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1 **Research Article**

2

3 **Biochemical characterization of the skeletal matrix of the massive coral,**  
4 ***Porites australiensis* - The saccharide moieties and their localization**

5

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25

26

27 **Abstract**

28 To construct calcium carbonate skeletons of sophisticated architecture, scleractinian corals  
29 secrete an extracellular skeletal organic matrix (SOM) from aboral ectodermal cells. The  
30 SOM, which is composed of proteins, saccharides, and lipids, performs functions critical for  
31 skeleton formation. Even though polysaccharides constitute the major component of the  
32 SOM, its contribution to coral skeleton formation is poorly understood. To this end, we  
33 analyzed the SOM of the massive colonial coral, *Porites australiensis*, the skeleton of which  
34 has drawn great research interest because it records environmental conditions throughout the  
35 life of the colony. The coral skeleton was extensively cleaned, decalcified with acetic acid,  
36 and organic fractions were separated based on solubility. These fractions were analyzed using  
37 various techniques, including SDS-PAGE, FT-IR, *in vitro* crystallization, CHNS analysis,  
38 chromatography analysis of monosaccharide and enzyme-linked lectin assay (ELLA). We  
39 confirmed the acidic nature of SOM and the presence of sulphate, which is thought to initiate  
40 CaCO<sub>3</sub> crystallization. In order to analyze glycan structures, we performed ELLA on the  
41 soluble SOM for the first time and found that it exhibits strong specificity to *Datura*  
42 *stramonium* lectin (DSL). Furthermore, using biotinylated DSL with anti-biotin antibody  
43 conjugated to nanogold, *in situ* localization of DSL-binding polysaccharides in the *P.*  
44 *australiensis* skeleton was performed. Signals were distributed on the surfaces of fiber-like  
45 crystals of the skeleton, suggesting that polysaccharides may modulate crystal shape. Our  
46 study emphasizes the importance of sugar moieties in biomineralization of scleractinian  
47 corals.  
48  
49

50 **Keywords**

51 *Porites australiensis*, biomineralization, coral, skeletal organic matrix, saccharide

52

53 **Abbreviations**

54 AIM: acid-insoluble matrix

55 ASM: acid-soluble matrix

56 DSL: *Datura stramonium* lectin

57 ELLA: enzyme-linked lectin assay

58 FT-IR: Fourier transform infrared spectroscopy

59 SOM: skeletal organic matrix

60

61

## 62 **Introduction**

63 Scleractinian corals are marine animals known for their capacity to elaborate calcium  
64 carbonate exoskeletons of complex shapes, many of which form reefs of great size (Spalding  
65 et al., 2001). Among metazoans, scleractinians are the main producers of biogenic aragonite in  
66 marine ecosystems (Milliman, 1993). From a geochemical viewpoint, scleractinian  
67 biomineralization can be considered as the uptake of inorganic ions from the environment and  
68 conversion of these ions into a spatially structured network of calcium carbonate biocrystals  
69 that contain trace elements (Beck et al., 1992; Tambutté et al., 2011). Because this process  
70 literally ‘freezes’ environmental information into skeletal tissues that fuel steadily  
71 sedimentary archives, scleractinian skeletons are often used to reconstruct sequences of  
72 paleoenvironmental conditions, in particular, past seawater temperatures via measures of  
73 Sr/Ca, Li/Ca, and  $\delta^{18}\text{O}/\delta^{16}\text{O}$  ratios (Beck et al., 1992; Druffel, 1997; Hathorne et al., 2013;  
74 Simkiss and Wilbur, 2012) or past ocean pH via boron isotopic composition ( $\delta^{11}\text{B}$ ) (Rollion-  
75 Bard et al., 2011). However, in spite of the increasing use of these geochemical proxies in  
76 corals, the molecular basis of scleractinian skeleton formation is far from understood.

77  
78 From a cellular viewpoint, scleractinian skeletal biomineralization is typically an epithelium-  
79 driven process, *i.e.*, all precursor components of the exoskeleton are secreted by the  
80 calicoblastic epithelium (also called the aboral ectoderm), a subset of the ectoderm (Allemand  
81 et al., 2004; Constantz and Weiner, 1988; Tambutté et al., 2011), in contact with the nascent  
82 mineral layer. These components comprise inorganic ions - calcium, bicarbonate and minor  
83 elements, *i.e.*, strontium and magnesium - that are extruded via membrane channels and  
84 pumps (transcellular pathway) or via intercellular space (paracellular pathway), and are  
85 combined into  $\text{CaCO}_3$  minerals in the sub-calicoblastic space (Tambutté et al., 2011). They  
86 also include a large set of organic macromolecules that constitute the skeletal organic matrix  
87 (SOM), which is allegedly released via a classical secretory pathway. This latter is however  
88 poorly documented for cnidarians in general.

89  
90 From a biochemical viewpoint, in many coral species examined to date, the SOM contains  
91 proteins, glycoproteins, lipids, and polysaccharides that are included in the skeleton during the  
92 calcification process (Constantz and Weiner, 1988; Cuif et al., 1999b; Dauphin, 2001; Farre et  
93 al., 2010; Puvarel et al., 2005). The SOM is distributed heterogeneously in the coral skeleton,  
94 being concentrated in “centres of crystallization” (Bryan, 1941; Ogilvie, 1896) or in “early

95 mineralization zones” (Cuif et al., 2003), from which fiber-like crystals distribute radially.  
96 The SOM is also detected on the top surface and growing steps in crystals (Cuif and Dauphin,  
97 2005). The complex topographical relationship between the organic and mineral phases  
98 suggests that the SOM plays an active role in regulating the initiation and growth of the  
99 calcium carbonate crystals. In particular, the SOM contains acidic macromolecules, usually  
100 considered as key-components for interacting with calcium carbonate crystals: in different *in*  
101 *vitro* functional assays, they have been shown to bind calcium ions (Isa and Okazaki, 1987)  
102 and modify the shapes of calcium carbonate crystal (Naggi et al., 2018; Ramos-Silva et al.,  
103 2014) similarly to what has been observed with mollusc SOM (Addadi and Weiner, 1985;  
104 Weiner and Hood, 1975). Amino acid analyses of SOMs of various coral species reported high  
105 contents of aspartic and glutamic acids (Constantz and Weiner, 1988; Cuif et al., 1999a;  
106 Gautret et al., 1997; Mitterer, 1978; Puvarel et al., 2005). More recently, aspartic acid-rich  
107 proteins in coral skeletons were predicted by whole genome sequencing of *Acropora*  
108 *digitifera* (Shinzato et al., 2011), and subsequently confirmed by transcriptomic and  
109 proteomic studies (Drake et al., 2013; Ramos-Silva et al., 2013b; Takeuchi et al., 2016).

110  
111 Contrary to these recent comprehensive surveys of skeletal proteomes, sugar moieties in coral  
112 SOM have not been well characterized, even though they are always detected in coral  
113 skeletons and are believed to play important, although undetermined functions in  
114 biomineralization (Albeck et al., 1996). The sugars in coral SOM include common  
115 monosaccharides, such as neutral (fucose, rhamnose, galactose, glucose, mannose, xylose),  
116 aminated (galactosamine and glucosamine) and acidic (glucuronic/galacturonic acids)  
117 hexoses. Previous studies showed that their proportions vary greatly among species (Cuif et  
118 al., 1999b; Naggi et al., 2018; Ramos-Silva et al., 2014), such that some present remarkable  
119 monosaccharide signatures. For example, in the *Acropora millepora* SOM, arabinose  
120 represents more than 60% of all monosaccharides, a finding correlated with its abundance in  
121 mucus of that species (Ramos-Silva et al., 2014; Wild et al., 2005). In addition, sulphated  
122 acidic sugars were detected in SOMs of several scleractinian coral species (Dauphin et al.,  
123 2008; Puvarel et al., 2005). However, polymeric structure and localization of saccharides in  
124 coral skeletons have not been studied.

125  
126 In this context, we have undertaken the overall biochemical characterization of the SOM of  
127 the massive colonial coral, *Porites australiensis*. By virtue of their longevity and ability to  
128 accumulate massive calcium carbonate skeletons, the genus *Porites* represents one of the most

129 important corals as a recorder of environmental conditions (Beck et al., 1992; Cobb et al.,  
130 2003; Linsley et al., 2000; Watanabe et al., 2011). We characterized the *Porites* SOM by using  
131 various molecular analyses, including SDS-PAGE, FT-IR, CHNS analysis, and *in vitro*  
132 calcium carbonate crystallization experiment. We confirmed the presence of sulphate, which  
133 contributes to the acidic nature of the *Porites* SOM. Furthermore, monosaccharides were  
134 quantified, and for the first time in corals, enzyme-linked lectin assay (ELLA) was performed.  
135 *In situ* localization of polysaccharide in coral skeleton was achieved using a biotinylated  
136 lectin (*Datura stramonium* Lectin - DSL) associated with anti-biotin antibody conjugated to  
137 nanogold. Our results showed that a DSL-reactive saccharide fraction was evenly dispersed  
138 on the surfaces of calcium carbonate crystals, suggesting that it may shape crystal growth  
139 during coral skeleton formation.

