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Hydrogen production in photosynthetic microbial mats in the Elkhorn Slough estuary, Monterey Bay

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Hydrogen (H_2) release from photosynthetic microbial mats has contributed to the chemical evolution of Earth and could potentially be a source of renewable H_2 in the future. However, the taxonomy of H_2-producing microorganisms (hydrogenogens) in these mats has not been previously determined. With combined biogeochemical and molecular studies of microbial mats collected from Elkhorn Slough, Monterey Bay, California, we characterized the mechanisms of H_2 production and identified a dominant hydrogenogen. Net production of H_2 was observed within the upper photosynthetic layer (0–2 mm) of the mats under dark and anoxic conditions. Pyrosequencing of rRNA gene libraries generated from this layer demonstrated the presence of 64 phyla, with *Bacteriodetes*, *Cyanobacteria* and *Proteobacteria* dominating the sequences. Sequencing of rRNA transcripts obtained from this layer demonstrated that *Cyanobacteria* dominated rRNA transcript pyrotag libraries. An OTU affiliated to *Microcoleus* spp. was the most abundant OTU in both rRNA gene and transcript libraries. Depriving mats of sunlight resulted in an order of magnitude decrease in subsequent nighttime H_2 production, suggesting that newly fixed carbon is critical to H_2 production. Suppression of nitrogen (N_2)-fixation in the mats did not suppress H_2 production, which indicates that co-metabolic production of H_2 during N_2-fixation is not an important contributor to H_2 production. Concomitant production of organic acids is consistent with fermentation of recently produced photosynthate as the dominant mode of H_2 production. Analysis of rRNA % transcript: % gene ratios and H_2-evolving bidirectional [NiFe] hydrogenase % transcript:% gene ratios indicated that *Microcoelus* spp. are dominant hydrogenogens in the Elkhorn Slough mats.
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Introduction

Microbial mats are among the most phylogenetically and physiologically diverse ecosystems on Earth (Ley *et al.*, 2006; Kunin *et al.*, 2008; Bolhuis and Stal, 2011). These systems undergo daily cycling of redox potential, increasing as oxygenic phototrophs supersaturate the upper strata with oxygen during the day and then decreasing as the mats become completely anoxic at night. Although the role of mats in global carbon and oxygen cycling is acknowledged and well studied (Nisbet and Fowler, 1999; Des Marais, 2003), their H_2 ecology has been underexplored.

H_2 release from microbial mats has likely been important for the chemical evolution of Earth, where its emission over long periods of geological time might be an important contributing factor to the oxidation of our atmosphere (Hoehler *et al.*, 2001). Today, H_2 is an important industrial chemical, where $10^8\,m^3$ (at 1 atm) are sold annually in the US alone (Lee *et al.*, 2010). Biological systems capable of producing industrially significant amounts of H_2 have been an important focus of alternative energy research. Mat ecosystems could thus have a potential role as a renewable source of H_2 in the future (Bender and Phillips, 2004). Knowledge of the metabolic processes that lead to H_2 generation and the phylogenetic identity of associated H_2-producing microorganisms (hydrogenogens) within highly complex microbial mat communities will be an important initial step towards realizing the potential of these systems as a viable H_2 source. This knowledge will be useful in optimizing system parameters,

such as light, carbon and nutrient concentrations, that enhance hydrogenogen activity and may facilitate future use of microbial mats as a commercial source of H_2.

The activity of phototrophs and diazotrophs is well established in photosynthetic microbial mats (Des Marais, 2003) and both groups are capable of producing H_2, either fermentatively or as a co-metabolic product of N_2-fixation (Bothe et al., 2010). Recent molecular ecology studies of microbial mats have focused on use of the H_2-evolving bidirectional [FeFe] hydrogenase as a biomarker for investigating hydrogenogens in photosynthetic mats (Boyd et al., 2009, 2010). These studies infer that non-phototrophic, anaerobes are important hydrogenogens in mat ecosystems. However, the class of hydrogenases targeted and the assays used in these studies did not include H_2-evolving bidirectional [NiFe] hydrogenases harbored by phototrophic microorganisms. To our knowledge, no studies have examined the diversity of H_2-evolving bidirectional [NiFe] hydrogenases in microbial mats that may be active in phototrophic hydrogenogens that produce H_2 by fermentative processes. Thus, our current understanding of the overall microbial ecology of H_2 cycling in microbial mats remains an incomplete picture.

Previous work, which employed biogeochemical approaches has served to constrain several aspects of H_2 cycling in microbial mats (Skyring et al., 1988, 1989; Hoehler et al., 2001). However, robust linkages between microorganisms and processes that generate H_2 remain to be established and the taxonomic identity of principal hydrogenogens is unknown. In this study we determined the location of hydrogenogens in Elkhorn Slough mats, the relative importance of co-metabolic production of H_2 during N_2-fixation vs fermentation and phototrophic vs heterotrophic H_2 production. In parallel we used pyrosequencing of SSU rRNA genes and transcripts and sequencing of H_2-evolving bidirectional [NiFe] hydrogenase genes and transcripts to obtain evidence for the taxonomic identity of dominant hydrogenogens.

Methods

Field site sampling and short-term maintenance of mats in a greenhouse
Photosynthetic microbial mats were sampled from a tidal zone in the Elkhorn Slough estuary at Monterey Bay, California (Latitude 36.830 °N, Longitude −121.785 °W) on November 9, 2009. The samples measured 18×23 cm^2 and were ~2 cm in depth. The samples were transported to the NASA Ames Research Center greenhouse within 1–2 h of collection from the slough. Upon arrival, mats were placed in acrylic aquaria transparent to ultraviolet radiation and covered with 4 l of water (~3 cm of overlying water column) from the field site. The salinity of the field site water was 35‰ and had a pH of 8.0. Mats were incubated under natural solar irradiance and a regulated temperature (~19 °C, in situ average) for up to 5 days, during which diel cycle experiments were carried out.

Analysis of H₂ and organic acids
Replicate vials were prepared identically for each control or manipulation experiment as follows: small sub-cores (11 mm diameter, 15 mm depth or vertically sectioned for depth profile analyses) were cut from whole sections of intact microbial mat and placed in serum vials with 4 ml of field site water. Serum vials were capped with butyl rubber stoppers. The 10.5 ml headspace of the serum vials was left as air for light/daytime incubations and was thoroughly flushed with N_2 (gas and liquid phase degassed) for dark/nighttime incubations. For H_2 analysis, at least six replicate vials were sampled at each time point for each control or manipulation experiment. Organic acid production was analyzed in each of three replicates for each time point for each control or manipulation experiment. Manipulations included sectioning of microbial mat to identify the location of H_2 production and suppression of diazotroph or phototroph (oxygenic and/or anoxygenic) activity to assess the roles of these microbial groups in H_2 production. See supplementary methods for further information regarding H_2 and organic acid analyses and details on how manipulation experiments were performed.

