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SASTAV ELEMENATA U OTOLITIMA: IZAZOVI I MOGUĆNOSTI ZA REKONSTRUIRANJE EKOLOŠKIH VEZA IZMEĐU STANIŠTA I VRSTA

DOKTORSKI RAD

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ELEMENTAL COMPOSITION OF OTOLITHS: CHALLENGES AND OPPORTUNITIES FOR RECONSTRUCTING ECOLOGICAL LINKS BETWEEN HABITATS AND SPECIES

DOCTORAL THESIS

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Ovaj je doktorski rad izrađen u sklopu Sveučilišnog poslijediplomskog doktorskog studija Oceanologije na Geološkom odsjeku Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu pod vodstvom dr. sc. Sanje Matić-Skoko. Istraživanje je provedeno u okviru projekta „Priobalna rastilišta: obrasci i procesi demografske varijabilnosti morskih riba uzduž istočnojadranske obale (NurseFish)“, koji podržava Hrvatska zaklada za znanost (projekt broj HRZZ-IP-2016-06-9884; glavni istraživač dr. sc. Sanja Matić-Skoko). Eksperimentalni dijelovi istraživanja provedeni su na Institutu za geoznanost Sveučilišta Johannes Gutenberg u Mainzu (JGU, Mainz, Institute for Geosciences, Njemačka); Odjelu za analizu stabilnih izotopa Sveučilišta Kalifornija u Davisu (UC, Davis, Stable Isotope Facility, SAD) i Odjelu za biomolekularnu fiziku Sveučilišta Babes-Bolyai u Cluju-Napoci (Babes-Bolyai University, Cluj-Napoca, Biomolecular Physics Department, Rumunjska), dok su se priprema uzoraka, analiza podataka i pisanje doktorskog rada obavili na Institutu za oceanografiju i ribarstvo, u Splitu, u Hrvatskoj.

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Sveučilište u Zagrebu Doktorski rad
Prirodoslovno-matematički fakultet
Geološki odsjek

SASTAV ELEMENATA U OTOLITIMA: IZAZOVI I MOGUĆNOSTI ZA REKONSTRUIRANJE EKOLOŠKIH VEZA IZMEĐU STANIŠTA I VRSTA

DARIO VRDOLJAK
Prirodoslovno-matematički fakultet, Geološki odsjek

Utvrđeni su elementni (kemijski) sastavi otolita odabranih vrsta riba porodice ljuskavki (Sparidae) pomoću laserske ablacije – induktivno spregnutom plazmatskom masenom spektrometrijom (LA-ICPMS), kristalna struktura otolita komarče, *Sparus aurata* pomoću konfokalne Ramanove mikrospektroskopije (CRM) te stabilni izotopi u mišićnom tkivu pica, *Diplodus puntazzo* i fratra, *Diplodus vulgaris*. Aragonit je glavna komponenta otolita komarče, a orijentacije kristala te tragovi kalcita i stroncijanita potvrdili su zone prirasta. Prikazan je obrazac korištenja estuarijskih i morskih rastilišta te povezanost koncentracije kemijskih elemenata i dijelova otolita. Inovativno kombiniranje elementnih sastava i stabilnih izotopa pokazalo je trofičke razlike kod pica i fratra pri korištenju morskog i estuarijskog rastilišta. Potencijalno je negativna izloženost onečišćenju na ušću Neretve utvrđena kod nedoraslih komarči s individualno povišenim koncentracijama Pb. Korištenjem multivarijatnih i kanonskih analiza uspješno su realocirani pic i fratar u poznata rastilišta na osnovi koncentracija Ba i Sr. Nešto je niža uspješnost postignuta u realokaciji nedoraslih komarči na osnovi koncentracija Sr, Zn i Mg. Daljnji razvoj sklerokronoloških istraživanja, uz utvrđene prepreke i nedostatke, dovest će do njihove šire primjene u Sredozemnome moru i omogućiti bolje razumijevanje ekoloških pokretača promjena u morskom okolišu.

(150 stranica, 4 slike, 359 literaturna navoda, jezik izvornika: hrvatski)
Ključne riječi: kemijski sastav otolita, stabilni izotopi, ribe, rastilišta, sklerokronologija
Mentor: dr. sc. Sanja Matić-Skoko, znanstvena savjetnica u trajnom zvanju
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dr. sc. Melita Peharda Uljević, znanstvena savjetnica u trajnom zvanju
doc. dr. sc. Zoran Marčić

University of Zagreb Doctoral thesis
Faculty of Science
Department of Geology

ELEMENTAL COMPOSITION OF OTOLITHS: CHALLENGES AND OPPORTUNITIES FOR RECONSTRUCTING ECOLOGICAL LINKS BETWEEN HABITATS AND SPECIES

DARIO VRDOLJAK

Faculty of Science, Department of Geology

The elemental (chemical) compositions of otoliths of selected Sparidae species using Laser ablation – Inductively coupled plasma mass spectrometry (LA-ICPMS), otolith crystal structure of gilthead seabream, *Sparus aura*ta using confocal Raman microscope (CRM) and stable isotope of muscle tissue of sharpsnout seabream, *Diplodus puntazzo* and common two-banded seabream, *Diplodus vulga*ris were studied. Aragonite is the main component of seabream otoliths while crystal orientations and calcite and strontianite traces have confirmed growth zones. The pattern of estuarine and marine habitats uses and relationships between the concentration of chemical elements and parts of otoliths are shown. An innovative combination of elemental compositions and stable isotopes indicates trophic differences in *D. puntazzo* and *D. vulgaris* when using marine and estuarine habitat. Potentially negative exposure to pollution at Neretva estuary was found in juvenile seabreams with individually elevated Pb concentrations. Using multivariate and canonical analyzes, *D. puntazzo* and *D. vulgaris* were successfully reallocated to known habitats based on Ba and Sr concentrations. Slightly lower success was achieved in the reallocation of juvenile seabream based on the concentrations of Sr, Zn and Mg. Further development of sclerochronological research, with identified obstacles and shortcomings, will enable its wider application in the Mediterranean Sea as a basis for a better understanding of the ecological drivers of change in the marine environment.

(150 pages, 4 figures, 359 references, original in Croatian)
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SADRŽAJ

POPIS ZNANSTVENIH RADOVA

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II. **Vrdoljak D**, Matić-Skoko S, Peharda M, Uvanović H, Markulin K, Mertz-Kraus R (2020) Otolith fingerprints reveals potential pollution exposure of newly settled juvenile *Sparus aurata*. Marine Pollution Bulletin 160: 111695. (IF = 5,553)

III. Lazar G, Firta C, Matić-Skoko S, Peharda M, **Vrdoljak D**, Uvanović H, Nekvapil F, Glamuzina B, Pinzaru S (2020) Tracking the growing rings in biogenic aragonite from fish otolith using confocal raman microspectroscopy and imaging. Studia Universitas Babes-Bolyai Chemia, LXV 1: 125-136. (IF = 0,447)

IV. **Vrdoljak D**, Matić-Skoko S, Peharda M, Uvanović H, Markulin K, Mertz-Kraus R, Grønkjær P (2021) Otolith fingerprints and tissue stable isotope information enable allocation of juvenile fishes to different nursery areas. Water 13: 1293. (IF = 3,103)

1. UVOD

1.1 Tvrde strukture morskih organizama

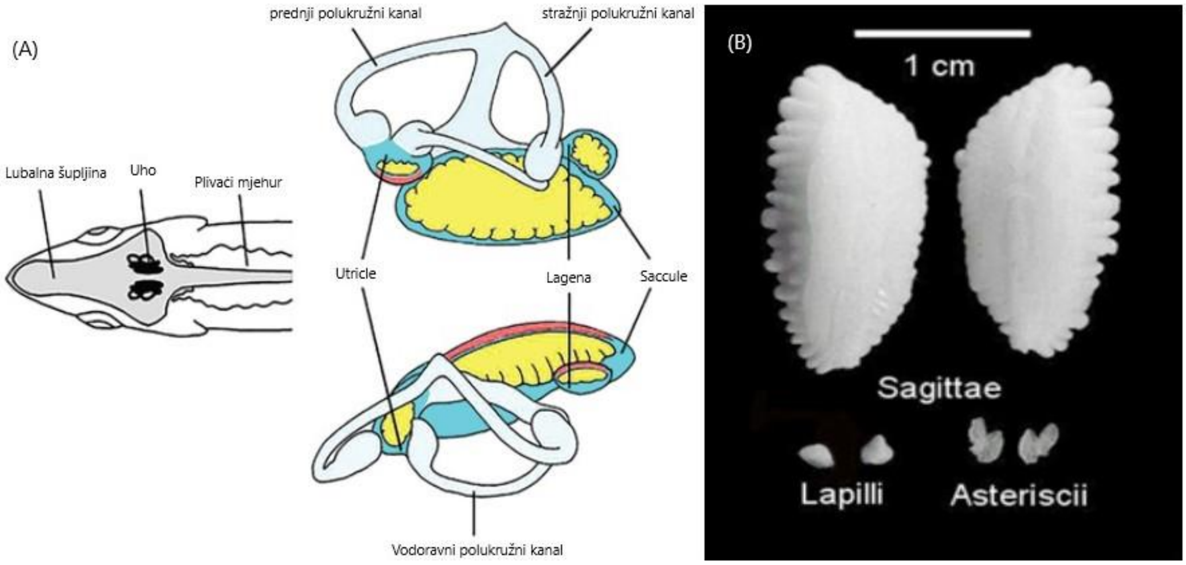
Kalcificirane, tvrde, strukture riba, koraligenih algi i nekih beskralježnjaka, poput koralja i ljuštura školjkaša, stvaraju se neprekidno tijekom života organizama i predstavljaju trajne zapise koji se mogu koristiti kao alat za rekonstruiranje okolišnih promjena u prostoru i tijekom vremena (npr. Hudson i sur., 1976.; Jones, 1983.; Black i sur., 2008.). Istraživanja tvrdih struktura morskih organizma ubrzano se razvijaju posljednja dva desetljeća i uključuju utvrđivanje njihovih morfoloških značajki te geokemijskog sastava. Dosad su provedena uglavnom na sjedilačkim organizmima, ponajprije na ljušturama školjkaša, a najistraživanija je vrsta školjkaša *Arctica islandica* (Linnaeus, 1767) (npr. Schöne, 2013.; Marali i sur., 2017.; Reynolds i sur., 2018.).

Ribe imaju nekoliko tvrdih struktura zanimljivih za morfološke i geokemijske analize, uključujući kralješke, otolite, ljuske i šipčice peraja (npr. Chilton i Beamish, 1982.; Panfili i sur., 2002.; Khan i Khan, 2009.). Iako je švedski svećenik Hederstörm (1759.) još u 18. stoljeću predložio brojenje prstenova na kralješcima riba za utvrđivanje starosti, ozbiljniji se pokušaji procjene starosti riba koristeći kralješke ipak nisu dogodili prije kraja 19. stoljeća (Panfili i sur., 2002.). Intenzivnija su istraživanja na tvrdim strukturama kod riba, posebice otolitima, započela prije 40 godina (Tzadik i sur., 2017.) i trenutno predstavljaju vrlo zanimljivu znanstvenu problematiku (npr. Panfili i sur., 2002.; Black i sur., 2005.; Grønkjær i sur., 2013.). U priručniku autora Panfili i sur. (2002.) detaljno su opisana morfologija i kemijski sastav tvrdih struktura kod riba te povezani mehanizmi regulacije taloženja i rasta. Poseban je doprinos za sve navedene aspekte kod otolita moguće pronaći u radovima Campana (1999.), Campana (2001.) i Campana (2005.). U ranijim radovima ovog autora i suradnika jasno je opisana mikrostruktura otolita (Campana i Neilson, 1985.; Campana i Jones, 1992.), dok su u kasnijim radovima detaljno pojašnjene mogućnosti primjene analize kemijskog sastava otolita u ribarstvene svrhe (npr. Campana i sur., 2000.; Campana i Thorrold, 2001.).

Od navedenih tvrdih struktura kod riba, otoliti se smatraju najpouzdanijima zbog njihova lakšeg načina pripreme za analizu, nedostatka resorpcije i činjenice da su metabolički inertne, aragonitne strukture sastavljene od kalcijeva karbonata ($CaCO_3$). Navedene osobine znače da organizam neće istaložene minerale ponovno koristiti čak ni u doba gladovanja (Campana i Neilson, 1985.; Campana i Thorrold, 2001.). Otoliti mogu sadržavati zapis koji u potpunosti odražava njihovu izloženost temperaturi i kemijskom sastavu okolne vode. Stoga je višegodišnjim proučavanjem rasta otolita prepoznat i njihov velik potencijal za otkrivanje detaljanog kronološkog zapisa okoliša kojem je riba bila izložena tijekom života (Campana, 1999.). Ostale se tvrde strukture kod riba, poput ljusaka, kralježaka i šipčica sve manje koriste i zbog njihova zahtjevnijeg načina pripreme za daljnje analize (Panfili i sur., 2002.) i zbog otežanog očitavanja prvog i kasnijih prstenova rasta (Tzadik i sur., 2017.), što ove strukture čini manje pouzdanima. Naime, upotreba kralježaka i šipčica zahtijeva prethodnu demineralizaciju i lipidnu ekstrakciju bjelančevina (Ankjærø i sur., 2012.), što usložnjava cijeli proces pripreme tih struktura za daljnje analize. S druge strane, ljuske riba koriste se više od stoljeća za određivanje starosti riba zbog relativne lakoće uzorkovanja i rukovanja (Tzadik i sur., 2017.). Velika je prednost uzorkovanje bez ranjavanja odnosno usmrćivanja organizma, što posebno dolazi do izražaja pri istraživanju ugroženih vrsta (Cano-Rocabayera i sur., 2014.), stoga su se ljuske riba počele koristiti kao prikladne strukture u pružanju biokemijskih oznaka riba te za rješavanje podrijetla i obrazaca kretanja populacija (Adey i sur., 2009.; Šegvić-Bubić i sur., 2020.).

1.2 Morfološke značajke otolita

Kod svih koštunjavih riba nalazimo tri para otolita koji se razlikuju po mjestu gdje su smješteni, funkciji, veličini, obliku i mikrostrukturi. Otoliti su smješteni u tri šupljine unutarnjeg uha (utriculus, sacculus i lagena). Najveći su par sagitalni otoliti (sagitae) smješteni u sacculusu (Slika 1a). Dva su manja para otolita lapilli (utricilus) i asteriscii (lagena) (Stevenson i Campana, 1992.; Elsdon i sur., 2008.) (Slika 1b). Svaka je šupljina obložena trepetljikavim osjetilnim stanicama unutar koje otoliti slobodno plutaju u endolimfatičkoj tekućini. Struktura i oblik otolita znatno kolebaju među vrstama. Dodatno, iako imaju trodimenzonalnu strukturu, otoliti ne rastu istom brzinom u svim smjerovima, stoga nisu ni pravilni ni simetrični po obliku (Campana i Thorrold, 2001.; Mendoza, 2006.).

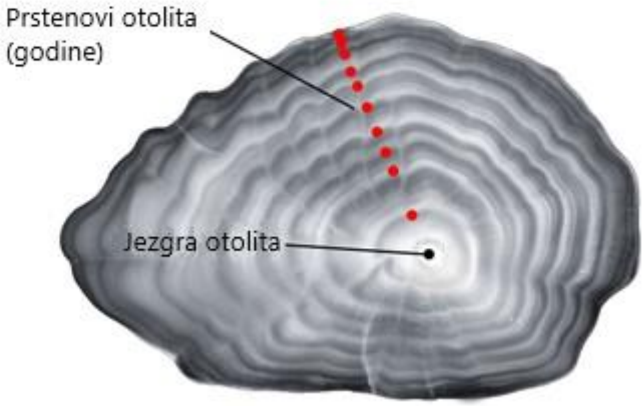


Slika 1. Otoliti koštunjavih riba razlikuju se po smještaju u šupljinama unutarnjeg uha (A) (prilagođeno iz rada Popper i Hawkins, 2019. za vrstu bakalar, *Gadus morhua* Linnaeus, 1758) te po vrsti otolita (B) kao npr. za saruna *Trachurus capensis*, Castelnau, 1861 (preuzeto iz rada Shivute, 2016.)

Morfološke karakteristike otolita znatno se razlikuju među pojedinim vrstama, ali i unutar istih vrsta koje žive u različitim geografskim područjima. Dvije su osnovne morfološke značajke otolita oblik i veličina (Slika 2). Oblik otolita izrazito je specifičan za vrstu i manje je promjenjiv tijekom rasta, odnosno života ribe, vjerojatno zbog dvostruke funkcije otolita kao organa ravnoteže i sluha (Popper i Zhonbmin, 2000.). Oblik koleba od relativno jednostavih kružnih oblika kao kod nekih vrsta plosnatica iz porodice Pleuronectidae do vrlo nepravilnih oblika koje nalazimo kod vrsta bodečnjaka iz porodice Sebastes (Hunt, 1992.). Zato je oblik otolita idealan prirodni zapis pojedine riblje populacije, stoga se široko koristi za idetinifikaciju vrsta i stokova riba (Campana, 1999.; Stransky i sur., 2005.). Veličina otolita, odnosno njegov rast, povezana je s rastom ribe, odnosno povećanjem veličine ribe i uglavnom slijedi alometrijski porast jedinke. Varijacije u veličini otolita mogu biti znatne i varirati u rasponu od svega nekoliko milimetara (npr. 0,4 mm za vrste iz porodice Coryphaenidae) do otolita velikih nekoliko centimetara (npr. 31,4 mm za vrste porodice sjenki, Scianidae) (Paxton, 2000.).

Slika 2. Oblici i veličina otolita kod vrsta porodice ljuskavki (*Sparus aurata*, *Diplodus puntazzo* i *Diplodus vulgaris*) prikupljenih u Jadranskom moru

Otolit se sastoji od niza koncentričnih kružnica različitih radijusa (Campana, 1999.; Morales-Nin, 2000a.; 2000b.; Black i sur., 2008.; Elsdon i sur., 2008.). Ovisno o količini organskog materijala istaloženog u pojedinoj zoni, zone otolita variraju od iznimno neprozirnih (svijetlih) pa do potpuno prozirnih (tamnih) (Mendoza, 2006.). Prva zona predstavlja jezgru otolita, nakon čega se izmjenjuju prozirne i neprozirne zone ovisno o razdoblju nastanka, a njihov broj ovisi o starosti ribe (Mendoza, 2006.) (Slika 3.). Neprozirne, svijetle zone u otolitu nastaju u razdoblju najvećeg rasta, dok prozirne, tamne zone nastaju za vrijeme najsporijeg rasta. Stoga je temeljna značajka otolita periodična izmjena svijetlih i tamnih zona rasta koje se nazivaju prstenovi rasta (Mendoza, 2006.) te predstavljaju dnevne ili godišnje priraste (Pannella, 1974.). Rast otolita uzrokovan je zajedničkom ovisnošću o metaboličkim procesima koji upravljaju rastom ribe i rastom otolita, što se objašnjava ontogenetskim promjenama u intezitetu hranjenja i ponašanja kroz stopu rasta i rizika od predacije (Bang i Grønkjær, 2005.). Na rast otolita utječe i genotip jedinke te njegov odgovor na promjenjive uvjete okoliša (Ross i sur., 2013.; Martens i sur., 2014.).



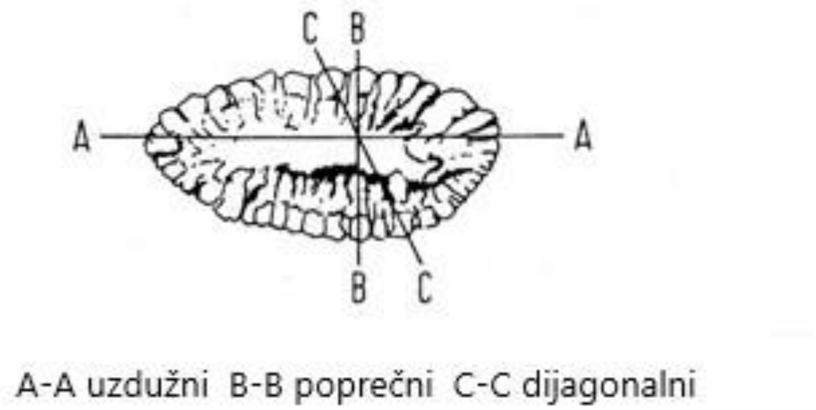
Slika 3. Otoliti sa zonama prirasta (prilagođeno iz https://www.offthescaleangling.ie/the-science-bit/fish-hearing/; Bill Brazer, 2017.)

Kod vrsta riba koje žive u različitim geografskim područjima specifični su procesi i čimbenici odgovorni za stvaranje zona prirasta. Većina se autora slaže kako i unutarnji i vanjski procesi utječu na njihovo stvaranje (Elsdon i Gillanders, 2002.; 2004.; Neuheimer i sur., 2011.) te kako je temperatura glavni i najsnažniji pokretač tih varijacija (Campana, 1999.; Grønkjær, 2016.). Razdoblje stvaranja zone prirasta kod neke određene vrste može varirati unutar njene geograske rasprostranjenosti te zbog demografskog stanja pojedinih subpopulacija unutar određenog areala (Grønkjær, 2016.). Pannella (1974.) prvi predlaže da dnevni prirasti u otolitima nastaju kod nekih umjerenih vrsta te u velikom broju kod otolita tropskih vrsta riba. Za određene vrste riba umjerenog pojasa, Pannella (1974.) je uspio pokazati postojanje dvotjednih, mjesečnih i godišnjih prirasta što potvrđuje periodične promjene debljine prirasta otolita. Tako u umjerenom pojasu neprozirna zona u otolitima nastaje sezonski, tijekom toplijeg dijela godine, kada ribe potaknute visokom temperaturom i pojačanim unosom hrane rastu brzo, dok prozirna zona nastaje u hladnom dijelu godine kao odgovor na smanjene stope hranjenja i niske temperature okoliša (Neat i sur., 2008.). S druge strane, kod nekih riba koje žive u područjima s manjim godišnjim kolebanjem temperature, poput tropskih i polarnih regija, često ne postoje vidljive zone rasta u strukturi otolita (Mendoza, 2006.). Zanimljivo je kako kod nekih dubinskih vrsta riba nalazimo istaknute zone prirasta na otolitima iako na velikim dubinama temperatura i svjetlo imaju znatno manju ulogu u njihovu stvaranju (Allain i Lorance, 2000.). Svakako se rast i neprozirnost otolita smatraju funkcijama fiziološkog stanja jedinke i okoline te se može povezati rast i preživljavanje jedinke s okolišnim uvjetima u kojima jedinka obitava. To znači da se okolišni signal može drukčije zabilježiti u otolitima dviju jedinki, ali i da osobine otolita odražavaju fiziološko stanje jedinke i ekološke učinke fenotipskih i genotipskih varijacija među jedinkama (Grønkjær, 2016.).

Stvaranje prozirne zone može se povezati i s proživljenim energetskim stresom jedinke, koji se posljedično odražava na usporavanje rasta (Grønkjær, 2016.). Tako je, na primjer, u tropskom području stvaranje zona prirasta na otolitima ovisno o količini hrane, što je pak uvjetovano sezonskim poplavama. Tijekom sezone poplava hrana je obilna i lako dostupna ribama te određena jedinka raste vrlo brzo stvarajući široku neprozirnu zonu, dok u suhoj sezoni hrana postaje rijetka, riba gladuje i doživljava energetski stres koji ima utjecaj na stvaranje uske prozirne zone prirasta (Holden i Raitt, 1974.; Mendoza, 2006.; Grønkjær, 2016.). Istraživanje rasta i neprozirnosti stoga doprinosi našoj spoznaji kako jedinka uspijeva rasti i preživjeti u okružju koje se stalno mijenja.

1.3 Tehnike pripreme otolita, očitavanja i metode validiranja periodičnosti stvaranja prstenova rasta

Jednostavne, no ponekad i vrlo složene tehnike pripreme i obrade otolita koriste se za određivanje morfologije otolita. Najjednostavnija je tehnika uranjanje cijelog otolita u bistru tekućinu, osvjetljavanje odozgo i promatranje na tamnoj pozadini svjetlosnog mikroskopa ili lupe (Campana i Neilson, 1985.). Ta je tehnika prikladna samo ako su otoliti relativno tanki i prozirni i ako se jasno mogu razlučiti svi prstenovi (Campana i Neilson, 1985.; Campana, 2001.). Otoliti se mogu ugraditi u epoksidnu smolu te se istodobno mogu pripremiti presjeci otolita, i to uzdužni, poprečni ili dijagonalni, ovisno o prethodno utvrđenoj prikladnosti kod pojedinih porodica i/ili vrsta (Campana, 2005.; Elsdon i sur., 2008.) (Slika 4). Presjek otolita obavlja se pomoću precizne pile za otolite opremljene dijamantnim brusom različite debljine.



Slika 4. Uzdužni, poprečni i dijagonalni presjek otolita bakalara *G. morhua* (prerađeno iz rada Henderson, 2017.)

Brušenje cijelog otolita ili presjeka otolita, zalijepljenog na predmetno stakalce pomoću neprozirnog ljepila, pomaže u otklanjanju nepravilnih prstenova koji otežavaju točno očitavanje prstenova otolita. Jednostavni brusni stroj omogućuje pripremu širokog raspona otolitskih sekcija radi lakšeg i točnijeg očitavanja otolita različitih veličina. Većina otolita manjih promjera te presjek otolita polira se nakon brušenja radi lakšeg očitavanja uskih prstenova rasta koji okružuju jezgru (Campana i Neilson, 1985.; Campana i Jones, 1992.). Pritom svakako treba paziti da se ne pretjera s brušenjem i poliranjem kako se ne bi nepovratno izgubili pojedini prstenovi.

Nevidljivi prstenovi rasta na otolitu kod nekih vrsta riba predstavljaju problem pri očitavanju, pa se tada može primijeniti tehnika spaljivanja otolita. Cijeli se otolit ili njegov poprečni presjek blago spali iznad vrlo slabog plamena, pri čemu prstenovi postaju vidljiviji zbog koagulacije bjelančevina (Christensen, 1964.). Upotreba tehnike spaljivanja omogućuje veću točnost određivanja starosti mnogih vrsta, osobito kod starijih jedinki koje većinom imaju zgusnute rubne prstenove rasta, zbog čega je dob često podcijenjena pri korištenju drugih tehnika obrade (Campana, 2001.; Campana i sur., 2015.).

Nakon očitavanja potrebno je validirati periodičnost stvaranja očitanih prstenova rasta. Postoje različite metode validacije očitane apsolutne starosti i stvaranja zona prirasta i detaljno su opisane u radu Campana (2001.) te unutra citiranim radovima. Markiranje ribe poznate starosti i njeno puštanje u divljinu te zatim njen ponovni ulov (engl. *Release of known age and* *marked fish*) najčešće je korištena metoda za potvrdu starosti čitanjem dnevnih i godišnjih prirasta zbog jednostavnosti provedbe iako metoda ima određene nedostatke. Puštene su ribe uglavnom mlađe od jedne godine te do ponovnog ulova provedu dovoljno vremena u prirodnom okružju kako bi se stvorile zone prirasta. Ta metoda nije prikladna za dugoživuće vrste zbog male vjerojatnosti ponovnog ulova kao ni za vrste koje ne mogu živjeti u zatočeništvu prije puštanja u divljinu. Støttrup i sur. (2002.) koristili su tu metodu za ispitivanje povećanja populacije romba *Psetta maxima* (Linnaeus, 1758) puštanjem uzgojenih jedinki u divljinu.

Metoda markiranja ribe kemijskim tvarima ili spojevima koji se vežu na kalcij u tvrdim strukturama, njihovo puštanje u divljinu i ponovni ulov (engl. *Mark-Recapture of chemically-tagged fish*), jedna je od učinkovitijih dostupnih metoda za potvrđivanje periodičnosti stvaranja prirasta. Metoda se temelji na brzoj ugradnji takvih tvari ili spojeva kao što su oksitetraciklin, alizarin, kalcein ili stroncij u kosti, ljuske, šipčice peraja i otolite jer se vežu na kalcij (Campana, 1999.). Rezultat je te metode trajno vidljiv trag pod fluorescentnim svjetlom mikroskopa. Nedostatak je te metode taj što je broj stvorenih prstenova nakon označavanja često nizak, što može dovesti do potencijalno velike relativne pogreške u validaciji ako se jedan od prirasta (poput onog na rastućem rubu) pogrešno protumači (Campana, 2001.). Nedostatak su i ozbiljne lezije, u nekim slučajevima i velika oštećenja peraja, što dovodi u pitanje korištenje te metode za validiranje periodičnosti stvaranja prstenova (Brouwer i Griffiths, 2004.).

Metoda ispitivanja ugradnje radioaktivnog ^{14}C ugljika (engl. *Bomb Radiocarbon*) je validacijska metoda koja se koristiti za točnu procjenu starosti morskih riba (Kalish, 1993.). Ta se metoda koristi za provjeru starosti riba tijekom i nakon povećanja koncentracije atmosferskog ^{14}C. Godina rođenja jedinke procjenjuje se usporedbom koncentracija radioaktivnog ugljika u jezgri otolita s koncentracijama u referentnoj seriji biogenih karbonata, poput kostura koralja (Campana, 2001.). Sanchez i sur. (2019.) koristili su tu metodu za procjenu starosti dugoživućih organizama porodice kirnji kod kojih je dob bila jako podcijenjena zbog poteškoća u razlikovanju prstena. Ta metoda nije pogodna za kratkoživuće vrste kod kojih kronologija starosti nije dostupna ili za one jedinke koje su „rođene“ nakon šezedesetih godina prošlog stoljeća zbog porasta atmosferskog ^{14}C nakon 1950-ih kada se on počeo ubrzano ugrađivati u tvrde strukture morskih organizama (Campana, 2001.).

Metoda koja se temelji na radioaktivnom raspadanju radioizotopa koji se pojavljuju u prirodi (^{210}Pb:^{226}Ra, ^{228}Th:^{228}Ra) (engl. *Radiochemical Dating*) i njihovoj ugradnji u otolit tijekom rasta najprikladnija je za dugoživuće vrste poput vrsta iz porodica bodečnjaka (Sebastes) ili Hoplostethus s većim brojem starosnih skupina zbog različitog vremena raspadanja pojedinih radioaktivnih elemenata. Naime, radioizotopi se nakon ugradnje raspadaju u sestrinske produkte, a kako je vrijeme poluraspada i navedenih radioizotopa i njihovih sestrinskih produkata poznato i fiksno, njihov omjer predstavlja indeks proteklog vremena od ugradnje roditeljskog radioizotopa te predstavlja osnovu za procjenu periodičnosti prirasta i modeliranje rasta. Nedostatak je te metode procjena starosti među zemljopisno odvojenim ribljim stokovima (Andrews i sur., 2011.)

Metoda za validaciju prirasta kod nedoraslih riba koja se temelji na mjerenju udaljenosti od jezgre do svakog pojedinog dnevnog prstena i/ili ruba otolita (engl. *Discrete length modes*) rijetko se primjenjuje za provjeru očitanih dnevnih prstenova kod nedoraslih riba. Praćenjem napredovanja diskretnih duljina među pojedinim prstenovima tijekom mjeseci u jednoj godini, relativno je lako odrediti odgovaraju li te duljine starosnim razredima (McQueen i sur., 2019.).

Metoda koja se temelji na lovu divlje ribe s prirodnim markerom poznatog vremenskog obilježja nastanka ili pojave na široj prostornoj skali (npr. godina obilježena snažnim *El Ninom* koja će dovesti do poremećaja rasta u toj specifičnoj godini) (engl. *Natural, date-specific markers*) služi za provjeru stvaranja prirasta, a temelji se na činjenici da je taj prirodni marker ostavio obilježje na svim jedinkama u populaciji. Takvi su markeri nedvojbeno prepoznatljivi u svakoj jedinki i mogu se pratiti duži niz godina (Hüssy i sur., 2021.).

Metoda koja se temelji na određivanju i mjerenju rubnog prirasta otolita (engl. *Marginal increment analysis*) najčešće je korištena metoda za provjeru periodičnosti prirasta u godišnjem (dnevnom) ciklusu jer bi krajnji prirast trebao prikazivati godišnji ili dnevni sinusoidalni ciklus kada se nacrta u odnosu na vrijeme (godišnje doba, doba dana). Razlozi široke uporabe te metode leže u njenoj nezahtjevnosti i niskim troškovima. Ipak, to je po mnogo čemu jedna od najkompleksnijih validacijskih metoda za pravilnu provedbu zbog tehničkih poteškoća povezanih s određivanjem djelomičnog prirasta pod utjecajem promjenljivog loma svjetlosti kroz rub koji postaje sve tanji na svom samom kraju, kao i zbog refleksije svjetlosti sa zakrivljene površine ruba otolita (Okamura i sur., 2013.).

Metoda validacije vremena stvaranja prstenova držanjem ribe u zatočeništvu (engl. *Captive rearing*) postaje sve češće korištena tehnika koja se koristi u studijama oporavka ugroženih populacija riba (Snyder i sur., 1996.), ali je općenito slabo prihvaćena metoda validacije nastanka prstenova rasta jer je iznimno skupa (Beck i sur., 1994.). Uzgojna, laboratorijska sredina može oponašati prirodni okoliš, no ipak umjetni fotoperiod, temperatura, ciklus hranjenja i ograničeni prostor za kretanje stvaraju umjetni okoliš koji ima sposobnost stvoriti umjetne prstenove na otolitu (Kelley i sur., 2005.).

Validirani podaci o očitanim prstenovima koriste se dalje u brojnim metodama za interpretaciju precizno određene starosti kojima prethodi niz dostupnih metoda validacije prvog i marginalnog prstena, koje su također predstavljene u Campana (2001.). Validirana starost i rast služe kao važni ulazni parametri za proučavanje dinamike populacija (Campana i Jones, 1992.). Dodatno se koriste još analize za povezivanje kronologije rasta koje se očituju u otolitu zbog utjecaja okoliša (Campana i Jones, 1992.) te proučavanje fizikalnih i kemijskih varijacija u tvrdim strukturama organizama (Panfili i sur., 2002.).

1.4 Elementni (kemijski) sastav otolita

Elementni (kemijski) se sastav otolita prema dosadašnjim istraživanjima sastoji od najmanje 31 elementa bez radioaktivnih elemenata kao što su torij (Th), radij (Ra) i uran (Ur). U sastavu dominiraju kalcij (Ca), kisik (O) i ugljik (C) koji čine matricu kalcijeva karbonata ($CaCO_3$) i gotovo 90 % cjelokupnog sastava otolita. Ostatak sastava čine elementi poput natrija (Na), stroncija (Sr), kalija (K), sumpora (S), dušika (N), klora (Cl) i fosfora (P), prisutni u niskoj koncentraciji (> 100 ppm) te elementi u tragovima s koncentracijom manjom od 10 ppm poput magnezija (Mg), silicija (Si), bora (B), željeza (Fe), mangana (Mn), barija (Ba), nikla (Ni), bakra (Cu), aluminija (Al), broma (Br), litija (Li), olova (Pb), arsena (As), cinka (Zn), srebra (Ag), kobalta (Co), kadmija (Cd), žive (Hg) i cezija (Cs) (Campana, 1999.).

Kemijskom analizom otolita nastoji se odvojiti kemijske elemente koji su dobar pokazatelj parametara okoliša nekog specifičnog područja, kao što su kemija vode, temperatura i slanost te elemente koji kontroliraju fiziologiju organizma. U morskom okružju Ba, Mg, U, B i Sr u različitim kalcitnim tkivima pokazuju snažne korelacije s temperaturom vode (Long i sur., 2014.), dok koncentracije elementa poput Sr i Ba odražavaju lokalnu dostupnost tih elemenata u morskoj vodi (Fowler i sur., 2016.). Tako su npr. Walther i Thorrold (2006.) objavili da 83 % Sr i 98 % Ba kod nedoraslih jedinki morske vrste *Fundulus heteroclitus* (Linnaeus, 1766), odnosno 88 % Sr kod jedinki slatkovodne vrste *Oreochromis niloticus* (Linnaeus, 1758) potječe iz okolne vode (Farrell i Campana, 1996.), pri čemu je preostali postotak vjerojatno nastao unosom hrane. Omjer Sr/Ca je u morskoj sredini slanosti 35 promila relativno konstantan, dok u slatkovodnom okolišu Sr i Ca pokazuju znatnu geografsku i vremensku varijabilnost. Općenito, omjer Sr/Ca je u slatkovodnom okolišu znatno niži od globalnog oceanskog omjera Sr/Ca, i to objašnjava činjenicu zašto je većina studija utvrdila pozitivnu korelaciju između omjera Sr/Ca u otolitu i slanosti okolne vode (Secor i Rooker, 2000.). U nekim je slučajevima omjer Sr/Ca u slatkoj vodi viši od omjera utvrđenog u morskoj vodi, što dovodi do negativne korelacije između omjera Sr/Ca u otolitu i slanosti (Limburg i Siegel, 2006.). Razlike u kemiji vode stoga mogu objasniti nepostojanje veze ili pozitivne odnosno negativne učinke slanosti okolne vode na koncentraciju Sr u otolitu (Elsdon i Gillanders, 2002.), što upućuje na važnost kritičkog vrednovanja kemije vode pri proučavanju različitih ekosustava.

