Supplementary Material on the Methodology Part IX

Microchemistry analysis of sardine otoliths

We focused on the analysis of the element of two otoliths parts: the core and the edge. The core region was considered to assess the plausible difference between individuals spawning in different areas, giving information as well of the first weeks of life. Furthermore, the clear discrimination of the element composition of the otolith core among regions suggest that mixing of fish derived from different juvenile source areas was minimal, supporting the existence of stock structure. The margins were analyzed to assess whether elemental chemistry of recently deposited otolith material varied among regions and to compare to that of otolith cores to assess the degree of spatial mixing

Data analysis

The data for the individual ablations were averaged across three margin region ablations (margins) to provide the margin elemental:Ca ratios used for all statistical analyses. Prior to formal statistical analyses, box plots of raw data were examined to identify extreme outliers. Data that were >3 inter-quartile ranges either above the 75^{th} percentile or below the 25^{th} percentile were disregarded from all further statistical analyses (Wilkinson *et al.*, 1996).

For the core and margin otolith regions, we conducted the same series of analyses. Firstly, we conducted univariate analysis of variance (ANOVA) to assess whether individual element:Ca ratios differed significantly among regions. Post-hoc Fisher LSD tests were used to determine the nature of significant differences among regions.

A forward stepwise linear discriminant analysis was built to classify each individual to one of the sites from which they were collected. Classification accuracies for each species and environment were evaluated through the percentage of correctly classified individuals using Jackknife classification. It should be noted that Jackknife classifications are particularly sensitive to low sample size, indeed the confidence in the degree of "true" group separation will be low. For this reason, we considered only the GSA as groups (>30 individuals per group) for all the analysis.

Results

The individual laser ablation of the otolith showed clear differences in the levels of Mg, Sr (higher in the core) and Ba (higher in the margin) between the core and margins of otoliths from the areas (Figure 1). For Mn, differences were not significant in the core.

