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Spatial and temporal variability in otolith elemental signatures of juvenile sardine off South Africa

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Abstract :

Otolith elemental signatures can be used to identify when individual or groups of fish are spending a significant amount of time in different environments. Elemental signatures of juvenile sardine Sardinops sagax caught in winter 2008 and 2009 around the coast of South Africa were measured using inductively-coupled plasma mass-spectroscopy. The otolith elemental signatures of 34 fish caught in 2008 and of 52 fish caught in 2009 were measured at the edge (to represent conditions 20-30 days prior to capture). Principal component analysis was used to visualise the relationships of individuals to each other, in terms of their otolith chemistry, in two-dimensional space, and multiple ANOVAs were used to investigate spatial and temporal variations among samples collected in 2008 and 2009. Significant differences among sites were found in MANOVAs, but the between-site differences varied among the elements. Magnesium concentration tended to decrease whereas barium concentration tended to increase from the west to the east coast. Barium indicate upwelling impact but for 2008 samples on the northern part of the west coast. Otolith microchemistry provides evidence of large and small-scale differentiation in sardine, but differences between years indicates that this is not necessarily temporally stable. The ocean off South Africa is a dynamic and variable environment and this is reflected in the inter-site, and also inter-annual, differences in elemental signatures of juvenile sardine.

Keywords : Microchemistry, Otoliths, Sardine, Stock structure, Upwelling

Introduction

In the inner ear of fish, there are three acellular, calcium carbonate ($CaCO_3$) structures known as otoliths (Tomás *et al.* 2004). Otoliths grow through the addition of material following a circadian rhythm that differs according to season, physiology of the fish, and environmental conditions (Jolivet *et al.* 2008). Although the majority (90-99%) of the otolith's matrix is $CaCO_3$, there are approximately 37 minor and trace elements that can be incorporated into the otolith during its growth, either as a substitute for calcium and/or a co-precipitate (e.g. magnesium, lithium, barium, strontium), or into the interstitial spaces of the crystal structure (e.g. sodium, chlorine, zinc, potassium) (Campana 1999, Thresher 1999, Tomás *et al.* 2004). Element uptake into the otolith matrix from surrounding water and food is a complex process involving transport of dissolved ions in the endolymph and over several membranes (Sturrock *et al.*, 2015; Thomas *et al.*, 2017). Therefore, otolith chemical composition is not a direct reflection of the composition of the surrounding waters and is also under physiological or genetic control. Nevertheless, several studies have shown that elemental incorporation in the otolith is influenced by pH, salinity, temperature and concentration gradients of trace elements (Mugiva and Tanaka, 1995; Campana 1999; Eldson and Gillanders 2002; Labonne *et al.*, 2009; Izzo *et al.* 2015). Thus, otoliths are useful natural tags of fish movement due to their continuous growth and metabolic inertness as well as the fact that incorporation of at least some elements is influenced by environmental conditions (Thomas *et al.*, 2017).

The elemental composition of otoliths is a useful tool for identifying geographic stock differentiation in fish that spend significant amounts of time in different environments. These chemical signatures provide insight into population structure, and to assess fish movement, including the dispersal of juvenile fish from their natal origin (Thresher 1999, Cook 2011; Tanner *et al.*, 2016; Avigliano *et al.* 2017).

The elemental signature of an otolith can also serve as a natural tag for stock differentiation studies (Campana 1999, 2005), should the population under study be distributed across environments that have sufficient spatial variation in water chemistry for this to be reflected in their otoliths. Turan (2006) was able to differentiate between Mediterranean horse mackerel, *Trachurus mediterraneus*, from the Black Sea and Aegean Sea using sodium, potassium, magnesium and barium concentrations in their otoliths, but found that fish from the eastern Mediterranean Sea and Marmara Sea (Turkey) were indistinguishable. Otolith microchemistry was used to distinguish among five groups of anchovy (*Engraulis encrasicolus*) in the Atlantic in one year, but the analyses failed to demonstrate differentiation the following year, suggesting that the differentiation is not temporally stable (Guidetti *et al.* 2013).

Along the South Africa coasts, sardine (*Sardinops sagax*) support a large pelagic fishery. The coastline is divided into three sections: the west coast (west of Cape Aghulhas), the south coast (east of Cape Agulhas) and the KwaZulu-Natal east coast. The west coast is characterised by a cool upwelling regime that is extremely productive during the austral summer when south-easterly, upwelling-driving winds prevail. The Agulhas Bank is a broad, irregular extension of the South African coastal plain that extends from Cape Point to East London and is bordered to the south by the Agulhas Current. Summer on the Agulhas Bank is characterised by easterly and westerly winds. The east wind results in subtropical surface waters becoming separated from a cool water ridge by a strong thermocline (Hutchings *et al.* 2002, 2009). The subsurface waters tend to flow east and the bottom current runs west, resulting in clockwise eddies forming and the local retention of fish eggs and nutrients. The summer thermocline breaks down in winter when westerly winds dominate and mixing occurs. The Agulhas Current retroflects on the edge of the continental shelf and flows back towards the central Indian Ocean (Hutchings *et al.* 2002, 2009). A strong jet current transports eggs and larvae that were spawned on the western Agulhas Bank to the west coast (Hutchings *et al.* 2002), although some are lost to filaments that extend off the shelf (Penven *et al.* 2001). The east coast is bordered by the warm waters of the Agulhas Current.

