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1 Are the large filamentous microfossils preserved in  
2 Messinian gypsum colorless sulfide-oxidizing bacteria?

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11 **ABSTRACT**

12 The thick gypsum deposits formed in the Mediterranean basin during the  
13 Messinian salinity crisis incorporate dense mazes of filamentous fossils, which were  
14 interpreted as algae or cyanobacteria, thus pointing to a shallow marine subtidal or  
15 intertidal environment. The data presented herein reveal that these filaments rather  
16 represent remains of colorless, vacuolated sulfide-oxidizing bacteria. This interpretation  
17 is supported by the presence of small crystal aggregates of iron sulfide (pyrite) and  
18 associated polysulfide within the filamentous fossils. Pyrite and polysulfide are  
19 considered to result from early diagenetic transformation of original zero-valent sulfur  
20 globules stored within the cells, which is a clade-diagnostic feature of living and  
21 degraded sulfur bacteria. Besides filamentous fossils, the studied gypsum crystals contain  
22 remains of eury- and stenohaline diatoms and clay-rich aggregates interpreted as

23 alteration products of marine snow floccules. This peculiar fossil assemblage reflects  
24 conditions of increased productivity in the water column, which was triggered by high  
25 fluxes of nutrients into the basin during phases of enhanced riverine runoff and fresh  
26 water discharge. This study confirms that gypsum evaporites have great potential to  
27 preserve the early stages of the taphonomic alteration of bacterial cells, shedding light on  
28 the paleoecology of ancient hypersaline environments.

## 29 INTRODUCTION

30 Being able to tolerate extreme, hypersaline conditions, prokaryotes are often the  
31 only fossils found in evaporites (Warren, 2010). The prokaryote remains are commonly  
32 exceptionally well-preserved because of fast and early growth of the evaporite minerals,  
33 allowing for the rapid entombment of cells (Lugli et al., 2010). Well known examples of  
34 fossiliferous evaporites are the thick gypsum sequences associated with halite and  
35 anhydrite that were deposited in the Mediterranean basin ~6 m.y. ago during the  
36 Messinian salinity crisis (MSC; Roveri et al., 2014). The Messinian gypsum incorporates  
37 dense mazes of filamentous fossils, which were originally interpreted as remains of  
38 benthic algae (Vai and Ricci Lucchi, 1977) or cyanobacteria (Rouchy and Monty, 2000).  
39 Should this assignment be correct, the depositional setting must have been shallow,  
40 situated within the photic zone. The extraction and amplification of cyanobacterial  
41 ribosomal RNA from filament-bearing gypsum from Italy supported this interpretation  
42 (Panieri et al., 2010). However, based on comparison with modern bacteria, Schopf et al.  
43 (2012) suggested that the filamentous fossils represent remains of colorless sulfide-  
44 oxidizing bacteria such as *Beggiatoa* and *Thioploca*. Similar filamentous fossils  
45 preserved in other lithologies than gypsum including chert (Schopf et al., 2015),

46 phosphorite (Bailey et al., 2013), and limestone (Peckmann et al., 2004) have previously  
47 been interpreted as members of the colorless sulfur bacteria. Here we present a  
48 petrographic, minerochemical, and Raman spectroscopy study of the fossiliferous  
49 gypsum from the Primary Lower Gypsum unit of the Piedmont Basin (northwest Italy;  
50 Fig. 1), focusing on the abundant filamentous fossils. The new results indicate that these  
51 enigmatic fossils are more likely to represent sulfur bacteria, agreeing with recent  
52 interpretations of the environmental conditions during the deposition of the Messinian  
53 gypsum.