Analysis of N₂-fixation
N_2-fixation activity was determined using the acetylene reduction (ethylene production) assay in triplicate incubations for each time point for each control or manipulation experiment using standard methods (Stewart et al., 1967; Bebout et al., 1993).

Statistical analysis
To determine significant differences between H_2 or organic acid production under control vs manipulated conditions, a one-way analysis of variance analysis was performed using the Tukey-HSD post hoc comparison within the JMP software package (v9; http://www.jmp.com/software/jmp9/). If a P-value of <0.05 was calculated between control and manipulated conditions, the difference was considered significant.

Nucleic acid isolation and cDNA synthesis
Microbial mat nucleic acids were isolated from at least three pooled mat cores, which were incubated in the same way as those analyzed for H_2 and organic acid production. Mat cores were sampled periodically from serum vials and immediately transferred to 2 ml tubes, flash frozen in liquid nitrogen and stored at −80 °C. DNA and RNA were

co-isolated from the upper photosynthetic layer (0–2 mm) of mat. DNA-free RNA was reverse transcribed to cDNA using random hexamers and a Superscript III first-strand synthesis kit (Invitrogen, Carlsbad, CA, USA). Further information regarding nucleic acid isolation, RNA purification and cDNA synthesis can be found in the supplementary methods.

Pyrotag sequencing of SSU rRNA genes and transcripts
The V8 hypervariable region of the SSU rRNA gene was amplified from DNA (rRNA gene) or cDNA (rRNA transcript) templates using the universal primer pair 926f/1392r, including the titanium adaptor sequences and a five-base barcode on the reverse primer according to the previously published protocols for pyrotag sequencing (Engelbrektson *et al.*, 2010). Amplicons were quality checked, trimmed to a 200-bp sequence length and clustered (97% similarity) using PyroTagger (Kunin and Hugenholtz, 2010). Representative sequences from each cluster (OTU) were compared with the Greengenes NAST-aligned database (DeSantis *et al.*, 2006) or the SILVA database (Pruesse *et al.*, 2007) using PyroTagger (Kunin and Hugenholtz, 2010). Taxonomic affiliations of OTUs were further verified by querying the NCBI non-redundant nucleotide sequence databases (Altschul *et al.*, 1990). If the best match was 100% identical over 200 bp, without multiple best matches to phylogenetically different taxa, then the OTU was conservatively assigned that taxonomic affiliation at the level of genus. If the OTU was <100%, but ⩾97% identical its taxonomic assignment was conservatively set to the level of order. OTUs with <97% identity matches were set to the level of phylum assigned by PyroTagger or if not assigned an identity by PyroTagger the OTU was unclassified (Kunin and Hugenholtz, 2010). Richness (Chao1 and ACE) and diversity (Simpson and Shannon) indices were calculated with the vegan package (v1.17–8) for R (v2.12; http://cran.r-project.org/web/packages/vegan/index.html). Hughes *et al.*, 2001 and Hill *et al.*, 2003 provide further information and critical review of these indices application in microbial ecology.

Design of degenerate bidirectional [NiFe] hydrogenase specific primers, PCR amplification, cloning and sequencing
A database of bidirectional [NiFe] hydrogenase gene sequences from a range of *Bacteria* and *Archaea* was compiled from the NCBI non-redundant nucleotide sequence database (Altschul *et al.*, 1990) and the Integrated Microbial Genomes database (Markowitz *et al.*, 2006). Gene sequences were converted to protein sequences to screen for the presence of bidirectional [NiFe] hydrogenase signature motifs L1 Ex[APV]xxxxRxCG[IL]Cxx[AS]Hx[IL][ACS][AGS] [AGNSV][KR][ATV]xD and L2 DPC[IL]SC[AS][AST] H[ASTV]x[AG]xx[APV] (PROSITE format; Vignais and Billoud, 2007). Sequences with >2 mismatches to signature motifs were not included in the database. The screened bidirectional [NiFe] hydrogenase gene sequences were aligned using MUSCLE (Edgar, 2004) and the resulting alignments checked manually in Geneious (v5.3, http://www.geneious.com). The database of aligned bidirectional [NiFe] hydrogenase gene sequences was queried against candidate degenerate primers using PRIMROSE to determine the number of mismatches between primers and templates and direct selection of the optimal degenerate primer pair (Ashelford *et al.*, 2002). The optimal degenerate primer pair that targeted the broadest diversity of *Cyanobacteria* and *Chloroflexales* corresponded to positions 13–19 (IEGHAKI) and positions 167–173 (WAVPGGV) in the *M. chthonoplastes* PCC7420 bidirectional [NiFe] hydrogenase protein sequence. Genomic DNA (gDNA) or cDNA was used as the template to amplify a ~480-bp fragment of the bidirectional [NiFe] hydrogenase gene using the degenerate primers HoxH_F37 (5′-ATHGARGGHCAYGCBAAR AT-3′) and HoxH_R518 (5′-ACNCCICCVGGNAYH GHCCA-3′) using the following PCR cycling conditions; 1×5 min, 95 °C (enzyme activation); 35×1 min, 95 °C (denature); 35×1 min, 56.5 °C (anneal); 35×1 min, 72 °C (extend) and 1×7 min, 72 °C (final extension). The 25 μl PCR reaction volume contained 12.5 μl GoTaq green master mix (contains 1.5 mM MgCl₂ and Taq enzyme; Promega, Madison, WI, USA), 8 μM of each primer (HoxH_F37/HoxH_R518), 20 μg bovine serum albumin and 1 μl template (10 ng genomic DNA or 1 μl of cDNA synthesis reaction). Positive and negative controls were also tested in PCR reactions (*M. chthonoplastes* PCC 7420 and *Crocosphaera watsonii* WH 8501, respectively) to evaluate the assay for efficiency and specificity. PCR products of the predicted size were excised from agarose gels after electrophoresis and purified using the Wizard SV gel and PCR clean-up system (Promega). Purified PCR product was ligated into the pCR2.1-topo vector (Invitrogen) and subsequently Sanger sequenced on a 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis of bidirectional [NiFe] hydrogenase clone sequences
Clone sequences were quality filtered, trimmed, translated to protein sequences, screened for L1 signature motif regions and aligned to a custom database of bidirectional [NiFe] hydrogenase protein sequences in Geneious (v5.3, http://www.geneious.com). Inferred hydrogenase amino-acid sequences were clustered at 97% similarity using CD-HIT (Huang *et al.*, 2010) and representative sequences were queried against the NCBI non-redundant peptide sequence database (Altschul *et al.*, 1990)