The elemental composition of otoliths has not been used previously in stock differentiation studies of marine fish in South Africa. In order for otolith microchemistry to be useful in stock differentiation studies, there needs to be temporally stable spatial variability in elemental composition in waters and among fish from different sites which could be found along the South Africa Coast.

Data on the otolith-edge microchemistry of juvenile sardine collected during 2 years around the South African coast will be described and used to test whether otolith microchemistry will be useful in testing the hypothesis of multiple stocks in South Africa (van der Lingen *et al.* 2006, 2010, 2015). Sardine are found throughout the South African coast and there is evidence of differences in meristics, morphology and parasite load that has led to the hypothesis of distinct stocks or functionally distinct adult assemblages on the west and south coast of South Africa, with a possible third stock on the east coast of South Africa that is characterised by the winter sardine run (de Moor and Butterworth 2013, Freon *et al.,* 2010). Although there has been evidence of spawning all along the South African coast throughout the year (Melo 1994), the bulk of spawning occurs on the

Agulhas Bank, away from the dispersive influence of the upwelling system, or on the west coast (Roel and Armstrong 1991, Penven *et al.* 2001). Eggs are generally found in water of temperatures between 15 – 21°C (Checkley *et al.* 2009). Hutchings *et al.* (2002) reported an eastward shift in the distribution of the bulk of spawning sardine. Spawning peaks in January with a second peak in September (Huggett *et al.* 1998).

Methods

Sample collection

Juvenile sardine were collected from inshore waters off the west, south and east coasts of South Africa. Sardine were collected from four midwater trawl samples taken during the 2008 pelagic recruit survey (RS) and from five samples taken during the 2009 RS conducted by the Department of Agriculture, Forestry and Fisheries (DAFF; see Barange *et al.* 1999). A tenth sample was collected from Warner Beach in KwaZulu-Natal in March 2009 using a beach-seine net (Figure 1, Table 1). Samples were frozen shortly after capture.

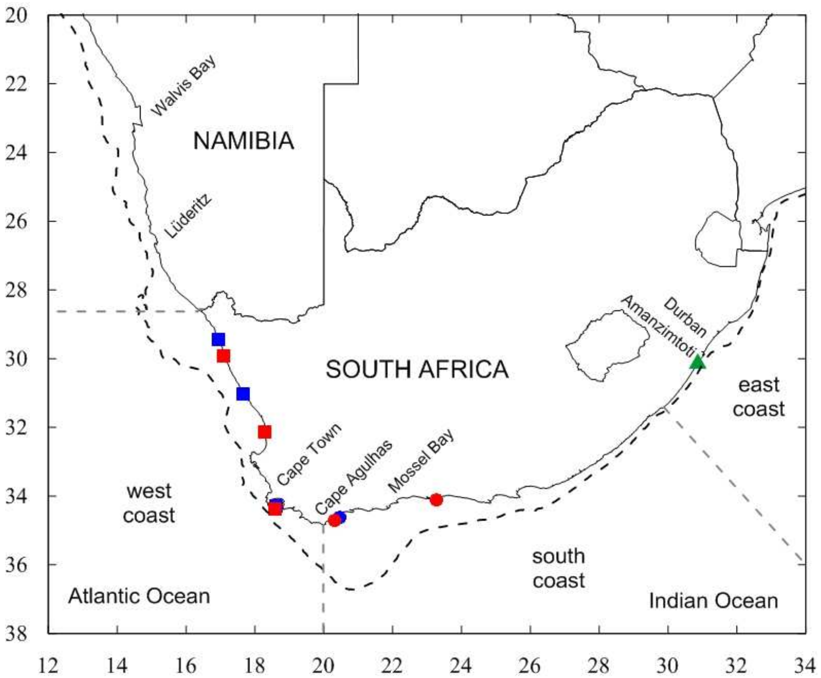


Figure 1: Approximate sampling site localities for juvenile sardine used in microchemistry analyses. West coast sites are shown as squares, south coast sites are shown as circles and the east coast site is shown as a green triangle; samples collected off the west and south coasts in 2008 are shown in blue whereas those collected during 2009 are shown in red. The 200m depth contour is shown by the dashed black line.

Table 1: Summary of samples of juvenile sardine collected in 2008 and 2009 for use in microchemistry analyses. The summary includes site code (summary of year, coast and sample number), number of individuals collected (N), average total length of fish (TL, mm) and standard deviation and the date of sample collection (Date). Sites are labelled in order from west to east within each region.

