

Preparation techniques alter the mineral and organic fractions of fish otoliths: insights using Raman micro-spectrometry

Aurélié Jolivet · Ronan Fablet · Jean-François Bardeau ·
Hélène de Pontual

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Abstract The high spatial resolution analysis of the mineral and organic composition of otoliths using Raman micro-spectrometry involves rigorous protocols for sample preparation previously established for microchemistry and trace elements analyses. These protocols often include otolith embedding in chemically neutral resin (i.e., resins which do not contain, in detectable concentration, elements usually sought in the otoliths). Such embedding may however induce organic contamination. In this paper, Raman micro-spectrometry reveals the presence of organic contamination onto the surface obtained from the use of epoxy resin, specifically Araldite. This contamination level varies depending on otolith structures. Core and checks, known

as structural discontinuities, exhibit the most important level of contaminations. Our results suggest that otolith embedding with resin affects the organic matrix of the otolith, probably through an infiltration of the resin in the crystalline structure. The interpretation of chemical otolith signatures, especially Raman otolith signatures, and stable isotope analyses should then be revised in light of these results. In this respect, we propose a method for the correction of Raman otolith signatures for contamination effects.

Keywords Epoxy resin · Otolith composition · Microstructure · Raman spectrometry

A. Jolivet · H. de Pontual
Département des Sciences et Technologies Halieutiques,
Ifremer, Z.I. Pointe du diable, CS 10070,
29280 Plouzané, France

A. Jolivet (✉)
Institut Universitaire Européen de la Mer, Laboratoire des Sciences
de L'Environnement Marin (UMR 6539),
Université de Bretagne Occidentale,
Technopôle Brest Iroise, Place Nicolas Copernic,
29280 Plouzané, France
e-mail: Aurelie.Jolivet@univ-brest.fr

R. Fablet
UMR 3192 LabSTICC, Institut Telecom/Telecom Bretagne,
Technopole Brest Iroise, CS83818,
29238 Brest Cedex 03, France

R. Fablet
Université Européenne de Bretagne, 5 Boulevard Laënnec,
35000 Rennes, France

J.-F. Bardeau
Institut des Molécules et Matériaux
du Mans (UMR CNRS 6283), LUNAM Université,
Avenue Olivier Messiaen,
72085 Le Mans Cedex 09, France

Introduction

Otoliths are calcareous concretions in inner ear of fish, consisting of calcium carbonate, mainly in form of aragonite (90–99 % of the total otolith mass) and an organic fraction [1]. Their accretional growth on a circadian rhythm is both physiologically controlled and influenced by environmental conditions. Otolith chemical signatures provide invaluable cues for marine ecology including individual life histories, population structure, larval dispersal, and population connectivity [2, 3]. Because they are metabolically inert (unlikely to be resorbed) and grow throughout the lifespan, otoliths are considered to be highly effective continuous natural markers of the past environmental history of the fish [2]. As previously reported, the chemical otolith composition may reflect modification of both temperature and elemental composition of the ambient water, but in practice, the quantitative analysis of the otolith composition remain a complicated task in part due to technical difficulties and experimental methodologies. The effect of possible chemical contamination has been a cause for concern [4–7], and several protocols based on contamination-free instruments

and environments have been proposed for the different preparation steps, i.e., extraction, cleaning, conservation, handling, resin embedding, and sectioning of the otoliths [8].

Very recently, new insights on the mineral and organic composition of otolith structures have been obtained from fine-scale analysis tools [9–12], especially confocal Raman micro-spectrometry. This technique, which is non-destructive and non-invasive, provides a qualitative and quantitative analysis of mineral and organic compounds [13–15]. This tool was intensively used for the identification of the polymorph of calcium carbonate [10] as well as for the discrimination of the calcium carbonate polymorphs (vaterite, aragonite, and calcite) in different species [16–19]. More recently, owing to its micrometric spatial resolution, Raman micro-spectrometry was used to characterize otolith microstructures [11, 12].

However, to our knowledge, the effects of preparation methods on the quantitative characterization have not been investigated yet. Among the different steps commonly involved in the preparation of otolith thin sections, resin embedding appears as a critical one that might directly affect the elemental composition of the otoliths, as already shown for bones [20, 21]. For microchemical analyses, epoxy resins such as Araldite are preferred as they are chemically inert (i.e., resin which do not contain, in detectable concentration, elements sought in the otoliths) and characterized by a low viscosity ensuring a good maintaining of the otolith during processing. Different requirements (viscosity, polymerization conditions, and organic composition) should however be considered when the focus is given to organic and mineral spectral signatures.

In this study, we evaluated (1) the spatial distribution and level of resin contamination in otolith thin sections and (2) the effects of resin embedding on their fine-scale mineral and organic signatures. Raman micro-spectrometry analyses were carried out to compare non-embedded samples to samples embedded with Araldite. As resin embedding does affect the otolith organic composition and thus modified the Raman spectral features of the otolith, we proposed a numerical method for contamination correction. We also discussed the extent to which previous works dedicated to the characterization of the organic and mineral fractions of the otolith including Raman micro-spectrometry analyses [11] and isotope analyses ([22] and references therein) should be revised in light of these results.

Materials and methods

Otolith preparations

We considered otoliths extracted from European hake (*Merluccius merluccius*, Linnaeus 1758) caught in the Northern Biscay or reared in aquaculture facilities [23] and measuring between 0.42 and 57 cm TL (Table 1).

Once extracted, otoliths were rinsed with MilliQ water (resistivity of $18.2 \text{ M}\Omega \text{ cm}^{-1}$), dried, and weighed. Only left sagittae were considered and thin sections were prepared as follows: (1) embedding in epoxy resin (Araldite 2020, Huntsman), (2) sectioning in transverse or sagittal plane, (3) embedding in epoxy resin on a glass slide previously covered with 1 mm layer of resin, (4) grinding with silicon carbide, and (5) polishing to the core with progressively finer grades of diamond suspensions (3 μm , 1 μm , and 0.25 μm grain size). Sections were ultrasonically cleaned with MilliQ water at the end of each polishing stage.

