS edge type. The following main information should also be considered: i) date of capture; ii) peak of spawning period; iii) periods of seasonal increment formation (ICES, 2013).

Table 9.2.1: Overall criteria used to age estimation based in the catch date and CS edge type.

Year period	Edge type	Age assignation
January-June	Opaque in adults	Years=(n+1) H annuli
	New Opaque in juveniles	Years=(n) H annuli
	Hyaline (H)	Years=(n) H annuli
July-December	New Opaque	Years=(n) H annuli
	New Hyaline	Years=(n-1) H annuli

9.3 MORPHOMETRIC MEASURES

CS measures are taken for corroboration studies and for a better application of the interpretation criteria and the observation of the growth pattern (Figure 8.4.2.1). The measures most frequently used are:

- CS total diameter (Dt),

- CS total radius (Rt),

- 1st annulus diameter (D1), 2nd annulus diameter (D2), 3rd annulus diameter (D3), ...

- 1st annulus radius (R1), 2nd annulus radius (R2), 3rd annulus radius (R3), ...

- False rings (or checks) radius (F1, F2, ...),

- Marginal increment (or best: relative marginal increment): measurement of the distance between the last hyaline annulus and the CS edge and the distance between the last two hyaline annuli.

Small otoliths can be measured under a binocular microscope using a millimetre reticule whether bigger otoliths (> 1 cm) can be measured with a gauge. However, image analyzers are used nowadays. Image analysis is a technique that allows the acquisition of the CS dimensions in an objective way and allows the calculation of the measurements (Dt, Rt, Di, Ri,...). The image is captured by a high resolution photographic camera attached to the binocular microscope and is transformed and analyzed using an image analysis system in a computer (see section 8).

9.4 QUALITY CONTROL AND AGE VERIFICATION

Verification confirms the consistency of the age interpretation, i.e. the repeatability and/or precision of a numerical interpretation that may be independent of the age. Considerable efforts are made by international committees to standardize the age interpretations (Panfili *et al*., 2002). ICES WKNARC-1 and WKNARC-2 recommended internal (within each research institute) and external (among institutes) quality controls to confirm the consistency of the age interpretation (ICES, 2011a; 2013a).

Age interpretation is a process that requires as much knowledge as experience. Age estimation process has to be internally determinate and the estimated ages must be internally and externally calibrated with other expert readers.

The age readers experience is an essential factor to obtain accurate age estimates and to reduce possible bias. Usually, there is more than one age reader for most species treated in IEO. In the case in which a single age reader is available, the age estimations are performed twice.

In addition to the estimated age, the quality (or credibility) of each age estimation is also assigned according to the "3 point grading system" recommended by WKNARC-1 and WKNARC-2 (ICES, 2011a; 2013a). Three possible results of age quality (AQ) are distinguished:

AQ1 (>75% reliability): *CS* easy to age whose estimated age was not doubtful.

AQ2 (25-75% reliability): *CS* difficult to age. The reader doubts between two ages, or only one age is estimated but with some doubts.

AQ3 (<25% reliability): *CS* impossible or very difficult to age, with doubts among three or more possible ages. These CS are excluded from further analysis.

When possible, two age readers by species estimate the age of each CS in IEO. This way if the age reader in charge of a species is absent, he/she can be replaced by the second reader. Independent age estimates of the same CS are performed by both age readers. This way, the accuracy between both readers can be estimated. The age estimation of a given CS is only accepted if both readers estimates agree. When a disagreement appears between both estimations, a new estimation is performed. If the disagreement persists, the CS is rejected. Illegible CS are also rejected.

When a single age reader is available, two estimates are performed at different times, following the same process than when two readers are available. The following analyses also may contribute to check the consistency of the age estimation:

1. Tendency in the growth pattern. This method is used to verify that the growth increments are consistent in their location inside the growth pattern of the otolith. The radius of each growth increment of the CS used in the CS-fish relationship is measured. The radius frequency of each translucent increment (to the exterior edge of the hyaline increment) is graphed for each age, by sex and area. This way it is possible to know if the increment formation pattern is constant or not, independently of the age.

2. Another method that uses the tendencies in the growth pattern is to compare between the length/age by the CS age estimations and the length/age obtained by back-calculation.

3. Different estimations of an age reader: Always it is necessary to read more than once the same CS to reduce some subjectivity. There are some statistic indexes and test to determinate the agreement degree of the age estimations.

9.4.1 INTERNAL CALIBRATIONS.

From time to time, internal calibrations between age readers of a species/stock are performed to secure the accuracy and quality of their age estimates. Each age reader estimates the age independently of the other readers, since the analysis of the CS exchange results gives a real view of the similarity and differences among age readers. The analysis results are set in a group of tables and figures where the results of the age readers are shown. The results are discussed and conclusions and recommendations

are obtained, paying special attention to those related to some corrective measures for improving the age estimation of the age readers.

9.4.2. EXTERNAL CALIBRATIONS (INTERNATIONAL EXCHANGES AND WORKSHOPS).

IEO age readers participate and have actively participated in every calibration exchange and workshop of this manual species that have been carried out in Europe (ICES, 2011b; 2012b; 2013a). Some of them have been coordinated by IEO personal. For pelagic and benthic species in ICES waters there are over 30 reports available in the repository of the ICES PGCCDBS (http://www.ices.dk/community/Pages/PGCCDBS-doc-repository.aspx)

9.5 TRAINING

When a new age reader is incorporated to an age estimation team, he/she is trained by the expert reader, performing internal calibrations between them, in collaboration with the scientist in charge. When the new reader reaches enough experience, as long as the internal calibrations with the expert reader are satisfactory, the age estimates of this new reader can be incorporated to the species data base.

9.6 AGE VALIDATION

The determination of the age structure of a fish population based in CS is founded in its uniform periodicity, the formation synchrony in a determined cohort of the population and in the deposition of a new growth structure at regular time intervals. At the beginning of a population study, it must be determined that the basic criteria of periodicity and synchrony are observed (Morales-Nin, 1992).

To corroborate that CS are valid for age estimation, it is fundamental to prove that there is a year period in which the hyaline edge is formed in the otolith (annuli are formed annually); that the frequency distribution of the distance between the otolith nucleus and each annulus is unimodal and their number increases with the fish age; and that there is a correlation between the fish length and the otolith length, and between the fish length and the annuli number. To prove all this, it is necessary to perform validation studies.

Validation of an absolute age is equivalent to determine the accuracy of an age estimate. The distinction between validating the periodicity of growth increment formation and absolute age is important (Campana, 2001).

It is necessary to check that the considered growth increments reflect the periodic variations of the growth rate. To validate the interpretation of the otolith annuli, it can be considered the following types of validation: direct, semi-direct and indirect validation. Most of these methods are summarized by Campana (2001) and Panfili *et al*. (2001).

9.6.1. DIRECT AGE VALIDATION.

Direct validation should be understood as the validation of the absolute age. The direct methods are not very often used because of the requirements needed to use them. The following are some of the main methods (see Campana, 2011, for more information).

1. Release of known age and marked fish
2. Bomb radiocarbon
3. Mark-recapture of chemically-tagged fish
4. Radiochemical dating

9.6.2. SEMI-DIRECT AGE VALIDATION.

1. Connection between otolith length and fish length: To study the connection between otolith length and fish length of a species, the total diameter and total radius of the otoliths are measured to a length rate of 1 cm, by sex and area, , always in the same otolith (the right or the left, but always the same).
2. Periodicity of the annuli formation: To determinate the periodicity of the annual growth increment formation, the otolith edge nature is observed (opaque/hyaline) monthly. It is the most commonly used of the validation methodologies. There are two methods:
   - Qualitative: The periodicity of the annuli formation is assigned according to the number of modes of the monthly percentage of otolith translucent (hyaline) edge along a year. This analysis also can be performed by age groups.
   - Quantitative: This method consists of the measure of the distance between the last annulus (hyaline annulus) and the otolith edge, and is called Marginal Increment (MI). Here, it can be seen the period in which the distance between the last winter annulus (hyaline annulus) and the otolith edge increases. Inversely, it can be seen when the marginal increment decreases. This will confirm the identification of the hyaline annuli as winter marks and their possible use as annual increments to age determination. The measures axis and marks description used have to be rigorously standardized (from the beginning, it has to be establish a criterion that has to be followed). The Absolute Marginal Distance (AMD) is the distance between the last hyaline annulusand the otolith edge; the Relative Marginal Distance (RMD) is the quotient between AMD and the distance between the last two hyaline annulus (see Panfili *et al.*, 2002): RMD=AMD/Di,i-1. It is recommended to use AMD only for each age class, this way the differences in the growth rate between individuals are taken into account. RMD, which is expressed in percentages, compensates the effects of the growth reduction with the age. It is less sensitive to the variability of the growth rate since it is a relative measure. According to Panfili *et al.*, 2002, it is more difficult to follow the cycle with AMD than with RMD. In general, quantitative methods are only used with fishes from 1 year-old onwards (not with 0-year-old fishes). With RMD all ages are mixed and this way the growth reduction with the age is not taken into account.

9.6.3. INDIRECT AGE VALIDATION OR AGE CORROBORATION.

These methods are based in information that corroborates the age interpretation but the periodicity of the growth increase is not validated by them. Methods for age corroboration are not equivalent to those for direct age validation, since corroboratory methods support or are correlated with a particular method of ageing, but are not directly or logically linked (Campana, 2001).

1. Tag-recapture analysis. The growth *rate*s estimated from recaptured specimens can be compared with those derived from annulus counts. The growth comparison is by inference since none of the recaptured fish are of known age (Campana, 2001).
2. Analisys of length distributions: The length composition of the catch is analyzed to identify the present age groups, based on the assumption that each age group has a normal length distribution and different modal lengths. Age 0 goes with the smallest mode present in the sample obtained after the spawning period, while subsequent modes go with age 1, 2, etc. This

method depends on the growth variability and the spawning period of the species, as lengths and modes can to overlap and only the first ages could be validated.

3. Tracking year-classes. This method compares the interval between periodic samples (i.e. yearly samples) and the increase in the apparent modal age of a recruitment pulse as determined through annulus counts (Campana, 2001). An age estimation method is accurate if the age composition of exceptionally good or weak year classes can be tracked over a long period of time (Panfili *et al*., 2002).
4. Daily growth studies: Daily increments counts between presumed annuli can provide strong corroboration of the frequency of the annuli formation (Campana 2001). The preparation and observation technique is a complex and time-consuming process that requires highly specialized staff. Difficulties may occur due to the misinterpretation of the daily increments, or an interrupted growth increment sequence.

10 MANUALS FOR SPECIFIC SPECIES: ANNEXES 1 - 11

11 PROTOCOLS SCHEMES: ANNEXES 12-14

Annex 1: ANCHOVY (*ENGRAULIS ENCRASICOLUS*)

Figure 1.1. Anchovy (*Engraulis encrasicolus*).

1.1 SAMPLING PROGRAM FOR AGE ESTIMATION

1.1.1 ANNUAL AGE

Samplings for age determination of anchovy are performed in IEO from the late 70s, from both, commercial catches and research surveys. The number of otoliths collected in 2012 is shown in Table 1.1.1.1

Table 1.1.1.1. Number of anchovy otoliths by stock collected from the commercial fleet and research surveys in 2012.

Stock	Commercial fleet	Research surveys	Total
Bay of Biscay (Subarea VIII)	1337	497	1834
Division IXa (IXa North)	90	99	189
Total	**1427**	**596**	**2023**

1.1.2 DAILY AGE

During the years between 2006 and 2009, juvenile anchovy otoliths were collected from research surveys for daily growth studies and otolith microstructure analysis (Table 1.1.2.1)

Table 1.1.2.1 Number of juvenile anchovy otoliths of the Bay of Biscay stock (Subarea VIII) collected for daily growth studies from research surveys during 2006-2009.

Year	Research surveys
2006	897
2007	1010
2008	788
2009	1303
Total	**3998**

1.2 OTOLITH EXTRACTION AND STORAGE

Anchovy otoliths are easy to extract from the fish by cutting through the top of the head (Figure 1.2.1, Figure 1.2.2). It is important to cut very carefully to avoid the otoliths damage as they are very fragile. The organic residues are very carefully removed with tweezers from the otoliths. Then, the otoliths are rinsed with distilled water and stored in black plastic plates with cover.

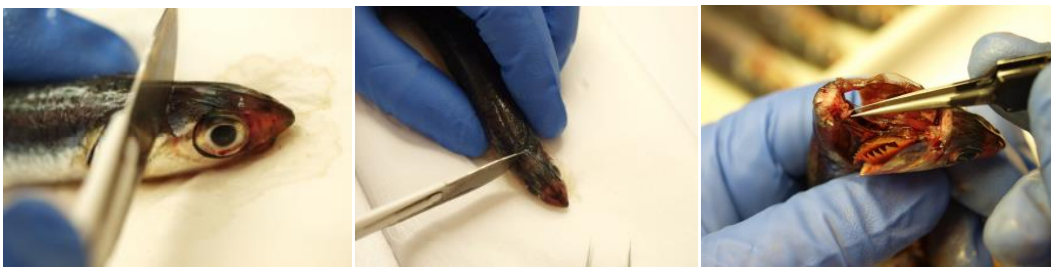


Figure 1.2.1. Extraction of anchovy otoliths.

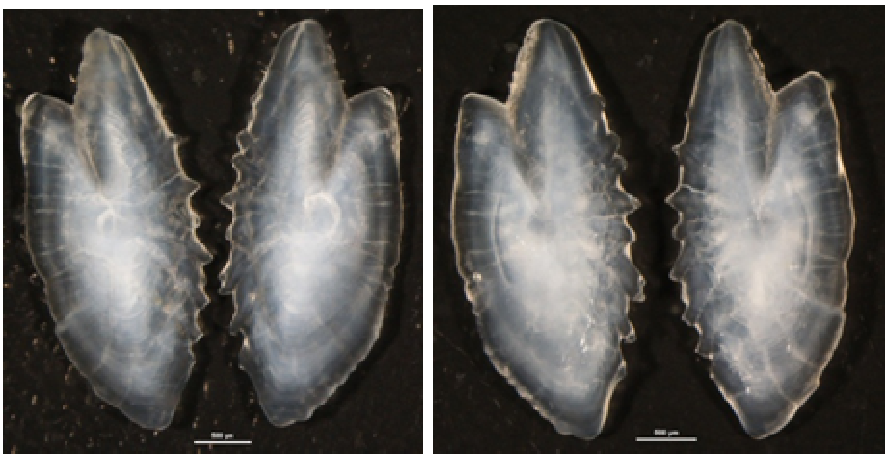


Figure 1.2.2. Anchovy otoliths: ventral and dorsal view.

1.3 OTOLITH PREPARATION METHOD

1.3.1 ANNUAL AGE

Like most small pelagic species, anchovy otoliths are observed under a binocular microscope inside black plastic plates, mounted on non-plastic transparent resin (Eukitt). In recent years, they are also observed immersed in water (see section 7.2).

1.3.2 DAILY AGE

For daily growth studies of juvenile anchovy, otolith sections are observed. In the case of larvae, whole otoliths are observed.

The sagitta otoliths extracted from juveniles are cut into sections by a sanding and polishing process (Secor *et al.*, 1992). Each otolith section is processed on the sagittal plane with respect to the fish (see section 7.4.2).

1.4 AGE ESTIMATION METHOD

1.4.1 ANNUAL AGE

Observation: Binocular microscope. Digital images are used for biometric measures and, in some cases, for age estimation.

Illumination: Reflected light (using fiber optic illuminators).

Magnification: Between 20x and 40x magnification, depending on the otolith size.

Reading axes: Translucent annuli (hyaline) are counted, preferably in the anterior (*rostrum*) and posterior (*post-rostrum*) part of the otolith.

Age estimation criteria: Anchovy age estimation criteria were recommended by ICES (2009). This method was adopted explicitly in the Workshop of anchovy age in 2002 (Uriarte *et al*., 2002) based in the validation study of Uriarte (2002a) and Uriarte *et al*. (2014) (Figure 1.4.1.1). This method is based on the knowledge of the standard pattern of annual growth in anchovy otoliths, the process of edge formation, and the most common false growth increments (*checks*) which are expected to be found. A set of an opaque and hyaline zone corresponds to an annual growth zone (annulus). The date of birth is conventionally assumed to be the 1st of January and the fish is assigned to a year class on this basis (if an otolith is collected during the first semester the age group correspond to the number of hyaline zones, if the otolith is collected from a fish caught during the second semester, the hyaline edge will not be considered as an annulus).