Site code	N	TL (sd) mm	Date
2008			
8WC1	10	79.23 (3.4)	23-May-08
8WC2	5	60.9 (5.1)	29-May-08
8WC3	9	80.6 (9.0)	07-June-08
8SC1	10	112.8 (8.9)	14-June-08
2009			
9WC1	9	69.8 (3.4)	18-May-09
9WC2	9	109.2 (2.7)	21-May09
9WC3	7	107.4 (8.8)	29-May-09
9SC1	8	103.2 (4.8)	04-June-09
9SC2	9	124.0 (8.7)	09-June-09
9EC	10	143.2 (10.2)	02-March-09

In the laboratory, fish were thawed and their total lengths (TL, to the nearest 1mm) recorded. Sampling was restricted to juvenile as they are supposed to move less than adults. The samples less than 136mm were classified as juveniles, less than one year, except for those from Warner Beach, where the maximum limit was 155mm and corresponded to one-year-old individuals (Thesis Malakia, 2015). This size limit aimed to restrict samples to juvenile fish since sardine length at 50% maturity has ranged between

170 and 191 mm caudal length (CL; approximately 190-211 mm TL) over the period 1953-2004 (van der Lingen *et al.* 2006).

Materials for otolith extraction, preparation, and analysis were decontaminated in 4% ultrapure nitric acid baths, rinsed with ultrapure water (18.2 MΩ) and dried under a Class 100 laminar flow hood (Fowler *et al.* 1995; Campana *et al.* 2000). Sagittal otoliths were removed with ceramic forceps, rinsed with ultrapure water and cleaned of tissue with a plastic toothbrush. They were sonicated for 5 min in ultrapure water and dried under the laminar flow hood.

Otolith microchemistry analysis

Samples were sent to the IRD laboratory in Brest, France, where chemical preparation and elemental analysis was conducted. Left otoliths were embedded in epoxy resin (Araldite 2020) and cut in a transverse plane including the core. Individual sections were glued to glass and the core was then exposed using sandpaper (4000 down to 500-grit) and its surface smoothed using a 1 µm polishing cloth (©Escil). A last sonication of 5 min and triple-rinsing with ultrapure water was performed for surface decontamination before drying and storing in dust-free conditions.

Trace elements were analysed using inductively-coupled plasma mass-spectroscopy (ICP-MS, X7 Thermo Electron ICP-MS coupled to a Cetac LSX-100 ultraviolet (UV) laser ablation) at the Pole Spectrometrie Ocean (IUEM, Plouzané, France).

A 60µm spot was analysed on the edge of the otoliths collected in 2008 and 2009, and this represented the average conditions in which fish were found in a short period prior to capture, approximately 20-30 days as the daily increments range between 3.08 and 1.9 µm in *Sardinops sagax* from South Africa (Waldron, 1998).

The laser conditions for the analyses were at 5Hz and 15Joules.cm^{-1}, and a gas blank was run between each otolith sample. After every 10 samples, a glass reference standard (NIST612, U.S. National Institute of Standards and Technology) was analysed and used to quantify and correct for mass bias and instrumental drift (Tournois et al, 2017, Martino *et al.*, 2017). At the beginning and conclusion of a run, a calcium carbonate standard (MACS-3: United States Geological Survey) was analysed as a measure of precision, with coefficient of variation (CVs) of elements being $< 5\%$ ($n = 18$).

Before laser analyses, a laser pre-ablation was used to clean the surface (spot 90, 4Hz, 15J). During acquisition, signal intensities were recorded for twenty elements but only lithium (^{7}Li), boron (^{11}B), magnesium (^{25}Mg), calcium (^{44}Ca), zinc (^{66}Zn), rubidium (^{85}Rb), strontium (^{88}Sr), barium (^{138}Ba), tin (^{118}Sn) and uranium (^{238}U) were above the detection limits, and these were not uniformly detectable across all sites and in both years. Ca was used as an internal standard to correct for laser beam energy drift, variation at the sample surface and trace elements results are expressed as ratio to Ca concentrations. In a group of samples, elements for which 50% of the measures were below LOD were removed from further analysis. When elements are presents in more than 50% of the samples but in some samples are below LOD, they are replaced by the average of their group for these samples.

Statistical analysis

All analyses were conducted on the log(x + 1) of the standardised values. Similarities in the elemental compositions of the otoliths for each of the data sets (2008 edge, 2009 edge) were analysed with principal component analysis (PCA), the advantages of which are explained by Agüera and Brophy (2011). Issues of colinearity are avoided because components are orthogonal to each other.

In order to disentangle the potential different fish stocks within the region, we investigated the difference in elemental concentrations of the otoliths from the different locations through an MANOVA with sampling sites and length of fish as potential predictors.

