



Sunken wood habitat for thiotrophic symbiosis in mangrove swamps

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ABSTRACT

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

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

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1. Introduction

Thiotrophic symbioses have long been associated with particular geological environments in the deep-ocean, such as hydrothermal vents or methane seeps. In these environments productive communities of invertebrates have been shown to rely on chemoautotrophic carbon fixation by microbes (Childress and Fisher, 1992). Among them, several species of bivalves and siboglinid tubeworms harbour endosymbiotic sulfide-oxidizing bacteria (Cavanaugh, 1985; Felbeck and Jarchow, 1998; Duperron et al., 2005). More recently, organisms relying on sulfide-oxidizing symbionts have been described in association with organic falls in the shallow and deep-sea, most of them being related to whale falls (Deming et al., 1997; Smith and Baco, 2003; Fujiwara et al., 2007; Lorion et al., 2008). Symbiotic associations were notably described for the mussel *Idasola washingtonia*, which lives attached to the bones, and the burrowing clam *Vesicomya* c.f. *gigas* found in the sediments surrounding whale skeletons (Deming et al., 1997). From fluorescence *in situ* hybridization analyses and transmission electron microscopy (TEM) observations, symbiotic associations were recently described for several mytilid species attached to the wood pieces (Gros and Gaill, 2007; Gros et al., 2007). While extracellularly located, comparative analyses of 16S rDNA and

adenosine-5'-phosphosulfate (APS) reductase gene sequences from these "epibionts" indicate that they are closely related to sulfide-oxidizing gill-endosymbionts of other *Bathymodiolinidae* (Duperron et al., 2008) providing the first molecular evidence for the occurrence of thiotrophic symbiosis in direct association with sunken wood.

Mangrove swamp is a shallow seawater environment known to offer a variety of habitats for thiotrophic symbioses. High organic carbon influx from the mangrove forest result in almost millimolar sulfide enrichment in the sediment, through anaerobic degradation processes. Some invertebrates like the lucinid bivalves *Lucina pectinata* and/or *Anodontia alba* living in these sediments were shown to harbour intracellular sulfide-oxidizing symbionts (Durand et al., 1996; Frenkiel et al., 1996; Gros et al., 2003). Moreover some ciliate colonies sampled from the mangrove swamp have also been shown to develop from artificial sulfide sources in the laboratory (Vopel et al., 2001), and were later confirmed to harbour sulfide-oxidizing epibionts (Rinke et al., 2006). Sulfide gradients characterized *in situ* in the environment of symbiotic ciliates confirmed their close association with sulfide "minivents" sustained by the degrading vegetable materials (Vopel et al., 2005). These ciliates have been found on disturbed bacterial mats at the peat surface, as well as on decaying leaves and rootlets from the mangrove peat (Bauer-Nebelsick et al., 1996a,b; Ott and Bright, 2004; Ott et al., 2004; Rinke et al., 2006), but have not been described associated with dead wood.

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

The questions we addressed in this study were twofold: (1) what characterizes these environments in terms of sulfide exposure? (2) Does sunken wood provide a suitable habitat for thiotrophic symbioses in a mangrove swamp? Our aim was to characterize biological and chemical habitat conditions at the interface of sunken wood and seawater. By deploying autonomous sulfide sensors on pieces of naturally sunken wood, we were able to directly assess (*in situ*) the daily variability of sulfide concentration at the surface of the wood. The associated organisms were subsequently observed and analyzed using scanning electron microscopy (SEM).

2. Materials and methods

2.1. Sampling

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

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

2.2. Sulfide concentration measurements

Autonomous probes were used to measure *in situ* hydrogen sulfide concentration and pH at the surface of sunken wood pieces (Fig. 1) and at the interface of mangrove swamp water and sediment. The sulfide measuring system composed of a custom-made Ag/Ag₂S sulfide electrode (0.8 mm diameter, 2 mm length) was combined with a 2 mm diameter pH glass electrode, both being connected to submersible potentiometric autonomous data loggers (NKE, France). These sensors have been used in a variety of deep-sea chemosynthetic habitats (Le Bris et al., 2008). The pH measuring system is similar to the one described by Le Bris et al. (2001). A series of successive short-term measurements were recorded on the surface of the wood, in the sediment and the overlying water with tightly attached sulfide and pH electrodes. Following these snapshot measurements, chemical conditions were continuously monitored over three days on a piece of wood about 1 m long and 10 cm wide (Fig. 1). One sulfide electrode was attached to the wood, in order to maintain its sensing part in close contact with the surface of the wood (Fig. 2). The sensing tip of the electrode was precisely positioned within a white patch of a few millimeters diameter on the wood surface that was suspected to indicate the presence of a ciliate colony. The 2 mm diameter pH electrode was positioned a few centimeters apart from the sulfide electrode on another white patch (Figs. 1 and 2). Our reason for decoupling the electrodes was to overcome a too large physical