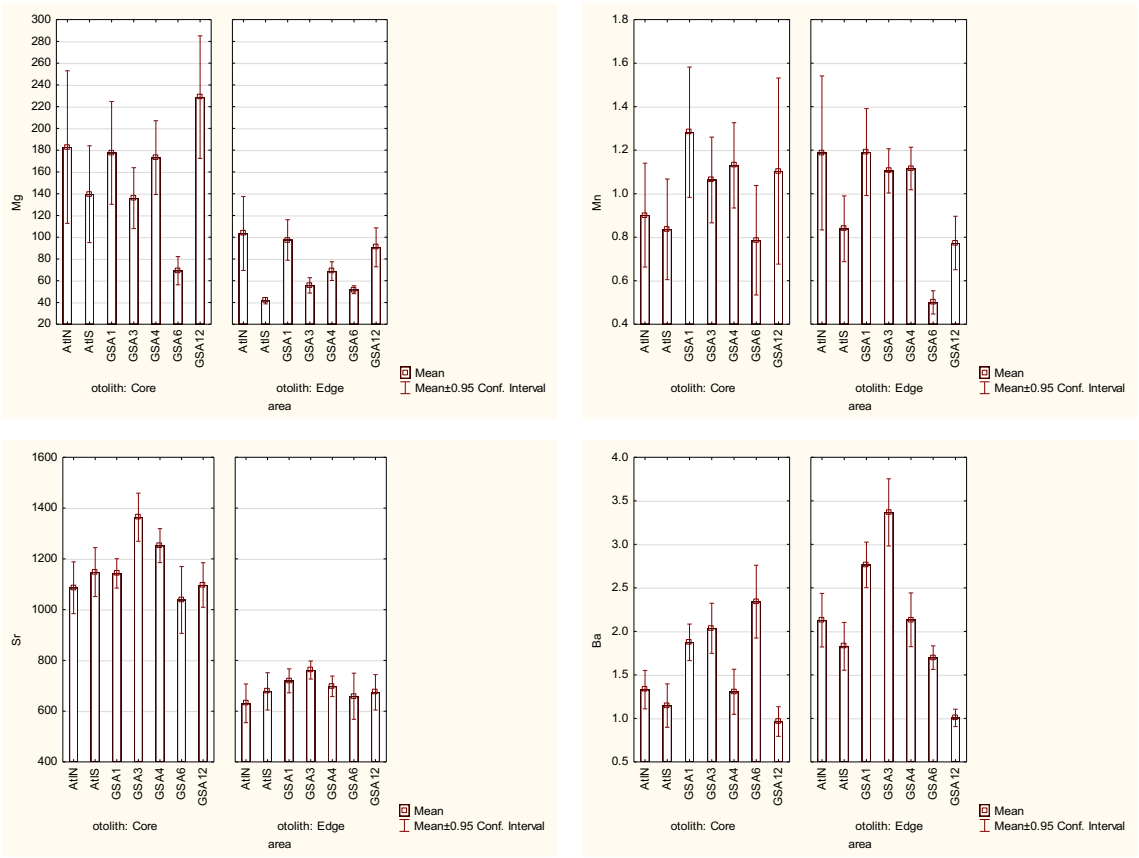

Figure 1. Comparison of individual element:Ca in otolith core and Margin of sardine collected from GSAs 1, 3, 4, 6 and 12, AtlN (atlantic North) and AtlS (atlantic South) for the following elements Mg (a), Mn (b), Sr (c) and Ba (d)

Element core

MANOVA of ratio Mg:Ca, Sr:Ca, Ba:Ca and Mn:Ca was highly significant ($F=5.615$, $p<0.000$**). Microchemistry analysis of the otolith core of sardine was found to be effective in detecting differences in elemental concentration across the different areas using ANOVA (Table 1). Magnesium Univariate ANOVA indicated significant differences in Mg:Ca ratio of otolith cores among the sampling areas ($p<0.05$) with the highest Mg:Ca ratio in the core observed in GSA 12. Post-hoc Fisher LSD test showed no difference in Mg:Ca between GSAs 4, 3 and 1 and AtlN and AtlS. For manganese, no-significant differences were observed between different areas in the Mediterranean and in the Atlantic. For strontium, specimens from GSA 3 and GSA 4 exhibited higher Sr:Ca ratios in the otolith cores compared to GSAs 1, 12 and 6 and AtlN and AtlS. Ba:Ca ratio showed significant differences in samples among collection locations. The Ba:Ca ratios, were the highest in GSAs 6, 3 and 1. The lowest concentration was observed in GSA 12 (Table 1)

Table 1. Results of univariate ANOVA and Post-hoc Fisher LSD tests comparing individual element ratio in otolith cores of sardine collected from GSAs 1, 3, 4, 6 and 12 and AtlN, AtlS.

Source	df	F	P	Post-hoc Fisher LSD test differences by areas
Mg:Ca Area	6	5.04	<0.00**	GSA6<AtlS, GSA6<GSA3, GSA6<GSA1 GSA6<AtlN,GSA6<GSA12, GSA6<GSA4 GSA3<GSA12 AtlS<GSA12, GSA1< GSA12
Mn:Ca Area	6	1.61	0.14	No significant differences
Sr:Ca Area	6	7.10	<0.00**	GSA3>AtlS GSA3>AtlN GSA3> GSA1 GSA3> GSA4, GSA3> GSA6, GSA4>AtlN GSA4> GSA1 GSA4> GSA6 GSA4< GSA3
Ba:Ca Area	6	12.03	<0.00**	GSA6>AtlS, GSA6>GSA4, GSA6>AtlN, GSA6>GSA1,GSA6>GSA12, GSA12<GSA3 GSA12<GSA1, GSA1>AtlS GSA1>AtlN GSA1>GSA4, AtlN>AtlS

Element edge

Otolith multi-element fingerprints of the margin in sampled areas showed high significant differences with MANOVA ($F = 13.57$, $p < 0.001$). Analysis of the otolith margin of sardine was also found to be effective in detecting differences in elemental concentration across the different areas sampled (ANOVA, Table 2). while the main pattern observe were partially different to those in the core.

