

1 **New highlights on phytolith structure and occluded carbon location: 3D X-ray microscopy**
2 **and NanoSIMS results**

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4 A. Alexandre¹, I. Basile-Doelsch¹, T. Delhaye², D. Borschneck¹, J.C. Mazur¹, P. Reyerson³, G.
5 M. Santos⁴

6 n phytolith structure and occluded carbon location: 3D X-ray microscopy and NanoSIMS
7 results

8 ¹ Centre Européen de Recherche et d'Enseignement des Géosciences de l'Environnement
9 (UMR 7330), CNRS, Aix-Marseille Université, Europôle méditerranéen de l'Arbois BP 80,
10 13545 Aix en Provence cedex 04, France

11 ² Plateforme NanoSIMS, OSUR, Université de Rennes 1, Campus de Beaulieu, 35042 Rennes
12 Cedex, France

13 ³ Department of Geography, University of Wisconsin-Madison, 550 North Park Street,
14 Madison, WI 53706, USA

15 ⁴ Earth System Science, University of California, Irvine, B321 Croul Hall, Irvine, CA 9269,
16 3100, USA

17

18 Correspondence e-mail : alexandre@cerege.fr

19 **Abstract**

20 Phytoliths contain occluded organic compounds called phytC. Recently, phytC content, nature,
21 origin, paleoenvironmental meaning and impact in the global C cycle has been the subject of
22 increasing debate. Inconsistencies were fed by the scarcity of in-situ characterization of phytC
23 in phytoliths. Here we reconstructed at high spatial resolution the 3-dimensional structure of
24 harvested grass short cell (GSC) phytoliths using 3D X-ray microscopy. While this technic has
25 been widely used for 3D reconstruction of biological systems it has never been applied in high
26 resolution mode to silica particles. Simultaneously, we investigated the location of phytC using
27 Nano-scale Secondary Ion Mass Spectrometry (NanoSIMS). Our data evidenced that the silica
28 structure contains micrometric internal cavities. These internal cavities were sometimes
29 observed isolated from the outside. Their opening may be an original feature or may result from
30 a beginning of dissolution of silica during the chemical extraction procedure, mimicking the
31 progressive dissolution process that can happen in natural environments. The phytC that may
32 originally occupy the cavities is thus susceptible to rapid oxidation. It was not detected by the
33 nanoSIMS technique. To the contrary another pool of phytC, continuously distributed in and
34 protected by the silica structure was evidenced. Its N/C ratio (0.27) is in agreement with the
35 presence of amino acids. These findings constitute a basis to further characterize the origin,
36 occlusion process, nature and accessibility of phytC, as a prerequisite for assessing its
37 significance in the global C cycle.

38

39 **1. Introduction**

40 When absorbing nutrients in the soil, plants roots also uptake a significant amount of silicon
41 (Si). The Si fluxes recycled by plants are substantial: as an example Si take up by tropical forests
42 or grasslands can reach twice to 10 times Si fluxes generated from the dissolution of soil
43 silicates that are exported to stream waters (e.g. Blecker et al., 2006; Struyf and Conley, 2009;
44 Cornelis, 2011; Alexandre et al., 2011). Inside the plant, Si is transported in the sap and
45 deposited inside the cells, in the cell walls and in extracellular spaces of stems and leaves as
46 micrometric hydrous amorphous silica particles called phytoliths. Upon plant decay, part of the
47 phytolith production can be incorporated into soils or sediments and preserved for up to millions
48 of years (Alexandre et al., 2011; Miller et al., 2012; Strömberg et al., 2013). Those fossil
49 phytolith assemblages can be used for reconstructing past vegetation and climate conditions via
50 their morphological and geochemical signatures (Piperno, 2006; Alexandre et al., 2012).
51 Phytoliths occlude small amounts of organic compounds, first evidenced by the production of

52 carbon (C) and nitrogen (N) during dry ashing (Jones and Beavers, 1963). Later on, scanning
53 transmission electron microscopy (STEM) and Energy Dispersive X-Ray (EDX) analyses of
54 phytoliths in the plant tissues confirmed that the occluded organic compounds contained C, N
55 and phosphorus (P) (Laue et al., 2007). By extension, these occluded compounds are here called
56 phytC. PhytC, which is assumed to be protected from natural oxidation by the siliceous
57 structure, has been the subject of increasing attention and debate.

58 Based on the assumption that phytC originated from the photosynthesis of atmospheric CO₂ in
59 the host plant, several studies used phytC ¹⁴C and δ¹³C signatures, respectively as a dating tool
60 (Piperno and Becker, 1996; Piperno and Stothert, 2003; McMichael et al., 2012) and a
61 paleoenvironmental proxy (Kelly, 1991; Smith and White, 2004; Carter, 2009; Webb and
62 Longstaffe, 2010; McInerney et al., 2011). However, very recently, ¹⁴C-AMS measurements of
63 phytC samples from modern grasses yielded ages of several thousand years, which suggested
64 that phytoliths may incorporate a substantial amount of old carbon, potentially from the soil
65 (Santos et al., 2010; Santos et al., 2012). Amino acids from soils have been shown to be taken
66 up by plants, and transported in small proportion to roots, stems and shoots (Paungfoo-
67 Lonhienne et al., 2008; Whiteside et al., 2009 ; Gao et al., 2010; Warren, 2012; Whiteside et
68 al., 2012). Thus it is not inconsistent to assume that C and N derived from these soil amino
69 acids have been trapped in phytoliths. Although the hypothesis still needs to be verified, it raised
70 the question of the molecular nature of phytC. Several techniques such as High-performance
71 liquid chromatography (HPLC), amino acid analyser, gas chromatography mass spectrometry
72 (GC-MS), protein staining, micro-Raman analysis or X-Ray photoelectron spectroscopy (XPS)
73 were used to characterize phytC and led to contradictory results, especially regarding the
74 presence or not of amino acids (Harrison, 1996; Pironon et al., 2001; Smith and Anderson,
75 2001; Elbaum et al., 2009; Watling et al., 2011). The problem is that these methods were applied
76 on phytolith concentrates that were not proven to be completely devoid of extraneous organic
77 remains. Chemical extractions leading to high purity phytolith concentrates are indeed difficult
78 to implement. Although the absence of organic particles can be checked by Scanning Electron
79 Microscopy (SEM) coupled with EDX (Corbineau et al., 2013), the presence of extraneous
80 organic remains on the phytolith surface cannot be accurately detected.