#### 54 **THE PRIMARY LOWER GYPSUM UNIT**

55       The Primary Lower Gypsum unit formed during the first stage of the MSC (5.97–  
56 5.60 Ma) in silled peripheral sub-basins of the Mediterranean (Roveri et al., 2014). The  
57 depth of these sub-basins is still a matter of discussion. As elsewhere in the  
58 Mediterranean, this unit shows a striking lithological cyclicity in the Piedmont Basin,  
59 defined by rhythmic alternation of shale and gypsum couplets. This cyclicity is  
60 interpreted to reflect precession-controlled humid (shale) to arid (gypsum) climate  
61 oscillations (Dela Pierre et al., 2014). The gypsum layers studied herein, up to 30 m thick  
62 (Fig. 2A), belong to the lowermost four cycles and are composed of dm-sized vertically  
63 oriented twinned selenite crystals (swallow-tail twins). The crystals nucleated at the  
64 sediment-brine interface with their vertical orientation reflecting competitive growth in a  
65 relatively deep basin permanently covered by brines (Lugli et al., 2010).

#### 66 **METHODS**

67       Petrographic sections of 20 samples collected from three outcrops were studied  
68 under an optical microscope and analyzed for their ultraviolet (UV) fluorescence (for

69 details see the GSA Data Repository<sup>1</sup>). Five representative samples were studied with a  
70 scanning electron microscope (SEM) coupled with an energy-dispersive X-ray  
71 spectrometer (EDS) and a Raman spectrometer. Three samples were dissolved in  
72 ultrapure water and the resulting residue and isolated fragments were analyzed by light  
73 microscopy, electron microscopy with coupled energy-dispersive X-ray spectroscopy,  
74 and microRaman. X-ray diffraction (XRD) analyses were performed on isolated  
75 filaments after dissolution.

## 76 **THE GYPSUM FILAMENTOUS FOSSILS**

77       The studied swallow-tail twins of gypsum display an internal lamination in the re-  
78 entrant angles marked by the rhythmic repetition of mm-thick clear and turbid laminae  
79 (Fig. 2B), possibly representing short term (annual?) climate oscillations between more  
80 humid (turbid lamina) and more arid (clear lamina) conditions. In the clear laminae solid  
81 inclusions are scarce or absent, whereas they are abundant in the turbid laminae. They  
82 include (1) rare stenohaline (*Navicula* sp., *Trigonium* sp.) and euryhaline (*Surirella* sp.)  
83 diatoms (Fig. 2C; Natalicchio et al., 2014), and (2) loosely packed, fluorescent clay-rich  
84 aggregates up to 500  $\mu\text{m}$  across and locally containing altered diatom frustules. Similar  
85 aggregates have already been reported from the shale layers interbedded with the gypsum  
86 and have been interpreted to represent marine snow floccules that originated by  
87 aggregation of clay and diatoms in the overlying water column during episodes of  
88 eutrophication and phytoplankton bloom (Dela Pierre et al., 2014). Other solid inclusions  
89 are (3) silt-sized terrigenous material (mica flakes and detrital mineral grains; Fig. DR2 in  
90 the Data Repository), and (4) curved and straight filaments (Figs. 2D and 2F). The  
91 filaments are up to 2 mm long and 60–80  $\mu\text{m}$  across, showing a rather uniform diameter