For magnesium, The Highest Mg:Ca ratios were observed in AtlN followed by GSA 1, and the lowest concentration was observed in AtlS. For manganese, Mn:Ca ratio was higher in GSA 1 and AtlN, contrariwise the lowest values were observed in GSAs 6 and 12.

Sr:Ca ratio in the sardine otolith edge was the highest in GSA 3 and the lowest in the AtlN Post-hoc Fisher LSD test showed no differences in concentration of Ba between GSAs 12, 6, 4 and 1 and AtlS. For barium, the highest Ba:Ca concentration was found in GSA 3 followed by GSA 1 and AtlN and the lowest concentrations were observed in GSAs 12 and 6.

Table 2. Results of univariate ANOVA and post-hoc Fisher LSD tests comparing individual element ratios in otolith edges of sardine collected from GSAs 1, 3, 4, 6 and 12 and AtlN, AtlS.

Source	df	F	P	Post-hoc Fisher LSD test differences by areas
Mg:Ca Area	6	16.73	<0.00**	AtlS<GSA6, AtlS<GSA12, AtlS<GSA4 AtlS<GSA3, AtlS<GSA1, AtlS<AtlN GSA4>GSA3 GSA4<GSA12 GSA4<GSA1

				AtlS<GSA12, GSA3< GSA1 GSA3<AtlN GSA6<AtlN, GSA3< GSA12
Mn:Ca Area	6	10.56	0.14	GSA6<AtlN GSA6<AtlS GSA6<GSA1 GSA6< GSA3 GSA6< GSA4 GSA6< GSA12 GSA12<GSA1,GSA12<GSA3 GSA12<GSA4 GSA12<GSA6 GSA12<AtlN, AtlS<GSA1 AtlS>AtlN, AtlS<GSA4 AtlS<GSA3
Sr:Ca Area	6	2.49	<0.02	GSA3>AtlS GSA3>AtlN GSA3> GSA12 GSA3> GSA4, GSA3> GSA6, GSA1>AtlN
Ba:Ca Area	6	23.94	<0.00**	GSA3>AtlS GSA3>AtlN GSA3> GSA12 GSA3> GSA4, GSA3> GSA6, GSA3> GSA1 GSA1>AtlS GSA1>AtlN GSA1> GSA12 GSA1>GSA4, GSA1>GSA6, GSA12<GSA1, GSA12<GSA3,GSA12<GSA4 GSA12<GSA6, GSA12<AtlN GSA12<AtlS

Analysis of sardine otolith microchemistry in the Mediterranean

Core

The discriminant analysis plot indicates some overlap especially among samples from GSAs 4 and 12 and between GSA 3 and GSAs 1 and 4. The samples from GSA 1 and GSA 4 were more variable than the other GSAs. However, the samples from GSA 6 were separated from the other GSAs along canonical variate 1 (Figure 2). This separation was due to the tendency for higher Ba:Ca and lower Mg:Ca in the core of sardine otoliths from GSA 6

