

# Elemental Fingerprints of Sparus Aurata Otoliths – Insight Into the Diversity of Nursery Habitats

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## Research Article

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## Abstract

This study aims to determine if otolith chemistry can differentiate between coastal lagoons and shallow coves and if it can effectively re-assign *Sparus aurata* specimens to the nearest nurseries. Young-of-the-year (Y-O-Y) and young adults were collected at fifteen sites within three nurseries along the eastern Adriatic. LA-ICP-MS was used to quantify the concentrations of 12 chemical elements in the otolith region corresponding to the juvenile nursery stage. The multivariate element concentration differed significantly among nurseries. Based on CAP analyses, using a suite of five trace elements (Sr, Mg, Zn, Ba and Pb), 41% *S. aurata* specimens were correctly re-allocated to the nurseries and a higher rate of success (46%) was achieved for shallow cove than for coastal lagoons. A separate CAP analysis explained 94% of element variance, with 100% discrimination for Sr:Ca, Mg:Ca and Pb:Ca, enabling re-allocation to shallow cove nurseries. The lowest success of Ba as a discriminant reduced re-allocation to shallow coves. The results suggested that number of shallow coves with continuous, submarine, freshwater springs along the coast, making them similar to coastal lagoons, could significantly contribute to the *S. aurata* recruitment, expanding attention from the protection of individual nursery towards a wider part of the coast.

## Introduction

Coastal habitats are among the most productive environments, as they receive nutrients and energy from both fresh and marine waters and can support a variety of life<sup>1,2</sup>. Heterogeneous coastal habitats can be colonized by a high diversity of fish species and it is particularly important to understand their nursery role in the overall metapopulation function<sup>2,3</sup>. Extensive work has been done on the importance of estuaries<sup>4-7</sup> and coastal lagoons<sup>3,8-12</sup> as nursery grounds for juvenile fish, highlighting that these transitional ecosystems may offer favourable developmental conditions with high primary productivity, lower salinity and some protection from predation. Non-estuarine habitats, including surf zones of sandy beaches<sup>13,14</sup>, and inshore shallow areas such as gulfs and shallow bays, have also been recognized as important fish feeding and nursery grounds<sup>15-18</sup>. However, shallow, sheltered, non-estuarine habitats have received less attention than estuaries and coastal lagoons for their role as nurseries for fishes<sup>2,19-21</sup>.

The gilthead seabream, *Sparus aurata* is a commercially important fish species in the Mediterranean Sea<sup>22</sup> both for the aquaculture and fisheries sectors<sup>23,24</sup>. However, although it is one of the most studied sparid species, its population characteristics and environmentally conditioned behaviour throughout the distribution range are still not clearly understood. It is reported that *S. aurata* performs ontogenetic and trophic migrations between coastal habitats, mostly lagoons, and the open sea<sup>3,25,26</sup>, that are associated with spawning<sup>27,28</sup>, and settlement and recruitment<sup>29-32</sup>. Juveniles colonise sheltered coastal areas in early spring to feed and grow during their critical first summer of life before migrating out to open sea in autumn<sup>22,25-27,33</sup>. There is evidence that the majority of the *S. aurata* adult populations originate from relatively small, shallow, brackish lagoons<sup>10,25-27,34</sup>, though the exact role of the various coastal habitats in local *S. aurata* metapopulation functions remains unclear<sup>3</sup>.

Over the last three decades, otolith microchemistry has been used extensively to study fish life cycles and identify their key lifetime habitats<sup>35</sup>, as otoliths provide great possibilities to record the passage of time and environmental conditions<sup>35-38</sup>. Otolith microchemistry can be used to estimate population structure<sup>39</sup> and discriminate between types of coastal nursery habitats<sup>12,26,40-42</sup>. Fish otolith chemistry reflects both local environmental chemistry and individual physiology, creating a unique specific signature that can be used as a natural tag<sup>35,37</sup> to distinguish location and infer ontogenetic change<sup>43,44</sup>. For *S. aurata*, the quality of lagoons as habitats for juveniles has been tested<sup>3,12,26</sup> and the contribution of Pb potentially contaminated nurseries to *S. aurata* stock dynamics<sup>45</sup> was evaluated. Šegvić-Bubić et al.<sup>46</sup> found that scale microchemistry yielded good resolution in identifying wild, farmed and wild farm-associated *S. aurata* among different habitats. There is good evidence that Mediterranean lagoons leave fingerprints on the otoliths of juveniles<sup>26</sup>, though it is still not clear whether these fingerprints are stable over time and therefore applicable for identifying nursery origin of adults<sup>12</sup>.

The condition and growth of juveniles in nurseries are expected to influence their recruitment success to the adult population<sup>47,48</sup>. Therefore, it is important to evaluate whether adults captured offshore have fingerprints indicating which coastal habitat they occupied as a nursery. In this study, we investigate the differences in otolith chemical composition among juveniles and young adults of *S. aurata* at different coastal habitats within three nurseries along the eastern Adriatic coast. We tested whether otolith elemental fingerprints of young adults could enable their proper allocation to their origin nursery. This knowledge can help to accurately identify the significance of coastal habitats colonized by juveniles, and to identify the nursery grounds among them that contribute to the maintenance of adult stocks.

