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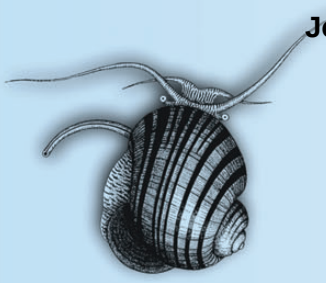

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THE DISTRIBUTION OF POLYENES IN THE SHELL OF *ARCTICA ISLANDICA* FROM NORTH ATLANTIC LOCALITIES: A CONFOCAL RAMAN MICROSCOPY STUDY

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ABSTRACT

Many of the pigments that are widely found in coloured parts of mollusc shells are polyenes, i.e. molecules with a central polyenic chain (carbon-carbon single and double bonds). Due to a resonant coupling of these molecules at wavelengths typically used in Raman spectroscopy, this method is well suited to investigate their occurrence in biogenic materials. Here we use confocal Raman microscopy to map the spatial distribution of polyenes within the shell of the bivalve *Arctica islandica* and to determine their chemical characteristics (chain length). Polyene chain length does not differ between shells from different localities (off Iceland, Baltic Sea and North Sea). We also show that the pigment polyenes are not only located at the outside of the shell, but also within the shell, developing the same layered pattern typical for growth bands. This finding raises the question as to whether polyenes may play a role in the biomineralization process itself.

INTRODUCTION

Pigment polyenes are polyunsaturated organic compounds that were recently described in several mollusc shells (Hedegaard, Bardeau & Chateigner, 2006). In general, pigments are substances that modify the colour of reflected or transmitted light by absorbing specific wavelengths and exist in many structures such as the human retina, bird feathers, hair and calcium carbonate shells (Britton, 2008). Pigment polyenes have been identified from coloured parts of mollusc shells and have previously been related to food source, although in general their origin and function are still unknown (Merlin & Delé-Dubois, 1986; Hedegaard *et al.*, 2006; Karampelas *et al.*, 2007; Evans, Camara & Langdon, 2009). In recent years mollusc shells have gained much attention in biomineralization research, primarily due to their long fossil record and their capacity to record environmental information as exemplified by *Arctica islandica* (Linnaeus, 1767) (Schöne *et al.*, 2004, 2005), but also because of their vulnerability to ocean acidification (e.g. Michaelidis *et al.*, 2005; Gazeau *et al.*, 2007).

Polyenes contain conjugated linear carbon-carbon single and double bonds, building a polyenic chain. Confocal Raman microscopy (CRM) allows determination of the spectral position related to the vibrational modes of the C–C single and C=C double bonds (Hedegaard *et al.*, 2006; Karampelas *et al.*, 2007; Nehrke & Nouet, 2011) and subsequently to estimate the number of C=C double bonds (Schaffer *et al.*, 1991). The shell of the pectinid *Chlamys senatoria* appears in varying colours with each colour corresponding to a different estimated amount of C=C double bonds of the polyenic chain as shown by Hedegaard *et al.* (2006). In their study these authors discussed the relation between colour variation and available food sources in various habitats.

So far, there have been only a few studies on polyenes in mollusc shells. These have mostly focused on the correlation between polyenes and the colour of the pigments (Barnard & de Waal, 2006; Hedegaard *et al.*, 2006) or on analytical methods (Withnall *et al.*, 2003; Karampelas *et al.*, 2007). The nature of polyene pigments, i.e. their origin and distribution within the shell, are still unknown.

We investigated shells of the burrowing heterodont bivalve *A. islandica*, one of the longest-lived solitary animals (Thompson, Jones & Dreibelbis, 1980; Ropes *et al.*, 1984; Schöne *et al.*, 2005; Wanamaker *et al.*, 2008; Butler *et al.*, 2013), from four different localities, to measure (1) whether polyenes are present and, if so, (2) to evaluate their origin. We estimated the number of conjugated C=C double bonds of the polyenes by determining the spectral position of the vibrational modes related to the C–C single and C=C double bonds using Raman spectroscopy. In addition we characterized, for the first time, the spatial distribution of polyenes within cross-sections of a bivalve shell.

