

**H<sub>2</sub>-saturation of high affinity H<sub>2</sub>-oxidizing bacteria  
alters the ecological niche of soil microorganisms  
unevenly among taxonomic groups.**

Sarah Piché-Choquette, Julien Tremblay, Susannah G Tringe, Philippe  
Constant

► **To cite this version:**

Sarah Piché-Choquette, Julien Tremblay, Susannah G Tringe, Philippe Constant. H<sub>2</sub>-saturation of high affinity H<sub>2</sub>-oxidizing bacteria alters the ecological niche of soil microorganisms unevenly among taxonomic groups.. PeerJ, PeerJ, 2016, 4, pp.e1782. <10.7717/peerj.1782 >. <pasteur-01351537>

**HAL Id: pasteur-01351537**

**<https://hal-riip.archives-ouvertes.fr/pasteur-01351537>**

Submitted on 3 Aug 2016

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.





# H<sub>2</sub>-saturation of high affinity H<sub>2</sub>-oxidizing bacteria alters the ecological niche of soil microorganisms unevenly among taxonomic groups

Sarah Piché-Choquette<sup>1</sup>, Julien Tremblay<sup>2</sup>, Susannah G. Tringe<sup>3</sup> and Philippe Constant<sup>1</sup>

<sup>1</sup> INRS-Institut Armand-Frappier, Laval, Quebec, Canada

<sup>2</sup> Biomonitoring, National Research Council Canada, Montreal, Quebec, Canada

<sup>3</sup> DOE Joint Genome Institute, Walnut Creek, California, United States of America

## ABSTRACT

Soil microbial communities are continuously exposed to H<sub>2</sub> diffusing into the soil from the atmosphere. N<sub>2</sub>-fixing nodules represent a peculiar microniche in soil where H<sub>2</sub> can reach concentrations up to 20,000 fold higher than in the global atmosphere (0.530 ppmv). In this study, we investigated the impact of H<sub>2</sub> exposure on soil bacterial community structure using dynamic microcosm chambers simulating soil H<sub>2</sub> exposure from the atmosphere and N<sub>2</sub>-fixing nodules. Biphasic kinetic parameters governing H<sub>2</sub> oxidation activity in soil changed drastically upon elevated H<sub>2</sub> exposure, corresponding to a slight but significant decay of high affinity H<sub>2</sub>-oxidizing bacteria population, accompanied by an enrichment or activation of microorganisms displaying low-affinity for H<sub>2</sub>. In contrast to previous studies that unveiled limited response by a few species, the relative abundance of 958 bacterial ribotypes distributed among various taxonomic groups, rather than a few distinct taxa, was influenced by H<sub>2</sub> exposure. Furthermore, correlation networks showed important alterations of ribotype covariation in response to H<sub>2</sub> exposure, suggesting that H<sub>2</sub> affects microbe-microbe interactions in soil. Taken together, our results demonstrate that H<sub>2</sub>-rich environments exert a direct influence on soil H<sub>2</sub>-oxidizing bacteria in addition to indirect effects on other members of the bacterial communities.

Submitted 13 January 2016  
Accepted 17 February 2016  
Published 10 March 2016

Corresponding author  
Philippe Constant,  
philippe.constant@iaf.inrs.ca

Academic editor  
Maria Rosaria Corbo

Additional Information and  
Declarations can be found on  
page 15

DOI 10.7717/peerj.1782

© Copyright  
2016 Piché-Choquette et al.

Distributed under  
Creative Commons CC-BY 4.0

OPEN ACCESS

**Subjects** Microbiology, Soil Science

**Keywords** Soil, Microbial ecology, Correlation network

## INTRODUCTION

Soil microbial communities are continuously exposed to molecular hydrogen (H<sub>2</sub>). Trace levels of H<sub>2</sub> (0.530 ppmv) diffuse into the soil from the global atmosphere, yet higher concentrations can be found in the rhizosphere of N<sub>2</sub>-fixing legumes (*Constant, Poissant & Villemur, 2009*). Indeed, H<sub>2</sub> is an obligate by-product of nitrogenase in N<sub>2</sub>-fixing free living or symbiotic bacteria, with an H<sub>2</sub> molecule produced for every reduced N<sub>2</sub> molecule (*Hoffman, Dean & Seefeldt, 2009*). It has been estimated that 240,000 L of H<sub>2</sub> are produced per hectare of legume crop over a growing season (*Dong et al., 2003*). At the microscale level, there is a steep H<sub>2</sub> concentration gradient starting at about 20,000 ppmv at the

soil-nodule interface and ending with sub-atmospheric levels a few centimeters away (*La Favre & Focht, 1983; Rasche & Arp, 1989; Witty, 1991*). Despite the high concentrations of H<sub>2</sub> found in legumes rhizosphere, a very small proportion escapes to the atmosphere due to H<sub>2</sub>-oxidizing bacteria (HOB) thriving in soil. These microorganisms also play a vital role in the global budget of H<sub>2</sub>, being responsible for about 80% (60 Tg H<sub>2</sub> yr<sup>-1</sup>) of the global losses of this trace gas from the atmosphere (*Constant, Poissant & Villemur, 2009; Ehhalt & Rohrer, 2009*).

Aerobic soil bacteria scavenging H<sub>2</sub> diffusing from the atmosphere and N<sub>2</sub>-fixing nodules encompass a broad range of taxonomic groups. These microorganisms generally possess one or up to four different types of [NiFe]-hydrogenases catalyzing the interconversion of H<sub>2</sub> into protons and electrons (H<sub>2</sub> ↔ 2H<sup>+</sup> + 2e<sup>-</sup>). These hydrogenases are classified into four distinct phylogenetic groups, each displaying particular physiological roles (*Constant & Hallenbeck, 2012; Vignais & Billoud, 2007*). Hydrogenases encompassing group 1h are generally characterized by a high affinity towards H<sub>2</sub> (<sub>(app)</sub>K<sub>m</sub> < 100 ppmv), conferring the ability to oxidize atmospheric H<sub>2</sub> (*Constant et al., 2010*). Genome database mining of *hhyL* gene encoding the large subunit of high affinity hydrogenases are differentially distributed in *Actinobacteria*, with few representatives of *Proteobacteria*, *Acidobacteria* and *Chloroflexi* (*Constant et al., 2011a; Constant et al., 2011b*). The energy yield associated with the oxidation of atmospheric H<sub>2</sub> is insufficient to support chemolithotrophic growth and compelling experimental evidence suggest that H<sub>2</sub> supplies maintenance energy requirements and mixotrophic growth in high affinity HOB (*Constant et al., 2011a; Constant et al., 2011b; Constant et al., 2010; Greening et al., 2014a; Greening et al., 2015*). The distribution of the other [NiFe]-hydrogenase groups is broader, including methanogenic archaea and *Cyanobacteria* (*Vignais & Billoud, 2007*). *Ralstonia eutropha* and *Bradyrhizobium japonicum*, part of the knallgas bacteria functional group, are able to scavenge H<sub>2</sub> diffusing from nodules in the presence of O<sub>2</sub>, yet they are unable to use atmospheric H<sub>2</sub> due to the low-affinity (<sub>(app)</sub>K<sub>m</sub> > 1,000 ppmv) and high H<sub>2</sub> threshold concentration of their [NiFe]-hydrogenases (*Conrad, Aragno & Seiler, 1983*). Knallgas bacteria can use H<sub>2</sub> as a sole or supplementary energy source in chemolithotrophic or mixotrophic growth, respectively. The co-occurrence of these two sub-populations of HOB, as defined by substrate affinity, is supported by the biphasic kinetics governing H<sub>2</sub> oxidation activity in soil (*Häring & Conrad, 1994*).

Laboratory incubations simulating H<sub>2</sub> fluxes from N<sub>2</sub>-fixing nodules demonstrated that soil bacterial community composition changed upon H<sub>2</sub> exposure (*Osborne, Peoples & Janssen, 2010; Stein et al., 2005; Zhang, He & Dong, 2009*). However, methods previously used provided a low taxonomic resolution and coverage of bacterial communities responding to H<sub>2</sub> exposure. Here, we revisited these experiments using a combination of high-throughput sequencing of the bacterial 16S rRNA gene and an H<sub>2</sub> metabolism analysis to compare microbial community structure in dynamic microcosm chambers simulating soil H<sub>2</sub> exposure from the atmosphere and from N<sub>2</sub>-fixing nodules, corresponding to unsaturating and saturating H<sub>2</sub> concentrations for high affinity hydrogenases, respectively. Elevated H<sub>2</sub> exposure was expected to activate and enrich HOB, consequently leading to

indirect impacts on the whole soil bacterial community through competitive and synergistic microbe-microbe interactions.