## Results

**Comparison of nursery areas.** Otolith elemental concentrations were significantly different between the 15 sites for Sr:Ca (Pseudo-F=1.840; P = 0.001) and Mg:Ca (Pseudo-F=1.617; P = 0.018), while no significant differences were found for Zn:Ca (Pseudo-F=1.347; P = 0.059), Pb:Ca (Pseudo-F=0.914; P = 0.602) or Ba:Ca (Pseudo-F=1.259; P = 0.087). As expected, the otolith elemental concentrations significantly differed among the sites (Pseudo-F=1.367; P=0.003) where Y-O-Y specimens were sampled (pair-wise test S1:S5, t = 1.31, P=0.017; S1:S10, t = 1.44, P = 0.019; S5:S10, t = 1.37, P=0.036). S1 and S5 also statistically differed in otolith elemental concentrations from almost all other sites, particularly those geographically distant from the coast (Table S1). However, S10 was significantly different in its elemental signature only with S1 and S5. There was no difference between sampling sites located proximal to S1, S5 and S10, situated near the coast, regardless of their affiliated nursery area (Table S1). In particular, the site S5 in N2 had reduced concentrations of Sr:Ca and Ba:Ca, while site S12 in the open sea and associated with N3 had elevated Sr:Ca, but the lowest values of Mg:Ca of all sites. Zn:Ca and Pb:Ca concentrations were greatest at S1 and S4 (both N1). Reduced values of Pb:Ca were recorded at the open sea site S7 (associated with N2) (Fig. 1). At the nursery level, significant differences were found for two pair-wise relations: between N1 and N3 for Zn:Ca (t=1.887; P=0.003), and between N2 and N3 for Mg:Ca (t=1.896; P = 0.018), while no significant differences were obtained between N1 and N2 for any ratio.

**Re-allocation to nursery habitats.** When element data were combined into two single matrixes, one for Y-O-Y specimens and one for young adults, forming the specific fingerprint of each individual, PERMANOVA analysis detected that both Y-O-Y (Pseudo-F=0.6624; P=0.812) and young adults (Pseudo-F=0.6821; P=0.759) did not differ significantly in their elemental signatures between “Nurseries”, although a difference was observed between nurseries and elements for young adults (Fig. 2). Since the Zn:Ca ratio was highly scattered, independent of nursery allocation (Fig. 2a), it is excluded to get more clear allocation of other elements. Thus on Fig. 2b is more clearly visible that Ba:Ca ratio was also scattered. Sr:Ca was clearly grouped for all three nurseries, while Mg:Ca and Pb:Ca were separated with a somewhat greater overlap between N1 and N2.

The incorporated concentration of each element per specimen in relation to their age, with the exception of Pb:Ca (Pseudo-F=1.48, P=0.125), was significantly different between Y-O-Y specimens and young adults (Table 1). Moreover, Mg:Ca, Sr:Ca and Zn:Ca were responsible for significant differences observed between nurseries. Finally, a strong significant difference in incorporated Sr:Ca between Y-O-Y specimens and young adults was obtained in each nursery (Pseudo-F=3.09, P=0.007).

Finally, we ran multivariate analyses considering data of all analysed elements as single individual fingerprint. The overall results for such multivariate analysis showed significant differences between Y-O-Y specimens and young adults (Pseudo-F=5.349; P=0.0001), nurseries (Pseudo-F = 1.694; P=0.027) and elemental fingerprints (Pseudo-F=38.13; P=0.0001) (Table 2; Fig. 3). The Zn:Ca concentrations were very scattered, though Y-O-Y specimens and young adults were clearly separated (Fig. 3a), this was followed by the Ba:Ca and Sr:Ca concentrations (Fig. 3b). Young adults were positioned on the left side of the mMDS due to higher concentrations of Zn:Ca, Ba:Ca and Sr:Ca, respectively.

Approximately four of ten (41.3%, n = 99/240) *S. aurata* were correctly allocated to the nursery area in which they were caught (Fig. 4; Table S2). More than 75% percent of the discriminating power from the CAP was captured in the first two canonical variates. The highest rates of success were achieved for N3 (45.6%), closely followed by N2 (42.5%), and were lowest in N1 (34.3%). The differences in trace-element signatures across different nursery areas were significant (Pseudo-F=1.606; P=0.009; Table 2) and these differences were not driven by different dispersions (PERMDISP; p=0.43, F=1.335).

A separate CAP analysis for the factor “Elements” generally gave high discrimination (CAP1 and CAP2 together explained 93.8% of the component variances captured in CAP), particularly for Sr:Ca (100%), Mg:Ca (100%) and Pb:Ca (100%), follow by Zn:Ca (93.75 %). The lowest rate of success was achieved for Ba:Ca concentrations (75.0%) (Fig. 5; Fig 6.).

The differences in trace-element signatures between Y-O-Y specimens and young adults were significant (PERMANOVA Pseudo-F = 5.349; P = 0.0001; Table 2) and these differences were not driven by different dispersions (PERMDISP; p = 0.253, F = 2.188). A separate CAP analysis for the factor “Age” gave better discrimination for young adults (86.86%) than Y-O-Y specimens (50.77%). However, CAP analysis conducted across each nursery area revealed that young adults were allocated to a specific nursery based on the high correctness of Sr:Ca (93.3–100.0%), Mg:Ca (88.9–100.0%) and Pb:Ca (88.9–100.0%). The correctness of Zn:Ca was around 70.0%, while Ba:Ca had the lowest percentage discriminating power, particularly at N1 (44.0%). Re-allocation of specimens to N1 and N2 was defined by five groups ordered along the CAP1 axis as follows: Sr>Ba>Mg>Pb>Zn (Fig. 6), while allocation to N3 was conducting with the opposite ordering: Zn>Pb>Mg>Ba>Sr (Fig. 6).

## Discussion

The results of this study demonstrated that the chemical composition of *Sparus aurata* otoliths have potential to differentiate between nursery areas, and between Y-O-Y specimens and young adults in the eastern Adriatic. Concentrations of five analysed elements (Mg, Ba, Sr, Zn and Pb) were above the detection and quantification limits and varied between sites. The most discriminant elements were Sr and Mg, while Zn and Pb spatial variations were visible but were not statistically significant for Pb. The concentration of Ba did not reveal any specific patterns.

