J Biol Phys (2018) 44:471–482
https://doi.org/10.1007/s10867-018-9502-y

ORIGINAL PAPER

Mn^{2+} concentrations in coastal fish otoliths: understanding environmental and biological influences from EPR

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Received: 6 June 2017 / Accepted: 22 May 2018 / Published online: 30 May 2018

Abstract The Mn^{2+} concentrations in the sagittae otoliths of 12 fish families (and 19 species) that co-occur in a coastal area of southeastern Brazil (~21°S) were quantified using electron paramagnetic resonance (EPR). Inferences were made about the relationship between fish habitat and trace element incorporation. Inferences were made on the relationship between trace element concentration and otolith shape. The differences in Mn^{2+} concentrations among the species suggest that habitat (and feeding habits) might drive the incorporation of this trace element into fish otoliths, with higher values in bottom-associated fish species than in surface-associated species. In surface-associated fish species, the correlation between trace element concentrations and otolith shape was stronger than in bottom-associated species. Thus, while the Mn bioavailability in a fish's habitat, especially from feeding resources, is a local driving influence of trace element incorporation in sagittae otoliths, species-specific requirements also have an influence. Quantitative EPR is a non-destructive technique that is very useful when the available samples cannot be damaged, like with otolith collections.

Keywords Coastal teleost · Electron paramagnetic resonance · Manganese · Sagittae otoliths

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10867-018-9502-y) contains supplementary material, which is available to authorized users.

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1 Introduction

Manganese (Mn) is a naturally occurring trace element and an essential nutrient that is actively assimilated and utilized by organisms. Mn is often transported in rivers adsorbed to suspended particles [1, 2], which is how it reaches marine coastal areas. In general, Mn concentrations are several orders of magnitude greater in marine sediments than in seawater. This trace element is concentrated in sediments in the deep ocean and in surface sediment on the continental shelf [3]. In aquatic ecosystems, the oxidized form of this trace element, Mn^{2+}, is bioavailable and can be taken up by organisms, especially by bottom-associated species [4].

The inner ear of teleost fish has three pairs of crystalline structures the otoliths (sagittae, asterisci, and lapilli), which are responsible for balance maintenance in the water column and for hearing [5]. The otoliths are biominerals that grow through calcium carbonate ($CaCO_3$) deposition, which can crystallize in vaterite, calcite or aragonite structure [6, 7]. The biomineralization process includes the incorporation of Mn^{2+} as a substitution impurity at calcium (Ca^{2+}) sites [8] and a substitution for other trace elements, like strontium and barium [9, 10].

Interspecific variations in fish otolith chemistry showed that the incorporation of trace elements might be species-specific [11–13]. The incorporation of trace elements into the $CaCO_3$ protein matrix of otoliths modifies its chemical composition and may be influenced by both exogenous (like water, sediment and food intake) and endogenous factors (like stress, embryological development, growth rate, and reproductive status), and it is useful as a fish population proxy [14–16]. Since the otoliths grow in layers that are continuously deposited during the fish's lifetime, trace elements incorporated into the otolith may reveal a long-term record of the trace element composition of the fish environment over time.

Electron paramagnetic resonance (EPR) detects non-paired electrons of paramagnetic species of chemical elements like Mn^{2+} [17]. The structure of the EPR spectrum of Mn^{2+} is related to the chemical and physical properties of $CaCO_3$ [18]. In polymorphs of $CaCO_3$, like otoliths, oxygen atoms coordinate the Mn^{2+}. The position, distance, and number of oxygen atoms around the Mn^{2+} define the spectrum shape. An EPR line has a shape of the first derivative of an absorption line. The double integration of an EPR line corresponds to the spectrum area, which is directly proportional to the total concentration of paramagnetic species detected [19].

EPR can be used to investigate biological systems at micro, meso, and macroscales, using different approaches [20]. Franco et al. [7] were the first to use the EPR technique as an innovative way to investigate fish otoliths. The authors analyzed the spectra of Mn^{2+} in the otoliths of 12 Sciaenidae fish species from southeastern Brazil (~ 21°S–23°S), describing the typical shape of this ion in the crystalline structure of aragonite and detecting differences in Mn^{2+} concentrations because of the otolith shape.