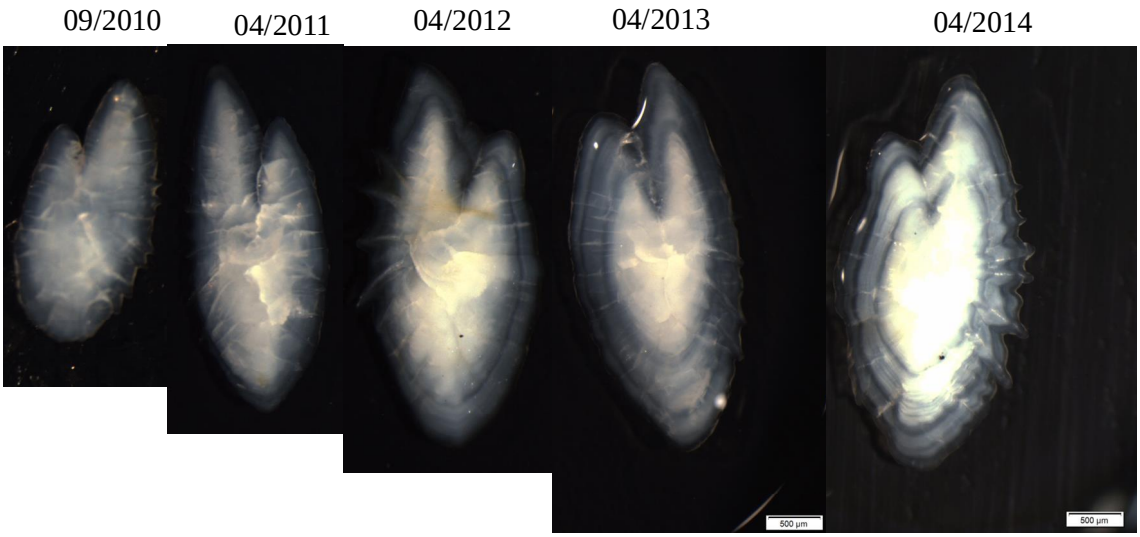


Figure 1.4.1.1. Anchovy otoliths (from left to right: ages 0 to 4) of the most recent year class (2010). The date of capture is detailed over the photos (Uriarte *et al*., 2014).

Interpretation difficulties: These difficulties could be explained by: 1) the first annulus position; 2) the otolith edge identification (opaque or hyaline); 3) the presence of false growth increments (checks) (Figure 1.4.1.2).

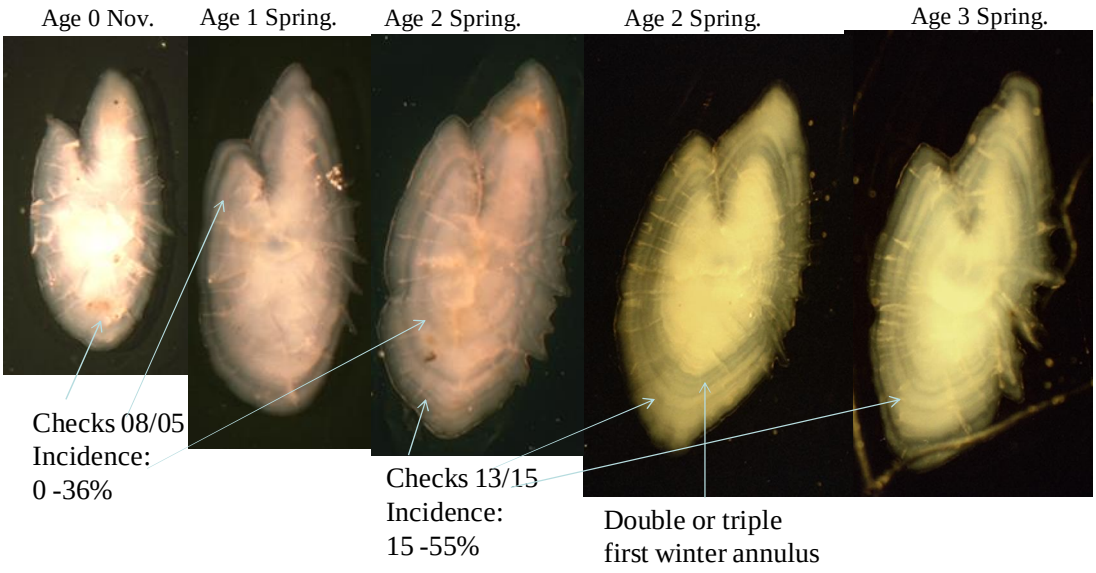


Figure 1.4.1.2. Anchovy otoliths with cheks (Uriarte *et al.,*2014).

1.4.2 DAILY AGE

Observation: Otolith sections viewed through a microscope connected to an image analyzer (Figure 1.4.2.1).

Magnification: In larvae, the whole otolith is observed at 1000x magnification along the maximum growth axis; in juveniles, the otolith nucleus is observed at 1000x magnification. The rest of the otolith is observed by two ways, depending on the reading criteria (see below): if the GBR criterion is applied, the otolith is observed at x100 or x200 magnification; if the IMR criterion is followed, x400 magnification is used. Close to the otolith margin, at the beginning of the formation of the first translucent zone, the magnification used is x630.

Reading axes: the age estimation is performed along the post-rostrum axis, which is generally the maximum growth axis in sagittal otoliths. The otolith radius is measured along the post-rostrum axis. Otolith microincrements are counted starting in the hatch check until the edge. The first evident increment corresponds to the hatch check, at a distance from the primordium between 3.5 and 5 µm (Aldanondo *et al*., 2008).

Age estimation criteria: For daily increment interpretation, two different criteria have been suggested: using the known as the group band reading (GBR) criterion, the reader counts every repetitive cyclic set of growth bands (usually two, but occasionally more) as a single daily increment, assuming that they are sub-daily marks. And, using the other criterion, known as the individual mark reading (IMR) criterion, each increment, regardless of its appearance, is counted as a single daily increment. According to Cermeño *et al*. (2008), the GBR criterion is the most reliable method for ageing European anchovy. In the Bay of Biscay, the application of this methodology (GBR) for anchovy otolith sections was agreed, irrespective of the season and geographical area (Morales-Nin *et al*., 2010; SARDONE, 2010; ICES, 2013b).

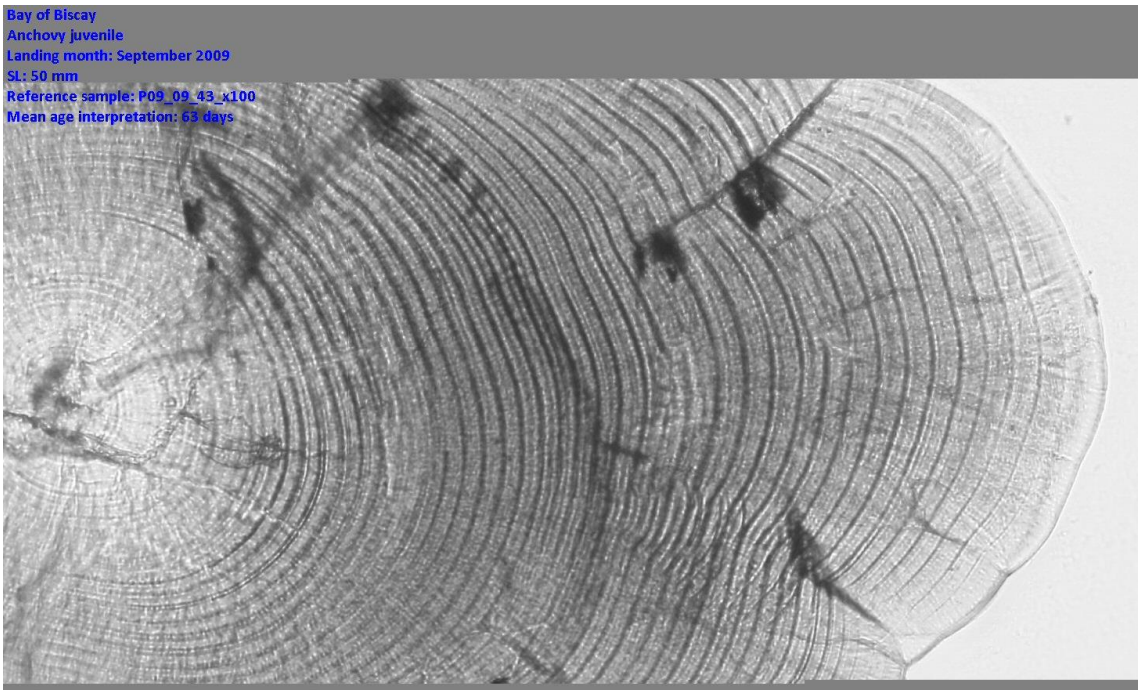


Figure 1.4.2.1. Image of an anchovy otolith section obtained for daily growth estimation.

Interpretation difficulties: These difficulties could be explain by: 1) difficulties in the interpretation of sub-daily increments, double structures or band zones; 2) unclear images, in which is difficult to interpret correctly the daily growth pattern due to under-or over-polishing, poor image acquisition or calibration problems. It is very important to obtain clear images to interpret properly daily micro increments in this species.

1.5 AGE ACCURACY, VALIDATION AND CORROBORATION

1.5.1 ANNUAL AGE

The periodicity of the growth increment formation in anchovy has been validated by the analysis of the otolith edge type in the Bay of Biscay (Uriarte, 2002a). The season in which the hyaline annuli are formed was determined by the examination of the frequency of the distribution of the otolith edge types throughout the year. The hyaline edge is mostly predominant from October to March-April while the opaque edge predominates in the rest of the year.

The age estimation criteria of anchovy were corroborated (or indirectly validated) by tracking the year-classes abundance indices between the years 1982-1989 in research surveys in the Bay of Biscay (Uriarte 2002a; Uriarte *et al.*, 2002; 2014).

Based on different daily growth studies, the position of the first annulus has been validated and the position of the first false ring or check has been corroborated in anchovy in the Bay of Biscay (ICES, 2013b). Annual increment deposition in the otoliths of young-of-the-year European anchovy has been validated (Aldanondo *et al.*, 2013). Early anchovy juveniles were maintained in captivity from October 2012 until April 2013 and the first annulus was validated using daily increments counts. According to that, the first opaque band is completed in October-November, whereas the translucent band is formed by March-April. As a reference point, the first annulus would be at 1156 µm (± 70 µm) from the

primordium. All hyaline growth increments that are at a distance of less than 850 µm (± 100µm) should be considered as a check (Hernandez *et al*., 2013).

1.5.1 DAILY AGE

The daily periodicity of micro-increment deposition has been validated in early life stages of anchovy, though in a few areas of distribution. As far as anchovy is concerned, validation studies have been carried out exclusively for individuals from Bay of Biscay. In particular, the daily increment deposition has been validated in hatched eggs and larvae reared in the laboratory under different temperature conditions (Aldanondo *et al*., 2008), as well as in wild juveniles marked by immersion in oxytetracycline hydrochloride (OTC) and reared until reaching adulthood over a period of 2 years (Cermeño *et al*., 2003).

1.6 AGE PRECISION, VERIFICATION AND QUALITY CONTROL

1.6.1 ANNUAL AGE

Anchovy age estimation criteria used by IEO readers have been externally verified by international otoliths exchanges and workshops (Astudillo *et al*., 1990; Villamor and Uriarte, 1996; Uriarte, 2002a; Uriarte *et al*., 2002, 2006 and 2007; ICES, 2009). Current IEO age readers have participated in recent otoliths exchanges and workshops, showing good results in agreement, precision and relative accuracy (ICES, 2009).

The age estimation of the same otolith is performed by two readers, or else, by one experienced reader, but then, the age of each otolith is estimated twice, in two separate occasions. The age estimation of a given otolith is accepted only if both estimations coincide. When a discrepancy between them is found, a third estimation is carried out. If the discrepancy persists, the otolith is rejected. Illegible otoliths are also rejected.

In addition to the age estimation, the quality (or credibility) of each age estimation is also assigned according to the "3 point grading system" recommended in WKNARC-1 and WKNARC-2 (ICES, 2011a; 2013a), as it is described in section 9.4.

1.6.2 DAILY AGE

To test the quality control of daily age estimations, internal (reading procedure) and external (planned otoliths exchanges and working groups) practices (SARDONE, 2010; ICES, 2013b) are developed by IEO. The internal practice concerns mainly to repeated age estimates performed independently by one or more readers, to check the precision in the estimations. Generally, otoliths are discarded when the reading precision shows an error higher than 5-10%.

Annex 2: SARDINE (*SARDINA PILCHARDUS*)

Figure 2.1. Sardine (*Sardina pilchardus*).

2.1 SAMPLING PROGRAM FOR AGE ESTIMATION

2.1.1 ANNUAL AGE

Samplings for age determination of sardine are performed in IEO since the early 80s, from both, commercial catches and research surveys. The number of otoliths collected in 2012 is shown in Table 2.1.1.1

Table 2.1.1.1. Number of sardine otoliths collected by stock from the commercial fleet and research surveys in 2012.

Stock	Commercial fleet	Research surveys	Total
Divisions VIIIc-IXa	3123	301	3424
Division VIIIb	-	80	80
Total	**3123**	**381**	**3504**

2.1.2 DAILY AGE

Daily growth studies in Atlantic Iberian sardine started in the early 90'. Studies were directed to validate the daily growth increments deposition in culture larvae and to estimate the birthdate in individuals from natural environments (Alvárez and Butler, 1992; Alemany and Alvárez, 1994; Alvárez and Alemany, 1997; Alvarez, 2005).

In recent years, larvae and juvenile (up to 18 months) of sardina have been obtained in captivity from fertilized eggs captured in the wild (Iglesias and Fuertes, 2014). Otoliths of known age from these individuals are being currently analyzed.

2.2 OTOLITH EXTRACTION AND STORAGE

Sardine otoliths are easy to extract from the fish by cutting through the top of the head (Figure 2.2.1; Figure 2.2.2). They are less fragile than mackerel or anchovy otoliths and, in comparison, are larger and tend to be exposed after the cut. The organic residues are very carefully removed with tweezers from the otoliths. Then, the otoliths are rinsed with distilled water and stored in black plastic plates with cover.

Figure 2.2.1. Extraction of sardine otoliths.

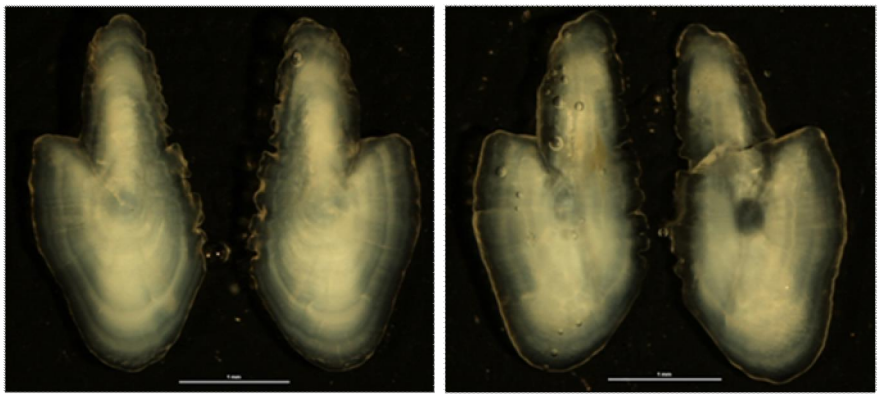


Figure 2.2.2. Sardine otoliths: ventral and dorsal view.

2.3 OTOLITH PREPARATION METHOD

2.3.1 ANNUAL AGE

Like most small pelagic species, sardine otoliths are observed under a binocular microscope inside black plastic plates, mounted on transparent resin (see section 7.1).

2.3.2 DAILY AGE

For daily growth studies of juvenile sardine, otolith sections are observed. In the case of larvae, whole otoliths are observed.

Sagitta otoliths extracted from juveniles and pre-recruits are cut into sections by a sanding and polishing process (Secor *et al*., 1992). Each otolith section is processed on the sagittal plane with respect to the fish (see section 7.4.2).

2.4 AGE ESTIMATION METHOD

2.4.1 ANNUAL AGE

Observation: Binocular microscope. Also, in some cases and for biometric measures, the age is estimated using digital images.

Illumination: Reflected light (using fiber optic illuminators).

Magnification: Using 20x magnification. The magnification can be increased near the otolith edge to improve the discrimination of narrow annuli in older individuals.

Reading axes: Translucent annuli are counted and the edge type is determined in the posterior (post-rostrum) otolith region, where annuli are generally clearer and the otolith growth is higher.

Age estimation criteria: The sardine age estimation criteria were recommended in last ICES sardine age reading workshop (ICES, 2011b). The method was adopted explicitly in FAO (1979) and can be summarized as follows: 1) a set of an opaque and hyaline zone corresponds to an annual growth zone (annulus); 2) the date of birth is conventionally assumed to be the 1st of January and the fish is assigned to a year class on this basis (if an otolith is collected during the first semester the age group correspond to the number of hyaline zones, if the otolith is collected from a fish caught during the second semester, the hyaline edge will not be considered) (Figure 2.4.2.1).

Interpretation difficulties: The main discrepancies in sardine age determination are the identification of the otolith edge type and the first annulus. Two problems related to the edge type were discussed at the last workshop: 1) difficulty in identifying the edge type (hyaline or opaque); 2) variation in the seasonality of the edge type (Figure 2.4.2.1).

