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H$_2$-saturation of high affinity H$_2$-oxidizing bacteria alters the ecological niche of soil microorganisms unevenly among taxonomic groups

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ABSTRACT

Soil microbial communities are continuously exposed to H$_2$ diffusing into the soil from the atmosphere. N$_2$-fixing nodules represent a peculiar microniche in soil where H$_2$ 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$_2$ exposure on soil bacterial community structure using dynamic microcosm chambers simulating soil H$_2$ exposure from the atmosphere and N$_2$-fixing nodules. Biphasic kinetic parameters governing H$_2$ oxidation activity in soil changed drastically upon elevated H$_2$ exposure, corresponding to a slight but significant decay of high affinity H$_2$-oxidizing bacteria population, accompanied by an enrichment or activation of microorganisms displaying low-affinity for H$_2$. 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$_2$ exposure. Furthermore, correlation networks showed important alterations of ribotype covariation in response to H$_2$ exposure, suggesting that H$_2$ affects microbe-microbe interactions in soil. Taken together, our results demonstrate that H$_2$-rich environments exert a direct influence on soil H$_2$-oxidizing bacteria in addition to indirect effects on other members of the bacterial communities.

Subjects Microbiology, Soil Science
Keywords Soil, Microbial ecology, Correlation network

INTRODUCTION

Soil microbial communities are continuously exposed to molecular hydrogen (H$_2$). Trace levels of H$_2$ (0.530 ppmv) diffuse into the soil from the global atmosphere, yet higher concentrations can be found in the rhizosphere of N$_2$-fixing legumes (*Constant, Poissant & Villemur, 2009*). Indeed, H$_2$ is an obligate by-product of nitrogenase in N$_2$-fixing free living or symbiotic bacteria, with an H$_2$ molecule produced for every reduced N$_2$ molecule (*Hoffman, Dean & Seefeldt, 2009*). It has been estimated that 240,000 L of H$_2$ are produced per hectare of legume crop over a growing season (*Dong et al., 2003*). At the microscale level, there is a steep H$_2$ 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_2 found in legumes rhizosphere, a very small proportion escapes to the atmosphere due to H_2-oxidizing bacteria (HOB) thriving in soil. These microorganisms also play a vital role in the global budget of H_2, being responsible for about 80% (60 Tg H_2 yr^{-1}) of the global losses of this trace gas from the atmosphere (*Constant, Poissant & Villemur, 2009*; *Ehhalt & Rohrer, 2009*).

Aerobic soil bacteria scavenging H_2 diffusing from the atmosphere and N_2-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_2 into protons and electrons ($H_2 \leftrightarrow 2H^+ + 2e^-$). 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_2 ($_{(app)}K_m < 100$ ppmv), conferring the ability to oxidize atmospheric H_2 (*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_2 is insufficient to support chemolithotrophic growth and compelling experimental evidence suggest that H_2 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_2 diffusing from nodules in the presence of O_2, yet they are unable to use atmospheric H_2 due to the low-affinity ($_{(app)}K_m > 1,000$ ppmv) and high H_2 threshold concentration of their [NiFe]-hydrogenases (*Conrad, Aragno & Seiler, 1983*). Knallgas bacteria can use H_2 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_2 oxidation activity in soil (*Häring & Conrad, 1994*).

Laboratory incubations simulating H_2 fluxes from N_2-fixing nodules demonstrated that soil bacterial community composition changed upon H_2 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_2 exposure. Here, we revisited these experiments using a combination of high-throughput sequencing of the bacterial 16S rRNA gene and an H_2 metabolism analysis to compare microbial community structure in dynamic microcosm chambers simulating soil H_2 exposure from the atmosphere and from N_2-fixing nodules, corresponding to unsaturating and saturating H_2 concentrations for high affinity hydrogenases, respectively. Elevated H_2 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® (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 ± 0.3%) and total nitrogen (0.3 ± 0.0%) content were determined using an elemental combustion system using the protocol described in *Khdhiri et al. (2015)*.