140

141

## 142 **Materials and methods**

### 143 **Sample collection and cleaning**

144 A living coral colony of *Porites australiensis* (approx. weight 50 grams) was collected at the  
145 Sesoko Marine Station, University of Ryukyus, Okinawa, Japan, under Okinawa prefecture  
146 permit (Number: 20–69). The organism was immersed overnight in 3L of 10x diluted  
147 household bleach solution and then extensively rinsed with water. This process, which was  
148 continued until the complete removal of animal tissue and other organisms on the surface,  
149 comprised the initial bleaching. The coral skeleton was rinsed, air-dried, and crushed into  
150 ~2mm fragments with a Jaw-crusher (Retsch BB200). Fragments were immersed in 0.1x  
151 sodium hypochlorite 10-15% (SIGMA ref. number 71696) for 50h (second bleaching). Then,  
152 the fragments were washed with twice-distilled water, dried, and powdered using a mortar  
153 grinder (Frisch Pulverisette 2). The powder (48 grams) was sieved (pore size <200 µm) and  
154 separated into two batches. The first was subsequently decalcified (see below), while the  
155 second was bleached overnight in NaOCl solution (third bleaching), thoroughly washed and  
156 air-dried at 37°C before decalcification.

157

### 158 **Extraction of skeletal organic matrices**

159 The cleaned powder samples (second or third bleaching) were suspended in cold water and  
160 decalcified overnight at 4°C by progressively adding (100 µL every 5 sec.) cold dilute acetic  
161 acid (10% vol/vol) with an electronic burette (Titronic Universal, Schott, Mainz, Germany).

162 After 15 hours (final pH 4), the clear solution was centrifuged (3900 G, 30 min.), in order to  
163 separate the pellet containing the acid-insoluble matrix or AIM from the supernatant, *i.e.*, the  
164 acid-soluble matrix (ASM). The AIM pellet was resuspended in Milli-Q water, centrifuged,  
165 and the supernatant discarded. After three cycles, the pellet was freeze-dried. The ASM  
166 solution was filtered (5  $\mu\text{m}$ ) on a Nalgene filtration apparatus and concentrated by  
167 ultrafiltration (Amicon stirred cell 400 mL) on a 10kDa cutoff membrane (Millipore, ref.  
168 PLGC07610). The concentrated solution (15 mL) was dialyzed 4 days against 1 L MilliQ  
169 water with several water changes, and freeze-dried.

170

### 171 **SDS-PAGE**

172 ASM lyophilisates were suspended in Milli-Q and an aliquot was added to an equal volume of  
173 2x Laemmli sample buffer containing  $\beta$ -mercaptoethanol. AIM pellets were gently  
174 fragmented with a scalpel and a part was directly resuspended in 1x Laemmli sample buffer.  
175 All preparations were denatured for 5 min at 99°C, cooled on ice and briefly centrifuged.  
176 While ASM was totally dissolved by denaturation, a part of AIM remained insoluble.  
177 Consequently, only the Laemmli-soluble fraction of AIM (referred to as LS-AIM) was further  
178 analyzed on gels. Proteins were run on precast 10%-20% gradient mini-gels (Bio-Rad) in  
179 mini-Protean III system. Gels were stained with silver nitrate (Morrissey, 1981), Stains-all  
180 (Campbell et al., 1983; Marin et al., 2005) and Alcian blue (Thornton et al., 1996) at pH 1.0 in  
181 order to detect sulphate groups.

182

### 183 **FT-IR spectroscopy**

184 FT-IR spectroscopy was used to check the overall chemical properties of the extracted  
185 matrices (ASM and AIM) after two or three bleaching steps. In each case, minute chips of  
186 lyophilized samples were analyzed with a Bruker Vector 22 instrument (Bruker Optics Sarl,  
187 Marne la Vallée, France) fitted with a Specac Golden Gate Attenuated Total reflectance (ATR)  
188 device (Specac Ltd, Orpington, UK) in the 4000–500  $\text{cm}^{-1}$  wavenumber range (twelve scans  
189 at a spectral resolution of 4  $\text{cm}^{-1}$ ). The choice for ATR mode was dictated by its reliability and  
190 reproducibility, as recently shown (Beasley et al., 2014). The qualitative assignment of  
191 absorption bands was performed by comparison with previously described spectra,  
192 determined by us or available in the literature (Dauphin, 2001; Dauphin et al., 2008; Kanold  
193 et al., 2015).

194

### 195 **Elemental analysis**

196 Elemental analyses were performed at the “Plateforme d’Analyse Chimique et de Synthèse  
197 Moléculaire de l’Université de Bourgogne (PACSMUB)” on a Fisons EA 1108 CHNS-O  
198 apparatus (M. Soustelle). As the technique requires 5 mg of material, only one AIM extract  
199 (AIM 3bl) was tested, and the measurement was performed twice.

200

### 201 ***In vitro* crystallization of CaCO<sub>3</sub> with ASM**

202 *In vitro* crystal growth experiments in the presence of ASM was tested as described in a  
203 previous paper (Kanold et al., 2015). In brief, 200 µL of 10 mM CaCl<sub>2</sub> containing ASM at  
204 increasing concentrations (1-32 µg/ml) was applied to a 16-well culture slide (Lab-Tek,  
205 Nunc/Thermo Scientific, Rochester, NY, USA). The glass slide was closed with a plastic  
206 cover in which 1-mm holes were pierced above each well to allow exposure of the solution to  
207 ammonium bicarbonate vapor, and sealed with Parafilm. The slide was placed in a desiccator,  
208 together with ammonium bicarbonate crystals, under vacuum and incubated at 4 °C for 3  
209 days. Subsequently, the solution was carefully removed by suction with a blunt needle  
210 connected to a vacuum pump and CaCO<sub>3</sub> crystals generated on the glass bottom were dried  
211 for 4h at 37°C. These were directly observed using a tabletop scanning electron microscope  
212 (TM 1000, Hitachi) without carbon sputtering.

213

### 214 **Monosaccharide analysis**

215 Monosaccharide quantification of ASMs and AIMs after two or three bleaching steps was  
216 performed according to the HPAE-PAD technology (High Pressure Anion-Exchange - Pulsed  
217 Amperometric Detection) on an ICS-3000, Dionex system equipped with a Dionex  
218 CarboPac™ PA-20 (3x150 mm) analytical column. In short, lyophilized samples were  
219 hydrolyzed in 2M trifluoroacetic acid at 105 °C for 4 h (100 µg/100 µL), and the solution was  
220 neutralized with sodium hydroxide. Hydrolytic conditions deacetylate N-acetyl-glucosamine  
221 and N-acetyl-galactosamine, which are subsequently analyzed as glucosamine and  
222 galactosamine, respectively. Filtered samples (20 µL) were eluted at 0.4 mL/min (35°C) using  
223 the following sodium hydroxide gradient: pure water 99.2% /250 mM NaOH 0.8% : 0→20  
224 min; pure water 75%/250 mM NaOH 20% /NaOAc (1M)- NaOH (20 mM) 5%: 20→37min ;  
225 pure water 40% /250 mM NaOH 20%/NaOAc (1M)-NaOH (20 mM) 40%: 37→41min. Each  
226 elution was followed by a wash and subsequent equilibration time. External sugar and uronic  
227 acids standards were used for calibration (7 points per curve): fucose, glucose, xylose,  
228 galactose, mannose, rhamnose, arabinose, glucosamine, galactosamine, galacturonic acid and  
229 glucuronic acid (all provided by Sigma-Aldrich).

230

231 **Enzyme-Linked Lectin Assay (ELLA)**

232 Enzyme Linked Lectin Assay (ELLA) was conducted as described previously (Kanold et al.,  
233 2015) on ASM fractions. This test, performed in solution, cannot be applied to AIM. Briefly,  
234 96-well plates (MaxiSorp, Nunc/Thermo Scientific, Nunc A/S, Roskilde, Denmark) were  
235 coated with ASM (50 ng/well) and incubated for 90 min at 37°C. They were washed three  
236 times with a solution of TBS/Tween-20 (0.5 mL Tween 20 per L) spread using a manual  
237 microplate 8-channel washer (Nunc Immuno Wash), and subsequently blocked with Carbo-  
238 free blocking solution (Vector Laboratories, ref. SP-5040) for 60 min at 37°C. Three sets of 7  
239 biotinylated lectins were tested (Vector Laboratories, Peterborough, UK, ref. BK-1000, BK-  
240 2000, BK-3000). They were applied to the wells (dilution to 10 µg/mL) and incubated for 90  
241 min at 37°C. Unbound lectins were removed by washing five times with TBS/Tween-20.  
242 Then, a solution containing alkaline phosphatase-conjugated avidin (Avidin-AP, Sigma  
243 A7294, St Louis, MO, USA) diluted 70,000 times was added (100 µL per well) and incubated  
244 for 90 min at 37 °C. Microplates were washed as before, and incubated with ELISA substrate  
245 solution (10% vol/vol diethanolamine in Milli-Q water, pH 9.8) containing phosphatase  
246 substrate (0.5 mg/mL, 4-nitrophenyl phosphate disodium salt hexahydrate (pNPP) tablet,  
247 Sigma, ref. UN3500-A) at 37 °C. They were incubated at 37°C and read every 15 minutes at  
248 405 nm using a Bio-Rad Model 680 micro-plate reader. Results were normalized and  
249 converted to percentage of reactivity by subtracting the background (negative control  
250 comprising ASM without lectin but with Avidin-AP) from all values and by considering the  
251 highest response as 100%. The test was repeated at least three times. For detailed information  
252 on the binding preference of each lectin, see Immel et al. (Immel et al., 2016).

