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Workshop on Ageing Validation methodology of *Mullus* species (WKVALMU)

15–19 May 2017

Conversano, Italy

International Council for the Exploration of the Sea

Conseil International pour l'Exploration de la Mer

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Executive summary

The Working Group on Biological Parameters (WGBIOP) meeting in 2016 (ICES, 2016) recommended a Workshop of Ageing Validation methodology for *Mullus* species (WKVALMU). This workshop (Co-chairs: Kélig Mahé, France; Pierluigi Carbonara, Italy and Chryssi Mytilineou, Greece) has been held in Conversano (Italy) from 15 to 18 May 2017. Five countries took part in this meeting (Italy, Spain, Greece, France, Croatia) for a total of 16 participants. This meeting was organized to try to clarify the rules which are applied on the ageing of mullet species (*Mullus surmuletus and M. barbatus*). At the beginning of the workshop, a lot of presentations were discussed focusing on the used ageing methodologies by each institute and all ageing validation studies. A synthesis of validation methods was conducted during the workshop aiming to identify a) the first false ring coinciding with the demersal check formation for both species (for the fish around 5 cm of total length), b) the period with opaque edge in otoliths (from May to October) and c) the mean length at the first age groups derived from the length distribution analysis. Various schemes for the age interpretation are used by the readers. This is a source of bias in the readings. As a consequence, schemes for the age interpretation have been discussed. Two main age interpretation schemes were decided to be appliedon a set of 40 images. These otoliths had also been used in the 2011 exchange. The bias between the two age interpretation schemes was estimated. According to this, the bias between readers was smaller for the scheme 1 (birthdate: 1st January) than scheme 2 (birthdate: 1st July). Differences in ageing were detected during the first semester. Moreover, these results showed the low agreement between readers for each interpretation scheme. Comparing the modal age obtained by each interpretation scheme, a significant difference was observed. For stock assessment and management purposes, it would be desirable that all countries use the same age interpretation scheme. Based on several discussions on the age interpretation results of this exercise, a new age interpretation scheme was proposed during this workshop. The WKVALMU proposed recommendations for the next exchange which will be organised in 2018. In 2019, the new workshop (WKCAM3; Split; co-chairs: P. Carbonara, Italy; K. Mahé, France; D. Medvesek, Croatia) will focus on the analysis of the new exchange results, validation studies and will formalise guidelines on the ageing of *Mullus surmuletus* and *M. barbatus*. During the present workshop, it was noted that the difficulty and the low agreement in age interpretation makes necessary the development of a European project on age validation methods. Consequently, a draft proposal has started to be written during this workshop.

1 Introduction

1.1 ToRs of the WKVALMU

The **Workshop on Ageing Validation methodology of *Mullus* species** (WKVALMU), chaired by Chryssi Mytilineou, Greece; Kélig Mahé, France and Pierluigi Carbonara, Italy will meet in Conversano Italy, 15–18 May 2017 to:

a) Analysis of the results of past exchanges and workshops;

b) Review of the age validation methods (direct, indirect and semi-direct) and their applicability on the *Mullus* species;

c) Examples of morphological and morphometric analysis in the context of the age validation;

d) Multi-parameters analysis on datasets with different ageing schemes/criteria (birthday, numbers of checks before the first winter ring, preparation method);

WKVALMU will report by 13 June 2017 for the attention of SSGIEOM and WGBIOP.

Supporting Information

Priority:	The age and growth (growth parameters, ALK) are essential input data for the models usually used in fish stock-assessment, mainly for the analytic ones, to establish a diagnosis on stock status. Many of the uncertainty on the stock evaluation could come from to the inconsistency on ageing analysis (otolith reading). In the last years, three exchanges and two workshops have been organized on the ageing calibration (ICES, 2009; ICES, 2012; Mahè et al., 2011; Mahè et al., 2016) of Mullus barbatus and Mullus surmuletus without substantial improvement of the age precision index (% agreement, CV and APE). The most important problems that affect the accuracy and precision are: • Identification of the first winter ring; • Different ageing schemes; • Ring overlapping in oldest specimens. The stock assessment groups for Mullus species continue to use the age data until now; however, without a substantial improvement on the ageing quality it would be better stop using the age data (otolith reading) as the input data for the stock assessment
Scientific justification:	This workshop will provide the opportunity for the ICES/GFCM community working on: • age validation methodology more appropriate to the Mullus species; • statistically evaluate the influence of the ageing protocol on the age data as well as effect of ageing scheme, ageing criteria preparation methods, birthday used etc. The workshop will provide an arena to discuss how it could help to overcome the uncertainty of otolith reading. The workshop will be based on the practical example on the application of the age validation methodology for the Mullus species.
Resource requirements:	In view of its relevance to the EU Data Collection Framework (DCF), the Workshop is expected to attract interest from ICES / GFCM Member States.
Participants:	In view of its relevance to the DCR, and ICES WG, the Workshop tries to join international experts on growth, age estimation and scientists involved in assessment in order to progress towards a common solution.

	Participants should announce their intention to participate in the WK no later than two months before the meeting.
Secretariat facilities:	None
Financial:	Additional funding will be required for facilitate the attendance of the scientists and technicians.
Linkages to advisory committees:	ACOM
Linkages to other committees or groups:	Outcomes from this Workshop will be of interest to all Assessment Working Group related to Mullus species. Moreover WGBIOP, ACOM, RCM, and scientific trawl survey working group like the IBTSWG, and WGMEGS and MEDITSWG.
Linkages to other organizations:	There is a direct link with the EU DCF.

2 Analyse the results of past exchanges and workshops (ToRa)

In September 2015, the Working Group on Biological Parameters (WGBIOP) recommended an otolith exchange for *Mullus surmuletus* and *Mullus barbatus* in 2016 (Otolith Exchanges proposals for 2016–2017; ICES, 2015). Two otolith exchanges (2008, 2011), and two age reading workshops (ICES, 2009; 2012), have been taken place until now (Mahé *et al.*, 2012). In 2016, a total of 13 readers from 5 countries (France, Spain, Italy, Cyprus and Greece) participated at the exchange. The otoliths of 465 individuals (345 *M. barbatus* & 120 *M. surmuletus*), sampled from 2011 to 2014 in the Mediterranean Sea (Central Adriatic Sea, Cyprus, Levantine Spain coasts, Balearic Islands) were used for this exchange. For both *Mullus* species, the precision values were very low, the PA ranged between 56 and 67% the CV ranged from 32 to 64% and the APE ranged from 1.9 to 3.6%. The results by area and species showed the same trend with the first age groups presenting the higher CV values and in some cases lower PA values. These results could be explained by the position of the first growth increment and the two different approaches of reading interpretation used by the readers (ICES, 2012).

The followed table (Tab. 1) presents the results during the exchanges and workshops on ageing of *Mullus* species. There is no observed trend on the data precision from 2008 to 2016.

Table 1: Readings precision (PA: percentage of agreement; CV: coefficient of variation) of mullets species during all workshops and exchanges by species and by areas. Green cases showed better precision than red cases.

Species	Area	Value	Exchange 2008	WKACM 2009	Exchange 2011	WKACM2 2012	Exchange 2016
M. surmuletus	Balearic island	PA			58,3	65	
	Bay of Biscay		64,3	72,6	74,8		
	Balearic Islands				52,2		56,2
M. barbatus	Southern Spain				58,7	76,5	
	Adriatic Sea		51,6		71,9		65
	Levantine Sea						62,7
	Cyprus						67
M. surmuletus	Balearic island	CV			23	17,4	
	Bay of Biscay		60,7	26,3	61,7		
	Balearic Islands				29,7		31,7
M. barbatus	Southern Spain				37,3	16,7	
	Adriatic Sea		68,5		59,5		64,6
	Levantine Sea						32,5
	Cyprus						60,9

3 Review of the age validation methods (direct, indirect and semi - direct) and their applicability on the *Mullus* species (ToRb) & Examples of morphological and morphometric analysis in the context of the age validation (ToRc)

So far, no age validation studies for both species have been based on marking/tagging methods. Only semi-direct age validation methods have been published to observe periodicity of formation of rings (opaque and transparent rings) and of marginal increment of otolith. A literature review is reported as follows. Moreover, during the WKVALMU, the results of the validation studies (indirect and semi-direct) from some lab were presented.

3.1 *Mullus barbatus* existing data in Mediterranean Sea

Two studies have been focused on age validation by marginal increment analysis. The first one measured annual increment formation all year long round and presented the lowest value of deposition in August and a highest in January, to conclude that the annual ring is formed after January and before August (EastMed, 2010 from Fisheries Laboratory, 1998).

The other study was based on monthly observations of the marginal increment in order to investigate its formation, which leads to a deposition of a translucent zone from November to May and an opaque zone from June to September (Fig. 1). Marginal Increment Analysis has shown both a translucent and an opaque increment laid down each year.

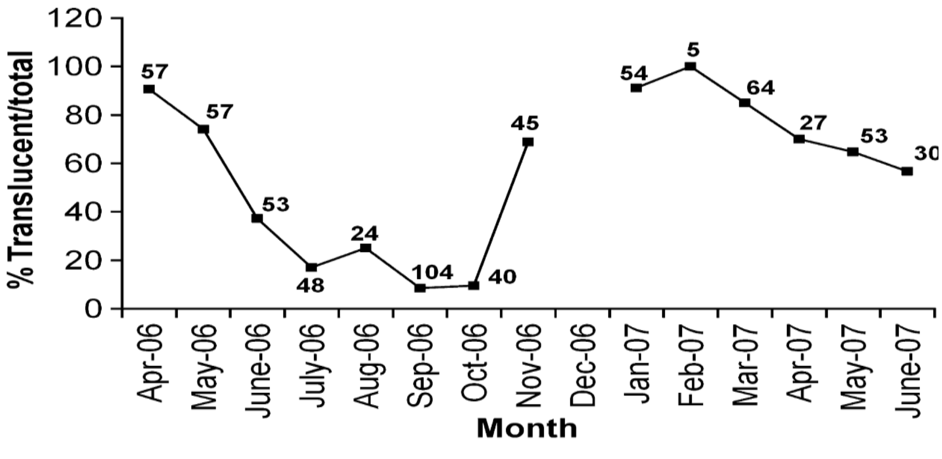


Figure 1. Percentage of occurrence (%) of translucent margins in *sagittae* of red mullet (*M. barbatus*). Number of specimens used to calculate the percentage by month (*In* Sieli *et al.*, 2011).

3.2 *Mullus surmulutus* existing data in the northeast Atlantic and Mediterranean Sea

Mahé *et al.*, (2005; 2013) analysed marginal increment to validate the periodicity of increment formation. Marginal-increment analysis (MI) was carried out on otoliths according to the following formula:

$$MI = (R-r_n)/(r_n-r_{n-1})$$

Where R is otolith ray, r_n is the ray of the last ring and r_{n-1} is the ray before the last ring.

It showed that months of the lowest values run from February to April (Fig. 2 and 3). Therefore, from winter to spring appeared as the possible period of annulus formation. Similar results were found by Reňones *et al*. (1995) and Pajuelo *et al.* (1997) analysing the percentage of individuals with opaque edge (Fig. 4 and 5).

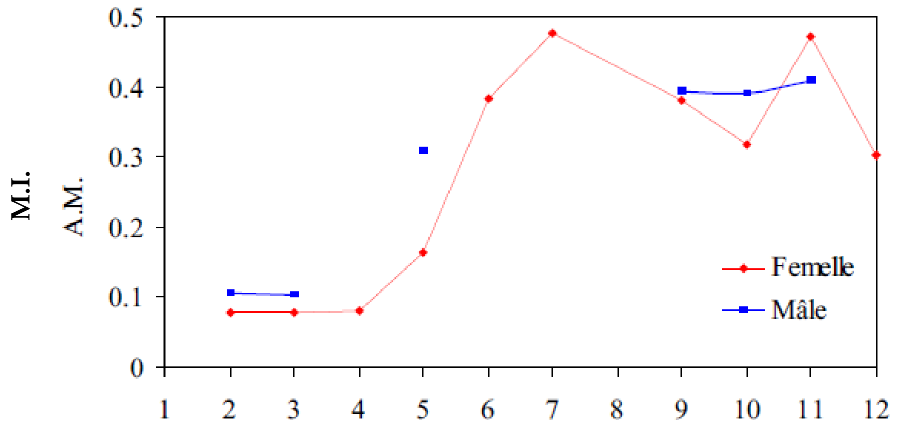


Figure 2: Marginal-increment (M.I.) per month of *M. surmuletus* in the Eastern English Channel and southern North Sea (*In* Mahé *et al*., 2005).

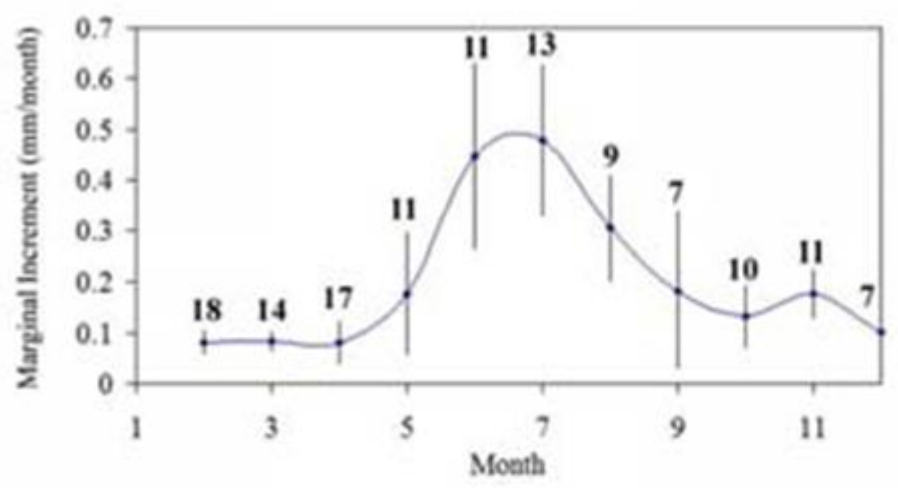


Figure 3: Monthly trend of average marginal increment (mm.month-1; mean±SD) on the sagittal otolith of the Striped red mullet in the eastern English Channel and southern North Sea (n=128) (*In* Mahé *et al*., 2013)

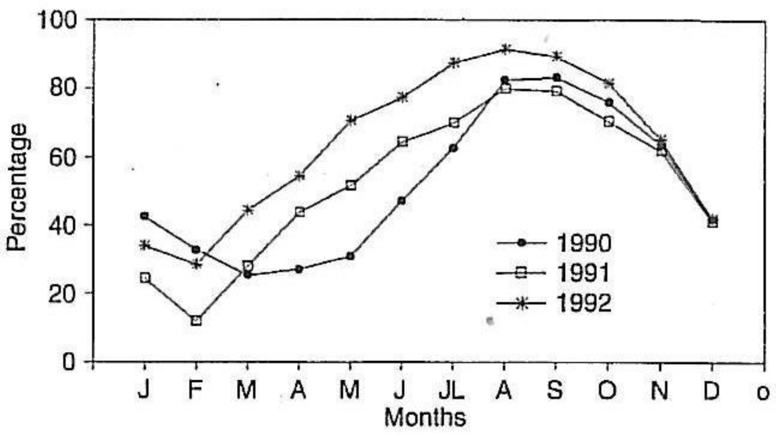


Figure 4: Monthly variations in percentage of individuals with opaque otolith marginal rings of *M. surmuletus* in 1990 (n=1220), 1991 (n=970), 1992 (n=1095) (*In* Renones *et al.*, 1995).

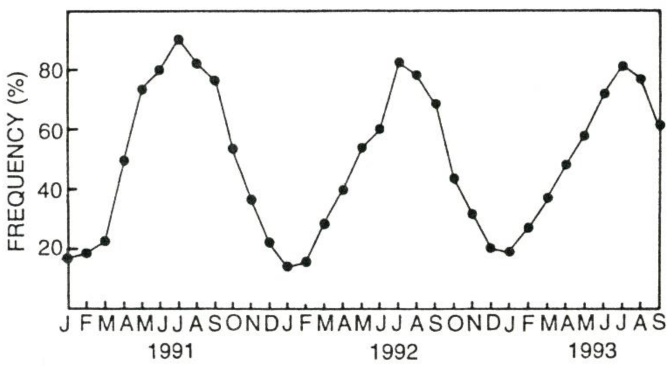


Figure 5: Monthly frequency of otoliths with opaque edges for *M. surmuletus* off the Canary Islands (*In* Pajuelo *et al.*, 1997).

3.3 Direct age validation methodology (F. Ordines, B. Guijarro, N. González)

We reviewed the direct validation methods that could be used in the ageing validation of *Mullus* spp. Particularly, the presentation focused on two methods: rearing of individuals and the possibility of a tagging experiment (both using T labels and chemical tagging for otoliths). The tagging experiment was considered as the most suitable direct age validation method. In this sense, the methodology used in a previous tagging experiment for hake in the Balearic Islands was overviewed. In that experiment, individuals were collected using a bottom trawl gear. In order to increase the survivorship of the individuals that were going to be labelled, a swimming pool codend was used instead of the usual codend used for fishing purposes (Fig. 6).

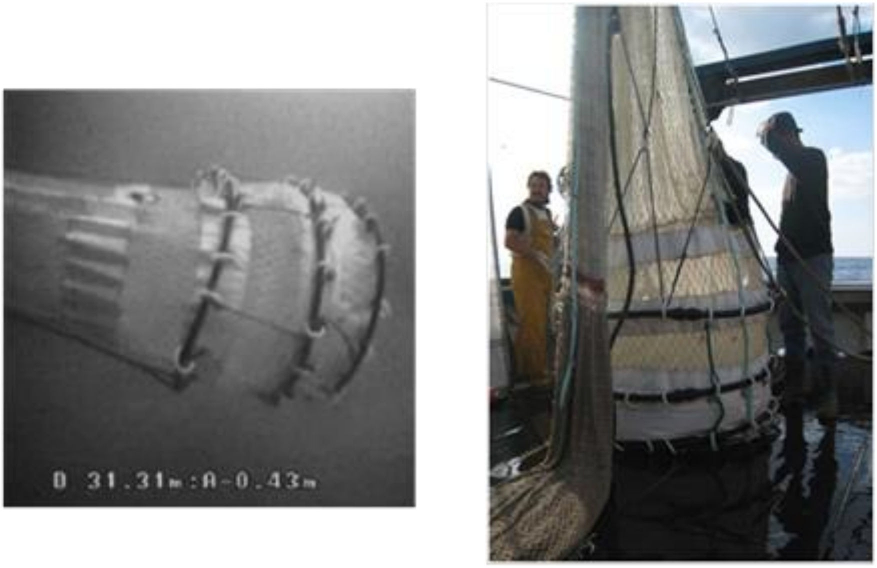


Figure 6: Swimming pool codend images. Left: during the hauling; right: once on deck, preserving the catch swimming in the retained water.

Once individuals are caught, they are kept alive on board in tanks with continuously renovated water. Other experiences carried out by the IEO and dedicated to the study of contamination, used the same methodology to obtain individuals alive of *Mullus* spp. that had to be studied. In that case, the survival rates were above 90% (MEDPOL project, J. A. Campillo pers. com.). Examples on how to decide on the best places to conduct the tagging experiments in a given GSA were described (Fig. 7).

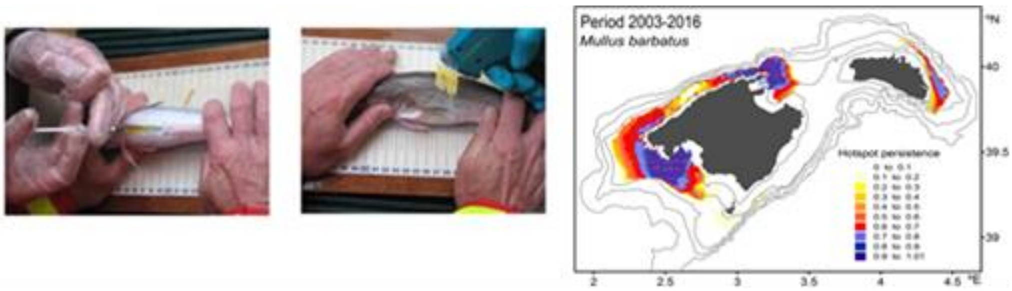


Figure 7: Left images: internal (oxytetracycline) and external labelling of individuals. Right image: results of the analysis for the detection of persistent abundance hostpot areas.

The discussion after the presentation ended up with the agreement of participants to make a proposal to conduct a tagging experiment in the Mediterranean. The proposal would include external and chemical tagging (i.e. oxytetracycline) of otoliths, which could be useful in the interpretation of otolith depositional pattern.

3.4 Holistic approach on the age validation for the red mullet (Mullus barbatus) in the Southern Adriatic Sea (Central Mediterranean) (P. Carbonara, L. Casciaro, M. Palmisano, I. Bitetto, G. Lembo and M.T. Spedicato)

Interpretation of otolith growth zones is challenging due to the occurrence of false rings along with those formed annually as well as the reproductive one (ICES, 2009; ICES 2012). Moreover the discrepancies between readers exist mostly on the identification of the first annual ring. Moreover in the otoliths of older individuals the winter rings are not always easy to read because of the overlapping of the annuli.

The deposition pattern of the ring on the otolith was analysed by a semi-direct (qualitative and quantitative) method: marginal analysis (MA). Regarding the marginal analysis, it was considered the monthly evolution of the type of edge (translucent or opaque) of the otolith. The two edge types are defined as translucent or opaque if more than ¾ of the margin appears as such. The otoliths in which about 50% of the edge is opaque or translucent are not considered in the analysis. The analysis was conducted in two separated groups: juvenile with a TL range between 3.5 – 8 cm and the adult one with a TL included between 11 and 20 cm (Carbonara *et al.*, 2015).

The monthly percentage of opaque margin in the adult specimens show a pattern where the opaque edge is prevalent (> 50%) between June to November while the transparent edge is prevalent from December to May (Fig. 8). The periodicity pattern of ring deposition showed that in red mullet there is a yearly deposition of one transparent ring followed by an opaque area. The same pattern has been shown by the edge analysis for juveniles, except in the case of July and August, where unlike adults, there is a prevalence of the specimens with transparent edge. Thus in juveniles the deposition of a transparent ring was also observed in the months of July and August. During the rest of the year, the adults and young specimens seem to have the same deposition annulus pattern (Fig. 8).

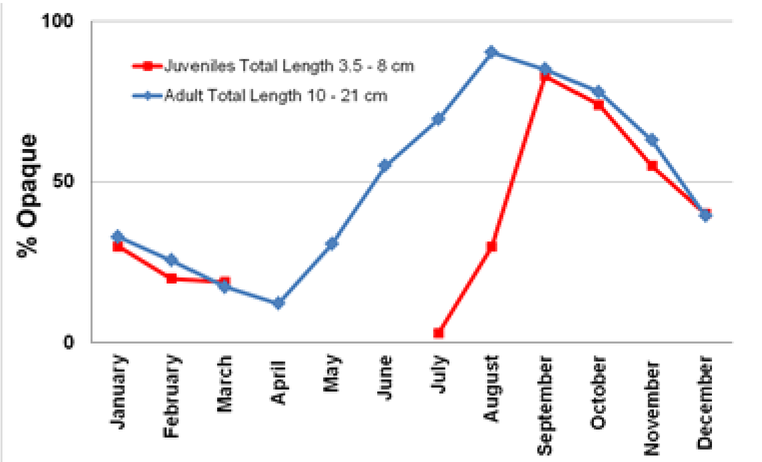


Figure 8 – Montly percentage (%) of opaque margins in red mullet sagittae. The blue line represents the adult, while the red one the juveniles. Numbers of specimens used to calculate the percentage by month is also indicated.