Controlled H_2 exposure in dynamic soil microcosm chambers

Dynamic microcosm chambers were designed to expose soil to controlled levels of H_2 (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^{-1}, 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_2 treatments were applied in parallel incubations. The first treatment (designated microcosms eH_2(a) and eH_2(b)), named elevated H_2 (eH_2), consisted of exposing soil to a dynamic headspace comprising 525 ppmv H_2 in synthetic air, simulating H_2 concentrations detected around N_2-fixing nodules in soil (*Hunt & Layzell, 1993*). The second was a control treatment (designated microcosms aH_2(a) and aH_2(b)), named atmospheric H_2 (aH_2), where soil was exposed to a dynamic headspace comprising 0.54 ppmv H_2 in synthetic air, representing H_2 concentrations found in the atmosphere. Both treatments were replicated, resulting in four soil microcosms in total. Each microcosm chamber contained 200 g$_{(dw)}$ 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_2 soil uptake rate measurements. Diffusion limitation was precluded since preliminary experiments using the same microcosm setup showed proportional H_2 uptake activity as a function of the amount of soil in the chamber using 150, 200 and 250 g$_{(dw)}$ 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_2

oxidation rate measurements (see below). A decrease in soil pH was observed over the course of the incubation (from pH 5.9 ± 0.2 to 5.1 ± 0.1) in all microcosms, without distinction between aH$_2$ and eH$_2$ 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$_2$ oxidation or production activity.

H$_2$ uptake activity

High affinity H$_2$ 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$_2$) and tightly closed with rubber septum caps. The 5-min flush was shown sufficient to avoid residual H$_2$ degassing that would otherwise have led to an underestimation of H$_2$ oxidation rate. A defined volume of H$_2$ gas mixture (525 ± 10 ppmv H$_2$ GST-Welco, PA, USA.) was injected to obtain an initial concentration of approximately 3 ppmv in the static headspace. Decrease of the H$_2$ 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®, DE, USA.) as previously described (*Khdhiri et al., 2015*). Considering the low level of H$_2$ added in the headspace, H$_2$ uptake reflected the activity of high affinity HOB, knallgas bacteria showing low affinity for H$_2$ being unable to use these trace amounts of H$_2$ (*Conrad, Aragno & Seiler, 1983*). The biphasic kinetic parameters governing H$_2$ 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$_2$ 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® (MP Biomedicals, Solon, OH, USA.). DNA was eluted in 100 µL nuclease-free water. Quality of the DNA was then examined on agarose gels and samples were quantified using Quantifluor dsDNA System® (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 (2×250 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.

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_2 uptake activities in soil microcosms incubated under aH_2 and eH_2 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
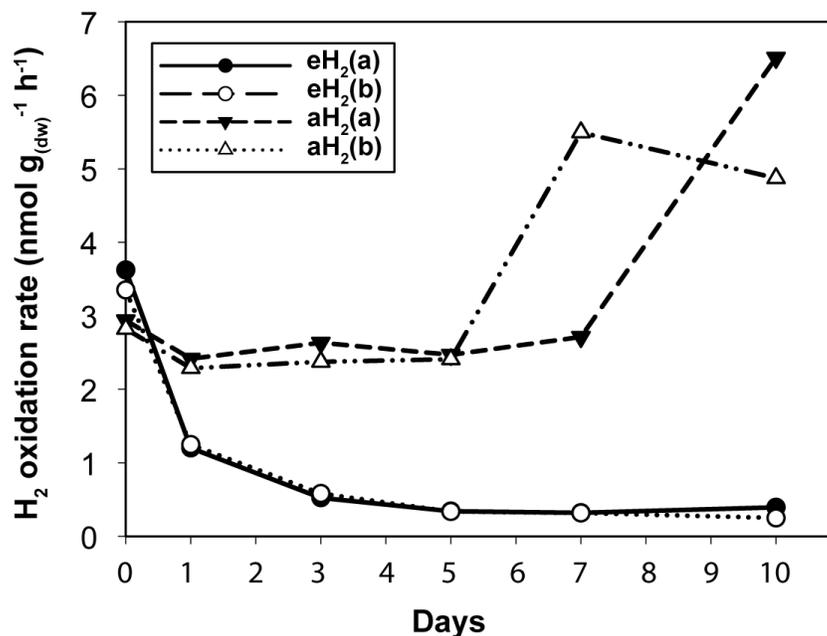
Figure 1 **High affinity H_2 oxidation rates.** Time series of the high-affinity H_2 oxidation rate measured in soil microcosms exposed to aH_2 or eH_2 throughout the incubation period.

