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Raman investigation of the pigment families in recent and fossil brachiopod shells

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ABSTRACT

Shells of the three subphyla of extant and extinct representatives of the phylum Brachiopoda display coloured patterns with diverse shapes and at different degrees. These colourations are readily visible in natural light but are best revealed under UV light for the fossils concerned. To identify these pigments, Raman spectroscopy has been used for the first time on brachiopod shells. The widespread identified pigments belong to the carotenoid family, best represented in all the animal kingdom, the second one concerns the melanin/melanin-like pigments and, surprisingly, additional molecules of the cytochrome family are revealed for the first time in one of the brachiopod shells studied. The putative functions of shell pigmentation, still under debate, are discussed.

1. Introduction

Brachiopods are marine benthic invertebrates, with a bivalved shell, present since the Lower Cambrian. Many extant species display a shell colouration, dispersed uniformly or present as patterns. Specimens of the three subphyla of the phylum Brachiopoda (Linguliformea, Craniiformea, Rhynchonelliformea) reveal this feature. The colouration is directly visible in natural light on shells of recent species. In the fossil world, colouration is more difficult to observe. The first descriptions of preserved colouration in fossil brachiopods date back to the middle of the 19th century [1–3]. The most ancient fossil brachiopods where residual colour patterns have been described go back to the Palaeozoic: the Ordovician [4], the Silurian [5], the Devonian (see [6,7] for partial reviews; see also [5,8,9]), the Carboniferous [2,6,7,10–12] and the Permian [7,8,13].

Shell colourations have also been observed on Mesozoic species: from the Triassic [14–19], the Jurassic [16] and the Cretaceous [16,18,19]. Finally, fossils from the Cenozoic also exhibited preserved colour patterns [16,20].

Whatever the fossil observed, the colouration under natural light revealed faded, even rub out. Similar observations emerged from mollusc shell analyses [21]. Nevertheless, Simpson [22] and, independently, Wagner [23] discovered the fluorescence ability of fossils and remains of vertebrates when excited by ultraviolet rays. This technique was then and is still commonly used for shell colouration studies on molluscs (see for example [24–26]). Regarding the brachiopods, this technique was used only very recently with similar results [19].

The next step of analysis was to tentatively identify the molecules responsible for these colourations. Raman spectroscopy was used for the first time in 1981 to allow identifying carotenoid pigments in a coral exoskeleton [27]. This technique was applied more recently on mollusc shells in a non-destructive manner [28,29]. On brachiopods, very little experiments have been performed to identify shell pigments. By using TLC (Thin Layer Chromatography) and mass spectrometry, Cusack et al. identified two carotenoids (canthaxanthin and astaxanthin) from the red shell of recent *Terebratella sanguinea* specimens (Rhynchonelliform) [30].

The aim of this work was to analyse the pigments involved in the colouration of the brachiopod shells from the three subphyla, on recent as well as on fossil species. Used for the first time on brachiopods, Raman spectroscopy has evidenced three families of pigments. Putative functions of shell pigmentation in brachiopods are discussed.

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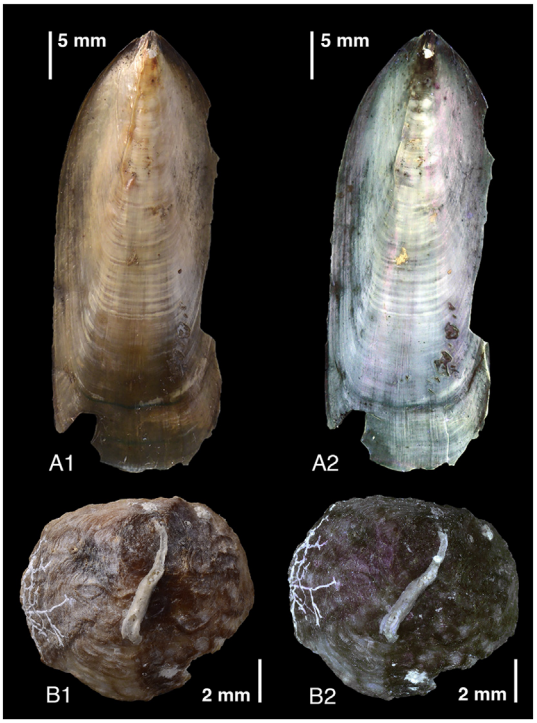

Fig. 1. A. *Lingula anatina* Lamarck, 1801 (Linguliformea Lingulidae). B. *Novocrania anomala* (Müller, 1776) (Craniiformea Craniidae). A1, B1: natural light pictures. A2, B2. UV light pictures emphasizing the growth lines and coloured radial patterns. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

2. Material and Methods

2.1. Brachiopod Specimens Analysed

Our work is based on the analysis of both extant (recent) and extinct (fossil) brachiopod species from different locations and stratigraphic ages. The different specimens analysed come from: the Zoothèque and Palaeontological collections of the Muséum National d'Histoire Naturelle (MNHN, Paris, France), the collections of Le Musée Vert, Musée d'Histoire Naturelle du Mans (MHNLM, Le Mans, France), the collections of Sorbonne Université (Paris, France) for the Triassic species, courtesy of members of the French Working Group on the Cretaceous (GFC) (University of Rennes, France), courtesy of brachiopod specialists from New-Zealand, D. Gaspard personal collections.

The extant species studied were:

- In the Linguliformea: *Lingula anatina* Lamarck, 1801 (Lingulidae), from the Japan coasts (MNHN Coll., n° MNHN.F.A69103).
- In the Craniiformea: *Novocrania anomala* Müller, 1776 (Craniidae), sampled from Gorringe bank (DW 37; 255–370 m), during the Seamount1 cruise (1987) (MNHN Coll., n° MNHN-IB-2014).
- In the Rhynchonelliformea: - *Notosaria nigricans* (Sowerby, 1846) (Notosariidae), from the New Zealand coasts (Tikoraki Point, 20 m depth) (MNHN Coll., n° MNHN.F.A69923), - *Neothyris lenticularis* (Deshayes, 1839) (Terebratellidae), from New-Zealand (Paterson Inlet, Steward Island, 15–25 m depth), (MNHN Coll., n° MNHN.F.A69930), - *Calloria inconspicua* (Sowerby, 1846) (Terebratellidae) from New Zealand (Tikoraki Point and Cape Wanbrow-Boatman Harbour at low-tide), (MNHN Coll., n° MNHN.F.A69929), - *Terebratella sanguinea* Leach, 1814 (Terebratellidae), from New Zealand (Doubtful Sound, 20 m depth), (MNHN Coll., n° MNHN.F.A69927), - *Terebratella haurakiensis* Allan, 1931 (Terebratellidae), from New-Zealand (Hauraki Gulf, 30–146 m depth), (MNHN Coll., MNHN.F.A69928), - *Frenulina sanguinolenta* (Gmelin, 1791) (Frenulinidae), from the Coral Sea (Chesterfield-Bellon Plate, 80 m depth), (MNHN Coll., n° MNHN.F.A60924) - *Dallinella occidentalis* (Dall, 1871) (Terebrataliidae), from Pacific Ocean (Californian coasts 100–205 m depth), (MNHN Coll., n° MNHN.F.A69925), - *Argyrotheca rubrocostata* Cooper, 1977 (Megathyrididae) from Caribbean Sea (26–55 m depth), (MNHN Coll., n° MNHN.F.A69926).