These results demonstrated the deposition of only one false ring.

During two hauls of the Medits survey (2012) both the two juvenile types (blue – pelagic and red – bottom) were caught; in total, 2202 specimens with TL included between 3.5 and 7.5 cm. In Figure 7, the percentage of the two juvenile types by length class (0.5 cm) is shown. The results of the logistic model analysis indicated that the size where the 50% of the specimens show the demersal coloration is 4.418 cm (Fig 9). The smallest demersal specimen observed was 4 cm in TL, while the first length class with 100% of demersal specimens was 5.5 cm.

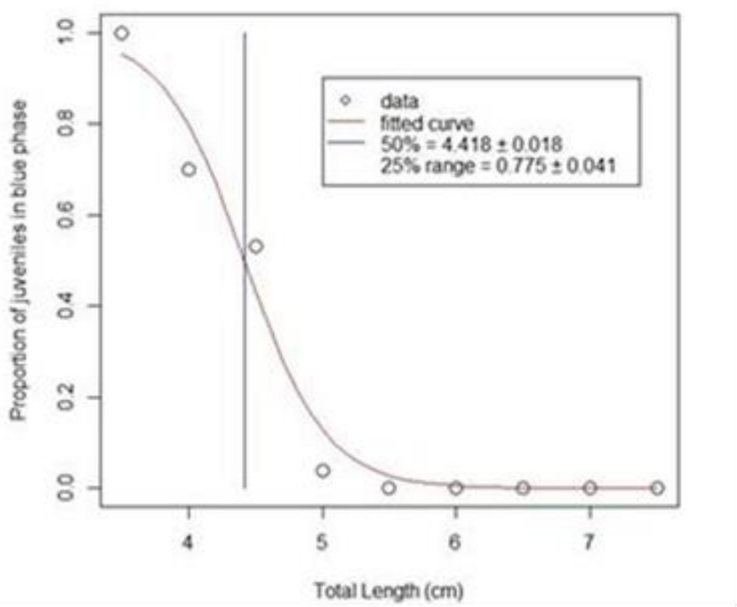


Figure 9: The logistic curve of the proportion of blue – pelagic juveniles of red mullet by length. The length at 50% of the specimens with the demersal colour is calculated.

The fish length at which different transparent rings were deposited was back-calculated using the modified Fraser-Lee formula (Campana, 1990) for the two sexes separately:

$$Li = Lc+(Lc-Lo)*(Si - Sc) / (Sc - So)$$

where Li and Si are respectively fish length and the otolith size at age i; Lc and Sc are respectively fish length and otolith size at capture; Lo and So are fish length and otolith size at hatching (biological intercept).

Taking into account the linear correlation between body length and otolith size, the size at hatching (Sabatés *et al.*, 2015) and the estimation of the otolith size at hatching, through modified Fraser-Lee formula (Campana, 1990) the fish lengths (Tab. 2 and 3) corresponding to the transparent rings were back calculated.

Table 2 – Mean back calculated length for each ring for females red mullet collected in the Southern Adriatic Sea.

		Rings								
N° Ring	**N° Specimens**	**1°**	**2°**	**3°**	**4°**	**5°**	**6°**	**7°**	**8°**	**8°**
1	97	4.47								
2	556	5.06	9.61							
3	652	5.21	9.25	12.31						
4	462	5.12	9.78	13.02	15.37					
5	233	4.58	9.94	13.09	15.59	17.39				
6	83	4.43	10.06	13.33	15.85	17.77	19.36			
7	26	5.13	10.02	13.21	15.58	17.50	19.10	20.58		
8	5	4.52	9.84	12.34	14.75	17.57	18.54	19.90	20.96	
9	3	4.62	9.70	13.26	15.17	17.55	20.23	21.79	22.91	23.90
Tot. number	2117	2109	2020	1464	812	350	117	34	8	3
mean (cm)		**4.51**	**9.32**	**12.73**	**15.49**	**17.49**	**19.29**	**20.58**	**21.69**	**23.90**
mean increment (cm)		5.71	3.61	3.41	2.76	2.00	1.80	1.30	1.11	2.20
sd		0.76	1.01	1.17	1.20	1.25	1.18	1.25	1.41	0.60
CV		13.35	10.78	9.21	7.77	7.13	6.10	6.09	6.50	2.49

Table 3 - Mean back calculated length for each ring for male red mullet collected in the Southern Adriatic Sea.

		Rings						
N° Ring	**N° Specimens**	**1°**	**2°**	**3°**	**4°**	**5°**	**6°**	**7°**
1	284	4.579						
2	542	4.236	8.3					
3	544	4.370	8.7	11.2				
4	372	4.780	9.1	11.7	13.4			
5	120	4.870	9.2	11.8	13.8	15.2		
6	16	4.050	8.8	11.4	13.5	15.1	16.3	
7	1	4.880	8.7	10.9	13.1	14.9	16.7	17.8
Tot. number	1879	1879	1595	1053	509	137	17	1
mean (cm)		**4.53**	**8.7**	**11.4**	**13.5**	**15.2**	**16.3**	**17.8**
mean increment (cm)		5.34	3.3	2.8	2.1	1.7	1.1	
sd		0.73	0.9	1.1	1.1	1.2	1.1	
CV		1.36	1.0	0.9	0.8	0.8	0.7	

The first ring back-calculated TL is comparable to the length (4.418 cm) where the morphological and ecological changes occur in the juveniles: changing from pelagic to demersal life. So the first ring, based on this evidence, can be considered as the false ring (demersal). Moreover considering that the back calculated rings are laid down during the winter time, the ages assigned to these rings is as follows: for the 2° ring 0.5 years, for the 3° ring 1.5 year, for the 4° ring 2.5 year and so on.

The statistical comparison (t-test) between the mean back-calculated length of the winter ring deposition and the mode (Bhattacharya method) of the LFD from the period of winter ring deposition shows no significant differences between the age-group identified through two independent methods (LFD analysis and direct age reading) (Tab. 4).

Table 4 – Results of t-test between average lengths at age (cm) obtained by back-calculation formula and by modal composition (Bhattacharya) for females and males.

MALE				FEMALE			
	Back-calculation	GRUND 2009			Back-calculation	GRUND 2009	
Age	mean TL (cm)	mean TL (cm)	*p> 0.05*	Age	mean TL (cm)	mean TL (cm)	*p> 0.05*
0.5	8.66	8.75	0.243	0.5	9.32	9.36	0.667
1.5	11.44	11.55	0.247	1.5	12.73	12.95	0.061
2.5	13.53	13.59	0.477	2.5	15.49	15.64	0.064
3.5	15.20	15.10	0.460	3.5	17.49	17.75	0.054
4.5	16.34	16.25	0.836	4.5	19.29	19.66	0.054

The statistical comparison (Chen-test) of the VBGF growth curves from the reading otolith and LFDA analysis by sex does not show significant differences (Fig. 10) (Fobs>Fcrit)

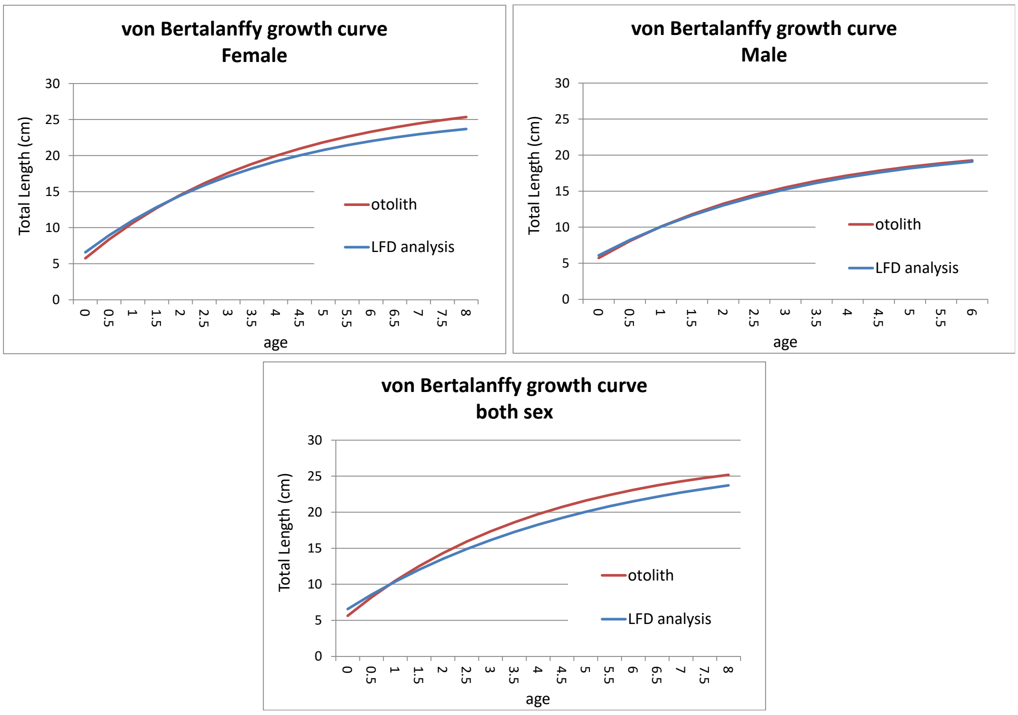


Figure 10: Growth curves from otolith reading (red line), LFDA (blue line) and back-calculation (green line) of male, female and both sex of *M. barbatus*.

The results from this study represent the first attempt of the age validation for the red mullet in the Mediterranean basin. The use of classical age validation methods as well as tag and recapture methods, chemical mark of the calcified structure, bomb radiocarbon dotation, radio chemical dating, captivity rearing (Campana 2001) are hindered considering the high mortality (Düzbastılar *et al.*, 2015) after the capture (stress, scale lost, wound) and the short life span of *M. barbatus* (Özbilgin *et al.*, 2004; Sieli *et al.*, 2011). To overcome these obstacles, it was necessary in this study to put together results from different approaches as well the Marginal Increment Analysis, Marginal Analysis, ring measurements and morphological analysis. This approach valid the ageing criteria partially identified in the ICES workshop (ICES, 2009; ICES, 2012):

- Distance from the core of the consecutive annuli should be decreasing
- Before the first winter ring was laid down only one false ring (demersal)
- Deposition of one opaque and one translucent zone per year
- Translucent true rings should be visible more or less around the whole otolith in order to be considered as annual rings.

Moreover the statistical comparison between the mean back-calculated length and the mean length mode (Bhattacharya method) of the LFD from the winter period (ring deposition months), provide an indirect validation of the detected age-group. Moreover the comparison of the growth curves from the otolith reading and the LFDA does not show any statistical differences. This result represents a corroboration of the otolith ageing criteria that were utilized.

3.5 Towards age validation of *Mullus surmuletus* in the South Aegean Sea (GSA22) (V. Kousteni, Ch. Mytilineou, P. Bekas, A. Anastasopoulou)

The aim of the present study was to validate the aging procedure for the striped red mullet *Mullus surmuletus* based on the identification of annual increments in the otoliths of the species. Complementary information derived from the reproductive period, the edge and marginal increment analysis, the examination of the annual, false and daily rings, the closure of the rostrum at the anterior part of the otolith and the length frequency distribution was also used. A total of 831 individuals were sampled within the Data Collection Framework Program in the South Aegean Sea between February and December 2016, ranging from 95 to 310 mm in total length (TL). The presence of a translucent ring at the otolith edge was observed mainly between February and June with highest values during March and April (Fig. 11). A translucent ring formation was also detected between August and October, however, in much lower percentages. The former translucent ring was considered as annual ring (winter ring), whereas the latter was supposed to be related to reproduction or environmental factors.

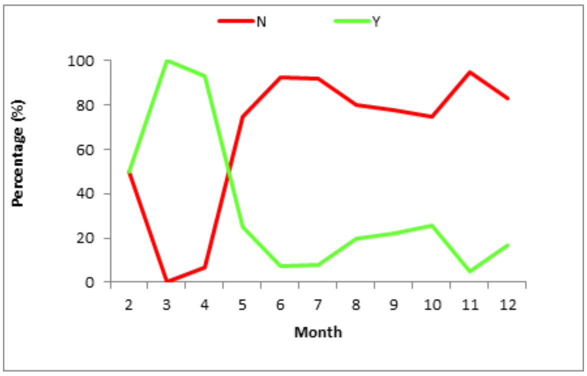


Figure 11: Percentage of translucent ring occurrence (Y) or not (N) at the edge of the otolith.

Further detailed study of the edge type revealed 5 categories of edge (A: Beginning of the appearance of a non-continuous translucent ring at the edge of the otolith, B: Continuous and thin (narrow) translucent ring at the edge of the otolith, C: Continuous and thick (wide) translucent zone at the edge of the otolith, D: Continuous translucent zone followed at the edge by a non-continuous thin and weak opaque zone, E: Continuous and obvious fully formed opaque zone) (Fig. 12).

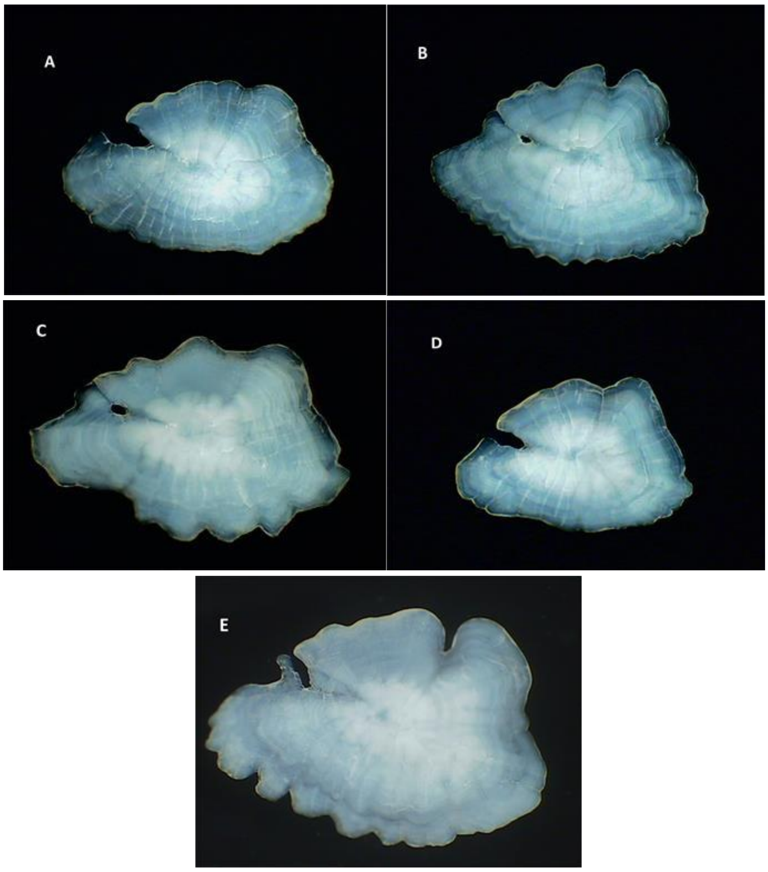


Figure 12: Different types of edge in the otoliths of *M. surmuletus* (see text for explanations).

The results were further confirmed for individuals with 1, 2 and 3 annual rings by their marginal increment analysis that showed an annual periodicity of increment formation, with lowest values specifically between February and April (Fig. 13).

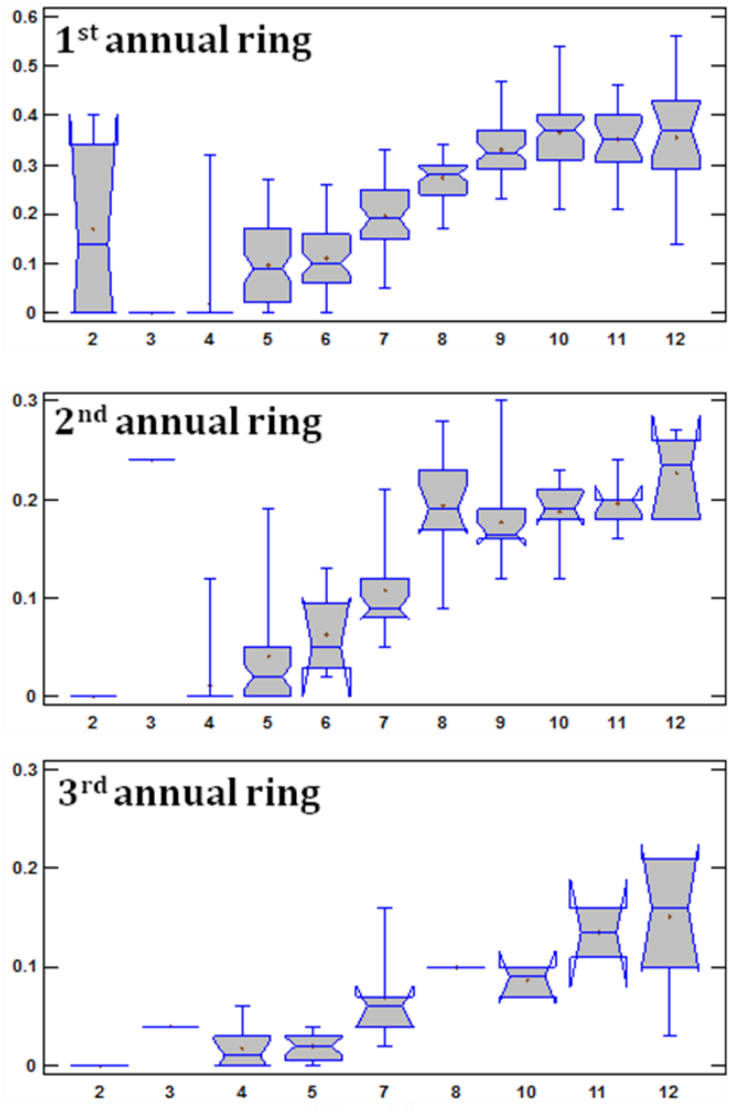


Figure 13: Marginal increment analysis for the 1st, 2nd and 3rd annual ring in *M. surmuletus* in the S. Aegean Sea.

False rings (FR) were present in all examined individuals (N=460), ranging in number from 1 to 8, with at least three rings being the most frequent case (30.4%). Moreover, it is worth mentioning that the formation of the first annulus coincided with the closure of the otolith rostrum in 65.7% of the otoliths examined, a characteristic that could be taken into consideration when aging the species. The mean value of the first annulus radius (R1) was close to the otolith radius of the young of the year (YOY), as estimated by the TL-R equation using as TL that of the mode of the YOY from the length frequency distributions of March and May; this also supported the identification of the first annulus. The radius of the ring considered as demersal was also used in the TL-R relationship to estimate the length of the species during settlement. Length frequency distributions revealed up to 3 different modes, followed in most of the months. On the other hand, identifying daily rings was proved to be time consuming with cases that were suspected of underestimating “true” age. Nevertheless, one specimen of 105 mm TL, caught in September and considered as 0+ group by otolith reading, presented 137 daily rings suggesting a birth date in April. This finding coincides with the peak of the reproductive period of the species occurring in April-May. In total, up to 5 annual increments were identified in the examined individuals. Age interpretation using both the 1st January and 1st June as birth dates resulted to different proportions of all five age groups, but to similar Von Bertalanffy growth parameters. The mean length of the three first age groups derived by V. Bertalanffy equation coincided with the modes of the length frequency distributions.

3.6 A first approach to validate the age of *Mullus barbatus* in the Eastern Ionian Sea (A. Chatzispyrou, A. Anastasopoulou, Ch. Mytilineou)

An accurate estimate of age structure of a fish population is an important requirement in fisheries stock assessment. Age derives usually by counting annual rings (zones of fast and slow growth) on some calcified structures of fish. The current work is a first approach to validate the age readings (mainly of the first annulus) of *Mullus barbatus* in the Eastern Ionian Sea using otoliths. Marginal increment and edge analysis along with the study of the reproductive period and the mode of the young of the year (YOY) in the length frequency distribution of the species were also used for this purpose. A total of 323 otoliths belonging to fish of age groups 0+, 1+ and 2+ according to a first reading were used to validate annual rings and record the following measurements: radius length (R) from the nucleus to the edge of the otolith, the distance of the annual rings from the nucleus and the distance of false rings observed before the first annulus. The closure of the rostrum at the anterior part of the otolith was also recorded in order to examine if this phenomenon coincides with the formation of the annual ring. The derived information was used for a second reading of the otoliths to verify the results of the first reading. For the age interpretation both dates of birth were used (1st of January and 1st of June) as well as the date of capture.

Otoliths reading indicated the presence of annual rings from January to June. Marginal increment analysis showed lowest values in February and March, which indicated the peak period of annuli formation (Fig. 14). Edge analysis confirmed that translucent ring formation starts from January to June, but fully formed translucent rings are mainly present from April to June. The same analysis indicated that in some cases translucent ring at the edge of the otolith existed also during the period from July to October. This ring was not considered as annulus in age readings, but it was supposed to be related with the reproduction of the species or other environmental conditions.

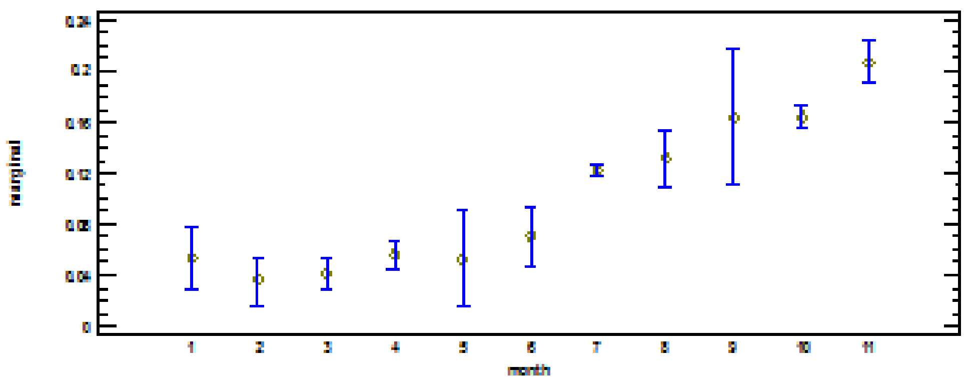
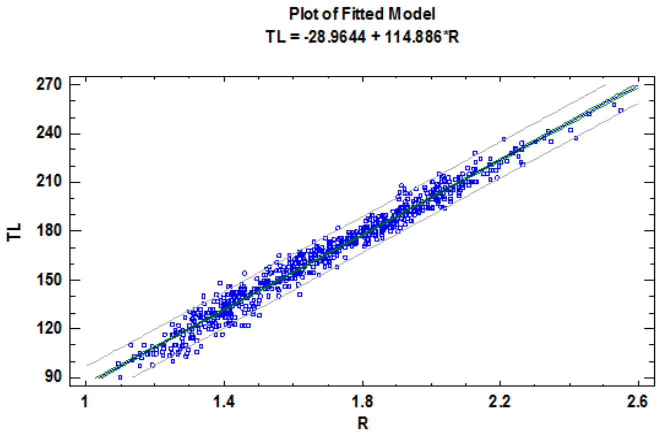


Figure 14: Marginal increment analysis by month for *M. barbatus* individuals with one annual ring.

