

Automated measurement of Magnesium/Calcium ratios in gastropod shells using Laser-Induced Breakdown Spectroscopy (LIBS) for paleoclimatic applications

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Abstract

The chemical composition of mollusk shells offers information about environmental conditions during the organism's lifespan. Shells are found in geological deposits and in many archaeological sites, enabling the reconstruction of past climatic conditions. For example, a correlation between the seawater temperature and the amount of some substituent elements (magnesium, strontium, ...) in the biogenerated calcium carbonate matrix of the shell has been found, although it is very species specific. Here we propose the use of the Laser-Induced Breakdown Spectroscopy (LIBS) technique to estimate Mg/Ca ratios in modern specimens of the common limpet *Patella vulgata*. An automated setup is able to obtain a sequence of Mg/Ca ratios across a sampling path that can be compared with the seawater temperatures recorded during the organism's lifespan. Results using four shells collected in different months of the year have shown a direct relationship between the Mg/Ca ratios and the seawater temperature, although the sequences also reveal small-scale (short term) variability and an irregular growth rate. Nevertheless, from the LIBS sequences, it is possible to infer the season of capture and the minimum and maximum seawater temperatures. This fact, along with the reduction

in sampling and measurement time compared to other spectrometric techniques (such as ICP-MS), makes LIBS useful for paleoclimatic studies.

Keywords: Laser-Induced Breakdown Spectroscopy; LIBS; laser spectroscopy; paleoclimate.

Introduction

The chemical composition of marine mollusk shells offers valuable information about environmental conditions during the organism's lifespan. This is due to the biomineralization process depending not only on the internal physiology but on external factors such as seawater temperature and salinity.¹ Mollusk shells are mainly composed of calcium carbonate in two different crystal forms: calcite and aragonite, along with traces of proteins and polysaccharides. Additionally, calcium ions can be easily substituted by magnesium, strontium and other elements, in a proportion of a few percent or less, which is heavily dependent on the seawater temperature, thus the amount of these elements in the shell being a proxy to estimate the surrounding temperature.² However, the relationship between the amount of a particular chemical element and external variables like salinity or temperature has been found to be species-specific, with direct, inverse or no relationships at all.^{3,4} Moreover, the shell composition is not homogenous, and two distinct layers of calcite and aragonite can be found in most species.

Recent studies have mainly used the Inductively Coupled Plasma Mass Spectrometry (ICP-MS) technique to obtain the relative concentration of such elements in the calcium carbonate matrix. Freitas found different relationships in mollusks: Mg/Ca ratios have a significant correlation with temperature in *Pinna nobilis*,³ but this is not the case for Sr/Ca ratios. A similar result was found in *Pecten maximus* but only in the inner calcite layers,⁵ while for *Mytilus edulis* the correlation of the Mg/Ca ratios was very weak for temperature, salinity or age.⁶ Several studies have analyzed the shells of the bivalve *Arctica islandica* due to its exceptional longevity (one specimen found alive in 2006 has the highest reported lifespan among animals: 507 years), but the results using Laser Ablation ICP-MS showed poor or no correlation with seawater temperature and salinity.^{7,8} However, a more recent study based on the same LA-ICP-MS technique⁹ that corrects the data for physiological effects due to aging, has found negative correlation of Mg/Ca and Sr/Ca ratios in aragonite

layers of this species with seawater temperature. These and other studies suggest that the use of Mg/Ca and Sr/Ca ratios as a temperature proxy, especially in bivalve mollusks, is still challenging.

However, elemental analysis can also be applied to ancient shells in order to reconstruct past seawater temperatures. This paleoclimatic application is of great interest in several fields, for example, in archaeology. The marine mollusks have supplied humans with essential nutrients for brain development and reproductive health,¹⁰ being routinely consumed in coastal areas around the world at least since 170 kiloyears (ky) before present. For example, these resources were exploited with intensity by human populations in Atlantic Europe during the Mesolithic period (around 10 ky before present).¹¹ In fact, huge accumulations of discarded shells have been found along coastal areas of the Cantabrian region (northern Spain). The common limpet *P. vulgata* Linnaeus, analyzed in this paper, and in lesser amounts, the topshell *Phorcus lineatus*, were highly consumed by human groups that inhabited this area during the Mesolithic. The structure and composition of those shells are the same as of modern specimens, as they are not yet fossilized and chemical alteration due to environmental factors (diagenesis) is usually negligible.¹² These characteristics enable the use of archaeological shells as paleoclimatic proxies.