92 throughout their length. All filaments are fluorescent when exposed to UV light (Fig. 2E),  
93 suggesting a high content of organic matter. While the filaments are mostly observed in  
94 the re-entrant angle of the crystals, they are also found on vertical growth bands with  
95 their long axis aligned to former crystal surfaces (Fig. 2D). All filaments are preserved as  
96 hollow tubes in the gypsum (Fig. 2F). Well-preserved ones are made of a sequence of  
97 cellular compartments of uniform shape and size (Fig. 2G). The surface of the filaments  
98 displays an irregular honey-comb structure (Fig. 2H; Fig. DR1), which—according to  
99 XRD data (Fig. DR4)—consists of clay minerals of the smectite group and traces of illite.  
100 The elemental composition of the clay minerals falls between the compositional fields of  
101 nontronite and montmorillonite (Fig. DR3), confirming the presence of smectite minerals.  
102 The composition of the smectite clay minerals is distinguishable from that of the detrital  
103 micas (Fig. DR2), which represent muscovite, phengite, and accessory Fe-Mg chlorite  
104 (Fig. DR3; Table DR1). The identification of clay minerals by micro-Raman was  
105 precluded due to their weak Raman scattering and the fluorescence of the filaments.  
106 However, micro-Raman revealed the scattered presence of carbonaceous material (Fig.  
107 3), possibly representing a remnant of the original biomass of the filamentous organisms.  
108 Some filaments are coated by a layer of anhedral dolomite microcrystals (2–5  $\mu\text{m}$   
109 across), which reveal a partially hollow core (Figs. 2I and 2J). The dolomite crystals  
110 apparently grew on the outer surfaces of filaments within a clayey matrix before the final  
111 incorporation of filaments within gypsum. In all studied samples, the filaments contain  
112 opaque, subspherical grains that are 1–2  $\mu\text{m}$  across (Fig. 2E), which were identified as  
113 iron sulfides by SEM-EDS and XRD. MicroRaman analyses identified the iron sulfides  
114 as aggregates of microcrystalline pyrite, revealing characteristic peaks at ~340, 376, and

115 426  $\text{cm}^{-1}$  (Fig. 3; see the Data Repository). In rare cases, a broad band at  $\sim 470 \text{ cm}^{-1}$  was  
116 observed (Fig. 3), which is best explained by the presence of polysulfide ( $\text{S}_n^{2-}$ ) that  
117 shows similar bands in the 440 and 480  $\text{cm}^{-1}$  wavelength region (main at 470; Berg et al.,  
118 2014).

## 119 **THE NATURE OF THE FILAMENTOUS FOSSILS**

120 The fact that the filaments were also observed along the vertical growth bands  
121 besides in the re-entrant angle of the crystals suggests that the microorganisms lived  
122 adhering to the crystal faces, thus representing fossils of benthic biota. After having being  
123 interpreted as fossils of algae (Vai and Ricci Lucchi, 1977) or cyanobacteria (Rouchy and  
124 Monty, 2000; Panieri et al., 2010), Schopf et al. (2012) suggested that the filamentous  
125 fossils represent remains of sulfide-oxidizing bacteria. The colorless, vacuolated sulfide-  
126 oxidizing bacteria like *Beggiatoa* and *Thioploca* oxidize hydrogen sulfide to sulfate with  
127 oxygen or nitrate, thus, requiring steep redox gradients and preferring microoxic  
128 environments (Schulz and Jørgensen, 2001). These and other closely related  
129 Gammaproteobacteria are able to grow to enormous sizes where the concentrations of  
130 their substrates are high enough to overcome size limitations posed by molecular  
131 diffusion (Schulz and Jørgensen, 2001). Such conditions are found in upwelling areas,  
132 silled basins, eutrophic lakes and bays, at hydrothermal vents, or at methane seeps  
133 (Schulz and Jørgensen, 2001). Different strains and populations of *Beggiatoa*, for  
134 example, reveal a range of filament widths from below 1–200  $\mu\text{m}$  (Teske and Nelson,  
135 2006). None of the studied Messinian filaments have the tapered ends that are observed  
136 in some *Thioploca* (Jørgensen and Gallardo, 1999), but this is no argument to exclude  
137 this genus, since tapering is not found in all of its members. The multicellular filaments