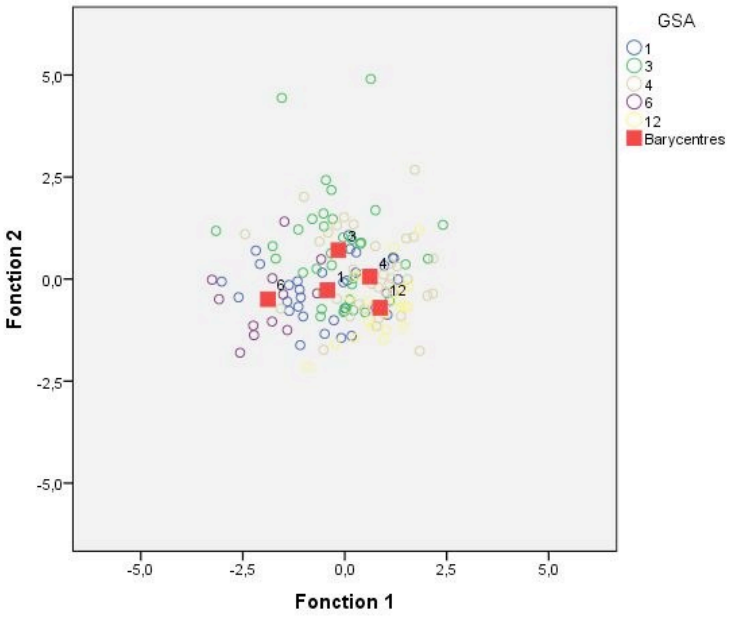

Figure 2. Scatter plot of scores obtained by DA of multi-element chemistry of otolith cores of sardine collected from GSAs 1, 3, 4, 6 and 12.

Table 3. Results of jackknife classification of individuals based on multi-element chemistry of otolith cores.

	Area classified to (% sample)				
Jacknife classification	GSA1	GSA 3	GSA4	GSA6	GSA12
GSA1	**34.4**	6.3	18.8	25	15.6
GSA 3	21.6	**43.2**	21.6	8.1	5.4
GSA 4	7.5	20	**40**	2.5	30
GSA6	8.3	16.7	0	**75**	0
GSA12	10	0	25	0	**65**

The jackknifed classification procedure involving all GSAs suggest a gradient from GSA 12 to GSA 6. Thus, the classification was moderately accurate in assigning individuals collected from GSA 6 and GSA 12 based on Ba:Ca, Mg:Ca, Sr:Ca and Mn:Ca ratios of their otolith cores, but performed poorly for GSAs 1, 4 and 3. Classification accuracy was highest for the GSA 6 at 75% followed by GSA 12 at 65% and then GSAs 3, 4 and 1 around 39%. Classification errors for GSA 1 samples were mostly due to their misclassifications in GSA 6. Classification errors in GSA 4 were almost entirely due to samples being misclassified in GSA 12. For the GSA 3 area classification errors were evenly divided between GSAs 4 and 1 (Table 3).

Edge

A forward stepwise linear discriminant analysis was performed using the GSAs as the grouping variable and the element:Ca ratio of otolith edge as explanatory variables. The Canonical variates plot discriminated four main areas; GSAs 12, 6 and 3 and an area composed of an overlapping between GSAs 4 and 1. The separation along the canonical variate 1 was due to the tendency for lower Ba:Ca ratio in GSA 12 compared to GSAs 3 and 1 but higher Mg:Ca in GSA 12 compared to GSA 3. Along the second variate the separation was due to higher Mn in GSA 1 compared to GSA 6 (Figure 3).

