# THE IMPACT OF MICROORGANISMS ON CONSUMPTION OF ATMOSPHERIC TRACE GASES

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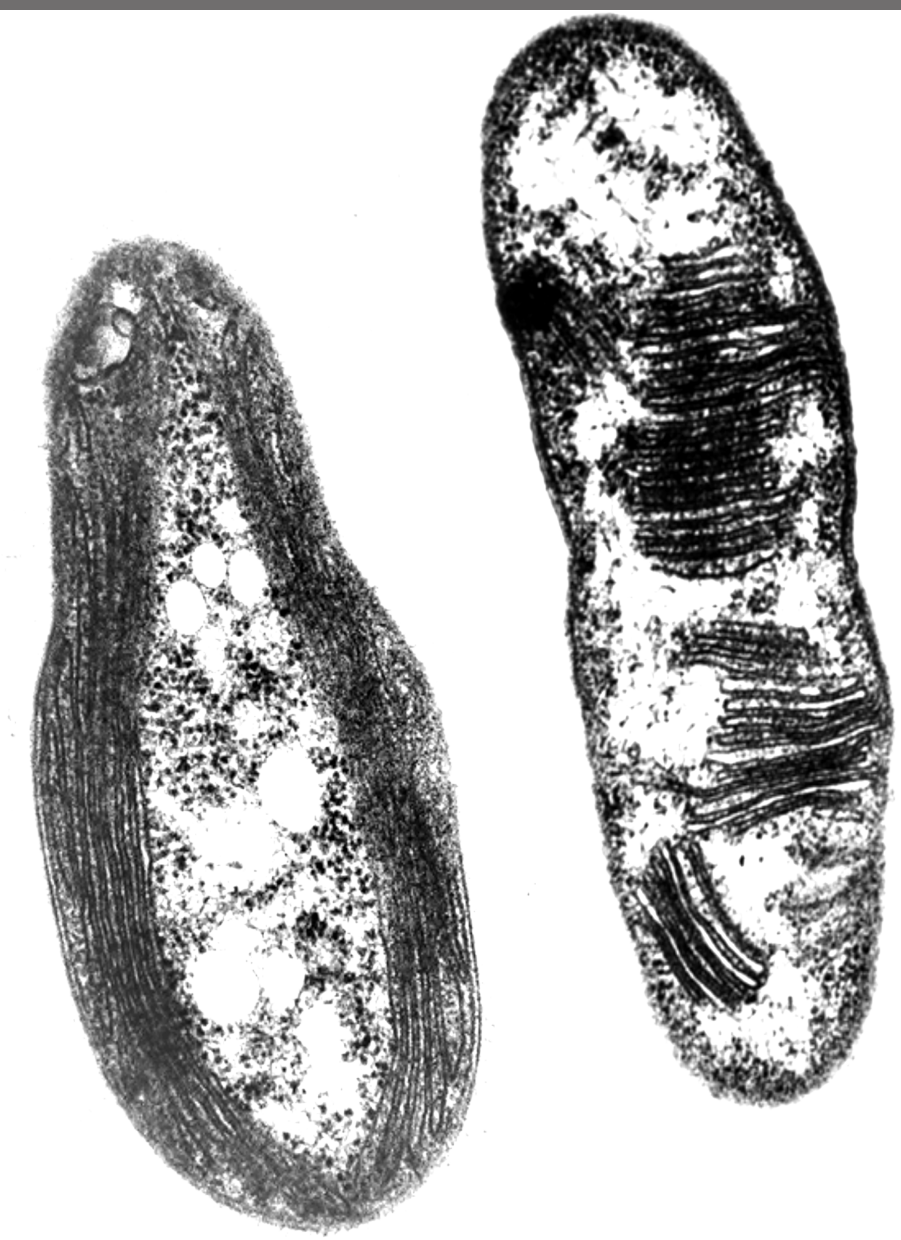

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# THE IMPACT OF MICROORGANISMS ON CONSUMPTION OF ATMOSPHERIC TRACE GASES

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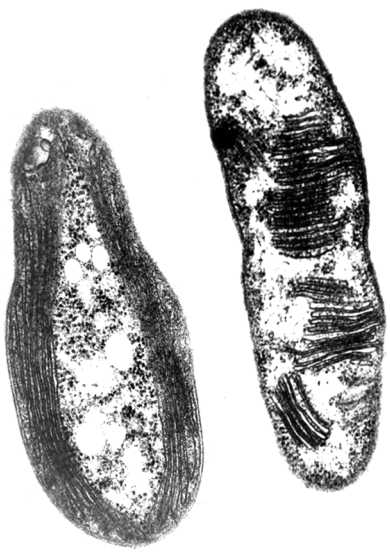

Electron micrographs of thin sections of the methane-consuming bacteria *Methylosinus trichosporium* (left) and *Methylomonas methanica* (right).

Image courtesy of Roger Whittenbury and the Late Howard Dalton.

Gases with a mixing ratio of less than one percent in the lower atmosphere (i.e. the troposphere) are considered as trace gases. Numerous of these trace gases originate from biological processes in marine and terrestrial ecosystems. These gases are of relevance for the climate as they contribute to global warming or to the troposphere's chemical reactive system that builds the ozone layer or they impact on the stability of aerosols, greenhouse, and pollutant gases.

These reactive trace gases include methane, a multitude of volatile organic compounds of biogenic origin (bVOCs) and inorganic gases such as nitrogen oxides or ozone. The regulatory function of microorganisms for trace gas cycling has been intensively studied for the greenhouse gases nitrous oxide and methane, but is less well understood for microorganisms that metabolize molecular hydrogen, carbon monoxide, or bVOCs. The studies compiled in this Research Topic reflect this very well. While a number of articles focus on nitrous oxide and methane or carbon monoxide oxidation, only a few articles address conversion processes of further bVOCs. The Research Topic is complemented by three review articles about the consumption of methane and monoterpenes, as well as the role of the phyllosphere as a particular habitat for trace gas-consuming microorganisms, and point out future research directions in the field.

The presented scientific work illustrates that the field of microbial regulation of trace glas fluxes is still in its infancy when one broadens the view on gases beyond methane and nitrous oxide. However, there is a societal need to better predict global dynamics of trace gases that impact on the functionality and warming of the troposphere. Upcoming modelling approaches will need further information on process rates, features and distribution of the driving microorganisms to fulfill this demanding task.

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EDITORIAL
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# Editorial: The Impact of Microorganisms on Consumption of Atmospheric Trace Gases

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**Keywords: methane, methanotroph, nitrous oxide, denitrification, volatile organic compounds, carbon monoxide, phyllosphere**

## Editorial on the Research Topic

## The Impact of Microorganisms on Consumption of Atmospheric Trace Gases

Gases with a mixing ratio of <1% in the atmosphere are considered as trace gases. Several of these trace gases originate from biological processes in marine and terrestrial ecosystems and are of relevance for the climate as they contribute to global warming, to the troposphere's chemical reactive system that builds the ozone layer, or they impact on the stability of aerosols, greenhouse, and pollutant gases (Conrad, 2009; Arneth et al., 2010; Penuelas and Staudt, 2010). These gases include methane ($CH_4$), a multitude of volatile organic compounds of biogenic origin (bVOCs) and inorganic gases such as nitrogen oxides or ozone (Conrad, 2009; Hewitt et al., 2011; Peñuelas et al., 2014; Fowler et al., 2016). The important role of microorganisms for trace gas cycling has been intensively studied for the greenhouse gases nitrous oxide ($N_2O$) and methane ($CH_4$), but is less well-understood for organisms that metabolize $H_2$, carbon monoxide (CO), or bVOCs. The studies compiled in this *Research Topic* reflect this very well. While a number of articles focus on $N_2O$ and $CH_4$ cycling or CO oxidation, only a few articles address conversion processes of bVOCs (Dixon et al.; Nadalig et al.). The *Research Topic* is complemented by three review articles about the consumption of $CH_4$ and monoterpenes, as well as the role of the phyllosphere as a particular habitat for trace gas-consuming microorganisms, and points out future research directions in the field (Marmulla and Harder; Bringel and Couee; Knief).

Articles in this research topic on $N_2O$ cycling are related to terrestrial environments and denitrification. Soil $N_2O$ emissions result from different biological processes with nitrification and denitrification being the most important ones. Denitrifiers are facultative aerobic microorganisms that reduce nitrate ($NO_3^-$) or nitrite ($NO_2^-$) to the gaseous products nitric oxide (NO), nitrous oxide ($N_2O$), or dinitrogen ($N_2$) under oxygen-limited conditions. Various environmental factors have been identified that affect the composition, abundance, and activity of these microbial groups (reviewed in Braker and Conrad, 2011). The study of Brenzinger et al. evaluates changes on the denitrifier microbiota and gene expression upon pH shifts. Their observation of changes in gene expression of specific taxa demonstrates that functional redundancy of the soil microbiome is important to maintain denitrification upon acidification. Functional redundancy and process partitioning among $NO_3^-$- and $N_2O$-reducing denitrifiers can also explain the findings that *in situ* $N_2O$ fluxes were largely unaffected upon manipulations of the water table in a wetland, although an increase in $N_2O$ reduction activity was observed upon flooding (Palmer et al.). In contrast, land-use change of an agroforest system to an open area converted a cork oak wood from

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a $N_2O$ source to a sink (Shvaleva et al.). This change was not reflected in the abundance of the denitrifier gene *nosZ*. All of these studies demonstrate that the underlying microbial processes that control $N_2O$ fluxes are complex and require a comprehensive analysis of all the different microbial groups that may be involved. Moreover, information about microbiome composition needs to be complemented by data on taxon abundance, activity, and ecophysiology of isolates in order to obtain an understanding of the mechanisms underpinning the observable net ecosystem $N_2O$ fluxes.

To better assess the biotic mechanisms driving $N_2O$ fluxes, *in vitro* analyses of pure cultures are helpful, as exemplified by two studies in this *Research topic*. Bueno et al. revealed that the well-known soil microorganism *Ensifer meliloti* strain 1021 displays a phenotype that enables it to function as a sink for $N_2O$ due to anaerobic $N_2O$ respiration. Remarkably, it appears that this strain is not able to grow *via* $NO_3^-$ respiration, although it can express all genes encoding a complete set of denitrification enzymes. More work is needed to elucidate this peculiarity. Kits et al. studied denitrification activity in the methanotroph *Methylomicrobium album* BG8. Although methanotrophs are considered to be the major biological sink for $CH_4$ in soil, some of them also form $N_2O$ through denitrification under hypoxic conditions. The authors prove in their study that *M. album* BG8 carries out denitrification using nitrite as electron acceptor while oxidizing a range of different one- and even two-carbon compounds, i.e., ethane and ethanol.

The microbial ecology of methanotrophs has been studied for decades, due to the importance of $CH_4$ as greenhouse gas. This has substantially increased knowledge on their ecophysiology and environmental distribution, as summarized in the review by Knief. Methanotrophic communities are often analyzed based on the marker gene *pmoA*, encoding a subunit of the membrane-bound methane monooxygenase. The extensive sequencing of *pmoA* has led to the identification of a number of new taxa. Based on this information, the occurrence of the different taxa in diverse ecosystems was analyzed within a meta-analysis, thus providing new knowledge about habitat preferences of specific methanotroph taxa. Some methanotroph taxa were identified as specialists, occurring only in specific ecosystems, while other genera appear to be generalists (Knief). Rather specific habitat preferences have for example been suggested for a group of uncultured atmospheric $CH_4$-consuming methanotrophs (termed USCα), with upland soils and especially forest soils being the preferred habitat (Kolb, 2009). The conversion of an Amazonian forest into a manioc (cassava) plantation resulted in drastically decreased abundance of this group of methanotrophs concomitant with the $CH_4$ sink activity (Lima et al.). Remarkably, this response was only observed in one of the two soils studied. In an Amazonian Dark Earth (syn. terra preta) soil, USCα was still abundant and $CH_4$ uptake rates remained high 5 years after deforestation and manioc cultivation. The causes for this difference are unclear and need further investigation.

Research on the biological cycling of other one-carbon VOCs such as CO or chloromethane has attracted less attention until now, despite the fact that these gases play a crucial role in atmospheric chemistry and impact on the global climate (Daniel and Solomon, 1998; Harper, 2000). The *Research Topic* includes three studies addressing CO oxidation, which demonstrate nicely the different experimental approaches that can be taken to study a specific functional guild in the environment. Lynch et al. studied trace gas consumers in dry high-elevation mineral soils originating from volcanic deposits in the Atacama Desert via a metagenomic analysis of the soil microbiome, and mined for genes and pathways known to be involved in trace gas conversion. The authors were able to reconstruct the genome of the dominant taxon, *Pseudonocardia* sp., and revealed genetic potential of this organism for hydrogen ($H_2$), CO, and some further one-carbon compounds oxidation. In the study by Quiza et al., aerobic CO oxidizing microorganisms were specifically detected by targeting a functional gene marker, *coxL*, encoding the large subunit of the CO-dehydrogenase. Highest activities, along with the detection of distinct aerobic CO oxidizers, were observed in a deciduous forest soil, compared to an adjacent afforested larch plantation and a maize field. Brady et al. applied stable isotope probing (SIP) in combination with 16S rRNA gene sequencing. SIP is a valuable method to specifically identify the metabolically active microbiome members but requires $^{13}CO_2$ controls to distinguish between CO- and $CO_2$-assimilating microorganisms. Brady et al. identified novel members of *Firmicutes* as autotrophic CO consumers in oxygen-limited geothermal springs using this experimental approach. The fact that heterotrophic carboxydotrophs, which use CO only as an energy source, will be missed by SIP highlights the need for the application of complementary approaches to comprehensively assess the role of CO consumers in the environment.

Chloromethane is a bVOC, and the most abundant halogenated compound in the atmosphere. The only known aerobic pathway for chloromethane oxidation is the *cmu* pathway, which has been well-investigated in aerobic *Alphaproteobacteria* (Nadalig et al.). Based on a genomic survey, Nadalig et al. show that the *cmu* pathway occurs in several other bacterial taxa, including obligate anaerobes. Interestingly, the authors found that the genome of the aerobe *Leisingeria methylhalidivorans*, i.e., a known chloromethane degrader, does not have the *cmu* pathway. Moreover, the authors proved that *L. methylhalidivorans* has diverging isotopic signatures of $^{13}C$ and $^{2}H$ when assimilating chloromethane compared to other alphaproteobacterial *cmu*-containing strains, suggesting the existence of a hitherto unknown pathway. This study demonstrates the limited knowledge we currently have about microbial bVOC metabolism. Another example is given by the review of Marmulla and Harder who provide an overview on the complex biochemistry and pathways of monoterpene degradation in microorganisms and conclude that these pathways need to be studied in more detail in the future. Considering the multitude of monoterpenes present in nature, it is evident that more research is needed. Knowledge about the biochemistry, the genetic makeup of the metabolic pathways, and their distribution among microorganisms is an indispensable prerequisite to perform environmental studies with the aim to improve our understanding of how microbiomes control trace gas fluxes.

The aforementioned studies of the *Research Topic* highlight that our current knowledge about the identity of microorganisms involved in the production or consumption of trace gases and, in particular bVOCs, is limited. For most bVOCs, the impact of microbial consumption on net fluxes between soil or marine ecosystems and the atmosphere is not clear to date. The study of Dixon et al. is one rare example on microbial acetone oxidation in marine waters with seasonal resolution and estimates the quantitative proportion of acetone carbon consumption compared to other bVOCs. Remarkably, the data suggest the existence of an unrecognized production mechanism for acetone during the winter. Besides soils and aquatic habitats, another major habitat for trace gas metabolizing microorganisms is the plant leaf surface, often referred to as the phyllosphere. Considering that plants are major producers of bVOCs (Penuelas and Staudt, 2010), their leaves represent an important interface to the atmosphere and are colonized by microorganisms. Bringel and Couee emphasize in their review the need to study this aspect in more detail. The authors summarize current knowledge about phyllosphere microorganisms metabolizing bVOCs, which is largely limited to one-carbon compounds.

Knowledge about microbial consumption of bVOCs is far from being compared to knowledge on microbial consumption of $CH_4$ or $N_2O$. This includes the quantitative relevance of microbial activities at the ecosystem level and globally, the identities of trace gas degrading microorganisms, the analysis of their degradation pathways, as well as the physiological traits that finally determining the activity of these microbes *in situ*. Thus, the research field of microbial trace gas consumption can be considered to be still in its infancy and necessitates future research to better quantitatively consider microbial trace gas conversions at an ecosystem level, e.g., in upcoming modeling efforts (Graham et al., 2016).

## AUTHOR CONTRIBUTIONS

All authors were involved in the set-up and design of this Research Topic. SK and CK wrote this editorial, MH and JM provided valuable comments.

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# pH-driven shifts in overall and transcriptionally active denitrifiers control gaseous product stoichiometry in growth experiments with extracted bacteria from soil

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Soil pH is a strong regulator for activity as well as for size and composition of denitrifier communities. Low pH not only lowers overall denitrification rates but also influences denitrification kinetics and gaseous product stoichiometry. $N_2O$ reductase is particularly sensitive to low pH which seems to impair its activity post-transcriptionally, leading to higher net $N_2O$ production. Little is known about how complex soil denitrifier communities respond to pH change and whether their ability to maintain denitrification over a wider pH range relies on phenotypic redundancy. In the present study, we followed the abundance and composition of an overall and transcriptionally active denitrifier community extracted from a farmed organic soil in Sweden ($pH_{H2O}$ = 7.1) when exposed to pH 5.4 and drifting back to pH 6.6. The soil was previously shown to retain much of its functioning (low $N_2O/N_2$ ratios) over a wide pH range, suggesting a high functional versatility of the underlying community. We found that denitrifier community composition, abundance and transcription changed throughout incubation concomitant with pH change in the medium, allowing for complete reduction of nitrate to $N_2$ with little accumulation of intermediates. When exposed to pH 5.4, the denitrifier community was able to grow but reduced $N_2O$ to $N_2$ only when near-neutral pH was reestablished by the alkalizing metabolic activity of an acid-tolerant part of the community. The genotypes proliferating under these conditions differed from those dominant in the control experiment run at neutral pH. Denitrifiers of the *nirS*-type appeared to be severely suppressed by low pH and *nirK*-type and *nosZ*-containing denitrifiers showed strongly reduced transcriptional activity and growth, even after restoration of neutral pH. Our study suggests that low pH episodes alter transcriptionally active populations which shape denitrifier communities and determine their gas kinetics.

**Keywords: pH, $N_2O$, denitrification, *nosZ*, *nirK*, *nirS*, transcriptionally active, extracted cells**

## Introduction

Soil $N_2O$ emissions from denitrification depend on environmental conditions that control the rates of denitrification and the $N_2O/N_2$ product ratio. Important soil and chemical factors are oxygen availability (as affected by soil moisture and respiration), temperature, nitrate availability and pH (Wijler and Delwiche, 1954; Nömmik, 1956; Firestone, 1982). Among these factors, soil pH is one of the most crucial ones, because it does not only affect overall denitrification rates, but more importantly seems to directly control the $N_2O/(N_2O + N_2)$ ratio of denitrification, and hence $N_2O$ emission rates from soils (Šimek and Cooper, 2002; Liu et al., 2010; Bakken et al., 2012). Denitrification rates increase with higher pH, whereas $N_2O/(N_2O + N_2)$ ratios decrease (Wijler and Delwiche, 1954; Nömmik, 1956; Dörsch et al., 2012). Direct inhibition of $N_2O$ reduction by low pH was demonstrated in laboratory experiments with *Paracoccus denitrificans* (Bergaust et al., 2010) and with soils from a long-term liming experiment in Norway (Liu et al., 2010) and may explain the negative correlation between soil pH and $N_2O$ emission found in certain field studies (e.g., Weslien et al., 2009; Van den Heuvel et al., 2011).

It is well known that pH also affects the composition and size of denitrifier communities in soil. Acidic soils harbor smaller and less diverse 16S rRNA and denitrification gene pools than neutral soils (Fierer and Jackson, 2006; Čuhel et al., 2010; Braker et al., 2012). Acidity seems to be particularly detrimental to *nirS*-type denitrifiers, resulting in a strong decrease of *nirS*/16S rRNA gene ratios (Čuhel et al., 2010). Whether pH-induced changes in taxonomic denitrifier community composition translate into functional differences is unclear. Several studies have linked potential denitrification rates or kinetics to size and composition of denitrifier communities in soils differing in pH (Cavigelli and Robertson, 2001; Bru et al., 2010; Dandie et al., 2011; Braker et al., 2012), suggesting that pH controls soil denitrification and its product stoichiometry via taxonomic differences. In some cases, the relative abundance of marker genes for $N_2O$-reducers (*nosZ*) vs. $N_2O$-producers (*nirS*, *nirK*, *norB*) explained the $(N_2O)/(N_2O + N_2)$ product ratio (Morales et al., 2010; Philippot et al., 2011; Billings and Tiemann, 2014), but this correlation seems to depend on habitat and environmental conditions (Morales et al., 2010; Philippot et al., 2011; Deslippe et al., 2014). In a recent study, Jones et al. (2014) proposed that soil pH controls the abundance of nitrite reductase genes as well as the abundance of the newly discovered *nosZ* Type II clade in soils with relevance to the soil's ability to reduce $N_2O$.

The direct effect of low pH on the transcription of denitrification genes has been studied in pure culture (Bergaust et al., 2010), soils (Liu et al., 2010) and cells extracted from soil (Liu et al., 2014). In general, low pH resulted in low numbers of transcripts encoding nitrite reductases (*nirS* and *nirK*) and $N_2O$ reductase (*nosZ*) (Bergaust et al., 2010; Liu et al., 2010), but the *nosZ*/*nirK* transcript ratio did not change. Interestingly, transcription of *nirS* seemed to be more suppressed by acidity than of *nirK* (Liu et al., 2010), but it is unclear how this affects $N_2O$ emissions. The underlying molecular mechanisms for direct pH control on $N_2O$ emissions are not fully resolved, but post-transcriptional impairment of nitrous oxide reductase ($N_2OR$) by pH $< 6.1$ has been suggested (Liu et al., 2014).

Together, this raises three basic questions: (i) is the ability of a soil denitrifier community to reduce $N_2O$ to $N_2$ entirely controlled by pH-impairment of $N_2OR$? (ii) do communities harbor organisms which can thrive over a wider pH range without losing $N_2O$ reductase activity? or (iii) are communities functionally redundant in that they contain distinct members with similar phenotypes adapted to different pH? In the present study, we approached these questions in a model community obtained by extracting microbial cells from a soil with neutral pH. The extracted cells were incubated in pH adjusted batch experiments and we followed the dynamics of denitrifying communities through the analysis of functional genes *nirK*, *nirS*, and *nosZ* and their gene expression while monitoring gas kinetics at high resolution. The community was extracted from a farmed organic soil in Sweden (SWE, native pH 7.1) which had been previously found to retain much of its functioning (low $N_2O/N_2$ ratios) in pH manipulation experiments (pH 5.4/7.1) (Dörsch et al., 2012). This finding was attributed to a species-rich denitrifier community, and hence to high functional diversity (Braker et al., 2012). Here, we revisited the pH manipulation experiment of Dörsch et al. (2012) and followed functional gene abundance and diversity of the overall denitrifier community (ODC) and the transcriptionally active denitrifier community (TADC) throughout anoxic growth, covering a transient pH range from 5.4 to 7.1. We hypothesized that the inherent alkalization ensuing anoxic growth of denitrifiers induces a succession of taxonomically distinct but, in terms of pH adaptation, functionally redundant denitrifier populations, thus supporting complete denitrification to $N_2$ over a wide pH range. Since gene expression does not necessarily result in functional enzymes at low pH (e.g., Bergaust et al., 2010), we compared shifts in transcripts to those in DNA over time, hypothesizing that only taxa expressing functional enzymes would propagate in the growing culture. In this way we assessed whether sustained function (here: complete denitrification to $N_2$) would be linked to structural changes in the underlying community.

## Materials and Methods

### Soil Sample

The soil was originally sampled from a Terric Histosol (FAO) in Sweden and has been used in several studies exploring functional characteristics of denitrification (Holtan-Hartwig et al., 2000, 2002; Dörsch and Bakken, 2004; Klemedtsson et al., 2009; Dörsch et al., 2012) and underlying denitrifier communities (Braker et al., 2012). The neutral pH of the organic soil is due to inclusion of lacustrine limestone from a former lake bottom. Detailed soil characteristics are given in Dörsch et al. (2012). By the time of the present study, the soil had been stored moist at 4°C for 15 years.

### Cell Extraction and Incubation Conditions

Cell extraction was performed as described previously (Dörsch et al., 2012) with the following modification: Instead of two portions of 50 g soils, four portions were used to recover a higher

total cell number. Pellets with extracted cells were resuspended in a total volume of 75 mL filter-sterilized bi-distilled water and stirred aerobically for 0.5–1 h to inactivate any existing denitrification enzyme prior to inoculation into a He-washed hypoxic mineral medium (0.7 μM $O_2$; see below).

The mineral media contained ($L^{-1}$): 200 mg $KH_2PO_4$, 20 mg $CaCl_2$, 40 mg $MgSO_4$, 3.8 mg Fe-NaEDTA, 0.056 mg LiCl, 0.111 mg $CuSO_4$, 0.056 mg $SnCl_2$, 0.778 mg $MnCl_2$, 0.111 mg $NiSO_4$, 0.111 mg $Co(NO_3)_2$, 0.111 mg $TiO_2$, 0.056 mg KI, 0.056 mg KBr, 0.1 mg $NaMoO_4$. The medium was buffered with 25 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethane-sulfonic acid) and was supplemented with 3 mM of the electron acceptor $KNO_3$ and 3 mM Na-glutamate as carbon and nitrogen source. The medium had an initial pH of 5.1. Two aliquots of sterile autoclaved medium were adjusted to pH 5.4 and pH 7.1, respectively, by adding 1 N NaOH to the medium. Two sets (15 each) of 120 mL-flasks were filled with 43 ml of medium of either pH 5.4 or pH 7.1, resulting in 30 sample flasks in total. Additional flasks were used as blanks without adding cells extracted from the soil. The serum flasks were crimp sealed with butyl septa and made near-anoxic (∼0.7 μM $O_2$) by six cycles of evacuation and He-filling using an automated manifold while stirring the suspension with magnetic stirrers at 500 rpm (Molstad et al., 2007).

## Incubation, Gas Analyses, and Sampling

Denitrification activity was measured directly after inoculation with the cells by denitrification product accumulation. Thirty serum flasks, three blanks, three calibration standards, and two flasks for $NO_2^-$ measurements were placed on a submersible magnetic stirring board (Variomag HP 15; H + P Labortechnik GmbH, Oberschleissheim, Germany) in a 15°C water bath. The water bath is an integrated part of an automated incubation system for the quantification of $O_2$ consumption and $CO_2$, NO, $N_2O$ and $N_2$ production in denitrifying cultures similar to that described by Molstad et al. (2007). After temperature equilibration, excess He was released by piercing the bottles with a syringe without plunger filled with 2 ml bi-destilled water to avoid entry of air. The bottles were inoculated with 2 mL of cell suspension, yielding approximate cell numbers of $2 \times 10^9$ cells per flask ($4 \times 10^7$ $mL^{-1}$). The headspace concentrations of $O_2$, $CO_2$, NO, $N_2O$, and $N_2$ were monitored every 5 h as described by Molstad et al. (2007) and Dörsch et al. (2012).

The incubation experiments were terminated after 210 h when $NO_3^-$-N added to flasks was recovered as $N_2$-N. After 0, 12, 26, 48, 70, 96, and 206 h, two to three sample flasks of each pH treatment were sacrificed. Cell densities were determined by spectrophotometry ($OD_{600}$) and $NO_2^-$ concentrations were measured by a spectrometer according to the international standard ISO 6777-1984 (E). The remaining suspension was centrifuged at 4°C and 8.400 × g and the cell pellet was immediately frozen in liquid nitrogen and stored at −80°C until further use. At each time point the pH in the supernatant was determined.

## Extraction of Nucleic Acids

DNA and RNA were extracted from the frozen cell pellets (−80°C) collected at each sampling point. For this, one or two frozen cell pellets were resuspended in 400 μL sterile water (Sigma-Aldrich, Taufkirchen, Germany). Nucleic acids were extracted using a modified SDS-based protocol (Bürgmann et al., 2003; Pratscher et al., 2011). In brief, the cells were disrupted in a FastPrep beat-beating system and nucleic acids were recovered from the supernatant using a phenol/chloroform/isoamyl alcohol extraction. Subsequently the nucleic acids were precipitated with polyethylene glycol (PEG) 6000 solution and redissolved in 100 μL of sterile (0.1 μm filtered) nuclease-free (DNase-, RNase-free) and protease-free bi-distilled water (Sigma-Aldrich). An aliquot of 20 μL was stored at −20°C for further DNA-based molecular analyses. The remaining 80 μL were treated with RNase-free DNase (Qiagen, Hilden, Germany) for removal of DNA. RNA was purified using the RNeasy Mini Kit (Qiagen), precipitated with 96% EtOH and resuspended in 15 μL nuclease-free water (Sigma-Aldrich) to increase the RNA concentration and stored at −80°C. The integrity of the RNA was checked on a 1.5% w/v agarose gel (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) and the concentration was determined by a NanoDrop1000 instrument (Thermo Fisher Scientific, Dreieich, Germany). The RNA was reverse transcribed with random hexamer primers (Roche, Mannheim, Germany) and M-MLV reverse transcriptase (Promega, Mannheim, Germany).

## Analysis of the Composition of *nirK*, *nirS*, and *nosZ* Genes and Transcripts

The composition of the denitrifier community was determined by terminal restriction fragment length polymorphism (T-RFLP). The nitrite reductase genes *nirK* and *nirS* as well as the nitrous oxide reductase gene *nosZ* were amplified from cDNA and DNA using the primer pairs nirK1F-nirK5R (∼516 bp), nirS1F-nirS6R (∼890 bp), and Nos661F-Nos1773R (∼1131 bp) and conditions described previously (Braker et al., 1998, 2000; Scala and Kerkhof, 1998). Details on primers and procedures are given in Table S1. These primers were chosen to allow for comparison of the results obtained in this study to previous ones (Braker et al., 2012), although different primers to target these genes have been published more recently (e.g., Green et al., 2010; Verbaendert et al., 2014). The forward *nirS* and *nosZ* primer and the reverse *nirK* primer were 5′-6-carboxyfluorescein labeled. The quantity and quality of the PCR product were analyzed by electrophoresis on a 1.5% w/v agarose gel after staining the gel with 3 × GelRed Nucleic Acid Stain (Biotium, Hayward, CA, USA). PCR products of the expected size were recovered from the gel using the DNA Wizard® SV Gel-and-PCR-Clean-up system (Promega). The PCR products of *nirK*, *nirS* and *nosZ* were digested using the restriction enzymes FastDigest *HaeIII*, FastDigest *MspI*, and FastDigest *HinP1I* (Thermo Fisher Scientific), respectively, following the manufacturer's specifications. The purified fluorescently labeled restriction fragments were separated on an ABI PRISM 3100 Genetic Analyzer sequencer (Applera Deutschland GmbH, Darmstadt, Germany) and the lengths of fluorescently labeled terminal restriction fragments (T-RFs) were determined by comparison with the internal standard using GeneMapper software (Applied Biosystems). Peaks with fluorescence of >1% of the total fluorescence of a sample and >30 bp length

were analyzed by aligning fragments to the internal DNA fragment length standard (X-Rhodamine MapMarker® 30–1000 bp; BioVentures, Murfreesboro, TN). Reproducibility of patterns was confirmed for repeated T-RFLP analysis using the same DNA extracts. A difference of less than two base pairs in estimated length between different profiles was the basis for considering fragments identical. Peak heights from different samples were normalized to identical total fluorescence units by an iterative normalization procedure (Dunbar et al., 2001).

### Quantitative Analysis of *nirK*, *nirS*, and *nosZ* Genes and Transcripts

The abundance of *nirK*, *nirS*, and *nosZ* genes and transcripts in the sample flasks was determined by qPCR using primers qnirK876-qnirK1040, qCd3af-qR3cd, and nosZ2F-nosZ2R (Henry et al., 2004, 2006; Kandeler et al., 2006). Details on primers and procedures are given in Table S1. The reaction mixture contained 12.5 μL SyberGreen Jump-Start ReadyMix, 0.5 μM of each primer, 3–4.0 mM MgCl2, 1.0 μL template cDNA or DNA and 200 ng BSA $mL^{-1}$ was added. All qPCR assays were performed in an iCycler (Applied Biosystem, Carlsbad CA, USA). Standard curves were obtained using serial 10-fold dilutions of a known amount of plasmid DNA containing the respective fragment of the *nirK-, nirS-, and nosZ*-gene. Negative controls were always run with water instead of cDNA or DNA. PCR efficiencies for all assays were between 80 and 97% with $r^2$-values between 0.971 and 0.995.

### Statistical Analyses

All statistical analyses and graphics were done using R version 3.0.1 (R Development Core Team, 2013). Significant differences of *nirK*, *nirS*, *nosZ*, bacterial 16S rRNA gene and transcript abundance as well as the calculated ratios were assessed using ANOVA ($P < 0.05$). All quantitative data were log-transformed prior to analysis to satisfy the assumptions of homoscedasticity and normally distributed residuals. The community composition changes in the overall and transcriptionally active denitrifier community by T-RFLP were analyzed using non-metric multidimensional scaling (NMDS) and overall differences were tested by ANOSIM ($P < 0.05$). Additionally, differences in the composition of transcriptionally active and overall denitrifier communities (ODC) at a given time point were tested by ANOSIM ($P < 0.05$). An ANOSIM R value near +1 means that there is dissimilarity between the groups, while an *R*-value near 0 indicates no significant dissimilarity between the groups (Clarke, 1993). NMDS analyses were performed with the Bray-Curtis similarity index (including presence and relative abundance of T-RF) which iteratively tries to plot the rank order of similarity of communities in a way that community point distances are exactly expressed on a two-dimensional sheet. The reliability of the test was calculated by a stress-value. Stress >0.05 provides an excellent representation in reduced dimensions, >0.1 very good, >0.2 good, and stress >0.3 provides a poor representation. All community composition data were Hellinger-transformed before analysis, in order to reach normal distribution. ANOSIM, ANOVA, and NMDS were done using package vegan version 2.0-5 (Oksanen et al., 2012).

## Results and Discussion

### Denitrification Kinetics and Shifts in Abundance and Composition of TADC and ODC at Native pH 7.1

At native pH 7.1, residual $O_2$ after He-washing was depleted and all nitrate was stoichiometrically converted to $N_2$ within 96 h of incubation (**Figures 1A,B**). Net accumulation of gaseous denitrification intermediates was low (<0.2% of initially present $NO_3^-$-N). Transcriptional activation of functional genes (**Figure 2A**) and proliferation of denitrifiers containing *nirK* and *nosZ* (**Figures 3A,C**) started instantly after the cells were transferred to the hypoxic medium. A maximum of relative transcription and community size was reached after 96 h (**Figures 3A,C**), ∼40 h after the start of exponential product accumulation ($CO_2$, $N_2$) (**Figures 1A,B**). The maximum relative transcriptional activity (cDNA/DNA ratio) was low with 0.077 for *nirK* (**Figure 3A**) and 0.002 *nosZ* (**Figure 3C**), but efficiently translated into denitrifier growth (**Figures 3A,C**). The strongest growth occurred for *nosZ*-containing denitrifiers (16,500-fold) while denitrifiers of the *nirK*-type grew 400-fold (Table S2). In contrast, growth of *nirS*-type denitrifiers showed a lag-phase of 49 h (**Figure 2A**, Table S2) after which they were transcriptionally activated (cDNA/DNA ratio of 0.11, Table S3) and increased in abundance, albeit only 50-fold (**Figure 3B**). Ratios (*nosZ*/[*nirK* + *nirS*]) of >50 after 96 h indicated a tendency of enhanced growth of *nosZ*-type denitrifiers compared to nitrite reducers (**Figure 4**, Table S4) which may explain the efficient conversion of $N_2O$ to $N_2$ (Philippot et al., 2011). However, PCR-based analyses of genes and transcripts may be biased. The primers used do for instance neither target *nirK* genotypes from *Rhodanobacter* species (Green et al., 2010) nor thermophilic Gram-positive denitrifiers (Verbaendert et al., 2014). The recently postulated *nosZ* clade II (Sanford et al., 2012; Jones et al., 2013) was also not analyzed in this study. Hence, *nosZ*/(*nirK* + *nirS*) ratios and their response to pH must be taken with caution.

Community composition data indicated selective transcriptional activity, followed by growth of only a few organisms (Figures S1A, S2A, S3A). Terminal restriction fragments (T-RFs) of 229 bp (representing *nirK* most closely related to *nirK* of *Alcaligenes xylosoxidans*) and of 37 bp length (38 bp *in silico* representing *nosZ* most closely related to *nosZ* of *Pseudomonas denitrificans*, *Ps. stutzeri,* and *Ps. aeruginosa*), (Table S5) which were of little abundance in or absent from the inocula, respectively, dominated the transcriptionally active *nirK*- and *nosZ*-containing denitrifier communities (Figures S1A, S3A). For *nirS*, a genotype most closely related to *nirS* of *Ps. migulae* (105-bp T-RF) was transcriptionally activated and proliferated that was not even detectable in the initial community (Figure S2A). Still, the composition of the transcriptionally active (TADC) and the overall denitrifier community (ODC) converged throughout the first 96 h of incubation as indicated by multi-dimensional scaling of T-RFs

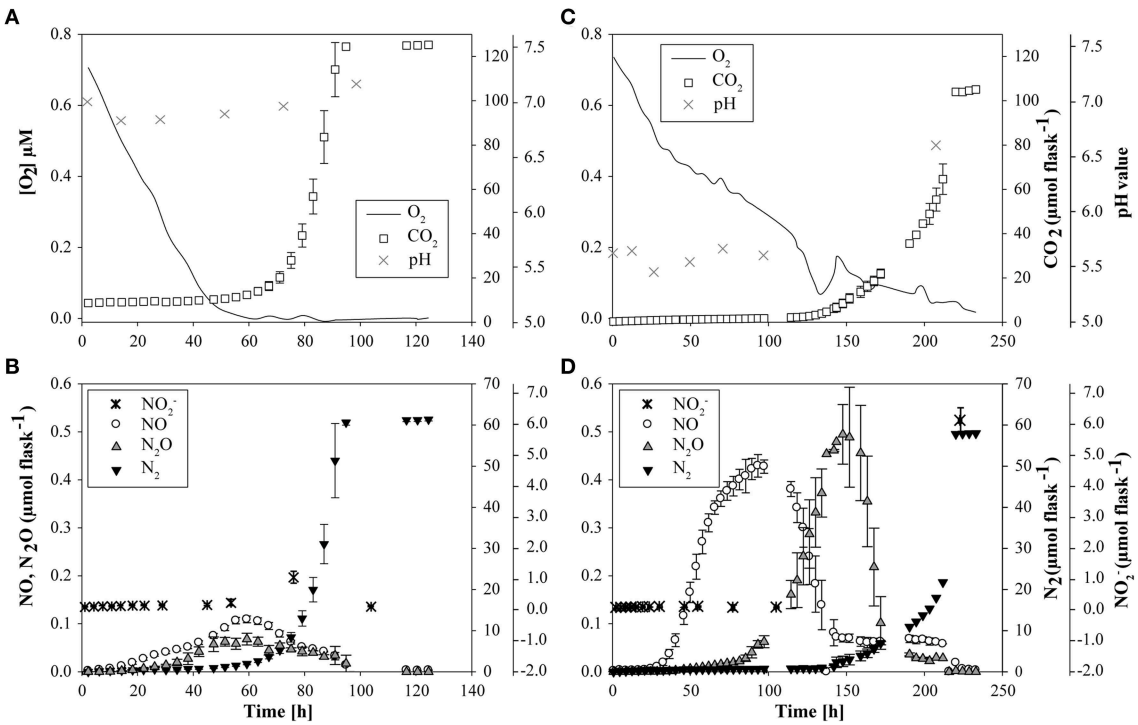


**FIGURE 1 | Accumulation of $O_2$, $CO_2$, NO, $N_2O$, $N_2$, $NO_2^-$ and changes in pH value in suspensions of cells extracted from a soil from Sweden at initially pH 7.1 (left panels) and at initially pH 5.4 (right panels) during incubation (0–206 h). (A)** $O_2$, $CO_2$ concentration and pH value at pH 7.1; **(B)** $NO_2^-$, NO, $N_2O$ and $N_2$ concentration at pH 7.1; **(C)** $O_2$, $CO_2$ concentration and pH value at pH 5.4; **(D)** $NO_2^-$, NO, $N_2O$ and $N_2$ concentration at pH 5.4.

(**Figures 5A–C**; $ANOSIM_{26-49h}$: $P < 0.05$; R between 0.423 and 0.873; $ANOSIM_{70-96h}$: $P > 0.05$; R between 0.142 and 0.275). The shifts in denitrifier community composition and the decrease in denitrifier diversity (Shannon index, Figures S1A–S3A) did not result in impairment of function, i.e., gaseous intermediates were efficiently taken up and reduced to $N_2$ (**Figures 1A,B**). This suggests that it was not the microbial diversity *per se* that mediated the community's functioning, but the specific metabolic capacities of the dominating denitrifying taxa. Transcription of denitrification genes decreased after all nitrogen oxides were depleted (**Figure 2A**) and the number of transcripts relative to gene copies became very low (**Figures 3A–C**). Hence, the increase in diversity and shift in cDNA composition observed for *nirK* and *nosZ*-containing denitrifiers at 206 h was presumably the result of transcript degradation following starvation (Figures S1A, S3A).

## Denitrification Kinetics and Shifts in Abundance and Composition of TADC and ODC When Exposed to Low pH

### Response of Denitrification to Incubation at Acid pH

Exposing the extracted cells to pH 5.4 showed that most of the functionality in denitrification (low accumulation of denitrification intermediates) was retained (**Figure 1D**). This was reported earlier for the denitrifying community of this soil (Dörsch et al., 2012). However, denitrification kinetics were clearly influenced by the initially low pH. Respiration activity (measured as $CO_2$ accumulation) at pH 5.4 was lower as compared to pH 7.1 (**Figure 1C**) and NO and $N_2O$ accumulation started approximately 15 h later (**Figure 1D**). Net production of NO and $N_2O$ was four- and nine-fold higher, respectively, than at neutral pH and due to slower denitrification kinetics, the reduction of intermediates occurred sequentially. This is in line with previous studies, finding clear pH effects on the accumulation of intermediates in denitrification (Bergaust et al., 2010; Liu et al., 2010, 2014). For instance, transient accumulation of $N_2O$ by *P. denitrificans* growing at pH 6.0 was 1500-fold higher than at neutral pH (Bergaust et al., 2010). Liu et al. (2010) found that the production of $N_2$ declined to zero with decreasing pH when comparing soils from a long-term liming experiment with *in situ* pH ranging from pH 4.0 to 8.0. Cells extracted from one of the neutral soils and incubated at pH levels between 7.6 and 5.7 for up to 120 h showed a peculiar pH threshold of 6.1, below which no functional $N_2O$-reductase was produced (Liu et al., 2014). In our study, nitrate was stoichiometrically converted to $N_2$ with less than 1% net $N_2O$-N accumulation when incubated at initially pH 5.4 (**Figure 1D**). However, complete N conversion coincided with a pH shift in the medium (from 5.4 to 6.6) which occurred between 150 and 206 h of incubation (**Figures 1C,D**). This shift was most likely driven by the strongly increasing denitrification activity during this period. Denitrification is an alkalizing reductive process, consuming 6 moles $H^+$ per mol $NO_3^-$ reduced to $N_2$. $CO_2$ production was clearly coupled to total N-gas production and came to a halt when all N-oxides

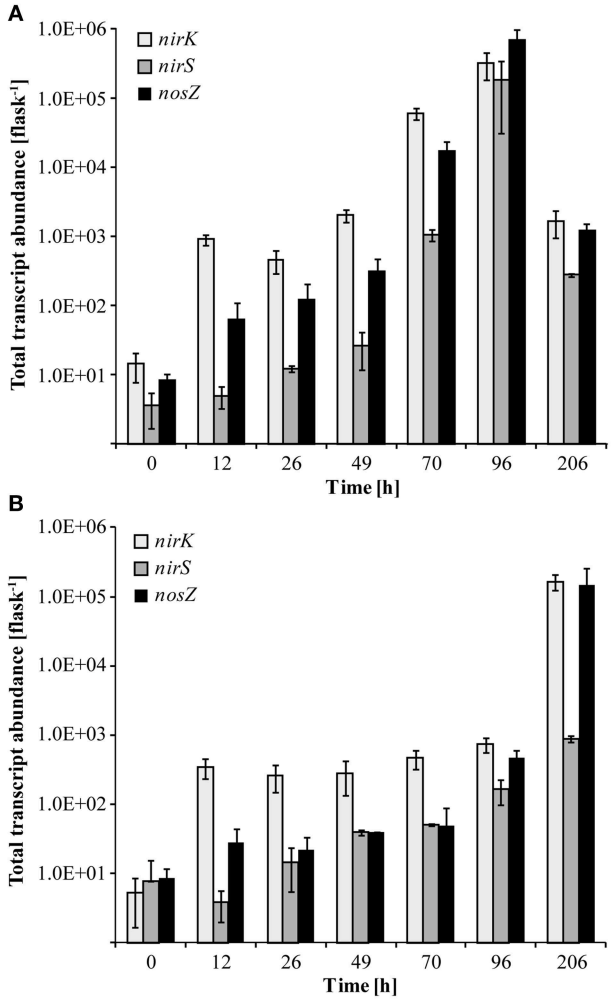


**FIGURE 2 | Transcript abundance of functional marker genes for denitrification (*nirK*, *nirS*, and *nosZ*) quantified by qPCR. (A)** Transcript copy numbers of the incubation at pH 7.1; **(B)** Transcript copy numbers of the incubation at pH 5.4 (Mean ± SD, $n = 3$).

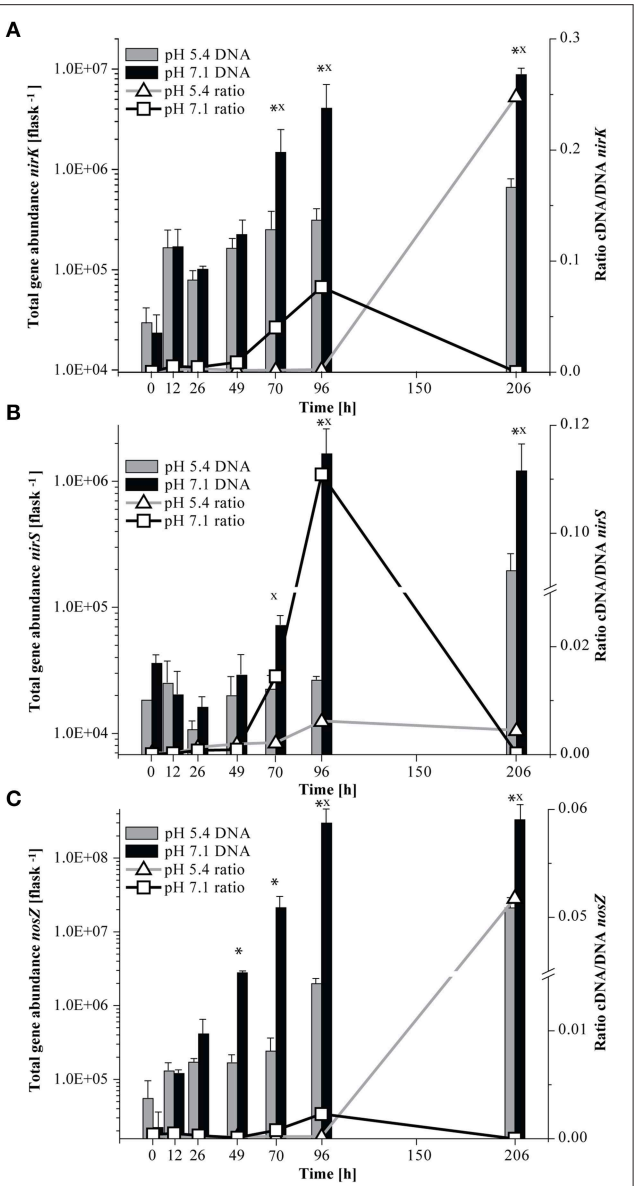


**FIGURE 3 | Abundance of functional marker genes for denitrification (*nirK*, *nirS*, and *nosZ*) quantified by qPCR and ratio of cDNA/DNA copy numbers.** Left axis, total gene abundance and right axis, ratio of cDNA/DNA copy numbers. Bars indicate the total gene copy numbers and the line the cDNA/DNA ratio. An asterisk indicates significant differences in gene abundance, x indicates significant differences in the ratio of cDNA/DNA copy numbers between incubation at pH 5.4 and pH 7.1 at a given time point (ANOVA: $P < 0.05$). **(A)** *nirK*; **(B)** *nirS*; **(C)** *nosZ* (Mean±SD, $n = 3$).

were reduced to $N_2$ (**Figure 1C**). This suggests that respiratory processes other than denitrification were absent and that the pH-threshold for $N_2O$ reduction in the medium was overcome by growing denitrifiers which consumed [$H^+$] (**Figure 1C**). This suggestion is further supported by the dominance (>90%) of phylotypes closely related to known denitrifiers at the end of the incubation (Table S6). These findings, together with the transient accumulation of NO at pH 5.4, led us to the conclusion that acid tolerant denitrifiers present in the native community must have been metabolically active at pH 5.4, illustrating the high functional versatility of this community with respect to pH.

## Response of *nirK* and *nosZ*-containing Denitrifier Communities to Incubation at Low pH

We studied how the denitrifier community responded to incubation at initially low pH in terms of growth and transcriptional activation of the denitrification genes *nirK*, *nirS*, and *nosZ*. Unfortunately, although functional data were collected for the period when the pH shift occurred, due to limitations in the number of samples that could be processed, no community data are available for the period of rapid pH shift. In general, incubation at low pH retarded the transcriptional activation of the functional marker genes (compare **Figure 2A** and **Figure 2B**, Table S2). As long as the pH remained stable at about 5.4 (until 96 h), copy numbers of *nirK* and *nosZ* cDNA increased in a range similar to the initial phase of the incubation at pH 7.1 (until 49 h). Moreover, transcriptional activation of *nirK* and

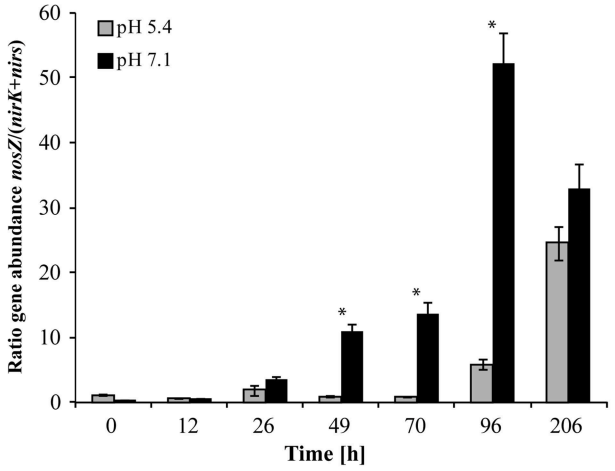


**FIGURE 4 | Ratio of *nosZ*/(*nirK* + *nirS*) gene copy numbers.** An asterisk indicates significant differences in ratios between incubation at pH 5.4 and pH 7.1 at a given time point (ANOVA: $P < 0.05$). (Mean ± SD, $n = 3$).

*nosZ* at pH 5.4 translated into growth of the communities albeit to a lesser extent than at neutral pH (**Figures 3A,C**). During the pH shift to 6.6 (96–206 h), presumably concomitant with the exponential accumulation of the $N_2$, transcript abundances increased reaching their highest densities at the end of the incubation (**Figure 2B**). However, the increase in denitrifier density was only 11-fold at most and hence less than at pH 7.1 (Table S2). Hence, although the relative transcriptional activity (ratio of cDNA/DNA copies) of *nirK* and *nosZ* exceeded levels at pH 7.1, transcription seemed not to translate into growth as efficiently.