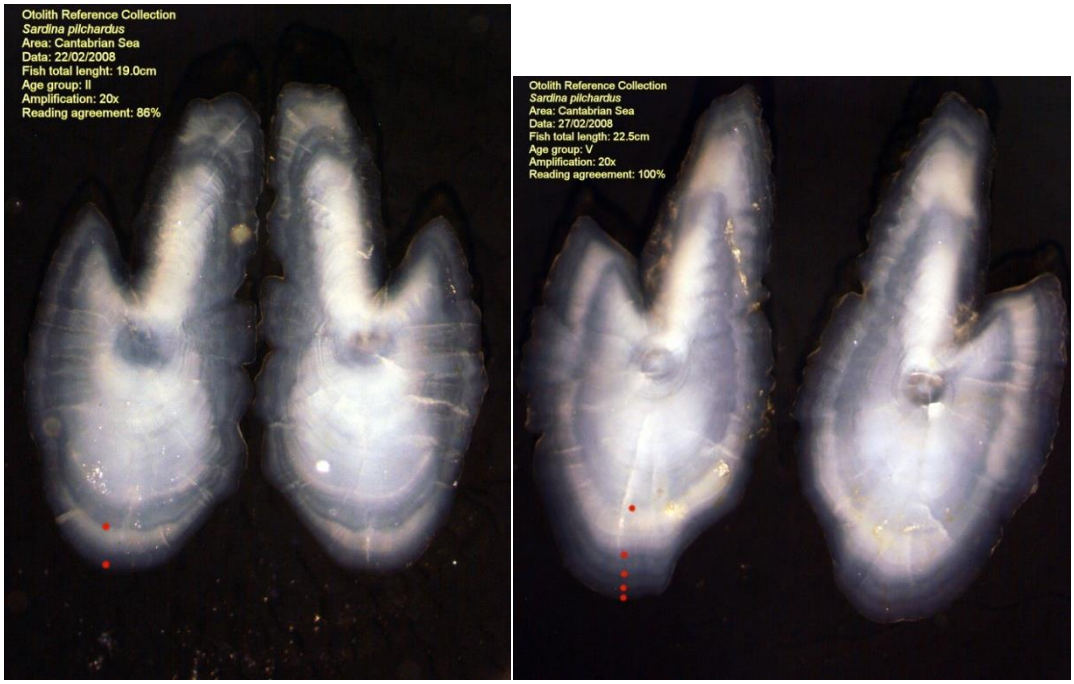


Figure 2.4.2.1 Otoliths of sardine (ICES, 2011b). The red dots indicate the hyaline (winter) annuli.

2.4.2 DAILY AGE

Observation: Microscope connected to an image analyzer

Magnification: In larvae, the whole otolith is observed at 1000x magnification along the maximum growth axis. In juveniles, the otolith nucleus is observed at 1000x magnification. The rest of the otolith is observed by two ways depending on the reading criteria (See section 1.4.2. in annex 1): if the GBR criterion is applied, the otolith is observed at x100 or x200 magnification; if the IMR criterion is followed, x400 magnification is used.

Reading axes: The age estimation is performed along the post-rostrum axis, which is generally the maximum growth axis of sagittal otoliths. The otolith radius is measured along the post-rostrum axis. Otolith micro-increments are counted starting in the hatch check until the edge. The first evident increment corresponds to the hatch check, at a distance between 5 and 7 µm from the primordium (Alemany and Alvarez, 1994).

Age estimation criteria: For daily increment interpretation of sardine otoliths, the same recommendations suggested for anchovy by ICES 2013b (ICES WKMIAS) are followed (See section 1.4.2. in annex 1). In the Bay of Biscay and Atlantic Iberian Peninsula, the application of the GBR criteria for sardine otolith sections was agreed, irrespective of the season and geographical area (Morales-Nin *et al*., 2010; SARDONE, 2010; ICES, 2013b).

Interpretation difficulties: These difficulties could be explain by: 1) difficulties in the interpretation of sub-daily increments, double structures or band zones; 2) unclear images, in which is difficult to interpret correctly the daily growth pattern due to under-or over-polishing, poor image acquisition or calibration problems. It is very important to obtain clear images to interpret properly daily micro-increments in this species.

2.5 AGE ACCURACY, VALIDATION AND CORROBORATION

2.5.1 ANNUAL AGE

All translucent annuli that are at a distance from the primordium of less than 1000 µm should be considered as checks (Silva *et al.,* 2012). That is based on 1) the validation of daily increments formation in sardine larvae and juveniles (Alemany and Alvarez, 1994), and 2) the corroboration of the position of the false annual ring (check) formed before its first hyaline (winter) annulus, through the micro-increment counts (ICES, 2011b; ICES, 2013b).

2.5.2 DAILY AGE

The daily periodicity of micro-increments deposition has been validated in early life stages of sardine in a few areas of distribution. In the Atlantic Iberian area, the daily deposition has been validated in sagitta otoliths of wild sardine larvae reared from birth until complete the absorption of the yolk sac (Re, 1984; Alemany and Alvarez, 1994). Similarly, the validation of daily increment formation of otoliths was carried out using a mesocosm experiment in sardine larvae grown on natural environmental conditions in the Adriatic Sea (Panfili, 2012).

2.6 AGE PRECISION, VERIFICATION AND QUALITY CONTROL

2.6.1 ANNUAL AGE

Sardine age estimation criteria used by IEO readers have been externally verified by international otoliths exchanges and workshops (ICES, 2005a; ICES, 2011b). Current IEO age readers have participated in recent otoliths exchanges and workshops, showing good results in agreement, precision and relative accuracy (ICES, 2011b).

The age estimation of each otolith is performed by two readers, or else, by one experienced reader, but then, the age estimation of each otolith is performed twice, in two separate occasions. The age estimation of a given otolith is accepted only if both estimations coincide. When a discrepancy between them is found, a third estimation is carried out. If the discrepancy continues, the otolith is rejected. Illegible otoliths are also rejected.

In addition to the age estimation, the quality (or credibility) of each age estimation is also assigned according to the "3 point grading system" recommended by WKNARC-1 and WKNARC-2 (ICES, 2011a; 2013a), as it is described in section 9.4.

2.6.2 DAILY AGE

To test the quality control of daily age estimations, internal (reading procedure) and external (planned otoliths exchanges and working groups) practices (SARDONE, 2010; ICES, 2013b) are developed by IEO. The internal practice concerned mainly to repeated age estimation performed independently by one or more readers, to check the precision in the age estimation. Generally, otoliths are discarded when the error in reading precision is higher than 5-10%.

Annex 3: MACKEREL (*SCOMBER SCOMBRUS*)

Figure 3.1. Mackerel (*Scomber scombrus*).

3.1 SAMPLING PROGRAM FOR AGE ESTIMATION

3.1.1 ANNUAL AGE

Samplings for age determination of mackerel are performed in IEO since 1982, from both, commercial catches and research surveys. The number of otoliths collected in 2012 is shown in Table 3.1.1.1

Table 3.1.1.1. Number of mackerel otoliths collected by stock from the commercial fleet and research surveys in 2012.

Stock	Commercial fleet	Research surveys	Total
Southern Component (Divisions VIIIc,IXa)	1032	1548	2580
Western Component (Divisions VIIIab, VII)	0	11	11
Total	**1032**	**1559**	**2591**

3.1.2 DAILY AGE

In 2000, otoliths of larvae, post-larvae and juveniles of mackerel were collected from research surveys and commercial fleet, for daily growth studies and otolith microstructure analysis within the SEAMAR Project (SEAMAR, 2002) (Table 3.1.2.1).

Table 3.1.2.1 Number of mackerel otoliths collected for daily growth studies from the commercial fleet and research surveys in 2000.

Stock	Commercial fleet	Research surveys	Total
Southern component (VIIIc,IXa)- Larvae	-	1126	1126
Southern Component (VIIIc,IXa)- Juveniles	160	218	378
Total	**160**	**1344**	**1504**

3.2 OTOLITH EXTRACTION AND STORAGE

Mackerel otoliths are more difficult to extract from the fish than in other small pelagic species, because they are very small compared to the fish. They are extracted by cutting through the top of the head (Figure 3.2.1; Figure 3.2.2). It is important to cut very carefully to avoid the otoliths damage as they are very fragile. The organic residues are very carefully removed with tweezers from the otoliths. Then, the otoliths are rinsed with distilled water and stored in black plastic plates with cover.

Figure 3.2.1. Extraction of mackerel otoliths.

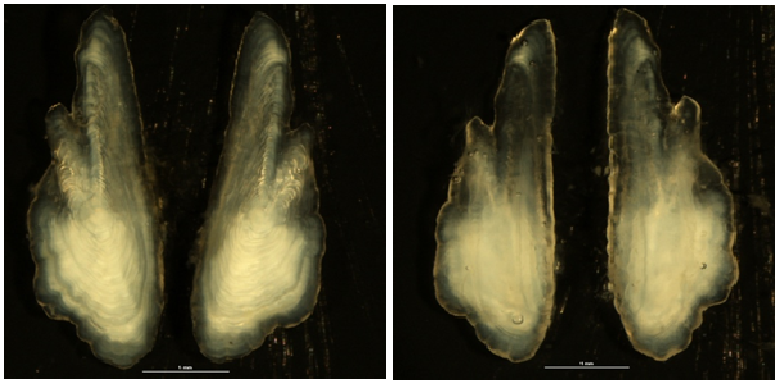


Figure 3.2.2. Mackerel otoliths: ventral and dorsal view.

3.3 OTOLITH PREPARATION METHOD

3.3.1 ANNUAL AGE

Like most small pelagic species, for age estimation, mackerel otoliths are observed under a binocular microscope inside black plastic plates, mounted on non-plastic transparent resin (see section 7.1).

3.3.2 DAILY AGE

In daily growth studies of juvenile mackerel, an otolith section is observed. In the case of larvae, whole otoliths are observed.

The sagitta otoliths are extracted from larvae and postlarvae using fine dissection needles under a binocular microscope 3-5 months after they have been preserved. Then, they are washed with distilled water, dried and mounted on glass slides within transparent synthetic enamel (Secor *et al*., 1992). Larvae and postlarvae otoliths are mounted whole and with the concave side upwards.

The sagitta otoliths extracted from juveniles and pre-recruits are cut into sections by a sanding and polishing process (Secor *et al*., 1992). Each otolith section is processed on the sagittal plane with respect to the fish.

3.4 AGE ESTIMATION METHOD

3.4.1 ANNUAL AGE

Observation: Binocular microscope. The age is also estimated using digital images in some cases, for biometric measures.

Illumination: Reflected light (using fiber optic illuminators).

Magnification: Between 20x and 40x magnification, depending on the otolith size.

Reading axes: Translucent annuli (hyaline) are counted, preferably in the anterior (*rostrum*) and posterior (*post-rostrum*) part of the otolith. When different ages are recorded in these two otolith areas, the older age estimate is then considered (ICES, 1995).

Age estimation criteria: Mackerel age estimation criteria were recommended by ICES (1995a; 2010). The method was adopted explicitly in the Workshop of mackerel ageing in 1995 (ICES, 1995a) based in the age validation of this species, by observing otoliths of known age (obtained from a tagging program).

The date of birth is conventionally assumed to be the 1st of January and the fish is assigned to a year class on this basis (if an otolith is collected during the first semester the age group correspond to the number of hyaline zones, if the otolith is collected from a fish caught during the second semester, the hyaline edge will not be considered).

Interpretation difficulties: These difficulties could be explained by: 1) different length of time for the opaque zone formation of the otolith between the different areas during the first year; 2) otolith edge interpretation; 3) possible presence of false annuli associated with the first maturity; 4) slowdown growth in older fish to such an extent that the opaque and translucent (hyaline) zones become confused and are more difficult to distinguish (Figures 3.4.1.1 and 3.4.1.2).

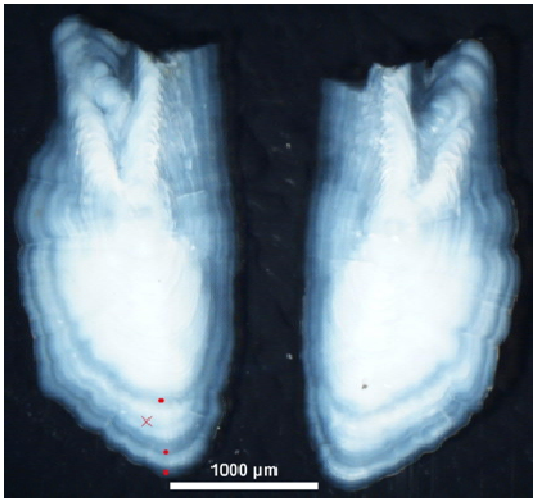


Figure 3.4.1.1 Otolith of *S. Scombrus* (TL: 32 cm, 3 years old, caught in January). The red dots indicate the hyaline (winter) annuli; X indicates the false ring.

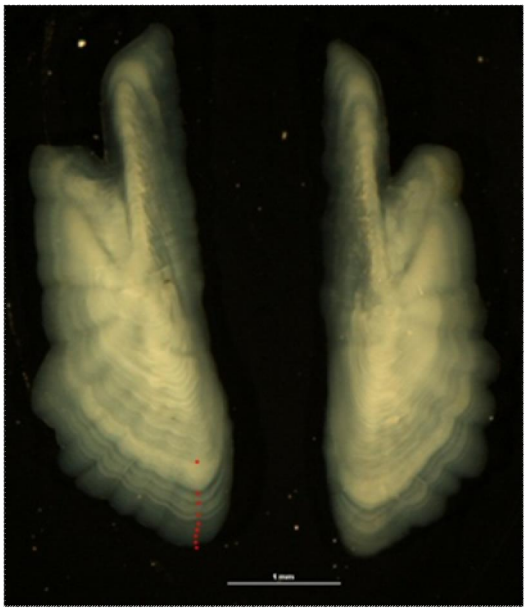


Figure 3.4.1.2 Otolith of *S. scombrus* (TL: 40 cm, 9 years old, caught in March). The red dots indicate the hyaline (winter) annuli.

3.4.2 DAILY AGE

Observation: Whole otoliths of larvae and otoliths sections of juvenile mackerels viewed through a microscope connected to a personal computer via a video camera.

Magnification: Otoliths are examined at 1000x. Growth increments are counted and measured using image analysis software (VISILOG/TNPC 3.1). In order to estimate the growth increments of both larvae otoliths and juveniles otolith sections, the objective (x100) is used with immersion oil.

Reading axes: Larvae otoliths are spherical, so that they can be read in any axis. However, otoliths sections of juveniles are processed along the short axis, and the growth increments are counted along the dorsal-ventral axis, as described by D'Amours *et al.* (1990). The numbering of growth increments on the otoliths is carried out within two triangular surfaces pointing towards the core on the dorsal-ventral axis relative to the fish; these two surfaces are defined as the standard reading fields, which correspond to the short axis of the otolith (Figure 3.4.2.1).

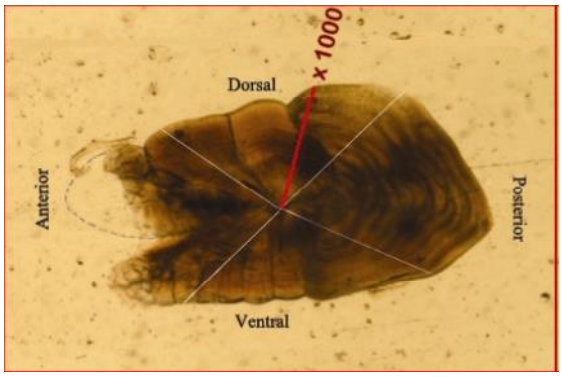


Figure 3.4.2.1. Sagittal section (at 5x) of mackerel sagitta otolith (164 mm standard length), showing: the standard fields of the growth rings interpretation in two triangular surfaces (blank lines) from the center of the otolith, in dorsal-ventral direction, and the area (red line) that is processed in the image analyzer at 1000 magnification.

Age estimation criteria: The numbering of otolith growth increments begins at the hatch check. The last increment is omitted because it is considered incomplete since it does not represent a full day. The deposition of daily growth increments in mackerel larvae, post-larvae and juveniles has been validated by Migoya (1989) and D'Amours *et al*. (1990) in the Northwest Atlantic, and by Mendiola and Alvarez (2008) in the Northeast Atlantic.

Interpretation difficulties: These difficulties could be explained by: 1) difficulties in the interpretation of subdaily increments, double structures or band zones; 2) difficulties in the interpretation of intermediate areas without growth increments in juvenile otoliths; 3) unclear images, in which is difficult to interpret correctly the daily growth pattern, due to under- or over-polishing, poor image acquisition or calibration problems. It is very important to obtain clear images to interpret properly daily micro-increments in this species.

3.5 AGE ACCURACY, VALIDATION AND CORROBORATION

3.5.1 ANNUAL AGE

The existing material of such work is rather limited, particularly the one related to the actual yearly age structures of mackerel otoliths. The validation of North East Atlantic mackerel annual age criteria was established in 1995 to age 11, using fish of known age, which were determined by mark-recapture experiments (ICES, 1995a). Older ages were not able to be validated by this method and it was assumed that the modal age of the age assignments performed by readers for the same otolith corresponded to the actual age, as recommended by ICES (1994).

3.5.2 DAILY AGE

The deposition of daily growth increments in larvae, post-larvae and juveniles of mackerel was validated by Migoya (1989) and D'Amours *et al.* (1990), in Northwest Atlantic, and by Mendiola and Alvarez (2008) in Northeast Atlantic. Direct transformation of the number of increments in age (days) is well justified.

Migoya (1989) and Mendiola and Alvarez (2008) incubated mackerel eggs in the laboratory and showed that the deposit of the first increment in the otolith occurred on the hatching day and that the increments were formed daily. In addition, D'Amours *et al*. (1990) performed a validation experiment on mackerel juveniles in captivity, marking their otoliths with a fluorescent substance and showing that the increments were deposited on a daily basis.