MATERIAL AND METHODS

Target specimens

To investigate pigment polyenes within the shell of *Arctica islandica* we used polished cross-sections of three shells from each of

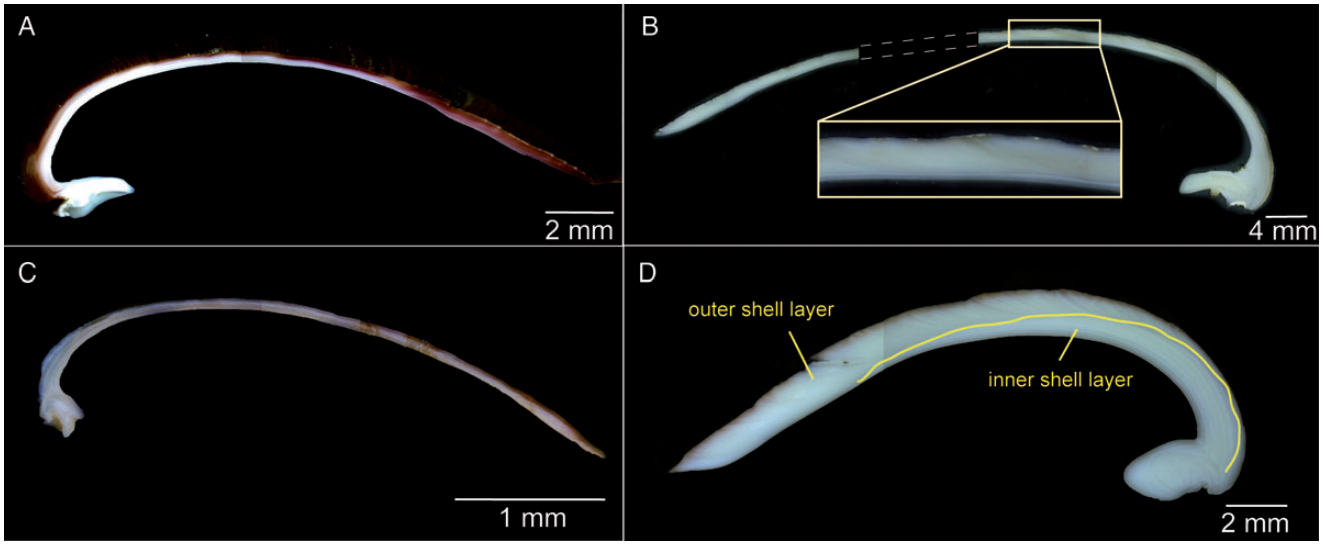

Figure 1. Shell cross sections of *Arctica islandica* from four different localities showing coloured areas in the outer shell layer and the distal part of the shell. **A.** Kiel Bight. **B.** North Sea, with enlarged shell area. **C.** Mecklenburg Bight. **D.** NE Iceland.

Table 1. Experimental parameters of Raman mapping in cross sections of shell of *Arctica islandica* from Kiel Bight shown in Figure 4B.

Label	Scan type	Size (μm)	Points per line	Objective	Integration time (s)
Scan 1	Large area scan	900 × 450	450 × 225	20× N.A. 0.4	0.05
Scan 2	Large area scan	700 × 400	350 × 200	20× N.A. 0.4	0.05
Scan 3	High resolution scan	90 × 90	360 × 360	50× N.A. 0.8	0.01

Wavelength of the laser was 488 nm with 600 gratings per mm.

four different localities (Fig. 1). Shell cross-sections from the western Baltic Sea (Mecklenburg Bight), North Sea and North Atlantic (NE Iceland) were obtained from the shell collection at the Alfred-Wegener-Institute (AWI, Bremerhaven, Germany). Shells from the Kiel Bight (western Baltic Sea) came from a previous culture experiment (Stemmer, Nehrke & Brey, 2013) and only these were used to study the details of the spatial distribution of polyenes within the shell. All material investigated in this study will be deposited in the AWI (Section of Functional Ecology).

The shell of *A. islandica* is composed of aragonite and is divided into an inner and outer shell layer (ISL and OSL), covered by a pale to dark organic periostracum (e.g. Brey *et al.*, 1990; Schöne *et al.*, 2005; Ridgway, Richardson & Austad, 2010). The ISL is responsible for the thickening of the shell, whereas the OSL provides additional growth in height and forms calcium carbonate increments and organic-rich growth lines that can be used for age determination (Fig. 1) (Thompson *et al.*, 1980; Ropes *et al.*, 1984; Witbaard *et al.*, 1994). It has been proposed that both these layers form in separate compartments between the mantle tissue and the inner shell surface (Wheeler, 1992). Shells vary in size and shape depending on the environment in which they grew, as can be seen from the specimens shown in Figure 1. *Arctica islandica* from the western Baltic Sea generally builds thinner shells and is generally smaller compared with those from the North Sea and around Iceland (Dunca *et al.*, 2009). Maximum ontogenetic age varies between populations and ranges from about 40 yr in the western Baltic Sea, over 125 yr in the North Sea and over 350 yr within the populations around Iceland (Schöne *et al.*, 2005; Begum *et al.*, 2010; Basova *et al.*, 2012), making *A. islandica* the longest-lived solitary animal yet known and a valuable study organism for several research disciplines (e.g. physiology, genetics and sclerochronology).

