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1 **SUNKEN WOOD HABITAT FOR THIOTROPHIC SYMBIOSIS IN MANGROVE**
2 **SWAMPS**

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19

20

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22 eukaryotes, ciliates, mangrove swamp.

23

24 Running title: Thiotrophic habitat on sunken wood?

25

26 **ABSTRACT**

27

28 Large organic falls to the benthic environment, such as dead wood or whale bones, harbour
29 organisms relying on sulfide-oxidizing symbionts. Nothing is known however concerning
30 sulfide enrichment at the wood surface and its relation to wood colonization by sulfide-
31 oxidizing symbiotic organisms.

32 In this study we combined *in situ* hydrogen sulfide and pH measurements on sunken wood,
33 with associated fauna microscopy analyses in a tropical mangrove swamp. This shallow
34 environment is known to harbour thiotrophic symbioses and is also abundantly supplied
35 with sunken wood. A significant sulfide enrichment at the wood surface was revealed. A
36 72 hour sequence of measurements emphasized the wide fluctuation of sulfide levels (0.1
37 to >100 μM) over time with both a tidal influence and rapid fluctuations. Protozoans
38 observed on the wood surface were similar to *Zoothamnium niveum* and to Vorticellids.
39 Our SEM observations revealed their association with ectosymbiotic bacteria, which are
40 likely to be sulfide-oxidizers. These results support the idea that sunken wood surfaces
41 constitute an environment suitable for sulfide-oxidizing symbioses.

42

43

44 **1 INTRODUCTION**

45

46 Thiotrophic symbioses have long been associated with particular geological
47 environments in the deep-ocean, such as hydrothermal vents or methane seeps. In these
48 environments productive communities of invertebrates have been shown to rely on
49 chemoautotrophic carbon fixation by microbes (Childress and Fisher 1992). Among them,
50 several species of bivalves and siboglinid tubeworms harbor endosymbiotic sulfide-
51 oxidizing bacteria (Cavanaugh 1985; Felbeck and Jarchow 1998; Duperron et al. 2005).
52 More recently, organisms relying on sulfide-oxidizing symbionts have been described in
53 association with organic falls in the shallow and deep-sea, most of them being related to
54 whale falls (Deming et al. 1997; Smith and Baco 2003; Fujiwara et al. 2007; Lorion et al.
55 2008). Symbiotic associations were notably described for the mussel *Idasola washingtonia*,
56 which lives attached to the bones, and the burrowing clam *Vesicomya* c.f. *gigas* found in
57 the sediments surrounding whale skeletons (Deming et al. 1997). From fluorescence *in Situ*
58 hybridization analyses and transmission electron microscopy (TEM) observations,
59 symbiotic associations were recently described for several mytilid species attached to the
60 wood pieces (Gros and Gaill 2007; Gros et al. 2007). While extracellularly located,
61 comparative analyses of 16S rDNA and adenosine-5'-phosphosulfate (APS) reductase
62 gene sequences from these “epibionts” indicate that they are closely related to sulfide-
63 oxidizing gill-endosymbionts of other *Bathymodiolinidae* (Duperron et al. 2008) providing
64 the first molecular evidence for the occurrence of thiotrophic symbiosis in direct
65 association with sunken wood.

66 Mangrove swamp is a shallow seawater environment known to offer a variety of
67 habitats for thiotrophic symbioses. High organic carbon influx from the mangrove forest
68 result in almost millimolar sulfide enrichment in the sediment, through anaerobic