to identify closest protein matches for phylogenetic analysis and tree building. Singletons were excluded from further analysis. Phylogenies were inferred using maximum-parsimony (PROTPARS; 1000 bootstraps) and maximum-likelihood (RAxML; PROTMIX rate distribution model; Dayhoff and WAG amino-acid substitution models; 1000 bootstraps) evolutionary models in ARB (Ludwig et al., 2004). Tree topologies from the different analyses were similar and the presented tree is based on a maximum-likelihood analysis, with bootstrap support of branch nodes indicated only when supported by all models. Representative clone sequences were deposited in GenBank (Accession numbers JF816258–JF816271).

Results

H$_2$ production
A time-course incubation of vertically sectioned mat was conducted to assess net H$_2$ production in space and time. H$_2$ production was observed predominantly at night under anoxic conditions within the upper photosynthetic layer (0–2 mm) of the mat and was an order of magnitude greater than that produced in lower layers (Figure 1a). H$_2$ produced in intact mat cores (0–15 mm) was similar to H$_2$ produced in the sectioned mat cores (0–2 mm; upper phototrophic layer) when calculated per surface area (exposed top side surface only; Supplementary Figure S1). This data further supported the observation that H$_2$ production is principally occurring within the upper layer.

Patterns of H$_2$ and organic acid production observed in control and manipulation experiments reported here (Figure 1) were reproducible in mats sampled at other times during December–January 2008/2009 and August–October of 2009 (data not shown). However, the magnitude of H$_2$ and organic acid production sometimes varied between diel cycle studies. This may have been due to community structure heterogeneity between different cores sampled from microbial mat pieces at different times or it may have been due to variable physiological states of hydrogenogens in the system.

H$_2$ production under phototroph suppressed conditions
Manipulations of photosynthetic activity were performed to assess the role of different photosynthetic pathways and organisms in H$_2$ production as explained below. In one set of experiments anoxygenic and oxygenic photosynthetic activity facilitated by photosystem (PSI) and photosytem II (PSII), respectively, was suppressed by depriving the mat of all light and thereby imposing continuously dark conditions over a complete diel cycle. In a second set of experiments performed under normal light conditions, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a well-known inhibitor of PSII was added to inhibit oxygenic photosynthesis specifically. Inhibition of PSII was confirmed using PAM fluorometry (Supplementary Table S1) and was further supported anecdotally by the absence of oxygen bubbles, which normally appear at the surface of the mat (Supplementary Figure S2). Light deprivation and the subsequent cessation of all photosynthetic activity during the day reduced nighttime H$_2$ production on the following night by an order of magnitude. However, specific and complete inhibition of oxygenic phototrophy did not significantly affect H$_2$ production under dark, anoxic conditions (Figure 1b). Together these results indicate that prior-day activity of photosynthetic microorganisms is the source of reducing equivalents for H$_2$ production and further suggest that anoxygenic phototrophy, facilitated by PSI, is fully capable of serving this function in Elkhorn Slough mats. However, these data alone do not distinguish between H$_2$ production occurring predominantly by (i) obligate oxygenic phototrophs that are replaced by anoxygenic phototrophs under the conditions of our experimental manipulation, (ii) oxygenic phototrophs capable of facultative anoxygenic phototrophy, (iii) anoxygenic phototrophs; or (iv) a combination of (i)–(iii).

Production of H$_2$ and organic acids under N$_2$-fixation suppressed conditions
To evaluate the relative importance of co-metabolic production of H$_2$ during N$_2$-fixation vs fermentative H$_2$ production, both of which are consistent with the observed pattern of H$_2$ production in space and time, we suppressed N$_2$-fixation. This was performed by addition of a combined nitrogen source (NH$_4$Cl). Night time N$_2$-fixation was completely suppressed when mats were incubated in the presence of NH$_4$Cl (Supplementary Figure S3), but H$_2$ production was not suppressed (Figure 1c). During the same nighttime period acetate, formate and propionate were produced with H$_2$ under dark and anoxic conditions (expected products of fermentation; Figure 1d). Lactate, butyrate and valerate were not detected ($<$200 nM). Organic acid accumulation was not significantly different when N$_2$-fixation was suppressed under the same conditions (Figure 1d), with the exception of one time point (0:00). Together these results indicate that N$_2$-fixation is a relatively insignificant contributor to H$_2$ formation and suggest that fermentation is the dominant mechanism of production.