This study continues the approach pioneered by Franco et al. [7], applying the EPR technique to quantify the Mn^{2+} concentration in the sagittae otoliths of other 12 fish families (and 19 species) that co-occur in a tropical coastal area of southeastern Brazil (~21°S). We wanted to understand both environmental (surface vs. sea bottom) and biological (otolith shape) influences on otolith growth through Mn incorporation. Additionally, constraints and benefits regarding the utilization of quantitative EPR for otolith analysis are discussed. We compare the EPR technique to typical spectrometric approaches.

2 Methods

2.1 Sampling

Twelve teleost fish families were obtained in 1998–2000 by local fishermen in artisanal fisheries using trawl nets and gillnets. A total of 19 species and 253 specimens were used for the analysis (Table 1). All specimens were obtained from fishing grounds used by fishermen in Atafona harbor (21°35'S), which are comprised of waters less than 20 nautical miles (approximately 1.5 km to 36 km) from shore. They were fished from depths ranging from 10 to 20 m and between 21°35'S and 21°50'S. The sampling area is under the permanent influence of the Paraíba do Sul River discharge, which is the main river runoff for southeastern Brazil. The river plume reaches the open ocean waters at velocities of 1.6 to 2.6 km day^{-1} [21].

After sampling, each fish was measured (to 0.1 cm). The sagittae otoliths were extracted, cleaned of adhering tissue, rinsed in ultrapure water, and dried at room temperature while being stored in individually labeled sealed plastic tubes at room temperature. The otoliths (left and right) were measured using a stereomicroscope with an ocular micrometer (to 0.1 mm) and weighed (to 0.0001 g). The otolith length was the distance between the anterior and posterior edges of the otolith. The width was the longest distance between the dorsal and ventral edges of the otolith, perpendicular to its length. The otolith samples used in the present study belong to the personal collection of one of the authors (APM Di Beneditto).

2.2 EPR measurements

The EPR spectra of the sagittae otoliths were obtained using a spectrometer Bruker E500 with a highly sensitive cylindrical cavity, operating with an X-band (9 GHz) at room temperature (300 K). The experimental settings were as follows: microwave power of 20 mW, modulation frequency of 100 kHz, modulation amplitude of 0.5 mT, receiver gain of 60 dB, and sweep time of 120 s.

The areas of Mn^{2+} EPR spectra, in arbitrary units (a.u.), were obtained using double integration of the set of six lines. The intensity was directly proportional to the number of resonance spins and to the concentration of the paramagnetic species. For the quantitative EPR analysis that considers the relative concentrations of paramagnetic elements, like in this study, it is not necessary to use a certified reference material to validate the results [19]. The Mn concentration in all fish specimens was normalized by otolith mass. This parameter, called the specific concentration of Mn^{2+}, was the following ratio: area of Mn^{2+} spectrum (a.u.)/otolith mass (g), representing the amount of Mn^{2+} per otolith, independent of the otolith dimension (size and weight).

The otolith samples were previously cleaned with pure acetone before spectral measurement. The preliminary spectra obtained from the left and right otoliths of some fish specimens verified the possible differences between the sides. Because both left and right otoliths produced similar spectra, we chose the left otolith for the EPR analysis. For nine fish families (15 species), the left otoliths were gathered into a composite sample and ground into a fine, homogeneous powder using mortar and pestle before analysis. This set of samples included otoliths with low Mn^{2+} concentration and a length lower than 4 mm, whose spectra shape of intact otoliths changed with sample orientation inside the quartz tube. For fish from the Carangidae and Paralichthyidae families, more than one species were gathered because of their small sample size. For the other three fish families (four species), the left otolith was intact for the EPR analysis (Table 1).

Table 1 Characteristics of the coastal fish species analyzed in this study. Values are expressed as the mean ± standard deviation. *Composite samples

