3. PROTOCOL FOR OTOLITH PREPARATION AND AGE READING

This protocol was developed to harmonize methodologies for otolith preparation and age reading within the framework of SUDOANG. It is based on Manual on Age reading (ICES 2009). The sex of individuals should be identified by macroscopic analysis (See *Protocol to sample gonads for sex ratio assessment*).

3.1. Extraction and storage

- Extract both sagittal otoliths from each eel;
- Clean them with water and dry them;
- Store the otoliths dry in small containers (e.g. *eppendorfs)*, but make sure they are fully dry to avoid deterioration.

3.2. Grinding and polishing

- Choose the right otolith for consistency. If not possible choose the left, but include this information in the observations;
- Otoliths can be observed whole (without preparation) with strong transmitted light or on a dark surface with strong incident light. If there are less than 4 to 5 annual marks, the age can be read without any other preparation, except being immersed into 96% ethanol to improve the visualization of the growth marks.
- If age is more than 5 years, otoliths must be embedded in resin;
- Sagittal sections are obtained by embedding directly the otolith in resin while transverse section requires embedding in two layers of resin, so that the otolith is in the middle of the resin block;
- The grinding process must be carefully checked until the midplane of the otolith has been reached. For that, the otolith is examined under a stereo dissecting microscope, with the largest magnification possible, using a variety of light types including transmitted, reflected or polarized light;
- The otolith is ground along the sagittal (if eel is up to 12 years, and otolith is not curved) or transverse plane (requires cutting a slice of otolith along the transverse axis with a diamond saw) (depending on the curvature of the otolith so depending on the size of the otolith) until the centre of the nucleus is reached;
- Grinding can be performed manually, or by using a grinding wheel with silicon carbide sandpaper, lubricated with distilled water;
- Polish the ground surface of the otolith using a decreasing range in coarseness (1200-4000 grit) of silicon carbide wet dry sandpapers, jewellery cloths or pastes made from aluminium or diamond powder, lubricated with distilled water.

For more details on the procedures, consult the Manual for Age Reading of Atlantic eel (link below in the references).

3.3. Age reading

- After preparation, you should acquire images of the otoliths to facilitate exchange among all readers.
- Sagittal sections require etching and dying to enhance winter marks. Transverse sections do not require that process;
- Read the age, i.e., count the number of winter marks. Translucent zones (winter) are bright, and opaque zones (summer) are dark, when the otolith is viewed with **transmitted light.** If viewed with **reflected light,** opaque zones (summer) are bright, and translucent zones (winter) are dark.

3.4. Material

- Stereo dissecting microscope (with digital camera and image capture and analysis software Image J);
- Glass microscope slides;
- Fine point forceps;
- Mounted needles;
- Slide container box.
- Reagents needed for the preparation:
  - Wax, epoxy resin, to embed the otolith;
  - Alumina and diamond powder pastes for grinding the otolith.

References

ICES. 2009. Workshop on Age Reading of European and American Eel (WKAREA). Bordeaux, France: ICES CM 2009\ACOM: 48, 66 pp.

ICES 2009. Manual for the Ageing of Atlantic Eel. In Workshop on Age Reading of European and American Eel, (WKAREA) Annex 4, 57 pp.

3. Age Reading

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Biometric data

(*Total Length - mm; Total Weight - g; Eviscerated Weight - g;* ***Sex*** *- Male/Female/Undifferentiated*)

Eel ID	Site code	Date	TL	TW	EW	Sex	Age	Remarks