Past seawater temperatures deduced from the shell elemental composition can be useful for paleoenvironmental reconstruction, but they can also be used to obtain information on subsistence strategies of human populations, such as for example the season of the year in which the shells were collected. Mollusks form their shells by precipitating calcium carbonate as growth increments during their lifespan. The season of collection is deduced from values obtained in the last growth increments, located at the shell edge and deposited immediately before the death of the mollusc. A comparison between these values and the annual maximum and minimum values recorded throughout the shell growth provides accurate data on shell collection. Information on the seasonality of shell collection is crucial for the interpretation of key issues such as availability of food resources, foraging practices and settlement patterns of prehistoric groups.¹¹

Mg/Ca and Sr/Ca ratios have been studied in the *Patella* family by Ferguson et al.,⁴ using ICP-MS. Before applying the technique to archaeological shells, they performed a study with modern specimens in order to validate the method. They found the highest correlation ($R^2 = 0.79$) between seawater temperature and the Mg/Ca ratios in the calcite layer of *Patella caerulea* and *Patella rustica* specimens. In contrast, elemental

analysis on *P. lineatus* has received less attention as a paleoclimatic proxy, and only oxygen isotope ratios measured in the aragonite layer of modern and archaeological shells have been used for environmental reconstruction and determination of season of collection.^{13,14}

One drawback of the commonly used ICP-MS or ICP-OES (and of the stable isotope analytical techniques) is the sampling process: small amounts of material need to be removed and collected from the appropriate layer of the shell at different points, typically along the whole growth axis from the shell to the apex, several centimeters long.¹⁵ In order to obtain enough temporal resolution, typical sampling distance is 100-150 μm , with many sampling points needed. The powder material from each sampling point follows a process of acid digestion, ultrasonic bath and centrifugation that is tedious and time-consuming. LA-ICP-MS, on the other hand, uses a laser to ablate the material, does not require sample preparation and it is able to automate some of the subsequent steps in the analytical process. Its main drawback is the high cost and complexity of the instrumentation and operation.¹⁶

However, the use of other techniques, such as Laser-Induced Breakdown Spectroscopy (LIBS), can be useful to determine elemental ratios in shells, as it needs little or no sample preparation, obtains fast results and can be easily automated with a simple instrumentation.^{17,18} LIBS has been used successfully for many kinds of archaeological analysis,¹⁹ but little attention has been paid to the analysis of biogenic carbonates in shells. Only very recently, the LIBS technique has been applied for the determination of Mg/Ca ratios in the shell of the marine gastropod *P. lineatus*.²⁰ They measured Mg/Ca ratios sequentially along the shell growth axis and found a correlation with oxygen isotope ratios measured at the same sampling points ($R^2=0.70$ and $R^2=0.65$ for two different shells). However, measurements on the shell edge (at the aperture) from shells collected monthly through a whole year produced a poor correlation with seawater temperatures recorded at the time of collection ($R^2=0.43$ and $R^2=0.44$ at the aragonite and calcite layers, respectively). In that study, the laser focal point for ablation was positioned manually at each sampling point, a complicated process due to the complex shape of the shell.

In this paper, we aim to establish whether correlation exists between Mg/Ca ratios from limpets *P. vulgata* collected alive in northern Spain, and seawater temperatures. For this purpose, shell samples will be measured using a fully automated LIBS setup to test the viability of the method.

Experimental

LIBS experimental setup. The LIBS setup is shown in Fig. 1. It is based on a Nd:YAG Q-Switched laser (Lotis LS-2147) with pulses lasting 16 ns at 1064 nm, 10 Hz repetition rate, and energy of 35 mJ per pulse. The laser produces a crater in the shell about 100 μm in diameter and 0.2 μm depth per pulse. The plasma is captured by a 600 μm -diameter optical fiber coupled to an Acton SP-300i spectrometer and a PIMAX-3 intensified CCD detector. Coaxially with the laser focusing optics, an industrial-grade color CCD camera allows a small area (7x5 mm) of the shell's surface to be seen. A contrast ratio analysis of this image allows the automatic focusing of the laser spot over non-flat surfaces. The shells are placed on a motorized XYZ positioner using a custom 3D-printed part so the exposed section is placed horizontally with its surface 2mm above the laser focal point, to prevent plasma formation in air. The setup is controlled by custom software in Matlab® and is able to automatically follow a sampling path over the calcite layer of the shell section.

For these experiments, a fixed distance between sampling points of 200 μm was chosen, with every spatial point sampled with 50 laser shots in order to improve the signal to noise ratio of the LIBS measurement.

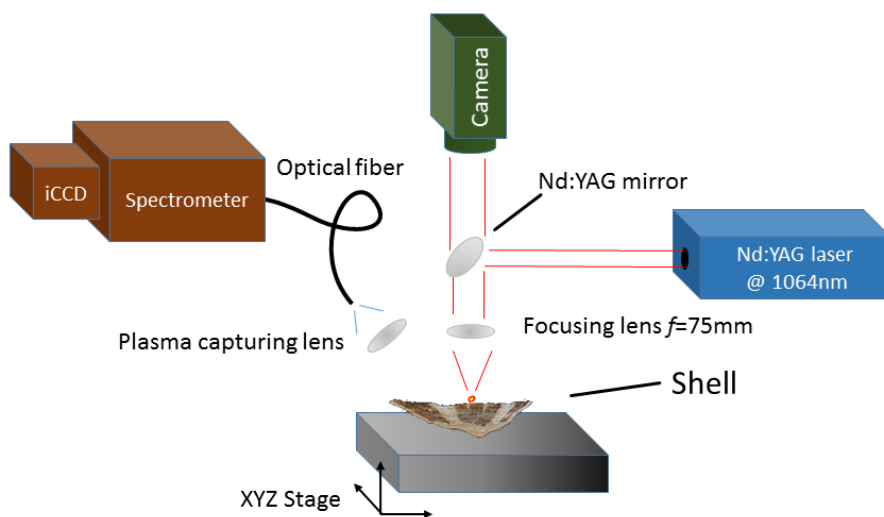


Fig. 1. LIBS setup. The high-energy pulsed laser is focused onto the sample surface with a mirror and a focusing lens, creating a plasma plume and ablating a tiny amount of shell's material. The light emission from the plasma is collected by a lens and sent to a spectrometer to obtain its spectral signature. The process is controlled and automated by a computer using a CCD camera and a XYZ motorized stage.