253

254 ***In situ* localization: lectin-gold assay.**

255 Sodium hypochlorite-bleached fragments of *P. australiensis* skeleton were cut with a diamond  
256 saw (Dremel) and one surface was flattened with fine sand paper. After rinsing with  
257 sonication, the surface was finely polished with an alumine suspension (0.05 µm) on a rotary  
258 polisher until a mirror polish was obtained. Samples were thoroughly cleaned by sonication  
259 and submitted to bleach (NaOCl, 0.26 % active chlorine) to remove contaminants that may  
260 have spread over the surface during polishing. They were rinsed twice with Milli-Q water, and  
261 gently dried on Whatman paper. The flat surface was then etched 5 minutes with a solution of  
262 1% EDTA (wt/vol) in the sonication bath to expose the skeletal matrix, then rinsed with Milli-  
263 Q water (5 min.).

264

265 All incubation steps described after were performed at room temperature. In brief, coral  
266 samples were incubated one hour in Carbo-free blocking solution alone ((Vector Laboratories,  
267 ref. SP-5040), then overnight in the same solution containing the biotinylated DSL (*Datura*  
268 *stramonium* lectin, diluted 100 times), and in the presence of the bactericidal agent, sodium  
269 azide (0.005% wt/vol). Samples were gently rinsed several times with TBS/Tween20, then  
270 incubated at least 90 minutes in a Carbo-free solution containing diluted (1/100) goat anti-  
271 biotin antibody conjugated to ultra-small gold particles (0.8 nm) (GABio, Ultra Small, ref.  
272 800.088; Aurion, Wareningen, The Netherlands). Samples were rinsed several times with  
273 TBS/tween20 and gently dried on filter paper. Enhancement of the gold signal was achieved  
274 by incubating samples in silver enhancement solution (British Biocell International, ref.  
275 SEKL15) for about 15-20 minutes. Samples were rinsed once in Milli-Q water, then dried at  
276 37°C.

277

278 Negative controls were performed in parallel under identical conditions, by omitting one or  
279 more incubation steps: no incubation step with DSL; incubation with gold-coupled anti-biotin,  
280 followed by silver enhancement; no incubation with DSL nor with biotin, but with silver  
281 enhancement. All samples were carbon-sputtered and observed either with a JEOL JSM 760 F  
282 field emission scanning electron microscope (JEOL, Tokyo, Japan) or with an ultra-high  
283 resolution, cold-field emission Hitachi SU8230 scanning electron microscope. Quick checks  
284 were performed with a tabletop SEM Hitachi TM1000. The experiment was repeated five  
285 times.

286

287

## 288 **Results**

### 289 **Cleaning of coral skeleton and total matrix quantification**

290 Because the *Porites* skeleton is porous, having a mesh-like structure, thorough cleaning of the  
291 skeleton was required to remove soft tissues of the coral and microorganisms present in the  
292 pores. We repeatedly cleaned pieces of coral skeleton by immersing them completely in  
293 bleach solution and rinsing exhaustively with ultrapure water until tissue and epibionts on the  
294 skeletal surface were completely removed (first bleaching). However, even though the  
295 skeleton looked superficially clean after this initial treatment, a careful check using SEM  
296 indicated that fibrous organic material was still present in the pores (Figure 1A, C). After a

297 second bleach treatment, these contaminants were removed (Figure 1B, D). Finally, to ensure  
298 the complete removal of contaminants, a third bleaching was performed on sieved skeletal  
299 powder (grain size < 200  $\mu\text{m}$ ). The total organic matrices were quantified after 2<sup>nd</sup> and 3<sup>rd</sup>  
300 bleaching as shown in Table 1. Interestingly, the amount of organic matrix - both soluble  
301 (ASM) and insoluble (AIM) - extracted after the 3<sup>rd</sup> bleaching was significantly lower  
302 (reduced by half) than that obtained after the 2<sup>nd</sup> one. The organic matrix represents 0.14%  
303 and 0.07% of the total skeleton by weight, after the 2<sup>nd</sup> and the 3<sup>rd</sup> bleaching, respectively.

304

### 305 **SDS-PAGE**

306 The ASM and the AIM (or, at least, its Laemmli-soluble moieties, referred to as LS-AIM)  
307 extracted from the 2<sup>nd</sup> and 3<sup>rd</sup> bleachings were analyzed by poly-acrylamide gel  
308 electrophoresis (SDS-PAGE) after staining with silver nitrate, Stains-all, and Alcian blue  
309 (Figure 2). Overall, broad and smeary staining was observed in all staining methods. Weak,  
310 blurred bands of high molecular weight (>170 kDa) and in the range of 50-70 kDa were  
311 detected in the silver stained gel, as well as negatively stained zones at the bottom of the lanes  
312 (<10 kDa for lane 2<sup>nd</sup> bleaching, and <17 kDa for the three other lanes) (Figure 2A). Stains-all  
313 staining showed a strong purple color in ASMs, while AIMs appeared more pinkish. (Figure  
314 2B). Such purple staining - between metachromatic blue and red - has already been observed  
315 in a previous study for ASM of other skeletal matrices, suggesting their ability to bind  
316 calcium (Marin et al., 2005). Alcian blue staining at low-pH (pH 1.0) stained both ASM and  
317 AIM (Figure 2C), suggesting the presence of sulphate groups in all four samples (Thornton et  
318 al., 1996). Low-molecular weight ASMs stained more intensely, while for AIMS, high-  
319 molecular weight compounds stained more strongly.

320

### 321 **FT-IR and CHNS analyses**

322 Fourier transform infrared (FT-IR) spectra were analyzed for SOMs (Figure 3). First, the  
323 absence of the typical carbonate peaks in the ASM and in the AIM demonstrated that both  
324 extracts did not contain any contaminating salts resulting from the decalcification. In all  
325 fractions, bands attributed to protein or sugar backbones were detected at around 3380  $\text{cm}^{-1}$ ,  
326 1650  $\text{cm}^{-1}$ , and 1540  $\text{cm}^{-1}$ , which correspond to amide A ( $\nu\text{N-H}$ ), amide I ( $\nu\text{C=O}$ ), and amide  
327 II ( $\nu\text{C-N}$ ) bands, respectively. The signal of amide I band was stronger than that of amide II in  
328 ASM samples, while they were equivalent in AIM fractions. Weak signals at 2921-2923  $\text{cm}^{-1}$   
329 in AIM and 2927-2937  $\text{cm}^{-1}$  in ASM, representing  $\nu\text{C-H}$  stretching vibrations, were also  
330 detected and may correspond to lipids. Signals around 1450 and 1410  $\text{cm}^{-1}$  may be related to

331 carboxylic groups. An absorption band specific to carbohydrate was observed near 1060-1070  
332  $\text{cm}^{-1}$  in all samples. In AIM fractions, this signal was significantly weaker after the third  
333 bleaching AIM with respect to the adjacent signal at  $1150 \text{ cm}^{-1}$ , which was sharply detected in  
334 both AIM samples. The band at  $1150 \text{ cm}^{-1}$ , together with signals at 961, 636 and  $554 \text{ cm}^{-1}$ ,  
335 corresponds to phosphate group (Panda et al., 2003; Rivera-Muñoz, 2011). These bands were  
336 hardly visible in ASM (shoulders at  $1150 \text{ cm}^{-1}$  and  $961 \text{ cm}^{-1}$ ); presumably because of their  
337 weak amplitudes, they were masked by other broad signals. The signal at  $1230\text{-}1250 \text{ cm}^{-1}$  is  
338 related to  $\nu\text{S=O}$  stretching vibration (Cabassi et al., 1978; Cael et al., 1976; Longas and  
339 Breitweiser, 1991). The presence of sulfur in the third bleaching AIM was also supported by  
340 CHNS analysis (Table 2).

341

### 342 ***In vitro* CaCO<sub>3</sub> crystallization with ASM**

343 Results of the *in vitro* crystallization assay (diffusion test) are shown in Figure 4. Crystals  
344 were grown in solution containing ASM, extracted after two (Figure 4A to E) and three  
345 (Figure 4G to L) bleach treatments. Addition of  $1 \mu\text{g/mL}$  ASM (Figure 4A, G) resulted in  
346 almost no difference with the control experiment (without ASM, Figure 4M), in which  
347 typical, rhombohedral calcite crystals were synthesized. The morphology of crystals was  
348 altered in the presence of  $4 \mu\text{g/mL}$  ASM (Figure 4B, H). Terraced structure appeared on the  
349 edges, and the crystal size was reduced. The increased concentration of ASM ( $16 \mu\text{g/mL}$ , 2<sup>nd</sup>  
350 bleach) resulted in polycrystalline structure (Figure 4C, D), and this effect was more  
351 significant at  $32 \mu\text{g/mL}$  of ASM (Figure E, F). In comparison, the alteration of crystal shape  
352 was more severe when high concentrations of ASM extracted after 3 bleaching steps were  
353 used: at  $16 \mu\text{g/mL}$ , crystal edges were rounded and the surface was rough-textured (Figure 4I,  
354 J). At the highest concentration ( $32 \mu\text{g/mL}$ ), flat crystal faces were completely destroyed, and  
355 aggregation of crystals was frequently observed (Figure 4K, L). In summary, higher  
356 concentrations of ASMs altered the morphology and size of CaCO<sub>3</sub> crystals, and this effect  
357 was more accentuated when using ASM extracted after 3 bleaching steps.

