In situ hydrogen dynamics in a hot spring microbial mat during a diel cycle

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Abstract

Microbes can produce molecular hydrogen (H_2) via fermentation, dinitrogen fixation or direct photolysis, yet the H_2 dynamics in cyanobacterial communities has only been explored in a few natural systems and mostly in the laboratory. In this study, we investigated the diel in situ H_2 dynamics in a hot spring microbial mat, where various ecotypes of unicellular cyanobacteria (Synechococcus sp.) are the only oxygenic phototrophs. In the evening, H_2 accumulated rapidly after the onset of darkness reaching peak values of up to 30 µmol H_2 L^{-1} at about 1 mm depth below the mat surface, slowly decreasing to about 11 µmol H_2 L^{-1} just before sunrise. Another pulse of H_2 production reaching a peak concentration of 46 µmol H_2 L^{-1} was found in the early morning under dim light conditions too low to induce accumulation of O_2 in the mat. The light stimulation of H_2 accumulation indicated nitrogenase activity to be an important source of H_2 during the morning. This is in accordance with earlier findings of a distinct early morning peak in N_2 fixation and expression of Synechococcus nitrogenase genes in mat samples from the same location. Fermentation might have contributed to the formation of H_2 during the night, where accumulation of other fermentation products lowered the pH in the mat to <pH 6 as compared to a spring source pH of 8.3.

Importance

Hydrogen is a key intermediate in anaerobic metabolism, and with the development of a sulfide-insensitive microsensor for H_2 it is now possible to study the microdistribution of H_2 in stratified microbial communities such as the photosynthetic microbial mat investigated here. The capability to measure H_2 profiles within the mat as compared to previous measurements of H_2 emission gives much more detailed information about sources and sinks of H_2 in such communities, and it was demonstrated that high rates of H_2 formation in the early morning when the mat was exposed to low light intensities could be explained by nitrogen fixation where H_2 is formed as a by-product.
Introduction

Cyanobacterial mats are found in various limnic, marine and hypersaline environments. The thickest and most homogeneous mats are found in extreme environments such as hypersaline habitats and geothermal springs with scarcity or total absence of grazers. Such microbial mats are important natural models systems for studying microbial interactions and fundamental links between structure, diversity and function in microbial communities (1,2). Another reason for the extensive interest in these mats is their strong apparent similarity to ancient microbial mats inhabiting the early Earth before grazers evolved and now preserved as stromatolites (3). Knowledge about the functioning of modern microbial mats may thus give insights into the functioning of early microbial ecosystems on planet Earth. Hydrogen (H₂) presumably played a major role in the metabolism of primitive microbial communities (4), and outgassing of H₂ originating or escaping from these microbial communities could have played a major role in the gradual oxidation of our planet (5).

Coastal and hypersaline cyanobacterial mats are known to be rather vigorous producers of H₂ during darkness (5-10), and some H₂ production may also be observed in the beginning of a light period due to photolysis (10). Microcoleus sp., which was a dominating cyanobacterium in the mat H₂ evolution studies listed above, is known to be a vigorous H₂ producer during fermentation (11). Burow et al. (7) showed that the H₂ production in the dark in a hypersaline microbial mat could be ascribed to cyanobacterial fermentation, whereas significant H₂ production from N₂-fixation as a by-product of nitrogenase activity (N₂ + 8 H⁺ + 8 e⁻ → 2 NH₃ + H₂) could not be verified. A detailed analysis of an intertidal cyanobacterial mat dominated by Microcoleus sp. using sulfide-insensitive H₂ microsensors showed that maximum H₂ concentrations reached up to 40 µmol H₂ L⁻¹ (depending on the previous light exposure history of the mat) within the first hour after darkening (10), whereafter the H₂ concentration decreased to near zero within about 7 h. A main reason for this decrease was consumption by sulfate reducing bacteria (SRB), as addition of the sulfate analogue and SRB inhibitor molybdate led to even higher H₂ concentrations that persisted for
a much longer time period. In another coastal microbial mat, SRB were also identified as the major hydrogenotrophic prokaryotes (12).