## Development of Transcriptionally Active and Overall *nirK*-type Denitrifier Communities When Exposed to Low pH

Contrary to the incubation at pH 7.1, the composition of the growing ODC in the initially acid incubation changed only marginally and thus differed significantly between the two pH treatments at the end of the experiment. While the development of the ODC at the native pH of the soil (7.1) reflected the composition of the TADC within the first 96 h (see above), this was not the case with initially acidic pH (**Figure 5A**, Figure S1B). Here, TADC patterns clustered separate (ANOSIM: $P < 0.05$; R between 0.742 and 0.841) from those of the ODC throughout the experiment due to the continuous predominance of the terminal restriction fragment (T-RF) of 229 bp length in the TADC which was of constantly low relative abundance in the ODC (Figure S1B). Thus, we conclude that transcriptional activation of the respective genotypes did not translate into denitrification activity and specific growth of these denitrifiers, suggesting regulation at the post-translational level. Such effects were previously suggested for *nosZ* gene expression in *P. denitrificans* by Bergaust et al. (2010) and confirmed by Liu et al. (2010, 2014) for soils and extracted cells. Bergaust et al. (2010) hypothesized that low pH (6.0) impairs the assembly of $N_2O$-reductase in *P. denitrificans*, leading to a dysfunctional enzyme and hence accumulation of $N_2O$.

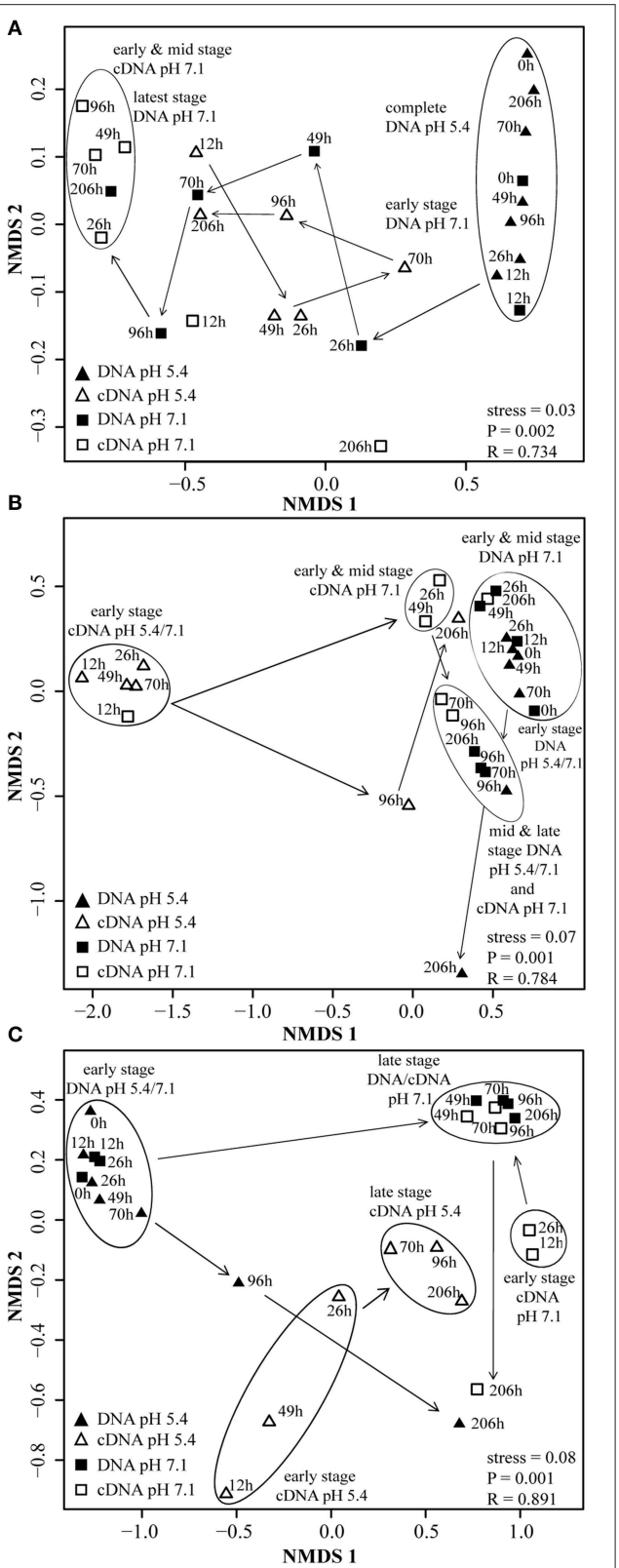


**FIGURE 5 | NMDS plots of denitrifier communities based on cDNA- and DNA-derived T-RFLP analysis of *nirK*, *nirS*, and *nosZ* from three pooled samples.** Data points represent averaged results of three replicate

*(Continued)*

**FIGURE 5 | Continued**
T-RFLP analyses. Community similarity was calculated by using the statistical program R and the Bray–Curtis similarity measurement, which includes presence and relative abundance of T-RF. Clusters and arrows were inserted manually to highlight clustering and community development. Significant differences in the composition of denitrifier communities at given time points were determined by ANOSIM ($P < 0.05$). **(A)** *nirK*; **(B)** *nirS*; **(C)** *nosZ*.

### Development of the Transcriptionally Active and Overall *nosZ*-containing Denitrifier Communities When Exposed to Low pH

Incubation at initially pH 5.4 altered the *nosZ*-TADC as well as the *nosZ*-ODC but they remained significantly different (**Figure 5C**; ANOSIM: $P < 0.05$; R between 0.712 and 0.831). During the first phase of the incubation (up to 70 h) at low pH, growth was small. However, $N_2O$-reducers present at very low abundance in the native community seemed to be functional. T-RFLP analysis revealed that after a lag phase of 26 and 70 h, T-RFs of 37 and 40 bp, respectively, that were present at undetectable levels in the ODC, became transcriptionally activated and increased in relative abundance (Figure S3B). After 96 h of incubation, the initial community started to be outcompeted by transcriptionally active *nosZ*-containing organisms. While $N_2O$-reducers (40 bp T-RF) were transcriptionally active in the low pH incubation only and started proliferating in the ODC toward the end of the incubation, the T-RF of 37 bp was detected at both pH levels and even dominated the community at neutral pH. Existence of acid-tolerant denitrifiers containing *nosZ* was previously demonstrated for a nutrient poor acidic fen by Palmer et al. (2010) and a riparian ecosystem (Van den Heuvel et al., 2011). Similar to pH 7.1, we observed a tendency of enhanced growth of *nosZ*-containing denitrifiers compared to nitrite reducers as reflected by a *nosZ*/(*nirK* + *nirS*) ratio >25 after 206 h (**Figure 4**, Table S4) when $N_2O$ was effectively reduced.

### Transcriptional Activity and Development of Transcriptionally Active and Overall *nirS*-type Denitrifier Communities When Exposed to Low pH

Transcription of *nirS* was not significantly inhibited by low pH and cDNA copy numbers increased slowly until 96 h (**Figure 2B**). The response in transcription of the community to incubation resembled that during the first 49 h at neutral pH (**Figure 2A**). When the pH started to shift back to near neutral (pH 6.6) and vigorous proliferation occurred (as judged from N gas kinetics), transcription of *nirS* was further enhanced but the high absolute and relative transcription levels observed for *nirK* and *nosZ* were never reached (**Figures 2B**, **3B**). This contrasts a recently published study with cells extracted from soil (Liu et al., 2014). Liu et al. (2014) observed constantly lower *nirK* and slightly increasing *nirS* and *nosZ* transcript numbers during incubation at pH 5.7 and 6.1, as compared to pH 7.6 where transcripts of all three denitrification genes increased equally. However, in that study, starting conditions were different; the community had a native pH of 6.1 and was preincubated under oxic conditions for several hours. Our findings also contrast other results of Liu et al. (2014), who found stable, pH-independent cDNA/DNA ratios for *nirS* and *nosZ*, whereas for *nirK* the ratio declined due to efficient growth of the *nirK*-type denitrifier community but constant level of transcription at higher pH. We observed persistently reduced relative *nirS* transcription at low pH compared to pH 7.1 and the growth of *nirS*-type denitrifiers was severely inhibited by low pH during the first 96 h of incubation (**Figure 3**). A previous pure culture study found that already at slightly acidic pH of 6.8, the *nirS*-type denitrifier *P. denitrificans* was unable to build up a functional denitrification pathway (Baumann et al., 1997). Although the nitrite reductase gene was properly induced, the enzyme could not be detected at sufficient amounts in the culture indicating that either translation was inhibited, or once synthesized, nitrite reductase was inactivated, possibly by high concentrations of nitrous acid ($HNO_2$). In our study, incubation at low pH did not increase $NO_2^-$ until 96 h (**Figure 1D**), and accumulation of NO was moderate within the nano-molar range (1 μmol NO in the bottle ∼ 730 nM in liquid). Moreover, Baumann et al. (1997) demonstrated that a functional nitrite reductase assembled at pH 7.5 was still active if the culture was shifted to acidic pH. The cells exhibited a reduced overall denitrification activity, but neither nitrite nor any other denitrification intermediate accumulated which is in agreement with our findings (**Figure 1D**). Despite the low levels of transcription, the *nirS* TADC shifted but only after 96 h of incubation and surprisingly, the ODC changed at the same time, although DNA copy numbers did not increase which cannot be explained. Only with the pH upshift between 96 and 206 h, a slight growth (one order of magnitude) occurred but the community developed distinctly from the TADC (**Figure 5B**; ANOSIM: $P < 0.05$; R between 0.671 and 0.912). Since the initial abundance of *nirK*- and *nirS*-type denitrifiers in the soil and hence in the inocula was equal, our results indicate a greater robustness of *nirK*-type vs. *nirS*-type denitrifier communities to acidity.

## Concluding Discussion

In this study of a model community, we linked transcriptional activation of denitrification genes (*nirK*, *nirS*, and *nosZ*) and growth of the communities to conversion of nitrogen oxides to $N_2$. We found a pronounced succession of TADC and ODC in batch incubations even at neutral pH, suggesting a strong selective pressure on the extracted community. Exposure to low pH (5.4) resulted in (i) sequential and slightly enhanced transient accumulation of denitrification intermediates (NO, $N_2O$), (ii) lower and/or retarded transcriptional activation of denitrification genes, together with selective activation of genotypes represented by certain T-RFs and (iii) impaired translation into functional enzymes, with consequences for growth of denitrifier communities. However, since only <1% of added N accumulated as $N_2O$ and NO at low pH, and growth of nitrite- (*nirK*-type) and $N_2O$-reducers was observed, we conclude that acid-tolerant denitrifier species maintained the functionality of the community as a whole although full conversion of nitrate to $N_2$ required extended incubation

periods. Experiments altering soil pH *in situ* or in laboratory experiments have repeatedly confirmed that denitrification rates and denitrifying enzyme activity are lower in acidic than in neutral or slightly alkaline soils (Šimek and Cooper, 2002).

Overall, our results show that different mechanisms may determine the response to low pH of a soil denitrifier community adapted to neutral pH:

(i) Activity and proliferation of *nirK*- and *nosZ*- but not of *nirS*-containing denitrifiers seemed to drive reduction of nitrogen oxides which in turn increased pH. Albeit not at the transcriptional level, growth of *nirS*-type denitrifiers was severely inhibited at low pH and occurred only during or after pH upshift. Acid pH has been repeatedly shown to impair nitrite and particularly $N_2O$ reduction in certain denitrifiers (e.g., *P. denitrificans*) (Baumann et al., 1997; Bergaust et al., 2010), in soils (Liu et al., 2014) and in cells extracted from soils (Liu et al., 2010), presumably by impairing the assembly of denitrification enzymes post-transcriptionally (Baumann et al., 1997; Bergaust et al., 2010). Here, we could show that expression of *nirK* in some denitrifiers may be affected as well.

(ii) These effects, however, might be compensated by acid-tolerant or acidophilic denitrifier species able to grow and actively denitrify at low pH. Denitrifiers of the *nirK*-type present in the native community of the soil seemed to tolerate a broad range of pH levels as the composition of the growing community remained unaltered during the incubation at low pH.

(iii) Low pH prompted growth of *nosZ*-containing denitrifiers of minor abundance in the native community that were acid-tolerant or even acidophilic. At low pH these *nosZ*-containing denitrifiers seem capable of functionally substituting $N_2O$-reducers that were more prevalent in the native community. This agrees well with the functional redundancy hypothesis that distinct species perform similar roles in communities and ecosystems at different environmental conditions, and may therefore be substitutable with little impact on ecosystem processes (Rosenfeld, 2002).

Previous studies have shown that pH-dependent responses in denitrification product ratios in soils were related to the size and composition of the underlying denitrifier communities (Čuhel et al., 2010; Braker et al., 2012). Large variations have been found in the specific activity of e.g., nitrite reductases (50-fold) even between strains of the same species (Ka et al., 1997). The higher susceptibility of *nirS*-type denitrifiers to environmental stressors (e.g., low pH, low C-content) has been repeatedly reported in other studies (Bárta et al., 2010; Čuhel et al., 2010; He et al., 2010). The abundance of *nirS* was also most strongly affected when the pH of a grassland was lowered experimentally for about one year resulting in a high *nosZ*/*nirS* ratio while the *nosZ*/*nirK* ratio remained unaffected (Čuhel et al., 2010). Hence, long-term exposure to low pH in the natural environment will shape soil microbial communities and predetermine a dominance of either *nirK* or *nirS* (Chen et al., 2015). This strongly suggests that taxonomic composition matters for the capability of a soil denitrifier community to effectively denitrify. On the other hand, bulk soil pH is unlikely to be homogeneous in structured soils, probably providing a range of pH habitats distributed throughout the soil matrix. Thus, the occurrence of e.g., $N_2O$ reduction in acidic soils can be explained by denitrification activity in neutral microsites as proposed by Liu et al. (2014) or by acid-tolerant denitrifiers being present in neutral soils. Consequently, soil denitrifier communities might be comprised of taxa differing in pH sensitivity, which jointly emulate the kinetic response of a soil to pH change.

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## Supplementary Material

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# Anoxic growth of *Ensifer meliloti* 1021 by $N_2O$-reduction, a potential mitigation strategy

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Denitrification in agricultural soils is a major source of $N_2O$. Legume crops enhance $N_2O$ emission by providing N-rich residues, thereby stimulating denitrification, both by free-living denitrifying bacteria and by the symbiont (rhizobium) within the nodules. However, there are limited data concerning $N_2O$ production and consumption by endosymbiotic bacteria associated with legume crops. It has been reported that the alfalfa endosymbiont *Ensifer meliloti* strain 1021, despite possessing and expressing the complete set of denitrification enzymes, is unable to grow via nitrate respiration under anoxic conditions. In the present study, we have demonstrated by using a robotized incubation system that this bacterium is able to grow through anaerobic respiration of $N_2O$ to $N_2$. $N_2O$ reductase ($N_2OR$) activity was not dependent on the presence of nitrogen oxyanions or NO, thus the expression could be induced by oxygen depletion alone. When incubated at pH 6, *E. meliloti* was unable to reduce $N_2O$, corroborating previous observations found in both, extracted soil bacteria and *Paracoccus denitrificans* pure cultures, where expression of functional $N_2O$ reductase is difficult at low pH. Furthermore, the presence in the medium of highly reduced C-substrates, such as butyrate, negatively affected $N_2OR$ activity. The emission of $N_2O$ from soils can be lowered if legumes plants are inoculated with rhizobial strains overexpressing $N_2O$ reductase. This study demonstrates that strains like *E. meliloti* 1021, which do not produce $N_2O$ but are able to reduce the $N_2O$ emitted by other organisms, could act as even better $N_2O$ sinks.

**Keywords: denitrification, dinitrogen, greenhouse gas, nitric oxide, nitrous oxide reductase**

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## Introduction

The presence of $N_2O$ in the atmosphere has been known since 1939 (Adel, 1939). However, its importance to the global environment was not recognized until the early 1970s when scientists hypothesized that $N_2O$ released into the atmosphere could activate reactions in the stratosphere that contribute to the depletion of the ozone layer (Crutzen, 1974). The fourth assessment report of the intergovernmental Panel on Climate Change (IPCC, 2007) estimated $N_2O$ emissions from both natural and anthropogenic sources to be 8.5–27.7 Tg $N_2O$/year. The terrestrial ecosystems are the main source of $N_2O$, accounting about 65% of total emissions. Agricultural activities are the major

sources of $N_2O$ emissions, accounting for 60–80% of the anthropogenic $N_2O$ sources, mostly as N inputs to agricultural soils (Smith, 2008; Smith et al., 2012). These $N_2O$ emissions are likely to increase with the predicted expansion in the use of nitrogenous fertilizers in order to satisfy the escalating demand for food of the growing world population.

A variety of biological pathways are involved in $N_2O$ emissions from soils, and it has been estimated that >65% of the atmospheric $N_2O$ derives from microbial N transformations, mainly through the processes nitrification and denitrification (Thomson et al., 2012). Of these, denitrification is generally considered to be the largest source of $N_2O$ and, depending on the type of microorganisms involved and the environmental conditions, this process can serve not only as source but also as sink for $N_2O$ (Thomson et al., 2012). Denitrification is the respiratory reduction of nitrogen oxides (NOx) which enables facultative aerobic bacteria to survive and multiply under oxygen-limiting conditions. During this process nitrate ($NO_3^-$) is converted into molecular nitrogen ($N_2$) via nitrite ($NO_2^-$) and the gaseous intermediates nitric oxide (NO) and nitrous oxide ($N_2O$) (Zumft, 1997).

In contrast to the variety of $N_2O$ sources in soils, removal of $N_2O$ is only achieved by the last step of the denitrification process which is catalyzed by the $N_2O$ reductase ($N_2OR$) enzyme encoded by the *nosZ* gene. Recent reports have demonstrated that diverse microbial taxa possess divergent *nos* clusters with genes that are related yet evolutionarily distinct from the typical *nos* genes of denitrifiers (Sanford et al., 2012). In fact, phylogenetic analyses of the *nosZ* gene revealed two distinct clades of *nosZ* differing in their signal peptides, indicating differences in the translocation pathway to the $N_2OR$ across the membrane (Jones et al., 2013). The expression and activity of $N_2OR$ is a natural target in the search for options to mitigate $N_2O$ emission from agricultural soils (Richardson et al., 2009). A promising mitigation strategy suggested recently is to stimulate $N_2O$ reductase by sustaining a high soil pH (Bakken et al., 2012). The latter is motivated by recent demonstrations that reduction of $N_2O$ is severely inhibited by suboptimal pH in the model organism *Paracoccus denitrificans* (Bergaust et al., 2010), in bacterial communities extracted from soils (Liu et al., 2014), and in intact soils (Raut et al., 2012; Qu et al., 2014). Another interesting option would be to alter the composition of the denitrifying community of soils, the objective being to enhance the growth of organisms with high $N_2O$ reductase activity. This would be a daunting task if the free-living soil bacteria were the target, but plant-associated bacteria appear more promising.

Rhizobia is a general term that describes bacteria that have the ability to establish $N_2$-fixing symbiosis in legume roots or on the stems of some aquatic leguminous plants. In addition to fixing $N_2$, many rhizobial strains have genes for enzymes of some or all of the four reductase reactions for denitrification. Several studies have reported that legume crops induce $N_2O$ emission by providing N-rich residues for decomposition (Baggs et al., 2000). In addition to soil denitrifiers, endosymbiotic bacteria may be partly responsible for this legume-induced $N_2O$ emission, since most rhizobia are able to denitrify under free-living and under symbiotic conditions (Bedmar et al., 2005; Delgado et al., 2007; Sanchez et al., 2011). Increased $N_2O$ emissions due to degradation of nodules were reported in soybean ecosystems (Inaba et al., 2012). Based on this, Itakura et al. (2013) hypothesized and proved that $N_2O$ emission from soil could be reduced by inoculating soybean plants with a *nosZ*-overexpressing strain of *Bradyrhizobium japonicum*. This suggests that root nodules of leguminous plants are net sources or sinks for $N_2O$. Thus, the investigation of denitrification among rhizobia may provide novel options for reducing $N_2O$ emissions from soils.

*Ensifer* (formerly *Sinorhizobium*) *meliloti* 1021 is a key model organism for studying the symbiotic interaction between rhizobia and plants of the genera *Medicago, Melilotus,* and *Trigonella*, that has also been extensively used in previous works to better understand the regulation and symbiotic characterisation of *E. meliloti* denitrification genes (Bobik et al., 2006; Meilhoc et al., 2010; Horchani et al., 2011). In fact, analysis of the *Ensifer meliloti* 1021 genome sequence revealed the presence of the *napEFDABC*, *nirK*, *norECBQD,* and *nosRZDFYLX* denitrification genes encoding a periplasmic nitrate reductase, a copper-containing nitrite reductase, a *c*-type nitric oxide reductase and a nitrous oxide reductase enzyme, respectively. The involvement of the *E. meliloti napA*, *nirK*, *norC,* and *nosZ* structural genes in nitrate respiration and in the expression of denitrification enzymes under specific growth conditions (initial oxygen concentrations of 2% and initial cell density of 0.2–0.25) was also demonstrated (Torres et al., 2014). However, this strain has for a long time been considered a partial denitrifier due to its apparent inability to grow under anaerobic conditions with nitrate or nitrite as final electron acceptors (Garcia-Plazaola et al., 1993; Torres et al., 2011a). In order to better understand the truncated denitrification phenotype of *E. meliloti* 1021, an accurate estimation of the efficiency of the denitrifying process is required. For that purpose, in this work we have used a robotized system which allowed us to simultaneously monitor the $O_2$ consumption, as well as the consumption and production of each NOx during the transition from oxic to anoxic respiration.

The results convincingly demonstrated that this strain (1021) was unable to reduce $NO_3^-$ or $NO_2^-$ to $N_2O$ or $N_2$. In contrast, this bacterium was capable to reduce externally supplied $N_2O$ to $N_2$, serving as a terminal electron acceptor during anoxic respiration. Thus, our study expands the current understanding of anaerobic respiration in rhizobia and explores the effect of pH, NOx and type of carbon source on $N_2O$ reduction in *E. meliloti*.

## Materials and Methods

### Bacterial Strains, and Growth Conditions in Batch Cultures

*Ensifer meliloti* 1021 ($Sm^r$, Meade et al., 1982), and *napA* (*napA*::mini-*Tn*5 $Sm^r$, $Km^r$, Pobigaylo et al., 2006) and *nirK* (*nirK*::mini-Tn5 $Sm^r$, $Km^r$, Pobigaylo et al., 2006) mutant strains were used in this study. *E. meliloti* strains were grown aerobically in 120 mL serum vials containing a triangular magnetic stirring bar and 50 mL of Triptone Yeast (TY) complete medium (Beringer, 1974) at 30°C. All cultures were continuously stirred

at 700 rpm to avoid aggregation and ensure complete dispersal of cells. These cultures were then used as inocula into vials containing minimal defined medium (Robertsen et al., 1981) supplemented with or without 10 mM of $KNO_3$ or 5 mM of $NaNO_2$. The influence of carbon susbtrates on $N_2O$ uptake capacity was analyzed in minimal medium where the carbon substrate was replaced with either 5 mM of succinate or 5 mM of butyrate as oxidized or reduced carbon sources, respectively. The effect of pH on $N_2O$ uptake capacity was also studied in minimal medium strongly buffered (50 mM phosphate buffer) at pHs 6, 7, and 8. In all the treatments the headspace was filled with an initial concentration of $O_2$ of 1 or 2% (12 or 24 μM dissolved $O_2$ at 30°C, respectively). The headspace of experimental vials used to study the $N_2O$ reduction capacity was additionally supplied with an initial concentration of $N_2O$ of 2% (0.42 mM) or 5% (1.2 mM). To avoid possible external contaminations, antibiotics were added to the cultures at the following concentrations (μg $mL^{-1}$); streptomycin, 200; kanamycin, 200.

## Preparation of Incubation Vials

120 mL vials containing 50 mL liquid medium were crimp-sealed with rubber septa (Matriks AS, Norway) and aluminum caps to ensure an airtight system. Oxygen from vials was removed by 6 cycles of air evacuation during 360 s and helium (He) filling during 40 s. Constant stirring (400 rpm) was kept to ensure optimal gas exchange between liquid and headspace. Then, vials were injected with the required concentrations of $O_2$ and $N_2O$.

## Gas Measurements

After inoculation, cultures, blanks, and gas standards were placed in a thermostatic water incubator containing a serial magnetic stirrer at 30°C, with continuous stirring at 700 rpm, and the gas kinetics were monitored in each vial (2 to 3 h intervals). The gas measurements were performed by monitoring the headspace-concentrations of relevant gases ($O_2$, $CO_2$, NO, $N_2O$, and $N_2$) by repeated gas sampling through the rubber septa of the incubation vials as described by Molstad et al. (2007). The gas samples were drawn by a peristaltic pump coupled to an autosampler (Agilent GC Sampler 80), and with each sampling an equal volume of He was pumped back into the vials. This secured that the gas pressure was sustained near 1 atm despite repeated sampling, but diluted the headspace atmosphere (with He). This dilution was taken into account when calculating rates of production/consumption for each time increment (Molstad et al., 2007). The sampling system was coupled to a gas chromatograph (GC) (Agilent GC -7890A) with two 30 m × 0.53 mm id columns: a Porous Layer Open Tubular (PLOT) column for separation of $CH_4$, $CO_2$ and $N_2O$, and a Molsieve column for separation of $O_2$ and $N_2$ (and Ar, Ne). The GC had three detectors: a flame ionization detector (FID), a thermal conductivity detector (TCD), and an electron capture detector (ECD). $N_2O$ was detected by both the ECD and TCD, thus securing accurate measurements at near-ambient concentrations (ECD, linear range 0–4 or 0–20 ppmv, depending on detector temperature) and linear response for higher concentrations (TCD). NO concentrations were determined by a Chemoluminiscence NOx analyser (Model 200A, Advanced Pollution Instrumentation, San Diego, USA).

## $OD_{600}$, Nitrate and Nitrite Measurements

Cell densities ($OD_{600}$), nitrate and nitrite concentrations were measured for each sample. Samples were taken from the liquid phase of the vials throughout the experiment to measure $OD_{600}$ (0.7 mL sample), $NO_3^-$ (0.1 mL sample), and $NO_2^-$ (0.1 mL sample) using sterile syringes. For determination of $NO_3^-$, a 10 μL aliquot was injected into a purge vessel with heating jacket and condenser (ASM 03292) containing 1 M HCl and vanadium (III) chloride. Temperature of vessel was controlled by a circulating water bath at 95°C and cold water for the condenser. In addition, a gas bubble/NaOH trap with Teflon sleeve (ASM 04000) was used to avoid the corrosive effects of HCl. Vanadium (III)/HCl converts nitrite and S-nitrosocompounds to NO, which is transported (by $N_2$) to a chemiluminescence detector Nitric Oxide Analyzer NOA 280i (General Electric). $N_2$ was continuously bubbled through the reducing agent to maintain an anaerobic environment in the system and to transport the NO through the NO analyzer (Walters et al., 1987). The approximate detection limit was 1 pmol NO, equivalent to 0.1 μM (when injecting 10 μL). For determination of $NO_2^-$, a 10 μL subsample was injected into a purge vessel (gas bubble/NaOH trap is not needed) containing acetic acid with 1% vol NaI where $NO_2^-$ is converted to NO.

## Analyses of Kinetics of Aerobic and Anoxic $NO_3^-$, $NO_2^-$, or $N_2O$ Respiration

Experimental dataset obtained from the series of incubations were used to determine the kinetics of $O_2$, $NO_3^-$ $NO_2^-$, or $N_2O$ respiration and NO, $N_2O$, and $N_2$ production in order to provide the most accurate information on *E. meliloti* physiology during the transition from aerobic to NOx anoxic respiration. $O_2$ and NO concentration in the liquid, determined as μM and nM, respectively, was estimated taking into account the partial pressure of these gases at headspace, their solubilities and transport coefficients between headspace and liquid. Additionally, $O_2$ concentration in liquid was estimated respective the $O_2$ respiration rate for each time increment (see Molstad et al., 2007 for details). $N_2O$ was analyzed as μmol $N_2O$ $vial^{-1}$, whereas $N_2$ was determined as cumulative net production of $N_2$. All data were corrected for dilution rates and losses by gas sampling, and leaks due to gas diffusion through the rubber septa. The concentrations of $NO_3^-$ and $NO_2^-$ were determined at different times compared to the gas sampling. However, we needed values for $NO_2^-$ concentrations at the same time as the gas sampling in order to estimate electron flow rates. For this reason, polynomial functions [f(t)] were fitted to the measured $NO_3^-$ and $NO_2^-$ concentrations, and used to estimate $NO_2^-$ concentration at the time of gas samplings. Graphical presentations for $NO_3^-$ and $NO_2^-$ concentrations include both measured data points and the polynomial function.

The apparent growth rates based on $O_2$ consumption ($\mu_{ox}$), and reduction of any NOx during the anoxic phase ($\mu_{anox}$) were estimated by regression [ln ($V_{e-}$) against time] for the phases with exponentially increasing rates. Yield (cells $pmol^{-1}$ $e^-$) calculation was based on the number of cells rendered per pmol electron used by the respiratory terminal oxidases to reduce $O_2$ to $H_2O$ during oxic phase ($Yield_{ox}$) or by the complete set

of denitrifying reductases to reduce $NO_3^-$ $NO_2^-$ or $N_2O$ to $N_2$ during anoxic phase ($Yield_{anox}$). $V_{max}$ is an useful parameter that can tell us the efficiency for $O_2$ and NOx respiration per cell. It estimates the maximal velocities per cell and per hour for the reduction of $O_2$ and $NO_x$. This parameter is based on the fmol of electrons used by the terminal oxidases and denitrifying enzymes to reduce $O_2$ or NOx, respectively, per cell and per hour. For further details regarding these calculations, see Molstad et al. (2007) and Nadeem et al. (2013).

## Results

### Kinetics of Aerobic Respiration

*E. meliloti* strain 1021 was grown aerobically for 30 h with vigorous stirring (700 rpm) until a maximal optical density at 600 nm ($OD_{600}$) of ∼0.3 to avoid generation of localized anoxic conditions due to cell aggregation. Then, an aliquot was used to inoculate the culture vials to an initial $OD_{600}$ of 0.01 ($8 \times 10^6$ cells $mL^{-1}$). The medium contained either 10 mM of nitrate (**Figure 1**), 5 mM $NO_2^-$ (**Figure 2**) or 10 mM nitrate plus 5% $N_2O$ (1.2 mM $N_2O$ concentration in the liquid when in equilibrium with the headspace) (**Figure 3**). In all the treatments for studying the kinetics of aerobic respiration, the initial $O_2$ concentration in the headspace was 2%. **Figure 1A** shows the measured $OD_{600}$, $O_2$, NO, $N_2O$, and $N_2$ concentrations in the medium for a single vial throughout the 40 h incubation in the presence of nitrate. $NO_3^-$ depletion and production of $NO_2^-$ is also shown (**Figure 1A**, insert). In nitrate-treated cells, oxygen was consumed within the first 15 h, $OD_{600}$ increased linearly with the cumulative $O_2$ consumption ($r^2$) = 0.9877, and remained practically constant throughout the anoxic phase. Rates of $O_2$ consumption for each time increment between two samplings were used to calculate electron ($e^-$) flow rates to oxygen ($V_{e-O2}$). As shown in **Figure 1B**, $V_{e-O2}$ increased exponentially throughout the first 7 h in proportion with the increase in $OD_{600}$ ($r^2 = 0.9105$), and declined gradually in response to diminishing $O_2$ concentrations. The initial exponential increase in electron flow during oxic respiration can be taken as an indirect measure of growth rate ($\mu_{ox}$) (Liu et al., 2013). Thus, the apparent $\mu_{ox}$ estimated by linear regression of ln ($V_{e-O2}$) against time was 0.30 (±0.03) $h^{-1}$ (**Figure 1B**, **Table 1A**). The final $OD_{600}$ was 0.15 (±0.02) ($1.60 \times 10^8$ cells $mL^{-1}$, **Table 1B**) resulting in a yield of 24.6 (±2.8) cells $pmol^{-1} e^-$ to $O_2$ (**Table 1A**). The apparent maximum specific respiration rate, $V_{max}$, which is a useful indicator of the respiration per cell, was 11.6 (±0.5) fmol $e^-$ $cell^{-1}$ $h^{-1}$ for oxygen respiration in cells grown in the presence of nitrate (**Table 1A**).

$O_2$ uptake and growth kinetics were also analyzed in cells grown in the presence of 5 mM $NO_2^-$ as final electron acceptor (**Figure 2**). For this treatment, $O_2$ was consumed within the first 30 h of incubation showing a delay in comparison to $NO_3^-$ treatment (**Figure 2A**). As observed in nitrate-treated cells, $OD_{600}$ also increased during the oxic phase in proportion with $O_2$ consumption, and remained constant during the anoxic phase. The estimated oxic growth rate in the presence of nitrite (linear regression of ln($V_{e-O2}$) against time was $\mu_{ox}$ = 0.11 (±0.02)$h^{-1}$ (**Figure 2B**, **Table 1A**) and the estimated cell yield was only 14.1 (±1.1) cells $pmol^{-1}$ $e^-$ (**Table 1A**). The estimated $V_{max}$ for oxygen respiration in cells grown in the presence of nitrite was 8.2 (±0.7) fmol $e^-$ $cell^{-1}$ $h^{-1}$ (**Table 1A**). Thus, the presence of $NO_2^-$ in the medium appeared to exert an inhibitory effect on the oxygen respiration by terminal respiratory oxidases, resulting in lower $V_{max}$ and cell yield per mol electron compared to cells grown in the presence of nitrate.

Finally, kinetics of $O_2$ respiration were also analyzed when cells were incubated in vials containing minimal medium with 10 mM of $NO_3^-$, and an initial concentration of 5% $N_2O$ and 2% $O_2$ in the headspace. **Figure 3A** shows the measured $O_2$, NO, $N_2O$, and $N_2$ for a single vial throughout the 40 h incubation, as well as the $OD_{600}$. In this case, oxygen was consumed within the first 15 h and the $OD_{600}$ increased in proportion with the cumulative $O_2$ consumption and continued increasing throughout the anoxic phase. Electron flow rate to $O_2$ increased exponentially with an apparent growth rate ($\mu_{ox}$) = 0.28 (±0.03) $h^{-1}$ (**Figure 3B**, **Table 1A**). Cell yield resulting from $O_2$ respiration was very similar to that observed in nitrate-treated cells [23.1 (±6.2) cells $pmol^{-1}$ $e^-$ with a $V_{max}$ of 8.9 (±0.13) fmol $e^-$ $cell^{-1}$ $h^{-1}$] (**Table 1A**).

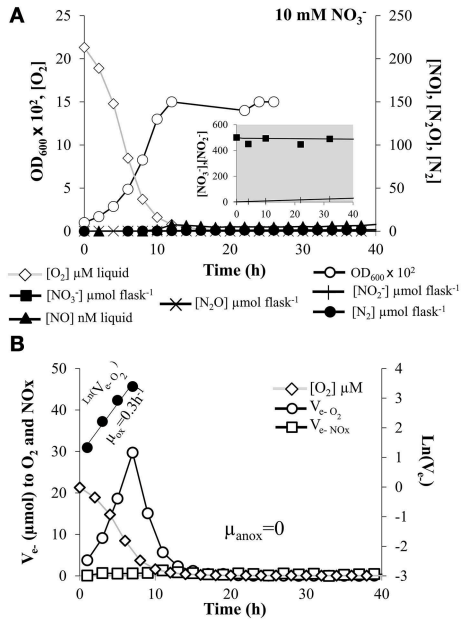


**FIGURE 1 | Kinetics of $O_2$ and $NO_3^-$ respiration. (A)** Absorbance at 600 nm ($OD_{600}$), $O_2$ consumption, $NO_3^-$ depletion (insert), and production of $NO_2^-$ (insert), NO, $N_2O$, and $N_2$ by *E. meliloti* 1021 when incubated in the presence of 10 mM $NO_3^-$ in the medium and an initial $O_2$ concentration of 2% in the headspace. **(B)** The electron flow rate to $O_2$ is shown as log-transformed values for the phases with exponential increase (filled circle symbols). The slopes estimating apparent growth rates were 0.3 (± 0.03) $h^{-1}$ and 0 for oxic and anoxic phase, respectively. Cultures with an initial $OD_{600}$ of 0.01 were vigorously stirred at 700 rpm. The result shown is for a single vial. Several replicates were analyzed, with similar results, although the exact timing of events was not the same. However, the consistency of the observations is demonstrated in **Table 1** where averages of at least three different cultures are reported.

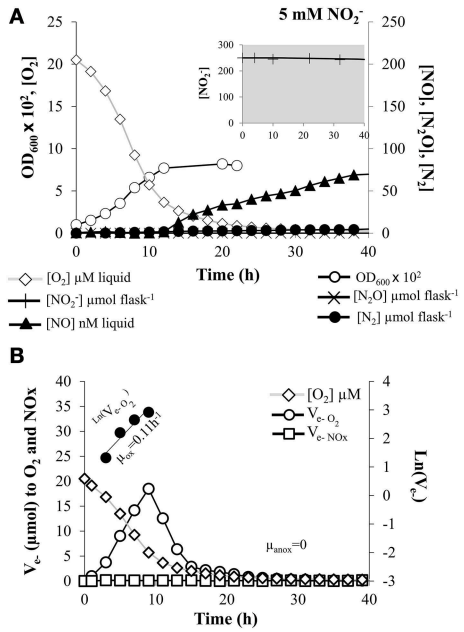


**FIGURE 2 | Kinetics of $O_2$ and $NO_2^-$ respiration. (A)** Absorbance at 600 nm ($OD_{600}$), $O_2$ consumption, $NO_2^-$ depletion (insert), and production of NO, $N_2O$, and $N_2$ by *E. meliloti* 1021 when incubated in the presence of 5 mM $NO_2^-$ in the medium and an initial $O_2$ concentration of2% in the headspace. **(B)** The electron flow to $O_2$ shown as log-transformed values for the phases with exponential increase (filled circle symbols). The slopes estimating apparent growth rates were 0.11 (±0.02) $h^{-1}$ and 0 for oxic and anoxic phase, respectively. Cultures with an initial $OD_{600}$ of 0.01 were vigorously stirred at 700 rpm. The result shown is for a single vial. Several replicates were analyzed, with similar results, although the exact timing of events was not the same. However, the consistency of the observations is demonstrated in **Table 1** where averages of at least three different cultures are reported.

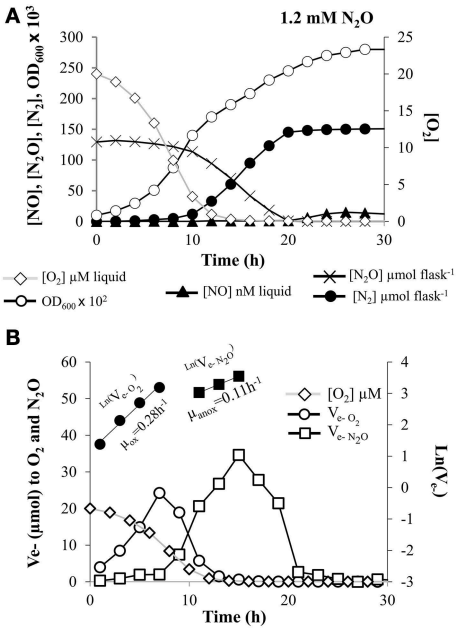


**FIGURE 3 | Kinetics of $O_2$ and $N_2O$ respiration. (A)** Absorbance at 600 nm ($OD_{600}$), $O_2$ and $N_2O$ depletion, and production of NO, and $N_2$ by *E. meliloti* 1021 when incubated in the presence of 10 mM $NO_3^-$ in the medium and an initial $O_2$ concentration of 2% in the headspace plus 5% $N_2O$. **(B)** The electron flow to $O_2$ or to $N_2O$ is shown as log-transformed values for the oxic (filled circle symbols) and anoxic phase (filled square symbols) with exponential increase. The slopes estimating apparent growth rates were 0.28 (±0.03) $h^{-1}$ for the oxic phase, and 0.11 (±0.03) $h^{-1}$ for the anoxic phase, respectively. Cultures with an initial $OD_{600}$ of 0.01 were vigorously stirred at 700 rpm. The result shown is for a single vial. Three replicates were analyzed in parallel with similar results. Consistency of the observations are demonstrated in **Table 1** where averages of at least three different cultures are reported.

## Kinetics of $NO_3^-$ and $NO_2^-$ Respiration

When cells were cultured with $NO_3^-$, there was a very low $NO_3^-$ consumption rate as well as very low progressive accumulation of $NO_2^-$ throughout the entire anoxic phase (**Figure 1A**, insert), reaching only ∼50 µmol $vial^{-1}$ (which accounts for 10% of the $NO_3^-$-N in the medium). Very low levels of NO were also observed (12.40 ± 2.10 nM) after 40 h incubation (**Table 1B**, **Figure 1A**). Production of $N_2O$ in the headspace was insignificant and the fraction of $NO_3^-$ reduced to $N_2$ at the end of the incubation was also extremely low (0.9 ± 0.3 %) (**Table 1B**, **Figure 1A**). When $NO_2^-$ was used as final electron acceptor, the first detection of NO occurred as the oxygen concentration in the liquid reached ∼3 µM (**Figure 2A**, **Table 1B**). During the subsequent anoxic phase, NO continued to accumulate, reaching 94.20 ±16.90 nM levels at the end of the incubation period (**Table 1B**, **Figure 2A**). Similarly as for nitrate-treated cells (**Figure 1A**), production of $N_2O$ was undetectable and the total, cumulative production of $N_2$ from the initially provided $NO_2^-$-N was also very low (0.18 ± 0.02 %) (**Figure 2A**, **Table 1B**). These data show that *E. meliloti* 1021 was clearly unable to shift effectively to $NO_3^-$ or $NO_2^-$ based anaerobic respiration. This inability was also confirmed by the lack of increase in measured $OD_{600}$ throughout the anoxic phase (**Figures 1**, **2**). Thus, the apparent growth rate during either $NO_3^-$ or $NO_2^-$ anoxic respiration ($\mu_{anox}$) was zero (**Figures 1B**, **2B**, **Table 1A**). Similar growth rates were observed by using 1 mM or 500 µM $NO_2^-$ as electron acceptor (data not shown). One possible explanation to the lack of efficient reduction of $NO_3^-$ and $NO_2^-$ could be that rapid depletion of the oxygen in these cultures may have resulted in entrapment of the bacteria in anoxia, as shown previously for *P. denitrificans* by Bergaust et al. (2010). To test this hypothesis, we performed a follow-up experiment where the stirring speed was reduced from 700 rpm (used in the experiments reported in **Figures 1**, **2**) to 200 rpm, in order to secure a slow transition from oxic to anoxic conditions in the liquid. These cultures showed the same lack of effective transition to denitrification as cultures with vigorous stirring, despite the fact that the cells with low stirring experienced a progressive $O_2$ limitation during 50 h prior to complete $O_2$ depletion (see **Supplementary Figure S1**).

## Kinetics of $N_2O$ Respiration

The capacity of *E. meliloti* 1021 to reduce $N_2O$ was examined in vials containing 10 mM $NO_3^-$ in the medium plus 5% $N_2O$ and 2% $O_2$ initially added to the headspace (**Figure 3**).

**TABLE 1 | Summary of oxic and anoxic growth parameters (A)[1] Depending on the presence of nitrogen oxides, and the subsequent conversion of the nitrogen oxides present (B)[2].**

| (A) | | | | | | |
|---|---|---|---|---|---|---|
| | Growth parameters for the oxic phase | | | Growth parameters for the anoxic phase | | |
| $NO_x$ present (mM) | $\mu_{ox}$($h^{-1}$) | $Yield_{ox}$ (cell $pmol^{-1}$ e-) | $V_{max}$(fmol e- $cell^{-1}$ $h^{-1}$) | $\mu_{anox}$ ($h^{-1}$) | $Yield_{anox}$ (cell $pmol^{-1}$ e-) | $V_{max}$(fmol e- $cell^{-1}$ $h^{-1}$) |
| $NO_3^-$(10) | 0.30(±0.03) a | 24.6(±2.8) a | 11.6(±0.5) a | 0 | 0 | 0 |
| $NO_2^-$(5) | 0.11(±0.02) b | 14.1(±1.1) b | 8.2(±0.7) b | 0 | 0 | 0 |
| $N_2O$(1.2)[3] | 0.28(±0.03)a | 23.1(±6.2) a | 8.9(±0.1) b | 0.11(±0.03) | 18(±0.6) | 5.7(±1.1) |
| **(B)** | | | | | | |
| $NO_x$present (mM) | $[O_2]$ at onset of $NO_x$-reduction ($\mu MO_2$) | | Max [NO−] in liquid (nM NO) | Fraction of $NO_x$reduced to $N_2$ (% of $NO_x$-N) | | Final OD ($OD_{600}$) |
| $NO_3^-$(10) | 2.7(±1.5) a | | 12.4(±2.10) a | 0.90(±0.30) a | | 0.15(±0.02) a |
| $NO_2^-$(5) | 3.0(±0.7) a | | 94.2(±16.9) b | 0.18(±0.02) b | | 0.08(±0.01) b |
| $N_2O$(1.2)[3] | 5.9(±2.6) b | | 15.0(±1.10) a | 100(±2.50) c | | 0.28(±0.05) c |

*The alternative respiratory substrate (NOx) present in the medium ($NO_3^-$ or $NO_2^-$) or at headspace ($N_2O$) for each analysis is indicated. All the experimental vials contained an initial $O_2$ concentration of 2% at headspace. Data are means with standard error (in parenthesis) from at least three independent cultures. Values in a column followed by the same lower-case letter are not significantly different according to One-Way ANOVA and the Tukey HSD test at $P \leq 0.05$.*

*[1]Apparent oxic growth ($\mu_{ox}$, $h^{-1}$) and anoxic growth ($\mu_{anox}$, $h^{-1}$) rates based on $O_2$ consumption during the oxic phase or reduction of $NO_3^-$, $NO_2^-$, or $N_2O$ during the anoxic phase. Yield (cells per mole electron) based on increase in OD vs. cumulated consumption of oxygen or reduction of $NO_3^-$, $NO_2^-$, or $N_2O$, and apparent maximum specific respiration rate ($V_{max}$, fmol electrons $cell^{-1}$ $h^{-1}$) during the initial phase (0–5 h) of the experiments (**Figures 1**, **2**).*

*[2]The oxygen concentration at the time of the first indications of anoxic respiration (i.e., appearance of NO in the treatments with $NO_3^-$ and $NO_2^-$, and appearance of significant $N_2O$ reduction to $N_2$ in the treatment with $N_2O$).*

*[3]5 % $N_2O$ (150 $\mu$mol $N_2O$ at 20°C) was injected into each vial, resulting in 1.1 mM $N_2O$ in the liquid when in equilibrium with the headspace.*

As shown in **Figure 3A**, $N_2O$ was consumed rapidly and $N_2$ production followed stoichiometrically the reduction of $N_2O$ to its complete depletion (100% of $N_2O$ was converted to $N_2$ gas) (**Figure 3A**, **Table 1B**). As shown in **Figure 3A**, $N_2O$ reduction was at first detected at an $O_2$ concentration of 5.9 (±2.6) μM (**Table 1B**). Traces of NO from $NO_3^-$ reduction were also detected (15 ±1.1 nM in the liquid; **Table 1B**). Final $OD_{600}$ of cells incubated with $N_2O$ was clearly higher than that obtained when cells were incubated only with $NO_3^-$ or $NO_2^-$ as alternative electron acceptors (**Table 1B**), demonstrating the capacity of *E. meliloti* to couple $N_2O$ reduction with growth.

Electron flow to $N_2O$ increased with an apparent growth rate ($\mu_{anox}$) of 0.11 (±0.03) $h^{-1}$ estimated by linear regression of ln ($V_{e-N2O}$)against time (**Figure 3B**, **Table 1A**). Although low rates of electron flow to $N_2O$ occurred after 3 h, it increased sharply after 7 h as the electron flow to oxygen decreased due to oxygen depletion. Thus, the cells were evidently able to shift gradually from respiring $O_2$ to $N_2O$, preserving the total electron flow rate essentially unaffected after the depletion of oxygen. As shown in **Table 1A**, the estimated cell yield from $N_2O$ reduction was 18 (±0.6) cells $pmole^{-1}$ $e^-$. Knowing the yield in cell number per hour and the electron flow rate per hour we could estimate the $V_{max}$ for $N_2O$ reduction to 5.7 (±1.1) fmol $e^-$ $cell^{-1}$ $h^{-1}$ (**Table 1A**).

## $NO_x$ Molecules Do Not Trigger $N_2O$R Activity in *E. meliloti*

To evaluate the effect of NOx molecules as inducers of $N_2O$R activity, we measured $N_2O$ uptake rates in cultures of *E. meliloti* 1021 strain that had received 10 mM $NO_3^-$ in the medium and compared this with cultures that were not supplemented with $NO_3^-$ (**Figures 4A,B**). The results showed similar $N_2O$ consumption as well as $N_2$ production rates for the two treatments. Furthermore, no differences in $N_2O$ respiration was found between wild-type cells and strains which were defective in the *napA* and *nirK* structural genes when cultured in a medium amended with 10 mM $NO_3^-$ (**Figures 4A,C,D**). The *E. meliloti napA* or *nirK* mutants were demonstrated previously to be unable to reduce nitrate and nitrite respectively, to any further NOx intermediary of the denitrification process (Torres et al., 2014). These results suggested that the ability to reduce $N_2O$ was not affected by the presence or absence of NO, $NO_2^-$, or $NO_3^-$.

## Low pH Severely Impaires $N_2O$ Uptake in *E. meliloti*

Since pH emerges as a master variable controlling the expression of $N_2O$ reductase, in this work we examined the pH effect on the kinetics of $N_2O$ reduction. For that purpose, *E. meliloti* cells were incubated in minimal medium strongly buffered with phosphate buffer, at pH 6, 7, and 8. Firstly, we grew *E. meliloti* 1021 cells aerobically to exponential (log) phase at pH 7. Then cells were transferred to the experimental vials containing 5% $N_2O$ and 2% $O_2$ in the headspace and 10 mM $NO_3^-$ in the medium. Rates of $O_2$ consumption were monitored until depletion and no differences were found between treatments. However, $N_2O$ reduction to $N_2$ was completely blocked at pH 6 (**Figure 5A**). Surprisingly, when cells were incubated at pH 8, a significant peak of NO was detected. A negative effect of high pHs on *nor* expression or Nor activity could explain that transient peak of NO.