Confocal Raman microscopy

For the investigation of the pigments we used a WITec alpha 300 R (WITec GmbH, Germany) Confocal Raman Microscope (CRM). The spatial distribution of the polyenes was mapped via intensity distribution of the Raman peaks related to vibrational modes induced by the C–C single and C=C double bonds. The experimental settings are given in Table 1 and a detailed description of the method applied to biogenic marine carbonates can be found elsewhere (e.g. Nehrke & Nouet, 2011; Nehrke *et al.*, 2012; Wall & Nehrke, 2012). For all Raman measurements a 488-nm diode laser was used. Single Raman spectra for the exact determination of the position of polyene peaks was done by integrating 10 spectra each measured for 0.5 s.

Spectral analysis and image processing was conducted using the WITecProject software v. 2.04 (WITec GmbH, Germany). Peak positions were determined using the 'multipeak fitting 2' routine of IGOR Pro v. 6.11 (WaveMetrics, USA), assuming a Gaussian shape for the Raman peaks (Fig. 2). Spectra were normalized to the peak position of the Rayleigh peak.

RESULTS AND DISCUSSION

Coloured shell areas

Shell sections of *Arctica islandica* from all four localities showed irregular colouring of brownish-pigmented areas (Fig. 1). The OSL and shell material towards the outer shell margin (distal part) were the most intensely coloured. The ISL was predominantly white. Qualitative differences in colour intensities between shell sections from the four different localities were observed. In shells from the Kiel Bight (Fig. 1A) the OSL showed a strong

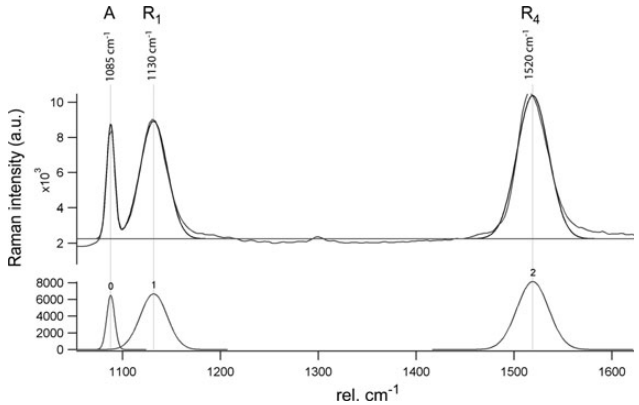

Figure 2. Examples of Raman spectra from *Arctica islandica* shell cross sections together with the peak fit assuming Gaussian peak shape as used to determine the exact peak position. The signal at 1,085 cm^{-1} (marked A) corresponds to the aragonite spectrum. R_1 and R_4 correspond to the major vibrational modes of polyene chains (stretching vibrations of C–C single bonds and C=C double bond, respectively).

red-brown colour with a distinct gradient between the latest-formed shell at the outer shell margin and the outermost OSL, decreasing in intensity towards the ISL and the ontogenetically older portions of the shell. Shells from Mecklenburg Bight (Fig. 1C) showed a similar proportion of coloured to noncoloured areas. However, the colour was yellow-brown, less intense and patchier in distribution than in shells from the Kiel Bight. Shells from the North Sea (Fig. 1B) and NE Iceland (Fig. 1D) were mostly white with coloured areas only in the outermost OSL.

During ontogeny, the colour of the organic periostracum changes from pale brown to dark brown (Murawski, Ropes & Serchuk, 1982), probably caused by iron depositions or by the degradation of certain proteins and consequent changes of their molecular structure (Brey *et al.*, 1990). In this study, we used only young specimens in which the colour of the periostracum was similar to the colour shown in the corresponding shell sections.