Otoliths from hake larvae extracted under polarized light and otoliths from fish measuring between 10 and 27 cm were also directly mounted on glass slides without any embedding. An analysis in the sagittal plane was carried out without any further preparation (Table 1).

Our investigations mainly concerned areas of interest on otoliths: (1) the core area, surrounding the primordium which is the initial otolith structure; (2) checks, i.e., structural discontinuities [24] mostly located in translucent zones of hake otoliths; and (3) microstructures, each consisting of a light zone (L-zone) and dark zone (D-zone) which deposition follows a circadian rhythm.

Raman micro-spectrometry

The experimental setting was the same as in Jolivet et al. [11], i.e., a micro-Raman spectrometer (Jobin-Yvon T64000) equipped with a confocal system and a motorized XY stage. The Raman spectra were recorded under a microscope (Olympus BX41) in the backscattering geometry with a $100\times$ objective (with a numerical aperture of 0.95) focusing the 514-nm line from an argon–krypton ion laser (Coherent, Innova) on samples with an estimated spot size of 0.8 μm . Raman measurements were carried out at very low laser power (0.06 mW on sample) to minimize possible sample deterioration. The Raman scattered light was filtered by a holographic Super Notch filter and analyzed by a spectrometer with a single monochromator (600 gratings mm^{-1}) coupled to a nitrogen-cooled CCD detector. Single spectra were recorded twice in the wavenumber region $100\text{--}5,000 \text{ cm}^{-1}$, with exposure times varying from 30 to 300 s depending on the sample. The depth of analysis was systematically set to 2 μm below the surface to minimize possible contamination due to the preparation process.

Analysis of organic and mineral fraction on Raman spectra

Organic and mineral vibrational signatures were extracted from Raman spectra following the protocol previously described [11]: (1) a data normalization was applied with respect to a reference acquisition setup of 50 s with a laser output power of 50 mW, and (2) the integrated intensities of

Table 1 Otoliths (sagittae) analyzed by Raman micro-spectrometry to evaluate resin embedding effects on vibrational spectra: fish origin, size (total length TL in cm), thickness of otolith sections (in μm), type of otolith section, preparation method with or without resin embedding, mean resin contamination measured on CH signatures (in %, see text) calculated on all spectra of each sample, and number (*N*) of spectra acquired on each otolith

Name	Origin	TL	Thickness	Section	Resin embedding	Contamination	<i>N</i>
Analysis of core							
S1	Reared	25	150	Transversal	Yes	100	1
S2	Wild	33	394	Transversal	Yes	90	1
S3	Wild	20	325	Transversal	Yes	84	1
S4	Reared	0.42		Sagittal	No	ND	1
Analysis of check							
S5	Wild	36	120	Transversal	Yes	80 \pm 4	2
S6	Reared	50	248	Transversal	Yes	83 \pm 12.5	2
S7	Reared	57	243	Transversal	Yes	74 \pm 8	2
Analysis of microstructures							
S1	Reared	25	150	Transversal	Yes	13 \pm 14	10
S5	Wild	36	120	Transversal	Yes	16 \pm 10	138
S8	Wild	21	90	Sagittal	Yes	65 \pm 12	64
S9	Wild	34	299	Sagittal	Yes	36 \pm 21	171
S10	Reared	40	210	Transversal	Yes	83 \pm 21	9
S11	Wild	28	210	Transversal	Yes	26 \pm 12	347
S12	Wild	35	90	Transversal	Yes	50 \pm 14	251
S13	Wild	42	162	Transversal	Yes	43 \pm 20	405
S14	Wild	40	204	Transversal	Yes	35 \pm 16	92
S15	Wild	30	221	Transversal	Yes	10 \pm 6	6
S16	Wild	33	418	Sagittal	Yes	57 \pm 14	20
S17	Wild	23	172	Transversal	Yes	26 \pm 16	22
S18	Wild	26	102	Transversal	Yes	38 \pm 10	13
S19	Wild	42	216	Transversal	Yes	2 \pm 5	40
S20	Wild	20	116	Transversal	Yes	3 \pm 5	4
S21	Wild	21	256	Transversal	Yes	9 \pm 8	2
S22	Wild	30	136	Transversal	Yes	2 \pm 6	10
S23	Wild	20	111	Transversal	Yes	7 \pm 8	8
S24	Wild	22	110	Transversal	Yes	3 \pm 6	76
S25	Wild	20	475	Transversal	Yes	51 \pm 14	54
S26	Wild	26		Sagittal	No	ND	3
S27	Wild	27		Sagittal	No	ND	3
S28	Wild	22		Sagittal	No	ND	4
S29	Wild	21		Sagittal	No	ND	3
S30	Wild	10.5		Sagittal	No	ND	150

ND not detectable

the observed vibrational bands were extracted using the LabSpec® software v.3.01 (Jobin-Yvon Horiba). With respect to Raman spectra of fish otoliths, the mineral fraction was characterized by 14 aragonite vibrational bands [14] and the organic fraction by 10 signatures identified on the spectra of the core, known to be a particularly organic-rich area (Table 2) [11, 12].

Contamination-free Raman signatures

The position and intensity of bands on Raman spectrum relate to the chemical composition and structure of the analyzed samples. Micrometric spatial resolution of Raman

spectroscopy combined with a confocal analysis makes this technique ideal for the detection of contamination agents on surfaces especially for low concentrated component analysis and chemical species inclusions. If known, the signatures of specific compounds such as resin or chemical markers (e.g., oxytetracycline used for age validation studies) can therefore, in theory, be revealed and identified in a Raman spectrum.

