Abstract: Integrated otolith chemistry and muscle tissue stable isotope analyses were performed to allocate juvenile Diplodus puntazzo and Diplodus vulgaris to nurseries in the Adriatic Sea. Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS) was used to quantify the concentrations of chemical elements in the otoliths. Fish muscle samples were analysed for δ13C and δ15N. In general, Ba/Ca and Sr/Ca ratios and isotopes varied between sites and species. Values of δ13C and δ15N were significantly different between species and sites. Multivariate analysis detected a significant difference in the element signature between species while there was no evidence for a significant interaction for sites. A clear pattern across the four groups of interest, D. puntazzo_Estuary > D. vulgaris_Estuary > D. puntazzo_Coastal > D. vulgaris_Coastal, following decreases in δ13C, and increases in δ15N were found. It seems that these species are feeding on the same local food web within more productive estuarine water while at costal site, feeding segregation among investigated species is evident. Both species were re-allocated correctly to the estuarine waters based on the otolith chemistry and stable isotopes information and higher value of δ13C and δ15N. Combining otolith chemistry with tissue isotope ratios of juvenile fish provided complementary information on nursery habitat use at different spatial scales and elucidated ecological and environmental linkages.

Keywords: Diplodus vulgaris; Diplodus puntazzo; geochemistry; trophic relation; essential habitats; Adriatic Sea

1. Introduction

Elucidating movement and life-history characteristics of marine organism is of crucial importance for their management and conservation [1–3] and the knowledge gap still represents a challenge to scientists working on this issue. Nearshore estuarine and marine ecosystems such as seagrass meadows, marshes and mangrove forests are often referred to as nursery grounds [4] due their positive effects on the diversity and productivity of fish and invertebrates in coastal waters. The greater food abundance and lower predation risk of these shallow habitats support high juvenile densities and may contribute juveniles or sub-adults to adult populations [5]. Coastal ecosystems are highly structured and fragile environments, and many valuable coastal systems are under high anthropogenic pressures, resulting in species loss and habitat degradation [6–8]. In particular, the highly populated
Mediterranean coastal areas are becoming progressively degraded, and increasing anthropogenic pressures and destructive and illegal fisheries are causing severe repercussions [9]. The Adriatic Sea, particularly its northern most part, is considered the most exploited basin of Mediterranean Sea [10].

The life history of many marine fishes begins with coastal spawning followed by larval ingress to nursery areas, which is influenced by physical oceanographic processes [4,11,12]. These areas provide critical habitats for larvae to settle and develop into juveniles, before leaving to join adult populations during their development to young adults [4,13,14]. Understanding how a specific nursery shapes juvenile behavior and consequently growth, and how connectivity determines the spatial scale between fish populations, population dynamics, and stock structure is ultimately necessary for conservation and management strategies [12]. This essential knowledge is increasingly being obtained from chemical analysis of fish otoliths.

The growing otoliths incorporate and store elements from the surrounding environment throughout the organism’s life [15]. The ambient concentrations of these elements are influenced by a range of external factors that vary at both spatial and temporal scales [12]. Consequently, the microchemistry of otoliths from different environmental conditions vary in their elemental composition [16]. These elemental fingerprints are widely used to successfully determine population structure [17], define estuarine nurseries [18] and assess connectivity between juvenile and adult populations [19,20].

In the marine environment, Ba/Ca, Mg/Ca, U/Ca, B/Ca, and Sr/Ca in various biological calcareous tissues (i.e., otolith) show strong correlations with ocean water temperatures [21–24]. Some elements (e.g., strontium and barium) are used successfully to reconstruct environmental and coastal-estuary migration histories for individual fish [25], as their concentrations reflect local availability in seawater. There are documented differences observed in the elemental ratios of otoliths of fish moving through freshwater, estuarine, and marine waters, with higher Sr/Ca found in marine and higher Ba/Ca found in freshwater [26,27]. A positive relationship between the Sr content of otoliths and ambient salinity has also been observed, though the magnitude of this effect varies with ambient water Sr concentrations [27–31]. Other elements, such as K, Na, Zn, and Mn, are likely to be mediated by the physiological regulation of organisms [32–34].

Additional information on the biotic environment can be obtained from stable isotope analysis of soft tissues and otoliths. These data reflect fish diet and can be used to determine movement from and within estuaries [35–37], migratory patterns [38,39], and habitat use [40,41]. Reis-Santos et al. [20] concluded existence of relationship between distinct isotope ratios of food sources and fish feeding in certain habitats primary producer groups exhibit distinctive isotope ratios that are propagated through local food webs. Thus, non-migratory individuals, such as juveniles within nurseries [33,42], are expected to exhibit stable isotope ratios in equilibrium with the local food webs, while transient individuals moving between habitats should display intermediate or greater isotope variation [36,43,44]. However, there are few studies that use both tissue stable isotopes and otolith chemistry to assess connectivity or population structure [39,45–47], though one study conducted an integral assessment using combined tissue isotope and otolith chemistry to determine connectivity within an estuary for two juvenile fish species [20].

Sparid fishes are highly valuable fish resources in the Mediterranean Sea [48]. Those of the genus Diplodus, including the common two-banded sea bream, Diplodus vulgaris (Geoffroy Saint-Hilaire, 1817) and the sharpsnout seabream, Diplodus puntazzo (Walbaum, 1792) inhabit coastal habitats from shallow waters to depths >50 m, with reproduction taking place in deep waters [49]. After one month of pelagic larval life, they settle in very shallow benthic habitats where they remain for several months before dispersing from the nurseries to join adults [50]. Settlement intensity varies spatially, temporally and among species, with D. puntazzo settling in October–November while D. vulgaris settles in two pulses, the first in November–December and the second in January–February [51,52]. How-
ever, these species are contemporaneous in nurseries [53], thus confirming the successful temporal partitioning of habitat use between different Diplodus species [51].

Juvenile fish from the genus Diplodus have been previously investigated in three studies. Correira et al. [33] applied solution-based analyses on whole otoliths and laser ablation analysis of otolith cores to obtain insight into the population structure of D. vulgaris. Di Franco et al. [54] investigated within-otolith variability in chemical fingerprints and found that individuals at the same site can show significant variability in elemental uptake. The possible use of otolith fingerprints as natural tags for the identification of juvenile D. sargus and D. vulgaris in ports were studied by Bouchoucha et al. [34]. However, there are no reports of any otolith chemistry studies using D. puntazzo. Other authors have recently conducted chemical analyses of juvenile fish otoliths [12, 20, 55–59].

The aim of the present study was to use both otolith chemistry and muscle stable isotope composition to allocate two closely related juveniles of D. vulgaris and D. puntazzo (age—zero) to two different nursery sites: an estuarine and a coastal (marine) nursery. We hypothesized that these closely related fish species, simultaneously present in the same nursery areas, exhibit different chemical signatures in estuarine and coastal waters as a reflection of their different behavior in foraging prey in specific nursery, which should consequently allow for the proper allocation of juveniles to a specific nursery. Such knowledge can help to accurately identify nursery origin and determine the relative contributions of individual nurseries to the coastal population of these species.

2. Materials and Methods

2.1. Study Locations and Fish Collection

Newly settled juveniles of sharpsnout seabream, Diplodus puntazzo and common two-banded sea bream Diplodus vulgaris were collected from two sites along the eastern Adriatic (Figure 1a, b): the estuarine site Pantan and coastal site Sovlja (Figure 1c), as sites known to be essential nursery areas for these species [60–62]. They are separated by a distance of 200 km and hydrologically represent different water types in the Adriatic Sea. The Pantan estuary is near Split, and receives the waters of the Pantan River, exhibiting variable salinity gradients during the year (transitional waters), with a muddy-sandy bottom partially overgrown with Zostera marina. Sovlja Cove is near Šibenik and is a typical coastal site, with a partially rocky-sandy bed with patches of Cymodocea nodosa meadows, and less influence of freshwater springs (Table 1).

| Site   | Pantan       | Sovlja       |
|--------|--------------|--------------|
|        | Bottom *     | Surface      | Bottom *    | Surface      |
| Temperature (°C) | 27.8 | 26.5 | 24 | 26.4 |
| Salinity     | 33.1 | 0.9 | 38.3 | 38 |
| Oxygen (mg/L) | 8.88 | 8.63 | 10.48 | 8.92 |

* Depth 1.5 m.