## MATERIALS AND METHODS

### Soil sample

Soil sample was collected in an agricultural land located in St. Claude (Québec, Canada) on the south shore of the St. Lawrence River (45.6809° N, -71.9969° W). The field is managed with fallow, potatoes and maize crop rotation. Potato seedlings (approximately 10 cm height) were present on the site during sampling in July 2013. The top layer of the A-horizon (0–10 cm depth) was collected, stored in plastic bags at 4 °C and processed within a week. Soil was air-dried for 48 h in the laboratory and sieved (2 mm mesh size) through a vibratory sieve shaker AS 200<sup>®</sup> (Retsch GmbH, Haan, Germany) before preparation of soil microcosms. Soil was classified as sandy clay loam according to soil textural class parameters identified with the hydrometer method (*Bouyoucos, 1936*). Soil pH was determined with 1:2 soil-water suspensions with an Accumet pH-meter (Fisher Scientific, Hampton, NH). Total carbon ( $3.1 \pm 0.3\%$ ) and total nitrogen ( $0.3 \pm 0.0\%$ ) content were determined using an elemental combustion system using the protocol described in *Khdhiri et al. (2015)*.

### Controlled H<sub>2</sub> exposure in dynamic soil microcosm chambers

Dynamic microcosm chambers were designed to expose soil to controlled levels of H<sub>2</sub> ([Fig. S1](#)). Microcosm chambers consisted of 0.9 L cell culture flasks (Corning, Tewksbury, MA, USA) equipped with a rubber stopper fitted with two 1/8" outside diameter PTFE (Teflon) tubes: the first supplied gas mixture to the microcosm chamber and the second was vented to the atmosphere. Synthetic gas mixtures supplied the microcosm chambers at a flowrate of 40 ml min<sup>-1</sup>, resulting in a dynamic headspace with a residence time of approximately 22 min in the enclosures. Gas mixtures were bubbled in water before entering in microcosms to prevent soil dryness. Two different H<sub>2</sub> treatments were applied in parallel incubations. The first treatment (designated microcosms eH<sub>2</sub>(a) and eH<sub>2</sub>(b)), named elevated H<sub>2</sub> (eH<sub>2</sub>), consisted of exposing soil to a dynamic headspace comprising 525 ppmv H<sub>2</sub> in synthetic air, simulating H<sub>2</sub> concentrations detected around N<sub>2</sub>-fixing nodules in soil (*Hunt & Layzell, 1993*). The second was a control treatment (designated microcosms aH<sub>2</sub>(a) and aH<sub>2</sub>(b)), named atmospheric H<sub>2</sub> (aH<sub>2</sub>), where soil was exposed to a dynamic headspace comprising 0.54 ppmv H<sub>2</sub> in synthetic air, representing H<sub>2</sub> concentrations found in the atmosphere. Both treatments were replicated, resulting in four soil microcosms in total. Each microcosm chamber contained 200 g<sub>(dw)</sub> soil at the beginning of the incubation period. This amount of soil ensured sufficient material for the monitoring of microbiological and physicochemical variables throughout the incubation period, while avoiding diffusion limitation of the H<sub>2</sub> soil uptake rate measurements. Diffusion limitation was precluded since preliminary experiments using the same microcosm setup showed proportional H<sub>2</sub> uptake activity as a function of the amount of soil in the chamber using 150, 200 and 250 g<sub>(dw)</sub> soil samples (raw data file). Soil water content was adjusted to 20% water holding capacity before incubation for 10 days at 28 °C in the dark. Synthetic gas mixture supply was continuously maintained, with the exception of routine soil subsamples collection and high affinity H<sub>2</sub>

oxidation rate measurements (see below). A decrease in soil pH was observed over the course of the incubation (from pH  $5.9 \pm 0.2$  to  $5.1 \pm 0.1$ ) in all microcosms, without distinction between aH<sub>2</sub> and eH<sub>2</sub> treatments. Soil moisture was monitored using standard gravimetric method and maintained at  $26 \pm 5\%$  throughout the incubation period. Blank (empty) microcosms incubated prior to the experiment did not show any H<sub>2</sub> oxidation or production activity.

### H<sub>2</sub> uptake activity

High affinity H<sub>2</sub> oxidation activity was routinely monitored throughout the incubation of the soil microcosms. Briefly, microcosms were disconnected from their respective gas supply, flushed 5 min with a synthetic gas mixture (0.54 ppmv H<sub>2</sub>) and tightly closed with rubber septum caps. The 5-min flush was shown sufficient to avoid residual H<sub>2</sub> degassing that would otherwise have led to an underestimation of H<sub>2</sub> oxidation rate. A defined volume of H<sub>2</sub> gas mixture ( $525 \pm 10$  ppmv H<sub>2</sub> GST-Welco, PA, USA.) was injected to obtain an initial concentration of approximately 3 ppmv in the static headspace. Decrease of the H<sub>2</sub> mixing ratio was monitored as a function of time by analyzing aliquots (10 ml) of the headspace air in a ta3000R gas chromatograph equipped with a reduction gas detector (Ametek Process Instruments<sup>®</sup>, DE, USA.) as previously described (Khdhiri *et al.*, 2015). Considering the low level of H<sub>2</sub> added in the headspace, H<sub>2</sub> uptake reflected the activity of high affinity HOB, knallgas bacteria showing low affinity for H<sub>2</sub> being unable to use these trace amounts of H<sub>2</sub> (Conrad, Aragno & Seiler, 1983). The biphasic kinetic parameters governing H<sub>2</sub> oxidation activity in soil (i.e.,  $(app)K_m$  and  $(app)V_{max}$ ) were measured at the end of the 10-day incubation period after the addition of specified amounts of pure H<sub>2</sub> into the headspace of the microcosms, as previously described (Schuler & Conrad, 1990).

### DNA extraction, qPCR and high-throughput sequencing of PCR-amplified bacterial 16S rRNA gene

Soil subsamples (approximately 10 g per subsample, without replacement) were collected in the four microcosms after 0, 1, 3, 5, 7 and 10 incubation days to investigate the taxonomic structure of microbial communities. The extraction of total genomic DNA was performed using the FastDNA SPIN kit for Soil<sup>®</sup> (MP Biomedicals, Solon, OH, USA.). DNA was eluted in 100  $\mu$ L nuclease-free water. Quality of the DNA was then examined on agarose gels and samples were quantified using Quantifluor dsDNA System<sup>®</sup> (Promega, Fitchburg, WI, USA). The V4 region of bacterial 16S rRNA gene was PCR amplified (Table S1) and sequenced on an Illumina MiSeq 2000 instrument as multiplexed libraries to generate paired-end reads ( $2 \times 250$  bp). Quantification of bacterial 16S rRNA and *hhyL* genes was done by qPCR following Khdhiri *et al.* (2015).

### Quality control of sequencing reads

Our sequencing analysis pipeline using QIIME 1.8.0 (Kuczynski *et al.*, 2011) software was based on the Itagger pipeline (Tremblay *et al.*, 2015). V4 region Illumina sequencing reads were first processed using the *split\_library* function implemented in QIIME for samples demultiplexing and forward primer removal. The assembly of paired-end reads was performed using FLASH software version 1.2.10 (Magoc & Salzberg, 2011). The software

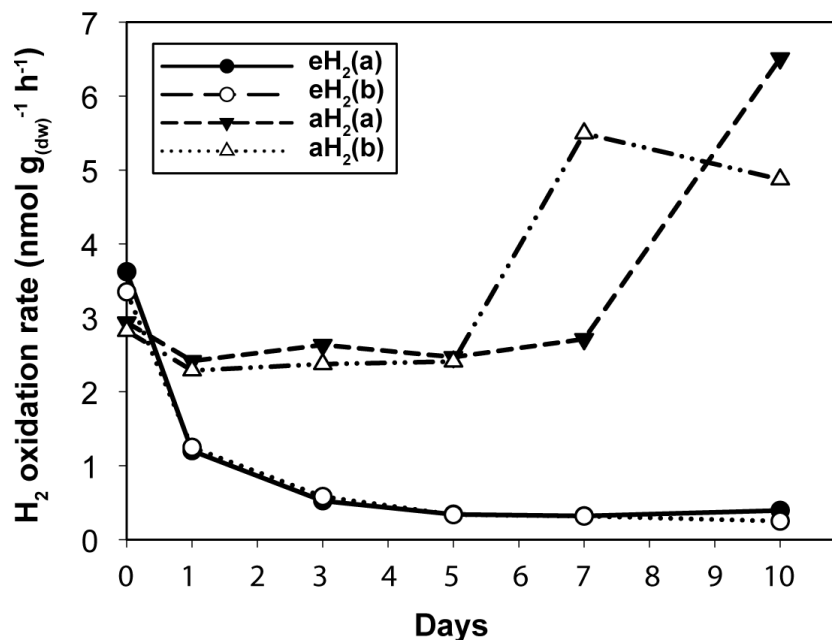
DUK (Li, Copeland & Han, 2011), a Kmer-based sequence matching tool, was then used to remove common sequencing contaminants as well as PhiX sequences added as control. Afterwards, sequences were trimmed to remove low quality ends and a general quality control was also applied. All reads containing at least a single *N* (ambiguous base), an average quality score of less than 30 or more than 15 bases with a quality score under 20 were discarded. The minimum and maximum read length were respectively set to 200 and 300 bases for the assembled paired-reads. Reverse primers were also removed using the *truncate\_reverse\_primer* function implemented in QIIME. Raw sequences were deposited in the Sequence Read Archive of the National Center for Biotechnology Information under the Bioproject [PRJNA295403](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA295403).