The Pillai trace test statistic from the MANOVAs was used to test significance; it is robust to violations of homogeneity of covariance and returns an approximate F value (Quinn and Keough 2002). Univariate ANOVAs and *post hoc* Tukey tests were used as procedures to investigate pairwise site differences of individual elements. The chemical elements were ordered by decreasing F value in the final MANOVA (Quinn and Keough 2002). Significance is set at $P < 0.05$ for all tests. Homogeneity of variances and normal distributions of residuals were tested visually with QQ plots, histograms and scatter plots of the residuals. Outliers were excluded once identified by Mahalanobi's Distance tests. All the above statistical analyses were done in R. The R script is available in Appendix 1.

Results

The concentrations of various elements in otoliths of juvenile sardine by year and site are shown in Figure 2. Elements were not at detectable limits in all sites and some were measured at much higher concentrations than others. For instance, magnesium and strontium were at higher concentrations than the rest of the elements (Figure 2). Only four elements were common to both 2008 and 2009, B, Mg, Sr and Ba. Sampling site and length of fish were predictor variables in the MANOVA but length of fish was not significant in any tests and was thus excluded from further analyses.

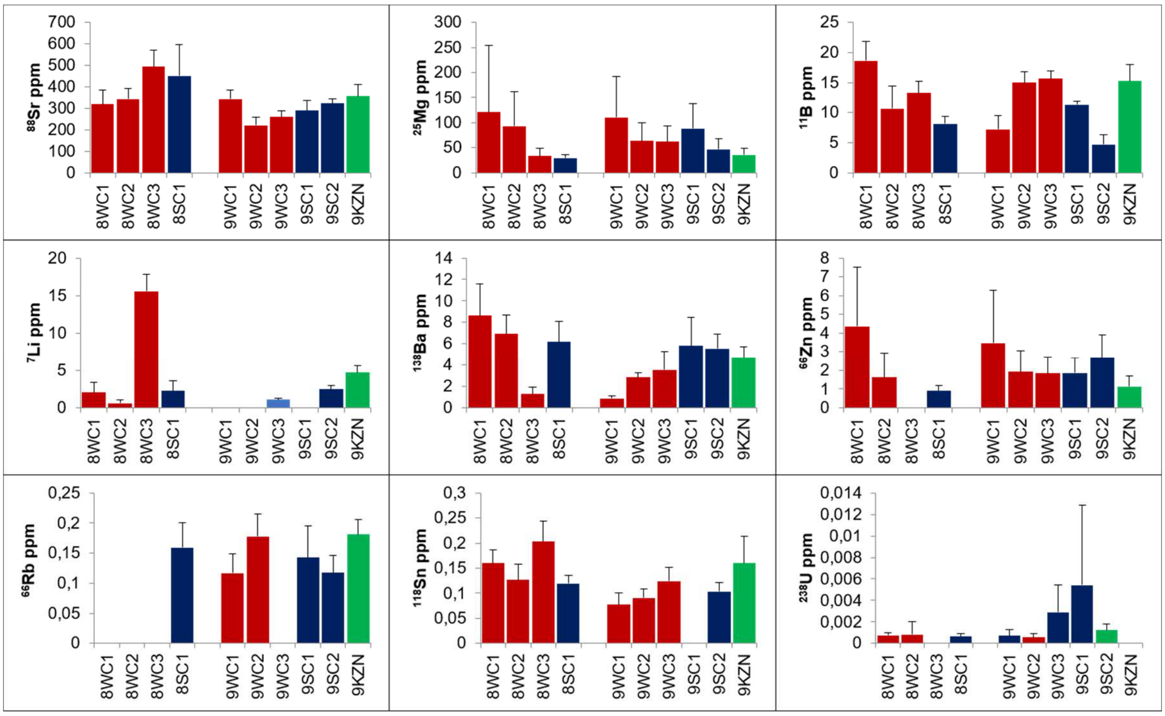
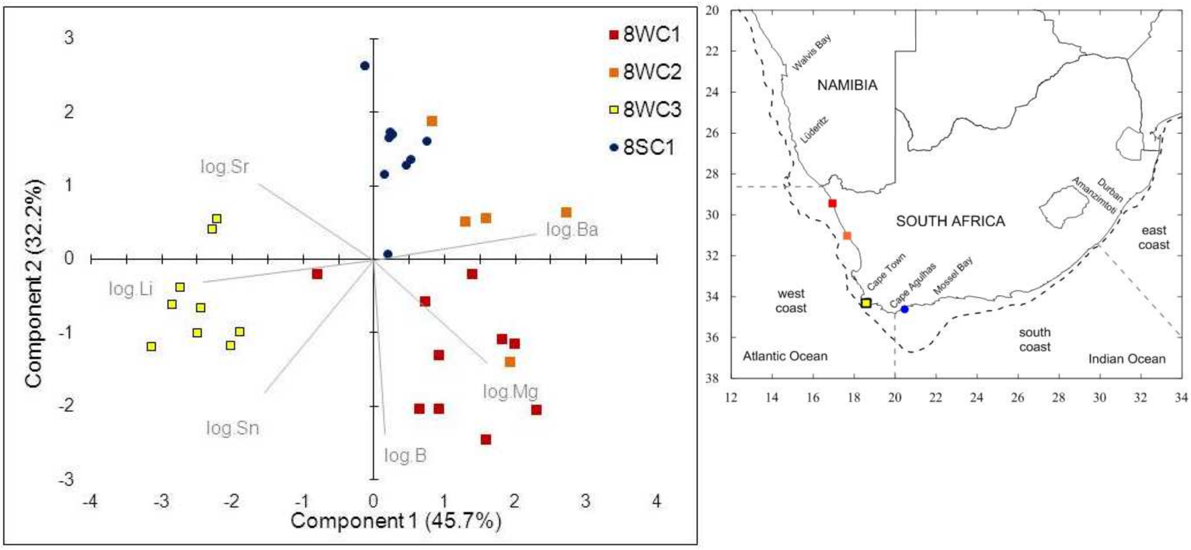