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_2 and eH_2 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_2 and eH_2 exposure treatments was tested using the likelihood ratio test using the package "edgeR." Covariation among OTUs during the incubation under controlled H_2 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_2 exposure on the distribution and activity of HOB

Agricultural soil microcosms were incubated under two different H_2 level treatments. The first treatment simulated soil exposure to atmospheric level of H_2 (aH_2; 0.54 ppmv), while the second simulated high affinity hydrogenases substrate saturation (eH_2; 525 ppmv). High affinity H_2 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_2 exposure could already be observed after 24 h. From hour 24 to day 10, a decline of high affinity H_2 uptake rate was observed in microcosms exposed to
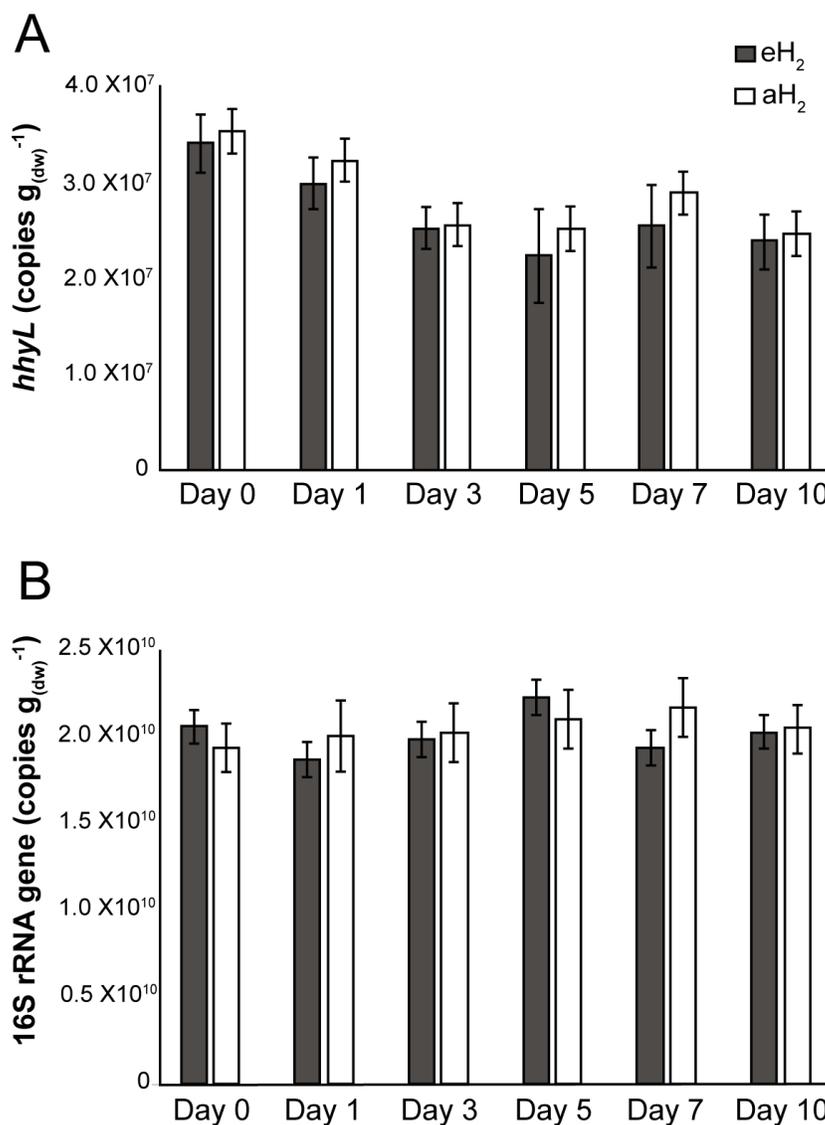
Figure 2 Time series of qPCR data. Time series of (A) *hhyL* and (B) 16S rRNA gene abundance in soil as determined by qPCR.

eH_2, while an increase of this uptake rate was measured in microcosms exposed to aH_2 levels. This trend was maintained over the course of the incubation, with an oxidation rate of 6.2 ± 1.9 and 0.3 ± 0.1 nmol $g_{(dw)}^{-1}$ h^{-1} in aH_2 and eH_2 treatments at the end of the experiment, respectively. This response of H_2 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_2 (exponential regression, $P = 0.06$), while a slight but significant exponential decay (exponential regression, $P = 0.02$) was observed under eH_2 (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_2 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_2 suggests that H_2 was not sufficient to promote growth and persistence of high affinity HOB.