138 may consist of a row of hundreds to a thousand disk-shaped cells and reach a length of  
139 several centimeters (Teske and Nelson, 2006). Consequently, the shape, the size, and the  
140 apparent segmentation (Fig. 2G) of the Messinian filaments agree with an assignment to  
141 the colorless sulfur bacteria. The presence of carbonaceous material in the filaments is of  
142 course not diagnostic for a group of prokaryotes, but is in accord with a biogenic origin.  
143 The recognition of dolomite coatings is remarkable, since early dolomite formation has  
144 been found to be driven by bacterial sulfate reduction (e.g., Vasconcelos et al., 1995).  
145 Dolomite formation occurred before the filaments were entombed by gypsum. The  
146 paleoenvironment was consequently conducive to dolomite formation; precipitation may  
147 have exclusively occurred post-mortem, but must have been a very early taphonomic  
148 process. Interestingly, some *Thioploca* benefit from the local production of hydrogen  
149 sulfide by sulfate-reducing bacteria of the genus *Desulfonema*, which grow on the outer  
150 surface of the *Thioploca* sheaths (Fukui et al., 1999). Such an association of sulfate  
151 reducers adhering to the filamentous sulfide oxidizers can explain the observed dolomite  
152 coatings.

153         A diagnostic feature of modern colorless sulfur bacteria is the presence of zero-  
154 valent sulfur globules stored within membrane-bounded vesicles, which represent an  
155 intermediate product of the oxidation of sulfide to sulfate (Teske and Nelson, 2006).  
156 Similar sulfur-rich inclusions are present in the microfossils studied here. Remarkably,  
157 colorless sulfur bacteria can sometimes retain elemental sulfur in the sheath after cell  
158 death and loss of cytoplasm (Bailey et al., 2013). Although no isolated elemental sulfur  
159 was detected, we observed aggregates of microcrystalline pyrite and associated  
160 polysulfide. The chemical nature of the sulfur stored by modern prokaryotes is

161 controversially discussed (Berg et al., 2014). Raman data indicate that this sulfur is  
162 extremely fine-grained and arranged in a stable S<sub>8</sub> ring configuration (Pasteris et al.,  
163 2001). Recently also polysulfide, possibly derived from the transformation of  
164 cyclooctosulfur, was reported in some *Beggiatoa* cultures (Berg et al., 2014). Therefore,  
165 the presence of polysulfide in the Messinian filaments is of interest. It may represent a  
166 remnant of elemental sulfur stored by the bacteria. The majority of the sulfur, however,  
167 reacted with iron, fostering the formation of pyrite (Berner, 1984). It is difficult to  
168 exclude that the polysulfide resulted from the reoxidation of pyrite during weathering, but  
169 the otherwise excellent preservation of the fossils in gypsum crystals—sealing off the  
170 solid inclusions from external influences—may be taken as an argument for a primary  
171 origin of polysulfide.

172         Although the Messinian filaments are unusually large for prokaryotes, bacteria  
173 other than colorless sulfur bacteria cannot be excluded based on size and shape alone.  
174 Some oscillatoriacean cyanobacteria, with sheaths up to 100 µm in diameter (Demoulin  
175 and Janssen, 1981) are virtually indistinguishable from colorless sulfur bacteria based  
176 solely on morphology. In Messinian gypsum from the Monte Tondo quarry, filaments  
177 with a width of up to 70 µm have been recognized by Schopf et al. (2012), which tempted  
178 the authors to suggest that the filaments were sulfur bacteria rather than cyanobacteria.  
179 Panieri et al. (2010) documented a range of diameters from 20 to 30 µm for filaments  
180 from the same quarry, which had been interpreted as cyanobacteria based on the  
181 extraction of ribosomal RNA from the gypsum. However, it cannot be excluded that this  
182 genetic material derived from planktic microorganisms that sunk to the seafloor (see  
183 Lugli et al., 2010) or from subrecent or recent endolithic cyanobacteria (cf. Ziolkowski et