The hot spring microbial mats in the siliceous alkaline Mushroom Spring and nearby Octopus Spring have been extensively studied for more than 40 years as natural model systems for investigating in situ interactions of metabolic processes and microbial diversity (1,2). In these hot springs, microbial mats growing at water temperatures >54°C show a restricted diversity, with various ecotypes of the cyanobacterium *Synechococcus* (13) being the only oxygenic phototrophs. Metagenomic and metatranscriptomic analyses have demonstrated a relatively low diversity of taxonomic groups, including several photo-heterotrophic or photo-mixotrophic Choroflexi (*Roseifexus, Choroflexus* and *Anaerolineae*-like taxa), photoheterotrophic acidobacteria (*Chloracidobacterium*), Chlorobi (*Candidatus Thermochlorobacter*), as well as two novel aerobic heterotrophic taxa (14-17). Such relatively limited diversity has made these hot spring mats attractive for study of metabolic interactions among taxa under in situ conditions and under a natural diel cycle. In the slightly alkaline 50-70°C Octopus Spring mat, for example, it was shown that the two most abundant taxa, *Synechococcus* and *Roseiflexus*, produce glycogen (18) (and in *Roseiflexus* polyhydroxyalkanoic acid, as well (17,19)), which they ferment at night time to produce large amounts of acetate and propionate that are photo-assimilated by filamentous, *Chloroflexus*-like organisms (probably related to *Roseiflexus* (20)) the next morning (19,21,22). Both Anderson et al. (22) and van der Meer (23) reported that H₂ was formed during darkness in the mats, but the accumulation was restricted by diffusion into the overlying water (19) as well as H₂ consumption by SRB (24,25) and methanogenic bacteria (21,26) in the 50-60°C mat. Rates of H₂ accumulation were much higher in the 65°C mat, which did not exhibit any methanogenesis.

Genomic and metagenomic analyses of microbial mats in Mushroom Spring and Octopus Spring have demonstrated that *Roseiflexus* (15,27), but not *Synechococcus* (28) possess hydrogenases, and could thus produce H₂ by fermentation. The lack of hydrogenases in *Synechococcus* means that any H₂ produced by *Synechococcus* nitrogen fixation is lost to the environment where other mat organisms may use it as
electron donor in anoxygenic photosynthesis or respiration. Metatranscriptomic studies conducted on a 60°C mat in Mushroom Spring, demonstrated that the expression of *Synechococcus* nitrogenase genes occurred in darkness and that N₂ fixation peaked in the early morning under dim light conditions below the compensation irradiance, keeping the mat largely anoxic (16,29,30). Based on these previous observations, we hypothesized that H₂ formation in Mushroom Spring and Octopus Spring cyanobacterial mats would exhibit a peak in the early morning due to N₂ fixation as well as a peak after sunset that could be caused by fermentation and N₂ fixation supplied with ATP from fermentation.

In this study we tested this hypothesis by quantifying the diel H₂ dynamics in a Mushroom Spring microbial mat at a hitherto unreached spatio-temporal resolution using a newly developed sulfide-insensitive H₂ microsensor (31). Earlier studies showed the potential for H₂ production in dark-incubated mats (22), including a few albeit un-calibrated H₂ microsensor measurements (23). The sensitivity of previous H₂ microsensors to hydrogen sulfide strongly limited their applicability for studying the zonation and dynamics of H₂ metabolism in microbial mats. By use of the improved microsensor for H₂ in combination with microsensors for O₂, H₂S and pH, it was possible to describe the chemical microenvironment in the mat in great detail enabling new insights into the regulation of H₂ dynamics.

**MATERIALS AND METHODS**

Mushroom Spring is a siliceous, slightly alkaline hot spring (2) located in the Lower Geyser Basin of Yellowstone National Park (Wyoming, USA). The spring has a large source pool that drains 68-70°C water through a single effluent channel that gradually widens, while the spring water is cooling down. Our study site had a water temperature throughout the diel cycle of 58±3°C and harbored a microbial mat with a ~1 mm thick green cyanobacterial layer on top of an orange under-mat. Field measurements were conducted on August 12-13, 2014, and the associated laboratory experiments were done a few days earlier.
Most measurements were done in situ, but a few cores (8 mm diameter, about 1 cm deep) were sampled and brought back to the laboratory for preliminary analysis. Samples were transported to the laboratory in a 1-L stainless steel thermos filled with in situ water and were then immediately placed in a 58°C bath filled with stirred in situ water. A fiber-optic halogen lamp equipped with a collimating lens (KL-2500, Schott, Germany) illuminated the mat samples with a downwelling photon irradiance of 1000 µmol photons m² s⁻¹, as determined with a PAR quantum irradiance sensor (MQS-B, Heinz Walz GmbH, Effeltrich, Germany).