Further detailed study of the edge type revealed 5 categories of edge (A: Beginning of the appearance of a non-continuous translucent ring at the edge of the otolith, B: Continuous and thin (narrow) translucent ring at the edge of the otolith, C: Continuous and thick (wide) translucent zone at the edge of the otolith, D: Continuous translucent zone followed at the edge by a non-continuous thin and weak opaque zone, E: Continuous and obvious fully formed opaque zone) (Fig. 15).

Figure 15: Various categories of the edge of *M. barbatus* otolith.

The mean value of the distance of the first annulus R1 (1.26mm) coincided with the radius of the otolith of the YOY (1.27mm), as estimated by the TL-R equation using as TL (115mm) that of the mode of YOY in the length frequency distribution of March (Fig. 16); that also corroborated the first annulus formation during this period. Closed rostrum did not seem to be related with the formation of the first annulus ring in *Mullus barbatus*. All specimens had at least one false ring before the first annulus, 68% of them had 2 false rings and only 6% of the specimens had a third one. False rings distance from the otolith nucleus differed significantly from R1. The use of the different birthdates for age interpretation resulted to different proportions of age groups 0+, 1+ and 2+ that leaves open the discussion for their use. The combination of the above analyses proved an effective tool towards the age validation in *M. barbatus*.



Mean estimated back calculated TL for the 1st annulus: 115.7mm
using R1= 1.26mm

Figure 16: TL-R relationship of *M. barbatus* in the E. Ionian Sea. Use of this equation for the calculation of mean TL for the specimens with one annual ring using the mean size of R1.

4 Multi-parameters analysis on datasets with different ageing schemes/criteria (birthday, number check before the first winter ring, preparation method) (ToR d)

4.1 Sources of bias in the age estimation: Interpretation criteria of the last growth ring

Achieving consistency between a group of age readers estimating the age of a certain species or a stock within a species has been the main objective for the age reader community since its beginning. The aim is to increase the adoption of procedures for age reading that include quality assurance and quality control mechanisms, for the improvement of stock assessment and environmental management techniques. The ultimate objective is to stimulate the achievement of a higher level of quality within, and integration between the partner institutes concerning fish age determination.

After the development of a European Fish Aging Network with two Concerted Actions, EFAN and TACADAR from 1997 to 2006, a lot of exchanges and workshops (14 from 2007 to 2012) were organized, most of them under the PGCCDBS umbrella.

A synthesis of 14 reports of the Workshop reports on Age Reading from 2006 to 2012 showed that the sources of bias in the age estimation between readers may origin in two primary reasons: Differences in preparation techniques and different interpretation of the growth structures, including the position of the first ring, the annual structure of growth rings and the interpretation of when to include a structure on the edge of the otolith.

Most workshops mediated the differences originating in the preparation methodology and also dealt with the divergence in interpretation of the first ring and definition of age structures. However only 3 out of 14 reports discuss action on how to interpret the last growth ring, which is a key issue when dealing with species displaying large variances in life-history, in particular for short-lived species.

When identifying the interpretation of the edge for specific stocks, additional information apart from the otolith structures should be considered:

- Date of capture
- Peak of spawning period for a given population i.e. date of birth which is not by default 1/1 for all stocks
- Main periods of seasonal increment formation for the species/stock
- Other features related to the growth characteristics of the calcified structure

These issues and the ecological characteristics of the individual stocks within a species must form the background for setting up schemes for interpretation of the edge structures over a year. As such, several schemes may be made for one species depending on the biology of the stocks within the species but also the agreed age estimation methodology of the species.

A way to ensure that all age readers will be able to do so, would be to have species-specific and stock-specific schemes for the interpretation of growth structures on the edge of the calcified structures. When having a varying date-of-birth within a species a decision should be made by either the Assessment WG or all the age-readers as how to assign to YCL based on the age-structures, as to ensure consistency in the ageing data. It does not rule out having several schemes for interpretation of the edge, however, it does require a calibration of the interpretations and an agreed methodology.

4.2 Choice of interpretation scheme of the age

During this workshop, the members are discussed around many schemes of the age interpretation. After 2 days and many presentations, it was decided to apply only two schemes (Fig. 17 and 18) on the same set of images from the exchange 2011.

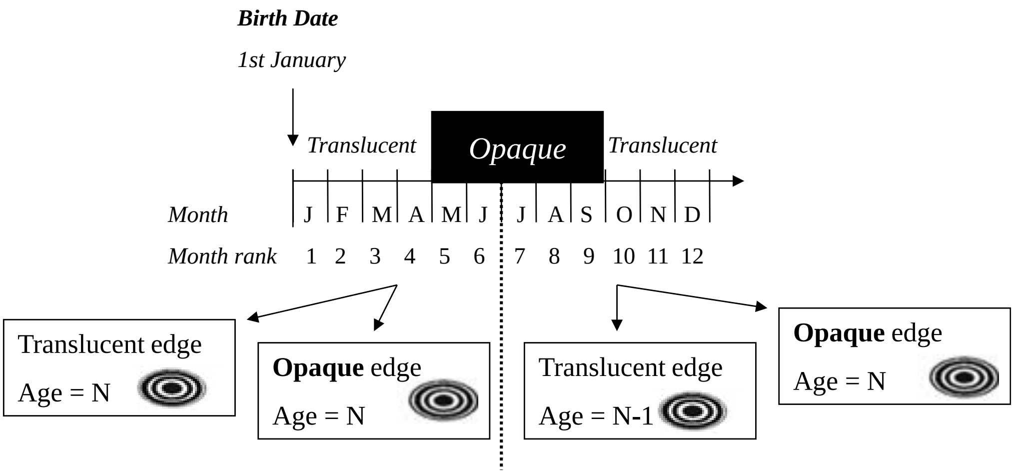


Figure 17: Scheme of age interpretation was the first January as date of birth (from WKACM2).

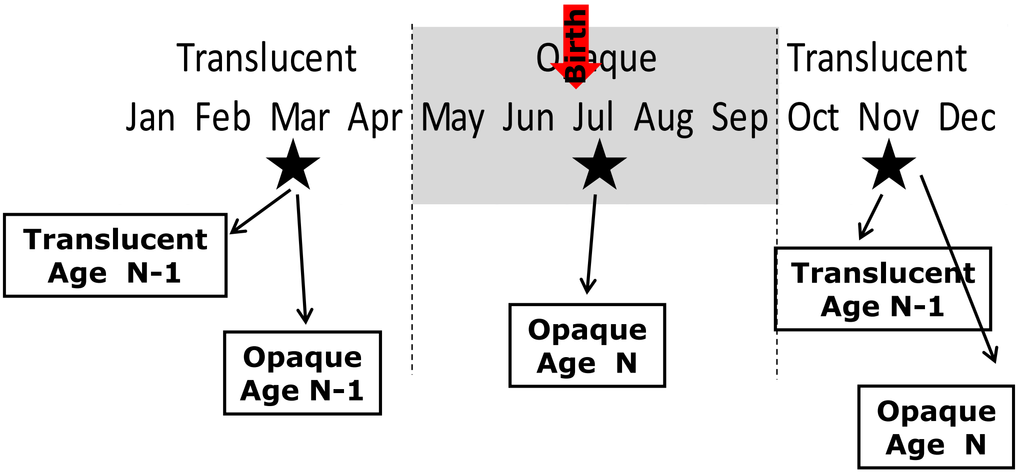


Figure 18: Scheme of age interpretation with the first July as date of birth (from IEO institute).

To analyse the use of only one scheme for both species in all GSAs in the Mediterranean Sea, the precision of age estimation has been tested from 40 images of red mullet in the Southern part of the Adriatic Sea (from the exchange 2011) from the spreadsheet of Eltink (2000) according to the instructions contained in Guidelines and Tools for Age Reading Comparisons by Eltink *et al.* (2000). Modal ages were calculated for each otolith red, with percentage agreement, mean age and precision coefficient of variation defined as:

- percentage agreement = 100x(no. of readers agreeing with modal age/total no. of readers).
- precision c. v. = 100x(standard deviation of age readings/mean of age readings).

To evaluate if there are some readings problems according to the rules of each interpretation scheme, the difference in age group for each image was tested for each reader. There is always 0 or 1 difference between two readings of the same reader. These results showed that the rules of each interpretation scheme were followed. Consequently, it is possible to compare the bias between the two interpretation schemes.

With the scheme 1 (birth date: 1st t January) as date of birth), the bias between readers was lower than that of the scheme 2 (birth date: 1st July) (Tab. 5). However, these results showed the low agreement between readers for both interpretation schemes.

Moreover in the otolith set utilized the CV seems not adequate at all to describe the variability. Indeed the value of CV is influenced by the mean of the age and standard deviation. In our case, the CV is smaller in the case of the first of January as birthday due the age resulting from the age-scheme. The specimens captured in June for an otolith with an opaque margin, following the age scheme of 1° January as birthday, gives N+1 years, increasing the mean and consequently decreasing the CV.

Table 5: Percentage of agreement and Coefficient of Variation (mean with range) between 11 readers from the scheme 1 (birth date: 1st January) and the scheme 2 (birth date: 1st July).

	Date of Birth	Parameter	Value
Scheme 1	1st January	% Agreement	56.8
		CV	59.3
Scheme 2	1st July	% Agreement	53.9
		CV	70.5

Comparing the modal age obtained by each interpretation scheme, there is a significant difference in the reading according to the scheme of the age interpretation ($P<0.05$). All reading differences were concentrated during the first semester. Therefore, it is necessary to use only one age interpretation scheme between all institutes which work on the same area. After many discussions on the results of this exercise, a new age interpretation scheme based on the publication of Morales-Nin and Panfili (2002) was agreed between participants (Fig. 19).

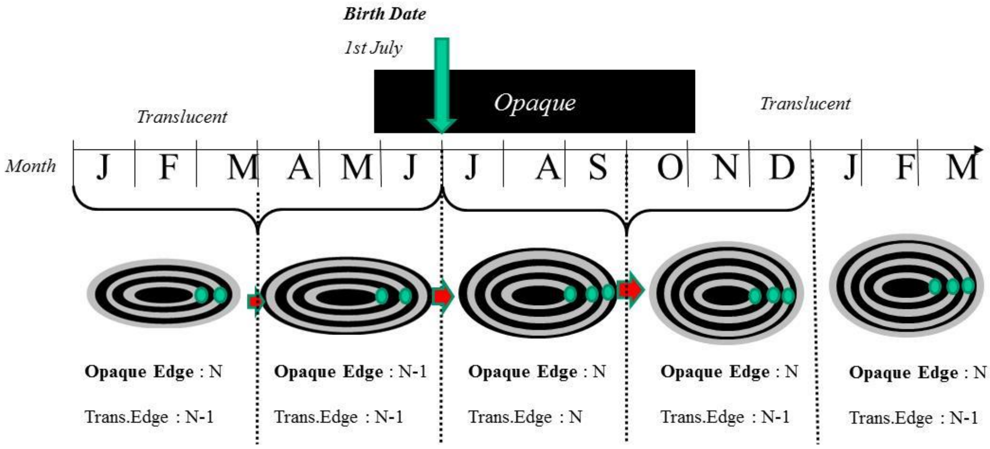


Figure 19: Age interpretation scheme based on the edge of the otoliths for the mullet species in the Mediterranean Sea (modified from Morales-Nin and Panfili, 2002). Green dots indicate translucent rings that according to the rules above have been considered. Annual increments are the sum of the translucent zone (grey rings) and opaque zone (black rings), equal to the age (years). N is the number of observed translucent rings (grey rings) and the estimated age is N or N-1 according to the sampling quarter and the nature of the edge.

Moreover it is important underline that regarding the resolution of the age if the ALK is used to transform the length of the capture / landing data in age it is recommended to use the resolution per year. While if it is used the age-slicing procedures (growth parameters) it is recommended to use a resolution of half year or more precisely (month). Indeed the more precisely resolution allow to obtain a better fitting of the growth parameters to be used in the age-slicing procedures.

5 Recommendations for the new exchange

5.1 One edge interpretation scheme

To limit the effect of the edge interpretation on the quality of the readings, only one age interpretation scheme must be used for the red mullets species in the Mediterranean sea (to see the above chapter).

5.2 The same magnification

When the exchange is organised, it is preferable to realise the blind readings (without the length information). However, with this methodology, it is very important to use only one magnification for all sampling to have some comparable images (Fig. 20).

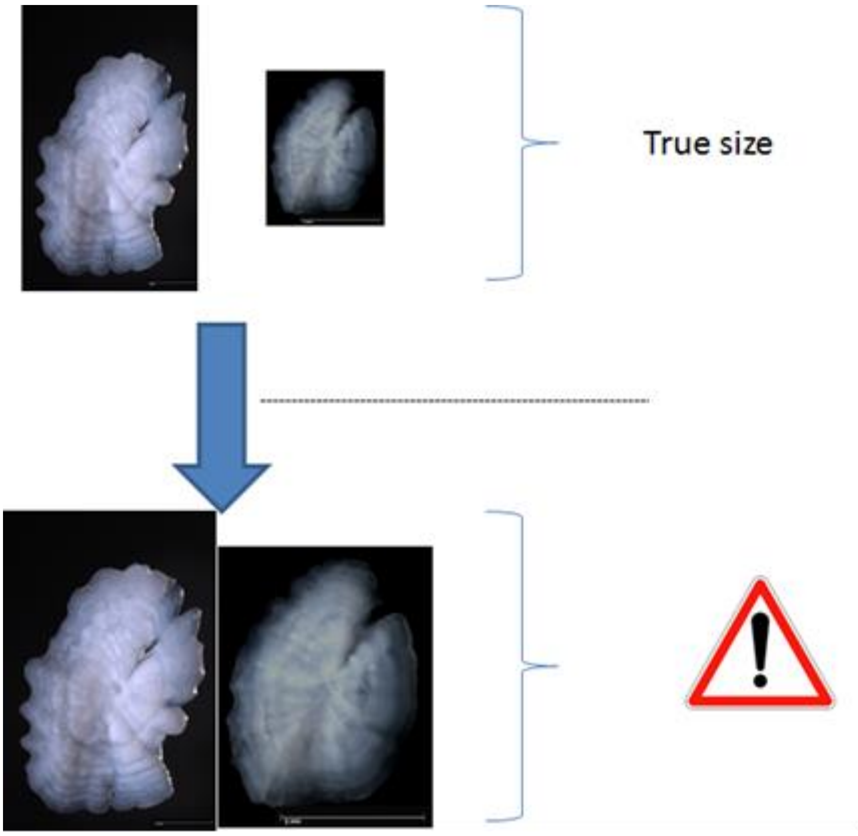


Figure 20: Illustration of the potential problem when two different magnifications are used.

5.3 Sampling around the year

To test the interpretation scheme, it must be important to have some fish (around 20) for each month to evaluate if the bias of reading due to the interpretation scheme is the same during the year.

5.4 Limit the number of different samplings

To discuss around the results during the workshop, it could be better to have one or two large samplings by species than a lot of small samplings to concentrate on the reading differences (than on the different growth patterns between geographical areas).

5.5 Edge classification

During the exchange, the images will be annotated. However, during the WKVALMU meeting, the edge classification (opaque or translucent) for the same images set was different between readers and so, it is a potential effect on the quality of reading. Consequently, during the exchange, the edge classification must be indicated by each reader.

6 Draft proposal for validation study of the ageing *Mullus* species

Age information is important in stock assessment studies since it has been considered among the most influential of biological variables. The impact of inaccurate age determination has contributed to serious overexploitation of stocks as in the case of orange roughy *Hoplostethus atlanticus* off New Zeeland and *Sebastes* spp off eastern anf western Canada (Campana, 2001 and references therein).

Mullus species ageing has been proven difficult until now because of the absence of accurate age estimation (lack of validation studies) as well as of low precision (inconsistency among readers) as proven in several dedicated exchanges and workshops (WKACM, 2009; WKACM2, 2012). The only validation studies related to *Mullus* species in the literature have been based on the edge type analysis and marginal increment analysis (Sieli *et al*., 2011; Mahé *et al*., 2009; Mahé *et al.*, 2013). However, marginal increment analysis is considered one of the most difficult validation methods to carry out properly, associated with problems in viewing an increment affected by variable light refraction through an edge that becomes thin as the margin in approached, as well as light reflections off the curved surface of the edge (Campana, 2000). Moreover, edge type identification is a component of age validation studies, but it is not a validation method. It is therefore essential to validate the absolute age and the first growth increment formation in these species. The two *Mullus* species consists one of the most commercial species in the Mediterranean Sea and their value in terms of landings as well as in terms of income is important for all the countries. Furthermore, since stock assessment is a requisite in management and age composition is necessary in stock assessment, it is of high importance to provide accurate age estimations for such a kind of analyses. Some corroborating methods for the age interpretation, such as modal progression analysis, length frequency analysis and daily growth increments indicating the formation of the demersal ring formation in *Mullus barbatus* have been presented during the last workshop (WKVALMU) as a first attempt towards the age validation of *Mullus* species. However, all participants of the workshop suggest that a research project targeting the accurate estimation of age of *Mullus* species is necessary using the most appropriate of the validation and corroboration the age interpretation methods. Many methods exist for these purposes, although, some of them have some limitations (e.g. bomb radiocarbon and radiochemical dating mainly for long-living species). However, mark-recapture of chemically-tagged wild fish could be applied as a direct very robust validation method. Among the indirect methods, daily increment widths and counts, although time consuming method, could also help the corroboration of the identification if the demersal ring, the first annulus formation and the identification of the type of the edge of the otolith; the latter seems to be one of the reasons of the low precision among the readers. All participants believe that these methods can be applied for *Mullus* species leading to results that will help the accuracy of these species ageing. Isotopic cycle study may also help the identification of growth variations, based on the fluctuations of calcium, phosphorus and oxygen isotopes in the calcified structure. Since these fluctuations need not to be annual, the method can be more helpful for the identification of false rings than for annual increment. Other indirect methods can also be used including information from the size of fish and otoliths or the length composition of the population studied and the progression of modes of cohorts in the length distribution that represent age groups.

The participant from Spain mentioned that the gear (swimming pool codend) for collecting alive specimens for tagging is available in their Institute as well as the know-how for all the process of the method. In addition the French participant mentioned that they have the know-how for isotopic cycle studies. Finally, participants for Italy and Greece showed their results on daily rings studies and length based analyses, mainly for *Mullus barbatus*, during the meeting and stated that they can undertake such a kind of work as well as a more detailed work based on length-based analyses for the two species using DCF and MEDITS data. All participants believe that DGMare, PGCCDBS, PGMED and GFCM as well as MEDSURMER, ADRIAMED and EASTMED could support this initiative in order to resolve the existing problems in *Mullus* species ageing, particularly in the Mediterranean Sea, taking into account the potential problems related with their assessment and management and their commercial importance in the whole Mediterranean Sea for European and non-European countries.

Therefore, the objectives of the present proposal can be summarized as follows:

1. Study of Direct validation methods for *Mullus* species ageing by mark-recapture chemically tagged wild specimens.

2. Study of Indirect validation and corroborating ageing methods by daily rings microstructure identification, isotopic cycles, modal progression and length frequency analysis as well as Length-Otolith radius relationship and Otolith weight-age relationship.

3. The selection of a big and accurately validated reference collection should be created to monitor ageing consistency and precision among readers as well as for new readers and training purposes.

Strengths and weaknesses of the proposed methods according to Campana (2001) can be as follows:

a) for mark-recapture of chemically-tagged wild specimens method, a low number of recaptures or recaptures before one year of the specimens at the sea can occur, not permitting accurate estimations. The time required is also long and depends on the longevity of the studied species. The samples can be used also for daily rings readings. The precision of the method is ±1 year and the cost is minimal except of the tagging cruise that however can be coinciding with MEDITS surveys or other kind of surveys that each Institute is caring out.

b) for daily increments readings, the method is valuable for identifying 1st annulus and possible false and demersal rings, but it is considered as time consuming and maybe difficult to be applied for *Mullus* species otoliths that are quite fragile. In addition, the otolith cannot be used after this process, so unsuccessful sections lead to the loss of the studied specimen. The method requires a specialization for the process and careful procedures. Low number of samples is required and the cost of the method is low compared to other methods.

c) for isotopic cycles studies, the main constrain is that it is difficult to satisfy the assumption that cycles and fluctuations are related to annuli, however, it can be useful for the identification of other kind of fluctuations in the growth of fish, related to environment, but confusing the accurate reading of the annual rings. The precision of the method is ±0-1 year and the cost of each sample (not required many) ranges from 50-500 €.

d) for modal progression analyses, the most appropriate class interval should be defined and representative length composition without mode overlaps. The mode of the young of the year (YOY) and that of the 1st and 2nd mode can be identified, but it is difficult to be applied for older ages. The cost of the method is minimal since DCF programs can offer this information.

e) Length-Otolith radius and Otolith weight-age relationships can also corroborate ageing validation studies with minimal cost.

All the above methods can help and support, if not resolve, the problems met until now by all readers in *Mullus* species ageing at least in the Mediterranean Sea, which have led to very low precision levels to date.

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Annex 2 Agenda

ICES Workshop on Ageing Validation methodology of *Mullus* species

Agenda

The meeting will start at 14.00 of May 15, 2017 and will end on May 18 (~13.00)

Meeting place: Conversano, ITALY,

Castle Acquaviva d'Aragona, Piazza della Conciliazione

Monday 15th May 2017

14.00-14.30

Welcome of the participants

Approval of the agenda

14.30-16.00

1. Results and Evidences from the last Exchanges and workshops on ageing of *Mullus* species (K. Mahè)

2. Benchmark session on *M. barbatus* and *M. surmuletus* biological parameters (GFCM Working Group on Stock Assessment of Demersal Species - WGSAD) (I. Bitetto)

16.00-16.30 Coffee break

16.30-17.30

1. Explorative analysis for a standardization ageing protocol: *Mullus barbatus* in the context of MEDITS working group (W. Zupa)

2. Sources of bias in the age estimation Interpretation criteria of the last growth ring (K. Mahé)

3. Ageing, growth and validation studies on *Mullus barbatus* and *Mullus surmuletus*: a review of existing data (P. Carbonara)

Tuesday 16th May 2017

9.00 – 11.00

1. Witness from each lab on: validation studies experience (deposition pattern, Length Frequency Distribution Analysis, Tagging etc) and ageing criteria utilized (theoretical birthday, false ring considered etc)

a. Input from Spain GSAs (X. Ordinas)

b. Otolith preparation methodology in France (R. Elleboode)

c. Input from GSA 9 (A. Massaro)

d. Ageing analysis of *Mullus barbatus* and *Mullus surmuletus* in GSA 10, GSA 18 and GSA 19 (L. Casciaro)

e. Input from GSA 11 (P. Pesci)

11.00-11.30 Coffee break

11.30 – 13.00

Continue on point 1.

f. Input from GSA 16 (S. Vitale, P. Rizzo, S. Gancitano)

g. Input from GSA 17 Croatian side (D. Medvesek)

h. Input from GSA 17 Italian side (M. Panfili)

j. Ageing criteria for *Mullus barbatus* in the western Ionian Sea (L. Sion)

j. Input from GSA 17 Italian side (M. Stagioni)

k. Input from Greece (Ch. Mytilineou)

13.00-14.30 Lunch break

14.30 – 16.00

1. Direct age validation methodology (X. Ordinas and B. Guijarro)

2. Indirect and semidirect age validation methodology: application on *Mullus barbatus* in Southern Adriatic Sea (P. Carbonara)

3. Towards the age validation of *Mullus surmuletus* in the South Aegean Sea (GSA 22) (Ch. Mytilineou)

4. A first approach to validate the age of *Mullus barbatus* in the Eastern Ionian Sea (GSA 20) (Ch. Mytilineou)

16.00-16.30 Coffee break

16.30-17.30

Constitution of working groups: 1) Theoretical Birthday; 2) Direct, Indirect and Semidirect age validation methodology

Working group

Wednesday 17 May 2017

9. 00 – 11.00

Working group

11.00-11.30 Coffee break

11.30-13.00

Working group

13.00-14.30 Lunch break

14.30 – 16.00

Working group

16.00-16.30 Coffee break

16.30-17.30

Synthesis and conclusions from working groups

Thursday 18 May 2017

9.00 – 11.00

Report

Synthesis and conclusions of the workshop

11.00-11.30 Coffee break

11.30-13.00

Planning of future work

Any Other Business and meeting closure

Annex 3 Otolith preparation methodology by institute and area

3.1 Spain in GSA 1, 2, 5 and 6

The procedures to extract, preserve, and read the otoliths in the Spanish GSAs is similar to those detailed by the colleagues from other GSAs during the workshop. The otoliths are stored dry and clean in plastic tubes. Reading of rings is done after immersion of the otoliths in a Glycerol/ethanol 50% mixture on a black background. The number of translucent rings and the nature of the marginal increment are recorded. It was tried to indirectly validate the annual nature of the rings by following the evolution throughout the year of the type of material deposited at the edge. Results did not confirm it due to the high uncertainty when trying to determine the type of material at the edge. First of all, in most otoliths we could observe both types of material (translucent and opaque in different regions of the margin, and secondly, the light incidence angle and the time that the otolith has been immersed in the glycerol/ethanol solution seems to affect the contrast of rings and, hence, the margin.