### Clustering and taxonomic identification of sequencing reads

Processed 2,554,543 high quality reads were clustered into OTUs (Operational Taxonomic Units) using the function *pick\_otus* implemented in QIIME along with the USEARCH (Edgar, 2010) clustering algorithm. Sequences dereplication was performed at 100% sequence identity and clusters were formed by denoising unique sequences at 99% identity. Singletons were removed to avoid diversity bias. Remaining clusters were filtered using UCHIME (Edgar et al., 2011) chimera filter in *de novo* mode, followed by a reference-based filter step against the Gold reference database. These 2 chimera scanning steps allow a better removal of chimeras by removing sequences flagged as chimeras by any of the two methods. Chimera-checked clusters were then clustered into OTUs at 97% identity threshold also using the function *pick\_otus* with the USEARCH software. Taxonomic identification of OTUs was performed using the *assign\_taxonomy* function along with the naïve Bayesian Ribosomal Database Project (RDP) classifier 2.7 (Wang et al., 2007). Taxonomy was assigned based on the Greengenes taxonomy with the 16S rRNA Greengenes reference database (DeSantis et al., 2006). A rarefaction step was applied to the OTU libraries to standardize all libraries, by random subsampling, to the lowest amount of sequences (74,316 reads) to avoid bias introduced due to unequal sequencing efforts of the samples. This rarefied OTU table comprising the frequency distribution of the OTUs in each sample was used in downstream statistical and co-occurrence network analyses.

### Statistical analyses

Statistical analyses were performed using the software R version 3.0.2 (R Development Core Team, 2008). Pairwise comparison of H<sub>2</sub> uptake activities in soil microcosms incubated under aH<sub>2</sub> and eH<sub>2</sub> exposure treatments was tested using one-way analysis of variance (ANOVA) and *post hoc* Tukey test. Shapiro–Wilk normality test was applied to confirm normal distribution of data before the ANOVA. The package “nlme” was used to compute nonlinear regression for modeling the first-order decay of *hhyL* gene abundance determined by qPCR. Discrimination of the samples according to their ribotyping profile was performed with the package “vegan.” Hellinger transformation of the OTU table (97% identity threshold) was computed due to the presence of zeros in the OTU table (Legendre & Gallagher, 2001). Euclidean distance matrix was used to generate an UPGMA agglomerative clustering of the samples. Identification of statistically different clusters



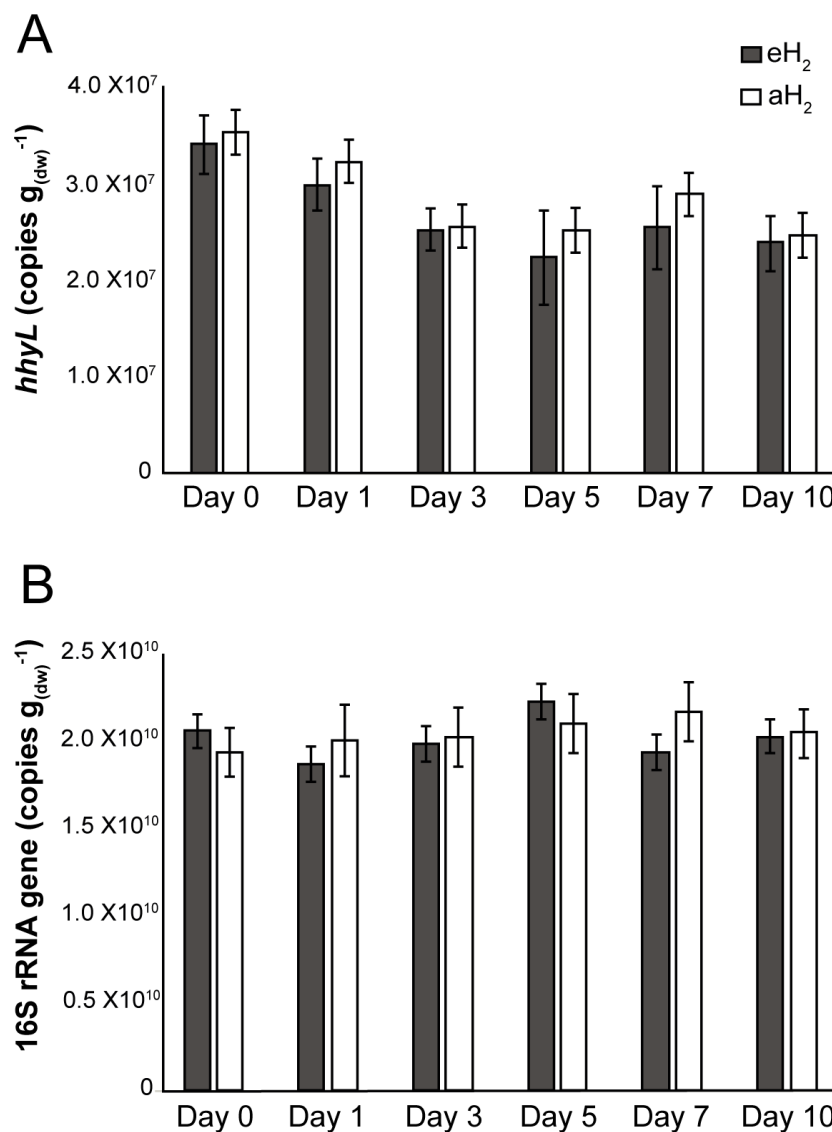
**Figure 1** High affinity H<sub>2</sub> oxidation rates. Time series of the high-affinity H<sub>2</sub> oxidation rate measured in soil microcosms exposed to aH<sub>2</sub> or eH<sub>2</sub> throughout the incubation period.

was done by performing 999 permutations of the OTU table dataset separately across the samples and comparing the observed similarity score of each cluster against the expected values under the null hypothesis using the similarity profile tool (SIMPROF) implemented in the package “clustsig” 1.1 (Clarke, Somerfield & Gorley, 2008). Pairwise comparison of relative abundance of OTUs having a higher contribution than average to explain the two dimensions of the PCA space in soil microcosms incubated under aH<sub>2</sub> and eH<sub>2</sub> exposure treatments was tested using Kruskal–Wallis and *post hoc* Tukey test. Pairwise comparison of relative abundance of all OTUs in soil microcosms incubated under aH<sub>2</sub> and eH<sub>2</sub> exposure treatments was tested using the likelihood ratio test using the package “edgeR.” Covariation among OTUs during the incubation under controlled H<sub>2</sub> levels was analyzed by correlation networks using the package “WGCNA” 1.41 (Langfelder & Horvath, 2008). A detailed methodology for the computation of correlation networks is provided in Text S1.