Concentrations of dissolved H₂ and O₂ as well as pH were measured in the mat and overlying spring water using microsensors. The H₂ microsensors (Unisense A/S) had tip diameters of 70 µm and were made H₂S insensitive by placing a ZnCl₂/propylene carbonate - based H₂S trap in front of the H₂ microsensor tip. The addition of the sulfide trap furthermore lowered the baseline current by lowering the H₂O influx to the anode so that the sensors could be used at temperatures up to about 60°C (31). The sensors optimally have a detection limit of about 20 nmol H₂ L⁻¹ (31), but at the high temperature of the spring and lack of continuous signal recording the detection limit was 0.2 µmol H₂ L⁻¹ corresponding to a 1 pA change in signal. The sensors were four-point calibrated (0-69 µmol H₂ L⁻¹) in the laboratory at 58°C verifying linearity of the signal, and two-point calibrated (0-34 µmol H₂ L⁻¹) in the field. The standards were prepared using dilutions of water saturated with H₂ at 20°C (805 µmol H₂ L⁻¹ at sea level pressure, but 693 µmol H₂ L⁻¹ at the barometric pressure of Bozeman, MT, where the bottle was prepared; 32). This procedure may induce some inaccuracy in the calibration, as the temperature of our supposedly 693 µmol H₂ L⁻¹ solution containing a H₂ headspace varied under field conditions causing changes in the equilibrium between gas-phase and dissolved H₂, and the temperature of the 34 µmol H₂ L⁻¹ standard was also affected by the dilution. We estimate that the potential error in the calibration could be as high as 15%. The sensors gave a linear response of about 4 pA per µM H₂ at the in situ temperature of 58°C. The sensors needed about 30 s of equilibration at each depth and concentration profiles we therefore only measured with 0.5 mm depth resolution.
The O₂ microsensors (33) had a tip diameter of 10 µm and were made in our laboratory at AU. It was linearly calibrated in the field based on sensor readings in anoxic parts of the mat (zero oxygen) and in spring water (58°C) equilibrated with air by vigorous shaking of a 50-ml centrifuge tube that was about 70% filled with spring water and with intermittent placement in the spring to keep the temperature near 58°C.

At the atmospheric pressure for the 2227 m elevation of Mushroom Spring it is necessary to multiply the saturations from standard tables (www.unisense.com) by a factor of 0.77, resulting in an O₂ concentration of 118 µmol L⁻¹. Due to non-perfect temperature control during air equilibration, we estimate a potential error in the calibration of up to 15%.

The pH microelectrode was made in our laboratory at AU and had a 250 µm-long pH sensitive glass tip and a tip diameter of 40 µm (34). It was calibrated in pH 6.96 and pH 9.76 buffers at 58°C using a commercial reference electrode (REF201, Radiometer, Denmark) and yielding a sensitivity of 58 mV per pH unit.

Laboratory profiles of H₂S were measured with an amperometric H₂S microsensor (35) having a tip diameter of 30 µm. The sensor was made in our laboratory at AU and was painted black to avoid light interference on the signal (35). The sensor was linearly calibrated from signal readings in H₂S-free water and in a standard prepared by dissolving a washed Na₂S ·9H₂O crystal in 10 µmol L⁻¹ anoxic HCl (10 mmol L⁻¹) to a final concentration of 200 µmol H₂S L⁻¹. The calibration was done within a few minutes of preparing the standard.

The signals from the O₂, H₂, and H₂S microsensors were read by a battery operated custom-built pA-meter with a resolution of 10⁻¹³ A. The signal from the pH sensor was read with a custom-built battery-operated high impedance voltmeter with an internal resistance of 10¹⁴ Ohms. Both meters were enclosed in plastic bags with silica gel during field measurements. In the laboratory and during daytime field measurements, the instruments were connected to a battery-operated strip-chart recorder (SE-110, Gossen-Metrawatt, Germany), while signals were read directly from the meter display during nighttime as steam from the hot spring water caused excessive condensation of water on all surfaces.
The microsensors were mounted and advanced vertically into the microbial mat with manually operated micromanipulators that could be read with better than 10 µm accuracy. The micromanipulators were attached to heavy metal stands.