69 degradation processes. Some invertebrates like the lucinid bivalves *Lucina pectinata*
70 and/or *Anodontia alba* living in these sediments were shown to harbour intracellular
71 sulfide-oxidizing symbionts (Durand et al. 1996; Frenkiel et al. 1996; Gros et al. 2003).
72 Moreover some ciliate colonies sampled from the mangrove swamp have also been shown
73 to develop from artificial sulfide sources in the laboratory (Vopel et al. 2001), and were
74 later confirmed to harbour sulfide-oxidizing epibionts (Rinke et al. 2006). Sulfide gradients
75 characterized *in situ* in the environment of symbiotic ciliates confirmed their close
76 association with sulfide “minivents” sustained by the degrading vegetable materials (Vopel
77 et al. 2005). These ciliates have been found on disturbed bacterial mats at the peat surface,
78 as well as on decaying leaves and rootlets from the mangrove peat (Bauer-Nebelsick et al.
79 1996a; 1996b; Ott and Bright 2004; Ott et al. 2004; Rinke et al. 2006), but have not been
80 described associated with dead wood.

81 To our knowledge, no study has been specifically dedicated to dead wood-
82 associated habitats in mangrove swamps. Nevertheless, naturally sunken dead wood is
83 abundant in mangrove swamps. These might constitute substantial habitats for organisms
84 relying on thiotrophic symbiosis in this environment. Furthermore, it was anticipated that
85 their study could provide some clues to address the relationship between sulfide
86 enrichment and the colonization by thiotrophic organisms.

87 The questions we addressed in this study were twofold: (1) what characterizes these
88 environments in terms of sulfide exposure? (2) does sunken wood provide a suitable
89 habitat for thiotrophic symbioses in a mangrove swamp? Our aim was to characterize
90 biological and chemical habitat conditions at the interface of sunken wood and seawater.
91 By deploying autonomous sulfide sensors on pieces of naturally sunken wood, we were
92 able to directly assess (*in situ*) the daily variability of sulfide concentration at the surface of

93 the wood. The associated organisms were subsequently observed and analyzed using
94 scanning electron microscopy (SEM).

95

96

97 **2 MATERIALS AND METHODS**

98

99 **2.1 Sampling**

100 For chemical measurements pieces of sunken wood from a few centimeters to 1
101 meter in length with a diameter up to 15 cm were analyzed. Such wood was located in < 1
102 meter depth in the mangrove swamp in Guadeloupe (16°N, 61.5°W) in the Caribbean area.
103 These pieces of wood, as well as the other collected vegetable material (leaves and seeds),
104 all derive from the dicotyledon species *Rhizophora mangle* (Linnaeus, 1753). A series of
105 these samples were transferred to the lab in order to analyze the associated microfauna
106 using SEM.

107 Collected samples were observed with a stereomicroscope before preparation for
108 SEM observations in order to detect possible symbioses among the organisms associated
109 with these items of vegetable debris. Underwater pictures were taken using a Nikon
110 Coolpix 5600 camera inside a DiCAPac bag.

111

112 **2.2 Sulfide concentration measurements**

113 Autonomous probes were used to measure *in situ* hydrogen sulfide concentration
114 and pH at the surface of sunken wood pieces (Fig. 1) and at the interface of mangrove
115 swamp water and sediment. The sulfide measuring system composed of a custom-made
116 Ag/Ag₂S sulfide electrode (0.8mm diameter, 2 mm length) was combined with a 2 mm
117 diameter pH glass electrode, both being connected to submersible potentiometric