The extinct species studied were:

- In the Linguliformea: - *Lingulepis pinnaformis* Owen, 1852 (Obolidae), from St Croix Falls (Minnesota/Wisconsin, USA), (Mid-Upper Cambrian, ~500 My) (MNHN Coll., n° MNHN.F.A68135), -

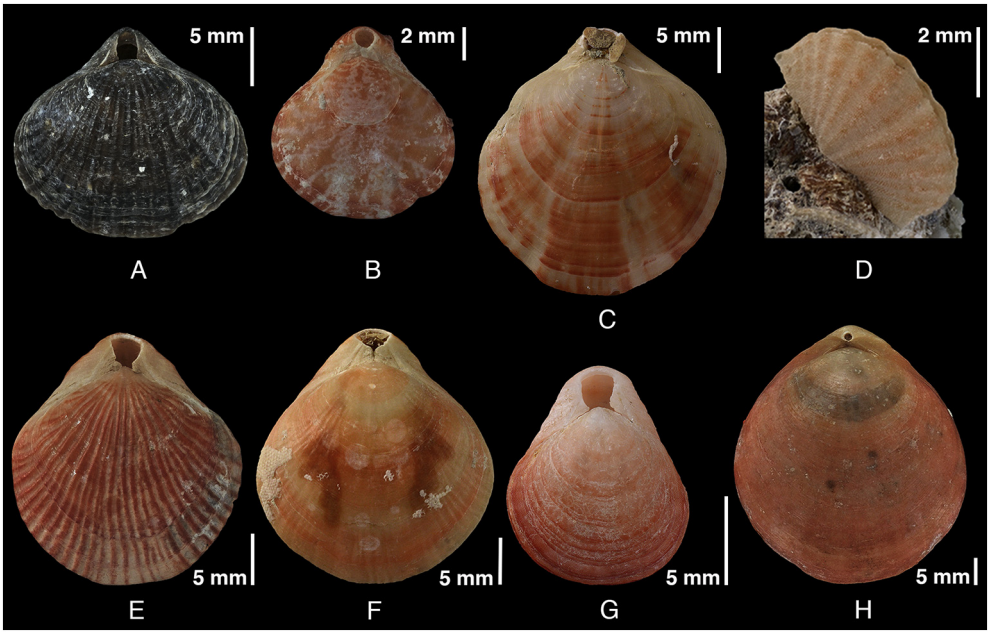

Fig. 2. Diversity of colouration and patterns in rhynchonelliform brachiopods (natural light pictures). A. *Notosaria nigricans* (Sowerby, 1846). B. *Frenulina sanguinolenta* (Gmelin, 1791). C. *Dallinella occidentalis* (Dall, 1871). D. *Argyrotheca rubrocostata* Cooper, 1977, E. *Terebratella sanguinea* Leach, 1814, F. *Terebratella haurakiensis* Allan, 1931. G. *Calloria inconspicua* (Sowerby, 1846). H. *Neothyris lenticularis* (Deshayes, 1879). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Lingula rauliniana d'Orbigny, 1847–51 (Lingulidae), from Roullet St.-Estèphe, Charentes, France (basal Upper Cenomanian, ~95 My), (Institut de Géologie de Rennes Coll., n° IGR.23221).

- In the Rhynchonelliformea: - *Coenothyris vulgaris* (Schlotheim, 1820) (Dielasmatidae), from the Muschelkalk of Lorraine and Var, France (Middle Triassic, ~235 My), (Sorbonne Université Coll., n° SU.PAL.AD.2305.1-SU.PAL.AD.2305.3), - *Sellithyris cenomanensis* Gaspard, 1982 (Sellithyrididae), from 'Les Sables et Grès du Mans", Sarthe, France (upper Middle Cenomanian, ~ 96 My), (MNHN Coll., n° F.A.51528, F.A.69931), - *Carneithyris carnea* (Sowerby, 1813) (Gibbithyrididae), from Northern Europe (Senonian, ~70–90 My) (MNHN Coll., n° MNHN.F.A70117), - *Rhynchonella* sp. Fischer de Waldheim, 1809, from Normandy cliffs (Middle Cenomanian, ~ 98 My) (MNHN Coll., n° MNHN.F.A70118).

2.2. Observation in Natural Light

Recent species that display a colouration, uniform at first sight, in all the shell or revealing patterns, were illustrated under natural light. The observations were made without filters, using the classical support of photography, equipped with 3 lamps Dedo-light DLH4 (Halogen 24 V/150 W) and diffusers. Fossil species are illustrated alike, but in that case, to emphasize the patterns observed, UV light observations have been also performed.

For the precise localization of pigmented areas on surface or cross-sections, complementary observations have been sometimes performed using a Leica MZ FLIII stereomicroscope (Leica Microscopy Systems Ltd., Heerbrugg, Switzerland).

2.3. Ultraviolet Light (UV) Light Observations

The complementary method of observation and illustration for coloured fossil brachiopod shells was the use of UV.

The shells were observed in a dark room. They were exposed under UV using two UV lamps Fluotest Forte (230 V, 50 Hz, 210 W) emitting at a wavelength of 360 nm. Details on the conditions of exposure were provided in a previous analysis [19]. Moreover, it should be noted that, contrary to Caze et al. [21], working on mollusc shells, and except for one species (for checking the real presence of a pigment as a shell component and not as a contaminant), brachiopod shells were not bathed in a sodium hypochlorite solution before UV light observations. This measure avoids the alteration/destruction of organic matrices of the biominerals observed, thus allowing further investigations in the same shells.

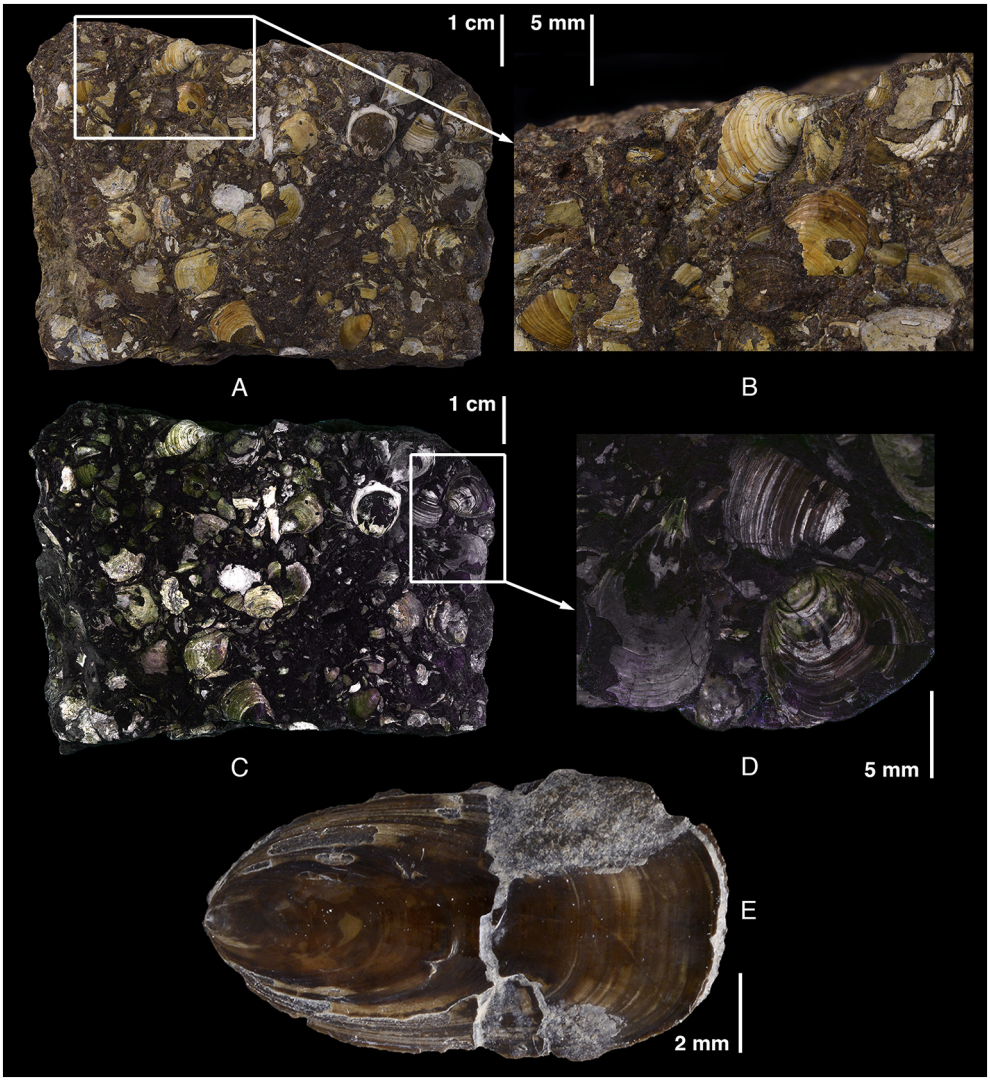

Fig. 3. Fossil linguliform brachiopods. A–D. *Lingulepis pinnaformis* Owen, 1852, from the Mid-Upper Cambrian (~500 My). B and D are magnifications of pictures A and C, respectively. E. *Lingula rauliniana* d'Orbigny, 1847–51, from the basal Upper Cenomanian (~95 My). A, B and E: natural light pictures. C and D: UV light pictures.