The field measurements were initiated in the late afternoon, but in the afternoon and evening only single profiles for all three parameters were measured. In the morning this was changed to triplicates for H₂, but still only single profiles for O₂ and pH. The three locations for measurement of H₂ were chosen at random within the 2.5 X 2.5 cm square covered by the x-y positioning of the micromanipulator.

Simultaneous with the diel microsensor measurements, 1 L bottles were filled to capacity with 58°C spring water and an 8 mL headspace of atmospheric air was introduced into the bottles through a butyl rubber septum. After equilibration of the water with the gas phase via 30 s of vigorous shaking, the H₂ gas concentration in the headspace was immediately analyzed on site using a portable Gas Chromatograph (490 Micro GC, Agilent Technologies, Santa Clara, CA, USA) using a thermal conductivity detector connected to a 10 m unheated MolSieve 5 Å column, running with a flow of 99.9999% pure Argon as carrier gas. A standard curve for the analysis was obtained by adding various volumes of H₂ saturated water to spring water sampled at daytime when there was no H₂ in the spring water. The detection limit of the GC was 4 nmol H₂ L⁻¹.

Temperature loggers (iButton DS1922L, Maxim Integrated Products, Inc., San Jose, CA, USA) were placed in the spring on the biofilm surface, logging the spring water temperature at 5 minute intervals. Downward photon irradiance of photosynthetically active radiation (PAR, 400-700 nm) was monitored continuously in air throughout the field measurements with a PAR quantum irradiance sensor (MQS-B, Heinz Walz GmbH, Effeltrich Germany) placed next to the spring and connected to a light meter (ULM-500, Heinz Walz GmbH, Effeltrich Germany).
Based on the in situ concentration profiles measured over a diel cycle, isopleths of pH, O₂, and H₂ concentration were generated using the software package Ocean Data View (Schlitzer R., Ocean Data View, http://odv.awi.de, 2015) based on weighted-average gridding.

RESULTS

The temperature of the spring water flowing above the mat was relatively constant over time, but lower air temperatures at night reduced the water temperature to \(-55^\circ\)C and solar exposure during daytime caused maximum temperatures of up to 60°C at noon (Fig. 1). The incident photon irradiance was fluctuating in the afternoon prior to 19:00 due to shifting cloud cover, but exhibited a very smooth curve in the interesting regions around sunset and sunrise. A photon irradiance of \(-1\%\) of maximum noon-time irradiance (1500 µmol photons m\(^{-2}\) s\(^{-1}\)) was reached at 20:20 in the evening, and at 06:28 in the morning.

The chemistry of the Mushroom Spring mat changed dramatically throughout the diel cycle. To illustrate this, pH, O₂ and H₂ concentration profiles measured at noon and at sunrise (07:00) down to 4-6 mm depth in the mat are shown in Fig. 2. At sunrise, the irradiance was too low (60 µmol photons m\(^{-2}\) s\(^{-1}\); Fig. 1) to cause a net production of O₂, and O₂ only penetrated to about 0.3 mm depth. At noon, oxygenic photosynthesis caused an oxygen peak in the surface layer of >800 µmol O₂ L\(^{-1}\), and O₂ was present in high concentrations down to the deepest analyzed layer at 5.25 mm. The pH values showed similar large changes with the water pH increasing from 7.4 at 07:00 to 8.5 at noon, and pH at 1 mm depth increasing from 6.1 to 9.4 in the same period. Hydrogen was present down to 3 mm depth at 07:00, and a maximum concentration of 46 µmol H₂ L\(^{-1}\) was found at 1.5 mm depth. At noontime, no H₂ could be detected in the mat.
Isopleths of pH, O₂ and H₂ concentration in the mat through the diel cycle (18.00 – 12:00) are shown in Fig. 4. The data-points used for construction of the H₂ isopleths are average values for three measurements in the period from 04:00 to 10:00 h.