118 autonomous data loggers (NKE, France). These sensors have been used in a variety of
119 deep-sea chemosynthetic habitats (Le Bris et al. 2008). The pH measuring system is
120 similar to the one described by Le Bris et al. (2001). A series of successive short-term
121 measurements were recorded on the surface of the wood, in the sediment and the overlying
122 water with tightly attached sulfide and pH electrodes. Following these snapshot
123 measurements, chemical conditions were continuously monitored over three days on a
124 piece of wood about 1 meter long and 10 cm wide (Fig. 1). One sulfide electrode was
125 attached to the wood, in order to maintain its sensing part in close contact with the surface
126 of the wood (Fig. 2). The sensing tip of the electrode was precisely positioned within a
127 white patch of a few millimeters diameter on the wood surface that was suspected to
128 indicate the presence of a ciliate colony. The 2 mm diameter pH electrode was positioned a
129 few centimeters apart from the sulfide electrode on another white patch (Fig. 1, 2). Our
130 reason for decoupling the electrodes was to overcome a too large physical disturbance that
131 would have resulted in both electrodes positioned in the same patch. For comparison, a
132 second sulfide electrode was positioned in the water a few centimeters from the wood
133 surface (Fig. 1, 2). The manipulation was performed very carefully avoiding displacing the
134 wood in order to preserve as much as possible the natural hydrodynamic conditions. The
135 electrodes were calibrated in the laboratory before deployment. The Nernstian sensitivity
136 of the silver sulfide electrode to sulfide ions offers the possibility to detect free sulfide over
137 a wide range of concentrations. Quantitative assessments of total free sulfide concentration
138 were done using the H_2S acidity constant of Rickard and Luther III (2007) and the pH
139 value simultaneously recorded with the glass electrode. It was assumed that the pH
140 variation is similar on both white patches, which might not be rigorously the case. As the
141 pH is needed to calculate the sulfide concentration from raw data, the results should be
142 regarded as first order estimates rather than high accuracy measurements. Nevertheless, the

143 continuous data provide access to the temporal variability of the medium, which is of
144 greater relevance to the addressed questions than would be a limited series of highly
145 accurate discrete measurements.

146

147 **2.3 Scanning Electron Microscopy observations**

148 Samples were fixed 2 hours at 4°C in 2% glutaraldehyde fixative solution in cacodylate
149 buffer (900 mOsm, pH 7.2). They were dehydrated in an ascending series of acetone,
150 critical point dried in CO₂, and sputter-coated with gold before viewed with a Hitachi S
151 2500 at 20 kV.

152

153

154 **3 RESULTS**

155

156 **3.1 Sulfide concentrations**

157 The mangrove sediments were confirmed to be highly enriched in sulfide while no
158 trace of sulfide was detected in the overlying water (Table 1). Concentrations of hydrogen
159 sulfide up to almost 1mM were detected in the mangrove sediment (data not shown),
160 consistent with the measurements of Ott et al. (1998) in the sediment of the mangrove
161 swamp of the Twin Cays (Belize). Sulfide variation with depth in the sediment appeared to
162 be very steep in the first centimeters. Microscale sediment profiling could not be achieved
163 with our sensors and was out of the scope of this study.

164 Short-term measurements within white patches on the surface of sunken wood
165 exposed to seawater also revealed local sulfide enrichments, up to at least 114 µM, while
166 no sulfide was detected in the surrounding water (Table 1). The 72 hour sequence,
167 additionally, showed that the sulfide levels and pH values on the surface of a naturally

168 sunken wood were very variable over time. Large fluctuations in the levels of sulfide were
169 observed, with concentration changes that can exceed 100 μM within 1 hour or less (Fig.
170 7a). In addition, a slower cycle was observed showing successive stages with low sulfide
171 maxima ($< 50 \mu\text{M}$ to undetectable) and high sulfide maxima ($>100 \mu\text{M}$) (Fig. 7a). This
172 periodicity was demonstrated by both the sulfide (Fig. 7a) and the pH (Fig. 7b) electrode
173 responses, even though the two measurements were completely independent, indicating
174 that this behavior was not a measurement artifact. A few centimeters from the wood
175 surface, the second sulfide sensor did not record any detectable sulfide in water. Our
176 measurement of sulfide therefore illustrates the environmental conditions at the surface of
177 the wood. Variations of 0.3 to 0.4 pH units in amplitude were observed over the 72 hour
178 period. Both pH and sulfide records correlated well with the water height at Pointe-à-Pitre,
179 suggesting a tidal influence on the local chemical conditions at the wood surface (Figs. 7 a
180 and b).