H_2 soil exposure exerted a significant impact on the kinetic parameters governing H_2 oxidation activity in soil. At the end of the incubation, microcosms exposed to aH_2 level displayed $_{(app)}K_m$ of 40 ± 5 ppmv (Fig. 3A), while microcosms exposed to eH_2 showed lower affinity towards H_2 with an $_{(app)}K_m$ of 838 ± 163 ppmv (Fig. 3B). Similar observations were obtained by *Dong & Layzell (2001)*, where soil exposed to elevated levels of H_2 (600 ppmv) displayed an $_{(app)}K_m$ of 1,028 ppmv H_2, while soil exposed to low H_2 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_2 uptake activities in both H_2 treatments. The H_2 oxidation activity was mainly catalyzed by bacteria demonstrating intermediate and high-affinity in aH_2 treatment (Fig. 3A, insert), while H_2-oxidation activity by bacteria displaying low- and high-affinity for H_2 were detectable in the eH_2 treatment (Fig. 3B, insert). Low H_2 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_2 oxidation activity under eH_2 treatment might be explained by knallgas bacteria such as *Proteobacteria* species encompassing *Ralstonia*, *Variovorax* and *Bradyrhizobium* using H_2 for mixotrophic growth (*Rittenberg & Goodman, 1969*), as they are known to exhibit a H_2 uptake threshold ranging from 1 to 200 ppmv H_2 (*Conrad, 1996*).

Impact of H_2 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_2 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_2 exposure, but a mistake in the reported H_2 concentration units by the authors (i.e., the use of a gas mixture of 2,000 ml H_2 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_2 exposure (ANOVA, $P > 0.05$), with Shannon indices of 9.38 ± 0.05 and 9.34 ± 0.17 for aH_2 and eH_2 treatment respectively. An UPGMA hierarchical clustering analysis was computed
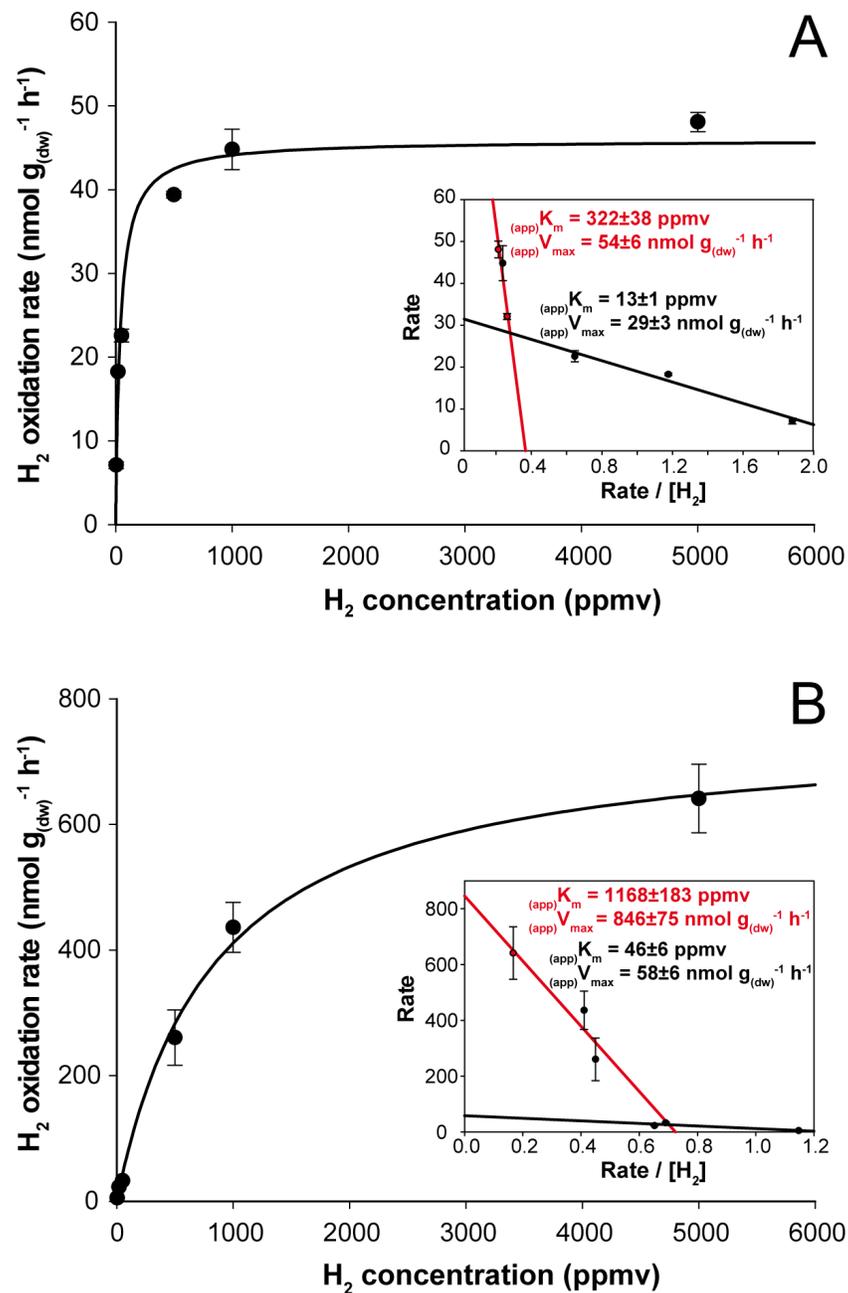
Figure 3 Kinetic parameters governing H_2 oxidation activity in soil microcosms incubated under (A) aH_2 and (B) eH_2 exposure. Michaelis-Menten graphs are presented with Eadie-Hofstee plots highlighting the biphasic kinetic of the reaction in the inserts.