Araldite, as all epoxy resins, is a polymer formed from reaction between an epoxide resin (Fig. 1a, component A) and a polyamine hardener (Fig. 1b, component B). The Raman spectrum of Araldite (Fig. 1b) presents specific signatures at 824, 1,607, 3,070, and between 1,200 and 1,300 cm^{-1} (not observed in non-embedded otoliths), which were considered

Table 2 Raman shifts and assignments of the Raman vibrational bands corresponding to the organic fraction of the core of sample S4 (resin-free sample), sample S2 (embedded sample), and Araldite (they are compared to the results reported by Zhang et al. [12] on small yellow croaker otoliths)

Raman shifts (cm ⁻¹)				Band attribution [12, 43–46]
Zhang [12]	Sample S4	Sample S2	Araldite	
765	771	771	771	Trp, amide IV, V
830		824	824	v-ring, Tyr, Ac Asp
853	856	856		δ(CCH) ring, Trp, Val, Hyp, Tyr
880	870	870		δ(CC) ring, Trp, Val, Hyp
		894	894	v(C–C)
		916	916	v(C–C) of Pro ring
		936	936	v(C–C) of protein backbone
940	950	950		Non-aromatic v(C–C), Lys, Val, Leu
1,003	1,005			C–C aromatic ring stretching, Phe
1,031	1,031			v(C–C) aromatic rings, Phe, Pro, Tyr, Thr
		1,185	1,185	Tyr
		1,229	1,229	Amide III
		1,254	1,254	d(NH ₂), amide III
1,272	1,275	1,275		d(NH ₂), amide III
1,297	1,297	1,297	1,297	δ(C=H), phospholipids
	1,377	1,377		CH ₂ wag
		1,387	1,387	CH ₂ wag
1,443	1,440	1,444		CH ₂ , CH, CH ₃ bending
1,461	1,461	1,461	1,457	CH ₂ wag, aragonite
1,555				v(C=C), Trp
		1,581	1,581	Trp v-ring, Pro, Hyp
	1,605	1,610	1,610	Trp, Phe, Tyr v-ring
1,660	1,665	1,665		v(C=O) amide I
	1,710	1,710		
2,852	2,855			v(CH ₂) sym lipids
		2,873	2,873	
2,882	2,888			v(CH ₂) asym lipids, v(CH ₂) sym proteins
		2,925	2,925	v(CH ₃) sym proteins and lipids,
2942	2939			v(CH ₂) asym proteins
		3,070	3,070	CH stretch
	3,082			

in the subsequent analysis to reveal and evaluate the contamination level of local otolith signatures due to resin embedding (identified by the gray area in Fig. 1b).

When the recorded Raman spectra present the specific signatures of the Araldite, corrected resin-free spectra was computed as follows: we first estimated the Araldite response from the intensity of the observed band at 3,070 cm⁻¹ and then removed the contribution of Araldite spectrum from the initial recorded Raman spectrum. The resin contamination was quantified from the integrated intensity of CH stretching band centered at 2,950 cm⁻¹ (I_{CH}) measured before and after correction and expressed as a percentage of contamination (P_C in %):

$$P_C = (I_{CH\text{-before}} - I_{CH\text{-after}}) / (I_{CH\text{-before}}) \quad (1)$$

Otherwise, we measured, on the corrected spectrum, the ratio between the CH stretching band and the strongest peak

of aragonite at 1,085 cm⁻¹ as a proxy of the ratio between the organic and mineral fractions of the otolith structures.

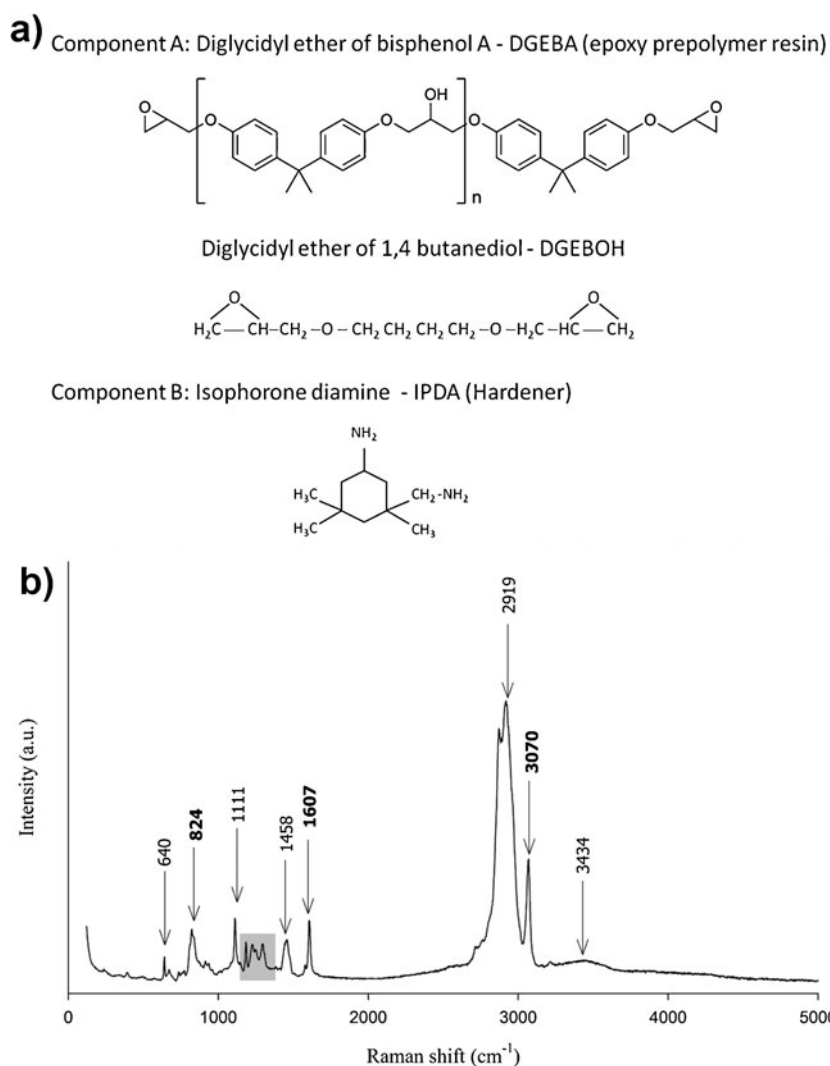
Results

Resin embedding contaminations

Global analysis

Raman experiments clearly evidenced the presence of resin in otoliths even after ultrasonic cleaning. The contamination was quantitatively evaluated by the percentage of the CH stretching bands assigned to the resin. Indeed, among the 1,905 spectra obtained on prepared otoliths (i.e., including

Fig. 1 **a** Molecular structure of the epoxy resin Araldite 2020 with its two compounds: the resin and the hardener (reported in [47]). **b** Raman spectra of the Araldite 2020 with the peak positions (in *bold*) used to detect the contamination and the Raman shift zone (cm^{-1}) (in the *gray area*) used to analyze the spatial distribution of the contamination



embedding step), 89 % of spectra show a contamination level of otolith greater than 10 %.