181

182 **3.2 Organisms analyzed**

183 All the wood samples analyzed were colonized by a very diversified fauna,
184 composed of organisms belonging to Annelida, Arthropoda, Ciliophora, Chordata,
185 Cnidaria, Kamptozoa, Mollusca, Nematoda, Plathelminthes, and Urochordata with
186 different abundances.

187 Among these organisms, the colonial ciliate *Zoothamnium niveum* (Hemprich &
188 Ehrenberg, 1831), which was attached to the surface of the wood, constituted the white
189 patches noticed during *in situ* sampling and chemical measurements. SEM observations
190 revealed the presence of numerous bacteria with 2 morphotypes covering the whole body
191 of the organisms similar to previous studies (Fig. 5) (Bauer-Nebelsick et al. 1996a; 1996b;
192 Rinke et al. 2006).

193 SEM observations of a vorticellid ciliate regularly recorded nearby *Zoothamnium*
194 *niveum* colonies on the wood samples, revealed the presence of a bacterial coat covering
195 the whole zooid and stalk surface (Fig. 6). The rod-shaped bacteria were organized in one
196 single layer on the surface of the organism.

197

198

199 **4 DISCUSSION**

200

201 **4.1 Wood as a suitable substrate to sustain thiotrophic symbioses**

202 Although our measurements did not achieve the sub-millimetric resolution of the
203 microelectrode used for the study of rootlets, they confirmed that sulfide is similarly
204 enriched at the surface of large pieces of sunken wood within the mangrove. Direct
205 degradation of wood material by microbes is likely to be the source for this sulfide
206 production, as the presence of a Teredinid burrow or a local accumulation of particulate
207 organic matter could be excluded. The mechanism that sustains sulfide production from
208 wood material still has to be detailed, but it is likely that microbial degradation of the wood
209 generates anoxic niches suitable for SRB activity as in organically enriched sediments. The
210 substantial pH decrease observed at the surface of the wood (7.3 to 7.6) compared to
211 mangrove swamp water (8.0) is also consistent with this assumption.

212 While *Z. niveum* and the vorticellid ciliates were already known in the mangrove
213 environment or on small pieces of decomposing organic substrate (Bauer-Nebelsick et al.
214 1996a; 1996b; Ott and Bright 2004; Ott et al. 2004), this study is the first to report their
215 presence on less easily degraded wood material. The sulfide enrichments measured on the
216 white patches of the sunken wood are comparable with those determined in the laboratory
217 by Vopel et al. (2001) on decomposing rootlets from peat pieces where *Z. niveum*

218 developed (110 and 360 μM H_2S) and *in situ* in the water contained in decomposing rootlet
219 tubes (up to 739 μM H_2S) (Vopel et al. 2005).

220 While the symbiotic association of *Z. niveum* with its sulfide-oxidizing symbionts has been
221 well described (Bauer-Nebelsick 1996 a and b; Ott et al. 1998; Ott and Bright 2004; Ott et
222 al. 2004; Rinke et al. 2006; 2007), there was no phylogenetic identification of the
223 vorticellid ciliate and its symbionts. In previous studies similar vorticellid ciliates,
224 identified as *Vorticella* sp., were able to develop on artificial sulfide sources (Vopel et al.
225 2001) and were observed close to *Z. niveum* colonies in the natural environment (Ott and
226 Bright, 2004). The vorticellid ciliate sampled in our study was identified as
227 *Pseudovorticella* sp. based on phylogenetic analyses (Muller, personal communication).

228 The presence of elemental sulphur, suggested by its white colour in incident light is
229 confirmed by Raman spectrometry (Maurin et al. 2008).

230 Regular observations of natural sunken wood pieces showed that *Z. niveum* colonies
231 persisted for several weeks on the surface of a single piece of wood, while their life span is
232 estimated at 7 days (Ott and Bright 2004), suggesting that these colonies find long-lasting
233 favourable conditions, implying that there is long-term sulfide availability.