**Comparisons of nursery areas.** Testing for natal homing is very challenging due to the difficulty in tracking all life stages<sup>44</sup>. Generally, otolith chemistry is affected by water chemistry<sup>35,49-51</sup>, and it has proved useful in examining movements among life stages in regions where there is detectable spatial variation<sup>37</sup>. Considering *Sparus aurata*, previous studies showed that juveniles occupy seasonally contiguous lagoons<sup>10,25,27,32,34</sup>, while adults inhabited open sea waters<sup>27,28,32</sup>. In this study, the most distinctive elemental signature was related to low salinity sites near the coast where Y-O-Y specimens were sampled. As expected, the chemical signatures of these three sites differed significantly, which can possibly be attributed to their specific relief features (fluvial or karstic relief), freshwater inflow (continuous or sporadic), water quality and/or surroundings (urban or sparsely populated area). However, the majority of sampling sites for young adults did not differ significantly, implying that the middle Adriatic coast generally provides uniform living conditions for *S. aurata* recruits. Additionally, sites situated far offshore exhibited certain specificities that resulted in significant differences from nursery sites, likely due to the higher and more stable hydrographic conditions and high salinity (>37 ppt) at these offshore sites.

In this study, Mg:Ca and Sr:Ca concentrations were significantly different between the analysed sites. Otolith Mg concentrations had elevated values in the coastal lagoon (S5), while the lowest values were recorded at an open-sea site (S12). Interesting, the opposite was found for Sr:Ca, where the lowest values were recorded at S5 and the maximum at S12. However, certain caution is needed when relating these values to environmental conditions. Higher values of Mg uptake into otoliths may more likely be related to endogenous processes<sup>52-54</sup>, and as such Mg is not a reliable environmental indicator<sup>55</sup>. Previously, Vrdoljak et al.<sup>45</sup> detected elevated but individually specific concentrations of the Mg:Ca ratio for juveniles of *S. aurata* from the Pantan lagoon (S5), suggesting that Mg incorporation is likely affected by an individual physiological mechanism. Two other elements analysed here, Sr and Ba, were previously successful in reconstructing environmental and coastal-estuary migrations<sup>56,57</sup>. Their ratios in otoliths are usually linked to concentrations of Sr:Ca and Ba:Ca in ambient water and salinity, with a higher Sr:Ca found in marine, and higher Ba:Ca found in freshwater<sup>58,59</sup>. Gillanders and Kingsford<sup>60</sup> reported higher Sr concentrations in otoliths of recruits from coastal reefs in comparison with those from estuarine seagrass habitats. However, no specific patterns of Ba incorporation were found in relation to salinity. Additionally, Zn:Ca and Pb:Ca concentrations were highest at the coastal lagoon Mala Neretva (S1) and a

proximal site (S4), while the most reduced values of these elements were recorded at open-sea sites (S7). Besides known fact about natural origin of Zn<sup>61</sup> and its individual physiological regulation<sup>62</sup>, the obtained elevated values of Zn can be also associated with anthropogenic pollution due to intensive marine traffic<sup>63,64</sup>. In addition, the highest Pb concentrations were found near known contamination sources<sup>45,60,65,66</sup>, i.e., at the Mala Neretva coastal lagoon (S1), though without prominent incorporation in specimen otoliths that would demonstrate a clear relationship with environmental contamination levels<sup>45</sup>.

**Re-allocation to nursery habitats.** The accurate discrimination by multivariate analyses indicated that *S. aurata* specimens originating from specific geographic areas in the Adriatic Sea have distinct elemental signatures that potentially enable their re-allocation to specific nursery habitats along the coast. Multivariate analyses have become a desirable tool for such purposes, since they are capable of separating different perturbations from natural spatio-temporal variability displayed by most populations<sup>67</sup> with an analytical procedure<sup>52,49,58</sup>. However, the lower rate of success (35–45%) achieved in differentiating between the analysed nursery areas may be driven by two potential mechanisms. The first by the intrinsic, physiological mechanisms of each individual that determine the supply of trace elements to the inner-ear and their subsequent incorporation into the otolith during its growth, and the second by differences in environmental exposure<sup>68</sup>. Using mixture models to estimate each nursery habitat contribution, Niklitschek and Darnaude<sup>69</sup> found that these results reflect the large interannual variability in nursery habitats<sup>12</sup>, which may be common to the shallowest water and estuarine nursery areas<sup>70</sup>, thereby making it difficult to find a clear pattern of overlap between different localities or ontogenetic groups. Since this is experimental study, no doubt the rate of success will increase with larger sample size.

The difference in element uptake by individual otoliths or intrinsic, physiological mechanisms, was most evident for Zn, which varied greatly in value from individual to individual but was clearly separated between the 0<sup>+</sup> and 2<sup>+</sup> aged specimens, with reduced values in young adults. That fact was previously proposed by Avigliano et al.<sup>71</sup> for the differentiation of cohorts, providing temporally constrained information on habitat, behaviour or nutrient supply for Y-O-Y specimens and young adults. Additionally, since cohorts occupy specific space-time frames, it is necessary to avoid mixing different cohorts in multivariate analyses due to the reliability of obtained results. On the other hand, the importance of environmental exposure is evident in the incorporation of Ba:Ca in *S. aurata* otoliths. This ratio gave the lowest discriminating power, which may suggest that the impact of freshwater inflow either from rivers or continuous or sporadic freshwater grounds along the eastern Adriatic coast is so strong that it prevents a clear salinity boundary between estuarine and marine waters. Therefore, all specimens caught in the sampling area exhibit Ba concentrations without any regular pattern, regardless of whether they were sampled in coastal lagoons, shallow coves or offshore. Therefore, the lowest percentage of Ba as a discriminant can also be considered to reduce the overall success of re-allocation, further highlighting that much of the marine waters along the middle Adriatic coast have lower than expected salinity. As mentioned above, there are a number of shallow, semi-protected bays with continuous submarine freshwater springs in the Adriatic Sea. These are karst phenomena created by geomorphological processes under the influence of recent sea-level changes<sup>72</sup>. Like coastal lagoons, these bays also comprise a diverse mosaic of environments with different physiochemical characteristics<sup>2,9</sup>.