With the available setup, which includes a Czerny-Turner spectrometer, one must choose either a high resolution or a wide spectral range. For this feasibility work, there is a conflicting interest in spectrally resolving the few Mg peaks from the abundant Ca ones, but also in using a wide spectral window that captures as many peaks as possible from the major constituents: Ca, C, O, Mg and Sr. The latter could enable the use of normalization techniques to improve the quantitative results based on the Saha-Boltzmann method²¹ or Calibration-Free LIBS.²² Two spectral measuring windows were finally used in this work: a narrow window from 274 to 306 nm using a 1200 grooves/mm grating; and a wide window from 277 to 563 nm using a 150 grooves/mm grating. In both cases, the spectra were captured in a gated temporal window starting 0.5 μ s after the laser pulse, and lasting 20 μ s. This is a compromise value with low contribution of the background radiation but strong atomic emission lines. A sample of the captured spectra is shown in Fig. 2. All of the unlabeled peaks in this figure correspond to calcium, a major constituent of the shell carbonate and with strong emission lines. Some of the calcium peaks are saturated due to the need to adjust the dynamic range of the spectrometer to the low emission intensities of the Mg I lines.

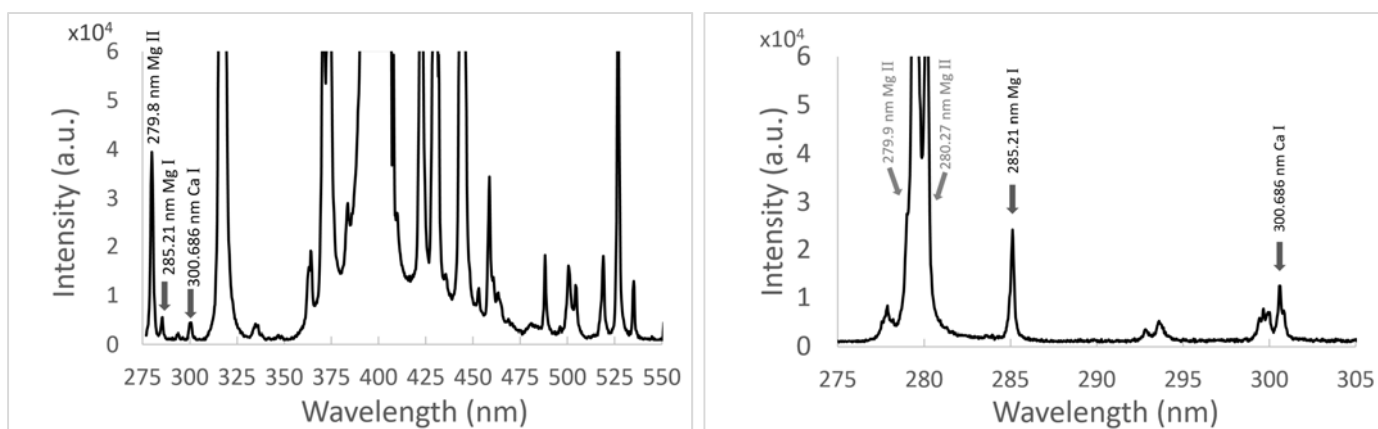


Fig. 2. Sample spectra in two spectral windows. The selected emission lines of Mg I and Ca I for the Mg/Ca ratios are highlighted (unlabelled peaks correspond to calcium lines).

From the captured spectra, simple Mg to Ca ratios were derived. The selected spectral lines were 285.21 nm (Mg I) and 300.686 nm (Ca I) in both windows. Due to the expected low concentration of Mg in the carbonate

shell and the weak intensity of that particular Ca line, both are expected to be free of auto-absorption. The ratio of the integrated area of the two emission peaks was calculated for every spectrum, and then the average ratio (50 spectra) at each sampling point was obtained. This simple processing scheme produces a Mg/Ca ratio in arbitrary units that is not the actual Mg/Ca molar concentration ratio, but the two should be linearly related. In order to verify this hypothesis, a calibration curve using a reference material with known concentration ratios has been obtained. It is well known that the spectral line intensities from a particular element in LIBS are heavily dependent on the physical and chemical properties of the sample (the so called matrix effect¹⁸), and therefore a reference material must have nearly identical properties to the sample. That kind of matrix-matched standard is almost impossible to find in the case of biogenic carbonates, while standard materials used in other spectrometric techniques for calibration purposes are not always appropriate for LIBS.²³ Nevertheless, this is not a drawback in this work as only the relative variation of the Mg/Ca ratios along different spatial points of the shell, and not the actual molar concentration, is needed for the proposed application. Additionally, the calcite layer in which the samples are taken is very homogenous in its physical (crystalline structure, density...) and chemical properties, so measuring artifacts from matrix effects are not expected. To verify that the raw line intensity ratio is linearly dependent with the Mg/Ca concentration ratio, a reference material has been created from a mix with different ratios of pure magnesium and calcium carbonate powders, embedded in Elastosil® M4601, a two-component silicone-based rubber with no calcium or magnesium on its composition, used as a binder for the carbonate powders. The emission spectra have been obtained and the Mg/Ca line intensity ratio calculated for six different Mg/Ca concentrations within the 2 mmol/mol to 100 mmol/mol range. The expected concentration values are not known *a priori*, but Ferguson *et al* have measured Mg/Ca ratios in the 15 to 30 mmol/mol range in other species of the *Patella* genus, using the ICP-MS spectrometric technique.⁴