Figure 2: Mean (standard deviation) concentrations (ppm) of nine elements that were above detection level, although not always in all sites, in the otoliths of juvenile sardine off South Africa. The first four bars show concentrations from four sites in 2008 (N = 10, 5, 9, 10) and measurements from six 2009 (N = 9, 9, 7, 8, 9, 10) sites. West coast samples

are in red, south coast in blue and east coast in green. Note the different scales of the y axes.

2008 Otolith data

The first four principal components explained 95% of the variance and the first two 78% of the variance in elemental composition of the edge of otoliths from juvenile sardine collected in 2008. Lithium and barium contributed the most to principal component one, although in opposite directions, whereas boron and tin contributed most to the second principal component in the same direction. (Figure 3). The sampling site 8SC1 separated from the west coast sites along the second axis, and 8WC3 separates along the first axis. One of the five samples from site 8WC2 groups with those from 8WC1 (Figure 3). There is some differentiation between west and south coast sites evident from the second



principal component.

Figure 3: a) Component 1 and 2 of the PCA of chemical elements Li, B, Mg, Sr, Ba and Sn for four sites from 2008 otolith samples from the west coast (8WC, red, orange and

yellow squares) and south coast (8SC, navy circles). Grey lines represent the direction of the component loadings for each log transformed element. A map with sampling sites is shown alongside.

The MANOVA was significant (Pillai's test = 2.089, $F = 10.318$, $df_1 = 18$, $df_2 = 81$, $P < 0.05$) and sites varied significantly in concentrations for all the elements from the 2008 otoliths. Lithium and barium contributed most to the MANOVA (Table 2). Mean lithium concentrations were significantly different among all but one pair of sites: 8WC1 and 8SC1 (Figure 4). The individual elements do not provide much clarity on which sites differ, because elements showed different patterns of differentiation (Figure 4). However, concentrations of Mg, Ba and B appear to decline and Sr increase from the west to south coast. The site, 8WC3, differs from the other sites in Li and Ba concentrations (Figure 4) and was the only 2008 site to have insufficient information of Zn and U concentrations to be included in the analyses (Figure 2).

Table 2: MANOVA results for 2008 data, degrees of freedom (df), sums of squares (SS), F and P values are shown for each of the elements.

Element		**df**	**SS**	**F**	**P**
^{7}Li	Site	3	8.175	66.626	P < 0.05
	Residuals	30	1.227		
^{138}Ba	Site	3	4.121	62.166	P < 0.05
	Residuals	30	0.663		
^{11}B	Site	3	0.674	38.317	P < 0.05
	Residuals	30	0.176		
^{118}Sn	Site	3	0.286	14.612	P < 0.05

	Residuals	30	0.196		
^{88}Sr	Site	3	0.216	8.679	0.0003
	Residuals	30	0.249		
^{25}Mg	Site	3	1.346	5.227	0.005
	Residuals	30	2.575		