## Reduced C-sources Attenuates $N_2O$ Uptake in *E. meliloti*

Carbon availability is another key environmental factor affecting $N_2O$ production in the field. However, information about the

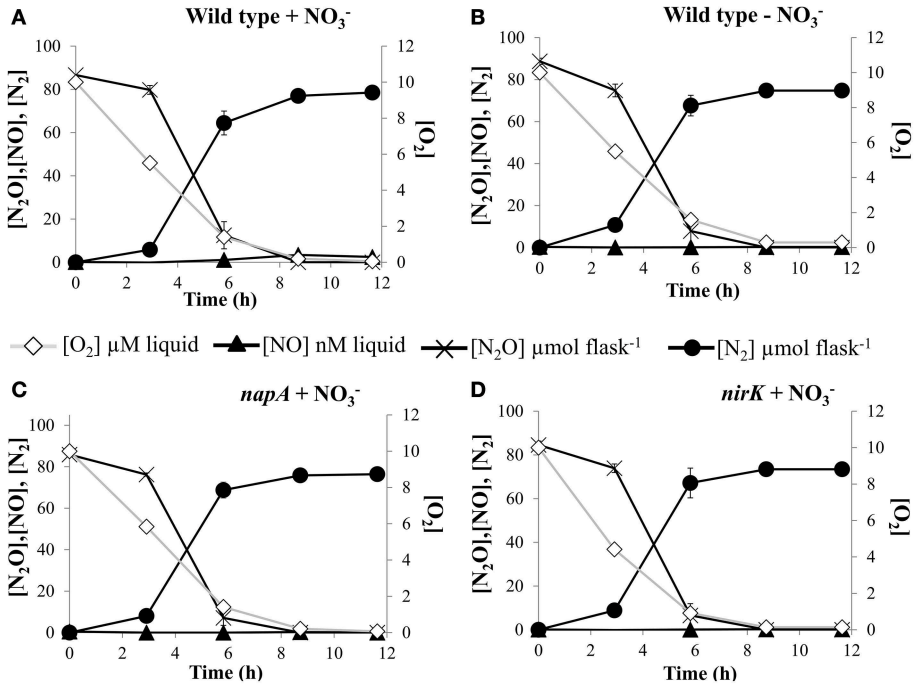


**FIGURE 4 | NOx effect on kinetics of $O_2$ and $N_2O$ depletion.** $O_2$ and $N_2O$ consumption, and NO and $N_2$ production by *E. meliloti* 1021 **(A,B)**, and *napA* **(C)** and *nirK* **(D)** mutant strains when incubated in the presence of 1% $O_2$ plus 2% $N_2O$ in the headspace. Cells were incubated in minimal medium with **(A,C,D)** or without **(B)** 10 mM $NO_3^-$. Cultures with an initial $OD_{600}$ of 0.01 were vigorously stirred at 700 rpm. Plotted values are average of three replicate flasks for each treatment, with standard deviation (SD) as vertical bars ($n = 3$).

implication of specific forms of reductants in $N_2O$ reductase activity is limited. Redox state of the C-sources might influence the amount of electrons available to reduce $N_2O$ to $N_2$. For that reason, we tested the capacity of *E. meliloti* 1021 to reduce $N_2O$ in the presence of C-substrates with different redox potential, from highly oxidized as succinate or highly reduced such as butyrate. Aerobically raised cells were collected and inoculated into experimental vials containing minimal medium where glycerol was substituted by either succinate or butyrate. By using the robotized incubation system, rates of $O_2$ respiration occurring previously to $N_2O$ consumption were also estimated. We found that $O_2$ respiration from cells incubated in the presence of butyrate was slightly decreased when compared to cells incubated in the presence of succinate (**Figures 6A,B**). However, rates of $N_2O$ consumption were largely dependent on the oxidized or reduced nature of the carbon source. Thus, when butyrate was used as electron donor, the $N_2O$ reduction to $N_2$ decreased about 3-fold compared to when succinate was used as the sole carbon substrate (**Figures 6A,B**).

## Discussion

In this work, we have used a robotized incubation system designed to simultaneously monitor with high sensitivity real-time changes in concentrations of $O_2$, $NO_3^-$, $NO_2^-$ NO, $N_2O$, and $N_2$ during the transition from oxic to anoxic respiration. By using this system, we found that *E. meliloti* 1021 is unable to reduce $NO_3^-$ or $NO_2^-$ to $N_2O$ or $N_2$ during the transition from oxic to anoxic conditions. Consequently, this bacterium was unable to sustain growth during anoxic conditions by using $NO_3^-$ or $NO_2^-$ as electron acceptors. This is in contrast to recent studies where growth of *E. meliloti* 1021 was observed during respiration of $NO_3^-$ as well as $NO_2^-$ (Torres et al., 2011a, 2014). This apparent discrepancy could be due to the different growth conditions and methodological approaches used by Torres et al. (2011a, 2014) and in this work. While they inoculated experimental vials with very high cell density ($OD_{600}\sim$ 0.2–0.25), which were shaken at 170 rpm, the initial cell density used in the present work was significantly lower ($OD_{600}\sim$ 0.01), and cultures were stirred at 700 rpm. The reason why we used different conditions in this work is to allow an efficient and controlled gas transfer from the headspace to the liquid and prevented cell aggregation and generation of localized micro-oxic spells during the aerobic phase previous to the transition to anaerobic respiration, as well as accumulation of toxic concentration of metabolites resulting from cell respiration. It might be possible that the growth conditions used by Torres et al. (2011a, 2014) provoked generation of anoxic micro-zones preceding total oxygen depletion due to cell aggregation and consequently the induction of *E. meliloti* 1021 denitrifying machinery would be facilitated. The present work extends the study of denitrification in *E. meliloti* by performing an estimation of the growth parameters (i.e., µ, yield, Vmax), as well as a precise quantification of NOx gases dynamics during the transition

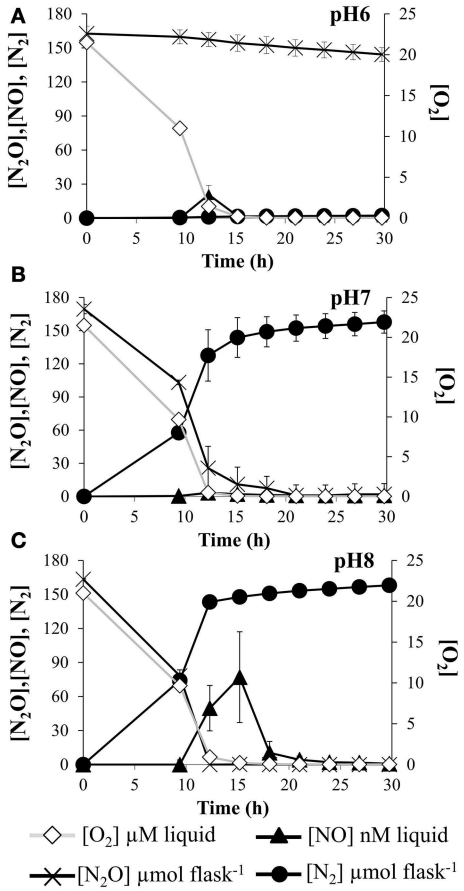


**FIGURE 5 | pH effect on kinetics of $O_2$ and $N_2O$ depletion.** $O_2$ and $N_2O$ consumption, and NO and $N_2$ production by *E. meliloti* 1021 when incubated in the presence of 10 mM $NO_3^-$ in minimal medium at pH 6 **(A)**, pH 7 **(B)** and 8 **(C)**, and an initial $O_2$ concentration of 02% in the headspace plus 5% $N_2O$. Cultures with an initial $OD_{600}$ of 0.01 were vigorously stirred at 700 rpm. Plotted values are average of three replicate flasks for each treatment, with standard deviation (SD) as vertical bars ($n = 3$). The decline in $N_2O$ concentration at pH = 6 is due to sampling loss, not biological reduction of $N_2O$ to $N_2$.

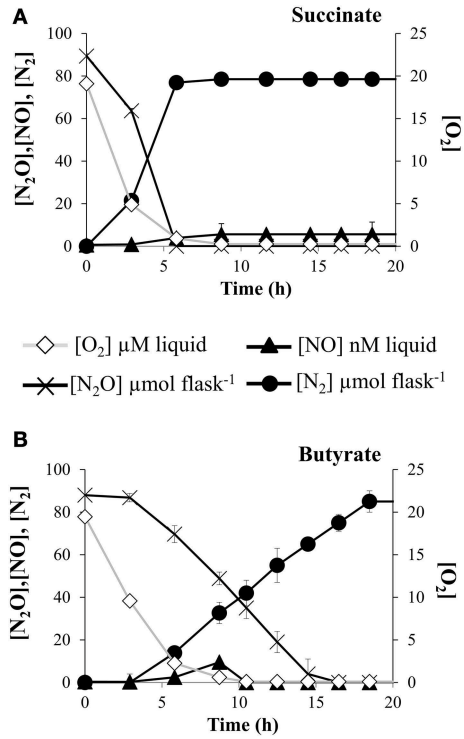


**FIGURE 6 | C-source effect on kinetics of $O_2$ and $N_2O$ depletion.** $O_2$ and $N_2O$ consumption, and NO and $N_2$ production by *E. meliloti* 1021 when incubated in the presence of 1% $O_2$ plus 2 % $N_2O$ in the headspace, and 10 mM $NO_3^-$ in Robertsen's medium which contained succinate **(A)** or butyrate **(B)** as carbon sources. Cultures with an initial $OD_{600}$ of 0.01 were vigorously stirred at 700 rpm. Plotted values are averages of three replicate flasks for each treatment, with standard deviation (SD) as vertical bars ($n = 3$).

from oxic to anoxic respiration. This approach, never used in rhizobia, allowed us to perform an accurate estimation of the efficiency of the denitrifying process, and is regarded to be more physiologically relevant than previously conducted growth experiments.

When $N_2O$ was provided as an alternative electron acceptor, anaerobic respiration, and growth was sustained by reducing $N_2O$ to $N_2$. In this context, a recent report showed the ability of *B. japonicum* USDA110 to grow anaerobically using exogenous $N_2O$ as the sole electron acceptor (Sanchez et al., 2013). Growth with $N_2O$ as electron acceptor has also been observed in *Anaeromyxobacter* (Sanford et al., 2012), and in *Wolinella*, *Campylobacter,* and *Geobacillus* (Liu et al., 2008; Kern and Simon, 2009) indicating that the atypical *nosZ* encodes a functional respiratory terminal $N_2O$ reductase in those bacteria. This is unlike *Pseudomonas aeruginosa* PAO1, which cannot grow on exogenous $N_2O$ as the only electron acceptor (Bryan et al., 1985; Zumft and Kroneck, 2007).

It is generally considered that low oxygen concentration is a requirement for expression of the denitrification machinery (van Spanning et al., 2007). Especially the $N_2OR$ has been considered as a very $O_2$ labile reductase which is inactivated by the presence of low amounts of $O_2$ (Alefounder and Ferguson, 1982; Coyle et al., 1985; Snyder and Hollocher, 1987). In contrast to these observations, our results suggest that expression of $N_2OR$ in *E. meliloti* might be subjected to a different regulation, in which $N_2O$ reduction occurs even in the presence of oxygen concentrations above 8 µM (**Figure 3A**).

It has been reported that expression and fine-tuning of the denitrification system also requires the presence of key molecules such as $NO_3^-$, $NO_2^-$, and NO which, through transcriptional factors and their protein-coupled sensory receptors, act as signals that trigger induction of the denitrification pathway (Zumft and Kroneck, 2007; Spiro, 2012). Our results suggested that oxygen limitation was the only prerequisite for maximal expression of $N_2OR$ in *E. meliloti*, although we cannot exclude that $N_2O$ is also necessary. The presence of a NOx (NO, $NO_2^-$, $NO_3^-$) was however not required, since $N_2OR$ activity remained at similar levels in the absence or in the presence of $NO_3^-$ in wild-type cells. Furthermore, in cells cultured with $NO_3^-$, no differences in $N_2OR$ activity were observed between wild-type, and the

*napA* or *nirK* mutant strains where the reduction of $NO_3^-$ or $NO_2^-$ is blocked, respectively. In fact, previous studies of gene expression proposed that limited oxygen tension alone resulted in induction of the expression of the whole *nos* operon in *E. meliloti* (Bobik et al., 2006). In contrast to these findings, transcriptional profile analysis suggested that induction of *nosR* and *nosZ* gene expression also requires the presence of nitric oxide (Meilhoc et al., 2010). In line with this, recent studies using qRT-PCR showed that maximal transcription of the *E. meliloti nosZ* gene occurred when cells were subjected to anoxic conditions in the presence of nitrate (Torres et al., 2014). Similarly to our observations, it was recently reported that *P. denitrificans* is fully able to reduce $N_2O$ in the absence of oxyanions and NO (Bergaust et al., 2012). In contrast, it was proposed that the inability of *Pseudomonas aeruginosa* PAO1 and *Bacillus vireti* to grow on exogenous $N_2O$ as the only electron acceptor was because these organisms need NO as an inducer of *nosZ* transcription (Arai et al., 2003).

Our results clearly showed that *E. meliloti* 1021 was unable to express $N_2O$R activity at pH 6. This difficulty in expressing $N_2O$R at low pH was observed in *P. denitrificans* (Bergaust et al., 2010) and in suspensions of extracted soil bacteria (Liu et al., 2014). The phenomenon is ecologically important since there is ample evidence that low soil pH results in high $N_2O/N_2$ product ratios of denitrification (Raut et al., 2012; Qu et al., 2014).

Among the environmental factors that influence $N_2O$ emissions, and specifically the bacterial $N_2O$R performance, very little is known about the mode in which availability and redox state of C-sources contribute. In this work, the observed attenuated $N_2O$R activity in the presence of highly reduced C-sources could be attributed to a reduced capacity of cells to metabolize more complex C-substrates such as butyrate, causing a lowered electron flow through the respiratory chain, resulting in a reduced electron availability to reduce $N_2O$ to $N_2$ by the $N_2O$R (Morley and Baggs, 2011). Alternatively, a reduced efficiency to metabolize butyrate could be due to the fact that its uptake into cell probably requires active transport, and consequently cells may be subjected to periods of reduced $N_2O$R activity (Schalkotte et al., 2000). Supporting this hypothesis, it was found that $N_2O$R activity was stimulated in the presence of artificial root exudates with easily metabolized C-sources such as glucose, as well as in soils amended with carbohydrates as glucose and starch (Murray et al., 2004; Henry et al., 2008). In addition, a regulatory control on *nos* transcription could also explain the dependence of the $N_2O$R activity on the redox state of C-sources. In accordance with this, it was recently reported that expression levels of the *B. japonicum* NorC component of the nitric oxide reductase in wild-type cells, incubated in minimal medium with succinate as the sole C-source, were significantly higher than those observed in cells incubated in the presence of butyrate (Torres et al., 2011b). Similarly, expression of the *B. japonicum fixNOQP* genes, encoding the high affinity terminal oxidase $cbb_3$, decreased when butyrate was the sole carbon source compared to when malate was used (Bueno et al., 2009).

Taken together, these results showed a novel denitrifying phenotype in *E. meliloti* 1021, for which the reduction of $NO_3^-$, or $NO_2^-$ was severely impaired, while $N_2O$ was actively reduced. We further demonstrated that the reduction of $N_2O$ sustained growth by *E. meliloti* 1021. To our knowledge this is the first time that it was demonstrated the capacity of *E. meliloti* to sustain anoxic respiration by using $N_2O$ as terminal electron acceptor. Since the effect of pH or C-sources on $N_2O$ reductase activity has never been examined in rhizobia, the relevance of this study is to demonstrate that both environmental factors affect $N_2O$ reductase activity in the model alfalfa endosymbiont, *E. meliloti* 1021. Although this strain is a model organism and is not commercially used as inoculant for alfalfa, the results obtained here could be expanded to more competitive and efficient $N_2$-fixers inoculants in order to develop strategies to reduce $N_2O$ emissions from alfalfa crops. In fact, despite the large research efforts invested in flux measurement of $N_2O$ emissions, progress in developing efficient mitigation options has hitherto been slow. An essential objective should be to understand the underlying mechanisms and factors that affect the regulation of $N_2O$ consumption and production, and consequently to improve the product stoichiometry of denitrification ($N_2O/N_2O + N_2$) in terrestrial ecosystems.

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## Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00537/abstract

**Supplementary Figure S1 | Kinetics of $O_2$ depletion and $N_2O$, NO, and $N_2$ production.** *E. meliloti* 1021 was incubated in the presence of 10 mM $NO_3^-$ in minimal medium and an initial $O_2$ concentration of 2% in the headspace. Cultures with an initial $OD_{600}$ of 0.01 were vigorously stirred at 200 rpm. Plotted values are averages of three replicate flasks for each treatment, with standard deviation (SD) as vertical bars ($n = 3$).

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# Environmental and microbial factors influencing methane and nitrous oxide fluxes in Mediterranean cork oak woodlands: trees make a difference

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Cork oak woodlands (montado) are agroforestry systems distributed all over the Mediterranean basin with a very important social, economic and ecological value. A generalized cork oak decline has been occurring in the last decades jeopardizing its future sustainability. It is unknown how loss of tree cover affects microbial processes that are consuming greenhouse gases in the montado ecosystem. The study was conducted under two different conditions in the natural understory of a cork oak woodland in center Portugal: under tree canopy (UC) and open areas without trees (OA). Fluxes of methane and nitrous oxide were measured with a static chamber technique. In order to quantify methanotrophs and bacteria capable of nitrous oxide consumption, we used quantitative real-time PCR targeting the *pmoA* and *nosZ* genes encoding the subunit of particulate methane mono-oxygenase and catalytic subunit of the nitrous oxide reductase, respectively. A significant seasonal effect was found on $CH_4$ and $N_2O$ fluxes and *pmoA* and *nosZ* gene abundance. Tree cover had no effect on methane fluxes; conversely, whereas the UC plots were net emitters of nitrous oxide, the loss of tree cover resulted in a shift in the emission pattern such that the OA plots were a net sink for nitrous oxide. In a seasonal time scale, the UC had higher gene abundance of Type I methanotrophs. Methane flux correlated negatively with abundance of Type I methanotrophs in the UC plots. Nitrous oxide flux correlated negatively with *nosZ* gene abundance at the OA plots in contrast to that at the UC plots. In the UC soil, soil organic matter had a positive effect on soil extracellular enzyme activities, which correlated positively with the $N_2O$ flux. Our results demonstrated that tree cover affects soil properties, key enzyme activities and abundance of microorganisms and, consequently net $CH_4$ and $N_2O$ exchange.

**Keywords: Mediterranean, oak woodland, methane, nitrous oxide, enzymes, *pmoA*, *nosZ***

## INTRODUCTION

Carbon dioxide ($CO_2$),methane ($CH_4$), and nitrous oxide ($N_2O$) are the most important greenhouse gasses (GHG) responsible for global warming. Methane and nitrous oxide contribute 17 and 6% to total global warming (Myhre et al., 2013), respectively. Climate change scenarios for the Iberian Peninsula suggest drier conditions (an average decrease of 20% in precipitation during both winter and summer) and an increase of 40% of the inter-annual variability in the dry period (Meehl and Tebaldi, 2004; Lionello, 2007). This will modify hydrological regimes in Mediterranean-type ecosystems, including the soil's wet-dry cycles. In the last decades, a decline in cork oak (*Quercus* sp.) has been observed (AFN, 2010) with an increase in tree vulnerability to abiotic and biotic stresses (Garcia-Herrera et al., 2007). Severe and recurrent droughts, as well as intensified wet-dry cycles due to changing climate will alter physical and chemical soil properties, which in turn will affect soil microbiological communities and their activity. Fluctuations of wet-dry cycles have been suggested to have a mechanistic interaction on denitrification through oxygen mediated derepression kinetics, which can contribute to peak $N_2O$ emissions (Smith and Tiedje, 1979; Betlach and Tiedje, 1981). Moreover, soil moisture can alter the induction time of $CH_4$ oxidation in forest soils (Bender and Conrad, 1995). However, relatively little is known about the influence of wet-dry cycles on the fluxes of greenhouse gasses (GHGs) such as $CH_4$ and $N_2O$ in Mediterranean oak forests.

Methane consumption in upland soils is mainly driven by soil methanotrophs, which are unique in their ability to use $CH_4$ as carbon and energy sources (Hanson and Hanson, 1996). Methanotrophs are traditionally classified into Type I (aerobic Gammaproteobacteria) and Type II (aerobic Alphaproteobacteria) groups (Hanson and Hanson, 1996). Methanotrophs have the functional gene *pmoA*, which encodes a subunit of particulate methane monooxygenase (pMMO). This gene exists in all methanotrophs with the exceptions of *Methylocella* sp. and *Methyloferula* sp., which have soluble MMO (sMMO; Theisen et al., 2005; Vorobév et al., 2011). Therefore, MMO genes are widely used as a biological marker in molecular ecological studies of methanotrophs (McDonald et al., 2008). Methanotrophs are widely distributed in various environments: such as paddy soils (Bodelier et al., 2000), upland forest soils (Knief et al., 2006; Lau et al., 2007; Mohanty et al., 2007; Kolb, 2009), landfill soils, wetlands (Einola et al., 2007; Siljanen et al., 2011), alpine grassland soils (Abell et al., 2009), and extreme thermoacidophilic environments (Pol et al., 2007; Islam et al., 2008). Soil moisture is important for induction of $CH_4$ oxidation and regulation of $CH_4$ uptake in soil (Bender and Conrad, 1995; Shrestha et al., 2012). However, methanotrophs are poorly known in temporally dry Mediterranean soils and little is known about how wet-dry cycles influence methanotroph activity and abundance under different vegetation covers (Castaldi and Fierro, 2005; Castaldi et al., 2007; Shvaleva et al., 2014).

The main biological sources of nitrous oxide in soil are nitrification and denitrification processes catalyzed by archaea, bacteria, and fungi (Braker and Conrad, 2011; Thomson et al., 2012; Stieglmaier et al., 2014). Although archaeal nitrifiers and fungal denitrifiers have the ability to produce NO and $N_2O$, they lack the capacity for complete $N_2O$ reduction to $N_2$ (Shoun et al., 1992; Kim et al., 2009; Bartossek et al., 2010; Walker et al., 2010; Stieglmaier et al., 2014). Production of $N_2O$ in forest soils depends on soil characteristics [e.g., moisture, temperature, aeration, pH, soil organic matter (SOM), nitrogen availability] as well as tree species composition (Butterbach-Bahl and Papen, 2002; Skiba et al., 2009; Weslien et al., 2009).

Biological consumption of nitrous oxide in soil is catalyzed by nitrous oxide reductase (NOR) of denitrifying bacteria, which reduces $N_2O$ to $N_2$. Whether soil acts as a sink or a source of nitrous oxide depends on the balance of $N_2O$ production (nitrification and denitrification) and abundance and activity of denitrifying bacteria carrying NOR. In recent years, the *nosZ* gene, which encodes the catalytic subunit of NOR, has been used as a common molecular marker for analysis of abundance and diversity of denitrifiers capable of $N_2O$ consumption in soil (Rich et al., 2003; Horn et al., 2006). Novel clade of denitrifiers, recognized as atypical *nosZ* (Sanford et al., 2012) or *nosZ* clade II (*nosZ*-II; Jones et al., 2013), have been recently found to dominate over previously known denitrifiers (Jones et al., 2013). These novel *nosZ*-II carrying denitrifiers have been suggested to contribute significantly to $N_2O$ consumption/sink activities, since these genes can be correlated with an $N_2O$ sink (Jones et al., 2014) and a major part of the genomes of these organisms lack genes for $N_2O$ production (Sanford et al., 2012). However, their respective contribution to the consumption of atmospheric $N_2O$ is yet to be clearly established.

The heterotrophic soil microbial community is largely responsible for the mineralization of SOM (Bardgett et al., 2002) and availability of carbon and nitrogen regulating microbial processes behind the $CH_4$ and $N_2O$ fluxes. Soil extracellular enzymes play a critical role in SOM decomposition regulating both carbon storage and nutrient supply (Burns and Dick, 2002). Human disturbance and changes in climate can substantially alter the availability of soluble carbon and nitrogen in soil (Nermani et al., 2003). The dry periods represent a significant physiological stress for soil microbial communities (Fierer et al., 2003; Jensen et al., 2003; Gordon et al., 2008; Kardol et al., 2011) and their extracellular enzyme activities (EEAs; Sardans and Penuelas, 2012), which results in reduced SOM turnover and soil nutrient availability (Schmidt et al., 2004; Allison and Treseder, 2008). This can then affect the specific microbial processes driving $CH_4$ and $N_2O$ dynamics.

Previously, we showed that oak trees influence soil properties by increasing the input of litter fall (increase in SOM) which together with changes in soil water content (SWC) can affect net $CH_4$ and $N_2O$ exchange in Mediterranean type ecosystems (Shvaleva et al., 2014). We hypothesize here that trees may affect soil microclimate and prolong influences of wet-dry cycles due to decreased evaporation rates and water uptake from deeper soil layers, which may in turn affect soil extracellular enzymatic activities and therefore have an impact on the functioning of methanotrophs and denitrifying bacteria. The specific hypotheses were: (1) plant cover (cork oak trees) has an effect on abundance of methanotrophs and $N_2O$ consuming microbes and moreover

on $N_2O$ and $CH_4$ fluxes, and (2) in addition to the effect of plant cover, seasonal variation in weather (temperature and precipitation) have an effect on the abundance of methanotrophs and $N_2O$ consuming bacteria.

# MATERIALS AND METHODS

## Site Description

The experimental site was located in Herdade da Machoqueira do Grou (39°08′18.29″ N, 8° 19′57.68″ W), 30 km northeast of Coruche, Portugal. The region has a typical Mediterranean climate with hot and dry summers, and moderately cold and mild wet winters. Long-term average meteorological data for this area show that more than 80% of annual precipitation (*ca* 669 mm) occurs between October and May and mean annual temperature is ∼15.9°C (Inst. of Meteorology, Lisbon). The study site is a typical evergreen cork oak open woodland with tree stand age of 50 years and a density of 177 trees $h^{-1}$. The site is certified as *montado and is part of a long-term ecological research project (LTER-Montado)*, which guarantees sustainable management. The natural understory consists of Mediterranean shrub species such as *Cistus salviifolius* L., *Cistus crispus* L., *Lavandula stoechas* L., and *Ulex* spp. and grasses. Two different areas (*ca* 25 $m^2$ each) were used to study $CH_4$ and $N_2O$ fluxes, soil properties and abundance of soil microbial communities. These areas were established in the natural understory: under projection of tree crowns (under canopy, hereafter named as UC area); and in large OAs not under projection of tree crowns (hereafter named as OA area). The soil is Cambisol (FAO). The distance between study areas was *ca* 100 m. Standard meteorological data for rainfall (ARG100, Environmental Measurements Ltd., Gateshead, UK), air humidity and temperature (CS215, Campbell, Inc., Logan, UT, USA) were collected over the study period at 30 min intervals and stored using a data logger (CR10X, Campbell Scientific, Inc., Logan, UT, USA).

## Soil Sampling and Temperature

Samples used for determination of seasonal heterogeneity of soil chemical and physical properties and abundance of microbial communities capable of $CH_4$ and $N_2O$ consumption were taken in 2011, May 23rd (end of spring rains), August 31st (dry extreme conditions), October 26th (after the first autumn rain event since August), November 9th (wet extreme) and December 15th (stabilized wet conditions) from triplicated study plots in the UC and OA areas. Soil cores (height 20 cm, diameter 2 cm) were collected from four randomly selected points in the UC and OA areas. For EEA determination, soil samples were additionally taken on July 6th, October 20th, and October 27th in order to increase the power of principal component analysis (PCA). Soil samples were packed in plastic bags and transported to the laboratory in an ice-cooled box. Soil samples for molecular biological analyses were immediately stored at −80°C. Soil temperature at 5 cm depth was measured near to soil gas flux collars by using a digital thermometer. The sample collection was always performed between 09:00 and 13:00 h.

## Soil Chemical Characteristics (C, N, P, SOM, pH, and Electrical Conductivity)

Soil samples for chemical analyses were first sieved (1 mm mesh) and then separated into three parts. One part was used to determine gravimetric SWC (%) by assessing weight loss after drying at 105°C for 24 h. A second part was used to determine nitrate ($NO_3^-$) and ammonium ($NH_4^+$) concentrations by spectrophotometry as described in Fangueiro et al. (2008). The third part of the soil samples was air-dried and analyzed for total soil organic carbon according to Nelson and Sommers (1996) using an Infrared Detection Promacs TOC Analyser (Skalar, Netherlands). SOM content was determined from the soil carbon data using the conventional Van Bemmelen factor of 1.72, i.e., SOM (%) = soil carbon (%) × 1.72 (Nelson and Sommers, 1996). Total nitrogen in the soil was quantified by the Kjeldahl method (Horneck and Miller, 1998), and total phosphorous was determined by the Egner–Rhiem method (Carreira and Lajtha, 1997) using molecular absorption spectrophotometry (Hitachi 2000, Tokyo, Japan). Soil pH was determined in a soil-water suspension (1:10, w/v) with a selective electrode (Micro pH 2001, Criston). Soil electrical conductivity (EC) was measured in a soil-water suspension (1:5, w/v), as described in Fangueiro et al. (2008).

## Enzyme Assays

Soil samples preserved at 4–6°C were used to determine EEA applying photometric and fluorometric micro-plate assays described by Pritsch et al. (2011). Seven EEA were measured, i.e., 1.4-β-xylosidase (Xyl, EC: 3.2.1.37) in presence of MU-xyloside; β-glucuronidase (Glr, EC: 3.2.1.31) in presence of MU-glucoronide; 1,4-β-cellobiosidase (Cel, EC: 3.2.1.91) in presence of MU cellobioydrofuran; *N*-acetyl-β-D-glucosaminidase (Nag, EC: 3.2.1.14) in presence of MU-*N*-acetylglucosamine; β-glycosidase (Gls, 3.2.1.21) in presence of MU-β-glycoside, acid phosphatase (Pho, EC: 3.1.3.2) and laccase (Lac, EC: 1.10.3.2) in presence of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ABTS. The main functions of these enzymes are listed in Supplementary Table S2.

## Tree Litter Fall and Root Density

Tree litter fall was determined as described in Shvaleva et al. (2014) with 16 litter baskets placed in two transects across the site with periodic sampling throughout 2011. Root density (dry mass $m^{-2}$) of soil was determined from triplicate soil samples of 0.2 m × 0.2 m × 0.2 m, collected in October 2011. In the laboratory, roots were separated from the soil, washed, and dried at 65°C for 48 h.

## Soil GHG Flux Measurement

Soil-atmosphere net GHG fluxes were measured from six cylindrical collars randomly installed in both UC and OA areas (three replicated study plots per area/treatment). Cylinder collars (polypropylene cylinders, Technical University of Lisbon, Portugal) of 0.3 m diameter were placed at 0.1 m depth into the soil, giving a headspace volume of 0.010 (±0.001) $m^3$. The collars were closed with a stainless-steel lid fitted with sample ports

(0.006 m diameter), which could be closed and opened by lock valves. The distance between replicates in UC and OA areas was *ca.* 5 m. Flux measurements were done as described in Shvaleva et al. (2014). The chamber was closed at time 0, and samples were taken immediately, at 30 min and after 60 min. Samples of 100 mL were taken from the chambers using a plastic syringe and stored in 20 mL gas vials stopped with butyl rubber septa. Nitrous oxide and $CH_4$ concentrations were analyzed at CEH (Edinburgh, UK) by a gas chromatograph (GC, HP5890 Series II, Hewlett Packard, Agilent Technologies UK Ltd., Stockport, UK) fitted with an electron capture detector (ECD) and a flame ionization detector (FID) for $N_2O$ and $CH_4$ analysis, respectively. The flux was calculated based on the slope of a linear regression fitted on data over the measurement time. Calibration of GC was performed with four standard gasses (concentration range: 0.205–1.008 ppm for $N_2O$ and 1.26–100.9 ppm for $CH_4$). GC precision was calculated based on standard gas measurements ($N = 2$–6, depending on number of samples in the GC run). Precision of $N_2O$ and $CH_4$ standards for each four standard gas concentration of all GC runs was $\pm 7$ ppb ($N = 44$) for $N_2O$ and $\pm 70$ ppb ($N = 44$) for $CH_4$. Minimum detectable fluxes based on precision of GC were 0.94 $\mu$g $N_2O$-N $m^{-2}$ $h^{-1}$ for $N_2O$ fluxes and, 11.11 $\mu$g $CH_4$-C $m^{-2}$ $h^{-1}$ for $CH_4$ fluxes with 60 min timescale in chamber volume of 0.010 $m^3$ and at 20°C temperature. Discarding these small fluxes (production or consumption) below minimum detectable fluxes would have lead on average to 220 and 53% overestimation of $CH_4$ and $N_2O$ fluxes, respectively. Nitrous oxide and $CH_4$ fluxes were compared to each other by calculating $CO_2$-equivalent values for both $CH_4$ and $N_2O$ fluxes for making overall comparison of both processes easier. This comparison was made based on radiative forcing of these gasses over 100 years time horizon, factor for $CH_4$ was 34 and 298 for $N_2O$ (Myhre et al., 2013).

## Soil DNA Extraction and Purification

Freeze-dried mortar-homogenized 100 mg soil (stored at −80°C) was used for DNA extraction as described in Siljanen et al. (2011) with slight modification. In brief, after phenol/chloroform/isoamylalcohol extraction, DNA was brownish and therefore it was further purified with PEG6000/NaCl precipitation as previously described by Griffiths et al. (2000). After purification DNA was eluted with 50 µl TE-buffer (Tris-Cl 10 mM, EDTA 1 mM, pH 8.0) and stored at −20°C.

## Quantitative PCR

Presence of PCR inhibiting substances were analyzed by dilution series of extracted DNA with Bacterial 16S rRNA quantitative PCR. It was shown that PCR reaction was not inhibited by undiluted DNA thus samples were used in further analyses. Supplementary Table S3 shows the complete list of primers and conditions used for quantification of microbial communities running $CH_4$ and $N_2O$ consumption. Primer combination A189q (5′-GGNGACTGGGACTTCTGG-3′) and Mb601 (5′-ACRTAGTGGTAACCTTGYAA-3′) targeting *pmoA* gene of Type Ia methanotrophs produced PCR product successfully. For analysis of nitrous oxide consuming bacteria primers targeting *nosZ* genes, nosZ2F (5′-CGCRACGGCAASAAGGTSMSSGT-3′) and nosZ2R (5′-CAKRTGCAKSGCRTGGCAGAA-3′; Henry et al., 2006) primers were used. Both genes were amplified with previously published cycling conditions with Bio-Rad iCycler iQ (Kolb et al., 2003; Henry et al., 2006). Reaction mixtures contained 2x Maxima SYBR Green master mix (Thermo Scientific) and 1 µM of each primer. The quantification of *pmoA* genes was done with cloned fragment of *pmoA* gene according to Siljanen et al. (2011). For quantification of *nosZ* gene genomic DNA of *Pseudomonas aeruginosa* was used. Quantification of both genes was based on a standard curve using 10-fold diluted positive control. Detection limits of qPCR assays were determined from dilution series of positive-control DNA (for *pmoA* $10^8$ to $10^1$ and for *nosZ* $10^6$ to $10^1$) target molecules per reaction. A minimum sensitivity of $10^1$ to $10^2$ target molecules per reaction for each assay was achieved. Amplified PCR products were confirmed by sequencing small clone libraries for both assays.

## Statistical Analyses

A mixed-effect model was used to evaluate the difference of measured variables between UC and OA areas over the timescale studied as previously described in Siljanen et al. (2012). When the data were not normally distributed, they were either square root transformed prior to analysis or non-parametric tests were carried out by performing a comparison on ranks and using Dunn's test was used for *post hoc* pairwise comparisons. The Pearson Product Moment Correlation coefficient was used to display the strength of the association between pairs of variables. All statistical relationships were considered significant at $P < 0.05$. Statistical analyses were carried out using SigmaStat (SigmaPlot for windows V 11, Dundas Software, Germany), SPSS 17.0 (SPSS, Inc., USA) and R statistical program (R Core Team, 2013).

# RESULTS

## Soil Properties

In 2011 the total annual precipitation was 883 mm and the average air temperature 15.5°C. August was an extremely dry (only 8 mm precipitation) and warm month (Supplementary Table S1). In October, mean air temperature (21°C) was higher than the long-term (1970–2000) average (16°C). Summer conditions extended until mid-October (first rain events occurred on DOY 296 – October 22nd). SWC at 10 cm depth ranged from 2 to 19.5% in the UC and from 0.6 to 16% in the OA (**Figure 1A**). The UC soil was significantly wetter ($P < 0.001$) than the OA soil in May, August, and November (**Table 1**). Soil temperature recorded in the upper 0.05 m varied between 13.7 and 23°C in the UC and between 12.7 and 27.9°C in the OA. The UC had lower soil temperatures than in the OA in May and August, but in December the reverse was true (**Table 1**).

SOM content in May, August, and December was higher in the UC than in the OA (**Table 1**). The presence of trees in the UC provided twice the input of dry mass $m^{-2}$ (litter fall) compared

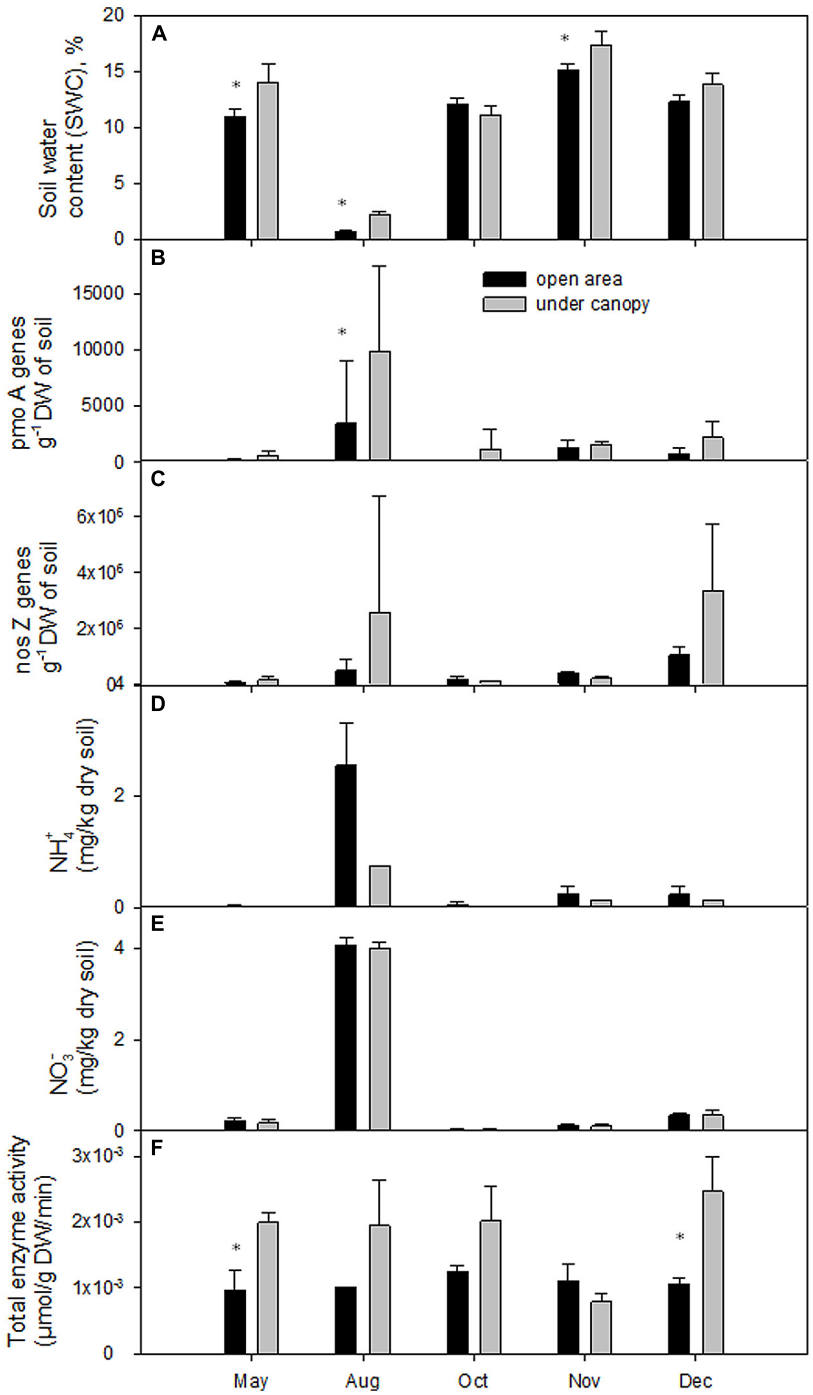


**FIGURE 1 | (A)** Soil water content (SWC,%), **(B)** *pmoA* gene abundance, **(C)** *nosZ* gene abundance, **(D)** soil $NH_4^+$ – N) concentration, **(E)** soil $NO_3^-$ – N concentration, and **(F)** total activities of studied enzymes in the 0–20 cm soil layers of the study areas. Statistical significance ($P < 0.05$) is shown with asterisk.

to the OA (290 g DW $m^{-2}$ $y^{-1}$ vs. 140 g DW $m^{-2}$ $y^{-1}$) and more than twice the root density in the OA (693 ± 70 g DW $m^{-2}$ $y^{-1}$ vs. 314 ± 58 g DW $m^{-2}$ $y^{-1}$). Similarly to SOM, soil electrical conductivity in UC was higher in May, August, and December compared to that in the OA (**Table 1**). No significant differences in soil pH between UC and OA areas were found. Soil total organic carbon and phosphorus ($P_2O_5$) contents were variable and ranged from 1 to 5.8%, and from 4.2 to 30.6 mg $kg^{-1}$ DW, respectively; these contents in the UC area were significantly higher in May, August, and December (**Table 1**). No differences in total soil N, and content of $NO_3^-$ – N and $NH_4^+$ – N content between the UC and OA were observed.

**TABLE 1 | Statistical significance of the effect of site [under canopy (UC) vs. OA as determined by pair wise comparison] on different soil related parameters: SWC (%), soil temperature, pH, soil organic matter (SOM), soil C/N ratio, carbon (C), nitrogen (N), phosphors ($P_2O_5$), electrical conductivity (EC), $NH_4^+$ – content, $NO_3^-$ – content, ß-glycosidase (Gls), cellobiosidase (Cel), glucuronidase (Glr), glucosaminidase (Nag), phosphatase (Pho), xylosidase (Xyl), total enzymes, *pmoA* gene, *nosZ* gene during the study period.**

| Parameters significance of effect (site/time) | May | August | October | November | December |
|---|---|---|---|---|---|
| SWC ***/*** | + *** | + *** | ns | + * | ns |
| Soil temperature */*** | − *** | − *** | ns | ns | + * |
| pH | ns | ns | ns | ns | ns |
| SOM ***/*** | + ** | + ** | ns | ns | + * |
| C/N | ns | ns | ns | ns | ns |
| C */** | + ** | + * | ns | ns | + * |
| N **/– | ns | ns | ns | ns | + * |
| $P_2O_5$ */– | + * | + * | ns | ns | + * |
| EC */*** | + ** | + ** | ns | ns | + * |
| $NH_4^+$ –/*** | ns | ns | ns | ns | ns |
| $NO_3^-$ –/*** | ns | ns | ns | ns | ns |
| Gls | ns | ns | ns | ns | ns |
| Cel | + * | ns | ns | ns | ns |
| Glr –/** | + * | ns | ns | ns | ns |
| Nag | ns | ns | ns | ns | + ** |
| Pho */* | + * | − * | ns | ns | + ** |
| Xyl –/* | + ** | ns | ns | − ** | ns |
| Total enzymes | + ** | ns | ns | ns | + ** |
| *pmoA* gene –/** | ns | + * | ns | ns | ns |
| *nosZ* gene –/* | ns | ns | ns | ns | ns |
| $CH_4$ flux –/* | ns | ns | + * | ns | − * |
| $N_2O$ flux */* | ns | ns | ns | ns | + * |

*Symbols: *,**,*** represent statistical significance at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively; and ns is not significant at $P = 0.05$, the "tree effect" is shown with +, if under canopy have higher value than open area, and − shows if the under canopy has lower value than the open area. Statistically significant differences of sites and timepoints across all sampling dates are marked with asterisk beside parameter name.*

## Quantification of *pmoA* and *nosZ* Genes

The methanotrophic *pmoA* gene abundance was detected throughout the study period in the UC and OA areas and ranged from $3 \times 10^2$ to $16 \times 10^3$ *pmoA* genes $g^{-1}$ DW and from $8 \times 10^1$ *pmoA* genes $g^{-1}$ DW to $10 \times 10^3$ *pmoA* genes $g^{-1}$ DW, respectively. In the UC site Type Ia *pmoA* gene copy numbers were more than 10 times higher in August compared to other periods of study (**Figure 1B**). Under the extreme dry conditions encountered in August, the abundance of methanotrophs in the UC was significantly higher than in OA (($P < 0.05$, **Table 1**). Our data showed positive correlations between *pmoA* gene abundance and soil $NH_4^+$ content in OA (Pearson's $r = 0.521$, $P < 0.05$) and in UC with $NO_3^-$ ($r = 0.65$, $P < 0.01$) content, i.e., the number of methanotrophs increased with increasing mineral nitrogen content (**Figures 1D,E**). Moreover, a negative correlation was observed between *pmoA* gene abundance and $CH_4$ flux ($r = -0.54$, $P < 0.05$) in the UC and with total nitrogen ($r = -0.52$, $P < 0.05$) in the OA.

Quantitative PCR with primers q189A/Mb601 targeting Type Ia methanotrophs was the only assay producing PCR products successfully. Other phylogenetic methanotroph groups (MOB amplified in nested PCR with A189/A682/mb661 primers and quantitative PCR with USCα, Type Ib, Type II and *Methylocella* sp. primers) showed only negligible PCR products.

The *nosZ* gene abundance in the UC and OA varied in range from $6 \times 10^4$ to $7.3 \times 10^6$ *nosZ* genes $g^{-1}$ DW and from $1 \times 10^5$ to $1.3 \times 10^6$ *nosZ* genes $g^{-1}$ DW, respectively (**Figure 1C**). Under summer drought (August) and stabilized wet conditions in winter (December, SWC around 15%) the number of *nosZ* gene abundance increased in the UC more than 18 times compared to other seasons. However, no differences in the *nosZ* gene abundance between the UC and OA were observed during the study. A negative correlation between *nosZ* gene abundance, and $N_2O$ flux ($r = -0.59$, $P < 0.05$) was observed in the OA site, but not in the UC site.

## Soil Enzyme Activities

Total enzyme activities were significantly higher in the UC area in May and December (**Figure 1F**). Enzyme activities did not correlate with gene copy numbers or $CH_4$ fluxes, but correlated with $N_2O$ fluxes. In the UC area, $N_2O$ flux correlated positively with total enzyme activity ($r = 0.60$, $P < 0.05$), with Glucuronidase activity ($r = 0.58$, $P < 0.05$), with Glucosaminidase activity ($r = 0.67$, $P < 0.01$), and with phosphatase activity ($r = 0.56$, $P < 0.05$), whereas in OA site, $N_2O$ fluxes had a positive correlation with phosphatase activity ($r = 0.58$, $P < 0.05$).

## Soil Net $CH_4$ and $N_2O$ Fluxes

Results showed that the soil acted mainly as a net sink for $CH_4$, however there were also periods of net $CH_4$ emissions. During the study period $CH_4$ fluxes ranged from −12.3 to 8.6 $\mu g\ C\ m^{-2}\ h^{-1}$. Methane emissions were observed in May in both UC and OA, and in August in the OA only (**Table 2**). The difference in $CH_4$ flux between areas was highest in October, when the $CH_4$ uptake in the OA was higher than in the UC, and in December, when on the contrary, $CH_4$ uptake in the OA was lower than in the UC. However, the tree cover had not a general effect on $CH_4$ flux when all time-points were included to the analysis (Mixed-effect model: $d.f._1 = 1$, $d.f._2 = 20$, $P = 0.655$). Methane fluxes correlated positively with soil temperature both in the OA ($r = 0.75$, $P < 0.01$), and UC areas ($r = 0.79$, $P < 0.001$). Methane fluxes also correlated positively with organic matter ($r = 0.55$, $P < 0.05$), CN-ratio ($r = 0.57$, $P < 0.05$) and total carbon ($r = 0.67$, $P < 0.01$) in the UC area. Mean $CH_4$ fluxes, shown as $CO_2$-equivalent fluxes were not different between areas (**Table 2**).

There was both net uptake and net release of $N_2O$ occurring and the flux varied from −6.5 to 6 $\mu g\ N_2O\text{-}N\ m^{-2}\ h^{-1}$ (**Table 2**). The most pronounced difference between areas was observed in December when the UC had $N_2O$ release but the OA showed $N_2O$ uptake. The tree cover had a general effect on $CH_4$ flux when

**TABLE 2 | Soil $CH_4$ ($\mu g\ CH_4$-C $m^{-2}h^{-1}$) and $N_2O$ ($\mu g\ N_2O$-N $m^{-2}h^{-1}$) fluxes measured at the study site from May to December, 2011.**

| Fluxes | Site | May | August | October | November | December | Mean of fluxes ($CO_2^{eq}$) |
|---|---|---|---|---|---|---|---|
| $CH_4$ [$\mu g\ CH_4$-C $m^{-2}h^{-1}$] | OA | 2.56 ± 4.4 | 1.09 ± 0.43 | −12.75 ± 0.99 [a] | −4.71 ± 0.32 | −6.68 ± 0.89 [a] | −4.10 ± 0.60 (−217 ± 32) |
| | UC | 2.22 ± 3.39 | −1.1 ± 0.57 | −8.29 ± 3.3 [b] | −5.69 ± 0.57 | −11.09 ± 0.73 [b] | −4.78 ± 1.21 (−186 ± 16) |
| $N_2O$ [$\mu g\ N_2O$-N $m^{-2}h^{-1}$] | OA | −0.77 ± 0.04 | 0.22 ± 0.48 | 0.78 ± 2.08 | −2.41 ± 0.63 | −5.5 ± 0.93 [a] | −1.54 ± 0.76 [a] (−720 ± 204) |
| | UC | −0.55 ± 0.34 | 1.35 ± 0.99 | −0.66 ± 1.32 | −1.4 ± 0.94 | 4.42 ± 1.12 [b] | 0.59 ± 0.64 [b] (276 ± 174) |

*Carbon dioxide equivalents ($CO_2^{eq}$, $\mu g\ CO_2^{eq}\ m^{-2}h^{-1}$) were calculated by multiplying the flux with Global Warming Potential in time-horizon of 100 years ($CH_4$ = 34; $N_2O$ = 298, Myhre et al., 2013). Values are mean ± SE (n = 3). December, 2011. Statistical significant differences (P < 0.05) between OA and UC area is shown with different letters.*

all time-points were included in the analysis (**Table 2**; Mixed-effect model: $d.f._1 = 1$, $d.f._2 = 20$, $P < 0.05$). Nitrous oxide fluxes correlated negatively with *nosZ* gene abundance ($r = -0.59$, $P < 0.05$) and soil pH ($r = 0.65$, $P < 0.01$), and positively with soil temperature ($r = 0.57$, $P < 0.05$) in OA. In the UC area, $N_2O$ fluxes correlated positively with total enzyme activity ($r = 0.60$, $P < 0.05$), with glucuronidase activity ($r = 0.58$, $P < 0.05$), with glucosaminidase activity ($r = 0.67$, $P < 0.01$), and with phosphatase activity ($r = 0.56$, $P < 0.05$). In the OA area $N_2O$ fluxes correlated positively with phosphatase activity ($r = 0.58$, $P < 0.05$). $N_2O$ fluxes in the UC area shown as $CO_2$-equivalents was higher than that in OA area (**Table 2**).

## DISCUSSION

The cork oak trees had a significant effect on soil properties and subsequent soil EEAs, on the abundance of microbes, and finally on the non-$CO_2$ net GHG fluxes. In this study soil $CH_4$ uptake was generally activated in autumn when soil moisture was higher and temperature lower than in summer. Trees are known to affect soil $CH_4$ consumption, but whether this is due to tree effects on microbial $CH_4$ oxidation or soil gas diffusivity is not known (Menyailo, 2007; Menyailo et al., 2010). Oak canopy increased soil moisture, which could explain the stronger negative correlation found between methane fluxes and *pmoA* gene abundance in the UC area compared to the OA area. It is possible, that the dryness in the OA area limited the activity and growth of methanotrophs. Thus, even at the highest water content, moisture did not limit the activity of methanotrophs indicating good availability of oxygen and methane. SWC and associated gas diffusivity are known to affect abundance and activity of methanotrophs (Borjesson et al., 2004; Einola et al., 2007). However, there is evidence for the presence of anaerobic microsites in the studied soils because net $CH_4$ emissions were also observed, showing that in some moisture and temperature conditions $CH_4$ production (activity of methanogens) exceeded $CH_4$ oxidation (activity of methanotrophs). The net release of $CH_4$ correlated positively with temperature and soil organic matter and carbon indicating that these factors favored methanogens over methanotrophs. However, Type Ia methanotrophs especially in the UC areas had a significant role in reducing of $CH_4$ emissions and in the consumption of atmospheric $CH_4$ since their abundance was affected by seasonal variation and correlated with $CH_4$ efflux. Input of organic carbon by trees in UC area increased $CH_4$ cycling, and therefore a positive correlation in UC area but not in OA area can be explained. An increase in organic matter supports the activity of heterotrophic microbes as seen here by the higher enzyme activities in the UC area as compared to the OA area. It is likely that the availability of low molecular weight organic substrates needed for methanogenesis was higher in the UC area resulting from the higher enzyme activities found there. An increase in soil temperature further supported net $CH_4$ release in the present study. This is associated with higher microbial decomposition processes and oxygen consumption at higher temperatures, which can create anaerobic microsites in the clay-rich soil. It is noteworthy that $CH_4$ fluxes in the UC and OA areas did not differ much. We would expect higher $CH_4$ production in UC area rather than in OA area. Evidently the higher $CH_4$ oxidation in the UC area discussed above counteracted the possible higher $CH_4$ production there.