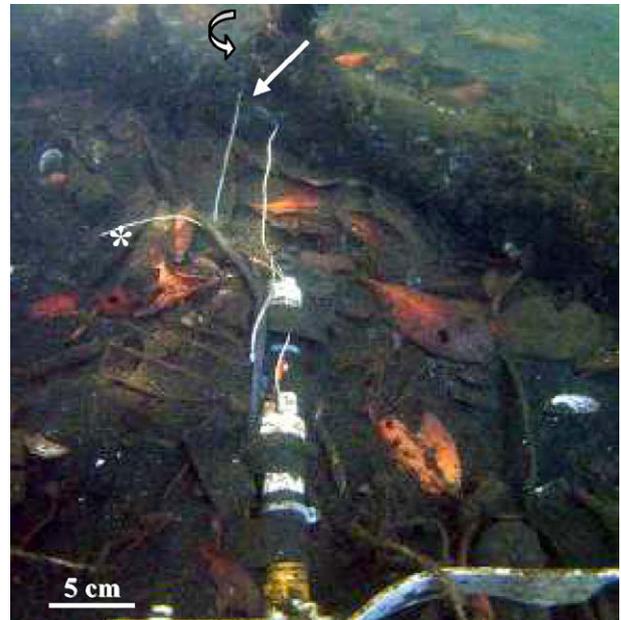


Fig. 1. *In situ* measurement set-up during the 3 day autonomous deployment. The pH electrode (straight arrow) and one of the sulfide electrodes (curved arrow) are positioned on the surface of a naturally sunken piece of wood, the second sulfide electrode is lying in the water above the mangrove sediment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

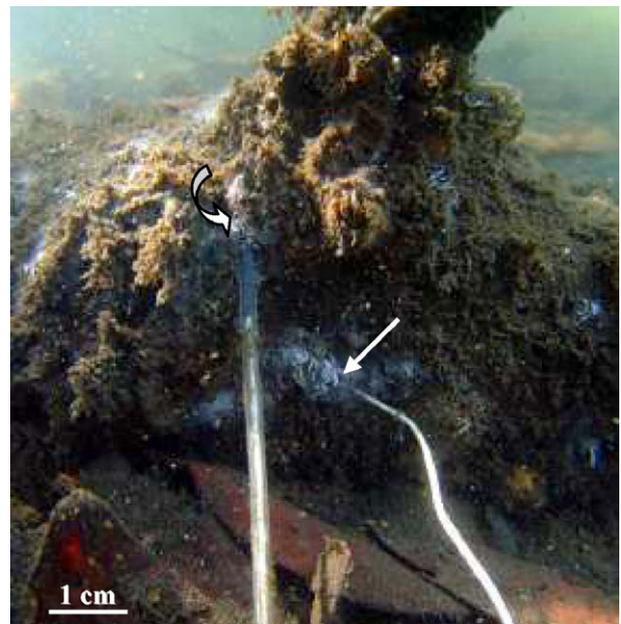


Fig. 2. Close up view of the sensing tips sulfide (curved arrow) and pH (straight arrow) electrodes at the surface of the wood. The electrodes are maintained in close contact with ciliate colonies visible as white patches on the wood surface.

disturbance that would have resulted in both electrodes positioned in the same patch. For comparison, a second sulfide electrode was positioned in the water a few centimeters from the wood surface (Figs. 1 and 2). The manipulation was performed very carefully avoiding displacing the wood in order to preserve as much as possible the natural hydrodynamic conditions. The electrodes were calibrated in the laboratory before deployment. The Nernstian sensitivity of the silver sulfide electrode to sulfide ions offers the possibility to detect free sulfide over a wide range of concentrations.

Quantitative assessments of total free sulfide concentration were done using the H_2S acidity constant of Rickard and Luther III (2007) and the pH value simultaneously recorded with the glass electrode. It was assumed that the pH variation is similar on both white patches, which might not be rigorously the case. As the pH is needed to calculate the sulfide concentration from raw data, the results should be regarded as first order estimates rather than high accuracy measurements. Nevertheless, the continuous data provide access to the temporal variability of the medium, which is of greater relevance to the addressed questions than would be a limited series of highly accurate discrete measurements.