to compare bacterial community profiles from soil subsamples collected in microcosms exposed to different 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 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_2 exposure. This discrimination was however transient as ribotyping profiles corresponding to the last incubation day could not be discriminated according to their respective H_2 treatments (Fig. 4A). The transient response was likely due to the fact that H_2 alone was not sufficient to maintain a sustained change in microbial community structure. Decrease of complex carbon sources and nutrients used along with H_2 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_2 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_2 until day 10, where they reached a similar relative abundance in aH_2 and eH_2 treatments. The transient response of these OTUs in eH_2 treatment could be a direct consequence of H_2 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_2 soil exposure on bacterial community structure based on 16S rRNA terminal restriction fragment profiles analysis. H_2 exerted no incidence on ribotyping profile in soil exposed to 250 nmol H_2 cm^{-3} h^{-1} (500 ppmv H_2 in artificial air, added at 45 ml min^{-1}), while a single ribotype related to *Mycobacterium* increased in soils upon elevated H_2 exposure (*Osborne, Peoples & Janssen, 2010*). On the other hand, an exposure rate of 33 nmol cm^{-3} h^{-1} (79 ppmv H_2 at 100 cm^3 min^{-1}) exerted a significant influence on bacterial community profile, with an increase of T-RFLP peaks belonging to γ-*Proteobacteria* and a decrease of peaks belonging to *Actinobacteria* and α-*Proteobacteria* upon elevated H_2 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_2 exposure. The relative abundance of OTUs responding to H_2 treatment ranged between 0.001 and 1.8%, suggesting an incidence of H_2 on members of the rare biosphere and abundant taxa. The influence of H_2 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_2 exposure (Table 1). Even though the uneven response among taxonomic groups impairs prediction of H_2 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
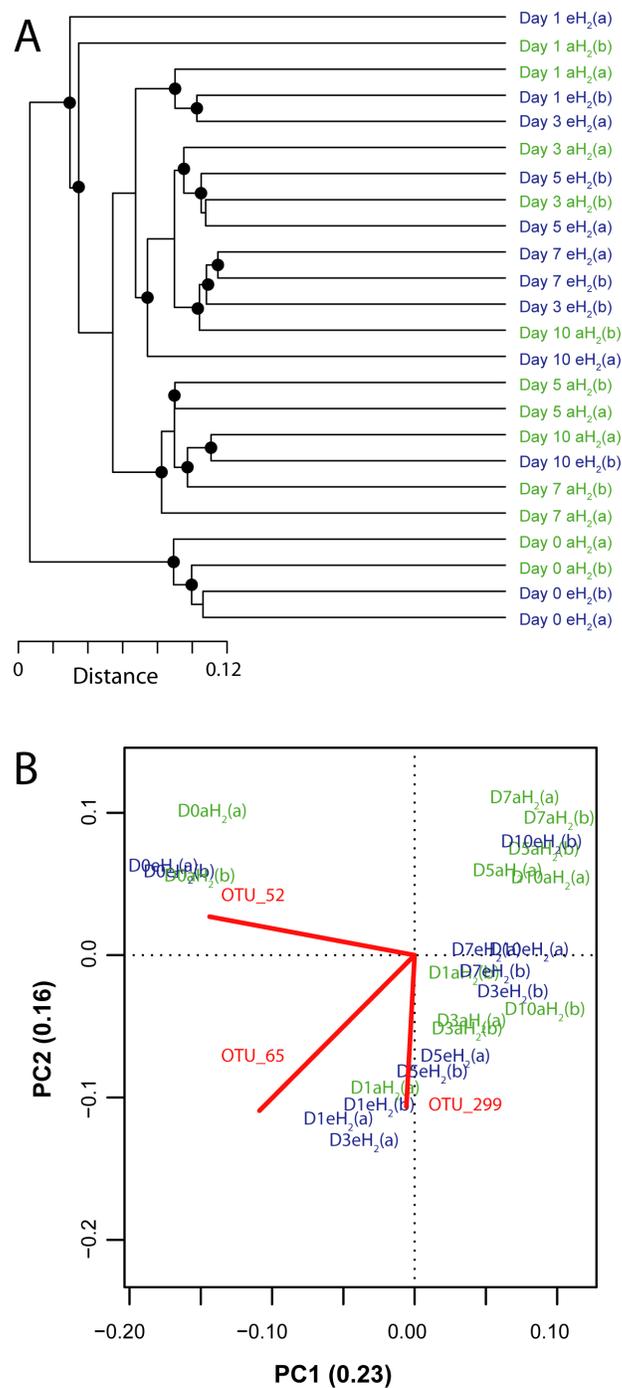
Figure 4 **Influence of H$_2$ exposure on bacterial ribotyping profile.** (A) UPGMA agglomerative clustering of soil samples derived from a matrix of Euclidean distance calculated after Hellinger transformation of OTU (97% identity) absolute abundance in soil microcosms exposed to eH$_2$ and aH$_2$ throughout a 10-day incubation period. The black circles ● 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.