Methanotrophs in the study site belonged to Type Ia methanotrophs. Type I methanotrophs are usually found in extreme conditions where competition survival strategy supports their fast response to improved substrate availability (Ho et al., 2013). Moreover, Type I methanotrophs grow in a wide temperature range, from thermophilic (Bodrossy et al., 1997; Tsubota et al., 2005) to psychrophilic (Liebner et al., 2009; Graef et al., 2011) conditions. In this site, soil temperature varied substantially from 12.7 to 27.9°C, which could favor the occurrence of Type I methanotrophs over the other types. In addition to the temperature related selection, potential internal methane source in the soil as reflected as $CH_4$ emissions, might have selected for presumably low affinity Type I methanotrophs in this site. However, the PCR assay used for USC(α) methanotrophs (Kolb et al., 2003) might not have recognized all high-affinity atmospheric $CH_4$ oxidizers living in this site. These methanotrophs could have been detected more recently generated primer set with broader specifity for USC(α) (Degelmann et al., 2010).

Nitrous oxide uptake from the atmosphere has been explored in few reports even under dry conditions when gas diffusivity is good (Rosenkranz et al., 2006; Goldberg and Gebauer, 2009). In theory, the dry conditions when oxygen availability is high should not support nitrous oxide reduction (Morley et al., 2008). Rosenkranz et al. (2006) linked negative fluxes in Mediterranean forest soil to very low N availability and high soil C content, and considered aerobic denitrification by heterotrophic denitrifiers as a possible pathway for $N_2O$ uptake. In our soil, higher soil moisture, higher *nosZ* gene abundance, higher total enzyme activities, and higher $N_2O$ fluxes (emissions) were concurrent

within UC area. Mineralization of SOM and exudates from tree roots in the UC area produced more soluble carbon to fuel denitrification. However, nitrate content was similar in both areas. We have no data on nitrification activity and nitrate uptake by plants, which hampers a concise conclusion about the nitrate turnover and availability in soils. In the OA area there was a positive correlation between $N_2O$ fluxes and *nosZ* gene abundance in contrast to the UC area. The primer set used for enumeration of *nosZ* genes did not cover *nosZ*-II genes. However, the typical $N_2O$ consuming *nosZ* genes detected in our study had a significant role in $N_2O$ consumption, since their abundance was correlated with $N_2O$ flux and affected by seasonal variation. We observed a positive correlation between SOM input in the UC area and catalase activity of four studied enzymes that degrade SOM and provide energy (C) and nutrients (N and P) for ecosystem functioning. These catalases also correlated positively with $N_2O$ flux in UC area. Since denitrifiers require organic carbon for growth, a correlative link between $N_2O$ flux and enzyme activities can be explained by their heterotrophic lifestyle.

Moreover, in this study $N_2O$ uptake was correlated with lower EEA, lower C and N supply and lower soil moisture. Positive correlations between $N_2O$ fluxes and soil enzyme activities, especially in UC area, could be explained by higher SOM input into UC area. However, in the UC area with higher water content and substrate availability for denitrification, more of the produced $N_2O$ could be reduced to $N_2$ and therefore gene abundance of *nosZ* did not reflect the overall denitrification. The EEAs are not connected directly to metabolism of nitrous oxide or bacterial denitrification. However, EEAs may provide a clue about the soil microbial activity in general, which is correlated with nitrous oxide fluxes. These correlations need to be evaluated critically since these linkages may be simply co-incidental without a real metabolic connection to each other. The impact of trees on soil properties (SWC, SOM, litter fall, root density) and a strong positive correlation between SOM and both $CH_4$ and $N_2O$ effluxes were previously reported (Shvaleva et al., 2014). The current study was able to link the abundance of methanotrophs with $CH_4$ fluxes in UC area, and the abundance of $N_2O$ consuming bacteria in OA area.

Nitrous oxide uptake was detected in 60% of all studied time-points. While measuring such small fluxes close to the detection limit of the gas chromatograph used, it is important to evaluate if the equipment is sensitive enough to detect $N_2O$ uptake. The GC and detectors used were accurate enough to measure such small $N_2O$ fluxes. Most of measured $N_2O$ fluxes were above minimum detectable flux. However, the measurements performed for non-$CO_2$ GHG fluxes didn't cover whole ecosystem GHG fluxes including processes in the phyllosphere. The tree stand itself contributes to the GHG balance by $CO_2$ sequestration through photosynthesis. In addition, trees are a transpiration channel from soil to atmosphere and it has been shown that plants are capable of $CH_4$ emissions (Keppler et al., 2006; Carmichael et al., 2014) and in some circumstances $CH_4$ uptake is possible by plants (Sundqvist et al., 2012). Moreover, $N_2O$ emissions from plants were reported recently, with a rate comparable with soil $N_2O$ emissions, by ammonia oxidizing bacteria on leaf surfaces (Bowatte et al., 2015). Therefore our measured soil-related non-$CO_2$ GHG balances between UC and OA areas might be underestimated, and we can't be completely certain of the total balance of all GHG produced and consumed in these sites. Similar non-$CO_2$ GHG balances were also earlier examined in this same study-site (Shvaleva et al., 2014). However, earlier in another montado site higher $CH_4$ uptake compensated $N_2O$ emission, which kept non-$CO_2$ balance negative (Shvaleva et al., 2011). This emphasizes spatial and seasonal variation of GHG effluxes in montado ecosystems. However, if future climatic conditions support tree decline, soil related nitrous oxide emissions might be reduced from Mediterranean montado ecosystems, provided that understory vegetation and soil conditions remain similar to OA area.

## CONCLUSION

Oak tree cover had an effect on soil properties, soil enzymatic activities, and the abundance of $CH_4$ and $N_2O$ metabolizing bacteria and as a consequence, on $CH_4$ and $N_2O$ fluxes. Correlation between soil-atmosphere $CH_4$ exchange and abundance of Type I *pmoA* genes under tree canopies, and correlation between $N_2O$ exchange and abundance of *nosZ* genes in OAs suggests that these microbial groups may contribute to most of the gasses consumed in evergreen oak woodlands. Oak trees exert these effects on a functional group of soil micro-organisms through the complex interactions between plants, microorganisms, and soil characteristics (SWC, SOM, root density, litter fall, and enzyme activities). Our results suggest that oak tree vegetation does not change mean soil $CH_4$ uptake, but significantly increases mean $N_2O$ fluxes and this neutralizes the soil non-$CO_2$ uptake in Mediterranean oak forests, and it can even turn the soil non-$CO_2$ GHG balance from negative to positive when compared to non-oak tree vegetated surfaces.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01104

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# Drying-Rewetting and Flooding Impact Denitrifier Activity Rather than Community Structure in a Moderately Acidic Fen

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Wetlands represent sources or sinks of the greenhouse gas nitrous oxide ($N_2O$). The acidic fen Schlöppnerbrunnen emits denitrification derived $N_2O$ and is also capable of $N_2O$ consumption. Global warming is predicted to cause more extreme weather events in future years, including prolonged drought periods as well as heavy rainfall events, which may result in flooding. Thus, the effects of prolonged drought and flooding events on the abundance, community composition, and activity of fen denitrifiers were investigated in manipulation experiments. The water table in the fen was experimentally lowered for 8 weeks in 2008 and raised for 5.5 months in 2009 on three treatment plots, while three plots were left untreated and served as controls. *In situ* $N_2O$ fluxes were rather unaffected by the drought treatment and were marginally increased by the flooding treatment. Samples were taken before and after treatment in both years. The structural gene markers *narG* and *nosZ* were used to assess possible changes in the nitrate reducer and denitrifier community in response to water table manipulations. Detected copy numbers of *narG* and *nosZ* were essentially unaffected by the experimental drought and flooding. Terminal restriction fragment length polymorphism (TRFLP) patterns of *narG* and *nosZ* were similar before and after experimental drought or experimental flooding, indicating a stable nitrate reducer and denitrifier community in the fen. However, certain TRFs of *narG* and *nosZ* transcripts responded to experimental drought or flooding. Nitrate-dependent Michaelis-Menten kinetics were assessed in anoxic microcosms with peat samples taken before and 6 months after the onset of experimental flooding. Maximal reaction velocities $v_{max}$ were higher after than before flooding in samples from treament but not in those from control plots taken at the same time. The ratio of $N_2O$ to $N_2O + N_2$ was lower in soil from treatment plots after flooding than in soil from control plots, suggesting mitigation of $N_2O$ emissions by increased $N_2O$-reduction rates after flooding. $N_2O$ was consumed to subatmospheric levels in all microcosms after flooding. The collective data indicate that water table manipulations had only minor effects on *in situ* $N_2O$ fluxes, denitrifier abundance, and denitrifier community composition of the acidic fen, while active subpopulations of denitrifiers changed in response to water table manipulations,

suggesting functionally redundant subpopulations occupying distinct ecological niches in the fen.

**Keywords: water table manipulation, climate change, wetlands, greenhouse gases, structural (functional) genes**

## INTRODUCTION

Peatlands cover about 3% of the earth's surface, are particularly important in mid- and high-latitudes, and store significant amounts of carbon and nitrogen (Gorham, 1991). Peatlands are sources and potential sinks of greenhouse gases such as methane ($CH_4$) and nitrous oxide ($N_2O$) (Christensen et al., 2003; Goldberg et al., 2008; Kolb and Horn, 2012). $N_2O$ is a major ozone-depleting substance and has a 300x higher global warming potential than $CO_2$, (Ravishankara et al., 2009). $N_2O$ emissions from peatland soils are controlled by microorganisms. In water saturated systems, $N_2O$ is almost exclusively produced by denitrification [i.e., the sequential reduction of nitrate and/or nitrite via nitric oxide (NO) to $N_2O$ and $N_2$; Zumft, 1997]. Nitrate or nitrite are used as terminal electron acceptors by denitrifiers, and are supplied to peatlands by aerial precipitation, surface runoff, groundwater inflow, or nitrification in oxic zones (Conrad, 1996; Mosier et al., 1998; Goldberg et al., 2010; Lohila et al., 2010; Palmer et al., 2010). The extent of the oxic zone, and thus the magnitude of the nitrification process as a substrate producer for denitrification, is largely dependent on the water table level in peatlands (Lipson et al., 2012). Even though many pristine peatlands are net sources of $N_2O$, (water-saturated) peatlands can be temporary sinks for $N_2O$ when nitrate/nitrite availability is low (Goldberg et al., 2008; Lohila et al., 2010; Palmer et al., 2010; Marushchak et al., 2011; Kolb and Horn, 2012; Palmer and Horn, 2012, 2015).

Peatland ecosystems are thought to be severely affected by future climate change (Gorham, 1991; Gong et al., 2012). Climate change is associated with increasing mean annual temperatures and an increased frequency of extreme weather events like prolonged dry periods and heavy rainfalls (Hartmann et al., 2013), which have the potential to lower and raise the water tables in soils, respectively (Gong et al., 2012). Those changes in watertable height will likely affect greenhouse gas emissions from peatlands. The effect of water table fluctuations on $N_2O$ emissions from wetlands is variably affected e.g., by the amplitude, frequency and duration of the water table fluctuations (Mander et al., 2011), ranging from enhanced emissions after long-term drainage (e.g., for forestry or agriculture), moderate short-term drainage or rapid flooding of dried peat soil (Martikainen et al., 1993; Goldberg et al., 2010; Maljanen et al., 2010; Jørgensen and Elberling, 2012) to reduced $N_2O$ emissions after flooding of peat soil (McNicol and Silver, 2014). Highly fluctuating water tables and rapid switching between water table heights lead to higher cumulative $N_2O$ emissions than stable water tables in wetland soils (Dinsmore et al., 2009; Mander et al., 2011; Jørgensen and Elberling, 2012; McNicol and Silver, 2014). In the past, much more attention has been paid to the effect of long-term (e.g., in multi-year drainage or peat restoration) than of short-term changes (i.e., on the basis of several weeks or months) in water table height on $N_2O$ fluxes from peatlands. Even fewer studies have focused on the effects of short-term intensive water table fluctuations on the denitrifier communities involved in $N_2O$ turnover in peatland soils (Kim et al., 2008). Kim et al. (2008) found a decline in *nirS* abundance in response to short-term drought in soil cores of bog and fen, suggesting a decline in proteobacterial *nirS*-hosting denitrifiers. Diversity of *nirS* was stable. However, effects of water table manipulations on denitrifier communities *in situ* are unclear to date.

Most denitrifiers are facultative aerobes and thrive under oxic as well as under anoxic conditions (Shapleigh, 2013). Indeed, oxygen rather than nitrate is the preferred electron acceptor for many denitrifiers, suggesting that oxic conditions will not impair denitrifiers and their genetic potential. Thus, we hypothesize that short-term water table fluctuations will change the denitrification activity of peat denitrifiers rather than their community composition. Thus, the aims of the present study were (i) to assess the effect of raised water tables on dentrification potentials in a model peatland, (ii) to determine the effect of lowered and raised water tables on the community composition of denitrifers, (iii) to detect possible changes in the active denitrifer communities, and (iv) to try to link the obtained results to observed *in situ* $N_2O$ fluxes.

## MATERIALS AND METHODS

### Study Site and Experimental Setup

The minerotrophic fen Schlöppnerbrunnen is located in the Lehstenbach catchment (Fichtelgebirge, Germany; N 50° 07′ 53″, E 11° 52′ 51″). Please refer to Palmer et al. (2010) for a more detailed description of the sampling site. Mean air temperature was 6.9 and 6.6°C, while annual precipitation was 957 and 972 mm in 2008 and 2009, respectively. Three treatment and three untreated control plots (size 7.2 × 5 m) were established on the site and water table manipulations were performed as described (Estop-Aragonés et al., 2012, 2013). In brief, treatment plots were subjected to experimental drought and flooding in the summers of 2008 and 2009, respectively. The height of the water table was measured continuously in treatment and control plots (**Figure S1**). Experimental drought was achieved by rain water exclusion and drainage ditches in the time period between June 10th and August 7th 2008. PlexiGlas® roofs allowing for light penetration and above ground air movement were temporarily installed during the drought period, thus minimizing potential side effects. After the experimental drought period, roofs were removed, drainage was stopped, and drought plots were rewetted with artificial rainwater (103 mm within 8 h). Experimental flooding was achieved by irrigating the treatment plots with water from the nearby creek "Lehstenbach." Creek water was spread onto the treatment plots via perforated tubes at an average rate of 70$m^3$ per day and plot in the time period between May 14th and October 30th 2009. Maximum temperatures of drought

and flooding plots in 5 cm of depth were ∼1°C higher and 1.5°C lower than in control plots at the same time, respectively, indicating a minor effect of water table manipulations on the peat temperature regime (Estop-Aragonés et al., 2012).

Samples for molecular analyses were collected in both years, while samples for microcosm studies were collected in 2009 only due to the need of minimizing destructive samplings in 2008. In 2008, soil was sampled for molecular analysis before drought (June 09th) and at the end of the drought phase (July 27th). In 2009, samples were taken before flooding (i.e., before the onset of irrigation; May 11th) and after flooding (i.e., after irrigation had been discontinued; November 16th). Soil samples were taken with a peat soil corer from depth 0 to 40 cm. Soil samples for molecular analyses were separated into four layers (0–10, 10–20, 20–30, 30–40 cm), frozen immediately in liquid nitrogen, and stored at −80°C until use. Soil for microcosm studies was separated into two layers (0–20 and 20–40 cm) and stored at 4°C for max. 24 h prior to microcosm studies. Potential anaerobic microbial activities were significantly higher in 0–20 than 20–40 cm depth, and dissolved oxygen in pore water was close to air saturation deeper than 30 cm of depth in drought plots during water level drawdown (Wüst et al., 2009; Palmer et al., 2010; Estop-Aragonés et al., 2012). Air filled pore space was greater than 12% (up to 50% at the end of the drought period) from 0 to 20 cm of depth in drought plots (Estop-Aragonés et al., 2012). After the rewetting of drought plots, dissolved oxygen decreased to lower than 20% air saturation in 0–10 cm depth and declined to ∼0 with increasing depth (Estop-Aragonés et al., 2012). In control plots, oxygen penetration was significant until 20 cm of depth (Estop-Aragonés et al., 2012). Thus, the results of (note: not the samples before) molecular analyses from 0 to 10 plus 10 to 20 cm, and from 20 to 30 plus 30 to 40 cm were pooled.

## Water Table Manipulations and Effects on Biogeochemistry

During the period of experimental drought (June 10th 2008 to August 7th 2008), water table heights ranged from −71 to −12 cm and from −90 cm (i.e., 90 cm below peat surface) to −14 cm in control and treatment plots, respectively (Estop-Aragonés et al., 2012; **Figure S1**). Average water table heights were −26.8 and −62.1 cm in control and drought plots, respectively, i.e., the water table was on average 35.4 cm higher in control than in treatment plots. Air filled pore space in 5 cm depth approximated 30% in control plots and 50% in drought plots. Water oxygen saturation approximated 0–1 and 80% in 30 cm depth of control and drought plots, respectively. Such strong lowering of the water table (i) increased dissolved oxygen levels close to saturation in more than 30 cm depth and (ii) significantly decreased concentrations of dissolved inorganic carbon in drought treatment relative to control plots (Estop-Aragonés et al., 2013). Nitrate concentrations were 0.02-0.15 mM in the pore water during the experimental period (Estop-Aragonés et al., 2013).

During flooding (May 14th 2009 to October 30th 2009), water table heights ranged from −49 to 1.6 cm and from −18 to 4.9 cm in control and treatment plots, respectively (Estop-Aragonés et al., 2012; **Figure S1**). Average water table heights were −15.4 and −0.7 cm in control and treatment plots, respectively, i.e., the water table was on average 14.7 cm higher in treatment than in control plots. Flooding (i) decreased dissolved oxygen (near 0 μmol/l in the final flooding phase), dissolved inorganic carbon and nitrate concentrations, and (ii) increased nitrate dependent electron turnover, acetate, and hydrogen concentrations relative to control plots (Estop-Aragonés et al., 2013).

## Assessment of *In situ* $N_2O$-Fluxes

*In situ* $N_2O$-fluxes were measured by the closed chamber technique from late May to early November 2008 and from mid-April to mid-October 2009. The measurements were conducted as described earlier (Goldberg et al., 2010). In brief, three collars (1.15 l volume) were installed on each plot, and $N_2O$ fluxes were measured in regular intervals (2–4 and 1–2 times per month in 2008 and 2009, respectively). For the measurements, chambers of 4 l volume were placed on top of the collars, and the $N_2O$ concentration in the chamber headspace was measured after 0, 8, 16, 24, and 32 min using a photoacoustic infrared gas analyzer (Multigas Monitor 1312, INNOVA, Denmark). $N_2O$ flux rates were calculated based on the linear increase or decrease in $N_2O$ concentration in the chamber headspace.

## Assessment of Denitrification Potentials in Soil Microcosms

Denitrification potentials of fen soil (0–20 and 20–40 cm) at its *in situ* pH were assessed in nitrate-supplemented anoxic microcosms as described earlier (Palmer et al., 2010). In brief, one volume of fen soil was diluted with three volumes of water in 125-ml infusion flasks, the flasks were sealed with butyl-rubber stoppers and the airspace was purged with argon to achieve anoxic conditions. The flasks were preincubated at 15°C for ∼16 h to reduce intially present nitrate. After preincubation, $NaNO_3$ was added to the flasks (0–100 μM nitrate). Flasks were incubated for up to 12 h in the dark at 15°C, and $N_2O$ headspace concentrations in each flask were quantified at three timepoints using a Hewlett-Packard 5980 series II gas chromatograph equipped with an electron capture detector, and a Porapak Q-80/100 (Supelco, Bellefonte, PA) column (length, 4 m; inner diameter, 3.2 mm) with Ar-CH4 (95:5) as the carrier gas. Acetylene inhibition was used to distinguish between $N_2O$ production and total denitrification and to estimate the ratio of $N_2O$ to ($N_2O + N_2$) as described earlier (Yoshinari and Knowles, 1976; Palmer et al., 2010). $N_2O$ production rates and apparent kinetic parameters [Michaelis-Menten constants ($K_M$), maximum reaction velocitites ($v_{max}$), $v_{max}/K_M$] were determined as described (Palmer et al., 2010).

## Extraction of Nucleic Acids and Reverse Transcription

Nucleic acids from all sampled soil layers taken in 2008 were extracted using a bead-beating protocol (Griffiths et al., 2000) followed by separation of DNA and RNA using the Qiagen RNA/DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Nucleic acids from all sampled soil layers taken in 2009 were extracted using

a similar bead-beating protocol with the exception that an additional aluminum precipitation was performed prior to bead beating to remove humic acids (Persoh et al., 2008; Palmer et al., 2012). Although cell lysis procedures that are regarded as critical for the DNA/RNA extraction bias were essentially identical, comparison of community structure between the two years might be biased. Identical DNA/RNA extraction procedures were applied within each year, thus allowing for a meaningful analysis of the effect of water table manipulations on microbial community structure. Reverse transcription of extracted RNA into cDNA was conducted using the SuperScript VILO cDNA Synthesis Kit (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol.

## Quantitative PCR

Quantitative kinetic real-time PCRs (qPCRs) of *narG* [narG1960f (TAY GTS GGS CAR GAR AA)/narG2650r (TTY TCR TAC CAB GTB GC); Philippot et al., 2002], *nosZ* [nosZF (CGC TGT TCI TCG ACA GYC AG)/nosZR (ATG TGC AKI GCR TGG CAG AA); Rich et al., 2003], and bacterial 16S rRNA genes [Eub341F (CCT ACG GGA GGC AGC AG)/Eub534R (ATT ACC GCG GCT GCT GG); Muyzer et al., 1993] from DNA samples were performed in three technical replicates per plot, sampling time point and soil depth as described (Zaprasis et al., 2010; Palmer et al., 2012). *narG* and *nosZ* amplified from cDNA obtained from the same fen samples during triplicate qPCRs yielded multiple bands on agarose gels and multiple melting points during melting curve analyses. Thus, *narG* and *nosZ* expression was not assessed. However, the bands with the correct size were excised from agarose gels and used for TRFLP analyses.

Obtained gene copy numbers were corrected for inhibition by spiking environmental DNA extracts with standard DNA as described earlier (Zaprasis et al., 2010; Palmer et al., 2012). Obtained inhibition factors ranged from 0.2–1.0, 0.1–1.0, and 0.1–1.0 for *narG*, *nosZ*, and 16S rRNA genes, respectively. Copy numbers of *narG*, *nosZ*, and 16S rRNA genes were compared between treatments and time points by Tukey's HSD test in IBM SPSS 22 after testing for normal distribution in R.

## Terminal Restriction Fragment Length Polymorphism

Triplicate qPCR reactions of *narG* and *nosZ* amplified from DNA or cDNA were pooled and gel purified using the Montage Gel Extraction Kit (Millipore Corporation, Bedford, MA, USA) prior to TRFLP analysis. The purified PCR products were digested with Mung Bean nuclease (New England Biolabs, Frankfurt am Main, Germany) to remove single-stranded DNA and reduce the probability of pseudo-terminal restriction fragments (Egert and Friedrich, 2003). The digested DNA was purified using the Millipore Multiscreen 96-well-Filtration System (Millipore Corporation, Bedford, MA, USA). *narG* and *nosZ* PCR products were digested with the restriction enzymes *Cfo*I and *Fnu4*HI (New England Biolabs, Frankfurt am Main, Germany), respectively. Polyacrylamide gel electrophoresis was performed as described previously (Palmer et al., 2010). Terminal restriction fragment (TRF) tables (i.e., relative fluorescence of TRFs per sample) were imported into Qiime 1.9 (Caporaso et al., 2010). Statistical differences between years, nucleic acid type and treatment were tested using the Qiime script compare_categories.py with the Adonis, anosim, mrpp, and permanova algorithms (for further details consult qiime.org). Results of the individual tests were compared. As obtained *P*-value estimations calculated by the different algorithms and the derived conclusions were similar, only *P*-values derived from Adonis are reported. Operational taxonomic units (OTUs as indicated by TRFs) that were differentially expressed between treatments and/or time points were identified using the Qiime script differential_otus.py. *In silico* digests of *narG* and *nosZ* obtained in an earlier study (Palmer et al., 2010) were used to identify TRFs. However, not all TRFs clearly affiliated with a taxon or sequencing based OTU of Palmer et al. (2010). Such TRFs were not linked to phylogeny.

# RESULTS

## Effect of Watertable Manipulations on Fen $N_2O$ Fluxes

$N_2O$ fluxes from Schlöppnerbrunnen fen were variable (**Figure 1**). In 2008 (drought experiment), mean $N_2O$ fluxes fluctuated between $-0.4\ \mu mol*h^{-1}*m^{-2}$ (net $N_2O$ uptake) and $1.2\ \mu mol*h^{-1}*m^{-2}$ (net $N_2O$ emission). Differences between drought and control plots were marginal and were detected after the rewetting had occurred (**Figure 1A**). Cumulative $N_2O$ fluxes were positive in 2008, i.e., the fen was a net source of $N_2O$ in both drought and control plots (**Figure 1B**). In 2009 (flooding experiment), mean $N_2O$ fluxes ranged from $-0.4\ \mu mol*h^{-1}*m^{-2}$ (net $N_2O$ uptake) to $0.4\ \mu mol*h^{-1}*m^{-2}$ (net $N_2O$ emission). Differences between treatment and control plots were marginal in both years, indicating that $N_2O$ fluxes were essentially unaffected by the experimental drying or flooding (**Figure 1A**). In 2009, cumulative $N_2O$ fluxes were negative in both plot types, indicating (i) net $N_2O$ uptake in both flooding and control plots and (ii) differences between the years 2008 and 2009 (**Figure 1B**).

## Effect of Watertable Manipulations on Copy Numbers of *narG, nosZ* and 16S rRNA Genes

Experimental drought successfully changed water levels and soil biogeochemistry relative to control plots as did experimental flooding (Estop-Aragonés et al., 2012; **Figure S1**; please refer to data from our colleagues presented in the Materials and Methods section for further details on the effect of water table manipulations on biogeochemistry). Inhibtion-corrected 16S rRNA gene copy numbers were averaged for depths of 0–20 and 20–40 cm, for different plot types, treatments, and time points. Averaged copy numbers ranged from $(7.8 \pm 3.0) \times 10^4$ to $(4.1 \pm 1.2) \times 10^5$ per ng DNA (**Figures 2A–D**). 16S rRNA gene copy numbers from both plot types and at all sampling time points were similar ($P > 0.1$). Inhibtion-corrected copy numbers of *narG* and *nosZ* amplified from extracted DNA ranged from 0.4 to 12% and from 0.01 to 0.25% of bacterial 16S rRNA gene copy numbers, respectively (**Figures 2E–L**). The following effects

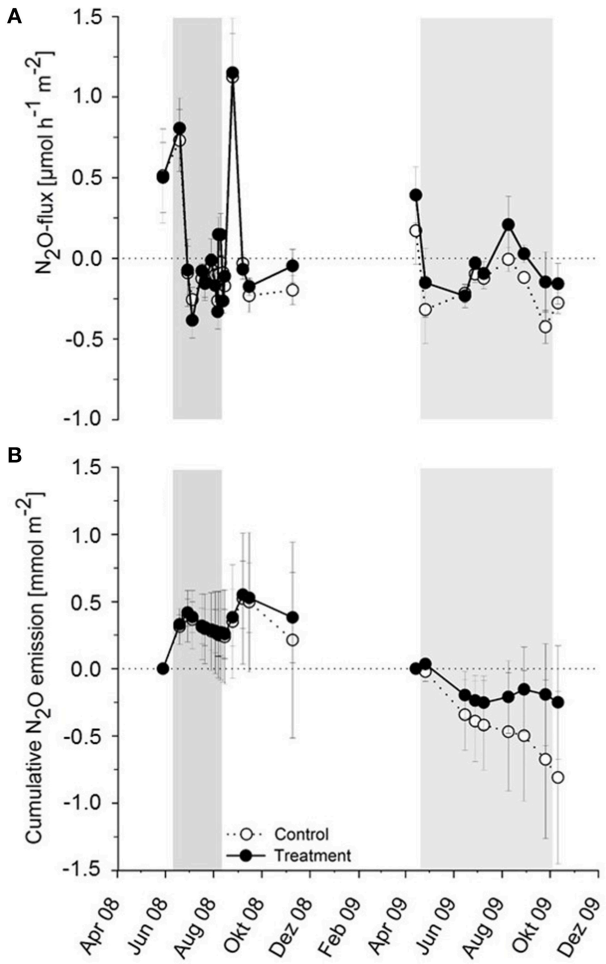


**FIGURE 1 | Effect of experimental drought (2008) and prolonged flooding (2009) on (A) Mean and (B) cumulative $N_2O$ fluxes in Schlöppnerbrunnen fen.** Means of three plots per plot type (three measurements per plot) and standard errors are displayed. The period of the manipulation experiments is shaded in gray for each year.

reflect tendencies rather than significant differences: In drought plots, relative abundances of *narG* and *nosZ* were similar ($P = 0.9$) and 3x higher ($P = 0.3$), respectively, after than before drought in 0–20 cm peat soil, while relative abundances of both genes were similar before and after drought in 20–40 cm soil (*narG*: $P = 0.7$, *nosZ:* $P = 0.9$; **Figures 2E–F,I–K**). When the same time points were compared, relative abundances of *narG* and *nosZ* from control plots were 2x lower ($P = 0.4$) and similar ($P = 0.99$), respectively, in 0–20 cm peat soil, and similar ($P = 0.99$) and 2.5x lower ($P = 0.8$), respectively, in 20–40 cm peat soil (**Figures 2E,F,I,K**, respectively).

Relative abundances of *narG* were marginally lower after than before flooding in treatment plots in both depths ($P \geq 0.9$), while relative abundances in control plots were about 3x lower ($P = 0.1$) in 0–20 cm peat soil when the same time points were compared (**Figures 2G,H**). Relative abundances of *nosZ* were similar at the after and before flooding time points in treatment plots and control plots at the same time in 0–20 cm peat soil ($P \geq 0.9$), and marginally lower after flooding of treatment plots in 20–40 cm peat soil from control plots ($P = 0.7$; **Figures 2K,L**). *narG* was on average 12x to 240x more abundant than *nosZ* (**Figures 2M–P**). The ratio of *nosZ* to *narG* was marginally higher in treatment than in control plots at both sampling time points during the drought experiment in 0–20 cm peat soil ($P \geq 0.7$), and no effect of the drought treatment on *nosZ* to *narG* ratios was observed (**Figure 2M**). In 20–40 cm peat soil the ratio was higher before than after drought in treatment plots ($P = 0.2$; **Figure 2N**). During the flooding experiment, a minor increase in the ratio of *nosZ* to *narG* in treatment plots was observed after relative to before flooding in 0–20 cm ($P = 0.7$) but not in 20–40 cm peat soil ($P = 0.99$; **Figures 2O,P**).

## Effect of Watertable Manipulations on Community Composition of *narG* Genes

Principal Coordinate Analysis of *narG* TRFLP patterns obtained from DNA samples indicative of the genetic potential for dissimilatory nitrate reduction revealed that the detected *narG* community composition was similar at all time points, as DNA samples clustered closely together in the PCoA plot (**Figures 3A,B**). This was observed in both depths, even though the clustering was slightly more pronounced in 0–20 cm depth (**Figure 3A**). Up to 9 and 13 major TRFs of *narG* were detected in DNA samples from both depths in 2008 and 2009, respectively, and the relative abundances of the individual TRFs were similar in treatment and control plots at all sampling time points. Detected TRFs were indicative of uncultured soil and sediment organisms related to *Deinococcus-Thermus* sp. of FEN CLUSTER 7, *Actinobacteria*-related uncultured soil bacteria of FEN CLUSTER 6, and uncultured Proteobacteria of FEN CLUSTERs 1–5 (Palmer et al., 2010) (**Figures S2A,B,E,F**). No statistically significant differences were detected between the treatments and time points ($P > 0.2$).

## Effect of Watertable Manipulations on Community Composition of *narG* Transcripts

Control plot samples obtained before and after treatments clustered more closely together than treatment plot samples based on TRFLP patterns of *narG* amplified from cDNA indicative of active nitrate reducers (**Figures 3A,B**). In 2008, when the drought experiment was performed, cDNA TRFLP patterns of *narG* differed already before drought in 0–20 and 20–40 cm peat soil (**Figures 3A,B**). The relative abundances of the two most abundant TRFs (23 and 57 bp) were similar in control plots in both soil layers ($P > 0.9$), while their relative abundances differed or tended to differ in treatment plots before and after drought ($P = 0.01$ and $P = 0.35$ for 23 and 57 bp TRF, respectively, in 0–20 cm soil; $P = 0.04$ for both 23 and 57 bp TRF in 20 to 40 cm soil; **Figures S2C,D**). This indicates that the activity of the groups behind those TRFs, i.e., *Deinococcus-Thermus* and *Actinobacteria* related uncultured fen nitrate reducers, is strongly affected by the experimental drought conditions (**Figures S2C,D**).

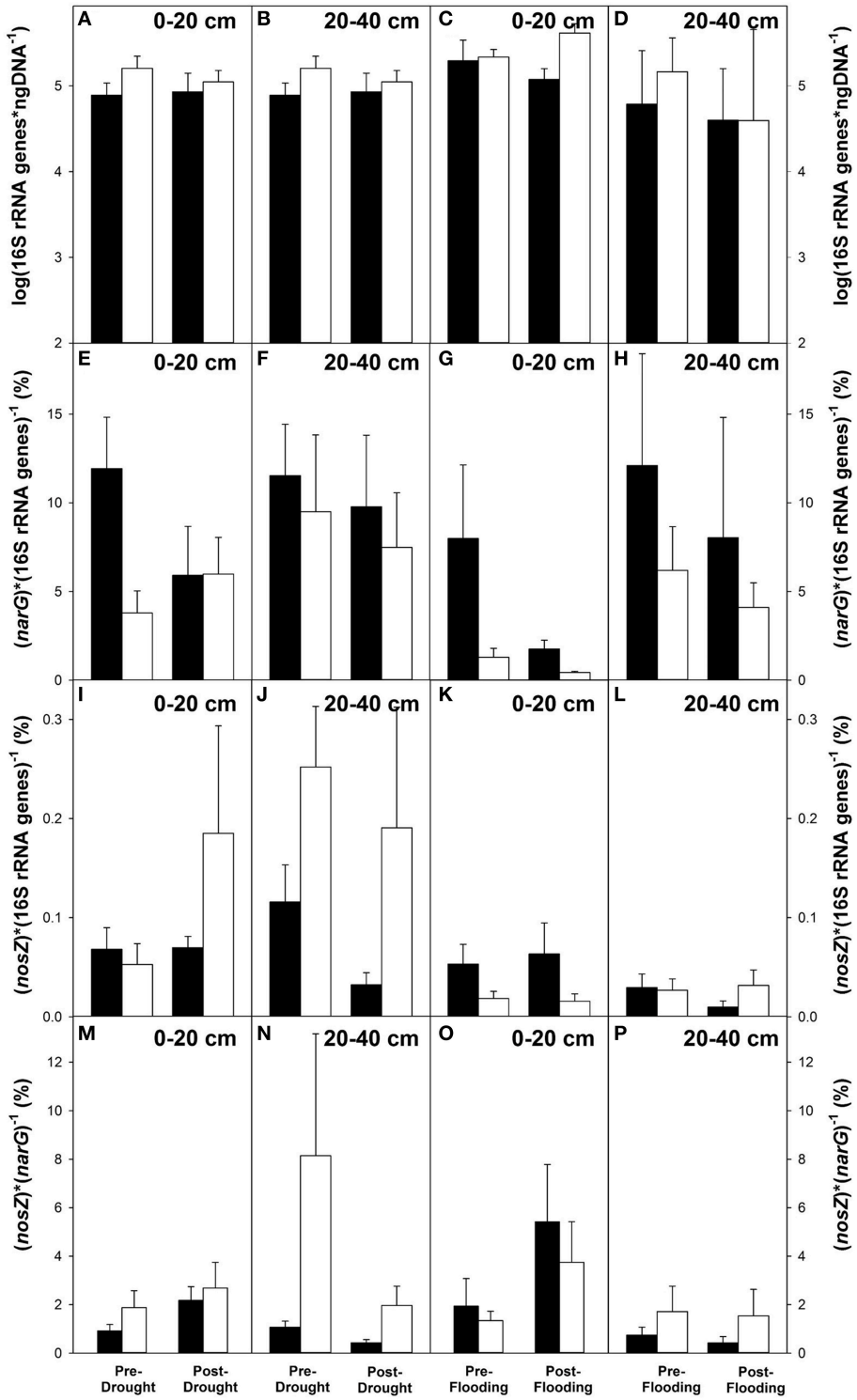


**FIGURE 2 | Effect of experimental drought (left) and prolonged flooding (right) on 16S rRNA gene copy numbers (A–D), relative abundances of *narG* (E–H), and *nosZ* (I–L) as well as on the ratio of *nosZ* to *narG* (M–P) in 0–20 and 20–40 cm fen soil.** Black bars represent control plots, white bars represent treatment plots. Mean values from triplicate analyses of all plots of one plot type and depth 0–20 or 20–40 cm were calculated and are displayed with error bars. Pre-drought samples were taken on June 09th 2008, post-drought samples were taken on July 27th 2008. Pre-flooding samples were taken on May 11th 2009, post-flooding samples were taken on November 16th 2009.

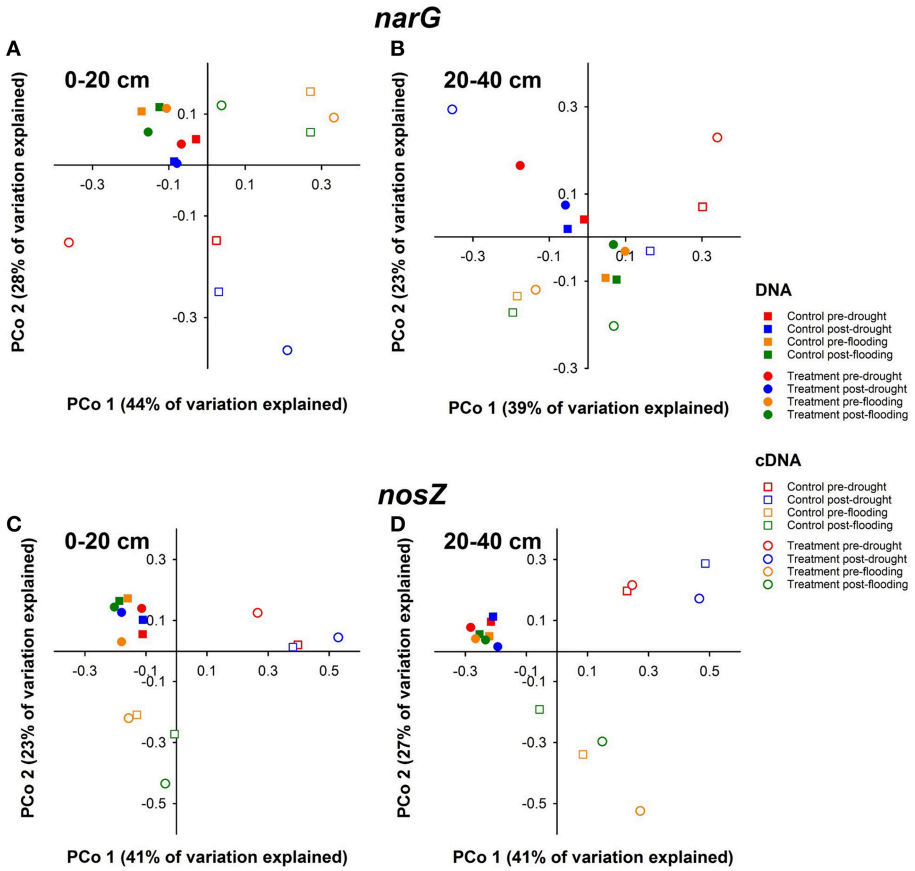


**FIGURE 3 | Effect of artificial drought and prolonged flooding on the community composition of *narG* (A,B) and *nosZ* (C,D) in DNA and cDNA samples from fen soil from 0 to 20 cm (A,C) and 20 to 40 cm (B,D) depth.** Principal coordinate analysis was conducted in Qiime using the average relative abundances of TRFs per plottype and time point as input. Pre-drought samples were taken on June 09th 2008, post-drought samples were taken on July 27th 2008. Pre-flooding samples were taken on May 11th 2009, post-flooding samples were taken on November 16th 2009.

In 2009 when the flooding experiment was performed, cDNA TRFLP patterns of *narG* from control plots clustered together in the PCoA plots (**Figures 3A,B**). The samples from control and flooding plots taken before the flooding treatment also clustered together, indicating that the active communities were rather similar in control and treatment plots before the onset of flooding. The samples from treatment plots after treatment clustered separately in the PCoA plots, suggesting an effect of flooding on active *narG* expressing microbes (**Figures 3A,B**). Marginal differences in the relative abundance of TRFs indicative of *Deinococcus-Thermus* and *Actinobacteria* related uncultured fen nitrate reducers suggest that those groups responded to flooding in upper peat soil (**Figure S2G**).

## Effect of Watertable Manipulations on Community Composition of *nosZ* Genes

TRFLP patterns of *nosZ* amplified from DNA were similar in 2008 (drought) and 2009 (flooding) and in both control and treatment plots ($P > 0.2$; **Figures 3C,D**). The five most prominent TRFs were indicative of uncultured soil organisms related to *Bradyrhizobiaceae* of FEN CLUSTER 1 and *Rhodospirillaceae* of FEN CLUSTERS 3–5 within the *Alphaproteobacteria* (Palmer et al., 2010) (**Figures S3A,B,E,F**).

## Effect of Watertable Manipulations on Community Composition of *nosZ* Transcripts

TRFLP patterns of *nosZ* obtained from cDNA differed from TRFLP patterns obtained from DNA in both years ($P = 0.001$; **Figures 3C,D**). The TRFs that were most prominent in DNA samples accounted only for 22–52 and 38–90% of the TRFs in cDNA samples in 2008 and 2009, respectively. In 2008, samples from control plots were rather similar in 0 to 20 cm depth at both sampling timepoints, while there were differences between samples taken before and after drought from treatment plots (**Figure 3C**). In 20 to 40 cm depth, samples from treatment plots taken before and after drought and those of control plots taken at the same time clustered together, and samples taken after drought were different from

the samples taken before drought as well as from each other (**Figure 3D**).

Significant differences between treatments and sampling time points were not detected ($P > 0.12$ in both soil layers) based on the overall TRFLP patterns obtained from cDNA samples in 2008 when the drought experiment was performed. Even though the overall community strucure was rather similar, 11 TRFs were expressed differentially in different plots or time points (**Table 1**; **Figure S3**). Those TRFs were indicative of uncultured *Bradyrhizobiaceae* and *Rhodospirillaceae* (**Figure S3**). Thus, data suggests that experimental drought affected activities of certain uncultured fen denitrifiers of the *Alphaproteobacteria*.

In 2009, when the flooding experiment was performed, samples taken before flooding of treatment plots from control and treatment plots clustered together in the PCoA plots in 0–20 cm depth, while they scattered in 20–40 cm depth (**Figures 3C,D**). Statistically, the overall TRFLP patterns were rather similar in both treaments and at both sampling time points in the upper layer ($P = 0.3$), indicating a minor effect of flooding on the active denitrifier community in the upper peat soil. On the contrary, the overall TRFLP patterns in the lower peat soil differed significantly between the plottypes and time points ($P = 0.02$). Seven TRFs were expressed differentially in different plots or time points (**Table 2**; **Figure S3**). The data suggests that activities of uncultered *Bradyrhizobia*-like denitrifiers were impaired by experimental flooding.

**TABLE 1 | Important *nosZ* cDNA TRFs responding to the drought treatment.**

| | | | Drought treatment | | Control | |
|---|---|---|---|---|---|---|
| | | | Pre- | Post- | Pre- | Post- |
| Drought treatment | Pre- | 0–20 | X | | 80 ↑ * | n.a. |
| | | | | | 390 ↑ *** | |
| | | | | | 700 ↓ *** | |
| | | 20–40 | X | 80 ↑ *** | 103 ↓ *** | n.a. |
| | | | | 103 ↓ *** | 149 ↑ *** | |
| | | | | 118 ↑ *** | 165 ↑ ** | |
| | | | | 135 ↑ *** | 419 ↑ ** | |
| | | | | 149 ↑ ** | | |
| | | | | 165 ↑ *** | | |
| | | | | 298 ↓ * | | |
| | Post- | 0–20 | | X | n.a. | |
| | | 20–40 | 80 ↓ *** | X | n.a. | 103 ↓ ** |
| | | | 103 ↑ *** | | | 118 ↓ *** |
| | | | 118 ↓ *** | | | |
| | | | 135 ↓ *** | | | |
| | | | 149 ↓ ** | | | |
| | | | 165 ↓ *** | | | |
| | | | 298 ↑ * | | | |
| Control | Pre- | 0–20 | 80 ↓ * | n.a. | X | 80 ↓ * |
| | | | 390 ↓ *** | | | 390 ↓ *** |
| | | | 700 ↑ *** | | | 700 ↑ *** |
| | | 20–40 | | n.a. | X | |
| | Post- | 0–20 | n.a. | | 80 ↑ * | X |
| | | | | | 390 ↑ *** | |
| | | | | | 700 ↓ *** | |
| | | 20–40 | n.a. | 103 ↑ ** | | X |
| | | | | 118 ↑ *** | | |

*Arrows indicate higher or lower relative abundances of TRFs when conditions are compared from left to right. *P < 0.10; **P < 0.05; ***P < 0.01. Empty fields indicate that no differentially expressed TRFs were detected in that comparison. n.a., not analyzed.*

## Effect of Experimental Flooding on Denitrification Potentials

Fen soil from both soil layers sampled before and after flooding of treatment plots from control and treatment plots produced $N_2O$ in anoxic microcosms. $N_2O$ production was minimal in all unsupplemented microcosms (**Figure S4**). $N_2O$ production was stimulated without apparent delay in microcosms supplemented with up to 100 μM nitrate (**Figure S4**). Observed $N_2O$ prodcution was always higher in the presence than in the absence of acetylene, indicating that part of the $N_2O$ was further reduced to $N_2$ in the absence of acetylene. Initial nitrate-dependent $N_2O$ production rates of fen soil microcosms amended with acetylene followed apparent Michaelis-Menten kinetics with soil from all soil layers and sampling time points (**Figure S5**). Apparent maximal reaction velocities ($v_{max,app}$) ranged from 7 to 68 nmol $h^{-1}g_{dw}^{-1}$ and were generally higher in 0–20 cm than in 20–40 cm soil ($P \leq 0.05$; **Figures 4A,B**). Apparent Michaelis-Menten constants $K_{M,app}$ ranged from 8 to 45 μM nitrate, but there was no statistically significant difference between the two soil layers ($P \geq 0.1$; **Figures 4C,D**). $v_{max,app}$ was significantly higher in treatment (i.e., flooded) plots after than before flooding in 20–40 cm soil ($p < 0.001$), while it was similar at both time points in control plots and in 0–20 cm soil from treatment plots ($p \geq 0.18$; **Figures 4A,B**). $K_M$ was significantly higher after than before flooding in 0–20 cm soil from treatment plots, ($p = 0.08$), while there were no significant differences between $K_M$ in 0–20 cm soil from control plots and in 20–40 cm soil from control and treatment plot at different sampling time points ($p \geq 0.2$; **Figures 4C,D**).

The ratio of $N_2O$ to ($N_2O + N_2$) ranged from 1.9 to 79% and from 38 to 99% for all supplied nitrate concentrations in microcosms with 0–20 and 20–40 cm fen soil, respectively. The average ratio of $N_2O$ to ($N_2O + N_2$) was 37 and 70% in 0–20 and 20–40 cm fen soil, indicating that more than half of the $N_2O$ produced from nitrate was further reduced to $N_2$ in the upper soil layer (**Figures 4G,H**). $N_2O$ to ($N_2O + N_2$) ratios were similar in control plots before and after flooding of treatment plots ($p \geq 0.13$). This was observed for both sampled soil layers. On the contrary, $N_2O$ to ($N_2O + N_2$) ratios were significantly lower in 0–20 cm fen soil after than before flooding from treatment plots or than in the samples taken after flooding of treatment plots from the control plots ($p = 0.01$ and $p < 0.001$, respectively). Thus, the results of the microcosm experiments indicate that prolonged flooding enhanced capacities for denitrification as well as $N_2O$ production concomittant to $N_2O$ reduction to $N_2$.

**TABLE 2 | Important *nosZ* cDNA TRFs responding to the flooding treatment.**

| | | | Flooding Treatment | | Control | |
|---|---|---|---|---|---|---|
| | | | Pre- | Post- | Pre- | Post- |
| Flooding treatment | Pre- | 0–20 | X | 90↓ *** | | n.a. |
| | | 20–40 | X | 165↓ ** | 118↑ *<br>309↓ *<br>700↓ * | n.a. |
| | Post- | 0–20 | 90↑ *** | X | n.a. | 135↓ ** |
| | | 20–40 | 165↑ ** | X | n.a. | 80↓ *<br>118↑ *<br>165↑ *** |
| Control | Pre- | 0–20 | | n.a. | X | 90↓ * |
| | | 20–40 | 118↓ *<br>309↑ *<br>700↑ * | n.a. | X | |
| | Post- | 0–20 | n.a. | 135↑ ** | 90↑ * | X |
| | | 20–40 | n.a. | 80↑ *<br>118↓ *<br>165↓ *** | | X |

*Arrows indicate higher or lower relative abundances of TRFs when conditions are compared from left to right. *P < 0.10; **P < 0.05; ***P < 0.01. Empty fields indicate that no differentially expressed TRFs were detected in that comparison. n.a., not analyzed.*

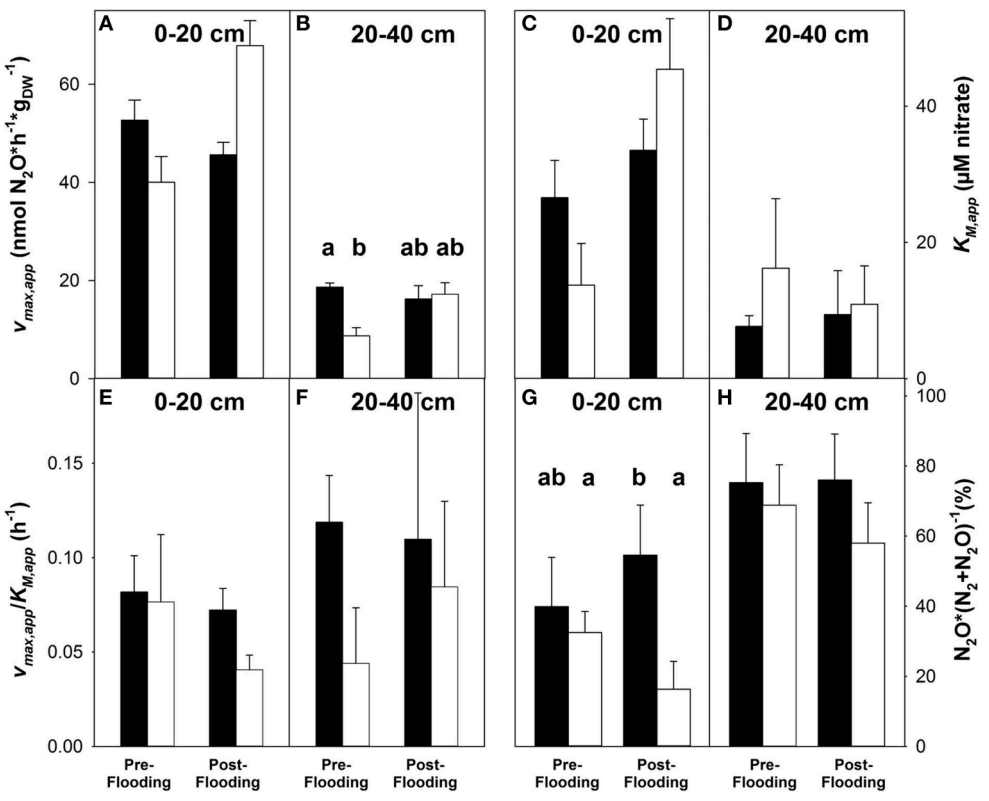


**FIGURE 4 | Effect of prolonged flooding on denitrification potentials in fen soil.** Effect on apparent maximal reaction velocities ($v_{max,app}$) **(A,B)**, apparent Michaelis-Menten constants ($K_{M,app}$) **(C,D)**, $v_{max,app}/K_{M,app}$ **(E,F)**, and $N_2O$ to ($N_2O + N_2$) ratios **(G,H)** in 0–20 and 20–40 cm fen soil. Values are obtained from apparent Michaelis-Menten kinetics based on mean values and standard errors of 3 replicate measurements (see **Figure S5**). Black bars represent control plots, white bars represent treatment plots. Siginificantly differing values are indicated by different lower-case letters. Pre-flooding samples were taken on May 11th 2009, post-flooding samples were taken on November 16th 2009.