By comparing the corrected spectra to the initial one, it was found that contamination can affect the observed spectral data with intensities variation ranging from 0 to 50 % depending on the band frequency (Table 3). These results illustrate that the contamination is mainly restricted to few mineral and organic otolith signatures.

Core

The comparison between the spectra obtained on the core of samples S2 and S4 and the Araldite signature depicts a strong correspondence of the organic signatures of the resin and the core of the embedded sample S2 (Fig. 2). Only the baseline and the two bands located at $1,710$ and $1,810 \text{ cm}^{-1}$ did not depict resin contamination (Fig. 2a). Several organic compounds of the otoliths core were clearly occluded by resin contamination (Fig. 2b). The Raman vibrational bands relative to Araldite and organic fraction of core on samples

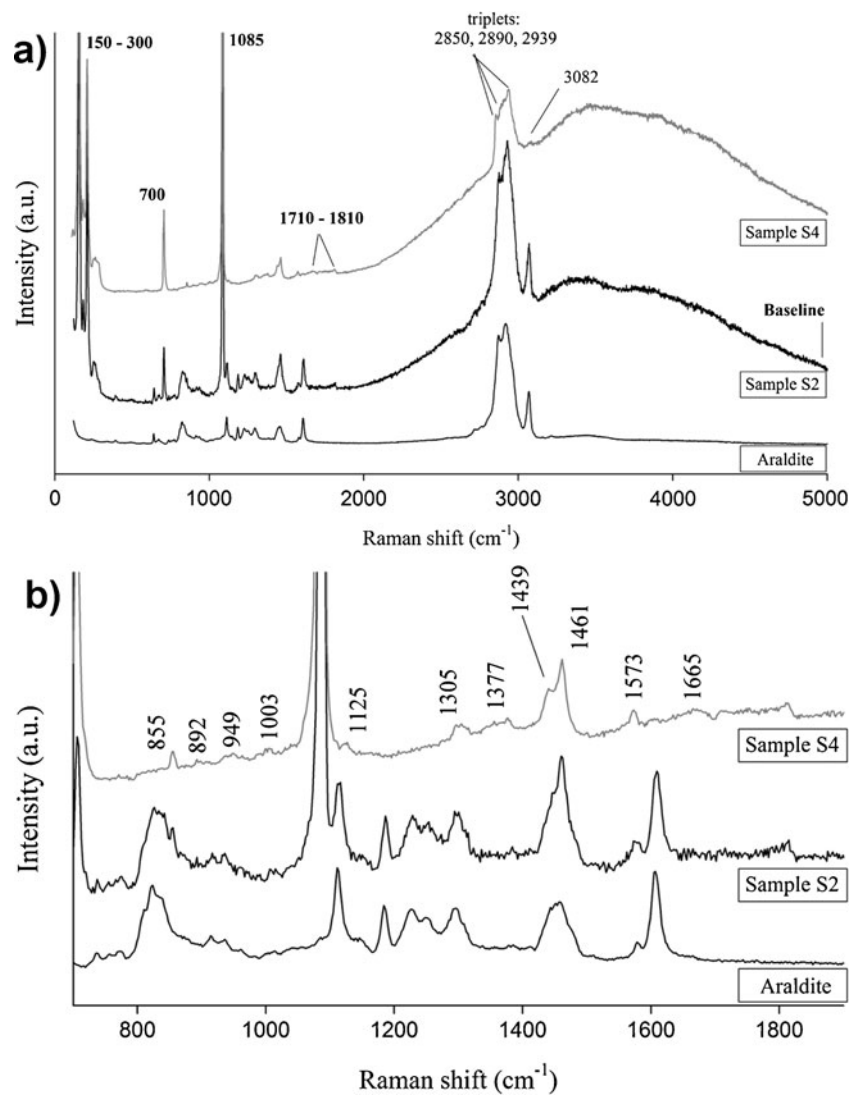
S2 and S4 are detailed in Table 2 with respect to those reported by Zhang et al. [12] for the core of the small yellow croaker otoliths.

The core appeared as a zone highly affected by resin embedding as the resin accounted for 80 % to 100 % of

Table 3 Intensity variations (in %) observed for the different aragonite (AR) and organic (OM) signatures on Raman spectra for a contamination level of 50 %

Raman shifts range (cm^{-1})	Assignments	Intensity variation (%)
Group 150	AR	0
Group 700	AR	0
855	OM	31.2
1,085	AR	0.6
1,461	AR/OM	13.4
1,571	AR/OM	4.6
Group 1,700	OM	0
2,950	OM	50
3,399	OM	2.6

Fig. 2 **a** Comparison between the spectra acquired on the core of sample S2 (contaminated), sample S4 (non-embedded), and the Araldite signature, with **b** a focus on the 700–1,900 cm^{-1} region. Peaks indicated in **bold** correspond to region non-impacted by the resin contamination. The other pointed peaks correspond to the organic signatures relative to the otolith composition and masked by the resin contamination



the Raman CH signature (Table 1). The spatial pattern of the Araldite contamination was characterized from a 2D mapping of the core of sample S2 (Fig. 3; 30 $\mu\text{m} \times 40 \mu\text{m}$ mapping with step fixed to 3 μm and 2.5 μm in the X and Y directions, respectively). The highest contamination was observed on the core and much less along the anti-sulcus, dorsal, and ventral directions. In contrast, the sulcus area depicted large contamination levels (Fig. 3).

Checks

Checks depicted Raman signatures similar to those observed in the core zone. As illustrated in Fig. 4a, compared to the surrounding points, checks depicted lower aragonite signatures except for the bands at 1,462 and 1,571 cm^{-1} and greater organic compounds signatures except for the peak at 1,710 cm^{-1} (Fig. 4a). It is worth noting that, contrary to the core, the signature at 3,399 cm^{-1} and the baseline did not vary between check and the adjacent points.