SSU rRNA genes and transcripts
To profile the identity and activity of microorganisms in Elkhorn Slough microbial mats, we compared rRNA gene and transcript pyrosequence tags from four different time points, including day and night samples (Table 1). For each time point DNA and RNA templates were co-isolated from the upper photosynthetic layer (0–2 mm), as our biogeochemical
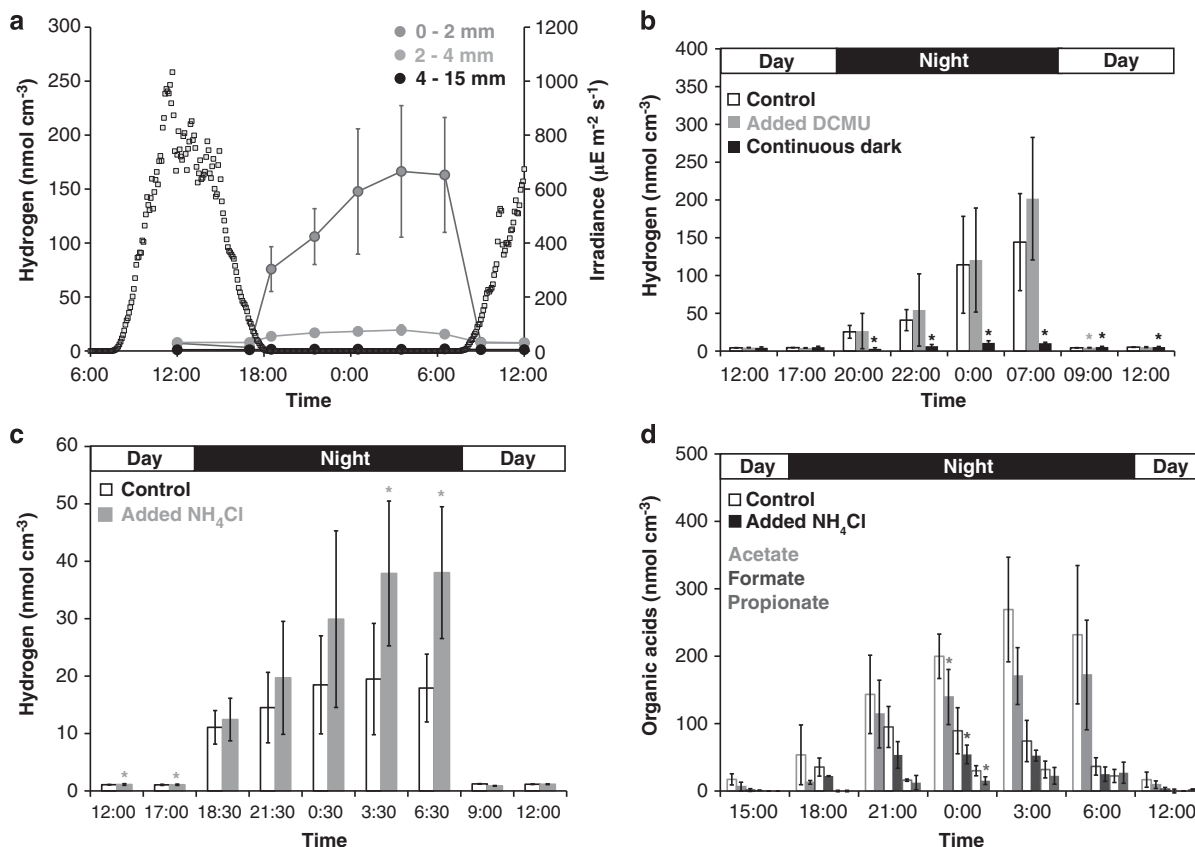
Figure 1 Diel production of H$_2$ or organic acids under manipulated conditions in Elkhorn Slough mats. Mat core incubations in vials commenced at dawn with H$_2$ and organic acids allowed to accumulate over a diel cycle with N$_2$ flushing of the vial at the end of the day and night periods. Vials in which organic acids were measured were reset at dawn by replacement with fresh field site water (amended as appropriate for manipulated conditions). Vertical error bars indicate the s.d. of six replicates (H$_2$) or three replicates (organic acids). Asterisks denote significant differences between control and manipulated conditions (P values <0.05). (**a**) H$_2$ production in different layers of microbial mat. Mat cores were sectioned at different depths, 0–2 mm, 2–4 mm and 4–15 mm. Yellow squares denote natural solar irradiance. µE, microeinsteins. (**b**) H$_2$ production under control, DCMU (20 µM) added and continuous dark conditions. (**c**) H$_2$ production under control and NH$_4$Cl (8.8 mM) added conditions. (**d**) Organic acid production under control and NH$_4$Cl (8.8 mM) added conditions.

Table 1 Sampling times and statistics of rRNA gene and transcript pyrotag sequencing

Library	Time and date	Pool	Filtered reads (+) singletons	Filtered reads (−) singletons	OTUs (97%) (+) singletons	OTUs (97%) (−) singletons
No. 1	Day, 1200, 10/11/2009	DNA	5796	5576	737	380
No. 2	Day, 1200, 10/11/2009	RNA	5756	5641	486	233
No. 3	Night, 0000, 11/11/2009	DNA	1191	1147	291	125
No. 4	Night, 0000, 11/11/2009	RNA	5310	5230	357	166
No. 5	Day, 1200, 12/11/2009	DNA	4487	4402	384	194
No. 6	Day, 1200, 12/11/2009	RNA	4825	4780	242	121
No. 7	Night, 0000, 13/11/2009	DNA	5573	5234	866	390
No. 8	Night, 0000, 13/11/2009	RNA	4091	4038	275	121

data demonstrated that H$_2$ was predominantly produced within this layer.

Richness estimates calculated using Chao1 and ACE indices suggested that we had not exhaustively sequenced the upper photosynthetic layer of the microbial mat community (Table 2). Microbial diversity was consistently estimated to be highest in the rRNA gene libraries as determined using either the Simpson (inverse) or Shannon indices,

with each rRNA gene library value higher than the value for the corresponding rRNA transcript library (Table 2).

Analysis of all four rRNA gene libraries ($n = 17$ 047 sequences) revealed that 64 phyla/divisions across all three domains of life were present in the upper photosynthetic layer of Elkhorn Slough microbial mats (Supplementary Table S2). Abundant phyla/divisions included, *Bacteriodetes* (29%),

Table 2 Richness and diversity estimates of OTUs (97% similarity)

Library	OTU richness[a]			OTU diversity[b]	
	Observed	Chao1	ACE	Simpson[c]	Shannon
No. 1 (Day 1[d], DNA)	737	1309 ($\pm$86)	1334 ($\pm$22)	52.84	5.08
No. 2 (Day 1, RNA)	486	880 ($\pm$70)	952 ($\pm$19)	5.30	3.15
No. 3 (Night 1[d], DNA)	291	633 ($\pm$81)	616 ($\pm$16)	29.55	4.52
No. 4 (Night 1, RNA)	357	645 ($\pm$59)	681 ($\pm$15)	3.88	2.63
No. 5 (Day 2[d], DNA)	384	723 ($\pm$72)	715 ($\pm$16)	14.10	3.77
No. 6 (Day 2, RNA)	242	469 ($\pm$62)	433 ($\pm$13)	3.38	2.18
No. 7 (Night 2[d], DNA)	866	1679 ($\pm$107)	1813 ($\pm$28)	35.20	4.96
No. 8 (Night 2, RNA)	275	543 ($\pm$63)	613 ($\pm$16)	3.93	2.50