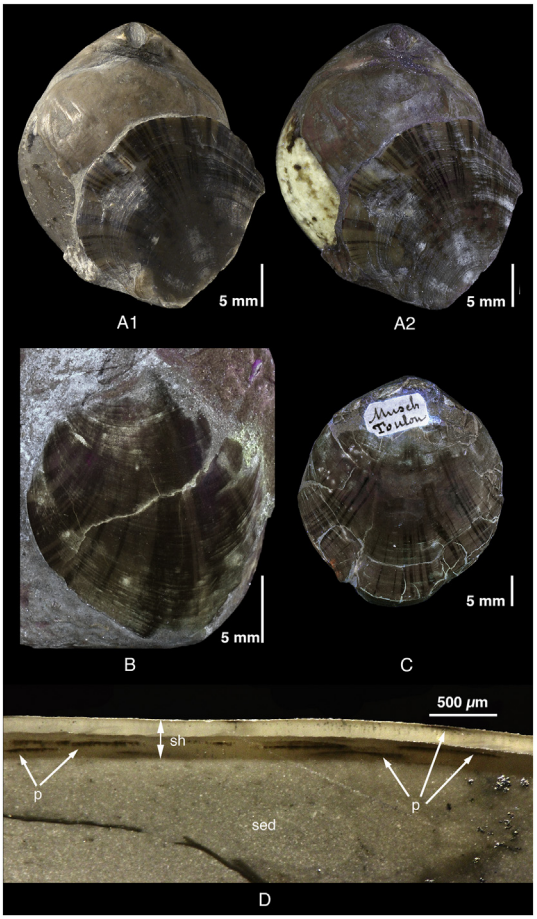

Fig. 4. *Coenothyris vulgaris* (Schlotheim, 1820) (Dielasmatidae), fossil rhynchonelliform brachiopod from the Muschelkalk (Middle Triassic). A, B and C are three different specimens. A1: natural light picture, A2: UV light picture; B and C: natural light pictures. D: Close-up view of a cross-section of a shell, still partly buried within a sediment (sed), showing pigmented bands (p) at different levels in the shell thickness (sh).

2.4. Raman Spectroscopy

The Raman spectra were recorded using two types of device according to the excitation wavelength used for the analyses:

- A LabRam HR 800 spectrometer (Horiba Jobin Yvon) using a Peltier cooled CCD detector and the 514 nm line of a water-cooled Ar^+ laser (Innova 90C, Coherent Inc.). Raman scattering is collected via a microscope (Olympus) equipped with a 50× long working distance objective, allowing a laser spot size of about 5 µm. The laser power at the sample is between 2 and 3 mW. The spectrometer is equipped with a 600 lines/mm grating and allowing a spectral resolution of 2 cm^{-1} on the spectral range 100–1900 cm^{-1}. Calibration has been checked with respect to the 520.7 cm^{-1} band of silicon. Typical recording times consist of 3 accumulations of 10 s.
- A Senterra Raman spectrometer (Bruker Optics) using a Peltier cooled CCD detector and an infrared diode laser for the 785 nm excitation wavelength. The laser is focused on the sample by an Olympus microscope, using a 100× long working distance objective, with a specific coating for the infrared excitation and giving an analytical spot size of approximately 2 µm of diameter. The laser power is about 3 mW on the sample. The grating used as 1200 lines/mm, giving a spectral resolution of about 3–5 cm^{-1} on the spectral range 100–1800 cm^{-1}. The acquisition time is of 2 × 100 s to obtain the best signal/noise ratio.

All the spectra shown in the paper have been obtained with the 514 nm excitation wavelength laser. The laser with the 785 nm excitation wavelength has been used on different shells (see Supplementary data, Appendices A and C), especially when a high Raman fluorescence was observed at 514 nm, but without any results regarding pigment detection for all the species analysed.

The Raman spectra illustrated have been baseline subtracted to simplify comparisons. Raman analyses have been made on external and internal surfaces of the brachial and/or the ventral valves and sometimes, for fossil species, on sections after inclusion in an epoxy resin, cutting and fine polishing.

3. Results

3.1. Natural and UV Light Observations

The colour patterns observed in natural light on brachiopod shells are various (Figs. 1–5).

Coloured ornamentations are most apparent on shells of recent species with radial bandings (on *Dalinella occidentalis* and *Terebratella sanguinea*, Fig. 2C and E, and lighlty on *Novocrania anomala* and *Argyrotheca rubrocostata*, Figs. 1A and 2D), rings (on *Lingulepis pinnaformis*, Fig. 3A), sometimes on recent species with both light bandings and rings, or totally coloured with marked rings (on *Terebratella haurakiensis*, Fig. 2F), as well as with reddish markings, also called divaricate patterns as introduced by Seilacher [31] (see *Frenulina sanguinolenta*, Fig. 2B). On species with a ribbed shell, the radial banding could be due to a more intense colouration linked to a difference in pigment concentration on the ribs (see for example, *Argyrotheca rubrocostata*, *Terebratella sanguinea*, Fig. 2D and E, respectively).

When uniform on the shell of certain recent species, the colouration is not clearly revealed by fluorescence (see for example Fig. 1A2 for *Lingula anatina*). Sometimes the uniformity is just apparent, but using UV light helps to refine light ornamentations (cf. *Lingula anatina*, Fig. 1A2; *Terebratella haurakiensis*, Fig. 2F).

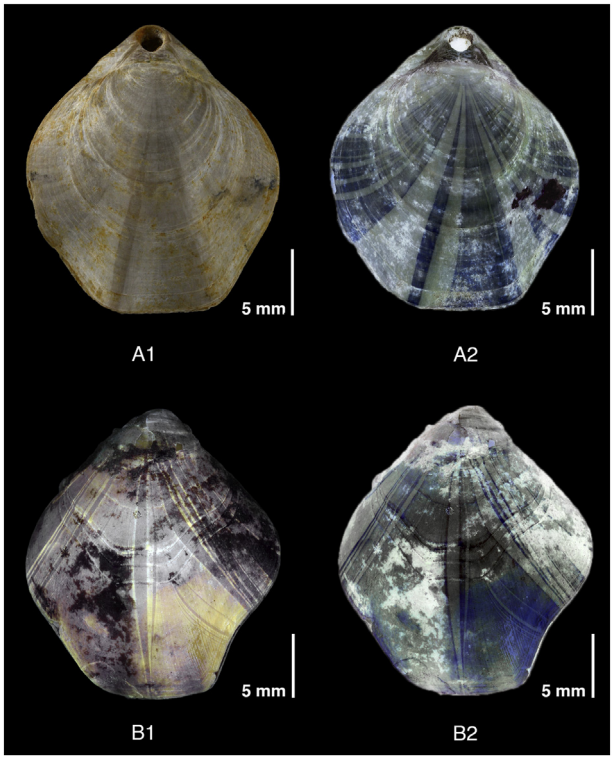

Fig. 5. *Sellithyris cenomanensis* Gaspard, 1982, fossil rhynchonelliform brachiopod from the upper Middle Cenomanian. A and B are two different specimens. A1: natural light picture, A2: negative UV light picture. B1: UV light picture, B2: negative UV light picture.