81 Differences in the efficiency of phytolith extraction protocols may also explain the
82 inconsistencies in phytC quantification. Accurately quantifying the phytC is important for the
83 assessment of its significance in the terrestrial C cycle. Multiple studies recently claimed that
84 phytC may play a role in atmospheric CO₂ sequestration and climate change mitigation (Parr

85 and Sullivan, 2005; Parr et al., 2010; Song et al., 2013 ; Huang et al., 2014; Li et al., 2014; Song
86 et al., 2014; Zuo et al., 2014), although the fluxes of phytC from vegetation to soils and the
87 residence time of phytC in soils are still largely unknown. PhytC content as high as 20% dry
88 weight was obtained when using a phytolith extraction method based on microwave digestion
89 (Parr and Sullivan, 2014). This value was more than 20 to 200 times higher than the values
90 obtained using a chemical method verified to be 100% efficient for removing extraneous
91 organic particles (from 0.1 to 1% dry weight; Smith and White, 2001). The difference was
92 somewhat justified by partial dissolution of phytC when using aggressive protocols. The
93 assumption that phytC may be located at different sites in the silica structure, with different
94 accessibility to oxidation, was put forward (Parr and Sullivan, 2014). This assumption
95 supplemented a previous one, widely found in the literature, that micrometric opaque areas
96 observed by Natural Light (NL) microscopy on some phytoliths, were holes containing the
97 phytC (Prychid et al., 2003; Piperno, 2006; Carter et al., 2009; Song et al., 2012; Parr and
98 Sullivan, 2014). No measurements were however performed to support any of these hypotheses.
99 Finally, the debates on content, location, nature, origin and paleoenvironmental meaning of
100 phytC were fed by the scarcity of in-situ characterization of phytC in phytoliths, despite few
101 seminal works (Harrison, 1996; Laue et al., 2007). Here we reconstructed, at high spatial
102 resolution, the 3-dimensional structure of grass phytoliths using 3D X-ray microscopy.
103 Simultaneously, we characterized the location of phytC using Nano-scale Secondary Ion Mass
104 Spectrometry (NanoSIMS).

105

106 **2. Material and methods**

107 Grasses are among the main producers of phytoliths. The leaves of *Triticum durum* wheat (TD-
108 F-L), were harvested in 2012 at the Genomics Research Centre in Fiorenzuola d'Arda (Italy).
109 Hundreds grams were made available to us for phytC investigation. Phytoliths were extracted
110 from 50g of dry leaves using a wet chemical protocol recently set up for geochemical analyses
111 of phytC. The protocol was described in detail in Corbineau et al. (2013). The organic matter
112 was oxidized with H₂SO₄, H₂O₂, HNO₃ and KClO₃, and potential remains on the phytolith
113 surfaces were dissolved using KOH (pH@11). Absence of residual extraneous organic particles
114 was checked using SEM-EDS (Corbineau et al., 2013). Dominant phytolith types were
115 recognized according to Madella et al. (2005) using NL microscopy at 600X and 1000X
116 magnifications. As expected, the Grass Short Cell group (GSC) and the Bulliform cell group
117 dominated the assemblage. These groups, that form in all grass epidermis, also dominate

118 phytolith assemblages produced by grasslands and recovered from soils (e.g. Alexandre et al.,
119 2011). Several NL microscopy and SEM pictures, illustrating the composition of the TD-F-L
120 phytolith assemblage, were taken. For the purpose of morphological comparison, pictures of
121 fossil GSC and bulliform phytoliths from available soil assemblages described in previous
122 papers, were additionally taken.

123 The 3D structure of the GSC phytoliths was reconstructed by X-ray imaging at the micro-scale,
124 using a 3D X-ray microscope Zeiss Ultra XRM L 200. A few phytoliths, randomly selected
125 from the bulk sample, were deposited on the inner surface of a bevel-cut Kepton tube of 50µm
126 of internal diameter. Five individual GSC phytoliths were recognized by optical microscopy at
127 200X magnification and their position located for 3D visualization. The principle of the 3D X-
128 ray microscopy technique consists in focusing the X-ray beam on a rotating sample using
129 optical lens; then transmitted x-rays are diffracted by a Fresnel zone plate on a scintillator in
130 front of an optical device to produce a 200X magnified image of the phytolith captured by a
131 CCD image sensor. Using a 1k x 1k detector, it leads to a voxel size of 63nm. X-ray beam path
132 is continuously flushed with helium to minimize the absorption of X-rays by air, the sample
133 and the optics excepted. While this technic has been widely used for 3D reconstruction of
134 biological systems it has never been applied in high resolution mode to silica particles. Analyses
135 of the phytoliths were proceeded at 150nm resolution for a 65µm field of view, in conventional
136 absorption contrast imaging mode at 8keV (copper rotating anode; power set at 40kV and
137 30mA). Using this mode, the contrast was generated both from the different x-ray attenuation
138 coefficients of the chemical elements composing the sample and from the density. Nine
139 hundreds one x-ray projections were recorded between -90° and +90° at an angle step of 0.2°
140 and an exposure time of 80s per view. After 20 hours of analysis, reconstruction of the phytolith
141 volume was performed using XMReconstructor (Zeiss Xradia software). The resulting stack of
142 2D grayscale slices was then exported to Avizo Fire (FEI group) for further image processing.

143 NanoSIMS analyses were performed on cross sections of TD-F-L phytoliths embedded in
144 epoxy resin. One mg of phytoliths was deposited on polytetrafluoroethylene (PTFE) filters
145 (9mm i.d.) stuck on double face tape. Polypropylene (PP) tubes (10mm i.d. and 15 mm long)
146 were placed on the tape, encircling the phytoliths. Epoxy resin (Araldite 100/hardener 16) was
147 slip into the tubes up to 3 mm height and left to dry 3H at 40°C. Seven mm height of resin was
148 added and left to dry 48H at 40°C. Those two steps prevented the resin to leak from the base of
149 the tube. Embedded samples were taken off the tubes and polished with diamond paste up to
150 0.1 µm, until the PTFE filter was completely removed and cross sections of phytoliths were

151 visible in NL microscopy. Samples were sawn into 4mm thick blocks. Dozens of GSC
152 phytoliths cross sections to be analyzed with the nanoSIMS were located by SEM. The
153 nanoSIMS technique is based upon the sputtering of a few atomic layers from the surface of a
154 sample induced by a primary ion bombardment. The primary ion impact triggers a cascade of
155 atomic collision. Atoms and atomic clusters are ejected. During the ejection process, some
156 atoms and clusters are spontaneously ionized. These secondary ions are characteristic of the
157 composition of the analyzed area. They are separated according to their mass and an image of
158 the intensity of the secondary ion beam is made for a selected mass (Cameca, 2014). Over the
159 past few years, the NanoSIMS technique was increasingly used in geosciences, to investigate
160 the elemental and isotopic composition of organic and inorganic materials (Herrmann et al.,
161 2007; Hatton et al., 2012; Mueller et al., 2012; Carsten W. Mueller, 2013). The NanoSIMS
162 technique was however scarcely used for measuring secondary ion emission from amorphous
163 silica. One study showed nanoSIMS images of a thin section of a giant siliceous sponge spicule
164 (several mm of diameter). A micrometric proteinaceous scaffold, which averaged 2% C dry
165 weight, could be detected in the siliceous structure (Müller et al., 2010). The NanoSIMS
166 technique was also used for identifying silicification sites in rice roots (Moore et al., 2011).
167 Here, we analyzed the intensities of $[^{28}\text{Si}]^-$, $[^{16}\text{O}]^-$, $[^{24}\text{C}_2]^-$ and $[^{26}\text{CN}]^-$ ions produced by selected
168 areas of the GSC phytoliths polished cross sections using a Cameca NanoSIMS 50. The section
169 was coated with 25nm gold and introduced in the NanoSIMS. A $[\text{Cs}]^+$ primary ion probe with
170 16kV primary ion impact energy and a 8kV secondary ion extraction voltage was used. The
171 best adjustment for obtaining secondary ion images of $[^{28}\text{Si}]^-$, $[^{16}\text{O}]^-$, $[^{12}\text{C}]^-$ and $[^{26}\text{CN}]^-$ was the
172 following: the selected phytolith surfaces were first pre-sputtered with a defocused primary
173 beam on a $60\mu\text{m}\times 60\mu\text{m}$ area during 3min. Then 256×256 pixel images were made using a 2.2
174 pA primary ion current (Primary Diaphragm Diameter = $300\mu\text{m}$), a counting time of 10 ms per
175 pixel for areas of $30\mu\text{m}\times 30\mu\text{m}$. Analyses with longer counting time, larger primary
176 diaphragm/higher primary beam intensity were also tested. Secondary ion images of $[^{28}\text{Si}]^-$,
177 $[^{16}\text{O}]^-$, $[^{12}\text{C}]^-$ and $[^{26}\text{CN}]^-$ were processed using the ImageJ software (<http://imagej.nih.gov/ij>).
178 Colors were assigned to different intensities of signal, increasing from black to red. Images of
179 the $[^{26}\text{CN}]^-/[^{12}\text{C}]^-$ ratio were also created. Line-scans were drawn across the analyzed surfaces
180 and ion intensity vs distance along the line were plotted.