Temperatura, neovisno o tome što utječe na stopu rasta, može utjecati i na asimilaciju nekih elemenata u otolitima. Temperatura općenito ima pozitivan utjecaj na omjer Sr/Ca u otolitu (Elsdon i Gillanders, 2002.; Martin i sur., 2004.). Učinak temperature na koncentraciju Ba, Mn i Mg varira od neznatnog utjecaja do izraženih pozitivnih ili negativnih korelacija u različitim ekosustavima. Utjecaj temperature na kemijski sastav otolita vjerojatno je posljedica fiziologije organizma (Townsend i sur., 1992.) i kinetičkih procesa u njemu (tj. temperature koji utječu na kristalografiju; Nielson i Christoffersen, 1982.). Drugi fiziološki važni elementi, kao što su P, Cu i S također ostaju neizmijenjeni naspram relativne koncentracije u okolišu (Campana, 1999.). Istovremeno, elementi poput K, Na, Zn i Mn su vjerojatno posredovani fiziološkom regulacijom (Bouchoucha i sur., 2018.). Glavni je izvor Zn u okolišu litosfera, posebno stijene u okolnoj vodi (Halden i sur., 2000.). Međutim, Zn je, poput Cu, Ni i Pb, također povezan i s antropogenim izvorima, uključujući odlagališta otpada, industrijske otpadne vode, odbačeni plastični otpad, protuobraštajne fosfatne boje za brodove, poljoprivredu i turističke aktivnosti (Cuevas i sur., 2019.; Nour i Nouh, 2020.), pa morski organizmi koji naseljavaju područja pogođena onečišćenjem vjerojatno akumuliraju elemente povezane sa zagađivalima te ih prenose u više karike hranidbenog lanca (Wang, 2002.). Iako priobalna staništa općenito nude veću kemijsku heterogenost zbog uzdizanja pridnene hladne vode, donosa rijeka te antropogenih unosa, često su onečišćivači koji bi pridonijeli takvim geografskim varijacijama (npr. Ni i Zn) fiziološki diskriminirani i obično ispod razine otkrivanja u otolitima, pa otoliti riba koje žive u priobalnom području ponekad ne odražavaju tu uočenu heterogenost u okolnoj vodi (Hanson i Zdanowicz, 1999.).

1.5 Stabilni izotopi u otolitima i mišićnom tkivu riba

Dodatne se informacije o tome kako morski organizmi, pa tako i ribe, koriste okoliš mogu dobiti analizom stabilnih izotopa (engl. *Stable isotope analysis*, SIA) njihovih tvrdih struktura, ponajprije otolita. Dodatno, analiza stabilnih izotopa mišićnog tkiva važan je alat za ekologe pri određivanju izvora energije i opisu strukture hranidbenih mreža (npr. Beaudoin i sur., 2001.). Najčešće su u otolitima analizirani stabilni izotopi kisika ($\delta^{18}O$), ugljika ($\delta^{13}C$), dušika ($\delta^{15}N$), stroncija ($\delta^{87}Sr$) i sumpora ($\delta^{34}S$), a u mišićnim tkivima stabilni izotopi ugljika ($\delta^{13}C$) i dušika ($\delta^{15}N$) te rjeđe kisika ($\delta^{18}O$) i sumpora ($\delta^{34}S$). Takvi podaci odražavaju ishranu riba i mogu se također koristiti za određivanje korištenja staništa (Vinagre i sur., 2008.; Green i sur., 2012.), kretanja iz ili unutar estuarija (Trueman i sur., 2012.) te otkrivanje migracijskih (Suzuki i sur., 2008.; Verweij i sur., 2008.) i okolišnih obrazaca (Disspain i sur., 2016.). Najčešće se određuju i njihove vrijednosti u okolišnoj vodi kako bi bilo moguće odrediti stopu i omjer njihova taloženja u otolitima i mišićnom tkivu.

Raspodjela stabilnih izotopa kisika u biogenim karbonatima poput otolita naširoko se koristi za procjenu paleotemperatura (Patterson, 1999.; Ivany i sur., 2000.; Carpenter i sur., 2003.). Omjer stabilnog izotopa kisika ($\delta^{18}O$) u otolitima u prvome se redu povezuje s temperaturom vode (Surge i Walker, 2005.; Rowell i sur., 2008.), a ovisi o izotopskoj vrijednosti omjera u okolnoj vodi u vrijeme ugradnje u otolit. Stoga posljedično može pružiti informacije o promjeni okoliša (Wurster i Patterson, 2001.; West i sur., 2011.; 2012.), sezonalnosti korištenja staništa (Hufthammer i sur., 2010.) i migracijskim obrascima (Torniainen sur., 2017). Kako se temperatura vode povećava, unos $\delta^{18}O$ u otolitima se smanjuje (Rowell i sur., 2008.) zbog smanjene topljivosti plinova pri povišenim temperaturama, a termodinamički odnosi rezultiraju činjenicom kako otoliti sadrže više lakših izotopa ^{16}O na višim temperaturama (Patterson i sur., 1993.). Stabilni izotopi kisika predstavljaju tragove pomoću kojih se mogu odrediti morski okolišni uvjeti u prošlosti jer postoje velike razlike između vrijednosti omjera izotopa u morskim i kopnenim vodama te vrijednosti $\delta^{18}O$ u otolitima ovisno o temperaturi i slanosti okoliša, dok uopće ne ovise o ishrani (Thorrold i sur., 1997.; Elsdon i Gillanders, 2002.). Slanost vode utječe na izotopski sastav kisika u otolitima (Gillanders i Munro, 2012.), dok u kombinaciji s temperaturom utječe i na međusobne omjere izotopskog sastava kisika u vodi i otolitima (Elsdon i Gillanders, 2002.). Uz to, isparavanje vode povećava površinsku vrijednost $\delta^{18}O$, dok je oborine smanjuju (Ashford i Jones, 2007.). Progresivno iscrpljivanje $\delta^{18}O$ u kiši i površinskim vodama događa se s povećanjem geografske širine, povećanjem nadmorske visine i udaljavanjem od oceana (Dansgaard, 1964.; Stewart i Taylor, 1981.; Nelson i sur., 1989.).

Za razliku od kisika, stabilni izotopi ugljika u otolitima talože se u neravnoteži s okolnom vodom (Iacumin i sur., 1992.). Ugljik u otolitnom aragonitu je smjesa otopljenog anorganskog ugljika dobivenog iz okolne vode i metaboličkog ugljika dobivenog iz hrane (Kalish, 1991.; Jamieson i sur., 2004.). Otopljeni anorganski ugljik ima različit izotopski sastav u odnosu na metabolički ugljik, dok su udjeli ugrađenog ugljika u otolitima kontrolirani metabolizmom ribe (Kalish, 1991.; Shephard i sur., 2007.). Stoga su vrijednosti δ^{13}C u otolitima osjetljive na promjene razine metaboličke aktivnosti, što može omogućiti uvid u ontogene promjene metabolizma jedinke (Jamieson i sur., 2004.; Ashford i Jones, 2007.; Shephard i sur., 2007.).

Stabilni izotopi dušika (δ^{15}N) u otolitima riba pokazali su se kao potencijalni izvor podataka o ishrani riba (Rowell i sur., 2010.; Lueders-Dumont i sur., 2018.). Uglavnom se koriste kod arhiviranih otolita kod kojih više nije dostupno tkivo organizma, ali je njihovo analiziranje dijelom ograničeno zbog niskog udjela dušika u otolitima (Lueders-Dumont i sur., 2018.). Prednost je korištenja otolita u odnosu na tkivo ribe što nema pretvorbe materijala. Ovo svojstvo omogućuje korištenje izotopske kemije otolita za praćenje promjena u okolišu i ishrani riba s mogućnošću rekonstrukcije trofičkih razina te tako pruža zapis o čitavoj životnoj povijesti organizma (Schwarzhans, 2007.; Rowell i sur., 2010.).

Izotopski se sastav stroncija (δ^{87}Sr) u otolitima pokazao korisnim u određivanju slatkovodnih rastilišta riba (Hobbs i sur., 2005.) i praćenju obrazaca kretanja (Kennedy i sur., 2000.) te trajanju migracije između morskog i slatkovodnog okoliša (Bacon i sur., 2004.; McCulloch i sur., 2005.). Ribe ugrađuju Sr izravno u kosti i otolite proporcionalno njihovoj dostupnosti u vodi bez modifikacija koje su posljedica promjena u ishrani, stresu, temperaturi ili drugim fiziološkim čimbenicima (Kennedy i sur., 2000.).

Stabilni izotopi sumpora (δ^{34}S) u otolitima potencijalno se koriste za razlikovanje mrijestilišta riba (Weber i sur., 2002.; Johnson i sur., 2012.) te određivanje različitih izvora hrane (Limburg i sur., 2015.). δ^{34}S nudi obećavajuću alternativu elementnom zapisu otolita budući da se organski sumpor u životinjskom tkivu dobiva iz organskog sumpora u njihovoj ishrani, a δ^{34}S se razlikuje između morskog i slatkovodnog okružja s malo ili bez trofičkog frakcioniranja i temperaturnog učinka (Hesslein i sur., 1993.; Barnes i Jennings, 2007.). Stoga izotopi sumpora u otolitima omogućuju razlikovanje anadromnih i katadromnih vrsta kao npr. vrste *Oncorhynchus nerka* (Walbaum, 1792) (Godbout i sur., 2010.).

Kako je već navedeno, analize stabilnih izotopa u mišićnom tkivu najčešće se koriste za određivanje izvora energije i strukture hranidbenih mreža. Stabilni se izotopi ugljika i dušika u mišićnom tkivu koriste za dobivanje podataka o ishrani i trofičkoj razini vrste u hranidbenoj mreži. Vrijednosti stabilnog izotopa $\delta^{13}C$ u tkivima koriste se za utvrđivanje doprinosa različitih stavki plijena u ishrani i pružaju podatke o trofičkoj strukturi upućujući na osnovni izvor ugljika u određenoj hranidbenoj mreži (Matley i sur., 2015.). Rekonstrukcija hranidbene mreže oslanja se na vrijednosti stabilnih izotopa ugljika i dušika između plijena i predatora (Fry, 1999.). Izotopi ugljika se razdvajuju, troše, odnosno frakcioniraju za manje od 1 ‰ između plijena i potrošača, a pretpostavlja se da se izotopi dušika frakcioniraju približno 3,5 ‰ među trofičkim razinama (Fry, 1999.; Elsdon i sur., 2010.).

Za razliku od stabilnih izotopa dušika ($\delta^{15}N$) u otolitima, njihove se vrijednosti u tkivu najčešće koriste u ekološkim studijama za određivanje trofičkih razina i struktura te duljine hranidbenog lanca (Vander Zanden i sur., 1997.; Post, 2002.) jer omjer ^{15}N do ^{14}N ($\delta^{15}N$) raste kako se prelazi s niže na višu razinu hranidbenog lanca (Rowell i sur., 2010.). Izotopski sastav dušika razlikuju se među vrstama, tkivima, različitim vrstama potrošača (npr. mesojed, biljojed) i tipu staništa (morski, slatkovodni ili kopneni) (Vander Zanden i sur., 1997.). Reis-Santos i sur. (2015.) su zaključili da postoji veza između različitih izotopskih omjera izvora hrane te ishrane riba na određenim staništima budući da primarni proizvođači pokazuju prepoznatljive izotopske omjere koji se šire putem lokalnog hranidbenog lanca. Stoga se očekuje da će nemigratorne ribe, poput nedoraslih riba (Correia i sur., 2011.) pokazivati manje varijacije omjera stabilnih izotopa u ravnoteži s lokalnim hranidbenim lancem, dok bi jedinke koje se neprestano kreću među staništima trebale pokazivati srednja ili veća kolebanja omjera stabilnih izotopa dušika (Herzka, 2005.).

Dok su razlike u omjerima stabilnih izotopa kisika u otolitima među različitim vrstama relativno dobro utvrđene, varijacije su stabilnih izotopa kisika u mišićnom tkivu riba slabo opisane i nedostupne (Coulter i sur., 2017.). Stoga se može zaključiti kako se kisik rijetko analizira u mišićnom tkivu.

Izotopski sastav sumpora ($\delta^{34}S$) u mekim tkivima morskih organizama pruža izravan dokaz o izvoru sumpora (Mizota i sur., 1999.; Yamanaka i sur., 2000.; Yamanaka i Mizota, 2001.). Dok su njegove uobičajene vrijednosti u morskoj vodi svih svjetskih oceana uniformne

(Rees i sur., 1978.), niže vrijednosti upućuju na njegovo vulkansko ili terestričko podrijetlo (Thode, 1988.) ili anoksični okoliš otvorenog mora u kojem su bakterije reducirale morske sulfate (Kajiwara, 1989.). Snižene vrijednosti stabilnog izotopa sumpora utvrđene su i u sedimentima eutrofiziranih i stratificiranih pridnenih voda (Carr i sur., 2017.).

1.6 Tehnike pripreme i metode određivanja kemijskog sastava otolita i mišićnog tkiva ribe

Važna su stavka kod određivanja kemijskog sastava otolita tehnike pripreme otolita za daljnje analize. Te tehnike uključuju i one koje su već navedene kod određivanja morfologije otolita te tehnike priprema za lasersko određivanje kemijskog sastava otolita te izotopskog sastava otolita i tkiva ribe.

Standarna je priprema za analizu kemijskog sastava ugradnja otolita u epoksidnu smolu te presjek otolita pomoću precizne pile opremljene dijamantnim brusom. Nakon toga se dijelovi otolita pričvršćuju na predmetno stakalce te bruse i poliraju i na kraju ispiru u ultrazvučnoj kupki određeno razdoblje (Stevenson i Campana, 1992.). Priprema otolita i mekog tkiva ribe za analizu stabilnih izotopa obavlja se tako da se otoliti i tkivo suše u peći na temperaturi od 60 °C do konstantne težine. Nakon toga slijedi usitnjavanje u fini prah te pohrana određene količine praha u kapsule (Post, 2002.)

Tehnike pripreme mogu biti ograničene na neki dio životnog ciklusa ribe (dnevni/godišnji prsten i/ili rub otolita) ili cijeli životni vijek ribe (cijeli otolit). Tehnike pripreme, ovisno o tome zahvaćaju li cijeli otolit ili njegov dio, daju odgovor jedinki na različite uvjete okoliša (Campana, 2005.). Analiza cijelih otolita daje kemijski zapis tijekom životnog vijeka ribe, od embrionalne faze do smrti, odnosno ulova, i okoliša u kojem je riba boravila i može služiti kao oznaka određene skupine za praćenje razdoblja migracije (Campana i sur., 1997.; Secor i sur., 2001.). Ako skupina riba migrira ili se pomiješa s drugom skupinom, kemijski zapis u otolitima ostat će različit i prepoznatljiv određeno vrijeme. Prirast otolita u tom razdoblju migracije ili miješanja imat će specifičan kemijski zapis koji se zato može koristiti za određivanje procesa tijekom migracija (Campana, 1999.; Secor, 1999.). Prednosti analize cijelog otolita uključuju jednostavnost pripreme, odsutnost pogreške povezane s uzorkovanjem ili određivanjem priraštaja te dostupnost točnog i preciznog protokola ispitivanja. Glavni je nedostatak povezan s nemogućnošću iskorištavanja slijeda kronološkog rasta zabilježenog u otolitu. Tehnika pripreme presjeka otolita omogućuje otkrivanje kemijskih zapisa manjih razmjera u otolitima koje bi inače bilo nemoguće otkriti analizom cijelog otolita, pa se stoga za različita razdoblja života mogu odrediti jedinstveni kemijski zapisi različite skupine riba ili životne sredine (Elsdon i Gillanders, 2002.).

Postoji cijeli niz kemijskih analitičkih metoda koje se koriste za utvrđivanje kemijskog sastava različitih struktura, pa tako i tvrdih struktura morskih organizama. Neke su od najpoznatijih i najčešće korištenih sljedeće. Atomska apsorpcijska spektrometrija (engl. *Atomic Absorption Spectrometry,* AAS) otkriva elemente u tekućim ili čvrstim uzorcima primjenom valnih duljina elektromagnetskog zračenja iz izvora svjetlosti (Grady i sur., 1989.; Hoff i Fuiman, 1993.; García i Báez, 2012.). Induktivno spregnuta plazmatska atomska emisijska spektrometrija (engl. *Inductively Coupled Plasma Atomic Emission Spectroscopy,* ICP-AES) (Edmonds i sur., 1995.) je analitička metoda koja pomoću emisijske spektroskopije koristi induktivno spregnutu plazmu za stvaranje pobuđenih atoma i iona koji emitiraju elektromagnetsko zračenje na valnim duljinama važnima za određeni kemijski element. Analiza neutronske aktivacije (engl. *Neutron activation analysis*) koristi se za određivanje koncentracija elemenata aktivacijom njihovih neutrona u velikoj količini materijala (Papadopoulou i sur., 1980.; Greenberg i sur., 2011.). Ramanova spektroskopija (engl. *Raman spectroscopy*) je spektroskopska tehnika koja se koristi za određivanje titraja molekula pojedinih kemijskih elemenata kako bi se dobio strukturni otisak pomoću kojeg se molekule mogu identificirati (Gauldie i sur., 1994.; Rygula i sur., 2013.). Masena spektrometrija je analitička tehnika za analizu proteinskih i molekularnih kompleksa koji se sastoje od proteina i spojeva male molekulske mase (Noda i sur., 2016.) i osnovna je tehnika za određivanje kemijskog sastava otolita (Matta i sur., 2013.). Tijekom godina korištenja, tehnika se usavršavala u smislu otkrivanja kemijskih elemenata niskih koncentracija i rada s malim uzorcima, pa tako danas postoji nekoliko varijanti ove tehnike ovisno o načinu pripreme i tretiranja uzorka. Induktivno spregnuta plazmatska masena spektrometrija (engl. *Inductively Coupled Plasma Mass Spectroscopy,* ICPMS) je masena spektrometrija induktivno spregnute plazme koja se koristi za ionizaciju uzorka (Edmonds i sur., 1991., Dove i sur., 1996.). Sve se navedene tehnike mogu koristiti za kemijsku analizu sastava otolita, ali ICPMS ima sposobnosti za brze i precizne izotopske i elementne testove na širokom rasponu elemenata i koncentracija. Inačica ove analitičke metode koja koristi izotopsko razrjeđivanje (engl. *Isotope Dilution ICPMS,* ID-ICPMS) često se koristi kako bi se odredila količina elemenata u uzorku i ispravila pretvorba elemenata koja se može dogoditi zbog uzorkovanja, pripreme i analize uzoraka (Campana i sur., 1995.; Campana, 1999.). Uzorci potrebni za većinu navedenih testova imaju od 5 do 10 mg otolitnog materijala, dok su ICPMS laseri opremljeni raspršivačima visoke učinkovitosti koji mogu obraditi mase otolita do 0,3 mg (Thorrold i sur. 1997.; Campana, 2005.). Laserska ablacija dodana na ICPMS (engl. *Laser ablation* - ICPMS, LA-ICPMS) je tehnika za brzu analizu kemijskih elemenata niskih koncentracija i stabilnih izotopa dobivenih iz prirodnih uzoraka. Prednost je te tehnike jednostavna priprema i mala potrebna količina uzorka uz korištenje protokola za analizu standardnih materijala poznatih osobina. Ta se metoda najčešće koristi za proučavanje točno određenih područja na tvrdim strukturama organizma (Serre i sur., 2018.). Stabilni izotopi ugljika i dušika najčešće se analiziraju pomoću elementnog analizatora povezanog s masenim spektrometrom za omjere izotopa (engl *Isotope Ratio Mass Spectrometer,* IRMS) koji su danas opremljeni vrhunskom elektronikom. Uzorci se mjere u odnosu na laboratorijske standarde koji su prilagođeni veličini uzorka i kalibrirani prema međunarodnim standardima (*Iso-Analytical*). Konačna delta jedinica izražena je u odnosu na međunarodne standarde uključujući VPDB (*Vienna PeeDee Belemnite*) za izotope ^{13}C i ^{15}N (Patterson i Caemichael, 2016.).

1.7 Primjena rezultata o elementnom sastavu otolita u ekološkim, sklerokronološkim i ribarstvenim istraživanjima

Morfološke analize kalcificiranih struktura koriste se za određivanje vrsta (npr. Charmpila i sur., 2021.), odjeljivanje spolova (npr. Basusta i Khan, 2021.), odjeljivanje stokova (npr. Morat i sur., 2012.; Mahé i sur., 2019.) i populacija (npr. Merigot i sur., 2007.), utvrđivanje različitih bioloških osobina i povezanosti (npr. sluha u radu Cruz i Lombarte, 2004.), rast i starost (npr. Reñones i sur., 2007.). Nadalje, u sinergiji morfološka i kemijska analiza otolita uspješno se primjenjuju radi utvrđivanja životnog ciklusa organizma te odgovora populacija na okolišne pritiske, bilo prirodne (okolišne varijacije) ili antropogene (npr. ribolov, zagađenje, razvoj obalnog područja, klimatske promjene) (Campana, 1999.; Panfili i sur., 2002.; Gillanders, 2005.; Oschmann, 2009.).

Kemijske analize kalcificiranih struktura omogućile su istraživanja poput korištenja staništa pojedinih jedinki (Secor i sur., 2001.; Gillanders i Kingsford, 2000.), obrasce migracija (Secor i sur., 2001.; Elsdon i Gillanders, 2002.; Elsdon i sur., 2008.; Adey i sur., 2009.; Šegvić-Bubić i sur., 2020.), strukture populacija (Campana i sur., 2000.; Patterson i sur., 2004.) te hranidbene obrasce (Campana, 1999.). Kako je već navedeno, korištenje kemijskog sastava otolita kao prirodnog markera moguće je jer su otoliti metabolički inertni, neprestano rastu i ugrađuju kemijske elemente u tragovima tijekom rasta (Campana, 1999.; Elsdon i sur., 2008.).

Pojam sklerokronologija predstavlja različita interdisciplinarna istraživanja (Peharda i sur., 2020.) i pojavio se u literaturi prije 45 godina kada su ga definirali Buddemeier i sur. (1974.) u vezi s istraživanjem povećanja koralja. Oschmann (2009.) ga detaljno definira kao proučavanje fizikalnih i kemijskih varijacija u tvrdim strukturama organizama te vremenski kontekst u kojem su te strukture nastale. Usredotočuje se ponajprije na obrasce rasta koji odražavaju godišnje, mjesečne, dvotjedne, plimne, dnevne i poludnevne priraste u vremenu kao rezultat okolišnih i astronomskih pokretača. Sklerokronologija je analogna dendrokronologiji koja proučava godišnje prstenove rasta na stablima (Black i sur., 2005.), a isto tako nastoji zaključiti obilježja povijesti života organizma te rekonstruirati zapise o promjenama okoliša i klime u prostoru i tijekom vremena (Morrongiello i sur., 2012.). Dugoročni su nizovi podataka starosti utvrđeni za brojne kopnene organizme i kopnene ekosustave, dok najčešće nedostaju za vodene sustave (Black i sur., 2005.). Kronologija tih sustava generirana je na temelju podataka zabilježenih u ljušturama školjkaša i koralja, ali i tvrdim strukturama riba (Black i sur., 2005.; 2008.) te je utvrđen relativno visok potencijal otolita za dobivanje dugoročnih zapisa (Morrongiello i sur., 2012.; Rountrey i sur., 2014). Za razliku od školjkaša i drveća koji mogu živjeti nekoliko stoljeća (npr. *Artica islandica*, 507 godina; Butler i sur., 2013.) ili čak tisućljećima (npr. *Pinus longaeva*, 4900 godina; Currey, 1965.), ribe imaju kraći životni vijek i predstavljaju veći izazov za statističku rekonstrukciju. Dobar su primjer vrste iz porodice bodečnjaka, Sebastes koje dosežu starost veću od 40 godina (Black i sur., 2008.; Matta i sur., 2018.).

Istraživanja kemijskog sastava otolita u Sredozemnom moru provedena su tek na nekoliko vrsta riba (Peres i Haimovici, 2004.; Consoli i sur., 2010.; Reñones i sur., 2010.), a upravo su vrste iz porodice Sparidae one koje su najčešće proučavane (Di Franco i sur., 2011., 2014.; Winkler i sur., 2019.). Vrste iz te porodice pripadaju redu Perciformes, spororastući su dugoživući organizmi (Hanel i Tsigenopoulos, 2011.) te su rasprostranjene u gospodarskom i rekreativnom ribolovu (Griffiths, 2002.; Morales-Nin i sur., 2005.; Comeros-Raynal i sur., 2016.). Sparidne vrste uglavnom imaju velike i lako čitljive sagitalne otolite koji su se najčešće koristili za očitavanje starosti i analizu tvrdih struktura (Buxton i Clarke, 1989.; 1991.; Brouwer i Griffiths, 2004.; Potts i sur., 2010.; Baudouin i sur., 2016.; Farthing i sur., 2018.) uz ljuske koje su se donedavno koristile jedino za očitavanje starosti (Kraljević i Dulčić, 1997.; Baudouin i sur., 2016.).

Vrlo se često specifičani kemijski sastav otolita neke vrste smatra njegovim otiskom (engl. *fingerprint*) i ti se otisci posljednjih 20 godina široko koriste za uspješnu procjenu strukture populacije (Tanner i sur., 2012.), definiranje rastilišta riba (Gillanders i Kingsford, 2000.) te međusobnu povezanost nedoraslih i odraslih populacija (Reis-Santos i sur., 2015.). Identifikacija rastilišta služi kao izravan dokaz o različitim doprinosima pojedinih rastilišta za odrasle subpopulacije određenih vrsta morskih riba, što je iznimno bitno za gospodarenje prelovljenim ribljim stokovima. Analizom različitih područja otolita mogu se prikupiti podaci o odvojenim razdobljima ontogenetskog razvoja jedinki, dok se točkasta mjerenja na prethodno određenim dnevnim ili godišnjim prirastima koriste za određivanje početka migracije i ostalih fiziološki važnih događaja tijekom života ribe (Secor i Rooker, 2000.). Analize podrazumijevaju kako rub otolita pruža podatke o okolišu u kojem su jedinke boravile neposredno pred ulov, dok jezgra otolita, s druge strane, predstavlja ličinačke i rane pelagijske nedorasle životne faze i može sadržavati podatke o prostornoj odvojenosti tih važnih životnih faza (Elsdon i sur., 2008.). Međutim, prije nego što se kemijski otisak otolita može primijeniti kao prirodna oznaka, mora se pokazati da se on razlikuje među geografskim područjima (Campana i sur., 2000.). Kako je već navedeno, kemijski sastav otolita iz različitih okolišnih uvjeta najčešće se razlikuje u sastavu kemijskih elemenata koji dodatno modificira niz čimbenika koji se razlikuju i na prostornoj i na vremenskoj skali (Elsdon i sur., 2008.) te ovisi o fiziološkom statusu i genetici svake jedinke (Grønkjær, 2016.; Rogers i sur., 2019.).

Razumijevanje je životnog ciklusa priobalnih vrsta morskih riba i identifikacija važnih rastilišnih staništa za nedorasle ribe iznimno kompleksno jer mnoge vrste riba imaju složene životne cikluse (Forrester i Swearer, 2002.; Gillanders i sur., 2003.; Fromentin i sur., 2009.). Većina riba započinje životni ciklus ličinačkim i poslijeličinačkim stadijima u pelagijalu, kao dio ihtioplanktona, a nakon toga slijedi proces preobrazbe i selidba u plitka priobalna (bentoska) staništa te zatim pridruživanje odraslim populacijama, najčešće u otvorenim vodama dalje od obale (Beck i sur., 2001.; Vasconcelos i sur., 2010.).

Preživljavanje ranih razvojnih stadija riba ima presudnu važnost u procesima naseljavanja i novačenja jer ono ima važan utjecaj na veličinu i stanje odraslih populacija. Pod procesom naseljavanja podrazumijeva se razdoblje u kojem jedinke mijenjaju stanište i način života prelaskom iz pelagijskog, ličinačkog i poslijeličinačkog stadija u pridneno stanište specifično za njihove kasnije nedorasle i odrasle stadije. Prelazak je kritično razdoblje razvoja (Victor, 1991.) koje će se odraziti na preživljavanje, novačenje te sastav i gustoću odraslih populacija (Sale i sur., 1984.).

Naseljenici (novopridošle jedinke) su uglavnom malih veličina (nekoliko centimetara dugi) i pri naseljavanju pokazuju sklonost prema točno određenim tipovima staništa, odnosno naseljavaju se u područja prema ontogenetskom predlošku vrste kojoj pripadaju (Macpherson, 1998.). S obzirom na vrijeme dolaska nedoraslih jedinki u plitka priobalna područja, može se unazad utvrditi vrijeme mrijesta pojedinih vrsta. Nedorasle jedinke u plitkim uvalama ostaju od nekoliko mjeseci do godine dana i kada dosegnu određenu specifičnu dužinu, napuštaju hranilišta i pridružuju se odraslim jedinkama, najčešće u dubljim vodama, u procesu koji zovemo novačenje (Dulčić i sur., 1997.; Macpherson, 1998.). Tako pojedine vrste iz porodice ljuskavki (Sparidae) kao pic (*Diplodus puntazzo* (Walbaum, 1792)), fratar (*Diplodus vulgaris* (Geoffroy Saint-Hilaire, 1817)) i salpa (*Sarpa salpa* (Linnaeus, 1758)) naseljavaju plitke uvale početkom zime te ih napuštaju nakon desetak mjeseci (Dulčić i sur., 1997.). Komarča (*Sparus aurata* Linnaeus, 1758) naseljava plitka priobalna područja početkom proljeća i napušta ih krajem ljeta (Bauchot i Hureau, 1986.), dok se šarag (*Diplodus sargus* Linnaeus, 1758) i kantar (*Spondyliosoma cantharus* (Linnaeus, 1758)), koji prvi put ulaze u plitka područja u lipnju, na njima zadržavaju kratko, tek 3 do 4 mjeseca (Dulčić i sur., 1997.). Za razliku od tih vrsta, nedorasle i odrasle jedinke špara (*Diplodus annularis* (Linnaeus, 1758)) stalno se nalaze zajedno u plitkim područjima, i to s prvim pojavljivanjem nedoraslih jedinki u srpnju prosječne starosti 2 mjeseca i napuštanjem plitkih uvala nakon 2 godine zajedničkog života u vrijeme prvog mrijesta (Matić-Skoko i sur., 2004.) kada, kao i druge sparidne vrste, odlazi prema otvorenom moru (Divanach, 1985.; Dulčić i sur., 1997.).

Dolazak novih nedoraslih jedinki u prikladna staništa koja su definirana kao rastilišta te njihova dostupnost ključni su čimbenici potrebni za uspješno obavljanje procesa naseljavanja i novačenja riba, koja ne samo da određuju obnovu populacija nego i oblik i strukturu odraslih zajednica riba (Cheminee i sur., 2011.). Rastilišta su definirana kao specifična mikrostaništa čije su osobine povoljne za naseljavanje nedoraslih riba, a doseljenici često imaju stroge, specifične potrebe za točno određenim mikrostaništima. Plitka se priobalna i estuarijska područja smatraju visokoproduktivnim i vrijednim ekosustavima koji se odlikuju raznolikom topografijom i predstavljaju esencijalna staništa velikom i raznovrsnom broju biljnih i životinjskih vrsta, među njima i riba (Guidetti i Bussotti, 2000a.; Beck i sur., 2001.). U takvim se staništima utvrđuje znatna brojnost ličinačkih, poslijeličinačkih i nedoraslih stadija, što naglašava važnost priobalnih staništa kao hranilišta i rastilišta mnogih vrsta riba (Vasconcelos i sur., 2013.). U brojnim je radovima naročito istaknuta rastilišna uloga estuarija (Gillanders i sur., 2003.; Dahlgren i sur., 2006.; Stierhoff i sur., 2009.; Vasconcelos i sur., 2015.) i obalnih laguna (Kjerfve, 1994.; Pauly i Yáñez-Arancibia, 1994.; Mouillot i sur., 2005.; Dulčić i sur., 2007.; Tournois i sur., 2013.; Isnard i sur., 2015.). Također, u istom smislu veliku važnost mogu imati i brojna druga morska priobalna staništa, poput plitkih uvala i zaljeva (Jug-Dujaković, 1988.; Dulčić i sur., 1997.), zaštićenih pješčanih (Nash i sur., 1994.; Gutiérrez-Martínez i sur., 2020.) i stjenovitih obala (Henriques i Almada, 1998.; Guidetti i Bussotti, 2000b.), a naročito ona koja su obrasla makrofitskom algalnom vegetacijom i livadama morskih cvjetnica (Guidetti i Bussotti, 2000b.). Ekološka je i ekonomska važnost ušća rijeka za obnovu priobalnih ribljih populacija općepriznata. Mnoge ribe koriste ušća kako bi zaokružile svoj životni ciklus, a ličinke ili rane nedorasle jedinke često provode mjesece, pa čak i godine, u tim sredinama prije novačenja u obalne odrasle populacije (Beck i sur., 2001.; Vasconcelos i sur., 2015.). U tim područjima prevladavaju pogodni uvjeti za rast, u smislu visoke dostupnosti hrane, odgovarajuće temperature vodene sredine i niskog biotičkog stresa (Beck i sur., 2001.). Nažalost, danas su ti morski ekosustavi često degradirani izravnim ljudskim aktivnostima do te mjere da u mnogim područjima kritična obalna staništa više nisu dostupna ili pogodna da bi mogla pružiti rastilišne, hranilišne ili reproduktivne funkcije, s negativnim posljedicama za proizvodnju i obnavljanje populacija (Barbier i sur., 2011.; Matić-Skoko i sur., 2020.). Stoga je identifikacija rastilišta vrlo važna za oblikovanja učinkovitih planova upravljanja ribarstvom i očuvanja bioraznolikosti i staništa. Planovi bi trebali biti definirani uzimajući u obzir ciljeve zaštite, odnosno jesu li ti ciljevi usmjereni na zaštitu metapopulacije pojedinih vrsta, nekoliko vrsta ili priobalno rastilište kao cjelinu (Isnard i sur., 2015.; Rogers i sur., 2019.).

CILJEVI I HIPOTEZE DOKTORSKOG RADA

Ova doktorska disertacija uključuje četiri znanstvena članka (I-IV) koji su obradili sljedeće postavljene ciljeve i hipoteze.

***Ciljevi** doktorske disertacije su:*

1. Utvrđivanje izazova i mogućnosti sklerokronoloških istraživanja na ribama u Sredozemnom/Jadranskom moru. (Članak I, II, IV)

2. Utvrđivanje strukture otolita u odnosu na prirast konfokalnom Ramanovom mikrospektroskopijom (CRM). (Članak III)

3. Utvrđivanje kemijskog sastava otolita fratra (*Diplodus vulgaris*), pica (*Diplodus puntazzo*) i komarče (*Sparus aurata*) iz porodice Sparidae (ljuskavki) u Jadranu. (Članak I, II, IV)

4. Identificiranje mogućeg estuarijskog podrijetla odabranih vrsta ili priobalnih rastilišta koje su koristile odabrane vrste iz porodice Sparidae. (Članak II, IV)

5. Određivanje relativnog doprinosa pojedinog rastilišta priobalnim populacijama odabranih vrsta riba porodice Sparidae. (Članak II, IV)

Ovim se istraživanjem želi realocirati odabrane sparidne vrste riba u specifična rastilišta te utvrditi obrasci kretanja nedoraslih i odraslih jedinki, uz ekološke veze vrsta i staništa na temelju kemijskog sastava elemenata u otolitama i stabilnih izotopa u mišićnom tkivu. Uspješna klasifikacija i razlikovanje među staništima trebali bi omogućiti identifikaciju podrijetla odraslih riba i relativan doprinos pojedinih rastilišta (stopu novačenja) odraslim populacijama u situaciji potencijalno visokog ribolovnog pritiska te razinu doprinosa onih rastilišta koja su karakterizirana relativno višim stupnjem onečišćenja zbog njihova potencijalno negativnog utjecaja na dinamiku populacija.