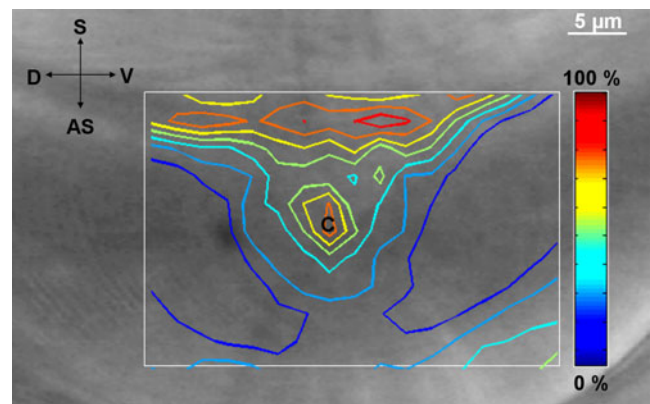
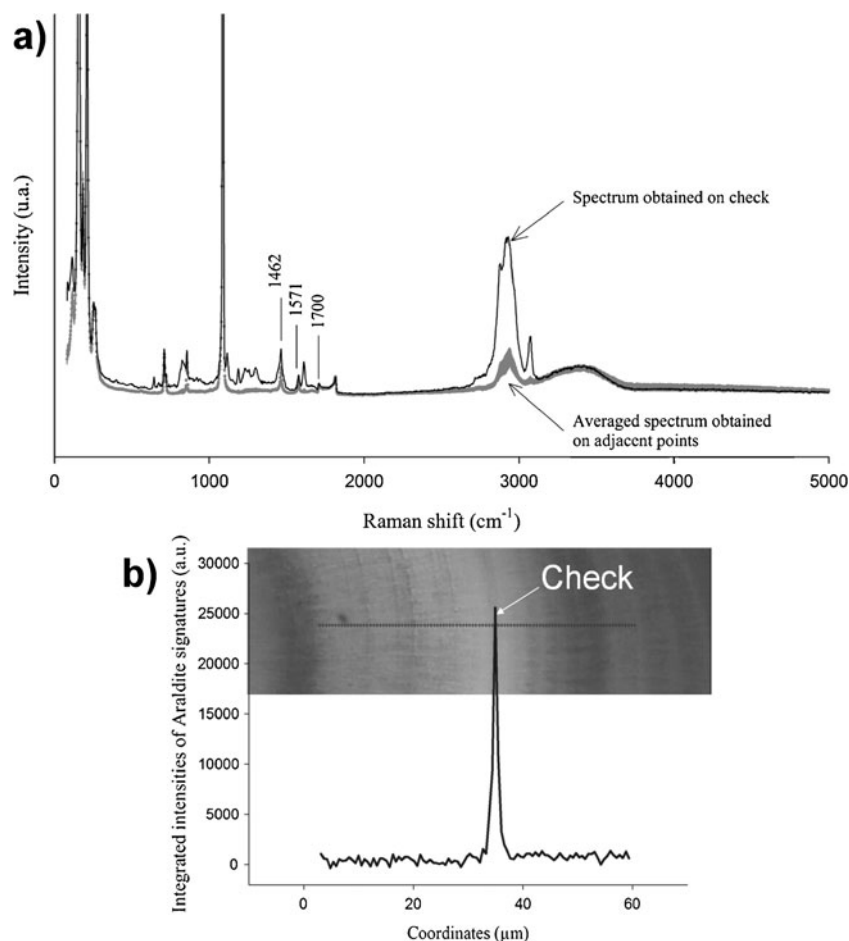


Fig. 3 Superposition of the view of S2 thin section under light microscopy (scale bar = 5 μm) and the 2D mapping (30 $\mu\text{m} \times 40 \mu\text{m}$) centered on the core with step increments fixed to 3 μm and 2.5 μm in the X and Y directions, respectively. The mapping shows the distribution of the percentage of Araldite contamination (D dorsal, V ventral, S sulcus, AS anti-sulcus, C core)

Fig. 4 Analysis of a transverse section of sample S5 presenting a check: **a** comparison of Raman spectra in the 100–5,000 cm^{-1} region for check (in *black*) and the average of the five adjacent points acquired every 1.2 μm on both sides of the check (in *gray*). The spectra thickness of the *gray line* corresponds to the standard deviation measured on the adjacent points. Pointed peaks correspond to region non-impacted by the resin contamination. **b** Distribution of the contamination determined from the integrated intensities of the bands characteristics of the Araldite resin located between 1,100 and 1,300 cm^{-1} characteristics of the Araldite resin



Checks also involved high contamination levels since the resin accounted for 60 % to 92 % of the Raman CH stretching bands (Table 1, Fig. 4a). This contamination effect was particularly concentrated as stressed by the measure of integrated intensities of Araldite signatures along a transverse section of sample S5 (Fig. 4b).

Otolith microstructures

The effects of resin embedding were analyzed on two sagittal sections embedded in Araldite (samples S8 and S9). Different levels of contamination were observed in spectra (Fig. 5a) varying from 35 % to 91 % for S8 and from 0 to 47 % for S9. The analysis along a transect covering several L- and D-zones of sample S8 showed that L-zones were significantly more affected than D-zones (*T* test, *df*=24, *P*<0.001, Fig. 5b).