181 For comparison with the NanoSIMS results, the C and N contents of the bulk TD-F-L phytolith
182 sample were measured by chemiluminescence after combustion at 1350° (for C) and 1000°C

183 (for N). The C and N contents of the Epoxy resin were measured with an Elemental Analyzer
184 (EA) after combustion at 1350°C.

185

186 3. Results

187 Three morphological categories of phytoliths, commonly found in grasses, constituted the bulk
188 sample. SEM pictures of phytoliths placed on the aluminum mount illustrate these categories
189 on figure 01. SEM pictures of cross sections of the same categories are shown on figure 02. For
190 each category, the mode of silica deposition is specified below when it has been previously
191 evidenced by MEB, MET, fluorescence microscopy or NanoSIMS images of plant cross
192 sections (Sangster and Parry, 1969; Sowers and Thurston, 1979; Harrison, 1996; Currie and
193 Perry, 2007; Law and Exley, 2011; Moore et al., 2011). The first phytolith category is
194 constituted of thin fragments of multi-cellular silica sheets, several tenths of μm long and wide
195 but less than a few micrometers thick (fig01a, 01b, 02a, 02b). These silica “skeletons”(Sangster
196 and Parry, 1969; Law and Exley, 2011) were shown to result from the silicification of the
197 middle lamella of the cells walls in grass epidermis and mesophyll, possibly as an early step of
198 silicification (Laue et al., 2007; Law and Exley, 2012). Although abundant in plants (Piperno,
199 2006), the multi-cellular silica sheets are rapidly subjected to fragmentation and dissolution and
200 are scarcely preserved in soils and sediments (Alexandre et al., 1996). The second phytolith
201 category is constituted of stellate silica particles, of 10-15 μm width (fig01c) that were shown
202 to form in the intercellular spaces of the grass epidermis (Lins et al., 2002). This mode of silica
203 precipitation was described as centripetal, starting as a narrow band lining the cavity, then
204 infilling partially or completely the intercellular space (Sangster and Parry, 1981). The third
205 category dominated the wheat phytolith assemblage. It is made of mono-cellular phytolith types
206 of 10-50 μm of length, width and thickness. Most of them are GSC phytoliths and belong to the
207 Rondel (Fig01d, 01e) and Polylobate (fig01f) types. The formation of the mono-cellular
208 phytolith type was also shown to be centripetal, starting in or against the cell walls and
209 progressively infilling the lumen (e.g. Zhang et al., 2013). The processes that leads to complete
210 silicification of the cells and to organic compounds occlusion are still unknown. Cellulose
211 fibrils from the cell wall may regulate the silica formation (Laue et al., 2007).

212 SEM observation of cross sections of tenths of GSC phytoliths evidenced one or two internal
213 cavities a few micrometers of diameter in the silica structure (fig02c, 02d, 02e). They were
214 similar in shape and size as the low electron density round area visible on one of the TEM image

215 of phytoliths shown by Laue et al. (2007) (Fig. 2 B of Laue et al., 2007). However, SEM
216 observation of the GSC phytoliths just placed on the aluminum mount did not evidence any
217 holes on the phytolith surfaces. When observed in NL microscopy (fig03) the internal cavities
218 were recognizable as opaque areas.

219 Two examples of reconstructed 3D X-ray microscopy volumes are presented in figures 04 and
220 05. The observed patterns were common to the five analyzed GSC particles. The siliceous
221 structure appeared porous at the sub-micrometer scale (fig0 4A and 05A). Inside the structure,
222 areas of a few micrometers of diameter, with significantly lower X-ray absorption than the
223 surrounding, were observed (fig04A). 2D-planes of the reconstructed volumes evidenced that
224 these heterogeneities were the cavities several micrometers wide previously identified on the
225 cross sections by SEM. The cavities were interconnected (fig04B, 05B). Some particles showed
226 cavities isolated from the phytolith surface by a few micrometers thick silica wall (fig04B).
227 Other particles showed cavities connected to the phytolith surface by small holes of one-tenth
228 micrometers of diameter only (fig05B). These cavities appeared filled with air (no X-ray
229 absorption), although the high contrast in X-Ray absorbance between silica and air may have
230 masked the presence of organic compounds.

231 The NanoSIMS results, common to the dozens of analyzed phytolith thin sections, are
232 illustrated in Figures 06, 07 and 08. Adjustments were done to find the pre-sputtering duration
233 (3 min), the primary ion beam intensity (L1=2kV), the primary diaphragm diameter (750 μ m)
234 and the duration of analyses (11 min) appropriate for obtaining sufficient total ion current (TIC)
235 and avoid charging effects (fig06A, 07A). When the primary ion beam intensity was increased
236 to L1=4kV (fig08A), when the primary diaphragm diameter was decreased to 300 μ m (fig08B),
237 or when a succession of analyses resulted in increasing the duration of sputtering (fig08C), a
238 zone devoid of secondary ion signal appeared at the center of the silica surface. This was
239 probably due to charging (Mueller et al., 2012) and/or to topographic heterogeneity
240 (Winterholler et al., 2008). As silica was more resistant to polishing than the Epoxy, silica
241 surfaces were often convex (fig08). The tests conducted here emphasized the importance of
242 looking for the most efficient adjustment (i.e. avoiding charging and topographic effects) before
243 performing NanoSIMS analyses on silica surfaces.