3.6 AGE PRECISION, VERIFICATION AND QUALITY CONTROL

3.6.1 ANNUAL AGE

Mackerel age estimation criteria used by IEO readers have been externally verified by international otoliths exchanges and workshops (Villamor and Meixide, 1994; Anon., 1995a; SAMFISH, 2002; ICES, 2010). Current IEO age readers have participated in recent otoliths exchanges and workshops, showing good results in agreement, precision and relative accuracy (ICES, 2010).

The age estimation of each otolith is performed by two readers, or else, by one experienced reader, but then, the age estimation of each otolith is performed twice, in two separate occasions. The age

estimation of a given otolith is accepted only if both estimations coincide. When a discrepancy between them is found, a third estimation is carried out. Unreadable otoliths are rejected.In addition to the age estimation, the quality (or credibility) of each age estimation is also assigned according to the "3 point grading system" recommended by WKNARC-1 and WKNARC-2 (ICES, 2011a; 2013a), as it is described in section 9.4.

3.6.2 DAILY AGE

To test the quality control of daily age estimations, internal (reading procedure) and external (planned otoliths exchanges and working groups) practices (SEAMAR, 2002) are developed by IEO. The internal practice concerned mainly to repeated readings performed independently by one or more readers, to check the precision in age estimation. Generally, otoliths are discarded when the error in reading precision is higher than 5-10%.

Annex 4: CHUB MACKEREL (*SCOMBER COLIAS*)

Figure 4.1. Chub mackerel (*Scomber colias*).

4.1 SAMPLING PROGRAM FOR AGE ESTIMATION

Routine samplings for age determination of chub mackerel began to be performed in IEO in 2011, as a new requirement of the DCF (UE) for the period 2011-2013. Samples are obtained from both, commercial catches and research surveys (Table 4.1.1) from Division VIIIc and Subdivision IXa North.

Table 4.1.1. Number of chub mackerel otoliths collected from the commercial fleet and research surveys in 2012.

Areas	Commercial fleet	Research surveys	Total
Division VIIIc	1049	246	1295
Division IXa North	1112	-	1112
Total	**2161**	**246**	**2407**

4.2 OTOLITH EXTRACTION AND STORAGE

Chub mackerel otoliths (Figure 4.2.1) are extracted and storage in the same way as the mackerel ones (see section 3.2)

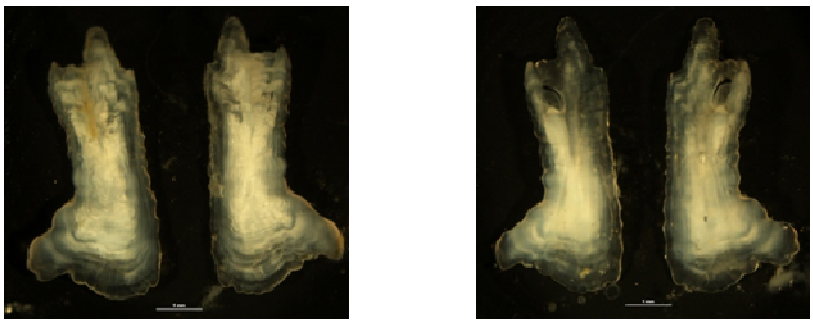


Figure 4.2.1. Chub mackerel otoliths: ventral and dorsal view.

4.3 OTOLITH PREPARATION METHOD

Like most small pelagic species, for the age estimation, chub mackerel otoliths are observed under a binocular microscope inside black plastic plates, mounted on non-plastic transparent resin (see section 7.1).

4.4 AGE ESTIMATION METHOD

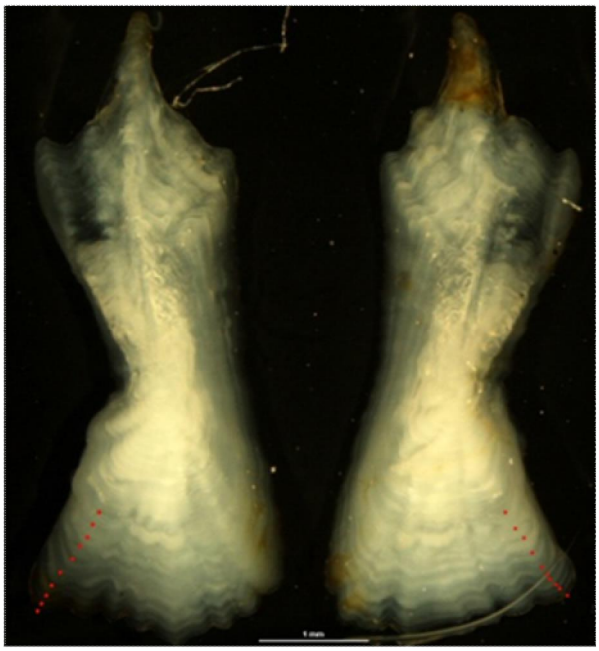
Observation: Binocular microscope. The age is also estimated using digital images in some cases, for biometric measures.

Illumination: Reflected light (using fiber optic illuminators).

Magnification: Between 20x and 40x magnification, depending on the otolith size.

Reading axes: Translucent rings (hyaline) are counted, preferably in the posterior (*post-rostrum*) part of the otolith.

Age estimation criteria: The criteria for the age determination of this species are still developing. Due to the similarity between both *Scomber* species, and while the criteria for the age determination of chub mackerel are not fully defined, the reading criteria applied to mackerel are recommended to be followed for chub mackerel (see section 3.4.1. in annex 3).



Although certain peculiarities are observed, such as: 1) higher presence of false growth increments; 2) priority is given to the post-rostrum in the age interpretation, as annuli tend to be better determined in this area than in the rest of the otolith; 3) usually, the otolith point provides little help in the age interpretation in chub mackerel, since the annuli are very crowded in this area and are difficult to determine, especially in otoliths of older specimens (Figure 4.4.1).

Figure 4.4.1. Otolith of *S. colias* (TL 38 cm, 10 years old, caught in June). The red dots represent the hyaline (winter) annuli.

Interpretation difficulties: These difficulties could be explained by: 1) difficulties in identifying the first annulus; 2) difficulties in differentiating between true annual rings (annuli) and false rings (checks); 3) insufficient annual growth pattern recognition; and 4) insufficient criterion regarding the otolith edge that can be expected to be seen along the year.

4.5 AGE ACCURACY, VALIDATION AND CORROBORATION

The available material of such work is rather limited. Currently, studies are underway to validate the age determination criteria of this species. First attempts in age corroboration (marginal increment analysis and edge zone analysis) are being performed in the Bay of Biscay (Navarro *et al*., 2014).

4.6 AGE PRECISION, VERIFICATION AND QUALITY CONTROL

Only one otolith exchange of chub mackerel has been carried out recently (Martins *et al*., 2014), where the IEO reader have participated, showing good results in agreement, precision and relative accuracy.

The age estimation of chub mackerel otoliths are performed by a specific experienced reader. The age estimation of each otolith is performed twice, in two separate occasions. The age estimation of a given otolith is accepted only if both estimations coincide. When there is a discrepancy between them, a third estimation is carried out. Unreadable otoliths are rejected.

In addition to the age estimation, the quality (or credibility) of each age estimation is also assigned according to the "3 point grading system" recommended by WKNARC-1 and WKNAR-2 (ICES, 2011a; 2013a), as it is described in section 9.4.

Annex 5: HORSE MACKEREL (*TRACHURUS TRACHURUS*)

Figure 5.1. Horse mackerel (*Trachurus trachurus*).

5.1 SAMPLING PROGRAM FOR AGE ESTIMATION

Samplings for age determination of horse mackerel are carried out in IEO since 1980s, from both, commercial catches and research surveys. The number of otoliths collected in 2012 is shown in Table 5.1.1.

Table 5.1.1. Number of horse mackerel otoliths collected by stock from the commercial fleet and research surveys in 2012.

Stock	Commercial fleet	Research surveys	Total
Southern Stock (IXa North)	583	240	823
Western Stock (VIIIc-VIIIb)	1515	1220	2735
Total	**2098**	**1460**	**3558**

5.2 OTOLITH EXTRACTION AND STORAGE

Horse mackerel otoliths can be easily extracted by cutting through the top of the head, at the operculum level (Figure 5.2.1, Figure 5.2.2). They are large and robust, so they are less easily broken during the extraction than other pelagic species otoliths. The organic residues are removed from the otoliths with tweezers. Then, the otoliths are rinsed with distilled water. Nowadays, horse mackerel otoliths are stored in micro-tubes, but until 2012, they were stored dry in envelopes (see sections 4.2. and 4.3.).

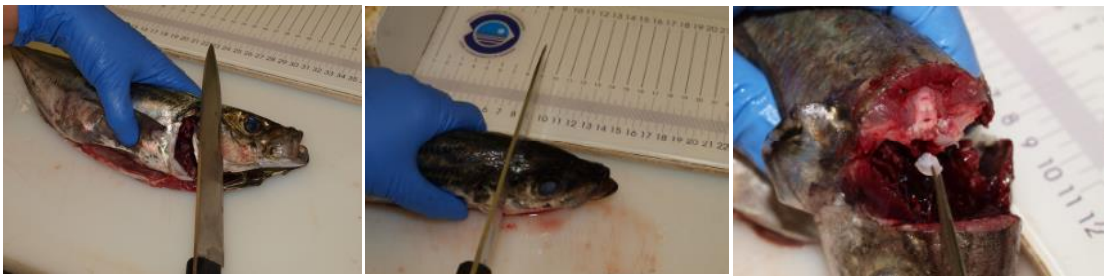


Figure 5.2.1. Extraction of horse mackerel otoliths.

Figure 5.2.2: Horse mackerel otoliths: ventral and dorsal view.

5.3 OTOLITH PREPARATION METHOD

Until a few years ago, in order to perform the age estimation, horse mackerel otoliths were burned (See section 7.3). Nowadays, there are two ways of determinate the age in horse mackerel otoliths depending on the size of the specimens. Usually, when the fish is below 26 cm, the whole otolith is directly observed under the binocular microscope. When the fish is over 26 cm, horse mackerel otoliths are cut in thin sections, due that they are very thick, which prevent a correct observation of its structural characteristics. Also, the use of otolith sections is mandatory with older ages (6-8).

After being extracted from each sampled fish, horse mackerel otoliths are stored in microtubes. Otoliths from specimens below 26 cm are placed in a recipient with alcohol and glycerin, and are observed directly under a binocular microscope. Otoliths from specimens over 26 cm are embedded in polyester resin in an aluminum mould. The resin blocks containing the embedded otoliths are removed from the moulds and cut into thin sections (0.5 mm) following the dorso-ventral plane of the otolith. With a cutting machine multiple otolith sections are obtained. The resulting sections are stuck in glass slides and properly labeled. (See section 7.4.).

5.4 AGE ESTIMATION METHOD

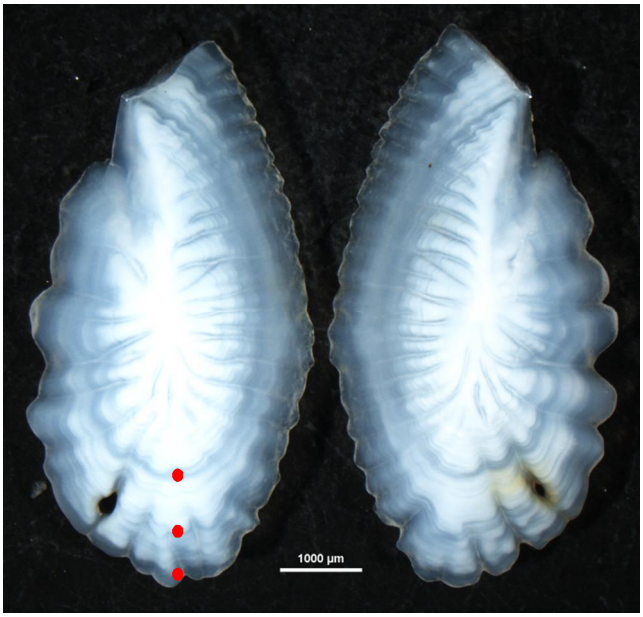
Observation: Whole horse mackerel otoliths (specimens below 26 cm) are placed in a recipient with alcohol and glycerin and examined by a binocular microscope. Slides with otolith sections (specimens over 26 cm) are also observed under a binocular microscope. Also, in some cases and for biometric measures, the age is estimated on digital images.

Illumination: Reflected light (using fiber optic illuminators).

Magnification: Between 15x and 50x magnification, depending on the otolith size. The examination method depends on the technique of preparation used. So, whole otoliths of young fishes are observed with low magnification (between 15x and 25x), otherwise, false rings may confuse inexperienced readers. Otoliths sections of older fishes are examined with a magnification between 30x and 50x.

Reading axes: Translucent rings (hyaline) are counted, preferably in the anterior (*rostrum*) and posterior (*post-rostrum*) parts of the otolith.

Age estimation criteria: Horse mackerel age estimation criteria were established by ICES (1999; and 2012a), based on direct age validation studies (Kerstan and Waldrom, 1995) and on indirect validation studies (ICES, 1999; Waldron and Kerstan, 2001; Abaunza *et al.*, 2003).



Interpretation difficulties: In general, the age of horse mackerel otoliths is very difficult to estimate in older fishes because they become thick with age. The first annuli interpretation in both, young and older fishes, appears to be the major cause of differences. The dissimilarity of the false rings and the variety of true annuli make difficult to follow the annuli formation. In some otoliths, problems are caused by the edge interpretation (Figures 5.4.1, 5.4.2).

Figure 5.4.1 Whole otolith of Atlantic horse mackerel (TL <26 cm, 3 years old, caught in March). The red dots represent the hyaline (winter) annuli.

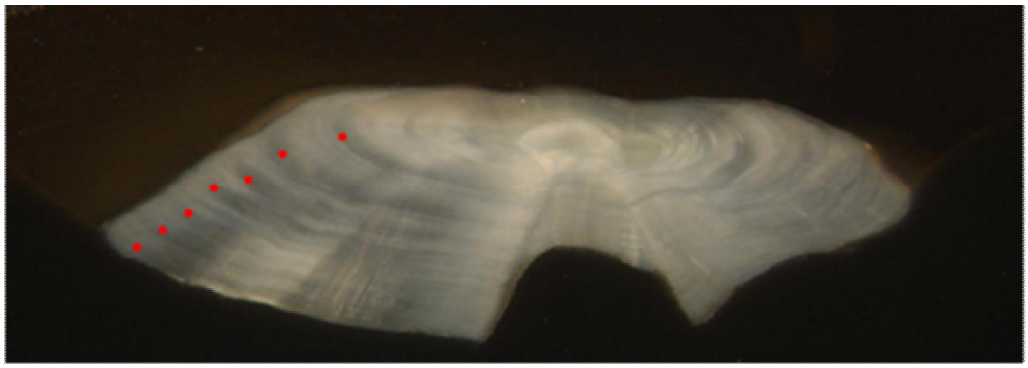


Figure 5.4.2 Thin section of Atlantic horse mackerel (TL 34.5 cm, 7 years old, caught in September). The red dots represent the hyaline (winter) annuli.

5.5 AGE ACCURACY, VALIDATION AND CORROBORATION

Direct age validation for northeast Atlantic horse mackerel has been carried out. It confirms that one opaque and one translucent zone constitute one annual growth zone (Kerstan and Waldrom, 1995).

Indirect age validation can be obtained from the comparison between the age estimation and the length-frequency distributions. This method confirmed the age estimation of the first years of life (up to age 4) (Letaconnoux, 1951). Other method is based on the occurrence of annual year-marks, and has been tested by following identifiable year classes through successive year's age compositions (Eltink and Kuiter, 1989). Indications that a correct age determination method has been applied can be obtained by such indirect validation technique. For example, in the catch in number of the western horse mackerel fishery, the extremely strong 1982 year class can be followed from 1984 to 1996 (ICES, 1999; Abaunza *et al*., 2003)

Waldron and Kerstan (2001) used two methods to validate the age determination of horse mackerel otoliths. In the first one, whole otoliths were examined; age was determined by identifying and counting annuli and marginal increment widths were also measured and used to estimate the age, which ranged from 0.6–4.3 years. In the second method, otoliths were examined with a scanning electron microscope and fish ages were determined by daily increment counts. Estimated ages agreed with ages derived by counting daily increments, thus validating the ages of horse mackerel up to four years.

5.6 AGE PRECISION, VERIFICATION AND QUALITY CONTROL

Horse mackerel age estimation criteria used by IEO readers have been externally verified by international otoliths exchanges and workshops (ICES, 1999; Bolle *et al*., 2006; ICES, 2012a). Current IEO age readers have participated in recent otoliths exchanges and workshops, showing good results in agreement, precision and relative accuracy (ICES, 2012a).

The age estimation of each otolith is performed by two readers, or else, by one experienced reader, but then, the age estimation of each otolith is performed twice, in two separate occasions. The age estimation of a given otolith is accepted only if both estimations coincide. When a discrepancy between them is found, a third estimation is carried out. Unreadable otoliths are rejected.In addition to the age estimation, the quality (or credibility) of each age estimation is also assigned according to the "3 point grading system" recommended by WKNARC-1 and WKNAR-2 (ICES, 2011a; 2013a), as it is described in section 9.4.