At the stage of assigning an age to the individuals, some inconsistencies appeared when applying the interpretation schemes proposed in the WKACM2. These inconsistencies are summarized in Figures 1 and 2, corresponding to the interpretation schemes that consider the first of January and the first of July as the birthday, respectively.

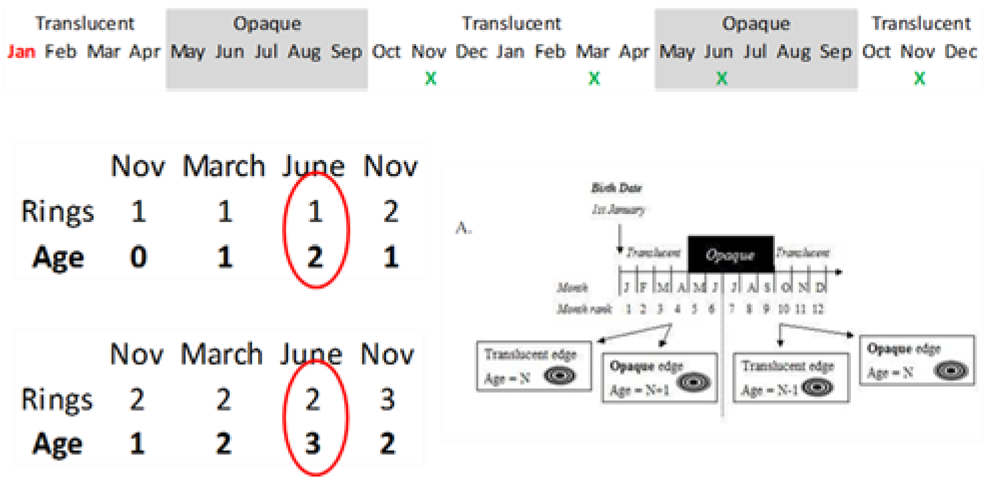


Figure 1. Top of the figure indicates possible catching dates and the nature of the material at the edge of the otolith. Down: Left hand side indicates the age interpretation from otoliths readings following the right hand interpretation scheme suggested in the WKACM2 when considering the first of January as the birthday.

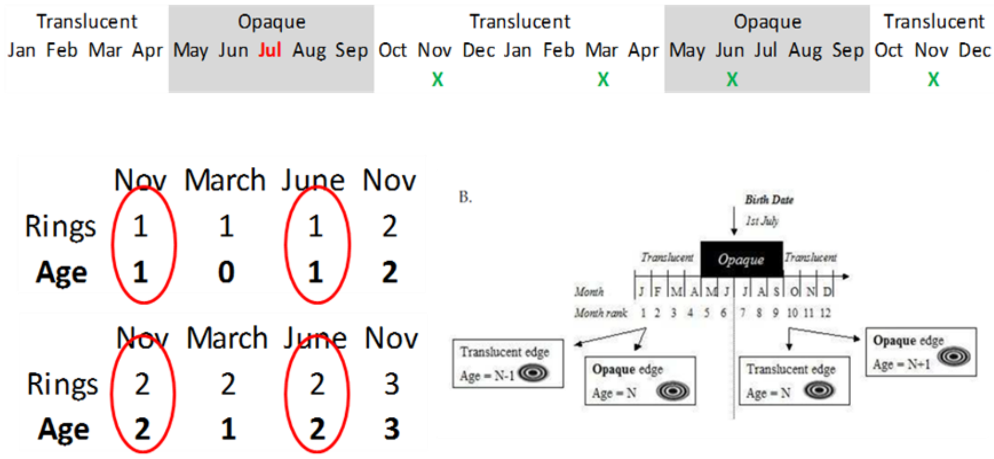


Figure 2. Top of the figure indicates possible catching dates and the nature of the material at the edge of the otolith. Down: Left hand side indicates the age interpretation from otoliths readings following the right hand interpretation scheme suggested in the WKACM2 when considering the first of July as the birthday.

A new interpretation scheme was proposed, which does not present those inconsistencies (Fig. 3). In the discussion the scheme was agreed as a possible interpretation scheme when the first of July is considered as the birthday. Afterwards, it was used as the interpretation scheme to be followed in the blind otolith reading performed during the workshop when the first of July was considered the birthday.

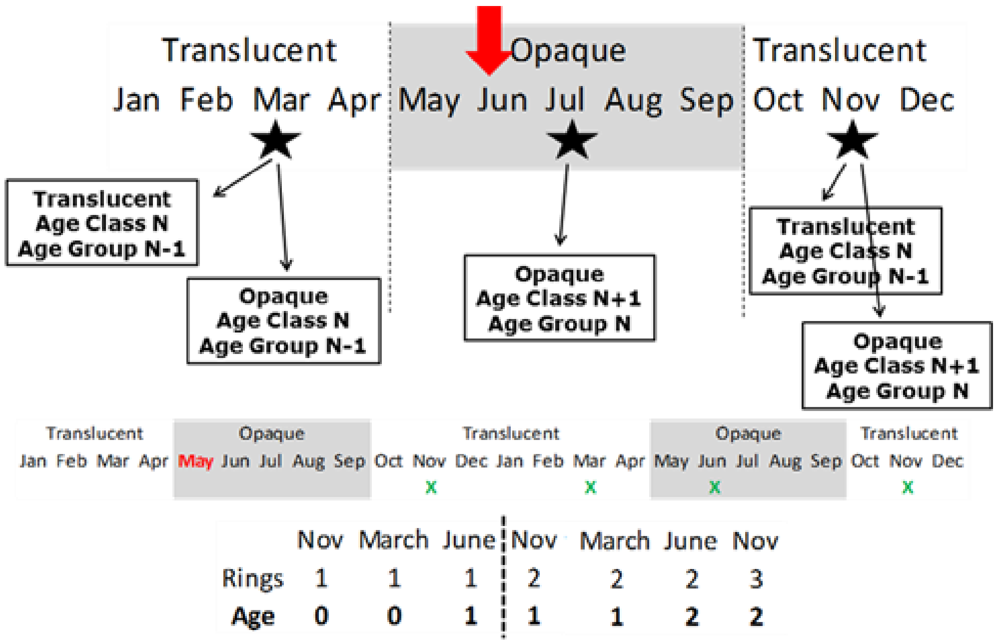


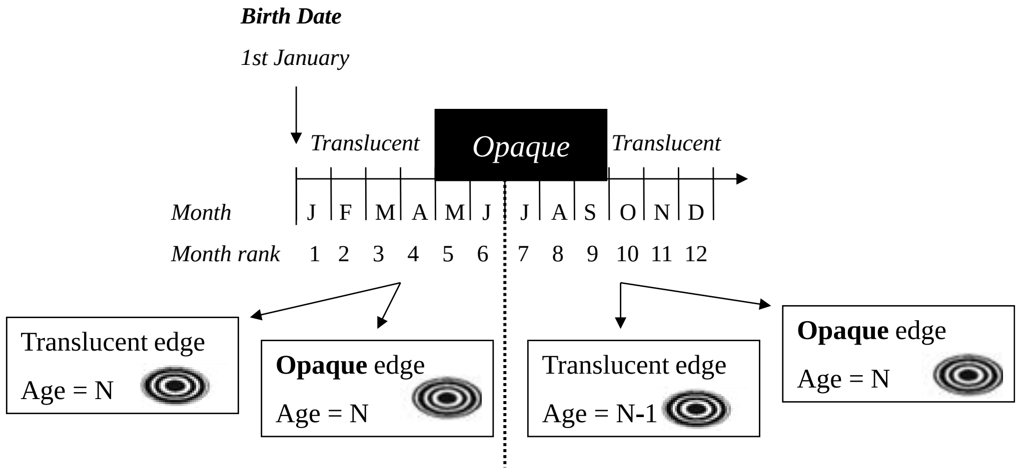
Figure 3. Top of the figure shows the new proposal of interpretation scheme, which was used in the WKVALMU2017 for blind reading when considering the first of July as the birthday (red arrow). Below, possible catching dates and nature of the material at the edge of the otolith. The results of the interpretation are indicated at the bottom.

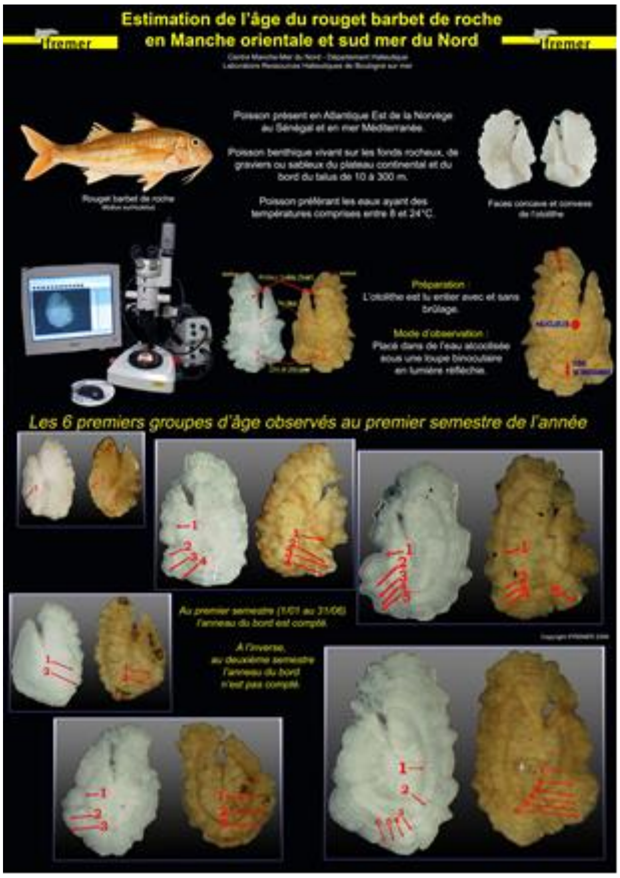
3.2 France in GSA 7 and 8

In France, for the red mullets and striped red mullets, the burning method described by Christensen (1964) is used. The result of the burning is that the translucent areas of slow growth (ZCL) take on a distinctly brown color which makes them more visible (Fig. 1).

Figure 1: Observations comparing whole otoliths of red mullet (the left without burning and the right with burning).

This technique may be carried out on an electric laboratory hot plate with indications of temperature. This is a procedure which had been used since 2007 because it uses a flat heating surface and a controlled temperature (250°C for a few seconds). The observation is realized by immersion in water with black bottom. Each otolith is analyzed using the TNPC software (digital processing of calcified structures, www.tnpc.fr). The images analysis could to have some tools to read the otoliths (comparison of individual growth curves; measure of all distances between the growth rings and the *nucleus*). In France, the readers used the same scheme of interpretation in the Mediterranean Sea than in Northern Atlantic Ocean (Fig. 2).





3.3 Italy (Coispa) in GSA 10, 18 and 19

In the GSAs 10, 18 and 19 the ageing criteria adopted are presented in the "Handbook for Fish Age Determination[1]". This volume was developed in order to standardize the ageing methodology used in Italy in the Data collection Framework (Carbonara et al., 2015).

The first step is the otoliths extraction, carried out through a transversal section slice performed transversely to the dorsal head of the fish in correspondence of the pre-opercula. After the extraction, the otoliths were cleaned, dried with paper, stored in plastic tubes and univocal coded. The plastic tubes have the advantage of being sufficiently rigid to protect the otoliths efficiently from breakages due to handling. The otoliths are analyzed: whole, by the binocular microscope, in sea-water (clarification medium), with reflected light, against a black background, with the distal surface turned up and the proximal surface (*sulcus acusticus*) turned down.

The otoliths of *M. barbatus* and *M. surmuletus* do not need a clarification phase before the age analysis except for the bigger specimens (> 20 cm) where a very short placement in the sea-water (2-4 minutes) could be enough. When they remain for too long time in sea water, the otolith, being very thin, could become translucent and therefore hardly readable.

For the *M. barbatus,* the theoretical birthday is the 1st July, because in the considered areas, the higher percentages of the mature/spawning females occur from April to June (Carbonara *et al.*, 2015) (Fig. 1).

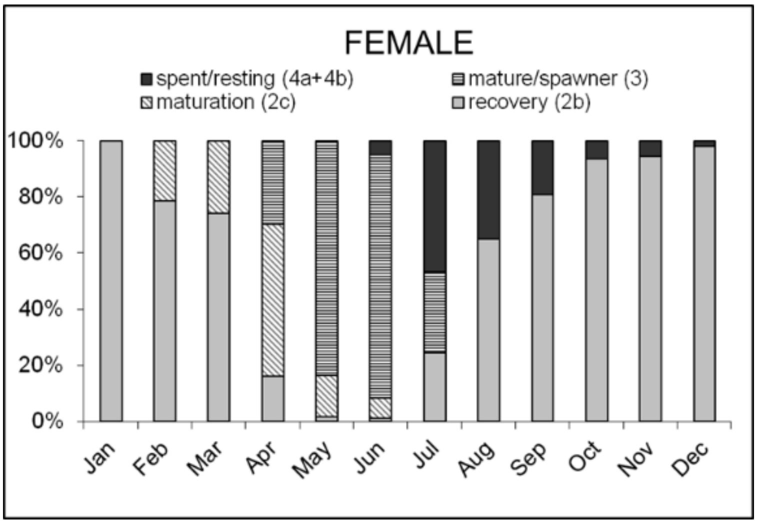


Figure 1 – Maturity stage percentage by month of red mullet females (Carbonara *et al.*, 2015).

The adopted ageing scheme is reported in the table xx with a resolution of half year. The scheme is divided in the quarter in order to include all case of marginal type (opaque, transparent) along the year. Take into account that the period of deposition of the opaque zone is included between Juneand November and the transparent between December and May (see the section of validation). The fish caught in the first part of the year (1st and 2st quarterly) present the transparent ring on the edge and considering the 1st July as the birthday the ring on the edge not represent one year spent, but half year so in this case the age will be equal to the number of the transparent ring included the edge less 0.5. In the first part of the year are also present specimens with an opaque edge. During the first quarter may be the case of specimens that not yet begun to lay down the transparent ring, while in the second quarterly specimens that they already started to lay down the summer ring (opaque). So in the first case (1st quarterly) the age will be equal to the number of transparent ring (n) plus 0.5, while in the case of 2st quarterly the age will be the number of transparent ring (n) less 0.5.

In the second part of the years (3st and 4st quarterly) the most of specimens present an opaque edge, the age, being the birthday (1st July) passed, will be equal to the number of the transparent ring (n). For the rest of the specimens with transparent ring on the edge in the second part of the year in the 3st quarterly may be specimen that not yet begun to lay down the opaque ring, while in the 4st quarterly specimens that already start to lay down the winter ring (transparent). So in the 3st quarterly the age will be equal to the number of transparent ring including the edge (N) and for the 4st quarterly to the number of ring N less 1.

Table 1 – The ageing scheme for *M. barbatus* with birthday 1st July. The n is the number of transparent ring excluded the edge (annulus); N is the number of transparent ring included the edge.

Date of capture	Otolith edge	Age
1st Quarterly	opaque	n + 0.5
	translucent	N - 0.5
2st Quarterly	opaque	n - 0.5
	translucent	N - 0.5
3st Quarterly	opaque	n
	translucent	N
4st Quarterly	opaque	n
	translucent	N-1

The ageing criteria utilized was defined in the ICES workshop (ICES, 2009):

- The winter rings should be visible around the whole otolith,
- The increments between consecutive winter rings should be decreasing with age.

Before the first winter ring, was consider as false only one ring (demersal), laid dawn during the passage from pelagic to demersal phase. In the following figures, there are some examples of ageing interpretation. Figure 1 represents an otolith of a specimen of 16 cm of TL, caught in March. The demersal ring, three winter rings, included the edge are recognized. The age, following the ageing scheme is of two years and an half. Figure 3 represent an otolith of a specimen of 17 cm of TL, caught in September. On the otolith are recognized: the demersal ring, two winter rings, an opaque edge, and between the two winter rings a false one (the reproductive one), so the assigned age in this case (Figure 3) is of two years. Figure 4 represents an otolith of a specimen of 26 cm of TL, caught in September, with the demersal ring, five true rings, and an opaque edge, so the assigned age is of five years.

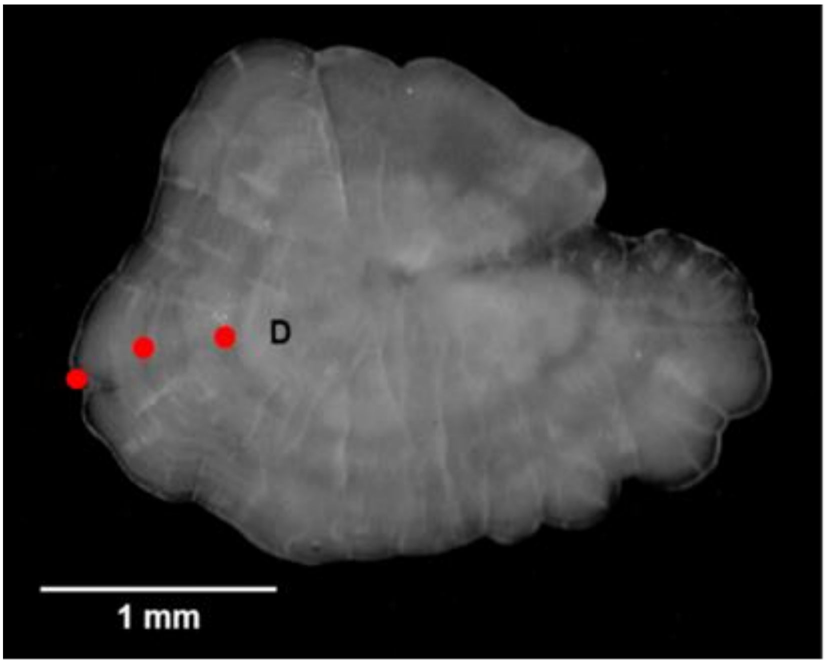


Figure 2 : Otolith of *M. barbatus* (age 2.5; TL 16 cm; sex M; month of capture March; D demersal ring).

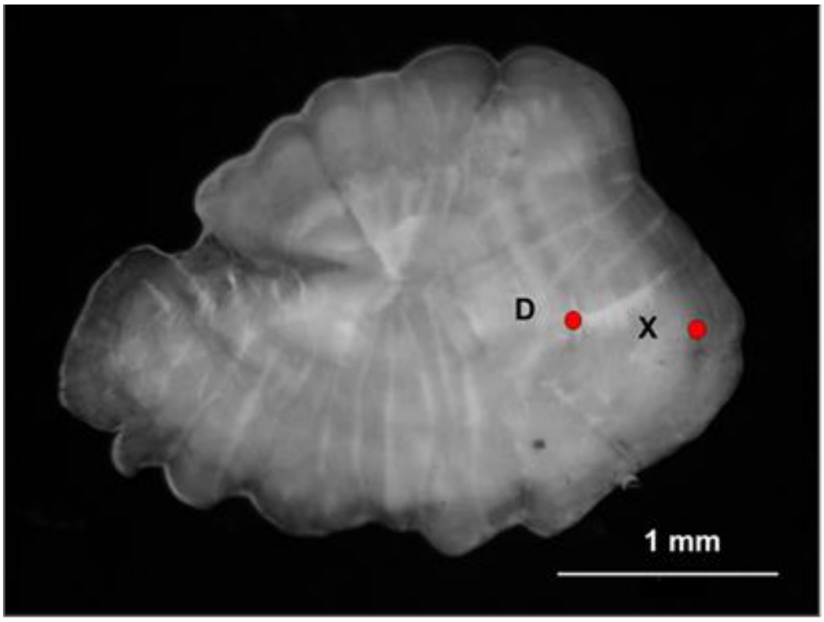


Figure 3 : Otolith of *M. barbatus* (age 2; TL 17 cm; sex F; month of capture September; D demersal ring; x reproductive check).

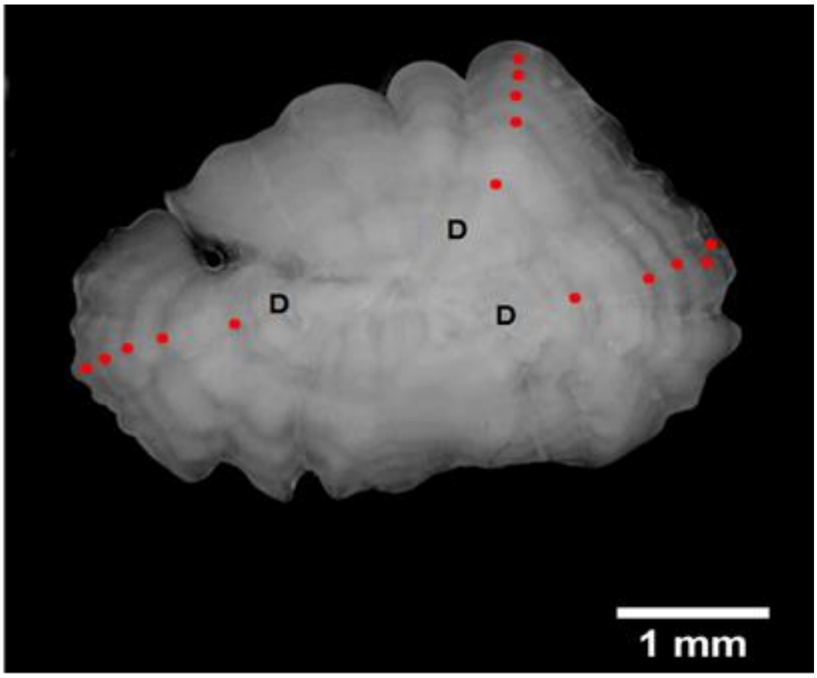


Figure 4 – Otolith of *M. barbatus* (age 5; TL 26 cm; sex F; month of capture September; D demersal ring).