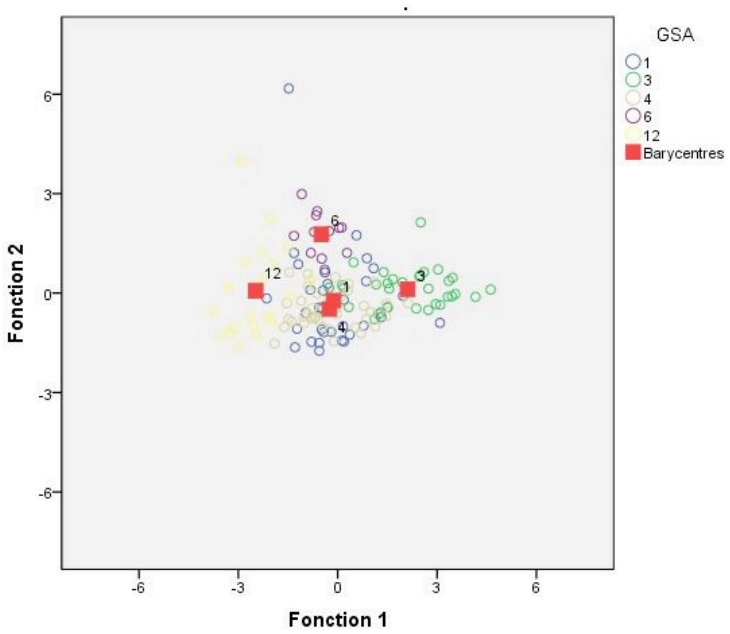

Figure 3. Scatter plot of scores obtained by DA of multi-element chemistry of otolith edges of sardine collected from GSAs 1, 3, 4, 6 and 12

The jackknifed classification procedure including all GSAs was moderate to highly accurate in assigning individuals to their collection region based on Mg:Ca, Ba:Ca and Mn:Ca ratios of their otolith edge (Table 4) Classification accuracy was again highest for individuals collected from GSA 6 at 91.7% followed by GSA 3 at 82.4% then GSA 12 (81%). The GSA 4 and GSA 1 samples showed relatively poor classification at respectively 57.5% and 40.6% reflecting the variability of the samples from these areas and their overlap between each other

Table 4. Results of jackknife classification of individual based on multi-element chemistry of otolith edges

	Area classified to (% sample)				
Jacknife classification	GSA1	GSA 3	GSA4	GSA6	GSA12
GSA1	**40.6**	15.6	28.1	12.5	3.1
GSA 3	11.8	**82.4**	2.9	2.9	0

GSA 4	20	10	**57.5**	0	12.5
GSA6	0	0	8.3	**91.7**	0
GSA12	0	0	4.8	14.3	**81**

Analysis of sardine otolith microchemistry in the Mediterranean and Atlantic

Core

A forward stepwise linear discriminant model analysis was built for core and edge otolith data to classify hake from Atlantic and Mediterranean. For the core, the canonical plot indicated that there is considerable overlapping among individual from all the areas whether Atlantic or Mediterranean causing a poor classification of samples. However a relatively large proportion of the sample from GSA 6 were separated from the other area along the first axis due to the tendency of higher Ba:Ca in the core of otoliths from sardine in GSA 6 (Figure 4).

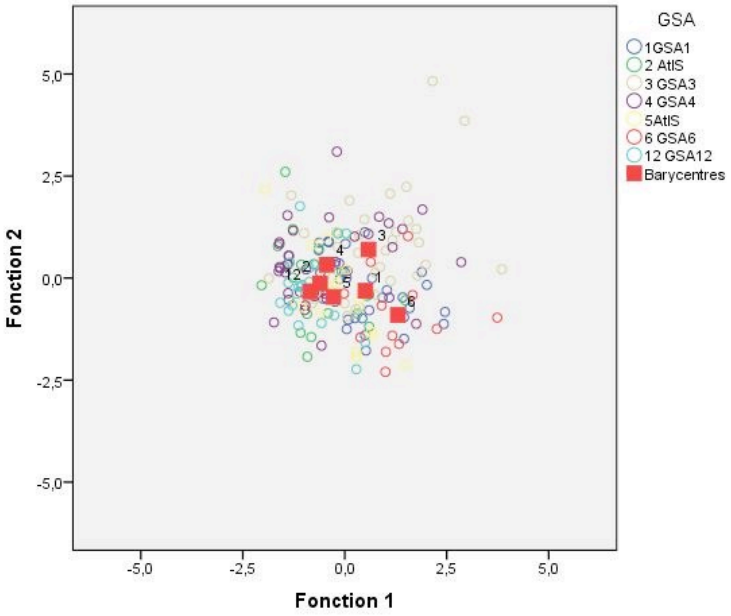

Figure 4. Scatter plot of scores obtained by DA of multi-element chemistry of otolith cores of sardine collected from GSAs 1, 3, 4, 6 and 12 and AtlN, AtlS

Edge

For the edge, classification accuracy was moderately accurate for GSAs 6, 12 and 3. The canonical variate plot indicated some overlap among individual sampled from Atlantic north, and GSAs 1 and 4. And individuals from south Atlantic overlap with those from GSAs 3 and 6 (Figure 5).