The obtained results revealed that the most specimens were correctly re-allocated to nursery area N3, which lacks specific coastal lagoons as potential nursery sites for *S. aurata*. This is a broad coastal area in the middle Adriatic with a number of coves that could be potential nursery sites, followed by two analysed coastal lagoons previously identified as nursery grounds for this species<sup>16,45,73</sup>. Namely, Mala Neretva and Pantan are small, shallow, brackish coastal lagoons that represent sites with obvious, optimal conditions required by this species. The specimens sampled within N3 were most correctly re-allocated (45.6%) based on their Mg:Ca, Sr:Ca and Pb:Ca ratios. Moreover, these elements were allocated with 100% accuracy. Contrary to what we expected, Ba had the lowest, but expressed percentage of correct allocation (75%). The shallow cove Prosika, although not a typical coastal lagoon, thus proved to have a role equivalent to a nursery. This is a shallow, semi-closed cove with an annual influence of freshwater springs, which is highest in the winter-spring period after snow melt and intensive rain<sup>74-76</sup>. These coves were previously identified as having higher fish species richness<sup>16</sup> than coastal lagoons, due to the wide connection with the sea that favours the entrance of fish eggs, larvae and young of-the-year by tides and currents from spawning areas<sup>70,77-79</sup>. Meanwhile, the entrance of marine fish eggs and larvae in coastal lagoons is limited because of the narrow sea connection, and therefore lagoons may suffer from severe conditions depending on precipitation and evaporation<sup>80,81</sup>. We do not claim that all young adults of *S. aurata* from nursery area N3 originated from a unique cove as a nursery habitat (e.g., S10). Moreover, we suggest that S10 is representative of a number of similar localities along the 120 km stretch of Adriatic coastline<sup>82</sup> sheltered by numerous islands that can serve as suitable nursery habitats for *S. aurata*. Morais et al.<sup>32</sup> suggested that *S. aurata* larvae displayed a cautionary behavioural strategy, relying on feeding plasticity and batch-spawning to compensate for the lack of apparent behavioural plasticity, to ensure metapopulation stability and resilience. This is reflected in the alterations in nursery selection from coastal lagoons, with reduced quality as habitats for juvenile fish due changed environmental conditions<sup>34,83,84</sup> and significant increase in eutrophication and anoxic crises<sup>85,86</sup> to other coastal habitats that could ensure the conservation of *S. aurata* local populations. The lowest correctness of re-allocation was associated with N1, due to the lowest correctness of all element allocation in young adults in comparison with specimens from N2 and N3. A possible explanation is that in the highly variable environment of the Mala Neretva coastal lagoon, individual physiological mechanisms prevail over environmental mechanisms due different diurnal movements, environmental preferences/tolerances, and prey-choice/availability across the habitat mosaic<sup>87,88</sup>.

Generally, the loss of biodiversity, ecosystem functions and vegetation in estuarine and coastal ecosystems highlight the need to better understand the environmental drivers in coastal habitats<sup>1,89</sup>. In this sense, conservation measures to maintain sustainable stock levels must be aimed not only at protecting coastal lagoons but at all essential habitats or even a part of the coast<sup>26</sup>. Mediterranean coasts have been significantly affected by anthropogenic destruction over the past 50 years. Matić-Skoko et al.<sup>90</sup> suggested that significantly modified juvenile fish communities in nurseries may be due more to constant human embankment and marine infrastructure construction along the coast in recent decades, than to climate change or fishing pressures, as generally considered. This indicates the necessity for accurate knowledge of fish lifetime movements and critical habitats<sup>91,92</sup>, and proper estimations of the contribution of each habitat to adult populations<sup>3</sup>.

## Conclusions

At the juvenile stage, a preference of *S. aurata* for shallow, coastal, low-salinity lagoons, like Pantan and Mala Neretva, was clearly seen and confirmed in the present study. However, though it has long been thought that these habitats are essential for the maintenance of *Sparus aurata* stocks, it seems that a whole range of complex shallow coves, such as Prosika, with continuous, submarine, freshwater springs along the eastern Adriatic coast, are equally important sources of recruits. These marine coves significantly contribute to recruitment, thus greatly ensuring the maintenance of adult *S. aurata* populations. Therefore, the importance of such habitats and the need to consider all coastal habitats as essential and sensitive in general are highlighted.

## Material And Methods

**Study sites and fish collection.** A total of 48 *Sparus aurata* specimens were collected at fifteen sites along the eastern Adriatic coast in May-June 2018 (Fig. 7; Table 3). Young-of-the-year (Y-O-Y) specimens were sampled at S1 (Mala Neretva), S5 (Pantan) and S10 (Prosika) located from Ploče to Zadar along the eastern Adriatic coast (Fig 7). These three sites were previously identified as essential nursery areas for *S. aurata* in the Adriatic<sup>73,93,94</sup>, and were the starting point for defining the three nursery areas N1, N2 and N3. The affiliation of the remaining twelve sites to a specific nursery area were determined according to their geographic position and prominent relief barrier (Ploče, Split, Šibenik-Zadar, respectively). Two sites, S1 situated in the estuary of the Neretva River, and S5 situated in the estuary of the Pantan River, receive fresh waters of nearby rivers and exhibit variable salinity gradients throughout the year<sup>73,95</sup> with a muddy-sandy bottom partially overgrown with *Zostera marina*. The third site, S10 is near Šibenik, and is characterized by a number of groundwater springs and indirectly influenced by the Krka River, with medium salinity<sup>16</sup>, a partially rocky-sandy bed with patches of *Cymodocea nodosa* and photophilic algae meadows. Other sites were classified based on salinity and closure according to obtained measurement (YSI 85) and expert knowledge.

In total, 13 Y-O-Y specimens of *S. aurata* specimens were collected using a specially constructed small shore seine (L=25 m; mesh size 4 mm). Collection of 35 young adults (2<sup>+</sup>–3<sup>+</sup>), of similar length and weight (Table 1), were aged based on previously established length at age relationships<sup>96</sup>. Specimens were collected using gillnets in collaboration with local professional fishermen. For the analysis, total length (TL) and weight (TW) were recorded and specimens were dissected to extract otoliths for chemical analysis.