***Hipoteze** doktorske disertacije su:*

Analiza kemijskog sastava elemenata u otolitima:

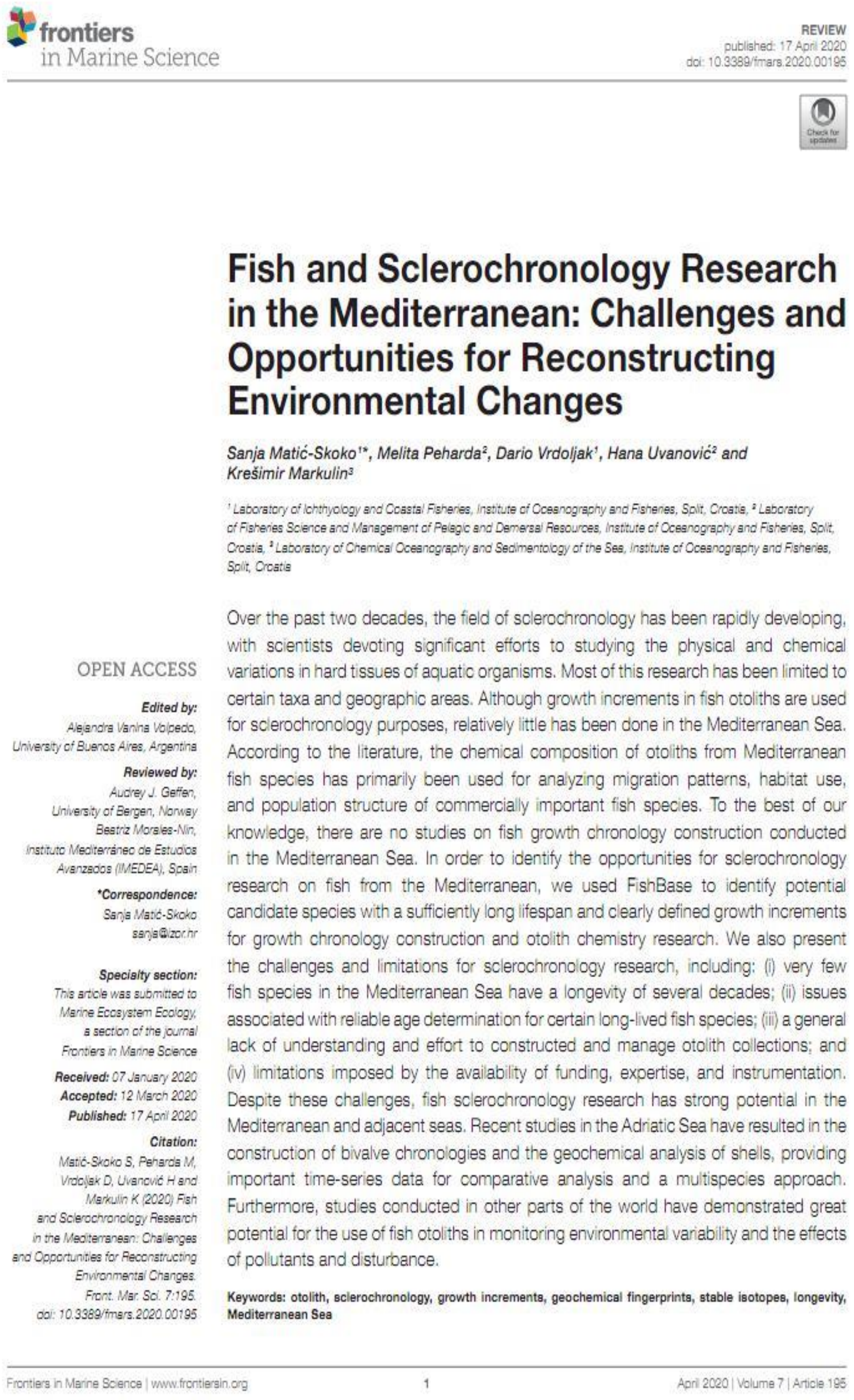
- omogućuje istraživanje tvrdih struktura i rekonstrukciju rasta riba (Članak I, II, III, IV)
- u kombinaciji sa strukturom otolita koristi se za validiranje periodičnosti prirasta odnosno prstenova otolita (Članak III)

- omogućuje prostornu alokaciju fratra (*D. vulgaris*), pica (*D. puntazzo*) i komarče (*S. aurata*) u specifična rastilišta (Članak II, IV)
- u kombinaciji sa stabilnim izotopima u tkivu fratra (*D. vulgaris*) i pica (*D. puntazzo*) pruža komplementarnu informaciju o korištenju rastilišta i pojašnjava ekološke veze između vrsta i staništa (Članak IV)
- omogućuje procjenu doprinosa odrasloj populaciji unovačenih nedoraslih komarča (*S. aurata*) koja potječe iz rastilišta koje je pod utjecajem onečišćenja (Članak II)

Prvi i treći cilj doktorske disertacije postignuti su u člancima I, II i IV gdje se istražuju mogućnosti sklerokronoloških istraživanja na ribama u Sredozemnom i Jadranskom moru te utvrđuje kemijski sastav odabranih vrsta riba. Drugi je cilj postignut u članku III gdje se opisuje sastav i strukura otolita komarče (*S. aurata*), dok su posljednja dva cilja postignuta u članacima II i IV gdje se detaljno identificiraju rastilišta i njihov relativan doprinos priobalnim populacijama fratra (*D. vulgaris*), pica (*D. puntazzo*) i komarče (*S. aurata*). Svi članci daju odgovor na prvu hipotezu, s time da članci I i III daju osnovu za treću hipotezu u kojima sastav elemenata u otolitima omogućuje (1) prostornu alokaciju navedenih vrsta u specifična rastilišta i određivanje relativnog doprinosa svakog pojedinog rastilišta odraslim populacijama fratra (*D. vulgaris*), pica (*D. puntazzo*) i komarče (*S. aurata*) (Članak II, IV); (2) u kombinaciji sa stabilnim izotopima u tkivu fratra (*D. vulgaris*) i pica (*D. puntazzo*) pruža komplementarnu informaciju o korištenju rastilišta i pojašnjava ekološke veze između vrsta i staništa (Članak IV) te (3) omogućuje procjenu doprinosa odrasloj populaciji nedoraslih komarči (*S. aurata*) unovačenih iz rastilišta koje je pod utjecajem onečišćenja (Članak II).

SAMOSTALNI ZNANSTVENI RADOVI

Članak I



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REVIEW
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Fish and Sclerochronology Research in the Mediterranean: Challenges and Opportunities for Reconstructing Environmental Changes

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Over the past two decades, the field of sclerochronology has been rapidly developing, with scientists devoting significant efforts to studying the physical and chemical variations in hard tissues of aquatic organisms. Most of this research has been limited to certain taxa and geographic areas. Although growth increments in fish otoliths are used for sclerochronology purposes, relatively little has been done in the Mediterranean Sea. According to the literature, the chemical composition of otoliths from Mediterranean fish species has primarily been used for analyzing migration patterns, habitat use, and population structure of commercially important fish species. To the best of our knowledge, there are no studies on fish growth chronology construction conducted in the Mediterranean Sea. In order to identify the opportunities for sclerochronology research on fish from the Mediterranean, we used FishBase to identify potential candidate species with a sufficiently long lifespan and clearly defined growth increments for growth chronology construction and otolith chemistry research. We also present the challenges and limitations for sclerochronology research, including: (i) very few fish species in the Mediterranean Sea have a longevity of several decades; (ii) issues associated with reliable age determination for certain long-lived fish species; (iii) a general lack of understanding and effort to constructed and manage otolith collections; and (iv) limitations imposed by the availability of funding, expertise, and instrumentation. Despite these challenges, fish sclerochronology research has strong potential in the Mediterranean and adjacent seas. Recent studies in the Adriatic Sea have resulted in the construction of bivalve chronologies and the geochemical analysis of shells, providing important time-series data for comparative analysis and a multispecies approach. Furthermore, studies conducted in other parts of the world have demonstrated great potential for the use of fish otoliths in monitoring environmental variability and the effects of pollutants and disturbance.

Keywords: otolith, sclerochronology, growth increments, geochemical fingerprints, stable isotopes, longevity, Mediterranean Sea

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INTRODUCTION

Hard structures of aquatic organisms, including mollusk shells, fish otoliths, corals, and coralline algae, are deposited continuously during the life of the organism, and thereby contain environmental information collected over the organism's life cycle (e.g., Hudson et al., 1976; Jones, 1983; Black et al., 2008). The field of sclerochronology utilizes these data archives by investigating their morphological (i.e., increment width) and geochemical composition to deduce organismal life history traits as well as to reconstruct records of environmental and climatic change through space and time (Oschmann, 2009). Although many sclerochronology studies have been conducted on sedentary organisms, primarily the bivalve *Arctica islandica* (e.g., Schöne, 2013; Marali et al., 2017; Reynolds et al., 2018), fish also present a very interesting target taxon for sclerochronology research (e.g., Panfili et al., 2002; Black et al., 2005; Grønkjaer et al., 2013). The objective of this review was to focus primarily on papers that analyzed otoliths in relation to environmental and climatic changes. There are numerous studies in the review that analyzed fish growth and age from growth increment structures in the otoliths (e.g., Gutiérrez and Morales-Nin, 1986; Morales-Nin and Moranta, 1997; Reñones et al., 2007), but they did not directly relate them to the environmental conditions and a detailed review of such studies is beyond the scope of a present paper.

One of the challenges in evaluating the status of sclerochronology research lies within the fact that this term is not always used in publications addressing the morphological and/or geochemical properties of hard structures in aquatic organisms (Gillikin et al., 2019). This is especially the case for research conducted on fish, despite the publication of the very comprehensive *Manual of Fish Sclerochronology* (Panfili et al., 2002).

Fish possess several hard structures interesting for sclerochronology analysis, including scales, the skeleton, and otoliths (e.g., Chilton and Beamish, 1982; Panfili et al., 2002). Of these, otoliths—calcium carbonate structures located in the inner ear of the fish—are considered the most reliable, as they are metabolically inert, hindering re-absorption (Campana and Neilson, 1985), unlike other structures, such as scales (Simkiss, 1974). Otoliths contain periodically deposited growth increments, from daily to annual, and can thereby provide high temporal resolution data (e.g., Campana, 1999; Morales-Nin, 2000; Black et al., 2008; Elsdon et al., 2008). As fish can attain a maximal life span of several decades, otolith analysis can provide an important window into the past (e.g., Campana, 1999; Black et al., 2008).

Chemical research on otoliths includes analysis of elemental and/or isotopic composition. In 1999, Campana published a review paper on the chemistry and composition of otoliths, presenting in detail the state of the art on this subject at that time and the applications and assumptions of this type of research. The applications of otolith chemistry for describing movements and life-history parameters of fish were comprehensively presented by Elsdon et al. (2008). Numerous publications followed, clearly demonstrating the potential for otolith chemistry as a natural tag of fish stocks (e.g., Trueman et al., 2012; Darnaude and Hunter, 2018; Izzo et al., 2018; Wright et al., 2018). Although most studies focus on stock identification and migration history, the elemental composition of otoliths can also be applied for identifying bioavailable contaminants and establishing long-term trends (e.g., Søndergaard et al., 2015; Andronis et al., 2017; Mounicou et al., 2019). Furthermore, as oxygen isotopes ($\delta^{18}O$) are considered a proxy of water temperatures, analysis of otolith isotopic composition can enable reconstruction of environmental conditions (e.g., West et al., 2012; Willmes et al., 2019).

Use of otolith growth increments to construct fish growth chronology and establishing the relationship with environmental conditions have received increasing attention over the past decade. The methodology for this research has been derived from dendrochronology—the study of growth rings in trees (Black et al., 2005). Primary target organisms are long-living fish species, such as yelloweye rockfish (*Sebastes ruberrimus*, >70 years; Black et al., 2008), and northern rockfish (*Sebastes polyspinis*, ~40 years; Matta et al., 2018). However, development of statistical methods and sample archives have also enabled growth chronology construction for shorter living species. For example, Tanner et al. (2019) constructed half a century chronology for a small, relatively short-lived (<16 years) pelagic fish (Atlantic horse mackerel, *Trachurus trachurus*).

The main objective of this paper is to present an overview of the sclerochronology related research in the Mediterranean Sea and to present its opportunities and challenges.

OVERVIEW OF PREVIOUS SCLEROCHRONOLOGY RELATED RESEARCH IN THE MEDITERRANEAN

In order to identify relevant publications on fish sclerochronology research in the Mediterranean Sea, we conducted a literature search through the Web of Science database. The keywords "Mediterranean" and "otolith" were used in combination with words "isotope," "element," "microchemistry," "chemistry," and "chronology." All publications obtained through this search were read in detail, and only those relating to otolith analysis were included (**Table 1** and **Supplementary Table S1**). Other structures, such as scales and vertebrae, were not considered for the purposes of this review.

Chemical analysis of otoliths has been conducted on over 41 fish species from the Mediterranean, and the most studied species are from the family Sparidae (**Table 1**). Published studies include data for the entire otolith (e.g., Iacumin et al., 1992; Gillanders et al., 2001; Marini et al., 2006; Arechavala-Lopez et al., 2016), data for a specific area of the otolith (e.g., Tanner et al., 2012; Mirasole et al., 2017; Rooker et al., 2019), and time series data (e.g., Correia et al., 2012; Mercier et al., 2012; Bouchoucha et al., 2018). In most reports, only a single species was analyzed, while Papadopoulou and Moraitopoulou-Kassimati (1977), Iacumin et al. (1992), Marini et al. (2006), Swan et al. (2006), Khemiri et al. (2014), Arechavala-Lopez et al. (2016), Mirasole et al. (2017), Bouchoucha et al. (2018), and Demirci et al. (2018) presented data for 2 to 24 different species.

TABLE 1 | List of Mediterranean fish species targeted in the otolith microchemistry research.

Family	Species	References
Anguillidae	*Anguilla anguilla*	Panfili et al., 2012; Capoccioni et al., 2014
Carangidae	*Trachurus mediterraneus*	Turan, 2006
	Trachurus trachurus	Iacumin et al., 1992
Centracanthidae	*Spicara maena*	Iacumin et al., 1992
Champsodontidae	*Chamsodon nudivittis*	Demirci et al., 2018
Clupeidae	*Sardina pilchardus*	Iacumin et al., 1992; Khemiri et al., 2014
Congridae	*Conger conger*	Papadopoulou and Moraitopoulou-Kassimati, 1977; Correia et al., 2011, 2012
Engraulidae	*Engraulis encrasicolus*	Iacumin et al., 1992; Marini et al., 2006; Guidetti et al., 2013; Khemiri et al., 2014
Gadidae	*Merlangus merlangus*	Papadopoulou and Moraitopoulou-Kassimati, 1977; Iacumin et al., 1992
	Trisopterus minutus	Iacumin et al., 1992
Gobiidae	*Gobius bucchichi*	Mirasole et al., 2017
	Gobius niger	Papadopoulou and Moraitopoulou-Kassimati, 1977
Labridae	*Coris julis*	Mirasole et al., 2017
	Symphodus ocellatus	Mirasole et al., 2017
Macrouridae	*Nezumia aequalis*	Swan et al., 2003, 2006
Moronidae	*Dicentrarchus labrax*	Iacumin et al., 1992; Traina et al., 2015; Arechavala-Lopez et al., 2016
Merlucciidae	*Merluccius merluccius*	Iacumin et al., 1992; Morales-Nin et al., 2005a; Swan et al., 2006; Tomas et al., 2006; Hidalgo et al., 2008; Tanner et al., 2012; Chang et al., 2012; Morales-Nin et al., 2014; Tanner et al., 2014; Vitale et al., 2016
Mugilidae	*Mugil cephalus*	Iacumin et al., 1992; Fortunato et al., 2017a,b
Mullidae	*Mullus barbatus*	Marini et al., 2006
Nemipteridae	*Nemipterus randalli*	Demirci et al., 2018
Pomacentridae	*Chromis chromis*	Mirasole et al., 2017
Sciaenidae	*Sciaena umbra*	Iacumin et al., 1992; Marini et al., 2006
	Umbrina cirrhosa	Marini et al., 2006
Scombridae	*Scomber japonicus*	Papadopoulou et al., 1978, 1980
	Thunnus alalunga	Davies et al., 2011
	Thunnus thynnus	Secor and Zdanowicz, 1998; Rooker et al., 2002, 2003, 2008a,b, 2014, 2019; Secor et al., 2002; Fraile et al., 2016
Sebastidae	*Helicolenus dactylopterus*	Swan et al., 2006
Serranidae	*Serranus scriba*	Iacumin et al., 1992
Sparidae	*Boops boops*	Iacumin et al., 1992
	Diplodus annularis	Papadopoulou and Moraitopoulou-Kassimati, 1977; Iacumin et al., 1992; Catalán et al., 2018
	Diplodus puntazzo	Iacumin et al., 1992
	Diplodus sargus	Iacumin et al., 1992; Di Franco et al., 2011, 2012, 2015b; Bouchoucha et al., 2018
	Diplodus vulgaris	Iacumin et al., 1992; Gillanders et al., 2001; Di Franco et al., 2014, 2015a; Mirasole et al., 2017; Bouchoucha et al., 2018
	Dentex dentex	Iacumin et al., 1992
	Lithognatus mormyrus	Iacumin et al., 1992
	Oblada melanura	Iacumin et al., 1992; Calò et al., 2016
	Pagellus erythrinus	Iacumin et al., 1992; Marini et al., 2006
	Sarpa salpa	Iacumin et al., 1992; Mirasole et al., 2017
	Sparus aurata	Iacumin et al., 1992; Mercier et al., 2012; Tournois et al., 2013; Arechavala-Lopez et al., 2016; Niklitschek and Darnaude, 2016
	Spondyliosoma cantharus	Iacumin et al., 1992
Soleidae	*Solea solea*	Dierking et al., 2012; Morat et al., 2012, 2013, 2014a,b

Species names listed as valid species according to World Register of Marine Species. More detail on these studies is available in ***Supplementary Table S1***.

Recently, Chang and Geffen (2013) summarized taxonomic and geographic influences on fish otolith microchemistry based on a number-published paper worldwide and all those related to the Mediterranean are also included in this study.

In total, 12 species from the family Sparidae are listed in **Table 1**, and most were addressed only by Iacumin et al. (1992). This study analyzed the oxygen and carbon isotope composition of aragonite in fish otoliths with regard to their possible suitability in paleoenvironmental and paleobiological work. This was the first attempt to apply stable isotope analysis to fish otoliths from the Mediterranean Sea. The mostly analyzed species are those from Diplodus genus, particularly *D. sargus*, and *D. puntazzo*, regarding possible environmental interpretation of otolith fingerprints related to spatial patterns of population connectivity and dispersal of marine fishes (Di Franco et al., 2011, 2015b), dispersal scales of fish at various life history stages, which is critical for successful design of networks of marine protected areas (Di Franco et al., 2012, 2015a), within-otolith variability enabling its usage as a marker for fish exposure to stressful conditions (Di Franco et al., 2014).

The majority of research on otolith chemical composition has been conducted on commercially important species. Atlantic Bluefin tuna (*Thunnus thynnus*) was the target of several studies on the element (Secor and Zdanowicz, 1998; Rooker et al., 2003) and isotope composition (Secor et al., 2002; Rooker et al., 2008a,b, 2014, 2019), aiming to reconstruct movement and population exchange. Fraile et al. (2016) observed the depletion in $\delta^{13}C$ in *T. thynnus* otoliths over time, associating this with the oceanic uptake of anthropogenically derived CO_2 from the Mediterranean Sea over the past two decades. These studies primarily focused on material deposited in the otolith core or during the first year of life.

The European hake (*Mercuccius merluccius*) is also an important target species for the analysis of otolith element and isotope composition, and different methodologies have been applied. Morales-Nin et al. (2005a) studied elements in different parts of the otoliths using laser ablation–spot analysis, while in a study from 2014, the same authors used the line scan approach. Laser ablation, as opposed to otolith dissolution that is applied in the analysis of the whole otolith, enables the collection of more data points that can be placed in time (e.g., Elsdon et al., 2008). Tomas et al. (2006) studied composition of the opaque and translucent bands with wavelength dispersive spectrometry (WDS) revealing that annual marks (translucent) were significantly richer in Sr and Ca and significantly poorer in Na than opaque bands. Swan et al. (2006) applied two methods—solution-based inductively coupled plasma mass spectrometry of the whole otolith and laser ablation analysis of the otolith nucleus on hake and bluemonth (*Helicolenus dactylopterus*). Chang et al. (2012) used hake otoliths to test different widths of ablation lines and evaluate the temporal resolution of data. Hidalgo et al. (2008) and Tanner et al. (2012) applied analysis of $\delta^{18}O$ and $\delta^{13}C$ to certain sections of the otolith, specifically the core and the edge zone, in determining hake movement and ecology. It is interesting that Tanner et al. (2012) combined stable isotope analysis with analysis of otolith element composition to obtain more comprehensive data. These studies on hake analyzed the migration, population structure, and ecology of this commercially important species. In recent papers, Tanner et al. (2014) accompanied genotype with otolith data to increase the classification accuracy of individuals to their potential natal origins, while Vitale et al. (2016) estimated longevity of 25 years of female hake by applying bomb radiocarbon dating.

Element and isotope composition of otoliths of the common sole (*Solea solea*) have been analyzed in a series of studies conducted in the Gulf of Lions, in the northwest Mediterranean (Dierking et al., 2012; Morat et al., 2012, 2013, 2014a,b). These studies analyzed dispersion between populations and the use of different habitats.

The chemical composition of otoliths as a proxy of environmental conditions has been analyzed in only a few studies in the Mediterranean Sea, including Traina et al. (2015). They investigated the metal content of European sea bass (*Dicentrarchus labrax*) otoliths from two fish aquaculture sites. Their results indicated variations in the concentrations of certain metals between locations that were likely due to industrial effluents.

To the best of our knowledge, there is no research in the Mediterranean Sea related to fish growth chronologies constructed from growth increment analysis. The literature search conducted through the Web of Science returned just one publication for the keyword combination "Mediterranean" and "fish" and "sclerochronology": a report by Prendergast and Schöne (2017) as the preface to the *Special Issue on Sclerochronology* containing research from different parts of the world including the Mediterranean, but not specifically on fish in the Mediterranean.

OPPORTUNITIES FOR FISH GROWTH CHRONOLOGY CONSTRUCTION

Over the past two decades, techniques developed in dendrochronology research have been applied for the construction of fish growth chronologies (Black et al., 2005, 2008). They clearly demonstrated the potential for obtaining long term data from growth patterns in otoliths and for identifying environmental drivers (Morrongiello et al., 2012; Rountrey et al., 2014). In a recent review, Black et al. (2019) presented a global list of fish species that have been the subject of sclerochronology studies that included growth chronology construction and applied cross-dating techniques. Their list includes 21 species, none of which were from the Mediterranean Sea. The most studied on the list are cold-water species, such as kelp greenling (*Hexagrammos decagrammus*) and black rockfish (*Sebastes melanops*) and other species from the genus *Sebastes* (*S. alutus*, *S. aurora*, *S. diploproa*, and *S. ruberrimus*). Other species were from the families: Girellidae, Labridae, Lethrinidae, Lutjanidae, Platycephalidae Pleuronectidae, Polyprionidae, Sciaenidae, and Scombridae. All of these are long-lived, non-migratory, nearshore residents with generalist diets that can be caught easily throughout a wide geographic range (Whitfield and Elliott, 2002).

Identifying target fish species with a sufficiently long life span and clearly defined growth increments is a prerequisite for statistically robust chronology construction. Unlike bivalves and trees that can live for several centuries (e.g., *A. islandica*, 507 years; Butler et al., 2013) or even millennia (e.g., *Pinus longaeva*, 4,900 years; Currey, 1965), fish have a shorter lifespan and present a greater challenge for constructing statistically robust chronologies. Another prerequisite for chronology construction is the availability of samples, which needs to take the conservation status of species into account. Although it is scientifically interesting to obtain data from endangered species, sampling such species should be clearly justified and ethically sound. Replication is essential for proper cross-dating that can yield annually resolved chronologies sensitive to environmental stressors (Hudson et al., 1976).

In order to identify possible candidates for fish growth chronology research in the Mediterranean Sea, we conducted a search of the FishBase database. This is a global, scientifically guided, biodiversity information system on fishes that provides a wide range of information (taxonomy, biology, trophic ecology, and life history) on all species currently known in the world, as well as historical data reaching back 250 years[1]. According to this database, a total of 755 fish species from 174 families inhabit the Mediterranean Sea. We made several reductions to obtain a reasonable pool of potentially interesting target species. Since the database provides data for maximal recorded total length (TL), maximal reported age, trophic level, and habitat p‘nces (demersal, pelagic-neritic, benthopelagic, bathypelagic, bathydemersal, pelagic-oceanic, and reef-associated) and status (endemic, introduced, and native or questionable), we first removed all short-lived (<2 years) and

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[1] fishbase.org

small fishes (TL < 30 cm). This resulted in the removal of species belonging to the families Apogonidae, Atherinidae, Blenniidae, Bregmacerotidae, Callionymidae, and Carapidae. Given their conservation status, Chondrichthyes and the primitive fishes (Myxinidae, Petromyzontidae, Chimaeridae, and Halosauridae) were also excluded. Furthermore, rare or poorly investigated species without any commercial interest or benthopelagic, bathypelagic, and bathydemersal fishes for which no age- related data were provided in FishBase were also excluded.

Finally, a pool of 263 fish species inhabiting Mediterranean Sea was obtained and used in the analysis. The estimated or determined maximum age of 31 fish species from 20 families was over 30 years. However, it is important to note that age was determined by age reading methods on specimens from the Mediterranean Sea for only 12 species (**Table 2**), while the age of other species was estimated based on growth equation parameters available for that specific species or closely related species from the same family, or age was reported for an area other than the Mediterranean Sea.

From these reports, the longest-living species in the Mediterranean Sea is the wreckfish, *Polyprion americanus* (see Peres and Haimovici, 2004). However, its maximum age of 76 years was reported for specimens from the continental shelf and slope off southern Brazil. Thus, its availability for sclerochronology studies in the Mediterranean is questionable, particularly since this species has been listed as Critically Endangered (CR) on the IUCN Red List (IUCN, 2017). The same is true for the red bream *Beryx decadactylus*, since the reported maximum age of 61 years was for specimens collected off the southeastern coast of the United States. To the best of our knowledge, there are no relevant data for the maximum age of either species in the Mediterranean.

The dusky grouper, *Epinephelus marginatus*, lives throughout the Mediterranean Sea and its maximum age of 60 years was reported for specimens from the Balearic Islands (Reñones et al., 2010). Most groupers are solitary, resident fishes. The Mediterranean is the upper limit of their northward distribution, and their growth in the Mediterranean is significantly slower than for groupers in tropical waters (Gracia_López and Castelló-Orway, 2003). Site specificity, a relatively slow growth rate (some species may not be mature until the age of 8 to 10 years) and spawning strategy (synchronic or protogynous hermaphrodites; Sadovy and Shapiro, 1987; Heemstra and Randall, 1993) make them particularly vulnerable (CITES/UNEP-WCMC, 2017). Although the long-life span and resident behavior makes *E. marginatus* an interesting candidate for construction of growth chronologies, its low abundance and protected status throughout the Mediterranean requires a strategic approach to sample collection extending over time, rather than single on-site sampling action.

Three families listed in **Table 2**—Sebastidae, Lutjanidae, and Sciaenidae—were identified earlier within the list of globally important fish taxa for sclerochronology research (Black et al., 2019). However, just two species from the Sebastidae family are listed in the Mediterranean Sea, and only *H. dactylopterus* can attain an age of over 40 years. Certain caution is needed, as this data was reported for individuals caught in the Northeast Atlantic and not in the Mediterranean Sea. According to the available data, *H. dactylopterus* grows faster and lives longer in the Northeast Atlantic than in the Mediterranean (Ragonese, 1989; Allain and Lorance, 2000; D'Onghia et al., 2004; Consoli et al., 2010). The maximum age reported for *H. dactylopterus* from the Mediterranean is 21 years (Consoli et al., 2010), questioning the availability of Mediterranean samples for growth analysis for this species.

Two species of Sciaenidae family are listed in **Table 2**. The maximum reported age for the meagre, *Argyrosomus regius*, in the Gulf of Cádiz (SW Iberian Peninsula) is 42 years (González-Quirós et al., 2011), while the brown meagre, *Sciaena umbra*, reached 31 years in the Gulf of Tunis (Chater et al., 2018). The dense calcium carbonate deposition of the large and very thick otoliths in Sciaenids reduces light transmission, making it almost impossible to distinguish hyaline and opaque zones (Arneri et al., 1998; Chater et al., 2018). According to Arneri et al. (1998), growth increments in otoliths of these taxa are more readable in cross-sections. Both species are commercially important and there is the potential for collection of representative otolith samples. However, further development of otolith reading techniques is needed to facilitate identification of growth increment boundaries and enable statistically robust chronology construction.

Two non-native species, yellowbar angelfish (*Pomacanthus maculosus*) and mangrove red snapper (*Lutjanus argentimaculatus*), have a lifespan of over 30 years (Grandcourt et al., 2004; Fry et al., 2006) and are interesting candidates for growth chronology construction. Both species entered the Mediterranean via the Suez channel and in recent years have established their populations in the eastern Mediterranean, along the coasts of Israel and Lebanon (Bariche, 2010; Sonin et al., 2019). The maximum reported age for *P. maculosus* is for specimens from the southern Arabian Gulf, while for *L. argentimaculatus* the maximum age data is reported for its native range—Papua New Guinea. These two species belong to long-living families (Grandcourt et al., 2004; Piddocke et al., 2015), and although determination of otolith growth patterns present certain challenges for the oldest specimens (Rezende and Ferreira, 2004; Steward et al., 2009), in the context of climate change they are interesting taxa for growth chronology research.

The remaining species listed in **Table 2** belong to the families Sparidae and Moronidae. There are total of 31 sparid species in the Mediterranean Sea, which are known to be slow-growing and long-lived (Hanel and Tsigenopoulos, 2011) and susceptible to over-exploitation due to their commercial importance (Comeros-Raynal et al., 2016). Sparid fishes generally have relatively large and easily readable sagittal otoliths, and despite the wealth of literature denouncing the use of whole, unsectioned otoliths in growth studies on sparid fishes (see Winkler et al., 2019), age determination using whole otoliths is still common. According to the information available in Fish Base, the maximum age reported for sparids in the Mediterranean ranges from 5 to 36 years (**Table 2**). Species with the greatest potential are common dentex *Dentex dentex* and zebra seabream *Diplodus cervinus*. Due to its commercial importance, wide distribution, clear growth patterns in otoliths, and lifespan of over 20 years (Kraljević et al., 1998), the gilt head seabream *Sparus aurata* is also an interesting candidate for sclerochronology research. From the Moronidae family, sea

TABLE 2 | The list of long-lived fish species in the Mediterranean Sea according to FishBase. Data sorted by descending maximum reported age.

Family	Species	Length (cm)	Maximum age (year)	Trophic level	Habitat	References
Serranidae	*Epinephelus marginatus*	150.0	60	4.1	Reef-associated	Reñones et al., 2010
Carangidae	*Pseudocaranx dentex*	122.0	49	3.9	Reef-associated	Kailola et al., 1993
Sebastidae	*Helicolenus dactylopterus*	50.0	43*	4.0	Bathydemersal	Allain and Lorance, 2000
Sciaenidae	*Argyrosomus regius*	230.0	42	4.3	Benthopelagic	González-Quirós et al., 2011
Moridae	*Mora moro*	80.0	39	3.8	Bathypelagic	Sutton et al., 2010
Pomacanthidae	*Pomacanthus maculosus*	61.0	36*	2.7	Reef-associated	Grandcourt et al., 2004
Sparidae	*Dentex dentex*	100.0	36	4.5	Benthopelagic	Baudouin et al., 2016
Sparidae	*Diplodus cervinus*	55.0	35	3.0	Reef-associated	Jarzhombek, 2007
Lutjanidae	*Lutjanus argentimaculatus*	150.0	31*	3.6	Reef-associated	Fry et al., 2006
Sciaenidae	*Sciaena umbra*	70.0	31	3.8	Demersal	Chater et al., 2018
Caproidae	*Capros aper*	30.0	30	3.1	Demersal	Hüssy et al., 2012
Moronidae	*Dicentrarchus labrax*	103.0	30	3.5	Demersal	Kottelat and Freyhof, 2007

**Data for specimens for locations other than Mediterranean Sea.*

bass, *Dicentrarchus labrax* can attain age of 30 years (Kottelat and Freyhof, 2007). The species mentioned in this paragraph are economically interesting, and EU Mediterranean countries collect relevant landing and biological data for them [data collection framework (DCF); Regulation (EU), 2017]. It is highly likely, either within monitoring programs or scientific research projects, that otoliths of these species are archived during several years or even decades by different institutions and could be used to extend time series data beyond the maximal reported age.

According to the data presented above, the availability of otoliths for long living species from the Mediterranean is quite limited, as there are only several species reaching a maximum reported age of over 30 years. However, development of statistical techniques enables construction of growth chronologies for shorter living fish species (<15 years) when samples are collected over several years or decades (Coulson et al., 2014). It is possible that, for certain fish species, adequate replicates for chronology construction can be obtained through archive collections.

OPPORTUNITIES FOR OTOLITH CHEMISTRY RESEARCH

Clarity of growth rings in otoliths is one of the main factors contributing to sclerochronology research, both for growth increment measurements and for otolith chemical analysis (Campana, 1999). Problems related to interpretation of increments in otolith, including age estimation and validation of periodicity, has been pointed out in number of studies in different parts of the world (e.g., Morales-Nin et al., 2005b; Stransky et al., 2005; Hüssy et al., 2016). This problem should not be underestimated, and interpretation of otolith increments needs to be carefully checked and validated. One of the most appreciated characteristics of otoliths is their lack of resorption. This means that once the material has been deposited, the organism will not use these minerals again, even in periods of starvation. Lack of resorption is not shared with other calcified structure (like scales and bones) in fishes or other vertebrates (Bilton, 1974; cited by Campana and Thorrold, 2001). Another special characteristic of otoliths is that they grow continuously throughout the lifetime of the fish (Campana, 1999).

In order to assign relevant chemistry data to a specific calendar year, it is crucial to distinguish growth increment boundaries (Black et al., 2005; Martino et al., 2019). However, many the most commercially important fish species living in the Mediterranean Sea do not have clearly distinguished growth patterns in their otoliths, which presents a challenge for this type of research. For example, it is still difficult to determine the growth boundaries for the first growth increments in otoliths of *Mullus barbatus* due slow growth and number of false-growth increments laid down before the annulus (Carbonara et al., 2018) and of *Merluccius merluccius* due to the fast growth (de Pontual et al., 2003; Piñeiro et al., 2007; Mellon-Duval et al., 2010) and long spawning period of the species (Morales-Nin and Aldebert, 1997) although a number of direct methods to validate age assessment were used, like mark-recapture (de Pontual et al., 2003; Mellon-Duval et al., 2010), first ring appearance (Belcari et al., 2006), or bomb radiocarbon dating (Vitale et al., 2016).

Species from the families Sparidae and Lutjanidae have annual growth rings, that although thin, are clearly visible (Piddocke et al., 2015; Winkler et al., 2019), and represent the most promising target taxa for sclerochronology studies. Interesting target species of Sparidae include *Dentex dentex*, *Diplodus cervinus*, and *Sparus aurata*. The latter species, together with *Dicentrarchus labrax* (Moronidae), are particularly interesting, as these are the most important fish aquaculture species throughout the Mediterranean region (Lacoue-Labarthe et al., 2016). In addition to these species and those listed in **Table 1**, another interesting taxon for chemical research of otoliths is *Seriola dumerili* (Carangidae), a species with a circumglobal distribution (Smith, 1997).