Contamination correction

Core and checks

The resin-free Raman spectra were computed for the core and check zones. Regarding the core, the correction leads to

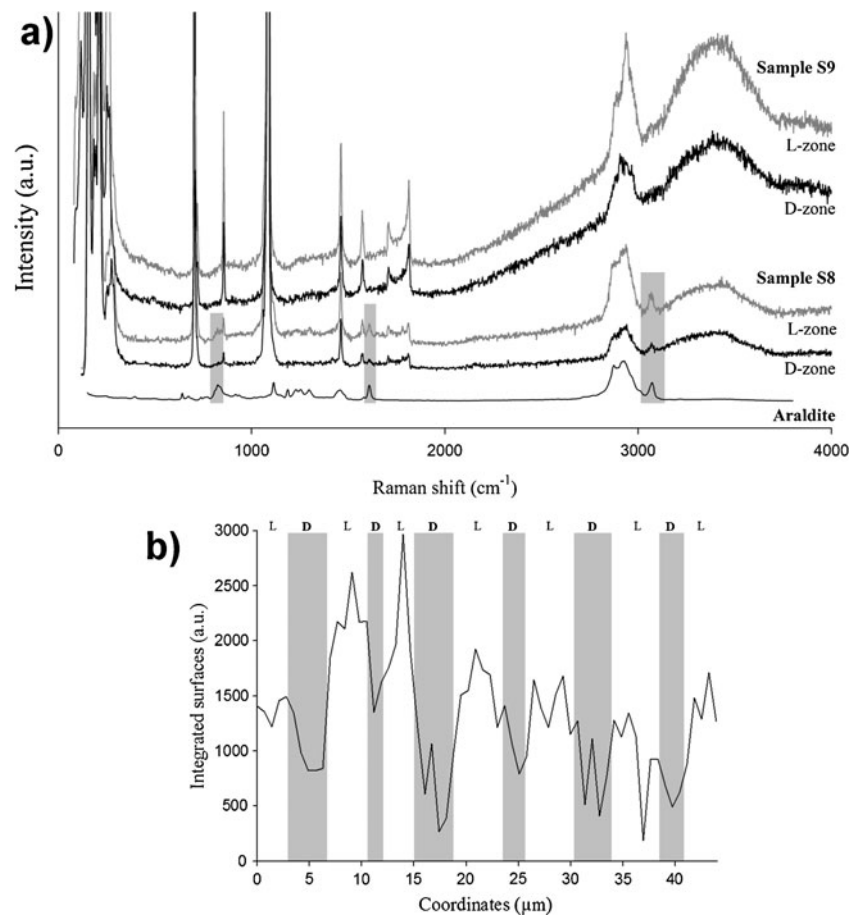
CH/AR intensity ratios lower than the one measured on the non-embedded core (Fig. 6a).

Regarding the checks zones, the resin-free Raman spectra depicted lower variability compared to non-corrected spectra ($\text{CH/AR}_{\text{corrected}}$ ranged from 0.22 to 0.50 vs. $\text{CH/AR}_{\text{contaminated}}$ ranged from 0.85 to 3.6). However, the corrected CH/AR intensity ratio remained significantly greater than those of otolith microstructures (*T* test, *df*=97, *P*<0.001, Fig. 6b).

Otolith microstructures

We analyzed the proposed resin-free otolith Raman spectra by comparing two groups of samples. The first group comprised 20 embedded samples (Table 1, S1, S5, S8–S25, 1,742 spectra) acquired in translucent and opaque zones and depicted 0 to 100 % contamination levels. The second group consisted of five non-embedded otoliths (Table 1, S26–S30, 163 spectra). The analysis showed a lower variability of CH/AR intensity ratios observed for contaminated samples (Fig. 7a). The corrected CH/AR intensity ratios were in the range of variations of the values obtained from non-contaminated spectra (Fig. 7b). For contamination

Fig. 5 **a** Comparison between Araldite spectrum and Raman spectra obtained on L-zones (gray) and D-zones (dark) of sample S8 (highly contaminated) and S9 (less contaminated). Specific bands corresponding to Araldite contamination are marked by gray areas. **b** Distribution of the contamination calculated from the Raman integrated intensities of the bands located between 1,100 and 1,300 cm^{-1} along a transect on S8 covering successive L- and D-zones. D-zones (D) are marked by gray areas



levels greater than 70 % of the CH signatures, a non-negligible number of corrected CH/AR intensity ratios (>5 % of analyzed samples) are below the minimum ratio observed on non-contaminated samples (values highlighted by the gray area on Fig. 7b).

Discussion

To our knowledge, the contamination issues on otolith signatures (and more broadly the signatures of other biominerals) due to resin embedding have already been addressed and were dealt with chemically neutral resin for microchemical analyses focusing on minor or trace elements [8]. As stressed here, it may, however, be questioned whether or not resin embedding modified the mineral and/or organic signatures of otolith thin sections previously reported in literature [11, 12]. Embedding is generally an exothermic reaction between both organic components (the resin and the hardener) which may affect otolith organic compounds as well as the aragonitic crystalline structure.

Contaminations due to resin embedding were here clearly evidenced from the Raman micro-spectrometry characterization of non-embedded and resin-embedded samples. The

comparison to the reference Raman spectrum of the resin provided a quantitative evaluation of contamination levels.

Spatial distribution and level of resin contamination in otolith thin sections

The surface contaminations were proven spatially inhomogeneous, and it may be suggested that the contaminations is correlated to the spatial distribution of the organic matrix of the otolith. Greater contamination levels were observed in the core (more than 80 %) and in structural discontinuities, such as checks (more than 60 %), known to be organic-rich structures [25, 26]. The contamination levels of otolith microstructures depicted greater variability ranging from 0 to 100 %. For the six organic signatures considered, the greater levels are depicted in L-zones, as shown by Jolivet et al. [11], as well as greater contamination levels than in D-zones. For the core and the checks, it might be argued that the greater contamination levels are due to less dense crystalline structure [11, 26, 27]. However, this statement does not hold for L- and D-zones, as L-zones depicted greater Raman signatures both for the organic and mineral fractions [11]. Differences in the crystalline organization of L- and D-zones [28] might explain the differences in the observed