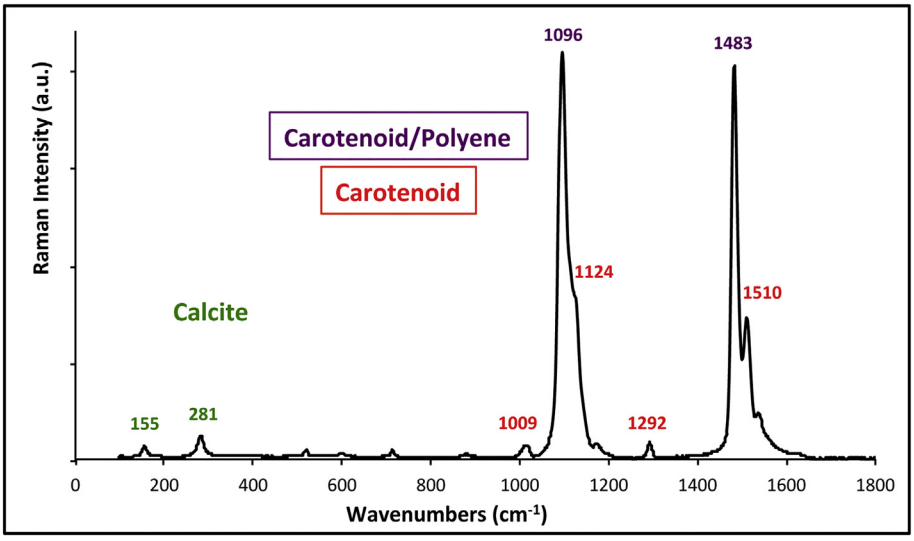

Fig. 6. Raman spectroscopy analysis of *Novocrania anomala* (Craniiformea) shell showing the presence of two types of carotenoid molecules in the same specimen (red and purple bands; note that the violet spectrum could be assigned to another polyene molecules than a carotenoid). The green labelled bands correspond to calcite. The bands at 1014 and 1292 cm^{-1} can be shared by the spectra of the two molecules. The main 1085 cm^{-1} band (carbonate group) is fused with the 1096 cm^{-1} band from one of the polyene molecules. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Furthermore, the pattern is not symmetrical on a valve whatever the valve observed (Fig. 5A2) and reveals also variations from the dorsal (brachial) to the ventral (pedicle) valve [19]. The coloured rings are often in the neighbourhood of growth marks: see *Lingula anatina* (Fig. 1A), *Terebratella sanguinea* (Fig. 2E), *Terebratella haurakiensis* (Fig. 2F), *Calloria inconspicua* (Fig. 2G) and *Lingulepis pinnaformis* (Fig. 3A–D).

As for colour patterns on fossils, differences were observed according to the specimens in the same population: see for example *Coenothyris vulgaris* (Fig. 4A, B and C), and *Sellithyris cenomanensis* (Fig. 5A and B).

UV light enhances the visibility of the colour patterns (*Coenothyris vulgaris*, Fig. 4; *Sellithyris cenomanensis*, Fig. 5). Note that the negative UV images (Fig. 5A2 and B2) generally emphasize the patterns observed on positive UV pictures. Nevertheless, in fossil species like *Lingula rauliniana* (Fig. 3E), with a brown colour, or *Carneithyris carnea* with a uniform brown/orange colour (see insert picture in Supplementary data, Appendix A), UV light observations do not highlight any particular pattern, except at the level of the growth rings. No fluorescence was obviously observed.

The colouration observed at the shell surface can be traced within the shell thickness in cross-sections (see for example *Coenothyris vulgaris*, Fig. 4D). This could be useful to access the pigments not localized sufficiently close to the surface to be accessible to the Raman laser beam. Moreover, we can observe on Fig. 4D that the pigmented bands responsible for the coloured pattern are not necessarily at the same level within the shell.

3.2. Raman Spectroscopy Analysis

In the field of biomineralization, micro Raman spectroscopy is mainly used for identifying the mineral composition of the samples studied. Nevertheless, we used also this technique for studying the pigmentation observed on/in the shell of brachiopods, as it was used for identifying pigments involved in mollusc shell colouration [28,29].

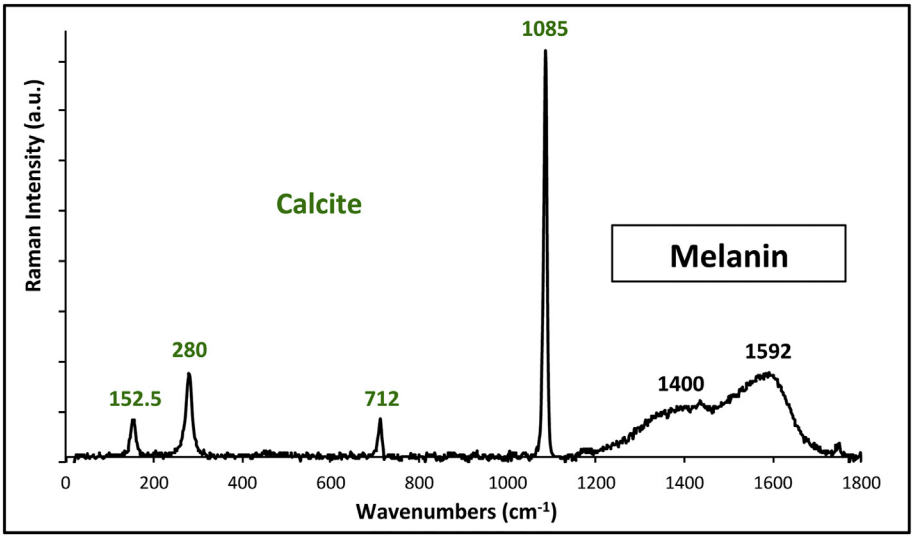

Fig. 7. Raman spectroscopy analysis of *Notosaria nigricans*. (Rhynchonelliformea) showing that the black colour of the shell is due to a melanin pigment. The green labelled bands correspond to calcite. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

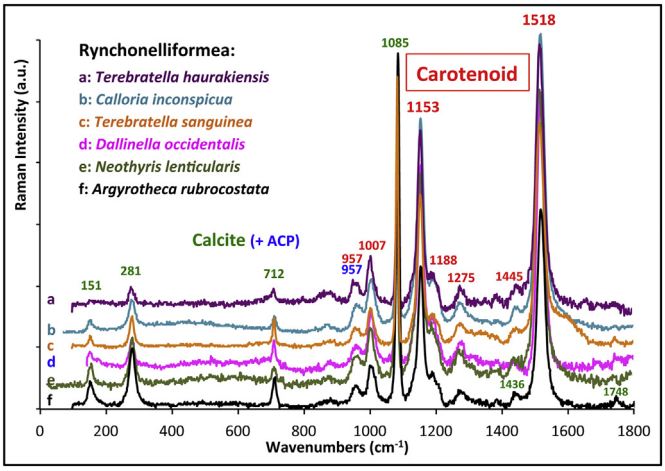

Fig. 8. Raman spectroscopy analysis of rhynchonelliform shells: a. *Terebratella haurakiensis*, b. *Calloria inconspicua*, c. *Terebratella sanguinea*, d. *Dallinella occidentalis*, e. *Neothyris lenticularis*, f. *Argyrotheca rubrocostata*. All the spectra are similar, corresponding to the same carotenoid molecule. The green labelled bands correspond to calcite. The band around 957 cm^{-1} can be assigned either to the carotenoid spectrum or to ACP. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In general, whatever the shell observed, analyses by Raman spectroscopy have been performed on the two valves (dorsal and ventral) on different spots. No difference has been observed from one area to another, from one valve to the other, in term of mineral composition, family of pigments and type of pigments of the same family.