Polyene peaks in CRM spectra

CRM spectra revealed strong peaks reflecting vibrational modes of polyene chains. Peaks marked R_1 and R_4 in Figure 2 resemble conjugated C–C single and C=C double bond vibrational modes of the major polyene peaks at ~1130 and 1520 cm^{-1}, respectively. We calculated the expected number of C–C single (N_1) and C=C double bonds (N_4) as done by Schaffer *et al.* (1991), where

$$N_1 = \frac{476}{R1 - 1,082} \quad \text{and} \quad N_4 = \frac{830}{R4 - 1,438}$$

The position of the Raman peak belonging to the C–C single and the C=C double bonds of the investigated *A. islandica* shells fall within a narrow range of ± 2 cm^{-1} and results in the same estimated number of $N = 10$ C=C double bonds. This suggests that the chain length of the polyenes in *A. islandica* is species-specific and independent of habitat. In general the variation in shell colour observed in *A. islandica* does not span the wide range observed for other species like, e.g. *Chlamys australis* (Barnard & de Waal, 2006). Hedegaard *et al.* (2006) investigated five differently coloured shells of *C. senatoria* and found that the peak position of the polyenes showed a range of up to 10 cm^{-1}. A comparison of our data with selected data from Hedegaard *et al.* (2006) is given in Table 2 and Figure 3. The narrower range of colours of *A. islandica* corresponds with its narrower range of peak positions. However, the colours are distinctly different (Fig. 1), although they contain polyene chains of the same length. The relation between habitat (e.g. food source) and colour of the pigments in bivalve shells (e.g. Hedegaard *et al.*, 2006) is evidently species-specific.

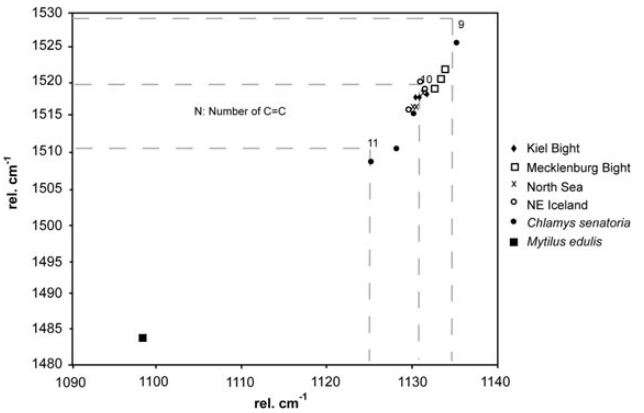

Figure 3. Plot of the relative wave numbers (after Hedegaard *et al.*, 2006) for pigment polyenes measured in the shell of *Arctica islandica* from different localities together with data from Hedegaard *et al.* (2006). The numbers within the plots represent the calculated number of C=C double bonds as given in Table 2 (the number of the double bonds of *Mytilus edulis* falls outside the plotted range).

Table 2. Shells of *Arctica islandica* investigated for pigment polyenes, giving locality ($n = 3$ from each), wave numbers of the two major polyene peaks and the calculated number of conjugated C=C double bonds (N_1/N_4; after Schaffer *et al.*, 1991), and comparison with data from Hedegaard *et al.* (2006).

Species	Colour	Location	R_1 (cm^{-1})	R_4 (cm^{-1})	N_1	N_4
Arctica islandia	Red browm	Kiel Bight	1,131 ± 0.7	1,519 ± 0.3	9.8 ± 0.1	10.3 ± 0
A. islandia	Pale brown	Mecklenburg Bight	1,133 ± 0.6	1,521 ± 1.4	9.3 ± 0.1	10.0 ± 0.2
A. islandia	Pale brown	North Sea	1,131 ± 0.7	1,518 ± 1.2	9.8 ± 0.1	10.4 ± 0.2
A. islandia	Pale brown	NE Iceland	1,130 ± 0.7	1,519 ± 2.0	9.8 ± 0.2	10.3 ± 0.3
Chlamys senatoria	Orange	Hedegaard *et al.* (2006)	1,135	1,526	9.0	9.4
C. senatoria	Yellow	Hedegaard *et al.* (2006)	1,125	1,509	11.1	11.7
C. senatoria	Red	Hedegaard *et al.* (2006)	1,130	1,517	9.9	10.5
C. senatoria	Purple	Hedegaard *et al.* (2006)	1,128	1,511	10.3	11.4
C. senatoria	Brownish	Hedegaard *et al.* (2006)	1,130	1,516	9.9	10.6
Mytilus edulis	Blue	Hedegaard *et al.* (2006)	1,098	1,484	29.8	18.0

Due to the nature of the resonance Raman signal even small amounts of polyenes within the sample (down to 10^{-8} M, Merlin, 1985) can induce detectable peaks. In a confocal system, like the Raman microscope, the intensity of the signal strongly depends on the roughness and tilt of the scanned sample surface. This sensitivity to surface features and the facts that each polished cross-section of a given sample is unique and that the distance between objective and sample cannot be exactly reproduced between scans, renders a quantification of the amount of pigments based on the measured intensity impossible. Nonetheless, qualitatively the intensity of the measured Raman signal can be attributed to the presence of polyenes.