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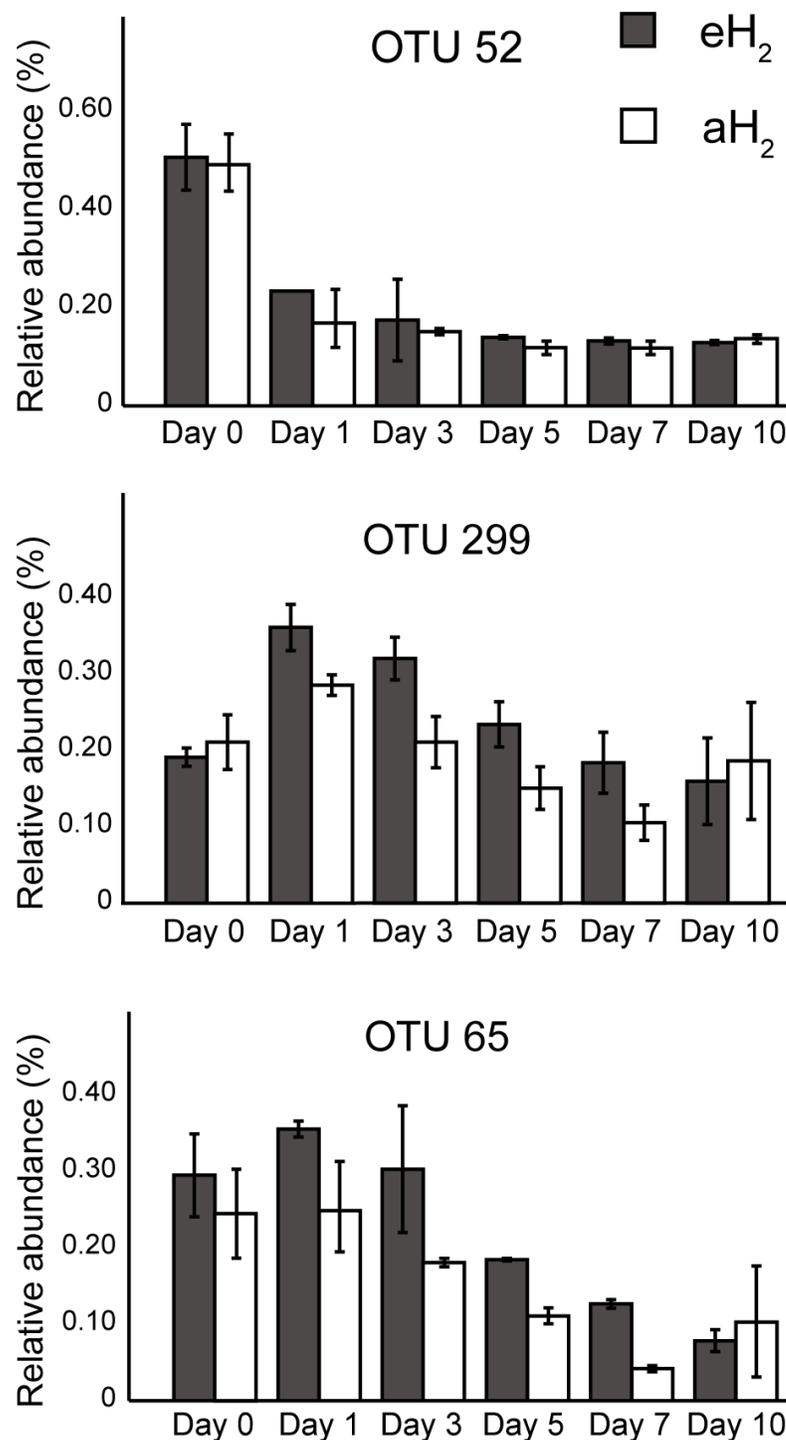
Figure 5 OTUs of interest to explain the PCA. Time series of the relative abundance of the 3 OTUs having a higher contribution than average to explain the two dimensions of the PCA space in soil microcosms. The average and standard deviation measured in replicated microcosms are represented. The closest 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_2 and aH_2 treatments (Likelihood ratio test, $P < 0.05$).** Altogether, 406 OTUs were more abundant in eH_2 and 552 were more abundant in aH_2. The eH_2 and aH_2 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_2 treatments is provided in the raw data file accompanying the article.