Regarding *M. surmuletus*, the theoretical birthday considered is the first of July in accordance with a deposition pattern (Fig. 5). Indeed the higher percentage of mature females occurs from March to May (Fig. 5), more close to the 1 July than 1 January. The same ageing scheme and the same ageing criteria of *M. barbatus* are adopted.

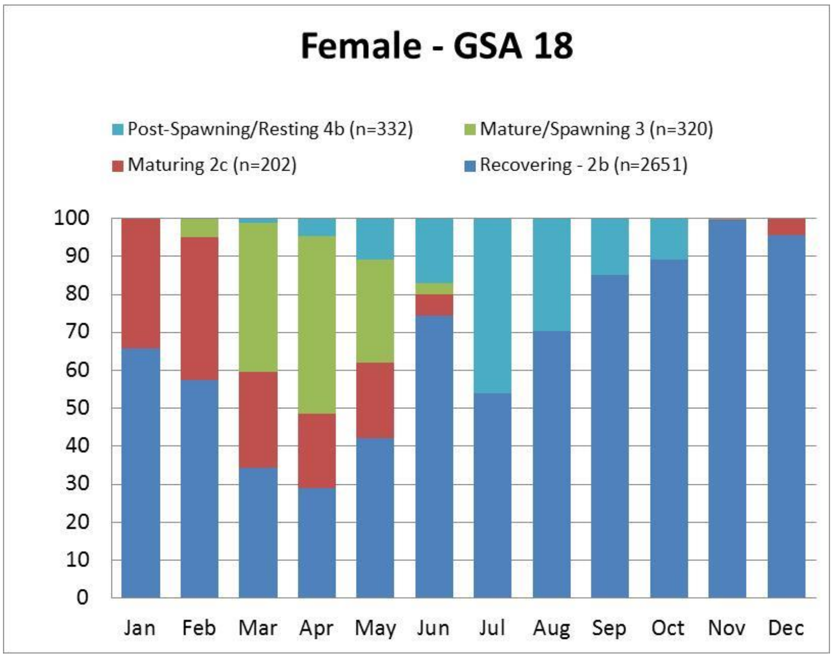


Figure 5: Maturation stage percentage by month of striped red mullet females in GSA 18.

In the Figure 6 is reported an otolith of a specimen of 33 cm of TL, caught in September, with the demersal ring, five true rings, and an opaque edge. So the age assigned in this case is of five years. Figure 7 report an otolith of a specimen of 34 cm TL, caught in

March, with the demersal ring, four winter rings, a translucent edge, and between the first two winter rings a false one (the reproductive ring) so the assigned age is 4.5 year. Figure 8 represents an otolith of a specimen of 20 cm of TL, caught in May, with the demersal ring, three winter rings, a translucent edge, so the assigned age is 3.5 year. Figure 9 report a picture of otolith of a specimen of 10 cm of TL, caught in February, with the demersal ring and a translucent edge, so the assigned age is 0.5 year.

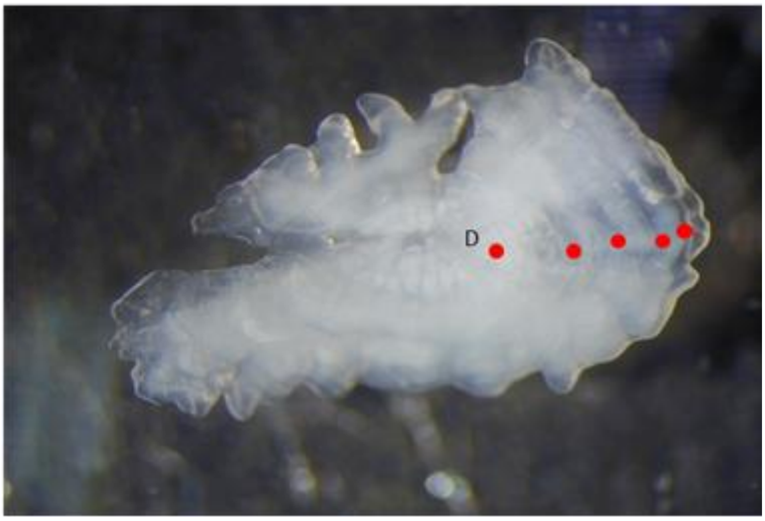


Figure 6 : Otolith of *M. surmuletus* (age 5 years; TL 33 cm; sex F; month of capture September; D demersal ring).

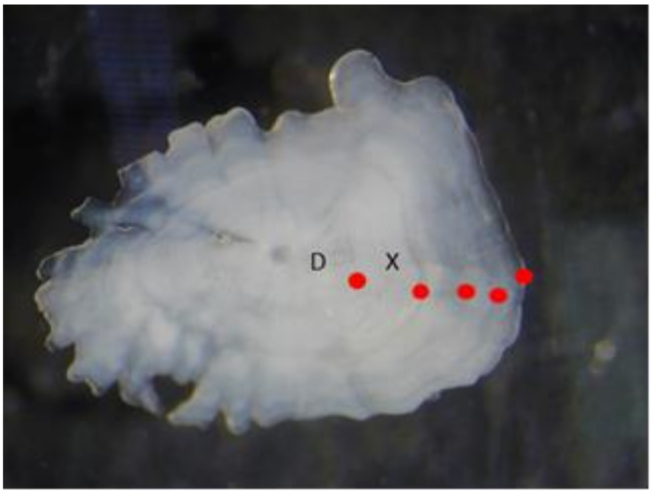


Figure 7: Otolith of *M. surmuletus* (age 4.5 years; TL 34 cm; sex F; month of capture March; D demersal ring; x reproductive check).

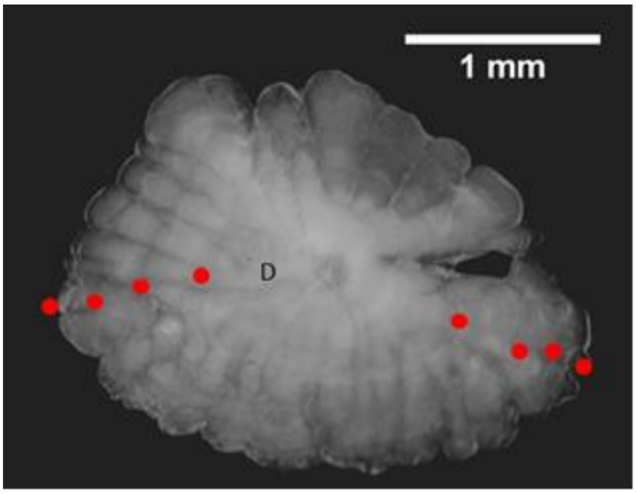


Figure 8 : Otolith of *M. surmuletus* (age 3.5 years; TL 20 cm; sex M; month of capture May; D demersal ring).

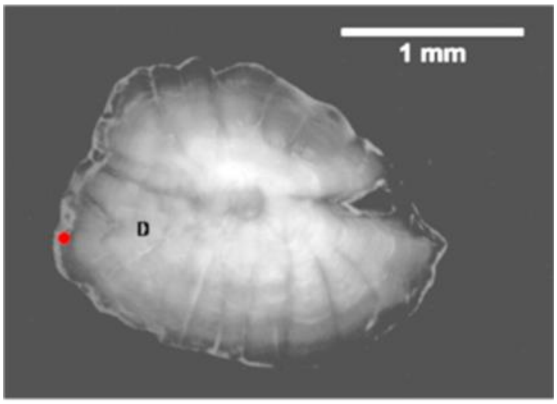


Figure 9 : Otolith of *M. surmuletus* (age 0.5 year; TL 10 cm; sex M; month of capture February; D demersal ring).

3.4 Italy in GSA 11

In GSA 11 (Sardinian Seas) red and striped red mullets are both collected for DCF, both from commercial catches and scientific survey (Medits). Data is collected on a length stratification scheme. Otoliths are removed from individuals, washed to remove all organic material, dried and stored in plastic tubes with a univocal code referring to the individual and number of haul.

Age determination is then made by observing the whole otoliths with a binocular microscope with reflected light against a black background. The otoliths orientation is the one with the sulcus acusticus down. A solution of glycerin and alcohol 1:1 is used as clarification medium. Each otolith is directly observed and photographed to permit subsequent reading and measurements. Transulcent rings are counted.

According to literature, the spawning period for *M. barbatus* in western Mediterranean sea shows a peak in June-July while for *M. surmuletus* the peak is generally reported earlier, around May. Also in Sardinian seas a summer spawning season has been identified for *M. barbatus* (with a peak in June) and in Spring-Summer for *M. surmuletus* (peak in May). For these reasons, the birthday considered for age determination in GSA 11 is July for both species.

A false ring is usually identified before the first winter ring. Age readings are made using indications from the "Handbook for Fish Age Determination" (2015). The ageing scheme used is reported below:

Months	Jan	Feb	Mar	Apr	May	Jun	July	Aug	Sep	Oct	Nov	Dec
Deposition pattern	T/O	T	T	T	T	O/T	O/T	O	O	O	O	T/O
Capture date												
Age with Edge T	N-0.5	N-0.5	N-0.5	N-0.5	N-0.5	N-0.5	N					N-1
Age with Edge O	N+0.5					N-0.5	N	N	N	N	N	N

Figure 10 - The age scheme for specie with 1st July as birthday. N represent the number of winter ring (transparent); T= transparent edge, O= opaque edge.

Figure 10. The age scheme for specie with 1st July as birthday. N represent the number of winter ring (transparent); T= transparent edge, O= opaque edge

Some examples of otoliths showed to the group to discuss are presented below :.

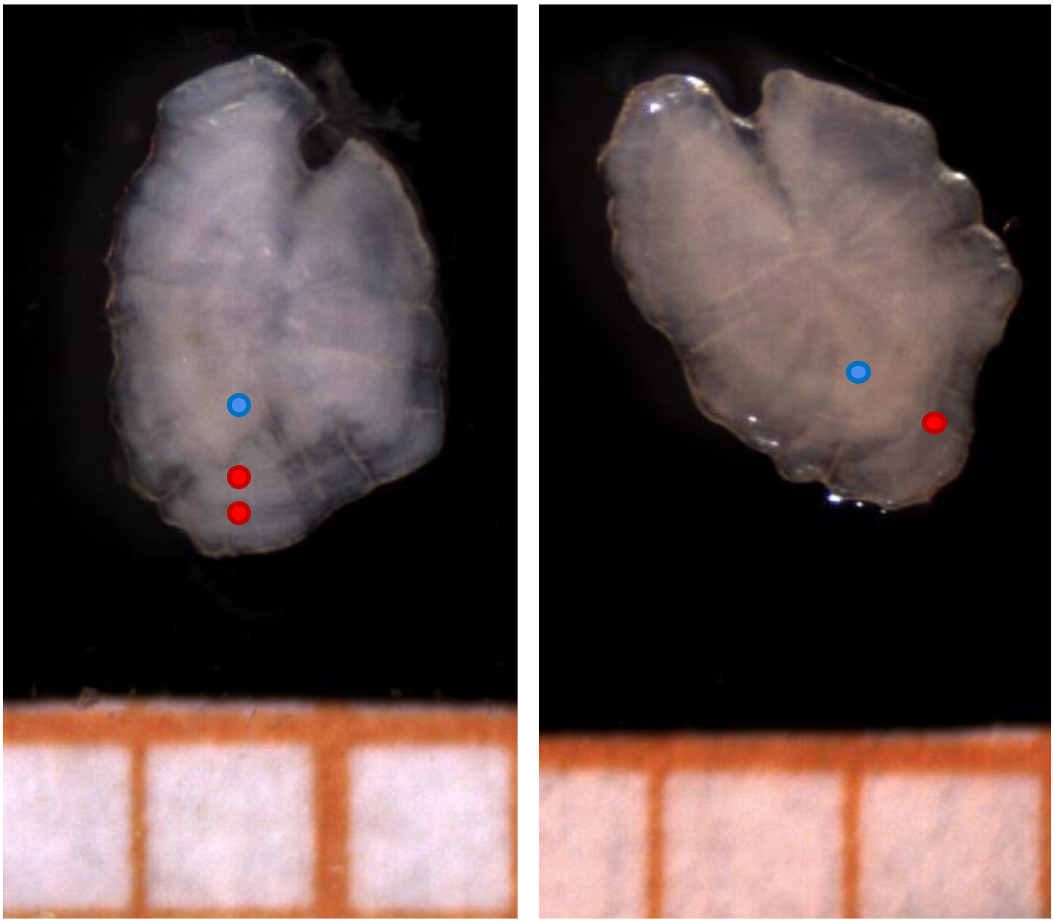


Figure : Examples of readings : ***M. surmuletus;*** **LT = 13.7 cm; month of capture: July (Left side),** ***M. barbatus;*** **LT = 12.2 cm; month of capture: July (Right side).A false ring (blue) and two yearly rings (red).**

3.5 Croatia in GSA 17

On Croatian side of Adriatic Sea (GSA 17), the reading of *Mullus barbatus* otoliths has recently been conducted from 2013 by experts of Laboratory of fisheries science and management of pelagic and demersal resources from the Institute of Oceanography and Fisheries (IOF).

The sampling of the otoliths is achieved during the MEDITS survey which is usually conducted during July. Figure X presents the map of MEDITS stations.

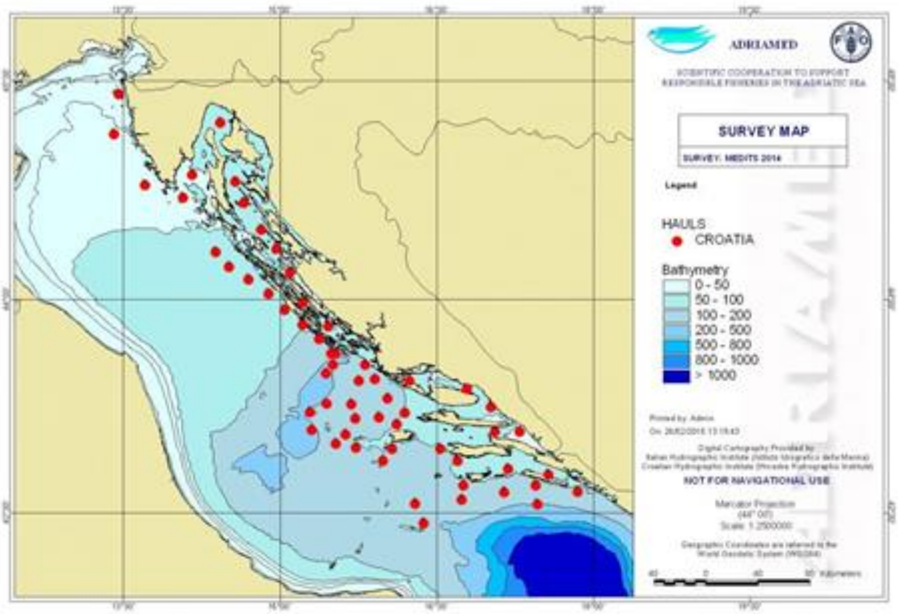


Figure 1. Map of stations during MEDITS survey in GSA 17.

Along with other biological data, otoliths are taken from *Merluccius merluccius* for five individuals by sex and length class (1 cm), *Trachurus trachurus* and *Trachurus mediterraneus* from 100 individuals each and *Mullus barbatus* for five individuals by sex and length class (0,5 cm). *Mullus surmuletus* in Croatian side of Adriatic is sporadic in trawl catches, especially during the MEDITS survey, therefore the sample is difficult to obtain and insufficient to be considered for age reading.

Besides the MEDITS survey the sample is collected from commercial landing biological sample (DC-MAP) randomly throughout the year to achieve good temporal distribution of the sample and the total of 500 otoliths for *M. merluccius* and 400 otoliths for *M. barbatus*.

Otoliths of *M. barbatus* are extracted from ventral side after gill removal. Small cut is made directly on the cranium and otoliths are removed, cleaned and stored in tubes with allocation number to ensure traceability.

The reading of the otoliths is conducted under Dino-Lite Digital Microscope against black background and the picture is taken for future reference.

Theoretical birthday considered for *M. Barbatus* is 1st of July and the interpretation of age is realised following the aging scheme purposed by Panfili *et al.* (2002) modified for 1st July as described in Table 1.

Table 1. Ageing scheme modified for 1st of July. n is the number of transparent ring excluding the edge and N is the number of transparent ring including the edge.

Date of capture	Edge	Age
1 JAN – 30 JUNE	Transparent	N – 0,5
	Opaque	n + 0,5
1 JULY – 31 DEC	Transparent	N - 1
	Opaque	n

Regarding the ageing protocol, two false rings are considered before the first winter ring, first occurring during the pelagic stage of juvenile life of the individual and the second occurring during the transition from pelagic to demersal stage (Fig. 2).

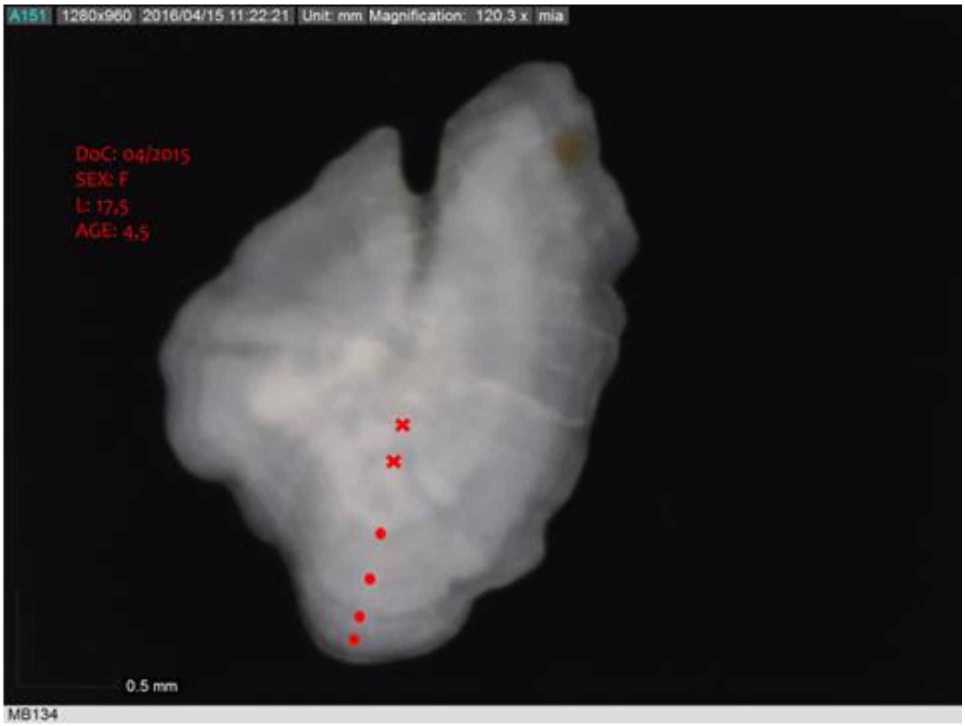


Figure 2. Example of false(X) and the growth rings (o).

3.6 Italy (University of Bologna) in GSA 17

Mullus species, in North-Middle Adriatic Sea, have different distribution and persistence (Fig. 1-2).

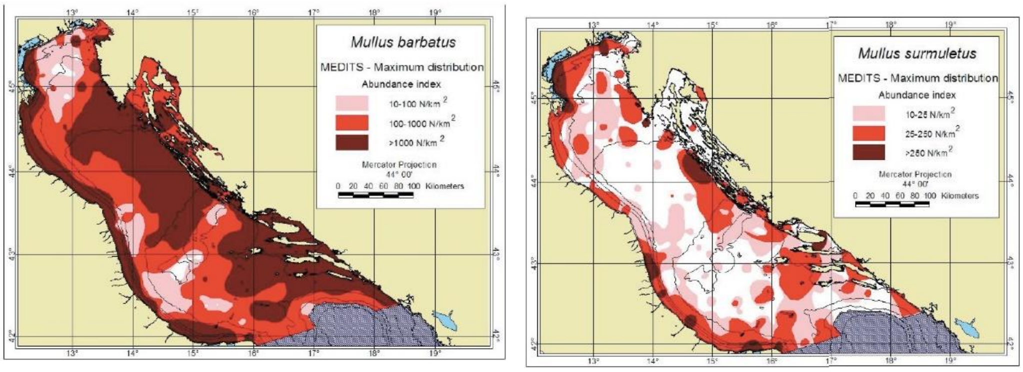


Fig. 1 Distribution of *Mullus* species in GSA17.

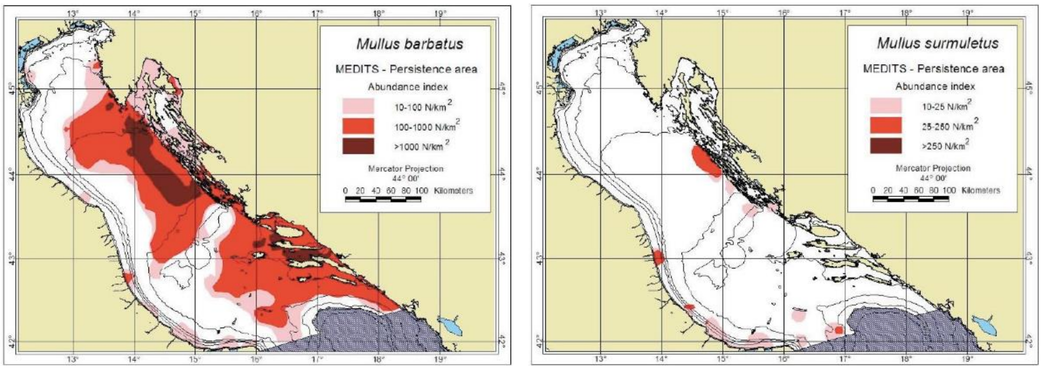


Figure 2. Persistence map of *Mullus* species.

Nursery area of *Mullus barbatus* is shoreline and shallower water in western side of GSA17 (Fig.3).

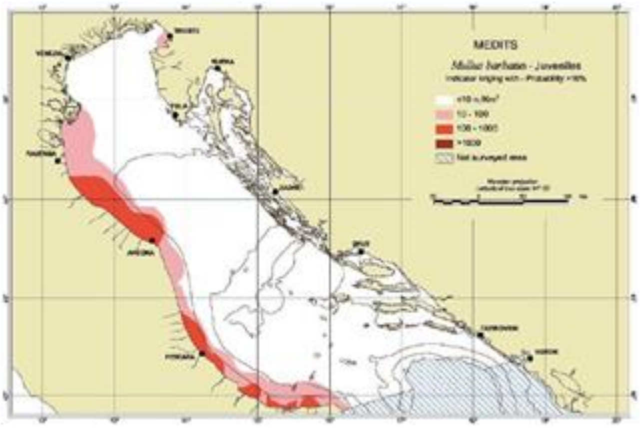


Figure 3 Nursery area of *Mullus barbatus*.

A subset of 600 *Mullus barbatus* otoliths were extracted from more of 3300 specimens caught on 2012 Medits survey in italian GSA17. After extraction with plastic forceps by transversal cut aligned with preopercular position (Fig. 4), otolith were cleaned from membrane and blood residuals with 3% hydrogen peroxide and wash in ultrasonic bath with distilled water . Before storing otolith in 2ml plastic microtube were been dried in oven at 35°C overnight.