**Sample preparation.** All fish were frozen until dissection in the laboratory. Prior to processing, the fish were defrosted. Both sagittal otoliths (hereafter: otoliths) were removed, rinsed with water, cleaned of soft tissue with plastic dissecting pins, washed with Milli-Q water, air-dried and stored in decontaminated labelled plastic vials. One otolith from each fish was embedded in epoxy resin and sectioned transversely through the core using a low-speed precision saw (Buehler Isomet 1000) equipped with a 0.4 mm thick diamond-coated blade. Otolith sections were affixed to glass slides using clear Crystalbond and subsequently ground (F800 and F1200 grit SiC powder). After grinding, otoliths were polished using a soft cloth impregnated with 3 µm diamond paste, then rinsed in distilled water and cleaned for 2 minutes in an ultrasonic bath (SONOREX SUPER RK 103 H) and left to air-dry.

**LA-ICP-MS analysis of otoliths.** Transverse sections were analysed at the Institute of Geosciences, JGU, Mainz, Germany, using an ESI NWR193 ArF excimer laser ablation system equipped with the TwoVol2 ablation cell, operating at 193 nm wavelength, coupled to an Agilent 7500ce quadrupole ICP-MS. Sections were preablated prior to each line scan to prevent potential surface contamination. The ICP-MS instrument was run at a frequency of 7 Hz and fluency of 3 J/cm<sup>2</sup>, carried out at a scan speed of 5 µm/s, using a rectangular beam of 50 x 40 µm (preablation beam 80 x 40 µm). Background intensities were measured for 15 s. Synthetic glass NIST SRM 612 (National Institute of Standards and Technology) was used to calibrate element concentrations of otolith samples. Quality control materials (QCMs) (USGS MACS-3, USGS BCR-2G, NIST SRM 610) were used to monitor accuracy and precision of the LA-ICP-MS analysis and calibration strategy. QCMs were assessed by measuring ca. 160 µm long line scans. Signals of all measurements were monitored in time-resolved mode and processed using an in-house Excel spreadsheet<sup>97</sup>. Details of the calculations are given in Mischel et al.<sup>98</sup>. Element concentrations of the otoliths were determined in line scan mode, along the axis of maximal growth, through the otolith core representing first six months of life. Monitored isotopes included <sup>7</sup>Li, <sup>23</sup>Na, <sup>26</sup>Mg, <sup>43</sup>Ca, <sup>55</sup>Mn, <sup>66</sup>Zn, <sup>88</sup>Sr, <sup>97</sup>Mo, <sup>137</sup>Ba, <sup>138</sup>Ba, <sup>208</sup>Pb and <sup>238</sup>U. <sup>43</sup>Ca was used as internal standard for the samples as Ca concentration of 38.8 wt.% or 388,000 µg/g<sup>99</sup>. The results for the QCMs agreed with the preferred values reported in the GeoReM data base (<http://georem.mpch-mainz.gwdg.de/>, application version 26; compare also Jochum et al.<sup>100-102</sup>). Concentrations of other elements in otoliths were converted to molar concentrations and estimated against the Ca concentration.

**Data analysis.** Data on 11 elements were obtained from all otolith samples. Element-to-Ca data of Li, Mn, Mo and U were below quantification and detection limits<sup>103</sup>. Moreover, for most elements, the signals during ablation on the otoliths were not higher than the noise of the background signal. Only Ba:Ca, Sr:Ca, Mg:Ca, Zn:Ca and Pb:Ca were mostly above the detection and quantification limits, and thus these elements were included in further analysis. For data visualization, an element linear raster was smoothed using a 31-pt arithmetic running average. Element concentration data exceeding the running averages by 5σ were considered outliers and were excluded from further analysis<sup>104,105</sup>. To enable comparison between samples and nurseries and to eliminate potential confounding effects due to temporal variation in the factors influencing otolith chemical composition<sup>41,106-110</sup>, all otoliths were reduced to the number of data points (526 points) from the core to the raster line length of the smallest sample. Statistical analysis was performed using PRIMER (V. 7.0.13; Auckland, NZ) and graphs were prepared using SigmaPlot (v. 13.0; Systat Software Inc, San Jose, CA, USA). Univariate PERMANOVA was used to test the difference of individual sites and nurseries on elemental data obtained from otoliths.

Differences in otolith elemental composition were evaluated via the permutational analysis of variance (PERMANOVA) using Manhattan distance dissimilarity matrices as it is sum of absolute differences between point across all the dimensions, since the elements were on very comparable measurement scales. Manhattan Distance is preferred over the usually used Euclidean distance metric as the dimension of the data increases<sup>111</sup>. We calculated the Manhattan measure separately for each element variable set and then transformed them to obtain a single overall matrix that measures the differences between specimens for the overall otolith signatures for elements. Differences in elemental composition (factor: "Elements") between sampling nurseries (factor:

“Nurseries”) and specimens defined as Y-O-Y or young adults (factor: “Age”) were visualized with metric multi-dimensional scaling (mMDS) based on the Manhattan distance dissimilarity matrices. Pairwise PERMANOVAs between all pair of group levels with corrections for multiple testing as a post-hoc test.

CAP analysis (CAP) was used to estimate the accuracy of otolith element signatures in classifying fish to the appropriate nursery (N1, N2 or N3). CAP is a routine for performing canonical analysis by calculating principal coordinates from the resemblance matrix among groups of samples to predict group membership, positions of samples with another single continuous variable, or finding axes having maximum correlations with another set of variables<sup>112</sup>. As result of cross validation, CAP provide % correctness and total correctness of allocations of observations to groups together with mis-classification error. CAP analyses were run separately for the factors “Nurseries” and “Elements” to reveal which element is mostly responsible for allocation to specific nursery. Additionally, to measure and test homogeneity of multivariate dispersions among a priori groups, PERMDISP analyses were used with use centroids and permutations, as recommended in Anderson<sup>111</sup>.

## Declarations

### Ethics statement

The methods involving animals in this study were conducted in accordance with the Laboratory Animal Management Principles of Croatia. All experimental protocols were approved by the Ethics Committee of the Institute of Oceanography and Fisheries.