244 [^{28}Si] $^-$, [^{16}O] $^-$, [^{12}C] $^-$ and [^{26}CN] $^-$ images clearly individualized phytoliths from the Epoxy resin.
245 The [^{28}Si] $^-$ and [^{16}O] $^-$ images and scan lines showed that phytoliths were made of a continuous
246 silica structure (fig06, 07) sometimes interrupted by central micrometric areas devoid of silica
247 (fig07). This is again in concordance with the central cavities identified in SEM and 3D-X-Ray

248 imaging. Carbon was present in the cavities and in the silica structure itself. However when
249 values of $[^{12}\text{C}]^-$ intensity were similar in the cavities and in the Epoxy resin, they were 10 to 20
250 times lower in the silica structure than in the Epoxy resin (fig06, 07). N was also present in the
251 silica structure and $[^{26}\text{CN}]^-$ intensity was 3 to 4 times lower in the silica structure than in the
252 cavities or the Epoxy (fig06, 07). Interestingly, the ratio $[^{26}\text{CN}]^-/[^{12}\text{C}]^-$ ranged between 20 and
253 30 in the silica structure and between 5 and 10 in the cavities and the Epoxy. The silica structure
254 was thus enriched in N by a 4 to 8 factor, relatively to the surrounding Epoxy. These features
255 were reproducible from a particle to another. Bulk C and N contents in phytoliths, measured by
256 chemiluminescence and EA (cf material and methods), were, for phytoliths 0.4 and 0.1% dry
257 weight respectively, and for the Epoxy resin 68.8 and 2.8% dry weight respectively. The N/C
258 ratio was 0.27 for the phytoliths and 0.04 for the Epoxy resin. The bulk phytolith sample was
259 thus enriched in N relatively to the Epoxy resin by a factor 6.8, in agreement with N enrichments
260 calculated from the NanoSIMS data. This consistency strengthened the accuracy of the $[^{12}\text{C}]^-$
261 and $[^{26}\text{CN}]^-$ relative intensities measured with the nanoSIMS. Finally, $[^{26}\text{CN}]^-/[^{12}\text{C}]^-$ NanoSIMS
262 images clearly showed that organic compounds, with content in N significantly higher than in
263 the resin, were continuously distributed (at the sub-micrometer scale) in the silica structure. To
264 the contrary, cavities appeared filled with the Epoxy resin.

265

266 4. Discussion

267

268 4.1. PhytC locations in the silica structure of GSC phytoliths

269 SEM, 3D X-Ray microscopy and NanoSIMS images showed that the silica structure of GSC
270 phytoliths was homogeneous at the micrometric scale, and systematically contained central
271 micrometric interconnected cavities. The fact that some particles contained cavities isolated
272 from the outside suggests that the opening to the outside can be either original or result from
273 dissolution posterior to the phytolith formation. Phytoliths often contain % to % dry weight of
274 aluminium (Al) (Bartoli and Wilding, 1980; Carnelli et al., 2004) co-precipitating with silica
275 (Hodson and Sangster, 1993). As Al dissolves in strong acids and in strong bases, the phytolith
276 chemical extraction procedure that included HNO_3 and H_2SO_4 steps, may have initiated
277 phytolith surficial dissolution and opened the few micrometers thick silica wall between the
278 cavities and the phytolith surface. The procedure also included a final alkaline step (KOH @
279 pH 11) that may also have increased the dissolution features on the silica surfaces. As phytoliths

280 were directly extracted from the plant, the surficial dissolution was revealed here at its
281 beginning. It is expected to reach higher degree over time in natural environment where
282 multiple dissolution factors come into play (Iler, 1979; Bartoli, 1983). Large dissolution
283 features were indeed often observed on fossil phytoliths and were quantified to assess the degree
284 of weathering of soil phytolith assemblages (Alexandre et al., 1999; Oleschko et al., 2004). To
285 illustrate this point SEM and NL microscopy images of whole and cross sections of fossil mono-
286 cellular phytoliths collected from soils are shown on Figure 09. The phytolith types are
287 characteristic of grass epidermis (GSC types and Cuneiform bulliform types; Madella et al.,
288 2005) (fig 09A, 09B) and wood parenchyma (Globular granulate type; Madella et al., 2005)
289 (fig9C). The dissolution of silica has made central depressions of several micrometers wide.
290 The particles appear empty inside, which is consistent with dissolution starting from the silica
291 walls located between the cavities and the phytolith surfaces, then slightly opening, or
292 increasing the opening of the cavities to the outside, then enlarging the cavities into dissolution
293 depressions. Such dissolution depressions are not limited to GSC phytoliths. They were
294 observed on many types of mono-cellular phytoliths from grasses and non-grasses extracted
295 from soils and sediments as illustrated in Figure 9A5 (Acicular type), 9B2 and 9B3 (Globular
296 granulate). This implies that the inner part of all these phytolith types was constituted of silica
297 less dense than the outer part, either due to phytC occlusion or to a lack of dissolved Si available
298 for precipitation during the phytolith formation.

299 Inside the internal cavities, no original organic compounds could be detected by NanoSIMS. If
300 initially present, they may have been squeezed out and replaced by the Epoxy resin during the
301 polishing step. To the contrary, the $[^{26}\text{CN}]^-/[^{12}\text{C}]^-$ images clearly evidenced the presence of
302 organic compounds rich in N continuously distributed in the silica structure and clearly
303 differentiated from the Epoxy resin. Absolute composition in $[^{26}\text{CN}]^-$ and $[^{12}\text{C}]^-$ were not
304 calculated. This would have required to include in the analyzed section standard materials with
305 known composition. However, the consistency of N enrichment of the organic compound in the
306 silica structure (measured by NanoSIMS) with N enrichment of the bulk phytC (measured by
307 chemiluminescences/EA), supports that the organic compound measured by NanoSIMS is
308 phytC. Finally, although our data cannot conclude on the presence or not of any phytC in the
309 internal cavities, they demonstrate that the phytC is, in a whole or in a part, continuously
310 distributed in the silica structure.

311

312 **4.2. Implications regarding phytC occlusion and phytC accessibility**

313 Evidences of the continuous distribution of phytC in the silica structure, at the sub-micrometric
314 scale, suggest that it was occluded since the early stage of silicification. SEM, environmental
315 scanning electron microscope (ESEM) and TEM-EDX analyses showed that silica first
316 precipitates in the inner cell wall, probably triggered by the presence of callose or lignin (Laue
317 et al., 2007; Law and Exeley, 2012; Zhang et al., 2013). Silica nanospheres are then organized
318 in a variety of structural motifs such as sheet-like, globular and fibrillar bundles that, from the
319 cell wall, infill the cell lumen in a centripetal way (e.g. Kaufman et al., 1970; Robert et al.,
320 1973; Sangster and Parry, 1981; Perry et al., 1984; Laue et al., 2007; Zhang et al., 2013), until
321 most of the cell becomes silicified (Motomura, 2004; Laue et al., 2006). As previously noted,
322 an organic template may participate to the silica formation (Harrison, 1996; Laue et al., 2007).
323 This organic template, progressively trapped in the silica structure may constitute the phytC
324 evidenced by NanoSIMS in the phytoliths. Its N/C value (0.27) is in the range of N/C values
325 characteristic of amino acids. Amino acids may originate either from the cell itself or from the
326 extra-cellular space. Different families of transporters have been identified for their import into
327 plant cells (Tegeder, 2012). In the same time, amino acids entering the cell simultaneously to
328 silica thanks to an invagination/vesicle formation mechanism previously evidenced (Neumann
329 and De Figueiredo, 2002) may occur.