Instrumental restrictions, related to the quantity of material required for the analysis of stable isotopes in otoliths, has been the main limitation for the development of isotope related research in otoliths (Sreemany et al., 2017). Due to the small size of the otolith, this resulted in time averaging of data, and analyses were limited to whole otoliths (e.g., Rooker et al., 2008a), or certain parts of otoliths, e.g., the core (Siskey et al., 2016;

Rooker et al., 2019) or edge (Hidalgo et al., 2008; Tanner et al., 2012), without the possibility of obtaining time series data. Development of instruments and methods, including high-resolution laser ablation systems (e.g., Sreemany et al., 2017) and continuous flow isotope ratio mass spectrometry system for ultra-microvolume carbonate samples (Kitagawa et al., 2013; Sakamoto et al., 2017), have opened new opportunities for obtaining time series data from otoliths. Although significant progress was made in instrument development, many are still available only to a limited number of scientists. Therefore, new opportunities for collaborations and research directions related to the Mediterranean are required.

CHALLENGES AND LIMITATIONS FOR FISH SCLEROCHRONOLOGY RESEARCH IN THE MEDITERRANEAN SEA

Alongside the constraints imposed by the biological characteristics of species, there are other challenges to conducting sclerochronology research in the Mediterranean region. Although otoliths are small structures that are easily archived, there still appears to be a general lack of understanding and effort to construct and manage otolith collections. Panfili et al. (2002) clearly indicated the importance of archiving otoliths, highlighting the need to evaluate, catalog, and conserve otolith collections in a way that will make both the otolith and corresponding fish life history information more accessible to all researchers. The Instituto de Ciencias del Mar-CSIC (Spain) maintains an otolith reference collection that includes samples from different parts of the world, including the Western Mediterranean Sea[2]. One example of an online searchable database of otolith collections from other parts of the world is the otolith collection database housed at the Burke Museum[3]. Although English is generally accepted as a global scientific language, the Mediterranean is highly politically, economically, cultural, and linguistically diverse region, which impacts sample, data, and knowledge storage and sharing. While online searchable otolith collections are not currently possible for a number of reasons, efforts should be made by different institutions or even scientists themselves to archive otoliths together with relevant collection and biology data. It is highly recommended that collections contain samples for different species, not only commercially most important ones, and that special efforts are made to archive otoliths of rare species. Furthermore, Disspain et al. (2016) pointed out the potential to use otoliths from archeological sites to analyze changes in the environment occurring through human history. Linking archeologists with fish biologist and environmental scientists can provide great potential for sclerochronology research.

In addition to the issues related to the availability of otolith collections, continuous sampling over several decades is also a challenge given the limited availability of funding for long-term studies and the logistics associated with field sampling. Long-term time series data are needed to estimate the real status of exploited resources and their evolution over time (Battaglia et al., 2010) and to analyze climate change effects on marine species and communities (Azzurro et al., 2019). Today, most scientific research projects are short in duration, resulting in difficulties related to securing funds needed for maintaining a longer data time series (Lleonart and Maynou, 2003; Rochet and Trenkel, 2003). Even when data collection takes place over longer periods, it often suffers from inconsistencies in sampling design or sampling methods (Rochet and Trenkel, 2003; Rochet et al., 2005). Sampling designs often tend to be incomplete, lacking either randomization or replication, and as such can never conclusively demonstrate the causes of the observed changes. Sampling and storage protocols are often specific to the institution or project. It is, however, encouraging that all the EU Member States bordering the Mediterranean Sea, eight in total, are required to collect fisheries data using unified methodology [Regulation (EU), 2017].

Human resources present another important segment in the development of all marine-related research in the Mediterranean, including sclerochronology. Although otoliths have been analyzed in relation to age and growth, very few attempts have been made to link the data derived from otoliths with environmental data. Education in methods associated with growth increments analysis, chemical composition of otoliths, and statistical methods related to sclerochronology research is strongly needed, either through personal or workshop-based interactions, to stimulate the involvement of fisheries scientists in sclerochronology studies. Furthermore, sclerochronology includes biological, chemical, and physical aspects, requiring an interdisciplinary research team.

Different types of instrumentation are required for sclerochronology analysis. Some instruments need to be readily and continuously available, such as saws and grinding and polishing machines, while those for chemical analysis of otoliths can be offsite. For example, the Laser Ablation System coupled with a High-Resolution Inductively Coupled Plasma Mass Spectrometer (HR-ICPMS) is a sophisticated tool for analysis of elemental composition that is both very expensive and requires specially trained personnel. Careful sample preparation and establishing collaboration with institutions possessing such an instrument can enable the processing of otolith samples at a reasonable cost. Efforts should be made to develop human and research capacities within the framework of different international projects, thereby promoting scientific collaboration and the education of young researchers.

CONCLUSION

The Mediterranean Sea is a hotspot of marine biodiversity and is also one of the most impacted ecoregions globally (Halpern et al., 2008; Costello et al., 2010), due to increasing levels of human threats that affect all levels of biodiversity (Mouillot et al., 2011; Coll et al., 2012; Micheli et al., 2013) and due to severe impacts from climate change (Lejeusne et al., 2010) and biological invasions (Katsanevakis et al., 2012).

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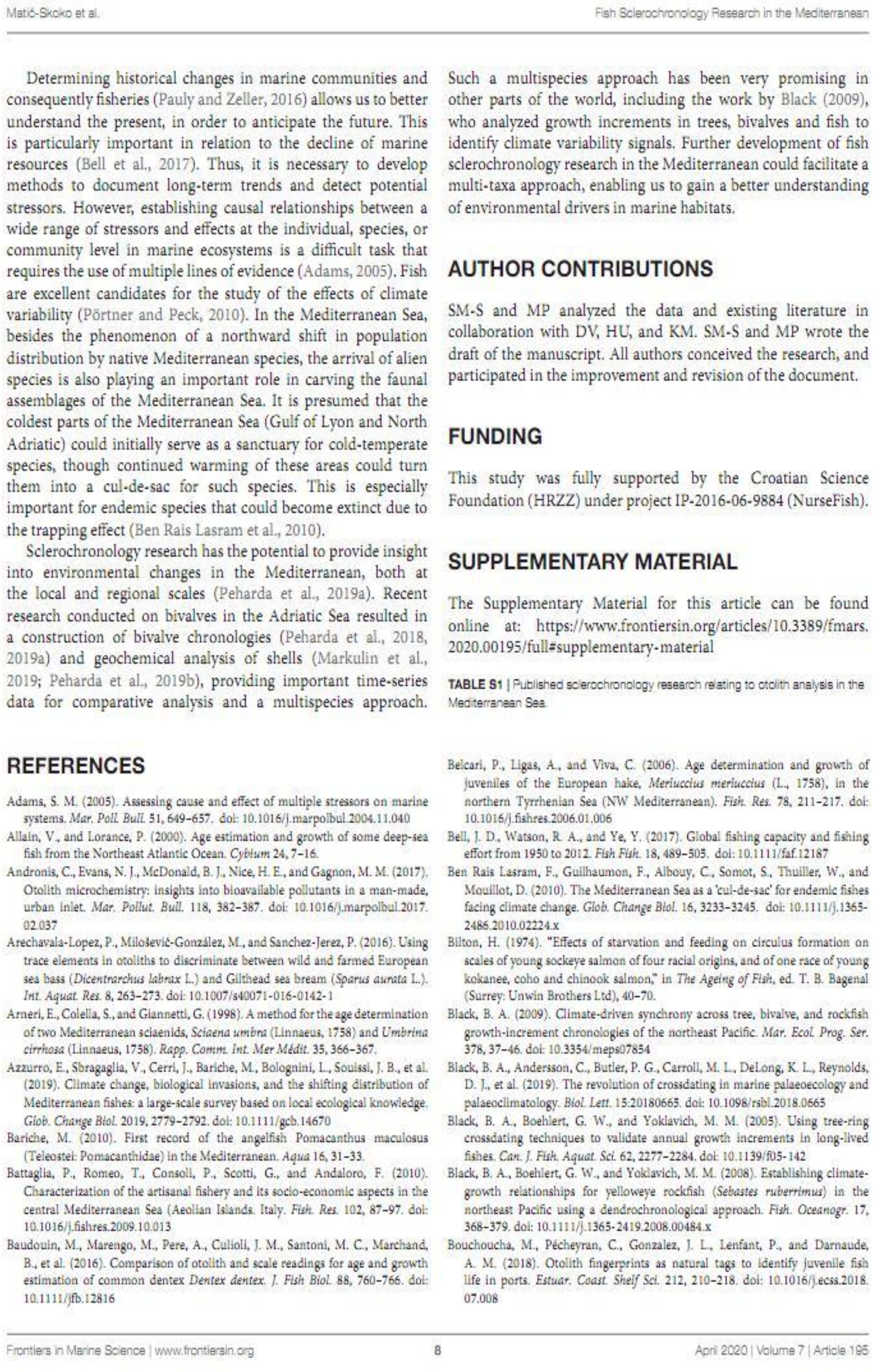
[2] ipt.vliz.be/eurobis

[3] www.burkemuseum.org



Determining historical changes in marine communities and consequently fisheries (Pauly and Zeller, 2016) allows us to better understand the present, in order to anticipate the future. This is particularly important in relation to the decline of marine resources (Bell et al., 2017). Thus, it is necessary to develop methods to document long-term trends and detect potential stressors. However, establishing causal relationships between a wide range of stressors and effects at the individual, species, or community level in marine ecosystems is a difficult task that requires the use of multiple lines of evidence (Adams, 2005). Fish are excellent candidates for the study of the effects of climate variability (Pörtner and Peck, 2010). In the Mediterranean Sea, besides the phenomenon of a northward shift in population distribution by native Mediterranean species, the arrival of alien species is also playing an important role in carving the faunal assemblages of the Mediterranean Sea. It is presumed that the coldest parts of the Mediterranean Sea (Gulf of Lyon and North Adriatic) could initially serve as a sanctuary for cold-temperate species, though continued warming of these areas could turn them into a cul-de-sac for such species. This is especially important for endemic species that could become extinct due to the trapping effect (Ben Rais Lasram et al., 2010).

Sclerochronology research has the potential to provide insight into environmental changes in the Mediterranean, both at the local and regional scales (Peharda et al., 2019a). Recent research conducted on bivalves in the Adriatic Sea resulted in a construction of bivalve chronologies (Peharda et al., 2018, 2019a) and geochemical analysis of shells (Markulin et al., 2019; Peharda et al., 2019b), providing important time-series data for comparative analysis and a multispecies approach. Such a multispecies approach has been very promising in other parts of the world, including the work by Black (2009), who analyzed growth increments in trees, bivalves and fish to identify climate variability signals. Further development of fish sclerochronology research in the Mediterranean could facilitate a multi-taxa approach, enabling us to gain a better understanding of environmental drivers in marine habitats.

AUTHOR CONTRIBUTIONS

SM-S and MP analyzed the data and existing literature in collaboration with DV, HU, and KM. SM-S and MP wrote the draft of the manuscript. All authors conceived the research, and participated in the improvement and revision of the document.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2020.00195/full#supplementary-material

TABLE S1 | Published sclerochronology research relating to otolith analysis in the Mediterranean Sea.

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Otolith fingerprints reveals potential pollution exposure of newly settled juvenile *Sparus aurata*

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ABSTRACT

Coastal ecosystems are increasingly threatened by a wide range of human activities. Fish otolith chemistry, by creating a unique specific signature, can be used as a natural tag for determining life stage dispersal, spatial connectivity and population structure. In this study, we tested whether differences in otolith composition among juveniles of gilthead sea bream, *Sparus aurata*, could enable their proper allocation to polluted areas based on higher concentrations of elements related to contaminants. Otoliths were embedded, sectioned and analysed by LA-ICP-MS in line scan mode. Multivariate analysis confirmed clear separation between sites and elements. Samples from the site under the strongest anthropogenic impact from industrial and agricultural river input were characterized by higher values of Pb/Ca and Zn/Ca. However, these relatively low values likely do not have a negative effect on *S. aurata* recruitment, though they could serve for identifying the contribution of polluted nurseries to stock dynamics.

1. Introduction

The gilthead seabream, *Sparus aurata* is one of the most studied and highly prized sparid species in the Mediterranean Sea (Bauchot and Hureau, 1986). Although it is a commercially important species, both for the aquaculture and fisheries sectors (Farrugio et al., 1994), its population characteristics throughout the distribution range are still not clearly resolved. Reliable knowledge of natal origin, population structure, habitat connectivity and vulnerability to anthropogenic pressure are still lacking. Some recent studies indicate positive changes in species abundance and distribution, implicating ocean warming as a potential factor behind increasing abundance (Glamuzina et al., 2014; Žužul et al., 2019). It could be argued that wild gilthead seabream, as a subtropical sparid, is taking advantage of the current climate change in terms of enhanced spawning, increased larval survival and subsequent recruitment success, but also in new establishment at the northern limits of its distribution range (Davis, 1988; Somarakis et al., 2013; Glamuzina et al., 2014; Avignon et al., 2017; Žužul et al., 2019). Another possible reason for the observed changes in population dynamics of *S. aurata* is escape from sea-cage aquaculture and escapes through spawning (Šegvić-Bubić et al., 2011, 2014, 2018). Escaped fish may affect native stocks through competition for resources, the spread of disease and alteration of genetic diversity due to hybridisation (Arechavala-Lopez et al., 2018), which can reduce the competitive ability and overall fitness of wild populations (McGinnity et al., 2003). Throughout the Mediterranean, it is known that *S. aurata* performs ontogenetic and trophic migrations between coastal lagoons and the sea (Lasserre, 1976; Mercier et al., 2012; Isnard et al., 2015) that are related to spawning (Audouin, 1962; Houde, 1989), settlement and recruitment (Cowen et al., 2000; Beck et al., 2001; Cowen and Sponaugle, 2009; Morais et al., 2017). Juveniles that colonise sheltered coastal areas in early winter migrate out to sea the following autumn (Audouin, 1962; Lasserre, 1976; Bauchot and Hureau, 1986).

Potential nurseries for juvenile fish in the coastal zone are of primary interest for resource sustainability (Lotze et al., 2006). Since most large-scaled industry and big cities are located in coastal areas, those ecosystems are under high and expanding impacts from human activities (Yanagi, 2015). Due to the intensification of climatic and anthropogenic pressures in these areas (Vitousek et al., 1997; Boesch et al., 2001), coastal areas are increasingly threatened by pollution, eutrophication, and habitat degradation (Nixon, 1995; Kemp et al., 2005), resulting in habitat loss, population fragmentation, and loss of biodiversity (Levin et al., 2001). Without a doubt, these alterations of coastal environmental conditions have reduced their quality as nurseries for

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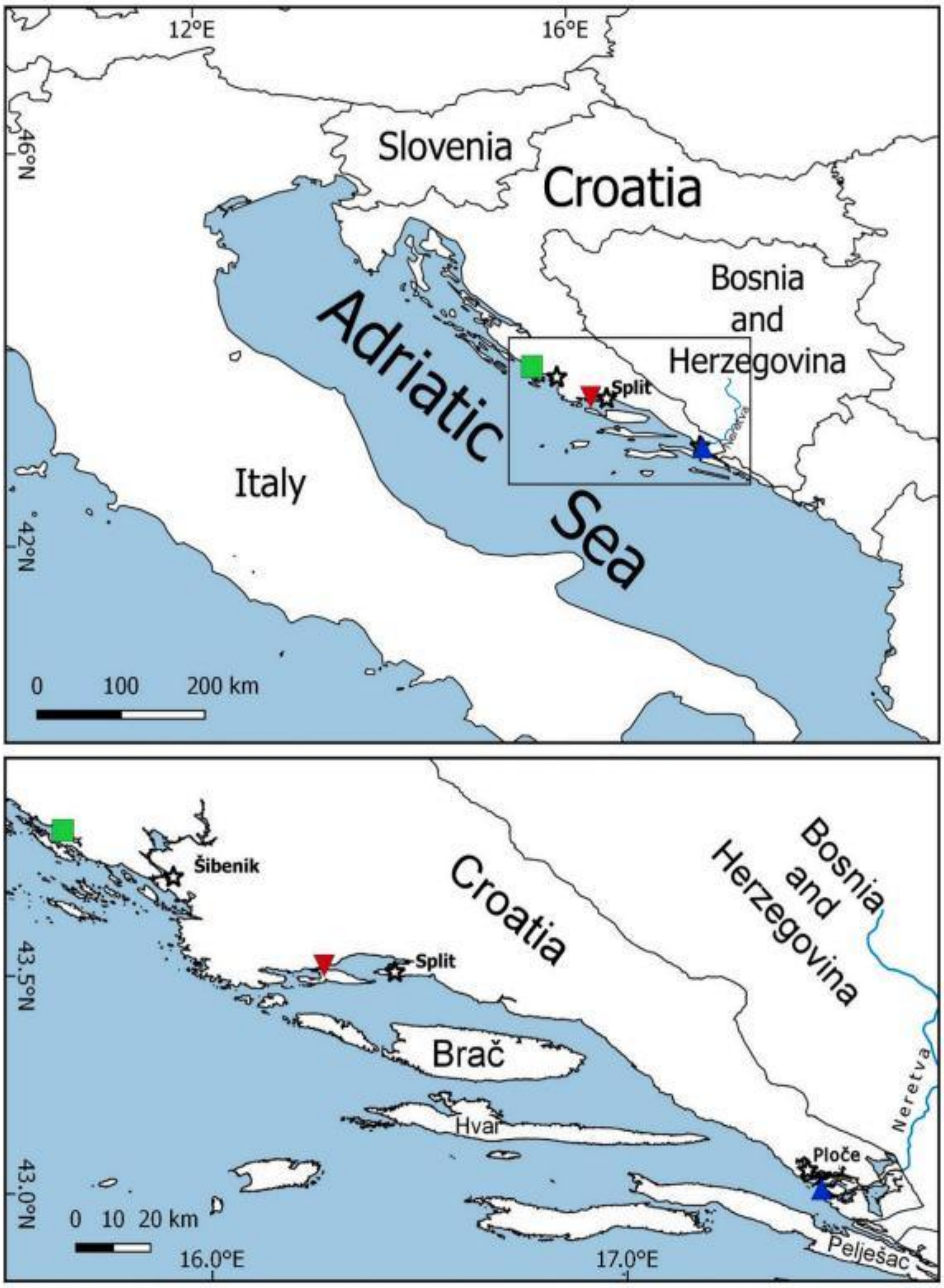

Fig. 1. Sampling area along the eastern Adriatic coast with selected sites: Prosika (green), Pantan (red) and Mala Neretva (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

juvenile fish (Quignard et al., 1984; Labourg et al., 1985; Brusle and Cambrony, 1992) and thus might have affected *S. aurata* migratory behaviour (Šegvić-Bubić et al., 2018), and consequently recruitment and stock dynamics (Madeira et al., 2016).

Otolith microchemistry is a powerful tool for examining life stage dispersal, connectivity in regions (Gillanders and Kingsford, 1996; Campana et al., 2000; Thorrold et al., 2001; Vasconcelos et al., 2007), assessing population structure (Tanner et al., 2012), defining estuarine nurseries (Gillanders and Kingsford, 2000), assessing connectivity between juvenile and adult populations (Reis-Santos et al., 2013, 2015) and to a lesser extent, as an indicator of environmental pollution (Saquet et al., 2002; Søndergaard et al., 2015; Cuevas et al., 2019). Different elements from the local environment are permanently incorporated into the crystalline matrix of the otolith that accrete continuously, as otoliths are metabolically inert and their increments are unlikely to be subject to resorption (Wright, 1990; Powles et al., 2006). Although otolith microchemistry can reflect a combination of local environmental chemistry and individual physiology, the resulting elemental composition can create a unique fingerprint that can serve as a natural tag (Campana and Thorrold, 2001; Elsdon et al., 2008) to distinguish location and infer ontogenetic change (Gibb et al., 2017). Consequently, the incremental microstructures of otoliths from different environmental conditions or locations vary in their elemental composition (Campana et al., 2000). Thus, marine organisms inhabiting impacted areas likely accumulate metals including anthropogenic ones from the surrounding environments and transfer/transport them into higher links of the food chain (Wang, 2002).

The ambient concentrations of trace elements are influenced by a range of external factors that vary at both the spatial and temporal scales (Campana and Thorrold, 2001; Rogers et al., 2019). Most studies of the elemental composition of fish otoliths have reported levels of elements, such as Ca, Sr, Na, K, Mg, Ba, P, S, and Cl (Edmonds et al., 1989; Kalish, 1991; Gunn et al., 1992; Edmonds et al., 1995; Fuiman and Hoff, 1995; Limburg, 1995; Secor et al., 1995), or the concentrations of trace elements like Cu, Zn, Fe and Sn (Campana et al., 1994; Campana and Gagne, 1995; Kalish et al., 1996; Thorrold et al., 1997). In the marine environment, Ba, Mg, U, B, and Sr in various calcitic tissues show strong correlations with ocean water temperatures (Sadekov et al., 2005, 2009; Montagna et al., 2007; Long et al., 2014), while other elements like Sr and Ba are used successfully to reconstruct environmental and coastal-estuary migration histories for individual fish (Fowler et al., 2016), as their concentrations reflect local availability in seawater. Other elements, like K, Na, Zn, and Mn, are likely to be mediated through physiological regulation (Correira et al., 2011; Bouchoucha et al., 2018).

Coastal areas generally offer greater chemical heterogeneity due to upwelling, fluvial and anthropogenic inputs, but often elements can originate both from natural and anthropogenic sources (Jiang et al., 2017). Also, elements related to potential contaminants that would contribute to such geographic variation (e.g. Ni and Zn) are physiologically discriminated against and typically below detection levels in otoliths (Hanson and Zdanowicz, 1999). We hypothesised that juveniles from polluted nursery areas can be allocated properly according to the accumulation of trace elements in otoliths related to possible sources of contamination in a certain area. Thus, the aim of this study was to investigate differences in otolith chemical composition among juveniles of *S. aurata* after several months of residency in selected nurseries in the eastern Adriatic Sea. We tested whether otolith elemental fingerprints from polluted areas could enable proper allocation of juveniles into a specific site based on higher concentrations of certain elements. This would allow the reliable assessment of polluted area recruitment contributions to adult fish populations and would draw attention to the possible effects of accumulated pollutants to stock dynamics.

2. Materials and methods

2.1. Study sites and fish collection

In this study conducted in the eastern Adriatic Sea, newly settled juveniles of *Sparus aurata* were collected from three estuarine sites previously known as essential nursery areas for this species (Dulčić et al., 2002, 2005; Matić-Skoko et al., 2007): Prosika (N 43.834162°, E 15.639217°), Pantan (N 43.523234°, E 16.268753°) and Mala Neretva (N 43.011862°, E 17.468263°; Fig. 1). The distance between the furthest sites is 200 km, and hydrologically they represent similar water types in the Adriatic Sea. Prosika is near Šibenik, characterized by a number of groundwater springs and indirectly influenced by the Krka River, with a partially rocky-sandy bed with patches of *Cymodoea nodosa* and photophilic algae meadows. The Pantan estuary is near Split, and receives the waters of the Pantan River, exhibiting variable salinity gradients during the year with a muddy-sandy bottom partially overgrown with *Zostera marina*. The Mala Nerteva site is situated in the estuary of the Neretva River near Ploče, with a similar bottom type as the Pantan site.

Samples of juvenile *S aurata* specimens were collected using a specially constructed small shore seine (L = 25 m; mesh size 4 mm) in May–June 2018. Sampling was carried out in the shortest possible time to avoid temporal variation in otolith chemistry analysis. Three hauls from each site were performed to collect specimens. In total, 13 juveniles of *S. aurata* were collected. Upon collection, specimens were transported to the laboratory and frozen until analysis. For the analysis, total length (TL) and weight (TW) were recorded and specimens were dissected to extract otoliths for chemical analysis.

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 and cut 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 glued 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 in distilled water and cleaned in an ultrasonic bath (SONOREX SUPER RK 103H) for 2 min and left to air-dry.

2.3. LA-ICP-MS analysis of otoliths

Element concentrations of the otoliths were determined by LA-ICP-MS in line scan mode, along the axis of maximal growth, through the otolith core. 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/cm^2. 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 × 40 μm (preablation beam 80 × 40 μm). Synthetic glass NIST SRM 612 (National Institute of Standards and Technology) was used to calibrate element concentrations of otolith samples and 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 applying the preferred values available from the GeoReM database (http://georem.mpch-mainz.gwdg.de/, application version 26; compare also Jochum et al., 2005, 2011, 2012). Monitored isotopes included ^{7}Li, ^{23}Na, ^{26}Mg, ^{43}Ca, ^{55}Mn, ^{66}Zn, ^{88}Sr, ^{97}Mo, ^{137}Ba, ^{138}Ba, ^{208}Pb and ^{238}U. Signals were monitored in time-resolved mode and processed using an in-house Excel spreadsheet (Jochum et al., 2007). Details of the calculations are given in Mischel et al. (2017). The concentration of Ca as an internal standard in otoliths was taken as 38.8% by weight or 388,000 μg/g following the determination of otolith Ca concentration (Yoshinaga et al., 2000). Concentrations determined on otoliths were converted to molar concentrations and standardised to calcium.

2.4. Data analysis

Element-to-Ca data for 11 elements were determined for all specimens. Most of these element-to-Ca data were below quantification and detection limits (Forootan et al., 2017). Moreover, for most elements the signals during ablation on the otoliths were not higher than the noise of the background signal. Ba/Ca and Sr/Ca ratios were above the detection and quantification limits while ratios for Mg/Ca, Zn/Ca and Pb/Ca exceeded the detection and quantification limit in several samples and thus those elements were included in further analysis. When element concentration data for these elements exceeding 31-point (31-pt) running averages by 5σ, they were considered outliers and were excluded from further analysis (see Marali et al., 2017a, 2017b). For data visualization, element linear raster was smoothed using a 31-pt arithmetic running average.

To enable comparison between samples and sites and to eliminate potential confounding effects due to temporal variation in the factors influencing otolith chemical composition (Patterson et al., 1999; Gillanders and Kingsford, 2000; Rooker et al., 2002, 2003; Gillanders, 2002; Hamer et al., 2003; Guido et al., 2004; Gibb et al., 2007; Correira et al., 2011), all otoliths were reduced on a raster line length of the smallest sample (1137 points) by cutting the same number of data points from the edge sections of otoliths (left and right).

Univariate PERMANOVA was used to test the difference of sites on elemental data obtained from otoliths. 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).

Differences in otolith elemental composition were evaluated via the permutational analysis of variance (PERMANOVA) using Manhattan distance dissimilarity matrices because elements were on very comparable measurement scales. We calculated the Manhattan measure separately for each element variable sets and then averaged the resulting Manhattan distance matrices to obtain a single overall matrix that measures the differences between specimens for the overall otolith signatures for elements. Differences in elemental composition between sampling sites were visualized with metric multi-dimensional scaling (mMDS) on the basis of the Manhattan distance dissimilarity matrices.

CAP analysis was used to estimate the accuracy of otolith element signatures 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 with another single continuous variable, or finding axes having maximum correlations with some other set of variables (Anderson and Willis, 2003). CAP analyses were run separately for each of the two factors: "Sites" and "Elements". The CAP routine output scores were then merged for both factors.

3. Results

At all sites, *S. aurata* specimens were from 3.1–6.0 cm TL. At the Prosika site, specimens ($n = 5$) ranged in TL from 3.1 to 3.4 cm (mean 3.27 ± sd 0.15 cm) and weight from 0.34 to 0.44 g (0.4 ± 0.06 g), at the Pantan site ($n = 5$) from 4.0 to 6.0 cm (5.18 ± 0.82 cm) and 0.79 to 2.66 g (1.75 ± 0.81 g), and at the Mala Neretva site ($n = 3$) from 4.5 to 5.9 cm (5.22 ± 0.53 cm) and weight from 0.65 to 2.82 g (1.75 ± 0.81 g).

3.1. Otolith trace element chemistry

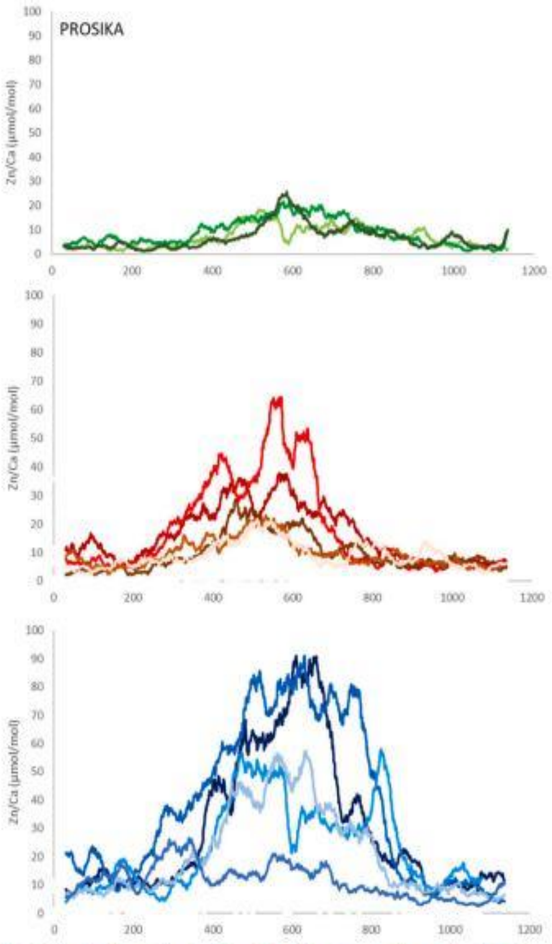

Fig. 2. Zn/Ca data series for all analysed *S. aurata* specimens for three sites, Prosika (green), Pantan (red) and Mala Neretva (blue). Individual specimens are presented in different shades of colour. Sampling conducted through the otolith core, along the axis of maximal growth. Results are displayed as 31-pt arithmetic running averages (x-axis). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Mg and Zn were accumulated in the highest concentration in the examined otoliths. Concentrations of all monitored elements (Mg, Ba, Sr, Zn and Pb) varied between sites (Table 1). Values of the Zn/Ca ratio were highest at Mala Neretva, and this ratio was significantly different between Mala Neretva and Pantan, while no significant difference was detected between Mala Neretva and Prosika or Pantan and Prosika. The Mg/Ca ratio was significantly different between all three sites ($p < 0.05$) with the highest median value noted at the Pantan site.

Table 1
Median values of Mg/Ca, Ba/Ca, Sr/Ca, Zn/Ca and Pb/Ca ratios observed in *Sparus aurata* otoliths from three study sites (Prosika, Pantan and Mala Neretva) with minimum and maximum values indicated in brackets. Significant differences in otolith signatures between sites were tested by univariate PERMANOVA.

Element/Ca	Prosika	Pantan	Mala Neretva	Pseudo-F	p
Mg/Ca (mmol/mol)	0.177 (0.009–1.546)	**0.393** (0.019–1.834)	0.207 (0.017–1.488)	3.841	0.016*
Ba/Ca (μmol/mol)	1.351 (0.364–9.068)	1.203 (0.337–6.830)	**1.376** (0.323–11.132)	0.961	0.536
Sr/Ca (μmol/mol)	**1.945** (0.797–3.357)	1.496 (0.736–3.934)	1.723 (0.802–3.484)	4.021	0.002*
Zn/Ca (μmol/mol)	7.047 (5.891–53.171)	8.224 (4.787–112.470)	**15.734** (5.622–160.383)	2.238	0.029*
Pb/Ca (μmol/mol)	0.382 (0.290–1.812)	0.470 (0.237–3.015)	**0.621** (0.298–5.404)	1.489	0.095

* significant $p < 0.05$.

However, the pairwise test did not reveal significant variations in the Mg/Ca ratio for either the Mala Neretva and Prosika, or Prosika and Pantan comparisons. Sr/Ca significantly differed between all three sites (pair-wise test, $p < 0.05$) with the highest value in otoliths sampled from specimens caught at the Prosika site. There were no significant differences among sites for Ba/Ca or Pb/Ca ($p > 0.05$). However, the median value of Pb/Ca was highest at the Mala Neretva site.

Ba/Ca, Pb/Ca and Zn/Ca ratios in otoliths of specimens from Mala Neretva were higher and showed a wider range than at the other two sites, although only Zn/Ca was significantly different.

Scatter plots indicate that the highest values of Zn/Ca ratios were accumulated in the otoliths in the Mala Neretva site and lowest at the Prosika site. All otoliths from Prosika had a similar pattern of Zn accumulation along the axis of maximum growth in the otolith. The most prominent variations between all samples are evident in the core of the otolith (Fig. 2).

Although Pb concentrations did not differ significantly between sites, scatter plots within sites clearly showed higher Pb accumulation at the Mala Neretva site. The lowest concentrations without a difference between samples were seen at the Prosika site. The highest concentrations of Pb at the Mala Neretva site were in the core, while such a clear pattern was not obtained at the Pantan site (Fig. 3).

Table 2
Summary of PERMANOVA results for the multivariate analysis of overall element compositions in otoliths for juvenile *Sparus aurata* collected at three sites (Prosika, Pantan and Mala Neretva) in the Adriatic Sea.

	df	MS	Pseudo-f	P (perm)
Sites	2	$1.862*10^6$	2.330	0.0012
Elements	4	$1.614*10^7$	20.185	0.0001
Sites x Elements	8	$1.564*10^6$	1.957	0.0009
Residuals	50	$7.992*10^5$		
Total	64			

Fig. 3. shows visible differences in Pb concentrations in otoliths from the Mala Neretva and other two sites. The linear raster suggests that Zn is continuously accumulated at each point while Pb is accumulated discreetly, since Pb had zero values at more than 50% of the linear raster points. This likely affected the statistical analyses, resulting in no spatial significant differences in Pb concentrations among sites.

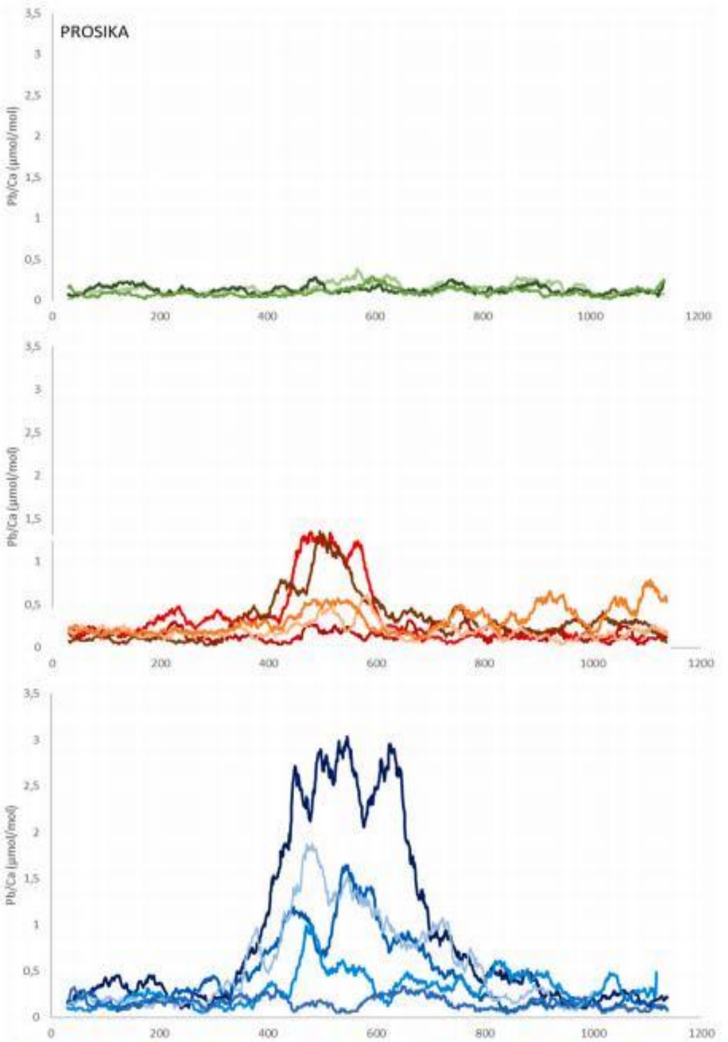

Fig. 3. Pb/Ca data series for all *S. aurata* specimens for three sites, Prosika (green), Pantan (red) and Mala Neretva (blue). Sampling conducted through the otolith core, along the axis of maximal growth. The results are displayed as 31-pt arithmetic running averages (x-axis). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

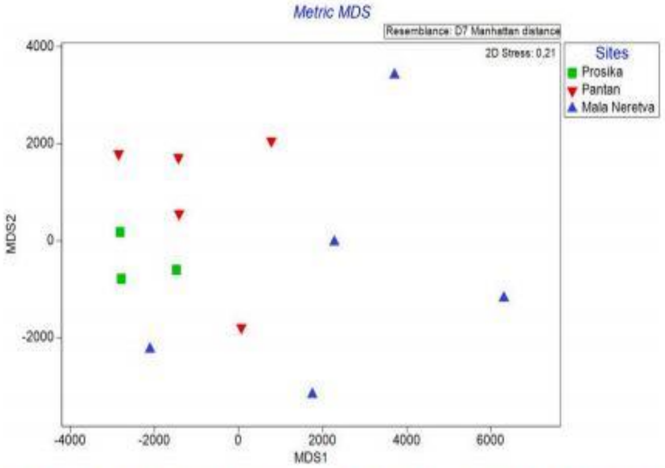

Fig. 4. Metric MDS for otolith fingerprint of juvenile *Sparus aurata* observed at the three sites tested (Prosika, Pantan and Mala Neretva) in the Adriatic Sea.