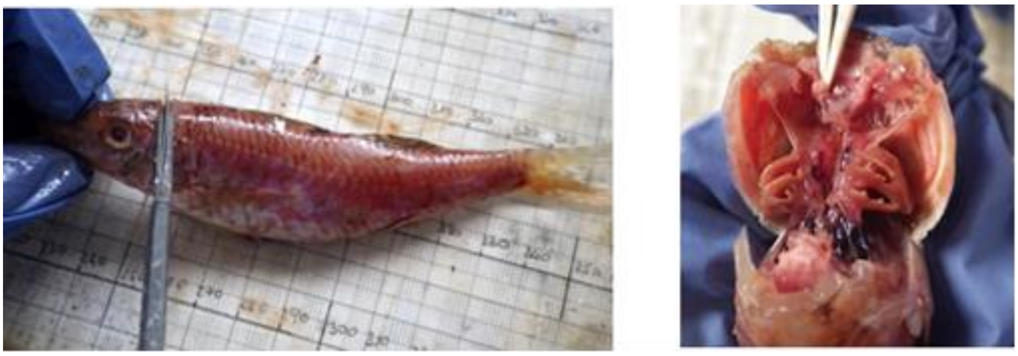


Figure 4 Transversal cut for otoliths extraction.

Digital image acquired by digital camera attached to binocular microscope were used for calibration, image analysis and age reading. Dried otoliths were immersed in sea water medium for 1-2 minutes before imaging. To avoid strong shadows otoliths were put on plastic petri disc lifted by 2 cm thick white foam drilled to the centre and illuminated with 2 led lamp at 45 degree on black background (Fig. 5).

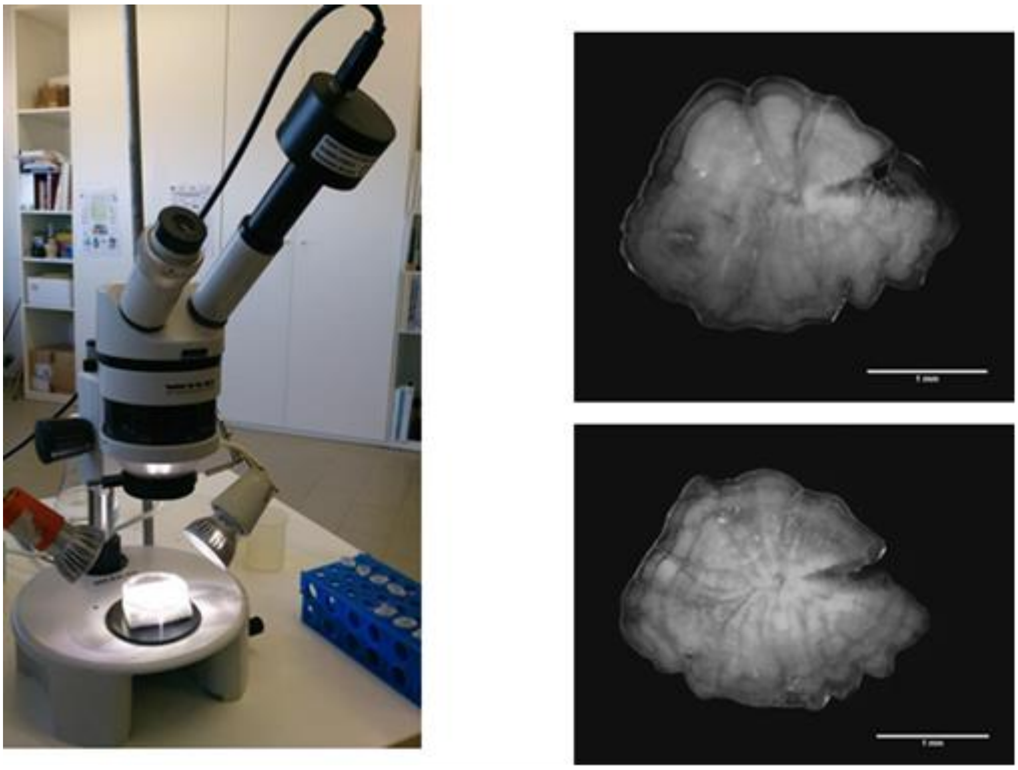


Figure 5 Digital image acquisition system (left) and otoliths (right).

Birth date is set to first june and rejected translucent ring shorter of 1.1 mm radius from core to postrostrum. Hard to read otoliths were burned whole at 250°C for 30 seconds and/or embedded, sectioned, polished and stained with neutral red for 15 min. (1% in acetic acid solution 0.1%) and toluidine blue for 15 min (1% solution in water) to enhance band contrast. A small number of sample (19) were grinded with alumina lapping film (9-5-1 micron) and polished with alumina paste (0.05 micron) on sagittal plane after embedding them in a drop of cold curing epoxy resin over a microscope glass slide to count daily rings (Fig. 6).

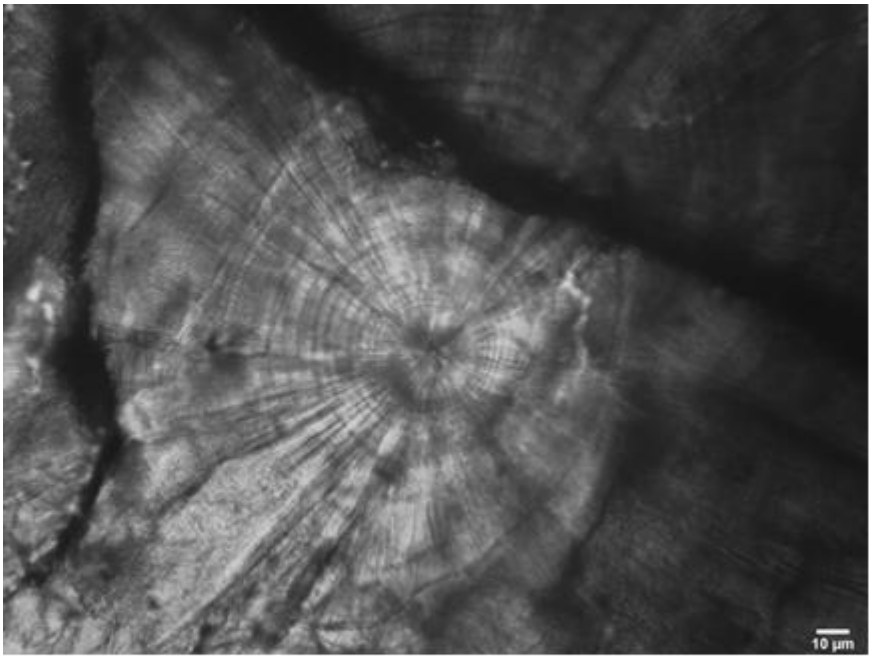


Figure 6 : Daily increment visible after that the otolith was grinded and polished.

Age structure is dominated by 0+ and 1+ groups and the mean length at age is shoved in Tab. 1.

Table 1: Mean length at age.

Age	Mean (mm)	sd
0+	97.838	18.840
1	125.607	13.549
2	156.544	15.551
3	180.578	16.531
4	201.333	22.053

The first false ring average radius occur at 1.08 mm, first real winter ring radius at 1.36, second at 1.57 and third at 1.76. Average marginal increment from first to second winter radius is 0.220 mm ± 0.074 with no significant difference by sex, average marginal increment from second to third winter radius is 0.170 mm ± 0.045 with significant difference by sex. Daily ring analysis showed that the first check occurs on 25±3 days, corresponding to a radius of 0.57mm.

3.7 Italy (CNR-ISMAR) in GSA 17

In GSA 17 (North and Central Adriatic Sea; Fig.1) the individuals of *Mullus barbatus* and *Mullus surmuletus* are collected for Data Collection Framework and are mainly sampled from commercial catches carried out by trawl net and gill net. About 600 individuals of *Mullus barbatus* and 250 of *Mullus surmuletus* are annually analysed for age determination.

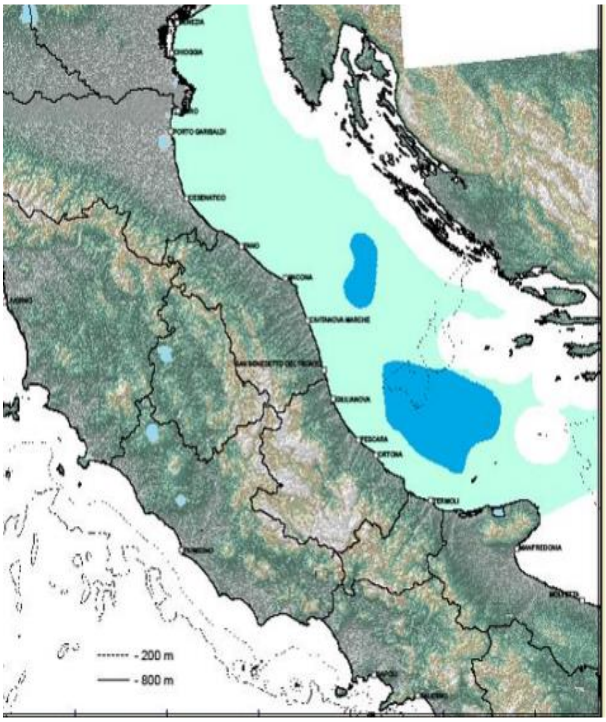


Figure 1: GSA 17 area corresponding to North and Central Adriatic Sea.

The *sagittae* extraction is made by posterior section: a slice is made posteriorly on the head, the skull is opened, the brain is removed and, finally, the otoliths are extracted with tweezers. After the extraction, the otoliths arecleaned of any residual organic tissue, washed, dried with paper and stored in plastic tubes with an identification number.

In order to be more readable, otoliths need to have a clarification in sea water for 2-4 minutes, then the otoliths are analysed to binocular microscope with reflected light against a black background. The microscope is connected to a video camera and to an image analysis system (LEICA Microsystem; Fig. 2).

The best otolith orientation for the analysis is with the distal surface turned up and the proximal surface (*sulcus acusticus*) down (Fig. 3). In this way, the dark rings can be counted in the *antirostrum* area (*radius*) as translucent growth rings (slow).

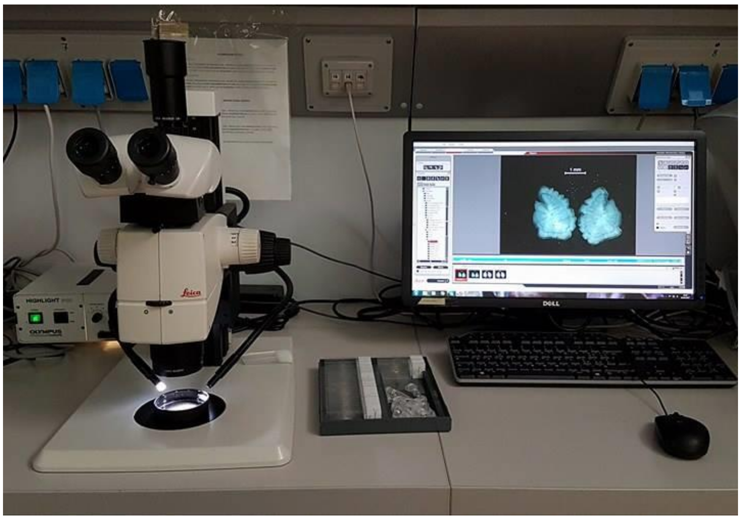


Figure 2: Digital image analysis system.

Measures and the distances between the rings are also taken on the post-rostrum along the axis joining the sulcus and the nucleus (Fig. 3). The edge discrimination (opaque or transparent) is finally noted.

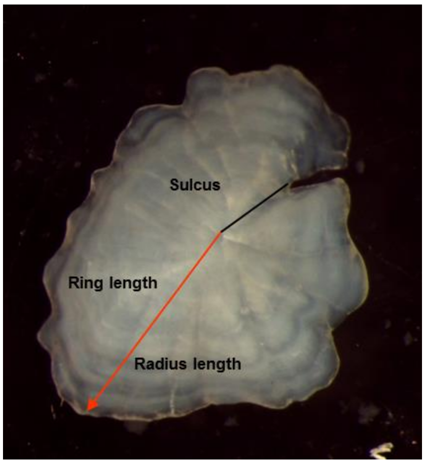


Figure 3: The mullet otolith with the indication of morphometrics measures.

For *Mullus barbatus,* a preliminary analysis of the distances between every ring to and core is performed. The distances (especially for the first rings) seem to be quite constant in both species, taking into account both the different areas (North Adriatic: Chioggia and Central Adriatic: Ancona) and different years of sampling. However, it is necessary to improve the number of individuals to have better results (Tab. 1; Fig. 4).

Table 1: Mean distances ± standard deviation between every ring and the core in *Mullus barbatus* caught in two different areas (Ancona e Chioggia) and in two different years (2015 and 2016). P = Pelagic check; D = Demersal check.

		P	D	1	2	3	4	5	n ind
Ancona 2015	mean (mm)	0,58	0,82	1,05	1,26	1,44	1,63	1,69	362
	sd	0,07	0,05	0,06	0,06	0,07	0,10	0,05	
Chioggia 2015	mean (mm)	0,48		1,11	1,29				41
	sd	0,01		0,07	0,07				
Ancona 2016	mean (mm)	0,53	0,81	1,07	1,31	1,53	1,72		23
	sd	0,04	0,08	0,07	0,08	0,11	0,06		

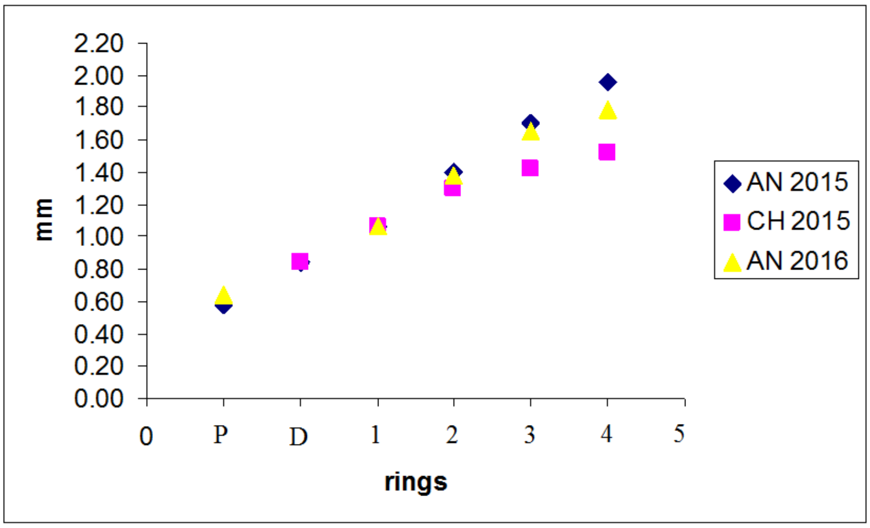


Figure 4 : Mean distances between every ring and the core in *Mullus barbatus* individuals caught in two different areas (Ancona e Chioggia) and in two different years (2015 and 2016). P = Pelagic check; D = Demersal check.

For age determination of *Mullus barbatus* and *Mullus surmuletus* in GSA 17, the following criteria are adopted:

The birthday for both species is set on **1st July**, according to their spawning period in the Adriatic Sea and in the Mediterranean basin. It usually comes from late spring-early summer with a peak in June-July for *M. barbatus* and a little bit earlier (May) for *M. surmuletus*. The transparent rings are counted taking into account the scheme (Fig. 5) from ICES, 2012 (Morales-Nin and Panfili, 2002; Mahé *et al.*, 2009).

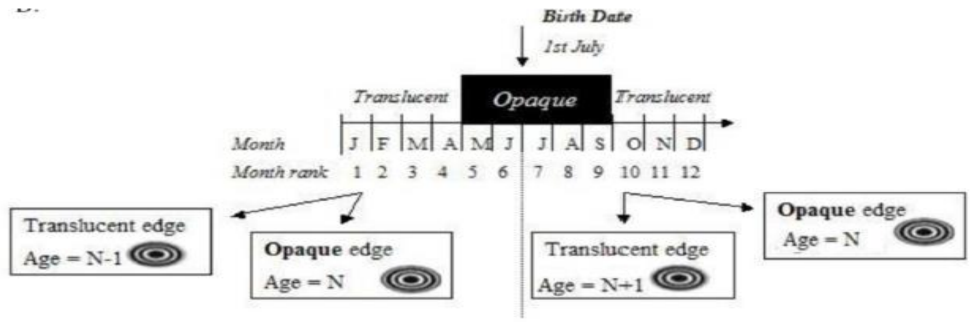


Figure 5 : Ageing scheme for *Mullus barbatus* and *M. surmuletus*. N = number of transparent rings.

Two checks before the first true winter ring are usually considered: **pelagic check** (P) closed to the core and **demersal check** (D; Fig. 6). The first real winter ring is set about 1.0 mm from the core (Fig. 7).

The other translucent rings are considered real if they are visible and continuous around the whole otolith and if the increments between the consecutive annuli decrease with age (Fig. 8 and 9).

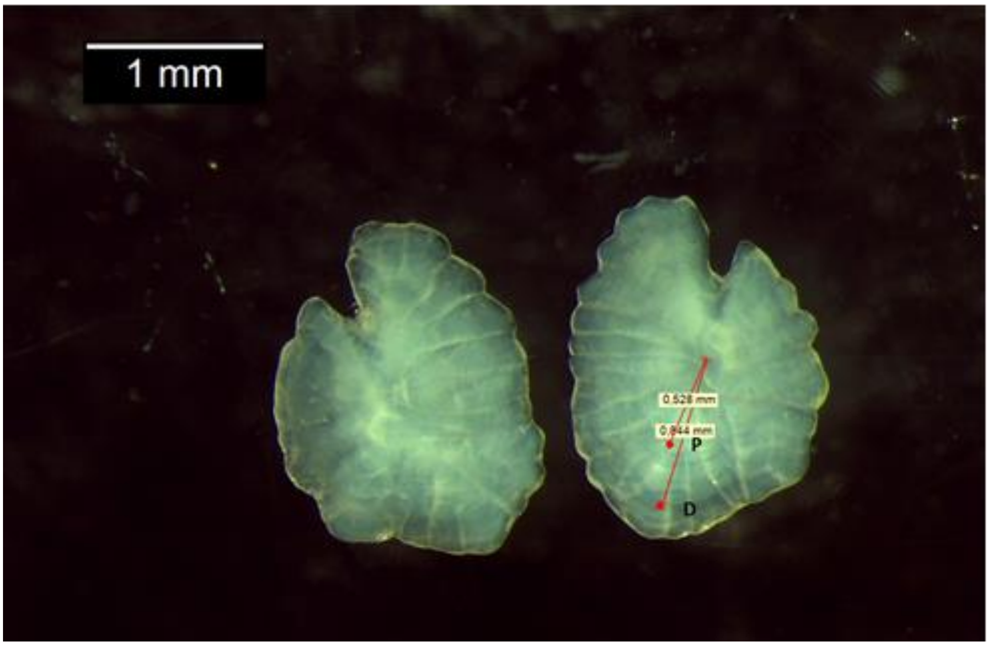


Figure 6 : Otolith of *M. barbatus*. TL: 9 cm, capture: March, Age: 0. P: pelagic check, D: demersal check.

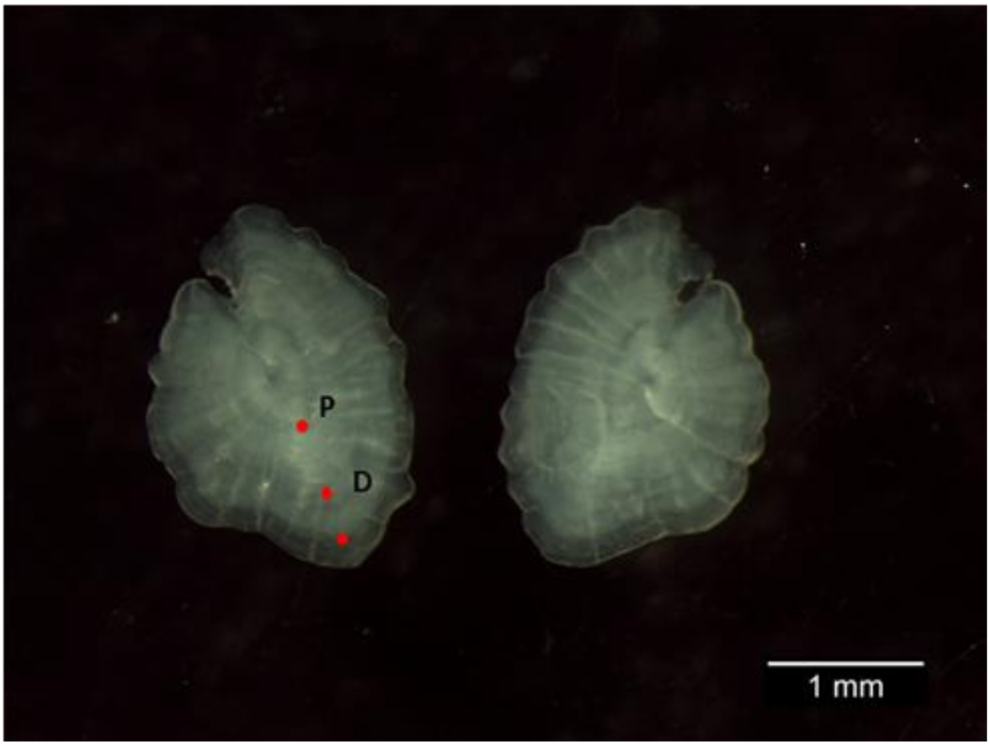


Figure 7 : Otolith of *M. barbatus*. TL: 11 cm, capture: January, Age: 1. P: pelagic check, D: demersal check.

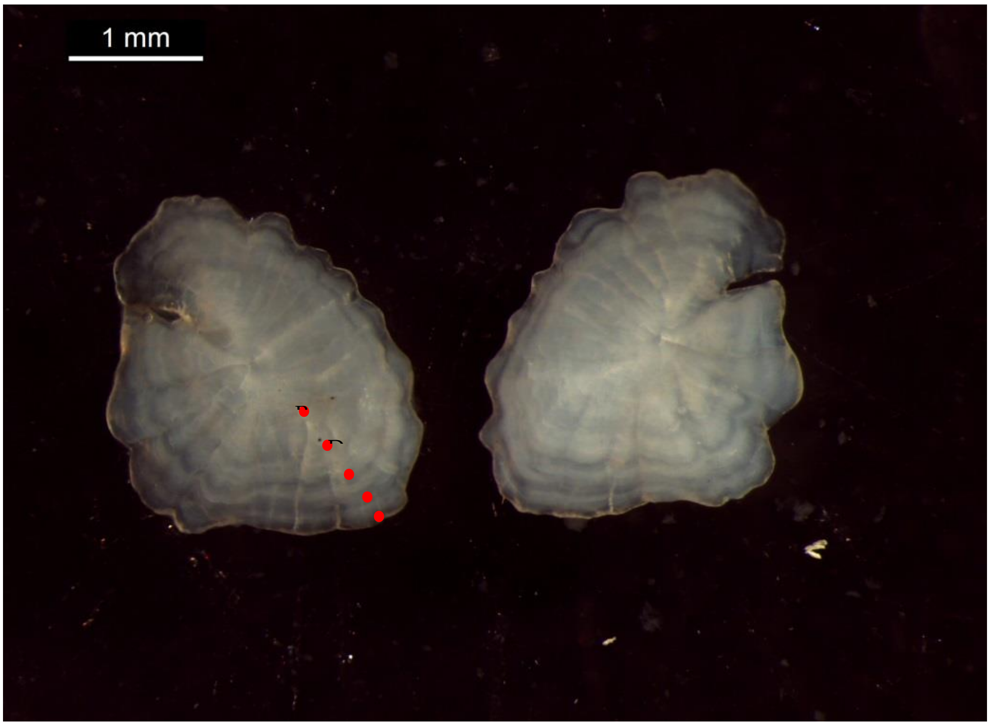


Figure 8 : Otolith of *M. barbatus*. TL: 15.5 cm, capture: July, Age: 3. P: pelagic check, D: demersal check.