## RESULTS AND DISCUSSION

### Impact of H<sub>2</sub> exposure on the distribution and activity of HOB

Agricultural soil microcosms were incubated under two different H<sub>2</sub> level treatments. The first treatment simulated soil exposure to atmospheric level of H<sub>2</sub> (aH<sub>2</sub>; 0.54 ppmv), while the second simulated high affinity hydrogenases substrate saturation (eH<sub>2</sub>; 525 ppmv). High affinity H<sub>2</sub> oxidation rates were not significantly different (ANOVA,  $P > 0.05$ ) between the two pairs of microcosms at the beginning of the incubation (Fig. 1). The influence of H<sub>2</sub> exposure could already be observed after 24 h. From hour 24 to day 10, a decline of high affinity H<sub>2</sub> uptake rate was observed in microcosms exposed to



**Figure 2** Time series of qPCR data. Time series of (A) *hhyL* and (B) 16S rRNA gene abundance in soil as determined by qPCR.

eH<sub>2</sub>, while an increase of this uptake rate was measured in microcosms exposed to aH<sub>2</sub> levels. This trend was maintained over the course of the incubation, with an oxidation rate of  $6.2 \pm 1.9$  and  $0.3 \pm 0.1$  nmol  $g_{(dw)}^{-1} h^{-1}$  in aH<sub>2</sub> and eH<sub>2</sub> treatments at the end of the experiment, respectively. This response of H<sub>2</sub> oxidation rate was accompanied by an alteration in the abundance of presumptive high affinity HOB in soil. The abundance of *hhyL* gene determined by qPCR did not change over the course of the incubation under aH<sub>2</sub> (exponential regression,  $P = 0.06$ ), while a slight but significant exponential decay (exponential regression,  $P = 0.02$ ) was observed under eH<sub>2</sub> (Fig. 2A). Indeed, the physiological role of high affinity hydrogenases differs within various taxonomic groups of bacteria. In streptomycetes, the enzyme is primarily expressed in spores to support a seed

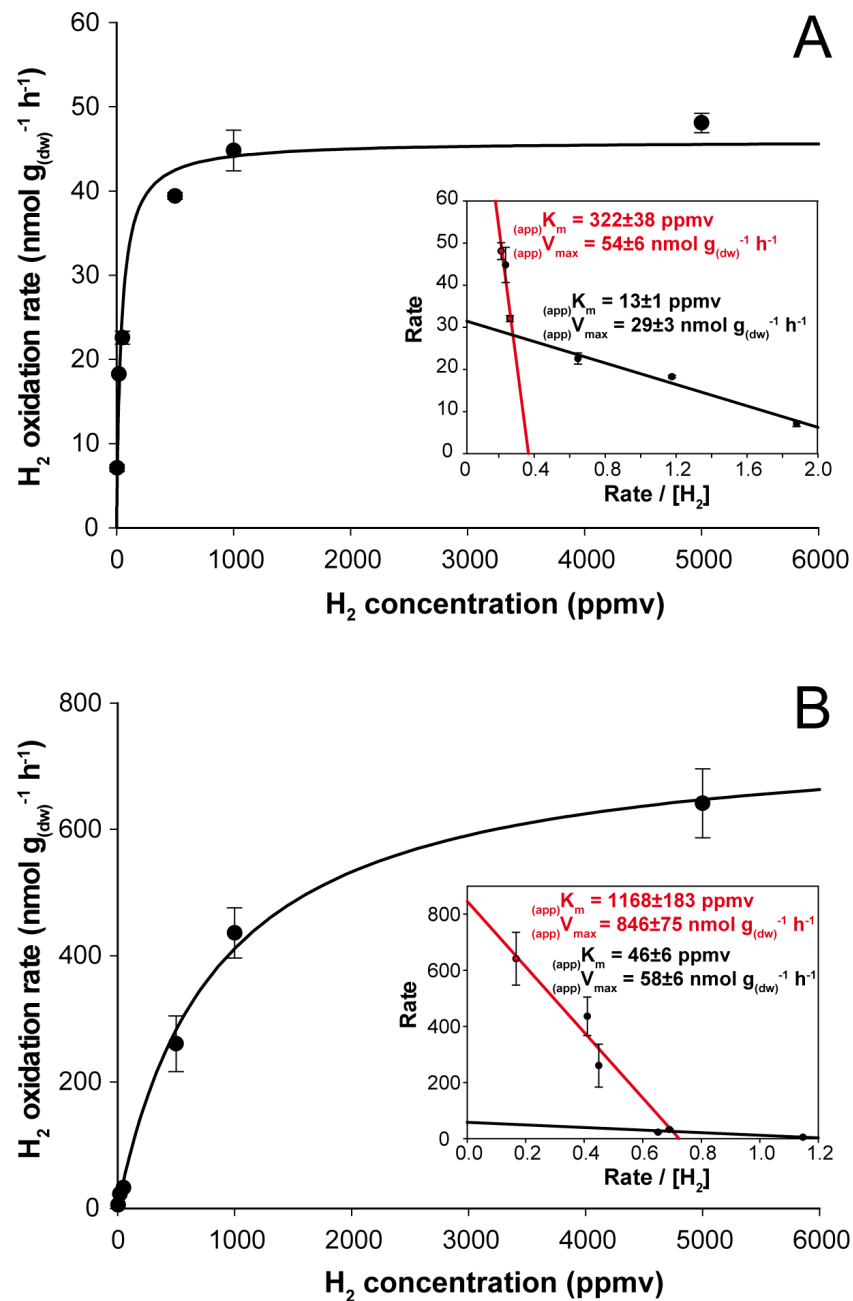


bank under a mixotrophic survival energy mode (Constant et al., 2010; Constant, Poissant & Villemur, 2008; Liot & Constant, 2015), while *Mycobacterium* express the enzyme in the exponential and stationary phases for mixotrophic growth as well as survival (Berney & Cook, 2010; Greening et al., 2015b; Greening et al., 2014b). Under axenic cultivation conditions, eH<sub>2</sub> level would be expected to favour an increase of *Mycobacterium* biomass and persistence of streptomycetes spores. It should be noted that our qPCR data cannot differentiate between active and inactive cells but the decreasing trend of *hhyL* copy number in soil exposed to eH<sub>2</sub> suggests that H<sub>2</sub> was not sufficient to promote growth and persistence of high affinity HOB.

H<sub>2</sub> soil exposure exerted a significant impact on the kinetic parameters governing H<sub>2</sub> oxidation activity in soil. At the end of the incubation, microcosms exposed to aH<sub>2</sub> level displayed  $(_{app})K_m$  of  $40 \pm 5$  ppmv (Fig. 3A), while microcosms exposed to eH<sub>2</sub> showed lower affinity towards H<sub>2</sub> with an  $(_{app})K_m$  of  $838 \pm 163$  ppmv (Fig. 3B). Similar observations were obtained by Dong & Layzell (2001), where soil exposed to elevated levels of H<sub>2</sub> (600 ppmv) displayed an  $(_{app})K_m$  of 1,028 ppmv H<sub>2</sub>, while soil exposed to low H<sub>2</sub> level (0.55 ppmv) was characterized by an  $(_{app})K_m$  of 40 ppmv. The biphasic kinetic parameters characteristics of low and high affinity HOB were also computed using Eadie-Hofstee plots to highlight high- and low-affinity H<sub>2</sub> uptake activities in both H<sub>2</sub> treatments. The H<sub>2</sub> oxidation activity was mainly catalyzed by bacteria demonstrating intermediate and high-affinity in aH<sub>2</sub> treatment (Fig. 3A, insert), while H<sub>2</sub>-oxidation activity by bacteria displaying low- and high-affinity for H<sub>2</sub> were detectable in the eH<sub>2</sub> treatment (Fig. 3B, insert). Low H<sub>2</sub> exposure supported the metabolism of intermediate- to high-affinity HOB, such as some *Actinobacteria* species of *Streptomyces*, *Rhodococcus* and *Mycobacterium* (Berney et al., 2014; Constant et al., 2011b; Golding et al., 2012; Liot & Constant, 2015; Meredith et al., 2014; Schäfer, Friedrich & Lenza, 2013). Triggering of low-affinity H<sub>2</sub> oxidation activity under eH<sub>2</sub> treatment might be explained by knallgas bacteria such as *Proteobacteria* species encompassing *Ralstonia*, *Variovorax* and *Bradyrhizobium* using H<sub>2</sub> for mixotrophic growth (Rittenberg & Goodman, 1969), as they are known to exhibit a H<sub>2</sub> uptake threshold ranging from 1 to 200 ppmv H<sub>2</sub> (Conrad, 1996).