Family	Species	Number of specimens	Habitat	Fish length (cm)	Elongation (otolith length/width)	Specific concentration of Mn^{2+} (a.u.)
Pristigasteridae	*Chirocentrodon bleekerianus* (Poey 1867)	30*	Pelagic	8.3 ± 0.8	1.6 ± 0.1	2.5 ± 0.3
	Odontognathus mucronatus (Lacepède 1800)	21*	Pelagic	10.8 ± 3.1	1.4 ± 0.1	3.1 ± 0.3
	Pellona harroweri (Fowler 1917)	20*	Pelagic	6.2 ± 1.8	1.4 ± 0.1	5.8 ± 0.6
Engraulidae	*Anchoa filifera* (Fowler 1915)	4*	Pelagic	7.9 ± 0.1	1.7 ± 0.1	8.8 ± 0.8
	Lycengraulis grossidens (Spix & Agassiz 1829)	4*	Pelagic	10.6 ± 1.2	1.7 ± 0.1	6.4 ± 0.6
Trichiuridae	*Trichiurus lepturus* (Linnaeus 1758)	12*	Pelagic	69.0 ± 52.8	2.4 ± 0.2	8.6 ± 0.9
Gerreidae	*Eucinostomus argenteus* (Baird & Girard 1855)	14	Demersal	8.4 ± 1.0	1.4 ± 0.1	176.8 ± 54.2
Haemulidae	*Conodon nobilis* (Linnaeus 1758)	20	Demersal	9.4 ± 1.7	1.6 ± 0.1	80.3 ± 24.0
	Orthopristis ruber (Cuvier 1830)	4	Demersal	7.7 ± 1.6	1.5 ± 0.1	43.0 ± 9.4
Carangidae	*Selene spixii* (Castelnau 1855), *S. vomer* (Linnaeus 1758), *Chloroscombrus chrysurus* (Linnaeus 1766)	17*	Demersal	8.0 ± 2.3	1.6 ± 0.2	7.1 ± 0.7
Stromateidae	*Peprilus paru* (Linnaeus 1758)	23*	Demersal	4.3 ± 2.0	1.3 ± 0.1	62.9 ± 6.3
Triglidae	*Prionotus punctatus* (Bloch 1793)	21*	Benthic	8.2 ± 1.5	1.5 ± 0.1	30.2 ± 3.0
Batrachoididae	*Porichthys porosissimus* (Cuvier 1829)	8	Benthic	21.4 ± 2.1	1.7 ± 0.2	22.9 ± 12.0
Paralichthyidae	*Citharichthys spilopterus* (Günther 1862), *Etropus* sp. (Jordan & Gilbert 1882)	19*	Benthic	7.2 ± 1.2	1.3 ± 0.1	29.7 ± 3.0
Cynoglossidae	*Symphurus plagusia* (Bloch & Schneider 1801)	26*	Benthic	13.0 ± 2.1	1.1 ± 0.1	5.6 ± 0.5
Achiridae	*Trinectes* sp. (Rafinesque 1832)	10*	Benthic	9.3 ± 1.0	1.2 ± 0.1	369.1 ± 40.0

The specific concentration of Mn^{2+} (a.u.) was obtained using nine scans (spectra) from each otolith sample (a single species sample, a composite sample of a single species or a composite sample of gathered species), and the values were expressed as the mean ± standard deviation (Table 1).

2.3 Data analysis

For data analysis, the fish species were grouped into categories according to their preferred habitat: (i) pelagic fish that were surface-associated, and (ii) demersal and benthic fish that were bottom-associated. After verifying the assumptions of normality and homoscedasticity, a Mann–Whitney *U* test was used to compare the specific concentration of Mn^{2+} in fish from different habitats. A Spearman's rank correlation verified the relation between specific concentrations of Mn^{2+} and otolith shape, referred to here as 'elongation' (ratio: length/width), to check whether more elongated otoliths have higher trace element concentration (Table 1). The statistical analysis was performed using Statistica 7.0 for Windows (StatSoft, Inc., 1984–2004). The *P* values were interpreted as the strength of evidence for the null hypotheses, rather than for the dichotomic scale of significance testing [22].

3 Results

All otolith spectra were similar, regardless of species, fish length and otolith size, characterized by a sextet with lines not equally spaced typical of the Mn^{2+} hyperfine structure, which is the interaction between the electronic spin and the nuclear spin $I = 5/2$ of the Mn, resulting in $(2*I + 1)$ lines $(2*5/2 + 1 = 6)$ in the spectrum. The complete sextet of Mn^{2+} spectrum of the *Conodon nobilis* otolith is shown at the top of Fig. 1. Each sextet line is distorted, and this distortion is increased from the low field to the high field side of the spectrum. The last line, indicated by a dashed square, shows the highest distortion. Figure 1 indicates the distortion of the last line in six otoliths measured by the distance between the two dotted vertical lines. This parameter is a reference to identify the Mn^{2+} site in the sample. For all samples, the observed distance was 8.86 mT, which corresponds to the zero-field splitting parameter D equal to 22.4 mT [18]. This value is characteristic of Mn^{2+} when occupying the Ca^{2+} site in the aragonite structure [7, 18], where the Mn^{2+} is coordinated with nine oxygen atoms [8]. Therefore, all otoliths studied had the crystalline structure of aragonite with Mn^{2+} as an impurity.