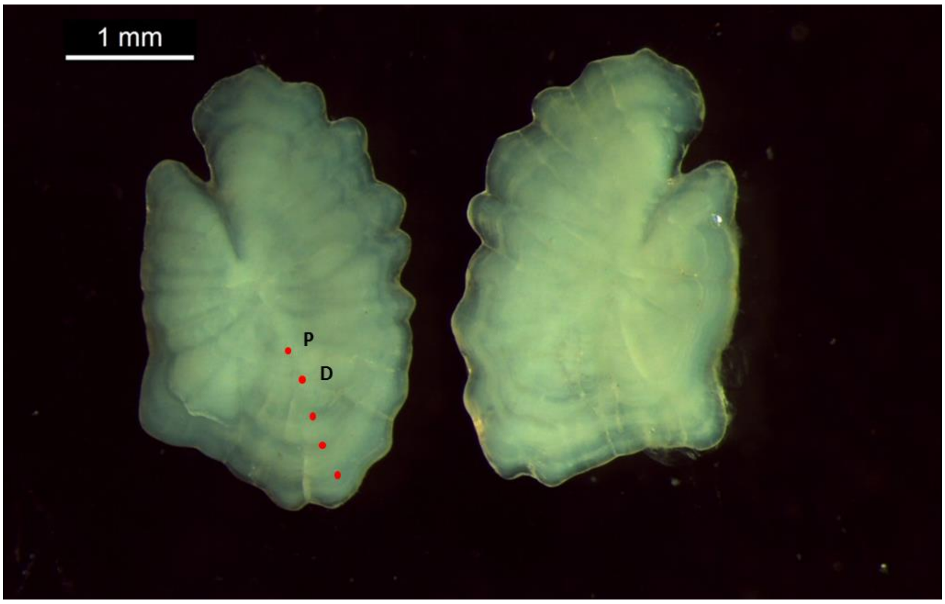


Figure 9 : Otolith of *M. barbatus.* TL: 18 cm, capture: June, Age: 3. P: pelagic check, D: demersal check.

On the contrary, in individuals of *Mullus surmuletus* a greater individual variability can be observed in the distances between the rings as you move away from the nucleus (Tab. 2; Fig. 10).

Table. 2 - Mean distances ± standard deviation between every ring and the core in *Mullus suruletus* caught in two different areas (Ancona e Chioggia) and in two different years (2015 and 2016). P = Pelagic check; D = Demersal check.

		P	D	1	2	3	4	n ind
Ancona 2015	mean (mm)	0,58	0,84	1,06	1,40	1,70	1,96	31
	sd	0,06	0,07	0,06	0,08	0,11	0,09	
Chioggia 2015	mean (mm)		0,85	1,06	1,30	1,42	1,52	50
	sd		0,00	0,08	0,09	0,13	0,04	
Ancona 2016	mean (mm)	0,64		1,06	1,38	1,65	1,79	66
	sd	0,06		0,07	0,10	0,13	0,14	

For the otolith in the Figure 11, two annuli are visible and the specimen has finished lying down the deposition of the opaque ring. Also in Figure 12 it is possible to distinguish two winter rings and the pelagic and demersal checks are very clear. In Figure 13 four winter rings are visible and the age determination is 4, in according with the scheme of age assignment.

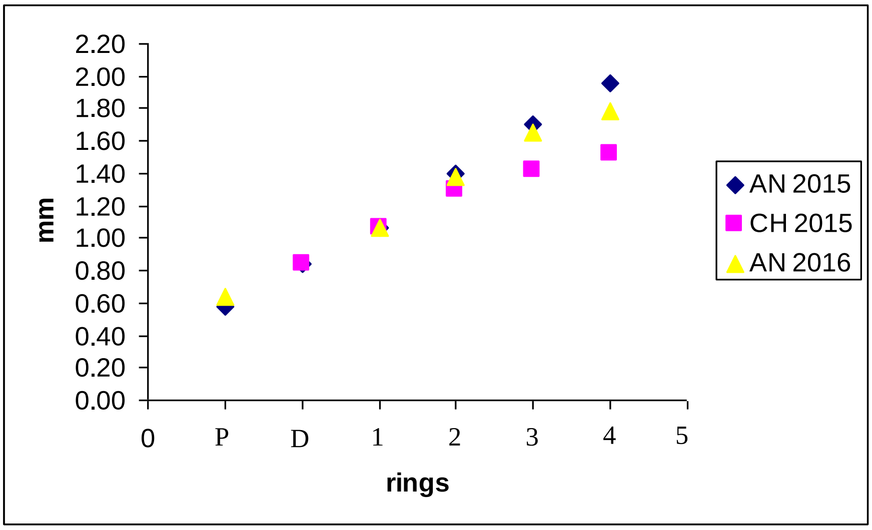


Fig. 10 – Mean distance between every ring and the core in *Mullus surmuletus* individuals caught in two different areas (Ancona e Chioggia) and in two different years (2015 and 2016). P = Pelagic check; D = Demersal check.

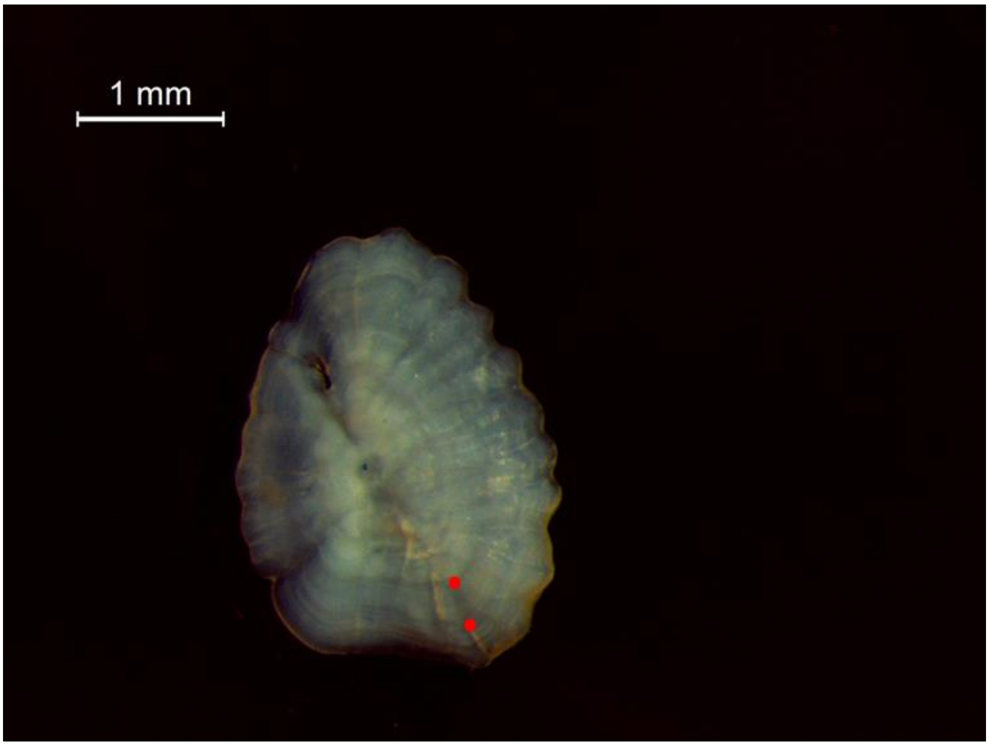


Figure 11: Otolith of *M. surmuletus*. TL: 18.5 cm, capture: December, Age: 2.

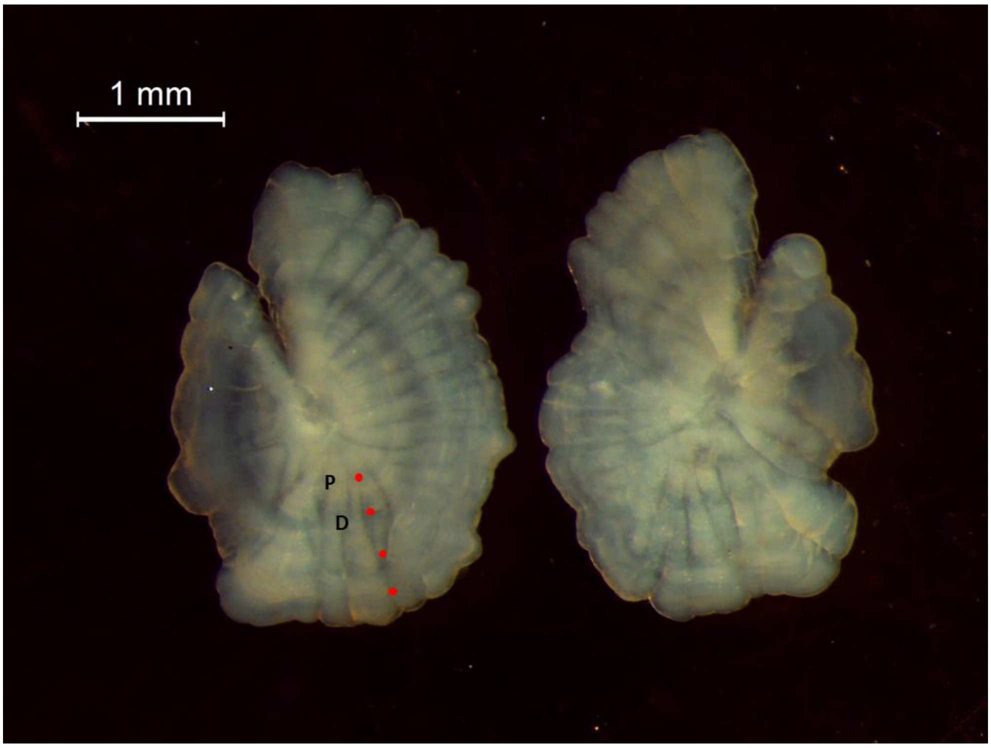


Figure 12 : Otolith of *M. surmuletus.* TL: 19.5 cm, capture: December, Age: 2. P: pelagic check, D: demersal check.

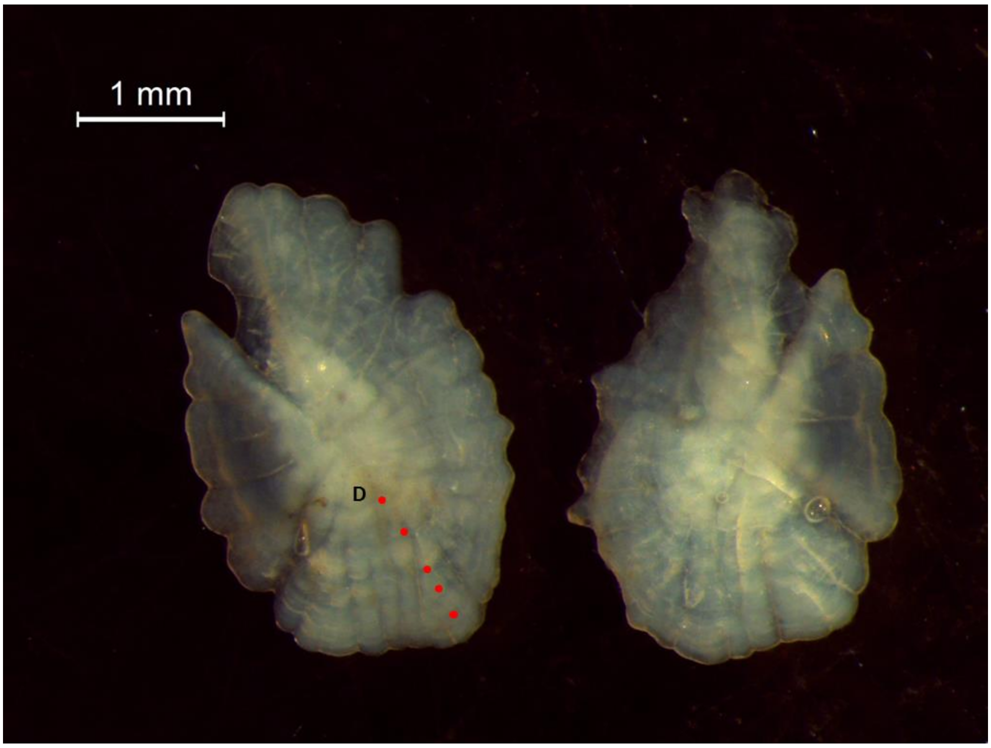


Figure 13 : Otolith of *M. surmuletus.* TL: 23 cm, capture: December, Age: 4. D: demersal check.

3.8 Greece in GSA 20, 22, 23

Otolith removal, cleaning and storing

Each individual is measured, weighted and the related biological data (e.g. sex, maturity stage, gonad weight) are recorded. Otoliths are removed carefully with the use of stainless steel forceps and kept dry in plastic vials after cleaned. Two ways of otolith extracting are used: a) a perpendicular cut on the dorsal side of the head, on the level of the otolith's cavity just behind the eyes and b) an horizontal-transverse cut on the ventral side of the head after the removal of gills (Fig. 1).

Figure 1: Otolith removal from the ventral location of fish head.

Subsequently, otoliths are cleaned in water and the remained organic attachments are removed carefully with the use of thin brushes. Both otoliths of each individual are stored in plastic vials before reading. In each vial, the id code of the specimen, the date of sampling and the area of capture are recorded (Fig. 2). This will allow cross-referencing of the sample with the biological data taken from each fish.

Figure 2: Store of both otoliths in plastic vials.

Otolith preparation for age reading

Whole otolith preparation technique is mainly used for both *Mullus* species age reading. Sections have been tested in the past in order to validate the first annual ring of the species.

Preparation, examination and analysis of otoliths for daily rings reading

The otolith preparation required for otolith microstructure and daily rings reading includes the following: a) Epoxy embedding: the right otolith from each pair is imbedded in a small block of resin (EpoFix Kit Struers) with the sulcus acusticus side up with the long axis of the otolith parallel to the long axis of the epoxy pool. All bubbles around the otolith are removed with a fine probe. The epoxy is left to solidify for two days before sectioning; b) Sectioning: thin sections of 600 μm are cut using a Buehler® Isomet low-speed diamond bladed saw (Fig. 3). By fitting the saw with two blades separated by a spacer, a thin section is prepared from an epoxy-embedded otolith with a single cut in less than two minutes. Each otolith is cross sectioned towards the anterior-posterior axis, where the daily increments are wider and appear more clearly than in the dorso-ventral axis. Each section is then mounted onto a microscope slide with a mounting medium with caution to avoid bubbles underneath and spreading a layer of epoxy on top of the section. The sections are allowed to dry overnight; c) Grinding and polishing: each section is manually grind with lapping paper of decreasing thickness (2400 and 4000) to be thinner, and further polished with decreasing grain size diamond suspensions (3 mm and 1 mm) to remove surface material and coarse scratches; d) Microscopical observation of the sections: General observations: the use of daily increments is time-consuming if the complete increment sequence from the core to the edge is required. The time required for a skilled person to mount, grid and polish an otolith to obtain a thin slice is about two hours. Preparing large numbers of thin slices may therefore limit the usefulness of the method.

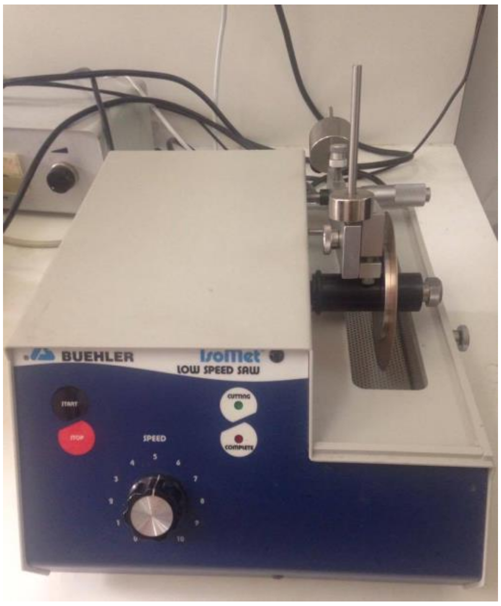


Figure 3: Otolith sectioning with a low-speed diamond bladed saw.

Otolith images and reading

Both otoliths (left and right) are placed in a petri dish with the *sulcus acusticus* facing down and immersed for 1-5 minutes in water. The purpose of liquid in the petri dish is to reduce glare on the three dimensional surface of the otolith and/or improve the visibility of the growth bands on the otolith surface (clearing technique). The petri dish is placed under a stereoscope with reflected light against a black background. Digital images of otoliths are taken by a camera using the Image Pro Plus Analysis System (Fig. 4). Images are taken with the same magnification; however different magnification could be taken for further help in the reading. If it is necessary, we should test and adjust the reflected light and focus of the stereoscope/microscope in order that the dark and translucent rings should be visible in the images (Fig. 5). Age estimation and measurements (e.g. for R, Ri, R of false rings) are taken from the left otolith of each individual, however the right otolith (WKACM, 2009) can also be used if any problems are presented in the left one. In this case, only the number of rings is taken into account without any measurement. Each measurement is performed on a defined growth axis (the longest axis from nucleus to the posterior area of the otolith) and kept in an excel file next to the id of each individual.

In case of daily rings readings, the slide is placed under a microscope and the same procedure is followed. Examination of the daily growth increments is always made with a compound microscope. Images and measurements are taken under the microscope by a digital camera using the Image Analysis Pro Plus software. Consecutive images are taken for each otolith along the axis of reading. Each image is stored with a unique name based on the information of each individual and the number of the photo taken for each otolith. Images are collated carefully to reproduce the whole image of the otolith in the axis of reading. Counting of daily rings are based on the entire axis as well as on part of it and then extrapolated to the R dimension. Daily increments are also measured to identify fast and low growth periods according to their width and their vicinity.

Figure 4 : Image Analysis Laboratory.

Figure 5: Otolith reading of *M. surmuletus* with two translucent rings.

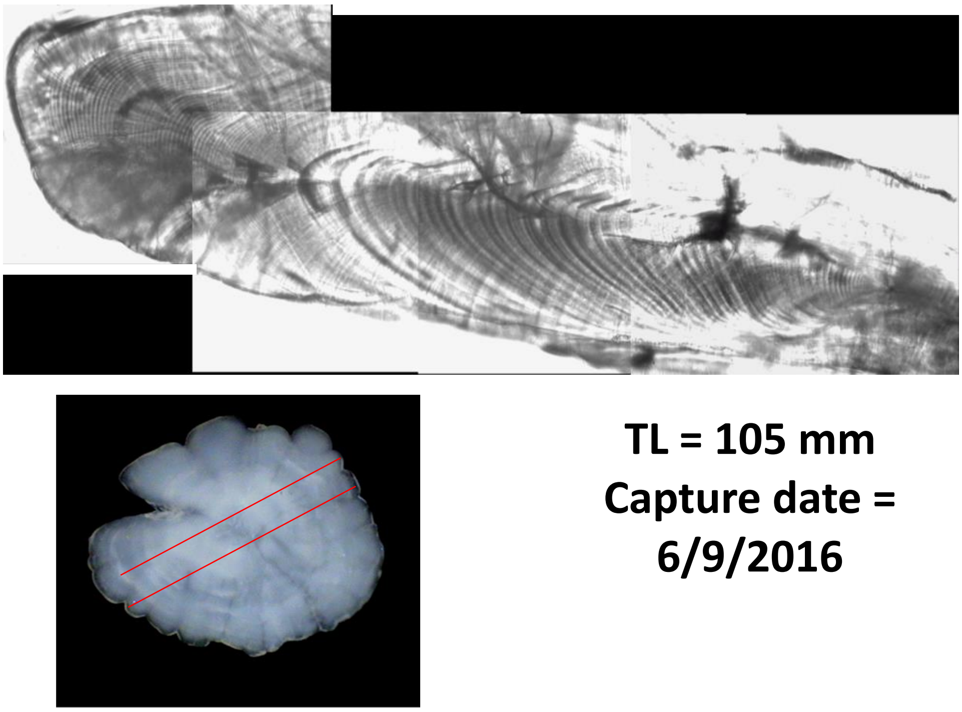


Figure 6 : Daily rings in *M. surmuletus* otolith and area of otolith section.

3.9 Ageing criteria for Mullus barbatus in the western Ionian Sea (GSA 19) (Sion L. and Indennidate A.)

In this presentation, ageing criteria adopted for Mullus barbatus in the Western Ionian Sea are presented, comparing direct method by otolith readings and indirect methods by back calculation and Modal Progression Analysis (MPA). Samples were collected during experimental trawl survey carried out in the spring-summer season 2015, as part of the MEDITS project. A total of 4817 individuals were collected; the Total Length (TL) to the nearest mm and the weight (g) were taken for each individual; the sex and gonads maturity stages were determined. The size-frequency distribution was calculated and size-classes of 5 mm TL were considered.

Sagittal otoliths were removed from 616 specimens cleaned and stored dry in eppendorf tube and 525 samples were used for reading purposes. Left sagittae were immersed in sea water and always observed by stereomicroscope. A reflected light against a black background was used to better distinguish alternating opaque and translucent. These zones deposition pattern were considered as annual events; the translucent zones were considered as annuli, counted and an Age Length Key (ALK) was inferred. The counts were converted into age assuming the 1st of July as the birthday data (Morales, Panfili, 2002) in convergence with red mullet reproduction peaks, generally observed in June and July, in the Western Ionian Sea.

On the post-rostrum, along the axis joining the sulcus and the nucleus, morphometric measures and ring distances were taken in an otoliths subsample (Domínguez-Seoane *et al*., 2006). Total Length (TL) and radius otolith (R) relationship was calculated by linear regression. A total of 515 measurements were collected in specimens between 87 and 229 mm TL. Growth rate was estimated using back calculation Fraser-Lee equation: TLi = c + (TLc - c) * (Ri/Rc), where TLc is the Total Length of the captured fish, Rc is the related otolith radius, SLi and Ri are the length at the i-annulus formation and the relative radius at the same time. The c value was calculated as the regression intercept between total length and radius otolith. Modal Progression Analysis (MPA) was performed by means of Bhattacharya method using FiSAT II software (Gayanilo et al., 2005) for survey carried out during September 2014 in which the recruitment fraction was observed. Each representative component, with a separation index (SI) greater than 2, was assumed to represent a different cohort. Mean lengths at age from otolith readings, back calculation method and MPA were compared by means of Kruskal-Wallis rank test.

The presence of false rings was detected during the reading of otoliths, in particular one or two false rings were observed in about 80% and 20% respectively. Subsequently the winter rings (translucent rings) were identified. A hypothesis could be that the 1st and 2nd false rings represent the pelagic and demersal rings respectively.

In order to corroborate the direct method by otolith readings, sagittae radius measurements were also carried out, in particular mean values of 1st false ring radius and 1st winter ring radius were 0.94 mm ± 0.06 and 1.17 mm ± 0.05 respectively.

A significant linear relationship ($p < 0.01$) was found between TL and otolith radius (R): TL = 123.30 mm, R = -48.80 (R2 = 0.89). These parameters were used in the back calculation Fraser-Lee equation. The mean lengths at age coming from back calculation method are showed in Tab. a.