3.2. Multi-parameter comparison

When the otolith chemistry data were combined into a single matrix, PERMANOVA analysis detected that values differed significantly with respect to the factor "Sites" (Pseudo-F = 3.841, p = 0.016). When element data per sample were analysed separately, PERMANOVA analysis detected that "Sites" and "Elements" of juvenile *S. aurata* differed significantly in their element signatures (Table 2). After running the pairwise test, elemental concentrations differed significantly between all elements ($p < 0.05$).

Spatially, after running the pairwise test, no significant differences were determined between the Pantan and Prosika sites ($p > 0.05$). However, significant differences in otolith composition were found between Mala Neretva and Pantan (t = 1.454, p = 0.018) and Mala Neretva and Prosika (t = 1.808, p = 0.002), revealing the distinction of the Mala Neretva site.

On the mMDS plot derived from the Manhattan distance for otolith samples, the points for Mala Neretva progressed toward the right and upward, confirming the multivariate statistic outputs (Fig. 4). Pantan and Prosika sites were positioned to the left with more gathered otolith data for Prosika.

A more detailed metric MDS showed a rather clear separation of Zn/Ca ratios (Fig. 5). It is apparent that all three sites can be distinguished for Zn concentrations. Although all data for Zn concentrations were dispersed, Mala Neretva samples were the most variable, making them difficult to congregate.

A separate CAP analysis for each of the two factors ("Sites" and "Elements") gave successful discrimination for both elements and sites. In particular, Sr/Ca ratios were correctly allocated based on the otolith chemistry information to Pantan (100%) and Prosika (100%), and to a lesser degree to Mala Neretva (80%) (Fig. 6A). Moreover, all Zn/Ca ratios corresponding to Pantan specimens were correctly allocated (100%), while two-thirds of Prosika specimens and four-fifths of Mala Neretva specimens were correctly assigned by their Zn/Ca ratios (Fig. 6B). On the other hand, Mg/Ca concentrations were correctly allocated to Mala Neretva (100%), while 80% of Pantan and only one-third of Prosika specimens could be correctly allocated based on this element (Fig. 6C).

4. Discussion

The results of this study clearly demonstrated that the chemical

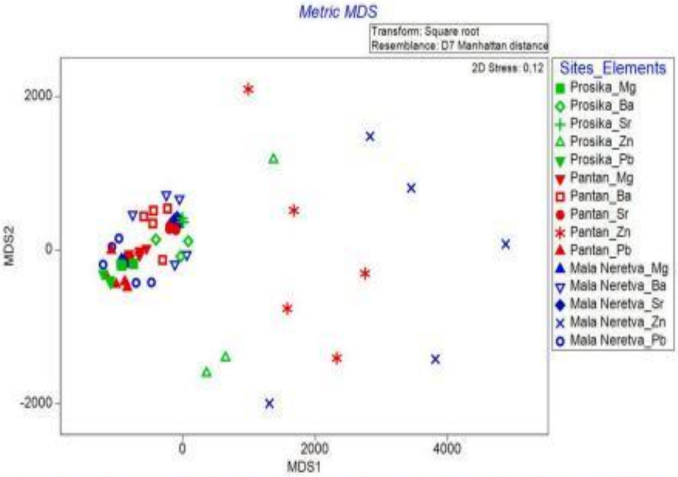

Fig. 5. Metric MDS plot for elements (Ba/Ca, Sr/Ca, Mg/Ca, Zn/Ca and Pb/Ca) chemistry of the otolith of juvenile *Sparus aurata* sampled in 2018, grouped by "Sites" and "Elements".

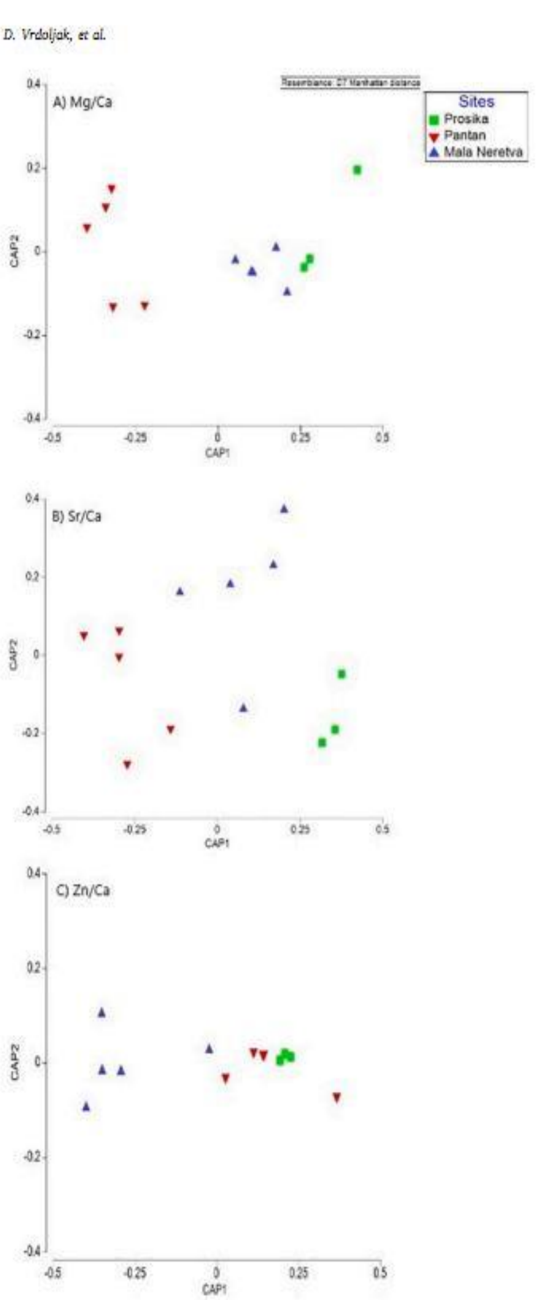

Fig. 6. Canonical variate plot (CAP) for Mg/Ca (A), Sr/Ca (B) and Zn/Ca (C) element chemistry of the otolith of juvenile *Sparus aurata* grouped by factor "Sites".

composition of juvenile *Sparus aurata* otoliths varied between nurseries in the eastern Adriatic. Concentrations of five elements (Mg, Ba, Sr, Zn and Pb) were above the detection and quantification limits, and varied between sites. The most discriminant element was Zn, while Pb spatial variations were visible but not statistically significant.

4.1. Otolith trace element chemistry

The incorporation of all elements in otoliths is more complex than their mere concentration in ambient water as this is affected by their bioavailability (Walther and Thorrold, 2006; Bouchoucha et al., 2018), the physiological state of individual fish (Sturrock et al., 2015; Grønkjær, 2016), ontogeny and growth rate (Correira et al., 2011), and the synergetic/concomitant effects of temperature, salinity and water chemistry (Sadovy and Severin, 1994; Bath et al., 2000; Campana et al., 2000; Elsdon and Gillanders, 2004; Elsdon et al., 2008). In this study, Mg, Sr and Zn concentrations were significantly different between sites while Pb, showed increased, though not significant, concentrations at the Mala Neretva site. The otolith concentrations of Mg from Pantan samples were well distinguished from the other two sampling sites. However, high variation was detected in the Mg/Ca ratio between all otoliths from the Pantan site, suggesting that incorporation of Mg is likely affected by the physiological state of individual fish. Higher values of Mg in otoliths was previously reported by Martin and Thorrold (2005), Martin and Wuenschel (2006) and Correira et al. (2011) who discussed that no studies have been able to clearly link the incorporation of Mg into otoliths with specific exogenous environmental factors, and that the uptake of this element into otoliths appears more likely to be related to endogenous processes.

Ratios of Sr/Ca and Ba/Ca in the otoliths are usually linked to their concentrations in ambient water and salinity, with a higher Sr/Ca found in marine and higher Ba/Ca found in freshwater (Gillanders, 2005; Gillikin et al., 2019). Thus, Sr and Ba can be used to successfully reconstruct environmental and coastal-estuary movement histories for individual fish (Fowler et al., 2016; Izzo et al., 2018). Since the Prosika site is not under direct river influence and is a typical coastal marine site, unlike the other two sites, it was expectedly characterized by the highest Sr/Ca ratio among the analysed otoliths. Significantly higher Sr concentrations in otoliths of recruits from coastal reefs than in estuarine seagrass habitat was reported by Gillanders and Kingsford (1996). Unfortunately, lack of water and sediment samples for investigated location disable relevant comparison and establishment between element concentration in the water column and otolith microchemistry. For sure, such limitations have to be consider in future sampling designs.

4.2. Zn/Ca

The main source of Zn in the environment is the lithosphere, particularly the rocks in the surrounding water (Halden et al., 2000). However, Zn, like Cu, Ni, and Pb, is also related to anthropogenic sources including landfill, plastic rubbish, fishing boats, phosphate operations, and tourist activities (Nour and Nouh, 2020). Some authors consider that Zn is more physiologically regulated, and less of a reflection of the surrounding environmental conditions (Campana, 1999; Halden et al., 2000). According to Willis and Sunda (1984), the primary mode of zinc uptake in seawater appears to be via food. Ranaldi and Gagnon (2008) corroborated this, concluding that zinc concentrations in otoliths are influenced by fish diet, which they later demonstrated experimentally (Ranaldi and Gagnon, 2010). They concluded that the availability of zinc, food-chain biomagnification and the flux of zinc depends on the thermal regime, pH, alkalinity and amount of particulate matter in water, all of which vary with the type of aquatic environment. In that line, Zn concentrations tend to be higher in urban coastal zones due to anthropogenic sources (Sturrock et al., 2012). Interestingly, we found that the median value of the concentration of Zn in the otolith was two times higher at the Mala Neretva site, and that it accumulated continually with the highest values in the core, declining toward the edges. Ontogenetically, the analysed otoliths correspond with 5-month old individuals and the first spring in their lives. It is known that the first growth ring in *S. aurata* in the Adriatic Sea appear in autumn, after the rapid growth during the summer months (Kraljević et al., 1998). Halden et al. (2000) reported that zinc, in addition to strontium, shows a systematic distribution in otoliths related to annular structure. According to Avigliano et al. (2015) the otolith Zn/Ca ratio tended to decrease with age. Thus, Zn concentrations could be expected to be higher around the growth rings, with descending values with the next age. Potentially, this could be used for the differentiation of cohorts as suggested by Avigliano et al. (2015), providing temporally constrained information on habitat, fish behaviour or nutrient supply within the life cycle.

4.3. Pb/Ca

The otolith signature of Pb within the Mala Neretva samples was more prominent than in the otoliths from the other two sites, though the signal was not of the same strength in all analysed otoliths and did not differ significantly between sites. It was previously reported that some elements, like Pb, exhibit variations in their incorporation into otoliths (Geffen et al., 1998; Hamer and Jenkins, 2007). However, the relationship between exposure level and Pb concentrations in otoliths is complex. Selleslagh et al. (2016) experimentally found that concentrations in *Platichthys flesus* were correlated with those measured in the water. Bouchoucha et al. (2018), studying otolith fingerprints of juvenile *Diplodus* species in ports, confirmed that Pb concentration in otoliths was species-dependent, but did not directly reflect environmental contamination levels, and as such, they concluded that Pb concentrations in the otolith could not provide reliable information capable of discriminating ports from other nursery areas. However, Søndergaard et al. (2015) reported the highest Pb concentrations in otoliths from the most Pb-polluted sites near a mine (up to 0.6 μg/g), with a decreasing gradient with increasing distance from the mine. Similarly, the greater concentrations of Pb and Zn recorded in anchoveta, *Engraulis ringens* otolith cores from central Chile were traced to nearby iron, steel and petrochemical industries (Cuevas et al., 2019). The Pb concentrations reported here from Mala Neretva samples revealed relatively high variability between individuals, with higher values in the core area of three specimens but relatively low values in two others. Gillanders and Kingsford (1996) also reported higher concentrations of Pb in otoliths of recruits from estuarine seagrass habitat under more intense anthropogenic influence than in otoliths of recruits from coastal reefs. However, Edmonds et al. (1992) highlighted that changing environmental conditions in coastal areas have the potential to cause substantial yearly differences in elemental fingerprints, both for Zn and Pb. Further, Søndergaard et al. (2015) reported that Pb and Sr variations in the otoliths of *Myoxocephalus scorpius* were closely correlated and showed an annual oscillatory pattern with peaks consistently found in the winter zones, without a clear explanation if such an effect is a consequence of high winter-time exposure of Pb through diet or water and/or to physiological processes such as growth. In this study, the highest value of Pb/Ca were found in the otolith core corresponding to the first winter in these juveniles, ontogenetically representing the period of fastest growth in their life cycle (Kraljević et al., 1998). For certain, the detected concentrations in this study are likely the consequence of *S. aurata* site fidelity to inshore nursery areas (Abecasis and Erzini, 2008), since such areas provide larvae or early juveniles with prey and shelter availability, enabling growth and completing the life cycle before recruiting to coastal adult populations (Beck et al., 2001).

Vasconcelos et al. (2011) reported that juvenile fish leaving the estuaries to join off-shore stocks during the first year of life export low levels of contamination, due to the low levels of Cd and Pb accumulating in their first months of life in estuarine nurseries (Durrieu et al., 2005). Nevertheless, many juveniles spend increased periods in estuaries and are exposed to pollution loads for longer periods (Usero et al., 2003; Vinagre et al., 2004; França et al., 2005), which could affect further life processes. Since juvenile *S. aurata* spend around nine months in nurseries, there is no indication that accumulated Pb and Zn concentrations at the Mala Neretva site could have a negative impact on the southern Adriatic stock dynamics.

4.4. Multivariate approach to chemical analysis of otoliths

The highly accurate discrimination by multi-parameter analyses indicated that the analysed nursery grounds have distinct elemental signatures. Recently, multivariate analyses have become a desirable tool for such purposes (e.g. Gillanders, 2005; Correira et al., 2011; Bouchoucha et al., 2018) due to the capability of this analytical procedure to separate different perturbations from natural spatio-temporal variability displayed by most populations (Terlizzi et al., 2005). In this study, multivariate analyses confirmed differences between sites but also between elements when each element was analysed separately. The three sites showed high contrast in contaminant concentrations, which are known to influence fish otolith composition (Campana, 1999; Sturrock et al., 2012). The obtained results revealed that all significant differences were attributed to the Mala Neretva site, while there were no prominent differences between the two more northern sites, Pantan and Prosika. Moreover, the otolith signatures from the Pantan and Mala Neretva sites were correctly allocated (100%) according to their Zn/Ca and Sr/Ca ratios.

The obtained results are consistent with those from similar studies conducted in the last ten years (e.g., Di Franco et al., 2011; Fortunato et al., 2017). Marine habitats differ in their environmental conditions, due to either variation in anthropogenic influences or natural variations (Barnes and Gillanders, 2013; Sturrock et al., 2015; Bouchoucha et al., 2018). In seeking out similarities between the sites, variation based on the type of river influence and main river characteristics could be compared. The Prosika site is under indirect influence of Krka River runoff and groundwater inputs in Prosika Bay, while the Mala Neretva (Oskoruš et al., 2019; Krvavica and Ružić, 2020) and Pantan sites (Matić-Skoko et al., 2005; Fistanić, 2006) sites are both under direct river influence.

In 2011, the Neretva River was proposed (Hrvatske vode, 2015) as a heavily modified water body; i.e. according to the EU legislation, its surface water has been substantially changed in character as a result of physical alterations by human activity (WFD, 2000). In the Neretva Delta, massive drainage and irrigation networks have been established over the past 50 years, and inter-basin water transfer projects implemented to produce agricultural land (Skoulikidis, 2009). Additionally, Croatia's second largest seaport is located at the river's mouth, and handles the import and export of commodities for neighbouring Bosnia and Herzegovina, including terminals for general cargo, containers, dry bulk cargo, ro-ro ships, passenger ships and tankers (Lušić et al., 2008). Some data are available regarding the metal contamination of sediment (Jurina et al., 2015; Giglio et al., 2020) and fish tissue (Bogut and Bukvić, 2003; Has-Schön et al., 2006; Has-Schön et al., 2008; Djedjibegovic et al., 2012) in the Neretva River area. This is an important agricultural region in southern Croatia, and pesticides and metals often used in agriculture are therefore discharged to nearby estuarine and marine areas (Filimonova et al., 2018). Elements, as Al, Cd, Ba, Mn, Pb, Co and Zn usually found as components in paints and fertilisers and occur naturally in freshwater runoff (Gillanders and Kingsford, 1996), often are occurring in greater concentrations in otoliths of recruits from estuarine regions and extensive industrial areas (Ranaldi and Gagnon, 2010). Recently, Bouchoucha et al. (2018), suggested that ports are well suited for studies on elemental fingerprints in otoliths as natural tags, because they are recipients of industrial and domestic wastes (Darbra et al., 2004). Unfortunately, there is a lack of data regarding water and sediment concentrations of elements related to contaminants that disable reliable otolith microchemistry interpretation in this study.

4.5. Conclusions

The determined concentrations of trace metals in this study were well below the European standards and guidelines for trace metals in fish and shellfish tissue (Commission Regulation EC No 1881/2006). However, human health risk assessment is needed to establish if these values represent a human health risk due to consumption. Otolith chemical composition yields valuable information on potential exposure that can be used to assess trends in the health of aquatic ecosystems and provide baseline information for risk assessment regarding the disruption of specific ecological functions. Also, it can serve as an indicator of how specific polluted nurseries contribute to stock or population dynamics. Gilthead sea-bream is one of the most important commercial fish species in the Mediterranean Sea, and comprehensive knowledge on its behaviour in the context of climate change and anthropogenic impacts through high fishing pressure and habitat modifications is crucial.

CRediT authorship contribution statement

All of the authors conceived the research. Dario Vrdoljak, Sanja Matić-Skoko, Melita Peharda, Hana Uvanović, Krešo Markulin and Regina Mertz-Kraus contributed to the sample design, collecting and preparing otoliths and muscle tissue for analyses, and running analyses. Dario Vrdoljak and Sanja Matić-Skoko wrote the draft of the paper and all authors participated in the improvement and revision of the document.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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TRACKING THE GROWING RINGS IN BIOGENIC ARAGONITE FROM FISH OTOLITH USING CONFOCAL RAMAN MICROSPECTROSCOPY AND IMAGING

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ABSTRACT. Fish otoliths pose increasing interest due to their potential of rendering information about environmental changes, underlined in their non-linear time-dependent biogenic crystal growth. Otoliths are acellular, metabolically inert and continuously grow in a complex process which still needs to be understood. In the present work confocal Raman micro-spectroscopy (CRM) and imaging is employed to investigate the growth pattern in otoliths from *Sparus aurata* of Mediterranean provenance. CRM signal acquired from otolith sagittal section is exploited to associate it with the periodic growth increments denoted as rings. Raman signal collected from the core to the margins with micrometer spatial resolution invariably revealed characteristic signal of aragonite. Relative intensity variability was observed particularly for the lattice modes, indicating changes in crystalline orientation relative to incident laser. Bands associated with organic fraction were absent in the 90-1840 cm^{-1} spectral range. Daily growth rings were further studied using the Raman mapping of main aragonite bands intensities at 1083, 704 and the lattice modes in the 100-350 cm^{-1} range. The spectral intensity pattern closely follows the daily growth pattern. Traces of toxic or heavy metals incorporated in biogenic carbonate mineral were spuriously detected in the mapped areas, according to the position and width of the Raman bands of witherite ($BaCO_3$), strontianite ($SrCO_3$), along with the main aragonite and trace of its calcite polymorph.

***Keywords**: Confocal Raman micro-spectroscopy, fish otoliths, Raman mapping, growth rings, aragonite.*

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INTRODUCTION

Otoliths are crystalline $CaCO_3$ biominerals with a small fraction of organic matrix, located in the inner ear of vertebrates. They serve as a balance organ for equilibrium and contribute to hearing [1], [2]. Fish otoliths are increasingly investigated due to the potential correlation of their lifetime development with the environmental changes they encountered. Defined as opaque zones delimiting the translucent ones, the otoliths growing rings provide valuable tool for the fish age determination. The use of otoliths as chronometric structures to track the recorded environmental conditions along the organism life-time is still in its infancy. However, increasing number of Raman spectroscopy studies already refer to the otolith sclerochronology as valuable tools for tracking environmental changes [3-4], employing the characteristic Raman signature of calcium carbonate polymorphs [5] which could occur in otoliths complex biomineralization process. Their growth pattern is composed of a number of concentric rings with different radii. Depending on the amount of organic material in each shell or zone, its appearance will vary from extremely opaque to complete hyaline (transparent) with the first zone being the nucleus (core) of the otolith. These zones are also called growth rings [4]. Previous Raman studies showed that the characteristic polymorph of calcium carbonate biomatrix, present in otoliths, is aragonite with the typical Raman vibration modes ν_1 (1085 cm^{-1}) and ν_4 (701 cm^{-1} and 705 cm^{-1}) as well as lattice modes (8 bands between 142 cm^{-1} and 282 cm^{-1}) [3, 6]. Moreover, some studies have reported two different $CaCO_3$ crystalline structures, aragonite and vaterite, in the otoliths of different fish species [7] [8] [9], which can differ dramatically in their trace elemental composition [10, 11]. Some of the studies reported the coexistence of three polymorphs of calcium carbonate (calcite, aragonite, vaterite) in the shell of Antarctic bivalves having the same growing pattern (rings) as fish otoliths [12]. Gauldie et al (1997) [13] reported first vaterite polymorph signature in the low wavenumber range of the micro-Raman spectra of otoliths core from the coho salmon *Oncorhynchus kisutch* (Teleostei: Salmonidae) and highlighted the aragonite nature of the otolith first ring.

On the other hand, the polymorphs of calcium carbonate may interfere with other, mixed carbonate minerals, whose Raman analysis relies on correct interpretation of the specific lattice modes [14, 15]. Furthermore, strict similarity of the biogenic with geogenic mineral Raman feature must be treated with caution, since their environment and mechanism of formation is different. Wehrmeister et al (2010) showed that the structure of vaterite contains three crystallographic independent carbonate groups and similar carbonate group layers, and firstly assigned a band at 263 cm^{-1} to vaterite [16].

The aim of the current study is to probe the potential of the confocal Raman micro-spectroscopy (CRM) technique for detailed investigation of otolith morphology and growing pattern in Mediterranean ***Sparus aurata*** and to probe the versatility of the Raman technique to upscale the study for larger otoliths groups. Optimized conditions for screening would generate robust Raman data which could be further exploited for any correlation with environmental conditions encountered during fish otoliths lifetime period.

RESULTS AND DISCUSSIONS

From the larger set of otoliths, we randomly selected one as subject for the present detailed study, as a prerequisite for the forthcoming comparative Raman analyses of otolith sets. The investigated otolith fragment is shown in the Figure 1 along with the optical micrographs taken with the Raman microscope in transmission (b) and direct illumination (c) using a 20x objective (NA 0.35).

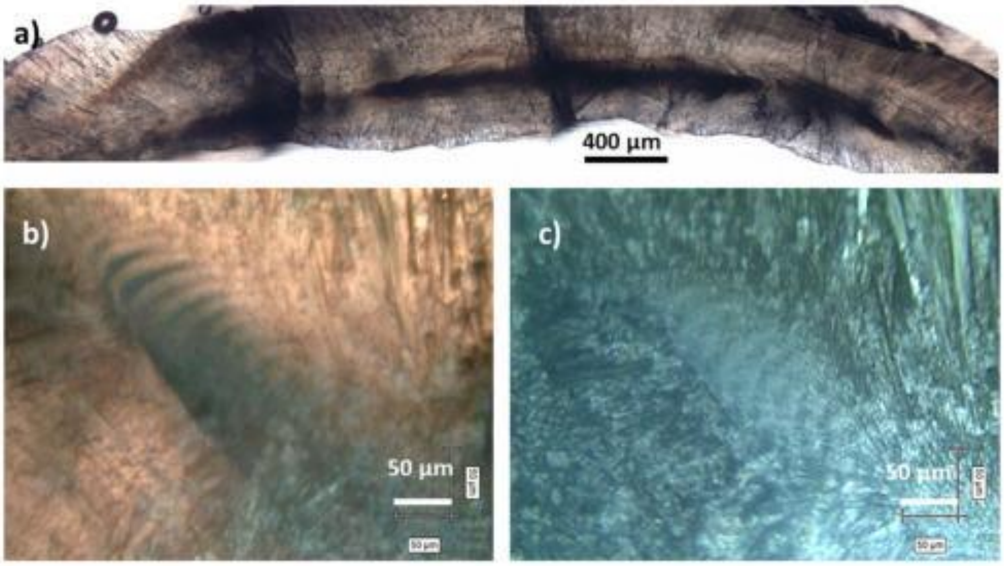

Figure 1. Photo-stitch of the otolith fragment in sagittal section (a), together with the light microscopy images taken with the Raman microscope in transmission (b) or direct illumination (c), using a 20x objective. Scale bars: 400 µm (a) and 50 µm (b and c).

A series of CRM spectra presented in the Fig. 2 acquired from otolith along a line starting from core toward edge, with a 50 µm step and corresponding to the growing direction, indicated by the orientation of the micro-crystallites, clearly depict aragonite as the main mineral component of the otolith across its sagittal section. These spectra were collected using the lowest magnification objective available (5x, NA 0.12). We noted lower background of the recorded signal when higher magnification objectives are employed. However, for gross screening of the whole otolith while preserving the screening line direction, the 5x objective was optimal. Bearing in mind that high background in the Raman spectra could potentially masque additional, low intensity bands from other trace minerals, further analysis is devoted later.

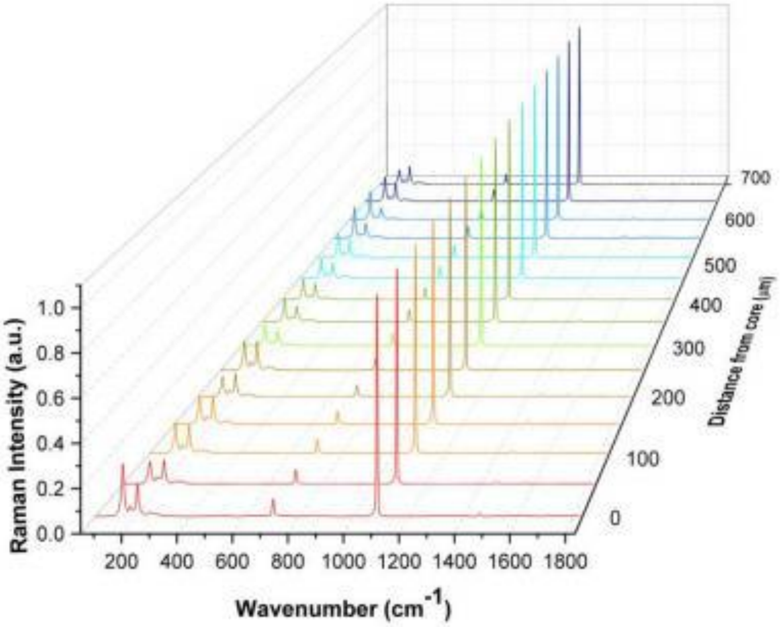

Figure 2. 3D display of the normalized, background subtracted CRM spectra collected along a line direction starting from otolith core toward edge with a 50 µm step. Excitation: 532 nm, 5x objective (NA 0.12).

In geogenic aragonite, the unit cell of crystalline orthorhombic aragonite comprises 20 atoms (four fomula units in a crystalline cell). Its 57 vibrational modes are classified [19] according to the irreducible representations of point groups theory, as follows:

$$\Gamma_{total} = 9A_g + 6A_u + 6B_{1g} + 8B_{1u} + 9B_{2g} + 5B_{2u} + 6B_{3g} + 8B_{3u}.$$

The g (*gerade*) modes are all Raman active while u (*ungerade*) modes are IR active, with the exception of Au modes, which are silent [19]. Thus, Raman spectrum of aragonite is expected to show 30 fundamental modes. However, due to the fact that many of the vibrational modes are of very weak intensity, it is extremely difficult to detect experimentally all the active modes [19], particularly in natural samples. In synthetic aragonite [13] the lattice modes detected in Raman spectra were reported at 113 (w), 142 (s), 153 (s), 180 (w),190(w), 206 (m), 242 (w), 261 (w), 284 cm^{-1} (w), the symmetric stretching mode ν_1 at 1085 cm^{-1} (vs), the ν_3 at 1462 (w) and 1574(w) and the ν_4 at 701(s) and 705 cm^{-1} (m). Table 1 summarized the observed Raman bands in otolith core and first ring, in comparison with the reported Raman data of geogenic aragonite [18, 19] calcite [19], vaterite [17] and two other minerals from the aragonite group, witherite [14] and strontianite [17], along with their assignment.

Table1. The Raman bands observed in spectra collected from otolith core and first ring along with the characteristic bands of geogenic aragonite polymorphs (calcite, vaterite) and other carbonate minerals from aragonite group, witherite, ($BaCO_3$) or strontianite ($SrCO_3$)

Otolith Core	Otolith 1st ring	Aragonite [19]	Aragonite [18]	Calcite [19]	Vaterite [17]	Whiterite [14]	Stronitianite [17]	Assignment (Symmetry) [19]
1461	1457 1571	1462.2 1573.9	1463 1576	1435.8		1420	1445	ν_3 (B_{1g})
1083	1081	1085.3	1085	1086.2	1080 1090	1059	1071	ν_1 (A_{1g})
		853.3	853					ν_2 (A_g)
685						690	510	ν_4 (A_g) (B_{2g})
690						699	710	
699	700	701.8	701					
704	704	706.1	705					
716	714	717.1	716	712.4				
727					739			
731					749			
		113.5	115	154.9 281.2	106 206 265 299	100 135 153 179.67 224.9	148 180 214 236 244 258	Lattice Modes
		125	125					
		142.9	145					
152	153	153	155					
		162.2	164					
180	180	180	182					
		190.7	192					
206	206	206.3	208					
		214.7	216					
		225.5						
	247	248.3	250					
	260	260.1	263					
		272	274					
		283.6	285					

The strong aragonite signal in the otolith (Fig. 2) was identified by the presence of the symmetric stretching mode of the carbonate ν_1 at 1083 cm^{-1} and the ν_4 vibrational mode at 701 and 704 cm^{-1} as well as lattice modes in the low wavenumbers region: 152, 180 and 206 cm^{-1}. The aragonite signal has proved to be the strongest along the entire scan line with apparent traces [14] of other calcium carbonate polymorphs, while the organic matrix was not detectable using the 5x objective (NA 0.12) for excitation and collecting optics in 1 s acquisition.

Taking a closer microscopy look while taking Raman spectra with higher magnification objectives, such as 20x (NA 0.35) or 100x (NA 0.9), respectively, subtle details have been observed on much lower background. The spectral details as showed in the Figure 3. Micro-Raman spectra collected from the first ring of otolith using three different objectives, 5x, 20x and 100x respectively, are showed in the Fig. 3 a) in comparison with the Raman spectra from RRUFF database of geogenic aragonite (RRUFF ID: R080142), calcite (RRUFF ID: R150075) and strontianite (RRUFF ID: R040037). Their spectra showed subtle differences, particularly in the lattice modes range (50-300 cm-^{1}), and in the main stretching modes around 1083 and 704 cm^{-1}. Spectral details are highlighted in the Fig. 3 b, c and d, which allowed us to argue that trace of calcite, witherite and strontianite could be present [14]. In addition, the main Raman stretching mode at 1083 cm^{-1}, which is slightly shifted from those of geogenic aragonite observed at 1085 cm^{-1}, showed a narrower band for the otolith core than for the first ring. The shoulder at 1077 cm^{-1} (Fig. 3 b) slightly higher in spectrum from the first ring than that from the core, and further different from that of geogenic aragonite, may suggest the presence of other carbonate mineral, such as strontianite (amorphous calcium carbonate was excluded, due to the clear evidence of the lattice modes). Zoom of the 680-740 cm^{-1} spectral range (Fig. 3 d) revealed aragonite modes at 704 cm^{-1} with additional weak band (shoulder) at 700 cm^{-1}, and other weak bands, potentially indicating calcite and witherite traces [14]. In the lattice modes region (Fig. 3 c) subtle differences among biogenic and geogenic aragonite are observed; the band at 180 cm^{-1} could be attributed to either aragonite or to a certain mixture of different carbonates [14]. Strontianite $(Sr\ 0.91\ Ca\ 0.09)CO_3$ exhibited main Raman band at 1077 cm^{-1} while in the lattice modes range it shows bands at 150 (strong) and 183 cm-^{1} (weak) which are overlapped with the aragonite lattice modes. Therefore, in an aragonite crystalline matrix, trace of Sr to replace Ca ions is difficult to reveal via Raman spectroscopy although the shoulder at 1077 cm^{-1} may suggest strontianite trace presence. Moreover, the 699 cm^{-1} mode of witherite [14] or strontianite [15] could be responsible for the observed shoulder in the ν_4 Raman mode of aragonite centered at 704 cm^{-1} (Fig. 3).

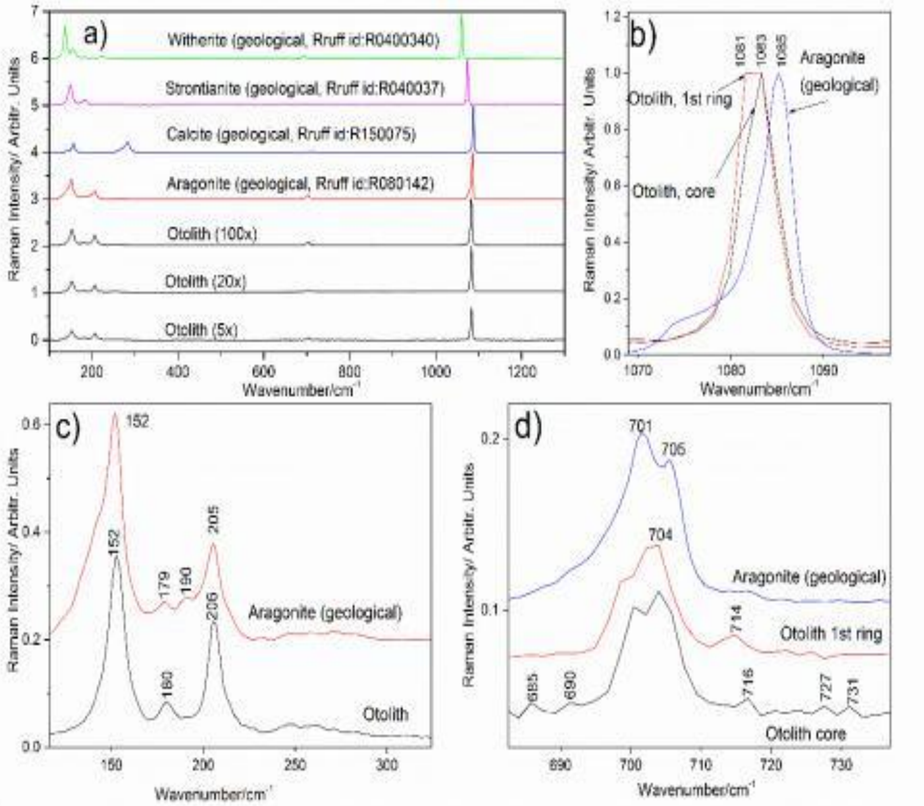

Figure 3. Micro-Raman spectra collected from the otolith in the optical region of the first yearly ring, using three different objectives, as indicated (a), in comparison with the geological aragonite (red, RRUFF ID: R080142), calcite (blue, RRUFF ID: R150075), strontianite (magenta, RRUFF ID: R040037) and witherite (green, RRUFF ID: 040040); (b) zoom of the main stretching mode at 1083 cm^{-1} showing narrower band for the otolith core than for the first ring; (c) lattice modes highlighting subtle differences among Raman signal of biogenic and geologic aragonite; (d) spectral zoom of the characteristic mode of aragonite at 704 cm^{-1} with additional weak bands potentially indicating calcite / strontianite trace; Excitation: 532 nm. Otolith spectra from (b, c, d) are collected with 20x objective.