The calibration curves obtained are shown in Fig. 3, for the wide (left) and narrow (right) spectral windows. It can be seen that the Mg/Ca ratios derived from the two selected emission lines show a linear relationship with the actual Mg/Ca molar concentration for a wide range of concentrations. The differences in the absolute value of the Mg/Ca ratio in the two spectral windows are explained by the different spectral transmissivity curves of the gratings at the two selected wavelengths. From the calibration curves, an estimation of the limit

of detection (LoD) of the experimental setup can be derived, using the expression recommended by Hahn & Omenetto:¹⁸

$$LoD = \frac{3 \cdot s_B}{b} \quad (1)$$

In Eq. 1, s_B is the standard deviation of the background, and b is the magnitude of the slope of the calibration curve (Fig. 3). A LoD value of 0.122 mmol/mol (122 ppm) is obtained for the wide window and 0.043 mmol/mol (43 ppm) for the narrow window. These results confirm the suitability of the proposed experimental configuration and of the two selected emission lines to estimate the Mg/Ca ratios.

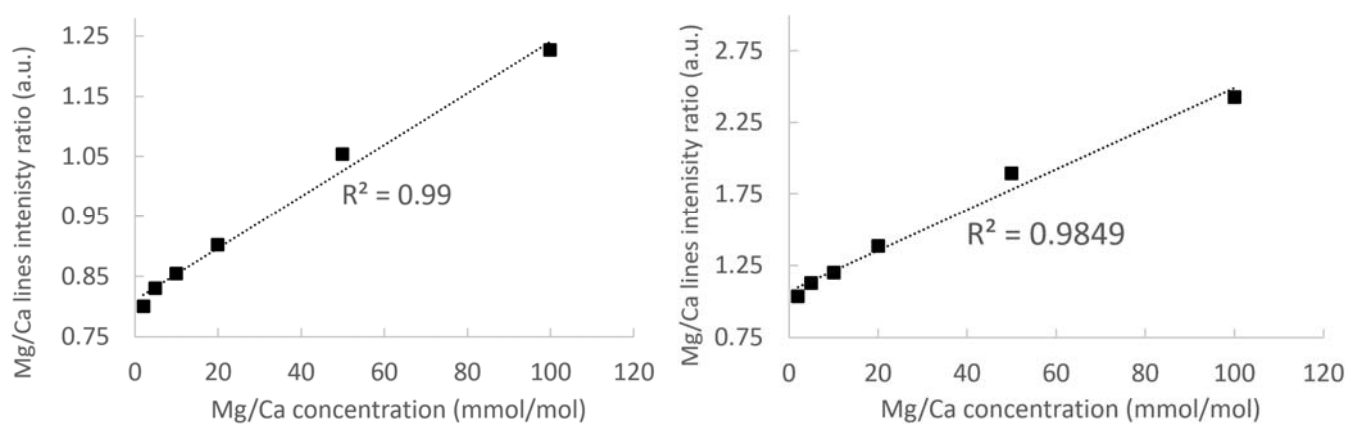


Fig. 3. Calibration curves using a reference material of known Mg/Ca concentration and measuring the ratio of the 285.21 nm (Mg I) and 300.686 nm (Ca I) emission lines captured in the wide spectral window (left) and the narrow one (right).

Preparation of shell samples.

Mg/Ca ratios were measured in four specimens of the limpet *P. vulgata* collected in February, May, August and November 2006, in the intertidal zone of Langre Beach (Cantabria, northern Spain). After collection, the meat was removed from the shell using a bone spatula. The shells were gently cleaned with a brush and water and then placed in an ultrasonic bath with water for two minutes and dried at ambient temperature. Shells were cut in half with a diamond Buehler® low-speed saw in order to expose the cross section. A shell and a detailed view of the exposed section are shown in Fig. 4. Limpet shells are formed through precipitation of calcium

carbonate in the form of growth increments, with the most recent biogenerated material in the underside of the shell, the so-called shell edge. The increments can be used to reveal the mollusk growth rates, which can be variable as a consequence of growth cessation or slowdown. Growth increments have usually a minute thickness of a few microns, but can be made visible using staining techniques. Some are highlighted close to the edge in Fig. 4. An optimum sampling path with the aim of verifying a chemical correlation with the seawater temperature should therefore cross the growth increments in areas of maximum growth rates to achieve sufficient temporal resolution. This corresponds roughly to the midpoint of the concentric crossed-foliated calcite layer,²⁴ as shown by the suggested sampling path highlighted in Fig. 4. We used this layer because Mg/Ca ratios obtained from the concentric crossed-foliated calcite layer of *Patella* using other spectrometric techniques have shown high correlation with seawater temperatures.⁴