234

235 **4.2 Sulfide variability in wood microhabitats and potential importance for** 236 **chemolithoautotrophic growth of symbiotic organisms**

237 The fact that the ciliates are concentrated on small patches suggests that suitable
238 conditions only occur on some particular features of the wood surface, presumably where
239 specific topographic features prevent the medium being totally flushed with oxygenated
240 seawater. Another important outcome of this study lies in the fact that at a single point the
241 sulfide concentration fluctuates over time. Sharp transitions commonly exceeding 100 μM

242 H₂S in amplitude over a period of < 1 hour and a marked periodicity of sulfide
243 concentration maxima were observed over longer time periods.

244 This study showed that periodicity was a characteristic feature of the sunken wood
245 habitat and reflected a tidal influence, and this is the first time that this has been recorded
246 in such a microhabitat. The lowest sulfide maxima were recorded during low tide and high
247 values corresponded with high tide. A likely explanation for this fluctuation is the tidal
248 variability of current intensity in the area (Bouchereau, personal communication). As
249 shown by Vopel et al. (2005) on shorter timescales, higher velocities may induce a
250 reduction of the thickness of the boundary layer at the surface of the wood and result in the
251 replacement of the sulfidic layer with an oxygenated layer. If we assume that the pH
252 increase at low tide is indicating the advection of mangrove water in the ciliate micro-
253 environment, the reduction of sulfide levels should be related to an increase in the oxygen
254 concentration. The fact that pH never goes up to the mangrove water value, even when
255 sulfide is not detectable, indicates that the chemical microenvironment remains
256 permanently under the influence of wood degradation processes and is thus likely to
257 remain hypoxic.

258 The tidal modulation of oxygen and sulfide exerts significant constraints on the
259 availability of chemical substrates for chemolithoautotrophic growth, with periods of low
260 electron donor availability. Conversely, the rapid and sharp fluctuations can be considered
261 to create optimal conditions for growth of epibionts, providing them with both sulfide and
262 oxygen. The concomitant pH increase suggests that oxygenated seawater is periodically
263 supplied to the microhabitat. These pulsations could be hydrodynamically-driven
264 fluctuations under the influence of local flow speed, as described in Vopel et al. (2005).

265

266

267 **5 CONCLUSION**

268

269 Fluctuating chemical conditions characterizing microhabitats on the surface of the
270 wood are likely to be a prerequisite for the settlement of sulfide-oxidizing symbioses.

271 Unlike peat rootlets, decaying leaves or disturbed peat surfaces, the production of
272 sulfide from the large piece of wood is potentially sustained over much longer period. This
273 would have important consequences on the capacity of thiotrophic organisms to colonize
274 sunken wood after its transport to the benthic habitat. From the analysis of wood-
275 associated fauna diversity, Distel et al. (2000) reported close relationships with vent and
276 seep species and suggested that these habitats might have served as stepping stones, at
277 evolutionary scale, for the colonization of deep-sea sulfidic habitats. This idea is consistent
278 with the fact that sulfide production can be supported by bacteria during the wood
279 degradation process. The potential for long-term evolution of colonization at the surface of
280 the wood needs to be further studied, particularly monitoring sulfide levels and
281 colonization over the course of the wood degradation.

282

283

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292

293

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413

414 **FIGURE CAPTIONS**

415

416 Figure 1: *In situ* measurement set-up during the 3 day autonomous deployment. The pH
417 electrode (straight arrow) and one of the sulfide electrodes (curved arrow) are
418 positioned on the surface of a naturally sunken piece of wood, the second sulfide
419 electrode is lying in the water above the mangrove sediment a few centimeters from the
420 wood (asterisk) (sensors data loggers are not visible on this picture).

421

422 Figure 2: Close up view of the sensing tips sulfide (curved arrow) and pH (straight arrow)
423 electrodes at the surface of the wood. The electrodes are maintained in close contact
424 with ciliate colonies visible as white patches on the wood surface.