The presence of a weak band at 690 cm^{-1} suggests the presence of barium carbonate (witherite) [14]. The supposition is further sustained by the preliminary scanning electron microscopy and energy dispersive X-Ray analyses (SEM-EDX) preliminary data (not shown here) on the otoliths set (manuscript in preparation), although the Sr trace randomly appeared at 0.2 Wt%, thus, at the limit of EDX detection. The Raman spectra obtained with higher magnification objectives, describes a smaller area with better focus on the otolith saggital section surface and the presence of the new bands could be attributed to different crystal orientations or to the trace presence of other carbonates in that spot. Potential accumulation of heavy or toxic metals from environment at a certain moment of the fish otolith evolution is random and could result in random deposit of carbonate mineral with replaced calcium by the respective metal. Powdering the whole otolith for X-ray powder difraction (XRPD) would be an alternative solution for analysis, provided that the trace metal form other carbonate minerals is sufficient for the XRPD detection limit. The drawback of such analysis is the complete loss of the spatial location to track the moment (or ring age) the event occurred during the fish lifetime. Another isssue related to the fish age and the counting rings is connected to the non-linear growth and development of the otolith, where plethora of factors can compete.

Choosing appropriate optics, vaterite trace could be presumably detected according to the weak bands observed at 263 cm^{-1} [16], although the main vaterite bands at 740-750 cm^{-1} as well as the split of the main ν_1 mode of carbonate is rather expected for typical vaterite polymorph presence. However, the major bands of vaterite were absent in the recorded spectra, sugesting its absence or trace occurence (vaterite being a metastable polymorph of calcium carbonate) compared to other dminant minerals. Also, the presence of other cations may promote the formation of the aragonite crystals. [17]

The daily rings or increments have been Raman mapped following the fast streamline imaging and signal-to-baseline criteria of the Wire 3.4 software. An area of 260 μm x 96 μm has been selected over the light microscopy image of the otolith viewed with the 20x objective. A scan step of 4 μm was used, resulting 65x24 pixels with distinct spectral information from 1560 spectra. For map analysis, signal-to-baseline criteria for representative modes of aragonite (696-707 cm^{-1}, 240-265 cm^{-1}), calcite (279-285, 712-716 cm^{-1}), strontianite (1073-1079 cm^{-1}) distribution have been selected. Further, overlay maps were generated.

Examination of the mapping results (Fig. 4) in terms of Raman intensity distribution over the selected area provides an overview of the chemical and crystalline composition. The intensity distribution over the mapped area showed consistent feature supporting aragonite main component but trace of calcite and strontianite could be detected. Mapping over the main Raman mode at 1083 cm^{-1} resembled similar distribution feature. The fact that the growth rings are visible both in optical microscopy images and in Raman maps is a clear sign that the Raman signal intensity is strongly correlated to certain activity patterns of the daily rings fish development. The maps clearly show an alternation of minima and maxima in all cases which can be linked to the activity of the

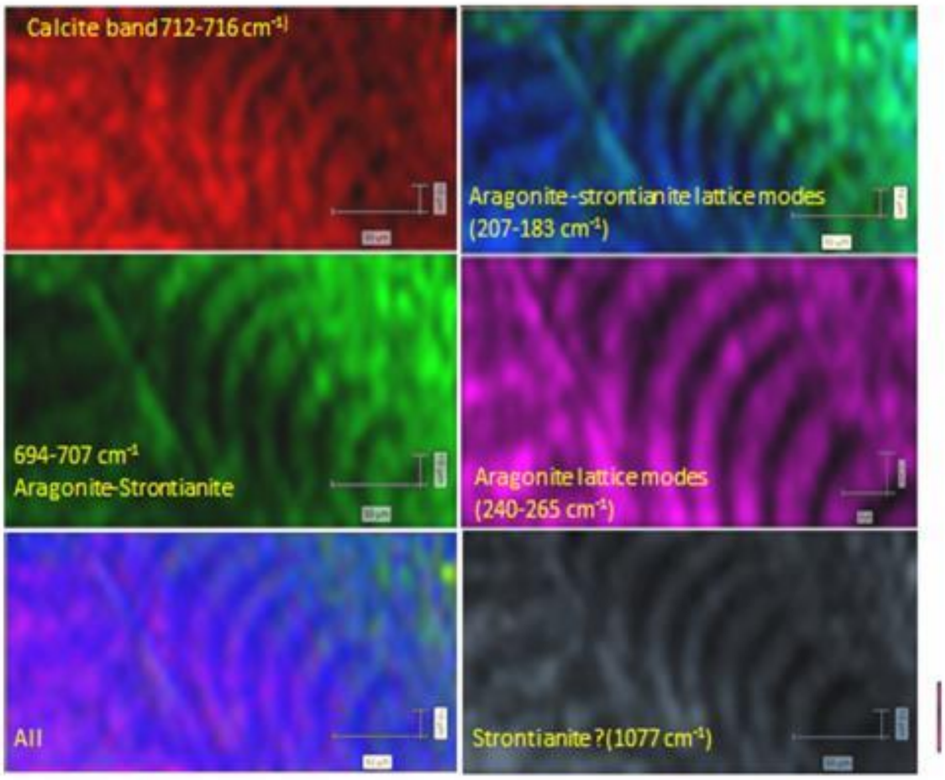

Figure 4. Raman mapping of otolith increments or "daily rings" following the signal-to-baseline criteria, as indicated in each map for trace of calcite (weak band between 712-716 cm^{-1}, top left), aragonite-strontianite (lattice modes - top right), v_4 mode - midde left), aragonite lattice modes (middle, right), trace of strontianite (1077 cm^{-1} bottom right) and all the overlapped images (bottom left).

fish on a daily cycle. The Raman intensity variation patterns follows the opacity patterns from light microscopy. The more intense color zones represeting areas with highest signal-to-baseline, suggest more deposited, highly ordered crystalline material and dark zones areas with less crystalline deposited material. This feature clearly suggests non-uniform otolith crystal growth which may be due to the genetic factors, water chemistry, temperature, migration habits, depth, and other variable conditions occuring in fish environment. Otolith spectroscopic data and their correlation in fish populations still needs to be understood. For such approaches, Raman versatility seems to be optimal for fast tracking information on the otolith gowth patterns.

CONCLUSIONS AND OUTLOOK

The current study provides additional insight into the Raman spectroscopy of otoliths and the dependencies of the Raman spectral features on the selected optics for otolith screening. Confocal Raman micro-spectroscopy is suitable for tracking the otoliths development and potential heavy or toxic metals intake in chemical composition. Thus, Raman techniques proved to be important tools for tracing biomineralization. An integrated, larger study on otolith sets from similar species and habit could use the present results for comparative Raman analysis. For long term, we aim to probe the correlation of otolith morphology and composition revealed from Raman data with environmental parameters. Further environmental changes could be potentially tracked according to the otoliths spectral characteristics, provided that robust data sets are available. Understanding the effects of environmental factors on the chemical composition and structure of otoliths could be very useful in the field of fisheries science, biomineralization or sclerochronology. Their use in reconstructing fish migratory paths or in analyzing population structure could prove to be very important in future approaches.

EXPERIMENTAL

Materials

One otolith was randomly selected from a stock of 16 adults of gilthead sea bream, *Sparus aurata*, collected from four locations in the eastern Adriatic Sea during 2017 (open sea, coastal waters, estuary and aquaculture rearing cages). The studied otolith is from a female captured from estuary area of Novigrad - Karinsko Sea, (Zadar County, Croatia), weighting 274 g and 27 cm in length with an estimated age of 2.5 years. For standardization purposes, the left sagittae were systematically considered. The otoliths were washed in 30% hydrogen peroxide solution for 2-4 minutes and rinsed in distilled water. Samples were then cleaned in an ultrasonic bath (SONOREX SUPER RK 103 H) for 2 minutes and left to air-dry. Epoxy resin (MEGAPOXY H) was prepared by mixing three parts of resin and one part of hardener. These two components were stirred together for 2-3 minutes until the mixture changed color to translucent. The otoliths were embedded in the molds which were lightly coated with Struers Silicone and they were left to dry in the fume cupboard for one day.

Isomet low-speed diamond bladed saw was used for preparing otolith section. Saw was fitted with two blades separated by a spacer (500 µm), producing a 400-500 µm otolith thin section. Each thin section was carefully grounded with Struers Labopol-5 using Struers wetted silicon carbide paper

(4000 grit) at the speed of 50 rpm. Thin sections were then polished using a soft cloth sprayed with diamond paste (3 µm) and washed again in ultrasonic bath for 2 minutes.

Stitched photography of otolith section has been achieved using a series of images taken with a ZEISS microscope equipped with AXIO camera and ZEN 2 (blue edition) program. Stitched image has been obtained with Image-Pro Premier 9.1 software. All sections (core and growth marks) were observed via optical microscopy. Opacity data were acquired on transects from the core to the ventral edge with black areas corresponded to opaque zones.

Methods

Confocal Raman spectra were acquired using a Renishaw InVia Confocal Raman System and a Cobolt DPSS laser emitting at 532 nm. During Raman microscopy the, 5X (NA 0.12 WD 13.2 mm), 20X (NA 0.35, WD 2 mm) and 100X (NA 0.9, WD 3.4 mm) collecting objectives were used with theoretical spatial resolutions of 2.7 µm, 0.927 µm and 0.36 µm respectively, and for single spectra acquisitions at controlled distances from otolith core, the acquisition parameters were 1 s, 1 acquisition, 200 mw laser power. An edge filter has been employed to record spectra in the 90-1840 cm^{-1} spectral range with 0.5 cm^{-1} resolution. Signal has been detected using a Rencam CCD and data acquisition and processing has been achieved with WIRE 3.4 and Origin 8.1 software. Micrographs of the morphological details have been acquired along with spectral data acquisition using the video image facility of the WIRE software. Yearly growing rings have been observed via optical microscopy before Raman measurements and subtle incremental rings have been observed and mapped using the "signal-to-baseline" or "intensity-at-point" options of the Wire 3.4 mapping software.

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Otolith Fingerprints and Tissue Stable Isotope Information Enable Allocation of Juvenile Fishes to Different Nursery Areas

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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 $\delta^{13}C$ and $\delta^{15}N$. In general, Ba/Ca and Sr/Ca ratios and isotopes varied between sites and species. Values of $\delta^{13}C$ and $\delta^{15}N$ 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 $\delta^{13}C$, and increases in $\delta^{15}N$ were found. It seems that these species are feeding on the same local food web within more productive estuarine site 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 $\delta^{15}N$. 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

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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 asses' 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]. However, 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).

Table 1. Summary of hydrographic characteristic during collecting juveniles of *Diplodus puntazzo* and *Diplodus vulgaris* at sampling sites (June 2018).

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.

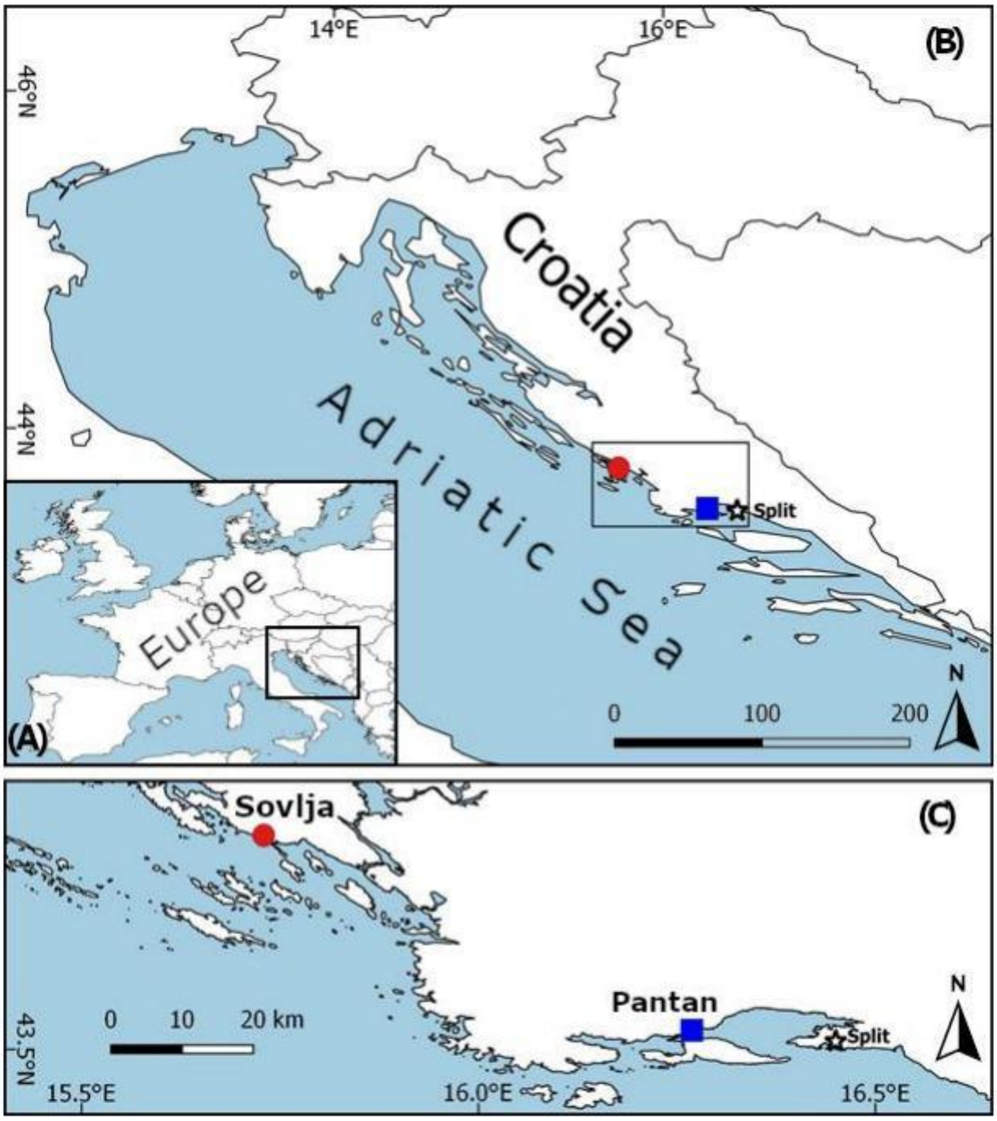

Figure 1. Sampling area in the Europe (**A**) along the eastern Adriatic coast (**B**) with selected sites: Pantan (square) and Sovlja (circle) (**C**).

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. *Element and Stable Isotopes Analyses*

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.

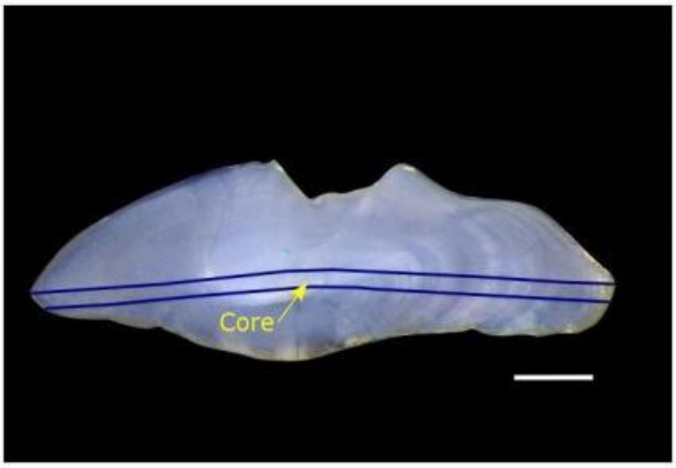

Figure 2. Otolith of juvenile *Diplodus vulgaris*. The blue line represents the line scan through the otolith core from one edge to the opposite edge. Scale bar = 100 µm (Magnification 3x). Data for each otolith were selected approximately on distance 200 µm from the core.

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/cm^2. 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 × 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;

compared with [67–69]). 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 (± 1σ) 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 (± 1σ 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	**USG MACS-3**		**USG BCR-2G**		**NIST SRM 610**	
	Measured values (µg/g)	Reference values (µg/g)	Measured values (µg/g)	Reference values (µg/g)	Measured values (µg/g)	Reference values (µg/g)
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 δ^{13}C and δ^{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 (δ) 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 5σ 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 $\delta^{13}C$ and $\delta^{15}N$ 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 ($t = 1.247$; $p = 0.137$).

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 ($t = 1.412$; $p = 0.093$).

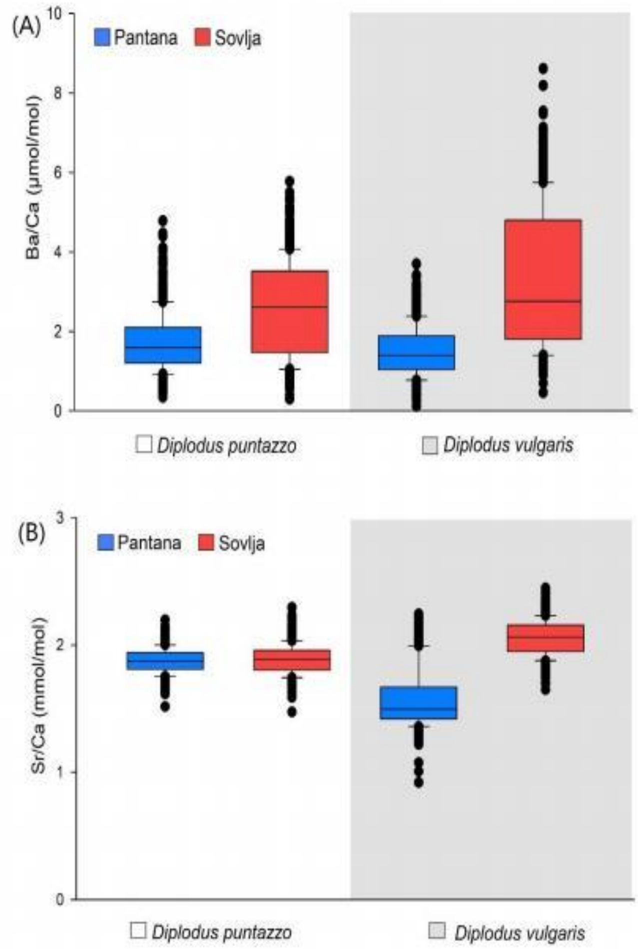

Figure 3. Box plots of median (±standard deviation) Ba/Ca (**A**) and Sr/Ca (**B**) otolith ratios (mmol/mol) of *Diplodus puntazzo* and *Diplodus vulgaris* collected from the Pantan estuarine site and Sovlja coastal site. Black dots present linear raster of Ba/Ca and Sr/Ca otolith ratios for each site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

3.2. Stable Isotope Analyses

Differences were observed in stable isotope composition of muscle tissue (δ^{13}C and δ^{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*, δ^{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 δ^{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 δ^{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 δ^{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 δ^{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).

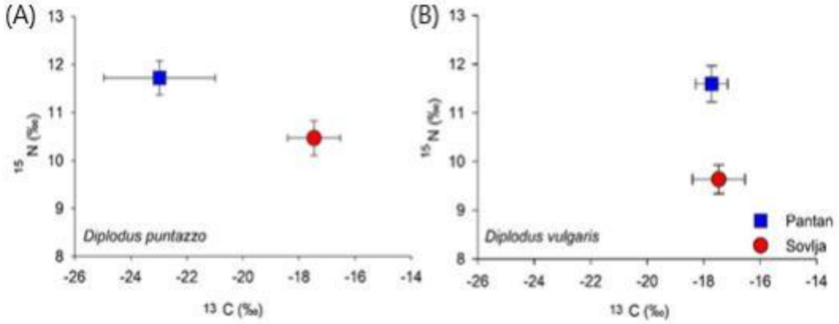

Figure 4. Plots of median (and standard deviation) of δ^{13}C and δ^{15}N ratio muscle tissue of *D. puntazzo* (**A**) *and D. vulgaris* (**B**) collected from the Pantan estuarine site and Sovlja coastal site.

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 (δ^{13}C) and nitrogen (δ^{15}N) stable isotope values in muscle tissue (**b**) for juvenile *Diplodus puntazzo* and *Diplodus vulgaris* collected at different sites.

Factors			(a) Sr and Ba			(b) δ^{13}C and δ^{15}N	
	df	MS	Pseudo-F	P (perm)	MS	Pseudo-F	P (perm)
Species	1	9.588E + 05	2.239	0.049	6.517	19.325	0.0005
Site	1	6.134E + 05	1.432	0.227	19.936	59.111	0.0001
Sp x Site	1	6.495E + 05	1.157	0.199	3.394	10.064	0.0001
Residauls	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 δ^{13}C (Figure 5A) and increase in δ^{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.

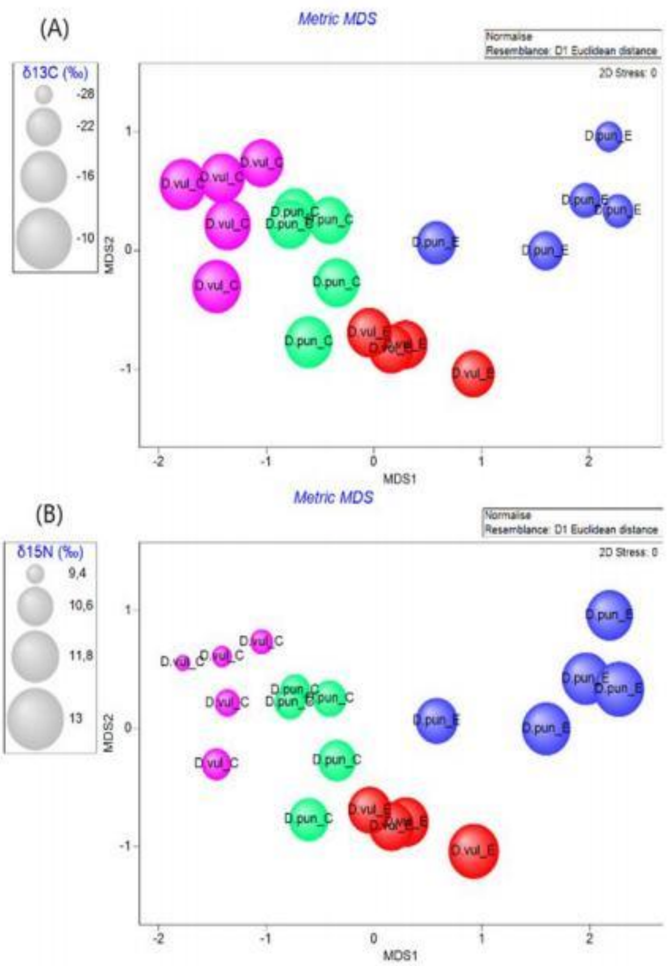

Figure 5. Metric MDS for juvenile *Diplodus puntazzo* and *Diplodus vulgaris* as a bubble plot for stable isotopes **(A)** δ^{13}C and **(B)** δ^{15}N for the coastal waters (C) and estuarine (E).

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.

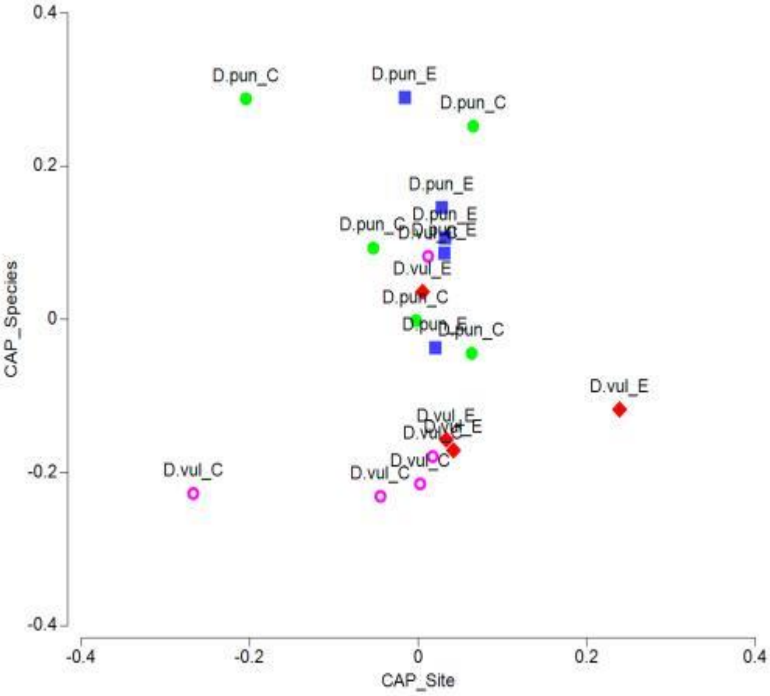

Figure 6. Canonical variate plot (CAP) for Ba and Sr element chemistry of the otolith of juvenile *Diplodus puntazzo* and *Diplodus vulgaris* sampled in 2018, grouped by "Site" and "Species".

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 $\delta^{15}N$ to estuarine waters.

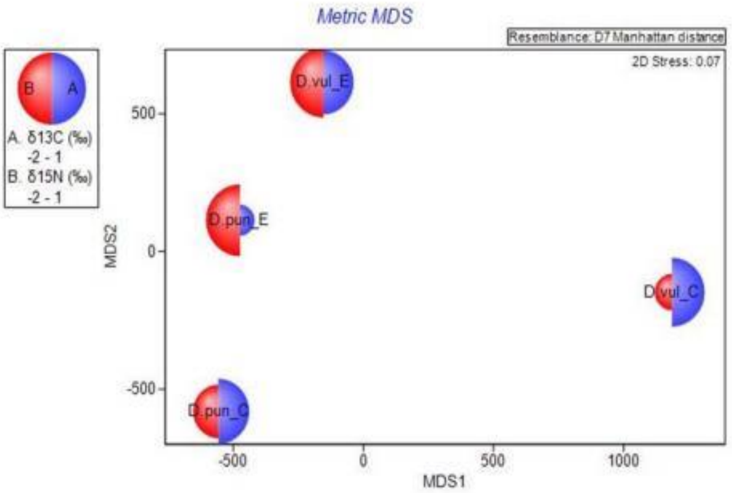

Figure 7. The metric MDS of the bivariate isotope data performed on Euclidean distances of normalized isotope values of δ^{13}C and δ^{15}N showing the patterns across the four groups of interest. Each half or circle correspond to δ^{13}C and δ^{15}N and its size reflects the contribution of each element isotope composition to the obtained distance.

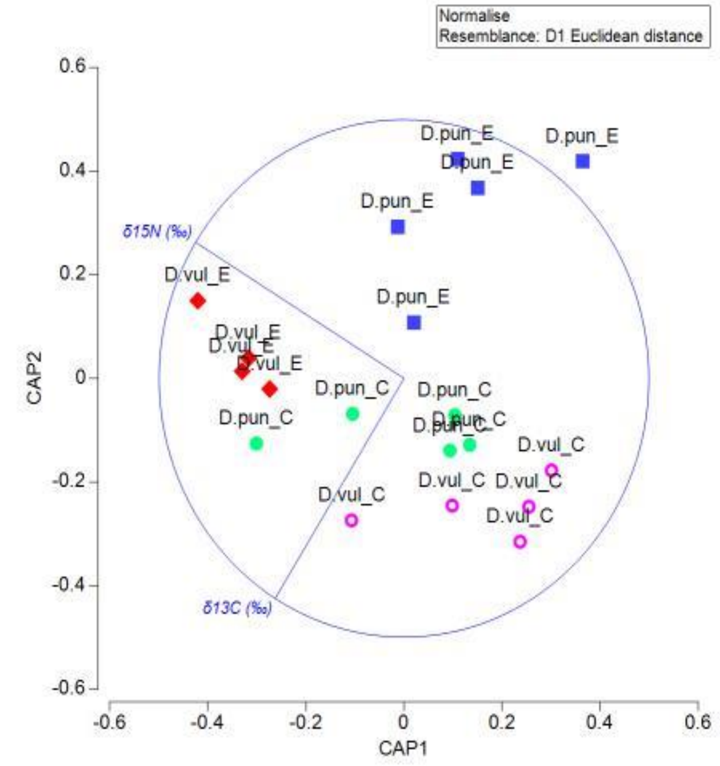

Figure 8. Canonical variate plot (CAP) of the otolith distance matrix on the isotope (continuous quantitative) values. Previously, the distance matrix based on otoliths was related to the distance matrix based on isotopes.

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, $\delta^{13}C$ and $\delta^{15}N$ differed significantly between sites and species. The median of $\delta^{15}N$ was higher for *D. puntazzo* while *D. vulgaris* had higher values of $\delta^{13}C$ at both sites. For soft tissue stable isotopes, lower $\delta^{13}C$ values were found at Pantan, which agrees with the expected natural patterns of $\delta^{13}C$ variation and displays an enrichment trend along the terrestrial–estuarine–marine gradient [99]. In addition, the overall richer $\delta^{15}N$ 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 δ^{13}C and increases in δ^{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 δ^{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 δ^{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.

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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.

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RASPRAVA

Tvrde, kalcificirane strukture morskih organizama često se istražuju multidisciplinirano, među ostalim, istražuju ih geolozi, biolozi, kemičari, paleontolozi i mnogi drugi (Peharda i sur., 2020). Neki se bave temeljnim pitanjima poput biomineralizacije, fiziologije i interpretacije prirasta rasta, a drugi primijenjenim istraživačkim pitanjima kojima nastoje utvrditi najvažnije znanstvene probleme koji se mogu rješiti primjenom znanja o tvrdim strukturama (Trofimova i sur., 2020). Osim klasičnih tvrdih struktura morskih organizama kao što su otoliti riba, statoliti glavonožaca, statociste žarnjaka, bodlje, ljuske, kosti, sipina kost, ljušture školjkaša, u novije se vrijeme počinju analizirati i druge tvrde strukture hrskavičnih riba i mekušaca (Avigliano i sur., 2020.). Te se tvrde strukture sve češće koriste za rekonstrukciju kretanja organizma i svojstva vode u mnogim različitim okružjima (Walther i sur., 2014.; Tzadik i sur., 2017.; Thomas i Swearer, 2019.; Avigliano i sur., 2020.; Peharda i sur., 2020). Kemijski elementi koji se talože u tim strukturama mogu pružati uvid u način na koji morski organizmi koriste stanište tijekom svog života te u endogene procese tih morskih organizama poput rasta i metabolizma (Hüssy i sur., 2020.). Okoliš kojem su organizmi izloženi tijekom svog života jedan je od glavnih egzogenih pokretača jer varijable poput temperature, pH, slanosti, dubine i otopljenog kisika mogu izravno modificirati kemijski sastav vode te izmijeniti fiziologiju i dinamiku unosa i ugradnje kemijskih elemenata u tvrde strukture organizma (Limburg i sur., 2015.; Loewen i sur., 2016.; Crichton, 2018.; Thomas i Swearer, 2019.; Hüssy i sur., 2020.; Nishida i sur., 2020.). Dodatno, ishrana, pa čak i različiti ribolovni pritisak (koji izravno utječe na parametre rasta) također mogu igrati važnu ulogu u kreiranju specifičnog kemijskog sastava tvrdih struktura (npr. Ranaldi i Gagnon, 2010.; Catalán i sur., 2018.; Nishida i sur., 2020.). Ništa manje nisu kompleksni ni čimbenici koji utječu na morfologiju tvrdih struktura (Lombarte i Lleonart, 1993.; Vignon i Morat, 2010.).

Kemijski sastavi i morfološka obilježja tvrdih struktura predstavljaju individualne uzorke ili otiske svake pojedine jedinke (Campana i sur., 1995.). Za precizno tumačenje tih uzoraka potrebno je zavidno znanje o pokretačima koji utječu na kemijski sastav i oblik te potencijalnu interakciju među njima (Avigliano i sur., 2020.). Mnoge studije predstavljene u uvodnom dijelu implicitno pretpostavljaju da je kemijski sastav vode glavni pokretač sastava tih tvrdih struktura. Međutim, novija su istraživanja otkrila složenu mrežu endogenih i egzogenih čimbenika koji mogu uzajamno djelovati i kontrolirati unos i ugradnju elemenata u tvrde strukture (Hüssy i sur., 2020.; Matta i sur., 2020.). Neka od novijih istraživačkih pitanja koja su povezana s odgovorima morskih organizama na klimatske promjene, izumiranjem vrsta ili promjenom raspona rasprostranjenosti te razlučivanjem učinaka ribolova u prošlosti od prirodne varijabilnosti morske faune kako bi se bolje moglo procjeniti kako promjene u obilju resursa utječu na ribare pripadaju vrhunskim istraživanjima današnjice (Trofimova i sur., 2020).

Ima li sklerokronologija na otolitima riba svoje mjesto u budućim istraživanjima promjene okoliša i klime u Sredozemnom i Jadranskom moru?

Sklerokronologija je znanost koja se bavi istraživanjem biologije i ekologije organizama te rekonstrukcijom čimbenika u okolišu tijekom životnog vijeka analiziranog organizma na temelju podataka prikupljenih iz kalcificiranih struktura. Čak i među znanstvenicima koji se bave tim područjem postoji nekonzistencija u korištenju pojma sklerokronologija, koja se istraživanja podrazumijevaju pod tim pojomom te koje se metode smatraju sklerokronološkim metodama. U posljednjih je 15 godina uložen velik trud kako bi se konsolidirali različiti pristupi, primjena i istraživačka pitanja unutar sklerokronologije (Trofimova i sur., 2020.) te kako bi se okupili znanstvenici različitih disciplina i zajednički razvijali tu znanstvenu disciplinu (Peharda i sur., 2020.). Najrazumljivija je definicija pojma sklerokronologija ona koju prenosi Oschmann (2009.), a prvi je put daju Buddemeier i sur. (1974.). Širok opseg istraživanja unutar sklerokronologije podrazumijeva procjenu starosti organizma i procjenu trajanja pojedinih životnih događaja upotrebom metoda analize različitih vrsta signala koji predstavljaju vremenske i prostorne zabilješke na tvrdim strukturama, bilo da su one strukturne, kemijske i/ili optičke naravi (Panfili i sur., 2002.). Te su se tehnike istraživanja počele primjenjivati za proučavanje kronologije rasta riba prije dvadesetak godina (Black i sur., 2005.; 2008.). No ponekad se među ta istraživanja uvrštavaju i samo analize kemijskog sastava tvrdih struktura bez relevantnih podataka o kronološkom vremenu polaganja materijala te istraživanja određivanja starosti organizama čitanjem zona prirasta, što zasigurno ne pripada sklerokronološkom istraživanju u užem smislu. Takva istraživanja rijetko koriste pojam sklerokronološka (Peharda i sur., 2020.).