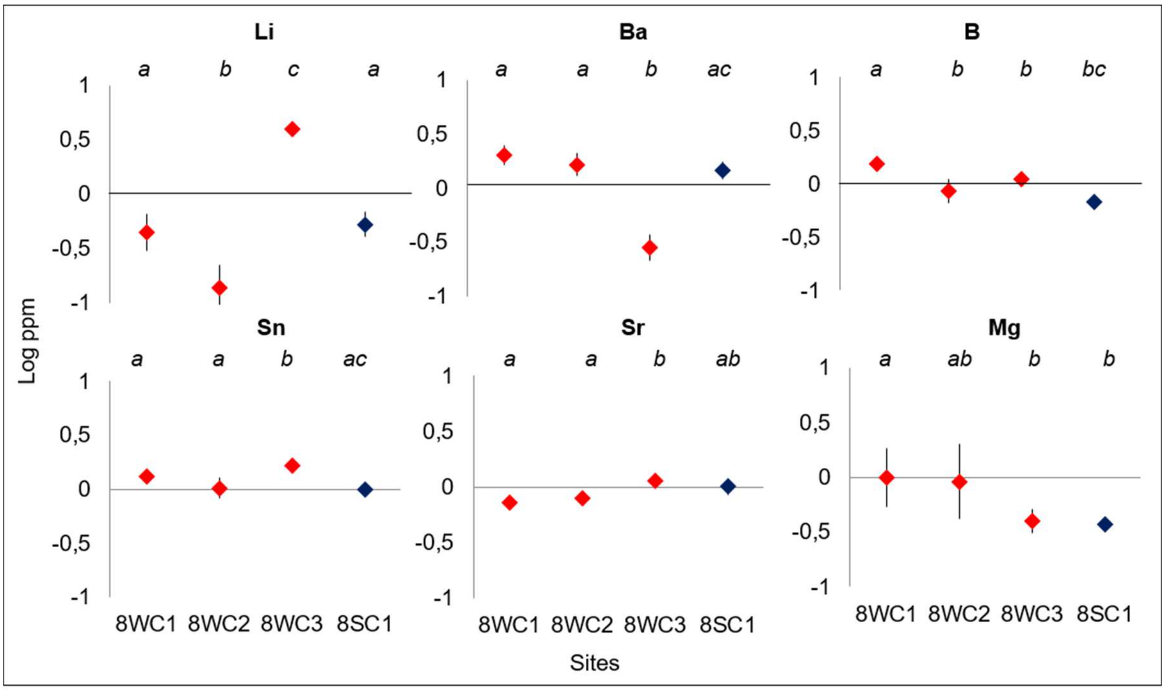


Figure 4: Log of standardised mean and confidence intervals of each element for sardine otoliths in each sampling site included in the 2008 MANOVA. *Post hoc* pairwise comparisons between sites for each element are represented with letters. When sites share a letter, (indicated above the graph) there is no significant difference between them.

2009 Otolith data

The first four principal components explained 93% of the variance and the first two 64% of the variance (Figure 5). The first principal component loadings were relatively evenly spread across four of the elements; magnesium and zinc contributed the most to principal component one (both positively), and barium and boron contributed to the first component in a negative direction. Strontium contributed most to the second principal component. The sampling site 9WC1 separates along the first axis and 9EC and 9SC2 separate along the second axis (Figure 5).

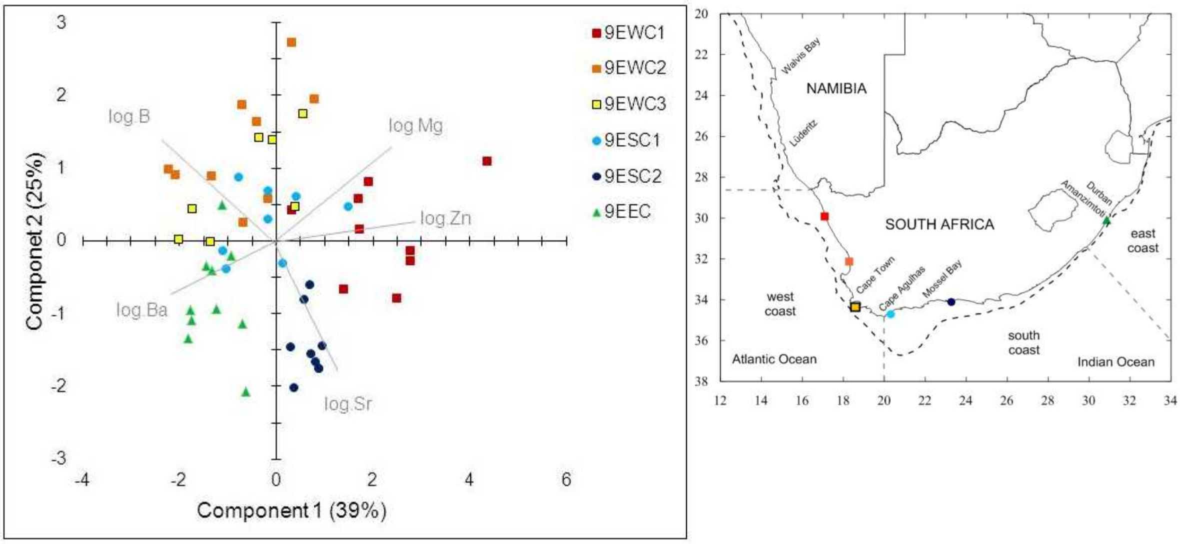


Figure 5: Axis 1 and 2 of the PCA of chemical elements B, Mg, Sr, Ba and Zn for six sites from 2009 otolith samples from the west coast (9WC, red, orange and yellow squares) and south coast (9SC, turquoise and navy circles) and east coast (9EC, green triangle). Grey lines represent the direction of the component loadings for each log transformed element. A map with sampling sites is shown alongside.