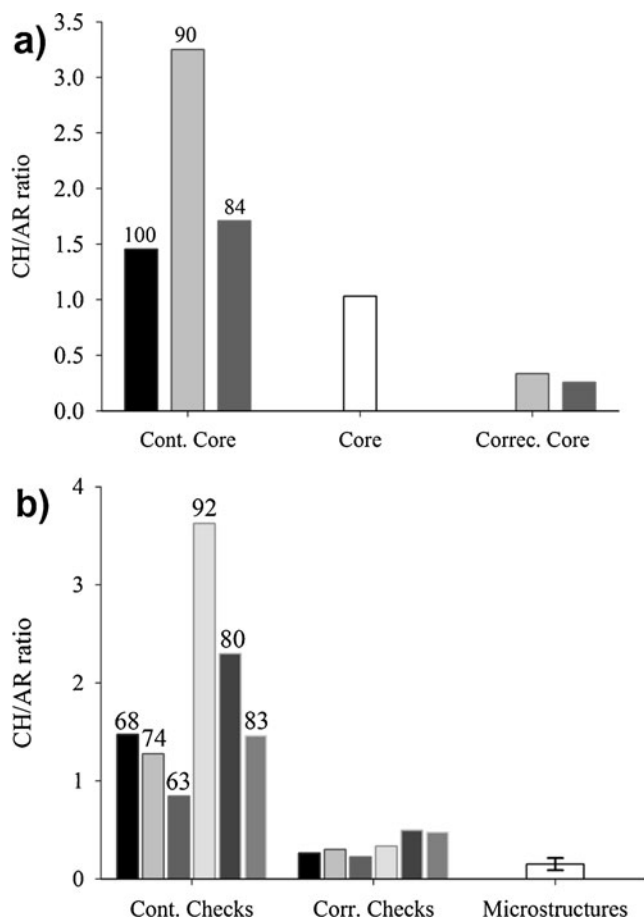


Fig. 6 Evaluation of the contamination effect on the intensity ratio between CH signature (at $2,950\text{ cm}^{-1}$) and the aragonite reference (at $1,085\text{ cm}^{-1}$) for the core (a) and the check (b) zones by comparison between contaminated data, corrected data, and non-contaminated data. Numbers correspond to the percentage of contamination

resin embedding effects and might be confirmed by coupling Raman micro-spectrometry with a micro-scale analysis of the crystalline structure by atomic force microscopy [16, 29] and scanning electron microscope.

A post-processing procedure is thus proposed to generate resin-free Raman spectra. Based on the presence of specific signatures of the resin, in particular peaks between $1,200$ and $1,300\text{ cm}^{-1}$, Raman spectra were corrected from the estimated resin contribution and the contamination level was evaluated as the relative contribution of the resin to the CH Raman signature. The direct validation of this procedure is not feasible as it would require analyzing the same microstructures prior and after resin embedding. We thus investigated the comparison between the distribution of corrected CH/AR intensity ratios of resin-embedded samples to those of non-embedded samples. It should be stressed that CH/AR intensity ratios are highly variable across samples, microstructures (L- vs. D-zones), and macrostructures (translucent vs. opaque zones). Overall, the higher the contamination level, the lower the corrected CH/AR intensity ratios.

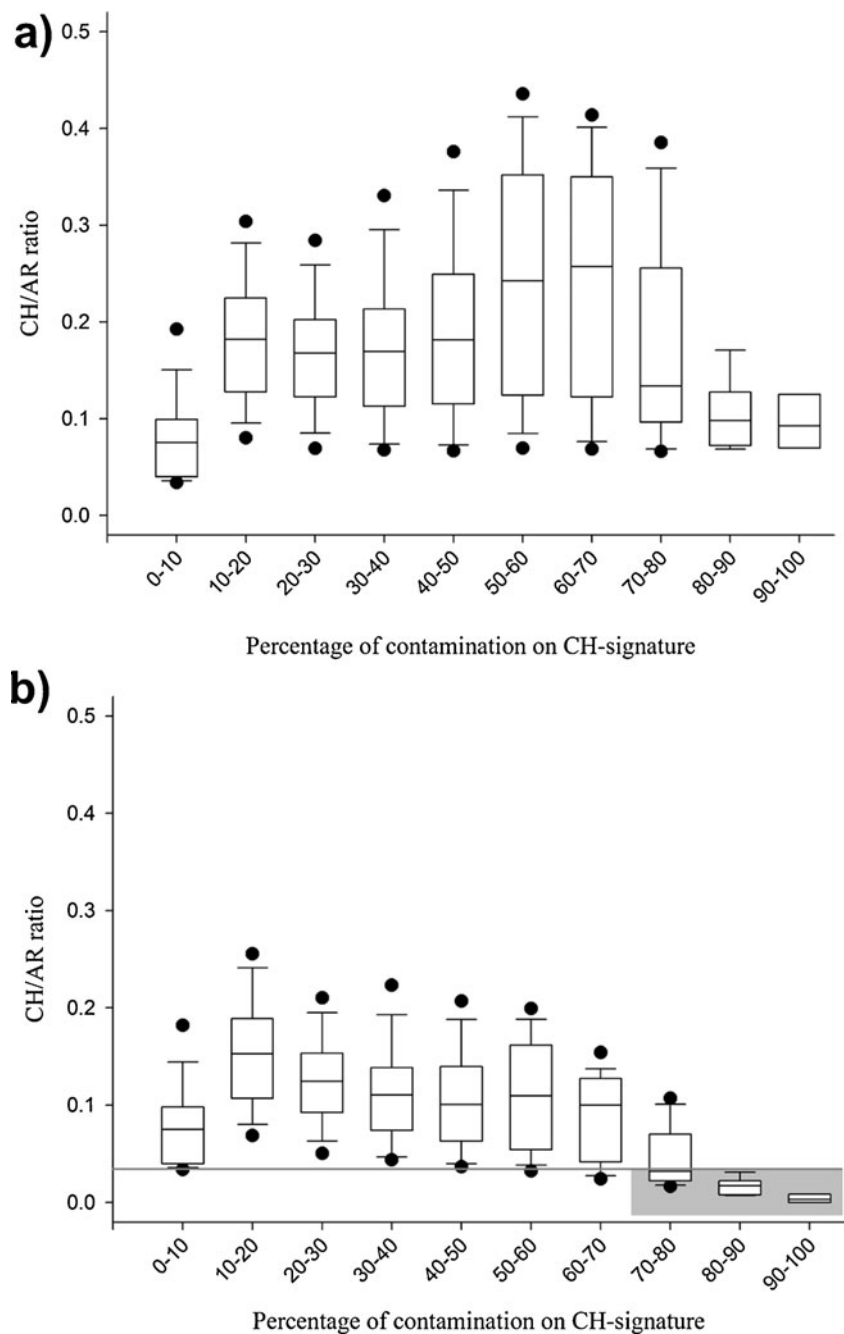
This suggests an infiltration of the resin in the crystalline structure of the otolith and a possible modification of the characteristics of the organic matrix as a result of the exothermic polymerization of the resin. Overall, for contamination levels above 70 %, the Raman signatures were shown to be altered as corrected CH/AR intensity ratios were mainly inferior to those measured on non-embedded samples.