330 At the end of the cell silicification, residual cell organic compounds that were not already
331 occluded may gather in a remaining space and delimitate the micrometric central cavities. This
332 second pool of phytC should be rapidly oxidized when phytoliths start to dissolve after their
333 deposition in litter, soil or sediment (fig.09). This suggests that this phytC pool participates in
334 a limited extent to long term atmospheric CO₂ sequestration. These considerations rise the need
335 to further estimate the respective contributions to C contents measured from bulk phytolith
336 concentrates of (i) phytC in the silica structure, of (ii) phytC in the central cavities, and of (iii)
337 extraneous C that may remain on porous phytolith surfaces. This is a prerequisite for reliable
338 assessments of the significance of phytC in atmospheric CO₂ sequestration. For that purpose,
339 phytC contents measured from phytolith assemblages characterized by 3D X-Ray microscopy
340 as dominated by phytoliths with closed internal cavities or by phytoliths with open cavities
341 should be compared.

342 **4.3. Reassessment of NL microscopy observations**

343 Several studies have speculated that opaque areas observed by NL microscopy in fossil
344 phytoliths from soils and sediments were burnt organic remains indicative of past fire
345 occurrence (Piperno, 1998; Kealhofer and Penny, 1998; Elbaum et al., 2003; Parr, 2006;

346 Piperno, 2006). However, when observed by NL microscopy, the empty dissolution depressions
347 evidenced by SEM on mono-cellular phytoliths from soils (fig09A) also appeared as opaque
348 areas, especially when they were oriented downwards (fig09C). This is probably due to trapped
349 air in the dissolution depressions that caused optical artifact at the place where air met the
350 mounting medium. This feature implies that opaque areas in fossil phytoliths should not be
351 considered as unequivocal evidence of burnt organic compounds. Similarly, internal cavities
352 may also appear as opaque spots due to the occurrence of trapped air, independently of the
353 presence of organic compounds.

354

355 **5. Conclusion**

356 3D-XRay microscopy reconstructions of GSC phytoliths from harvested grasses, and SEM
357 observations of their cross sections, showed that the silica structure contains micrometric
358 internal cavities. These cavities were sometimes observed isolated from the outside. Their
359 opening may be an original feature or may result from the silica dissolution during the chemical
360 extraction procedure, mimicking the beginning of dissolution process that may happen in
361 natural environments. The phytC that may originally occupy those cavities is thus susceptible
362 to rapid oxidation. It was not detected by the nanoSIMS technique. To the contrary another pool
363 of phytC, continuously distributed in and protected by the silica structure, was evidenced by
364 nanoSIMS. Its N/C ratio (0.27) is in agreement with the presence of amino acids. **These findings**
365 **constitute a basis to further characterize the origin, occlusion process, nature and accessibility**
366 **of phytC, necessary for assessing its significance in the global C cycle.** The authors would like
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369

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- 538

539 **Figures captions**

540 **Figure 1.** SEM images of TD-F-L wheat phytolith assemblage, deposited on aluminum mount.

541 Three categories are illustrated: 1) silica sheets (a,b), 2) stellate type from intercellular space
542 (c) and 3) GSC phytoliths including Rondel type (d,e) and Polylobate type (f).

543 **Figure 2.** SEM images of the thin section of the TD-F-L wheat phytolith types including silica
544 sheets (a,b), and GSC phytoliths of the Rondel type (c,d,e). GSC types show micrometric
545 internal cavities (IC).

546 **Figure 3.** NL microscopy images of grass short cell (GSC) phytolith types from the wheat TD-
547 F-L sample showing opaque areas (O).

548 **Figure 4:** 3D X-ray microscopy of a GSC phytolith from wheat (TD-F-L): **A:** four view of the
549 reconstructed volumes; internal cavities (IC) are distinguishable. **B:** 2D x-ray slices
550 superimposed on the phytolith volume rendering showing from front to back the internal
551 cavity (IC). No connection to the surfaces was evidenced. The blue area corresponds to the
552 thresholding of the phytolith grayscale values.

553 **Figure 5:** 3D X-ray microscopy of a GSC phytolith from wheat (TD-F-L): **A:** reconstructed
554 volume. **B:** 2D X-ray images from back to front of the phytolith showing the internal cavity
555 (IC) and its connection to the surfaces forming holes (H). The blue area corresponds to the
556 thresholding of the phytolith grayscale values.

557 **Figure 6.** NanoSIMS images and intensities of a first typical GSC phytolith (Rondel type) from
558 TD-F-L (wheat) embedded in Epoxy resin (polished section). Pre-sputtering: L1=2kV
559 defocused (60X60mm) [Cs]⁺ primary beam, during 3min **A:** [²⁸Si]⁻, [¹⁶O]⁻, [¹²C₂]⁻, [¹²C
560 ¹⁴N]⁻ and [¹²C ¹⁴N]⁻/ [¹²C₂]⁻ images obtained with a [Cs]⁺ primary beam with L1=2kV, D1-
561 1 primary diaphragm (750µm), during 11min; **B:** Secondary ion intensities along line scans
562 (red line in Fig. 6A).

563 **Figure 7.** NanoSIMS images and intensities of a second typical GSC phytolith (Rondel type)
564 from TD-F-L (wheat) embedded in Epoxy resin (polished section). Pre-sputtering: L1=2kV
565 defocused (60X60mm) [Cs]⁺ primary beam, during 3min **A:** [²⁸Si]⁻, [¹⁶O]⁻, [¹²C₂]⁻, [¹²C
566 ¹⁴N]⁻ and [¹²C ¹⁴N]⁻/ [¹²C₂]⁻ images obtained with a [Cs]⁺ primary beam with L1=2kV, D1-1
567 primary diaphragm (750µm), during 11min; **B:** [²⁸Si]⁻ image obtained with a [Cs]⁺ primary
568 beam increased with L1=4kV, D1-1 primary diaphragm, during 11min; **C:** Secondary ion
569 intensities along line scans (red line in Fig. 5A).

570 **Figure 8.** SEM images of the polished section showing convex silica surfaces (Si) in the Epoxy
571 resin (r). Associated NanoSIMS [^{28}Si] $^{-}$ images showing central areas devoid of secondary
572 ion signal. **A:** [Cs] $^{+}$ primary beam with L1=4kV, D 1-1 primary diaphragm (750 μm), 11min;
573 **B:** [Cs] $^{+}$ primary beam with L1=2kV, D 1-2 primary diaphragm (300 μm), 11min; **C:** [Cs] $^{+}$
574 primary beam with L1=2kV, D1-1 primary diaphragm (750 μm), 3min analyses for
575 successively 1,2 and 3.