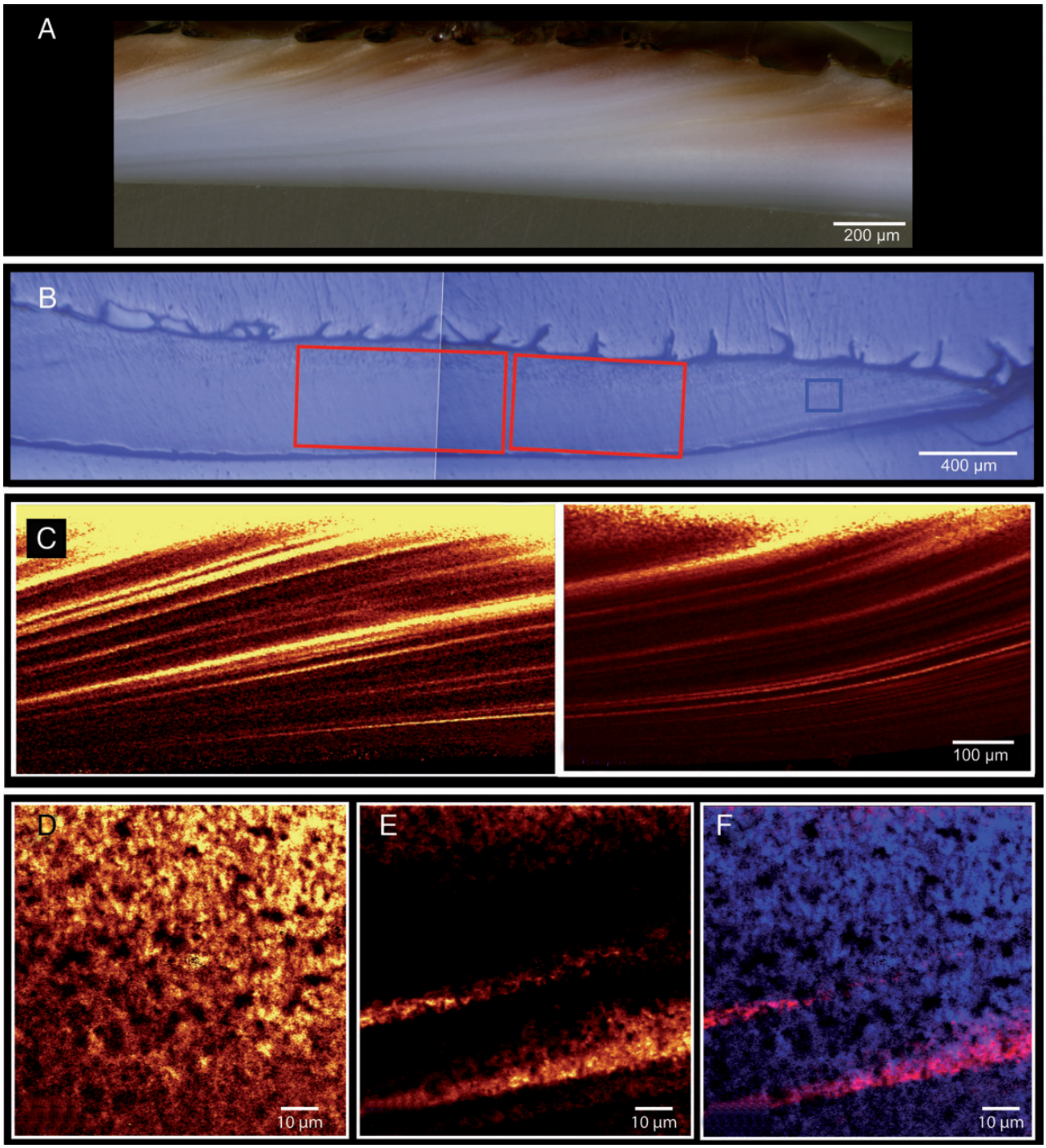

Figure 4. Distribution of pigment polyenes in a shell cross section of *Arctica islandica* from the Kiel Bight. **A.** Colour gradient from the outer to the inner shell layer. **B.** Distal part of shell cross section indicating areas scanned by CRM (red squares: scans 1 and 2 shown in **C**; blue square: scan 3 shown in **D–F**; see Table 1 for scan parameters). **C.** Polyene distribution showing banding pattern corresponding with growth lines. **D–F.** High resolution (~300 nm) Raman scan. **D.** Aragonite distribution. **E.** Polyene distribution in two lines of the growth pattern shown in **C. F.** Overlay of **D** and **E** showing polyenes integrated into aragonitic granules.