2.3. Scanning electron microscopy observations

Samples were fixed 2 h at 4 °C in 2% glutaraldehyde fixative solution in cacodylate buffer (900 mOsm, pH 7.2). They were dehydrated in an ascending series of acetone, critical point dried in CO_2 , and sputter-coated with gold before viewed with a Hitachi S 2500 at 20 kV.

3. Results

3.1. Organisms analyzed

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

Among these organisms, the colonial ciliate *Zoothamnium niveum* (Hemprich and Ehrenberg, 1831) (Fig. 3), which was attached to the surface of the wood, constituted the white patches noticed during *in situ* sampling and chemical measurements. SEM observations revealed the presence of numerous bacteria with two morphotypes covering the whole body of the organisms similar to previous studies (Fig. 5) (Bauer-Nebelsick et al., 1996a,b; Rinke et al., 2006).

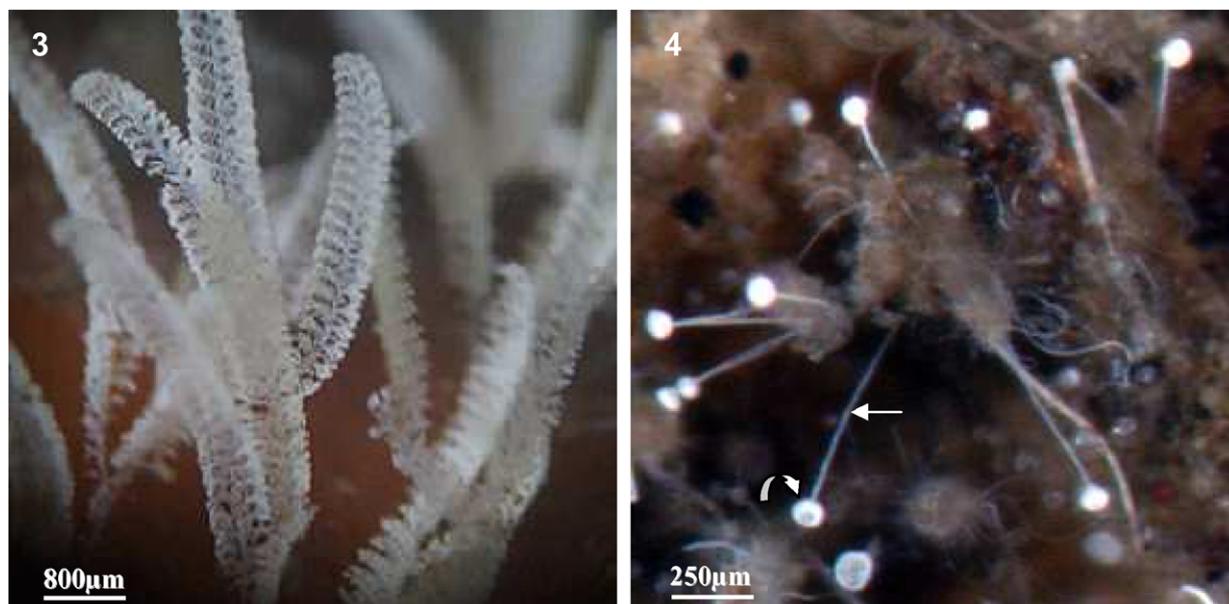
SEM observations of a vorticellid ciliate regularly recorded nearby *Z. niveum* colonies on the wood samples (Fig. 4), revealed

the presence of a bacterial coat covering the whole zooid and stalk surface (Fig. 6). The rod-shaped bacteria were organized in one single layer on the surface of the organism.

3.2. Sulfide concentrations

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

Short-term measurements within white patches on the surface of sunken wood exposed to seawater also revealed local sulfide enrichments, up to at least 114 μM , while no sulfide was detected in the surrounding water (Table 1). The 72 h sequence, additionally, showed that the sulfide levels and pH values on the surface of a naturally sunken wood were very variable over time. Large fluctuations in the levels of sulfide were observed, with concentration changes that can exceed 100 μM within 1 h or less (Fig. 7a). In addition, a slower cycle was observed showing successive stages with low sulfide maxima (<50 μM to undetectable) and high sulfide maxima (>100 μM) (Fig. 7a). This periodicity was demonstrated by both the sulfide (Fig. 7a) and the pH (Fig. 7b) electrode responses, even though the two measurements were completely independent, indicating that this behavior was not a measurement artifact. A few centimeters from the wood surface, the second sulfide sensor did not record any detectable sulfide in water. Our measurement of sulfide therefore illustrates the environmental conditions at the surface of the wood. Variations of 0.3–0.4 pH units in amplitude were observed over the 72 h period. Both pH and sulfide records correlated well with the water height at Pointe-à-Pitre, suggesting a tidal influence on the local chemical conditions at the wood surface (Fig. 7a and b).