576 **Figure 9.** NL microscopy and SEM images of dissolution depressions (DD) affecting fossil
577 phytoliths from soils. **A:** Grass epidermis monocellular phytoliths (Cuneiform Bulliform
578 types and Acicular type) from Mascareignite (MSG 70, La Réunion, France) (Crespin et al.,
579 2008); NL microscopy phytolith surface (1, 2), SEM phytolith volume (3) and polished
580 section (4, 5). **B:** Grass epidermis monocellular phytoliths from a ferruginous soil (Salitre,
581 Brazil) (Alexandre et al., 1999); NL microscopy phytolith **C:** Phytoliths from palms and
582 trees from a ferallitic soil (Dimonika, RDA) (Alexandre et al., 1997); SEM Globular
583 granulate type volumes (1, 2) and polished section (3). **D:** Opaque areas observed in NL
584 microscopy on bulliform cell phytoliths from MSG 70 (1,2) and Salitre (3,4). Scale bars:
585 10 μm .

586

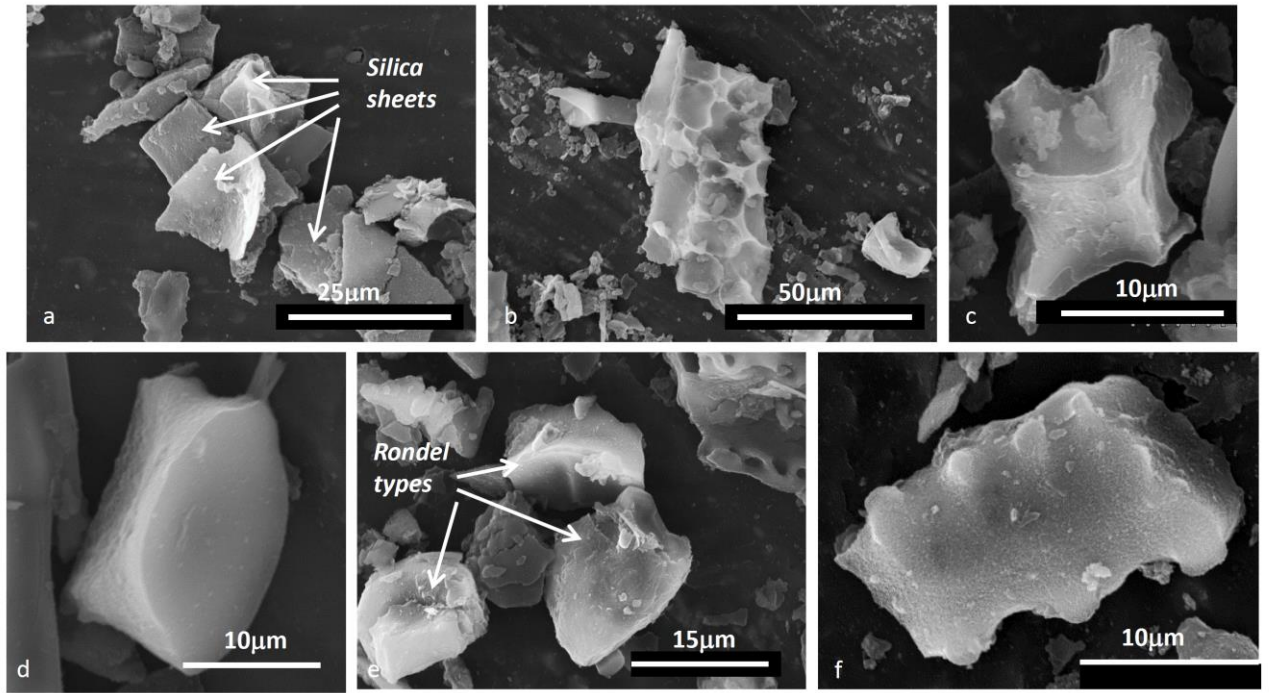


Figure 1.

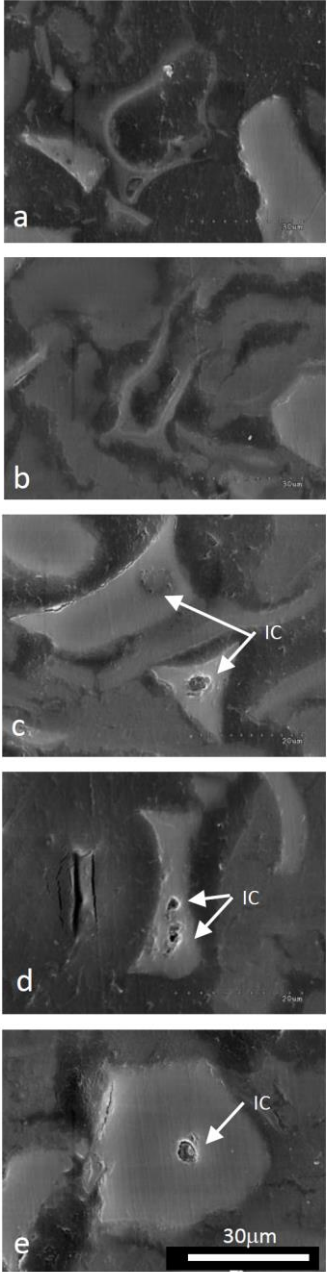


Figure 2.

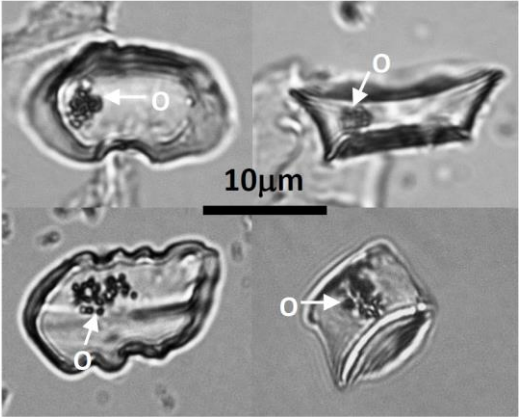


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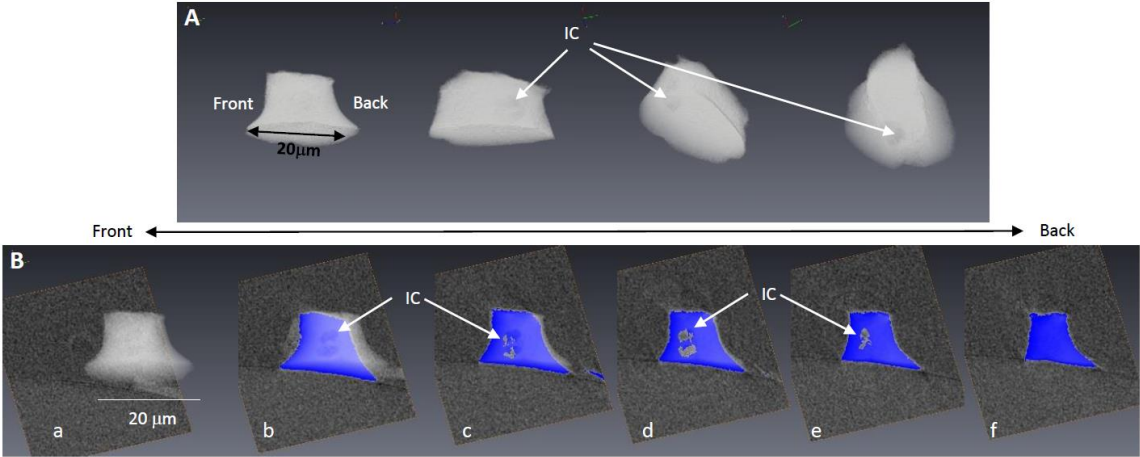