Istraživanja su tvrdih struktura morskih organizama, s naglaskom na otolite riba, u Sredozemnom moru započela davne 1978. godine istraživanjem koncentracije cinka u otolitima skuše (*Scomber japonicus colias* Gmelin, 1789) iz Egejskog mora (Papadopoulou i sur., 1978.). Punih 14 godina nije bilo objavljenog rada koji bi se mogao uvrstiti u sklerokronološka istraživanja do trenutka kad su Iacumin i sur. (1992.) odredili koncentracije stabilnih izotopa kisika i ugljika u otolitima riba iz nekoliko sredozemnih porodica riba, među kojima i devet vrsta iz porodica ljuskavki. No važno je napomenuti da u tom istraživanju autori nisu analizirali kronološki aspekt ugradnje stabilnih izotopa u otolite. U članku I predstavljen je pregled svih istraživanja na otolitima morskih riba u Sredozemnom moru od 1978. do 2020. godine te su predstavljeni izazovi i mogućnosti takvih istraživanja na navedenom području u budućnosti. Uz radove koji su predmet ove disertacije, novoobjavljeni radovi u posljednjoj godini potvrđuju postojanje interesa za ovu disciplinu i upućuju na nove smjerove primjene. Tako su npr. Traina i sur. (2021.) izvijestili o regionalnom kolebanju kemijskog sastava otolita nedorasle plavoperajne tune, *Thunnus thynnus* (Linnaues, 1758), u Sredozemnom moru. Agiadi i Albano (2020.) su koristeći otolite riba nađene u površinskom sedimentu rekonstruirali morsku faunu riba uz plitku južnoizraelsku obalu prije otvaranja Sueskog kanala. Kvantificirali su starost koristeći datiranje radiaktivnim ugljikom i opisali taksonomski sastav, geografski afinitet i trofičku strukturu. Dodatno, istom su metodom pobili hipotezu da je *Bregmaceros nectabanus* Whitley, 1941 pleistocenska reliktna vrsta (Gadiformes (Cods), Bregmacerotidae (Codlets)) i potvrdili da je riječ o lesepsijskoj vrsti te dokazali invazivnost još nekoliko vrsta iz Indijskog oceana. Zanimljivo je i nedavno objavljeno istraživanje koje je kombiniralo morfologiju otolita i mikrokemijski otisak otolita jegulje *Anguilla anguilla* (Linnaeus, 1758) za odjeljivanje stokova u Hrvatskoj i Crnoj Gori (Milošević i sur., 2021.).

Sklerokronološka istraživanja imaju nekoliko osnovnih preduvjeta za dobivanje pouzdanih rezultata. Jasna vidljivost zona prirasta u otolitima jedan je od osnovnih preduvjeta, bilo za mjerenja širine zona prirasta potrebnih za izradu kronologija rasta ili za kemijske analize otolita po specifičnim dijelovima otolita i dobivanje vremenskog niza podataka (Campana, 1999.) Problemi povezani s identifikacijom, validiranjem i tumačenjem zona prirasta otolita istaknuti su u brojnim radovima (npr. Morales-Nin i sur., 2005.; Stransky i sur., 2005.; Hüssy i sur., 2016.). Nažalost, ni za mnoge gospodarski važne vrste ribe u Sredozemnom moru kao npr. trlju od blata, *Mullus barbatus* Linnaeus, 1758 i oslića, *Merluccius merluccius* (Linnaeus, 1758) nisu točno određene zone prirasta iako se njihovi otoliti masovno i dugotrajno skupljaju i očitavaju. Otoliti navedenih vrsta analiziraju se za potrebe utvrđivanja dinamike stokova na području EU-a (Uredba (EU), 2017.), no nepostojanje konsenzusa oko validacije zone prirasta otežava postizanje zacrtanih ciljeva oko zajedničkog gospodarenja ribolovnim resursima. Jasna i pažljiva identifikacija zona prirasta, potrebnih zbog daljnjih (kemijskih i izotopskih) analiza otolita, izazov je za sklerokronološka istraživanja te se taj problem ne smije podcijeniti.

Osim problema određivanja zona prirasta, velik su problem i ograničenja korištenih analitičkih metoda zbog relativno male veličine otolita i često nedostatno male količine materijala koja se može dobiti iz jednog otolita, a zahtijeva se za analitičko određivanje stabilnih izotopa u otolitima (Matta i sur., 2013.; Sreemany i sur., 2017.), stoga su te analize ograničene na cijele otolite (npr. Rooker i sur., 2008.) ili određene veće dijelove otolita, npr. jezgru (Siskey i sur., 2016.; Rooker i sur., 2019.) ili rub otolita (Hidalgo i sur., 2008.; Tanner i sur., 2012.). No tada nije moguće dobiti podatke o vremenskim nizovima. Ipak, znatan napredak u razvoju analitičkih instrumenata i metoda za analizu uključuje nadogradnju postojećih instrumenata s visokorezolucijskim laserskim ablacijskim sustavom te sustav masene spektrometrije s kontinuiranim protokom izotopa za volumenski ultramale karbonatne uzorke (Kitagawa i sur., 2013.; Sakamoto i sur., 2017.; Sreemany i sur., 2017.), što omogućuje dobivanje podataka i iz manje količine materijala, a time i željenih vremenskih nizova. Nishida i sur. (2020.) su primijenili mikroanalitičku tehniku za analizu stabilnih izotopa ugljika ($\delta^{13}C$) i kisika ($\delta^{18}O$) u otolitima japanske srdele (*Sardinops sagax* (Jenyns, 1842)) i inćuna (*Engraulis japonicus* Temminck & Schlegel, 1846) i uspjeli dobiti visokorezolucijske podatke o padu koncentracije $\delta^{18}O$ pri migraciji ličinki i nedoraslih riba iz mrijestilišta u rastilišta, i to s većom ovisnošću o slanosti okolne vode nego o kolebanju temperature.

Black i sur. (2019.) predstavili su svjetski popis koji uključuje dvadesetak, uglavnom hladnovodnih vrsta riba kao npr. *Hexagrammos decagrammus* (Pallas, 1810) i *Sebastes (Sebastosomus) melanops* Girard, 1856 kojima su izrađene usporedno datirane (engl. *crossdated*) kronologije rasta (usporedno datiranje je tehnika kojom se svakom prstenu pridružuje točno određena godina nastajanja traženjem podudaranosti između širokih i uskih prstenova rasta kod jedinki s iste lokacije i različitih lokacija). Uz porodicu Sebastes za koju se smatra kako ima najdugovječnije vrste riba na svijetu, autori navode i niz drugih potencijalno zanimljivih porodica riba koje uključuju dugoživuće, nemigratorne, širokorasprostranjene, priobalne ribe s generalističkom ishranom (Whitfield i Elliott, 2002.) poput porodica Girellidae, Labridae, Lethrinidae, Lutjanidae, Platycephalidae Pleuronectidae, Polyprionidae, Sciaenidae i Scombridae. Uvjet dugovječnosti važan je za izradu kronologija rasta upotrebom statističkih metoda preuzetih iz dendrokronoloških istraživanja. Važan je preduvjet povezan s pogodnosti neke vrste za izradu kronologije tijekom određenog razdoblja dostupnost većeg broja uzoraka koji su nužni za pravilno unakrsno datiranje (Hudson i sur., 1976.).

U članku I pretragom znanstvene baze podataka o biološkoj raznolikosti riba (FishBase) izdvojene su ukupno 263 vrste riba koje su zatim detaljno analizirane prema osnovnim ekološkim osobinama (stanište, rezidentnost, trofički status, primjerice kako je navedeno za ljuskavke u Tablici 1) kao potencijalno moguće za sklerokronološka istraživanja na području

Sredozemnog mora. Ipak, osnovni je postavljen uvjet o starosti iznad 30 godina zadovoljilo tek tridesetak vrsta. Uvjet od 30 godina uzet je na osnovi činjenice da je to 30 % od najstarije poznate određene starosti kod riba (Lackman i sur., 2019.).

Iako su u Članku I kao potencijalno najdugovječnije vrste u Sredozemnom moru istaknute dubinska kirnja, *Polyprion americanus* (Bloch & Schneider, 1801) (Peres i Haimovici, 2004.) te vrsta iz porodice Berycidae, *Beryx decadactylus* Cuvier, 1829, s maksimalnom starošću od 76 odnosno 61 godine, za obje vrste ne postoje relevantni podaci o maksimalnoj starosti u Sredozemnom moru. Kirnje su prema Black i sur. (2019.) globalno potencijalne vrste za sklerokronološka istraživanja, a Sredozemno more predstavlja njihovu gornju (sjevernu) granicu rasprostranjenosti i ovdje rastu sporije nego u tropskim područjima (Gracia-López i Castelló-Orway, 2003.). One imaju složene cikluse razmnožavanja (Sadovy i Shapiro, 1987.; Heemstra i Randall, 1993.), što ih čini posebno ranjivima s obzirom na stupanj zaštite (CITES / UNEP-WCMC, 2017.). Kirnja golema, *Epinephelus marginatus* (Lowe, 1834) je zanimljiv kandidat za izradu kronologije rasta jer doseže starost od 60 godina (Reñones i sur., 2010.), ali zbog prelovljenosti i zaštićenog statusa diljem Sredozemnog mora, istraživanje bi zahtijevalo strateški pristup u prikupljanju uzoraka tijekom dužeg razdoblja. U skorije je vrijeme upitna mogućnost takve realizacije.

Na globalnom popisu zanimljivih vrsta za sklerokronološka istraživanja, Black i sur. (2019.) naveli su još vrste iz porodica Sebastidae, Lutjanidae i Sciaenidae. No samo su dvije vrste iz porodice Sebastidae zastupljene u Sredozemnom moru, i jedino vrsta *Helicolenus dactylopterus* (Delaroche, 1809) prema literaturnim podacima može doseći starost veću od 40 godina, no čini se da za analizirano područje nema naznake o starosti iznad 21 godine (Consoli i sur., 2010.). Dvije gospodarski važne vrste iz porodice Sciaenidae koje su zastupljene i u Jadranu, hama, *Argyrosomus regius* (Asso, 1801) i kavala, *Sciaena umbra* Linnaeus, 1758) mogu doseći maksimalnu starost od 42 godine (González-Quirós i sur., 2011.), odnosno 31 godine (Chater i sur., 2018.). Zbog navedene starosti i velikih otolita obje vrste predstavljaju određen potencijal za razmatranje iako su utvrđeni problemi s validiranjem periodičnosti stvaranja prstenova rasta (Arneri i sur., 1998.; Chater i sur., 2018.). Zanimljive su vrste za sklerokronološka istraživanja u Sredozemnom moru, u kontekstu klimatskih promjena, i invazivne lesepsijske vrste *Pomacanthus maculosus* (Forsskål, 1775) i *Lutjanus argentimaculatus* (Forsskål, 1775). Obje imaju životni vijek iznad 30 godina (Grandcourt i sur., 2004.; Piddocke i sur., 2015.) i nedavno su uspostavile održive populacije uz obale Izraela i Libanona (Bariche, 2010.; Sonin i sur., 2019.).

Vrste s najvećim potencijalom za sklerokronološka istraživanja u Sredozemnom moru, zbog potencijalno visoke starosti su zubatac, *Dentex dentex* (Linnaeus, 1758) i *Diplodus cervinus* (Lowe, 1838) iz vrstama vrlo brojne porodice Sparidae (Tablica 1). Obje su pod izrazitim ribolovnim pritiskom sportskih ribolovaca, posebice zubatac (Marengo i sur., 2014.). I komarča, *Sparus aurata* je zbog svoje visoke gospodarske vrijednosti, široke rasprostranjenosti, dobro vidljivih zona prirasta u otolitima i utvrđene starosti iznad 20 godina (Kraljević i Dulčić, 1997.) zanimljiv kandidat, stoga je analizirana kao ciljana vrsta u ovoj disertaciji.

Tablica 1. Potencijal vrsta iz porodice ljuskavki (Sparidae) za sklerokronološka istraživanja prema osnovnim ekološkim obilježjima u Sredozemnom moru prema www.fishbase.org. Vrste su poredane padajućim nizom od najstarije, abecednim redom.

Vrsta	**Uk. dužina (cm)**	**Starost**	**Status**	**Stanište**	**Autor**
Sparus aurata	70,0	54	nativna	demerzalna	Kalish, 1993.
Dentex dentex	100,0	36	nativna	bentopelagična	Baudouin i sur., 2016.
Diplodus cervinus	55,0	35	nativna	grebenska	Jarzhombek, 2007.
Pagrus Auriga	97,6	32	nativna	bentopelagična	procijenjena
Pagrus major	122,0	26	unesena	demerzalna	Druzhinin, 1976.
Acanthopagrus bifasciatus	44,2	21	unešena	grebenska	Grandcourt i sur., 2004.
Pagellus bogaraveo	85,4	20	nativna	bentopelagična	Druzhinin, 1976.
Pagellus erythrinus	73,2	20	nativna	bentopelagična	Druzhinin, 1976.
Pagrus pagrus	91,0	18	nativna	bentopelagična	Potts i Manooch, 2002.
Sarpa salpa	62,2	15	nativna	bentopelagična	Tsikliras i Stergiou, 2015.
Dentex gibbosus	117,7	14	nativna	bentopelagična	Druzhinin, 1976.
Pagrus caeruleostictus	109,8	14	nativna	bentopelagična	procijenjena
Dentex macrophthalmus	65,0	13	nativna	bentopelagična	Yasuda, 1950.
Centracanthus cirrus	34,0	12	nativna	bentopelagična	procijenjena
Lithognathus mormyrus	55,0	12	nativna	demerzalna	Kraljević i sur., 1996.
Oblada melanura	36,6	12	nativna	bentopelagična	Mahmoud, 2010.
Rhabdosargus haffara	35,0	12	unesena	grebenska	procijenjena
Crenidens crenidens	30,0	11	unešena	demerzalna	procijenjena
Diplodus bellottii	36,6	11	nativna	bentopelagična	procijenjena
Diplodus vulgaris	45,0	11	nativna	bentopelagična	Dulčić i sur., 2011.

Dentex maroccanus	45,0	10	nativna	demerzalna	Nguyen i Wojciechowski, 1972.
Diplodus sargus	45,0	10	nativna	demerzalna	Gordoa i Molí, 1997.
Evynnis ehrenbergii	43,0	10	nativna	bentopelagična	Giret, 1974.
Pagellus acarne	43,9	9	nativna	bentopelagična	Druzhinin, 1976.
Diplodus puntazzo	60,0	8	nativna	bentopelagična	Druzhinin, 1976.
Pagellus bellottii	51,2	8	nativna	demerzalna	Druzhinin, 1976.
Boops boops	40,0	6	nativna	demerzalna	Druzhinin, 1976.

Prema predstavljenim podacima, broj dugoživućih vrsta riba u Sredozemnom moru prilično je nizak i postoji samo nekoliko onih koje dosežu maksimalnu utvrđenu starost iznad 30 godina. Međutim, ubrzani razvoj statističkih metoda koje omogućuju konstrukciju kronologije rasta i za kratkoživuće vrste riba (<15 godina), ako se prikupljaju podaci tijekom nekoliko godina ili desetljeća (Coulson i sur., 2014.; Rountrey i sur., 2014.), djeluje vrlo obećavajuće u tom kontekstu. Tako su Matta i sur. (2020.) razvili vremensku seriju od 28 godina iz zona prirasta na otolitima vrste *Pleurogrammus monopterygius* (Pallas, 1810) primijenivši pristup mješovitog numeričkog modeliranja kako bi otkrili unutarnje i vanjske čimbenike rasta. Vrlo interesantno, njihovi rezultati upućuju na zaključak kako temperatura vode nije bila prediktor rasta kako bi se moglo očekivati, već biotički čimbenici poput brojnosti predatora (vrsta lososa) i plijena (kopepodni račići).

Uz spomenuta ograničenja koja proizlaze iz bioloških i ekoloških osobina vrsta u Sredozemnom i Jadranskom moru, postoje i drugi izazovi za sklerokronološka istraživanja poput nepostojanja kontinuiranog prikupljanja, arhiviranja otolita te nedostatka skupe, moderne analitičke opreme za njihovu analizu i obučenog tehničkog osoblja za rad na takvim instrumentima. Kontinuirano je prikupljanje otolita izazov s obzirom na ograničenu dostupnost financiranja i kratko vrijeme trajanja pojedinih stručnih studija i projekata. Dodatno, protokoli uzorkovanja i pohrane često su specifični za instituciju ili pojedini projekt, što rezultira nedosljednošću u prikupljanju podataka, a time i njihove neprikladnosti za statističke analize (Rochet i Trenkel, 2003.; Rochet i sur., 2005.). Panfili i sur. (2002.) jasno su uputili na važnost arhiviranja otolita te na činjenicu kako otoliti arhivirani u zbirkama moraju biti više dostupni svim zainteresiranim znanstvenicima. Iako su otoliti male strukture i time pogodni za arhiviranje, još je uvijek prisutno institucionalno nerazumijevanje za potrebu stvaranja zbirki otolita na ovom području i upravljanja njima. Preporučuje se kako bi zbirke svakako trebale sadržavati otolite većeg broja, posebice onih rijetkih, a ne samo gospodarski važnih vrsta. Disspain i sur. (2016.) ističu i visok potencijal korištenja otolita s arheoloških nalazišta, kakvih je u Sredozemnom moru poveći broj na obalama Grčke, Male Azije, ali i Jadranskog mora, radi utvrđivanja okolišnih promjena tijekom geološke i ljudske povijesti (npr. Agiadi i sur., 2018.).

Uza sva navedena ograničenja, sklerokronološka istraživanja imaju svoju budućnost globalno u cijelom nizu novih smjernica (Peharda i sur. 2020.) i istraživačkih pitanja (Trofimova i sur., 2020.), pa se to odnosi i na Sredozemno i Jadransko more. Istraživanja na otolitima svakako su doprinos sklerokronologiji i s daljnjim razvojem analitičkih metoda ona će se u narednim godinama povećavati, vjerojatno u smjeru povezivanja sklerokronoloških studija s ostalim znanstvenim poljima numeričkim modeliranjem promjene klime i ekosustava u priobalnom području (Peharda i sur., 2020).

Može li konfokalna Ramanova spektroskopija (CRM) biti učinkovita metoda za validiranje periodičnosti prirasta odnosno prstenova otolita

Konfokalna Ramanova mikrospektroskopija pogodna je za praćenje razvoja i rasta karbonatnih tvrdih struktura (Tomás i Geffen, 2003.), pa tako i otolita te ugradnje potencijalno teških ili otrovnih metala u kemijski sastav otolita te se tako ta tehnika nametnula kao vrijedan alat za praćenje biomineralizacije (Casselman i Gunn, 1992.). Naime, osim kalcijeva karbonata, tvrde strukture sadrže i neznatan matriks drugih kemijskih elemenata koji imaju važnu ulogu u kalcifikaciji (Guzman i sur., 2007.). Konfokalna Ramanova mikroskopija kombinira spektralne informacije iz Ramanove spektroskopije s prostornim filtriranjem konfokalnog optičkog mikroskopa za kemijsku sliku uzoraka visoke rezolucije i omogućuje dobivanje kvantitativnih podataka potrebnih za bolje razumijevanje biomineralizacije otolita (Jolivet i sur., 2008.). Spektralni Ramanovi podaci osjetljivi su na vibracijske načine pripreme uzorka i pružaju opsežan kemijski, fizički i strukturni uvid, dok konfokalna optika mikroskopa omogućuje prostorno filtriranje volumena unutar uzorka u bočnoj (XY) i aksijalnoj (Z) osi (Gauldie i sur., 1997.). Sinergija između spektralnih i prostornih informacija omogućuje kemijsku analizu pojedinačnih čestica, diskretnih uzoraka ili slojeva do veličine manje od 1 μm. Visoko prostorno razlučivanje mineralnog i organskog sastava otolita pomoću Ramanove mikrospektrometrije uključuje stroge protokole za pripremu uzoraka. Ti protokoli često uključuju ugrađivanje otolita u kemijski neutralnu smolu (tj. smole koje ne sadrže istraživane elemente u koncentracijama iznad granica otkrivanja), ali takvo ugrađivanje može izazvati organska površinska onečišćenja koja kolebaju ovisno o otolitnim strukturama. Jezgra i prstenovi, kao strukturni diskontinuiteti, pokazuju višu razinu onečišćenja utječući na organsku matricu otolita jer smole vjerojatno prodiru u kristalnu strukturu (Jolivet i sur., 2013a). Također, upotreba jetkanja i bojanja prilikom pripreme može dovesti do razlika u sastavu otolitnih mikrostruktura (Jolivet i sur., 2008.) pa prilikom razmatranja rezultata navedena saznanja o onečišćenju uzorka treba uzeti u obzir. U članku III predstavljena je ova analitička metoda za utvrđivanje načina rasta otolita kod komarče, *Sparus aurata* i ovisnost Ramanovih spektralnih obilježja na odabranoj optici za probir (analizu) otolita.

Prema objavljenim podacima, ta se metoda dosad nije upotrebljavala za analiziranje otolita u Sredozemnom i Jadranskom moru. Ispitivanje rezultata mapiranja u smislu raspodjele intenziteta Ramanova signala na odabranom području pružila su uvid u kemijski i kristalni sastav otolita komarče. Prema konfokalnoj Ramanovoj mikrspektroskopiji (CRM), orijentacija mikrokristalita jasno pokazuje kako je aragonit glavna mineralna komponenta kod otolita ove vrste. Jaki aragonitni signal određen je prema prisutnosti simetričnog načina istezanja karbonata. U većini je objavljenih radova na drugim vrstama riba upravo aragonit glavna komponenta otolita (vidi Maisey, 1987.), no noviji radovi upućuju na utjecaj ontogenetskog razvoja i temperature na polimorfni sastav kalcijeva karbonata u otolitima (Loeppky i sur., 2021.). No, osim mineralne komponente, otoliti sadrže i organsku frakcija, a omjer aragonita i organskih frakcija (tzv. Ramanov potpis) je snažno povezan s otolitnom neprozirnošću (Jolivet i sur., 2013b.) ukazujući na postojanje dvije kategorije organskih potpisa sa suprotnim učincima na neprozirnost uslijed razlika u organskim spojevima i (ili) varijacije u njihovim relativnim količinama.

Otoliti riba su gusta tijela od kalcijeva fosfata i/ili kalcijeva karbonata u mineraloškoj formi aragonita, vaterita i/ili kalcita, i mogu biti nekristalični ili slabo kristalični ili stvarati polikristalične agregate ili čak jedan kristal (npr. kod gnatostoma). Na manjem povećanju objektiva (5 x) vide se i tragovi drugih polimorfa kalcijeva karbonata (Kaabar i sur., 2011.), dok organski matriks nije moguće odrediti. No s većim povećanjem (20 x ili 100 x) otkrivaju se osim aragonita i tragovi kalcita i stroncijanita, što se podudara s objavljenim podacima za nedorasle haringe, *Clupea harengus* Linnaeus, 1758 (Tomás i Geffen, 2003.). Uz upotrebu prikladnije optike, vjerojatno bi se mogao otkriti i vaterit. On nije u uzorcima utvrđen ili zato što je stvarno odsutan ili ga ima u tragovima jer je on metastabilan polimorfni oblik kalcijeva karbonata (Donnelly i sur., 2017.). Naime, prisutnost vode, ne samo u organskom matriksu otolita nego i među kristalima, esencijalna je u održavanju kristalične stabilnosti aragonita, a posebice vaterita, koji se brzo pretvara u druge kalcitne polimorfe u slučaju dehidracije

(Maisey, 1987.). U članku III, kod otolita *S. aurata* vide se razlike u biogeničnom i geogeničnom aragonitu te tragovi ugradnje Sr i Ba na mjestu Ca. Viši su pikovi Sr utvrđeni posebice na otolitima iz otvorenog mora srednjeg Jadrana (otok Šćedro), što može biti povezano s povišenom slanošću.

Dnevni prstenovi utvrđeni su na analiziranim otolitima komarče primjenom CRM metode prateći promjene kriterija signala i usmjeravanja. Ramanovi spektri dobiveni većim uvećanjem objektiva opisuju manja područja spektra s boljim fokusom (usmjerenjem) na površini presjeka sagitalnog otolita. Prisutnost novih linija rasta pripisuje se ili otkriva različitim orijentacijama kristala ili prisutnosti drugih karbonata u tragovima na tom mjestu i time je potvrđeno njihovo postojanje. Analizirano je ukupno 16 odraslih jedinki te potvrđena njihova starost od 2^{+}-3^{+}. Činjenica da su prstenovi rasta otkriveni i optičkim mikroskopiranjem i pomoću Ramanove spektrometrije jasan je dokaz da je intenzitet Ramanova signala snažno povezan s obrascima nastanka prstenova i prikladna metoda za njihovo otkrivanje i validiranje. Najčešće se u sklekronološkim radovima ova tehnika i koristi za validaciju periodičnosti nastanka prstenova rasta (Pierre i sur., 2014.). Jasno se izmjenjuju ciklusi minimalnih i maksimalnih vrijednosti aragonita koji se mogu povezati s dnevnim ciklusom prirasta. Varijacija intenziteta prema CRM-u jasno slijedi uzorak svijetlih i tamnih prstenova dobivenih pomoću svjetlosne mikroskopije. Intenzivnije obojene zone predstavljaju područja s najjačim signalom i upućuju na više istaložen, visokousmjeren kristalinični materijal, dok tamne zone imaju manje istaloženog kristaliničnog materijala. Ta značajka jasno upućuje na nejednolik rast kristala otolita koji bi mogao biti posljedica ontogenije organizma, kemije vode, temperature, migracijskih navika, dubine i drugih promjenjivih uvjeta koji se javljaju u okolišu u kojem riba živi (Mendoza, 2006.).

Iako se u neposrednoj blizini Novigradskog mora, koje je mjesto uzorkovanja otolita iz estuarijskog područja, nalaze ostaci nekadašnje tvornice glinice u Obrovcu s poznatim problemom uskladištenog crvenog mulja, teški metali i potencijalno otrovni metali nisu utvrđeni. Naime, crveni mulj nastaje kad se aluminijev oksid iz boksita odvaja rastvaranjem u natrijevu hidroksidu, pri čemu nastaju silicijevi, željezni i titanijevi oksidi (Kutle i sur., 2004.; Šeremet, 2013). Potencijalno je ugrađivanje teških ili otrovnih metala iz okoliša u nekom trenutku razvoja otolita sasvim slučajno, ali može rezultirati nasumičnim taloženjem karboniziranog minerala u kojem je kalcij zamijenjen određenim metalom (Behrens i sur., 1995.; Brown i Severin, 1999.). Kako bi se otkrili ovakvi kemijski elementi koji su zastupljeni s niskim koncentracijama, moguća je primjena alternativnih analiza koje uključuju mljevenje cijelog otolita u prah za rendgensku (X-ray) difrakciju (XRPD), pod uvjetom da je koncentracija metala u tragovima koji tvori karbonatni mineral dovoljna za granicu otkrivanja pomoću XRPD-a. Nedostatak takve analize predstavlja potpuni gubitak prostornog razmještaja (podatka) za praćenje onog trenutka (odnosno prstena otolita) u kojem se ta ugradnja tijekom života dogodila (Wehrmeister i sur., 2009.; Donnelly i sur., 2017.) odnosno vremenskog obilježja ugradnje. Ta nam analiza nije bila dostupna, pa kako je već navedeno, nema naznake ugradnje bilo kakvih otrovnih ili teških metala u analizirane otolite. U dostupnoj literaturi nije moguće pronaći primjere o rezultatima takvih analiza na otolitima.

Buduća bi istraživanja otolita srodnih vrsta te njihovih ponašanja pomoću konfokalne Ramanove spektrometrije mogla koristiti rezultate naših istraživanja za usporedbu te dugoročno ispitivanje sastava i morfologije otolita u odnosu na parametre okoliša. U dužem bi razdoblju bilo poželjno utvrditi korelaciju morfologije i sastava otolita otkrivene iz Ramanovih podataka s parametrima okoliša. Daljnje bi se promjene u okolišu mogle potencijalno pratiti prema spektralnim značajkama otolita, da su nam dostupni robusni skupovi podataka. Kako je već naglašeno, razumijevanje učinaka čimbenika okoliša na kemijski sastav i strukturu otolita može se korisno iskoristiti u području ribarstvene znanosti, biomineralizacije ili sklerokronologije.

Omogućuje li kemijski sastav elemenata u otolitima prostornu alokaciju fratra (*Diplodus vulgaris*), pica (*Diplodus puntazzo*) i komarče (*Sparus aurata*) u pojedino rastilište i određivanje relativnog doprinosa svakog pojedinog rastilišta odraslim populacijama navedenih vrsta?

Geokemijski markeri u otolitima koji zapravo predstavljaju elementni sastav sve se više koriste kako bi se retrospektivno utvrdilo podrijetlo, migracije i povezanost među populacijama različitih vrsta riba (npr. Tanner i sur., 2012.; Rooker i sur., 2019.). U članku II i IV utvrđeni su kemijski sastavi otolita nedoraslih jedinki komarče (*Sparus aurata*) (Članak II), fratra (*Diplodus vulgaris*) i pica (*Diplodus puntazzo*) (Članak IV) pomoću laserske ablacije induktivno spojene masene spektrometrijske plazme, engl. *Laser Ablation Inductively Coupled Plasma Mass Spectrometry* (LA-ICP-MS) u linijskom skeniranju, duž osi maksimalnog rasta, kroz jezgru otolita. LA-ICP-MS analiza je u posljednje vrijeme najčešće korištena metoda koja daje kemijski zapis (otisak) otolita cjelokupnog života ribe (npr. Di Franco i sur., 2014.).

U oba su članka utvrđeni odnosi elemenata, litija (^{7}Li), natrija (^{23}Na), magnezija (^{26}Mg), mangana (^{55}Mn), cinka (^{66}Zn), stroncija (^{88}Sr), molibdena (^{97}Mo), barija (^{137}Ba; ^{138}Ba), olova (^{208}Pb) i urana (^{238}U) prema kalciju (Ca). Navedeni elementi u tragovima (npr. Ba, Li, Mg, Mn, Sr) i teški metali (npr. Pb, Cu, Zn) ugrađuju se u otolite riba tijekom cijelog života organizma (Miller, 2011.; Herrera-Reveles i sur., 2013.; Reis-Santos i sur., 2013.; Sturrock i sur., 2014.). Ipak, nakon provedene laserske ablacije, utvrđeno je kako su ugrađene koncentracije većine analiziranih elemenata bile ispod granice otkrivanja (engl. *Limit of detection*; LOD) i granice kvantifikacije (engl. *Limit of quantification*; LOQ) (Forootan i sur., 2017.). Takvi su elementi isključeni iz daljnjih analiza zbog malih koncentracija. Odnosi Ba/Ca i Sr/Ca u otolitima su bili iznad granice otkrivanja i kvantifikacije tijekom cijele osi rasta otolita (Članak II i IV), dok su odnosi za Mg/Ca, Zn/Ca i Pb/Ca bili iznad granice otkrivanja i kvantifikacije u nekoliko segmenata uzorka te su uključeni u daljnje analize (Članak II). Za razliku od njih, odnosi Na/Ca, Mg/Ca, Zn/Ca, Mn/Ca i Li/Ca su bili iznad granice otkrivanja samo u jednom segmentu uzorka, iako su kod većine uzoraka bili ispod granice kvantifikacije, stoga nisu uvršteni u daljnje analize (Članak IV). Naime, ugrađivanje je elemenata u otolite složen proces i osim dostupnih koncentracija elemenata u okolnoj vodi, dodatno ovisi o bioraspoloživosti (Walther i Thorrold, 2006.; Bouchoucha i sur., 2018.), fiziološkom stanju jedinki (Sturrock i sur., 2015.; Grønkjær, 2016.), ontogeniji i stopi rasta (Correia i sur., 2011.) te popratnim učincima temperature, slanosti i kemijskog sastava vode (Sadovy i Severin, 1994.; Bath i sur., 2000.; Campana i sur., 2000.; Elsdon i Gillanders, 2004.; Elsdon i sur., 2008.).

U članku II istraživali smo razlike u sastavu otolita među nedoraslim jedinkama komarče s različitih rastilišta s mogućnostima povezivanja jedinki i područja (rastilišta) na temelju koncentracija kemijskih elemenata. Rezultati članka II pokazali su kako je kemijski sastav otolita nedoraslih komarči varirao među različitim rastilištima u istočnom Jadranu. Koncentracije elemenata Mg, Sr i Zn su se statistički znatno razlikovale među rastilištima za razliku od Ba i Pb. U članku IV istraživane su razlike u kemijskom sastavu otolita među nedoraslim jedinkama pica i fratra iz poznatog estuarijskog i morskog rastilišta. Također, testirana je točnost odnosno relevantnost realokacije u jedno od ta dva bitno različita staništa na osnovi dobivenog elementnog sastava. Rezultati su pokazali da se kemijski sastav otolita kod obje vrste razlikuje u koncentracijama elemenata Sr i Ba u morskom i estuarijskom rastilištu. Omjeri tih elemenata u otolitima najčešće se povezuju s njihovim koncentracijama u okolnoj vodi i njezinoj slanosti te su utvrđeni viši omjeri Sr/Ca u morskim vodama, a viši Ba/Ca u slatkim vodama (Gillanders, 2005.; Gillikin i sur., 2019.). To je i osnovni razlog zbog kojeg se koncentracije Sr i Ba u otolitima mogu koristiti za uspješnu rekonstrukciju okolišnih parametara i korištenja različitih staništa tijekom života pojedinih vrsta riba (Fowler i sur., 2016.; Izzo i sur., 2018.).

U članku II omjeri Sr/Ca su se statistički znatno razlikovali među postajama te su, očekivano, najveće koncentracije Sr u analiziranim otolitima nedoraslih komarči bile s postaje Prosika koja nije pod izravnim riječnim utjecajem i tipično je obalno-morsko područje. Gillanders i Kingsford (1996.) su također izvijestili o znatno višim koncentracijama Sr u otolitima nedoraslih riba s obalnih grebena u odnosu na one iz estuarijskih područja. U članku IV omjeri Sr/Ca nisu se statistički znatno razlikovali, ali su vrijednosti Sr/Ca varirale među vrstama i postajama. Kako na ugradnju Sr u otolite, osim same koncentracije Sr u okolnoj vodi i slanosti, utječu i drugi čimbenici, pokazuje *D. vulgaris* koji je na obje postaje ugradio više Sr, a to može biti povezano s endogenim čimbenicima kako je već istaknuto (npr. Elsdon i sur., 2008.; Correia i sur., 2011.; Grønkjær, 2016.).

Rezultati članka II pokazali su da nema statistički znatne razlike s podjednakim koncentracijama omjera Ba/Ca među istraživanim postajama s najvećim vrijednostima Ba na postaji Mala Neretva koja je pod izravnim utjecajem obližnje rijeke, što potvrđuje navedene tvrdnje o višim koncentracijama Ba pri sniženoj slanosti (Davis, 1993.; Patterson i sur., 1999.; 2004.; Elsdon i Gillanders, 2006.; Hamer i sur., 2006.; Walther i Thorrold, 2006.). Iako se u članku IV omjeri Ba/Ca nisu znatno statistički razlikovali među vrstama i rastilištima, ipak su istaknutije razlike dobivene među vrstama. Premda je uvala Sovlje inicijalno izabrana kao morska, a Pantan kao estuarijska postaja, nepostojanje statistički znatnih razlika u Ba/Ca među rastilištima može se objasniti vjerojatnim obogaćivanjem Ba na postaji Sovlja unosom slatke vode putem podzemnih (vrulja) i oborinskih voda. Naime, svako dotjecanje slatke vode posljedično povećava Ba iznad očekivanih koncentracija za to područje, a slično objašnjavaju Correia i sur. (2011.). Nadalje, vrijednosti omjera Ba/Ca bile su različite kod pica i fratra, što potvrđuje varijabilnost ugradnje elemenata kod različitih vrsta koje obitavaju u istom području (Di Franco i sur., 2014.; Bouchoucha i sur., 2018.).

Koncentracije omjera Mg/Ca u uzorcima otolita s postaje Pantan znatno su viših vrijednosti te su se jasno razlikovale od onih s Male Neretve i Prosike, kako je navedeno u članku II. Ipak, zabilježene su i izražene razlike ugrađenih koncentracija Mg među jedinkama s postaje Pantan, što upućuje na to da je ugradnja Mg vjerojatno uvjetovana endogenim čimbenicima kako prethodno predlažu i Martin i Wuenschel (2006.) te Correia i sur. (2011.)

predlažući kako se više vrijednosti Mg u otolitima riba ne mogu jasno povezati sa specifičnim egzogenim čimbenicima okoliša.

U članku II, u otolitima nedoraslih jedinki komarče srednja je vrijednost koncentracije omjera Zn/Ca bila dva puta veća na postaji Mala Neretva u odnosu na postaje Pantan i Prosika. Koncentracije Zn imaju tendenciju da budu više u urbanim obalnim područjima zbog antropogenih izvora (Sturrock i sur., 2012.). Naime, Zn je, osim prirodnog litosfernog izvora (Halden i sur., 2000.), povezan s antropogenim izvorima kao i neki drugi elementi poput Cu, Ni i Pb (Nour i Nouh, 2020.). Ipak, neki autori smatraju da je Zn više fiziološki reguliran, a manje odražava vrijednosti parametara okoliša (Campana, 1999.; Halden i sur., 2000.). U tom kontekstu, Willis i Sunda (1984.) smatraju kako je primarni izvor unosa cinka u morsku vodu putem hrane, što su potvrdili i Ranaldi i Gagnon (2008.; 2010.).