The MANOVA was significant (Pillai's test = 2.517, F = 9.326, df_1 = 25, df_2 = 230, P< 0.05) and sites varied significantly in all of the elements from the 2009 otolith edges. Boron and barium contributed most to the MANOVA (Table 3). Once again, individual elements did not show the same patterns for how sites differed from each other, although

Mg concentrations seem to decline from west to east while Ba concentrations appear to increase (Figure 6).

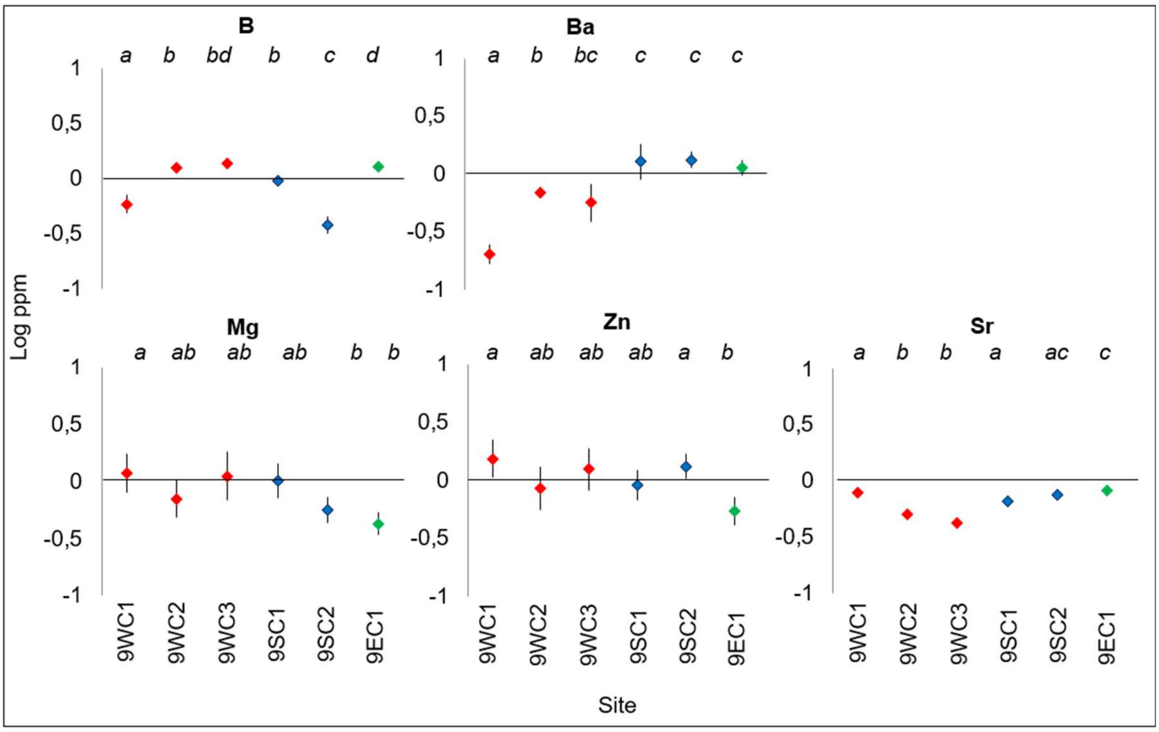


Figure 6: Log standardised mean and standard deviations for each sampling site of each element included in the 2009 MANOVA. *Post hoc* pairwise comparisons between sites for each element are represented with letters. When sites share a letter, (indicated above each graph) there is no significant difference between them. Sites are ordered from west to east coast.

Table 3: MANOVA results for 2009 data, degrees of freedom (df), sums of squares (SS), F statistic and P values are shown for each of the elements

Element		**df**	**SS**	**F**	**P**
^{11}B	Site	5	2.188	59.341	P < 0.05
	Residuals	46	0.339		

^{138}Ba	Site	5	4.197	37.593	P < 0.05
	Residuals	46	1.027		
^{88}Sr	Site	5	0.284	17.435	P < 0.05
	Residuals	46	0.150		
^{25}Mg	Site	5	1.235	4.820	0.001
	Residuals	46	2.357		
^{66}Zn	Site	5	1.193	4.657	0.002
	Residuals	46	2.357		

Discussion

The South African coastline is subject to both temporal and spatial variability in oceanographic conditions which manifests in small scale variability embedded in large regional differences in coastal oceanography. This environmental instability means that species that have extensive distributions in coastal waters, such as small pelagic fish, experience a range of environmental conditions across their habitat. By comparing groups of individuals from different geographical regions, it can be seen whether the groups of fish have experienced different environmental conditions (Campana 1999).

In this study, the otolith elemental chemistry results showed evidence of small scale differences among fish collected at different sites (particularly in samples collected in 2008), and some large regional patterns of differentiation between the west, south and east coasts. In particular, certain elements appear to increase (Ba, Sn) or decrease (Mg) from west to east. Multivariate statistics that incorporate all elements were more useful at showing differences between sites than individual elements, which showed conflicting patterns of differentiation. Boron was the element that contributed most to the MANOVA results from the 2009 edge, and the third most important (after lithium and barium) contributor to the 2008 MANOVA results, the pairwise comparisons of which showed the most conclusive differentiation among sites.