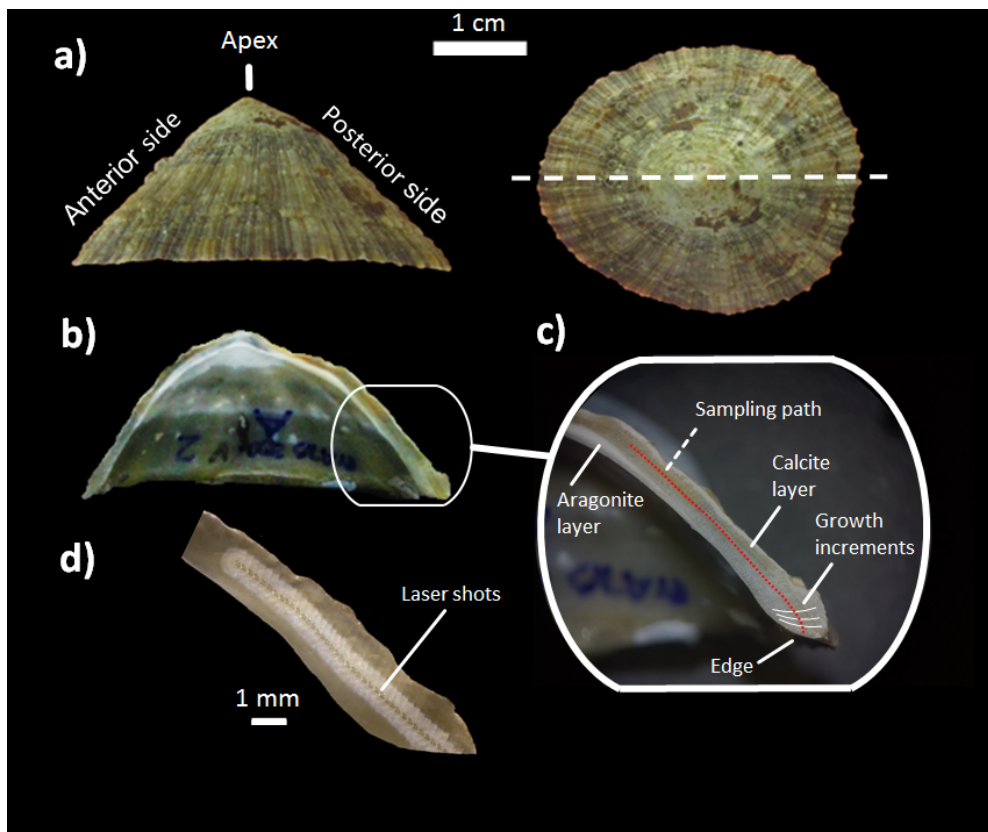


Fig. 4. One of the *P. vulgata* shells measured in this work. a) view of the shell. b) one of its halves after cutting the shell. c) detailed view of the exposed section, showing a sampling path over the calcite layer, starting from the shell's edge. Some growth increments (see text) with their approximate shape are highlighted. d) laser craters along the sampling path.

Although an exact estimation of the age of the captured specimens is difficult from the shell size and morphology due to the unpredictable growth rests and other environmental and physiological variables, previous studies have demonstrated that shells with similar size to those analyzed in this study present around a two-year lifespan.²⁵ Therefore, several cycles of the seasonal seawater temperature variations should then be revealed in the Mg/Ca ratios measured along the sampling path. The instrumental seawater temperatures were measured near Langre beach by the Aquaculture Facility of Santander's Oceanographic Centre, belonging to the Spanish Institute of Oceanography.

Results and discussion

Four limpets collected in February, May, August and November, 2006, were measured following a sampling path through the selected calcite layer, as previously described. Measurements provided a sequence of Mg/Ca ratios throughout the growth of every shell. Fig. 5 shows sequences corresponding to February (top) and November (bottom).