[a]Chao1 and ACE (abundance-based coverage estimators) indices are non-parametric estimators of species (OTU) richness (total number of different OTUs in a given sample).
[b]Shannon and Simpson diversity indices consider both species richness and evenness (relative abundance).
[c]Inverse simpson values are reported.
[d]Day 1 is 1200 hours, on 10 November, 2009; Night 1 is 0000 hours, on 11 November, 2009; Day 2 is 1200 hours, on 12 November, 2009; Night 2 is 0000 hours, on 13 November, 2009.
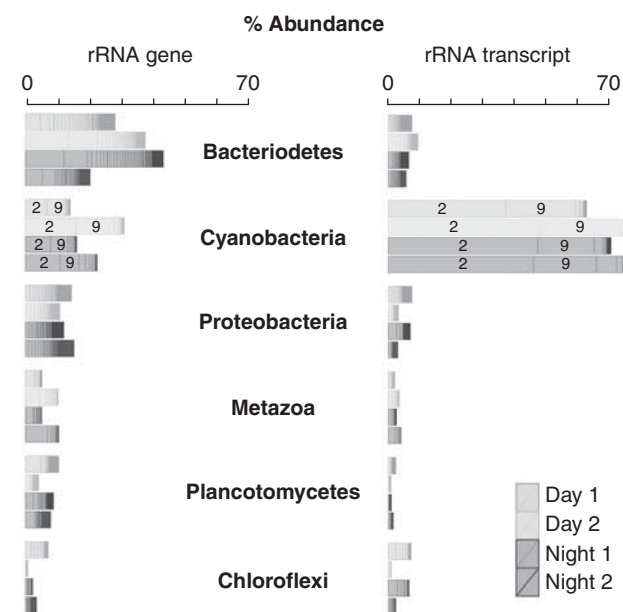

Figure 2 Ranked barplot of the six most abundant phyla of the rRNA gene (left) and rRNA transcript (right) libraries. Day 1 is 12:00 on 10 November, 2009, Day 2 is 12:00 on 11 November, 2009, Night 1 is 0:00 on 11 November, 2009 and Night 2 is 0:00 on 13 November, 2009. OTUs within the same phyla are stacked in order of relative abundance and separated graphically into segments. The most abundant OTUs no. 2, *Microcoleus* spp. and no. 9, *Cyanobacteria*) are labeled according to their (arbitrary) OTU identification number assigned during PyroTagger clustering.

Cyanobacteria (22%) and *Proteobacteria* (14%) (average of four libraries; Figure 2). A lower diversity of phyla/divisions were recovered from the upper photosynthetic layer of all four rRNA transcript libraries ($n = 19\,982$ sequences), with 49 phyla/divisions represented (Supplementary Table S3). *Cyanobacteria* dominated these libraries, representing 71% of all reads (average of four libraries; Figure 2). The rRNA transcript libraries were dominated by *Microcoleus* spp. affiliated OTU 2

(Library no. 2, Day 1, 37%; Library no. 4, Night 1, 47%; Library no. 6, Day 2, 48%; Library no. 8, Night 2, 46%; Figure 2) and *Cyanobacteria* affiliated OTU 9 (Library no. 2, Day 1, 22%; Library no. 4, Night 1, 18%; Library no. 6, Day 2, 27%; Library no. 8, Night 2, 20%; Figure 2). These OTUs are likely important members of the active microbial mat community during the day and also at night.

Relative activity of microbial populations was inferred at the transcript level from the ratio of the fraction of tags in the rRNA transcript library (active microorganisms) to those in the paired rRNA gene library (active + inactive microorganisms) for a given time point. OTUs were considered relatively active if the % rRNA transcripts recovered was greater than the % rRNA genes recovered that is, % rRNA transcripts:% rRNA genes ratio >1 (Rodriguez-Blanco *et al.*, 2009; Jones and Lennon, 2010). These ratios may give additional insight into active microorganisms beyond those that we previously identified from our rRNA transcript libraries (the 'abundant and active' OTUs 2 and 9) and identify those organisms that are relatively 'rare and active' that are not obvious from examination of rRNA transcript libraries alone. We were specifically interested in detecting microorganisms correlated with H₂ production and thus we report in Table 3 only those OTUs that were most relatively active at night and present in all our nighttime libraries. *Microcoleus* spp. (OTU 2) had the highest nighttime % transcript:% gene ratios of all OTUs analyzed (present in all libraries), 6.0 (Nov/11/2009) and 4.2 (Nov/13/2009). An OTU (225) with the second highest nighttime ratios was affiliated with the *Desulfobacterales* (Table 3). Bacteria within this order are exclusively sulfate-reducing bacteria (SRB) and these microorganisms may also be H₂-consumers (hydrogenotrophs; Widell, 1987). The most relatively active nighttime OTUs present in all nighttime libraries were predominantly microbial groups within either the *Cyanobacteria* or

Table 3 rRNA % transcript :% gene ratios of the most relatively active OTUs observed in all nighttime pyrotag libraries

OTU[a]	Most related sequences[b]	Accession number	Identities[c]	E-value[d]	OTU taxonomic affiliation[e]	Ratio[f] night 1, 11/11/2009	Ratio[f] night 2, 13/11/2009
2	*Microcoleus chthonoplastes*	AM709630	200/200	6e-97	*Microcoleus* spp.	6.0	4.2
	Microcoleus chthonoplastes	GQ402023	200/200	6e-97			
225	*Desulfococcus* sp.	EF442980	194/200	9e-89	*Desulfobacterales*	5.5	2.6
	Desulfococcus multivorans	AF418173	194/200	9e-89			
5	*Leptolyngbya fragilis*	HQ832925	198/200	3e-94	*Oscillatoriales*	4.1	3.2
	Coral bacterium clone	FJ203604	193/200	4e-87			
9	Mat bacterium clone	EU246323	200/200	7e-97	*Cyanobacteria*	3.3	3.3
	Phormidium cf. *formosum*[b]	EU196640	190/200	2e-83			
31	Mat chloroflexales clone	AJ308496	200/200	7e-97	*Chloroflexales*	4.8	1.5
	Oscillochloris sp.[b]	AF149018	188/200	2e-78			
387	Microbialite nematoda clone	GQ483712	200/200	7e-97	*Nematoda*	2.1	3.7
	Eumonhystera cf. *simplex*[b]	AY284692	177/200	6e-66			
379	Wetland cytophagales clone	FJ516772	188/200	1e-80	*Bacteriodetes*	1.6	3.8
	Aquiflexum balticum[b]	NR025634	186/200	7e-78			
1176	Mat chloroflexi clone	DQ329977	199/200	3e-95	*Chloroflexi*	1.9	2.9
	Oscillochloris sp.[b]	AF149018	178/200	5e-67			