A comparison between fish habitats (surface vs. sea bottom) regarding the specific concentration of Mn^{2+} in otoliths showed higher concentrations in bottom-associated species (Mann–Whitney *U* test: $U = 6.00$, $Z = -2.20$, $P = 0.0075$) (Fig. 2).

The relationship between the specific concentration of Mn^{2+} and otolith shape (elongation) varied among fish species from different habitats (Fig. 3). In the surface-associated species, the correlation between these parameters was stronger (Spearman's rank correlation: $r_s = 0.72$, $N = 6$, $P = 0.1032$) than in the bottom-associated species (Spearman's rank correlation: $r_s = -0.14$, $N = 10$, $P = 0.6983$).

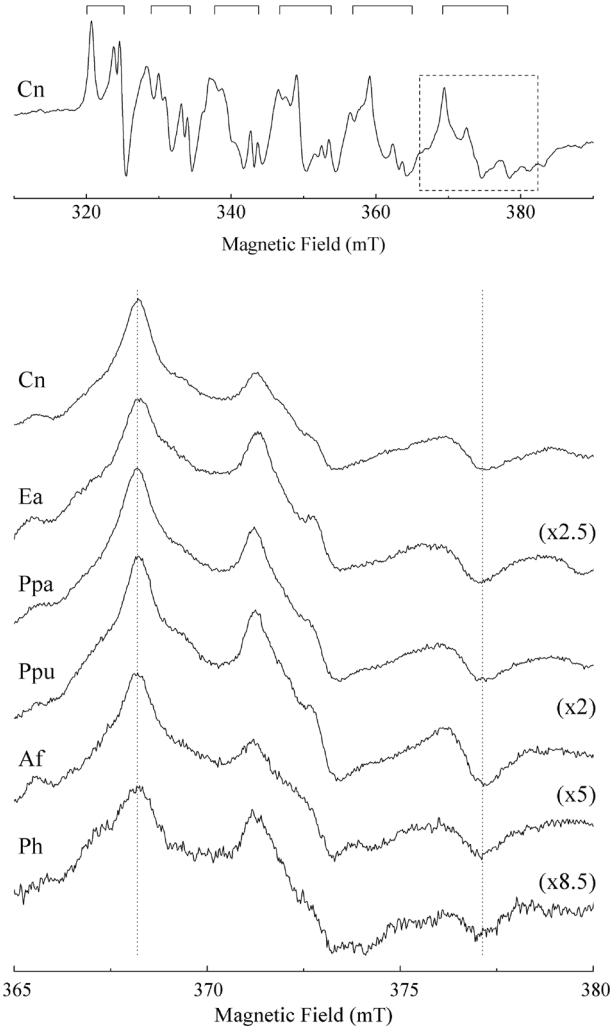

Fig. 1 Electron paramagnetic resonance spectra of coastal fish otoliths. At the *top*, the spectrum of the Mn^{2+} sextet is indicated by a *dashed square*. The *numbers in parentheses* represent the magnification factors of the axis intensity. The splitting of *dotted vertical lines* is correlated with the Mn^{2+} in the aragonite structure (Cn, *Conodon nobilis*; Ea, *Eucinostomus argenteus*; Ppa, *Peprilus paru*; Ppu, *Prionotus punctatus*; Af, *Anchoa filifera*; Ph, *Pellona harroweri*)

4 Discussion

4.1 Mn^{+2} concentrations in otoliths: environmental and biological influences

The differences in the Mn^{2+} concentration among the coastal fish species, as demonstrated by EPR measurements, suggests that the preferred fish habitat (surface or sea bottom) is the primary driver of the incorporation of this trace element into their sagittae otoliths. The bottom-associated fish species had higher Mn^{2+} concentrations compared to the surface-associated ones, and this is consistent with the higher Mn^{2+} availability on the sea bottom [4].