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### Author Contributions

All of the authors conceived the research. SMS, DV, HU contributed to sample design, otolith collection and preparation, MK and RM assisted in running analyses. SMS and DV wrote the draft of the paper and all authors participated in the improvement and revision of the manuscript.

### Competing interests

The authors declare no competing interests.

### Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

### Contribution to the Field

This paper reveals the potential of otolith chemistry in the interpretation of *Sparus aurata* adult re-allocation to origin nurseries according to otolith fingerprints. The results suggest there are more suitable nursery habitats than only coastal lagoons, previously considered the only essential habitat for that purpose for this species, namely, a number of shallow coves with continuous, submarine, freshwater springs along the eastern Adriatic coast have been recognized as potentially suitable habitats, and significantly contribute to the *S. aurata* recruitment process.

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## Tables

**Table 1.** Summary of PERMANOVA results for the multivariate analysis of elemental composition for Y-O-Y specimens and adults *Sparus aurata* collected at three nursery areas (N1, N2, N3).

Element/Ca	Mg:Ca (mmol/mol)				Ba:Ca (µmol/mol)			Sr:Ca (µmol/mol)			Zn:Ca (µmol/mol)		
	df	MS	Pseudo-F	P (perm)	MS	Pseudo-F	P (perm)	MS	Pseudo-F	P (perm)	MS	Pseudo-F	P (perm)
Age	1	20024	3.55	<b>0.009*</b>	1.41E+06	3.20	<b>0.008*</b>	54149	3.09	<b>0.007*</b>	5.91E+07	3.24	<b>0.006*</b>
Nursery	2	12645	2.24	<b>0.033*</b>	4.90E+05	1.10	0.298	45552	2.60	<b>0.006*</b>	3.86E+07	2.11	<b>0.016*</b>
Age x Nursery	2	9590.6	1.70	0.093	3.46E+05	0.78	0.724	61162	3.50	<b>0.0004**</b>	2.53E+07	1.38	0.128
Residuals	42	5751			4.44E+05			17500			1.83E+07		
Total	47	3.02E+05											

\*p<0.05; \*\*p<0.005

**Table 2.** Summary of PERMANOVA results for the multivariate analysis of overall elemental composition for Y-O-Y specimens and adults *Sparus aurata* collected at three nursery areas (N1, N2, N3).

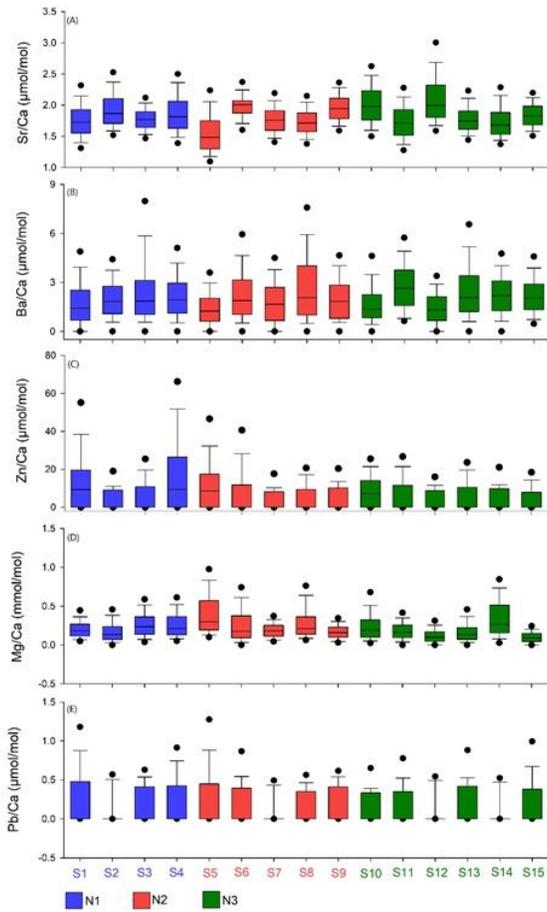
	df	MS	Pseudo-F	P (perm)
Nurseries (Nu)	2	2.375E+05	1.694	<b>0.027</b>
Elements (El)	4	5.348E+06	38.13	<b>0.0001</b>
Age (Ag)	1	7.502E+05	5.349	<b>0.0001</b>
Nu x El	8	2.252E+05	1.606	<b>0.009</b>
Nu x Ag	2	1.825E+05	1.302	0.128
El x Ag	4	5.929E+05	4.228	<b>0.0001</b>
Nu x El x Ag	8	1.578E+05	1.126	0.255
Residuals	210	1.403E+05		
Total	239			