425

426 Figures 3-4: Colonies of ciliates are restricted to specific areas of wood surface.

427 *Zoothamnium niveum* feather-shaped colonies (Fig.3) and *Vorticella* sp. (Fig. 4) appear
428 white-colored in incident light. The little zooid (curved arrow) of *Vorticella* sp. is on a
429 long stalk (straight arrow).

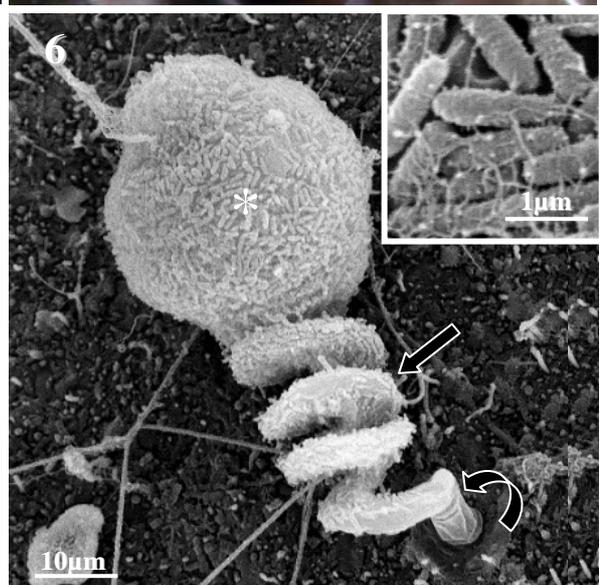
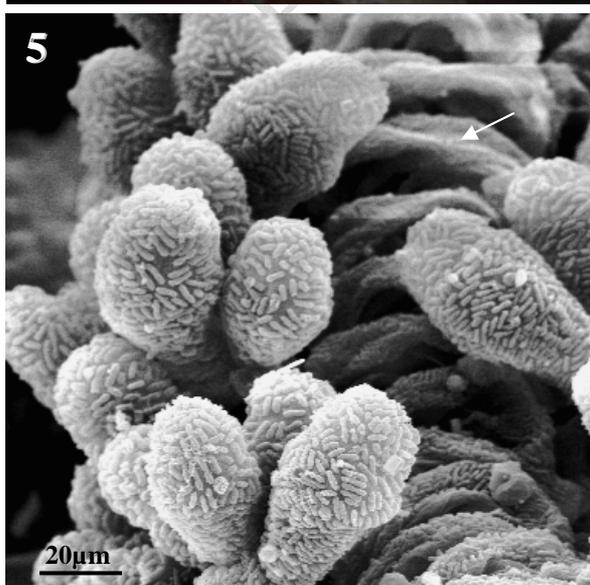
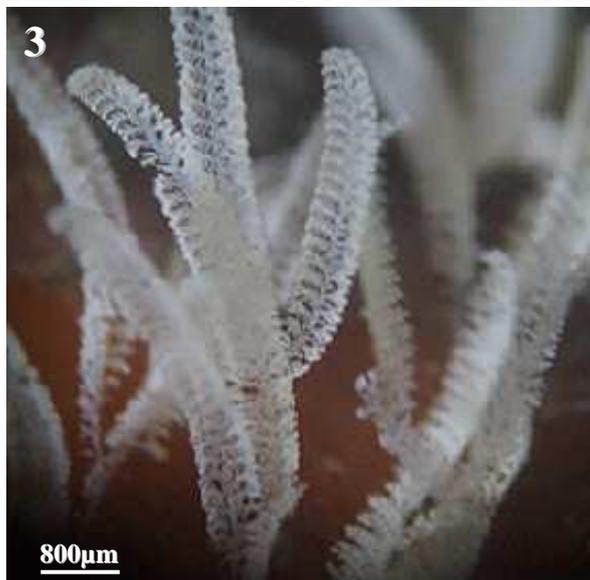
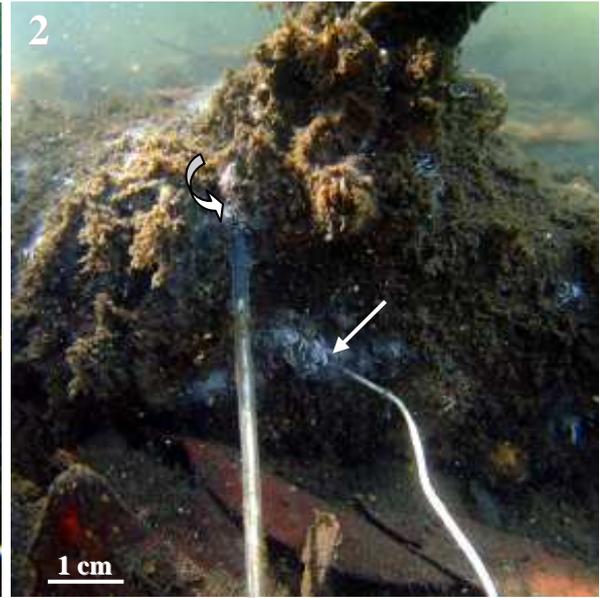
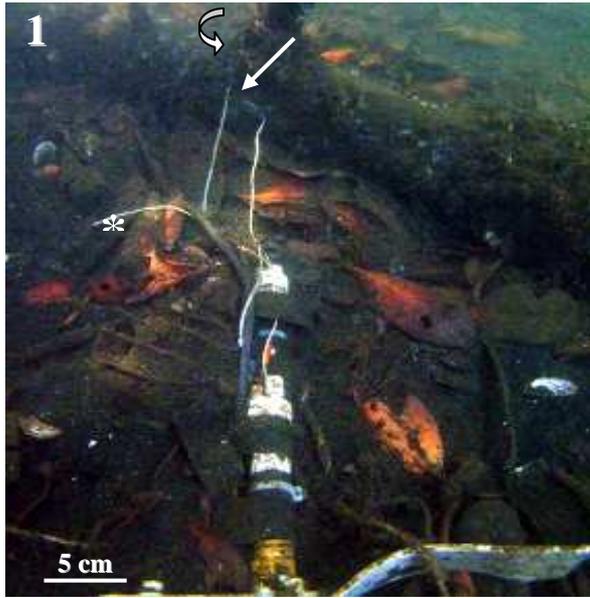
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431 Figures 5-6: SEM views of the protozoan ciliates collected on wood samples. The retracted
432 coiled central stalk (arrow) of the feather-shaped colonial ciliate (*Zoothamnium*
433 *niveum*) with numerous zooids. Both, zooids and stalk, are covered by bacteria (Fig. 5).
434 The solitary vorticellid ciliate is composed of a single inverted bell-shaped zooid
435 (asterisk) borne by a long spring-shaped stalk when contracted (straight arrow) (Fig. 6).
436 Both, zooids and stalks, are also covered by bacteria of about 1.5 μ m long (insert). Only
437 the adhesive disk and the more basal part of the central stalk are devoid of bacteria
438 (curved arrow).