[a]OTU identification number is arbitrary and wa designated by PyroTagger.
[b]The NCBI non-redundant nucleotide sequence database was queried to identify closest matches to OTU sequences; closest pure culture match where a given OTU has its closest matches to only uncultured clone sequences.
[c]BLASTN uses an identity matrix for nucleotide comparisons, see http://www.ncbi.nlm.nih.gov/BLAST/; total length of query was 200 bp.
[d]E-value or expect value describes the significance of the match between two sequences, see http://www.ncbi.nlm.nih.gov/BLAST/.
[e]For OTU taxonomic affiliation designation see methods (*Pyrotag sequencing of SSU rRNA genes and transcripts*).
[f]rRNA % transcript:% gene ratios were calculated for sequences recovered from DNA or cDNA template-based libraries constructed from Night 1 (0000 hours) on 11 November, 2009 or Night 2 (0000 hours) on 13 November, 2009 samples by dividing the percentage abundance of pyrotags in the rRNA transcript library by the percentage abundance in the rRNA gene library.

Chloroflexi. These data correlate with our biogeochemical data and generally support the hypothesis that *Cyanobacteria*, including *Microcoleus* spp. (oxygenic phototrophs) and/or members of the *Chloroflexi* (anoxygenic phototrophs) are hydrogenogens in Elkhorn Slough mats.

H₂-evolving bidirectional [NiFe] hydrogenase genes and transcripts

H₂-evolving bidirectional [NiFe] hydrogenases are found in a diversity of *Cyanobacteria*, *Chloroflexi* and *Proteobacteria* (also other *Bacteria* and *Archaea*) and are the only known hydrogenases in these taxonomic groups capable of evolving H₂ via fermentation (Tamagnini *et al.*, 2007; Vignais and Billoud, 2007). Thus, genes encoding for these enzymes represent useful biomarkers for evaluating the diversity of hydrogenogens in photosynthetic microbial mats. [NiFe] hydrogenases are quite diverse (Vignais and Billoud, 2007) and therefore it is impossible to design degenerate primers that capture the entire diversity that may be present in highly complex ecosystems such as microbial mats. However, as our biogeochemical and pyrotag sequencing data indicated that the important hydrogenogens in this system are potentially members of the *Cyanobacteria* and *Chloroflexales*, we focused on amplifying bidirectional [NiFe] hydrogenases from these two groups, for which it was possible to design a single PCR-based assay.

Amplicons were generated that spanned the L1 signature motif region of the large subunit of the bidirectional [NiFe] hydrogenase. The primer pair HoxH_F37/HoxH_R518 designed in this study generated an amplicon of ~480 bp, shorter than the bidirectional [NiFe] hydrogenase-specific primer pair HoxHf/HoxHr that produces an ~1190-bp amplicon (Barz *et al.*, 2010). However, HoxH_F37 and HoxH_R518 have substantially fewer primer-template mismatches to publicly available bidirectional [NiFe] hydrogenase genes than the bidirectional [NiFe] hydrogenase-specific primer pair HoxHf and HoxHr (Supplementary Table S4). Of the 39 publicly available *Cyanobacteria* or *Chloroflexales* bidirectional [NiFe] hydrogenases 38 had ≤2 mismatches to HoxH_F37/HoxH_R518 compared with 9 of 39 for HoxHf/HoxHr, indicating that HoxH_F37/HoxH_R518 targets a larger portion of bidirectional [NiFe] hydrogenases from *Cyanobacteria* and *Chloroflexales*. Analysis of a selection of uptake [NiFe] hydrogenases showed that all had ≥3 primer-template mismatches to HoxH_F37/HoxH_R518 (Supplementary Table S4). Amplicons of the expected size were generated from a selection of *Cyanobacteria* strains and one *Chloroflexales* strain that have a bidirectional [NiFe] hydrogenase, but these amplicons were not generated from *Crocosphaera watsonii* WH 8501, a cyanobacterium that does not have a bidirectional [NiFe] hydrogenase but has an uptake [NiFe] hydrogenase (Supplementary Table S5).

A total of 205 bidirectional [NiFe] hydrogenase gene and transcript fragments were amplified from DNA (91 clones) and RNA (cDNA; 114 clones) templates obtained from the upper photosynthetic

Table 4 % transcript:% gene ratios of bidirectional [NiFe] hydrogenase sequence clusters

Sequence cluster	Most related sequences[a]	Accession number	% ID	Transcript[b] clones	Gene[c] clones	Ratio[d]
HoxH1	*Microcoleus chthonoplastes* PCC7420	ZP_05027024	98	70 (61%)	28 (31%)	2.0 (61%:31%)
	Arthrospira maxima CS328	ZP_03273562	92			
HoxH2	*Microcoleus chthonoplastes* PCC7420	ZP_05027024	96	17 (15%)	3 (3%)	4.5 (15%:31%)
	Cyanothece sp. PCC7822	YP_003888687	93			
HoxH3	*Arthrospira platensis* FACHB439	AAQ63960	87	2 (2%)	0 (0%)	—[e]
	Arthrospira maxima CS328	ZP_03273562	87			
HoxH4	*Cyanothece* sp. PCC8802	YP_003135898	70	10 (9%)	44 (48%)	0.2 (9%:48%)
	Cyanothece sp. PCC8801	YP_002370357	70			
HoxH5	*Roseiflexus castenholzii* DSM13941	YP_001431482	73	0 (0%)	4 (4%)	—
	Anaerolinea thermophila UNI1	YP_004173889	73			
HoxH6	*Anabaena variabilis* ATCC29413	YP_325153	68	0 (0%)	6 (7%)	—
	Synechococcus sp. PCC7335	ZP_05039794	68			
HoxH7	*Cyanothece* sp. PCC8801	YP_002370357	68	3 (3%)	1 (1%)	2.4 (3%:1%)
	Solibacter usitatus Ellin6076	YP_826256	68			
HoxH8	*Roseiflexus* sp. RS1	YP_001277847	80	0	2 (2%)	—
	Nostoc sp. PCC7120	NP_484809	80			
HoxH9	*Desulfurispirillum indicum* S5	YP_004113284	78	10 (9%)	0 (0%)	—
	Nostoc sp. PCC7120	NP_484809	75			