Samples of juvenile fish specimens were collected using a special constructed small shore seine net (L = 25 m; mesh size 4 mm) in June 2018. Three hauls for each site were performed to collect an adequate number of specimens. To avoid temporal variation in otolith chemistry and stable isotope analysis, sampling was carried out in the shortest possible time. At both sites, Pantan and Sovlja, both species, Diplodus vulgaris and Diplodus puntazzo, were present with similar abundance (up to 7 specimens in each haul) and similar sizes (from 38 to 71 mm and 31 to 72 mm, respectively). Additionally, 5 individuals per site of blue mussel, Mytilus galloprovincialis were sampled. Upon collection, specimens were transported to the laboratory and frozen until analysis. For the analysis, total length (TL;
cm) and weight (TW; g) were recorded and specimens were dissected to extract white fish muscle tissue and otoliths for stable isotope analyses and otolith chemistry, respectively.

Figure 1. Sampling area in the Europe (A) along the eastern Adriatic coast (B) with selected sites: Pantan (square) and Sovlja (circle) (C).

2.2. Sample Preparation

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 labelled plastic vials. The otoliths were embedded in epoxy resin (Buehler EpoThin 2) 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. Otoliths sections were affixed to glass slides using clear Crystalbond and subsequently ground (F800 and F1200 grit SiC
powder) and polished using a soft cloth impregnated with diamond paste (3 μm). After polishing, otoliths were rinsed and cleaned ultrasonically (2 min).

Muscle tissue of *M. galloprovincialis* was used as appropriate baseline since sedentary bivalves can be useful indicators of isotopic baseline [63] in the coastal ecosystem. That is needed to integrate the variation in isotope values at the base of food webs [64] when trophic status of specific marine organisms is requested while data of prey spectra trophic status is unknown.

Standard preparation for stable isotope analysis consisted of oven drying samples at 60 °C until constant weight. Tissues were then ground to a fine powder with a mortar and pestle and approximately 1 mg of sample was weighed into tin cups. Lipid extraction of fish muscle samples was not performed as individuals were juveniles and body lipid was uniformly low (<5%) and insufficient to bias carbon stable isotope analysis or require corrections as suggested by Post et al. [65].

### 2.3.1. LA-ICP-MS Analysis of Otoliths

The concentrations of Li, Na, Ca, Mg, Mn, Zn, Sr, Mo, Ba, Pb, and U were determined using laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) in line scan mode, through the otolith core from edge to edge (Figure 2). Each point on the otolith corresponds to a specific point on laser trajectory enabling selection of the otolith part to be analyzed.

![Figure 2. Otolith of juvenile *Diplodus vulgaris*. The blue line represents the line scan through the otolith core from one edge to the opposite edge. Scale bar = 100 μm (Magnification 3x). Data for each otolith were selected approximately on distance 200 μm from the core.](image)

Analyses were performed 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. Sample surfaces were preablated prior to each line scan to prevent potential surface contamination. The laser repetition rate was 7 Hz and laser energy on samples was about 3 J/cm². Background intensities were measured for 15 s. Line scans were 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). Synthetic glass NIST SRM 612 (National Institute of Standards and Technology; Gaithersburg, Maryland, United States) was used to calibrate element concentrations of otolith samples and quality control materials (QCMs) (USGS MACS-3, USGS BCR-2G, NIST SRM 610) (Table 2) were used to monitor accuracy and precision of the LA-ICP-MS analysis applying the preferred values available from the GeoReM database ([66], application version 26;
Signals were monitored in time-resolved mode and processed using an in-house Excel spreadsheet [70]. Details of the calculations are given in Mischel et al. [71]. The concentration of $^{43}$Ca as an internal standard in otoliths was taken as 38.8% by weight or 388,000 ppm following the determination of otolith Ca concentration [72]. Concentrations determined on the otoliths were converted to molar concentrations and standardized to calcium.

Table 2. Average concentrations and standard deviations ($\pm 1\sigma$) of strontium and barium in reference materials USGS MACS-3, USGS BCR-2G and NIST SRM 610 as determined during the LA-ICP-MS analysis. Reference values ($\pm 1\sigma$ uncertainties) for USGS BCR-2G and NIST SRM 610 are available from the GeoReM database (application version 26; preferred values). Values for MACS-3 are from Jochum et al. ([68] Table 1, “Preliminary reference values”, “prel. RV”). Reference values given as oxide wt% in the GeoReM database have been calculated into element concentrations applying the respective stoichiometric factor.

| Element | USGS MACS-3 | USGS BCR-2G | NIST SRM 610 |
|---------|-------------|-------------|--------------|
|         | Measured values | Reference values | Measured values | Reference values | Measured values | Reference values |
| Sr      | 6181 ± 174 | 6760 ± 350 | 345.6 ± 1 | 342 ± 4 | 530.5 ± 7 | 515.5 ± 1 |
| Ba      | 57.9 ± 2 | 58.7 ± 2 | 647.1 ± 5 | 683 ± 7 | 438.8 ± 9 | 452 ± 9 |

2.3.2. Stable Isotope Analyses of Muscle Tissue

Muscle samples were analyzed for $\delta^{13}$C and $\delta^{15}$N using a PDZ Europa ANCA-GSL elemental analyser interfaced to a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) at the UC Davis Stable Isotope Facility. Samples were combusted at 1000 °C in a reactor packed with chromium oxide and silvered copper oxide. Following combustion, oxides were removed in a reduction reactor (reduced copper at 650 °C) and the helium carrier was released through a water trap (magnesium perchlorate and phosphorous pentoxide). N$_2$ and CO$_2$ were separated on a Carbosieve GC column (65 °C, 65 mL/min) before entering the Isotope-ratio mass spectrometry (IRMS). Stable isotopes were expressed in standard delta ($\delta$) notation as parts per thousand (‰).

During analysis, samples were interspersed with several replicates of at least four different laboratory reference materials, previously calibrated against international reference materials, including: IAEA-600, USGS-40, USGS-41, USGS-42, USGS-43, USGS-61, USGS-64, and USGS-65 reference materials. A sample’s provisional isotope ratio was measured relative to the reference gas peak analyzed against each sample. These provisional values were finalized by correcting the values for the entire batch based on the known values of the included laboratory reference materials. The long term standard deviation is 0.2 ‰ for $^{13}$C and 0.3 ‰ for $^{15}$N [73].

2.4. Data Analysis

Element-to-Ca data for Li, Na, Mg, Ba, Sr, Mn, Zn, Mo, Pb, and U were determined for all specimens. Most of these element-to-Ca data were below quantification and detection limits. Some ratios including Na/Ca, Mg/Ca, Zn/Ca, Mn/Ca, and Li/Ca exceeded the detection limit in several otoliths, although they were below the quantification limit in most samples. Ba/Ca and Sr/Ca ratios were above the detection and quantification limits [74] and thus subjected to further analysis. Element concentration data Ba/Ca and Sr/Ca ratios for D. vulgaris and D. puntazzo samples exceeding 31-point (31-pt) running averages by 5$\sigma$ were considered outliers and excluded from further analysis (see [75,76]). For data visualization, element linear raster was smoothed using a 31-pt arithmetic running average.

Differences in otolith chemistry composition were evaluated via the permutational analysis of variance (PERMANOVA) using Manhattan distance dissimilarity matrices [77], since both elements were on very comparable measurement scales. The metric Multi-
dimensional Scaling (mMDS) ordination were used for showing the patterns across the four groups of interest and the contribution of each element isotope composition to the obtained distance. Starting point for data selection on linear raster was 200 µm which corresponds approximately to the third month of fish juvenile life according to settlement mark [34,51,78,79]. We calculated the Manhattan measure separately for each of the barium and strontium variable sets and then averaged the resulting Manhattan distance matrices to get a single overall matrix that measures the differences between fish species for the overall otolith signatures for both elements. Differences in muscle δ^{13}C and δ^{15}N isotope ratios were normalized and evaluated via PERMANOVA using Euclidean distance dissimilarity matrices.

Canonical analysis of principal coordinates (CAP) was used to estimate the accuracy of otolith element signatures and muscle stable isotopes in classifying fish to their collection site. 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 along another single continuous variable or finding axes having maximum correlations with some other set of variables [77].

CAP analyses were run separately for each of the two factors: “Site” and “Species”. The CAP routine output scores were then merged for both factors. Finally, we relate the distance matrix based on otoliths to the distance matrix based on isotopes and performed CAP as a canonical correlation analysis of the otolith distance matrix on the isotope (continuous quantitative) values [77].