358

### 359 **Monosaccharide analysis**

360 Results of the monosaccharide analysis after mild hydrolysis (with TFA) of both ASM and  
361 AIM, after 2 or 3 bleaching steps, are shown in Figure 5, as relative percentages of the total  
362 sugar composition. The data allow a double comparison, between AIM and ASM on the one  
363 hand, and between the 2<sup>nd</sup> and 3<sup>rd</sup> bleaching on the other hand. First, in both ASMs, the most  
364 abundant saccharides are galactose>arabinose>fucose, with the two first representing  $\geq 50\%$

365 of all sugars. Xylose, glucosamine, glucose and mannose are minor monosaccharides  
366 (between 4 and 10%) while rhamnose and galactosamine are quantitatively negligible (below  
367 3%). The sugar profiles of both AIMs are somewhat similar to those of ASMs, except that  
368 galactose and glucose are the dominant sugars ( $\sim \geq 20\%$  each), with  $\sim 15\%$  arabinose. Xylose  
369 and fucose ( $>10\%$ ) are next most abundant, followed by glucosamine and mannose (5-10%).  
370 As in ASMs, rhamnose and galactosamine are extremely minor. Galacturonic and glucuronic  
371 acids were not detected in any of our samples. Second, the third bleaching treatment had a  
372 more pronounced effect on ASMs than on AIMs: while it did not modify the sugar  
373 abundances in AIMs, resulting in two superimposable AIM histogram patterns, it induced in  
374 ASM a large decrease of arabinose (from 36.2 to 20.7%) and slight increases in galactose  
375 (28.6 to 29.2%), fucose (13.2 to 16.2%), glucosamine (4.2 to 8.4%), glucose (5.1 to 7.4%) and  
376 xylose (5.6 to 8.6%). However, in spite of these few percentage changes, the 3rd bleaching  
377 did not fundamentally modify the monosaccharide composition pattern of ASM.

378

#### 379 **ELLA**

380 In order to determine the types of glycan structures present in ASM (extracted after two and  
381 three bleaching steps), the affinity of these two extracts for biotinylated lectins was examined  
382 by enzyme-linked lectin assay (ELLA) (Kanold et al., 2015). The results, expressed as  
383 percentages of reactivity with respect to the most reactive lectin, are shown in Figure 6. It is  
384 noteworthy that the two ASM extracts reacted almost identically to the 21 different lectins: in  
385 both cases, the strongest signal by far was observed with *Datura stramonium* lectin (DSL),  
386 which preferentially binds to ( $\beta$ -1,4) linked GlcNAc oligomers, LacNAc, and poly-LacNAc  
387 (Sondej et al., 2009). Greatly reduced affinities (20-25% of those obtained with DSL) were  
388 recorded with Concanavalin A (ConA), *Lycopersicon esculentum* lectin (LEL), and *Solanum*  
389 *tuberosum* lectin (STL). Other lectins including, SBA, DBA, PSA, LCA, RCA<sub>120</sub>, PHA-E,  
390 ECL, UEA I, SJA, PNA, Jacalin, WGA, GSL I, PHA-L, Succinylated WGA, VVA, and GSL  
391 II, exhibited very weak reactivity (less than 15% of DSL) for the two ASM extracts.

392

#### 393 ***In situ* localization study using gold-conjugated DSL**

394 Since DSL showed the strongest affinity to *Porites* ASM in ELLA, an *in situ* assay was  
395 developed to localize the target saccharide moiety in the coral skeleton. Figure 7 shows SEM  
396 images of the coral skeleton treated with biotinylated DSL, which was subsequently targeted  
397 with an anti-biotin antibody coupled with gold nanoparticles. Figure 7A, acquired in  
398 secondary electron (SE) mode, shows tips of the fibers that radiate from the early

399 mineralization zone (not shown), where crystallization occurs. Figure 7B presents the same  
400 area in back-scattered electron (BSE) mode. It shows tiny bright spots that represent gold  
401 particles, and consequently sugar-bound biotinylated lectins. Since an SEM image in SE  
402 mode (Figure 7A) does not show any signal, this confirms that the signals detected in BSE  
403 mode come from the gold particles. In Figure 7B, there is no particular concentration of gold  
404 signals at the apex of the fibers, as one might have expected. Rather, the signals are dispersed  
405 along the crystal surfaces.

406 Figures 7C, D and E (in BSE mode) display successive enlargements of the same area. Here  
407 again, one notices that signals are homogeneously distributed on the surfaces of the fibers,  
408 irrespective of the growth lines (belt-like structures) that are perpendicular to the direction of  
409 crystal growth (Figure 7C and D). At high magnification (Figure 7E), the density of spots  
410 reaches about 10-15 per  $\mu\text{m}^2$ . At very high magnification (x100 000), gold particles can be  
411 measured (diameter  $\sim 50$  nm) (Figure 7F). In none of our experiments did we detect  
412 concentrations of gold spots near the centers of the spherulites, a finding that may suggest  
413 that DSL-binding sugar moieties are not involved in crystal nucleation, but that they have  
414 other functions related to crystal growth. Negative controls are shown on Figure 7G (SE  
415 mode) and H (BSE mode). Very few signals were obtained when gold-coupled anti-biotin was  
416 added to the skeleton in the absence of biotinylated DLS, confirming the low background of  
417 the sample and the specificity of sugar labeling.

418

419

## 420 **Discussion**

421 In the present study, we have characterized the skeletal organic matrix (SOM) of the massive  
422 coral, *Porites australiensis*, using various biochemical techniques, including FT-IR, CHNS,  
423 ELLA, gel electrophoresis, monosaccharide analysis by chromatography, and *in vitro*  
424 crystallization. We have also localized sugar components directly in the coral skeleton, with a  
425 technique that we adapted for the first time for corals. Our discussion focuses on three points:  
426 the influence of the bleaching, the presence of sulphate and the properties of the sugar  
427 moieties.

428 As discussed in one of our earlier papers (Ramos-Silva et al., 2013a), bleaching coral skeletal  
429 tissues with sodium hypochlorite is crucial for obtaining a SOM free of organic contaminants,  
430 namely soft tissues originating from the ectoderm, or microorganisms accumulated in skeletal  
431 pores and pockets. We chose a cleaning strategy consisting of successive bleaching treatments

432 to eliminate these contaminants, and characterized the extracted SOMs (ASMs, AIMS) after  
433 the second and third bleaching steps. Depending on the technique used, we found either that  
434 the third bleaching did not produce any modification of the biochemical properties of the  
435 SOMs, or that it induced some significant changes.

436 Among the techniques that did not detect any change, SDS-PAGE, regardless of the staining  
437 used, showed smeared patterns in all SOMs, with no differences between the two ASMs and  
438 the two AIMS. Similarly, ELLA on the two ASMs showed that both exhibit very similar  
439 lectin-binding signatures featuring strong reactivity with DSL, moderate affinity with ConA  
440 and LEL, and very low affinity with all 18 other lectins. Last, monosaccharide analyses of the  
441 AIMS showed almost no differences in their composition. Taken together, these techniques  
442 suggest that the third bleaching did not alter the qualitative biochemical characteristics of the  
443 saccharidic moieties of the SOM.

444 However, other techniques employed in our study evidenced noticeable effects that were  
445 induced by the third bleaching step. First, the SOM quantity (ASM and AIM) was reduced by  
446 half. Second, FT-IR detected a decrease of the sugar peak around 1000-1050  $\text{cm}^{-1}$  for both  
447 ASM and AIM. Third, monosaccharide analysis of the ASMs showed that arabinose was  
448 significantly reduced. Finally, the *in vitro* crystallization assay evidenced a noticeable  
449 difference: the ASM extracted after the third bleaching induced stronger effects on crystal  
450 morphologies than the ASM extracted after the second bleaching. These results suggest that  
451 some sugar-containing macromolecules of the SOM (either polysaccharides or glycoproteins)  
452 are peripheral to the mineral grains and destroyed by the third bleaching step, while some  
453 others are protected from the harsh treatment and should be considered as 'intracrystalline', to  
454 employ the old terminology of Crenshaw (Crenshaw, 1972). It is significant that the  
455 'intracrystalline' ASM fraction, which is supposed to have a strong affinity for calcium  
456 carbonate, induces stronger effects *in vitro* than the ASM extracted after two bleaching steps.  
457 To summarize this point, we reemphasize the importance of cleaning skeletal tissues with  
458 sodium hypochlorite and think that applying successive bleaching steps to the skeletal powder  
459 may be an elegant manner to pre-select SOM macromolecules that have the highest affinity to  
460 the mineral phase.