Spatial distribution of polyenes

The shells of *A. islandica* used to analyse the spatial distribution of polyenes had been collected and cultured within a project related to ocean acidification. That study showed that the microstructural features did not differ between natural and cultured shells (Stemmer *et al.*, 2013). The animals were originally collected from Kiel Bight and cultured for 3 months under ambient conditions (Stemmer *et al.*, 2013). Polyenes present in shell cross-sections produced Raman signals well within the detectable range. To visualize the polyene distribution we plotted the intensity distribution of the major peak at 1530 cm^{-1} (Fig. 4), following the method of Nehrke & Nouet (2011). Raman scans performed across shell cross sections showed that polyenes were inhomogeneously distributed. The signal was strong within the OSL and at the outer shell margin. However, polyene pigments could not be detected in the inner shell layer, which leads to the conclusion that only the outer extrapallial fluid (Wheeler, 1992), located between the commissure and the pallial line, deposits polyenes which become incorporated in the OSL.

Cross sections of *A. islandica* reveal growth lines (Thompson *et al.*, 1980) that form during slowing of growth in the OSL and contain a high amount of organic material (Lutz & Rhoads, 1977; Schöne *et al.*, 2004). The intensity distribution of the polyenes is shown in Figure 4C and reveals a banding pattern congruent with the characteristic growth lines. In addition, an enhanced abundance of polyenes occurs in the coloured areas of the OSL adjacent to the periostracum. The presence of polyenes in the outermost shell of other taxa has been documented previously (Barnard & de Waal, 2006; Hedegaard *et al.*, 2006), but the banding pattern within cross sections is described here for the first time.

Annual growth lines of bivalve shells are often used to determine the age of the bivalve when the date of death is known (Jones, 1980; Schöne *et al.*, 2005). The shell increments (shell material between growth lines) can be further investigated for biochemical signatures and are a valuable tool for environmental reconstruction (Schöne *et al.*, 2005). The investigated shell material was obtained from culture experiments in which the growth increments were determined by means of labelling at the beginning of the experiment (Stemmer *et al.*, 2013), from which it is revealed that the growth bands are intra-annual; about 10 bands were formed during the 3-month culture period (Fig. 4C). The potential of this information to sclerochronology and palaeoclimatic research has to be further investigated.

High resolution (~300 nm) Raman scans of growth lines (Fig. 4D–F) were used to investigate how the polyenes are incorporated into the shell material. Figure 4D shows a Raman map based on the intensity distribution of the aragonite peak at ~1,085 cm^{-1}. This map illustrates an homogeneous crystal fabric built of small aragonitic granules (as previously described for *A. islandica* by Schöne *et al.*, 2013). From the same shell region the intensity distribution of the polyene-related peak at ~1,530 cm^{-1} is plotted in Figure 4E, showing two bands related to growth lines (Fig. 4C). An overlay of both maps (Fig. 4F) indicates that the polyenes are incorporated within the aragonitic crystals and do not surround them. For many biogenic carbonates it is known that organic molecules surround the aragonitic (or calcitic) granules like an envelope (Cuif, Dauphin & Sorauf, 2011; Cuif *et al.*, 2012). We cannot determine from the Raman scans if organic envelopes are present around the aragonite granules, since most of the organic molecules expected within the organic matrix do not exhibit a resonance Raman signal, as required to detect the very low concentrations in which they are likely to be present.

Two questions arise from the spatial distribution of polyenes determined in this study.

First, why are the polyenes present in the outer part of the shell but not in the older parts of the shell? Within the framework of this study we cannot determine whether the polyenes have never been present in the older parts of the shell or if alteration processes led to their disappearance; this should be resolved by further studies on very young specimens. Second, does the association of polyenes with growth bands in shells of *A. islandica* indicate an important role of the polyenes in the biomineralization process itself?

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