Treatments	Phyla	Most abundant OTU (order level)
eH_2	*Proteobacteria* (27.6%)	*Myxococcales*
	Planctomycetes (10.6%)	*Gemmatales, Phycisphaerales*
	Bacteroidetes (9.6%)	*Sphingobacteriales*
	Chloroflexi (9.1%)	*Ktedonobacteria*
	Acidobacteria (7.1%)	*Acidobacteriales, Solibacterales*
	Verrucomicrobia (6.2%)	*Verrucomicrobiales*
	Actinobacteria (5.9%)	*Actinomycetales, Solirubrobacterales*
	Elusimicrobia (3.0%)	*FAC88*
	Gemmatimonadetes (2.7%)	*Gemmatimonadales*
	Firmicutes (1.5%)	*Clostridiales, Bacillales*
	Archaea (1.0%)	*Methanobacteriales*
	Armatimonadetes (0.7%)	*CH21*
	Chlorobi (0.2%)	*SM1B09*
	Others (14.8%)	–
aH_2	*Proteobacteria* (33.9%)	*Rhodospirillales, Myxococcales*
	Acidobacteria (11.4%)	*Solibacterales*
	Planctomycetes (11.1%)	*Gemmatales*
	Chloroflexi (6.2%)	*Ktedonobacteria, A4b*
	Bacteroidetes (5.1%)	*Sphingobacteriales*
	Actinobacteria (4.3%)	*Actinomycetales, Acidimicrobiales*
	Firmicutes (3.8%)	*Clostridiales*
	Verrucomicrobia (3.4%)	*Spartobacteriales*
	Gemmatimonadetes (2.7%)	*Gemmatimonadales*
	Archaea (1.8%)	*SD-NA (Crenarchaeota)*
	Elusimicrobia (1.4%)	*Elusimicrobiales, MVP-88*
	Armatimonadetes (1.3%)	*Chthonomonadales, CH21*
	Chlorobi (0.9%)	*SM1B09*
	Others (12.7%)	–