461

462 Our second focus is the presence of sulphate in the SOM of *Porites australiensis*,  
463 demonstrated by Alcian blue staining at low pH, FT-IR, and CHNS analysis. First, Alcian  
464 blue binds polyanionic groups, whatever they are, in particular those found in  
465 mucopolysaccharides. At low pH (=1), Alcian blue specifically binds sulphate-containing

466 mucopolysaccharides (Thornton et al., 1996). In the present case, both ASM and LS-AIM are  
467 stained, but not identically: stronger staining occurred in the lower two-thirds of the gel for  
468 ASM, and the upper third for LS-AIM, indicating that the distribution of sulphate differs in  
469 these two fractions. Bulk analysis by FT-IR spectroscopy confirms the presence of sulphate  
470 groups, characterized by a peak in the range 1200-1250 cm<sup>-1</sup>. Interestingly, pairwise  
471 comparisons of sulphate peak amplitudes from the second and third bleaching steps showed  
472 that the additional cleaning treatment did not degrade sulphate. This result is consistent with  
473 the CHNS analysis, which unambiguously detected sulfur (>1%) in AIM after three bleaching  
474 steps. Our data consequently suggest that the sulphate-containing macromolecules - likely  
475 polysaccharides - are truly intracrystalline.

476 More generally, the presence of sulfur in coral skeletons is a recurrent finding that leaves the  
477 question of its role open (Dauphin et al., 2008). Previous studies demonstrated that sulfur was  
478 present in the SOM of *Stylophora*, *Pavona*, *Hydnophora*, and *Merulina* (Dauphin et al., 2008;  
479 Puvarel et al., 2005). Furthermore, sulphated sugars were found to be predominant over  
480 sulfur-containing amino acids (methionine and cysteine) in ASM (Dauphin et al., 2008). As a  
481 general rule, sulphated groups on sugars (ester sulphate) increase the acidic nature of the  
482 SOM. They can bind calcium ions or exert an ionotropic effect by creating a very localized  
483 anionic environment to concentrate calcium ions in the vicinity of the nucleation spots.  
484 Sulphate is concentrated in early mineralization zones, suggesting that it may also promote  
485 crystal formation (Cuif et al., 2003; Lloyd et al., 1961). Interestingly, in other calcified tissues,  
486 such as nacre of the cephalopod *Nautilus pompilius*, a chemical mapping of single nacre  
487 tablets showed that sulphate-containing macromolecules were localized in a central annular  
488 zone in close association with carboxylate-rich macromolecules that are supposed to be the  
489 calcium carbonate nucleators (Nudelman et al., 2006). This peculiar localization suggests a  
490 cooperative role of both categories of macromolecules. Clearly, future work on coral  
491 biomineralization should elucidate the precise function of sulphated sugars, either as Ca<sup>2+</sup>  
492 concentrators or as mineral nucleators.

493

494 The third focus of our discussion concerns some specific properties of the saccharidic  
495 moieties of *Porites australiensis* SOM, including the lectin-binding signature, the  
496 monosaccharide composition and the *in situ* localization of DSL-binding saccharide. At first,  
497 the peculiar lectin-binding signature of the ASM deserves mention. Among all tested lectins,  
498 DSL gave by far the strongest signal. DSL, a lectin extracted from the jimson weed, binds  
499 chitotriose, chitobiose, and N-acetyl-D-glucosamine, with decreasing affinity. Its

500 carbohydrate-binding site recognizes oligomers of N-acetyl-D-glucosamine; thus, it exhibits  
501 strong affinity for chitin. DSL also binds the disaccharide N-acetyllactosamine (formed from  
502 the condensation of beta-galactose and N-acetyl-glucosamine) and its oligomers. It is  
503 remarkable that other lectins that typically bind chitin / chitin-like motifs, such as WGA, STL,  
504 or LEL give very little or no signal with the ASM of *P. australiensis*. This may suggest that  
505 the saccharidic motif predominant in ASM is not N-acetyl-glucosamine, but rather N-  
506 acetyllactosamine or its oligomers. This is consistent with the high amount of galactose, by  
507 far the most abundant monosaccharide identified in ASM and AIM. On the other hand, none  
508 of the galactose-binding lectins, such as jacalin, PNA, SBA, GSL-1, RCA, and SJA give  
509 signal with *P. australiensis* ASM, but their lack of reactivity may reflect individual  
510 recognition specificities for each of them, as listed by Immel and coworkers (Immel et al.,  
511 2016). Beside this, additional useful structural information can be extracted from lectin  
512 signature: for instance, jacalin interacts with most O-linked glycoproteins, but is unreactive  
513 with ASM, while ConA, a lectin that binds mannose of N-linked glycopeptides, gives the  
514 second highest signal (although moderate). This may suggest that most ASM glycoproteins of  
515 *P. australiensis* skeleton are of the N-linked-type. Fucose is the third most abundant  
516 monosaccharide in ASM, but UEA-1, which binds strongly to  $\alpha(1,2)$ -linked fucose, does not  
517 react. This suggests either that fucose residues present in the ASM are predominantly of the  
518  $\alpha(1,3)$  or  $\alpha(1,6)$ -types, or that they occupy internal positions in the saccharidic chains, or that  
519 they form fucose-containing trisaccharides, a motif that inhibits UEA-1.

520 Finally, the three most abundant sugar residues of *P. australiensis* ASM, namely galactose,  
521 arabinose, and fucose, represent the residues that are predominantly represented in the soluble  
522 SOM of two acroporid corals, *Montipora caliculata* and *Astreopora myriophthalma* (Cuif et  
523 al., 1996). Galactose and fucose are also abundant in *Heliastrea curta* and *Fungia rependa*  
524 while galactose and arabinose predominate in *Acropora danae* (Cuif et al., 1996).  
525 Interestingly, the monosaccharide composition of *P. australiensis* ASM is in agreement with  
526 that of zooxanthellate coral ASMs in general, which was statistically distinguished from that  
527 of non-zooxanthellate coral ASMs by the presence of arabinose - a monosaccharide exclusive  
528 to zooxanthellate corals - the abundance of galactose, and low amounts of galactosamine and  
529 glucosamine (Cuif et al., 1999b). This monosaccharide composition contrasts singularly with  
530 that recently published by Naggi and coworkers (2018) who identified by other techniques  
531 (chromatography and mass spectrometry) glucuronic acid as the main saccharide residue  
532 present in the SOM of three stony corals, *Astroides calycularis*, *Balanophyllia europaea*, and  
533 *Stylophora pistillata*. We did not detect this acidic sugar in our samples and then reason of the

534 discrepancy between our data and theirs is not known.  
535 Last, using gold-conjugated DSL, we localized sugar components that have a strong affinity  
536 to DSL lectin. In all experiments, we obtained a homogeneous distribution of gold particles on  
537 the surface of radiating aragonite fibers. We did not observe signals concentrated along  
538 growth lines, perpendicular to fiber elongation nor signals at the tips of the fibers. Finally, the  
539 centers of spherulites are enriched in organics that are supposed to be the nucleating  
540 macromolecules. However, we did not detect any high concentration of gold spots around  
541 these centers, *i.e.*, at the bases of the fibers that are the closest to the center. In this latter case,  
542 we cannot exclude that the treatment of the polished surface - prior to lectin incubation -  
543 which consists in cleaning with bleach followed by slight etching with EDTA, may have  
544 eliminated most of the organics located at the centers of spherulites. In spite of these potential  
545 preparation artifacts, our observations suggest that the DSL-binding saccharide fraction is  
546 neither involved in crystal nucleation nor in stopping crystal growth (where it would be  
547 distributed along the growth lines). Because the signal homogeneously covers the aragonite  
548 fibers, and because DSL-reactive ASM exerts a significant *in vitro* effect on crystal size and  
549 shape, we put forward the hypothesis that this fraction modulates size, shape, and/or fiber  
550 growth kinetics. In order to better understand the role of these sugar moieties in *P.*  
551 *australiensis* biomineralization, further experiments, such as *in vitro* crystallization  
552 experiments with purified DSL-binding polysaccharide, should be conducted.

553

## 554 **Conclusions**

555 Our study represents the first biochemical characterization of the skeletal organic matrix of  
556 the massive coral, *Porites australiensis*, under different cleaning conditions. We focused on  
557 the sugar moieties. Besides obtaining peculiar signatures of monosaccharide composition and  
558 of lectin profile, we localized *in situ*, for the first time, a DSL lectin-binding fraction. Our  
559 results re-emphasize the role of saccharides in coral biomineralization and aims at identifying  
560 sugar signatures of coral SOMs, by waiting for understanding the structure-function  
561 relationships of this class of macromolecules in biomineralization. Further investigation of  
562 this organic fraction, together with the ongoing proteomic analysis of *Porites* SOM, is  
563 essential to obtain the most complete picture of macromolecular constituents required for  
564 building the skeleton of these major reef-forming corals.

565

566



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579

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731

Table 1. Quantification of AIMs and ASMs extracted from *P. australiensis* skeleton.