Figure 4

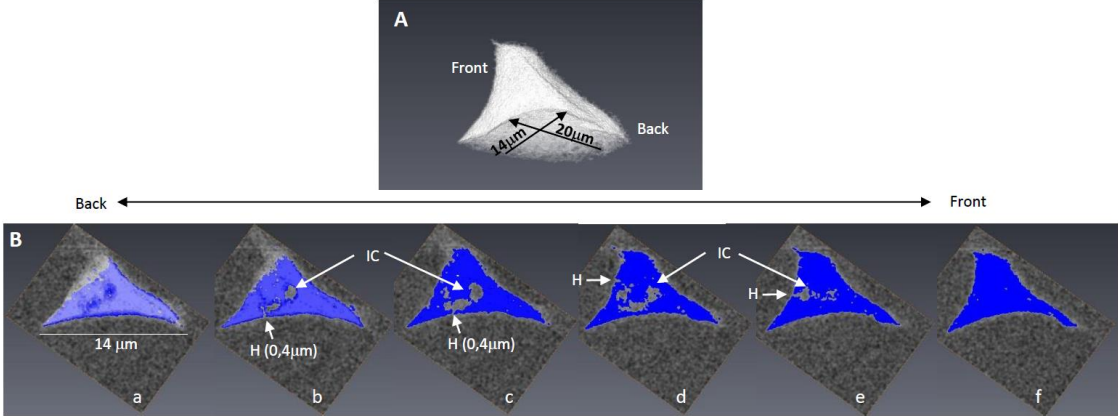


Figure 5

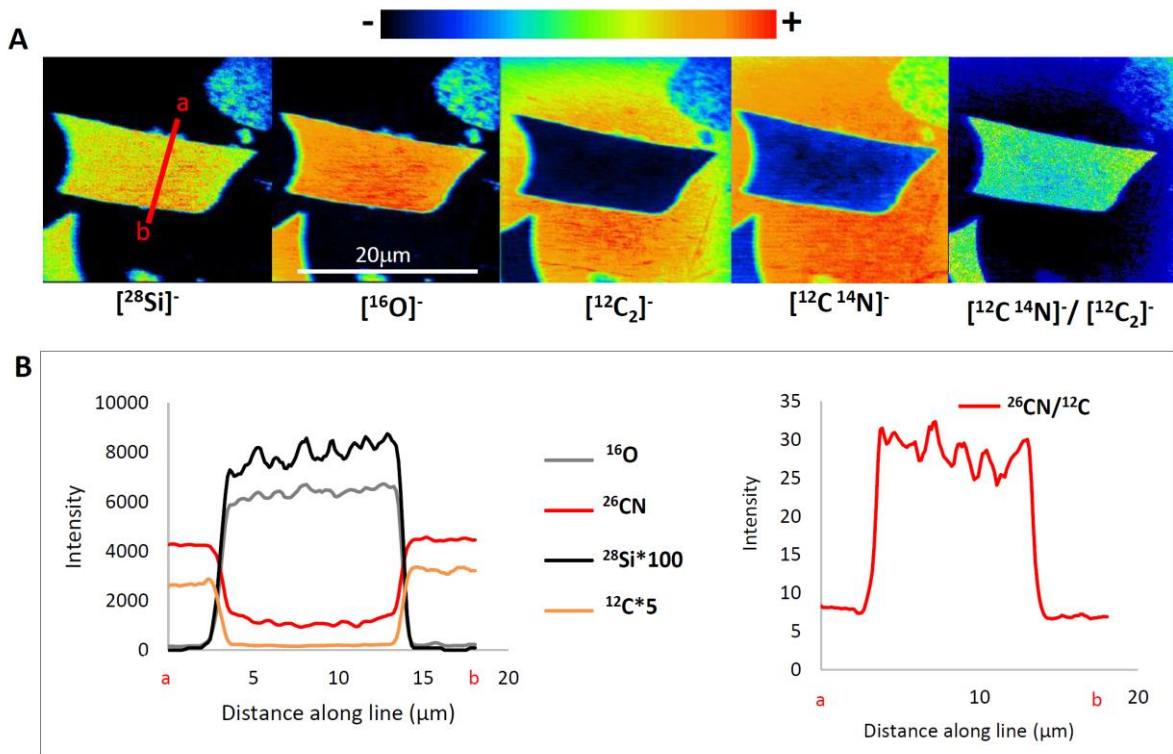


Figure 6.

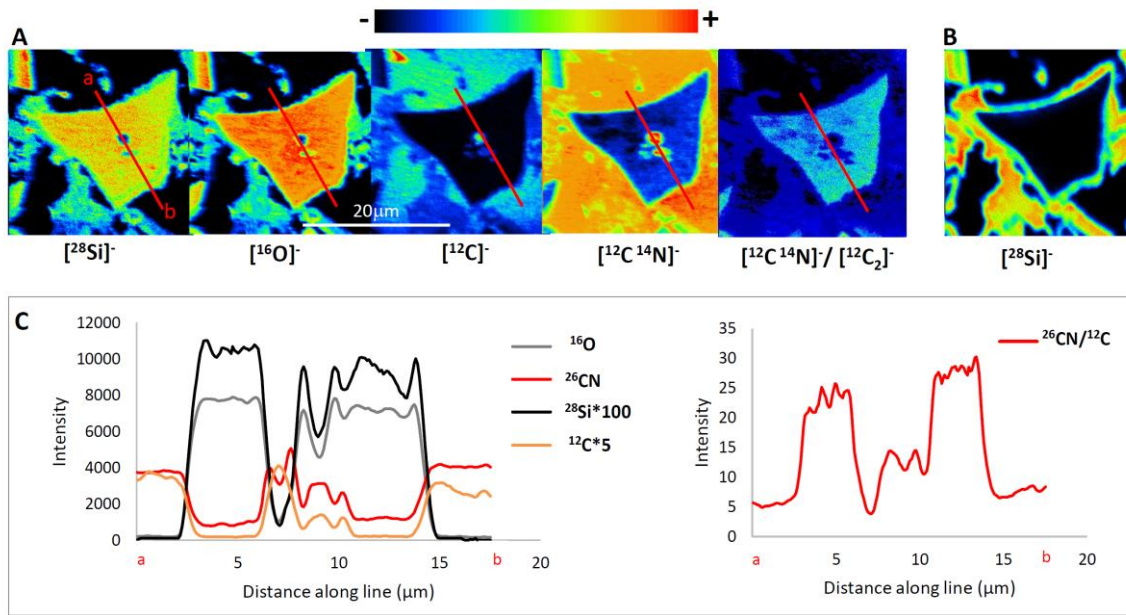


Figure 7.