Regarding the mineral composing the brachiopod shells, all the Raman spectra confirm the presence of calcite with characteristic bands at around 152–155, 280–285, 710–715 and 1085 cm^{-1} as the main band and weaker bands around 1436 and 1748 cm^{-1} (Figs. 6–10 and 12, [32]), for the rhynchonelliforms and the craniiforms except for the linguliform species (extant and extinct), the mineral of which is predominantly calcium phosphate [33], characterized by a specific main band at around 963 cm^{-1} (Fig. 11) corresponding to the ν_1 P—O vibration of the phosphate group present in a crystalline network [34]. The linguliform shell mineral has been more precisely identified as a carbonate fluorapatite [35].

In extant Linguliformea, Raman spectroscopy analysis performed on *Lingula anatina* shell (Fig. 1A) has not evidenced any pigment (a high Raman fluorescence was observed whatever the wavelength used, 514 or 785 nm; data not shown). This is in accordance with the absence of fluorescence when observed under UV light (Fig. 1A2). The brown colouration of the shell remains unexplained by using these techniques.

The spectrum of the recent craniiform, *Novocrania anomala* (Fig. 6), is characteristic of the presence of a carotenoid pigment revealed by the two main bands at 1124 and 1510 cm^{-1}, which are due to C—C stretching (ν_2) and in-phase C═C (ν_1) vibrations of the polyene chain, respectively, and two weaker bands at 1009 (ν_4) and 1292 cm^{-1} (ν_3) due to in-plane rocking modes of CH_3 groups attached to the polyene chain and coupled with C—C bonds as observed in β-carotene, for example [29,36,37].

Furthermore, it seems that another pigment (probably of the same carotenoid family) coexists simultaneously, evidenced by two intense bands at 1096 and 1483 cm^{-1}, close to the two main bands of the first mentioned carotenoid. In this case, the two weaker carotenoid bands could be shared by the two molecules.

In the Rhynchonelliformea, three families of pigments have been evidenced. First, in *Notosaria nigricans* which exhibits a black shell (Fig. 2A), the Raman spectrum obtained (Fig. 7) evokes a melanin spectrum, which is very similar to amorphous carbon and well-known broad D and G bands around 1400 cm^{-1} and 1590 cm^{-1} [38], as shown for synthetic and *Sepia* melanin [39]. They are assigned to the in-plane stretching of the aromatic rings and the linear stretching of the C—C bonds.

The second family of pigments found in the rhynchonelliform brachiopods with pink/orange/red coloured shell is the carotenoid family, as shown on the spectra of 6 of the studied species (Fig. 8). Apparently all the species have the same carotenoid molecule, with two main bands at 1153 and 1518 cm^{-1} and weaker bands at 1007, 1188, 1275 and 1445 cm^{-1}. The position of the two main bands appear different compare to those observed for the two carotenoids found in *N. anomala*. Note that the weakest band visible at around 957 cm^{-1} can be assigned either to the ν(C═C) and ν(C—C) vibrations from the carotenoid molecule (but more particularly when using a 632 or 785 nm excitation

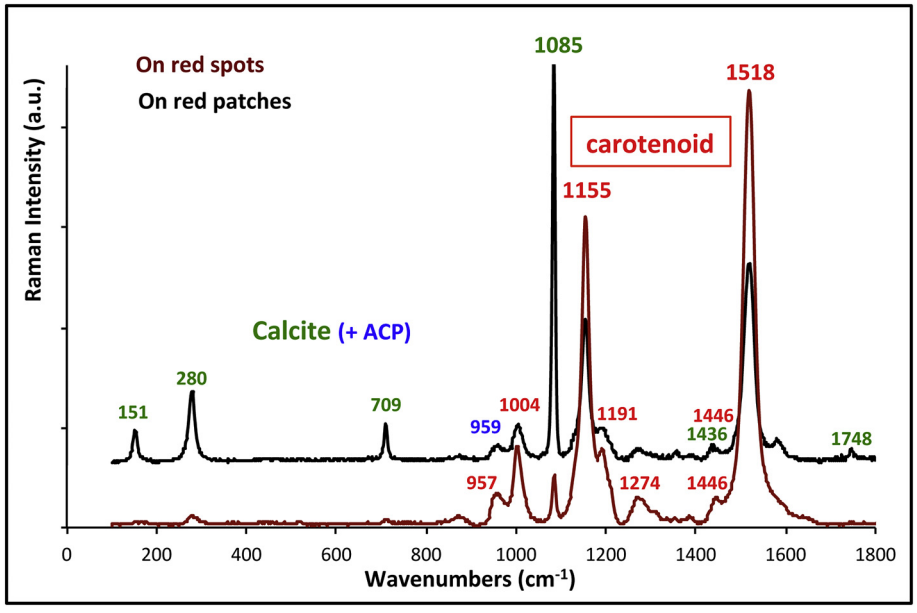

Fig. 9. Raman spectroscopy analysis of *Frenulina sanguinolenta* (Rhynchonelliformea) performed on red spots (brown curve) or on larger red area (dark curve) on the shell. The two spectra are typical of a carotenoid pigment. The spectrum obtained on a red spot (brown curve) shows enrichment in carotenoid compare to the spectrum obtained from red patches (well visible at the level of the main 1085 cm^{-1} band). The green labelled bands correspond to calcite. Note that the band around 957 cm^{-1} can be assigned either to carotenoid or ACP. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

wavelength laser [40]), or to the ν_1 P—O vibration from phosphate groups of calcium phosphate mineral [34]. The shift observed for this band towards lower wavenumbers is characteristic of calcium phosphate under its amorphous form (ACP) [34]. This weak band is present in all the carotenoid-exhibiting spectra except for the spectrum of the only craniiform species studied (Fig. 6). The presence of this band, if associated to ACP, could be subphylum-dependent, only visible in rhynchonelliforms. We must remember that the shell of all the linguliforms is made up of carbonate fluorapatite, the shell of all the craniiforms studied so far only of calcite.

A seventh rhynchonelliform species, *Frenulina sanguinolenta*, has been studied showing the presence of 2 families of pigments in the same shell. First, the same carotenoid pigment than the one observed for the 6 other studied rhynchonelliforms is detected (Fig. 9). The brown spectrum present in this figure has been obtained by focusing the laser beam on a red spot visible at the shell surface, the black curve by analysing a larger red area (see the *F. sanguinolenta* shell colouration on Fig. 2B). On the red spot, calcite seems weakly detected, whereas carotenoid bands are enhanced. This corresponds to a variation of the ratio calcite/carotenoid. On the same shell, at the level of pink regions localized around the red areas and spots, another type of pigment has been detected (series of small bands from 1009 to 1620 cm^{-1}; Fig. 10A).

A magnification of this region (on the 900–1800 cm^{-1} range) allowed us to clearly assign a wavenumber to each band and to identify a cytochrome-type spectrum (Fig. 10B).

Furthermore, it seems that two types of cytochrome molecules coexist here. The red labelled bands fit with cytochrome *c* Raman bands [41,42]. The very strong band at 1584 cm^{-1} corresponds to C═N vibration (π conjugation in the porphyrin ring), the strong band at 1527 cm^{-1} to νC═C vibration, the bands at 1395 and 1358 cm^{-1} to (═C—N) vibration and the band at 1308 cm^{-1} to porphyrin stretching. The band at 1223 cm^{-1} can arise from in-plane bending vibration of the methine hydrogens (δ(C—H) vibration). The other bands at 1170, 1125 and 1009 cm^{-1} have also been observed in previously reported results on cytochrome *c* [42]. Regarding the bands obtained at 1395, 1434, 1490, 1527, 1555, 1584 and 1620 cm^{-1}, they could be assigned to other forms of hemoprotein of the cytochrome family as for example the ones identified from neutrophils cells, cytochromes p30 and *b*-558, close to each other [43]. Some bands in these last cytochromes spectra are shared by the cytochrome *c* spectrum.