Figs. 3–4. Colonies of ciliates are restricted to specific areas of wood surface. *Zoothamnium niveum* feather-shaped colonies (Fig. 3) and the solitary vorticellid ciliate (Fig. 4) appear white-colored in incident light. The little zooid (curved arrow) of the vorticellid is on a long stalk (straight arrow).

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 <5 cm ($n = 7$)	<0.1–375	nd
Mangrove sediment 20–35 cm ($n = 4$)	71–850	7.33–7.78
Sunken wood surface ($n = 8$)	<0.1–114	7.42–7.92

nd, not determined.

4. Discussion

4.1. Wood as a suitable substrate to sustain thiotrophic symbioses

Although, our measurements did not achieve the sub-millimetric resolution of the microelectrode used for the study of rootlets, they confirmed that sulfide is similarly enriched at the surface of large pieces of sunken wood within the mangrove. Direct degradation of wood material by microbes is likely to be the source for this sulfide production, as the presence of a Teredinid burrow or a local accumulation of particulate organic matter could be excluded. The mechanism that sustains sulfide production from wood material still has to be detailed, but it is likely that microbial degradation of the wood generates anoxic niches suitable for SRB activity (Leschine, 1995) as in organically enriched sediments. The substantial pH decrease observed at the surface of the wood (7.3–7.6) compared to mangrove swamp water (8.0) is also consistent with this assumption.

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

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

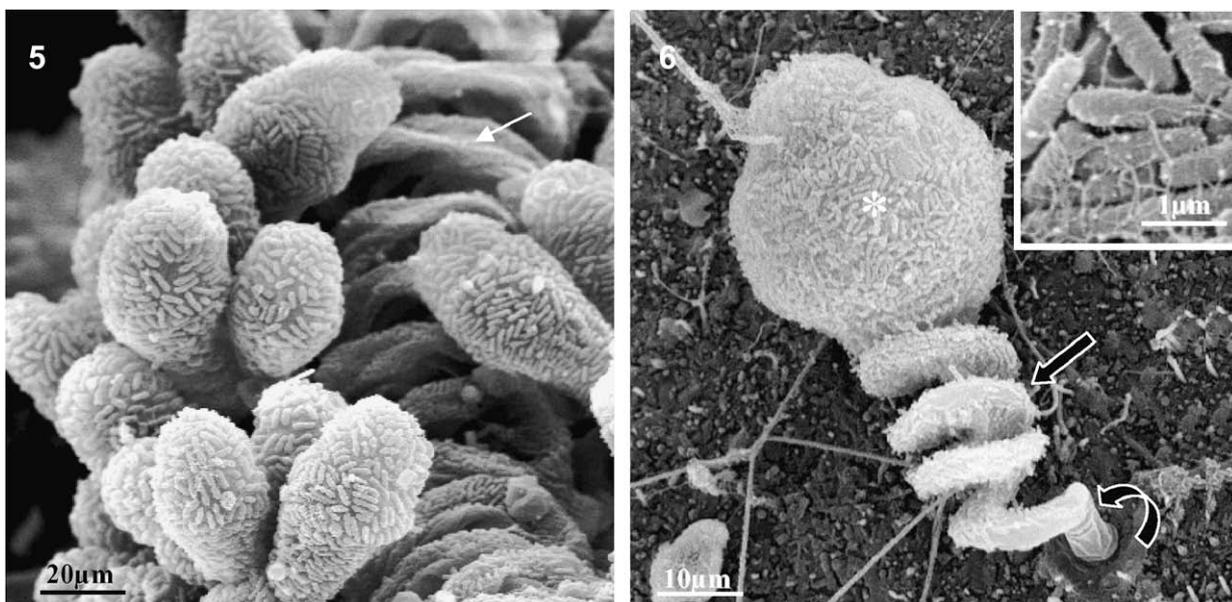
While the symbiotic association of *Z. niveum* with its sulfide-oxidizing symbionts has been well described (Bauer-Nebelsick et al., 1996a,b; Ott et al., 1998, 2004; Ott and Bright, 2004; Rinke et al., 2006, 2007), there was no phylogenetic identification of the vorticellid ciliate and its symbionts. In previous studies similar vorticellid ciliates, identified as *Vorticella* sp., were able to develop on artificial sulfide sources (Vopel et al., 2001) and were observed close to *Z. niveum* colonies in the natural environment (Ott and Bright, 2004). The vorticellid ciliate sampled in our study was identified as *Pseudovorticella* sp. based on phylogenetic analyses (Muller, personal communication). The presence of elemental sulfur, suggested by its white color in incident light is confirmed by Raman spectrometry (Maurin et al., 2008).