184 al., 2013). Another group that could be considered as producers of the filaments are iron-  
185 oxidizing bacteria. Interestingly, the mineral composition of the filaments, which is  
186 clearly different from that of the associated detrital micas, is consistent with microbially-  
187 mediated clay authigenesis (cf. Konhauser and Urrutia, 1999). Chamosite and illite are  
188 typical products of this process, but smectites (particularly nontronite) are also found  
189 (Ueshima and Tazaki, 2001). Unfortunately, the mineralogy of the studied filaments is  
190 not diagnostic of a particular group of bacteria (cf. Konhauser and Urrutia, 1999).  
191 However, in an environment that sustained bacterial iron oxidation, the nucleation of  
192 clays with high iron contents ought to be expected (cf. Peckmann et al., 2008). The  
193 absence of such clay minerals argues against an assignment of the Messinian filaments to  
194 iron-oxidizing bacteria. Such an attribution is further unlikely, because known  
195 filamentous iron oxidizers are much smaller than the studied filaments (5–6  $\mu\text{m}$ ; Crosby  
196 et al., 2014). Based on the different lines of evidence, we interpret the filaments  
197 preserved in Messinian gypsum as fossils of colorless sulfide-oxidizing bacteria.

## 198 **IMPLICATIONS FOR MESSINIAN GYPSUM DEPOSITION**

199       Modern colorless sulfur bacteria occur in a wide range of water depths from  
200 bathyal to peritidal settings (Bailey et al., 2009) and show a phobic response to light  
201 (Nelson and Castenholz, 1982). The assignment of the Messinian filaments to this group  
202 of bacteria indicates that the gypsum locally formed at greater water depth than  
203 previously suggested, which was partly based on the assumption that the filaments reflect  
204 benthic phototrophs. The revised scenario agrees with the findings of Ochoa et al. (2015),  
205 who reported that gypsum deposition was not limited to shallow depth. The large sulfur  
206 bacteria inhabit diverse environments, including those in which bacterial sulfate

207 reduction produces hydrogen sulfide in organic-rich sediments (Teske and Nelson, 2006).  
208 Deposition of organic-rich sediments, commonly containing abundant diatoms and  
209 marine snow floccules, is favored by eutrophication of the water column caused by  
210 increased nutrient influx in the course of enhanced riverine runoff (Graco et al., 2001).  
211 And indeed, recent work confirms that the early stages of the MSC were typified by algal  
212 blooms caused by eutrophication (Dela Pierre et al., 2014). Similarly, a local increase of  
213 riverine runoff has been demonstrated for the early stage of the MSC by gypsum fluid  
214 inclusion data, indicating influx of sulfate-rich waters that mixed with seawater  
215 (Natalicchio et al. 2014). The algal blooms enhanced organic matter degradation by  
216 bacterial sulfate reduction in an oxygen-depleted sedimentary environment, which  
217 provided the high hydrogen sulfide flux required for the growth of colorless sulfur  
218 bacteria. A steep gradient between anoxic, sulfide-rich sediments and oxygen-depleted  
219 but probably nitrate-rich bottom water supposedly favored these bacteria. Such an  
220 eutrophication scenario agrees with our reinterpretation of the Messinian filaments as  
221 sulfide-oxidizing bacteria, similar to those that are found in association with diatoms and  
222 marine snow floccules in modern eutrophic settings.

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334 **FIGURE CAPTIONS**

335 Figure 1. Distribution of Messinian evaporites (gypsum and halite) in the Mediterranean  
336 basin (after Lugli et al., 2010). PB—Piedmont basin.

337

338 Figure 2. A: Outcrop view of the Banengo section (northwest Italy) with underlying pre-  
339 Messinian salinity crisis marls (Pre-MSC) and three tilted Primary Lower Gypsum cycles

340 composed of shale (S) and gypsum. Arrows indicate upward gypsum growth direction. B:  
341 Gypsum twin showing the alternation of turbid and clear laminae within the re-entrant  
342 angle. The turbid laminae are rich in filamentous fossils. C: The euryhaline diatom  
343 *Surirella* sp. D: Gypsum twin with curved filaments aligned to the vertical growth bands  
344 (solid lines). Lamination in the re-entrant angle is indicated by dotted lines. E:  
345 Fluorescent filament with small opaque pyrite inclusions. F: Hollow filamentous fossils  
346 within gypsum. G: Isolated filament; a sequence of cellular compartments (outlined by  
347 dashed lines) can be recognized. H: External surface of an isolated filament with a honey-  
348 comb structure. I: Isolated filament coated by dolomite microcrystals. J: Detail of I:  
349 rounded dolomite microcrystals. B–D and F are plane-polarized light photomicrographs;  
350 E is UV-light photomicrographs; G–J are scanning electron microscopy images.