**Table 3.** Summary of sampling data.

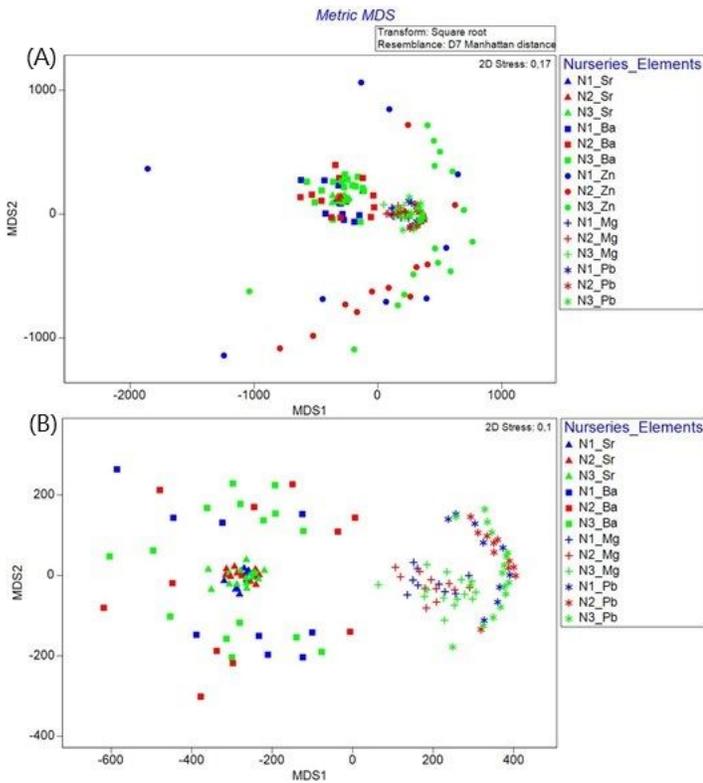
No	Sites	Abbreviation	Geographic position	Nursery affiliation	Proximity to nursery	Salinity*	Site closure	Age	Sample size	Total length (range in cm)
S1	Mala Neretva	MNE	south	N1	Close	Low	Yes	0+	5	4.5-5.9
S2	Bistrina	BIS	south	N1	Far	Medium	Yes	>2+	3	22.8-26.2
S3	Gradac	GRA	south	N1	Far	Medium	Yes	>2+	3	26.2-28.6
S4	Viganj	VIG	south	N1	Far	High	No	>2+	3	25.7-26.8
S5	Pantan	PAN	middle	N2	Close	Low	Yes	0+	5	4.0-6.0
S6	Nečujam	NEC	middle	N2	Far	High	No	>2+	3	24.0-27.0
S7	Splitska vrata	SVR	middle	N2	Close	High	No	>2+	3	24.0-25.2
S8	Kaštelanski zaljev	KZA	middle	N2	Close	Medium	Yes	>2+	4	23.9 - 28.1
S9	Šćedro	SCE	middle	N2	Far	High	No	>2+	2	23.1-29.0
S10	Prosika	PRO	north	N3	Close	Medium	Yes	0+	3	3.1-3.4
S11	Dugi Otok	DOT	north	N3	Far	High	No	>2+	3	24.1-26.8
S12	Murtersko more	MMO	north	N3	Far	High	No	>2+	4	23.5-26.5
S13	Primošten	PRI	north	N3	Far	High	No	>2+	3	23.5-28.5
S14	Virski Brak	VBR	north	N3	Far	High	No	>2+	2	24.2-25.3
S15	Šibenik	SIB	north	N3	Close	Medium	Yes	>2+	3	27.5-29.7

\*Low: < 15 ppt low; Medium: 15-25 ppt; High > 25 ppt

## Figures

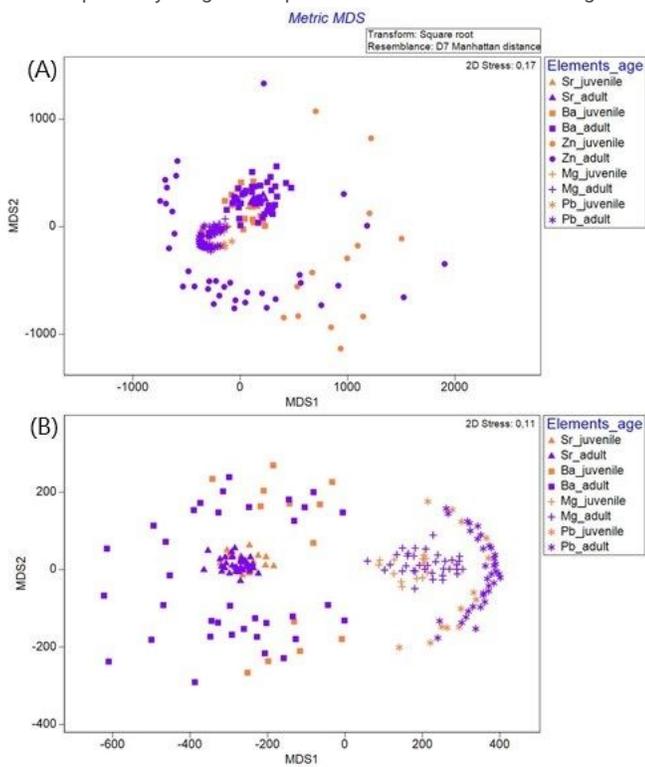


**Figure 1**  
 Box plots of median ( $\pm$  standard deviation) Sr/Ca, Ba/Ca, Zn/Ca, Mg/Ca, Pb/Ca otolith ratios (all  $\mu\text{mol/mol}$  except Mg/Ca mmol/mol) of *Sparus aurata* collected from 15 sites within nurseries N1 (blue), N2 (red) and N3 (green). Black dots present min and max values of element ratios for each site.



**Figure 2**

Metric MDS plot for young adult *Sparus aurata* for their element signatures in the nurseries N1, N2 and N3, a) Zn:Ca included; b) Zn:Ca excluded.



**Figure 3**

Metric MDS plot for overall elemental composition of Y-O-Y specimens and young adult *Sparus aurata* in the nurseries N1, N2 and N3, a) Zn:Ca included; b) Zn:Ca excluded.

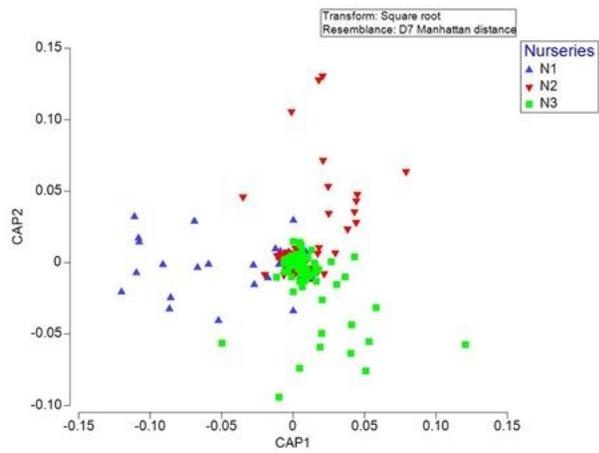


Figure 4

Canonical variate plot (CAP) of element otolith chemistry of *Sparus aurata* grouped by the factor "Nurseries".