The diel variation in H₂ concentration within the mat exhibited two peaks (Fig. 4): i) a broader peak with maximum concentrations of about 30 µmol H₂ L⁻¹ after sunset (20:30 to 22:15) that faded away to reach about 11 µmol H₂ L⁻¹ just before sunrise, and ii) a sharp peak in the early morning at 07:00 under a low diffuse photon irradiance of ~60 µmol photons m⁻² s⁻¹. The average maximum H₂ concentration from the three profiles measured at 07:00 was 46 µmol H₂ L⁻¹, but the profiles were very different with maximum concentrations at 1-1.5mm depth of 49, 27, and 64 µmol H₂ L⁻¹, respectively. The depth penetration of H₂ was also variable, with two profiles extending down to 2 mm, whereas one profile had a H₂ penetration of 4 mm. The deep H₂ penetration seen in Fig. 3 at 7:00 it thus due to one atypical profile and should not be over-interpreted. Similar variations among sites were also found in the other morning H₂ concentration profiles as evident from the large standard deviations in Fig. 4.

The first trace of H₂ (0.7 µmol H₂ L⁻¹) in the evening was found at 19:25 when there was still an irradiance of 170 µmol photons m⁻² s⁻¹. An O₂ profile was recorded 10 minutes later (19:35, at an irradiance of 100 µmol photons m⁻² s⁻¹) and at that time the O₂ peak had disappeared and the mat was anoxic below 0.5 mm depth. A similar high photon irradiance was needed to cause a significant build-up of O₂ in the mat in the morning, where a photon irradiance of 250 µmol photons m⁻² s⁻¹ at 07:58 did not cause significant change, whereas the following O₂ concentration profile measured at 08:22 (350 µmol photons m⁻² s⁻¹) exhibited an O₂ peak and a 0.75 mm increase in O₂ penetration depth as compared to the dark profiles. The H₂ concentration decreased to one third from 07:00 to 07:36 although we could not detect a change in the O₂ concentration profile until 07.58, where no H₂ was detected in the mat. The depth resolution of our measurements was, however, rather crude with O₂ only being measured every 0.25 mm, and pH and H₂ every 0.5 mm. While a slight increase in O₂ penetration between 07:00 and 07:58 that went undetected cannot be ruled out, the O₂ concentration in the overlying water remained constant at 61-63 µmol O₂ L⁻¹.
during the measurements at 05:53, 06:26, and 06:53, before showing a slight increase to 91 µmol O₂ L⁻¹ at 07:58. Consequently, there cannot have been any change in the O₂ profile until after 06:53 (and possibly considerably later), while the morning peak in H₂ concentrations in the mat was measured from 06:32 – 07:40.

The standard deviations on the measured H₂ concentrations are substantial, and based on the data from the H₂ microprofiles alone we cannot conclude that the two peaks in H₂ accumulation are representative. However, the efflux of H₂ from the mat was investigated by GC analysis, and the data from these measurements also showed a two-peak pattern (Fig. 4). However, the H₂ content in the spring water was probably strongly affected by the H₂ emission from mats further up-stream and thus presents some kind of integration of the H₂ efflux from mats growing at 58-70°C.

Concentration profiles of O₂ and H₂S were measured in mat cores that were brought back to the laboratory. We also attempted H₂S measurements in the field, but insufficient optical insulation of the H₂S microsensors (35) destroyed these before H₂S might have accumulated during the night. The laboratory measurements showed a maximal concentration of 70 µmol H₂S L⁻¹ (non-dissociated H₂S, total sulfide is higher) -1.5 mm below the mat surface after 2 hours of dark incubation, decreasing to 40 µmol H₂S L⁻¹ at 6 mm depth (data not shown). An earlier study of the Mushroom Spring mat found even lower free H₂S concentrations (23), while Dillon et al. (25) reported maximum concentrations of 250 µmol H₂S L⁻¹ and reported substantial sulfate reduction rates in the upper mat layers. The H₂ sensor applied in the field was only tested for H₂S insensitivity up to ~200 µmol H₂S L⁻¹, but H₂S interference on the H₂ signal would show up as a high signal increasing with depth (9,10), while all H₂ profiles showed zero concentration in deeper layers.

**DISCUSSION**
Earlier studies have also reported H₂ formation in hot spring microbial mats, where H₂ production may be due to fermentation (18,19,22,36) or formation as a by-product of nitrogenase activity during N₂ fixation (30). The relative importance of these processes for H₂ dynamics in hot spring cyanobacterial communities has remained unclear, while studies of hypersaline and coastal cyanobacterial mats have identified fermentation as the major H₂ source (7-9). In this study we show that the H₂ production/accumulations in the Mushroom Spring mat correlate well with the patterns of nitrogenase expression and activity described in previous studies.