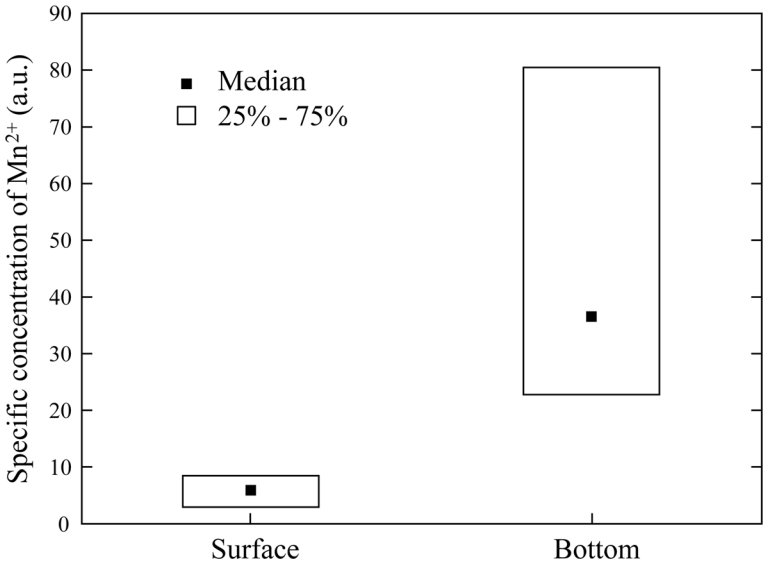

Fig. 2 Specific concentration of Mn^{2+} (a.u.) in otoliths of surface and bottom-associated coastal fish. Data are shown as the median, 75 and 25% values

In our sampling, all fish specimens were collected in a marine coastal area under permanent influence of Paraíba do Sul River discharge (~21°S). At first, all these specimens would be susceptible to the same environmental influence. The river discharge greatly influences the seawater chemistry and oceanographic conditions along the coastal area, especially during the rainy season (October to March) and during low tides [21]. This influence decreases the salinity and pH levels and increases the water temperature in adjacent marine coastal areas. These conditions might favor Mn incorporation by coastal organisms [23, 24] and influence otolith chemistry.

The extent to which the seawater chemistry and oceanography conditions influence the presence of Mn in fish bodies is still a controversial matter. Arai and Hirata [25] demonstrated the high potential for use of this trace element in distinguishing Japanese eel (*Anguilla japonica*) habitats (marine vs. freshwater), with higher concentrations from otoliths of freshwater specimens. Hägerstrand et al. [26] also inferred the relation between higher Mn concentrations in otoliths of whitefish *Coregonus lavaretus* and river discharges in Baltic Sea. On the contrary, Franco et al. [7] found that the same fish species from the Sciaenidae family under different environmental influences (marine vs. freshwater discharge) exhibited different Mn values in their otoliths, with greater values associated with marine influences. In some studies, Mn concentrations in otoliths were reported to not be associated with salinity [27, 28] or water chemistry [29].

Bioavailable trace elements in the sediment could enter fish eggs incubated on the bottom, thereby becoming incorporated into the otolith core [30]. Brophy et al. [14] verified that otoliths from both pelagic and demersal spawning fish contain elevated Mn concentrations, which does not support this assumption. In this study, most fish families undergo pelagic spawning, even though juvenile and adult fish are demersal or benthic [31–33]. Thus, the Mn transfer from sediment to the otolith during the egg phase is not a plausible explanation for the higher concentrations in bottom-associated species compared to surface-associated ones.