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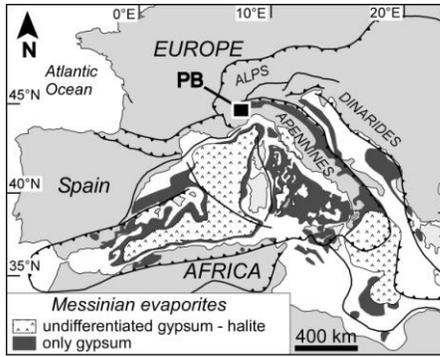
352 Figure 3. From the bottom to the top, Raman spectra of gypsum with filaments, pyrite  
353 (rectangles), pyrite with polysulfide (circle), and carbonaceous material (dotted  
354 rectangles).

355

356 <sup>1</sup>GSA Data Repository item 2015 xxx, sampling, methodology, minerochemical and  
357 XRD data of Messinian filamentous fossils (Table DR1 and Figures DR1–DR4) is  
358 available online at [www.geosociety.org/pubs/ft2015.htm](http://www.geosociety.org/pubs/ft2015.htm), or on request from  
359 [editing@geosociety.org](mailto:editing@geosociety.org) or Documents Secretary, GSA, PO Box 9140, Boulder, CO,  
360 80301, USA.

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363 Dela Pierre et al. Fig. 1 jpeg.

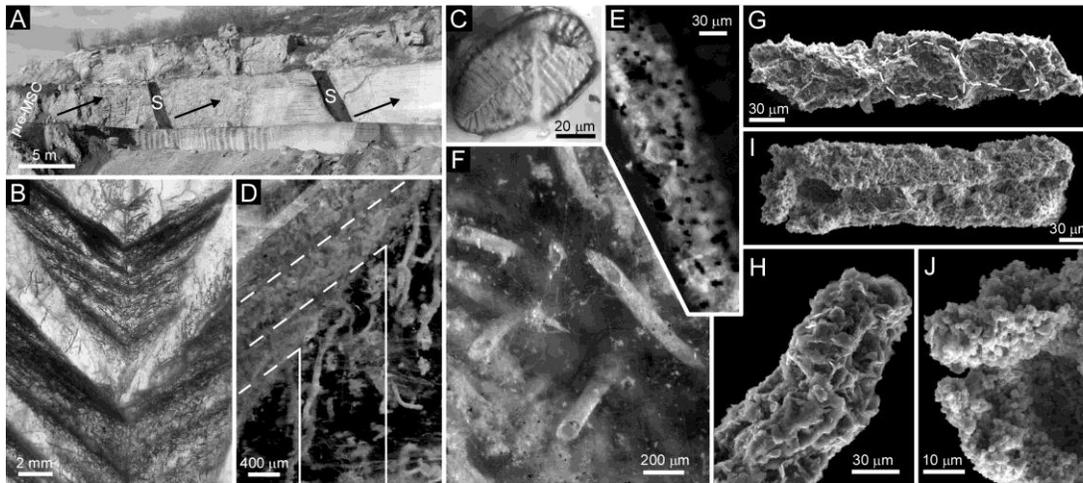
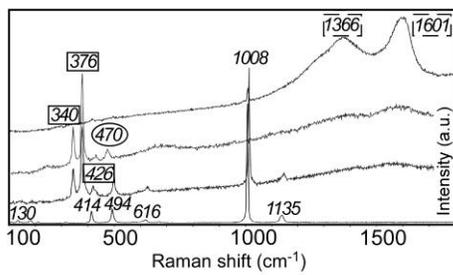


Fig. 2

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365 Dela Pierre et al Fig. 3 jpeg.