Most non-heterocystous cyanobacteria are prone to O₂ inhibition of the nitrogenase enzyme catalyzing N₂ fixation (37), although some can perform the process under fully oxic conditions (38-40). In cyanobacterial mats found at ~55 to 65°C in Mushroom Spring and nearby Octopus Spring, N₂ fixation is limited to conditions of hypoxia or anoxia in the mat during low irradiance and nighttime (29,30). Dinitrogen fixation is a very energy-demanding process, and the finding of the highest levels of nitrogenase activity at sunrise was explained by the presence of light for ATP production, while the mat was still largely anoxic due to the consumption of O₂ within the mat being higher than the production (30).

We observed a peak in H₂ production exactly in the period where the cyanobacterial layer of the mat was still largely anoxic under a low incident photon irradiance of about 60 µmol photons m⁻² s⁻¹. However, it cannot be ruled out that the H₂ production continued to be high also later in the morning, but that autotrophic or mixotrophic anoxygenic phototrophy by Chloroflexus-like organisms (17,23) and oxic metabolism based on cryptic oxygenic photosynthesis consumed this H₂. Prokaryotes having high-affinity terminal oxidases are not restricted in their O₂ consumption until they experience concentrations of a few nmoles O₂ L⁻¹ (41) and an environment with simultaneous presence of H₂ and a cryptic O₂ production by oxygenic photosynthesis would select for knallgas bacteria (i.e., bacteria oxidizing H₂ with O₂) having such high affinity terminal oxidases.

High levels of nitrogenase gene expression (16,29,30) as well as N₂ fixation activity (30) have been observed at sunset in the Octopus and Mushroom Spring mats. While we cannot rule out that all H₂ production in the
afternoon-evening transmission was due to nitrogenase activity, the massive accumulation of fermentation products shown in dark-incubated mats from Mushroom Spring (19,22) and the very low nighttime pH values measured in this study provide evidence that fermentation could be another source of H₂ accumulation in the mat. Previously measured accumulations of acetate + propionate in dark incubated vials containing a 1-cm deep core of the mat (22) thus indicate that volatile fatty acids may build up to tens of millimolar concentrations in the upper millimeters of the mat during the night. Furthermore, metagenomic analysis of the mat has demonstrated the presence of many taxa that possess hydrogenase genes and should be capable of producing H₂ during fermentation (42).

The balance between nitrogenase- and fermentation-mediated H₂ production in a hypersaline mat dominated by Microcoleous sp. was investigated by Burow et al. (7), and they only found evidence of fermentation as a source of H₂, which was further supported in a recent study of H₂ dynamics in intertidal cyanobacterial mats (9). Diel changes in N₂ fixation in Mushroom Spring were investigated by Steunou et al. (30), who found early morning peak rates of nitrogenase activity (i.e., acetylene reduction) in the illuminated but largely anoxic mat that were >10 times higher than the nitrogenase activity measured just before sunset, indicating a pronounced potential stimulation of H₂ formation by low light. There are no known mechanisms whereby light could stimulate fermentation, but low irradiance might provide energy for cyanobacterial N₂ fixation (and associated H₂ production) in three different ways: 1) by cryptic oxygenic photosynthesis producing both ATP and NADH, but where O₂ does not build up as the consumption in the mat is larger than the production, 2) by respiratory use of the O₂ produced by cryptic oxygenic photosynthesis, and 3) via PSI-driven ATP formation without O₂ evolution by use of the Mehler reaction (43). We conclude that the high H₂ concentration measured in the Mushroom Spring mat during the morning was most likely due to light-driven alleviation of energy limitation fueling intense N₂-fixation. Other diazotrophs than Synechococcus could in principle produce H₂, but the light stimulation of H₂ formation indicates that the dominating N₂-fixing organisms should be phototrophs, and the abundant anoxygenic phototrophs in the mat (Roseiflexus and Chloroflexus) have not been shown to express genes

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for N₂ fixation (30). Instead of producing H₂ these organisms may use H₂ as electron donor (30). The quantitative contribution of fermentation to H₂ production in the Mushroom Spring mat remains unclear. Steunou et al. (30) found that N₂-fixation (nitrogenase activity) in the evening just after sunset was only 20% of the peak morning values at low light intensities, whereas we found only 1½ times higher (H₂ profiles, Fig. 4) or similar (overlying water H₂, Fig. 4) H₂ concentrations in the morning peak as compared to the evening. Just before sunrise the nitrogenase activity was <5% of the peak rates found just 2 h later. The discrepancy between nitrogenase activity pattern and H₂ accumulation indicates that fermentation could be a major factor responsible for the H₂ accumulation in the evening and night.