[a]Amino-acid sequences inferred from nucleotide sequences.
[b]Total transcript clones = 114, including two singletons. Percentage abundance (rounded to nearest whole number) of a given clone recovered from the transcript library is denoted in parenthesis.
[c]Total gene clones = 91, including three singletons. Percentage abundance (rounded to nearest whole number) of a given clone recovered from the gene library is denoted in parenthesis.
[d]% transcript:% gene ratios calculated by dividing percentage abundance of transcript clones by the percentage abundance of gene clones.
[e]Ratio was not calculated as numerator or denominator was 0%.

layer (0–2 mm) of Elkhorn Slough mats under dark anoxic conditions at 0000 hours, on 11 November, 2009. The gene and transcript fragments formed nine distinct clusters (including five singleton clusters) at the 97% similarity threshold (Table 4; Figure 3). The majority of the sequences were affiliated with *M. chthonoplastes* PCC7420 (HoxH1 and HoxH2 clusters). The HoxH1 cluster represented 31% of gene clones and 61% of transcript clones (98% identity; >65% bootstrap support for branch point) and the HoxH2 cluster represented 3% of gene clones and 15% of transcript clones (96% identity; >50% bootstrap support for branch point). Another highly represented cluster, HoxH4 (48% of gene clones; 9% of transcript clones), was most closely related to *Cyanothece* sp. PCC8802 (70% identity; unresolved branch point). Clusters HoxH1 and HoxH2 were relatively active, with a % transcript:% gene ratio >1, whereas HoxH4 was relatively inactive with a % transcript:% gene ratio <1.

Discussion

Analyses of rRNA and H$_2$-evolving bidirectional [NiFe] hydrogenase genes and transcripts combined with biogeochemical measurements across a range of manipulations strongly suggest that fermentation of stored photosynthate by *Microcoleus* spp. is a major source of H$_2$ in Elkhorn Slough mats.

H$_2$ production occurred predominantly in the upper photosynthetic layer (0–2 mm) of Elkhorn Slough mats. Similar results have been reported in other photosynthetic mats from Guerrero Negro, Mexico (Hoehler *et al.*, 2001), suggesting that quantitatively important hydrogenogens are active in the upper layer of geographically diverse mats. Elkhorn Slough mats also harbored SRB (*Desulfobacterales*; Table 3), a group known to be actively reducing sulfate over the full diel cycle in photosynthetic microbial mats (Canfield and Des Marais, 1991). These microorganisms include hydrogenotrophs (Widdel, 1987) and thus H$_2$ production observed in our experiments is likely a measurement of net H$_2$ production. Our observations cannot rule out that enhanced H$_2$ consumption in deeper layers accounts for the lower net H$_2$ production relative to the upper photosynthetic layer. Indeed, we anticipate that more stable physico-chemical conditions within the permanently dark, anoxic portion of the mat should allow for the development of closer associations between hydrogenogens and hydrogenotrophs, and thus a lower net efflux of H$_2$ than in the highly dynamic upper phototrophic layer. However, we hypothesize that the observed rates of H$_2$ and organic acid production are unlikely to be sustained in lower aphotic layers, which lack daily photosynthetic replenishment of the fixed carbon pool and therefore have a discrete reservoir of carbon that would be rapidly depleted. Further investigation of hydrogenotrophs is needed to better understand the complete H$_2$ cycle and controls on net H$_2$ production in microbial mats.

Previous research of microbial mats from Shark Bay, Australia had concluded that co-metabolic
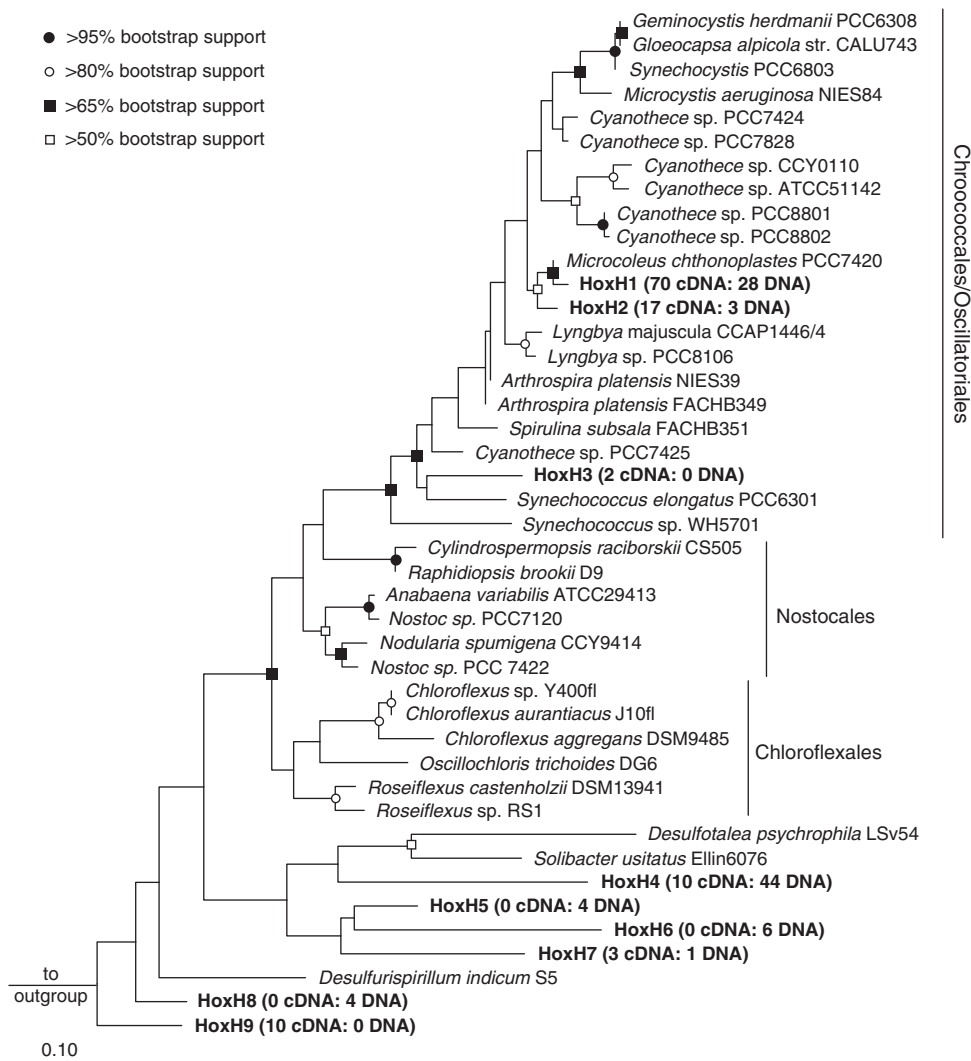
Figure 3 Maximum-likelihood phylogenetic tree of bidirectional [NiFe] hydrogenases. Branch points supported by all phylogenetic analyses (maximum likelihood using Dayhoff or WAG amino-acid correction and maximum parsimony evolutionary models) and their bootstrap support are denoted. The bar represents 0.1 changes per amino acid and the outgroup was *Methanococcus voltae* A3 HoxH.