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

4.2. Sulfide variability in wood microhabitats and potential importance for chemolithoautotrophic growth of symbiotic organisms

The fact that the ciliates are concentrated on small patches suggests that suitable conditions only occur on some particular features of the wood surface, presumably where specific topographic features prevent the medium being totally flushed with oxygenated seawater. Another important outcome of this study lies in the fact that at a single point the sulfide concentration fluctuates over time. Sharp transitions commonly exceeding 100 μM H_2S in amplitude over a period of <1 h and a marked periodicity of sulfide concentration maxima were observed over longer time periods.

This study showed that periodicity was a characteristic feature of the sunken wood habitat and reflected a tidal influence, and this



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

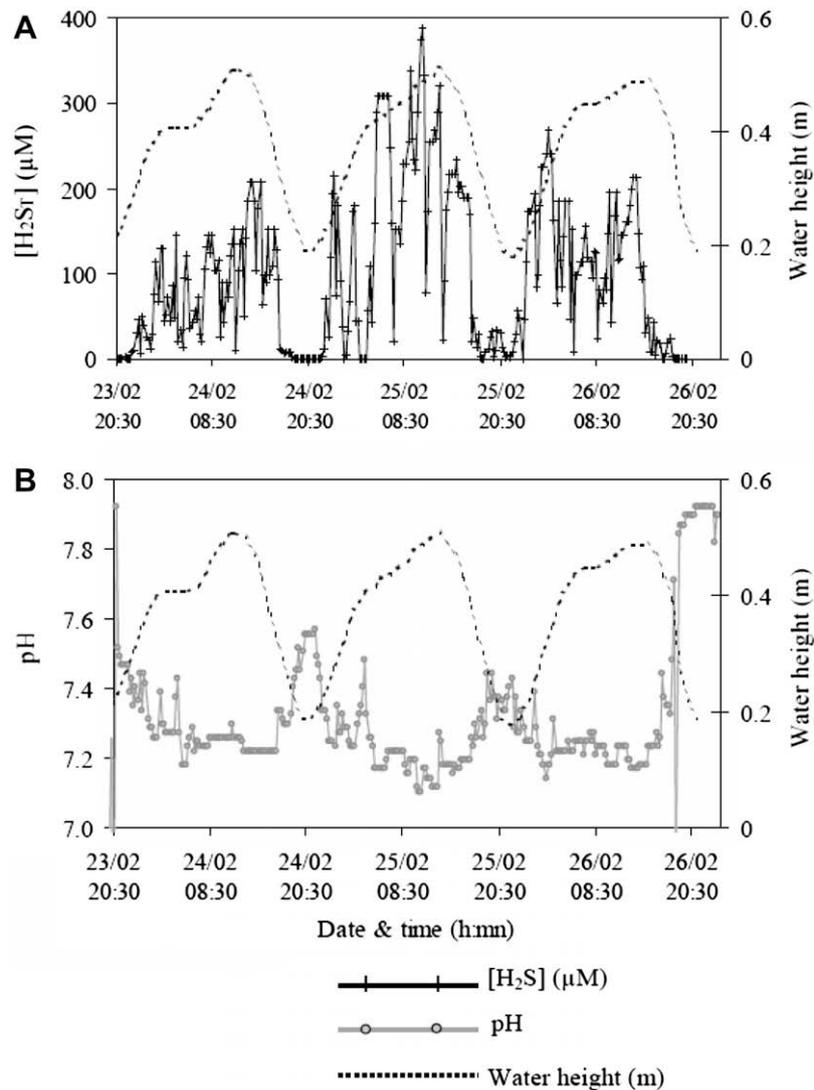


Fig. 7. Variation of the total H_2S concentration (a) and pH (b) over 72 h on the surface of a sunken piece of wood, together with predicted water height at Pointe-à-Pitre.

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

The tidal modulation of oxygen and sulfide exerts significant constraints on the availability of chemical substrates for chemolithoautotrophic growth, with periods of low electron donor availability. Conversely, the rapid and sharp fluctuations can be considered to create optimal conditions for growth of epibionts, providing them with both sulfide and oxygen. The concomitant pH increase suggests that oxygenated seawater is periodically supplied to the microhabitat. These pulsations could be hydrodynam-

ically driven fluctuations under the influence of local flow speed, as described in Vopel et al. (2005).

5. Conclusion

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

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

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