Consequences regarding previous interpretations of Raman otolith signatures

Given the exhibited effects of resin embedding on the Raman signatures of organic and mineral fraction of the otoliths, one may question the conclusions drawn in previous works [11, 12, 30, 31]. The samples considered in Jolivet et al. [11] were part of the current study. Eighty-nine percent of these samples involved contamination levels greater than 10 %. However, more than 78 % of the sampled points were associated with a contamination level below 70 %, and the key contributions drawn in Jolivet et al. [11] were confirmed: (1) the core is a particular point with a strong concentration in organic compounds, (2) L-zones depict stronger concentrations in mineral and organic fractions than D-zones, and (3) the relative proportion of CH versus AR is greater in L-zones than in D-zones. However, we cannot verify the Raman features of the checks on non-embedded samples given the systematic high level of contamination of these areas (ranged from 64 % to 92 %). Checks were described as depicting lower aragonite fractions and greater organic signatures similarly to the core [25, 32, 33]. The agreement between the core and check signatures for resin-embedded sample supports this statement.

The contamination occurred at the otolith–resin interface particularly when fixing the otolith section ($850\text{ }\mu\text{m}$ thickness) onto the glass slide. The section was embedded between two layers of resin, one between the otolith and the glass slide, necessary whenever the samples should be turned over to reach the core plane, and another one above the otolith. The latter was removed during grinding to obtain $100\text{ }\mu\text{m}$ thickness sections for microstructure analysis, however, at the expense of a resin-embedding contamination of otolith physico-chemical signatures. This was particularly stressed for sample S8 which depicted a high contamination level. In contrast, thicker sections, such as sample S9 with a $299\text{ }\mu\text{m}$ thickness, depicted lower contamination levels. Obviously, the analysis of non-embedded samples should be preferred when possible and relevant. The preparation of otolith sections is however a prerequisite in most cases to proceed to a fine-scale analysis of otolith structures. Two main guidelines may be proposed in this context: (1) resins with a rapid and endothermic polymerization should be preferred, and (2) the resin layer inserted between the sample and the glass slide should be avoided whenever possible.

Fig. 7 Distribution of contaminated (a) and corrected (b) values of CH/AR intensity ratio with respect to the percentage of contamination measured from CH signatures acquired on microstructures. A box plot representation was used, with the boundary for the 25th and 75th percentiles, the line within the box for the median, the whiskers (error bars) above and below the box for the 90th and 10th percentiles, and the black points for the 5th and 95th percentiles. Corrected CH/AR intensity ratios inferior to the minimum ratio observed on non-contaminated samples (horizontal gray line) are marked in the gray area



Consequences for the interpretation of chemical otolith signatures

The impact of resin embedding on the physico-chemical signatures of fish otoliths was shown here to depend on various factors: the preparation protocol, and the thickness of the samples and the analyzed zones. In all cases, however, one should carefully consider potential resin embedding effects in the characterization of otolith thin sections, especially when the structures of interest may depict high contamination levels, for instance the core, checks, as well as the edge of the otolith. This recommendation obviously

includes studies of the organic fraction of the otolith and also the analysis of isotopic signatures of chemical elements such as carbon, oxygen, and nitrogen as well as chemical elements which bind to proteins synthesized in the endolymph such as strontium, copper, and zinc.

The reconstruction of life history traits from carbon and oxygen isotope signatures typically involves micromilling resin-embedded sections along transects from the core to the edge [34–37]. Under this protocol, one should be cautious with drilling effects that may resort the transformation of aragonite into calcite under heating conditions [38]. To our knowledge, resin contamination effects have been widely

overlooked. For instance, the core typically depicted carbon isotopic ($\delta^{13}\text{C}$) values significantly lower than the edge of the otolith, generally interpreted as a metabolism-induced signature ([22] and references therein). Our results may suggest other interpretations as the core was characterized by high contamination level (greater than 80 %) and the resin depicts low $\delta^{13}\text{C}$ values (typically, -28.05 ‰ for epoxy resin, [39]). One may then question whether or not low $\delta^{13}\text{C}$ values resulted from resin contamination. This hypothesis is supported by studies reported on other biominerals. In an experimental analysis of $\delta^{13}\text{C}$ signatures of resin-embedded and non-embedded beluga teeth, Stewart et al. [39] have shown that the resin accounted for 5 % to 30 % of the $\delta^{13}\text{C}$ signature of the embedded samples. Our results strongly suggest undertaking new experiments to perform a quantitative evaluation of resin embedding effects on otolith isotopic signatures and subsequently on the reconstruction of individual life traits.

Resin contamination should also be considered with care when the focus is given to chemical elements, such as strontium or other metals, which bind to proteins of the organic matrix of the otolith. Such metal–protein interactions were reported to account for significant metal fractions in the otoliths: for instance, copper and zinc in the EDTA-soluble fraction of the organic matrix of cod otoliths accounted respectively for 70 % to 100 % and 40 % to 60 % of the total mass of copper and zinc found in whole otoliths [40]. The analysis of the concentration of such chemical elements along transects on otolith sections are of great interest to reconstruct, for instance, migrations patterns from environmental variability [2, 41, 42]. They often depict high inter- and intra-individual variability. As suggested here, this variability may not only reveal environmental and metabolic conditions but also resin embedding effects on the organic matrix of the otolith and associated chemical elements. Raman micro-spectrometry appears as a powerful non-destructive technique; these questions could be addressed by coupling this technique to a laser ablation inductively coupled plasma mass spectrometry analyses.

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