### Impact of H<sub>2</sub> exposure on soil bacterial community structure

Soil subsamples were collected in the microcosms during the incubation period to evaluate the temporal variation of soil bacterial community structure. H<sub>2</sub> treatment did not influence the abundance of 16S rRNA gene in soil (Fig. 2B). This is in contrast with another study that reported more than twofold increase of the amount of bacterial cells upon elevated H<sub>2</sub> exposure, but a mistake in the reported H<sub>2</sub> concentration units by the authors (i.e., the use of a gas mixture of 2,000 ml H<sub>2</sub> per L of synthetic air) impairs a sound comparison with the present investigation (Stein et al., 2005). High-throughput sequencing of PCR-amplified 16S rRNA gene unveiled that bacterial communities were dominated by *Proteobacteria* (34%), *Acidobacteria* (20%), *Actinobacteria* (10%), *Verrucomicrobia* (8%) and *Bacteroidetes* (5%) (Fig. S2). Species richness was not affected by H<sub>2</sub> exposure (ANOVA,  $P > 0.05$ ), with Shannon indices of  $9.38 \pm 0.05$  and  $9.34 \pm 0.17$  for aH<sub>2</sub> and eH<sub>2</sub> treatment respectively. An UPGMA hierarchical clustering analysis was computed



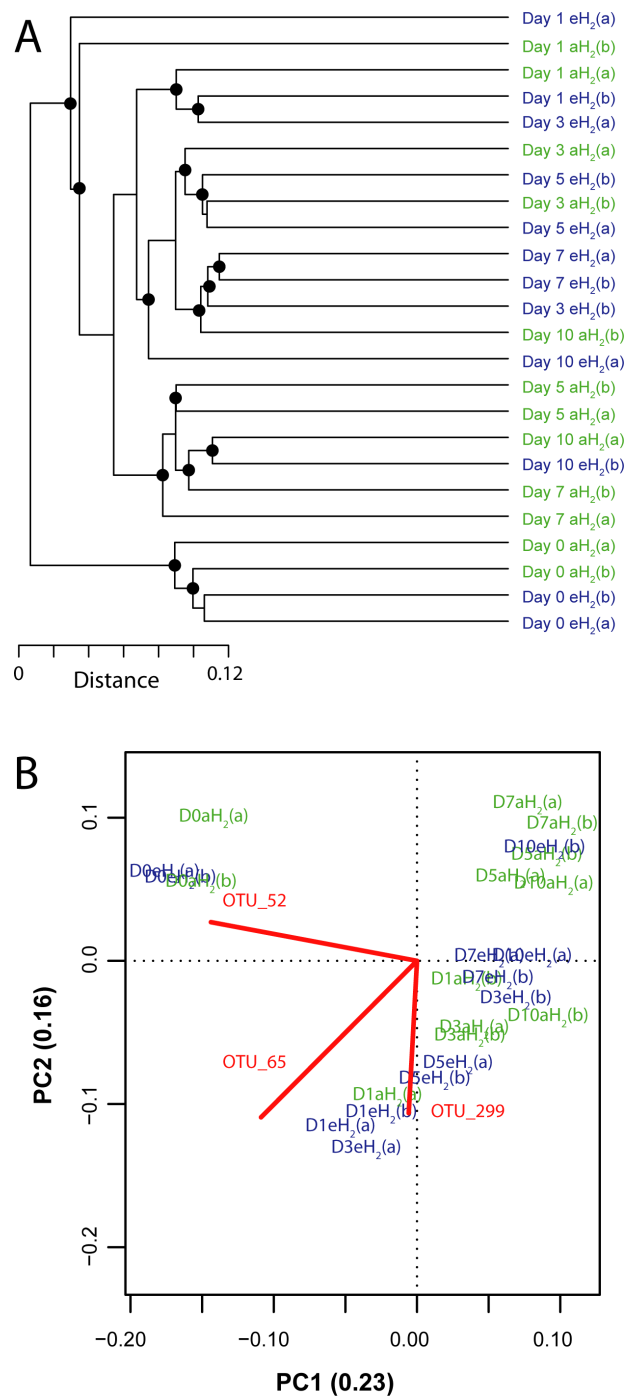
**Figure 3** Kinetic parameters governing  $\text{H}_2$  oxidation activity in soil microcosms incubated under (A)  $\text{aH}_2$  and (B)  $\text{eH}_2$  exposure. Michaelis-Menten graphs are presented with Eadie-Hofstee plots highlighting the biphasic kinetic of the reaction in the inserts.

to compare bacterial community profiles from soil subsamples collected in microcosms exposed to different  $\text{H}_2$  concentrations. The four microcosms encompassed the same cluster before the incubation, indicating high similarity of their initial ribotyping profile (Fig. 4A). No coherent impact of  $\text{H}_2$  exposure on microbial community structure was observed after 1 and 3 incubation days. Ribotyping profiles obtained for sub-samples

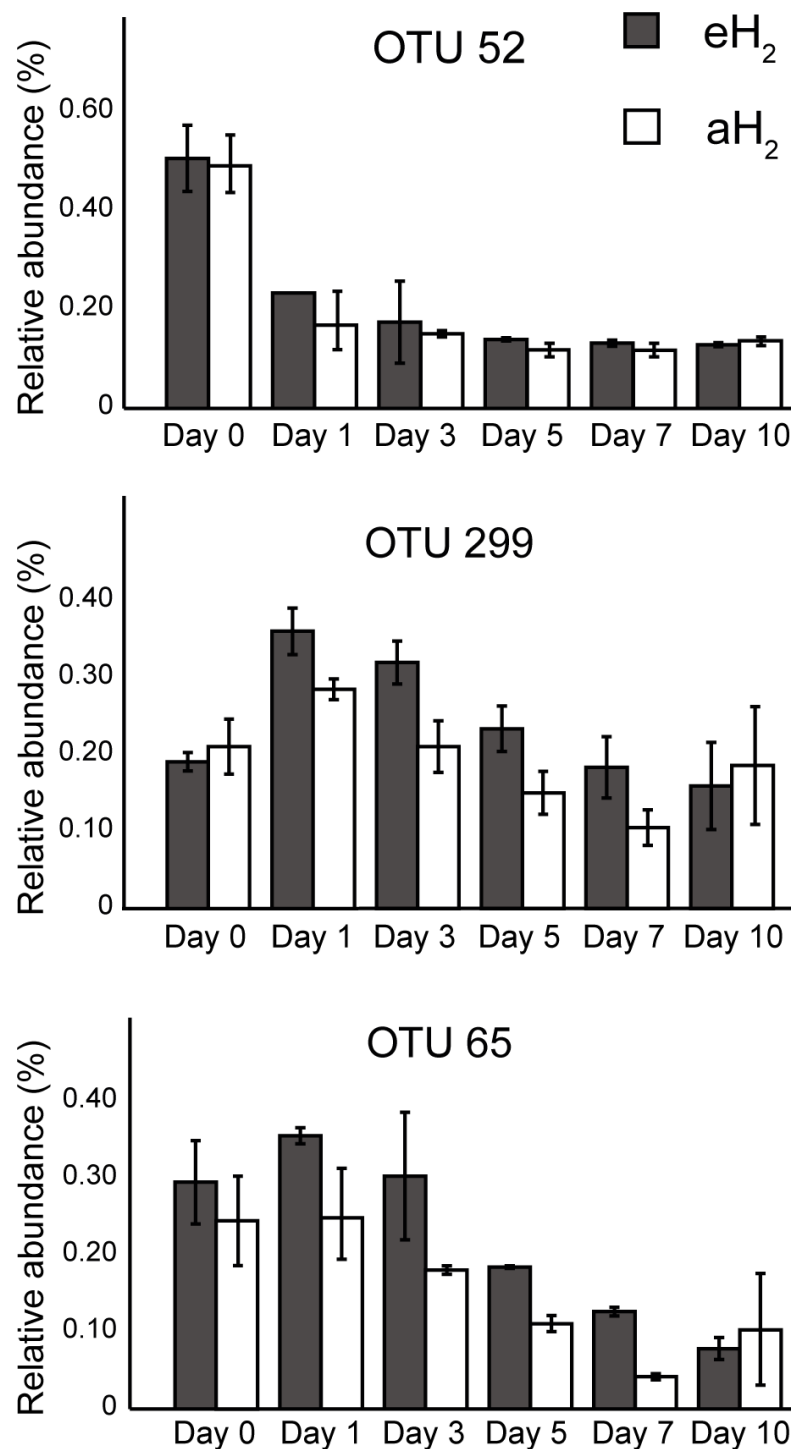
collected after 5 and 7 incubation days were separated with confidence in different clusters, suggesting a response of microbial communities to H<sub>2</sub> exposure. This discrimination was however transient as ribotyping profiles corresponding to the last incubation day could not be discriminated according to their respective H<sub>2</sub> treatments (Fig. 4A). The transient response was likely due to the fact that H<sub>2</sub> alone was not sufficient to maintain a sustained change in microbial community structure. Decrease of complex carbon sources and nutrients used along with H<sub>2</sub> for mixotrophic growth in soil is a potential explanation for the convergence of the ribotyping profiles after 7 incubations days.

A PCA was computed to identify OTUs contributing to the clusterization of ribotyping profiles. The ordination space defined by the first two components explained 38.6% of the variation observed (Fig. 4B). Three OTUs defined an important proportion of the reduced space represented by both axes (Fig. 5). The relative abundance of OTU 52 (classified as a member of the class *Betaproteobacteria*) contributed to distinguish the four soil subsamples collected before the incubation from the 20 other subsamples collected after 1, 3, 5, 7 and 10 incubation days along the first axis as it decreased after the beginning of the incubation period (Kruskal–Wallis,  $P < 0.05$ ). Two OTUs classified as members of the order *Bacillales* (OTUs 65 and 299) contributed to discriminate both H<sub>2</sub> treatments in the ordination space after 5 and 7 incubation days (Kruskal–Wallis,  $P < 0.05$ ). These OTUs were more abundant in microcosms exposed to eH<sub>2</sub> until day 10, where they reached a similar relative abundance in aH<sub>2</sub> and eH<sub>2</sub> treatments. The transient response of these OTUs in eH<sub>2</sub> treatment could be a direct consequence of H<sub>2</sub> exposure as representatives of the genus *Bacillus* possess putative membrane-bound type 1a and 1d [NiFe]-hydrogenases (Greening *et al.*, 2016).