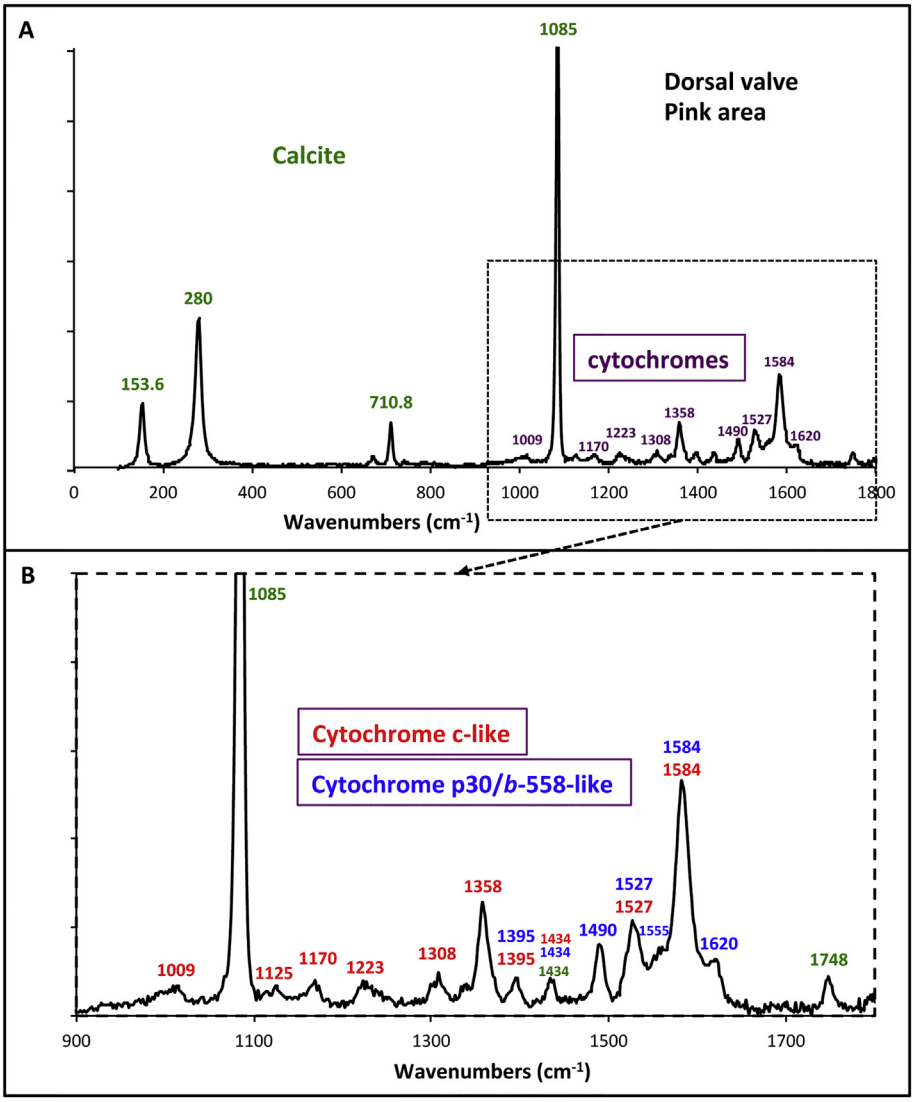

Fig. 10. Raman spectroscopy analysis of *Frenulina sanguinolenta* (Rhynchonelliformea) performed on pink area localized between the red spots and area on the same dorsal valve of the shell analysed in Fig. 9. A. Whole spectrum, from 100 to 1800 cm^{-1} where, beside calcite, a lot of bands are visible from 1008 to 1620 cm^{-1}. B. Magnification on the 900–1800 cm^{-1} spectral range allowing a precise wavenumber attribution to the bands, all identified as originating from molecules of the cytochrome family: cytochrome *c* for the red labelled bands and cytochrome *p*30-*b*558 for the blue labelled bands. The green labelled bands correspond to calcite. Note that the band around 1434 cm^{-1} can be assigned either to calcite or cytochromes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

To check if these unusual pigments are really part of the shell and not the result of an external contamination, we have first analysed shells from four different specimens and obtained similar results. Then, the shell of one specimen has been washed with sodium hypochlorite solution to remove putative organic contaminants, and analysed. The two spectra obtained from this shell, before and after bleaching, revealed completely identical (Supplementary data, Appendix B).

Note that, in the spectra showing the presence of carotenoids, the small band at 957 cm^{-1} characteristic of ACP is present, whereas the spectra exhibiting cytochromes-like pigments seem devoid of ACP.

All the living brachiopods shells are not necessarily coloured, some shells appearing white or cream. The Raman spectra obtained in this case reveal only the mineral, calcite (as observed for example for the two rhynchonelliforms, *Aerothyris kerguelenensis* and *Liothyrella neozelanica*; data not shown).

Regarding the fossil species, only few results have been obtained. The spectra obtained from *Lingulepis pinnaformis* (Linguliformea) and *Coenothyris vulgaris* (Rhynchonelliformea) (Figs. 11 and 12) are similar to the melanin spectrum [39], as the one shown on Fig. 7. As they are fossil shells, the dark colour that we first assigned to melanin could not be the original one. We keep in mind that these two broad bands could be assigned to disordered graphite (containing hexagonal carbon rings as melanin) present in the shell because of possible alteration occurring during the fossilization process [38,39]).

All the results, including UV observations and Raman spectroscopy analyses, are summarized in Table 1.

4. Discussion

Many marine invertebrates exhibit coloured ornamentations, notably on the skin or the exoskeleton [44]. The most studied phylum is Mollusca [26,45,46]. Brachiopods, Lophotrochozoans with a bivalved shell, have also coloured shells, described since a long time. The colour patterns observed are as diversified as the ones observed on/in mollusc shells, going from a complete uniform colouration to the presence of more or less important geometrical patterns: bands, rings, patches, zig zag, or dots [6,7,9,31].

Regarding the fossil species, if the presence of colour patterns or traces has been mentioned since the 19th century, the recent use of UV light allowed better observations of the remaining colouration, although faded with time and during fossilization.

Nevertheless, if many descriptions of different types of colour ornamentations have been made, only a few studies have dealt with molecular identification of the pigments responsible for these colourations, whatever the phylum considered [28,29,46].

First used in molluscs, Raman spectroscopy technique applied to brachiopods for the first time in this work allowed the identification of three families of pigments.

Most of the shell of the recent brachiopods, more particularly in the Rhynchonelliformea, appears completely or partially pink/orange/red coloured. As carotenoids are the most widely distributed family of pigments found in the living world, being present from microorganisms to plants and animals, it is not surprising to identify carotenoid pigments as responsible for this colouration range. Unfortunately, by Raman spectroscopy it is not possible to precisely identify the carotenoid molecule present for different reasons developed below.

Note that only one kind of carotenoid molecule seems to be present in the shell of all the rhynchonelliform species studied. Only one studied species, *Novocrania anomala*, of the Craniiformea subphylum, shows simultaneously two different carotenoid spectra from its shell analysis.

Regarding these two carotenoid-like spectra in the same shell, different hypotheses may be advanced. This may be assigned to the presence of two unidentified molecules of the carotenoid family (astaxanthin, cantaxanthin, zeaxanthin or others) [12,30], as also observed for seashell [46]. Indeed, it is known that the wavenumber positions of the two main bands of a carotenoid molecule are dependent of the length of the chain [37]. More particularly, the band in the 1400–1600 cm^{-1} region is known to have a more or less "pronounced shift to lower wavenumbers as the number of double bonds increases" [47]. Another explanation is that the two spectra could correspond to a racemic mixture of two stereoisomers of the same carotenoid [48], as it was demonstrated for astaxanthin in shrimps of the genus *Penaeus* [49].