Annex 6: MEDITERRANEAN HORSE MACKEREL (*TRACHURUS MEDITERRANEUS*)

Figure 6.1. Mediterranean horse mackerel (*Trachurus mediterraneus*).

6.1 SAMPLING PROGRAM FOR AGE ESTIMATION

Routine sampling for the age determination of Mediterranean horse mackerel began to be performed in IEO in 2011, as a new requirement of the DCF for the period 2011-2013. Samples are taken from both, commercial catches and research surveys (Table 6.1.1.1), mainly from ICES Division VIIIc.

Tabla 6.1.1.1. Number of Mediterranean horse mackerel otoliths collected from the commercial fleet and research surveys in 2012.

Areas	Commercial fleet	Research surveys	Total
Division IXa North	1	-	1
Division VIIIb	-	6	6
Division VIIIc	422	196	618
Total	**423**	**202**	**625**

6.2 OTOLITH EXTRACTION AND STORAGE

It is the same as in the case of horse mackerel (see section 5.2 in annex 5).

6.3 OTOLITH PREPARATION METHOD

It is applied the same as in the case of horse mackerel otolith (see section 5.3 in annex 5).

6.4 AGE ESTIMATION METHOD

The same protocol as in the case of horse mackerel is followed to estimate the age of Mediterranean horse mackerel.

Observation, illumination, magnification and reading axes are the same as in horse mackerel (see section 5.4 in annex 5)

Age estimation criteria: They were recommended by ICES (2012a), the same as in the case of horse mackerel (see section 5.4. in annex 5).

Interpretation difficulties: Mediterranean horse mackerel otoliths are difficult to interpret, in a similar way as in horse mackerel otoliths, whose age determination for older individuals is particularly imprecise. However, Mediterranean horse mackerel otoliths present specific problems when assigning ages to younger individuals, related to the first annulus interpretation.

6.5 AGE ACCURACY, VALIDATION AND CORROBORATION

Accuracy cannot be evaluated in the Atlantic area for Mediterranean horse mackerel since validation data are not available at the moment and the true age determination in this species is not possible. However, in the Eastern Mediterranean the time of annulus completion was estimated by the study of monthly marginal increments (Karlou-Riga, 2000).

6.6 AGE PRECISION, VERIFICATION AND QUALITY CONTROL

Only one otolith exchange and workshop of Mediterranean horse mackerel have been carried out recently (ICES, 2012a), where the current IEO reader has participated.

The age estimation of Mediterranean horse mackerel otoliths are performed by a specific experienced reader. The age estimation of each otolith is performed twice, in two separate occasions. The age estimation of a given otolith is accepted only if both estimations coincide. When there is a discrepancy between them, a third estimation is carried out. Unreadable otoliths are rejected.

In addition to the age estimation, the quality (or credibility) of each age estimation is also assigned according to the "3 point grading system" recommended by WKNARC-1 and WKNARC-2 (ICES, 2011a; 2013a), as it is described in section 9.4.

Annex 7: BLUE WHITING (*MICROMESISTIUS POUTASSOU*)

Figure 7.1. Blue whiting (*Micromesistius poutassou*).

7.1 SAMPLING PROGRAM FOR AGE ESTIMATION

Samplings for age determination of blue whiting are carried out in IEO since 1980s, from both, commercial catches and research surveys. The number of otoliths collected in 2012 is shown in Table 7.1.1.

Table 7.1.1. Number of blue whiting otoliths collected from the commercial fleet and research surveys in 2012.

Areas	Commercial fleet	Research surveys	Total
Division VIIIc	629	1722	2351
Division IXa North		275	275
Total	**629**	**1997**	**2626**

7.2 OTOLITH EXTRACTION AND STORAGE

Blue whiting otoliths can be easily extracted by cutting through the top of the head. They are large and robust, so they are not easily broken during the extraction. Once the otoliths are extracted (Figure 7.2.1, Figure 7.2.2), they are rubbed with the fingers to eliminate the remains of organic material and washed with water. Then, they are stored dry in microtubes (since 2012). Originally, blue whiting otoliths were stored in distilled water with thymol; later on until 2012 they were stored dry in envelops (see section 4.2. and 4.3.).

Figure 7.2.1. Extraction of blue whiting otoliths.

Figure 7.2.2. Blue whiting otoliths: ventral and dorsal view.

7.3 OTOLITH PREPARATION METHOD

For age estimation, whole blue whiting otoliths are observed under a binocular microscope, immersed in water. In the past, these otoliths were mounted on a block of resin and cut in sections before being observed under the binocular microscope.

It is recommended to estimate the age of blue whiting otoliths immediately after their removal from the fish. Blue whiting otoliths are stored dry in microtubes, but being soaked in water for 24 hours beforehand is recommended. This will enhance the winter rings. If the otoliths are stored in water longer than 7 days, the shape/composition of the otolith seems to change (due to the unstable pH of the water), so the storage is recommended to be done dry. The otolith should not be soaked in water for more than 48 hours each time, as it could possibly affect the annuli structure due to the freshwater composition.

7. 4 AGE ESTIMATION METHOD

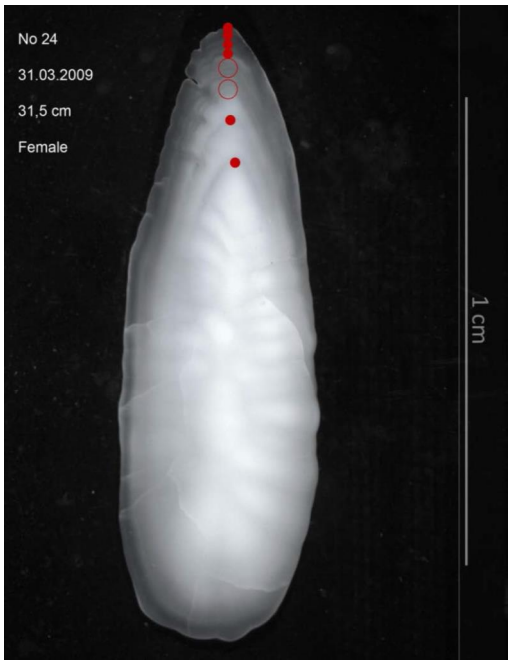
Observation: Whole otoliths immersed in water under a binocular microscope.

Illumination: Reflected light (using fiber optic illuminators).

Magnification: 6/6.4x magnifications against a black background, where 12 e.p.u (eyepiece units) are equal to 2 mm. Amplification and light intensity can be adjusted by each reader. The magnification is increased with the otolith size.

Reading axis: Blue whiting otoliths are interpreted by counting the translucent annuli (hyalines) up the *rostrum* area and using the whole otolith pattern as a guide. Usually, the clearest pattern is seen when the convex side of the otolith (sulcus side) is facing down. However, handling the otolith, turning it in various directions, may be a way of assuring the estimated age. With difficult otoliths, the observation of both otolith sides (concave and convex) is recommended to gain a better age estimation.

Age estimation criteria: Blue whiting age estimation criteria were recommended by ICES (2005b; 2013c). The correct annulus identification can be induced by measuring the inner annulus length, thereby avoiding the inclusion of the Bailey's zone (Bailey, 1970) as the first annulus. A 'false' ring known as the 'Baileys zone' may appear inside the first winter ring, confusion can be eliminated keeping in mind that if a ring is less than 48 e.p.u. it probably is a Bailey's zone. Usually, a growth increment in the length range of 50 to 56 e.p.u (8.33 to 9.33 mm) can be considered as the first annulus (ICES 2005b).



Interpretation difficulties: The interpretation of blue whiting otoliths is generally difficult. Even in well-marked otolith annuli, there are subjective decisions to be made, which are highly dependent on each reader's experience. These difficulties could be explained by: 1) the difficulty in the interpretation of the first annulus position, where the Bower zone is clear; 2) the presence of false and split growth increments, which is a severe problem that causes large differences in age; 3) the edge interpretation, which is another source of error that produces a difference of one year in the assigned age. (Figure 7.4.1)

Figure 7.4.1.Blue whiting otolith (TL: 31.5 cm, 7 years old, caught in March). The red dots represent the hyaline (winter) annuli and the empty circles show split rings. Each empty circle should be counted only as one year (from ICES WKARBLUE 2013, ICES, 2013).

7.5 AGE ACCURACY, VALIDATION AND CORROBORATION

Little has been performed to validate the blue whiting age estimations. There is only one study on southern blue whiting (Hanchet and Uozumi, 1996), where ages of adults were validated up to at least 10 years by following strong year classes both from otolith based age frequency distributions and from length frequency data.

7.6 PRECISION, VERIFICATION AND QUALITY CONTROL

Blue whiting age estimation criteria used by the IEO readers have been externally verified by international otoliths exchanges and workshops (Monstad and Linkowski, 1988; Meixide, 1990; Anon,

1993; ICES, 2005b; ICES, 2013c). Current IEO age readers have participated in recent otoliths exchanges and workshops, showing good results in agreement, precision and relative accuracy (ICES, 2013c).

The otoliths of blue whiting are aged by a specific experienced reader. The age estimation of each otolith is performed twice, in two separate occasions. The age estimation of a given otolith is accepted only if both estimations coincide. When there is a discrepancy between them, a third estimation is carried out. Unreadable otoliths are rejected.

In addition to the age estimation, the quality (or credibility) of each age estimation is also assigned according to the "3 point grading system" recommended by WKNARC-1 and WKNARC-2 (ICES, 2011a; 2013a), as it is described in section 9.4.

Annex 8: MEGRIM (*LEPIDORHOMBUS WHIFFIAGONIS*)

Figure 8.1. Megrim (*L. whiffiagonis*)

8.1 SAMPLING PROGRAM FOR AGE ESTIMATION

8.1.1 ANNUAL AGE

Samplings for age determination of megrim are performed in IEO from the late 80s, from both, commercial catches and research surveys. The number of otoliths collected in 2012 is shown in Table 8.1.1.1.

Table 8.1.1.1. Number of megrim (*L. whiffiagonis*) otoliths by stock collected from the commercial fleet and research surveys in 2012.

Stock	Commercial fleet	Research surveys	Total
ICES Div. VIIb-k, VIIIabd	1427	699	2126
ICES Div. VIIIc, IXa	493	396	889
Total	**1920**	**1095**	**3015**

8.2 OTOLITH EXTRACTION AND STORAGE

8.2.1 ANNUAL AGE

To extract the otoliths, the megrim is held and, taking its operculum as a reference, a path is followed up vertically up to its dorsum where a cut is performed with a sharp knife, sectioning its skull (Figure. 8.2.1.1).

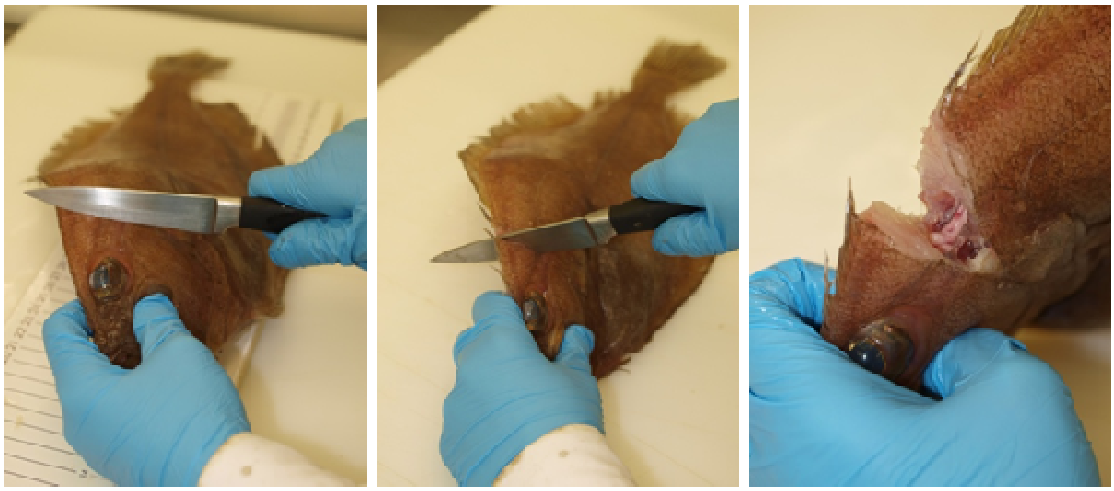


Figure 8.2.1.1. Cut of a megrim head for the otoliths extraction.

The section is held open, observing that the otoliths are located within the skull, in two sinuses on each side of the brain. The tips of straight metal laboratory tweezers are introduced smoothly to extract each otolith from those spaces. The megrim otoliths are robust, and depending on the accuracy of the cut, sometimes they will be more in sight and other times they will be more hidden, so they have to be searched with tweezers sensing them by touch (Figure 8.2.1.2).

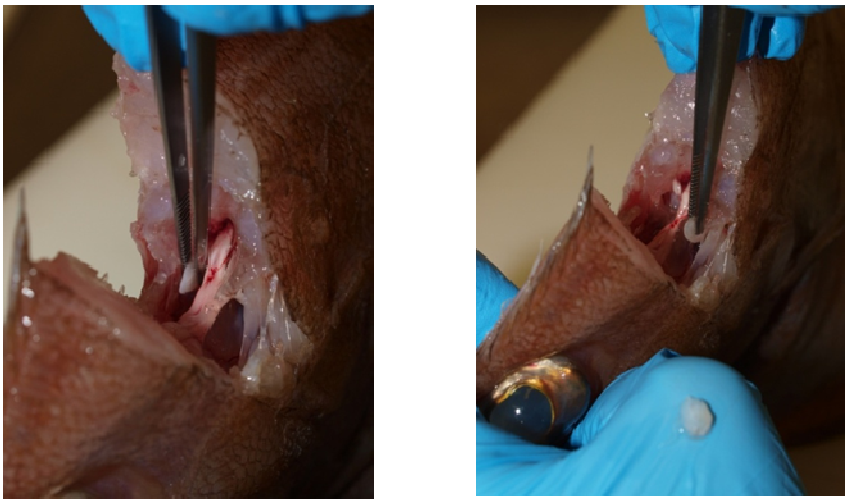


Figure 8.2.1.2. Extraction of megrim otoliths

After both otoliths have been extracted from a megrim, the accompanying organic remains are removed carefully with tweezers, to avoid the otolith breakage. Both otoliths from each megrim are stored in a paper envelope), correctly labeled with the data of the sampled specimen (Figure. 8.2.1.3).

Figure 8.2.1.3.Cleaning a pair of megrim otoliths (left) and storing them inside an envelope (right).

8.3 OTOLITH PREPARATION METHOD

For age estimation, the two otoliths are removed from their respective storage envelope and placed in a black plastic container immersed in water, in order to highlight their growth increments and facilitate the age estimation. Years ago they were immersed in an aqueous solution of glycerol to 40%, for a period of approximately 24 hours before being observed under a binocular microscope, but the age interpretation was not substantially improved by using that methodology. Once estimated the age, otoliths are dried and deposited back into their respective storage envelopes.

8.4 AGE ESTIMATION METHOD

Observation: Binocular microscope.

Illumination: Under reflected light (using fiber optic illuminators), on a black background.

Magnification: 8-15x.

Reading axes: The age is estimated by observing both otoliths. The anterior area (*rostrum*) of the left otolith, which has a higher distance from the center to its edge, is where the annuli are better distinguished and it is the first area to be observed to estimate the age. After that, the right otolith is observed in both areas, anterior and posterior (*post-rostrum*) areas (Figure 8.3.1.1). The anterior area of the left otoliths is usually broken, due to problems in the otolith extraction or handling, and therefore, that area is not usually available for age estimation.

Age estimation criteria: The age estimation criteria for megrim otoliths are basically that of Anon (1997b) for *L. whiffiagonis*, being similar for both *Lepidorhombus* species. The age estimation criteria have remained basically the same from the earliest age estimations (eg. Conan *et al.*, 1981). The translucent (hyaline) increments that are clearly marked and comparatively wider than the others are considered as annuli and are, therefore, counted. For the otolith edge interpretation, additional otolith information is considered, such as the date of capture, the spawning season (peak mainly from February to April) and the main period of seasonal increment formation (ICES, 2013a). Table 8.3.1.1 shows the overall agreed scheme of Anon (1997b) used for edge interpretation. Hyaline edge in the 2nd or 3rd quarter is unusual, being mostly predominant from October to April (Rodríguez and Iglesias, 1985).

Table 8.3.1.1. Overall scheme used for otolith age interpretation of megrim (*L. whiffiagonis*). N= number of hyaline annuli.

Edge type	Quarter 1	Quarter 2	Quarter 3	Quarter 4
Hyaline	Age = N	Age = N	Age = N-1	Age = N-1
Opaque	Age = N+1	Age = N	Age = N	Age = N

Interpretation difficulties: the age estimation of *L. whiffiagonis* is not especially difficult compared with other species. True annuli can be easily observed in most megrim otoliths (Figure 8.3.1.1).