Univariate permutational analysis of variance (PERMANOVA) was used to test the difference of site or species effects on elemental data obtained from otoliths and stable isotope data obtained from white muscle. Statistical analysis was done using PRIMER (V. 7.0.13; Auckland, NZ) and graphs were prepared using SigmaPlot (v. 13.0; Systat Software Inc, San Jose, CA, USA).

3. Results

Juveniles of *D. puntazzo* ranged in TL from 5.2 to 6.1 cm (mean 5.58 ± SD 0.35 cm) and weight from 2.32 to 3.78 g (mean 2.95 ± SD 0.6 g), and from 3.1 to 7.2 cm (mean 5.40 ± SD 1.66 cm) and 0.42 to 5.68 g (mean 2.51 ± SD 2.48), at sites Pantan and Sovlja, respectively. Juveniles of *D. vulgaris* ranged in TL from 4.3 to 7.1 cm (mean 5.37 ± SD 1.23 cm) and weight from 1.17 to 5.50 g (mean 2.70 ± SD 1.95 g), and from 3.8 and 6.2 cm (mean 4.74 ± SD 1.08 cm), and 0.8 to 4.19 g (mean 1.82 ± SD 1.43 g), at sites Pantan and Sovlja, respectively.

3.1. Otolith Trace Element Chemistry

Ba/Ca and Sr/Ca ratios varied between sites and species. Data for 31-pt moving averages for Ba/Ca in *D. puntazzo* ranged from 0.30 to 5.78 µmol/mol (median 2.6 µmol/mol) and 0.35 to 4.78 µmol/mol (median 1.59 µmol/mol) for Sovlja and Pantan, respectively (Figure 3A). For *D. vulgaris*, 31 pt moving averages for Ba/Ca ranged from 0.46 to 8.61 µmol/mol (median 2.76 µmol/mol) for Sovlja and 0.12 to 3.7 µmol/mol (median 1.4 µmol/mol) for Pantan (Figure 3A). The median values of Ba/Ca were higher for both species, *D. vulgaris* and *D. puntazzo*, at Sovlja while spatial differences in Ba concentration was not significant neither between species (t = 1.345; p = 0.066), neither between sites (t = 1.247; p = 0.137).

For 31 pt moving averages for Sr/Ca values in *D. puntazzo* ranged from 1.47 to 2.3 mmol/mol (median 1.89 mmol/mol) and 1.52 to 2.20 mmol/mol (median 1.87 mmol/mol) for Sovlja and Pantan, respectively (Figure 3B). The Sr/Ca value in *D. vulgaris* ranged from 1.65 to 2.45 µmol/mol (median 2.06 mmol/mol) and 0.92 to 2.24 mmol/mol (median 1.50 mmol/mol) for Sovlja and Pantan, respectively (Figure 3B). Although the median Sr/Ca was higher for *D. vulgaris* at Sovlja, spatial differences in the Sr/Ca ratio were not significant between species (t = 1.126; p = 0.271) and also not between sites (t = 1.412; p = 0.093).
not significant neither between species ($t = 1.345; p = 0.066$), neither between sites ($t = 1.247; p = 0.137$).

Data for 31 pt moving averages for Sr/Ca values in *D. puntazzo* ranged from 1.47 to 2.3 mmol/mol (median 1.89 mmol/mol) and 1.52 to 2.20 mmol/mol (median 1.87 mmol/mol) for Sovlja and Pantan, respectively (Figure 3B). The Sr/Ca value in *D. vulgaris* ranged from 1.65 to 2.45 µmol/mol (median 2.06 mmol/mol) and 0.92 to 2.24 mmol/mol (median 1.50 mmol/mol) for Sovlja and Pantan, respectively (Figure 3B). Although the median Sr/Ca was higher for *D. vulgaris* at Sovlja, spatial differences in the Sr/Ca ratio were not significant between species ($t = 1.126; p = 0.271$) and also not between sites ($t = 1.412; p = 0.093$).

**Figure 3.** Box plots of median (±standard deviation) Ba/Ca (A) and Sr/Ca (B) otolith ratios (mmol/mol) of *Diplodus puntazzo* and *Diplodus vulgaris* collected from the Pantan estuarine site and Sovlja coastal site. Black dots present linear raster of Ba/Ca and Sr/Ca otolith ratios for each site and species.

### 3.2. Stable Isotope Analyses

Differences were observed in stable isotope composition of muscle tissue ($\delta^{13}C$ and $\delta^{15}N$) between sites and species. Values for carbon stable isotope in *D. puntazzo* ranged from $-18.41$ to $-15.43\%$ (median $-17.59\%$) and from $-25.17$ to $-20.06\%$ (median $-23.60\%$) for Sovlja and Pantan, respectively (Figure 4A). For *D. vulgaris*, $\delta^{13}C$ values ranged from $-18.11$ to $-15.14\%$ (median $-16.41\%$) in Sovlja and from $-19.61$ to $-17.25\%$ (median $-17.73\%$) in Pantan (Figure 4B). Median values of $\delta^{13}C$ were higher for *D. vulgaris* at both sites, and differences were significant both between species ($t = 5.134; p = 0.0002$) and sites ($t = 5.550; p = 0.0003$). Data for $\delta^{15}N$ in *D. puntazzo* ranged from $10.12$ to $10.93\%$ (median $10.37\%$) and $11.16$ to $12.11\%$ (median $11.84\%$) for Sovlja and Pantan, respectively (Figure 4A). For *D. vulgaris*, data for $\delta^{15}N$ ranged from $9.26$ to $10.04\%$ (median $9.63\%$) and $11.25$ to $12.12\%$ (median $11.47\%$) for Sovlja and Pantan, respectively (Figure 4B). Although the median of $\delta^{15}N$ was higher for *D. puntazzo* at both sites, this difference was not statistically significant between species ($t = 2.994; p = 0.011$), though it was between sites ($t = 10.039; p = 0.0001$).
3.3. Multi-parameter Comparison

When the otolith chemistry data were combined into a single matrix, PERMANOVA analysis detected that “Species” differed significantly in their element signatures, although significant level is not high (P = 0.049) while “Site” did not (Table 3). There was also no evidence for a significant interaction, as PERMANOVA analysis conducted after pooling the Site x Species interaction term did not change this result.

Table 3. Summary of PERMANOVA results for the multivariate analysis of overall elemental composition of strontium (Sr) and barium (Ba) in otoliths (a) and overall carbon (δ¹³C) and nitrogen (δ¹⁵N) stable isotope values in muscle tissue (b) for juvenile Diplodus puntazzo and Diplodus vulgaris collected at different sites.

| Factors       | df   | MS      | P (perm) | MS      | P (perm) |
|---------------|------|---------|----------|---------|----------|
| Species       | 1    | 9.588E+05 | 0.049    | 6.517   | 0.0005   |
| Site          | 1    | 6.134E+05 | 0.227    | 19.936  | 0.0001   |
| Sp x Site     | 1    | 6.495E+05 | 0.199    | 3.394   | 0.0001   |
| Residuals     | 15   | 4.283E+05 | 0.337    |         |          |
| Total         | 18   |         |          |         |          |

After pooling the isotope data, the plot clearly showed effects for each of the four “Species x Site” groups and for each of the stable isotopes (Figure 5). PERMANOVA showed that both factors (“Species” and “Site”) had main effects and a significant interaction term (Table 3). The metric MDS of the bivariate isotope data showed patterns across the four groups, with an evident pattern with a decrease in δ¹³C (Figure 5A) and increase in δ¹⁵N (Figure 5B) (going from left to right). The four groups ordered along this axis as follows: Diplodus puntazzo_Estuary > Diplodus vulgaris_Estuary > Diplodus puntazzo_Coastal > Diplodus vulgaris_Coastal.
showed that both factors (“Species” and “Site”) had main effects and a significant interaction term (Table 3). The metric MDS of the bivariate isotope data showed patterns across the four groups, with an evident pattern with a decrease in $\delta^{13}C$ (Figure 5A) and increase in $\delta^{15}N$ (Figure 5B) (going from left to right). The four groups ordered along this axis as follows: D. puntazzo _Estuary > D. vulgaris _Estuary > D. puntazzo _Coastal > D. vulgaris _Coastal.