Furthermore, the shift observed for certain bands between purified or synthetic carotenoids and carotenoids present within shells could be due to the chemical environment of the last ones. Indeed, in a general way, it is suggested that the pigments are trapped in the organic matrix and probably enter the shell bound to a protein carrier (forming a so-called carotenoprotein complex, as reported [29,50]).

For example, astaxanthin is considered as one of the dominant carotenoids in aquatic animals [51,52]. In crustaceans, astaxanthin is the major carotenoid pigment found in the carapace, widely distributed as free, esterified and protein-complexed forms (bound non covalently to a hexadodecameric protein complex, crustacyanin [52,53].

In brachiopods, Cusack et al. [30] have shown the presence of astaxanthin but also cantaxanthin in the shell of *Terebratella sanguinea*, *Neothyris lenticularis* and *Calloria inconspicua*. They have also evidenced

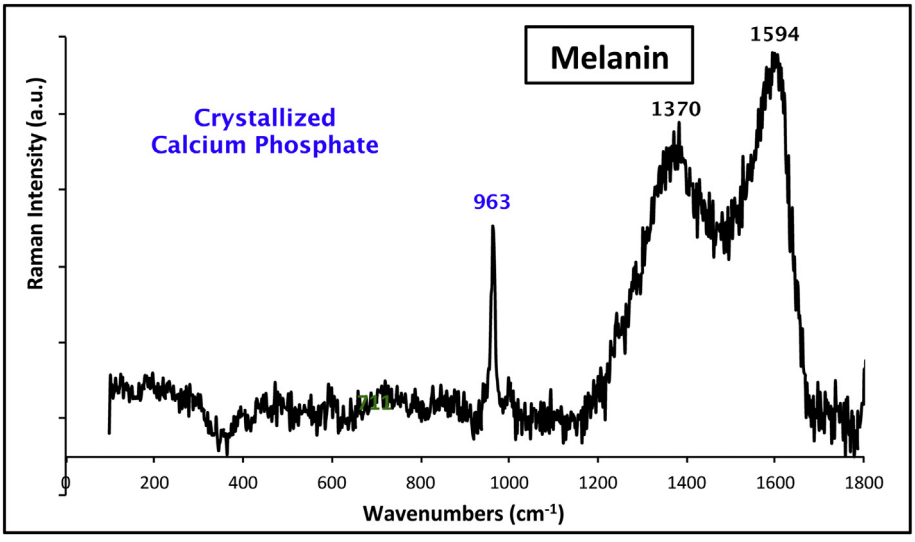

Fig. 11. Raman spectroscopy analysis of *Lingulepis pinnaformis* shell (fossil linguliform). The spectrum obtained, with two broad bands at around 1370 and 1594 cm^{-1}, could correspond to either melanin or graphite (amorphous carbon). The sharp band at 963 cm^{-1} is assigned to the presence of a crystalline polymorph of calcium phosphate.

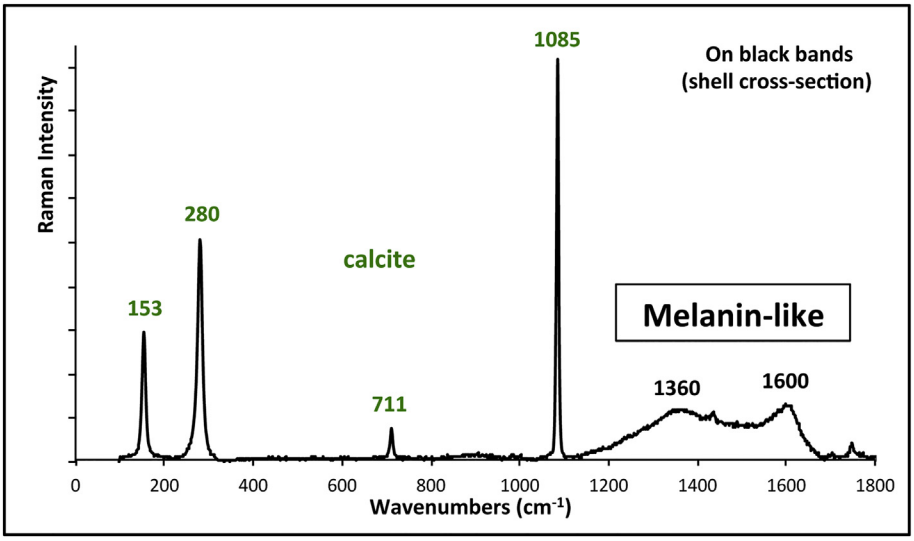

Fig. 12. Raman spectroscopy analysis of *Coenothyris vulgaris* shell (fossil rhynchonelliform). The spectrum obtained on the black bands visible in shell cross section (see Fig. 4D) shows two broad bands at around 1360 and 1600 cm^{-1}, which can be assigned to either melanin or graphite (amorphous carbon).

a small chromoprotein, the function of which is the formation of a carotenoid-protein complex enabling the incorporation of the pigment within the organic network of the shell.

Note also that in craniiform species, the calcitic shell is richer in magnesium as well as in organic matter than in rhynchonelliforms [54]. This could also explain the shifts observed for the main carotenoid bands, even if the same carotenoid is present.

Finally, we cannot exclude that these two bands around 1096 and 1483 cm^{-1} could arise from other polyenic molecules than those belonging to the carotenoid family.

For these different above-mentioned reasons, whatever the carotenoid considered, the four principal bands exhibit small shifts from one to another molecule, so that the precise identification of the carotenoid present by using Raman spectroscopy cannot be achieved. Furthermore, for the same reasons, using the shell colouration as a taxonomic criterion seems inappropriate.

An outstanding observation is the presence of two completely different categories of pigments in the same shell, mentioned here for the first time. Indeed, in *Frenulina sanguinolenta* (Rhynchonelliformea), beside the most frequently found carotenoid, cytochrome-like molecules have been detected. To our knowledge, the presence of this category of molecules is mentioned here for the first time as involved in an exoskeleton colouration. It seems moreover that at least two molecules of this family are simultaneously present. In mollusc shells, porphyrin pigments (Uroporphyrin I as the major one), which has a tetrapyrrole cyclic structure similar to the one found in cytochromes, have been evidenced [26]. Another category of closely related pigments called bile pigments, in which the pyrrole rings are arranged in a linear form, has also been identified [45]. Nevertheless, the Raman spectrum we have obtained is not typical of porphyrins, notably because of the absence of bands in the low wavenumbers [55]. Moreover, porphyrins are known to develop a red fluorescence under UV light [26,56], which is not observed for *Frenulina* (data not shown).

Another possibility is that this "mixed" spectrum observed arises from another yet unknown member of the pyrrole-containing family of pigments [45,46].

As for the dark colour, the spectrum obtained in the extant black-shelled brachiopod analysed, *Notosaria nigricans*, can be credited to (eu)melanin, even if the pigment has not been chemically identified. Melanin is a pigment common in invertebrates [57], evidenced in

Table 1
Summary of the results obtained from UV and Raman analyses of recent and fossil briachopod shells. b: broad; d.: detected; m: medium; n. d.: not detected; s: strong; w: weak.