Conflicting results were obtained in two different studies reporting the impact of H<sub>2</sub> soil exposure on bacterial community structure based on 16S rRNA terminal restriction fragment profiles analysis. H<sub>2</sub> exerted no incidence on ribotyping profile in soil exposed to 250 nmol H<sub>2</sub> cm<sup>-3</sup> h<sup>-1</sup> (500 ppmv H<sub>2</sub> in artificial air, added at 45 ml min<sup>-1</sup>), while a single ribotype related to *Mycobacterium* increased in soils upon elevated H<sub>2</sub> exposure (Osborne, Peoples & Janssen, 2010). On the other hand, an exposure rate of 33 nmol cm<sup>-3</sup> h<sup>-1</sup> (79 ppmv H<sub>2</sub> at 100 cm<sup>3</sup> min<sup>-1</sup>) exerted a significant influence on bacterial community profile, with an increase of T-RFLP peaks belonging to  $\gamma$ -*Proteobacteria* and a decrease of peaks belonging to *Actinobacteria* and  $\alpha$ -*Proteobacteria* upon elevated H<sub>2</sub> exposure (Zhang, He & Dong, 2009). A likelihood ratio analysis, fitting a negative binomial generalized log-linear model to the sequencing data (McCarthy, Chen & Smyth, 2012; Robinson, McCarthy & Smyth, 2010), unveiled that distribution of 958 OTUs was influenced by H<sub>2</sub> exposure. The relative abundance of OTUs responding to H<sub>2</sub> treatment ranged between 0.001 and 1.8%, suggesting an incidence of H<sub>2</sub> on members of the rare biosphere and abundant taxa. The influence of H<sub>2</sub> was uneven among different taxonomic groups as different representatives of the same taxa (i.e., OTUs classified at the phylum and the order taxonomic levels) were found to be favored or disfavored in response to eH<sub>2</sub> exposure (Table 1). Even though the uneven response among taxonomic groups impairs prediction of H<sub>2</sub> exposure on metabolic functions in soil (Langille *et al.*, 2013), this observation is sufficient to demonstrate that previous investigations relying on low-resolution community fingerprinting techniques



**Figure 4** Influence of  $H_2$  exposure on bacterial ribotyping profile. (A) UPGMA agglomerative clustering of soil samples derived from a matrix of Euclidean distance calculated after Hellinger transformation of OTU (97% identity) absolute abundance in soil microcosms exposed to  $eH_2$  and  $aH_2$  throughout a 10-day incubation period. The black circles  $\bullet$  represent significant nodes ( $P \leq 0.05$ ). (B) Principal component analysis showing the distribution of soil subsamples in a reduced space defined by the relative abundance of 16S rRNA gene sequences classified at the OTU level (97% identity). Only the OTUs (represented by red lines) having a higher contribution than average to explain the two dimensions of the PCA space are shown to facilitate visualization of the analysis. Soil subsamples collected in microcosms exposed to  $eH_2$  and  $aH_2$  are shown in blue and green, respectively.



**Figure 5** OTUs of interest to explain the PCA. Time series of the relative abundance of the 3 OTUs having a higher contribution than average to explain the two dimensions of the PCA space in soil microcosms. The average and standard deviation measured in replicated microcosms are represented. The closest taxonomic affiliations of OTUs 52, 299 and 65 are, respectively, the bacterial order *MND1* (*Betaproteobacteria*), genus *Bacillus* (*Firmicutes*) and the species *Bacillus cereus* (*Firmicutes*).

**Table 1** Summary of OTUs showing different relative abundance in eH<sub>2</sub> and aH<sub>2</sub> treatments (Likelihood ratio test,  $P < 0.05$ ). Altogether, 406 OTUs were more abundant in eH<sub>2</sub> and 552 were more abundant in aH<sub>2</sub>. The eH<sub>2</sub> and aH<sub>2</sub> rows indicate the treatment in which the identified phylotypes are more abundant. A single or the two most abundant OTUs are identified for each phylum. A list of the 958 OTUs whose distribution was influenced by H<sub>2</sub> treatments is provided in the raw data file accompanying the article.

Treatments	Phyla	Most abundant OTU (order level)
eH <sub>2</sub>	<i>Proteobacteria</i> (27.6%)	<i>Myxococcales</i>
	<i>Planctomycetes</i> (10.6%)	<i>Gemmatales</i> , <i>Phycisphaerales</i>
	<i>Bacteroidetes</i> (9.6%)	<i>Sphingobacteriales</i>
	<i>Chloroflexi</i> (9.1%)	<i>Ktedonobacteria</i>
	<i>Acidobacteria</i> (7.1%)	<i>Acidobacteriales</i> , <i>Solibacterales</i>
	<i>Verrucomicrobia</i> (6.2%)	<i>Verrucomicrobiales</i>
	<i>Actinobacteria</i> (5.9%)	<i>Actinomycetales</i> , <i>Solirubrobacterales</i>
	<i>Elusimicrobia</i> (3.0%)	FAC88
	<i>Gemmatimonadetes</i> (2.7%)	<i>Gemmatimonadales</i>
	<i>Firmicutes</i> (1.5%)	<i>Clostridiales</i> , <i>Bacillales</i>
	<i>Archaea</i> (1.0%)	<i>Methanobacteriales</i>
	<i>Armatimonadetes</i> (0.7%)	CH21
	<i>Chlorobi</i> (0.2%)	SM1B09
	Others (14.8%)	–
aH <sub>2</sub>	<i>Proteobacteria</i> (33.9%)	<i>Rhodospirillales</i> , <i>Myxococcales</i>
	<i>Acidobacteria</i> (11.4%)	<i>Solibacterales</i>
	<i>Planctomycetes</i> (11.1%)	<i>Gemmatales</i>
	<i>Chloroflexi</i> (6.2%)	<i>Ktedonobacteria</i> , A4b
	<i>Bacteroidetes</i> (5.1%)	<i>Sphingobacteriales</i>
	<i>Actinobacteria</i> (4.3%)	<i>Actinomycetales</i> , <i>Acidimicrobiales</i>
	<i>Firmicutes</i> (3.8%)	<i>Clostridiales</i>
	<i>Verrucomicrobia</i> (3.4%)	<i>Spartobacteriales</i>
	<i>Gemmatimonadetes</i> (2.7%)	<i>Gemmatimonadales</i>
	<i>Archaea</i> (1.8%)	SD-NA ( <i>Crenarchaeota</i> )
	<i>Elusimicrobia</i> (1.4%)	<i>Elusimicrobiales</i> , MVP-88
	<i>Armatimonadetes</i> (1.3%)	<i>Chthonomonadales</i> , CH21
	<i>Chlorobi</i> (0.9%)	SM1B09
	Others (12.7%)	–

have considerably underestimated the response of soil microbial communities to H<sub>2</sub> exposure.

### Impact of H<sub>2</sub> exposure on the co-occurrence of OTUs

Correlation networks were computed to investigate the impact of H<sub>2</sub> exposure on the covariation of OTUs throughout the incubation period and identify OTUs for which the distribution is influenced by H<sub>2</sub>. Two separate networks were computed: the first for elevated H<sub>2</sub> exposure (eH<sub>2</sub> network) and a second for low H<sub>2</sub> exposure (aH<sub>2</sub> network). Both networks contained 43 modules and module preservation statistics demonstrated that module composition significantly changed across H<sub>2</sub> treatments (Text S1). The

significances of measured H<sub>2</sub> oxidation rate in explaining network structure were 0.33 and 0.98 in aH<sub>2</sub> and eH<sub>2</sub> networks, again pointing out a significant impact of H<sub>2</sub> on soil microbial community structure. Indeed, module eigengenes of five modules were significantly correlated with high-affinity H<sub>2</sub> oxidation rate time series in eH<sub>2</sub> network, while no module was related to the activity in aH<sub>2</sub> network (Fig. S3). Together, these 5 modules represent 1,140 OTUs, which is more than a third of the whole eH<sub>2</sub> network (3,154 OTUs). OTUs belonging to one module were hindered by eH<sub>2</sub> exposure, while members of the four other modules were favored by eH<sub>2</sub> treatment. The OTUs belonging to these five modules were members of the rare biosphere as well as more abundant ribotypes, with relative abundance ranging between 0.001% and 4.3% encompassing *Proteobacteria*, *Chloroflexi*, *Acidobacteria* as well as other phyla (Fig. S4). Clustering of these OTUs at the class and order levels unveiled that none of the taxonomic groups were restricted to the five modules correlated with H<sub>2</sub> oxidation rate, supporting the previous observation that response to H<sub>2</sub> exposure and the distribution of hydrogenase genes is uneven within each taxonomic group. The 38 modules in eH<sub>2</sub> network whose eigengene showed no significant correlation with H<sub>2</sub> oxidation rate are ecologically relevant observations since they represent indirect impacts of H<sub>2</sub> exposure on soil microbial communities and highlight a previously overlooked role of H<sub>2</sub> in shaping potential microbe-microbe cooperation and competition interactions. Deciphering the impact of these complex interactions on soil biogeochemical processes was beyond the scope of this study but will deserve attention in future investigations.