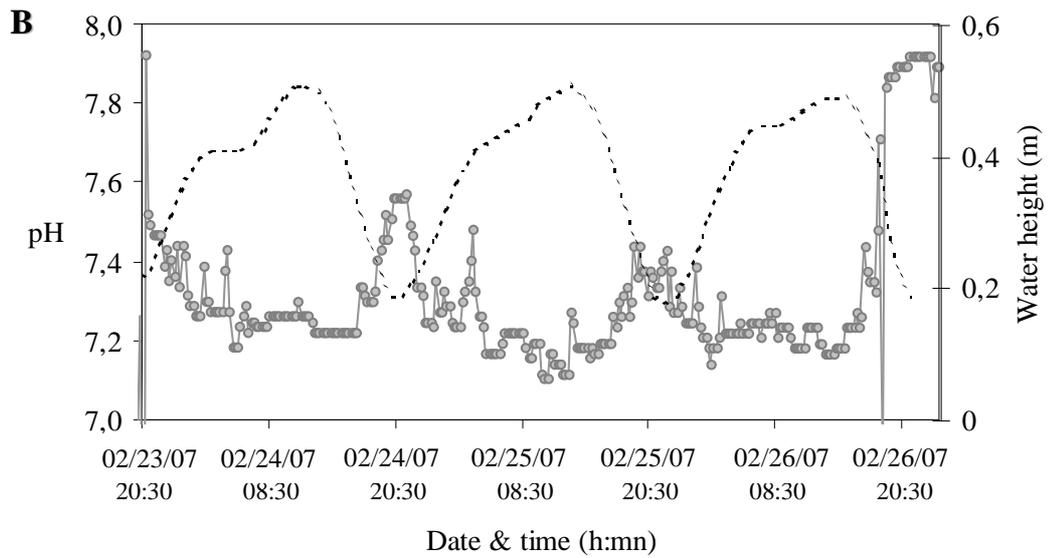
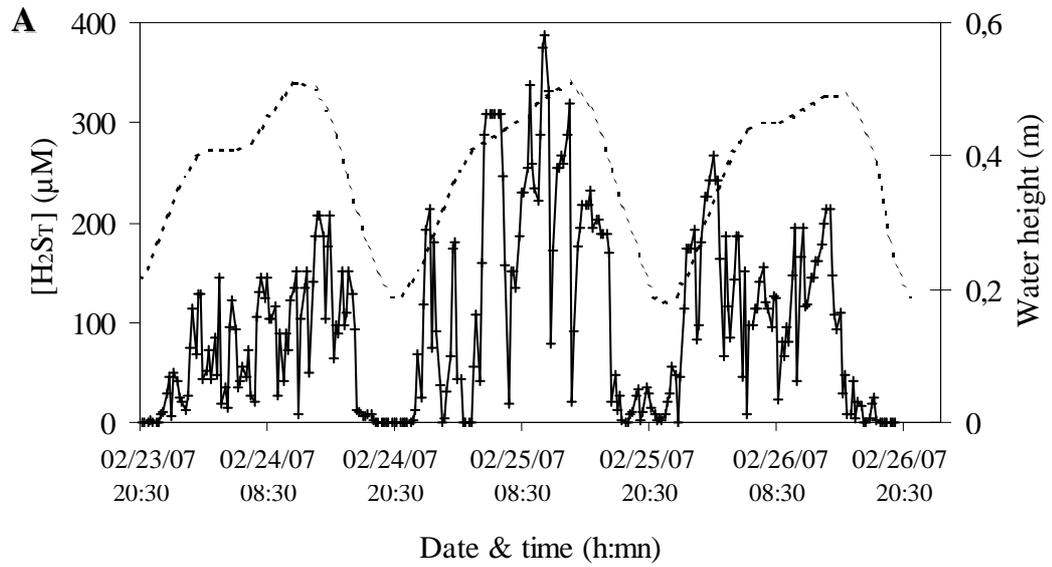
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440 Figure 7: Variation of the total H₂S concentration (a) and pH (b) over 72 hours on the
441 surface of a sunken piece of wood, together with predicted water height at Pointe-à-
442 Pitre.

443



7



—+—+—+— [H₂S] (µM)
—○—○—○— pH
·········· Water height (m)

Table 1: Sulfide content measured from discrete measurements in the mangrove water, sediment pore water and sunken wood surface.

Environment	Sulfide concentration (μM)	pH
Mangrove water (n=4)	< 0.1	7.86 – 7.99
Mangrove sediment < 5cm (n=7)	<0.1 - 375	nd
Mangrove sediment 20-35cm (n=4)	71 - 850	7.33 – 7.78
Sunken wood surface (n=8)	< 0.1 - 114	7.42 – 7.92

nd = not determined