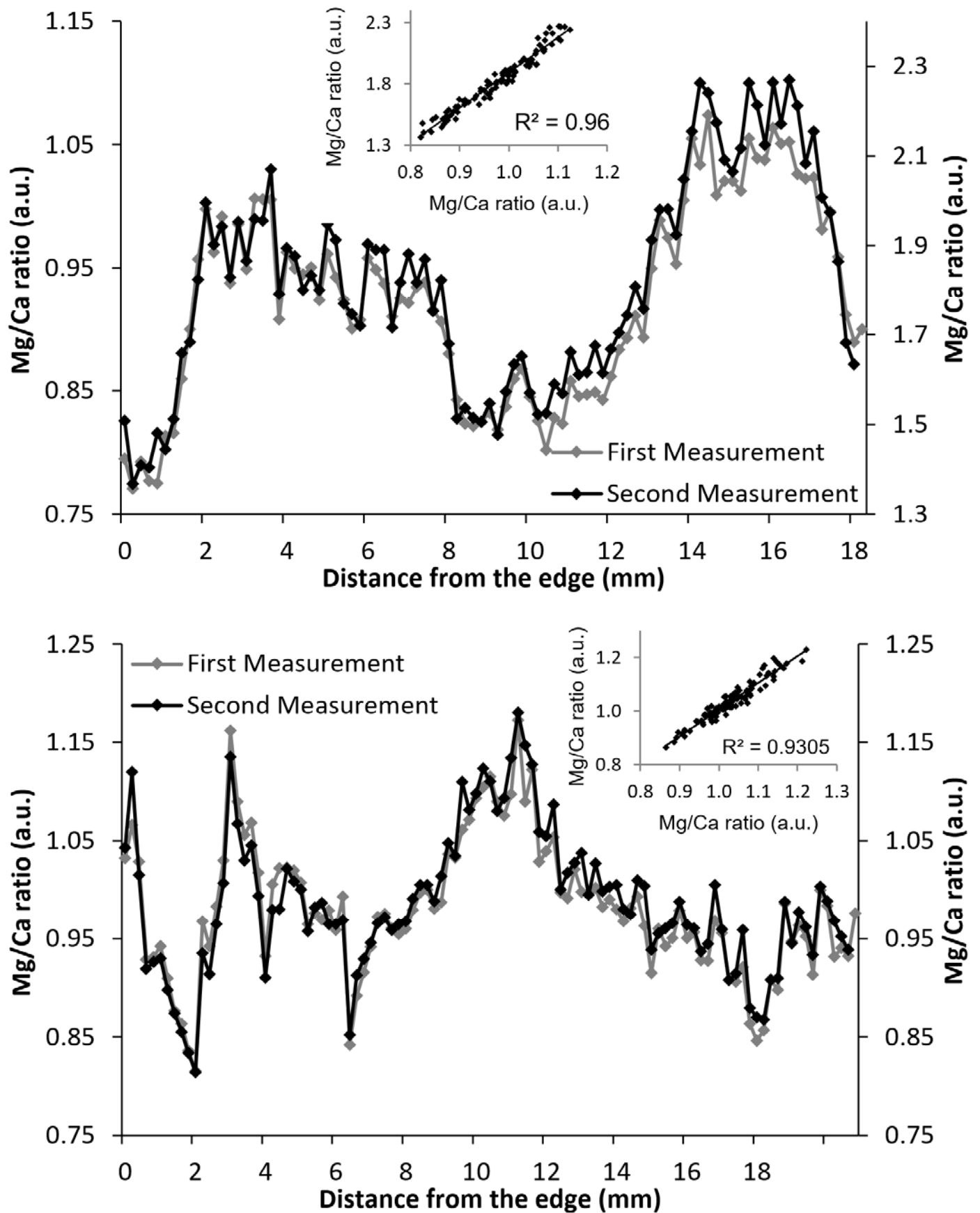


Fig. 5. Mg/Ca ratio sequences from limpets collected in February (top) and November, 2006 (bottom).

Fig. 5 (top) shows the sequence from the specimen collected in February. The two superimposed sequences correspond to two measurements along the same path, using the wide and narrow spectral windows already commented. Despite some differences, both sequences show similar trend and variability. It must be noted that both sequences were obtained in two consecutive experiments on the same sampling points, so the ablated material was not strictly the same. Both sequences show a clear long-term variation in the Mg/Ca ratios that could correspond to two years of growth. However, there is also a clear short-term variability, in contrast with sequences obtained with other spectrometric techniques (see for example Ferguson et al, 2011⁴ for HR-ICP-MS ratio measurements).

The same effect can be seen in Fig. 5 (bottom) for another specimen collected in November. In this case, Mg/Ca ratios were measured using the same spectral window in two consecutive experiments on the same sampling points. The resulting sequences are almost identical. This confirms the repeatability of the LIBS measurements and suggests the existence of local spatial variability in Mg/Ca ratios, not identified in previous studies using alternative spectrometric techniques. Our hypothesis is that physiological mechanisms affecting the magnesium substitution in the biogenesis of the calcium carbonate (in addition to the temperature dependence) create small-scale variations not observed by other analytical methods due to the difference in the sampled volume. Typical ICP procedures involve the extraction of about 100-150 μg of material per sampling point, while each ablated crater in our LIBS experiments removes around 0.2 μg of carbonate for 50 laser shots, a 500:1 difference. This fact opens up the possibility for LIBS to study these biomineralization mechanisms with higher spatial resolution.

However, the seasonal changes in seawater temperature can be masked by this small-scale variability. For this reason, we propose to post-process the Mg/Ca sequences using the Singular Spectral Analysis (SSA) algorithm. This method is widely used in climatic sciences to decompose time series into fast variation components, related to noise or weather events, and into slow variation components, related to climatic trends.²⁶ In this work, we used a Matlab® SSA implementation²⁷ to decompose Mg/Ca sequences into 8 components. Then, the sequences were reconstructed using only the first 4 components to remove the small-scale fluctuations. This algorithm has proved to be very effective in removing the unwanted local variability in the sequences while preserving not only the long-term annual temperature trends but also the abrupt

variations of the Mg/Ca ratios associated with resumed growth after prolonged cessation. An overview of the SSA-processed sequences compared to instrumental seawater temperatures is shown in Fig. 6.