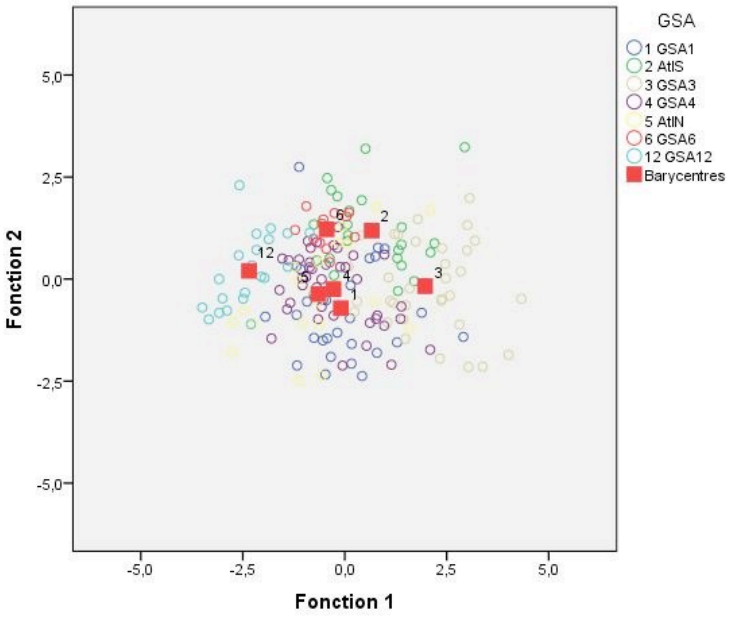

Figure 5. Scatter plot of scores obtained by DA of multi-element chemistry of otolith edges of sardine collected from GSAs 1, 3, 4, 6 and 12 and AtlN, AtlS

Discussion and conclusions

The ablation over the otolith core region would have provided an element signature for a period of time within the first weeks of life when individuals would have been approximately 10 mm total length, and pelagic in the upper water column. The comparison of the otolith core and edge chemistry is important in relation to assessing stock structure. The chemical analysis of the sardine otolith edge was compared with the aim of detecting whether 'recently' deposited otolith material differed among fishing regions, assuming that the fish from the different regions had remained separated for the recent period of time over which the otolith edge material was precipitated.

Four elements demonstrated variation among the different fishing regions. However, the only elements that showed relatively consistent variation for both otolith sampling position were Ba and Mg

For Ba there is a great evidence across multiple species that incorporation into otolith is driven principally by ambient concentration (Bath *et al*., 2000; Hamer *et al*., 2003, 2006). Differences in otolith Ba can therefore be assumed to be indicative of variation in the ambient Ba level that the fish was exposed to. This element is often associated with high primary productivity and exhibits a nutrient-type profile, with surface depletion and enrichment at depth and potential to be enriched in inshore coastal waters and marine dominated bays and estuaries.

The patterns of otolith Ba variation between sampled areas were relatively similar for both otolith core and margin. Indeed, for the otolith cores and margins Ba:ca ratio was the highest in GSAs 3, 1 and 6 and the least in GSA 12. Otolith Ba variation among areas could be indicative of variation in the amount of Ba water in these areas**.** Also, the different level of otolith Ba

between the core and the margin among GSA 1, 3 and 4 and the Atlantic area could be related to shifts in the spatial and or depth distribution between larvae and adult life stage indeed sardine larvae is mainly distributed in the upper 20-25 m of the water column (Santos *et al*., 2006) whereas adult sardine reaches deeper water up to 100m deep (Pilar Tugores *et al*., 2011). This could not be the case for GSA 12 where the level of Ba is practically same in the core and the margin.