production of H₂ during N₂-fixation could be an important contributor to net H₂ production as N₂-fixation was observed to occur contemporaneously with H₂ production (Skyring *et al.*, 1989). Similar patterns of N₂-fixation and H₂ production were observed in mats from Elkhorn Slough. However, further examination of the role of N₂-fixation in H₂ production using N₂-fixation suppression experiments indicated that H₂-production is independent of N₂-fixation and most likely fermentative due to the simultaneous production of organic acids (expected fermentation products) under dark and anoxic conditions.

Inhibition of phototrophic activity generally or oxygenic phototrophy specifically, by respectively, depriving the microbial mats of solar irradiance or by inhibiting with DCMU suggested that phototrophs were important contributors to the H₂ production observed during the subsequent night-

time period. However, biogeochemical data alone did not provide evidence to identify the type of phototroph or the taxonomic identity of microorganisms producing H₂.

Our analysis of rRNA genes and transcripts demonstrated that *Cyanobacteria*, specifically *Microcoleus* spp. were important members of the active microbial community. We obtained results similar to an investigation of photosynthetic mats from Guerrero Negro, Mexico by Ley and colleagues (2006). In both studies, *Cyanobacteria* dominated the active microbial communities as determined by rRNA transcript analysis despite being substantially less significant within the total (active + inactive) community as determined by rRNA gene analysis. These observations underscore the need to investigate both the presence and activity of microorganisms in complex microbial ecosystems to gain a better understanding of their likely metabolic importance.

Some cyanobacterial strains isolated in pure culture from microbial mats are capable of fermentative H$_2$ production from stored photosynthate, including glycogen (Stal and Moezelaar, 1997). No studies have been carried out on true *Microcoleus* sp. isolates to ascertain their capabilities of producing H$_2$ (Garcia-Pichel et al., 1996; Moezelaar et al., 1996; Bolhuis et al., 2010). However, recent analysis of publicly available cyanobacterial genomes have shown that all *Cyanobacteria* (including *M. chthonoplastes* PCC7420) harboring a bidirectional [NiFe] hydrogenase also harbor a pyruvate:flavodoxin/ferredoxin oxidoreductase, leading to speculation that the bidirectional [NiFe] hydrogenase is used to shunt electrons (as H$_2$) via a pyruvate:flavodoxin/ferredoxin oxidoreductase-like enzyme during fermentation (Barz et al., 2010). H$_2$-evolving bidirectional [NiFe] hydrogenase clone libraries constructed from the upper photosynthetic layer of Elkhorn Slough mats using degenerate primers designed to equally target many *Cyanobacteria* and *Chloroflexales* were dominated by sequence clusters most closely affiliated with *M. chthonoplastes* PCC7420 (Figure 3). Overrepresentation of hydrogenases in the transcript library for clusters most closely affiliated with *M. chthonoplastes* PCC7420 (Table 4) provides further evidence that *Microcoleus* spp. are the main hydrogenogens in Elkhorn Slough mats.

Data obtained in this study indicate that anoxygenic phototrophy can drive H$_2$ production in microbial mats. Facultative anoxygenic phototrophy in *Cyanobacteria*, including *Microcoleus* sp. has been reported (Garlick et al., 1977; Cohen et al., 1986; De Wit et al., 1988). However, misidentification of cyanobacterial strains as *Microcoleus* sp., including 'strain 11' used by de Wit and colleagues (1988) has been documented (Garcia-Pichel et al., 1996; Bolhuis et al., 2010) and thus it is unclear if *Microcoleus* spp. are capable of anoxygenic phototrophy. The capability of *Microcoleus* spp. to switch between oxygenic and anoxygenic phototrophy might be important to their survival and proliferation in microbial mats where sulfurous compounds can be prevalent and inhibit PSII activity (Jorgensen et al., 1986). Alternatively, anoxygenic phototrophic *Chloroflexi* strains may have a role in H$_2$ production. Analysis of nighttime rRNA gene and transcript libraries revealed that OTU 31 affiliated with *Chloroflexales* and OTU 1176 affiliated with *Chloroflexi* were overrepresented in the rRNA transcript libraries. These OTUs had % transcript:% gene ratios >1 in both nighttime samples (Table 3), indicating that they were consistently relatively active when H$_2$ production was pronounced. Cloning of H$_2$-evolving bidirectional [NiFe] hydrogenases did not indicate that *Chloroflexales* bidirectional [NiFe] hydrogenases were expressed. It should be noted that although degenerate primers were designed to amplify bidirectional [NiFe] hydrogenases from a diversity of *Cyanobacteria* and *Chloroflexales*, a relatively high number of mismatches ($\geqslant 2$) between both forward and reverse primers to the bidirectional [NiFe] hydrogenase gene was determined for one of the *Chloroflexales* members, *Oscillochloris trichoides* DG6 (Supplementary Table S4). Thus, it is possible that *Oscillochloris*-like spp. or other as yet unknown *Chloroflexales* may contribute to the observed H$_2$ produced in Elkhorn Slough mats without being represented in the hydrogenase gene and transcript libraries. In this regard it is noteworthy that the closest pure culture match to OTUs 31 and 1176, was *Oscillochloris* sp. BM (Table 3).

Although we do not rule out a role for *Chloroflexales* in H$_2$ production, analysis of rRNA pyrotags and bidirectional [NiFe] hydrogenases indicates that *Cyanobacteria*, including *Microcoleus* spp., were relatively more active than *Chloroflexales*. Also, the dominance of *Microcoleus* spp. within the active fraction of the microbial community in the H$_2$ producing layer suggests that the contribution of microorganisms other than *Cyanobacteria* to H$_2$ production is likely minimal. Together, these biogeochemical data, manipulations and molecular data demonstrate that *Microcoleus* spp. are dominant hydrogenogens in Elkhorn Slough mats.

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