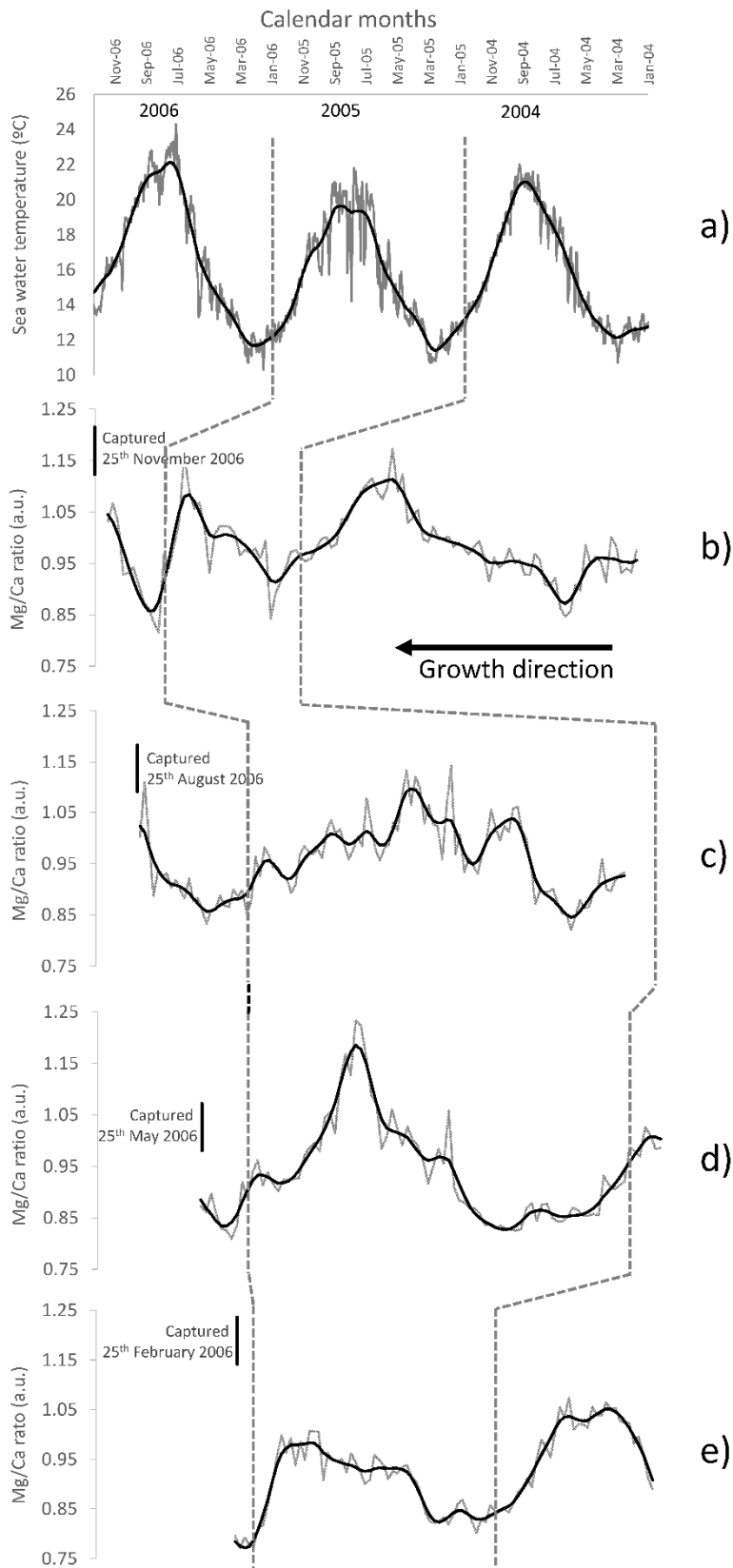


Fig. 6. Comparison between instrumental temperatures and Mg/Ca ratios: (a) Instrumental seawater temperatures recorded during the lifespan of the shells; (b-e) Mg/Ca ratios obtained with the proposed LIBS procedure (gray line) and the same data post-processed with the SSA algorithm (black line).

Instrumental seawater temperatures during the period 2004-2006 ranged between 10°C in winter and 23°C in summer (Fig. 6a). Seasonal variations observed in Mg/Ca ratios from all samples followed the same pattern as instrumental temperatures, although some differences were identified due to the variable growth rate of each individual (Fig. 6b-e). For example, the shell collected in November 2006 (Fig. 6b) shows a lifespan of three years and a temporal compression of the sequence for the most recent years. This is probably due to a slower growth rate, which is in agreement with the observed phenomenon of ontogenic metabolic slowdown in mature mollusks.²⁸ A clear seasonal variation and a shorter lifespan were deduced from Mg/Ca sequences belonging to the rest of the samples (Fig. 6c-e).

Despite the variable growth rates exhibited by the analyzed shells, Mg/Ca ratios also enable the estimation of season of shell collection, which has important implications for the interpretation of past human behavior. Mg/Ca ratios obtained from growth increments located close to the shell edge, and therefore generated immediately before the death of the mollusk, provide information on the season of collection. Thus, Mg/Ca ratios from samples collected in August, May and February (Fig. 6c-d-e) are in agreement with collection in summer, spring and winter, respectively. However, shell edge values from the sample collected in November 2006 indicate collection in late spring or early summer (Fig. 6b). The disagreement with the actual collection date is probably due to growth cessation during warmer months, as demonstrated by isotopic analyses from modern specimens of *P. vulgata* collected on the Cantabrian coast.²³ Growth cessation precludes recording environmental signals, such as that of seawater temperature, during a short time in the summer before growth is resumed again in autumn, and this could be promoted in this sample by the ontogenic growth slowdown mentioned above.