Upwelled waters are known to be enriched in trace elements (e.g. Ba, Cu, Cd), which can then be incorporated into calcified structures such as corals (e.g. Lea *et al.,* 1989) and, otoliths (Clarke *et al*., 2007). Due to biological uptake, particle sinking, and then remineralization, Ba has a 'nutrient like' profile with concentrations which can be three times higher in deep waters than in surface waters in the Pacific Ocean (Lea *et al.*, 1989; Nozaki, 2001; Esser & Volpe, 2002).

The high concentrations found in 2008 samples from the northern part of west coast could be a signature of upwelling, as other studies show it (Kingsford *et al.*, 2009, Wheeler *et al.*, 2016). Upwelling events caused by seasonal winds (Eckman transport) cause Ba-rich deep waters to rise along the west coast. These high concentrations are not visible in 2009 samples perhaps due to a weaken upwelling during the period before fish caught.

The pattern found in this study, of small scale differences in one year, but not the next, is similar to what was found in European anchovy, in the Ligurian Sea, where it was suggested that elemental differentiation occurred in some cohorts but not others (Guidetti *et al.* 2013). D'Avignon and Rose (2013) suggest that, where signatures differ among years, a long-term study is required to determine temporal stability and, thus, whether the elemental signatures can be used as natural tags of stock differentiation.

Inter-annual variability in elemental composition could represent differences in environmental conditions experienced by different cohorts at the time of spawning, as hypothesised for reef fish (Cook 2011). It is unlikely that, in a dynamic environment such as the southern Benguela, elemental condition will be stable over long periods of time. The temporal instability and small-scale variability found in the element signatures of this study suggest that local retention of sardine in areas in some years, but not others, could be responsible for the elemental signatures found in South African sardine.

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Appendix 1

```
#Adapted from code from various sources.

setwd("F:/microchem")
edge2008 = read.csv("2008test.csv", header=T) #T=true, take headers as headers

summary(edge2008) #check
names(edge2008) #check
attach(edge2008)

#Viewing the data
#e.g.
plot(Site, logLi, xlab= "Site", ylab="Li")

#subset the data to include elements present in all samples
data <- cbind(logLi, logB, logMg, logSr, logBa, logSn)
#data in order of descending F values
```

```
datanew <- cbind(logLi, logBa, logB, logSn, logSr, logMg)

#add column names
colnames(data) <- c("log.Li", "log.B", "log.Mg", "log.Sr", "log.Ba", "log.Sn")
colnames(datanew) <- c("log.Li", "log.Ba", "log.B", "log.Sn", "log.Sr", "log.Mg")

#pairwise scatterplots to check for correlations
pairs(data)

#Principal components Analysis
arc.pcal <- princomp(data, scores=TRUE, cor=TRUE)
summary(arc.pcal)
plot(arc.pcal)
biplot(arc.pcal)
arc.pcal$scores
arc.pcal$loadings

require(FactoMineR)
result <- PCA(data)

#means model predicting colour and data shape PCA with sites
arc.pcal <- princomp(data, scores=TRUE, cor=TRUE)
summary(arc.pcal)
plot(arc.pcal)

clus = kmeans(arc.pcal$scores[,1:2], centers=5)
key = data.frame(Site=edge2008$Site,
                 shape=as.numeric(edge2008$Site),
                 color=clus$cluster)
plot(arc.pcal$scores[,1:2],
     col=clus$cluster,
     pch=14+as.numeric(edge2008$Site))
```

```
#MANOVA Site
MANOVAS <- manova(datanew ~ Site)
summary(MANOVAS, test="Pillai")
summary(MANOVAS, test="Wilks")
summary(MANOVAS, test="Hotelling-Lawley")
summary.aov(MANOVAS)

#testing assumptions - identify multivariate outliers by plotting the ordered squared robust Mahalanobis distances of observations against the empirical distributional function of the MD
#testing assumptions
require(mvoutlier)

#testing univariate assumptions
#e.g.
par(mfrow=c(3,2))
qqnorm(logLi, ylab="Li")
qqline(logLi)

#testing homogeneity of variance
#e.g.:
bartlett.test(logLi~Site)
#e.g.
fligner.test(logLi~Site)

#testing multivariate normality
require(mvnormtest)

#graphically testing normality QQ plot
par(mfrow=c(1.1))
x <- as.matrix(data)
center <- colMeans(x)
n <- nrow(x)
```

```
p <- ncol(x)
cov <- cov(x)
d <- mahalanobis(x,center,cov)
qqplot(qchisq(ppoints(n), df=p), d, main="QQ Plot assessing Multivariate Normality",
ylab = "Mahalanobis D2")
abline(a=0, b=1)
```