Separate CAP analysis for each of the two factors (“Site” and “Species”) gave successful discrimination for species but not for sites. In particular, 80% D. puntazzo specimens were correctly allocated based on the otolith chemistry information, as opposed to 77.8% of D. vulgaris specimens. The two-way CAP plot obtained by merging output scores for the CAP analysis of “Site” and “Species” showed separation of the two species (Figure 6). It is apparent that the site differences (“E” estuary vs. “C” coastal) were able to distinguish for D. vulgaris. In contrast, the D. puntazzo samples from the estuary were consistently clustered, while coastal samples were more variable, making them difficult to classify.

**Figure 5.** Metric MDS for juvenile Diplodus puntazzo and Diplodus vulgaris as a bubble plot for stable isotopes (A) $\delta^{13}C$ and (B) $\delta^{15}N$ for the coastal waters (C) and estuarine (E).
It is apparent that the site differences ("E" estuary vs. "C" coastal) were able to distinguish for *D. vulgaris*. In contrast, the *D. puntazzo* samples from the estuary were consistently clustered, while coastal samples were more variable, making them difficult to classify.

**Figure 6.** Canonical variate plot (CAP) for Ba and Sr element chemistry of the otolith of juvenile *Diplodus puntazzo* and *Diplodus vulgaris* sampled in 2018, grouped by “Site” and “Species”.

The mean isotope values for the four groups of factors (Species x Site) were plotted as distances among centroids based on otolith data (Figure 7), which showed a clear separation of the coastal and estuarine sites. This was confirmed by CAP as a canonical correlation analysis of the otolith distance matrix on the isotope (continuous quantitative) values (Figure 8). According to our results, based on the otolith chemistry and stable isotope information, correct re-allocation of *D. vulgaris* individuals to the estuarine waters were confirmed. Samples of *D. puntazzo* were correctly re-allocated due to the higher value of δ¹⁵N to estuarine waters.
Figure 7. The metric MDS of the bivariate isotope data performed on Euclidean distances of normalized isotope values of $\delta^{13}$C and $\delta^{15}$N showing the patterns across the four groups of interest. Each half or circle correspond to $\delta^{13}$C and $\delta^{15}$N and its size reflects the contribution of each element isotope composition to the obtained distance.

Figure 8. Canonical variate plot (CAP) of the otolith distance matrix on the isotope (continuous quantitative) values. Previously, the distance matrix based on otoliths was related to the distance matrix based on isotopes.
4. Discussion

This study investigated the potential of otolith chemistry and tissue stable isotope analyses to distinguish between two different nursery areas of two closely related fish species of the genus Diplodus. Juveniles of D. puntazzo and D. vulgaris from the Pantan and Sovlja sites have similar reproductive and early life characteristics [52], inhabiting nursery habitats and leaving them in early summer [53,60]. The larvae of D. puntazzo settle in these shallow sites earlier as they hatch several weeks before D. vulgaris, so their juveniles are larger at both sites [51].

A commonly used method is laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS), which produces an elemental fingerprint at a discrete time-point in the life of a fish [80]. Trace elements (e.g., Ba, Li, Mg, Mn, and Sr) and heavy metals (e.g., Pb, Cu, and Zn) are acquired by fish during the life history and preserved within the otolith structure [19,80–82]. In addition to these typically analyzed elements, we also examined Na, Mo, and U in line with the protocol of the Institute of Geosciences, JGU [83]. Unfortunately, as most of the analysed element/Ca ratios were below the quantification and detection limits, only Ba/Ca and Sr/Ca were analysed in this study. A number of factors, such as salinity, temperature, water chemistry, age and growth, physiology, and metabolism may be responsible for the incorporation of trace elements into otoliths, though this is a complex process and remains poorly understood for most elements (with the exception of Ba and Sr) [27,33,84–92].

Data for Ba/Ca elemental composition were not significant between species and sites, although more prominent differences were obtained between species. Generally, Ba incorporation into otoliths appears largely determined by ambient concentrations, which are spatially variable and typically higher in inshore waters, estuaries, and upwelling zones [1,91–96]. Although, both sites are inshore, Pantan is estuarine and Sovlja is coastal, and therefore the hydrological conditions differ. Though not substantial, there is some enrichment of Ba in the coastal Sovlja site, likely influenced by local fluvial runoff and groundwater input, as suggested by Correira et al. [33] which consequently raise this concentration of above expected. The Ba/Ca concentration ratios were different in both species at both sites, confirming variability in element uptake of different species at same site [34,74]. Further on, Bouchoucha et al. [34] studying life of juvenile D. vulgaris and D. sargus reported that Ba was systematically the most discriminating element, since its concentrations in otoliths were generally higher outside ports than inside, probably due to river runoff. The Sr/Ca ratio was also more variable between species and sites but this difference was not significant for site neither for species. Sr incorporation is also influenced by ambient concentration, and has been linked to salinity, though temperature, ontogeny and growth rate may also influence patterns of Sr incorporation into otoliths [1,16,33,87,92,97,98]. The higher Sr levels from Sovlja are likely related to exogenous factors (marine site with higher salinity and temperature), though there may also be certain endogenous causes since D. vulgaris incorporated more Sr at both sites but this influence is too weak to make a significant difference. However, the variability with at each otolith concentrations have to be discussed with attention due different sampling size and site.

Since the investigated species are closely related and show no temporal segregation in nursery areas, we hypothesized that foraging behavior and diet composition may have contributed to the observed differences in the element incorporation between species and sites. Both, δ^{13}C and δ^{15}N differed significantly between species and sites. The median of δ^{15}N was higher for D. puntazzo while D. vulgaris had higher values of δ^{13}C at both sites. For soft tissue stable isotopes, lower δ^{13}C values were found at Pantan, which agrees with the expected natural patterns of δ^{13}C variation and displays an enrichment trend along the terrestrial–estuarine–marine gradient [99]. In addition, the overall richer δ^{15}N values at Pantan than at the coastal Sovlja site were likely due to anthropogenic nitrogen inputs in the estuary (e.g., wastewater, and fertilizers) [20,36,46,100]. The observed intra-species differences in fish muscle stable isotopes reflected the isotope composition of local food webs and available prey [20,101]. It seems that in estuarine Pantan, both species feed on the
same local food web for a longer period and do not disperse widely around the sampling site. Since targeted specimens in this study are juveniles representing similar growing stage and values obtained for stable isotopes were adjusted to blue mussel baseline, one should consider that in general for fish muscle the turnover rate is around months [102,103], while short-living consumers, such as zooplankton, have high tissue turnover rates, similar to that of phytoplankton [104]. Abecasis et al. [42] reported that in estuarine waters, juvenile *D. vulgaris* make only short movements and typically remain in the same areas for extended periods, and this is likely also the case for *D. puntazzo*. Higher isotope values in *D. puntazzo* may reflect that these specimens are possible several weeks older and, thus, larger and are likely to forage on bigger prey. Estuarine areas are often highly productive with a narrow prey spectrum, but with high prey availability and abundance [105]. The marked differences in isotope concentration of muscle tissue in specimens from the coastal site Sovlja suggest that these two species feed on different local food webs, with *D. vulgaris* foraging at a higher trophic level [106]. In coastal areas, the availability and abundance of prey are usually lower though the prey spectrum is wider [107].

PERMANOVA clearly confirmed the different element signatures of *D. vulgaris* and *D. puntazzo*. Although the incorporation of Ba and Sr is largely influenced by environmental factors (temperature and salinity), these differences in the otolith fingerprints likely resulted from the homeostatic apparatus of the individual fish, i.e., its physiology and ultimately its genetic makeup [98]. The fact that PERMANOVA did not reveal significant difference between sites raises the question of how these sites, defined as estuarine and coastal, really differ in the study area due to the specific oceanographic properties of the eastern Adriatic Sea, with many freshwater grounds in the coastal area [108]. Unfortunately, lack of water sample from both habitats disable relevant comparison and establishment of the relationship between Ba and Sr concentration and otolith microchemistry in this study. For sure, such limitations have to be consider in future sampling designs.

The metric MDS of the bivariate isotope data clearly shows patterns that can be interpreted as decreases in $\delta^{13}$C and increases in $\delta^{15}$N (*D. puntazzo_Estuary > D. vulgaris_Estuary > D. puntazzo_Coastal > D. vulgaris_Coastal*). Both species exhibited different behaviours in estuarine and coastal waters, which is likely related to foraging and feeding. *D. puntazzo* is more efficient in feeding in estuarine waters than *D. vulgaris*, and it grows faster, incorporating more $\delta^{15}$N in the more productive estuarine waters [105]. Moreover, this greater efficiency of *D. puntazzo* over *D. vulgaris* is even more prominent in coastal waters, where prey is generally less available and foraging time is longer [106,107].