# DISCUSSION

## Impacts of Extreme Weather Events/Short-Term Water Table Fluctuations on Fen Processes and $N_2O$ Source and Sink Strength

The present study extends existing data on the effects of water table manipulations obtained from peatlands by providing insights into process-associated microbial communities (Martikainen et al., 1993; Silvola et al., 1996; Reiche et al., 2009; Goldberg et al., 2010; Maljanen et al., 2010; Elberling et al., 2011; Estop-Aragonés et al., 2013). Incubation studies indicated higher denitrification potentials but reduced $N_2O$ to ($N_2O$ + $N_2$) ratios after prolonged flooding (**Figure 4**). *In situ* $N_2O$ fluxes from flooded and from control plots differed marginally (**Figure 1**). Thus, the data indicates that the stimulation of denitrification-derived $N_2O$ production was essentially mitigated by improved $N_2O$ reduction capacities. In a recent $^{15}N$-tracer study, lower $N_2O$ to ($N_2O$ + $N_2$) ratios were reported under constantly flooded conditions than under fluctuating water tables in a transition bog, supporting the view that complete denitrification is stimulated in flooded peatlands (Tauchnitz et al., 2015). Similar findings were obtained with fresh water marsh, where $N_2O$ emissions are minimal when the water table is above the peat surface (Yang et al., 2013). Thus, the depletion of nitrate after prolonged flooding coupled with low $N_2O$ to ($N_2O$ + $N_2$) ratios might prevent higher $N_2O$ emissions from fen soil, while maintaining its capacity for nitrogen removal.

In Schlöppnerbrunnen fen, $N_2O$ emissions increased upon rewetting after moderate water table drawdown in 2007 (Goldberg et al., 2010). This was consistent with the literature indicating that rewetting peat sites coincides with high nitrate turnover (Russow et al., 2013). However, $N_2O$ emissions were rather stable during the more severe water table drawdown in 2008 (**Figure 1**). During severe water table drawdown, alternative electron acceptors can accumulate (Reiche et al., 2009; Estop-Aragonés et al., 2013) and accumulated nitrate is expected to stimulate denitrification derived $N_2O$ emissions upon rewetting. However, nitrate accumulation in drought plots was not dramatically higher than in control plots (Estop-Aragonés et al., 2013). Most denitrifiers are heterotrophs depending on organic electron donors (Shapleigh, 2013). Dissolved organic carbon concentrations strongly decreased in response to strong water table drawdown, suggesting that limitations of easily available electron donors did not allow for a stimulation of denitrification and associated $N_2O$ production upon rewetting relative to control plots (Estop-Aragonés et al., 2013).

## Regulators of Denitrification and $N_2O$ Turnover during Short-Term Water Table Fluctuations

*In situ* denitrifier activity is dependent on a variety of environmental factors such as soil temperature, water table height, and availability of N-oxides. Higher soil temperatures as well as higher soil moisture content generally promote denitrification (Stres et al., 2008; Palmer et al., 2010). Thus, highest sink functions of wetlands for nitrate and $N_2O$ are observed in summer (Jørgensen and Elberling, 2012). Oxygen availability in peat is mainly controlled by watertable height (Estop-Aragonés et al., 2012). Elevated dissolved oxygen concentrations in the pore water are well known to suppress synthesis of denitrification associated reductases (Tiedje et al., 1982; Shapleigh, 2013). Redox potential changes rather than water content itself impact $N_2O$ emissions (Liu et al., 2012). However, activities of nitrate, nitrite, nitric oxide, and nitrous oxide reductases display different sensitivities toward oxygen inhibition in model organsims such as *Paracoccus denitrificans* and *Pseudomonas fluorescens* (Davies et al., 1989; McKenney et al., 1994). Nitrate reduction is generally the least oxygen sensitive step. Nitrite, NO, and $N_2O$ reductions are each increasingly sensitive to oxygen inhibition. Indeed, only the $N_2O$ reductase is directly inhibited by oxygen (Zumft, 1997). Thus, increased oxygen concentrations due to lowered water tables explain initial increases in $N_2O$ production observed after moderate water table drawdown in Schlöppnerbrunnen fen and suggest a contribution of denitrifiers to increased $N_2O$ emissions due to impaired $N_2O$ reduction (Goldberg et al., 2010). $\delta^{15}N$ and $\delta^{18}O$ values of $N_2O$ suggest a minor contribution, if any, by nitrification (Goldberg et al., 2010). The observed $N_2O$ consumption of Schlöppnerbrunnen fen when water tables were lowered either naturally in control or experimentally in treatment plots (**Figure 1**) remains a phenomenon to date that necessitates more research in the future.

Heightened and lowered water tables decrease and increase peat temperature, respectively. In Schlöppnerbrunnen fen, maximal temperatures were 1.5°C lower in flooded than in control plots and about 1°C higher in drought than in control plots (Estop-Aragonés et al., 2012). Given the daily temperature amplitude of 17°C in control plots in 5 cm of depth, such differences appear to be minimal (Estop-Aragonés et al., 2012). Those moderate changes in temperature have the potential to affect denitrifier activities, but do not necessarily change denitrifier community composition (Stres et al., 2008). Indeed, denitrifier community composition in Schlöppnerbrunnen fen remained similar at all time points of the manipulation experiments (**Figure 3**; **Figures S2**, **S3**). During periods of drought, enhanced rates of nitrification are feasible due to elevated oxygen availability (Fromin et al., 2010). In Schlöppnerbrunnen fen soil, nitrate concentrations in the pore water increased upon moderate water level draw down up to 500 μM and decreased after rewetting (Herrmann et al., 2012). Although nitrate was not significantly increased due to strong water level drawdown, concentrations of other terminal electron acceptors such as $Fe^{3+}$ and sulfate increased during drying and decreased during rewetting, suggesting a buffering capacity for high redox potentials in the fen (Estop-Aragonés et al., 2013). Such a buffering capacity together with carbon limitation might have prevented a major stimulation of denitrification after rewetting.

During flooding, constant inputs of nitrate and sulfate raised their concentrations in the peat to ∼40 and 100 μM, respectively (Estop-Aragonés et al., 2013). The constant

supply of nitrate in low concentrations might lead to growth and activation of fen denitrifiers, which are often N-limited, and increased nitrate supply might lead to increased $N_2O$ emissions (Novak et al., 2015; Palmer and Horn, 2015). Along these lines, denitrification capacities of Schlöppnerbrunnen fen were higher after than before flooding in treatment plots (**Figures 4A–F**). Potential $N_2O$ to ($N_2O + N_2$) ratios (**Figures 4G,H**) tended to be lower after than before flooding, while the abundance of denitrification associated genes remained rather unaffected or tended to decrease (**Figure 2**). Model denitrifiers such as *P. denitrificans* and *P. fluorescens* are capable of minimizing $N_2O$-release during complete denitrification under stable anoxic conditions by a stable expression of denitrification associated reductases (McKenney et al., 1994; Baumann et al., 1996). Such data suggest that the nitrate input during flooding did not allow for massive growth of denitrifiers, and that the prevalent denitrifier community is regulated in a way that the conversion of $N_2O$ to $N_2$ *in situ* was efficient.

Water table manipulation studies in wetland soils indicate that the effect of short-term water table fluctuations on denitrifier abundance is variable, ranging from no effect to decreased or increased abundances (Kim et al., 2008; Song et al., 2010). Differences in denitrifier activity are observed after short-term water table manipulations in many wetland systems (Kim et al., 2008; Song et al., 2010). In Schlöppnerbrunnen fen, the relative abundance of detected *narG* was rather unaffected by the drought and flooding treatments (**Figures 2E–H**), while the relative abundance of detected *nosZ* was higher after drought in upper fen soil (**Figure 2I**). Song et al. (2010) concluded that short-term water table variations impact denitrifier activity rather than denitrifier community structure. Similar effects have been observed for methanogenic communities: While increased substrate availability increases methanogenic activity, the community composition of methanogens is rather unaffected by anthropogenic disturbances (Basiliko et al., 2013). Thus, the observed changes in denitrification potentials and $N_2O$ emission in Schlöppnerbrunnen fen appear to be caused by changes in denitrifier activity rather than by changes in denitrifier community size.

## Fen Denitrifiers Are Resistant to Climate Change Induced Short-Term Water Table Fluctuations and Are Capable to Adapt Their Activity to Changing Redox Conditions

Denitrifier community composition is rather unaffected by water table fluctuations in many soils (Stres et al., 2008; Song et al., 2010). Certain microbial communities are resistant to environmental stress, such as water table fluctuations, varying temperatures or freeze-thaw events (Griffiths and Philippot, 2013). Denitrifier communities are also rather stable to water table fluctuations in other wetland soils such as in Ohio wetlands, saltmarshes or wetland ponds (Fromin et al., 2010; Song et al., 2010; McKew et al., 2011). Indeed, *narG* and *nosZ* copy numbers were only marginally affected by the water table manipulations in Schlöppnerbrunnen fen (**Figures 2E–L**), indicating that facultative fen denitrifiers are able to cope with changing water tables and the resulting changes in oxygen supply. Moreover, DNA-based TRFLP analyses indicate a stable denitrifier community composition (**Figure 3**), i.e., resistance to water table changes is similar in most groups of fen denitrifiers. Earlier studies with Schlöppnerbrunnen fen soil indicate the presence of nitrate reducers including denitrifiers related to *Deinococcus-Thermus*, *Actinobacteria* as well as *Alpha-* and *Beta-Proteobacteria* (Palmer et al., 2010). Also the present study detected TRFs indicating the presence of such groups on both gene and transcript level (**Figures S2**, **S3**). Thus, based on transcript level TRFLP analysis, *Deinococcus-Thermus* related microbes, *Proteobacteria* as well as *Actinobacteria* were active in Schlöppnerbrunnen fen under variable environmental conditions. *Deinococcus - Thermus* related microbes represent a deep-branching group that are widespread in extreme environments and resistant to environmental stress (da Costa et al., 2006; Theodorakopoulos et al., 2013). *Proteobacteria* are found in most soil ecosystems and under a variety of environmental conditions due to their versatile metabolic capabilities (Dworkin et al., 2006). *Actinobacteria* are likewise common to many soils, frequently occur in more extreme habitats, and show high tolerance to environmental stress (Zenova et al., 2011). Many *Actinobacteria* possess a truncated denitrification pathway, and NO or $N_2O$ are often end products of *Actinobacterial* denitrification (Shapleigh, 2013). Schlöppnerbrunnen fen emits up to 1 $\mu$mol NO $m^{-2}$ $h^{-1}$, demonstrating significant production and stability of NO to act as biological signal molecule (Goldberg et al., 2010). Less than 1 nM concentrations of NO suffice to induce *norBC* expression and 5 nM of NO result in maximal expression of *norBC* as well as *nirS* in *Pseudomonas stutzeri* (Vollack and Zumft, 2001). Thus, the NO produced in peatlands (eventually by incomplete denitrifiers like *Actinobacteria*) might act as an activator for the denitrifying microbial community in peatland soils by inducing the expression of denitrification genes (i.e., those of detected *Proteobacteria*).

The versatility of such peatland denitrifiers likely contributed to the observed stability of the denitrifier community. The stability of the Schlöppnerbrunnen fen denitrifier community was corroborated by the absence of significant seasonal variations in control plots (**Figure 3**). Boreal lake sediments likewise host a rather stable denitrifier community throughout most sampling times during a year (Saarenheimo et al., 2015). However, such findings are in contrast to other studies of agricultural soils, drained peatlands and intertidal wetland ecosystems where season significantly impacted denitrifier community composition (Bremer et al., 2007; Marhan et al., 2011; Andert et al., 2012; Hu et al., 2014; Wang et al., 2014). During such studies a developing plant community, seasonally changes of environmental parameters such as pH, and sampling times that covered the whole year including winter contributed to the observed seasonal changes in denitrifier community. During our study, sampling was restricted to the time periods

from June until August (experimental drought) and from May to November (experimental flooding) and established plant communities were rather stable. Minor community changes might have escaped detection by TRFLP analysis and few species sensitive to water table manipulations might have been replaced by others yielding a similar TRF. Other factors such as nutrient availability might affect the resistance of the microbial community (Royer-Tardif et al., 2010; Liu et al., 2012). Fluctuating water tables and thus redox conditions occur frequently in Schlöppnerbrunnen fen soil, thus an adaptation and hence stability of microbial communities toward redox fluctuations and changing environmental conditions is likely. Indeed, laboratory studies on soil microbes lend support for such a conclusion (Pett-Ridge and Firestone, 2005).

Although denitrifier community structure was stable and the effect of water table manipulations on *in situ* $N_2O$ fluxes was low, water table manipulations affected potential activities and active denitrifiers (**Figures 2**, **3**). Microcosm experiments with Schlöppnerbrunnen fen soil indicate increased $v_{max}$ after flooding as well as decreased $N_2O$ to ($N_2O + N_2$) ratios (**Figure 4**), and cDNA-based TRFLP analyses of *narG* and *nosZ* indicate differences in the active denitrifier community at different water table regimes (**Figure 3**). Short-term water table fluctuations affect denitrifier activity (Fromin et al., 2010; Song et al., 2010). Denitrifier activities do not always correlate with denitrifier community structure (Andert et al., 2012). Thus, the observed stability of the fen denitrifier community composition during the vegetation period and short-term water table fluctuations might be an interesting feature that might be more common than previously thought. Observed differences in $N_2O$ emission are likely caused by changes in substrate availability and denitrifier activity rather than by changes in community composition.

## CONCLUSIONS AND LIMITATIONS

Denitrifier communities are diverse, the denitrification pathway is modular, and the knowledge on existing forms of N-oxide respiring enzymes is growing constantly. Recently, atypical *nosZ* belonging to the clade II have been described that occur in microbes lacking modules of the denitrification pathway and those atypical *nosZ* can account for more than half of the *nosZ* in soil (Jones et al., 2013; Orellana et al., 2014). Organisms hosting clade II *nosZ* can be denitrifiers or non-denitrifiers. Many non-denitrifying $N_2O$-reducers are obligate anaerobes rather than facultative aerobes, suggesting that they have a higher sensitivity toward redox fluctuations than denitrifiers. However, their importance in peatlands is unclear to date, and thus their role has to be further clarified in future studies. The present study focused on denitrifers, and the molecular analyses were conducted with primers targeting clade I *nosZ* of denitrifiers. Due to selectivities of primers, microbial abundance and diversity might be underestimated. However, even though the present study captures only part of the genetic denitrifier diversity, trends observed for detected genes and transcripts are valid, and the collective data of the study indicate (i) rather stable *in situ* $N_2O$ fluxes during drought and flooding experiments, (ii) increased potential activity of fen denitrifiers as well as a higher percentage of complete denitrification after prolonged flooding, (iii) a stable denitrifier community in Schlöppnerbrunnen fen soil that is resistant to short-term water table fluctuations, (iv) a potential of the core denitrifier community to react to fluctuating water tables by differential activation, and (v) the ability of fen denitrifiers and eventually non-denitrifying $N_2O$ reducers to consume $N_2O$ under moderately acidic conditions. Those findings support the hypothesis that short-term water table fluctuations affect denitrifier activity rather than their community composition. It is feasible that enhanced overall denitrification rates as they can be expected under certain conditions (e.g., after prolonged flooding) and enhanced $N_2O$ consumption rates equal out, thus leading to rather stable overall $N_2O$ fluxes.

## AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: KP, JK, GG, and MH. Performed the experiments: KP and JK. Analyzed the data: KP, JK, GG, and MH. Contributed reagents/materials/analysis tools: MH and GG. Wrote the paper: KP and MH.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00727

**Figure S1 | Effect of artificial drought (2008) and prolonged flooding (2009) on watertables in control and treatment plots.** Negative values indicate a watertable below the peat surface, positive values indicate a watertable above the peat surface. Averages of 18 piezometer measurements (6 per plot) are displayed. Error bars have been omitted to improve picture clarity. Sampling time points are indicated by vertical black lines.

**Figure S2 | Effect of experimental drought (A–D) and prolonged flooding (E–H) on community composition of *narG* in DNA (A,B,E,F) and cDNA (C,D,G,H) in fen soil from 0 to 20 cm (A,C,E,G) and 20 to 40 cm (B,D,F,H) depth.** PCR products were digested with *Cfo*I. Mean values of values from all plots of one plot type and soil depths 0–20 or 20–40 cm (i.e., $3 \times 2 = 6$) are displayed. TRFs with a relative abundance <5% in all samples are combined as "Rare." Pre-drought samples were taken on June 09th 2008, post-drought samples were taken on July 27th 2008. Pre-flooding samples were taken on May 11th 2009, post-flooding samples were taken on November 16th 2009. TRFLP patterns were dominated by TRFs of 23, 57, and 128 bp size, which were indicative of FEN CLUSTER 7 (*Deinococcus-Thermus* sp.-related uncultured soil bacteria), FEN CLUSTER 6 (*Actinobacteria*-related uncultured soil bacteria), and FEN CLUSTERS 1–5 (related to uncultured Proteobacteria).

**Figure S3 | Effect of experimental drought (A–D) and prolonged flooding (E–H) on community composition of *nosZ* in DNA**

**(A,B,E,F) and cDNA (C,D,G,H) in fen soil from 0 to 20 cm (A,C,E,G) and 20 to 40 cm (B,D,F,H) depth.** PCR products were digested with *Fnu4*HI. Mean values of values from all plots of one plot type and soil depths 0–20 or 20–40 cm (i.e., 3 × 2 = 6) are displayed. TRFs with a relative abundance <5% in all samples are combined as "Rare." Pre-drought samples were taken on June 09th 2008, post-drought samples were taken on July 27th 2008. Pre-flooding samples were taken on May 11th 2009, post-flooding samples were taken on November 16th 2009. In DNA samples, the five most prominent TRFs (149, 165, 215, 298, 700 bp) had cumulative relative abundances of 82–99% in all treaments and sampling time points in 2008 and 2009. In cDNA samples, those same TRFs accounted only for 22–52% and 38–90% of the TRFs in 2008 and 2009, respectively. The TRFs were indicative of FEN CLUSTER 1 (TRFs 165, 298; affiliated with *Bradyrhizobiaceae*-related uncultured soil bacteria) and FEN CLUSTERS 3–5 (TRFs 149, 215; affiliated with *Rhodospirillaceae*-related uncultured soil bacteria).

**Figure S4 | Effect of acetylene and supplemental nitrate on the production and consumption of $N_2O$ in anoxic microcosms with 0–20 cm (1) and 20–40 cm (2) fen soil.** Blue squares represent the pre-flooding control plot, red circles represent the pre-flooding treatment plot, black triangles represent the post-flooding control plot, green diamonds represent the post-flooding treatment plot. Closed symbols represent microcosms with acetylene, open symbols represent microcosms without acetylene. Supplied concentrations of nitrate were 0 μM **(A)**, 10 μM **(B)**, 20 μM **(C)**, 40 μM **(D)**, and 100 μM **(E)**. Mean values and standard errors of three replicate microcosms are shown.

**Figure S5 | Effect of prolonged flooding on apparent Michaelis-Menten kinetics in peat soil from 0 to 20 cm (A) and 20 to 40 cm (B) peat soil.** $N_2O$-production rates were measured at different nitrate concentrations in microcosms that were supplemented with acetylene. Averages of three measurements and standard errors are displayed. Open symbols = control plots, closed symbols = treatment plots. Squares = pre-flooding, circles = post-flooding.

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# Diverse electron sources support denitrification under hypoxia in the obligate methanotroph *Methylomicrobium album* strain BG8

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Aerobic methane-oxidizing bacteria (MOB) are a diverse group of microorganisms that are ubiquitous in natural environments. Along with anaerobic MOB and archaea, aerobic methanotrophs are critical for attenuating emission of methane to the atmosphere. Clearly, nitrogen availability in the form of ammonium and nitrite have strong effects on methanotrophic activity and their natural community structures. Previous findings show that nitrite amendment inhibits the activity of some cultivated methanotrophs; however, the physiological pathways that allow some strains to transform nitrite, expression of gene inventories, as well as the electron sources that support this activity remain largely uncharacterized. Here we show that *Methylomicrobium album* strain BG8 utilizes methane, methanol, formaldehyde, formate, ethane, ethanol, and ammonia to support denitrification activity under hypoxia only in the presence of nitrite. We also demonstrate that transcript abundance of putative denitrification genes, *nirS* and one of two *norB* genes, increased in response to nitrite. Furthermore, we found that transcript abundance of *pxmA*, encoding the alpha subunit of a putative copper-containing monooxygenase, increased in response to both nitrite and hypoxia. Our results suggest that expression of denitrification genes, found widely within genomes of aerobic methanotrophs, allow the coupling of substrate oxidation to the reduction of nitrogen oxide terminal electron acceptors under oxygen limitation. The present study expands current knowledge of the metabolic flexibility of methanotrophs by revealing that a diverse array of electron donors support nitrite reduction to nitrous oxide under hypoxia.

**Keywords: methanotroph, nitrous oxide, denitrification, hypoxia, *Methylomicrobium album* BG8, methane monooxygenase, nitrite reduction**

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## Introduction

Aerobic methane-oxidizing bacteria (MOB) form an important bridge between the global carbon and nitrogen cycles, a relationship impacted by the global use of nitrogenous fertilizers (Bodelier and Steenbergh, 2014). Ammonia ($NH_3$) and nitrate ($NO_3^-$) can stimulate the activity of methanotrophs by acting as a nitrogen source for growth and biomass production (Bodelier et al., 2000; Bodelier and Laanbroek, 2004). Further, some methanotrophs such as *Methylomonas denitrificans* utilize $NO_3^-$ as an oxidant for respiration under hypoxia (Kits et al., 2015).

Evidently, denitrification in aerobic methanotrophs functions to conserve energy during oxygen ($O_2$) limitation (Kits et al., 2015). Alternatively, $NH_3$ and nitrite ($NO_2^-$) can act as significant inhibitors of methanotrophic bacteria (King and Schnell, 1994). $NH_3$ is a competitive inhibitor of the methane monooxygenase enzyme and $NO_2^-$, produced by methanotrophs that can oxidize $NH_3$ to $NO_2^-$, is a toxin with bacteriostatic properties that is known to inhibit the methanotroph formate dehydrogenase enzyme that is essential for the oxidation of formate to carbon dioxide (Dunfield and Knowles, 1995; Cammack et al., 1999; Nyerges et al., 2010).

In spite of the recent discovery that aerobic methanotrophs can denitrify, the energy sources, genetic modules, and environmental factors that govern denitrification in MOB are still poorly understood. *M. denitrificans* FJG1 respires $NO_3^-$ using methane as an electron donor to conserve energy. However, it is not known whether $C_1$ energy sources other than $CH_4$ (methanol, formaldehyde, and formate) can directly support denitrification. Another possibility, which has not yet been investigated, is that $C_2$ compounds (such as ethane and ethanol) and inorganic reduced nitrogen sources ($NH_3$) support methanotrophic denitrification. Previous work shows that several obligate methanotrophs, including *Methylomicrobium album* strain BG8, oxidize ethane ($C_2H_6$) and ethanol ($C_2H_6O$) using particulate methane monooxygenase (pMMO) and methanol dehydrogenase (MDH), respectively, even though neither substrate supports growth (Whittenbury et al., 1970; Dalton, 1980; Mountfort, 1990). $NH_3$ may be able to support methanotrophic denitrification because many aerobic methanotrophs are capable of oxidizing $NH_3$ to $NO_2^-$: a process facilitated by the presence of a copper-containing monooxygenase (CuMMO) enzyme and, in some methanotrophs, a hydroxylamine dehydrogenase homolog (Poret-Peterson et al., 2008). The ability to utilize alternative energy sources to support denitrification would augment the metabolic flexibility of methanotrophs and enable them to sustain respiration in the absence of $CH_4$ and/or $O_2$.

*Methylomicrobium album* strain BG8 is an aerobic methanotroph that belongs to the phylum Gammaproteobacteria; the genome lacks a soluble methane monooxygenase but does contain one particulate methane monooxygenase operon (*pmoCAB* – METAL_RS17430, 17425, 17420) and one operon encoding a putative copper monooxygenase (*pxmABC* – METAL_RS06980, 06975, 06970) with no known function. The genome also contains gene modules for import and assimilation of $NH_4^+$ (*amtB* – METAL_RS11045/*gdhB* – METAL_RS11695/*glnA* – METAL_RS11070/*ald* – METAL_RS11565), assimilation of $NO_3^-$ (*nasA* – METAL_RS06040/*nirB* – METAL_RS15330, *nirD* – METAL_RS15325), oxidation of $NH_2OH$ to $NO_2^-$ (*haoA* – METAL_RS13275), as well as putative denitrification genes – cytochrome $cd_1$ nitrite reductase (*nirS* – METAL_RS10995), and two copies of cytochrome *c*-dependent nitric oxide reductase (*norB1* – METAL_RS03925, *norC1* – METAL_RS03930/*norB2* – METAL_RS13345). The recent release of several genome sequences of aerobic methanotrophs, including *M. album* strain BG8, points to the frequent presence of putative nitrite and nitric oxide reductases, while only three cultivated methanotrophs possess a respiratory nitrate reductase (Stein and Klotz, 2011; Stein et al., 2011; Svenning et al., 2011; Khadem et al., 2012b; Vuilleumier et al., 2012; Kits et al., 2013). It is also unclear whether methanotrophs that lack a respiratory nitrate reductase but possess dissimilatory nitrite and nitric oxide reductases are still capable of denitrification from $NO_2^-$. Moreover, due to the significant divergence of the methanotroph *nirS* from known sequences, it is not known, whether *nirS* is the operational nitrite reductase in the methanotrophs that lack a *nirK* (Wei et al., 2015). While the genome of the nitrate respiring *M. denitrificans* FJG1 encodes both *nirS* and *nirK* nitrite reductases, transcript levels of only *nirK* increased in response to denitrifying conditions (Kits et al., 2015).

The goal of the present study was to test whether a variety of $C_1$, $C_2$, and inorganic energy sources can directly support denitrification, characterize the environmental factors that regulate $NO_2^-$-dependent $N_2O$ production in *M. album* strain BG8 and to assess the expression of its putative denitrification inventory.

## Materials and Methods

### Cultivation

*Methylomicrobium album* strain BG8 was cultivated in 100 mL of nitrate mineral salts medium containing 11 mM $KNO_3$ (NMS) or 10 mM $KNO_3$ plus 1 mM $NaNO_2$ (NMS + $NO_2^-$) in 300 mL glass Wheaton bottles topped with butyl rubber septa (Whittenbury et al., 1970). The NMS media was buffered to pH 6.8 using a phosphate buffer (0.26 g/L $KH_2PO_4$, 0.33 g/L $Na_2HPO_4$). The final concentration of copper ($CuSO_4$) was 5 μM. Using a 60 mL syringe (BD) and a 0.22 μm filter/needle assembly, $CH_4$ (99.998%) was added into the sealed bottles as a sole carbon source. The initial gas-mixing ratio in the headspace was adjusted using $O_2$ gas (99.998%, Praxair) to 1.6:1, $CH_4$ to $O_2$ (or ca. 28% $CH_4$, 21% $O_2$). The initial pressure in the gas tight bottles was adjusted to ca. 1.3 atm to prevent a vacuum from forming during growth as gas samples and liquid culture samples were withdrawn every 12 h for analysis. Cultures were incubated at 30°C and shaken at 200 rpm. To track growth, the cultures were periodically sampled using a needle fitted syringe (0.5 mL) and cell density was determined by direct count with phase contrast microscopy using a Petroff–Hausser counting chamber. Six biological replicates were grown on separate days and data was collected on each replicate ($n = 6$). Culture purity was assessed by 16s rRNA gene sequencing, phase contrast microscopy, and plating on nutrient agar and TSA with absence of growth indicating no contamination. We assessed purity of the cultures prior to beginning all of the experiments and then assessed it again for each replicate at the conclusion of each experiment.

### Gas Analysis

Concentrations of $O_2$, $CH_4$, and $N_2O$ were determined by sampling the headspace of each culture using gas chromatography (GC-TCD, Shimadzu GC8A; outfitted with

a molecular sieve 5A and a Hayesep Q column, Alltech). The headspace of each batch culture was sampled with a 250 μL gastight syringe (SGE Analytical Science; 100 μL/injection) at 0 (immediately post inoculation), 6, 12, 16, 20, 24, 30, 36, 42, 48, 60, 72, 96, and 120 h. A total of 200 μL was sampled from each replicate at every time point. We determined the bottles were gastight by leaving a replicate set of bottles uninoculated throughout the experiment and measuring headspace gas concentrations; leakage was <1% over 120 h. Standard curves using pure gases $O_2$, $CH_4$, and $N_2O$ (Praxair) were generated and used to calculate the headspace concentrations in the batch cultures.

## Instantaneous Micro-sensor Assays

*Methylomicrobium album* strain BG8 was grown in NMS + $NO_2^-$ medium as described above. At 96 h of growth, when denitrification activity was highly evident, $4 \times 10^{10}$ cells were harvested using a filtration manifold onto 0.2 μm filters (Supor 200, 47 mm, Pall Corporation). The biomass was washed three times with sterile, nitrogen-free mineral salts medium – identical to the mineral salts medium used for cultivation but devoid of $NH_4Cl$, $KNO_3$, or $NaNO_2$. For data presented in **Figures 2** and **4,** the washed biomass was resuspended in the same nitrogen-free medium and transferred to a gastight 10 mL micro-respiration chamber equipped with an OX-MR $O_2$ micro-sensor (Unisense) and an $N_2O$-500 $N_2O$ micro-sensor (Unisense). For data presented in **Figure 3**, biomass was resuspended in mineral salts medium amended with 100 μM $NaNO_2$. Data was logged using SensorTrace Basic software. $CH_4$ gas, 0.001% $CH_3OH$ (HPLC grade methanol, Fisher Scientific), 0.01% $CH_2O$ (Methanol free 16% formaldehyde, Life technologies), 10 mM $HCO_2H$, $C_2H_6$ gas (99.999%), 0.01% $C_2H_6O$ (Methanol free 95% ethyl alcohol, Commercial Alcohols), 200 mM $NH_4Cl$, and/or 1 M $NO_2^-$ was injected directly into the chamber through the needle injection port with a gas-tight syringe (SGE Analytical Science). In **Figures 3B–E**, the dissolved $O_2$ was decreased to <100 μmol/L (**Figure 3B**) and <25 μmol/L (**Figures 3C–E**), respectively, with additions of $CH_4$ (**Figure 3A**), $CH_3OH$ (**Figure 3B**), $CH_2O$ (**Figure 3C**), $HCO_2H$ (**Figure 3D**), $C_2H_6$ (**Figure 3E**), $C_2H_6O$ (**Figure 3F**) before data logging was enabled to limit the traces to <100 min and to reduce the number of sampling points. $NO_2^-$ concentration was determined using a colorimetric method (Bollmann et al., 2011). Experiments were performed 3–4 times to demonstrate reproducibility of results and a single representative experiment was selected for presentation.

## RNA Extraction

Total RNA was extracted from ca. $10^9$ *M. album* strain BG8 cells grown in NMS or NMS + $NO_2^-$ medium at 24, 48, and 72 h using the MasterPure RNA purification kit (Epicentre). Briefly, cells were harvested by filtration through a 0.22 μm filter and inactivated with phenol–ethanol stop solution (5% phenol, 95% EtOH). Total nucleic acid was purified according to manufacturer's instructions with the following modifications: 6 U proteinase K (Qiagen) were added to the cell lysis step and the total precipitated nucleic acid was treated with 30 units of DNase I (Ambion). The total RNA was then column-purified using RNA clean & concentrator (Zymo Research). RNA quality and quantity was assessed using BioAnalyzer (Agilent Technologies) and Qubit (Life Technologies). Residual genomic DNA contamination was assessed by quantitative PCR (qPCR) targeting *norB1* or *nirS* genes (primers listed in **Table 1**). PCR conditions are described below. The total RNA samples were deemed free of genomic DNA if no amplification was detected after 40 cycles of qPCR. High quality RNA (RIN number >9, no gDNA detected) was converted to first strand cDNA using Superscript III reverse transcriptase (Life Technologies), according to manufacturer's instructions.

## Quantitiative PCR

Gene copy standards were created using the genomic DNA of *M. album* strain BG8 using universal and gene-specific primers

**TABLE 1 | qPCR Primers used in this study.**

| Gene target | Locus tag[1] | Primer set | Sequence (5′→3′) | qPCR efficiency | Standard curve – $R^2$ | Reference |
|---|---|---|---|---|---|---|
| *pxmA* | METAL_RS06980 | QpxmA-FWD-3 | GCTTGTCAGGGCTTACGATTA | 97.7% – 101.8% | 0.9989 | This study |
| | | QpxmA-REV-3 | CTTCCAGTCCACCCAGAAATC | | | |
| *pmoA* | METAL_RS17425 | QpmoA-FWD-7 | GTTCAAGCAGTTGTGTGGTATC | 95.1% – 97.2% | 0.9999 | This study |
| | | QpmoA-REV-7 | GAATTGTGATGGGAACACGAAG | | | |
| *nirS* | METAL_RS10995 | QnirS-FWD-1 | GTCGACCTGAAGGACGATTT | 95.1% – 98.8% | 0.9999 | This study |
| | | QnirS-REV-1 | GTCACGATGCTGTCGTCATA | | | |
| *norB1* | METAL_RS03925 | QnorB-FWD-2 | ACTGGCGGTGCACTATTT | 97.2% – 97.4% | 0.9998 | This study |
| | | QnorB-REV-2 | CATCCGGTTGACGTTGAAATC | | | |
| *norB2* | METAL_RS13345 | QnorB-F-1 | CACCATGTACACCCTCATCTG | 96.2% – 102.2% | 0.9999 | This study |
| | | QnorB-R-1 | CCAAAGTCTGCGCAAGAAAC | 96.1% – 101.8% | 0.9999 | This study |
| 16S rRNA | METAL_RS04240 | 341F | CCTACGGGAGGCAGCAG | 96.9% – 102.2 % | 0.9997 | Muyzer et al., 1993 |
| | | 518R | ATTACCGCGGCTGCTGG | | | |

[1]*The complete genome sequence of M. album strain BG8 is deposited in Genbank (http://www.ncbi.nlm.nih.gov/genbank/) under the accession NZ_CM001475 (http://www.ncbi.nlm.nih.gov/genome/?term=methylomicrobium%20album%20Bg8).*

(**Table 1**). A 10-fold dilution series ($10^0$–$10^8$ copies/20 μl reaction) of purified amplicons was prepared and used to establish an optimized qPCR condition. Each 20 μl reaction contained 10 μl of 2X qPCR SYBR based master mix (MBSU, University of Alberta), 0.2 μM of forward and reverse primer, 1 μl diluted cDNA, and nuclease-free water. Amplification was performed on a StepOne Plus qPCR system (Applied Biosystems) with an initial activation at 95°C for 3 min and fluorescence emission data collected from 40 cycles of amplification (95°C for 15 s, 60°C for 15 s, and 72°C for 15 s). Target specificity was assessed by melt curve analysis, which ensured that a single peak was obtained. Gene copy number was estimated from cDNA diluted from $10^{-3}$ to $10^{-5}$ copies for 16S rRNA and *pmoA* transcript analyses and dilutions from $10^{-1}$ to $10^{-3}$ copies for *nirS*, *norB1*, *norB2*, and *pxmA* transcript analyses. The transcript abundance of each functional gene was normalized to that of 16s rRNA to yield a copy number of transcripts per one billion copies of 16s rRNA. Then, to calculate the N-fold change, we divided the transcript abundance (per one billion copies of 16s rRNA) in the NMS + $NO_2^-$ cultures by transcript abundance (per one billion copies of 16s rRNA) in the NMS cultures. Samples were run in triplicate with three dilutions each on at least three biological replicates from cells grown and processed on separate dates. Quantitative PCR efficiencies ranged from 95–102% with $r^2$-values of at least 0.99 for all assays (**Table 1**).

### Statistics

A Student's *t*-test (two tailed) was used to calculate the P-level between the control (NMS alone) and experimental (NMS + $NO_2^-$) replicates as indicated for each experiment. Equal variance between the control and experimental groups was determined using a two sample *F* test for variance. The doubling time, $O_2$ and $CH_4$ consumption, cell density, and total headspace $O_2$ and $CH_4$ consumed (Supplementary Table S1) all had equal variance between the control and experimental ($F < F_{crit}$); thus a homoscedastic *t*-test was calculated for the aforementioned comparisons. For qPCR, comparisons between NMS + $NO_2^-$ and NMS alone at 48 h for *pmoA*, *pxmA*, *nirS*, and *norB1*, as well as for *pxmA* and *nirS* at 72 h showed unequal variance ($F > F_{crit}$); thus a heteroscedastic *t*-test was used to calculate the P-level for these comparisons. The variance between NMS + $NO_2^-$ and NMS alone for all other genes at all other time points was equal ($F < F_{crit}$).

## Results

### Growth Phenotype of *Methylomicrobium album* Strain BG8 in the Absence or Presence of $NO_2^-$

*Methylomicrobium album* strain BG8 was cultivated in NMS or NMS supplemented with $NO_2^-$ over 120 h to determine the effect of $NO_2^-$ on growth, $O_2$ and $CH_4$ consumption, and $N_2O$ production (**Figure 1**). The total amount of nitrogen was kept constant to eliminate a difference in N-availability and salt concentration between treatments. All of the cultures were initiated at an oxygen ($O_2$) tension of 19.5 ± 0.7% (**Figure 1B**). As observed previously (Nyerges et al., 2010), $NO_2^-$ amendment (1 mM) did not have an inhibitory effect on growth or substrate consumption of *M. album* strain BG8 (**Figures 1A–C** and Supplementary Table S1). The limiting substrate in all treatments was $O_2$, as demonstrated by supplementing cultures with additional $O_2$ (20 mL) after 48 h of growth and observing a significant increase in optical density in comparison to cultures not receiving additional $O_2$ (Supplementary Figure S1). $N_2O$ production occurred only in the NMS plus $NO_2^-$ cultures (**Figure 1D**). $N_2O$ production was first apparent in the headspace of $NO_2^-$ amended cultures at 72 h of growth when $O_2$ reached ca. 1.8% of the headspace and continued up to the termination of the experiment (120 h) at a rate of $9.3 \times 10^{-18}$ mol $N_2O$ per cell per hour (**Figure 1D**). After 120 h of growth, the $N_2O$ yield percentage from the added $NO_2^-$ (100 μmol) was 5.1 ± 0.2% (5.1 ± 0.2 μmol) in the NMS + $NO_2^-$ cultures.

### $O_2$ Consumption and $N_2O$ Production by Resting Cells of *M. album* Strain BG8 with Single or Double Carbon Substrates or Ammonium under Atmospheric and Hypoxic $O_2$ Tensions

To determine which conditions govern $N_2O$ production in *M. album* strain BG8, we measured instantaneous $O_2$ consumption and $N_2O$ production by *M. album* strain BG8 with $CH_4$ as the sole carbon and energy source in a closed 10-mL micro-respiratory (MR) chamber outfitted with $O_2$ and $N_2O$-detecting microsensors. Introduction of $CH_4$ (300 μM) into the chamber led to immediate $O_2$ consumption; $O_2$ declined to below the detection limit of the sensor (<50 nM $O_2$) after ca. 3 min (**Figure 2A**). Addition of $NO_2^-$ to the chamber led to production of $N_2O$ shortly after $O_2$ declined below the detection limit at a rate of $7.9 \times 10^{-18}$ mol cell$^{-1}$ h$^{-1}$ (**Figure 2B**). In the absence of $NO_2^-$, we observed no measureable $N_2O$ production (**Figure 2A**). Though the $O_2$ concentration is <50nM $O_2$ when $N_2O$ production is evident, it is important to note that *M. album* strain BG8 still requires $O_2$ for methane oxidation and cannot grow on $CH_4$ anaerobically.

Using the same setup described above, we supplemented resting cells in the MR chamber with $CH_3OH$, $CH_2O$, $HCO_2H$, $C_2H_6$, or $C_2H_6O$ to experimentally address whether carbon-based reductant sources other than $CH_4$ support denitrification in *M. album* strain BG8. Also, to substantiate that the one- and two-carbon sources we tested can all serve as direct electron donors for denitrification by *M. album* strain BG8 under hypoxia, we provided resting cells only enough reductant to consume the dissolved $O_2$ (ca. 234 μmol/L) present in the MR chamber sparing no reductant to support denitrification (**Figure 3**). We then measured instantaneous $N_2O$ production through serial addition of small quantities of $CH_4$, $CH_3OH$, $CH_2O$, $HCO_2H$, $C_2H_6$, or $C_2H_6O$ to the MR chamber, which contained medium supplemented with $NaNO_2$ (100 μM; **Figure 3**). For all six substrates, $N_2O$

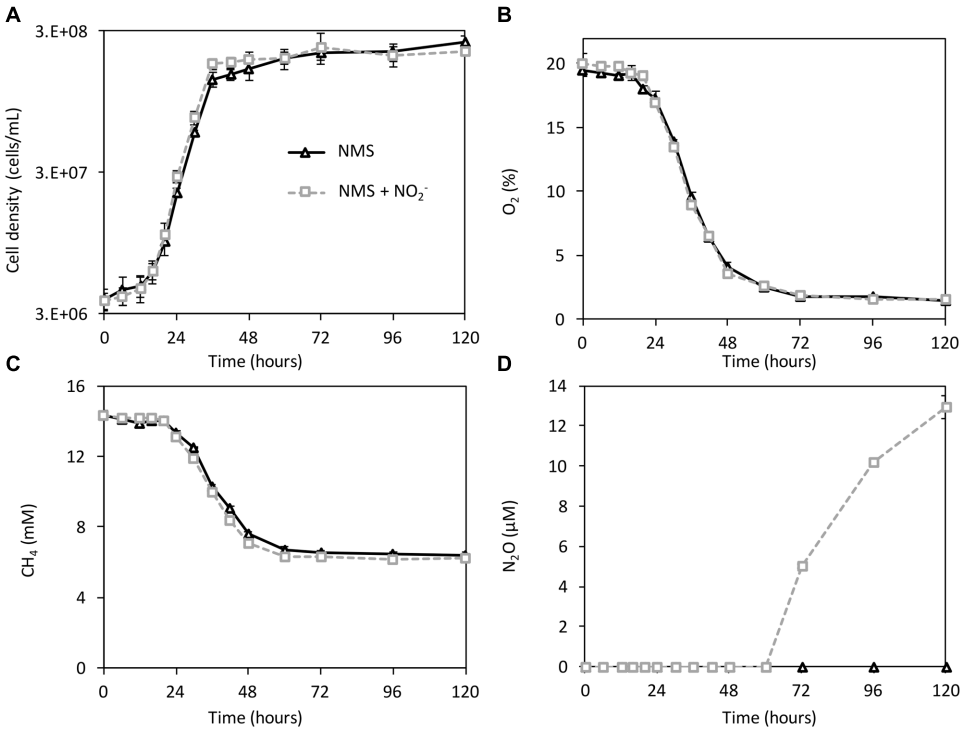


**FIGURE 1 | Growth, $CH_4$ and $O_2$ consumption, and $N_2O$ production by *Methylomicrobium album* strain BG8 cultivated on NMS and NMS plus 1 mM $NaNO_2$.** *Methylomicrobium album* strain BG8 was cultivated for 5 days in 100 mL of NMS (black triangles) or NMS + 1 mM $NO_2^-$ (gray dashed squares) media in 300 mL closed glass Wheaton bottles sealed with butyl rubber septum caps. The initial headspace gas-mixing ratio of $CH_4$ to $O_2$ was 1.6:1. Cell density **(A)** was measured using direct count with a Petroff–Hausser counting chamber and headspace gas concentrations of $O_2$ **(B)**, $CH_4$ **(C)** and $N_2O$ **(D)** were measured using GC-TCD. All data points represent the mean ± SD for six biological replicates ($n = 6$).

production was stoichiometric with the amount of added substrate (**Figure 3**).

Many methanotrophs, including *M. album* BG8, can oxidize $NH_3$ to $NO_2^-$ due to homologous inventory to ammonia-oxidizing bacteria (Yoshinari, 1985; Bedard and Knowles, 1989; King and Schnell, 1994; Holmes et al., 1995; Poret-Peterson et al., 2008; Campbell et al., 2011; Stein and Klotz, 2011). We aimed to test whether reductant and $NO_2^-$ from $NH_3$ oxidation could also drive denitrification by *M. album* strain BG8. Resting cells in the MR chamber consumed the dissolved $O_2$ promptly after $NH_4Cl$ (200 µM) was injected into the chamber (**Figure 4**). After ca. 70 min, the biomass depleted the dissolved $O_2$ to <50 nM and $NO_2^-$ concentration reached 163 ± 5 µM. The rate of $N_2O$ production following $O_2$ depletion was $1.2 \times 10^{-18}$ mol cell$^{-1}$ h$^{-1}$.

## Expression of Predicted Denitrification Genes in *M. album* Strain BG8 under Denitrifying Conditions

The genome of *M. album* strain BG8 encodes several genes predicted to be involved in denitrification. The first step in respiratory denitrification is the one-electron reduction of $NO_3^-$ to $NO_2^-$; a reaction performed by one of two membrane-associated dissimilatory nitrate reductase enzymes, neither of which is encoded in the *M. album* strain BG8 genome (Kits et al., 2013). The second step in denitrification, the one-electron reduction of $NO_2^-$ to NO is carried out by one of two non-homologous nitrite reductases, either a copper containing (*nirK*) or a cytochrome $cd_1$ containing (*nirS*) nitrite reductase, of which the latter was annotated in the genome (Kits et al., 2013). The genome of *M. album* strain BG8 also contains two copies of a putative cytochrome *c*-dependent nitric oxide reductase (*norB1* and *norB2,* respectively). We also investigated expression of the *pxmA* gene of the *pxmABC* operon that encodes a CuMMO with evolutionarily relatedness to particulate methane monooxygenase (Tavormina et al., 2011). We chose to examine expression of *pxmA* in *M. album* strain BG8 to determine whether this gene responded similarly to that of *M. denitrificans* FJG1; expression of the *pxmABC* operon in *M. denitrificans* FJG1 significantly increased in response to denitrifying conditions (Kits et al., 2015).

To assess the effect of $NO_2^-$ amendment on gene expression, we used cultures grown in NMS alone as the control. The $O_2$ concentration in the headspace of NMS and NMS + $NO_2^-$ cultures after 24 h growth was ca. 17.2 and 16.9%, respectively (**Figure 1B**). The transcript levels of *pmoA, pxmA, nirS,* and *norB1* were significantly higher at the 24 and 48 h time points in the $NO_2^-$ amended cultures when compared to the

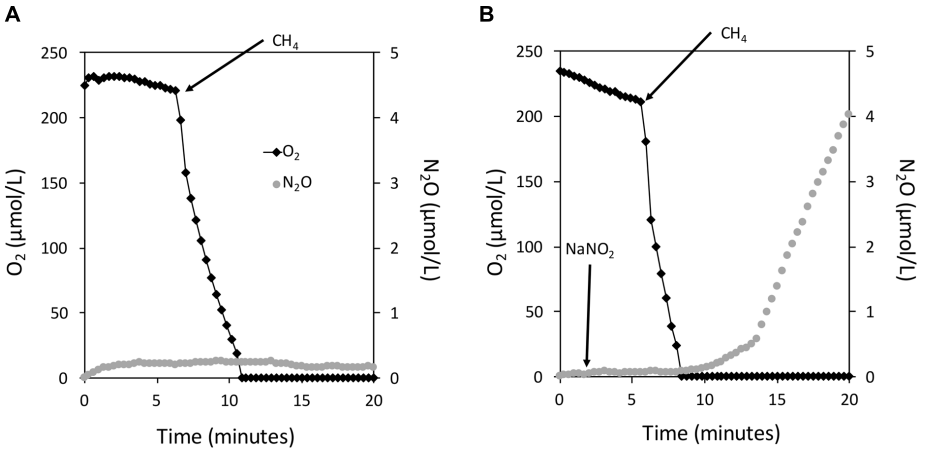


**FIGURE 2 | The instantaneous coupling of $CH_4$ oxidation to $NO_2^-$ reduction in *Methylomicrobium album* strain BG8 under hypoxia.** Experiments were performed in a closed 10 mL micro-respiratory chamber outfitted with an $O_2$ and $N_2O$ microsensor and logged with Sensor Trace Basic software. $O_2$ (black diamonds) and $N_2O$ (gray circles) were measured using microsensors. Cells of *M. album* strain BG8 were harvested as described in the materials and methods and resuspended in nitrogen free mineral salts medium. Arrows mark the addition of $CH_4$ (~300 µM) and $NaNO_2$ (1 mM) to the micro-respiratory chamber in all panels. There is no measureable denitrification activity in the absence of $NO_2^-$ **(A)**; denitrification activity is dependent on $CH_4$ and $NO_2^-$ **(B)**.

NMS alone (**Figure 5**). At the 72 h time point, levels of *pmoA* and *nirS* transcript levels remained significantly elevated in the NMS + $NO_2^-$ relative to the NMS only cultures, whereas expression of *norB1* was no longer significantly elevated (**Figure 5**). Most interestingly, the transcript abundance of *pxmA* at 72 h was 19.8-fold higher in NMS + $NO_2^-$ relative to NMS only cultures (**Figure 5**). The second copy of *norB* (*norB2*) was unresponsive (below twofold) to $NO_2^-$ amendment at all time points sampled.

## Discussion

### *Methylomicrobium album* Strain BG8 Produces $N_2O$ Only as a Function of Hypoxia and $NO_2^-$

Batch cultivation of *M. album* BG8 clearly revealed that both $NO_2^-$ and low $O_2$ were required for denitrification, as measured by $N_2O$ production. Although batch cultures of *M. album* strain BG8 have been shown to produce $N_2O$ previously in end-point assays (Nyerges et al., 2010), the mechanism and required conditions for denitrification by this strain were not determined until now. $N_2O$ production by *M. denitrificans* FJG1 was also shown to be dependent on hypoxia (Kits et al., 2015); however, this strain was able to respire $NO_3^-$ in addition to $NO_2^-$ likely due to the presence of a *narGHJI* dissimilatory nitrate reductase that is absent in the genome of *M. album* strain BG8. The genome of *M. album* strain BG8 encodes putative dissimilatory nitrite (*nirS*) and nitric oxide (*norB*) reductases (Kits et al., 2013) like *M. denitrificans* FJG1; hence, it is likely that $N_2O$ by *M. album* strain BG8 is from the enzymatic reduction of $NO_2^-$ to $N_2O$ via the intermediate NO.

The correlation between $N_2O$ production and low $O_2$ tension is similar to two other microbial processes, aerobic denitrification in heterotrophic bacteria such as *Paracoccus denitrificans* and nitrifier denitrification in ammonia-oxidizing bacteria (Richardson et al., 2001; Kozlowski et al., 2014). Aerobic denitrification in chemoorganoheterotrophs and nitrifier-denitrification in ammonia-oxidizing bacteria is a tactic used to maximize respiration during $O_2$ limitation or to expend surplus reductant (Richardson et al., 2001; Stein, 2011). Utilization of $NO_2^-$ in combination with or instead of $O_2$ in the respiratory chain of *M. album* strain BG8 would reduce the overall cellular $O_2$ demand, thus conserving $O_2$ for additional $CH_4$ oxidation. Thus, it is possible that *M. album* strain BG8 uses $NO_2^-$ as a terminal electron acceptor under $O_2$ limitation to maximize total respiration. The $N_2O$ yield percentage from $NO_2^-$ by *M. album* strain BG8 (5.1 ± 0.2%) is similar to that of *Nitrosomonas europaea* ATCC 19718 (ca. 4.8%) and one order of magnitude higher than that of *Nitrosospira multiformis* ATCC 25196 (0.27 ± 0.05%; Kozlowski et al., 2014; Stieglmeier et al., 2014).