U članku II je utvrđeno kako se Zn kontinuirano akumulirao s najvišim vrijednostima u jezgri i kako su te koncentracije opadale prema rubu otolita potvrđujući prethodno utvrđenu sustavnu raspodjelu tog elementa uz prstenastu strukturu otolita s tendecijom opadanja od središta otolita prema rubu (Halden i sur., 2000.; Avigliano i sur., 2015.). Kako analizirani otoliti komarče odgovaraju petomjesečnim jedinkama, i kako će dobiti prvi godišnji prsten ujesen (Kraljević i sur., 1998.), može se očekivati kako će koncentracije Zn rasti do pojave prvog prstena i imati povišene vrijednosti na području samih godišnjih prstenova, a zatim padati s između dva prstena rasta i dodatno sa svakim sljedećim prstenom, idući od jezgre prema rubu. Avigliano i sur. (2015.) su predložili da bi se ta značajka Zn mogla koristiti za razlikovanje kohorti riba pružajući vremenski dostupne informacije o korištenju staništa, ponašanju riba ili opskrbi hranjivim tvarima u životnom ciklusu.

Za razliku od Zn/Ca, u članku II, koncentracije omjera Pb/Ca u uzorcima otolita nedoraslih komarči nisu se statistički znatno razlikovale među istraživanim postajama. Na postaji Mala Neretva, blizu Ploča, koncentracije Pb bile su znatno izraženije, ali su i uočene znatno različite koncentracije među analiziranim jedinkama, iako su sakupljene u istom trenutku na istoj postaji. To upućuje na endogenu uvjetovanost ugradnje Pb u otolite komarče kako je već prije predloženo (Geffen i sur., 1998.; Hamer i Jenkins, 2007.). Međutim, veza između izloženosti i same ugrađene koncentracije Pb u otolitima je dosta složena i ponekad je povezana s dostupnošću u okolnoj vodi (npr. Selleslagh i sur., 2016.), no ponekad izravno ne održava razinu onečišćenja okoliša (npr. Bouchoucha i sur., 2018.). Najčešće su koncentracije Pb najviše u otolitima riba vezanim za izvor onečišćenja te su utvrđene opadajuće vrijednosti s povećanjem udaljenosti od izvora onečišćenja (Søndergaard i sur., 2015.; Cuevas i sur., 2019.).

Relativno su visoku vrijednost i varijabilnost u koncentracijama Pb u jezgri otolita među uzorcima s istog područja, kao što je slučaj kod Male Neretve, utvrdili i Gillanders i Kingsford (1996.) u otolitima nedoraslih jedinki vrste *Achoerodus viridis* (Steindachner, 1866) istraživanih uz obale jugoistočne Australije. U njihovu istraživanju, otoliti riba iz estuarijskih rastilišta pod intenzivnijim antropogenim utjecajem (s višim stupnjem onečišćenja) u odnosu na otolite riba iz obalnog područja pod manjim utjecajem onečišćenja upućuje na već spomenutu činjenicu kako ovisnost ugradnje elemenata ovisi o fiziološkom stanju svake pojedine jedinke.

Odnedavno su višeparametrijske analize (engl. *multivariate analyses*), (PERMANOVA) postale iznimno vrijedan alat za razlikovanja pojedinih područja/rastilišta riba (npr. znansteveni radovi od Gillanders, 2005.; Correia i sur., 2011.; Bouchoucha i sur., 2018.) zbog sposobnosti analitičkog postupka razdvajanja različitih poremećaja prirodnih, prostorno-vremenskih varijabilnosti koje pokazuje većina populacija (Terlizzi i sur., 2005.). U članku II iznimno je točna diskriminacija korištenjem višeparametrijskih analiza podataka pokazala kako analizirani otoliti iz različitih rastilišta imaju različite elementne zapise (otiske). Analize su potvrdile razlike među postajama Mala Neretva, Pantan i Prosika i elementima te su iste bile dostatne za statistički uspješnu realokaciju važnih rastilišta za komarču. Rastilišta su pokazala visok kontrast u smislu onečišćenja, i ono se, kako je i očekivano, odrazilo na kemijski sastav otolita (Campana, 1999.; Sturrock i sur., 2012.). Dobiveni su rezultati potvrdili specifičnosti rastilišta Mala Neretva i sličnosti između rastilišta Pantan i Prosika. Za uspješnu su realokaciju bile najvažnije koncentracije Zn/Ca i Sr/Ca u otolitima jer su oni omogućili 100-postotnu točnost odjeljivanja jedinki pridošlih iz rastilišta Pantan i Mala Neretva. Kako su u članku IV analizirane razlike između dvaju poznatih različitih staništa, morskog i estuarijskog, razlika u kemijskom sastavu otolita pridonijela je uspješnoj realokaciji iako se kemijski sastav otolita nije znatno statistički razlikovao. Dobivene su razlike vjerojatno uvjetovane endogenim uzrokom, odnosno razlikama u načinu održavanja homeostaze u okolišu sniženog, odnosno povišenog saliniteta kod jedne odnosno druge analizirane vrste kako savjetuje i Grønkjær (2016.).

U ovom trenutku zbog tehničkog napretka u analitičkim i statističkim metodama, kemijski sastav otolita predstavlja najrelevantniji pristup za utvrđivanje podrijetla riba (npr. Gillanders i Kingsford, 2000.), posebice kod migratornih vrsta, ali i uobičajenih morskih vrsta čije su populacije odraslih i nedoraslih riba prostorno odijeljene (npr. Rogers i sur., 2019.). U ovom je radu korištena kanonska analiza glavnih koordinata (CAP), kao i kod niza drugih autora (npr. Thorrold i sur., 2001; Gillanders, 2009.; Jenkins i sur., 2016.). CAP se koristi kao neparametarska alternativa za ograničavajuće analize diskriminirajuće funkcije (engl. *Discriminant function analaysis*, DFA) koja se prije najčešće koristila u ovu svrhu (Neubauer i sur., 2013.; Rogers i sur., 2019.). DFA zahtijeva normalnu raspodjelu multivarijatnih podataka, što najčešće nije ispunjeno kod ovih podataka. Postavljanje početnih uvjeta, odnosno ograničavanje ordinacija (engl. *Constrained ordination*) povećava vjerojatnost alokacije zbog unaprijed postavljenih postavki za razliku od neograničenih metoda koje uključuju multidimenzionalno skaliranje (MDS) s klasterskim analizama (npr. Tanner i sur., 2012.) ili neuvjetovane Bayesove mješovite modele (enlg. *Bayesian mixture models* (npr. Neubauer i sur., 2013.) korištene za ovu svrhu. No ipak treba naglasiti kako je mala veličina uzorka korištena u člancima relevantna za pokazivanje potencijala kemijskog zapisa otolita za utvrđivanje podrijetla odraslih jedinki, ali ne i za apsolutnu procjenu doprinosa pojedinog rastilišta. Ona je u planu u budućim istaživanjima, posebice za komarču. U ovom radu smo eksperimentalno pokazali visok potencijal tavog pristupa koji omogućuje nove smjernice u proučavanju korištenja rastilišta i obrasce kretanja vrsta među staništima tijekom života.

Može li kemijski sastav elemenata u otolitima u kombinaciji sa stabilnim izotopima u tkivu pružiti komplementarnu informaciju o načinu na koji različite vrste koriste rastilište i pojasniti ekološke veze između vrsta i staništa?

Unatoč povećanoj primjeni prirodnih markera, studije koje koriste zajedno stabilne izotope u tkivima i kemijske otiske otolita kako bi se procjenila povezanost ili struktura populacija rijetke su (npr. Dierking i sur., 2012.; Fodrie and Herzka, 2013.) i gotovo su nepostojeće one koje razmatraju ove prirodne markere u integrativnom smislu (npr. Reis-Santos i sur., 2015). Kretanja među staništima te oblici korištenja pojedinog staništa variraju među vrstama kao funkcija životnih strategija, specifičnih zahtjeva za stanište s obzirom na veličinu ili starost i uvjetovani su dodatno kombinacijom ekoloških i okolišnih čimbenika te ponašanja svake pojedine vrste (vidi Vasconcelos i sur., 2010.), a mogu se otkriti analizom kemijskog sastava otolita (Elsdon i sur., 2008.). S druge strane, analizom stabilnih izotopa tkiva mogu se dobiti važni podaci o korištenju staništa, kretanju među staništima i ishrani riba (Green i sur., 2012.; Disspain i sur., 2016.). Iz navedenog je jasno preklapanje tih dvaju prirodnih markera u ciljevima, što otvara mogućnost za komplementarni pristup njihova korištenja. U članku IV je zato pokazan potencijal kombiniranja podataka o kemijskom sastavu otolita i podataka o koncentracijama stabilnih izotopa $\delta^{13}C$ i $\delta^{15}N$ mišićnog tkiva riba za utvrđivanje obrasca korištenja različitih rastilišta srodnih vrsta. Odabrane vrste, fratar, *Diplodus vulgaris* i pic, *Diplodus puntazzo* imaju slične reproduktivne strategije i ekologiju ranih životnih stadija (Mouine i sur., 2012.), naseljavaju priobalna područja u slično vrijeme te ih napuštaju i priključuju se odraslim populacijama početkom ljeta (Dulčić i sur., 1997.; Dulčić i sur., 2002.). Kako istraživane vrste ne pokazuju vremensku i prostornu segregaciju u rastilištima, što znači da žive izložene istim egzogenim čimbenicima okoliša, pretpostavljeno je kako ishrana i pronalaženje hrane mogu biti važni čimbenici koji će pridonijeti različitom ugrađivanju kemijskih elemenata u otolite kod obiju vrsta u različitim rastilištima. I zaista su rezultati analize stabilnih izotopa $\delta^{13}C$ i $\delta^{15}N$ tkiva riba pokazali statistički znatne razlike unutar postaja i vrsta. Općenito, estuariji su često visokoproduktivna područja s uskim spektrom plijena, ali s visokom dostupnošću i brojnosti svakog pojedinog plijena (Elliot i Quintino, 2007.), dok je uobičajeno produktivnost te dostupnost i brojnost plijena u obalnim područjima niža iako je spektar plijena širi (van Leeuwen i sur., 2013.). Niže vrijednosti $\delta^{13}C$ tkiva ribe pronađene su na postaji Pantan, što se slaže s prirodnim obrascima varijacije $\delta^{13}C$ i pokazuje trend obogaćivanja duž kopneno-estuarijsko-morskog gradijenta (Fry, 1999.). S druge strane, povećane vrijednosti $\delta^{15}N$ tkiva ribe na ovoj postaji upućuju na višu produktivnost estuarijskog područja i vjerojatno su uvjetovane antropogenim unosom dušika na širem području ušća rijeke Pantan (npr. otpadne vode iz domaćinstva, umjetna gnojiva zbog ispiranja poljoprivrednih površina) kako su već predlagali brojni autori (npr. Herzka, 2005.; Schlacher i sur., 2005.; Dierking i sur., 2012.; Reis-Santos i sur., 2015.). Iako su uočene razlike u vrijednostima stabilnih izotopa kod obje vrste te je medijan stabilnog izotopa $\delta^{15}N$ tkiva ribe bio veći kod pica i na postaji Pantan i Prosika, razlike među vrstama nisu bile statistički važne. Kako te razlike vjerojatno odražavaju izotopski sastav lokalnih hranidbenih mreža i dostupnost plijena (França i sur., 2011.; Reis-Santos i sur., 2015.), izgleda da je u estuariju Pantan za obje vrste dostupan isti hranidbeni lanac i da se vrste hrane istim spektrom plijena tijekom dužeg razdoblja bez velikog prostornog udaljavanja. S druge strane, izraženije razlike u koncentraciji stabilnih izotopa u mišićnom tkivu pica i fratra s postaje Sovlje upućuju na to da se na toj postaji pic i fratar hrane različitim lokalnim hranidbenim mrežama, i da je moguće da se fratar hrani na nešto višoj trofičkoj razini, odnosno organizmima koji su smješteni na višem mjestu u hranidbenom lancu. Budući da su ciljane vrste članka IV nedorasle jedinke starosti nekoliko mjeseci koje imaju sličnu brzinu rasta, te rezultate treba razmatrati tako kao da je stopa pretvorbe tkiva riba niska i traje nekoliko mjeseci (Hesslein i sur., 1993.; MacNeil i sur., 2006.). Više vrijednosti stabilnih izotopa kod *D. puntazzo* mogu biti i posljedica ishrane većim, energetski bogatijim plijenom (Nunn i sur., 2012.) s obzirom na to da se smatra da obje vrste čine tek kratka gibanja unutar rastilišta (Abecasis i sur., 2009.). Dodatno, nedorasle jedinke pica nekoliko su tjedana starije, te su stoga većih ukupnih dužina i masa u oba rastilišta, i to može donekle utjecati na dobivene rezultate.

Dobiveni rezultati pokazuju obrasce koji se mogu protumačiti kao smanjenje vrijednosti $\delta^{13}C$ tkiva ribe i povećanje vrijednosti $\delta^{15}N$ tkiva ribe idući iz obalnih, morskih rastilišta u estuarijska rastilišta. Obje se istraživane vrste roda *Diplodus* različito ponašaju u estuarijskim i obalnim vodama, što je vjerojatno povezano s ishranom i učinkovitošću pronalaženja hrane. Naši rezultati pokazuju da je pic, *D. puntazzo* vjerojatno učinkovitiji u ishrani u produktivnijim estuarijskim vodama, što je već opisano (Elliot i Quintino, 2007.) i raste brže te pritom ugrađuje znatno više vrijednosti $\delta^{15}N$ za razliku od fratra koji je nešto učinkovitiji u obalnim rastilištima. Štoviše, veća je učinkovitost ugradnje stabilnih izotopa dušika u mišićno tkivo pica još više izražena u obalnim morskim vodama, gdje je plijen općenito manje dostupan, a pronalaženje prikladnije hrane traje duže (Nunn i sur., 2012.; van Leeuwen i sur., 2013.). Pic po svemu sudeći ima sličan obrazac ugradnje kemijskih elemenata u otolite i stabilnih izotopa u tkiva u različitim staništima i stoga je moguća visoka točnost realokacije pica prema višoj vrijednosti $\delta^{15}N$ u estuarijskim vodama. S druge strane, kod fratra se uočava obrazac manje učinkovitosti ugradnje u estuarijskim vodama.

Dodatno, rezultati kanonske analize glavnih koordinata (CAP) pokazuju da će s vremenom razlike kemijskog sastava otolita kod fratra iz različitih staništa postati znatnije i stoga će se fratar moći još točnije razvrstati u estuarijske vode na osnovi specifičnog sastava elemenata otolita i podataka o stabilnim izotopima. Takav kombinirani pristup pokazuje kako jedinke reagiraju na procese koji se događaju na određenim prostorno-vremenskim skalama i kako on može donijeti vrijedne rezultate o povezanosti populacija i različitom korištenju rastilišta. Stoga se očekuje kako će korištenje višestrukih prirodnih markera povećati vjerodostojnost procjene povezanosti (npr. Abaunza i sur., 2008.; Fodrie i Herzka, 2013.; Lawton i sur., 2010.; Perrier i sur., 2011.; Thorrold i sur., 2001.; Verweij i sur., 2008.). Dodatno, očekuje se da će stabilni izotopi u tkivima pružiti informacije o dnevnim odnosno mjesečnim promjenama (Herzka, 2005.), dok otoliti uključuju vremenski povezane informacije o cijelom životu jedinke (Campana, 1999.; Elsdon i sur., 2008.).

Može li kemijski sastav elemenata u otolitima omogućiti procjenu doprinosa odrasloj populaciji unovačenih jedinki komarče (*Sparus aurata*) koje potječu iz rastilišta koje je pod utjecajem onečišćenja?

U cijelom je Sredozemnom moru poznato kako komarča, *Sparus aurata* ontogenetski migrira između priobalnih rastilišta i otvorenog mora (Mercier i sur., 2012.; Isnard i sur., 2015.) koje su povezane s razmnožavanjem (Audouin, 1962.; Houde, 1989.) te selidbom i novačenjem (Cowen i sur., 2000.; Beck i sur., 2001.; Cowen i Sponaugle, 2009.; Morais i sur., 2017.). Nedorasle komarče koje nastanjuju različita priobalna rastilišta početkom zime najvećim se dijelom iz njih sele sljedeće jeseni (Bauchot i Hureau, 1986.). Morska se staništa, pa tako i potencijalna rastilišta, razlikuju po okolišnim uvjetima, bilo zbog varijacija antropogenih utjecaja ili zbog prirodnih varijacija (Barnes i Gillanders, 2013.; Sturrock i sur., 2015.; Bouchoucha i sur., 2018.). U traženju sličnosti među rastilištima mogu se usporediti prirodne varijacije temeljene na glavnim značajkama rijeka kada se razmatraju estuarijska rastilišta. Tako je postaja Prosika pod neizravnim utjecajem rijeke Krke s pojačanim dotokom podzemnih voda (vrulja) u zimskom i proljetnom dijelu godine, dok su postaje Mala Neretva (Oskoruš i sur., 2019.; Krvavica i Ružić, 2020.) i Pantan (Matić-Skoko i sur., 2005.; Fistanić, 2006.) pod izravnim utjecajem rijeka, koje se nalaze u neposrednoj blizini, tijekom cijele godine. No specifični otisci koji su rezultat antropogenog onečišćenja nekog područja i time povezanih povišenih vrijednosti kemijskih elemenata blisko povezanih s poznatim izvorom onečišćenja mogu biti važno razlikovno sredstvo za alokaciju rastilišta, a time i pružaju mogućnost za izračun doprinosa novaka iz tog rastilišta odrasloj populaciji istraživane vrste.

Dobiveni su rezultati relativnog doprinosa pojedinog rastilišta u članku II u skladu s onima iz sličnih studija provedenih posljednjih godina (npr. Di Franco i sur., 2011.; Fortunato i sur., 2017.). Nedorasle jedinke komarče iz rastilišta Mala Neretva imaju statistički znatno više koncentracije Zn/Ca te povišene vrijednosti Ba/Ca i Pb/Ca u odnosu na druga dva analizirana rastilišta. Uočena je znatna razlika među jedinkama u njihovoj ugradnji Pb na ovoj postaji, što je već raspravljeno. Budući da nedorasle jedinke komarče *S. aurata* provode oko devet mjeseci u rastilištima, nema naznaka da bi ugrađene relativno niske koncentracije Pb i Zn na postaji Mala Neretva mogle imati negativan utjecaj na dinamiku populacija komarče u južnom Jadranu, stoga se smatra da je negativan doprinos tog rastilišta neznatan. Utvrđene su koncentracije elemenata u tragovima bile znatno ispod europskih standarda i smjernica za elemente u tragovima u tkivima riba i školjakaša (Uredba Komisije EC br. 1881/2006.). Inače, dostupni su neki podaci u vezi s onečišćenjem sedimenata metalima (Jurina i sur., 2015.; Giglio i sur., 2020.) i tkiva riba (Bogut i Bukvić, 2003.; Has-Schön i sur., 2006.; 2008.; Djedjibegovic i sur., 2012.) na području rijeke Neretve koja su najvjerojatnije posljedica poljoprivrednih djelatnosti na tom području, pa se pesticidi i kemijski spojevi metala koji se često koriste u poljoprivredi ispuštaju u obližnja estuarijska i morska područja (Filimonova i sur., 2018.). Elementi, kao što su Al, Cd, Ba, Mn, Pb, Co i Zn, obično se nalaze kao sastojci u bojama i gnojivima, a prirodno ih nalazimo u slatkovodnim vodotocima (Gillanders i Kingsford, 1996.), zato se često javljaju u povećanim koncentracijama u otolitima nedoraslih riba iz regija ekstenzivnih industrijskih područja i ušća rijeka (Ranaldi i Gagnon, 2010.). Vasconcelos i sur. (2011.) su utvrdili kako nedorasle jedinke gospodarskih vrsta *Solea solea* (Linnaeus, 1758), *Solea senegalensis* Kaup, 1858, *Platichthys flesus* (Linnaeus, 1758), *Diplodus vulgaris* i *Dicentrarchus labrax* (Linnaeus, 1758) napuštaju ušća rijeka tijekom svoje prve godine života radi pridruživanja obalnim adultnim populacijama, prenose niske razine onečišćenja zbog niskih razina Cd i Pb koje su ugradili tijekom prvih mjeseci života u estuarijskim područjima (Durrieu i sur., 2005.). No mnoge nedorasle ribe provode poduža razdoblja u estuarijima, stoga mogu biti duže razdoblje izložene onečišćenju (Usero i sur., 2003.; Vinagre i sur., 2004.; França i sur., 2005.), što bi moglo imati utjecaja na daljnje životne procese. Valja očekivati kako će one komarče koje prve jeseni ne napuste rastilište Mala Neretva imati u svom kasnijem otisku otolita izraženije vrijednosti koncentracije Pb od onih koje su prije unovačene.

Ipak, ukupan doprinos rastilišta Mala Neretva populaciji komarči u Jadranskom moru tek treba biti određen kada se budućim istraživanjem na većem broju uzoraka uzduž obale, uzimajući u obzir sva potencijalna rastilišta, analizira ukupan godišnji doprinos te onda na osnovi specifične realokacije odredi i postotni doprinos svakog pojedinog rastilišta odrasloj populaciji. Moguće je da će specifičan otisak otolita koji predstavlja kombinaciju povišenih koncentracija Zn/Ca, Ba/Ca i Pb/Ca biti odlučujući za realokaciju novaka koji potječu iz rastilišta Mala Neretva.

Smjernice za buduća istraživanja

U ovoj doktorskoj disertaciji prikazan je potencijal kemijskog zapisa otolita za proučavanje korištenja rastilišta odabranih vrsta iz porodice ljuskavki. Dodatno, kemijski zapis otolita u kombinaciji sa stabilnim izotopima u tkivu pruža komplementarne informacije o korištenju rastilišta. Takav je pristup istaživanju, bez obzira na mali broj uzoraka, obećavajući te omogućuje bolji uvid u obrasce kretanja vrsta među različitim staništima tijekom života te utvrđivanje podrijetla odraslih jedinki. Broj uzoraka otolita korišten u člancima nedovoljan je za procjenu doprinosa pojedinog rastilišta te ukupnog doprinosa svih analiziranih rastilišta odraslim populacijama odabranih vrsta riba iz porodice Sparidae. Stoga u budućim istraživanjima treba povećati broj uzoraka te obratiti pozornost na uravnoteženu raspodjelu uzoraka sa svake lokacije. Procjena doprinosa svakog pojedinog rastilišta bit će napravljena u budućim istraživanjima usporedbom kemijskih otisaka nedoraslih i odraslih jedinki određene vrste. Uzorkovanjem se treba obuhvatiti istu kohortu ribe, stoga je nužno uzorkovati odrasle ribe starosti 2 godine s vremenskim odmakom uzorkovanja od minimalno 2 godine. U budućim istaživanjama svakako treba uzorkovati morsku vodu u svakom staništu zbog različitih koncentracija kemijskih elemenata koji se ugrađuju u otolite i na taj način utvrditi vezu između dostupnosti pojedinog kemijskog elementa u vodi i njegove ugradnje u otolit. Također, poželjno je u istu svrhu analizirati omjere stabilnih izotopa u morskoj vodi i tkivu riba. Istraživanjem su utvrđene različite veličine jedinki u istom trenutku uzorkovanja na različitim postajama. To upućuje na različitu produktivnost te korištenje staništa i u tom smislu različite stope rasta nedoraslih jedinki neke vrste u različitim staništima. Stoga će se u budućim istraživanjima odrediti dnevni prirast riba brojenjem dnevnih prstenova na otolitima radi točnog određivanja vremena ulaska i napuštanja rastilišta te odrediti postoje li razlike u rastu jedinki među rastilištima na različitoj prostornoj i ekološkoj skali. Svakako uzorkovanje odraslih jedinki starosti 2^+ godina treba obavljati u neposrednoj blizini i dalje od rastilišta radi utvrđivanja broja jedinki koje se zadržavaju oko rastilišta tijekom dužeg razdoblja te koliko im dugo funkcija rastilišta pogoduje. Nažalost, u posljednje su vrijeme mnoga priobalna staništa degradirana zbog nasipavanja uvala krutim materijalom radi stvaranja plaža. Takvim su zahvatima nepovratno uništena neka plitka priobalna područja koja predstavljaju rastilišta određenim vrstama. Ona zato gube funkciju rastilišta zbog nanosa novog materijala i smanjenog strujanja morske vode te postaju nedostupna nedoraslim ribama. Budućim je istraživanjima nužno procijeniti smanjenja doprinosa iz utvrđenih, poznatih rastilišta i onih novih koji su preuzeli funkciju rastilišta zbog degradacije okoliša te dalekosežne posljedice takvih aktivnosti u priobalju.

ZAKLJUČCI

Rezultati predstavljeni u četiri članka te raspravljeni s novim podacima upućuju na sljedeće:

1. Provedena su istraživanja u sva 4 članka pokazala dobar potencijal korištenja kemijskog sastava otolita za praćenje prirodne i antropogene varijabilnosti staništa i istraživanih vrsta.
2. Globalni razvoj sklerokronoloških istraživanja na otolitima omogućit će širu primjenu i u Sredozemnom i Jadranskom moru uz sve trenutno utvrđene prepreke i nedostatke koji uključuju mali broj dugoživućih vrsta, nepostojanje arhiviranih otolita, kratkoročna istraživanja neujednačenih protokola i nedostatak analitičke opreme i obučenog osoblja. Navedena bi istraživanja trebala stvoriti podlogu za bolje razumijevanje ekoloških pokretača prirodnih i antropogenih promjena u morskom okolišu.
3. Analizom elementnog sastava otolita ciljanih vrsta porodice ljuskavki, Sparidae pomoću LA-ICPMS metode (Laserska ablacija – Induktivno spregnuta plazmatska masena spektrometrija) dobiven je preliminarni uvid korištenja niza estuarijskih i morskih rastilišta uzduž istočne obale Jadrana te prostorna povezanost koncentracije kemijskih elemenata i izraženih dijelova otolita (jezgra, prstenovi rasta, rub).
4. Kod otolita komarče, *Sparus aurata* prema CRM (konfokalnoj Ramanovoj mikrspektroskopiji) i orijentaciji mikrokristalita, aragonit je glavna mineralna komponenta uz tragove kalcita i stroncijanita. Prisutnost zona prirasta potvrđena je različitim orijentacijama kristala ili prisutnosti drugih karbonata u tragovima na mjestu nastanka prstenova. To jasno upućuje na prikladnost ove metode za validiranje periodičnosti stvaranja prstenova rasta.
5. Kemijski sastavi otolita kombinirani s koncentracijama stabilnih izotopa mišićnog tkiva kod pica, *Diplodus puntazzo* i fratra, *Diplodus vulgaris* (uz upotrebu mediteranske dagnje, *Mytilus galloprovincialis* kao izotopske osnove) upućuju na razlike u trofičkom statusu tih vrsta i njihovu različitom korištenju estuarijskog i morskog rastilišta. Pic je učinkovitiji u ishrani u produktivnijim estuarijskim vodama i raste brže od fratra. Brži rast može nagovijestiti ranije napuštanje rastilišta i ranije pridruživanje odraslim populacijama. To naglašava potrebu za razmatranjem parametara okoliša koji dodatno oblikuju ponašanja, kretanja i povezanosti vrsta na temelju otisaka otolita. Kemijski zapis (otisak) otolita u kombinaciji sa stabilnim izotopima u tkivu pruža

komplementarne informacije o korištenju rastilišta i predstavlja inovativan pristup u ovakvim istraživanjima.

6. Dobiveni rezultati kemijskog sastava otolita nedorasle komarče, *S. aurata* odražavaju okolišne čimbenike na tri različita rastilišta (Prosika, Pantan i Mala Neretva) te omogućuju vrijedne informacije o potencijalnoj izloženosti nedoraslih jedinki komarče na onečišćenje okoliša na području rastilišta Mala Neretva gdje su zabilježene povećane koncentracije Zn i Pb u otolitima.
7. Korištenjem multivarijatnih i kanonskih analiza pokazan je relativno visok postotak uspjeha (80 % i 77,8 %) u realokaciji pica *D. puntazzo* i fratra, *D. vulgaris* u poznato morsko odnosno estuarijsko rastilište na osnovi koncentracija Ba i Sr. Kod nedoraslih komarči, *S. aurata* uspješnost postignuta u realokaciji u različita rastilišta ovisna je o elementima, stoga je veća realokacija postignuta u rastilištu Pantan prema Sr (100 %), Zn (100 %) i Mg (80 %) i manja u rastilište Prosika prema Sr (100 %), Zn (66,6 %) i Mg (33,3 %). Rastilište Mala Neretva karakterizirano je sa 100 %, odnosno 80 % uspješnosti realokacije prema koncentraciji Mg i Zn, i individualno specifičnim povišenim koncentracijama Pb. Uspješna realokacija koja je rezultat specifičnih razlika u elementnom sastavu otolita u ovisnosti o pojedinim staništima omogućit će buduću identifikaciju podrijetla odraslih riba i relativni doprinos nedoraslih jedinki s točno određenih rastilišta odraslim populacijama (stopa novačenja) te doprinos onih rastilišta koja su karakterizirana relativno višim stupnjem onečišćenja.
8. Za potpunu kvantitativnu i kvalititavnu procjenu doprinosa svakog pojedinog rastilišta odraslim populacijama potrebno je specifično uzorkovanje većeg broja jedinki na širem području uz uzorkovanje okolne vode te uključiti istraživanje dnevnog prirasta otolita, što će omogućiti određivanje vremenskih odrednica selidbe i novačenja (ulazak i izlazak iz svakog pojeding rastilišta).

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ŽIVOTOPIS

Dario Vrdoljak rođen je u Splitu, u Hrvatskoj 1. listopada 1989. godine. Završio je III. gimnaziju (matematičku) u Splitu 2008. godine, nakon čega je upisao Svučilišni odjel za studije mora na Sveučilištu u Splitu, gdje je završio preddiplomski studij 2011. godine te postao prvostupnik inženjer morskog ribarstva. Diplomirao je 2014. godine te stekao titulu magistra inženjera morskog ribarstva. Zaposlio se na mjesto stručnog suradnika u Institutu za oceanografiju i ribarstvo u Splitu 2015. godine, gdje je obavljao poslove povezane s priobalnim ribolovom, biologijom i ekologijom riba, terenskim istraživanjima, prikupljanjem, obradom i analizom podataka. Od kolovoza 2018. godine doktorand je na projektu „Priobalna rastilišta: obrasci procesi demografske varijabilnosti morskih riba uzduž istočnojadranske obale (NurseFish)", čiji je voditelj dr. sc. Sanja Matić-Skoko. Upisao je poslijediplomski doktorski studij (Oceanologija) na Geološkom odsjeku Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu 2016. godine pod mentorstvom dr. sc. Sanje Matić-Skoko, znanstvene savjetnice u trajnom zvanju (Institut za oceanografiju i ribarstvo). Sudjelovao je na nekoliko nacionalnih i međunarodnih usavršavanja povezanih s biološkim analizama podataka, metodama i pripremama otolita za kemijsku analizu te pripremama otolita i tkiva ribe za analizu stabilnih izotopa. Aktivni je suradnik na brojnim nacionalnim i međunarodnim projektima. Prvi je autor na dva objavljena znanstvena rada te koautor u osam znanstvenih radova. Sudjelovao je u međunarodnim znanstvenim konferencijama kao prvi autor s jednom usmenom prezentacijom i šest poster prezentacija te kao koautor na 20 prezentacija.

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Elasmobranch Association 23rd annual conference, Rende, Italy, October 1-18, 2019, Session II: Ecology, Migration and Feeding, p52.

SUDJELOVANJE NA PROJEKTIMA

Uprava za ribarstvo: Kvalitativno-kvantitativne analize ulova obalnih potegača prema propozicijama koje je definirao STECF, suradnik projekta (2015)

Uprava za ribarstvo: Istraživanje trostrukih mreža stajaćica: sezonska raspodjela ribolovnog napora i ulova s kvalitativno-kvantitativnim sastavom lovina, suradnik projekta (2015)

Uprava za ribarstvo: Procjena stanja hlapa (*Homarus gammarus*) na zapadnoj obali Istre, suradnik projekta (2016)

Uprava za ribarstvo: Provedba istraživanja i prikupljanja podataka za stvaranje stručne podloge o inventarizaciji morskih zajednica i stanju ribolovnih resursa te socio-ekonomsko istraživanje lokalnog ribarstva na području Paškog zaljeva, suradnik projekta (2016)

Uprava za ribarstvo: Procjena štete od predatora (dupina) u hrvatskom ribarstvu, suradnik projekta (2017)

HRZZ Istraživački projekti IP – 06-2016 Priobalna rastilišta: obrasci i procesi demografske varijabilnosti morskih riba uzduž istočnojadranske obale (NurseFish), suradnik projekta (2017. – 2021.)

Uprava za ribarstvo: DCF – Nacionalni program za prikupljanje podataka o ribarstvu, suradnik projekta (2015. –)

Sustavno ispitivanje kakvoće prijelaznih i priobalnih voda, suradnik projekta (2015. –)

ADRION Interreg ARIEL: Promoting small scale fisheries and aquaculture in Adriatic-Ionian macro region, suradnik projekta (2018. – 2020.)

INTERREG Italija – Hrvatska CBC Programa 2014. – 2020: Smanjivanje i sprječavanje, integrirani pristup gospodarenju otpadom iz mora u Jadranu (REducing and Preventing, an integrated Approach to Marine Litter Management in the Adriatic Sea) – akronim ML-REPAIR, suradnik projekta (2018. – 2019.)

Uprava za ribarstvo: Provedba projekta ocjene stanja priobalnog ribolova i ribolovnih resursa uz sjeverozapadnu obalu Istre s posebnim osvrtom na Savudrijsku uvalu, suradnik projekta (2018. – 2021.)

Program praćenja ciljanih vrsta priobalnih riba kao bioloških indikatora klimatskih promjena u Nacionalnom parku „Brijuni“, suradnik projekta (2016. – 2019.)

INTERREG Italija – Hrvatska Adri.SmArtFish: Valorisation of SMall-scale ARTisanal FISHery along the Adriatic coasts, in a context of sustainability, suradnik projekta (2019. – 2021.)

Uprava za ribarstvo: Provedba istraživanja i prikupljanja podataka za stvaranje stručne podloge vezane uz trostruke mreže stajaćice u kontekstu promjene u konstrukciji sipara i povećanja minimalne veličine oka mreža listarica i ocjena stanja lovišta u akvatoriju oko otoka Paga za ludar, suradnik projekta (2019. – 2020.)

USAVRŠAVANJA

GCP/RER/010/ITA – FAO AdriaMed Project - Participation in Training Course on the use of the “R” system. FAO HQ, Rome, September 7-11, 2015.

PRIMER 7 Workshop, UK Multivariate Analysis in Ecology (& other Sciences). Plymouth, UK, June 19-23, 2017.

lsotopes in marine and fisheries ecology. Institute of Oceanography and Fisheries, Split, Croatia, October 17, 2018.

Methods and analysis in dendochronology and sclerochronology. Institute of Oceanography and Fisheries, Split, Croatia, September 24, 2018.

Otolith Decontamination for Elemental and Isotopic Analysis. Bioscience Departement, University of Aarhus, Aarhus, Denmark, February 4-13, 2019.

Application of laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) to determine element concentrations in otoliths. (sample preparation for LA_ICP_MS, sample processing on the instrument, data anarysis and interpretation). Johannes Gutenberg-Universitiit Mainz (JGU), Laboratory at the lnstitute for Geosciences, University of Mainz, Germany, March 18-22, 2019.

PERMANOVA+ WORKSHOP Multivariate Analysis in Ecology (& other Sciences). University of Trieste, Italy, September 16-20, 2019.

Ecological modelling" ECHO Group Oceanography Division. OGS (National Institute of Oceanography and Applied Geophysics), Trieste, Italy, February 11, 2020.

An overview of otolith microchemistry: an introduction into some of the analytical challenges and statistical approaches. Institute of Oceanography and Fisheries, Split, Croatia, June 11, 2021.

SUDJELOVANJE U ORGANIZACIJSKIM ODBORIMA

5th International Sclerochronology Conference, ISC 2019, Split, Croatia, 16-20 June 2019, organizacijski odbor.

2nd Southeast European Ichthyological Conference (SEEIC), Supetar, Croatia, October 2021, organizacijski odbor.