Furthermore, we attempted to correctly allocate these species to the estuarine or coastal environments through CAP analyses. 80% *D. puntazzo* and 77.8% of the *D. vulgaris* specimens were allocated correctly based on the otolith chemistry information. However, the results suggested that over time, the otolith fingerprint differences observed in *D. vulgaris* in different waters will become more significant and thus it can be allocated correctly in estuarine water using otolith chemistry and stable isotope information. *D. puntazzo* incorporates elements into otoliths in different environments in a similar way and therefore can be allocated according to the higher value of $\delta^{15}$N in estuarine waters.

The present study provides preliminary insight into juvenile fish nursery use at different spatial scales in the Adriatic Sea by combining otolith chemistry with tissue isotope ratios of the same individuals to determine distinct ecological and environmental linkages [20]. Although, conducted on relatively small sampling size, otolith chemistry results reflected the environmental characteristics of the juvenile *Diplodus* nursery areas, while muscle stable isotope analysis indicated the isotope differences between species and between sites, accentuating the need to consider both environmental gradients and species behaviour in movement and connectivity studies based on otolith fingerprints. Such knowledge can help to accurately identify nursery origin and determining the relative contributions of individual nursery areas to the adult coastal populations of species [18,39,46]. Moreover, better understanding of settlement and recruitment processes, and nursery habitat use and movement patterns between juveniles and adults enables more sustainable
management of fishery resources and essential habitat conservation based on ecological principles.

Author Contributions: All of the authors conceived the research. D.V., S.M.-S., M.P., H.U., K.M., and R.M.-K. contributed to the sample design, collecting, and preparing otoliths and muscle tissue for analyses, and running analyses. P.G. helped to define the research questions and sampling design. D.V. and S.M.-S. wrote the draft of the paper and all authors participated in the improvement and revision of the document. All authors have read and agreed to the published version of the manuscript.

Funding: This study was fully supported by the Croatian Science Foundation (HRZZZ) under project IP-2016-06-9884 (NurseFish).

Institutional Review Board Statement: 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 Ethics Committee of the Institute of Oceanography and Fisheries.

Informed Consent Statement: Not applicable

Data Availability Statement: Data used in this manuscript are available from the corresponding author upon reasonable request.

Acknowledgments: The authors are grateful to Distinguished Professor Marti J. Anderson (Director PRIMER-e) for all knowledge sharing with us during the PERMANOVA workshop at the University of Trieste, Italy (September 2019) and special thanks for assisting with the creation of the PERMANOVA design and run analyses for this study.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Elsdon, T.S.; Wells, B.K.; Campana, S.E.; Gillanders, B.M.; Jones, C.M.; Limburg, K.E.; Secor, D.H.; Thorrold, S.R.; Walther, B.D. Otolith chemistry to describe movements and life-history parameters of fishes: Hypotheses, assumptions, limitations and inferences. *Oceanogr. Mar. Biol.* 2008, 46, 297–330.

2. Catalán, I.A.; Alós, J.; Díaz-Gil, C.; Pérez-Mayol, S.; Basterretxea, G.; Morales-Nin, B.; Palmer, M. Potential fishing-related effects on fish life history revealed by otolith microchemistry. *Fish. Res.* 2018, 199, 186–195. [CrossRef]

3. Darnaude, A.M.; Hunter, E. Validation of otolith δ18O values as effective natural tags for shelf-scale geolocation of migrating fish. *Mar. Ecol. Prog. Ser.* 2018, 598, 167–185. [CrossRef]

4. Beck, M.W.; Heck, K.L.; Able, K.W.; Childers, D.L.; Egglestoon, D.B.; Gillanders, B.M.; Halpern, B.; Hays, C.G.; Hoshino, K.; Minello, T.J.; et al. The identification, conservation, and management of estuarine and marine nurseries for fish and invertebrates. *Bioscience* 2001, 51, 633–641. [CrossRef]

5. Dahlgren, C.P.; Todd Kelisson, G.; Adams, A.J.; Gillanders, B.M.; Kendall, M.S.; Layman, C.A.; Ley, J.A.; Nagelkerken, I.; Serafy, J.E. Marine nurseries and effective juvenile habitats: Concepts and applications. *Mar. Ecol. Prog. Ser.* 2006, 312, 291–295. [CrossRef]

6. Lotze, H.K.; Lenihan, H.S.; Bourque, B.J.; Bradbury, R.H.; Cooke, R.G.; Kay, M.C.; Kidwell, S.M.; Kirby, M.X.; Peterson, C.H.; Jackson, J.B.C. Depletion degradation, and recovery potential of estuaries and coastal seas. *Science* 2006, 312, 1806–1809. [CrossRef]

7. Worm, B.; Barbier, E.B.; Beaumont, N.; Duffy, J.E.; Folke, C.; Halpern, B.S.; Jackson, J.B.C.; Lotze, H.K.; Micheli, F.; Palumbi, S.R.; et al. Impacts of biodiversity loss on ocean ecosystem services. *Science* 2006, 344, 787–790. [CrossRef]

8. Waycott, M.; Duarte, C.M.; Carruthers, T.J.B.; Orth, R.J.; Dennison, W.C.; Olyarnik, S.; Calladine, A.; Fourquarean, J.W.; Heck, K.L.; Jr.; Hughes, A.R.; et al. Accelerating loss of seagrasses across the globe threatens coastal ecosystems. *Proc. Natl. Acad. Sci. USA* 2009, 106, 12377–12381. [CrossRef]

9. Claudet, J.; Fraschetti, S. Human-driven impacts on marine habitats: A regional meta-analysis in the Mediterranean Sea. *Biol. Conserv.* 2010, 143, 2195–2206. [CrossRef]

10. Barausse, A.; Ducu, A.; Mazzoldi, C.; Artioli, Y.; Palmeri, L. Trophic network model of the Northern Adriatic Sea: Analysis of an exploited and eutrophic ecosystem. *Est. Coast. Shelf Sci.* 2009, 83, 577–590. [CrossRef]

11. Teodósio, M.A.; Paris, C.B.; Wolanski, E.; Morais, P. Biophysical processes leading to the ingress of temperate fish larvae into estuarine nursery areas: A review. *Estuar. Coast. Shelf Sci.* 2016, 183, 187–202. [CrossRef]

12. Rogers, T.A.; Fowler, A.J.; Steer, M.A.; Gillanders, B.M. Spatial connectivity during the early life history of a temperate marine fish inferred from otolith microstructure and geochemistry. *Estuar. Coast. Shelf Sci.* 2019, 227, 106342. [CrossRef]

13. Cowen, R.K.; Lwiza, K.M.M.; Sponaugle, S.; Paris, C.B.; Olson, D.B. Connectivity of marine populations: Open or closed? *Science* 2000, 287, 857–859. [CrossRef]

14. Cowen, R.K.; Sponaugle, S. Larval dispersal and marine population Connectivity. *Ann. Rev. Mar. Sci.* 2009, 1, 443–466. [CrossRef]
15. Campana, S.E.; Thorrold, S.R. Otoliths, increments, and elements: Keys to a comprehensive understanding of fish populations. *Can. J. Fish. Aquat. Sci.* 2001, 58, 30–38. [CrossRef]

16. Campana, S.E.; Chouinard, G.A.; Hanson, J.M.; Fréchet, A.; Brattey, J. Otolith elemental fingerprints as biological tracers of fish stocks. *Fish. Res.* 2000, 46, 343–357. [CrossRef]

17. Tanner, S.E.; Vasconcelos, R.P.; Cabral, H.N.; Thorrold, S.R. Testing an otolith geochemistry approach to determine population structure and movements of European hake in the northeast Atlantic Ocean and Mediterranean Sea. *Fish. Res.* 2012, 125, 198–205. [CrossRef]

18. Gillanders, B.M.; Kingsford, M.J. Elemental fingerprints of otoliths of fish may distinguish estuarine “nursery” habitats. *Mar. Ecol. Prog. Ser.* 2000, 201, 273–286. [CrossRef]

19. Reis-Santos, P.; Tanner, S.E.; Vasconcelos, R.P.; Elsdon, T.S.; Cabral, H.N.; Gillanders, B.M. Connectivity between estuarine and coastal fish populations: Contributions of estuaries are not consistent over time. *Mar. Ecol. Prog. Ser.* 2013, 491, 177–186. [CrossRef]