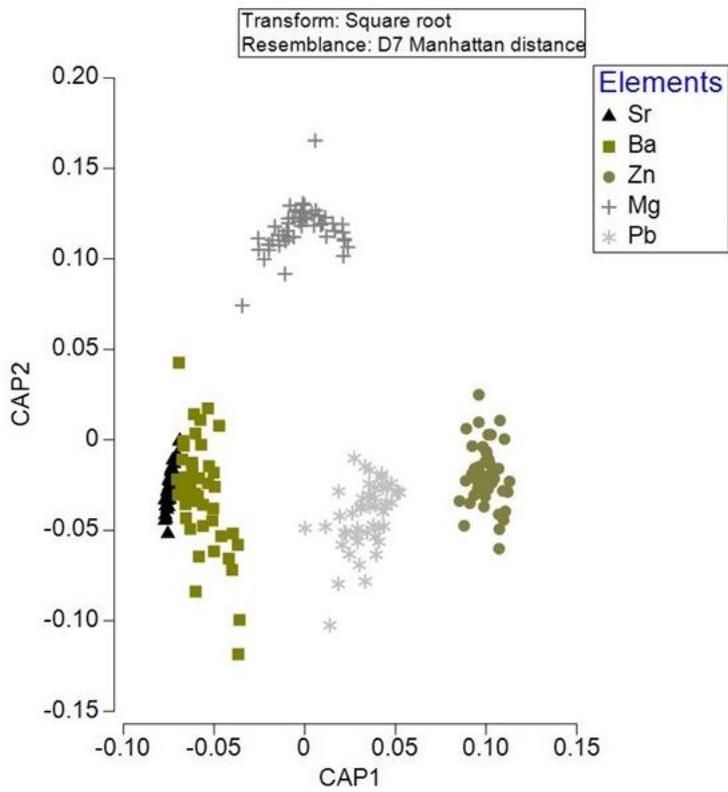
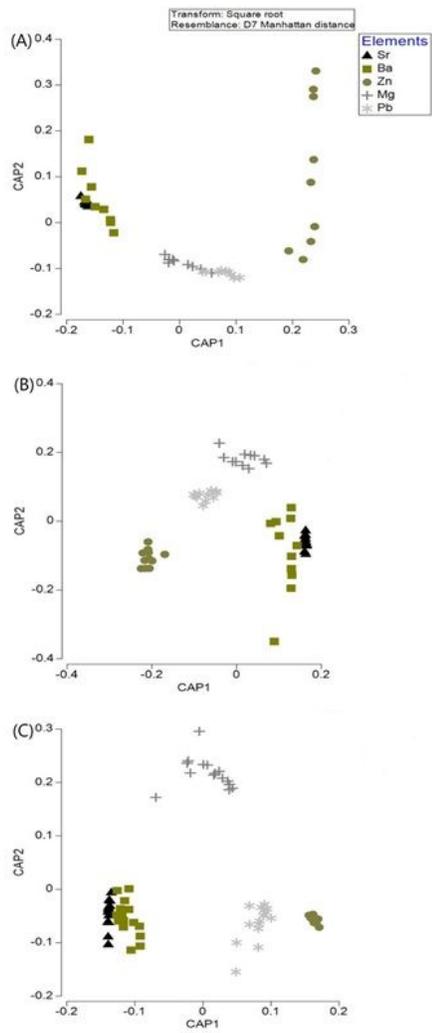


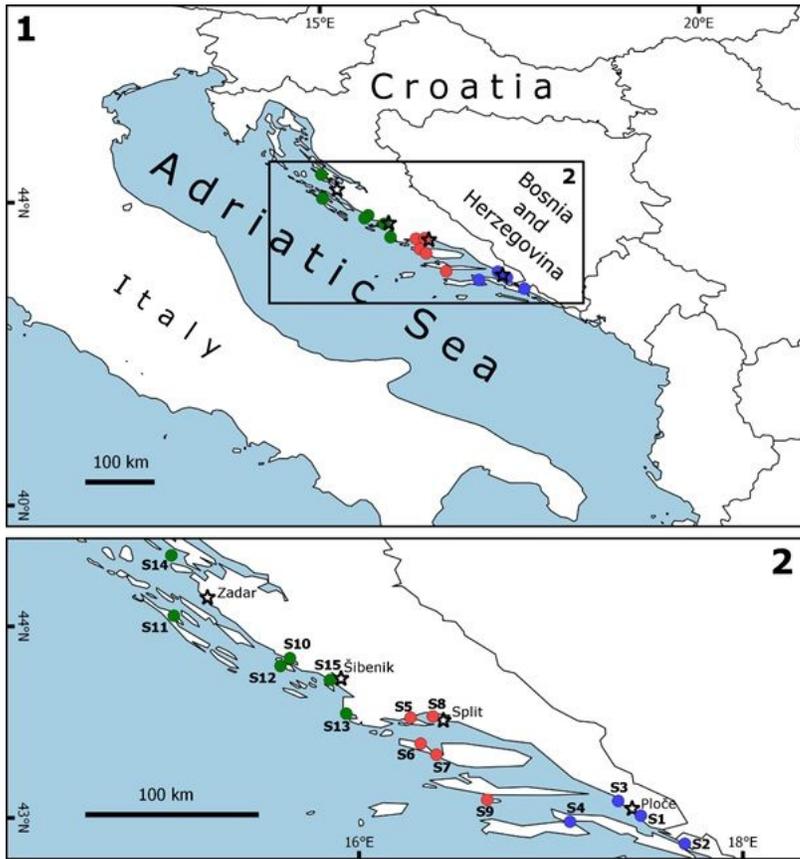
Figure 5

Canonical variate plot (CAP) of element otolith chemistry of *Sparus aurata* grouped by the factor "Elements".



**Figure 6**

Canonical variate plot of element otolith chemistry (Sr, Mg, Ba, Zn and Pb) of *Sparus aurata* for each nursery area: a) N1; b) N2 and c) N3.



**Figure 7**

Sampling area along the eastern Adriatic coast with selected nurseries: N1 (blue), N2 (red) and N3 (green). Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

## Supplementary Files

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- [MatiSkokoetal.2021SupplementaryInformation.docx](#)