The Bhattacharya's method was applied for the length distribution analysis and 6 modal groups were detected. Tab. B shows the identified modes in the size-frequency distribution related to the trawl survey carried out in September 2014.

In Tab. b are also presented ALK results between direct and indirect methods. The differences among the three different methods, evaluated by means of Kruskal-Wallis rank test, resulted not statistically significant ($p > 0.05$).

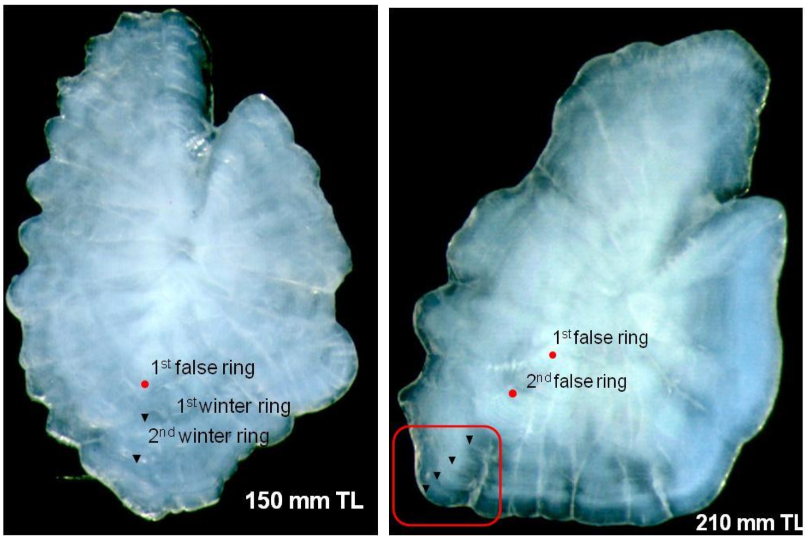


Fig. A - One or two false rings observed in red mullet sagittae.

Tab. a - The mean lengths at age estimated using back calculation Fraser-Lee equation.

N	Rings	False ring	1	2	3	4	5
42	False ring	71.75					
244	1	65.88	94.81				
165	2	64.74	96.80	131.30			
43	3	71.43	96.59	130.36	160.68		
17	4	67.20	99.61	131.25	160.32	189.31	
4	5	66.32	91.36	121.39	148.85	171.31	196.44
515	**Mean TL (mm)**	66.74	95.81	130.94	159.84	185.88	196.44
	SD	9.91	9.70	11.72	11.76	11.97	7.14

Tab. b - The mean lengths at age from otolith readings, MPA and back calculation.

	Mean TL (mm)		
Age class	**Otolith reading**	**MPA**	**Back calculation method**
0	70	56	67
1	115	94	96
2	134	136	131
3	154	167	160
4	173	191	186
5	189	223	196

Considerations:

- The occurrence of high percentage of mature individuals during the MEDITS trawl survey confirms the choice of the birthday data on the 1st of July;
- the correspondence in the growth pattern shown between the otolith readings, back calculation and MPA, makes reliable the identification of winter rings;
- some doubts remain on the false ring before the of first winter ring because its measurement corresponds, in the Bhattacharya method, with the length of zero age class in the other methods (MPA and back calculation) respectively;
- the point above could depend because it is not clear the interpretation of annual growth ring, particularly the first winter ring, so it is fundamental to approve a common scheme of reading followed from age validation methods to improve the growth rings interpretation.

Annex 4 Benchmark session on *M. barbatus* and M*. surmuletus* biological parameters (GFCM Working Group on Stock Assessment of Demersal Species - WGSAD) (I. Bitetto)

Isabella Bitetto (COISPA) reported the presentation of Charis Charilou, made during the benchmark session of mullet species at the last GFCM working group on stock assessment of demersal species (WGSAD) held in Rome in November 2016. Firstly, she reported the concern of the 2015 WGSAD experts about the variability of biological parameters across the Mediterranean regions that are considered due to non-standardized age reading procedure rather than geographical difference. The experts expected the outcomes of ICES otoliths exchange 2015-2016; they recommended including in the agenda of the GFCM WGSAD 2016 a session dedicated to the revision of biological parameters for mullets species.

During the first ageing workshop in 2009 (WKACM), following results of the Otolith exchange exercise in 2008, significant differences in age estimates were identified. During this meeting a first attempt of developing agreed guidelines for ageing of Mullus species. In the second ageing workshop in 2012 (WKACM2) that followed the results of otolith exchange exercise in 2011, the integrated use of validation methods is recommended and the guidelines were upgraded about:

- Blind reading as first step;
- Selection of measurement axis;
- Annotation of all (false/true) rings and collection of their measure;
- Translucent true rings visible around whole otolith;
- Date of birth in Med: 1st of January or 1st of July (with different reading interpretations);
- Evaluation of blind reading;
- Use validation methods (back-calculation, marginal increment analysis, examination of growth increment between consecutive rings).

During the last ICES Mullus Exchange 2015-2016 a new tool has been introduced, WebGR (http://webgr.azti.es/ce/search/myce), as suggested by ICES working groups, in order to collect different age readings from different readers for a set of 345 otoliths of M. barbatus and 102 of M. surmuletus. The level of agreement among the readers was estimated using the Guus Eltink spreadsheet. This otolith s exchange exercise showed the low level of agreement: only three otoliths of M. barbatus and 1 of M. surmuletus got 100% of agreement; this is a clear signal of the need of standardization of the ageing procedure across Mediterranean regions.

Isabella Bitetto pointed out the importance of a standardized ageing procedure, because strictly linked to the biological parameters, natural mortality and maturity at age used in the stock assessment process during the STECF and GFCM working groups.

STECF stock assessment working groups recommended using slow and fast growth set of von Bertalanffy parameters in the cases of unreliable age-length key. The parameters associated to the two growth hypothesis for each species are reported in tables below.

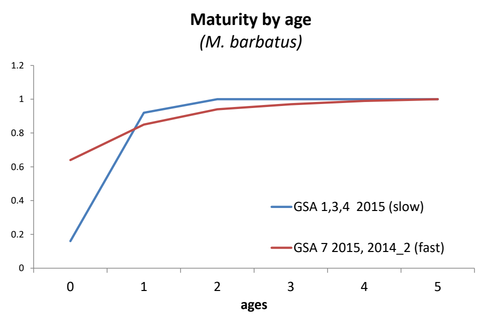
Species	***Param. set***	***L∞ (cm)***	***K***	***T_0***	***Source***
M. barbatus	*Fast*	*34.5*	*0.34*	*-0.143*	*Length*
	Slow	*26.0*	*0.41*	*-0.4*	*Otoliths*

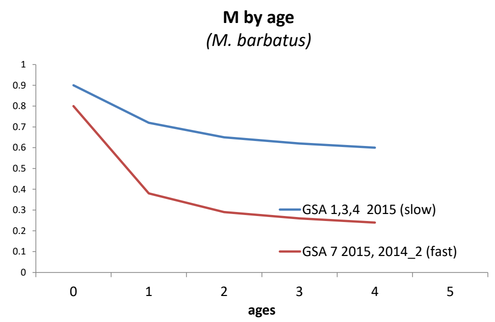
Species	***Param. Set***	***L∞ (cm)***	***K***	***T_0***
M. surmuletus	*Fast*	*38*	*0.31*	*-0.4*
	Slow	*36*	*0.22*	*-0.7*

The same working groups underlined the need of testing different hypothesis of natural mortality, applying different methods (Gislason, Prodbiom, etc…), as also suggested by GFCM stock assessment working groups. Indeed, during the last GFCM WGSAD 2016 the variability of the natural mortality has been taken into account in each stock assessment presented.

The age reading influences even the estimation of the natural mortality by age as well as the estimation of the maturity by age. Indeed, different growth parameters could produce very different maturity and mortality vectors by age, as shown in the graphs below).

Consequently, an unreliable estimation of biological parameters could produce a wrong estimation of maturity and natural mortality and, thus, respectively, of spawning stock biomass and fishing mortality, invalidating the diagnosis of the stock which the management measures are based on.





Annex 5 Explorative analysis for an ageing protocol standardization: *Mullus barbatus* (W. Zupa and P. Carbonara

The objective of the analysis is to measure the influence of geographic variables and reading protocols in the aging of Mullus barbatus. This represents a first step to harmonize reading protocols among the groups in the Mediterranean basin in the perspective to obtain unbiased ALK.

This analysis was performed using data collected during MEDITS surveys. In particular, one year data in the range comprised among 2013-2015 from 12 GSAs and 11 laboratories were used. This is just a preliminary dataset and data from other GSAs will be added in the future analysis.

In the analysis were used ageing data from MEDITS survey because samples were collected using a sampling protocol common for all the groups and the data were obtained from the same ageing method: otolith reading. The analysis wants to highlight the differences in data collected by different groups. Differences in reading protocol taken in count: the theoretical birthday (1st July or 1st January) and the number of false rings before the first winter ring. Other differences could be generated by the reading experience of the reader. Intrinsic differences of the sample are dependent to the geographical position of the sample collection place.

A former Principal Component Analysis (PCA) was carried out taking in count all the age groups together. The analysis was performed using fish total length (TL) and Age as quantitative variables, while number of false rings, sex, birthday, reader experience and Geographic Sub-Area (GSA) of origin were used as qualitative variable.

Other PCA analyses were carried out for each sex and age class using TL and both GSA's latitude and longitude as quantitative variables. The qualitative variables considered were: number of false rings, birthday and reader experience.

The variables that resulted mostly correlated with the first two Principal Components in the analysis with all age groups are the number of false rings and the GSA. In the other analyses the experience of the readers seems to be one of the most important factors influencing the age data variability. In all the sexes a clear segregation from the High-Medium experienced reader group from the Low experienced readers it was highlighted.

The choice of the birthday shows in the first age groups (0 and 1) a lower influence in comparison with readers experience and number of false rings considered, while in the other age group this variable has a greater influence. In opposition, the number of false rings seems one of the most important factors influencing the ageing of the first age groups (0 and 1), while in the age 2 group it has a lower influence.

Also the geographical factor has a significant influence in the age data variability: longitude shows the stronger correlation with the two principal components.

All these results show that a standardized ageing protocol for Mullus barbatus should take in account primarily the readers experience choosing opportune threshold limits of either the percentage of agreement or the CV or APE. Another important factor to be considered is the number of the false rings before the first winter ring considered, that results critical in the ageing of the first age classes. Finally also a fixed birthday it is desirable to reduce the reading differences amongst the groups in the perspective of ageing protocol standardization.

Table 1 - Results of the PCA analysis performed with all the sexes and age classes

	% of variance	
	Dim 1	Dim2
all the age classes	82.234	17.766
variable	R^2	
TL	0.934	-0.356
AGE	0.934	0.356
GSA	0.162	0.521
No. Rings	0.136	0.101

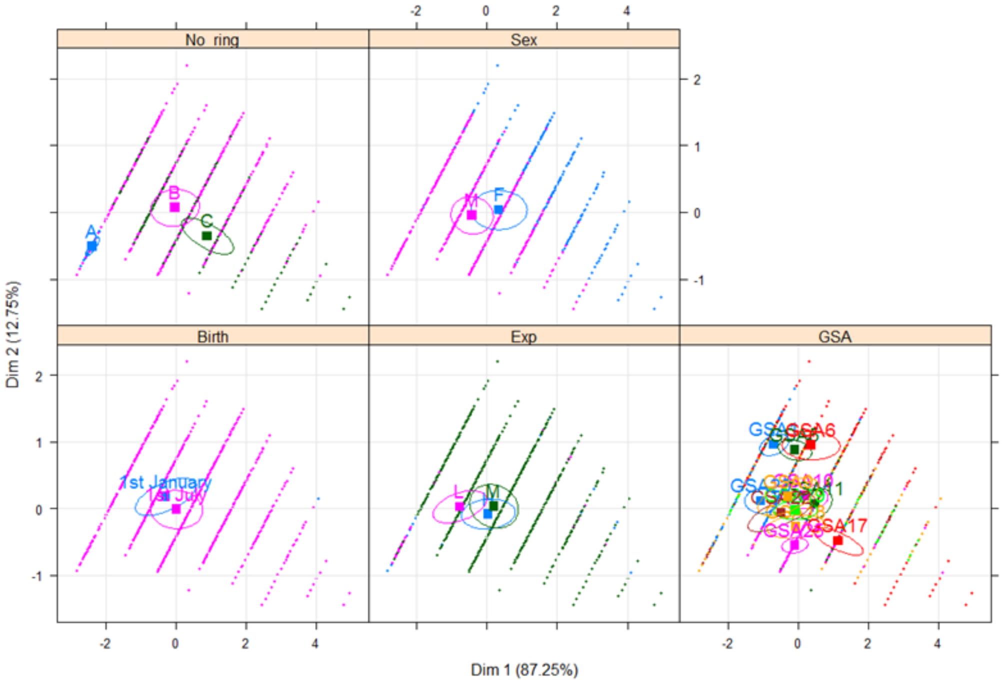


Figure 1 – PCA plot of ageing data in the first two dimensions. Number of rings: A=0 ring; B= 1 ring; C= 2 rings; Reader experience: L< 2000 otoliths; M 2000-6000 otoliths; H> 6000 otoliths

Table 2 - Results of the PCA analysis performed on the age class 0

	% of variance	
	Dim 1	Dim2
AGE 0 - M	55.29	37.83
variable	R^2	
TL	0.858	-0.426
Longitude	-0.936	-0.174
Latitude	0.211	-0.426
Exp	0.692	0.488
No. Rings	0.346	0.069
Birthday	-	0.381

	% of variance	
	Dim 1	Dim2
AGE 0 - F	54.216	40.428
variable	R^2	
TL	0.823	-0.46
Longitude	-0.933	-0.249
Latitude	0.165	0.969
Exp	0.588	0.542
No. Rings	0.322	0.103
Birthday	-	0.382

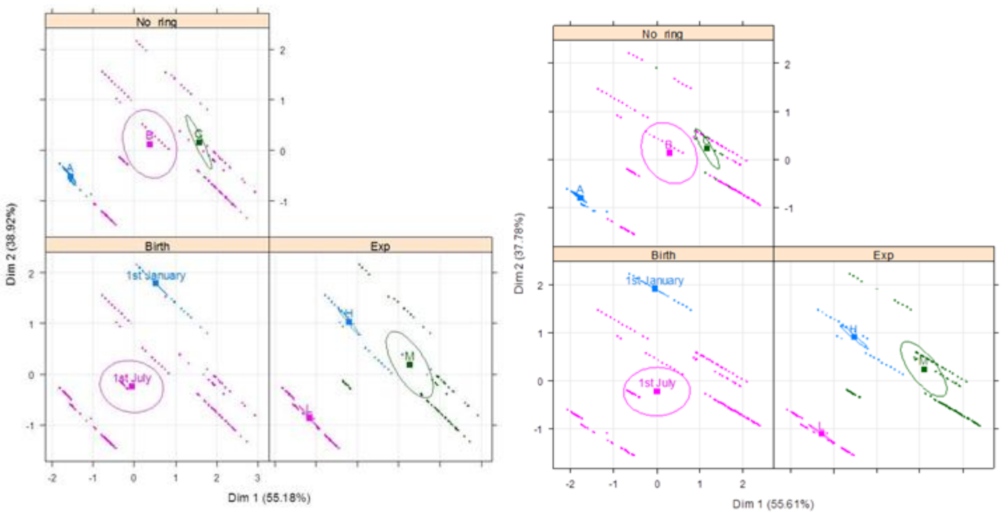


Figure 2 – PCA plot of the females (right) and male (left) of age 0 in the first two dimensions. Number of rings: A=0 ring; B= 1 ring; C= 2 rings; Reader experience: L< 2000 otoliths; M 2000-6000 otoliths; H> 6000 otoliths

Table 3 - Results of the PCA analysis performed on the age class 1

	% of variance	
	Dim 1	Dim2
AGE 1 - M	57.963	32.308
variable	R^2	
TL	0.495	0.85
Longitude	-0.922	-
Latitude	0.802	-0.497
Exp	0.343	0.245
No. Rings	-	-
Birthday	0.023	-

	% of variance	
	Dim 1	Dim2
AGE 1 - F	63.684	27.317
variable	R^2	
TL	0.694	0.686
Longitude	-0.921	-
Latitude	0.76	0.589
Exp	0.303	0.216
No. Rings	-	0.025
Birthday	0.072	0.031

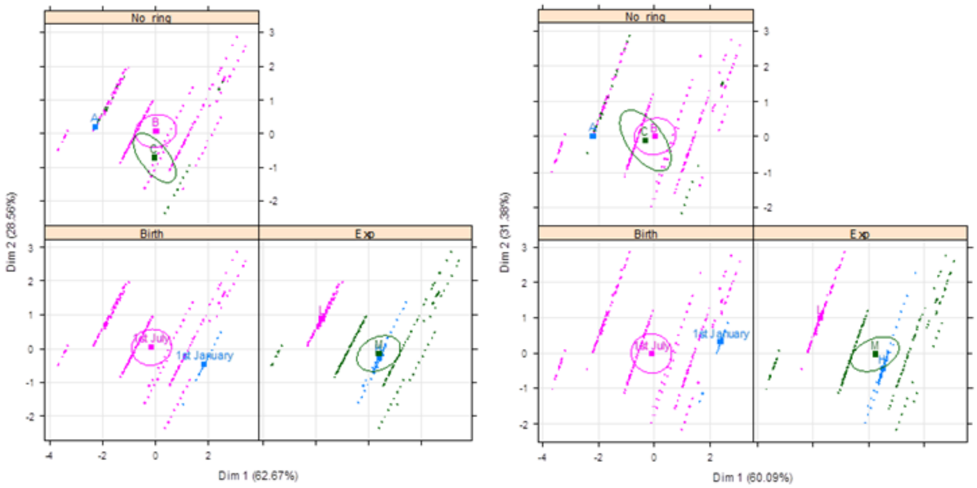


Figure 3 – PCA plot of the females (right) and male (left) of age 1 in the first two dimensions. Number of rings: A=0 ring; B= 1 ring; C= 2 rings; Reader experience: L< 2000 otoliths; M 2000-6000 otoliths; H> 6000 otoliths

Table 3 - Results of the PCA analysis performed on the age class 2

	% of variance	
	Dim 1	**Dim2**
AGE 2 - M	65.279	28.947
variable	**R^2**	
TL	0.538	0.838
Longitude	-0.946	-
Latitude	0.879	-0.391
Exp	0.13	0.156
No. Rings	0.035	0.149
Birthday	0.056	-

	% of variance	
	Dim 1	**Dim2**
AGE 2 - F	66.535	25.344
variable	**R^2**	
TL	0.737	0.641
Longitude	-0.928	-
Latitude	0.769	-0.59
Exp	0.1	0.137
No. Rings	0.024	0.146
Birthday	0.056	0.013

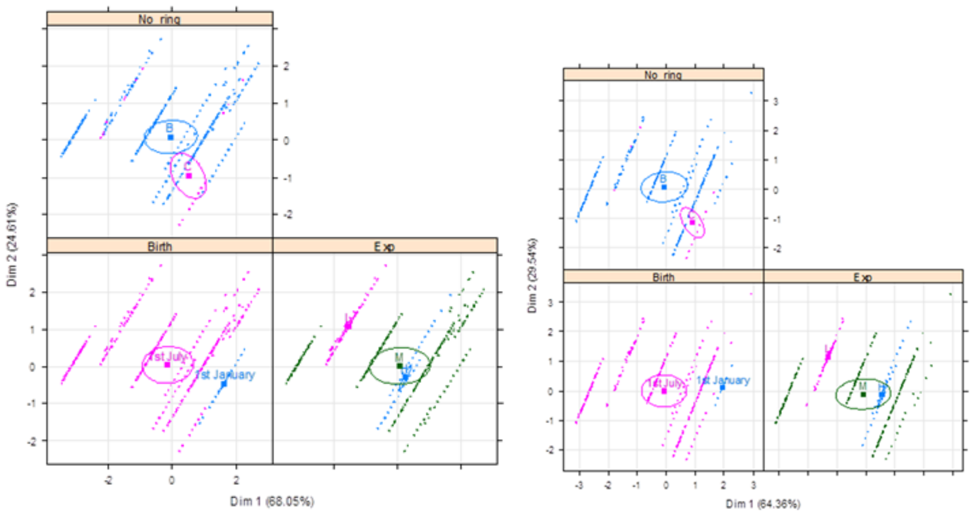


Figure 4 – PCA plot of the females (right) and male (left) of age 2 in the first two dimensions. Number of rings: A=0 ring; B= 1 ring; C= 2 rings; Reader experience: L< 2000 otoliths; M 2000-6000 otoliths; H> 6000 otoliths

Annex 6 WKACM3 terms of reference for the next meeting

The **Workshop on Age reading red mullet (*Mullus barbatus*) and striped red mullet (*Mullus surmuletus*) 3** (WKACM3), co-chaired by Pierluigi Carbonara, Italy; Kélig Mahé, France and Damir Medvesek, Croatia) will meet in Split, Croatia, from 18 to 22 March 2019 to:

Review of available data through new validation studies

Analysis of the results of last exchange between ageing labs, according to the information from the WKVALMU;

Clarify the interpretation of annual growth rings particularly the first growth ring ;

Improve the age reading protocols produced during the WKACM2;

Increase existing reference collections of otoliths and improve the existing data base of otolith images during the WKACM2;

Address the generic ToRs adopted for workshops on age calibration (see WGBIOP Guidelines for Workshops on Age Calibration').

WKACM3 will report by DATE to the attention of ACOM.

Supporting Information

Priority:	Essential. Age determination is an essential feature in fish stock assessment to estimate the rates of mortalities and growth. Age data is provided by different countries and are estimated using international ageing criteria which have not been validated. There is necessary to continue to clarify this guideline of age interpretation in the Mediterranean sea for *Mullus* species. Therefore, an appropriate otolith exchange programme will carry out in 2018 for the purpose of intercalibration between ageing labs according to the results of the WKVALMU meeting. Results of this otolith exchange will discuss during WKACM3 (2019).
Scientific justification:	The aim of the workshop is to identify the current ageing problems between readers and standardize the age reading procedures in order to improve the accuracy and precision in the age reading of this species.
Resource requirements:	No specific resource requirement beyond the need for members to prepare for and participate in the meeting.
Participants:	In view of its relevance to the DC-MAP, and ICES WG, the Workshop try to join international experts on growth, age estimation and scientists involved in assessment in order to progress towards a solution. Participants should announce their intention to participate in the WK no later than two months before the meeting.
Secretariat facilities:	
Financial:	
Linkages to advisory committees:	ACOM
Linkages to other committees or groups:	WGBIOP, MEDITS Group, RCMED, PGMED, ACOM,GFCM, WGSAD
Linkages to other organizations:	There is a direct link with the EU DCF.

Annex 7 Recommendations

Recommandations	Addressed to
1.WKACM3 Workshop in 2019	WGBIOP, ACOM
2. Otoliths Exchange of *M. surmuletus* and *barbatus* from the Mediterranean sea in 2018	WGBIOP, ACOM
3. Age validation study to solve the growth rings interpretation	WGBIOP, MEDITS Group, RCMED, RCM NS-EA, RCM NA, PGMED, ACOM,GFCM, WGSAD
4. Ageing protocol must follow the new ageing scheme in the Mediterranean sea	WGBIOP, GFCM, WGSAD, MEDITS Group, EASTMED,ADRIAMED, MEDSUDMED