20. Reis-Santos, P.; Tanner, S.E.; França, S.; Vasconcelos, R.P.; Gillanders, B.M.; Cabral, H.N. Connectivity within estuaries: An otolith chemistry and muscle stable isotope approach. *Ocean Coast. Manag.* 2015, 118, 51–59. [CrossRef]

21. Sadekov, A.; Eggins, S.M.; De Deckker, P. Characterization of Mg/Ca distributions in planktonic foraminifera species by electron microprobe mapping. *Geochim. Geophys. Geosyst.* 2005, 6. [CrossRef]

22. Montagna, P.; McCulloch, M; Mazzoli, C.; Silenzi, S.; Odorico, R. The non-tropical coral *Cladocora caespitosa* as the new climate archive for the Mediterranean: High-resolution (~weekly) trace element systematics. *Quat. Sci. Rev.* 2007, 26, 441–462. [CrossRef]

23. Sadekov, A.; Eggins, S.M.; De Deckker, P.; Ninnemann, U.; Kuhnt, W.; Bassinot, F. Surface and subsurface seawater temperature reconstruction using Mg/Ca microanalysis of planktonic foraminifera *Globigerinoides ruber*, *Globigerinoides sacculifer*, and *Pulleniatina obliquiloculata*. *Paleoecol.* 2009, 24, PA3201.

24. Long, K.; Stern, N.; Williams, I.S.; Kinsley, L.; Wood, R.; Sporici, K.; Fallon, S.; Kokkonen, H.; Motflh, I.; Grün, R. Fish otolith geochemistry, environmental conditions and human occupation at Lake Mungo, Australia. *Quaternary Sci. Rev.* 2014, 88, 82–95. [CrossRef]

25. Fowler, A.M.; Smith, S.M.; Booth, D.J.; Stewart, J. Partial migration of grey mullet (*Mugil cephalus*) on Australia’s east coast revealed by otolith chemistry. *Mar. Environ. Res.* 2016, 119, 238–244. [CrossRef]

26. Gillanders, B.M. Using elemental chemistry of fish otoliths to determine connectivity between estuarine and coastal habitats. *Estuar. Coast. Shelf Sci.* 2005, 64, 47–57. [CrossRef]

27. Gillikin, D.P.; Wanamaker, A.D.; Andrus, C.F.T. Chemical sclerochronology. *Chem. Geol.* 2019, 526. [CrossRef]

28. Secor, D.H.; Rooker, J.R. Is otolith strontium a useful scalar of life-cycles in estuarine fishes? *Fish. Res.* 2000, 46, 359–371. [CrossRef]

29. Kraus, R.T.; Secor, D.H. Dynamics of white perch *Morone americana* population contingents in the Patuxent River estuary, Maryland, USA. *Mar. Ecol. Prog. Ser.* 2004, 279, 247–259. [CrossRef]

30. Tabouret, H.; Lord, C.; Bareille, G.; Pavillon, F.; Monti, D.; Keith, P. Otolith microchemistry in *Sicydium punctatum*: Indices of environmental condition changes after recruitment. *Aqua. Lim. Res.* 2011, 24, 369–378. [CrossRef]

31. Izzo, C.; Reis-Santos, P.; Gillanders, B.M. Otolith chemistry does not just reflect environmental conditions: A meta-analytic evaluation. *Fish Fish.* 2018, 19, 441–454. [CrossRef]

32. Green, B.C.; Smith, D.J.; Earley, S.E.; Hepburn, L.J.; Underwood, G.J.C. Seasonal changes in community composition and trophic structure of fish populations of five salt marshes along the Essex coastline, United Kingdom. *Estuar. Coast. Shelf. Sci.* 2009, 85, 1–10. [CrossRef]

33. Correira, A.T.; Pipac, T.; Gonçalves, J.M.S.; Erzini, K.; Hamer, P.A. Insights into population structure of *Diplodus vulgaris* along the SW Portuguese coast from otolith elemental signatures. *Fish. Res.* 2011, 111, 82–91. [CrossRef]

34. Bouchoucha, M.; Pécheyran, C.; Gonzalez, J.L.; Lenfant, P.; Darnaude, A.M. Otolith fingerprints as natural tags to identify juvenile fish life in ports. *Estuar. Coast. Shelf Sci.* 2018, 212, 210–218. [CrossRef]

35. Hobson, K.A. Tracing origins and migration of wildlife using stable isotopes: A review. *Oecologia.* 1999, 120, 314–326. [CrossRef]

36. Herzka, S.Z. Assessing connectivity of estuarine fishes based on stable isotope ratio analysis. *Estuar. Coast. Shelf Sci.* 2005, 64, 58–69. [CrossRef]

37. Trueman, C.N.; Mackenzie, K.M.; Palmer, M.R. Identifying migrations in marine fishes through stable-isotope analysis. *J. Fish Biol.* 2012, 81, 826–847. [CrossRef]

38. Suzuki, K.W.; Kasai, A.; Ohta, T.; Nakayama, K.; Tanaka, M. Migration of Japanese temperate bass *Lateolabrax japonicus* juveniles within the Chikugo River estuary revealed by δ13C analysis. *Mar. Ecol. Prog. Ser.* 2008, 358, 246–256. [CrossRef]

39. Verweij, M.C.; Nagelkerken, I.; Hans, I.; Ruseler, S.M.; Mason, P.R.D. Seagrass nurseries contribute to coral reef fish populations. *Limnol. Oceanogr.* 2008, 53, 1540–1547. [CrossRef]

40. Green, B.C.; Smith, D.J.; Grey, J.; Underwood, G.J.C. High site fidelity and low site connectivity in temperate salt marsh fish populations: A stable isotope approach. *Oecologia* 2012, 168, 245–255. [CrossRef]

41. Vinagre, C.; Salgado, J.; Costa, M.J.; Cabral, H.N. Nursery fidelity, food web interactions and primary sources of nutrition of the juveniles of *Solea solea* and *S. senegalensis* in the Tagus estuary (Portugal): A stable isotope approach. *Estuar. Coast. Shelf Sci.* 2008, 76, 255–264. [CrossRef]
42. Abecasis, D.; Bentes, D.; Erzini, K. Home range, residency and movements of Diplodus sargus and Diplodus vulgaris in a coastal lagoon: Connectivity between nursery and adult habitats. *Estuar. Coast. Shelf Sci.* 2009, 85, 35–529. [CrossRef]

43. Fry, B. Using stable isotopes to monitor watershed influences on aquatic trophodynamics. *Can. J. Fish. Aquat. Sci.* 1999, 56, 2167–2171.

44. Rubenstein, D.R.; Hobson, K.A. From birds to butterflies: Animal movement patterns and stable isotopes. *Trends Ecol. Evol.* 2004, 19, 256–263. [CrossRef] [PubMed]

45. Lawton, R.J.; Wing, S.R.; Lewis, A.M. Evidence for discrete subpopulations of sea perch (*Helicolenus eucnidos*) across four fjords in Fjordland, New Zealand. *New Zealand J. Mar. Freshw. Res.* 2010, 44, 309–322. [CrossRef]

46. Dierking, J.; Morat, F.; Letourneur, Y.; Harmelin-Vivien, M. Fingerprints of lagoon life: Migration of the marine flatfish *Solea solea* assessed by stable isotopes and otolith microchemistry. *Estuar. Coast. Shelf Sci.* 2012, 104, 23–32. [CrossRef]

47. Fodrie, F.J.; Herzka, S.Z. A Comparison of Otolith Geochemistry and Stable Isotope Markers to Track Fish Movement: Describing Estuarine Ingress by Larval and Post-Larval Halibut. *Estuar. Coast.* 2013, 36, 906–917.

48. Marenco, M.; Durieux, E.D.H.; Marchand, B.; Francour, P. A review of biology, fisheries and population structure of *Dentex dentex* (Sparidae). *Rev. Fish. Biol. Fisheries* 2014, 24, 1065–1088. [p. 479]

49. Vanegas, L.A.; Rolls, H.J. Using otolith microchemistry to assess nursery habitat contribution and function at a fine spatial scale. *Mar. Ecol. Prog. Ser.* 2018, 606, 151–173. [CrossRef]

50. Ley, L.A.; Rolls, H.J. Using otolith microchemistry to assess nursery habitat contribution and function at a fine spatial scale. *Mar. Ecol. Prog. Ser.* 2018, 606, 151–173. [CrossRef]

51. Dimanach, P. Contribution de la Biologie et de l’élevage de 6 Sparidés Mediterranéens: *Sparus aurata*, *Diplodus sargus*, *Diplodus vulgaris*, *Diplodus annularis*, *Lithognathus mormyrus*, *Puntazzo puntazzo* (Poissons Téléostéens). Thèse d’Etat, Université des Sciences et Techniques de Languedoc, Montpellier, France, 1985; p. 479.