	AIM (mg·g <sup>-1</sup> )	ASM (mg·g <sup>-1</sup> )	Total organic matrix (% w/w)
2nd bleaching	1.31	0.12 ± 0.01	0.143
3rd bleaching	0.64	0.06 ± 0.01	0.07

732

733

Table 2. CHNS analysis of 3rd bleaching AIM.

	N (wt%)	C (wt%)	H (wt%)	S (wt%)
3bl AIM	1.42	21.63	3.15	1.05

734

735

## 736 **Figure Legends**

737

738 **Figure 1. SEM images of *Porites australiensis* skeleton before and after second bleaching.**

739 (A) Surface of a skeleton fragment before the second bleaching. Fibrous structures of  
740 microorganisms (red arrowheads) are visible. (B) The same skeleton surface of (A) after the  
741 second bleaching. Contaminating fibers were completely removed. (C) Magnified image of  
742 (A). (D) Magnified image of (B).

743

744 **Figure 2. SDS-PAGE of skeletal organic matrices.** (A) Silver staining, (B) Stains-all  
745 staining, (C) Alcian blue staining. Twenty  $\mu\text{l}$  of sample solution was loaded in each lane for  
746 silver staining and 15  $\mu\text{l}$  for Stains-all staining and Alcian blue staining. ASM: Acid-soluble  
747 matrix. LS-AIM: Laemmli-soluble acid-insoluble matrix. 2bl: second bleaching. 3bl: third  
748 bleaching. MM: Molecular weight markers.

749

750 **Figure 3. FT-IR spectra of skeletal organic matrices.** Major peaks discussed in the main  
751 text are colored. These signals indicate the presence of proteins or saccharides (i), lipids (ii),  
752 sulphates (iii), phosphates (iv), and saccharides (v). ASM: Acid-soluble matrix. AIM: Acid-  
753 insoluble matrix. 2bl: second bleaching. 3bl: third bleaching.

754

755 **Figure 4. SEM images of  $\text{CaCO}_3$  crystal formed in the presence of *Porites* ASM.** (A-F)  
756 Second bleaching ASM:  $1\mu\text{g}\cdot\text{mL}^{-1}$  (A),  $4\mu\text{g}\cdot\text{mL}^{-1}$  (B),  $16\mu\text{g}\cdot\text{mL}^{-1}$  (C,D),  $32\mu\text{g}\cdot\text{mL}^{-1}$  (E,F).  
757 (G-I) Third bleaching ASM:  $1\mu\text{g}\cdot\text{mL}^{-1}$  (G),  $4\mu\text{g}\cdot\text{mL}^{-1}$  (H),  $16\mu\text{g}\cdot\text{mL}^{-1}$  (I,J),  $32\mu\text{g}\cdot\text{mL}^{-1}$  (K,L).  
758 (M)  $\text{CaCO}_3$  crystals generated in ASM-free solution. Morphology of the generated crystals  
759 are affected by the presence of ASM in dose-dependent manner. The effect of third bleaching  
760 ASM is more pronounced than that of second bleaching ASM.

761

762 **Figure 5. Monosaccharide composition of ASM and AIM after two (left) or three (right)**  
763 **bleachings.** While the third bleaching did not produce any important change in the relative  
764 percentages of monosaccharides in AIMS, it was more effective for ASMs, where it induced a  
765 noticeable decrease of arabinose percentage.

766

767 **Figure 6. Enzyme-linked lectin assay (ELLA) on ASMs.** ELLA was performed with 21  
768 lectins after the second (left) and third (right) bleaching steps. Absorbance values at 405 nm  
769 were normalized to the highest value (DSL), corresponding to 100% reactivity ( $n = 3$ , means

770 ± S.D.).

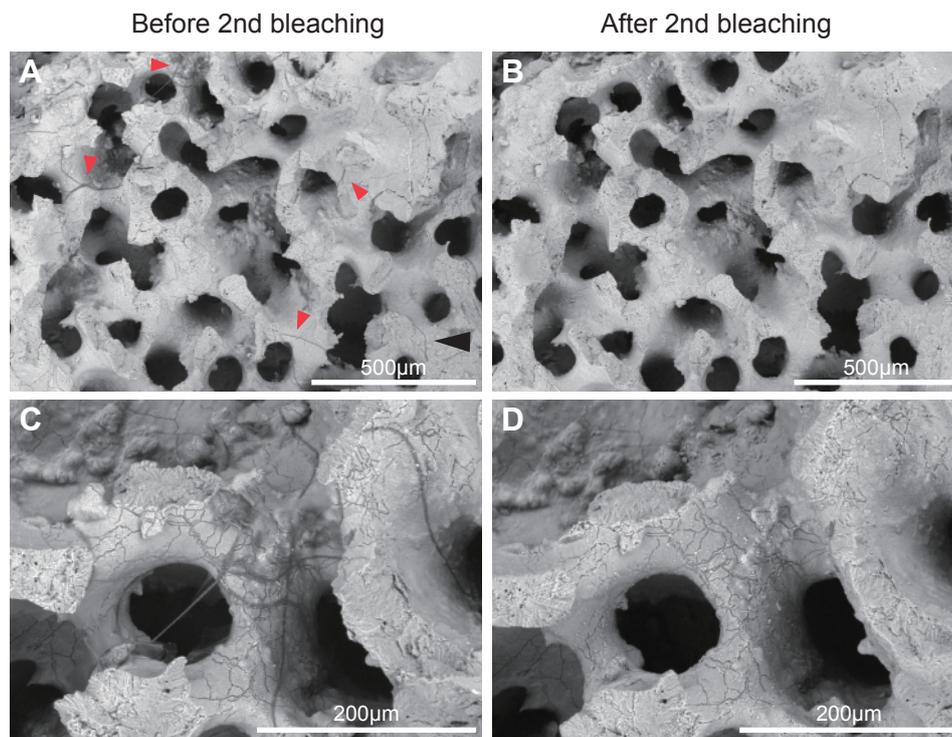
771

772 **Figure 7. *In situ* localization of the DSL-reactive saccharide fraction.** Observation with  
773 SEM in secondary electron (SE, A and H) and in back-scattered electron (BSE, B to G)  
774 modes. A to F: positive control, with *Datura stramonium* lectin (DSL). G, H: negative control,  
775 in the absence of DSL, but with gold-coupled anti-biotin. In (A) and (B), tips of needle-like  
776 crystals point to the early mineralization zone (front side of the pictures). The early  
777 mineralization zone is indicated in dotted circle in (C). Note that the gold particles,  
778 characterized by tiny bright spots in BSE mode, are distributed evenly on the aragonite fibers.

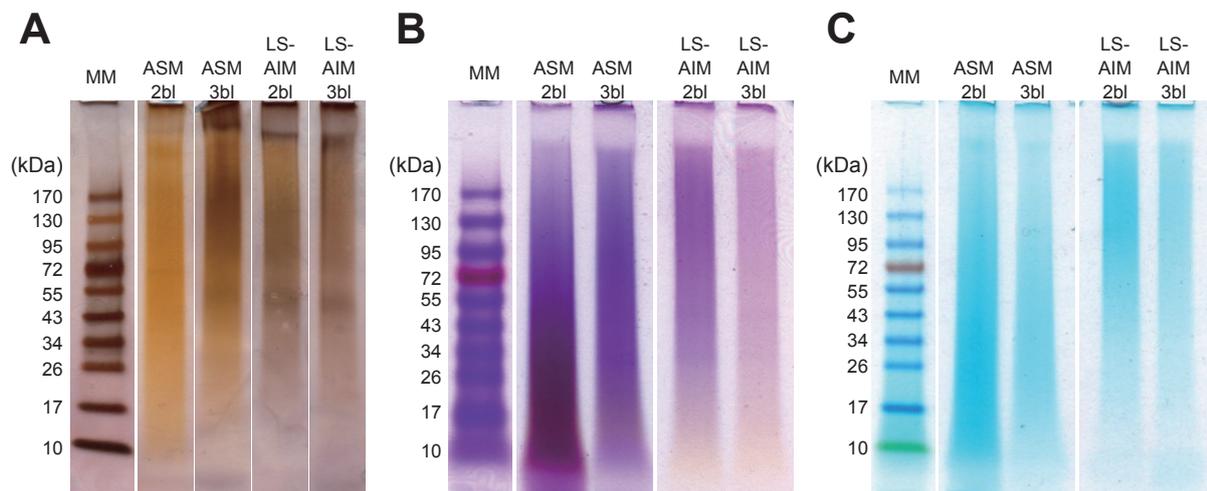
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**Figure 1.**