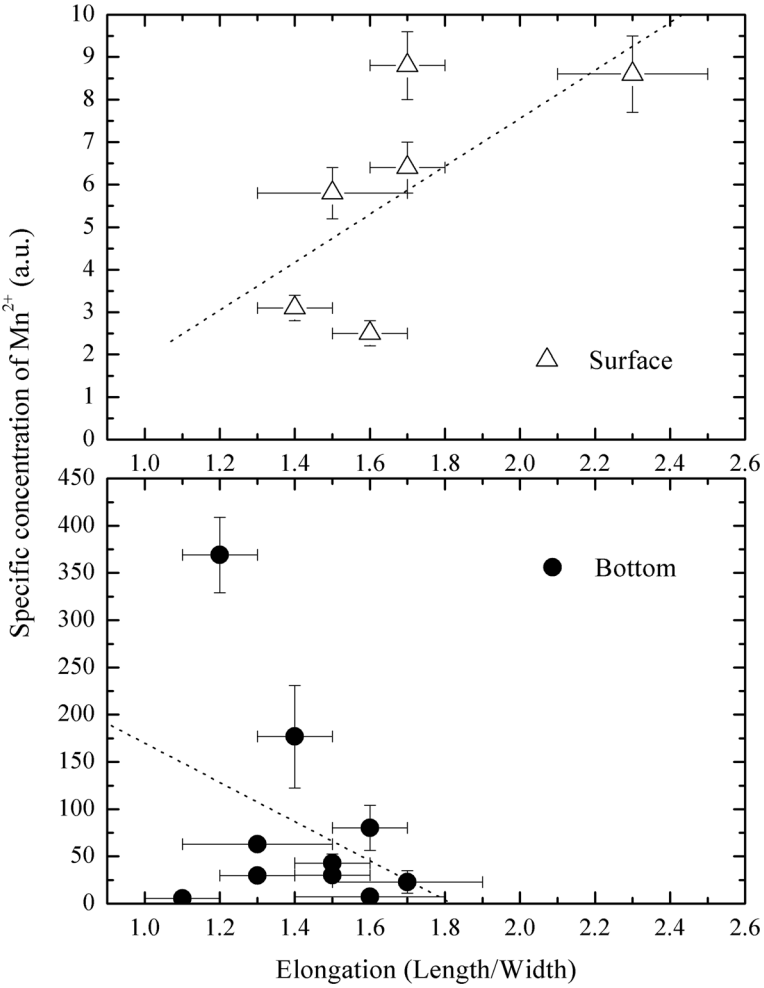

Fig. 3 Relationship between specific concentration of Mn^{2+} (a.u.) and otolith elongation (length/width) in surface and bottom-associated coastal fish

Food consumption is an important pathway for the incorporation of trace elements into animals' bodies [34, 35]. Thus, the ingestion of different prey with different trace element concentrations influences the accumulation in an animal's soft tissues and hard structures, including otoliths. Most of the analyzed pelagic fish are zooplanktivorous, while all demersal and benthic fish species are carnivorous bottom feeders [33]. The cutlassfish, *Trichiurus lepturus*, is a carnivorous predator whose habitat is demersal-pelagic. This species spends the day near the sea-bottom and swims along the water column during the night, reaching the sea-surface [33]. Along the sampling site, the feeding habits of *T. lepturus* are well known, and the preferred prey species are pelagic fish from the Engraulidae and Pristigasteridae families [36, 37]. Thus, we considered this fish to be a pelagic species because of its local preferred feeding habit.

At the sampling site, most of the Mn^{+2} incorporated into the otoliths of the bottom-associated fish probably came from ingested items. Bottom-associated prey species, which are the main food

items for demersal and benthic fish, act as a source of trace elements for the predators [38, 39]. Furthermore, the direct intake of sediment by many bottom-associated fish species, regardless of whether the species is a detritus feeder or carnivorous feeder that ingests sediment together with prey, is also an important pathway for trace element incorporation into fish [40, 41].

The correlation between the specific concentration of Mn^{2+} in the otoliths and otolith shape (elongation) differed from the results obtained by Franco et al. [7] for Sciaenidae fish species at the same sampling site, in which higher concentrations were detected in the more elongated otoliths. The coefficient of correlation adjusted to the surface-associated species was 0.72, which is close to that of the Sciaenidae species (0.69). For the bottom-associated species that share a habitat with Sciaenidae fish, this relationship was weaker (− 0.14). In this way, it can be argued that the concentration of Mn in the sagittae otoliths is independent of the otolith shape in bottom-associated species, except for Sciaenidae species.

The role of otoliths in teleost fish hearing, especially in sound reception, is well known. Many fish species use sound to attract reproductive mates, perform courtship displays, defend territory, and perceive obstacles [42]. Although many fish communicate using sound, Sciaenidae fish are unique in the diversity of their sound mechanisms, as described by Ramcharitar et al. [43]. These fish, known as croakers and drums because of their sonorous characteristics, are probably the most active sound producers among fish, exhibiting wide diversity in the morphology of their sound structures in the form of swim bladders, ears, and otoliths. The sagittae otolith in all Sciaenidae fish is enlarged compared to other fish species, with interspecific variation in their morphology. The biological and ecological importance of hearing sensitivity in Sciaenidae fish can lead to differences in physiological processes related to otolith accretion compared with other fish. These differences can govern the deposition pattern of Mn^{+2} into the otoliths of the $CaCO_3$ matrix and otolith growth, leading to differences in shape.