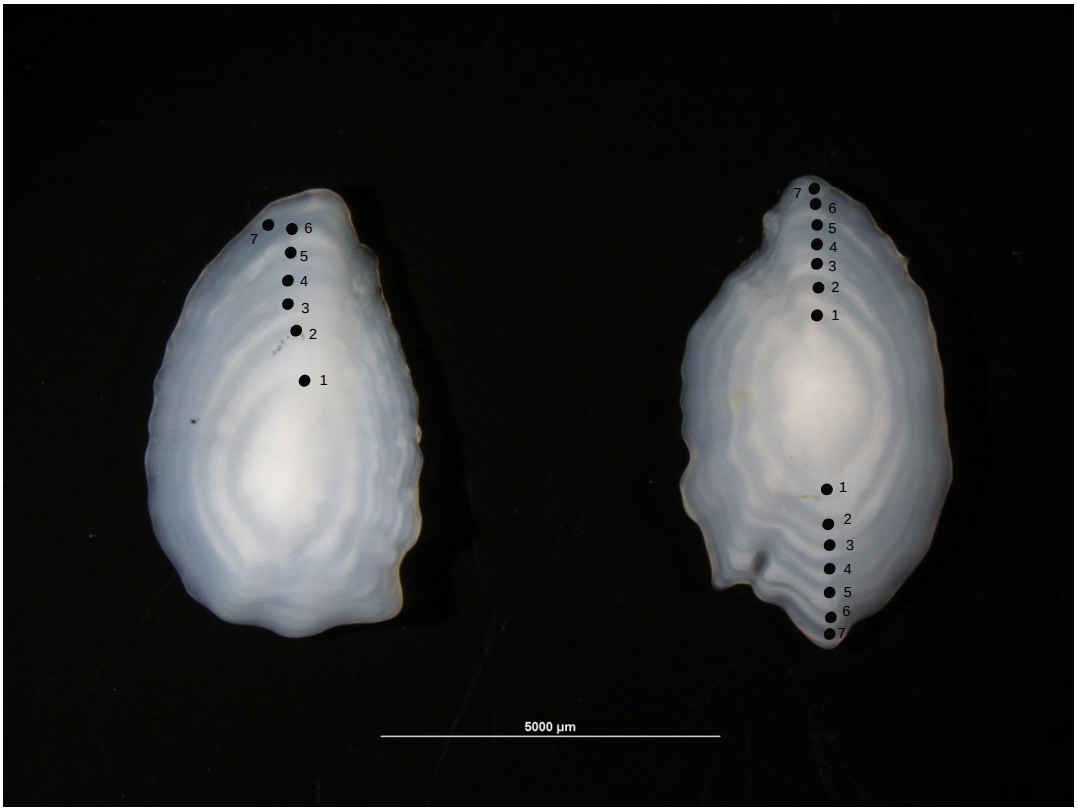


Figure. 8.3.1.1. Megrim (*L. whiffiagonis*) otoliths of 7 years of estimated age, captured in the 4th quarter. The three main reading areas are showed. Each counted hyaline annulus is marked by a spot and a number.

8.4 AGE ACCURACY, VALIDATION AND CORROBORATION

8.4.1 ANNUAL AGE

The periodicity of growth increment formation in *L. whiffiagonis* otoliths has been validated by edge analysis in several areas of northeast Atlantic (ICES Div. VII, VIIIabc, IXa) (Rodríguez and Iglesias, 1985; Landa and Piñeiro, 2000). The season in which the hyaline annulus is formed was determined by examination of the frequency distribution of otolith edge types throughout the year. The hyaline edge is mostly predominant from October (Rodríguez and Iglesias, 1985) and during the first four months of the

year, while the opaque edge predominates in the rest of the year (Rodríguez and Iglesias, 1985; Landa and Piñeiro, 2000). April is the month in which the hyaline annulus formation terminates.

The age estimation criterion of *L. whiffiagonis* was preliminary corroborated (or indirectly validated) by tracking year-classes abundance indices in the research surveys in the north and northwestern Iberian Peninsula (ICES Divisions VIIIc and IXa) (ICES, 1995b; Sánchez *et al.*, 1998).

8.5 AGE PRECISION, VERIFICATION AND QUALITY CONTROL

8.5.1 ANNUAL AGE

The age estimation criterion of *L. whiffiagonis* used by IEO readers was externally verified in international otoliths exchanges and workshops (Anon, 1991a; Dawson, 1991; Anon, 1997b; Egan *et al*., 2004; Etherton, 2011). The current IEO age readers participated in the recent otoliths exchanges and workshops, showing good results in agreement, precision and relative accuracy (Egan *et al*., 2004; Etherton, 2011).

In addition to the estimated age, the quality (or credibility) of each age estimation is also assigned according to the "3 point grading system" recommended WKNARC-1 and WKNARC-2 (ICES, 2011a; 2013a), as it is described in section 9.4.

Annex 9: FOUR SPOT MEGRIM (LEPIDORHOMBUS BOSCII)

Figure.9.2.1. Four spot megrim (*L. boscii*), showing the four spots in the dorsal and ventral fins.

9.1 SAMPLING PROGRAM FOR AGE ESTIMATION

9.1.1 ANNUAL AGE

Samplings for age determination of four spot megrim are performed in IEO since the late 80s, from both, commercial catches and research surveys. The number of otoliths collected in 2012 is shown in Table 9.1.1.1.

Table 9.1.1.1. Number of four spot megrim (*L. boscii*) otoliths collected by stock from the commercial fleet and research surveys in 2012.

Stock	Commercial fleet	Research surveys	Total
ICES Div. VIIIc, IXa	257	709	966

9.2 OTOLITH EXTRACTION AND STORAGE

9.2.1 ANNUAL AGE

The extraction procedure of four spot megrim otoliths is the same as that described for megrim otoliths (see section 8.2., in annex 8).

9.3 OTOLITH PREPARATION METHOD

The preparation method of four spot megrim otoliths is the same as that described for megrim otoliths (see section 8.3., in annex 8).

9.4 AGE ESTIMATION METHOD

The observation methodologies and age estimation criteria followed are basically that of Anon (1997b) for *L. whiffiagonis*, as the otoliths of both *Lepidorhombus* species are so similar (see section 8.3. in annex 8). The age estimation criterion has not substantially shifted from when its age began to be estimated (eg. Fuertes, 1978). The age estimation of *L. boscii* is not especially difficult compared with that of other species, the annuli can be observed in many otoliths (Fig. 9.3.1.1).

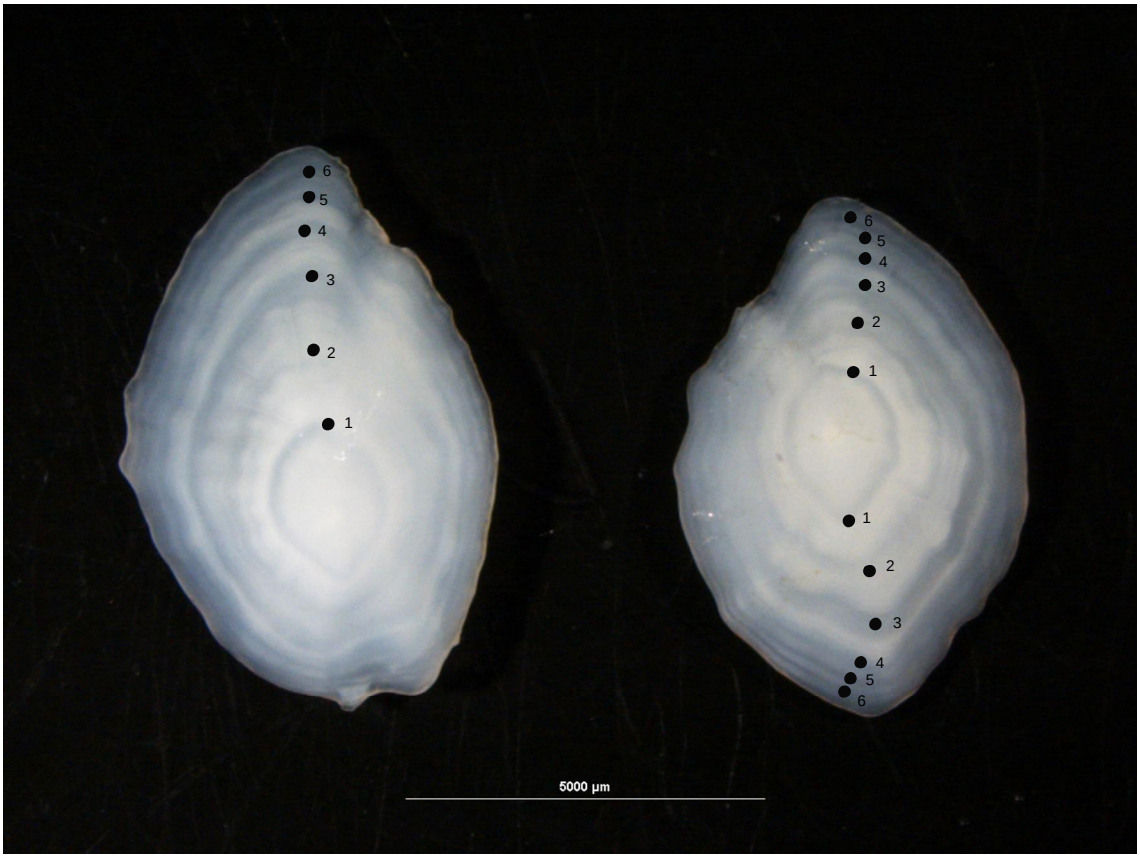


Figure. 9.3.1.1. 6 years old four spot megrim (*L. boscii*) otoliths captured in the 4th quarter. The three main reading areas are showed. Each counted hyaline (winter) annulus is marked by a spot and a number.

9.4 AGE ACCURACY, VALIDATION AND CORROBORATION

9.4.1 ANNUAL AGE

The periodicity of growth increment formation in *L. boscii* otoliths has been validated by edge analysis in northwestern Iberian Peninsula (ICES Div. VIIIc, IXa) (Fuertes, 1978). The season in which the hyaline

annulus is formed was determined by the examination of the frequency distribution of the otolith edge types throughout the year. The hyaline edge is mostly predominant from November to April, while the opaque edge predominates in the rest of the year. April is the month in which the hyaline annulus formation terminates (Fuertes, 1978).

The age estimation criteria of *L. boscii* was preliminary corroborated (or indirectly validated) by tracking year-classes abundance indices in research surveys in the north and northwestern Iberian Peninsula (ICES Divisions VIIIc and IXa) (ICES, 1995b; Sánchez *et al.*, 1998).

9.5 AGE PRECISION, VERIFICATION AND QUALITY CONTROL

9.5.1 ANNUAL AGE

The age estimation criteria are similar for both *Lepidorhombus* species. Although international otoliths exchanges and workshops have not yet been held for *L. boscii*, the current IEO age readers have participated in recent *L. whiffiagonis* ones (see section 8.5. in annex 8) showing good results in agreement, precision and relative accuracy (Egan *et al.*, 2004; Etherton, 2011).

In addition to the estimated age, the quality (or credibility) of each age estimation is also assigned according to the "3 point grading system" recommended by WKNARC-1 and WKNARC-2 (ICES, 2011a; 2013a), as it is described in section 9.4.

Anexx 10: WHITE ANGLERFISH (LOPHIUS PISCATORIOUS)

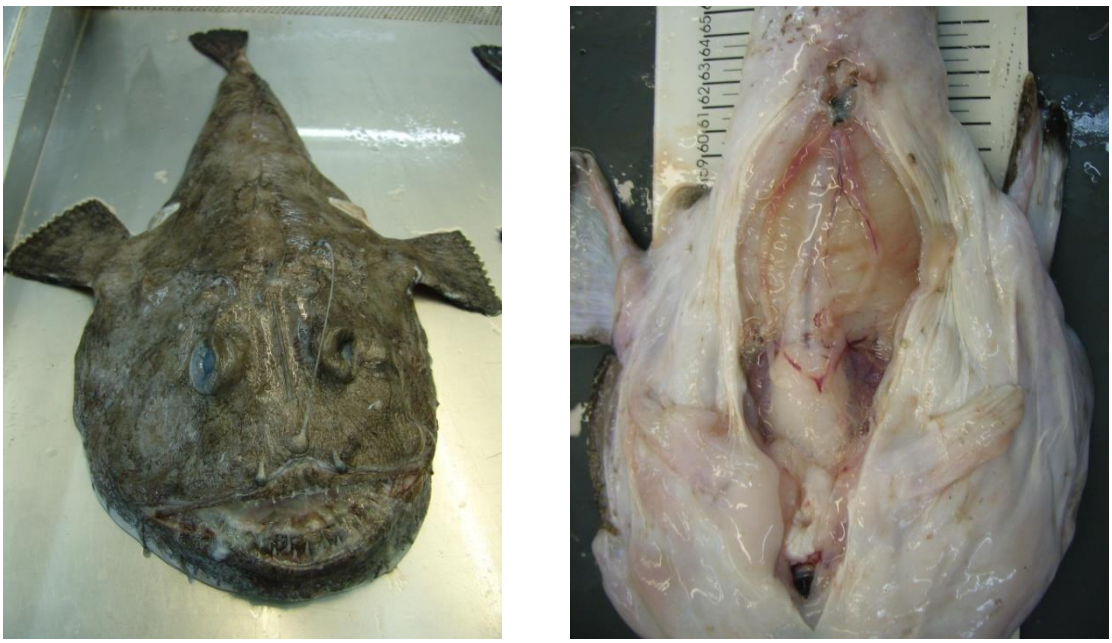


Figure. 10.1. Dorsal and ventral view of *L. piscatorius*, showing the white peritoneum.

10.1 SAMPLING PROGRAM FOR AGE ESTIMATION

10.1.1 ANNUAL AGE

Routine samplings for age determination of *L. piscatorius* are performed in IEO since the mid 90s, from both, commercial catches and research surveys. The number of *illicia* collected in 2012 is shown in Table 10.1.1.1.

Table 10.1.1.1. Number of *L. piscatorius illicia* collected from the commercial fleet and research surveys in 2012.

Stock	Commercial fleet	Research surveys	Total
ICES Div. VIIb-k, VIIIabd	408	235	643
ICES Div. VIIIc, IXa	925	151	1076
Total	**1333**	**386**	**1719**

10.1.2 DAILY AGE

Lapilli otoliths from juvenile *L. piscatorius* for daily growth studies and otoliths microstructure analysis, were caught in research surveys in 2010 and 2012 (Table 10.1.2.1).

Table 10.1.2.1. Number of *L. piscatorius lapilli* otoliths collected for daily growth studies in research surveys during 2010 and 2012 in ICES Div. VIIIc, IXa.

Year	Research surveys
2010	134
2012	55
Total	**189**

10.2 ILLICIA/OTOLITH EXTRACTION AND STORAGE

10.2.1 ANNUAL AGE

The *illicium* is cut from the fish at its base, lifting its apical end to facilitate the cutting (Figure 10.2.1.1). The *illicium* of each anglerfish is placed inside an envelope, labeled with the data of the sampled specimen, with its base first, and the top part of the illicium that stand out of the envelope is cut. Only the bottom of the *illicium* is enough (Figure 10.2.1.2). The illicium skin is not retired (see section 4.2.).

Figure 10.2.1.1. Cutting an *illicium* of anglerfish at its base.

Figure 10.2.1.2. *Illicium* section to keep on an envelope.

10.2.2 DAILY AGE

To extract the *lapilli* otoliths, a dorso-ventral cut (parallel to the margin of the orbit) is made using a sharp knife, thus exposing the brain which is carefully extracted (Figure 10.2.2.1). Through a careful observation, the semicircular canals are located along the lateral walls of the brain cavity, and the *lapilli* (Figure 10.2.2.2) are found at the confluence of the canals (Secor *et al*., 1992). *Lapilli* are removed using tweezers, cleaned and stored in black plastic plates labeled with the appropriate sample information.

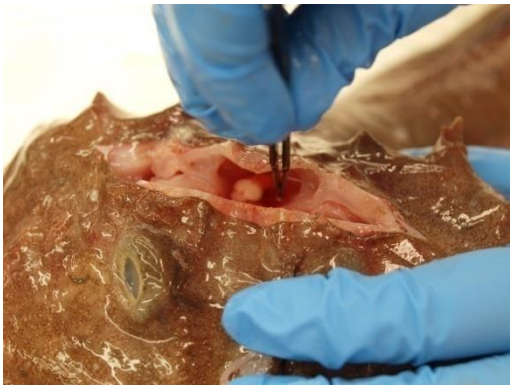


Figure 10.2.2.1. Macroscopic dissection technique used on juvenile anglerfish to extract otoliths.

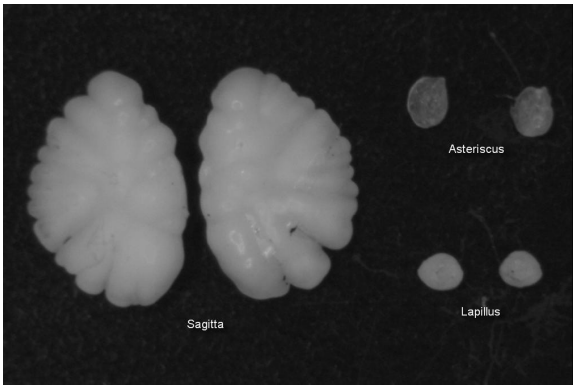


Figure 10.2.2.2. The three pairs of anglerfish otoliths. The *lapilli* otoliths are used for daily increment analysis.