## CONCLUSION

In conclusion, this exploratory study validated our hypothesis that elevated H<sub>2</sub> exposure influences the activity of HOB, leading to direct impacts on the HOB but also indirect impact on the whole soil bacterial community through competitive and synergistic microbe-microbe interactions. Indeed, H<sub>2</sub> soil exposure has an impact on the ecological niche of bacteria unevenly distributed among taxonomic groups beyond the alteration of HOB reported in early investigations. Our study was limited to a single farmland soil, with two replicated microcosms per treatment. Nonetheless, considering the significant impact that H<sub>2</sub> exposure has brought to soil bacterial communities, it is reasonable to expect a similar effect on other soil types. Considering the steep H<sub>2</sub> concentration gradient surrounding N<sub>2</sub>-fixing nodules, from 20,000 ppmv to sub-atmospheric levels along a 4.5 cm radius, the response of microorganisms might vary as a function of the distance from H<sub>2</sub> diffusing sources as well as soil types, physicochemical conditions and the structural and functional structure of microbial community (Hartmann *et al.*, 2015). The impact of H<sub>2</sub> and HOB on soil microbial communities will definitely deserve more attention in soil microbiology. There is a need to further study whether elevated H<sub>2</sub> exposure also exerts noticeable changes in soil ecological functions other than H<sub>2</sub> oxidation, such as carbon metabolism, nutrient cycling, trace gas exchanges and xenobiotic degradation. Such a significant impact of a gas molecule on soil was observed in a study investigating the impact of a doubling atmospheric CO<sub>2</sub> concentration, simulating climate change, on microbial communities

(Zhou *et al.*, 2011). A metagenomics approach on the matter might provide further answers on this rather ubiquitous microniche as well as the succession of hydrogenases along H<sub>2</sub> concentration gradients in the environment.

## ADDITIONAL INFORMATION AND DECLARATIONS

### Funding

This work has been supported by a Natural Sciences and Engineering Research Council of Canada Discovery grant to PC and by the Community Sequencing Program of the Joint Genome Institute (US Department of Energy) to PC, JT and SGT. The work conducted by the US Department of Energy Joint Genome Institute, a DOE Office of Science User Facility, is supported by the Office of Science of the US Department of Energy under Contract No. DE-AC02-05CH11231. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

### Grant Disclosures

The following grant information was disclosed by the authors:

Natural Sciences and Engineering Research Council of Canada Discovery.

Community Sequencing Program of the Joint Genome Institute (US Department of Energy).

US Department of Energy Joint Genome Institute.

DOE Office of Science User Facility.

Office of Science of the US Department of Energy: DE-AC02-05CH11231.

### Competing Interests

The authors declare there are no competing interests.

### Author Contributions

- Sarah Piché-Choquette conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Julien Tremblay and Susannah G. Tringe analyzed the data, contributed reagents/materials/analysis tools, reviewed drafts of the paper.
- Philippe Constant conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.

### DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

Raw sequences were deposited in the Sequence Read Archive of the National Center for Biotechnology Information under the Bioproject [PRJNA295403](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA295403).

### Data Availability

The following information was supplied regarding data availability:

Raw data can be found in the [Supplemental Information](#).



## Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.1782#supplemental-information>.

## REFERENCES

- Berney M, Cook GM. 2010.** Unique flexibility in energy metabolism allows mycobacteria to combat starvation and hypoxia. *PLoS ONE* 5:e8614 DOI 10.1371/journal.pone.0008614.
- Berney M, Greening C, Hards K, Collins D, Cook GM. 2014.** Three different [NiFe] hydrogenases confer metabolic flexibility in the obligate aerobe *Mycobacterium smegmatis*. *Environmental Microbiology* 16:318–330 DOI 10.1111/1462-2920.12320.
- Bouyoucos G-J. 1936.** Directions for making mechanical analyses of soils by the hydrometer method. *Soil Science* 42:225–230 DOI 10.1097/00010694-193609000-00007.
- Clarke KR, Somerfield PJ, Gorley RN. 2008.** Testing of null hypotheses in exploratory community analyses: similarity profiles and biota-environment linkage. *Journal of Experimental Marine Biology and Ecology* 366:56–69 DOI 10.1016/j.jembe.2008.07.009.
- Conrad R. 1996.** Soil microorganisms as controllers of atmospheric trace gases (H<sub>2</sub>, CO, CH<sub>4</sub>, OCS, N<sub>2</sub>O, and NO). *Microbiological Reviews* 60:609–640.
- Conrad R, Aragno M, Seiler W. 1983.** The inability of hydrogen bacteria to utilize atmospheric hydrogen is due to threshold and affinity for hydrogen. *FEMS Microbiology Letters* 18:207–210 DOI 10.1111/j.1574-6968.1983.tb00479.x.
- Constant P, Chowdhury SP, Hesse L, Conrad R. 2011a.** Co-localization of atmospheric H<sub>2</sub> oxidation activity and high affinity H<sub>2</sub>-oxidizing bacteria in non-axenic soil and sterile soil amended with *Streptomyces* sp. PCB7. *Soil Biology and Biochemistry* 43:1888–1893 DOI 10.1016/j.soilbio.2011.05.009.
- Constant P, Chowdhury SP, Hesse L, Pratscher J, Conrad R. 2011b.** Genome data mining and soil survey for the novel group 5 NiFe -hydrogenase to explore the diversity and ecological importance of presumptive high-affinity H<sub>2</sub>-oxidizing Bacteria. *Applied and Environmental Microbiology* 77:6027–6035 DOI 10.1128/AEM.00673-11.
- Constant P, Chowdhury SP, Pratscher J, Conrad R. 2010.** Streptomycetes contributing to atmospheric molecular hydrogen soil uptake are widespread and encode a putative high-affinity [NiFe]-hydrogenase. *Environmental Microbiology* 12:821–829 DOI 10.1111/j.1462-2920.2009.02130.x.
- Constant P, Hallenbeck PC. 2012.** Hydrogenase. In: Pandey A, Chang J-S, Hallenbeck PC, Larroche C, eds. *Biohydrogen*. San Diego: Elsevier B.V, 75–102.
- Constant P, Poissant L, Villemur R. 2008.** Isolation of *Streptomyces* sp. PCB7, the first microorganism demonstrating high-affinity uptake of tropospheric H<sub>2</sub>. *ISME Journal* 2:1066–1076 DOI 10.1038/ismej.2008.59.
- Constant P, Poissant L, Villemur R. 2009.** Tropospheric H<sub>2</sub> budget and the response of its soil uptake under the changing environment. *Science of the Total Environment* 407:1809–1823 DOI 10.1016/j.scitotenv.2008.10.064.

- DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Applied and Environmental Microbiology* 72:5069–5072 DOI 10.1128/AEM.03006-05.
- Dong Z, Layzell DB. 2001. H<sub>2</sub> oxidation, O<sub>2</sub> uptake and CO<sub>2</sub> fixation in hydrogen treated soils. *Plant and Soil* 229:1–12 DOI 10.1023/A:1004810017490.
- Dong Z, Wu L, Kettlewell B, Caldwell CD, Layzell DB. 2003. Hydrogen fertilization of soils—is this a benefit of legumes in rotation? *Plant, Cell & Environment* 26:1875–1879 DOI 10.1046/j.1365-3040.2003.01103.x.
- Edgar RC. 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26:2460–2461 DOI 10.1093/bioinformatics/btq461.
- Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. 2011. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27:2194–2200 DOI 10.1093/bioinformatics/btr381.
- Ehhalt DH, Rohrer F. 2009. The tropospheric cycle of H<sub>2</sub>: a critical review. *Tellus B* 61:500–535 DOI 10.1111/j.1600-0889.2009.00416.x.
- Golding AL, Zou Y, Yang X, Flynn B, Dong Z. 2012. Plant growth promoting H<sub>2</sub>-oxidizing bacteria as seed inoculants for cereal crops. *Agricultural Sciences* 3:510–516 DOI 10.4236/as.2012.34060.
- Greening C, Berney M, Hards K, Cook GM, Conrad R. 2014a. A soil actinobacterium scavenges atmospheric H<sub>2</sub> using two membrane-associated, oxygen-dependent NiFe hydrogenases. *Proceedings of the National Academy of Sciences of the United States of America* 111:4257–4261 DOI 10.1073/pnas.1320586111.
- Greening C, Biswas A, Carere CR, Jackson CJ, Taylor MC, Stott MB, Cook GM, Morales SE. 2016. Genomic and metagenomic surveys of hydrogenase distribution indicate H<sub>2</sub> is a widely utilised energy source for microbial growth and survival. *ISME Journal* 10:761–777 DOI 10.1038/ismej.2015.153.
- Greening C, Constant P, Hards K, Morales SE, Oakeshott JG, Russell RJ, Taylor MC, Berney M, Conrad R, Cook GM. 2015. Atmospheric hydrogen scavenging: from enzymes to ecosystems. *Applied and Environmental Microbiology* 81:1190–1199 DOI 10.1128/AEM.03364-14.
- Greening C, Villas-Boas SG, Robson JR, Berney M, Cook GM. 2014b. The growth and survival of *Mycobacterium smegmatis* is enhanced by co-metabolism of atmospheric H<sub>2</sub>. *PLoS ONE* 9:e103034 DOI 10.1371/journal.pone.0103034.
- Häring V, Conrad R. 1994. Demonstration of two different H<sub>2</sub>-oxidizing activities in soil using an H<sub>2</sub> consumption and a tritium exchange assay. *Biology and Fertility of Soils* 17:125–128 DOI 10.1007/BF00337744.
- Hartmann M, Frey B, Mayer J, Mader P, Widmer F. 2015. Distinct soil microbial diversity under long-term organic and conventional farming. *ISME Journal* 9:1177–1194 DOI 10.1038/ismej.2014.210.
- Hoffman BM, Dean DR, Seefeldt LC. 2009. Climbing nitrogenase: toward a mechanism of enzymatic nitrogen fixation. *Accounts of Chemical Research* 42:609–619 DOI 10.1021/ar8002128.

- Hunt S, Layzell DB. 1993. Gas exchange of legume nodules and the regulation of nitrogenase activity. *Annual Review of Plant Physiology and Plant Molecular Biology* 44:483–511 DOI 10.1146/annurev.pp.44.060193.002411.
- Khdhiri M, Hesse L, Popa ME, Quiza L, Lalonde I, Meredith LK, Röckmann T, Constant P. 2015. Soil carbon content and relative abundance of high affinity H<sub>2</sub>-oxidizing bacteria predict atmospheric H<sub>2</sub> soil uptake activity better than soil microbial community composition. *Soil Biology and Biochemistry* 85:1–9 DOI 10.1016/j.soilbio.2015.02.030.
- Kuczynski J, Stombaugh J, Walters WA, González A, Caporaso JG, Knight R. 2011. Using QIIME to analyze 16S rRNA gene sequences from microbial communities. *Current Protocols in Bioinformatics* 36:10.7.1–10.7.20.
- La Favre JS, Focht DD. 1983. Conservation in soil of H<sub>2</sub> liberated from N<sub>2</sub> fixation by Hup<sup>-</sup> nodules. *Applied and Environmental Microbiology* 46:304–311.
- Langfelder P, Horvath S. 2008. WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics* 9:559 DOI 10.1186/1471-2105-9-559.
- Langille MGI, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, Clemente JC, Burkepile DE, Vega Thurber RL, Knight R, Beiko RG, Huttenhower C. 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nature Biotechnology* 31:814–821 DOI 10.1038/nbt.2676.
- Legendre P, Gallagher E. 2001. Ecologically meaningful transformations for ordination of species data. *Oecologia* 129:271–280 DOI 10.1007/s004420100716.
- Li M, Copeland A, Han J. 2011. DUK-A Fast and efficient Kmer based sequence matching tool. Available at <https://publications.lbl.gov/islandora/object/ir%3A155200> (accessed 17 February 2016).
- Liot Q, Constant P. 2015. Breathing air to save energy—new insights into the eco-physiological role of high-affinity [NiFe]-hydrogenase in *Streptomyces avermitilis*. *MicrobiologyOpen* 5:47–59 DOI 10.1002/mbo3.310.
- Magoc T, Salzberg SL. 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27:2957–2963 DOI 10.1093/bioinformatics/btr507.
- McCarthy DJ, Chen Y, Smyth GK. 2012. Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. *Nucleic Acids Research* 40:4288–4297 DOI 10.1093/nar/gks042.
- Meredith LK, Rao D, Bosak T, Klepac-Ceraj V, Tada KR, Hansel CM, Ono S, Prinn RG. 2014. Consumption of atmospheric hydrogen during the life cycle of soil-dwelling actinobacteria. *Environmental Microbiology Reports* 6:226–238 DOI 10.1111/1758-2229.12116.
- Osborne CA, Peoples MB, Janssen PH. 2010. Detection of a reproducible, single-member shift in soil bacterial communities exposed to low levels of hydrogen. *Applied and Environmental Microbiology* 76:1471–1479 DOI 10.1128/AEM.02072-09.
- Rasche ME, Arp DJ. 1989. Hydrogen inhibition of nitrogen reduction by nitrogenase in isolated soybean nodule bacteroids. *Plant Physiology* 91:663–668 DOI 10.1104/pp.91.2.663.

- R Development Core Team. 2008.** *R: a language and environment for statistical computing*. Vienna: R Foundation for Statistical Computing.
- Rittenberg SC, Goodman NS. 1969.** Mixotrophic growth of *Hydrogenomonas eutropha*. *Journal of Bacteriology* **98**:617–622.
- Robinson MD, McCarthy DJ, Smyth GK. 2010.** edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**:139–140 DOI [10.1093/bioinformatics/btp616](https://doi.org/10.1093/bioinformatics/btp616).
- Schäfer C, Friedrich B, Lenza O. 2013.** Novel, oxygen-insensitive group 5 [NiFe]-hydrogenase in *Ralstonia eutropha*. *Applied and Environmental Microbiology* **79**:5137–5145 DOI [10.1128/AEM.01576-13](https://doi.org/10.1128/AEM.01576-13).
- Schuler S, Conrad R. 1990.** Soils contain two different activities for oxidation of hydrogen. *FEMS Microbiology Letters* **73**:77–83 DOI [10.1111/j.1574-6968.1990.tb03927.x](https://doi.org/10.1111/j.1574-6968.1990.tb03927.x).
- Stein S, Selesi D, Schilling R, Pattis I, Schmid M, Hartmann A. 2005.** Microbial activity and bacterial composition of H<sub>2</sub>-treated soils with net CO<sub>2</sub> fixation. *Soil Biology & Biochemistry* **37**:1938–1945 DOI [10.1016/j.soilbio.2005.02.035](https://doi.org/10.1016/j.soilbio.2005.02.035).
- Tremblay J, Singh K, Fern A, Kirton ES, He S, Woyke T, Lee J, Chen F, Dangl JL, Tringe SG. 2015.** Primer and platform effects on 16S rRNA tag sequencing. *Frontiers in Microbiology* **6**:1–15 DOI [10.3389/fmicb.2015.00771](https://doi.org/10.3389/fmicb.2015.00771).
- Vignais PM, Billoud B. 2007.** Occurrence, classification, and biological function of hydrogenases: an overview. *Chemical Reviews* **107**:4206–4272 DOI [10.1021/cr050196r](https://doi.org/10.1021/cr050196r).
- Wang Q, Garrity GM, Tiedje JM, Cole JR. 2007.** Naïve bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology* **73**:5261–5267 DOI [10.1128/AEM.00062-07](https://doi.org/10.1128/AEM.00062-07).
- Witty JF. 1991.** Microelectrode measurements of hydrogen concentrations and gradients in legume nodules. *Journal of Experimental Botany* **42**:765–771 DOI [10.1093/jxb/42.6.765](https://doi.org/10.1093/jxb/42.6.765).
- Zhang Y, He X, Dong ZM. 2009.** Effect of hydrogen on soil bacterial community structure in two soils as determined by terminal restriction fragment length polymorphism. *Plant and Soil* **320**:295–305 DOI [10.1007/s11104-009-9894-3](https://doi.org/10.1007/s11104-009-9894-3).
- Zhou J, Deng Y, Luo F, He Z, Yang Y. 2011.** Phylogenetic molecular ecological network of soil microbial communities in response to elevated CO<sub>2</sub>. *mBio* **2**: e00122–00111.