have considerably underestimated the response of soil microbial communities to H_2 exposure.

Impact of H_2 exposure on the co-occurrence of OTUs

Correlation networks were computed to investigate the impact of H_2 exposure on the covariation of OTUs throughout the incubation period and identify OTUs for which the distribution is influenced by H_2. Two separate networks were computed: the first for elevated H_2 exposure (eH_2 network) and a second for low H_2 exposure (aH_2 network). Both networks contained 43 modules and module preservation statistics demonstrated that module composition significantly changed across H_2 treatments (Text S1). The

significances of measured H_2 oxidation rate in explaining network structure were 0.33 and 0.98 in aH_2 and eH_2 networks, again pointing out a significant impact of H_2 on soil microbial community structure. Indeed, module eigengenes of five modules were significantly correlated with high-affinity H_2 oxidation rate time series in eH_2 network, while no module was related to the activity in aH_2 network (Fig. S3). Together, these 5 modules represent 1,140 OTUs, which is more than a third of the whole eH_2 network (3,154 OTUs). OTUs belonging to one module were hindered by eH_2 exposure, while members of the four other modules were favored by eH_2 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_2 oxidation rate, supporting the previous observation that response to H_2 exposure and the distribution of hydrogenase genes is uneven within each taxonomic group. The 38 modules in eH_2 network whose eigengene showed no significant correlation with H_2 oxidation rate are ecologically relevant observations since they represent indirect impacts of H_2 exposure on soil microbial communities and highlight a previously overlooked role of H_2 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_2 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_2 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_2 exposure has brought to soil bacterial communities, it is reasonable to expect a similar effect on other soil types. Considering the steep H_2 concentration gradient surrounding N_2-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_2 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_2 and HOB on soil microbial communities will definitely deserve more attention in soil microbiology. There is a need to further study whether elevated H_2 exposure also exerts noticeable changes in soil ecological functions other than H_2 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_2 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_2 concentration gradients in the environment.

ADDITIONAL INFORMATION AND DECLARATIONS

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.

Data Availability

The following information was supplied regarding data availability:
Raw data an 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_2, CO, CH_4, OCS, N_2O, 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_2 oxidation activity and high affinity H_2-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_2-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_2. *ISME Journal* **2**:1066–1076 DOI 10.1038/ismej.2008.59.

Constant P, Poissant L, Villemur R. 2009. Tropospheric H_2 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 Microbioly* **72**:5069–5072 DOI 10.1128/AEM.03006-05.

Dong Z, Layzell DB. 2001. H_2 oxidation, O_2 uptake and CO_2 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_2: 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_2-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_2 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 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_2. *PLoS ONE* **9**:e103034 DOI 10.1371/journal.pone.0103034.

Häring V, Conrad R. 1994. Demonstration of two different H_2-oxidizing activities in soil using an H_2 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_2-oxidizing bacteria predict atmospheric H_2 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_2 liberated from N_2 fixation by Hup$^-$ 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.

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.

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.

Stein S, Selesi D, Schilling R, Pattis I, Schmid M, Hartmann A. 2005. Microbial activity and bacterial composition of H_2-treated soils with net CO_2 fixation. *Soil Biology & Biochemistry* **37**:1938–1945 DOI 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.

Vignais PM, Billoud B. 2007. Occurrence, classification, and biological function of hydrogenases: an overview. *Chemical Reviews* **107**:4206–4272 DOI 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.

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.

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.

Zhou J, Deng Y, Luo F, He Z, Yang Y. 2011. Phylogenetic molecular ecological network of soil microbial communities in response to elevated CO_2. *mBio* **2**: e00122–00111.