### Denitrification by *M. album* Strain BG8 is Enzymatically Supported by Diverse Reductant Sources

Resting cells of *M. album* strain BG8 reduced $NO_2^-$ to $N_2O$ at the expense of any of four tested $C_1$ substrates ($CH_4$, $CH_3OH$, $CH_2O$, $HCO_2H$), the two $C_2$ substrates ($C_2H_6$, $C_2H_6O$), and $NH_4Cl$. These data show that intermediates of the methanotrophic pathway and co-substrates of pMMO, MDH, and likely hydroxylamine dehydrogenase support respiratory denitrification. These results agree with previous work on the methanotroph *Methylocystis* sp. strain SC2, which couples $CH_3OH$ oxidation to denitrification under anoxia (Dam et al., 2013). Remarkably, both $C_2$ compounds we tested – $C_2H_6$ and $C_2H_6O$ – supported denitrification. The ability of $C_2$ compounds to support denitrification in

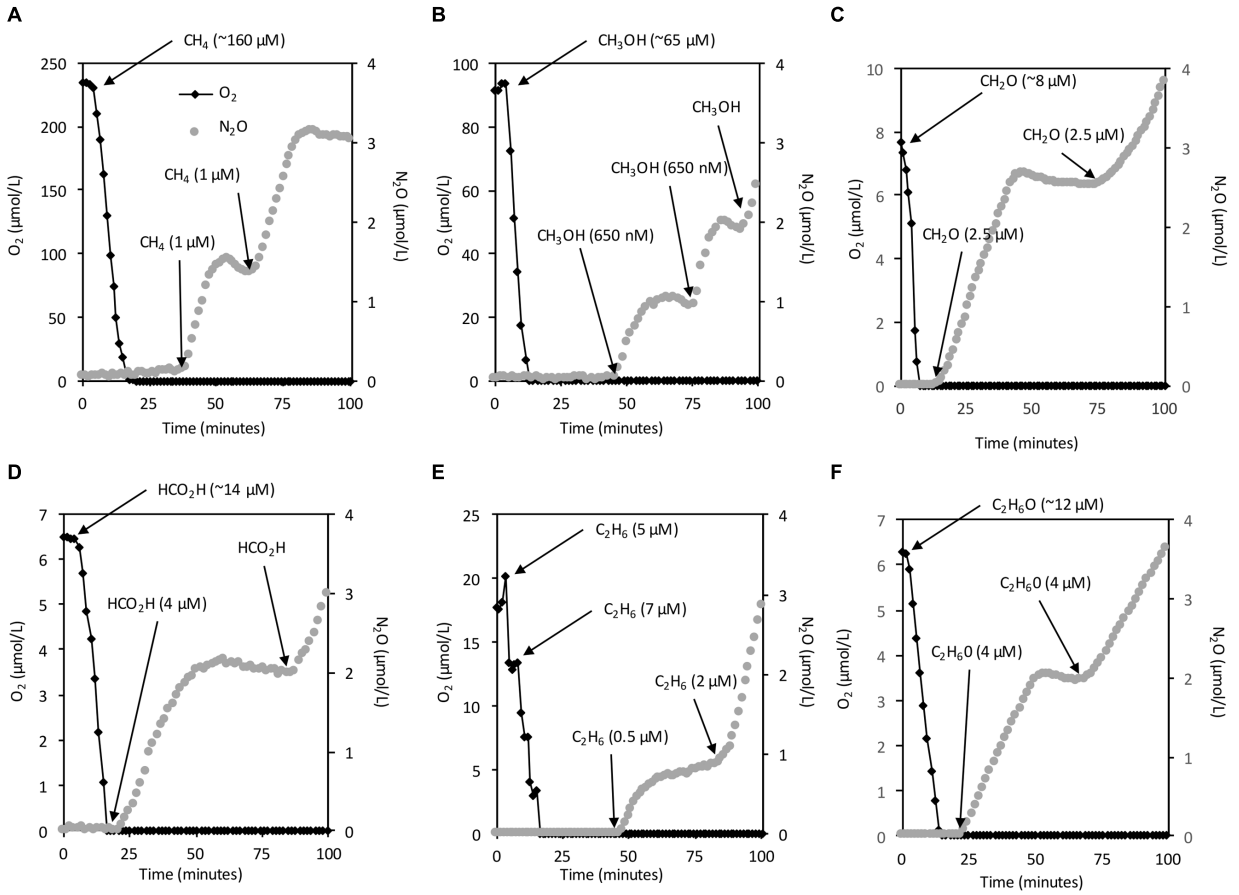


**FIGURE 3 | $NO_2^-$ reduction to $N_2O$ by *M. album* strain BG8 is dependent on an energy source at <50 nM $O_2$.** Experiments were performed in a closed 10 mL micro-respiratory chamber outfitted with an $O_2$ and $N_2O$ microsensor and logged with Sensor Trace Basic software. $O_2$ (black diamonds) and $N_2O$ (gray circles). Cells of *M. album* strain BG8 were harvested as described in the materials and methods and resuspended in mineral salts medium containing 100 µM $NO_2^-$. Arrows mark the addition of either $CH_4$ **(A)**, $CH_3OH$ **(B)**, $CH_2O$ **(C)**, $HCO_2H$ **(D)**, $C_2H_6$ **(E)**, $C_2H_6O$ **(F)**, in all panels. The right *y*-axis is identical in all panels. However, it should be noted that the left *y*-axis differs in all panels.

methanotrophs may have environmental significance as natural gas consists of ∼1.8–5.1% (vol%) $C_2H_6$ (Demirbas, 2010). Further, $C_2H_6O$ is a significant product of fermentation by primary fermenters during anoxic decomposition of organic compounds (Reith et al., 2002). The results also demonstrate that electrons derived from the oxidation of $NH_3$ to $NO_2^-$ were effectively utilized by nitrite and nitric oxide reductases in *M. album* strain BG8, which represents yet another pathway for methanotrophic $N_2O$ production that is not directly dependent on single-carbon metabolism, provided that the methane monooxygenase can access endogenous reductant (Dalton, 1977; King and Schnell, 1994; Stein and Klotz, 2011).

Instantaneous $O_2$ consumption and $N_2O$ production measurements (**Figures 2–4**) provide strong support that catabolism of $C_1$ – $C_2$ substrates and ammonia is directly coupled to $NO_2^-$ reduction under hypoxia in *M. album* strain BG8. Some aerobic methanotrophs ferment $CH_4$ and excrete organic compounds such as citrate, acetate, succinate, and lactate (Kalyuzhnaya et al., 2013). Some studies also suggest that methanotrophs only support denitrification within $CH_4$-fed consortia by supplying these excreted organics to denitrifying bacteria, since methanotrophs were thought incapable of denitrification by themselves (Costa et al., 2000; Knowles, 2005; Liu et al., 2014). Although *M. album* strain BG8 may excrete organic compounds under hypoxia when provided with $CH_4$, the ability of $CH_3OH$, $CH_2O$, $HCO_2H$, $C_2H_6$, $C_2H_6O$, or $NH_3$ oxidation to support denitrification unequivocally demonstrates the linkage between methanotroph-specific enzymology and denitrifying activity within a single organism.

## Transcription of Predicted Denitrification Genes, *nirS* and *norB1*, Increased in Response to $NO_2^-$ but not Hypoxia

The expression of a *nirS* homolog in an aerobic methanotroph has been investigated so far only in the $NO_3^-$ respiring *M. denitrificans* FJG1 (Kits et al., 2015). Interestingly, the genome of *M. denitrificans* FJG1 encodes both the copper-containing (*nirK*) and cytochrome $cd_1$ containing (*nirS*) nitrite reductases and only the steady state mRNA levels of *nirK* increased in

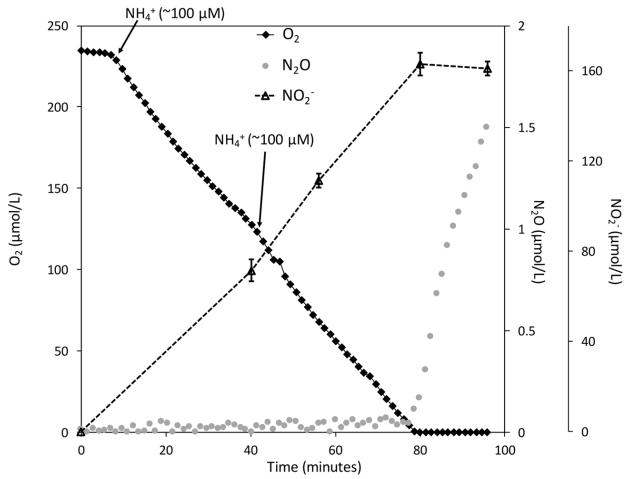


**FIGURE 4 | The coupling of $NH_3$ oxidation to $NO_2^-$ reduction in *Methylomicrobium album* strain BG8 under hypoxia.** Experiments were performed in a closed 10 mL micro-respiratory chamber outfitted with an $O_2$ and $N_2O$ microsensor and logged with Sensor Trace Basic software. $O_2$ (black diamonds), $N_2O$ (gray circles), $NO_2^-$ (black dashed triangles). Cells of *M. album* strain BG8 were grown and harvested as described in the materials and methods and resuspended in nitrogen free mineral salts medium. Arrows mark the addition of $NH_4^+$ (100 μM) to the closed micro-respiratory chamber. Traces ($O_2$ + $N_2O$) are single representatives of reproducible results from cultures grown on different days. $NO_2^-$ was measured using a colorimetric method as described in the Section "Materials and Methods" and data points represent the mean ± SD for three technical replicates.

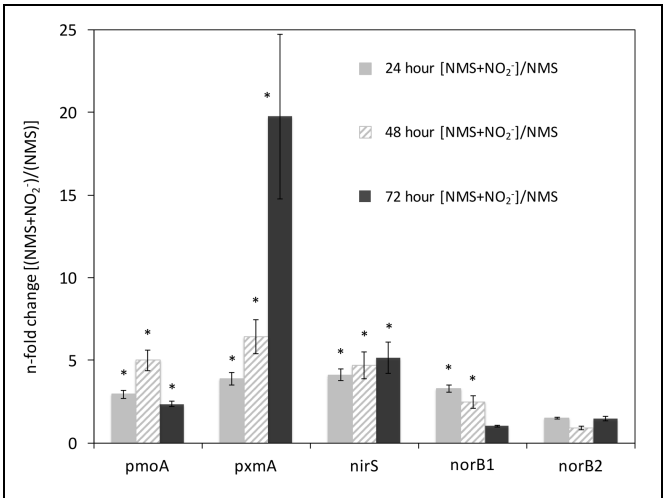


**FIGURE 5 | Expression of *pmoA*, *pxmA*, *nirS*, *norB1*, and *norB2* in *Methylomicrobium album* strain BG8 cultivated in NMS or NMS media amended with 1 mM $NaNO_2$.** Total RNA was extracted from *Methylomicrobium album* strain BG8 at 24, 48, and 72 h of growth (see **Figure 1**) from three separate cultures, converted to cDNA, and the abundance of *pmoA*, *pxmA*, *nirS*, *norB1*, and *norB2* transcripts was determined using quantitative PCR. The transcript abundance of each gene of interest was normalized to that of 16s rRNA. The n-fold change in transcript abundance of the $NO_2^-$ amended (1 mM $NaNO_2$) NMS cultures relative to the unamended NMS cultures at 24 h of growth (light gray), 48 h of growth (diagonal white/gray), and at 72 h of growth (black). Error bars represent the SD calculated for triplicate qPCR reactions performed on each of the three biological replicates for each treatment. The (*) above the bars designates a statistical significance ($P < 0.05$) as determined by *t*-test between NMS only and NMS + $NO_2^-$ for each time point.

this strain in response to simultaneous $O_2$ limitation and $NO_3^-$ availability (Kits et al., 2015). In the case of *M. album* strain BG8, which only possesses a *nirS* homolog, we showed that the abundance of this *nirS* transcript responded positively to $NO_2^-$ treatment but not to $O_2$ limitation. This suggests that $NO_2^-$ availability alone elicits the expression of *nirS*, even though hypoxia was required for $NO_2^-$ reduction to occur.

The cytochrome *c* dependent nitric oxide reductase (*norB*) is widely found in the genomes of aerobic methanotrophs (Stein and Klotz, 2011). This may in part be due to the need to detoxify NO that is produced during aerobic ammonia oxidation by reducing it to $N_2O$ (Sutka et al., 2003). The expression

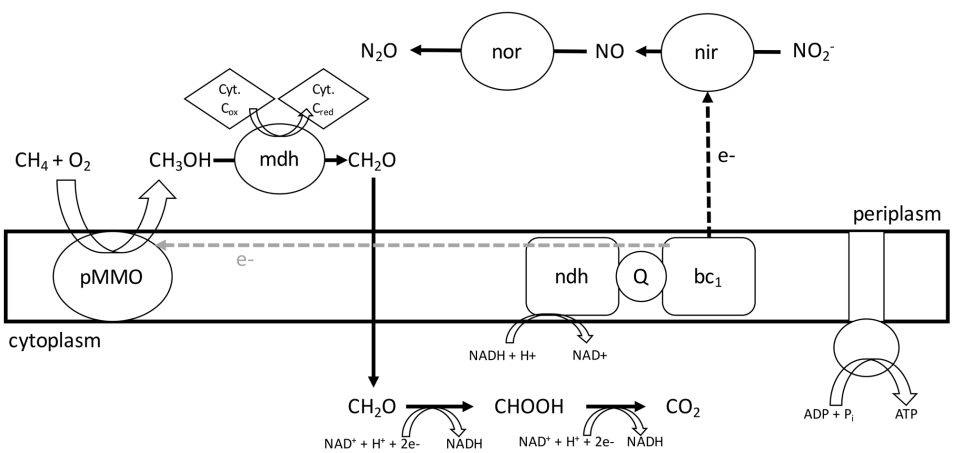


**FIGURE 6 | Proposed model for $NO_2^-$ respiration and central metabolism in *Methylomicrobium album* strain BG8.** During hypoxia, *M. album* strain BG8 utilizes electrons from aerobic $CH_4$ oxidation to respire $NO_2^-$. Abbreviations: pMMO, particulate methane monooxygenase; mdh, methanol dehydrogenase; Cyt, cytochrome; nor, nitric oxide reductase; nir, nitrite reductase; ndh, NAD(P)H dehydrogenase complex; Q, coenzyme Q; $bc_1$, cytochrome $bc_1$ complex.

of *norB* in *Methylococcus capsulatus* strain Bath increased 4.8-fold after treatment with 0.5 mM sodium nitroprusside, a NO releasing compound (Campbell et al., 2011). It is possible that the NorB protein is involved in detoxification of NO during $NH_3$ oxidation in *M. capsulatus* strain Bath, since the genome lacks a dissimilatory nitrite reductase. More recently, it was demonstrated in *M. fumariolicum* strain SolV that transcription of *norB* was upregulated during $O_2$ limitation during chemostat growth (Khadem et al., 2012a); however, it is unknown whether *M. fumariolicum* strain SolV can consume $NO_2^-$ or NO. The transcription of *norB* in *M. denitrificans* FJG1 increased 2.8-fold in response to $NO_3^-$ and hypoxia (Kits et al., 2015). While the genome of *M. album* strain BG8 encodes two copies of the *norB* gene, only one copy (*norB1*) is followed by *norC* – the essential cytochrome *c*-containing subunit (Mesa et al., 2002). Although some organisms like *Cupriavidus necator* possess two independent functional nitric oxide reductases (Cramm et al., 1997), the present work illustrates that expression of only *norB1* in *M. album* strain BG8 is responsive to $NO_2^-$ treatment. Although the function of NorB may differ between *M. album* strain BG8 and *M. capsulatus* strain Bath, both bacteria show a similar transcriptional response of *norB* genes to $NO_2^-$ (Campbell et al., 2011).

### Transcript Abundance of *pxmA* Significantly Increased in Response to both $NO_2^-$ and Hypoxia

Genomes of some aerobic methanotrophs belonging to the phylum *Gammaproteobacteria* have been shown to encode a sequence divergent CuMMO protein complex, pXMO (Tavormina et al., 2011). The function and substrate of the putative pXMO protein encoded by the *pxm* operon remains unknown. Previous studies on the *pxm* operon have shown that it is expressed at low levels during growth in *Methylomonas* sp. strain LW13 as well as in freshwater peat bog and creek sediment (Tavormina et al., 2011). Metagenomic sequencing of the SIP-labeled active community in an oilsands tailings pond revealed that *pxmA* sequences were present in the active methanotroph community (Saidi-Mehrabad et al., 2013). Analysis of the transcriptome of *M. denitrificans* FJG1 revealed that steady state mRNA levels of the *pxmABC* operon increased ∼10-fold in response to denitrifying conditions (Kits et al., 2015).

We now demonstrate that expression of *pxmA* in *M. album* strain BG8 is significantly increased in response to both $NO_2^-$ and hypoxia. We did not observe any increase in the expression of *pxmA* in $O_2$ limited NMS-only cultures where denitrification was not occurring, suggesting that hypoxia alone is not sufficient to illicit an increase in the steady state mRNA levels. This study adds further support to the observation that expression of *pxmA* is responsive to denitrifying conditions. However, it must be noted that at 72 h in the $NO_2^-$ amended media, absolute transcript abundance of *pxmA* ($1 \times 10^3$ copies *pxmA*/$1 \times 10^9$ copies 16s rRNA) was three orders of magnitude lower than absolute transcript abundance of *pmoA* ($1 \times 10^6$ copies *pxmA*/$1 \times 10^9$ copies 16s rRNA).

## Conclusion

The present study demonstrates that an aerobic methanotroph – *M. album* strain BG8 – couples the oxidation of $C_1$ ($CH_4$, $CH_3OH$, $CH_2O$, $HCO_2H$), $C_2$ ($C_2H_6$, $C_2H_6O$), and inorganic ($NH_3$) substrates to $NO_2^-$ reduction under $O_2$ limitation resulting in release of the potent greenhouse gas $N_2O$. The ability to couple $C_1$, $C_2$, and inorganic energy sources to $O_2$ respiration and denitrification gives *M. album* strain BG8 considerable metabolic flexibility. We propose a model for methane driven denitrification in *M. album* strain BG8 (**Figure 6**). This discovery has implications for the environmental role of methanotrophic bacteria in the global nitrogen cycle in both $N_2O$ emissions and N-loss. Comparing the genome and physiology of the $NO_2^-$ respiring *M. album* strain BG8 to $NO_3^-$ respiring *M. denitrificans* FJG1 suggests that the inability of *M. album* strain BG8 to reduce $NO_3^-$ to $N_2O$ is likely due to the absence of a dissimilatory nitrate reductase in the genome, but that expression of predicted denitrification genes, *nirS* and *norB1*, enable this aerobic methanotroph to respire $NO_2^-$.

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## Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01072

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# Diversity and Habitat Preferences of Cultivated and Uncultivated Aerobic Methanotrophic Bacteria Evaluated Based on *pmoA* as Molecular Marker

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Methane-oxidizing bacteria are characterized by their capability to grow on methane as sole source of carbon and energy. Cultivation-dependent and -independent methods have revealed that this functional guild of bacteria comprises a substantial diversity of organisms. In particular the use of cultivation-independent methods targeting a subunit of the particulate methane monooxygenase (*pmoA*) as functional marker for the detection of aerobic methanotrophs has resulted in thousands of sequences representing "unknown methanotrophic bacteria." This limits data interpretation due to restricted information about these uncultured methanotrophs. A few groups of uncultivated methanotrophs are assumed to play important roles in methane oxidation in specific habitats, while the biology behind other sequence clusters remains still largely unknown. The discovery of evolutionary related monooxygenases in non-methanotrophic bacteria and of *pmoA* paralogs in methanotrophs requires that sequence clusters of uncultivated organisms have to be interpreted with care. This review article describes the present diversity of cultivated and uncultivated aerobic methanotrophic bacteria based on *pmoA* gene sequence diversity. It summarizes current knowledge about cultivated and major clusters of uncultivated methanotrophic bacteria and evaluates habitat specificity of these bacteria at different levels of taxonomic resolution. Habitat specificity exists for diverse lineages and at different taxonomic levels. Methanotrophic genera such as *Methylocystis* and *Methylocaldum* are identified as generalists, but they harbor habitat specific methanotrophs at species level. This finding implies that future studies should consider these diverging preferences at different taxonomic levels when analyzing methanotrophic communities.

**Keywords: methanotrophic bacteria, *pmoA*, diversity, phylogeny, habitat specificity, ecological niche**

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## OCCURRENCE AND ROLE OF METHANE-OXIDIZING BACTERIA

The activity of methane-oxidizing bacteria contributes significantly to the global methane budget. Methane is the second most abundant carbon compound in the atmosphere with a current concentration of 1.8 ppmv and a 26-fold stronger radiative efficiency compared to carbon dioxide (IPCC, 2013). The major sink of atmospheric methane is its oxidation by OH radicals, but soils also serve as sink by about 5% due to the activity of methanotrophic bacteria (IPCC, 2013). Moreover,

methanotrophs are of particular importance in attenuating net fluxes of this greenhouse gas into the atmosphere in diverse ecosystems that are sources of atmospheric methane (De Visscher et al., 2007; Reeburgh, 2007; Conrad, 2009). Known sources are freshwater and permafrost ecosystems, some animal species and termites, and the release of methane from geological processes, wildfires and hydrates. Another 50–65% of the total emissions are due to anthropogenic activities including ruminant husbandry, fossil fuel extraction and use, rice paddy agriculture and emissions from landfills and waste, resulting in a current elevation of the atmospheric methane concentration by a factor of 2.5 compared to preindustrial times (IPCC, 2013). All these ecosystems with source function for atmospheric methane are typical habitats of methane-oxidizing bacteria. These include freshwater and marine sediments and water columns, aquifers, floodplains, peat bogs, high-arctic, and tundra wetlands, upland soils, rice paddies, landfill covers, and sewage sludge (Hanson and Hanson, 1996; Conrad, 2007; Bowman, 2014).

Besides their importance in the global methane cycle, aerobic methanotrophic bacteria are of biotechnological interest since a long time. They can be used for biodegradation processes of organic pollutants based on the fact that the key enzyme for methanotrophy in these organisms, the methane monooxygenase, catalyzes diverse non-specific oxidation reactions, e.g., of chlorinated solvents such as trichloroethylene (Hanson and Hanson, 1996; Smith and Dalton, 2004; Dalton, 2005; Jiang et al., 2010; Semrau et al., 2010; Strong et al., 2015). Moreover, methanotrophs have been studied in detail with regard to their potential to convert methane to complex organic molecules of higher value. Since the 1970s, methanotrophic bacteria have been studied for single cell protein production (Dalton, 2005). Besides, biopolymers such as polyhydroxybutyrate, metabolic products such as organic acids, vitamins, pigments or lipids (for biodiesel production) may be produced from methane by methanotrophs (Strong et al., 2015). Further possible applications for biosynthesis processes are based on the co-metabolic activities of the methane monooxygenase, e.g., for epoxide production via the conversion of propene to epoxypropane (Hanson and Hanson, 1996; Dalton, 2005). Moreover, researchers address the question to what extent methanotrophic bacteria can be used to increase reduction of methane emissions from anthropogenic sources such as landfills or coal mines (Jiang et al., 2010).

## DIVERSITY AND ECOPHYSIOLOGY OF CULTIVATED METHANOTROPHIC BACTERIA

### Brief History About the Cultivation of Aerobic Methanotrophic Bacteria and Current Diversity and Phylogeny of Cultivated Methanotrophs

Methanotrophic bacteria have been studied since the beginning of the last century, initiated by the work of Kaserer (1905) and Söhngen (1906) who reported for the first time the existence of methane-oxidizing bacteria. The first isolates were methanotrophic *Gammaproteobacteria*, among them *Methylomonas methanica*, initially referred to as "*Bacillus methanicus*" (Söhngen, 1906), and *Methylococcus capsulatus* (Foster and Davis, 1966). Extensive enrichment and isolation work by Whittenbury et al. (1970b) led to isolates of further *Gammaproteobacteria* and the genera *Methylocystis* and *Methylosinus*, i.e., the first methanotrophic *Alphaproteobacteria*. During the following years and with the availability of molecular methods for the rapid identification and classification of bacteria, several existing strains were reclassified and new genera were described (e.g., Bowman et al., 1993, 1995; Bodrossy et al., 1997). In particular the work of the last 10 years has resulted in a doubling of the number of known genera and species. Currently, 18 genera of cultivated aerobic methanotrophic *Gammaproteobacteria* and 5 genera of *Alphaproteobacteria* are known, represented by approx. 60 different species (**Table 1**). The number of *Gammaproteobacteria* increases to 20 if "*Candidatus* Crenothrix polyspora" and "*Candidatus* Clonothrix fusca" are included. These genera do not contain cultivated representatives but were only studied in natural enrichments so far (Stoecker et al., 2006; Vigliotta et al., 2007). To give an exact number of known methanotrophic taxa at species level is difficult because the taxonomic status of some species, e.g., "*Methylomonas rubra*," *Methylococcus chroococcus*, *Methylococcus mobilis* or *Methylococcus thermophilus* is unclear (**Table 2**). In addition to the species considered in this review, more species have been described in the (early) literature, in particular within the genera *Methylomonas* and *Methylocystis* (e.g. Whittenbury et al., 1970b; Gal'chenko et al., 1977), but these were never validated. Several of them will probably be members of species that have been described in the meantime. For an overview of non-validated species with uncertain taxonomic position the reader is referred to Green (1992) or the relevant chapters in taxonomic textbooks (Bowman, 2005a,b, 2014).

The known diversity of aerobic methanotrophic bacteria was further expanded by the detection of methanotrophic bacteria within the phylum *Verrucomicrobia* (**Table 3**). Their existence was described in three independent studies in 2007 and 2008 (Dunfield et al., 2007; Pol et al., 2007; Islam et al., 2008) and they were reported to represent distinct species of the genus "*Methylacidiphilum*" (Op den Camp et al., 2009). Recently, a second genus within the newly formed methanotrophic family *Methylacidiphilaceae* was proposed, "*Methylacidimicrobium*," also consisting of three species (van Teeseling et al., 2014).

Phylogenetically, the methanotrophic *Alphaproteobacteria* belong to two families, the *Methylocystaceae* and *Beijerinckiaceae* (**Figure 1**, **Table 1**). Both families include additional genera of non-methanotrophic bacteria. Nearly all methanotrophic *Gammaproteobacteria* are classified into the families *Methylococcaceae* or the recently delineated *Methylothermaceae* (Hirayama et al., 2014). These families do not contain any non-methanotrophic bacteria. "*Candidatus* Crenothrix polyspora" is the only exception as it belongs to a distinct family, the *Crenotrichaceae*, but this classification was put into question by Op den Camp et al. (2009), who proposed that *Crenothrix* could

**TABLE 1 | Taxonomic and physiological characteristics of aerobic methanotrophic *Alphaproteobacteria*.**

| Type strain[a] Culture collection numbers | Type | Isolation source[b] | (Eco-) physiology | pMMO present | *mmoX* sequence | References[c] |
|---|---|---|---|---|---|---|
| ***Methylocystaceae*** | | | | | | |
| *Methylocystis bryophila* H2s DSM 21852, VKM B-2545 | IIa | Peat bog lake | Facultative methanotrophic, moderate acidophilic | Yes | FN422005 | Belova et al., 2013 |
| *Methylocystis echinoides* 2 IMET 10491, LMG 27198, NCIMB 13100, UNIQEM 25, VKM B-2128 | IIa | Sewage treatment plant | Facultative methanotroph | Yes | Not detected | Gal'chenko et al., 1977; Bowman et al., 1993 |
| *Methylocystis heyeri* H2 DSM 16984, VKM B-2426 | IIa | Peat bog lake | Facultative methanotrophic, moderate acidophilic | Yes | AM283545 | Dedysh et al., 2007 |
| *Methylocystis hirsuta* CSC1 ATCC BAA-1344, DSM 18500, LMG 27832 | IIa | Groundwater | | Yes | DQ664498 | Lindner et al., 2007 |
| *Methylocystis parvus* OBBP * ACM 3309, ATCC 35066, IMET 10483, NCIMB 11129, UNIQEM 38, VKM B-2129 | IIa | (Soil and fresh water sediments) | | Yes | Not detected | Whittenbury et al., 1970b; Bowman et al., 1993; del Cerro et al., 2012 |
| *Methylocystis rosea* SV97 ATCC BAA-1196, DSM 17261, LMG 27835 | IIa | Arctic wetland soil | | Yes | Not detected | Wartiainen et al., 2006b |
| *Methylosinus sporium* 5 ACM 3306, ATCC 35069, DSM 17706, IMET 10545, NCIMB 11126, UNIQEM 60 | IIa | Rice paddy | | Yes | DQ386732 | Whittenbury et al., 1970b; Bowman et al., 1993 |
| *Methylosinus trichosporium* OB3b* ACM 3311, ATCC 35070, IMET 10543, NCIMB 11131, UNIQEM 75, VKM B-2117 | IIa | (Soil, fresh water sediments, groundwater) | | Yes | X55394 | Whittenbury et al., 1970b; Bowman et al., 1993; Stein et al., 2010 |
| ***Beijerinckiaceae*** | | | | | | |
| *Methylocapsa acidiphila* B2 * DSM 13967, NCIMB 13765 | IIb | Peat bog | Moderate acidophilic | Yes | Not detected | Dedysh et al., 2002; Tamas et al., 2014 |
| *Methylocapsa aurea* KYG DSM 22158, VKM B-2544 | IIb | Forest soil | Moderate acidophilic, facultative methanotrophic | Yes | Not detected | Dunfield et al., 2010 |
| *Methylocapsa palsarum* NE2 LMG 28715, VKM B-2945 | IIb | *Sphagnum* from a palsa | Moderate acidophilic | Yes | Not detected | Dedysh et al., 2015a |
| *Methylocella palustris* K* ATCC 700799 | IIb | Peat bog | Moderate acidophilic, facultative methanotrophic | No | AJ458535 | Dedysh et al., 2000 |
| *Methylocella silvestris* BL2 CIP 108128, DSM 15510, LMG 27833, NCIMB 13906 | IIb | Forest soil | Moderate acidophilic, facultative methanotrophic | No | AJ491848 | Dunfield et al., 2003; Chen et al., 2010 |
| *Methylocella tundrae* T4 DSM 15673, LMG 27838, NCIMB 13949 | IIb | Tundra peatland | Moderate acidophilic | No | AJ555245 | Dedysh et al., 2004 |
| *Methyloferula stellata* AR4 * DSM 22108, LMG 25277, VKM B-2543 | IIb | Peat bog | Moderate acidophilic | No | FR686346 | Vorobev et al., 2011; Dedysh et al., 2015b |

[a] *Type species representing the respective genus are marked with an asterisk.*

[b] *Information given in brackets refers to information obtained from the analysis of a related strain, since this information was not available for the type strain of the species.*

[c] *Listed publications describe the initial isolation, current classification and genome sequence of the strain as far as already available.*

**TABLE 2 | Taxonomy, isolation source, and physiological characteristics of aerobic methanotrophic *Gammaproteobacteria*.**

| Type strain[a] Culture collection numbers | Type | Isolation source[b] | (Eco-) physiology | *mmoX* sequence | Synonym/basonym | References[c] |
|---|---|---|---|---|---|---|
| ***Methylococcaceae*** | | | | | | |
| *Methylobacter luteus* 53v* ACM 3304, ATCC 49878, IMET 10584, NCIMB 11914, UCM 53B, VKM 53B | Ia | Sewage | | Not analyzed | *Methylococcus luteus*<br>*Methylobacter bovis*<br>*Methylococcus bovis*<br>*Methylococcus fulvus* | Romanovskaya et al., 1978; Bowman et al., 1993 |
| *Methylobacter marinus* A45 ACM 4717 | Ia | Seawater sediment | | Not detected | *Methylomonas marinus* | Lidstrom, 1988; Bowman et al., 1993 |
| *Methylobacter psychrophilus* Z-0021 VKM B-2103 | Ia | Tundra | Psychrophilic | Not analyzed | | Omelchenko et al., 1996 |
| *Methylobacter tundripaludum* SV96 ATCC BAA-1195, DSM 17260 | Ia | Tundra soil | | Not detected | | Wartiainen et al., 2006a; Svenning et al., 2011 |
| *Methylobacter whittenburyi* Y ACM 3310, ATCC 51738, NCIMB 11128 | Ia | Lake sediment | | Not analyzed | *Methylobacter capsulatus*<br>*Methylococcus whittenburyi*<br>*Methylobacter vinelandii*<br>*Methylococcus vinelandii* | Romanovskaya et al., 1978; Bowman et al., 1993 |
| *Methyloglobulus morosus* KoM1 * DSM 22980, JCM 18850 | Ia | Lake sediment | Perferentially microaerophilic | Not detected | | Poehlein et al., 2013; Deutzmann et al., 2014 |
| *Methylomarinum vadi* IT-4* DSM 18976, JCM 13665 | Ia | Marine hydrothermal system | Halophilic | Not detected | | Hirayama et al., 2013 |
| *Methylomicrobium agile* A30 * ACM 3308, ATCC 35068, NCIMB 11124 | Ia | Sewage | | Not detected | *Methylomonas agile*<br>*Methylobacter agilis* | Whittenbury et al., 1970b; Bowman et al., 1995; Hamilton et al., 2015 |
| *Methylomicrobium album* BG8 ACM 3314, ATCC 33003, NCIMB 11123, VKM-BG8 | Ia | Soil | | Not detected | *Methylomonas albus*<br>*Methylobacter albus* | Whittenbury et al., 1970b; Bowman et al., 1995; Kits et al., 2013 |
| *Methylomicrobium alcaliphilum* 20Z DSM 19304, LMG 27836, NCIMB 14124, VKM B-2133 | Ia | Soda lake | Moderate halophilic, alkaliphilic | Not detected | *Methylobacter alcaliphilus* | Khmelenina et al., 1997; Kalyuzhnaya et al., 2008; Vuilleumier et al., 2012 |
| *Methylomicrobium buryatense* 5B VKM B-2245 | Ia | Soda lake | Moderate halophilic | AOTL00000000 | | Kaluzhnaya et al., 2001; Khmelenina et al., 2013 |
| *Methylomicrobium japanense* NI FERM BP-5633, NBRC 103677, VKM B-2462 | Ia | Marine sediment | Slightly halophilic | AB253366 | | Kalyuzhnaya et al., 2008 |
| *Methylomicrobium kenyense* AMO1 DSM 19305, NCCB 97157, NCIMB 13566, VKM B-2464 | Ia | Soda lake | Moderate halophilic, alkaliphilic | Not detected | | Kalyuzhnaya et al., 2008 |
| *Methylomicrobium pelagicum* AA-23 ACM 3505, NCIMB 2265 | Ia | Marine | Moderate halophilic | Not detected | *Methylomonas pelagica*<br>*Methylobacter pelagicus* | Sieburth et al., 1987; Bowman et al., 1995 |
| *Methylomonas aurantiaca* JB103 ACM3406, UQM 3406 | Ia | Sewage | | Not analyzed | | Bowman et al., 1990 |
| *"Methylomonas denitrificans"* FJG | Ia | Soil | | Not detected | | Kits et al., 2015 |

*(Continued)*

**TABLE 2 | Continued**

| Type strain[a] Culture collection numbers | Type | Isolation source[b] | (Eco-) physiology | *mmoX* sequence | Synonym/basonym | References[c] |
|---|---|---|---|---|---|---|
| *Methylomonas fodinarum* LD2 ACM3268, UQM 3268 | Ia | Coal mine drainage water | | Not analyzed | | Bowman et al., 1990 |
| *Methylomonas koyamae* Fw12E-Y JCM 16701, LMG 26899, NBRC 105905, NCIMB 14606 | Ia | Rice paddy | | Not detected | | Ogiso et al., 2012 |
| *Methylomonas lenta* R-45377 LMG 26260, JCM 19378 | Ia | Manure | | Not detected | | Hoefman et al., 2014a |
| *Methylomonas methanica* $S_1$* ACM 3307, ATCC 35067, IMET10543, NCIMB 11130, UNIQEM 8, VKM B-2110 | Ia | (Freshwater sediment, lake, pond water, swamply soil) | | Not detected | *Bacillus methanicus*<br>*Methanomonas methanica*<br>*Pseudomonas methanica* | Söhngen, 1906; Whittenbury and Krieg, 1984 |
| *Methylomonas paludis* MG30 DSM 24973, VKM B-2745 | Ia | Peat bog | Acid-tolerant | Not detected | | Danilova et al., 2013 |
| *"Methylomonas rubra"* 15m[d] ACM3303, NCIMB11913, UCM B-3075, VKM-15m | Ia | Coal mine drainage water | | Not analyzed | *Methylomonas rubrum* | Whittenbury et al., 1970b; Romanovskaya et al., 2006 |
| *Methylomonas scandinavica* SR5 VKM B-2140 | Ia | Groundwater | Psychrotolerant | Not detected | | Kalyuzhnaya et al., 1999 |
| *Methyloprofundus sedimenti* WF1 * ATCC BAA-2619, LMG 28393 | Ia | Marine sediment | Moderate halophilic | Not detected | | Tavormina et al., 2015 |
| *Methylosarcina fibrata* AML-C10 * ATCC 700909, DSM 13736 | Ia | Landfill cover soil | | Not detected | | Wise et al., 2001; Hamilton et al., 2015 |
| *Methylosarcina lacus* LW14 ATCC BAA-1047, DSM 16693, JCM 13284 | Ia | Lake | | Not detected | | Kalyuzhnaya et al., 2005, 2015 |
| *Methylosarcina quisquiliarum* AML-D4 ATCC 700908, DSM 13737 | Ia | Landfill cover soil | | Not detected | | Wise et al., 2001 |
| *Methylosoma difficile* LC2 * DSM 18750, JCM 14076 | Ia | Lake sediment | Microaerobic | Not detected | | Rahalkar et al., 2007 |
| *Methylosphaera hansonii* AM6 * ACAM 549 | Ia | Lake | Psychrophilic, moderate halophilic | Not detected | | Bowman et al., 1997 |
| *Methylovulum miyakonense* HT12* ATCC BAA-2070, DSM 23269, NBRC 106162 | Ia | Forest soil | | AB501288 | | Iguchi et al., 2011; Hamilton et al., 2015 |
| *Methylocaldum gracile* 14L NCIMB 11912, VKM-14L | Ib | Fresh water mud | Thermotolerant | Not detected | *Methylomonas gracilis* | Romanovskaya et al., 1978; Bodrossy et al., 1997 |
| *Methylocaldum marinum* S8 DSM 27392, NBRC 109686 | Ib | Marine sediment | Thermotolerant, moderate halophilic | AB900160 | | Takeuchi et al., 2014 |
| *Methylocaldum szegediense* OR2* | Ib | Water from hot spring | Moderate thermophilic | Not detected | | Bodrossy et al., 1997 |
| *Methylocaldum tepidum* LK6 | Ib | Agricultural soil | Thermotolerant | Not detected | | Bodrossy et al., 1997 |
| *Methylococcus capsulatus* Texas* ACM1292, ATCC 19069, NCIMB 11853, UNIQEM 1 | Ib | Sewage sludge | Thermotolerant | AMCE00000000 | | Foster and Davis, 1966; Kleiveland et al., 2012 |

*(Continued)*

**TABLE 2 | Continued**

| Type strain[a] Culture collection numbers | Type | Isolation source[b] | (Eco-) physiology | *mmoX* sequence | Synonym/basonym | References[c] |
|---|---|---|---|---|---|---|
| *Methylococcus chroococcus* 9[e] | Ib | (Soil, fresh water sediments, groundwater) | | Not analyzed | *Methylobacter chroococcum*? | Whittenbury et al., 1970b; Romanovskaya et al., 1978 |
| *Methylococcus mobilis* LMD 77.28[e] NCCB 77028 | Ib | Active silt | | Not analyzed | | Hazeu et al., 1980 |
| *Methylococcus thermophilus* VKM-2Yu[f] ACM3585, IMV-B 3037 | Ib | (Mud, soils, pond sediment) | Thermophilic | Not analyzed | | Malashenko et al., 1975 |
| *Methylogaea oryzae* E10 * DSM 23452, JCM 16910 | Ib | Rice paddy | | Not detected | | Geymonat et al., 2010 |
| *Methylomagnum ishizawai* RS11D-Pr DSM 29768, JCM 18894, KCTC 4681, NBRC 109438 | Ib | Rice rhizosphere | | AB983338 | | Khalifa et al., 2015 |
| *Methyloparacoccus murrellii* R-49797 * LMG 27482, JCM 19379 | Ib | Pond water | | Not detected | | Hoefman et al., 2014b |
| "*Candidatus* Clonothrix fusca"[g] (no pure culture) | | Drinking water | | Not analyzed | | Vigliotta et al., 2007 |
| ***Methylothermaceae*** | | | | | | |
| *Methylohalobius crimeensis* 10Ki* ATCC BAA-967, DSM 16011 | Ic | Hypersaline lake sediment | Moderate halophilic | Not detected | | Heyer et al., 2005; Sharp et al., 2015 |
| *Methylomarinovum caldicuralii* IT-9 * DSM 19749, JCM 13666 | Ic | Marine hydrothermal system | Moderate thermophilic, moderate halophilic | Not detected | | Hirayama et al., 2014 |
| *Methylothermus subterraneus* HTM55 DSM 19750, JCM 13664 | Ic | Hot aquifer | Moderate thermophilic | Not detected | | Hirayama et al., 2010 |
| *Methylothermus thermalis* MYHT* IPOD FERM P-19714, VKM B-2345 | Ic | Hot spring | Moderate thermophilic, halotolerant | Not detected | | Tsubota et al., 2005 |
| ***Crenotrichaceae*** | | | | | | |
| "*Candidatus* Crenothrix polyspora"[g] (no pure culture) | | Drinking water | Facultative methanotrophic | Not detected | | Stoecker et al., 2006 |

[a] *Type species representing the respective genus are marked with an asterisk.*

[b] *Information given in brackets refers to information obtained from the analysis of a related strain, since this information was not available for the type strain of the species.*

[c] *Listed publications describe the initial isolation, current classification and genome sequence of the strain as far as already available.*

[d] *The type strain of Methylomonas rubra was reported to belong to the species "Methylomonas methanica" (Bowman et al., 1993), but later it was proposed to represent a separate species (Romanovskaya et al., 2006); this has not been validated yet.*

[e] *According to Bergey's manual of systematic bacteriology, strains of these species do not exist anymore and the species are not considered as valid (Bowman, 2005a), but at least for Methylococcus mobilis, this appears not to be correct, as the type strain is listed in the NCCB catalog.*

[f] *According to Bodrossy et al. (1997), the type strain is no longer available and the available strain M. thermophilus strain IMV-B3122 (NCIMB 13419) is actually a strain of Methylocaldum tepidum.*

[g] *Candidatus Crenothrix polyspora and Clonothrix fusca do not contain any cultured type strains and the genus Clonothrix is not validated according to the list of prokaryotic names with standing in nomenclature (Parte, 2014), therefore these genera are referred to as Candidatus; according to Op den Camp et al. (2009) the validated family Crenotrichaceae is phylogenetically a subset of the Methylococcaceae.*

**TABLE 3 | Isolation source and physiological characteristics of methanotrophic bacteria harboring *pmoA* outside the phylum *Proteobacteria*.**

| Type strain | Type | Isolation source | (Eco-)physiology | *mmoX* sequence | Synonym/basonym | References[a] |
|---|---|---|---|---|---|---|
| ***Verrucomicrobia, Methylacidiphilaceae*** | | | | | | |
| *"Methylacidimicrobium cyclopophantes"* 3B | III | Volcanic soil | Thermophilic, acidophilic | Not detected | | van Teeseling et al., 2014 |
| *"Methylacidimicrobium fagopyrum"* 3C | III | Volcanic soil | Thermophilic, acidophilic | Not detected | | van Teeseling et al., 2014 |
| *"Methylacidimicrobium tartarophylax"* 4AC | III | Volcanic soil | Thermophilic, acidophilic | Not detected | | van Teeseling et al., 2014 |
| *"Methylacidiphilum fumariolicum"* SolV | III | Themal mud pod | Thermophilic, acidophilic | Not detected | *"Acidimethylosilex fumarolicum"* | Pol et al., 2007; Op den Camp et al., 2009; Khadem et al., 2012 |
| *"Methylacidiphilum infernorum"* V4 | III | Soil, geothermal area | Thermophilic, acidophilic | Not detected | *"Methylokorus infernorum"* | Dunfield et al., 2007; Hou et al., 2008; Op den Camp et al., 2009 |
| *"Methylacidiphilum kamchatkense"* Kam1 | III | Hot spring | Thermophilic, acidophilic | Not detected | *"Methyloacida kamchatkensis"* | Islam et al., 2008; Op den Camp et al., 2009; Erikstad and Birkeland, 2015 |
| **CANDIDATE DIVISION NC10** | | | | | | |
| *"Candidatus* Methylomirabilis oxyfera" NC10 | – | River sediment | Anaerobic | Not detected | | Ettwig et al., 2010 |

[a]*Listed publications describe the initial isolation, current classification and genome sequence of the strain as far as available.*

be a member of the *Methylococcaceae,* based on its 16S rRNA gene phylogeny (**Figure 1**).

## Classification of Cultivated Methanotrophic Bacteria into Type I and Type II Methanotrophs?

The characterization of several new genera of methanotrophs in the 1970s and 1980s resulted in the classification into two major groups, type I and type II methanotrophs based on physiological, morphological, ultrastructural and chemotaxonomic traits (Whittenbury and Dalton, 1981). Major distinctive characteristics between type I and type II methanotrophs were the arrangement of internal membranes as vesicular discs (type I) or paired membranes aligned to the cell periphery (type II), the carbon fixation mechanism via the ribulose monophosphate pathway (type I) or serine cycle (type II), the capability of nitrogen fixation, the formation of resting stages, and the predominance of specific $C_{16}$ (type I) or $C_{18}$ (type II) fatty acids (Hanson and Hanson, 1996; Trotsenko and Murrell, 2008). In some studies, type X methanotrophs were further differentiated from type I methanotrophs based on characteristics such as the presence of ribulose-1,5-bisphosphate carboxylase, differences in nitrogen fixation capability or higher optimum growth temperatures (Green, 1992; Hanson and Hanson, 1996; Bowman, 2006). Phylogenetic analyses of 16S rRNA gene sequences confirmed this classification, whereby type I and type X methanotrophs correspond to the *Gammaproteobacteria* and type II to the *Alphaproteobacteria*. However, the characterization of several new genera and species during the last years has turned this distinction based on the mentioned criteria largely into question. While the major carbon fixation pathway is still a distinctive feature, several other characteristics are no longer exclusively found in one or the other group:

(1) Methanotrophic *Beijerinckia* species are not considered as typical type II methanotrophs as most of them lack the characteristic internal membrane system. Some may have vesicles instead, but only *Methylocapsa palsarum* has a well-developed membrane system. Furthermore, the genera *Methylocella* and *Methyloferula* do not possess the particulate methane monooxygenase (Dedysh et al., 2000, 2002, 2004, 2015a; Dunfield et al., 2003, 2010; Vorobev et al., 2011).

(2) All methanotrophic *Beijerinckia* species lack the classical 18:1ω8c signature fatty acid of type II methanotrophs (Dedysh et al., 2000, 2002, 2004, 2015a; Dunfield et al., 2003, 2010; Vorobev et al., 2011). Similarly, *Methylosinus sporium* does not possess this signature fatty acid (Bodelier et al., 2009).

(3) *Methylocystis heyeri* possesses with 16:1ω8c a signature fatty acid of type I methanotrophs (Dedysh et al., 2007).

(4) Most members of the *Methylothermaceae* have signature fatty acids of type II methanotrophs: *Methylohalobius crimeensis*, *Methylothermus subterraneaus*, and *Methylomarinovum caldicuralii* contain 18:1ω7c among their major fatty acids (Heyer et al., 2005; Hirayama

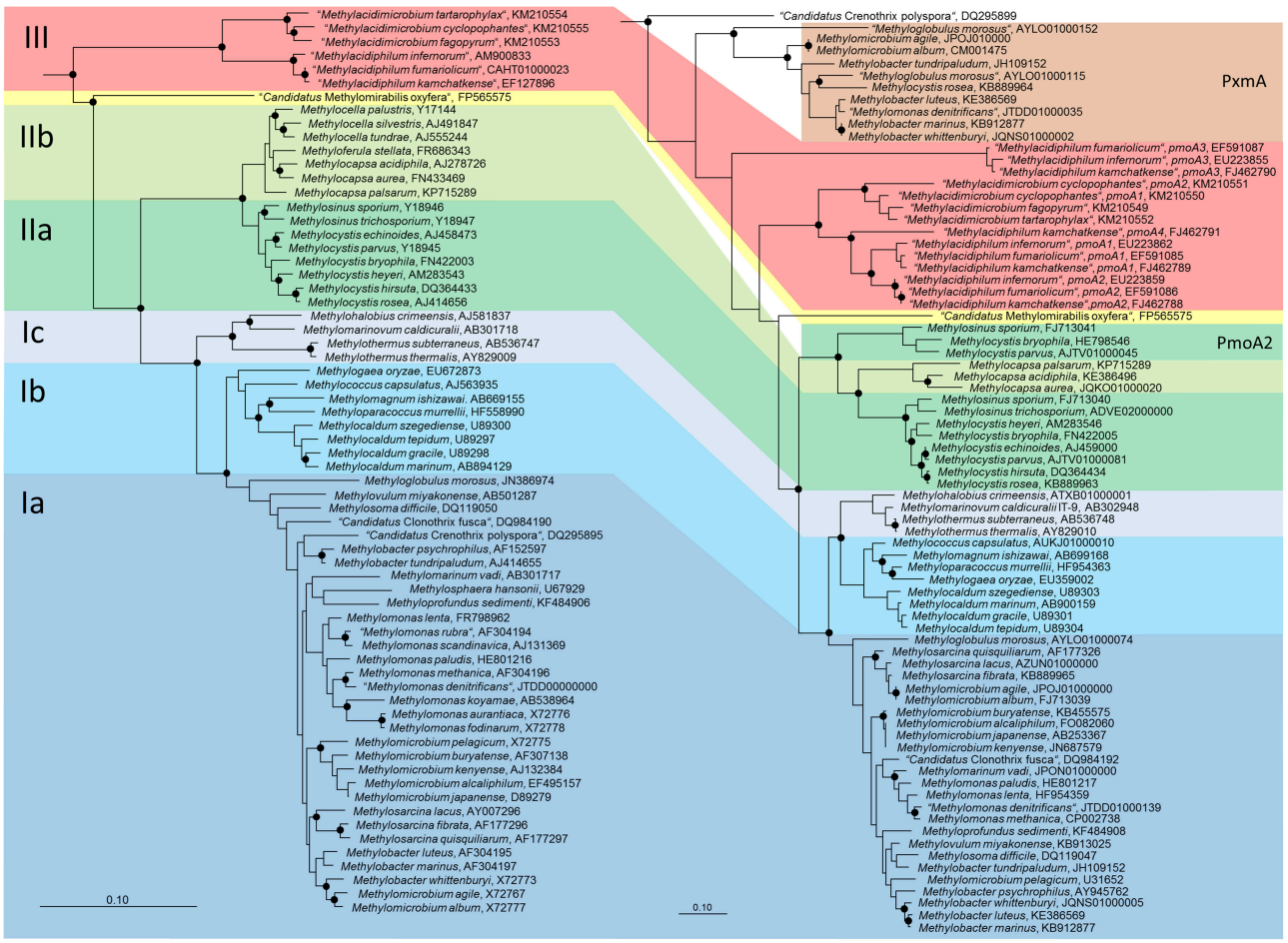


**FIGURE 1 | Phylogenetic trees showing the phylogeny of methanotrophic type strains based on 16S rRNA gene sequences (left tree) and PmoA sequences (right tree).** The neighbor joining trees were calculated using the ARB software package (Ludwig et al., 2004) based on 1556 nucleotide positions with Jukes Cantor correction or 160 amino acid positions with Kimura correction, respectively. PmoA sequences of *Methylobacter luteus, Methylobacter whittenburyi*, and *Methylomicrobium pelagicum* are not available from the type strains, but were taken from a different strain representing the species. The 16S rRNA gene based tree was rooted with sequences of methanogenic *Archaea* (AB301476, M60880, AB065296, AM114193, AB196288), the PmoA tree with AmoA sequences of ammonia-oxidizing bacteria (NC_004757, X90822). Dots label branch points that were confirmed in maximum likelihood trees. The scale bars display 0.10 changes per nucleotide or amino acid position.

et al., 2010, 2014). The fourth member of this family, *Methylothermus thermalis*, contains 18:1ω9c, a $C_{18}$ fatty acid neither abundant in the other *Methylothermaceae* nor in type II methanotrophs (Tsubota et al., 2005).