10.3 ILLICIA/OTOLITH PREPARATION AND AGE ESTIMATION METHOD

10.3.1 ANNUAL AGE

The traditional methodology of *illicia* mounting in resin plates was originally described by Dupouy *et al*. (1986) and, after several European age estimation workshops of anglerfish (Anon, 1997a; Anon, 1999; Landa *et al*., 2002), it was standardized and was included in an age estimation guide for anglerfish (Duarte *et al*., 2002). That methodology was used in most of the growth studies using *illicia* (Duarte *et al*., 1997; Quincoces *et al*., 1998a; Landa *et al*., 2001; Ofstad and Laurenson, 2007). However, several modifications in the traditional methodology of Dupouy *et al*. (1986) have been recently carried out for *illicia* preparation, observation and age interpretation (Landa *et al*., 2013). Those methodological modifications have been performed to allow a more clear observation of the growth pattern, showing mainly the most apparent growth marks, in order to allow the distinction of the annuli:

Section thickness. More annual increments are observed with thin illicia sections than with thicker ones. Transverse sections ~0.50–0.55 mm thick allow the observation of the most clearly marked increments, probably those that are annual. However, the observation of sections thinner than 0.5 mm (~0.4 mm) can show some false increments that can be wrongly counted as true annuli (Landa *et al*., 2013).

Magnification. Both, the use of a profile projector at 50 × (as it was initially used by Dupouy *et al*., 1986), or the use of a microscope at 40x, allow a better observation of the annuli. However, the use of higher magnification (100 x), which was the standard observation methodology used by Duarte *et al*. (2002) and subsequent studies, involves the observation and counting of both, true annuli and some false increments (Landa *et al*., 2013).

Age interpretation in *illicia* consists of identifying dark and light annual increments; although for age estimation only the dark annuli are counted (Fig. 10.3.1.1). The annuli in some *illicia* are clearly visible because they are well defined, but the increments appear doubled in others, which makes age estimation difficult.

The age estimation guide of anglerfish (Duarte *et al*., 2002) also included some characteristics inherent to the age interpretation using *illicia* that must be considered:

It is important to adjust and play with the light and focus of the microscope, to identify an overall pattern of growth. Unlike otoliths, where the annuli widths tend to decrease when approaching the edge, in *illicia*, the annuli remain with a similar width throughout all the section. Annuli close to the edge may even be wider than those closer to the nucleus (Duarte *et al*., 2002).

Annuli in *illicia* differ in composition. As a result, the surface appears rippled, alternating between high and low ridges. The differences in these levels relate directly to the dark and light rings. This characteristic is very apparent from research carried out using scanning electron microscopy (Duarte *et al*., 2002).

Annuli may not be visible in all the axes of the section. Defined annuli, which are clearly visible in one part of a section may be less defined or even appear to double in another part of the section. The counting should be based upon the area where good contrast between annuli exists (Duarte *et al*., 2002).

The next step in the age estimation process is the identification of the first annulus position, and its confirmation by measuring its diameter. The first well-marked growth increment observed is considered to be a consequence of a change in the life cycle of the fish (from planktonic to benthic living), and is therefore designated as the benthic growth increment. Although the next growth increment has been traditionally considered to be the first annulus, following the age estimation guide (Duarte *et al*., 2002), the study of Wright *et al*. (2002) based on micro-increment analysis of *L. piscatorius*, concluded that that growth increment (that oval shaped in Figure 10.3.1.1) should not be considered as an annulus. The following growth increment, with a diameter that tends to be around 300-380 µm (Figure 10.3.1.1), is then considered as a real annulus (Landa *et al*., 2013), while the named benthic growth increment (not an annual increment) tends to be around 160-220 µm. Although, there is not yet validation studies on the diameters of the first growth increments for *L. budegassa*.

To identify the outer annulus, it is very important to observe the *illicium* edge. For this, it is essential to know the quarter (or month) in which the sample was taken. This will determine whether or not the annulus at the edge is to be counted in the age estimation process. If the outer annulus is not visible throughout the whole *illicium* section, this may be because the section has not been cut perpendicularly and not because it is not a true annulus. When a dark annulus appears at the edge in Q1, it should be counted and included in the age estimation. If a similar annulus appears in Q4 it should not be counted or included in the age estimation.

To estimate the age of the *illicia* of a fish group with a similar length altogether is recommended, starting first with the clearest *illicia* sections. This is a good exercise to help train the eye in identifying the typical growth pattern of the *illicia*. Since the first annuli in young fish are often difficult to define it is easier to begin estimating the age of the *illicia* of a medium-size length fish to establish the growth pattern of these first annuli (Duarte *et al.,* 2002).

Fish length can be a useful piece of information in the *illicia* age estimation. Doing a first estimation of the age and, afterwards, checking that this age estimation lies within the possible mean length range of the fish at that age is recommended (Duarte *et al.,* 2002).

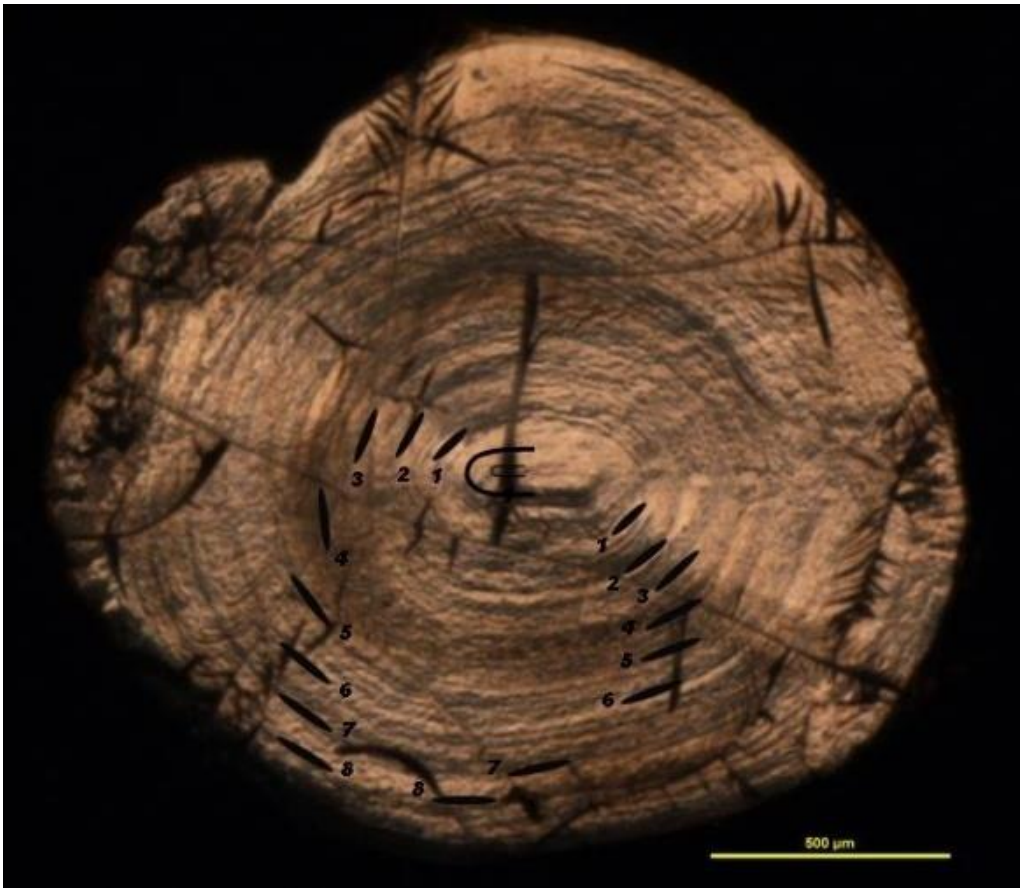


Figure 10.3.1.1. *Illicium* of *L. piscatorius* of 89 cm and an estimated age of 8 years old. The annuli are marked with numbers, and the two structures marked in the central area, lineal and oval in shape, are both considered checks (false annual increment) (Landa *et al.*, 2013).

Difficulties in age interpretation: The age estimation of anglerfish is not easy, mainly because annuli appear doubled or are not well defined in some *illicia* sections and false annual increments may also occur (Duarte *et al.,* 2002). As an example we can see that within the *illicia* exchange of *L. piscatorius* in 2011, the "medium" credibility level was the most frequent for most readers (50%). The "high" and "low" credibility levels were estimated in a similar proportion (around 21-25%) (Landa, 2012).

Doubts in age estimation of *illicia* of intermediate ages may be related to first maturation or any other unidentified life-history event, which causes changes in the growth pattern (Duarte *et al*., 2002).

The age of *L. piscatorius* is not routinely estimated by the IEO age readers, although the *illicia* are being collected in the routinely samplings (see section 10.1.1., in annex 10). Solid research steps are being

taken to improve the knowledge on the real annual growth pattern of this species using CS (see section 10.4.1, in annex 10).

10.3.2 DAILY AGE

The *lapilli* otoliths are used to examine daily increments, since they appear more clearly defined in these pair of otoliths. The *lapilli* otoliths of *L. piscatorius* are very small with a rounded shape and thickness, making them less fragile than the other two otoliths pairs.

Preparation: The *lapilli* otoliths extracted from juveniles are cut into sections by a sanding and polishing process (Secor *et al*., 1992) (see section 7.4.2). Each otolith section is processed on the sagittal plane with respect to the fish.

Observation: Juvenile *lapilli* otolith sections are viewed through a microscope connected to an image analyzer.

Magnification: To facilitate the observation of otolith sections, a drop of immersion oil is added to the surface of each otolith section, and they are viewed at x400 magnification (and x1000 for analyzing the primordium zone) by means of a light microscope equipped with a digital camera.

Reading axes: The widths of the increments are measured along the longest radius of the *lapilli*, starting at the hatch check (Campana and Jones, 1992) to the outer edge of *lapillus* as it gives the most unambiguous sequence of growth increments.

Age estimation criteria: The first increment is assumed to being formed on the hatching day and the other increments are assumed to be laid down daily, thus the increment number is considered an indicator of the age in days. The primordium of *lapilli* consists of one o two cores followed by a few increments surrounded by well defined increments that could be most likely the hatching check. A second less evident check, the possible first feeding check, is also observed.

Interpretation difficulties: These difficulties could be explain by: 1) difficulties in the interpretation of subdaily increments; 2) the localization of microstuctural checks in the area surrounding the primordium; 3) unclear images, in which is difficult to interpret correctly the daily growth pattern due to under-or over-polishing or poor image acquisition. The downside of this process is that a large number of samples are rejected.

10.4 AGE ACCURACY, VALIDATION AND CORROBORATION

10.4.1 ANNUAL AGE

The age estimation from *illicia* of a decadal time-series was performed for the southern stock (Iberian Atlantic) assessment of *L. piscatorius*, using the internationally standardized age estimation criterion of Duarte *et al*. (2002). A catch-at-age by year matrix was built, but inconsistencies in cohort tracking were found (Azevedo *et al*., 2008). Since then no age-structured model has been used for the assessment of both northern and southern stocks of the European Atlantic southern shelf of *L. piscatorius* (ICES, 2011c). A production model (ASPIC, Prager, 1994) has been used for the assessment as an alternative to the age-structured models. Length-based model (CASA, Sullivan *et al*., 1990; Dobby, 2002) including von Bertalanffy growth parameters is also being attempted for the stock assessment of *L. piscatorius*.

Growth studies alternative to the age estimates on CS of *L. piscatorius*, such as tagging-recapture (Laurenson *et al*., 2005; Landa *et al*., 2008), daily increment analysis (Wright *et al*., 2002) and length frequency distributions of catches (Dupouy *et al*., 1986; Thangstad *et al*., 2002; Jónsson, 2007), also showed that the growth pattern estimated using the traditional age estimation criterion based on *illicia* (Duarte *et al*., 2002) was partially underestimated and that that criterion was not accurate, although it was internationally standardized and used in several age estimation anglerfish workshops (Anon 1991b; Anon 1997a; Anon 1999; Landa *et al*., 2002; Duarte *et al*, 2005). Modifications in the methodology of *illicia* preparation and in the traditional age estimation criterion have allowed a new age estimation criterion on *illicia* (Landa *et al.,* 2013). The use of this new criterion allows a better cohort tracking of the catch at the age data from the survey data of Porcupine Bank and is consistent with the length-frequency analyses of those data (Landa *et al.,* 2013). Another study (Ofstad *et al*., 2013) has been recently presented on the age and growth of *L. piscatorius* in Faroese waters. These two studies presented a similar growth pattern from *illicia* and are also consistent with growth estimates from aforementioned length frequency analyses and tagging -recapture results.

Further studies on validation and corroboration of age and growth of *L. piscatorius* have been recommended (ICES, 2012b).

10.4.2 DAILY AGE

The daily periodicity of micro-increment deposition has not been validated in early life stages of *L. piscatorius* by any of the validation techniques available for daily age (Campana 2001). There are two studies on juvenile growth of this species in North-East Atlantic waters (Wright *et al*., 2002; Hernández *et al*., 2014).

10.5 AGE PRECISION, VERIFICATION AND QUALITY CONTROL

10.5.1 ANNUAL AGE

The first growth patterns of *L. piscatorius* in Atlantic waters estimated by using hard parts (*illicia* by Dupouy *et al*., 1986 and otoliths by Crozier, 1989) showed similarities, but with one age class of difference between them. The growth pattern is easier to distinguish in the *illicium* (Crozier, 1989; Woodroffe *et al.*, 2003) and it has become the standard structure for age estimation of *L. piscatorius* in most European countries (Landa *et al.*, 2008).

The age estimation criterion on both *Lophius* species used by IEO readers has been externally verified in international *illicia* and otoliths exchanges and workshops (Anon, 1997a; Anon, 1999; Landa *et al*., 2002; Duarte *et al*., 2005; Landa, 2012). The current age readers of IEO participated in the recent exchanges and workshops, showing good results in agreement, precision and relative accuracy for *illicia* (Duarte *et al*., 2005; Landa, 2012). However, improving the precision in the absence of accuracy cannot, under any account, guarantee data quality (de Pontual *et al*., 2006). Although the age criteria for this species were internationally standardized and the age estimation of the readers until 2007 was verified, it did not mean that this criterion was not biased, as previously explained.

The age of *L. piscatorius* illicia is estimated by two readers.

In addition to the estimated age, the quality (or credibility) of each age estimation is also assigned according to the "3 point grading system" recommended by WKNARC-1 and WKNARC-2 (ICES, 2011a; 2013a), as it is described in section 9.4.

10.5.2 DAILY AGE

To test the quality control of daily age estimates, internal (reading procedure) practices are developed by IEO. The daily age estimation of each *lapillus* is performed at least twice until a consistent increment count is obtained. When error in the daily age estimation precision is more than 10%, a third estimation is performed. If the discrepancy persists, the *lapillus* is discarded.

Annex 11: BLACK ANGLERFISH (LOPHIUS BUDEGASSA)

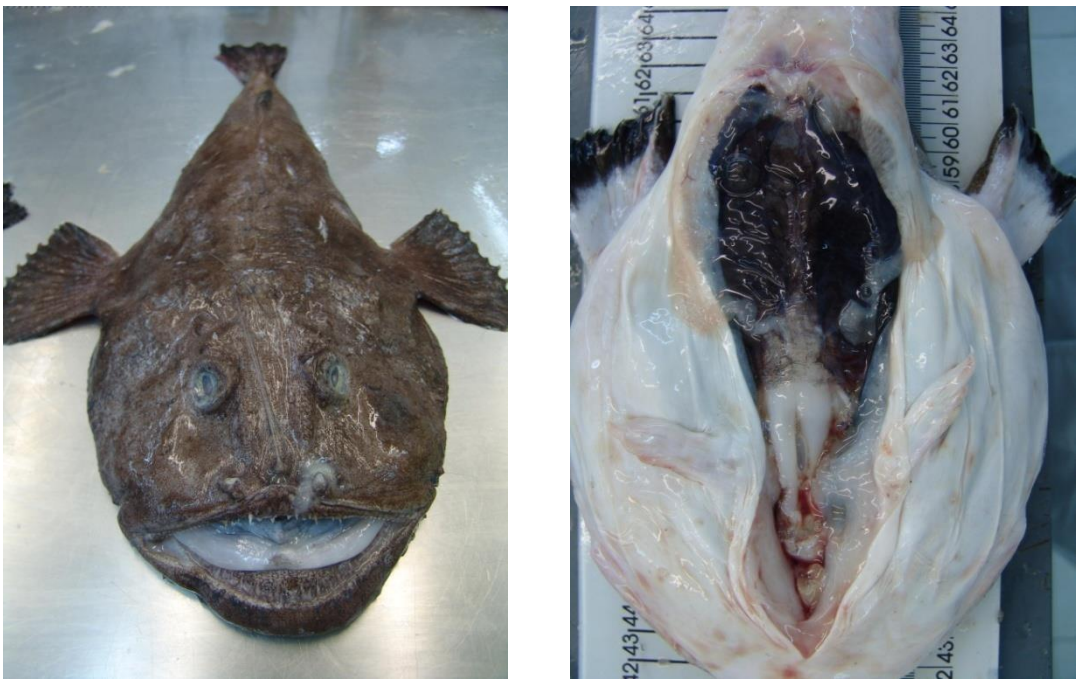


Figure11.1. Dorsal and ventral view of *L. budegassa*, showing the black peritoneum.