**Figure 2.**

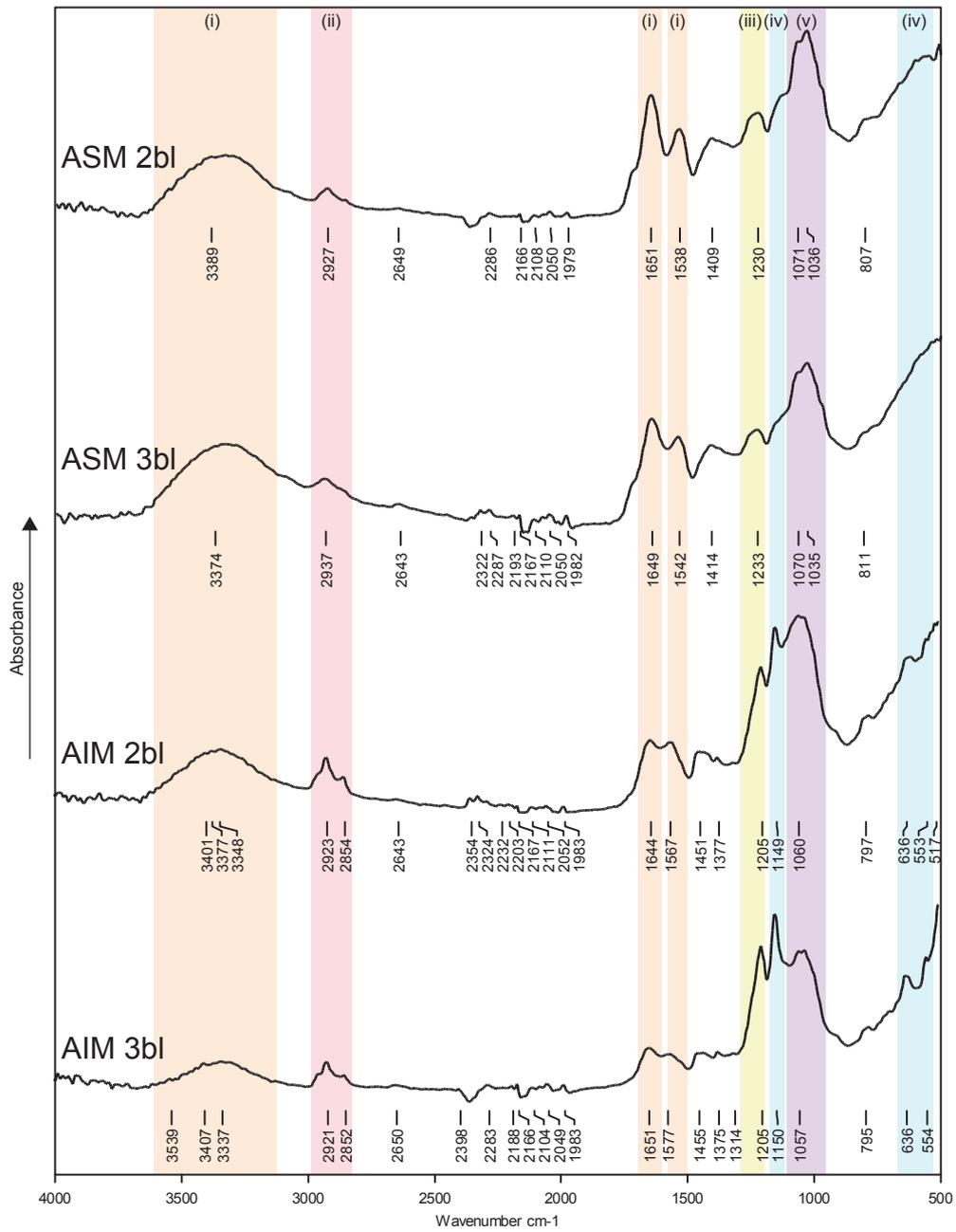
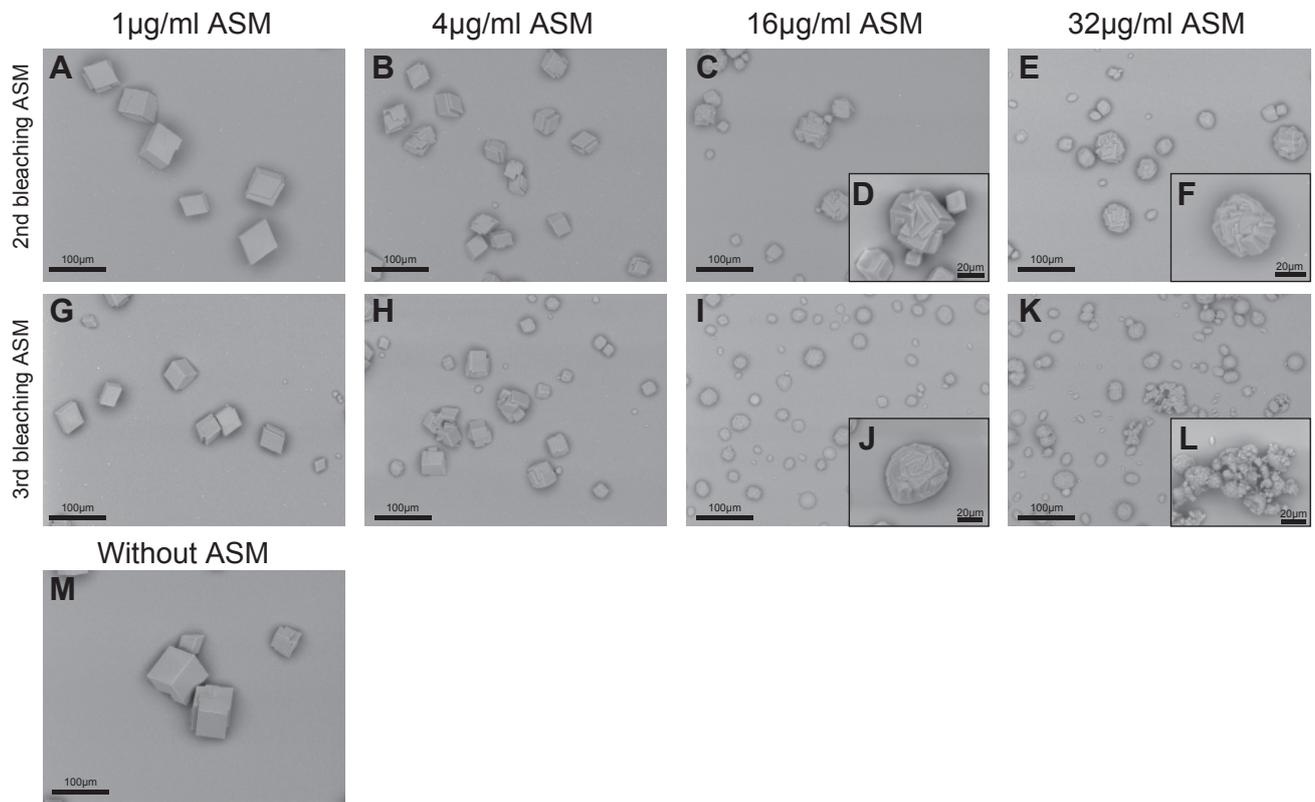
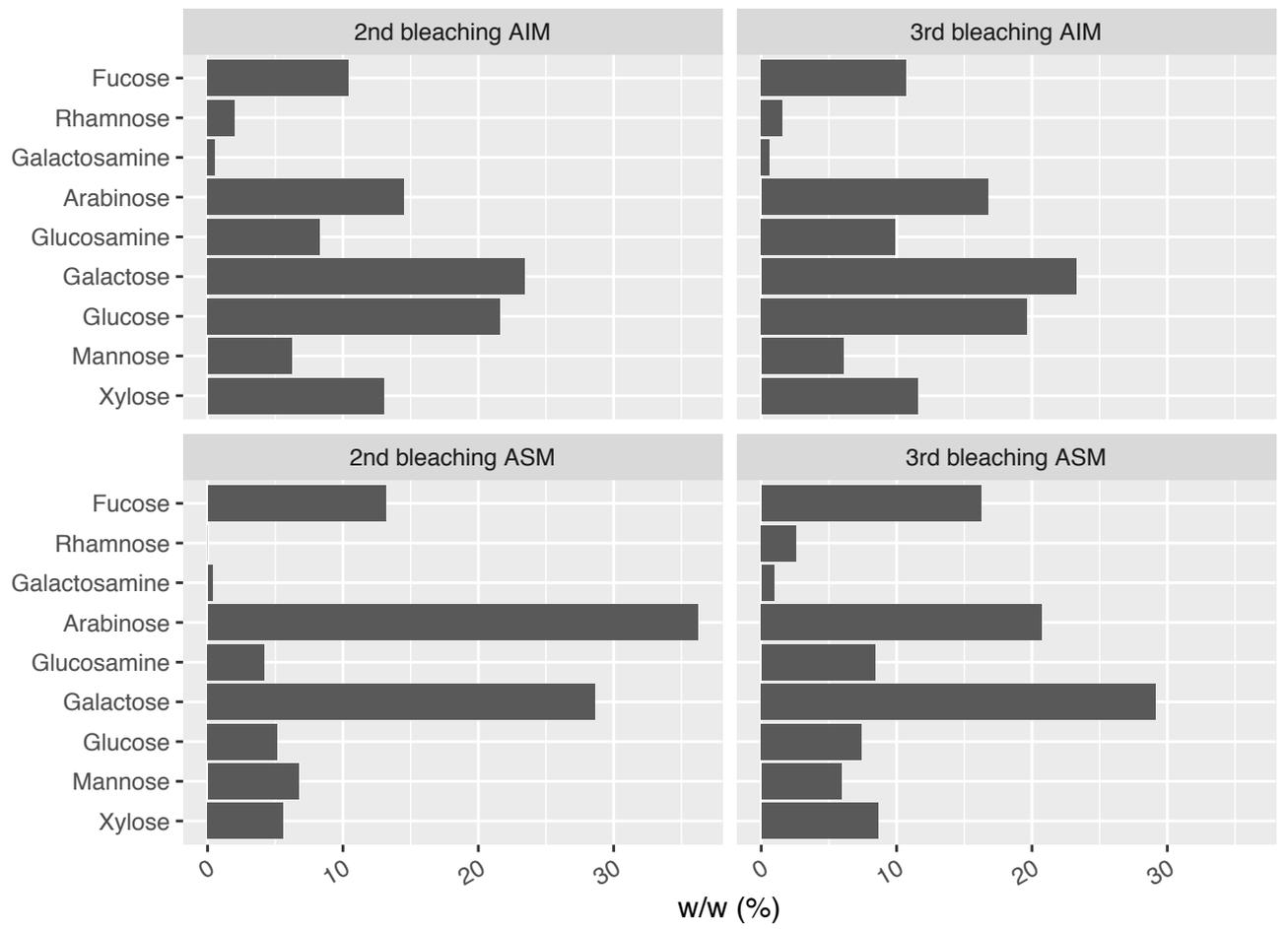