52. MacPherson, E. Ontogenetic shifts in habitat use and aggregation in juvenile sparid fishes. *J. Exp. Mar. Bio. Ecol.* 1998, 220, 127–150.

53. Vigliola, L.; Harmelin-Vivien, M.L.; Biagi, E.; Galzin, R.; Garcia-Rubies, A.; Harmelin, J.G.; Jouvenel, J.Y.; Le Direach-Boursier, L.; Macpherson, E.; Tunesi, L. Spatial and temporal patterns of settlement among sparid fishes of the genus *Diplodus* in the north-western Mediterranean. *Mar. Ecol. Prog. Ser.* 1998, 168, 45–56. [CrossRef]

54. Mouine, N.; Francour, P.; Kari, M.H.; Chakroun-Marzouk, N. Reproductive biology of four *Diplodus* species *Diplodus vulgaris*, *D. annularis*, *D. sargus sargus* and *D. puntazzo* (Sparidae) in the Gulf of Tunis (central Mediterranean). *J. Mar. Biol. Ass. UK.* 2012, 92, 623–631.

55. Dulčić, J.; Krkaljević, M.; Grbec, B.; Pallaoro, A. Composition and temporal fluctuations of inshore juvenile fish populations in the Kornati Archipelago, eastern Mediterranean. *Mar. Biol.* 1997, 129, 267–277. [CrossRef]

56. Di Franco, A.; Bulleri, F.; Pennetta, A.; De Benedetto, G.; Clarke, K.R.; Guidetti, P. Within-Otolith Variability in Chemical Fingerprints: Implications for Sampling Designs and Possible Environmental Interpretation. *PLoS ONE* 2014, 9, e101701. [CrossRef]

57. Vasconcelos, R.P.; Reis-Santos, P.; Maia, A.; Fonseca, V.; França, S.; Wouters, N.; Costa, M.J.; Cabral, H.N. Nursery use patterns of commercially important marine fish species in estuarine systems along the Portuguese coast. *Estuar. Coast. Shelf Sci.* 2010, 86, 613–624. [CrossRef]

58. Zeigler, J.M.; Whitledge, G.W. Otolith trace element and stable isotopic compositions differentiate fishes from the Middle Mississippi River, its tributaries, and floodplain lakes. *Hydrobiologia* 2011, 661, 289–302. [CrossRef]

59. Gibb, F.M.; Régnier, T.; Donald, K.; Wright, P.J. Connectivity in the early life history of sandeel inferred from otolith microchemistry. *J. Sea Res.* 2017, 119, 8–16. [CrossRef]

60. Avigliano, E.; Pisonero, J.; Doméncio, A.; Silva, N.; Sánchez, S.; Vanina Volpedo, A. Spatial segregation and connectivity in young and adult stages of *Megaleporinus obtusidens* inferred from otolith elemental signatures: Implications for management. *Mar. Biol.* 2018, 204, 239–244. [CrossRef]

61. Ley, L.A.; Rolls, H.J. Using otolith microchemistry to assess nursery habitat contribution and function at a fine spatial scale. *Mar. Ecol. Prog. Ser.* 2018, 606, 151–173. [CrossRef]

62. Dulčić, J.; Matić, S.; Krkaljević, M. Shallow covens as nurseries for non-resident fish: A case study in the eastern middle Adriatic. *J. Mar. Biol. Ass. U.K.* 2002, 82, 991–993. [CrossRef]

63. Dulčić, J.; Matić-Skoko, S.; Krkaljević, M.; Fencil, M.; Glamuzina, B. Seasonality of a fish assemblage in shallow waters of Dučė- Glava, eastern middle Adriatic. *Cybium* 2005, 29, 57–63.

64. Matić-Skoko, S.; Krkaljević, M.; Dulčić, J.; Pallaoro, A.; Lučić, D.; Glamuzina, B. Growth of juvenile sharpnose seabream, *Diplodus puntazzo* (Teleostei: Sparidae) in the Kornati Archipelago, eastern Adriatic Sea. *Vie Milieu.* 2007, 57, 13–19.

65. Fukumori, K.; Oi, M.; Doi, H.; Takahashi, D.; Okuda, N.; Miller, T.W. Bivalve tissue as a carbon and nitrogen isotope baseline indicator in coastal ecosystems. *Estuar. Coast. Shelf Sci.* 2008, 79, 45–50. [CrossRef]

66. Post, D.M. Using stable isotopes to estimate trophic position: Models, methods, and assumptions. *Ecology* 2002, 83, 703–718. [CrossRef]

67. Post, D.M.; Layman, C.A.; Arrington, D.A.; Takimoto, G.; Quattrochi, J.; Montaña, C.G. Getting to the fat of the matter: Models, methods and assumptions for dealing with lipids in stable isotope analyses. *Ecology* 2007, 152, 179–189. [CrossRef]

68. Available online: [http://geoem.mpch-mainz.gwdg.de/](http://geoem.mpch-mainz.gwdg.de/) (accessed on 15 May 2020).

69. Jochum, K.P.; Nohl, U.; Herwig, K.; Lammel, E.; Stoll, B.; Hofmann, A.W. GeoReM: A new geochemical database for reference materials and isotopic standards. *Geostand. Geoanalytical Res.* 2005, 29, 333–338. [CrossRef]
68. Jochum, K.P.; Weis, U.; Stoll, B.; Kuzmin, D.; Yang, Q.; Raczek, I.; Jacob, D.E.; Stracke, A.; Birbaum, K.; Frick, D.A.; et al. Determination of reference values for NIST SRM 610-617 glasses following ISO guidelines. Geostand. Geoanalytical Res. 2011, 36, 397–429. [CrossRef]

69. Jochum, K.P.; Scholz, D.; Stoll, B.; Weis, U.; Wilson, S.A.; Yang, Q.; Schwalb, A.; Börner, N.; Jacob, D.E.; Andreae, M.O. Accurate trace element analysis of speleothems and biogenic calcium carbonates by LA-ICP-MS. Chem. Geol. 2012, 318–319, 31–44. [CrossRef]

70. Jochum, K.P.; Stoll, B.; Herwig, K.; Willbold, M. Validation of LA-ICP-MS trace element analysis of geological glasses using a new solid-state 193 nm Nd:YAG laser and matrix-matched calibration. J. Anal. At. Spectrom. 2007, 22, 112–121. [CrossRef]

71. Mishel, S.A.; Mertz-Kraus, R.; Jochum, K.P.; Scholz, D. TERMITE: An R script for fast reduction of laser ablation inductively coupled plasma mass spectrometry data and its application to trace element measurements. Rapid Commun. Mass Spectrom. 2017, 131, 1079–1087. [CrossRef]

72. Yoshinaga, J.; Nakama, A.; Morita, M.; Edmonds, J.S. Fish otolith reference material for quality assurance of chemical analyses. Mar. Chem. 2000, 69, 91–97. [CrossRef]

73. Sharp, Z. Principles of stable isotope geochemistry. Choice Rev. Online. 2007. [CrossRef]

74. Vrdoljak, D.; Matić-Skoko, S.; Peharda, M.; Edmonds, J.S. Fish otolith reference material for quality assurance of chemical analyses. Mar. Chem. 2000, 69, 91–97. [CrossRef]

75. Marali, S.; Schöne, B.R.; Mertz-Kraus, R.; Griffin, S.M.; Wanamaker, A.D., Jr.; Butler, P.G. Reproducibility of trace element analysis of speleothems and biogenic calcium carbonates by LA-ICP-MS. Palaeogeogr. Palaeoclimatol. Palaeoecol. 2017, 484, 109–128. [CrossRef]

76. Marali, S.; Schöne, B.R.; Mertz-Kraus, R.; Griffin, S.M.; Wanamaker, A.D., Jr.; Matras, U. Ba/Ca ratios in shells of Arctica islandica—a LA-ICP-MS line scan study. Palaeogeogr. Palaeoclimatol. Palaeoecol. 2000, 160, 111695. [CrossRef] [PubMed]