Thus, while Mn bioavailability in a fish's habitat, especially in food, is a local driving influence of trace element incorporation into the sagittae otoliths, species-specific requirements also have an influence, as previously described in the literature [11–13].

4.2 Comparison between usual spectrometric techniques and quantitative EPR for otolith analysis: constraints and benefits

For more than two decades, otolith microchemistry has been a widely used approach for elucidating life history patterns in fishes [15, 44–46]. It allows for the determination of trace elements in specific sections of the otoliths (core or edge, for instance) or even in each otolith annuli. It also avoids the loss of concentration information for trace elements, averaging the entire fish life history and highlighting differences among larval, juvenile, and adult phases. The most used technique regarding this approach is laser ablation—inductively coupled plasma mass spectrometry (LA-ICP-MS) that involves sectioning and polishing the otolith, destructive sampling, and collecting trace elemental signatures via laser ablation with results validation by certified reference materials (precision of measurement and recovery rates efficiency) (see references above for details).

Recently, a new method of laser ablation was adjusted for otolith microchemistry analysis—depth-profiling laser ablation [47]. The authors argued that this technique offers reduced sample handling and lower contamination risks compared to LA-ICP-MS, which should improve accuracy and reduce costs. However, it requires a more appropriate instrument, including a long-focus short-wavelength laser and modern low-volume ablation chamber,

besides some analytical caveats regarding the samples, which limit the application of the depth-profiling to smaller otoliths.

The advances that the laser ablation technique provides in understand fish life histories are unquestionable. However, the otolith solution methodology to determine the total concentration of trace elements with inductively coupled plasma-optical (atomic) emission spectrometer (ICP-OES) is still used in many studies [26, 48–50]. This technique is a destructive sampling that requires otoliths dissolution using chemical digestion and certified reference materials to validate the results (see references above for details). Regardless, the loss of information on the fish's life phases, the total concentration of trace elements may clarify about preferred habitats, nursery areas, feeding areas and environmental pollution levels with reliable results (see references above for details).

Considering the above arguments, what are the constraints and benefits of the quantitative EPR compared to spectrometric techniques? Unfortunately, quantitative EPR does not have the same sensitivity as the spectrometric techniques to determine the many trace elements that occur in otoliths but only paramagnetic elements, like Mn^{2+}. Additionally, EPR does not allow for Mn determination in specific sections of the otoliths. It is possible using LA-ICP-MS, but only for total concentration measurements, indicating the element accumulation throughout the entire fish life without age or ontogenetic phase distinctions.

Quantitative EPR allows for both relative and absolute concentration measurements of a given paramagnetic element in a sample. In the last measurement, a certified reference material is necessary to validation results in spins/g, which is not the case in relative concentration measurements [19]. Despite lack of accuracy, the relative concentration is a precise measurement, as demonstrated by the specific concentrations of Mn^{+2} in otoliths calculated in the present study and in Franco et al. [7]. Moreover, the relative concentrations of Mn^{2+} in fish otoliths provided answers about the species preferred habitat, allowed for inferences about the main pathway for the incorporation of Mn into fishes' otoliths and corroborated species-specific requirements in otoliths growing. Thus, the understanding of some ecological and biological aspects of fish species from quantitative EPR analysis of their otoliths can be reached by the Mn relative concentration measurements. It should be noted that the costs are reduced because the certified reference material is not necessary.

In conclusion, the EPR is a non-destructive technique, in contrast with spectrometric techniques, which is very useful when the available samples cannot be damaged, as in otolith scientific collections [7]. The technique is not suitable for otoliths less than 4 mm in length (weak resonance signal) for which composite samples are more appropriate. The EPR technique also offers reduced sample handling since it does not require prior sample treatment except for cleaning. Although this technique is not a novel approach to investigate biological systems (see Section 1) [51, 52], its utilization in fish otoliths is still recent but viable and reliable, depending on the questions raised by the study.

Acknowledgements This study was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq (grant n° 301.405/2013-1) and Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro - FAPERJ (grant n° E-26/201.161/2014). The EPR spectrometer was supported by FAPERJ (grant n°. E-26/112.219/2008).

Compliance with ethical standards

Conflict of interest Ana Paula Madeira Di Beneditto declares that she has no conflict of interest. Roberto Weider de Assis Franco declares that he has no conflict of interest.

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