(5) Methanotrophic *Verrucomicrobia* do not fit well into the scheme as most of them lack the typical intracytoplasmic membranes (only exception "*Methylacidimicrobium fagopyrum*") and have distinct dominant fatty acids (i14:0, a15:0) (Op den Camp et al., 2009; van Teeseling et al., 2014).

(6) Further differentiation criteria such as nitrogen fixation capability, the formation of resting stages, or the optimum growth temperature, which were initially applied, are not indicative for one or the other type anymore.

Based on these exceptions, the initial concept of type I and II methanotrophs is no longer useful to categorize all known aerobic methanotrophic bacteria and it has been proposed to abandon it (Op den Camp et al., 2009; Semrau et al., 2010). Nevertheless, the terms are still frequently used and adapted to the increasing diversity of methanotrophs, but should only be considered as synonyms for the phylogenetic groups of methanotrophic *Alpha-* and *Gammaproteobacteria*. In this way the terms will be used in this review.

The methanotrophic *Alphaproteobacteria* were recently divided into type IIa (*Methylocystaceae*) and type IIb (*Beijerinckiaceae*) methanotrophs (Deng et al., 2013; Dumont et al., 2014). Likewise, the methanotrophic *Gammaproteobacteria* are frequently differentiated into subgroups. Often they are divided into two groups, whereby the genera *Methylococcus*, *Methylocaldum*, *Methylogaea* and the *Methylothermaceae* form type 1b methanotrophs, while the remaining gammaproteobacterial genera are grouped as type 1a methanotrophs (Chen et al., 2008; Deutzmann et al., 2011; Dumont et al., 2011; Siljanen et al., 2011; Krause et al.,

2014). Some recent studies differentiated the methanotrophic *Gammaproteobacteria* into three type I subgroups, but this categorization is not consistent among different publications. A taxonomic review referred to the clade consisting of *Methylococcus, Methylocaldum, Methylogaea, Methyloparacoccus* as type Ia and to members of the family *Methylothermaceae* as type Ic, while the remaining *Gammaproteobacteria* represented type Ib methanotrophs (Bowman, 2014). In contrast, in some cultivation independent studies the above mentioned frequent grouping into type Ia and Ib was applied and extended by introducing type Ic, comprising *pmoA* sequences of uncultivated taxa (USC$\gamma$, JR2, JR3, OPU1) and the *amoA* sequence of *Nitrosococcus* (Lüke and Frenzel, 2011; Henneberger et al., 2012; Dumont et al., 2014). It is thus referring to a group of uncultivated methanotrophs. Such a further differentiation of the methanotrophic *Gammaproteobacteria* appears useful to refer to the specific subgroups of cultivated and uncultivated methanotrophs easily. In this review, the nomenclature of type Ia and Ib methanotrophs as applied in diverse cultivation-independent studies is kept, while the *Methylothermaceae* are referred to as type Ic methanotrophs (**Table 2**). The clade of *Nitrosococcus* and related uncultivated clusters represent type Id organisms when discussing diversity based on *pmoA* phylogeny. Methanotrophic *Verrucomicrobia* are referred to as type III.

## Ecophysiology of Aerobic Methanotrophic Bacteria

Aerobic methanotrophic bacteria occur in terrestrial, aquatic and marine ecosystems, typically at oxic-/anoxic interfaces, where oxygen is available as electron acceptor and methane as carbon and energy source, which is released as end product from the anaerobic degradation of organic matter. They are likewise present in diverse upland soils where they are responsible for atmospheric methane oxidation or become temporarily active when higher concentrations of methane are available (Knief et al., 2006; Dunfield, 2007; Kolb, 2009). The ecology of methanotrophic bacteria has been reviewed in diverse articles and will not be discussed in detail here (e.g., Hanson and Hanson, 1996; Conrad, 2007; Semrau et al., 2010; Chowdhury and Dick, 2013; Bowman, 2014). The focus in this article is on physiological adaptations to particular environmental conditions in relation to phylogeny.

In terms of metabolic adaptations, some methanotrophic bacteria show higher versatility than initially thought. They are capable of growing on carbon compounds with C-C bond, while most methanotrophic bacteria are obligate methanotrophs. The existence of such facultative methanotrophs had been debated for a long time (reviewed in Theisen and Murrell, 2005; Semrau et al., 2011), until it was rigorously proven for *Methylocella silvestris* BL2 (Dedysh et al., 2005). This strain has the broadest versatility currently known among methanotrophs; besides $C_1$-compounds, it can use a variety of organic acids including acetate, pyruvate, propionate, succinate, malate, and gluconate, alcohols such as ethanol and 2-propanol and the gaseous compounds ethane and propane (Crombie and Murrell, 2014). Growth on acetate is more efficient than on methane and methane monooxygenase expression is down-regulated in the presence of acetate (Dedysh et al., 2005; Theisen et al., 2005). In contrast, methane and propane are consumed simultaneously in this strain (Crombie and Murrell, 2014). A facultative lifestyle with a much narrower substrate range has been reported for other members of the genus *Methylocella* and for *Methylocapsa aurea* (**Table 1**), but it is not a general feature of all methanotrophic *Beijerinckiaceae*. Moreover, several *Methylocystis* strains including diverse type strains are able to grow on acetate or ethanol, but with growth rates 3–10-fold lower compared to growth on methane (Belova et al., 2011; Im et al., 2011). Gene expression of methane monooxygenase appears not to be regulated by acetate in these methanotrophs (Belova et al., 2011; Yoon et al., 2011). It remains to be proven whether the capability to grow on acetate is linked to phylogeny within this genus. *Crenothrix polyspora* is the only methanotrophic gammaproteobacterium for which uptake of acetate and, to lesser extent, glucose, has been reported (Stoecker et al., 2006), but besides evidence from fluorescence *in situ* hybridization experiments coupled to microautoradiography (FISH-MAR) performed on natural enrichments, this phenomenon has not been further proven. It is obvious that a facultative lifestyle can provide a benefit for methanotrophic bacteria. However, the relevance of facultative methanotrophy in nature remains little understood, and linked to this the question to what extent a facultative life style may influence methane emissions in the environment. Only few studies have analyzed the consumption of methane and alternative substrates under *in situ* conditions so far. In mire samples, acetate addition resulted in a reduction of methane emission rates and decreased *pmoA* expression rates of *Methylocystis* (Wieczorek et al., 2011). Likewise, acetate addition decreased methane oxidation rates and stimulated growth of *Methylocystis* in paddy soil samples. Stable isotope probing with $^{13}$C-labeled acetate under aerobic conditions resulted in a labeling of *Methylocystis* in these samples, demonstrating that the labeled carbon was somehow metabolized and incorporated by the cells (Leng et al., 2015).

Another aspect that has repeatedly been addressed is the adaptation to low methane concentrations. The observations made in competition experiments with isolates grown in continuous culture and in incubations with rice field soils resulted in the frequently cited conclusion that type I methanotrophs are more competitive under low methane concentrations compared to type II methanotrophs (Graham et al., 1993; Henckel et al., 2000b; Macalady et al., 2002). This seems to apply to ecosystems as long as methane supply remains at a rather high level, but when methane concentrations drop below 1000 or even 100 ppmv for prolonged periods of time, *Methylocystaceae* have the better potential to remain active (Knief and Dunfield, 2005).

Most methanotrophic bacteria are mesophilic and neutrophilic organisms, but several isolates were obtained from more extreme habitats and are specifically adapted to lower or higher pH, temperature, salt or oxygen concentrations (Trotsenko and Khmelenina, 2002). Methanotrophic bacteria adapted to warmer or colder temperatures are found in a couple

of distinct genera of *Gammaproteobacteria*, often side by side with mesophilic species (**Table 2**). Among the methanotrophic *Alphaproteobacteria* adaptations to temperatures outside the mesophilic range appear to be uncommon. Outstanding are the verrucomicrobial methanotrophs, which represent the most thermophilic methanotrophs (optimum temperature 55–60°C) (Op den Camp et al., 2009). These are at the same time acidophiles, with pH optima for growth between 2.0 and 4.3. All isolates were obtained from geothermally influenced environments (Op den Camp et al., 2009; van Teeseling et al., 2014). The occurrence of these thermoacidophilic methanotrophs appears to be largely restricted to such geothermal environments, in particular to acidic conditions, while they seem to have a broader temperature range, as revealed by cultivation-dependent and -independent analyses (Sharp et al., 2014; van Teeseling et al., 2014).

An adaptation to mildly acidic pH values (growth optima between 5.0 and 6.0) is characteristic for methanotrophic *Beijerinckiaceae* and some *Methylocystis* strains, which were mostly isolated from acidic peatlands or forest soils (**Table 1**). Cultivation-independent analyses suggest that the occurrence of *Methylocella* is not limited to these acidic environments (Rahman et al., 2011). Less common are acidophilic methanotrophs among the *Gammaproteobacteria*. Members of the species *Methylomonas paludis* have been described as acid-tolerant and are inhabitants of acidic peatlands (Danilova et al., 2013; Danilova and Dedysh, 2014). Methanotrophs that are adapted to high pH values are found within the *Gammaproteobacteria*, in particular within the genus *Methylomicrobium*. The occurrence of alkaliphilic methanotrophs is not restricted to the class of *Gammaproteobacteria*, the isolation of an alkaliphilic *Methylocystis* isolate has also been reported (Eshinimaev et al., 2008). Some alkaliphilic *Gammaproteobacteria* are at the same time halophiles (*Methylomicrobium alcaliphium* and *Methylomicrobium kenyense*), isolated from soda lakes (Kalyuzhnaya et al., 2008). Methanotrophic bacteria that were isolated from marine ecosystems are also adapted to higher salt concentrations and are likewise found among methanotrophic *Gammaproteobacteria*. High salt tolerance is not necessarily a characteristic of all members of a genus, as exemplified by *Methylocaldum* and *Methylomicrobium* (**Table 2**). In the last few years, the first methanotrophic isolates were described that live preferentially under lower oxygen concentrations (*Methylosoma difficile* and *Methyloglobulus morosus*). They were enriched in systems with opposing gradients of methane and oxygen, thus mimicking the conditions in sediments (Rahalkar et al., 2007; Deutzmann et al., 2014).

In conclusion, a broad versatility in terms of adaptation to different environmental conditions can be found among the methanotrophic *Gammaproteobacteria* (low and high temperatures, low and high pH, high salt, low oxygen), which comes along with a high diversity of methanotrophs within this group. Cultivated methanotrophic *Alphaproteobacteria* are less diverse and show less and different adaptations (low pH, low methane availability) based on current knowledge. At genus level, the occurrence of methanotrophic bacteria that are adapted to a specific environmental condition is not necessarily limited to one phylogenetic lineage, but can often be found within different genera of methanotrophs side by side with species that show different adaptations and habitat preferences. Thus, some genera have a broad ecological niche, though the individual species or strains have smaller niches, while others are less diverse in term of ecophysiological adaptations and have a rather narrow niche. Habitat adaptation and specialization appear to occur at different taxonomic levels. Consequently, the distribution of methanotrophic bacteria in the environment should be evaluated at these different taxonomic levels in order to better understand distribution and community composition. Such a detailed evaluation is undertaken in this review, based on a meta-analysis including the large diversity of uncultivated methanotrophs (see Sections Description of Major Uncultivated Groups of Methanotrophic Bacteria and Their Habitat Specificity and Habitat Specificity of Methanotrophic Taxa Evaluated at Higher Taxonomic Resolution).

# CULTIVATION-INDEPENDENT DETECTION OF AEROBIC METHANOTROPHIC BACTERIA BASED ON MOLECULAR MARKERS

Tools for the cultivation-independent detection of aerobic methanotrophic bacteria exist since 20 years and have been used in diverse studies. The most frequently targeted gene in environmental studies, the 16S rRNA gene, can be used for the detection of methanotrophic bacteria using taxon specific primers and probes that are available for several different groups (compiled by McDonald et al., 2008). While the analysis of this gene provides valuable information about the phylogenetic placement of methanotrophic bacteria detected in environmental samples, it does not allow the identification of methanotrophic bacteria beyond the well-known families.

## Functional Marker Genes as Molecular Markers

Such a limitation is of less relevance when functional genes are used as markers, such as the methane monooxygenase encoding genes (McDonald et al., 2008). The methane monooxygenase is the key enzyme responsible for the initial conversion step of methane to methanol. Two forms of this enzyme are known, the soluble methane monooxygenase (sMMO) and a membrane-bound enzyme, the particulate methane monooxygenase (pMMO). The *pmoA* gene encoding the β-subunit of the particulate methane monooxygenase is the most frequently used marker, as it is present in most aerobic methanotrophic bacteria with exceptions among the *Beijerinckiaceae* (**Table 1**). It is also present in anaerobic denitrifying bacteria, represented by an enriched culture of "*Candidatus* Methylomirabilis," a bacterium of the NC10 phylum (Ettwig et al., 2010).

To include *Beijerinckiaceae* and to obtain a more complete picture about the methanotrophs present in a sample, the *mmoX* gene encoding the α-subunit of the soluble methane monooxygenase hydroxylase component has been used in

addition to *pmoA* in some studies (e.g., Morris et al., 2002; Chen et al., 2008; Deng et al., 2013). However, due to its limited occurrence in methanotrophs (**Tables 1**–**3**), *mmoX* is much less frequently used as marker. It is not uniformly present or absent within the same genus and variation exists even at species level, as evident from studies with *Methylocystis, Methylosinus,* and *Methylomonas* strains (Shigematsu et al., 1999; Heyer et al., 2002).

Further gene markers that can be used for the detection of methanotrophs are not unique to this metabolic guild, but shared with other organisms. Among those are the *mxaF* gene, which encodes the large subunit of the methanol dehydrogenase, and a couple of other markers of the methylotrophic metabolism (reviewed by Kolb and Stacheter, 2013; Dumont, 2014).

## *pmoA* as Molecular Marker

Both, *pmoA* and *mmoX* have been shown to produce phylogenies that are largely congruent with those of the 16S rRNA gene (Auman and Lidstrom, 2002; Heyer et al., 2002; Kolb et al., 2003), which allows to draw conclusions about the phylogenetic placement of methanotrophs possessing genes with novel sequence types. Updated trees (**Figure 1**) show that this is still the case, but research of the last few years has revealed that this congruency includes more and more exceptions. The presence of paralogous gene copies in methanotrophic bacteria as well as the detection of evolutionary related monooxygenases in non-methanotrophic bacteria contribute to sequence diversity in cultivation-independent studies (see next section). Hence, conclusions about the taxonomic identity of bacteria detected based on their *pmoA* sequences have to be drawn with care, in particular if sequences cluster distantly to those of well-known methanotrophs. This is also exemplified by the *pmoA* sequence of the gammaproteobacterial "*Candidatus* Crenothrix polyspora," which is highly divergent from those of all other methanotrophic *Gammaproteobacteria*. Besides these issues, inconsistency exists among the type Ia methanotrophs (**Figure 1**). Tree reconstructions within this group are in general not highly robust, but both, *Methylobacter* and *Methylomicrobium* species do not form monophyletic clusters, independent of the applied treeing method and the phylogenetic marker. *Methylobacter psychrophilus* and *Methylobacter tundripaludum* appear to be distinct from the other *Methylobacter* species, likewise as *Methylomicrobium album* and *Methylomicrobium agile* cluster with *Methylobacter whittenburyi* in 16S rRNA gene based trees and with *Methylosarcina* species in *pmoA* based trees rather than with the other *Methylomicrobium* species. Elaborate taxonomic analyses including information derived from whole genome sequencing projects of these and further reference strains will be necessary to ensure the taxonomic placement of these species.

A couple of different primer sets were developed for the amplification of *pmoA* gene fragments, but remarkably, the first published primer pair (A189/A682) is still most frequently used (Holmes et al., 1995). Only one alternative system (A189/mb661) is often used instead or in addition to the before mentioned system (Costello and Lidstrom, 1999). This second primer combination is more specific for methanotrophic bacteria as it does not amplify the *amoA* gene of ammonia-oxidizing bacteria (Costello and Lidstrom, 1999). However, it fails to detect some of the clusters that have a phylogenetic position between *pmoA* and *amoA* sequences, such as the RA21 or the *pxmA* cluster, it largely discriminates USCα and amplifies type IIb methanotrophs less efficiently (Bourne et al., 2001; Deng et al., 2013). A third primer, A650 does not show this limitation while excluding *amoA*, but has less frequently been used (Bourne et al., 2001; Shrestha et al., 2012). Because primer system A189/A682 results in the production of additional unspecific PCR products in some cases, a semi-nested approach was used in these studies. After a first PCR using primers A189/A682 a second PCR with primers A189/mb661 or A189/A650 was applied (Singh et al., 2007; Qiu et al., 2008; Kip et al., 2011; Siljanen et al., 2011; Barbier et al., 2012). Alternatively, a combination of both reverse primers in a multiplex PCR was used in the second PCR to overcome the detection limitations of primer mb661 (Horz et al., 2005). Some further general and several specific primers for the detection of subgroups were developed, as compiled in review articles (McDonald et al., 2008; Dumont, 2014). Many of them were developed for qPCR assays targeting subgroups (Kolb et al., 2003, 2005; Degelmann et al., 2010; Wieczorek et al., 2011; Sharp et al., 2014). Moreover, specific primers are needed to amplify *pmoA* genes of *Verrucomicrobia* (Erikstad et al., 2012; Sharp et al., 2012), the homologous *pmoA2* gene (Tchawa Yimga et al., 2003), or the *pmoA* genes of anaerobic methanotrophic bacteria of the NC10 phylum (Luesken et al., 2011).

# *pmoA* PARALOGS AND EVOLUTIONARY RELATED MONOOXYGENASES

Paralogous copies of the *pmoA* gene and evolutionary related monooxygenases in non-methanotrophic bacteria are sometimes detected in cultivation-independent studies, depending on the primers used to amplify the target gene. They can thus contribute to the diversity of detected sequence types in environmental studies, but do not represent distinct methanotrophs. A couple of sequence clusters in *pmoA* based phylogenetic trees have meanwhile been identified as paralogs or alternative monooxygenases.

## *pmoA* Paralogs in Methanotrophic Bacteria

Many methanotrophs have multiple copies of the *pmo* operon and initially it appeared that these copies are (nearly) identical (Auman et al., 2000; Gilbert et al., 2000), so that they do not affect diversity studies that are based on *pmoA* gene detection. *Methylocystis* sp. SC2 was the first methanotrophic strain in which two different *pmoA* genes were discovered, the conventional and a second copy, referred to as *pmoA2*, with only 73% identity to the well-known *pmoA* gene of *Methylocystaceae* (Dunfield et al., 2002). The application of specific primers for the detection of the *pmoA2* gene revealed that this second gene copy is present in diverse though not all *Methylocystis* and *Methylosinus* strains (Tchawa Yimga et al., 2003). The *pmoA2* gene is localized in the *pmoCAB2* operon, which encodes a functional methane monooxygenase, enabling *Methylocystis* SC2 to oxidize methane at lower mixing ratios compared to the conventional monooxygenase, which is downregulated under these conditions in strain SC2 (Baani and Liesack, 2008). This

finding was taken as explanation for the previously described capability of *Methylocystis* species to oxidize methane at very low mixing ratios down to atmospheric level over a period of several months and their capability to grow at mixing rations as low as 10–100 ppmv (Knief and Dunfield, 2005). Moreover, this corresponds very well to the observation that *Methylocystis* strains are frequently detected in upland soils and hydromorphic soils, where they face low methane supply almost constantly (Dunfield, 2007). However, the *pmoA2* gene of *Methylocystis* and *Methylosinus* has not been detected very frequently in upland soils, but rather in different other ecosystems (Tables S1–S4). Either the commonly applied primers are not well suited to amplify *pmoA2* genes of those *Methylocystaceae* that occur in upland soils, or the *pmoA2* gene is more important for survival of methanotrophs residing in habitats with fluctuating methane supply at higher concentrations.

In methanotrophic *Verrucomicrobia*, multiple different *pmoA* gene copies are present (**Figure 1**). All genes are highly divergent from those of proteobacterial methanotrophs and quite different to each other (Op den Camp et al., 2009). The strains "*Methylacidiphilum fumariolicum*" SolV and "*Methylacidiphilum infernorum*" V4 possess three complete *pmoCAB* operons, while "*Methylacidiphilum kamchatkense*" Kam1 has a fourth distinct copy of *pmoA*, localized in a truncated *pmoCA* operon. An expression study performed with this strain revealed that the methane monooxygenase encoded by *pmoCAB2* is strongly expressed when cells are grown under laboratory conditions (Erikstad et al., 2012). The function of the other copies and regulatory mechanisms that may control the expression of these genes remain currently largely unknown.

## The *pxmA* gene

In methanotrophic *Gammaproteobacteria* of the genera *Methylomonas, Methylobacter* and *Methylomicrobium* another homolog of *pmoA* has been detected, the *pxmA* gene (Tavormina et al., 2011). Recent genome sequencing projects reveal that *pxmA* genes occur more widespread in methanotrophs. They are present in further *Methylococcaceae* strains, which are distantly related to the known genera but have so far not been further described in the literature. A *pxmA* copy is also present in an alphaproteobacterial strain, *Methylocystis rosea*. In *Methyloglobulus morosus* an additional *pxmA* like gene is present besides *pmoA* and *pxmA*. All *pxmA* gene sequences form a monophyletic cluster that is clearly distinct from *pmoA* sequences of methanotrophic *Proteobacteria* and *Verrucomicrobia* (**Figure 1**). Already before their description by Tavormina et al. (2011), *pxmA* genes were detected in environmental samples, they were referred to as "*pmoA/amoA* like" sequences or as Cluster WC306-54 (Nold et al., 2000; Lau et al., 2007; Dörr et al., 2010). The presence of *pxmA* appears not to be closely linked to phylogeny, similarly to the occurrence of *pmoA2* among *Methylocystaceae* or *mmoX* among the methanotrophic Proteobacteria. The function of the gene product and regulation of gene expression remain currently largely unknown. So far, it could be shown that the gene, which is localized in the *pxmABC* operon, is expressed under environmental and *in vitro* conditions (Tavormina et al., 2011; Kits et al., 2015).

## Evolutionary Related Monooxygenases

It is well known that the particulate methane monooxygenase and the ammonia monooxygenase of nitrifying bacteria and archaea are evolutionary related (Holmes et al., 1995). Meanwhile, further monooxygenases of the superfamily of copper-containing membrane-bound monooxygenases have been identified, involved in the oxidation of short chain hydrocarbons, but not methane (Redmond et al., 2010; Sayavedra-Soto et al., 2011; Coleman et al., 2012; Suzuki et al., 2012). In phylogenetic trees, the sequences of these genes form clusters that are distantly related to those of the known *pmoA* and *amoA* genes. Due to the high sequence divergence, most of these sequence types have not frequently been detected in cultivation-independent PCR-based studies using current *pmoA* primers, but some of them have been found in metagenomic or metatranscriptomic datasets, e.g., in hydrocarbon-rich marine ecosystems (Li et al., 2014).

The existence of a butane monooxygenase in *Nocardioides* sp. CF8 related to the particulate methane monooxygenase was already postulated by Hamamura and Arp (2000), but molecular evidence was provided only recently when the whole genome of the strain was sequenced (Sayavedra-Soto et al., 2011). The butane-oxidizing monooxygenase is encoded by the genes in the *bmoCAB* operon, which have less than 50% amino acid similarity to the genes of the methane and ammonia monooxygenase. Similar genes were also detected in *Mycobacterium smegmatis* strains NBB4 and NBB3 (Coleman et al., 2012). The enzyme in strain NBB4 was shown to oxidize ethane, propane, butane and ethylene. Due to the broader substrate spectrum of the enzyme in *Mycobacterium*, the enzyme was referred to as hydrocarbon monooxygenase, encoded in the *hmoCAB* operon. Genome sequencing projects suggest that similar monooxygenases exist in *Mycobacterium chubuense* B4, *Nocardioides luteus* FB or the uncultured deltaproteobacterial SAR324 clade, which is ubiquitous in the ocean (Sheik et al., 2014).

Redmond et al. (2010) described another cluster of putative hydrocarbon monooxygenases (*emoA*), detected upon stable isotope probing with $^{13}$C-ethane at a hydrocarbon seep. The authors speculate that the labeled organisms are members of the *Methylococcaceae*, which seem to be incapable of methane oxidation. These assumptions can currently only be confirmed by sequence data from isolates referred to as *Methylococcaceae* ET-SHO and ET-HIRO, which were deposited in the NCBI database in an independent study, but remain to be published. Based on the entries in the NCBI database it appears that these *Methylococcaceae* isolates, which were also obtained from a marine habitat, could grow on ethane, but not on methane.

Further types of monooxygenase genes related to *pmoA* and *amoA* are found in *Gammaproteobacteria* of the genus *Haliea* and in the genome of the alphaproteobacterium *Skermanella aerolata* KACC 11604 (= 5416T-32$^{T}$). Strains *Haliea* ETY-M and ETY-NAG grow on ethylene and oxidize in addition ethane, propane and propylene, but not methane (Suzuki et al., 2012). In case of *Skermanella aerolata* KACC 11604 growth on hydrocarbons has

not yet been studied. The sequence of their monooxygenase is different from the *hmoA* and *emoA* genes, but related to the *pmoA* sequences of type II methanotrophs.

## A COMPARISON OF CULTIVATION-DEPENDENT AND –INDEPENDENT DIVERSITY OF METHANOTROPHS BASED ON *pmoA* AS PHYLOGENETIC MARKER

### Classification of *pmoA* Sequences Based on Phylotyping or OTU Clustering

Using *pmoA* as molecular marker for the detection of methanotrophic bacteria it turned out that there is a huge diversity of methanotrophs present in nature that is not represented by isolates in the laboratory. Approximately 15,000 *pmoA* and *pmoA*-like sequences can be found in the Genbank database. To describe and discuss the current diversity of aerobic methanotrophic bacteria based on this data resource, sequences have to be grouped based on similarity. In many studies such groups are defined based on their clustering in phylogenetic trees in relation to known phylotypes, which are represented by sequences of type strains or other well-studied reference strains as well as selected sequences of uncultivated clades. Dumont et al. (2014) recently defined 53 representative sequences for major cultivated and uncultivated phylogenetic clusters.

Another approach is the grouping of similar sequences into operational taxonomic units (OTUs) using a predefined cut-off value. Some studies applied a 3% cut-off without explicitly linking this to a specific phylogenetic resolution (Saidi-Mehrabad et al., 2013; Sharp et al., 2014). Other studies determined and used cut-off values with the aim to reflect genus and species resolution. These values were determined in correspondence to the routinely used cut-off values known from 16S rRNA gene sequence analyses, i.e., 3% sequence difference to distinguish between species and 5% to differentiate genera (Schloss and Handelsman, 2005). For *pmoA* sequences, Lüke et al. (2010) defined cut-off values at 10 and 17% sequence dissimilarity for species and genus delineation, respectively, based on the fact that the nucleotide substitution rate of *pmoA* is 3.5 times higher than that of 16S rRNA genes. The factor 3.5 was derived by correlation of 16S rRNA and *pmoA* gene sequence identities of approx. 75 *Methylocystis* and *Methylosinus* strains (Heyer et al., 2002). Degelmann et al. (2010) included *Gammaproteobacteria* in the comparative analysis and compiled 16S gene sequence identity values of 22 methanotrophs. They correlated 16S rRNA gene to *pmoA* gene as well as to deduced PmoA protein sequence identity values and defined a cut-off of 13% at DNA level for species delineation, corresponding to 7% cut-off at protein level. When comparing these cut-off values to the sequence differences observed between methanotrophic type strains within the same and of different genera, it is apparent that they reflect the average sequence difference between type strains so that genera and species will not be fully resolved using these values (**Figure 2**). At the same time the diagrams, which display minimum and maximum sequence difference of each type strain to another type strain within the same genus and family, reveal that it will be impossible to find cut-off values that differentiate perfectly well all genera without already differentiating species within a genus. Similar difficulties in determining cut-off values that correspond to a certain phylogenetic resolution are known from 16S rRNA gene based analyses (Schloss and Westcott, 2011).

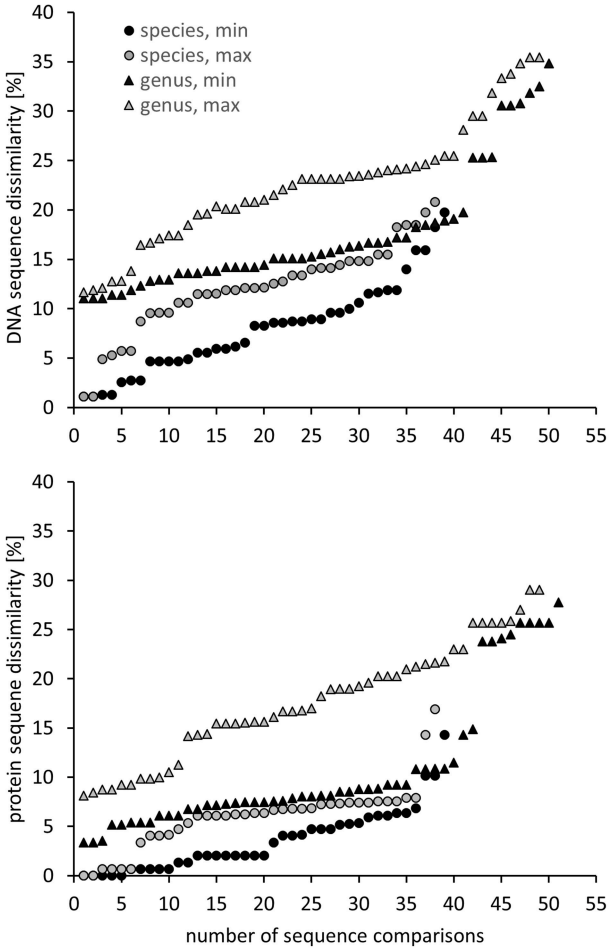


**FIGURE 2 | Minimum and maximum *pmoA* sequence dissimilarity at DNA (upper panel) and protein level (lower panel) between a type strain and its most closely and most distantly related type strain within the same species, as well as to the most closely and distantly related type strain of a different genus within the same family or type (according to Table 1).** DNA and protein distance matrices were calculated in ARB based on 480 aligned nucleotide positions or 160 deduced amino acid positions. *Methylomicrobium album* and *Methylomicrobium agile* were not included, due to the very distant clustering from the other *Methylomicrobium* strains (**Figure 1**), while "*Candidatus* Crenothrix polyspora" was excluded due to the fact that it contains a highly divergent *pmoA* sequence compared to all other *Gammaproteobacteria*.

For the evaluation of the diversity of methanotrophic bacteria in this review article, OTU clustering was performed based on cut-off values that reflect a higher resolution compared to the published values to resolve the distinct genera and species as good as possible. The compilation of minimal DNA

sequence differences between genera reveals that a cut-off value of 11% is necessary to differentiate all genera (**Figure 2**). Indicative for an adequate resolution is the separation of the two most closely related genera, *Methylocystis* and *Methylosinus*. To further evaluate the 11% cut-off value, it was applied to cluster all available high quality *pmoA* sequences using the Mothur classification tool with average neighbor algorithm. Sequences of at least 400 bp length and without accumulation of evident sequencing errors were considered as high quality here and the dataset is referred to as "large *pmoA* dataset" in the following. When performing OTU clustering using different cut-off values it turned out that not 11% but 12% cut-off is sufficient for nearly full resolution at genus level (**Figure 3**). At the same time, type strains belonging to the same genus were grouped into distinct clusters in five cases: "*Methylacidiphilum*," "*Methylacidimicrobium*," *Methylocapsa, Methylomicrobium,* and *Methylobacter*. In case of *Methylobacter* and *Methylomicrobium*, this finding corresponds to the polyphyletic clustering in *pmoA* trees (**Figure 1**). To fully prevent the formation of more than one OTU for these genera, a much higher cut-off value of >20% would be necessary.

The differentiation of *pmoA* sequences at species level is affected by similar difficulties. A cut-off value of 1% is necessary to resolve all species (except *Methylomicrobium album* and *Methylomicrobium agile*, which have even more similar *pmoA* sequences), while such a low value will classify at the same time many strains belonging to the same species into distinct taxonomic units. A higher cut-off value of 3 or 4% leaves only some species unresolved (**Figure 2**), namely the two *Methylothermus* species, *Methylocystis hirsuta*, and *Methylocystis rosea*, as well as some of the *Methylomicrobium* species. OTU clustering applied to the "large *pmoA* dataset" confirmed these findings and shows that a cut-off value of 4% is sufficient to differentiate the majority of species.

Phylogenetic analysis of functional genes is frequently based on protein sequences. This excludes sequence variability at nucleotide positions that are not under evolutionary selection pressure, but provides at the same time less information, so that resolution of closely related taxa becomes more difficult. OTU clustering of sequences with a cut-off that roughly reflects genus level resolution can be achieved at 6% sequence dissimilarity (**Figure 2**). It only fails to resolve *Methylomarinovum* from *Methylothermus*, but a lower value should nevertheless not be selected as the 6% value already provides higher resolution compared to the 12% cut-off value at DNA level when considering the large PmoA dataset including sequences of uncultivated methanotrophs (**Figure 3**). Differentiation of species based on protein sequences is even more difficult. Full resolution cannot be obtained as *Methylomicrobium* and *Methylothermus* species are not even separated at 1% cut-off. A cut-off of 2% already fails to resolve the majority of type strains within the genera *Methylocystis*, *Methylomicrobium,* and *Methylothermus*, although it still gives a higher number of OTUs compared to the 4% cut-off at DNA level when sequences from cultivation-independent studies are included (**Figure 3**).

Due to the difficulties in finding appropriate cut-off values at protein level, *pmoA* sequence diversity was evaluated based on DNA sequences but not protein sequences in the present work. The 12% cut-off was applied to differentiate sequences at a level that allows resolution of most methanotrophic genera and a 4% cut-off was used to differentiate species reasonably well. To distinguish in the following OTU classification done with 12% cut-off from classification with 4% cut-off, the OTUs are referred to as $OTU_{12}$ and $OTU_4$, respectively.

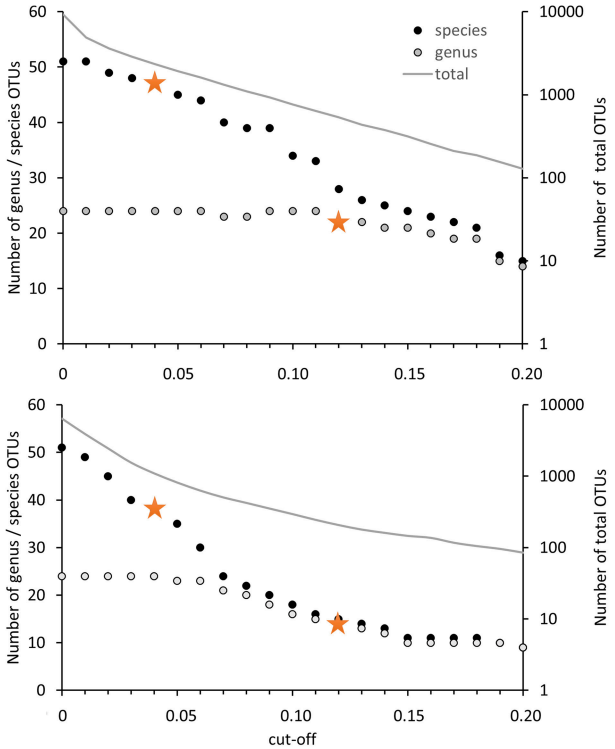


**FIGURE 3 | Number of OTUs in dependence on the cut-off value applied for OTU differentiation.** The number of OTUs containing type strains of different genera or species are displayed on the left axis, the number of OTUs formed based on all high quality sequences (= total) is presented on the right axis at logarithmic scale. Clustering was performed with 12502 high quality *pmoA* sequences (upper panel) or the deduced amino acid sequences (lower panel) available from Genbank. Sequences with at least 400 bp sequence length and without accumulation of sequencing errors were included. Distance matrices were calculated in ARB based on 480 aligned nucleotide positions or 160 deduced amino acid positions. OTU clustering was done using Mothur by applying the average neighbor algorithm. Orange stars denote the cut-off values applied in this review.

## How well do Cultivated Strains Cover the Diversity of Methanotrophic Bacteria as Seen Based on Cultivation-Independent Studies?

Of the 15,000 *pmoA* sequences that have been deposited in the Genbank database, the vast majority was derived from cultivation-independent studies. Slightly less than 3% were obtained from cultured methanotrophic strains. Most of them belong to the well-known genera *Methylocystis, Methylosinus, Methylomonas, Methylobacter, Methylocaldum*,

or *Methylomicrobium* (**Table 4**). Approximately 20 sequences represent isolates that cannot be assigned to a specific known genus; at least some of them may represent new genera. At species level, isolates that are similar to *Methylocystis rosea, Methylocystis hirsuta, Methylocystis echinoides, Methylosinus sporium,* and *Methylosinus trichosporium* or "*Methylomonas denitrificans*" have most frequently been obtained (**Table 5**). In contrast, more than half of the described species are represented by only one single strain at the moment.

To evaluate how well cultivated strains cover the diversity of methanotrophic bacteria as seen in cultivation-independent studies, the distribution of their *pmoA* sequences upon OTU clustering was assessed based on the above mentioned "large *pmoA* dataset" containing 12,502 high quality sequences. The dataset includes different homologs of *pmoA* that have been detected in methanotrophs. Clustering of the sequences applying the 12 and 4% cut-off value resulted in 522 and 2287 OTUs, respectively (**Table 6**). In both cases, there was a rather low number of clusters with high read numbers, while one third of the $OTUs_{12}$ or even half of the $OTUs_4$ were represented by just one read (singletons). This demonstrates the existence of a very high number of taxa that are rarely detected. The percentage of OTUs that contained sequences of cultivated strains was 12 and 6%, respectively, at the different cut-off levels,

**TABLE 4 | Detection frequency of methanotrophic genera in cultivation-dependent and -independent studies.**

| Genus | Number of reads from isolates | Reads in $OTUs_{12}$ | Reads in $OTUs_4$ |
|---|---|---|---|
| *Methylocystis* | 141 | 2754 | 1743 |
| *Methylosinus* | 95 | 173 | 141 |
| *Methylomonas* | 43 | 690 | 98 |
| *Methylobacter* | 34 | 743 | 153 |
| *Methylocaldum* | 16 | 283 | 254 |
| *Methylomicrobium* | 13 | 67 | 78 |
| *"Methylacidiphilum"* | 10 | 10 | 10 |
| *Methylococcus* | 7 | 320 | 282 |
| *"Candidatus* Crenothrix*"* | 6 | 43 | 9 |
| *"Methylacidimicrobium"* | 6 | 6 | 4 |
| *Methylosarcina*[a] | 3 | 457 | 50 |
| *Methylothermus* | 3 | 44 | 30 |
| *Methylocapsa* | 3 | 39 | 7 |
| *Methyloglobulus* | 3 | 7 | 3 |
| *Methylohalobius* | 3 | 5 | 3 |
| *Methylomarinum* | 2 | 2 | 2 |
| *Methyloparacoccus*[b] | 2 | 422 | 6 |
| *Methylovulum* | 2 | 30 | 3 |
| *Methylosoma*[c] | 1 | 252 | 1 |
| *"Candidatus* Methylomirabilis*"* | 1 | 51 | 1 |
| *Methylogaea* | 1 | 24 | 11 |
| *Methyloprofundus* | 1 | 8 | 3 |
| *Methylomarinovum* | 1 | 2 | 1 |

*The number of isolates assigned to a genus is given and the total number of pmoA sequence reads in the OTUs that harbor these isolates. A strong decrease in read numbers from 12% cut-off to 4% cut-off means that isolates are different from the most frequently detected pmoA sequence types in the environment that are classified into the same OTU at genus level resolution.*

[a] *Includes Methylomicrobium album and Methylomicrobium agile at 12% cut-off.*

[b] *Includes Methylomagnum ishizawai at 12% cut-off.*

[c] *Includes Methylobacter tundripaludum at 12% cut-off.*

**TABLE 5 | Representativeness of methanotrophic type strains at species level resolution.**

| Species | Cultivation-independent studies | Further isolates |
|---|---|---|
| *Methylocystis echinoides* | 694 | 13 |
| *Methylocystis rosea, hirsuta* | 330 | 53 |
| *Methylococcus capsulatus* | 273 | 3 |
| *Methylocaldum tepidum* | 91 | 1 |
| *Methylocaldum szegediense* | 74 | 2 |
| *Methylosinus sporium* | 49 | 10 |
| *Methylosarcina lacus* | 49 | 0 |
| *Methylosinus trichosporium* | 46 | 10 |
| *Methylocaldum gracile* | 45 | 8 |
| *Methylobacter tundripaludum* | 39 | 1 |
| *Methylomicrobium buryatense, alcaliphilum, japanense* | 20 | 1 |
| *Methylomicrobium pelagicum* | 20 | 0 |
| *Methylomicrobium agile, album* | 19 | 3 |
| *Methylocystis parvus* | 17 | 3 |
| *Methylocaldum marinum* | 17 | 0 |
| *Methylobacter whittenburyi* | 14 | 4 |
| *Methylobacter marinus* | 14 | 2 |
| *Methylobacter luteus* | 13 | 2 |
| *Methylothermus thermalis, subterraneaus* | 10 | 0 |
| *Methylogaea oryzae* | 10 | 0 |
| *"Methylomonas denitrificans"* | 6 | 10 |
| *Methylomonas methanica* | 8 | 0 |
| *Methylocystis bryophila* | 5 | 9 |
| *Methyloparacoccus murrellii* | 4 | 1 |
| *Methylomicrobium kenyense* | 4 | 0 |
| *Methylomagnum ishizawai* | 4 | 0 |
| *Methylocystis heyeri* | 3 | 1 |
| *"Candidatus* Crenothrix*"* | 3 | 0 |
| *Methylocapsa acidiphila* | 3 | 0 |
| *Methylomonas lenta* | 2 | 0 |
| *Methyloprofundus sedimentii* | 2 | 0 |
| *Methyloglobulus morosus* | 1 | 0 |
| *Methylohalobius crimeensis* | 1 | 0 |
| *Methylovulum miyakonense* | 1 | 0 |
| *Methylobacter psychrophilus* | 1 | 0 |
| *Methylosarcina fibrata* | 1 | 0 |
| *Methylomarinum vadi* | 0 | 2 |
| *Methylocapsa aurea* | 0 | 0 |
| *Methylomarinovum caldicuralii* | 0 | 0 |
| *Methylomonas paludis* | 0 | 0 |
| *Methylosoma difficile* | 0 | 0 |

*The number of reads derived from cultivation-independent studies and of further isolates that were assigned to the same $OTU_4$ as the respective type strain are given.*

**TABLE 6 | Statistics about OTU clustering and distribution of *pmoA* sequences of cultivated methanotrophic strains within these clusters.**

| Cut-off: | 12% | 4% |
|---|---|---|
| **STATISTICS OF OTU CLUSTERING** | | |
| Number of OTUs | 522 | 2287 |
| Number of reads in largest cluster | 2666 | 708 |
| % of clusters with ≥ 100 reads | 4 | 0.5 |
| % of clusters with < 100 reads but ≥ 10 reads | 20 | 10 |
| % of singletons | 36 | 54 |
| **OTUs CONTAINING CULTIVATED STRAINS** | | |
| % of OTUs with cultivated strains | 11.9 | 6.2 |
| % of OTUs that contain a type strain | 8.2 | 3.0 |
| % of OTUs that contain only cultivated strains | 5.7 | 3.4 |
| % of singletons represented by a cultivated strain | 2.5 | 1.8 |
| **REPRESENTATIVENESS OF OTUs CONTAINING CULTIVATED STRAINS** | | |
| % of sequences in clusters with cultivated strains | 52 | 24 |
| % of sequences in clusters with type strains | 50 | 17 |

which means that only a small fraction of the methanotrophic diversity is represented by cultivated strains. But remarkably, when considering the size of the $OTUs_{12}$, it turned out that 52% of all available sequences fall into clusters that contain *pmoA* sequences of isolates. This demonstrates that half of the sequences that have been detected in cultivation-independent studies are closely related to or represented by cultivated genera. At species level, still 24% of all sequences fall into the same $OTU_4$ as a cultivated strain. In conclusion, a surprisingly high number of sequence reads that are detected in environmental studies are closely affiliated to cultivated genera or species, despite the fact that the total diversity of methanotrophs that is present in nature is substantially higher than the cultured diversity.

To further evaluate the representativeness of the cultivated genera and species, the size of the OTUs harboring isolates was evaluated. The most frequently detected genera of methanotrophic bacteria in environmental studies are the alphaproteobacterial genera *Methylocystis* and *Methylosinus* and the gammaproteobacterial genera *Methylomonas*, *Methylobacter*, *Methylosarcina, Methylomicrobium*, *Methylococcus*, *Methylocaldum*, *Methylosoma* as well as the recently described genus *Methyloparacoccus* (**Table 4**). At higher taxonomic resolution, the isolated *Methyloparacoccus* species remains distinct from the related sequences that have been frequently detected in environmental samples. The same applies to *Methylosoma* and "*Candidatus* Methylomirabilis." Further methanotrophic genera that have very rarely or not yet been detected in environmental samples via cultivation-independent methods comprise *Methylomarinovum, Methylomarinum, Methylohalobius, Methyloglobulus* and the verrucomicrobial lineages "*Methylacidiphilum*" and "*Methylacidimicrobium*" (**Table 4**). At lower phylogenetic resolution, the genera *Methyloglobulus* and *Methylomarinum* do serve as cultivated representatives for major uncultivated clusters (see Section Cluster 2 (CL2) or TUSC). In case of the verrucomicrobial lineages, the limited detection in environmental samples is explained by their highly divergent *pmoA* sequences, which prevents PCR amplification using the standard *pmoA* primers. At species level, the frequently detected taxa in cultivation-independent studies are *Methylocystis echinoides*, *Methylocystis rosea,* and *Methylocystis hirsuta*, the two *Methylosinus* species, *Methylococcus capsulatus* and most species of the genus *Methylocaldum* (**Table 5**). Nearly half of the validly described methanotrophic species have only rarely been detected in environmental samples based on cultivation-independent studies (<10 reads), showing that our culture collections contain many strains of which the ecological relevance in their natural ecosystems remains unknown. Remarkably, *Methylosinus* strains have very frequently been isolated, but not that frequently been detected by cultivation-independent studies. This is evident from the fact that 54% of all *Methylosinus* sequences in the database are from isolates, while most other frequently detected genera have only about 5% cultivated representatives (**Table 4**).

## DESCRIPTION OF MAJOR UNCULTIVATED GROUPS OF METHANOTROPHIC BACTERIA AND THEIR HABITAT SPECIFICITY

Clusters of *pmoA* sequences representing uncultivated methanotrophs have been defined in diverse studies mostly at a taxonomic resolution above genus level. They are often named according to the habitat in which they are predominantly found, the sampling site from which they were obtained, or derived from the name of the first described clones of a cluster. The assignment of sequences to a characteristic cluster is usually done in the context of phylogenetic tree reconstruction, guided by a few characteristic reference sequences that are given in the literature as representatives.

The same approach was used here to assign OTUs to described clusters of uncultivated methanotrophic bacteria. Neighbor joining and maximum likelihood trees were constructed using one representative sequence for each $OTU_{12}$. These representative sequences were selected within each OTU based on the following criteria: OTUs harboring a cultivated strain were represented by the sequence of this strain. For OTUs consisting of sequences from uncultivated bacteria only, the most representative sequence from the first dataset reporting about this sequence type was taken. All representative sequences are listed along with their cluster assignment and accession number in Table S1. Uncultivated clusters were identified in the phylogenetic trees based on the position of published reference sequences. Several OTUs showed inconsistent clustering (in particular among the type I methanotrophs), they were excluded from clusters and are referred to as "incerta sedis" or by their family names and are displayed as "unknown" in **Figure 4**.

To integrate habitat preferences of methanotrophic lineages into the phylogenetic tree (**Figure 4**), information about the habitat from which sequences were obtained was collected from the literature and the NCBI database. The definition of categories was largely guided by the terminology used in the literature and the number of sequence reads obtained for

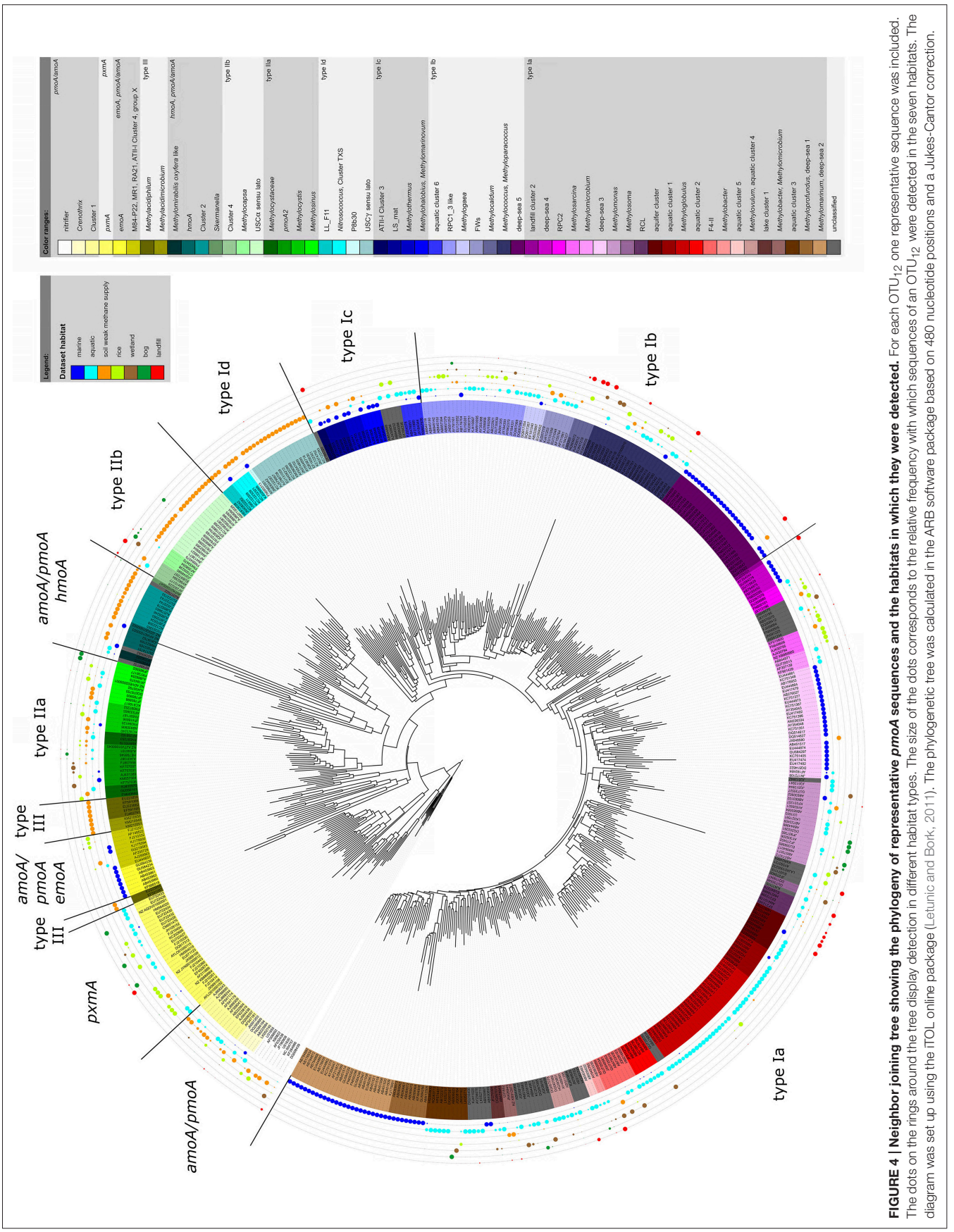


**FIGURE 4 | Neighbor joining tree showing the phylogeny of representative *pmoA* sequences and the habitats in which they were detected.** For each $OTU_{12}$ one representative sequence was included. The dots on the rings around the tree display detection in different habitat types. The size of the dots corresponds to the relative frequency with which sequences of an $OTU_{12}$ were detected in the seven habitats. The diagram was set up using the iTOL online package (Letunic and Bork, 2011). The phylogenetic tree was calculated in the ARB software package based on 480 nucleotide positions and a Jukes-Cantor correction.