Subphylum	Species	Shell colour	UV fluo	Pigments	Band wavenumbers (cm^{-1})
Recent specimens					
Craniiformea	*Novocrania anomala*	Orange/brown	d.	Carotenoid	1124 (s), 1510 (s), 1009 (w), 1292 (w)
				Carotenoid/polyene	1096 (s), 1483 (s)
Linguliformea	*Lingula anatina*	Brown	n. d.	n. d.	n. d.
Rhynchonelliformea	*Notosaria nigricans*	Black	n. d.	Melanin	1400 (b,s), 1592 (b,s)
	Frenulina sanguinolenta	Pink/red	d.	Carotenoid	1155 (s), 1518 (s), 1004 (w), 1191 (w), 1274 (w), 1446 (w)
	" "	"		Cytochromes-like	1009 (w), 1125 (w), 1170 (w), 1223 (w), 1308 (w), 1358 (m) 1395 (w), 1434 (w), 1490 (w), 1527 (w), 1584 (m), 1620 (w)
	Dalinella occidentalis	Orange	d.	Carotenoid	1153 (s), 1518 (s), 1007 (w), 1188 (w), 1275 (w), 1440 (w)
	Argyrotheca rubrocostata	Orange	d.	Carotenoid	1153 (s), 1518 (s), 1007 (w), 1188 (w), 1275 (w), 1440 (w)
	Terebratella sanguinea	Pink/red	d.	Carotenoid	1153 (s), 1518 (s), 1007 (w), 1188 (w), 1275 (w), 1440 (w)
	Terebratella haurakiensis	Orange	d.	Carotenoid	1153 (s), 1518 (s), 1007 (w), 1188 (w), 1275 (w), 1440 (w)
	Calloria inconspicua	Pink/red	d.	Carotenoid	1153 (s), 1518 (s), 1007 (w), 1188 (w), 1275 (w), 1440 (w)
	Neothyris lenticularis	Orange	d.	Carotenoid	1153 (s), 1518 (s), 1007 (w), 1188 (w), 1275 (w), 1440 (w)
Fossil specimens					
Linguliformea	*Lingulepis pinnaformis*	Brown rings	d.	Melanin/graphite	1500 (b,s), 1594 (b,s)
	Lingula rauliniana	Black/brown	n. d.	n. d.	n. d.
Rhynchonelliformea	*Coenothyris vulgaris*	Black bands	d.	Melanin/graphite	1360 (b,s), 1600 (b,s)
	Sellithyris cenomanensis	Yellow bands	d.	n. d.	n. d.
	Rhynchonella sp.	Orange/brown	n. d.	n. d.	n. d.
	Carneithyris carnea	Orange/brown	n. d.	n. d.	n. d.

many molluscs [45,46]. The presence of melanin in brachiopod shells has already been mentioned, more particularly in Linguliformea [5,7]. It has been also suggested that the outer chitin-protein periostraca could be rich in iron-containing proteins and melanin conferring an apparent shell colouration [58].

But regarding the fossil species, the conclusions are less evident. Indeed, the spectra obtained (Figs. 11 and 12) may be assigned to a melanin-like pigment but also to disordered graphite (amorphous carbon), both dark-coloured carbon-rich compounds containing hexagonal carbon rings, the structural elements that generate two identical broad (D and G) Raman bands [38,39]). Nevertheless, black bands are clearly visible within sections of the shell of *Coenothyris vulgaris* (Fig. 12) entrapped in the biomaterial, the inorganic compound of which is still clearly calcite (only partly altered), so that the fossilization process has not been drastic, and the remaining pigment could be the original one.

Further, pigments are not necessarily responsible of a fossil (or recent) shell colouration. It has been mentioned that beside colour patterns produced by pigments, a second class of colouration is of structural origin, "resulting from diffraction or differential absorption of light by materials of contrasting composition or crystal structure" such as is iridescence observed on some mollusc shells and crustacean carapaces [7,59–61].

It is impossible to know the original colour of the shell of the extinct species. Some fossil species exhibit dark-brown colours whereas others have lighter colourations such as red. The observed colour may originate from a diagenetic process, as observed for the shell of a *Rhynchonella* specimen from the Cretaceous (Supplementary data, Appendix C). This shell exhibits a brown part, where we identified quartz, whereas we have detected the presence of quartz and moganite, both polymorphs of SiO_2, on the other red part [61,62]. In addition to this kind of drastic diagenetic event replacing calcite by (an)other mineral(s), oxidation processes could occur [63] with, as a result, a shell with an orange-brown colour. This colouration can be uniformly dispersed, as observed on the shell of *Carneithyris carnea* (Rhynchonelliformea from the Senonian), the colour of which could be due to the presence of goethite, a hydrated iron oxide, as for the sample analysed (Supplementary data, Appendix A; [64]). It is considered for example that iron oxide deposition may have replaced the original pigmentation in some cases [44].

Other parameters, which have to be taken into account and which introduce bias, are linked to their conditions of preservation. Modifications occurring during taphonomic processes and after fossilization could have not only faded but also destroyed the pigments responsible for the colouration. This could have otherwise modified the original pigment molecules, rendering the molecule impossible to identify [58,65,66]. For example, in a fossil population, all the specimens are not similarly exposed to light (under direct sunlight, in the shadow or in the dark when they are buried). Regarding specimens of the same species collected from different localities all over the world, even similarly exposed, differences of colouration may be due to their geolocalization (weather, sunshine duration, light transmittance rate, etc.) [67]. These variations may be at play on extant as well as on fossil specimens.

Because of possible more or less drastic diagenetic transformations with time, added to a great individual variability of coloured patterns observed on shells (of both extant and fossil species), it seems very hazardous to use residual colours as additional taxonomic characters for fossils, as proposed for molluscs [68,69].

The suggestion that animal colourations represent evolutionary adaptations (produced by natural selection) has been long the subject of a strong debate [70]. The current idea regarding the origin of a colouration of a specimen is that it is probably the result of the expression of several parameters such as the genome [71], the diet [7,46,72,73], and circumstantial events (epigenetic factors). As an example of the impact of the last parameters, the shell colour polymorphism in a population may be due to environmental gradients related to climate, light exposure, salinity, considered separately or in combination [74].

In the brachiopods, as in the other coloured shell-bearing animals, it is supposed that epithelial cells of the mantle secrete shell pigments. They are sometimes considered as the result of disposal of dietary or metabolism wastes, which could be at the origin of shell colour patterns [7,46]. The current functions assigned to these colourations would be secondarily acquired by selection pressure.

The functions of the pigments in brachiopods shells are probably as diversified as the putative functions evoked for all the other shell-bearing animals like molluscs. Reported hypotheses regarding these functions have been debated over a long time [7,69].

First they are considered as playing a protective function, against predators through mimicry or camouflage (crypsis) linked to the evolution of vision in predators, against microbial and parasites (physiological resistance) and against light variability [19]. Regarding their involvement in photoprotection, they can act as spectral filters to reduce light intensity penetrating thin shells or to absorb (blue-violet) light in deep water to help blend with the environment. On the other hand, in deep-sea species, the loss of pigments is generally observed [5,7]. Melanin pigments are thought to harden materials but their presence can be also related to light availability [7,46].

Furthermore, pigments could be involved in physiological mechanism such as osmoregulation (salinity) [71] or thermoregulation [75,76]. Carotenoids as well as cytochromes are known to serve not only as colorants but also as anti-oxidant agents [51,77].

5. Conclusion

Three families of pigments have been identified by micro Raman spectroscopy, in recent and fossil species, among which the most common are carotenoids and melanin. Interestingly, molecules of the cytochrome family, evidenced for the first time in all the shell-bearing animals, have been characterized in recent specimens; the reason of their presence is unclear.

Unfortunately, the apparent colouration of some brachiopod shell reveals unexplained by using these techniques. Experiments using a combination of techniques such as surface-enhanced Raman scattering (SERS), HPLC chromatography and mass spectrometry, could allow a precise identification of the pigments observed and maybe of other pigments, undetectable so far. Other analyses could lead to precisely determine their localization within the shell: are they organic matrix-associated appearing as pigment-protein complexes, as generally admitted, or linked to the mineral phase? Further investigations would be necessary to decipher their function relative to the physiology, the behaviour and the geolocalization (location, depth) of the specimens studied.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.saa.2018.09.050.

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