Mg is essential for a range of cellular process and its level in fish tissue and plasma is subject to significant physiological regulation that explain its incorporation in the otolith.

Otolith Mg is not affected by either temperature and/or salinity but is correlated with otolith precipitation and somatic growth rate (Artetxe-Arrate *et al*., 2019; Bath, Martin and Thorrold, 2005). The patterns of otolith Mg variation among areas were similar for both core and margin of the otolith. The mean otolith cores Mg was higher in GSA 12 than all the other areas fom Atlantic and Mediterranean. Among the areas, the levels of Mg in the otolith core were practically the double of those of otolith margins. This is probably related to the slowdown of growth and or metabolic rate with age.

Similar to Mg, Mn incorporation into the otolith is physiologically regulated and sensitive to growth effects. Environmental influences seem also to affect otolith Mn concentration. According to Ruttenberg *et al*. (2006), high level of Mn near the otolith primordium was related to maternal transfer. Interpretation of variation in otolith Mn was complicated due to the differing patterns of variation among areas depending on otolith sampling position.

Like Ba the, Sr incorporation into otoliths has been shown to be correlated with ambient concentration, and thus appear to be reliable ‘geographical marker’ (Zimmerman, 2005). The concentrations of Sr reflect salinity changes, while deposition of Sr in the otoliths might diminish with age (Weatherley and Gill, 1987). The patterns of otolith Sr variation among areas were similar for both otolith core and margin. Indeed, for the otolith cores and margins the mean level of Sr was higher in GSAs 3, 4 and 12. Relatively low levels of Sr:Ca were observed in GSAS 6,1 and the Atlantic area which may be due to lower salinity in these areas, given that Sr concentration in the water is positively correlated with ambient salinity.

Although, analysis of MANOVA and ANOVA among areas showed in a statistical sense that sardines from the different areas would be from different stocks, discriminant analysis showed that there is a considerable overlapping between individual from the different areas.

The canonical variate plots of the otolith core chemistry indicated relative tighter clustering of the samples from GSA 6 and GSA 12 compared to those from GSAs 4, 1 and 3. This clustering was behind the relatively high classification accuracy of these two areas samples. The overlapping with the other areas was especially with GSA 1 where 25% of the samples were classified in GSA 6 and with GSA 4 where 30% of the samples were classified in GSA 12. Two possible causes of misclassifications which are: (1) similarity of the environmental condition influencing otolith chemistry and or (2) mixing of the individual between areas. The otolith margin chemistry indicated tighter clustering of the samples from GSAs 6, 12 and 3 with the highest classification accuracy of 91.7%, 82.4% and 81% and 75.4% than GSA 1 and 4 that overlapped between each other and seemed to be closer. The high separation of GSA 6, 12 and

3 samples from the other areas suggests that there is limited or no mixing of the adult sardines from these areas with the other population even though there is some overlapping of GSA 12 with GSA 6 and GSA 3 with GSA 1. The overlapping distribution of the samples from these areas may be attributable to extensive migration in these waters. Given that the sampling was done during the summer feeding season. During this period sardines undertake feeding migration involving broader areas.

Overall, the results of the core otolith chemistry were not sufficiently different for consistent discrimination between sardine stock from the different sampling sites as well as from Atlantic or Mediterranean. This could be related to a common origin of the sardine or due to limited variability of the chemical composition of their ambient environment owing to the interchange of water between Atlantic and Mediterranean. On the other hand, the composition of the otolith margin showed some separation in adult sardine mainly for GSAS 6, 3 and 12 from the other areas. This separation assumes that these sardines remained separated for the recent period of time over which the otolith margin material was precipitated. However, the overlapping observed between GSAs 1 and 4 and sometimes with GSA 3 is probably related to extensive migration in search of optimal feeding condition so their marginal otolith chemistry become comparable.

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