High morning rates of N₂-fixation and associated H₂ production could also be significant in other types of cyanobacterial mats. Diel variations in N₂-fixation in marine cyanobacterial mats have been subject to investigation (44), but expression of nitrogenase genes and rates of N₂ fixation were not found to correlate and no morning peak in N₂-fixation was detected. However, if high morning rates would be present, it is unlikely that they would result in massive H₂ accumulation as H₂ produced by nitrogenase activity in the early morning may be consumed rapidly by SRB that are much more abundant in marine and hypersaline mats, and which may be very active after several hours of anoxia. It was thus shown that high concentrations of H₂ were found in a marine cyanobacterial mat throughout an 8-h dark period when molybdate was added as an inhibitor of sulfate reduction, while mat without inhibitor addition was depleted of H₂ within a few hours (10). We note that SRB could potentially also contribute significantly to N₂ fixation in such mats but a recent study only showed a minor contribution of SRB to N₂-fixation in a hypersaline mat (45).

We only found measurable H₂ levels down to 4.5 mm below the mat surface, and H₂ never accumulated in the deeper parts of the Mushroom Spring mat. High rates of sulfate reduction have been measured in the Mushroom Spring mat (25), and sulfate reduction could thus cause depletion of H₂ in deeper layers. In nearby Octopus Spring, methanogenesis was shown to be the major sink for H₂ in dark-incubated 55°C mats.
(22), where addition of the methanogenesis inhibitor bromoethane sulfonic acid (BES) led to massive efflux of \( \text{H}_2 \) from mat samples. An efflux of \( 0.2 \, \mu\text{mol H}_2 \, \text{cm}^{-2} \, \text{h}^{-1} \) can be calculated from the profile in Fig. 2 using Fick’s first law of diffusion and the diffusion coefficient for \( \text{H}_2 \) at 58°C in water (9.7 x 10^{-5} \, \text{cm}^2 \, \text{s}^{-1}; \) tables at www.unisense.com), and that is essentially identical to the \( \text{H}_2 \) formation rate after BES addition found earlier. Similar rates of methanogenesis (0.04 \, \mu\text{mol CH}_4 \, \text{cm}^{-2} \, \text{h}^{-1} \) corresponding to a consumption of 0.16 \, \mu\text{mol H}_2 \, \text{cm}^{-2} \, \text{h}^{-1} \) were measured in the 60°C Mushroom Spring mat (46), i.e., close to the site analyzed in this study (58°C). Both sulfate reducers and methanogens could thus have contributed to keeping \( \text{H}_2 \) concentrations below detection limit in deeper mat layers (>4.5 mm depth). High-sensitivity gas chromatography on anoxic mat material from deeper layers would probably have shown \( \text{H}_2 \) concentrations of 1-10 nmol L^{-1} as typically found in methanogenic and sulfate reducing environments (47), but such concentrations are more than an order of magnitude lower than the detection limit of our sensors which at the high temperature of the mat was 0.2 \, \mu\text{mol L}^{-1}. The mat was oxic far below 4.5 mm depth during the day (Fig. 3), but analysis of mat samples (from Octopus Spring) that were oxic during daytime showed abundant presence of methanogenic archaea (\( 10^7 \text{-} 10^8 \) cells g^{-1} wet weight) and pure cultures isolated from the samples survived hours of oxic conditions (24; NP Revsbech and DM Ward, unpublished).

We found lower early morning pH levels (<pH 6) in the mats than the pH minimum of 7 earlier measured in Octopus Spring (48) and Mushrooms Spring (49) mats, and we speculate whether a change in sensor calibration during the measuring period could be the explanation. A change in the reference potential could cause such effects, while it is not possible to obtain an increase in pH microsensor sensitivity significantly above the 58 mV per pH unit determined in the calibration. The measured pH at 2 to 3 mm depth was 1.4 pH unit below the water reading at 04:00, and as other investigations (48-50) found early morning pH values in the overlying water from 7.7-7.9 in similar settings, it can be concluded that the pH in the mat must have been below 6.5. Our site was far outside the main flow channel, and the overlying water pH might have been further lowered as seen in our data (pH 7.1 at 04:00) by a long contact time with the mat, resulting in a pH minimum in the mat considerably below 6.5. Extensive effects of mat-water exchange on
overlying water chemistry are also evident from the O$_2$ measurements showing minimum O$_2$ concentrations of 61 µmol O$_2$ L$^{-1}$ from 06:00 to 07:00 and up to 185 µmol O$_2$ L$^{-1}$ at 12:00.