11.1 SAMPLING PROGRAM FOR AGE ESTIMATION

11.1.1 ANNUAL AGE

Routine samplings for age determination of *L. budegassa* are performed in IEO since the mid 90s, from both, commercial catches and research surveys. The number of *illicia* collected in 2012 is shown in Table 11.1.1.1.

Table 11.1.1.1. Number of *L. budegassa illicia* collected from the commercial fleet and research surveys in 2012.

Stock	Commercial fleet	Research surveys	Total
ICES Div. VIIb-k, VIIIabd	404	35	439
ICES Div. VIIIc, IXa	446	98	544
Total	**850**	**133**	**983**

11.1.2 DAILY AGE

Lapilli otoliths from juvenile *L. budegassa* for daily growth studies and otolith microstructure analysis were caught in research surveys in 2010 and 2012 (Table 11.1.2.1).

Table 11.1.2.1 Number of *L. budegassa lapilli* otoliths collected for daily growth studies from research surveys during 2010 and 2012 in ICES Div. VIIIc, IXa.

Year	Research surveys
2010	37
2012	21
Total	**58**

11.2 ILLICIA/OTOLITH EXTRACTION AND STORAGE

The methods for obtaining the *illicium* (for annual age) and the otoliths (for daily age) are the same as those described for *L. piscatorius* (section 10.2., in annex 10).

11.3 ILLICIA/OTOLITH PREPARATION AND AGE ESTIMATION METHOD

11.3.1 ANNUAL AGE

The traditional methodology of *illicia* mounting in resin plates of *L. budegassa* is the same as that of *L. piscatorius* (see section 10.3.1., in annex 10). It was originally described by Dupouy *et al*. (1986) and, after several European age estimation workshops of anglerfish (Anon, 1997a; Anon, 1999; Landa *et al*., 2002), it was standardized and was included in an age estimation guide for anglerfish (Duarte *et al*., 2002). That *illicia* methodology was used in most of the growth studies (Duarte *et al*., 1997; Quincoces *et al*., 1998b; Landa *et al*., 2001).

The age of *L. budegassa* is not routinely estimated by the IEO age readers, although the *illicia* are being collected in the routinely samplings (section 11.1.1). The aforementioned methodology of *illicia* preparation is available, but an age estimation criterion validated / corroborated is not currently available for this species. There is not enough knowledge on the real annual growth pattern of *L. budegassa*, but some solid research steps are being taken to estimate it (see section 11.4.1, bellow). Thus, the recent advances in its otolith microstructure analysis (La Mesa and De Rossi, 2008; Hernández *et al*., 2015) can help to locate the first annulus more precisely. Further research using several methods, as length frequency analysis, tagging-recapture studies, etc, is also necessary to obtain a better knowledge about the true growth pattern of *L. budegassa*.

11.3.2 DAILY AGE

The otoliths preparation and age estimation method for daily growth studies is the same as that described for *L. piscatorius* (see section 10.3.2., in annex 10).

11.4 AGE ACCURACY, VALIDATION AND CORROBORATION

11.4.1 ANNUAL AGE

The age estimation from *illicia* of a decadal time-series was performed for the southern stock (Iberian Atlantic) assessment of *L. budegassa*, using the internationally standardized age estimation criterion of Duarte *et al*. (2002). A catch-at-age by year matrix was built, but inconsistencies in cohort tracking were found (Azevedo *et al*., 2008). Since then no age-structured model has been used for the assessment of both northern and southern stocks of the European Atlantic southern shelf of *L. budegassa* (ICES, 2011c). A production model (ASPIC, Prager, 1994) has been used for the assessment as an alternative to the age-structured models.

The micro-increments studies of La Mesa and De Rossi (2008) and Hernández *et al*. (2015) showed a faster growth pattern in the early stages of *L. budegassa* in Mediterranean and Atlantic waters respectively. Both studies also showed that juveniles at least up to 20 cm collected in September or October were born in the same year and, therefore, belong to age class 0. As the traditional annual age estimation criterion based on *illicia* that was used in the assessment of Atlantic stocks was being underestimated, those results may be also basic to establish a new and corroborated age estimation criterion using hard parts, in a similar way to that occurred in *L. piscatorius*.

11.4.2 DAILY AGE

The daily periodicity of micro-increment deposition has not been validated in early life stages of *L. budegassa* by any of the validation techniques available for daily age (Campana 2001). There are two reports on daily growth increment counts for this species performed on *lapilli* otoliths of juveniles captured in the Adriatic Sea (Mediterranean waters) (La Mesa and De Rossi, 2008) and in the Cantabrian Sea (Atlantic waters) (Hernández *et al*., 2015).

11.5 AGE PRECISION, VERIFICATION AND QUALITY CONTROL

11.5.1 ANNUAL AGE

As in its congener *L. piscatorius*, the growth pattern of *L. budegassa* is also easier to distinguish in the *illicium* (Anon, 1991) and it has become the standard structure for age estimation in most European countries.

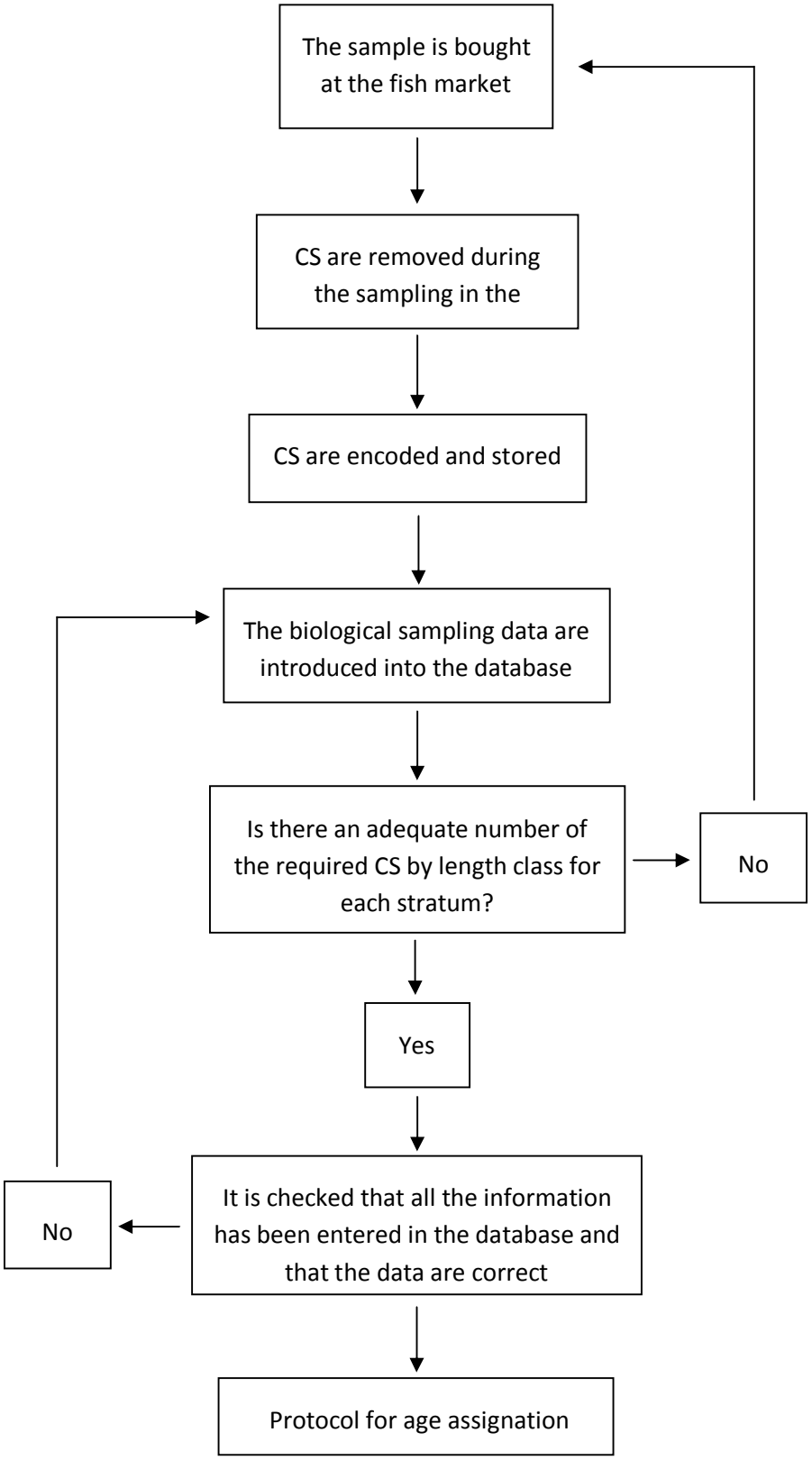
The age estimation criterion on both *Lophius* species used by IEO readers has been externally verified in international *illicia* and otoliths exchanges and workshops (Anon, 1997a; Anon, 1999; Landa *et al*., 2002; Duarte *et al*., 2005). The current age readers of IEO have participated in the recent exchanges and workshops, showing good results in agreement, precision and relative accuracy for *illicia* (Duarte *et al*., 2005). However, improving the precision in the absence of accuracy cannot, under any account, guarantee data quality (de Pontual *et al.,* 2006). Although the age criteria for this species were internationally standardized and the age estimation of the readers until 2007 was verified, it did not mean that this criterion was not biased, as previously explained.

In addition to the estimated age, the quality (or credibility) of each age estimation is also assigned according to the "3 point grading system" recommended by WKNARC-1 and WKNARC-2 (ICES, 2011a; 2013a), as it is described in section 9.4.

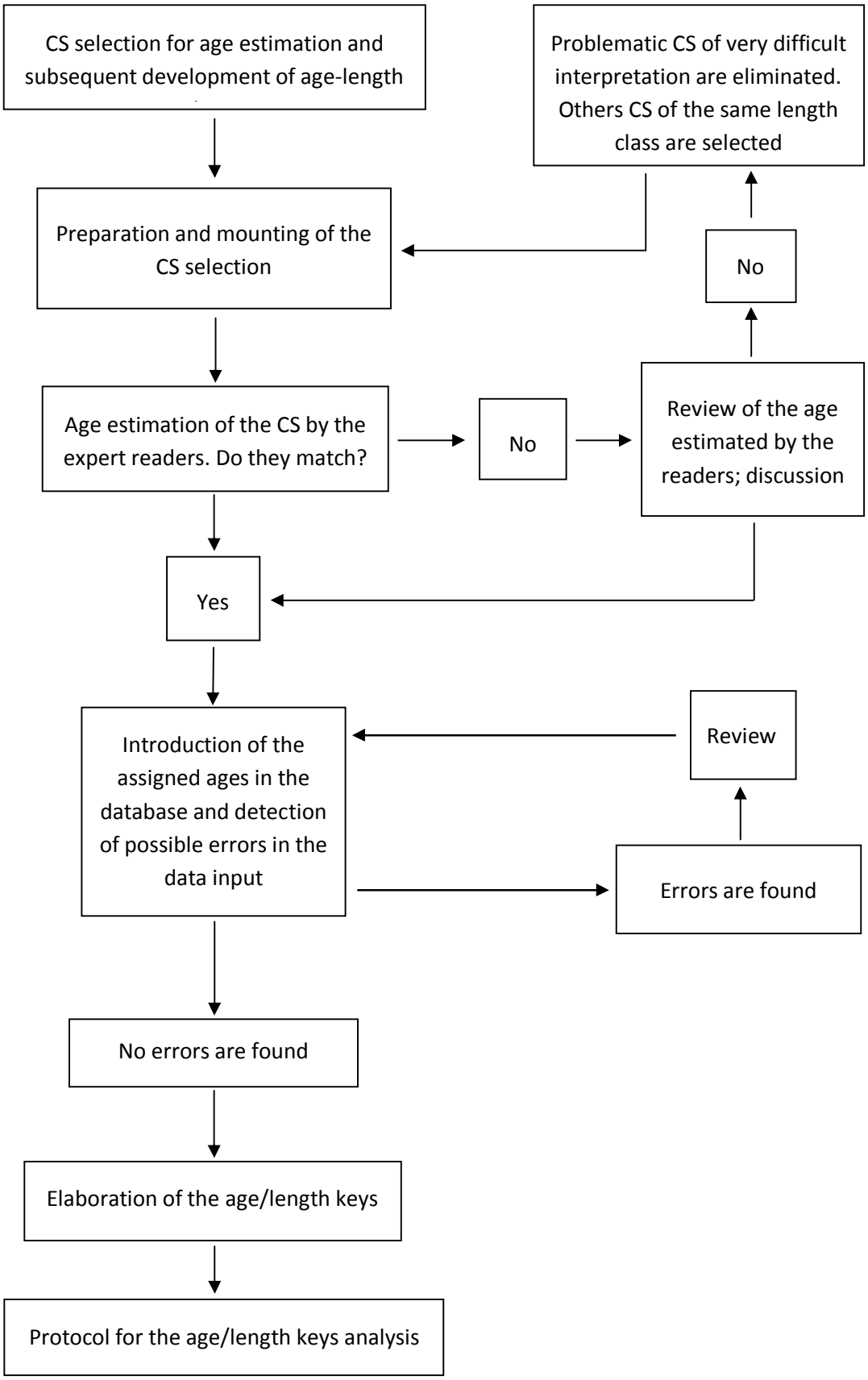
11.5.2 DAILY AGE

To test the quality control of daily age estimates, the same internal (reading procedure) practices as those described for *L. piscatorius* (see section 10.5.2., in annex 10) are applied in *L. budegassa.*

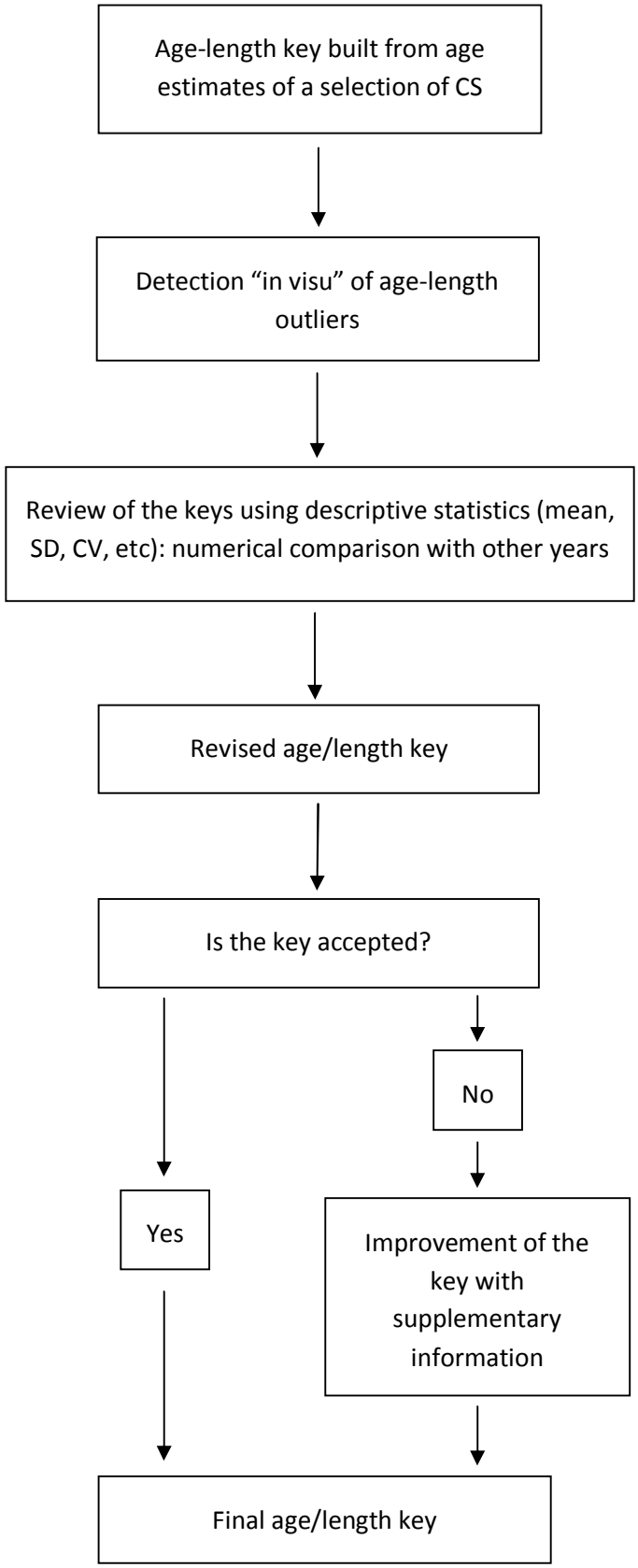
ANNEX 12. PROTOCOL FOR DATA COLLECTION AND STORING



ANNEX 13. PROTOCOL FOR AGE ESTIMATION



ANNEX 14. PROTOCOL FOR AGE-LENGTH KEYS ANALYSIS



ACKNOWLEDGEMENTS

This work was funded through the Spanish Institute of Oceanography (IEO, BIOPEL and BIOBENTON projects) and European Union (Data Collection Framework Program). We would like to thank all those who collaborated in that project. The authors wish also to thank the IEO scientific teams in CO Santander, CO Vigo y CO A Coruña, as well as all the participants in the IEO research surveys that made the fish samplings. We also thank the samplers in harbors and fish markets, as well as the fishers, crews and ship-owners of commercial vessels, for their collaboration in the fish samplings.

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