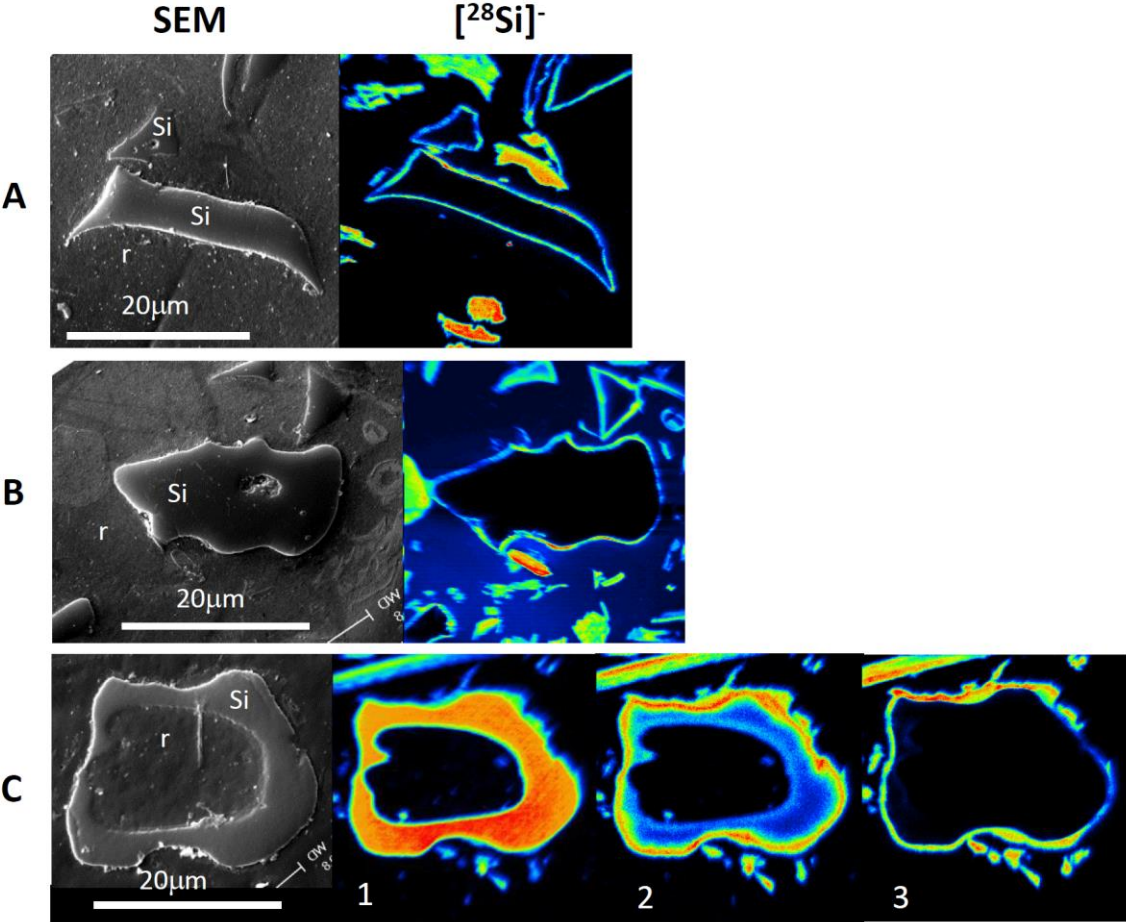


Figure 8.

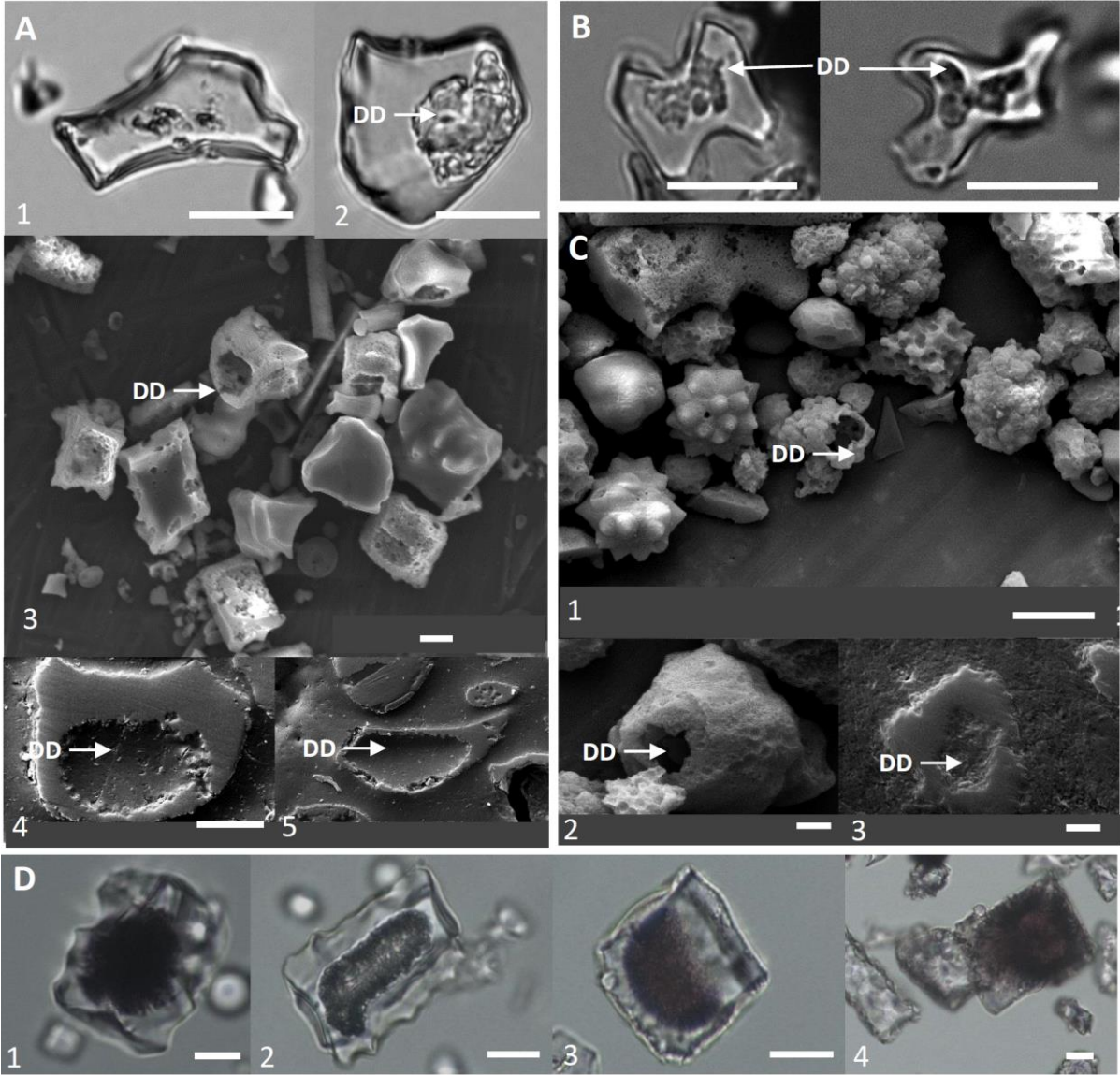


Figure 9.