Although we could not observe any change in O$_2$ profile in the mat until after 08:00 in the morning, with the first significant change observed at 8:22, a substantial change in the pH profile could be observed. At 04:00, the overlying water was at pH 7.1, and the minimum pH at 1.5 mm depth was -pH 5.7. At 07:12, coinciding with the maximum H$_2$ accumulation, the pH in the water had increased to pH 7.7, and the pH at 1.5 mm depth was pH 6.4. At 07:43, the pH in the water was 8.0, and the pH at 1.5 mm depth was 6.8.

These rather large deviations in overlying water pH as compared to the source water (pH 8.3; 23) indicate that there was a high photosynthetic activity in the mat prior to 08:22 although this did not result in an accumulation of O$_2$ as the incident photon irradiance was at or below the compensation irradiance, where O$_2$ production and consumption processes balanced each other. It is not possible to estimate how much of this photosynthetic activity was due to the oxygenic cyanobacteria or the anoxygenic phototrophs in the mat, but photo-mixotrophic anoxygenic metabolism has been documented for the filamentous anoxygenic phototrophic bacteria *Chloroflexus* spp. and *Roseiflexus* spp. that are abundant in the Mushroom Spring Mat (19), and these organism may thus both fix CO$_2$ and photoassimilate VFAs (22).

Both the N$_2$ fixation by *Synechococcus* and the photoassimilation of fermentation products are facilitated by the diel changes in chemistry and light regime of the mat. While *Chloroflexus aurantiacus* and *Roseiflexus castenholzi* can be cultured in laboratory media supplied with for example acetate (27,51), high concentrations of acetate are usually not found in static environments. Microbial specialization to utilize the metabolic opportunities created by cyclic changes in environmental conditions (e.g., 19) may be more common than generally realized. Other well-known examples are the accumulation of polyphosphate by e.g., *Candidatus Accumulibacter phosphatis* during the oxic part of oxic/anoxic cycles (52), and the accumulation of elemental sulfur in the beginning of a light period by purple sulfur bacteria (53).
In conclusion, our in situ measurements of the diel H$_2$, O$_2$ and pH dynamics in the Mushrooms Spring microbial mat showed strong evidence for nitrogenase activity being a major source of H$_2$ accumulating in the mat during the evening and especially early morning, when dim irradiance alleviated energy limitation in the upper mat layers. Photosynthetic activity at such times with low irradiance may have profound effects on microbial communities by supplying energy to anoxic types of metabolisms, and possibly also oxic metabolism occurring at non-detectable O$_2$ concentrations.

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Figure 1. Photon irradiance (400-700 nm), air temperature and water temperature at the mat surface measured throughout the diel experimental period. The dip in irradiance at 18:00 was due to cloud cover.

Figure 2. In situ vertical profiles of O₂, pH and H₂ levels in the Mushroom Spring mat measured during the period with maximum H₂ concentrations (07:00) and at noontime. The conversion factor from micromolar \( H_2 \) to ppmv is \( 1 \mu mol \, H_2 \, L^{-1} = 1613 \) ppmv (at the elevation of Mushroom Spring).

Figure 3. Isopleths of O₂, pH and H₂ depth distribution over a diel cycle in the 58°C Mushroom Spring mat. The measured profiles on which the interpolations were based are shown as dots. The last O₂ profile in the morning was recorded at 08:22 when the first peak of O₂ was recorded, the upper 1 mm of the mat became oxic, and H₂ became non-detectable. Notice that the interpolation for H₂ between about 23:00 and 03:00 is uncertain due to a lack of measuring points during this time interval.

Figure 4. Maximum in situ H₂ concentrations in the 58°C mat as measured with microsensors, and H₂ concentrations in the overlying spring water as measured by GC during the diel cycle. Only one profile was measured for each time point in the evening, whereas three profiles at random positions were measured in the morning. Standard deviations are thus shown for the morning values.