77. Kraljević, M.; Matić-Skoko, S.; Dulčić, J.; Pallaoro, A.; Jardas, I.; Glamuzina, B. Age and growth of sharpsnout seabream (Pseudopleuronectes americanus) from the eastern Adriatic Sea (Croatian coast). J. Appl. Ichthyol. 2011, 27, 1254–1258. [CrossRef]

78. Dulčić, J.; Pallaoro, A.; Matić-Skoko, S.; Dragičević, B.; Tutman, P.; Grgićević, J.; Kovač, Ž.; Janev, B. Age and growth of sharpsnout seabream Diplodus puntazzo (Cetti, 1777) in the eastern Adriatic Sea. Cah. Biol. Mar. 2007, 48, 145–154. [CrossRef]

79. Anderson, M.J.; Willis, T.J. Canonical analysis of principal coordinates: A useful method of constrained ordination for ecology. Ecology 2003, 84, 511–525. [CrossRef]

80. Miller, J.A. Effects of water temperature and barium concentration on otolith composition along a salinity gradient: Implications for migratory reconstructions. J. Exp. Mar. Bio. Ecol. 2011, 405, 42–52. [CrossRef]

81. Herrera-Reveles, A.T.; Lemus, M.; Marín, B.; Prin, J.L. Trace metal incorporation in otoliths of a territorial coral reef fish (Abudelfalu saxatilis) as an environmental monitoring tool. E3S Web Conf. 2013, 1, 34007. [CrossRef]

82. Sturrock, A.M.; Trueman, C.N.; Milton, J.A.; Waring, C.P.; Cooper, M.J.; Hunter, E. Physiological influences can outweigh environmental signals in otolith microchemistry research. Mar. Ecol. Prog. Ser. 2014, 500, 245–264. [CrossRef]

83. Markulin, K.; Peharda, M.; Mertz-Kraus, R.; Schöne, B.R.; Uvanović, H.; Kovač, Ž.; Janev, B. Age and growth of sharpsnout seabream Diplodus puntazzo (Cetti, 1777) in the eastern Adriatic Sea. Cah. Biol. Mar. 2007, 48, 145–154. [CrossRef]

84. Kalish, J.M. Otolith chemistry: Validation of the effects of physiology, age and environment on otolith composition. J. Exp. Mar. Biol. Ecol. 1989, 132, 151–178. [CrossRef]

85. Kalish, J.M. Determinants of otolith chemistry: Seasonal variation in the composition of blood plasma, endolymph and otoliths of bearded rock cod Pseudophycis barbatus. Mar. Ecol. Prog. Ser. 1991, 74, 137–159. [CrossRef]

86. Radtké, R.L.; Shafer, D.J. Environmental sensitivity of fish otolith microchemistry. Aust. J. Mar. Freshwater Res. 1992, 43, 935–951. [CrossRef]

87. Sadovy, Y.; Severin, K. Elemental patterns in Red Hind (Epinephelus guttatus) otoliths from Bermuda and Puerto Rico reflect growth rate, not temperature. Can. J. Fish. Aquat. Sci. 1994, 51, 133–141. [CrossRef]

88. Tzeng, W.N. Temperature effects on the incorporation on strontium in otoliths of Japanese eel Anguilla japonica. J. Fish Biol. 1994, 45, 1055–1066. [CrossRef]

89. Campana, S.E. Chemical and composition of fish otoliths: Pathways, mechanisms and applications. Mar. Ecol. Prog. Ser. 1999, 188, 263–297. [CrossRef]

90. Elsdon, T.S.; Gillanders, B.M. Reconstructing migratory patterns of fish based on environmental influences on otolith chemistry. Rev. Fish Biol. Fisher. 2003, 13, 219–235. [CrossRef]

91. Hamer, P.A.; Jenkins, G.P.; Coutin, P. Barium variation in Pagrus auratus (Sparidae) otoliths: A potential indicator of migration between an embayment and ocean waters in south-eastern Australia. Estuar. Coast. Shelf Sci. 2006, 68, 686–702. [CrossRef]

92. Walther, B.D.; Thorrold, S.R. Water, not food, contributes the majority of strontium and barium deposited in the otoliths of a marine fish. Mar. Ecol. Prog. Ser. 2006, 311, 125–130. [CrossRef]

93. Davis, W.J. Contamination of coastal versus open ocean surface waters: A brief meta-analysis. Mar. Pollut. Bull. 1993, 26, 128–134. [CrossRef]

94. Patterson, H.M.; Thorrold, S.R.; Shenker, J.M. Analysis of otolith chemistry in Nassau grouper (Epinephelus striatus) from the Bahamas and Belize using solution-based ICP-MS. Coral Reefs 1999, 18, 171–178. [CrossRef]
95. Patterson, H.M.; Kingsford, M.J.; McCulloch, M.T. Elemental signatures of Pomacentrus coelestis otoliths at multiple spatial scales on the Great Barrier Reef, Australia. *Mar. Ecol. Prog. Ser.* 2004, 270, 229–239.

96. Elsdon, T.S.; Gillanders, B.M. Temporal variability in strontium, calcium, barium, and manganese in estuaries: Implications for reconstructing environmental histories of fish from chemicals in calcified structures. *Estuar. Coast. Shelf Sci.* 2006, 66, 147–156.

97. Bath, G.E.; Thorrold, S.R.; Jones, C.M.; Campana, S.E.; McLaren, J.W.; Lam, J.W.H. Strontium and barium uptake in aragonitic otoliths of marine fish. *Geochim. Cosmochim. Acta.* 2000, 64, 1705–1714. [CrossRef]

98. Grønkjær, P. Otoliths as individual indicators: A reappraisal of the link between fish physiology and otolith characteristics. *Mar. Fresh. Res.* 2006, 57, 881–888. [CrossRef]

99. Fry, B.; Baltz, D.M.; Benfield, M.C.; Fleeger, J.W.; Gace, A.; Haas, H.L.; Quiñones-Rivera, Z.J. Stable isotope indicators of movement and residency for brown shrimp (Farfantepenaeus aztecus) in coastal Louisiana marshscapes. *Estuaries* 2003, 26, 82–97. [CrossRef]

100. Schlacher, T.A.; Liddell, B.; Gaston, T.F.; Schlacher-Hoenlinger, M. Fish track wastewater pollution to estuaries. *Oecologia* 2005, 144, 570–584. [PubMed]

101. França, S.; Vasconcelos, R.P.; Tanner, S.; Maguas, C.; Costa, M.J.; Cabral, H.N. Assessing food web dynamics and relative importance of organic matter sources for fish species in two Portuguese estuaries: A stable isotope approach. *Mar. Environ. Res.* 2011, 72, 204–215. [CrossRef]

102. Hesslein, R.H.; Hallard, K.A.; Ramlal, P. Replacement of sulfur, carbon, and nitrogen in tissue of growing broad whitefish (Coregonus nasus) in response to a change in diet traced by δ34S, δ13C, and δ15N. *Can. J. Fish. Aquat. Sci.* 1993, 50, 2071–2076. [CrossRef]

103. MacNeil, M.A.; Drouillard, K.G.; Fisk, A.T. Variable uptake and elimination of stable nitrogen isotopes between tissues in fish. *Can. J. Fish. Aquat. Sci.* 2006, 63, 345–353.

104. Yoshioka, T.; Wada, E. A stable isotope study on seasonal food web dynamics in a eutrophic lake. *Ecology* 1994, 75, 835–846.

105. Elliot, M.; Quintino, V. The Estuarine Quality Paradox, Environmental Homeostasis and the difficulty of detecting anthropogenic stress in naturally stressed areas. *Mar. Poll. Bull.* 2007, 54, 640–645.

106. Nunn, A.D.; Tewson, L.H.; Cowx, I.G. The foraging ecology of larval and juvenile fishes. *Rev. Fish Biol. Fisheries* 2012, 22, 377–408. [CrossRef]

107. van Leeuwen, A.; Huss, M.; Gårdmark, A.; Casini, M.; Vitale, F.; Hjelm, J.; Persson, L.; de Roos, A.M. Predators with multiple ontogenetic niche shifts have limited potential for population growth and top-down control of their prey. *American Naturalist.* 2013, 182, 53–66. [CrossRef]

108. Buljan, M.; Zore-Armanda, M. Oceanographic properties of the Adriatic Sea. *Oceanogr. Mar. Biol. Ann.* 1976, 14, 11–98.