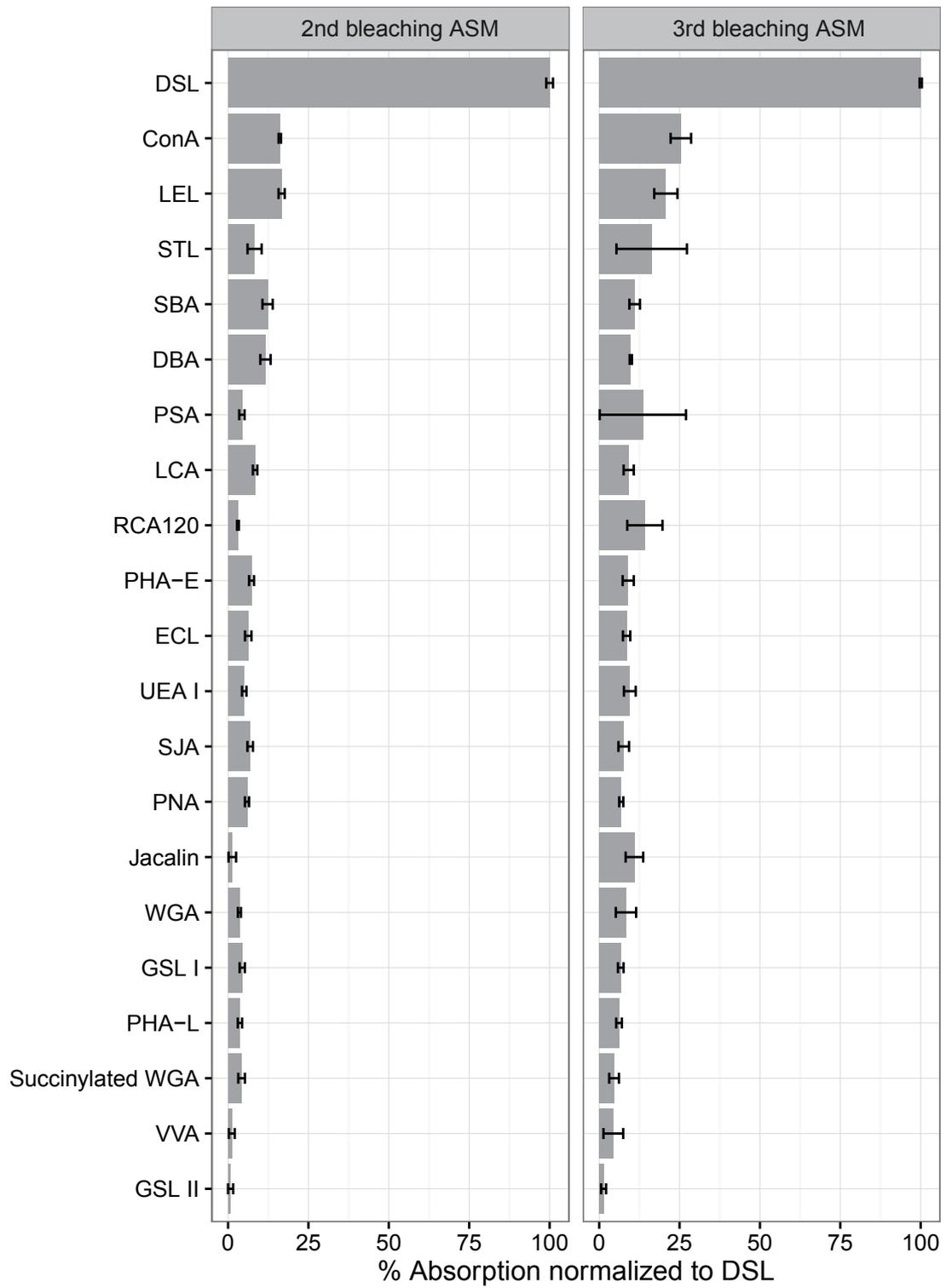
Figure 3.



**Figure 4.**



**Figure 5.**



**Figure 6.**

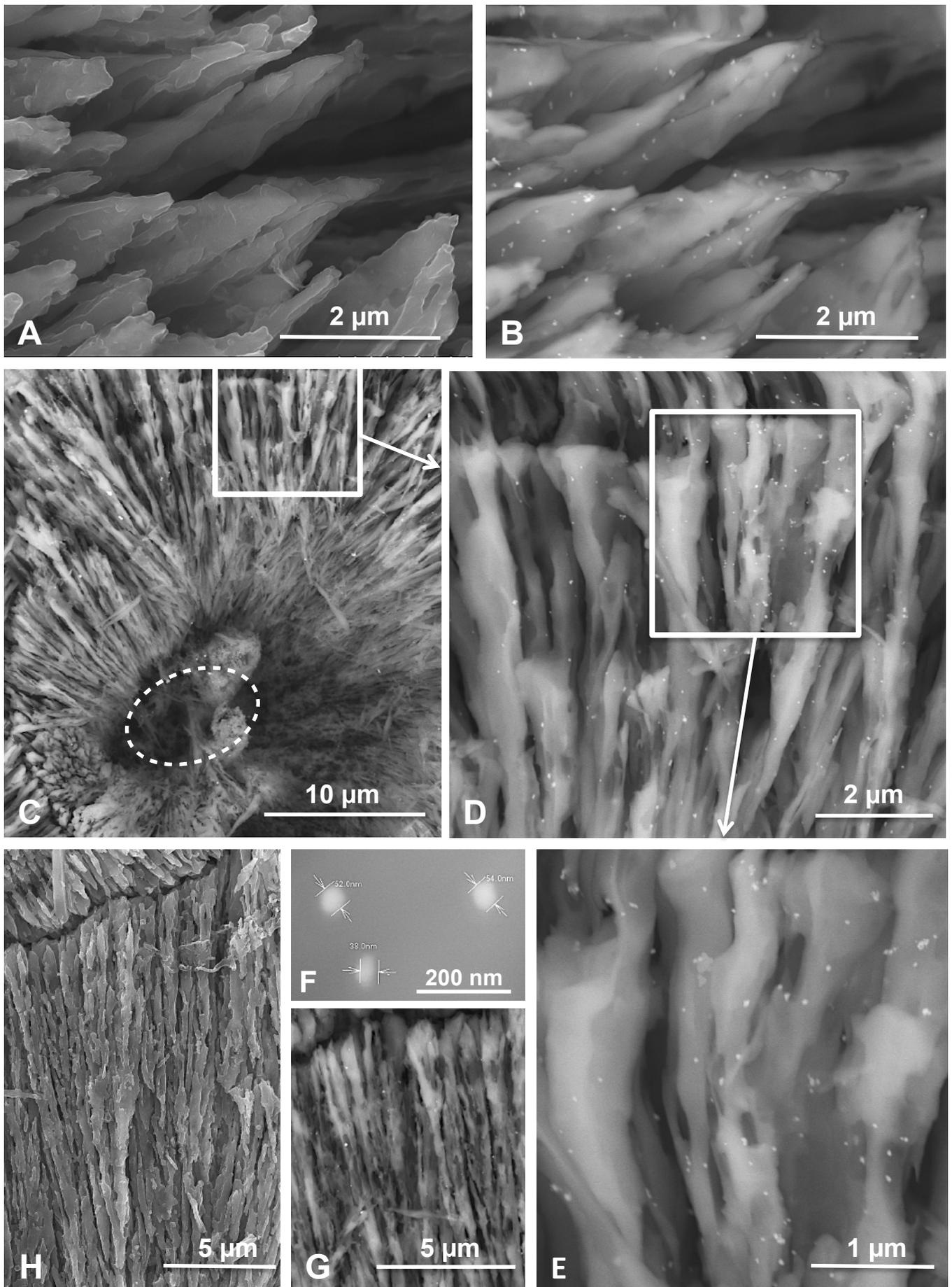


Figure 7.