In addition to the general evolution of seawater temperatures, Mg/Ca ratios can also be used for temperature reconstruction, with reproducible results in different individuals of the same species. Fig. 7 shows the minimum, mean and maximum values of the four Mg/Ca sequences, plotted against the minimum, mean and maximum seawater temperature during the mollusk lifespans. Results suggest that there is good correlation between absolute Mg/Ca values and instrumental temperatures. Therefore, data obtained using modern limpets living under known environmental conditions provide a reference relationship between Mg/Ca ratios and instrumental seawater temperature, thus allowing the reconstruction of past seawater temperatures from the elemental sequences contained in archeological shells. The results from this investigation suggest that this methodology could be employed on those archaeological shells that have not suffered any fossilization or diagenesis processes. Our aim is the analysis of shells collected by the prehistoric humans during the Mesolithic, period dated around 8.000 year before present, a time span, that in this case and considering the type of conservation of the ancient samples recovered by us, is not sufficient to alter the shell composition.

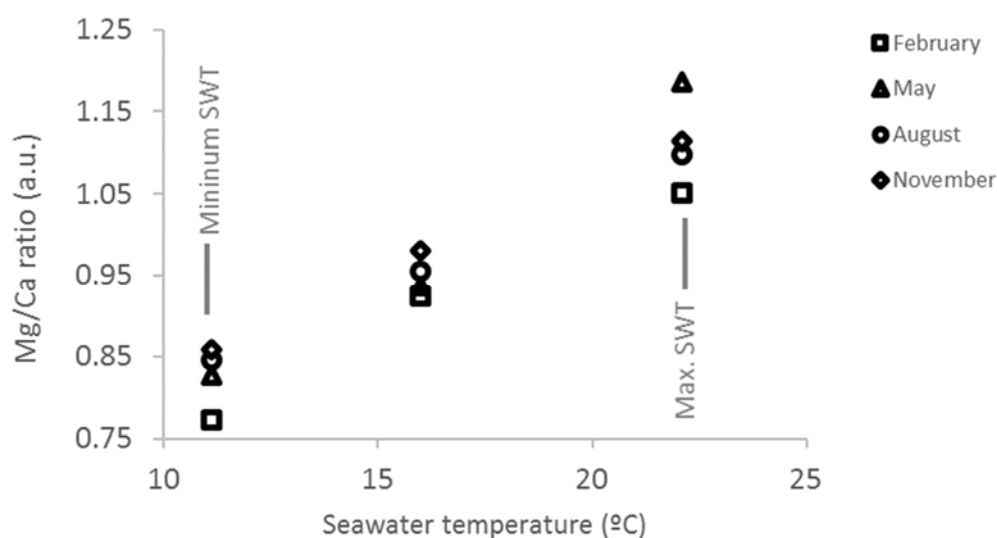


Fig. 7. Measured minimum, mean and maximum Mg/Ca ratios for each specimen in relation to the minimum, mean and maximum of instrumental seawater temperature (SWT).

It should be noted that the reliability of the studies on collection habits and past seawater temperatures is very dependent on the number of shells analyzed for each stratigraphic unit. The proposed LIBS technique could

therefore enable new statistically significant studies due to the great reduction in the total measurement time in comparison with other spectrometric techniques (for example, a 20:1 reduction compared with ICP-OES has been verified by the authors), along with less expensive and simpler instrumentation and operation.

Conclusion

The feasibility of LIBS to measure magnesium incorporation in biogenic calcium carbonate shells has been evaluated. It has been applied for the first time in shells of *P. vulgata* to obtain sequences of Mg/Ca ratios, that is, the evolution of Mg/Ca ratios throughout the mollusk's lifespan. As in previous studies with other species and other spectrometric techniques, a direct correlation between seawater temperature and Mg/Ca ratios has been found. The sequences obtained, from four modern shells collected in different months, revealed variable growth rates in the analyzed shells, but also a small-scale variability of Mg/Ca ratios due to the small sampling volume (< 1 µg in weight) used in the LIBS experiments. To reduce this small-scale variability while preserving the seasonal temperature-related variations, the sequences have been post-processed with the SSA smoothing algorithm. The sequences allow the estimation of the seasonal variations of the seawater temperature, its maximum and minimum annual values, and the season of collection for each limpet. This is particularly interesting in archaeological studies to infer the behavior of prehistoric human groups and in paleoclimatic sciences to reconstruct past environmental conditions. In addition to a simpler and less expensive instrumentation, LIBS offers a huge reduction in working time and thus enables statistically significant studies with a large number of shells. Those studies are being carried out at the moment with archeological samples from the Mesolithic shell midden site of El Mazo (Asturias, northern Spain), using the proposed methodology. Further research will be also focused on two main improvements: 1) chemical mapping of the shell layers to obtain new insight about the small-scale variations in Mg/Ca ratios; and 2) using the LIBS ablation process to obtain Mg/Ca ratios throughout the outer layer of the shell, thus avoiding the need of cutting it.

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