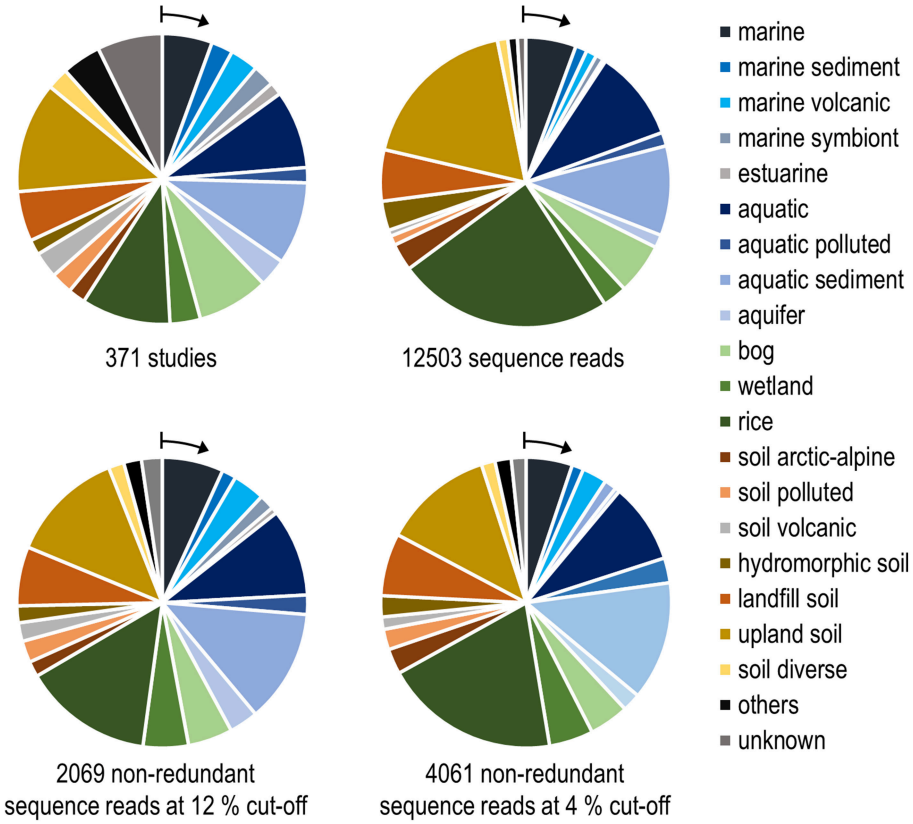


**FIGURE 5 | Number of habitats that were analyzed in research studies (upper left) and grouping of *pmoA* sequences from the NCBI database according to the habitats in which they were detected.** The upper right diagram is based on all available high quality sequences, while the lower diagrams include only non-redundant sequence reads. Redundant reads are those that were detected in the same study and fall within the same OTU. Arrows denote the position of the group that is shown as first entry in the legend.

each of these categories. The majority of sequences that are currently stored in the public database are from studies that analyzed methanotrophic communities in rice fields, upland soils, aquatic or marine environments (**Figure 5**). 2.4% of the sequences remained unclassified, either because no information about the habitat was available or they were obtained from studies analyzing rather unusual and thus little studied habitats of methanotrophs (bioreactor, manure, rumen, waste water or plants). Seven major habitat types were defined based on this information and the relative detection frequency of each $OTU_{12}$ within these habitats calculated. The presentation of these data in combination with phylogeny allows the identification of major clusters with habitat preferences (**Figure 4**). Habitat specificity of individual OTUs cannot be inferred from this presentation, as a substantial number of OTUs are represented by just one sequence and thus displayed with 100% recovery from one single habitat. To evaluate this aspect, further data analysis is needed as described in Section Habitat Specificity of Methanotrophic Taxa Evaluated at Higher Taxonomic Resolution.

Remarkably, three-fourths of all $OTUs_{12}$ represent type I methanotrophs in the phylogenetic tree (**Figure 4**), with nearly 50% belonging to type Ia methanotrophs. This confirms that methanotrophic diversity is highest within the *Gammaproteobacteria*. Furthermore, it is evident from **Figure 4** that the methanotrophs that are found in upland soils, aquatic and marine environments form distinctive and large clusters, while the methanotrophs that are found in other habitats such as rice field soils, wetlands or landfill cover soils are found in smaller clusters that are often detected in different habitats. It is tempting to speculate that colonization of the rather young anthropogenic habitats such as rice field soils or landfill cover soils occurs via methanotrophs that evolved in the much older pristine habitats, so that evolutionary processes leading to diversification and specialization are still in a very early phase in these human made habitats. Moreover, rice field soils and wetlands may represent transitions between terrestrial and aquatic ecosystems and thus share more taxa with other habitats. The absence of specific clusters in wetlands may at least partially be the result of a rather small number of studies in which *pmoA* sequences were published for this ecosystem (**Figure 5**) leading to an underrepresentation of sequence reads from this habitat.

In the following, information about the major uncultivated clusters of methanotrophs residing in different habitats is compiled. A condensed phylogenetic tree shows the phylogenetic placement of these clusters in relation to each other and to cultivated type species (**Figure 6**).


**FIGURE 6 | Neighbor joining tree showing the phylogeny of uncultivated clusters in relation to methanotrophic type strains.** The tree includes *pmoA* sequences from all OTUs that were assigned to uncultivated clusters. It was calculated based on 480 nucleotide positions with Jukes Cantor correction. The scale bars display 0.10 changes per nucleotide or amino acid position.

## Rice Paddy Clusters (RPC) and Japanese Rice Clusters (JRC), Including the Lake Washington Cluster (LWs), and the Organic Soil Cluster (OSC)

Several different rice paddy clusters and Japanese rice clusters have been defined (Lüke et al., 2010; Stralis-Pavese et al., 2011), but only some of them are regularly detected in diverse studies and implemented in phylogenetic trees. These are RPC1, 2, 3, and JRC3 as well as JRC4, which has meanwhile a cultivated representative, *Methylogaea oryzae* (Geymonat et al., 2010). JRC3, RPC1, and RPC3 are distantly related to *Methylocaldum* and *Methylococcus* and thus part of the type Ib group (**Figure 6**). RPC2 was reported to show variable clustering either with type Ia or Ib, depending on the algorithm used for tree reconstruction (Lüke and Frenzel, 2011). It is composed of a high number of OTUs at species level resolution, but contains only four OTUs at genus level. RPC1, RPC3, and JRC3 were combined into a larger monophyletic cluster referred to as RPC1_3 in this review, because JRC3 did not form a monophyletic cluster and could not be clearly delineated from RPC1. The RPC1_3 like cluster consists of 25 $OTUs_{12}$, including in addition the clusters LWs and OSC. Similarly, a large cluster containing the sequences of RPC1, LWs, and OSC but without RPC3 was also formed in some other studies and referred to as freshwater lineage 1 (Lüke and Frenzel, 2011). The major habitat of the methanotrophs belonging to the RPC1_3 like cluster are rice field and aquatic ecosystems (**Figure 4**). RPC1 and JRC3 were initially exclusively detected in rice paddy associated habitats (Lüke et al., 2010; Lüke and Frenzel, 2011). Exceptional within the RPC1_3 like cluster is OSC, which occurs predominantly in bogs and in some upland soils (**Figure 4**, Tables S1–S4). Thus, the large RPC1_3 cluster is heterogeneous in terms of habitat preference, with some habitat-specific subgroups. In in-depth studies, biogeographic patterns have been shown for clusters RPC1 and JRC3 (Lüke et al., 2010). Moreover, they respond to the environmental factor rice genotype, either directly or possibly indirectly via altered physicochemical conditions in the plant rhizosphere (Lüke et al., 2011).

## Upland Soil Clusters (USCα and USCγ), Jasper Ridge Clusters (JR1, JR2 and JR3), Moor House Peat Cluster (MHP), and Cluster 5

Phylogenetically, the upland soil clusters form two major groups. Sequences of USCα, JR1, and MHP (also referred to as Cluster 5) are related to *Methylocapsa* (**Figure 6**). USCα was initially detected by Holmes et al. (1999) and termed RA14. The name USCα was proposed for this sequence type when a second group of sequences with preferential occurrence in upland soils but related to sequences of methanotrophic *Gammaproteobacteria*, USCγ, was discovered (Knief et al., 2003). USCγ as well as JR2 and JR3 belong to the type Id group (**Figure 6**). These sequences are related to methanotrophic *Gammaproteobacteria* and the *amoA* sequence of *Nitrosococcus oceani*.

It was proposed to refer to the large group of USCα, JR1/Cluster 5, and MHP sequences as USCα-like sequences or USCα sensu lato, while the initially discovered RA14 clade was defined as USCα sensu stricto (Shrestha et al., 2012). Based on the sequence dataset used in this study, USCα sensu lato consists of 18 $OTUs_{12}$ and shows an enormous diversity at lower resolution with 133 $OTUs_4$ (**Table 7**). In particular USCα sensu stricto shows a high diversity at species level resolution. In analogy to this differentiation of USCα sensu lato, sequence clusters USCγ, JR2, and JR3 will be referred to as USCγ sensu lato in this review, while USCγ sensu stricto refers specifically to the USCγ clade. The USCγ sensu lato group is less diverse compared to USCα, consisting of 15 $OTUs_{12}$ and 98 $OTUs_4$ with USCγ sensu stricto as most diverse group, especially at species level resolution (**Table 7**).

All upland soil cluster sequences occur in soils, predominantly in upland soils. USCα sensu lato has been identified as dominant *pmoA* type in different forest soils (Kolb et al., 2005; Degelmann et al., 2010; Dörr et al., 2010). Some USCα sequence types have additionally been detected in hydromorphic soils (**Figure 4**, Tables S1–S4) (Knief et al., 2006; Shrestha et al., 2012). USCγ sensu lato occurs in pH neutral and alkaline soils and has been reported to dominate in soils collected from an alpine meadow, an arid desert ecosystem and a former lake (Angel and Conrad, 2009; Zheng et al., 2012; Serrano-Silva et al., 2014). Moreover, USCγ OTUs have been detected sporadically in landfill cover soils (Kumaresan et al., 2009; Henneberger et al., 2012). The occurrence of the two upland soil clusters is clearly pH dependent. USCα sensu lato occurs in acidic to pH neutral soils, while USCγ is only detected in pH neutral and alkaline soils (Knief et al., 2003; Kolb, 2009).

The occurrence of the USC methanotrophs is in most soils reduced the more intensively a soil is agriculturally managed. The clusters are consistently found in forest soils, often as most abundant group, they are quite frequently detected in grassland soils, but rarely detected in intensively managed agricultural soils (Knief et al., 2006; Dunfield, 2007). It has been reported that populations decrease and become inactive when forest soils are converted into agricultural soils, or grasslands are subjected to grazing (Knief et al., 2005; Abell et al., 2009; Dörr et al., 2010; Lima et al., 2014). They recover in afforested or reforested sites and grassland soil in which nitrogen fertilization is reduced (Nazaries et al., 2011; Shrestha et al., 2012). The data of Degelmann et al. (2010) suggest that habitat specificity may exist within USCα sensu lato, as some OTUs occurred in deciduous but not in spruce forest soils.

The USC methanotrophs are assumed to be involved in the oxidation of atmospheric methane (Dunfield, 2007; Kolb, 2009), but this might be different for one specific OTU within USCα sensu lato. OTU 75 (USCα 5, MHP) has more frequently been detected in soils with higher methane supply, i.e., peatlands and wetland, than in typical upland soils (Tables S1–S4) (Chen et al., 2008; Liebner and Svenning, 2013; Yun et al., 2015). Initially it was assumed that the USC methanotrophs may obtain enough energy from atmospheric methane oxidation for cell maintenance and growth (Knief and Dunfield, 2005; Kolb et al., 2005), but later calculations based on methane uptake rates

**TABLE 7 | Characteristics of uncultivated clusters of *pmoA* and *pmoA*-like sequences[a].**

| Cluster | No. of OTUs (12% cut-off) | No. of OTUs (4% cut-off) | Related to[b] | Major habitat | Representative sequences[c] | References |
|---|---|---|---|---|---|---|
| **TYPE IA** | | | | | | |
| Aquatic cluster 1 (incl. *Clonothrix*) | 8 | 13 | Type Ia | aquatic | AB930877, AB844864, AB845005 | This study |
| Aquatic cluster 2 (incl. *Methylosoma*) | 6 | 16 | Type Ia | Aquatic | AB478795, KC188735, AB478808, JF811270 | This study |
| Aquatic cluster 3 | 9 | 21 | Type Ia | Aquatic | HQ383800, AB722373, JN591162 | This study |
| Aquatic cluster 4 | 4 | 8 | Type Ia | Aquatic | AY488060, HQ383801 | This study |
| Aquatic cluster 5 | 2 | 7 | Type Ia | Aquatic | AB563463, AB505022 | This study |
| Aquifer cluster | 9 | 13 | Type Ia | Diverse | AM410175, AB930937, AM410175 | Dumont et al., 2014 |
| Deep-sea 1 (incl. OPU2, *Methyloprofundus*) | 8 | 33 | Type Ia | Marine | AB089967 (OPU1), AM089968 | Hayashi et al., 2007; Lüke and Frenzel, 2011; Dumont et al., 2014 |
| Deep-sea 2 (incl. PS-80, *Methylomarinum*) | 24 | 68 | Type Ia | Marine | AY354047, AB176934, EU444860, AB176935, AF211872 (PS-80) | Lüke and Frenzel, 2011; Dumont et al., 2014 |
| Deep-sea 3 (incl. OPU3, EST) | 27 | 75 | Type Ia | Marine | AB276027 (OPU3), AF182484 (EST), AB276027, AB176933 | Hayashi et al., 2007; Lüke et al., 2010; Lüke and Frenzel, 2011; Dumont et al., 2014 |
| Deep-sea 4 | 6 | 9 | Type Ia (Ib) | Marine | GU584278, AY354044, GU115829, FN650295 | Lüke and Frenzel, 2011; Dumont et al., 2014 |
| F4-II | 8 | 18 | Type Ia | Diverse | AB478819, GU735534, HM216892 | This study, referring to Chauhan et al., 2012 |
| Lake cluster 1 | 3 | 17 | Type Ia | Diverse | AB478843, DQ067079 | Dumont et al., 2014 |
| Landfill cluster 2 | 1 | 2 | Type Ia | Landfill soil | EU275101 | Dumont et al., 2014 |
| RCL | 5 | 47 | Type Ia | Diverse | EF212356, EF212340, HM216885 | Chen et al., 2007 |
| RPC2 | 4 | 51 | Type Ia (Ib) | Rice, aquatic | FN600101, EU358980 | Lüke et al., 2010; Dumont et al., 2014 |
| **TYPE IB** | | | | | | |
| Aquatic cluster 6 (incl. *Methyloparacoccus* and *Methylomagnum*) | 6 | 15 | Type Ib | Aquatic | AF211880, JX184344, AB478851 | This study |
| Deep-sea 5 (incl. OPU1) | 28 | 54 | Type Ib/c/d | Marine | AB276025 (OPU1), AY354045, AB176940, FN650305, EU417532 | Hayashi et al., 2007; Lüke and Frenzel, 2011; Dumont et al., 2014 |
| FWs | 4 | 50 | Type Ib | Aquatic | AF211881, AF150764, EU131048 | Lüke and Frenzel, 2011; Dumont et al., 2014 |
| RPC1_3 like (incl. RPC1, RPC3, LWs, JRC3, OSC) | 25 | 178 | Type Ib | Diverse | AJ299956 (RPC1), EU193281 (RPC3), DQ067069 (LWs), AY355388 (JRC3), AY781161 (OSC), AB845118 | Lüke et al., 2010; Lüke and Frenzel, 2011; Dumont et al., 2014 |
| **TYPE IC AND ID** | | | | | | |
| ATII-I Cluster 3 | 2 | 2 | Type Ic/d | Marine | KJ175590, EU275114 | This study, referring to Abdallah et al., 2014 |
| LL_F11 | 3 | 5 | Type Id | Upland soil | HE613038, KC122282 | This study |
| LS_mat | 5 | 12 | type Ic/d | Marine, upland soil | JF780903, FR670562, JF780909 | This study, referring to Crépeau et al., 2011 |

*(Continued)*

**TABLE 7 | Continued**

| Cluster | No. of OTUs (12% cut-off) | No. of OTUs (4% cut-off) | Related to[b] | Major habitat | Representative sequences[c] | References |
|---|---|---|---|---|---|---|
| TXS | 4 | 21 | Type Id | Upland soil | KC122309, KJ026966, KC122329 | This study, referring to Serrano-Silva et al., 2014 |
| USCγ, sensu stricto | 7 | 63 | Type Id | Upland soil | AJ579667, AY662351, KC122280 | Knief et al., 2003 |
| USCγ, JR2 | 6 | 17 | Type Id | Upland soil | AY654695, KC122283, KC122301 | Horz et al., 2005 |
| USCγ, JR3 | 3 | 18 | Type Id | Upland soil | AY654702, KM390988 | Horz et al., 2005 |
| **TYPE IIB** | | | | | | |
| Cluster 4 = MO3 | 4 | 10 | Type IIb | Diverse | AF283229, AM410177 | Henckel et al., 2000b; Knief et al., 2006 |
| USCα, sensu stricto = RA14 | 4 | 72 | Type IIb | Upland soil | AF148521, EF015805 | Holmes et al., 1999; Knief et al., 2003 |
| USCα, MHP | 4 | 25 | Type IIb | Upland soil | EF644609, AJ868263 | Chen et al., 2008 |
| USCα, JR1 = Cluster 5 | 10 | 36 | Type IIb | Upland soil | AJ868264, AY662381 | Horz et al., 2005; Knief et al., 2005 |
| ***pmoA/amoA*** | | | | | | |
| ATII-I Cluster 4 | 2 | 2 | *pmoA/amoA* | Marine | KJ175600, KJ175594 | This study, referring to Abdallah et al., 2014 |
| Cluster 1 = *Crenothrix* related | 12 | 47 | *pmoA/amoA* | Upland soil | AF358041, AJ868244, AF547181, AF547179 | Kolb et al., 2005; Ricke et al., 2005; Lüke and Frenzel, 2011 |
| Cluster 2 = TUSC | 10 | 21 | *pmoA/amoA* | Upland soil | AJ579663, AJ868246, EU723743, KC122308 | Knief et al., 2005; Ricke et al., 2005; Lüke et al., 2010 |
| M84-P22 | 1 | 2 | *pmoA/amoA* | Diverse | AJ299963 | Horz et al., 2001 |
| MR1 | 2 | 3 | *pmoA/amoA* | Upland soil | AF200729, GQ219583 | Henckel et al., 2000a |
| RA21 | 3 | 29 | *pmoA/amoA* | Rice | AF148522, FJ210291, FJ210332 | Holmes et al., 1999 |

[a]*The number of different OTUs per cluster given in this table should be considered as estimate reflecting the diversity within each cluster. The assignment of sequences to clusters was evaluated by comparison of trees calculated based on different algorithms and sequence input datasets, but it cannot be excluded that further variation in treeing algorithms and input data may lead to slightly different results in terms of clustering. This applies in particular to type Ia clusters.*

[b]*Assignment to type Ia, Ib, Ic, or Id is in some cases uncertain as it varies depending on the tree reconstruction algorithm and sequence dataset.*

[c]*Reference sequences were selected from those OTUs that were most frequently detected in different studies, reflected the diversity of the cluster as good as possible and showed robust results during phylogenetic tree reconstruction. A complete list of sequences assigned to each cluster based on the results in* ***Figure 4*** *is given in Table S1.*

and estimated cell numbers in forest soils indicated that an additional energy source is needed for survival (Degelmann et al., 2010). Indeed, it could be proven that $^{13}$C-labeled acetate is incorporated into the biomass of USCα methanotrophs, suggesting that these are facultative methanotrophs (Pratscher et al., 2011).

## Cluster 4 (CL4) or MO3

Besides USCα sensu lato, only one further cluster of sequences representing an uncultivated group of methanotrophs is known among the type II group. This is Cluster 4, also known as MO3. It consists of only four $OTUs_{12}$, is related to *Methylocapsa* and was initially detected in rice field soil (Henckel et al., 2000b). Upon repeated detection it was defined as cluster 4 (Knief et al., 2006). The cluster has been detected quite frequently in diverse soil habitats including landfill cover, hydromorphic, upland and wetland soils (**Figure 4**, Tables S1–S4). Its growth was stimulated when rice field soil was incubated under high methane and oxygen concentrations (Henckel et al., 2000b).

## Cluster 1 (CL1) or *Crenothrix* Related Cluster

A sequence cluster related to *pmoA* of *Crenothrix*, *amoA* of nitrifying bacteria and hydrocarbon monooxygenases (*hmoA, emoA*) was described as cluster 1 (Kolb et al., 2005; Ricke et al., 2005; Knief et al., 2006; Lüke and Frenzel, 2011). It was later also referred to as *Crenothrix* related cluster (Lüke and Frenzel, 2011). Cluster 1 contains some sequences from methanotrophic isolates that were obtained from Canadian Arctic soils (Pacheco-Oliver et al., 2002). Based on their 16S rRNA gene sequences, these isolates are related to *Methylocystis* and *Methylosinus*. Unfortunately, the isolates have been lost and similar isolates could so far not be obtained again, so that the identity and characteristics of the bacteria harboring this *pmoA* sequence type remain unclear. It has been speculated that cluster 1 organisms are responsible for atmospheric methane uptake, as they were detected as dominant *pmoA* sequence type in some upland soils, in particular in pH neutral soils (Kolb et al., 2005; Ricke et al., 2005; Kolb, 2009). Experimental proof for this hypothesis is still missing. Further sequences assigned to Cluster 1 were detected in aquatic sediments and aquifers (**Figure 4**, Tables S1, S3). This corresponds well to the habitat of the related *Crenothrix* organisms, which were enriched from backwash water of sand filters fed with ground water (Stoecker et al., 2006). Thus, at least some Cluster 1 organisms may be similar to *Crenothrix* and the whole cluster appears to harbor methanotrophs adapted to different habitats.

## Cluster 2 (CL2) or TUSC

Another sequence cluster with *pmoA/amoA* like sequences was referred to as cluster 2 upon its recurring detection (Knief et al., 2003, 2005; Ricke et al., 2005). In later studies it was named tropical upland soil cluster (TUSC) (Lüke et al., 2010), though its occurrence is not restricted to tropical soils. Instead, it has been detected in diverse upland soils and some hydromorphic soils. It shows similarities in dispersal to USCγ, as it is largely absent in wetlands and acidic soils (Kolb, 2009; Martineau et al., 2014). Moreover, it shows reduced occurrence in intensively managed agricultural soils (Lima et al., 2014) with the exception that it has been found in some agricultural fields that are subjected to organic farming and/or that are characterized by higher carbon content (upon biochar or organic residue application; Dörr et al., 2010; Lima et al., 2014; Ho et al., 2015).

It has been speculated that the organisms harboring genes of this sequence cluster are involved in atmospheric methane oxidation, but this is solely based on the specific detection of this sequence type in upland soils. Further proof for this hypothesis is missing. It can currently not even be excluded that the genes of this sequence cluster encode a non-methane hydrocarbon monooxygenase, which is suggested by the fact that the sequences are related to those of hydrocarbon monooxygenases (**Figure 6**). The only evidence that supports the assumption that cluster 2 sequences may represent methanotrophic *Gammaproteobacteria* comes from a study of Kalyuzhnaya et al. (2006), who enriched methanotrophic bacteria from lake Washington sediment by cell sorting using 16S rRNA targeted fluorescent probes. Twenty percent of a *pmoA* clone library, constructed from a cell suspension enriched with a probe for type I methanotrophs, represented cluster 2 *pmoA* sequences. Unusual in this context remains the unique detection of this sequence type in a lake sediment.

## Deep-Sea Clusters 1 to 5 Including OPU1, OPU3, and PS-80

Sequences retrieved from marine environments can be grouped into five major clusters, referred to as deep-sea clusters 1 to 5 (Lüke and Frenzel, 2011). Deep-sea clusters 1, 2, and 3 belong to the type Ia methanotrophs (**Figure 6**). Deep-sea cluster 4 is distantly related to known type Ia and Ib methanotrophs. Depending on the subset of sequences and the method used for tree reconstruction this cluster falls within either type Ia or type Ib methanotrophs (Lüke and Frenzel, 2011). Deep-sea cluster 5 is a deeply branching lineage related to type Ib and Ic methanotrophs. The clustering is variable in different phylogenetic trees, so that an unambiguous assignment to one or the other type is difficult. In some studies, this cluster was even assigned to type Id (referred to as type Ic in those studies; Lüke and Frenzel, 2011; Henneberger et al., 2012).

Deep-sea clusters 1 and 2 have meanwhile cultivated representatives. Cluster 1 includes the cultivated genus *Methyloprofundus* and cluster 2 the genus *Methylomarinum*. These genera represent one single OTU within the respective clusters, while the clusters contain in total eight and 27 $OTUs_{12}$. Thus, it appears likely that they consist of more than one genus. Hence, the well-established names deep-sea cluster 1 and 3 are kept for these larger clusters of sequences in this review. Deep-sea cluster 2 includes the uncultivated PS-80 cluster, which is displayed as distinct cluster in some phylogenetic trees or given as alternative name for deep-sea cluster 2 (Deng et al., 2013; Dumont et al., 2014; Li et al., 2014). Likewise, deep-sea cluster 3 includes the sub-clusters OPU3 and EST, which are repeatedly mentioned in the literature and sometimes given as synonym for deep-sea cluster 3 (Lüke et al., 2010; Tavormina et al., 2010, 2013; Crespo-Medina et al., 2014; Li et al., 2014). The same

applies to deep-sea cluster 5, which includes or corresponds to OPU1.

Deep-sea clusters 1 and 4 are rather small with only 6 and 8 $OTUs_{12}$ and have less frequently been detected compared to the other three clusters, which contain between 20 and 30 $OTUs_{12}$ (**Table 7**). Most deep-sea clusters consist exclusively of sequences from marine habitats, the only exceptions are found in deep-sea clusters 3 and 5 (**Figure 4**). They contain one single $OTU_{12}$, which was retrieved from a terrestrial habitat, i.e., a mud volcano and a landfill cover soil (Henneberger et al., 2012). Furthermore, OTU 271 in cluster 3 contains some sequences from an aquatic habitat. These were detected in an estuarine sediment, which harbored otherwise sequences that are typical for aquatic habitats (McDonald et al., 2005).

Possible habitat preferences of the different deep-sea clusters remain currently largely unknown. In most studies, sequences of two or more deep-sea clusters have been detected in the same sample (Nercessian et al., 2005; Yan et al., 2006; Redmond et al., 2010; Ruff et al., 2013). Nevertheless, methanotrophic communities can differ substantially between sites (Ruff et al., 2013). Clear differences were also seen between sediment and water column within the same site (Tavormina et al., 2008), but overall, all five clusters have been detected in samples from the water column or the sediment with roughly equal frequency. Evidence for habitat specificity is only seen within deep-sea cluster 1, which harbors the majority of sequences that were found in association with marine animals (Zbinden et al., 2008; Wendeberg et al., 2012; Raggi et al., 2013). These methanotrophs live as endosymbionts in mussels, tube worms or shrimps and contribute to the food web of deep-water ecosystems (Petersen and Dubilier, 2009). Deep-sea cluster 2 and 4 sequences have also been detected as endosymbionts or epibionts of marine animals, but less consistently (Zbinden et al., 2008; Rodrigues et al., 2011; Watsuji et al., 2014).

## Lake Cluster 1, Aquifer Cluster, and Aquatic Clusters 1 to 6

Sequence types that have predominantly been detected in aquatic habitats are grouped into lake cluster 1 and 2 and the aquifer cluster (Dumont et al., 2014). Lake cluster 1 is a small group of sequences (3 $OTUs_{12}$) belonging to the type Ia methanotrophs (**Figure 6**). Most lake cluster 1 sequences were detected in aquatic ecosystems, while few were found in a wetland. Lake cluster 2 sequences represent also type Ia methanotrophs and were grouped by the Mothur classification tool into one single large OTU together with *Methyloparacoccus*. Thus, it is referred to as *Methyloparacoccus* here instead of lake cluster 2. This OTU was not only detected in aquatic ecosystems, but also in rice ecosystems and sporadically in other habitats (**Figure 4**).

The aquifer cluster consists of nine $OTUs_{12}$ and just a few more OTUs at species level resolution. It is also representing type Ia methanotrophs. The name refers to the initial detection in a petroleum-contaminated aquifer (Urmann et al., 2008), but sequences of this cluster occur in different habitats. Half of the $OTUs_{12}$ are common in aquatic ecosystems, while others were detected in landfill cover soils (**Figure 4**, Tables S1, S2). This applies even to the OTU harboring the aquifer sequences; it was also detected in landfill cover soils.

The evaluation of the relationship between phylogeny and habitat revealed the existence of possible further aquatic clusters that were defined in this work (**Figure 4**). The aquatic clusters 1 to 5 are related to type Ia methanotrophs, while aquatic cluster 6 is a member of the type Ib methanotrophs. Aquatic cluster 1 is related to *Clonothrix*, aquatic cluster 2 to *Methylosoma*, and cluster 4 often includes *Methylovulum* in phylogenetic trees. All clusters are rather small, consisting of two to nine $OTUs_{12}$ (**Table 7**). They contain dominantly sequences from aquatic ecosystems plus some sequences from other habitats, often from marine ecosystems (**Figure 4**). Most aquatic clusters and the lake cluster 1 OTUs were detected in samples from the water column as well as the sediment. Only aquatic cluster 4 shows a much higher detection frequency in studies of sediment samples, while cluster 2 shows a higher detection frequency in samples from the water column (Tables S1–S4). Similarly, the aquifer cluster has not yet been detected in aquatic sediment samples.

## Further Clusters of Uncultivated Gammaproteobacterial Methanotrophs

Two further clusters of uncultivated methanotrophs are related to type Ia methanotrophs, represented by cluster RCL and F4-II. Cluster RCL was named after the first clones, obtained during a study analyzing active methanotrophs in landfill cover soil (Chen et al., 2007). It consists of only five $OTUs_{12}$, but a much higher number of 47 $OTUs_4$ at higher taxonomic resolution (**Table 7**). It has been detected in different ecosystems, in particular in aquatic sediments and landfill cover soils (Tables S1–S4, **Figure 4**). Cluster F4-II was defined in this work, referring to the first study in which this sequence type was discovered (Chauhan et al., 2012). It consists of eight $OTUs_{12}$ and contains sequences from diverse habitats, especially aquatic and wetland ecosystems.

Cluster FWs is present within the type Ib methanotrophs and was defined recently (Dumont et al., 2014). It has a relatively high diversity at species level and has most frequently been detected in aquatic environments.

Two rather small clusters of uncultivated methanotrophs, clusters LS-mat and ATII-I cluster 3 can be assigned to the type Ic or Id methanotrophs, depending on the treeing approach (**Figures 4**, **6**). These clusters were named in this work in accordance with the sample and cluster names given in the studies in which they were first described (Crépeau et al., 2011; Abdallah et al., 2014). They are closely related to each other and were detected in different marine studies and with lower frequency in some terrestrial habitats.

Besides USCγ sensu lato one further cluster of uncultivated sequences is present within the group of type Id methanotrophs. The TXS cluster consists of four $OTUs_{12}$ and has been exclusively detected in upland soils so far, likewise as the other uncultivated type Id clusters (Serrano-Silva et al., 2014). Whether the organisms of this cluster are also involved in atmospheric methane oxidation is unknown.

## Further *pmoA*/*amoA* Like Clusters: MR1, RA21, and Others

Several further sequence types form small clusters that are distantly related to the well-known *pmoA* and *amoA* sequences as well as to those of *pxmA* and non-methane hydrocarbon monooxygenase genes. Cluster MR1 is represented by two $OTUs_{12}$ in this study and has only been detected in some upland soils (**Table 7**). In contrast, RA21, which has been more frequently retrieved and consists of three $OTUs_{12}$, is predominantly found in rice field soils. Some further clusters have been defined in this region of the phylogenetic tree, such as the two marine clusters referred to as group X (Tavormina et al., 2010) and ATII-I Cluster 4 (Abdallah et al., 2014), or cluster M84-P22 (Horz et al., 2001). These clusters have until now only been detected very rarely, so that it is too early to draw further conclusions about possible habitat preferences.

# HABITAT SPECIFICITY OF METHANOTROPHIC TAXA EVALUATED AT HIGHER TAXONOMIC RESOLUTION

To evaluate habitat specificity for cultivated and uncultivated taxa of methanotrophic bacteria in more detail and at higher taxonomic resolution, 19 different habitat types were defined, which contained at least 30 sequence reads. The assignment of sequences to one of these more specific habitat types was in most cases unambiguous, but for the soil categories an overlap between habitats may exist. This applies for instance to soils collected in arctic-alpine environments, which include samples from glacier forefields as well as alpine meadows and grasslands. Some of these soils may also represent the category "upland soil" or "hydromorphic soil." Likewise, a polluted soil may at the same time be an "upland soil." Soils in the category "polluted soils" were collected from areas with hydrocarbon pollution, near coal mines or above oil and gas reservoirs. Four percent of the soil derived sequence reads could not be assigned to a specific soil habitat since no further information about the type of soil habitat was available. These sequences are presented as "soil diverse" in **Figure 5**, but were excluded from subsequent analyses as they formed a very heterogeneous group. Certain overlap may also exist between wetlands and bog ecosystems, as it cannot be fully excluded that the term wetland was in some cases used by authors for the description of samples from bog ecosystems.

Due to the fact that methanotrophic communities were analyzed at very great depth in some studies, numerous redundant reads are present in the database and a high number of sequence reads assigned to certain OTUs may be the result of just a few studies rather than frequent detection in diverse studies. To correct for this possible artifact, replicate sequence reads, i.e., those that represent the same study and the same OTU, were excluded during the further analysis. This resulted in 2079 non-redundant reads at $OTU_{12}$ cut-off and 4061 reads at $OTU_4$ cut-off level. In particular at 12% cut-off, this caused a more even distribution of sequence reads across the different habitat types (**Figure 5**). The recovery of specific sequence types in different habitats was thus evaluated based on their presence or absence in individual studies, while the information from approximately 370 studies was used to estimate the detection frequency of each OTU quantitatively. Non-metric multidimensional scaling plots were calculated to visualize (dis-)similarities between habitats (**Figure 7**). The major pattern was largely similar, regardless of the applied OTU resolution, demonstrating that major differences between samples are indeed already manifest at genus level. Methanotrophic communities in marine habitats are most distinct from those of all other habitats, as evident from their clear separation along the first axis of the plot. This was also seen when applying other multivariate approaches (principal component analysis, hierarchical cluster analysis) and is in agreement with the existence of the very specific marine clades deep-sea clusters 1 to 5. The second axis separates volcanic soils from all other samples, which can be explained by the unique presence of *Verrucomicrobia* in several of these soils (Sharp et al., 2014). The high dissimilarities of methanotrophic communities in marine ecosystems and volcanic soil samples compared to all other ecosystems were verified by an analysis of similarity (ANOSIM), which revealed very high values of $R = 0.985$ ($P = 0.001$) at $OTU_{12}$ level and of $R = 0.926$ ($P = 0.001$) at $OTU_4$ level. To better evaluate dissimilarities between the remaining aquatic and terrestrial ecosystems, the marine and volcanic soil sample data were excluded from NMDS plots (**Figure 7**). These reduced datasets reveal that aquatic habitats including the estuarine habitat are again distinct from the other habitats, supported by ANOSIM values of $R = 0.444$ ($P = 0.006$) for $OTU_{12}$ and $R = 0.460$ ($P = 0.004$) for $OTU_4$. This agrees with the existence of different aquatic clades (**Figure 4**). Methanotrophic communities in aquifers appear to be somewhat different from those in aquatic habitats (**Figure 7**). The terrestrial samples did not show highly consistent patterns in the NMDS plots (or in other multivariate approaches), besides the observation that those soils that are exposed to low methane concentrations, i.e., upland soils, arctic-alpine soils, and hydromorphic soils, are often located close to each other. This is in agreement with the unique occurrence of the upland soil clusters and some other clades in these soils (**Figure 4**). The limited resolution of differences between the different soil sample types may be related to the fact that these categories may partially overlap, as explained above.

## Habitat-specific OTUs

To identify common and habitat specific groups at $OTU_{12}$ and $OTU_4$ level, the relative detection frequency of OTUs across habitats was determined based on non-redundant read counts. OTUs that were detected in at least five studies were included in this evaluation. Otherwise, OTUs may appear erroneously as habitat-specific based on the fact that they have been detected in a limited number of studies. The detection frequency of OTUs across habitats is displayed as heat map and reveals that a rather low number of OTUs is highly habitat specific (**Figure 8**). The identified habitat specific and common OTUs are listed in **Tables 8, 9**. The number of specific OTUs increases at species level resolution. This is to some extent the

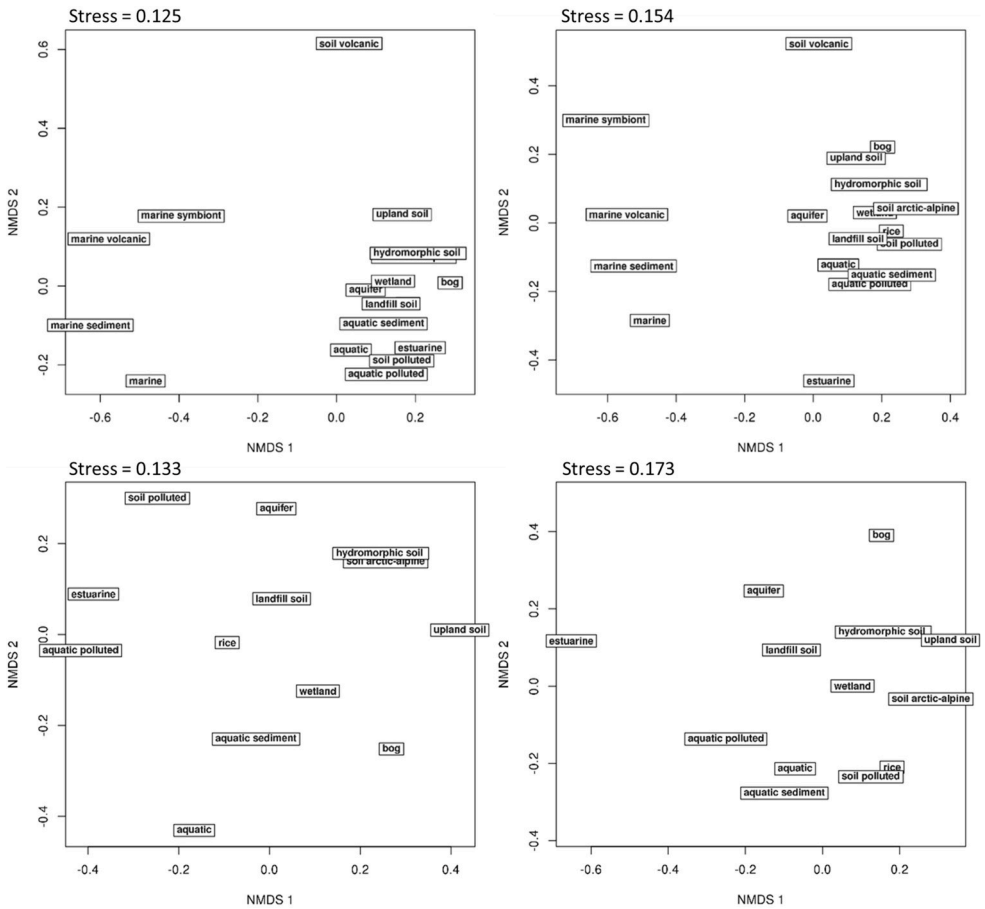


**FIGURE 7 | Habitat specificity of methanotrophic bacteria evaluated in non-metric multidimensional scaling (NMDS) plots.** Non-redundant sequence reads were used to calculate the relative detection frequency of all OTUs in a habitat. The upper plots show differences between all 18 different habitats, while the lower plots focus on the 13 most similar habitats. OTU clustering was done using 12% (left panels) and 4% dissimilarity cut-off (right panels). The NMDS plots were set up based on Bray-Curtis dissimilarities calculated from Hellinger transformed data using the online tool GUSTA ME (Buttigieg and Ramette, 2014).

result of splitting a habitat specific genus into several habitat specific species. Furthermore, it is based on the fact that some habitat specific species exist within genera that show a broad distribution, as observed for some *Methylocystis* species. The genus is commonly found in diverse environments, but some *Methylocystis* species show habitat specificity and appear to be characteristic for aquatic environments or landfill cover soils (**Tables 8, 9**). Likewise, the genus *Methylocaldum* has been detected in diverse habitats, while the species *Methylocaldum gracile* was found with very high frequency in landfill cover soils.

The marine habitats, which appeared most distinct in the NMDS plots, are not only characterized by the presence of very unique taxa that are mostly absent from all other ecosystems. Additionally, most taxa with broad distribution in diverse habitats are largely absent in marine ecosystems, in particular at species level resolution (**Figure 8**). The OTUs that are characteristic for marine habitats belong to the deep-sea clusters 1 to 5 (**Table 8**). Due to a high phylogenetic diversity within these clusters, most of the individual OTUs have so far only been detected in a few studies, so that many OTUs of these clades were excluded from this kind of analysis. This explains the unexpectedly low number of OTUs that are displayed for the marine samples at species level resolution in the heat map (**Figure 8**).

The different aquatic habitats have several OTUs at genus and species level in common (**Figure 8**). This includes the uncultivated clusters FWs, lake cluster 1 and LP20, which have already been described as habitat-specific before (Dumont et al., 2014), and most of the aquatic clusters that were defined in this article. Moreover, the genus *Methyloparacoccus murrellii* as well as specific $OTUs_4$ of the genera *Methylobacter, Methylomonas, Methylosoma,* and *Methylocystis* are characteristic for aquatic habitats (**Table 9**). Some OTUs are even more habitat specific and occur preferentially either in the water column or the sediment. Specific for the water column are the aquatic clusters 2b and 5a and lake cluster 1, while aquatic cluster 4a, the *Methyloglobulus* like cluster LP20 and some further OTUs related to M*ethylobacter psychrophilus, Methyloglobulus morosus, Methyloparacoccus murrellii* and *Methylosoma difficile*

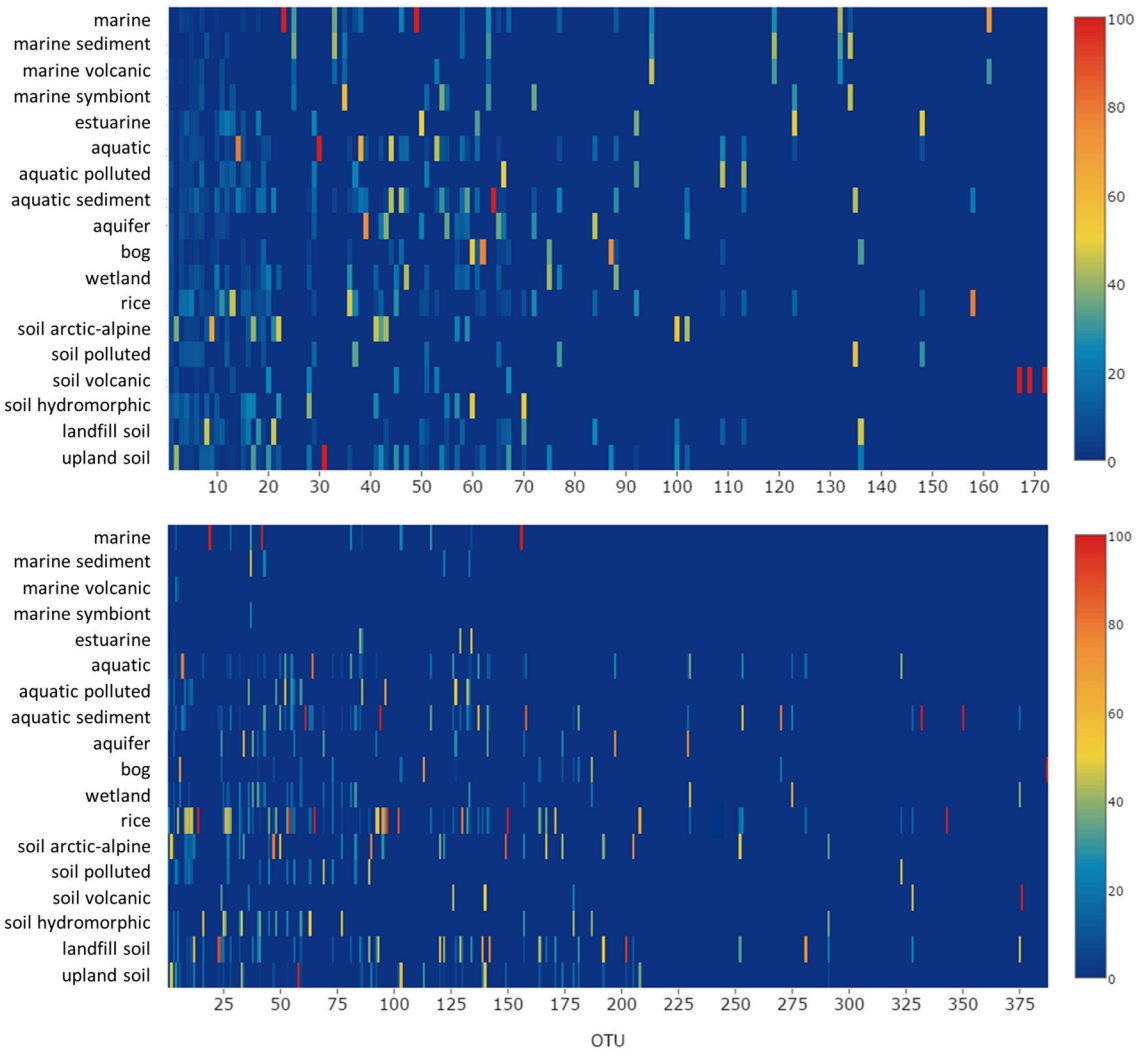


**FIGURE 8 | Relative detection frequency of OTUs across habitats.** Non-redundant reads were normalized by the number of studies available for each habitat and the relative frequency with which each OTU was detected across the different habitats was calculated. The upper panel shows the results at genus level resolution ($OTU_{12}$), the lower panel at species level resolution ($OTU_4$). OTUs displayed in red are highly specific for a certain type of habitat. OTUs that were detected in less then five studies were set to zero. The identity of the most habitat-specific and most common OTUs is given in **Tables 8**, **9**. A list including detailed information about all OTUs is provided as Supplementary Material.

are specific for the sediment (**Figure 8**, Tables S1–S4). In agreement with this preferential occurrence, the cultivated strains of these species were also obtained from aquatic habitats (**Table 2**).

The terrestrial habitats show a lower number of specific OTUs, in agreement with the weaker resolution in the NMDS plots. Rice associated habitats harbor no characteristic OTUs at genus level resolution, but some specific OTUs related to *Methyloparacoccus* and *Methylocystis* or the uncultivated lineages RPC1 and RPC2 at higher taxonomic resolution (**Table 9**). Characteristic in landfill cover soils are strains of *Methylocaldum gracile* and of an unclassified *Methylocystis* species, but no specific clusters of uncultivated methanotrophs were detected. As expected, different lineages of USCα and USCγ are specific for upland soils, while the genus *Methylocapsa* and a specific uncultivated *Methylocystis* species are typical inhabitants of bog ecosystems (**Tables 8**, **9**).

## Broadly Distributed Methanotrophic Taxa

Several $OTUs_{12}$ occur in diverse habitats. These include a number of cultivated genera, in particularly those that have been discovered and described quite early and that have been obtained as isolates frequently (**Tables 4**, **8**). Furthermore, some lineages of uncultivated methanotrophs are broadly distributed such as OTUs of the clusters FWs, RCL, RA21, or RPC1_3. At species level resolution, the number of common OTUs is lower (**Table 9**). This can be explained by habitat specialization with increasing taxonomic resolution, as observed for cultivated and uncultivated members of the genera *Methylocystis*, *Methylocaldum,* or *Methylobacter* (**Tables 8**, **9**).

**TABLE 8 | Broadly distributed and habitat-specific $OTUs_{12}$.**

| OTU | Name of cluster | Marine | Aquatic | Upland soils | Rice | Wetland | Bog | Soil, landfill | Number of non-redundant sequences |
|---|---|---|---|---|---|---|---|---|---|
| **COMMON, CULTIVATED** | | | | | | | | | |
| 1 | *Methylocystis* | 2 | 23 | 23 | 20 | 5 | 16 | 9 | 175 |
| 3 | *Methylomonas* | 6 | 29 | 11 | 31 | 4 | 8 | 11 | 81 |
| 4 | *Methylosarcina, Methylomicrobium album/agile* | 4 | 25 | 20 | 36 | 2 | 0 | 14 | 62 |
| 5 | *Methyloparacoccus* | 6 | 30 | 14 | 39 | 5 | 2 | 5 | 68 |
| 6 | *Methylobacter psychrophilus* | 4 | 51 | 16 | 2 | 8 | 6 | 12 | 51 |
| 7 | *Methylococcus* | 14 | 29 | 33 | 14 | 5 | 0 | 5 | 28 |
| 8 | *Methylocaldum* | 4 | 8 | 35 | 8 | 0 | 0 | 46 | 33 |
| 10 | *Methylosoma, Methylobacter tundripaludum* | 2 | 40 | 10 | 20 | 12 | 7 | 10 | 62 |
| 15 | *Methylosinus* | 2 | 36 | 21 | 19 | 2 | 7 | 12 | 49 |
| 18 | *Methylobacter luteus/whittenburyi/marinus* | 3 | 34 | 14 | 14 | 3 | 3 | 29 | 40 |
| 65 | *Methylovulum* | 8 | 38 | 23 | 8 | 8 | 8 | 8 | 14 |
| 66 | *Methylocystis, pmoA2* | 0 | 47 | 11 | 16 | 0 | 11 | 16 | 19 |
| 67 | *Methylomicrobium pelagicum* | 10 | 0 | 60 | 10 | 0 | 10 | 10 | 11 |
| **COMMON, UNCULTIVATED** | | | | | | | | | |
| 12 | FWs 1a | 4 | 50 | 14 | 21 | 7 | 0 | 4 | 29 |
| 16 | RCL a | 0 | 14 | 24 | 29 | 5 | 0 | 29 | 22 |
| 19 | RPC1_3 like 1, LWs | 0 | 44 | 12 | 12 | 8 | 20 | 4 | 25 |
| 21 | RA21 | 0 | 10 | 60 | 20 | 10 | 0 | 0 | 10 |
| 36 | *Methylocystaceae* 11 | 0 | 12 | 12 | 59 | 12 | 6 | 0 | 17 |
| 51 | RPC1_3 like 8, JRC 3 | 10 | 20 | 30 | 20 | 10 | 0 | 10 | 10 |
| 61 | RPC1_3 like 2, LWs | 0 | 58 | 8 | 8 | 8 | 17 | 0 | 12 |
| **SPECIFIC, CULTIVATED** | | | | | | | | | |
| 134 | *Methyloprofundus* | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 5 |
| 167 | *"Methylacidiphilum fumarolicum/kamchatkense," pmoA1* | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 5 |
| 169 | *"Methylacidiphilum fumarolicum/kamchatkense," pmoA2* | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 5 |
| 172 | *"Methylacidiphilum fumarolicum/kamchatkense," pmoA3* | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 5 |
| 158 | *Methylosinus trichosporium, pmoA2* | 0 | 20 | 0 | 80 | 0 | 0 | 0 | 5 |
| 60 | *Methylocapsa acidiphila* | 0 | 0 | 17 | 0 | 0 | 83 | 0 | 6 |
| **SPECIFIC, UNCULTIVATED** | | | | | | | | | |
| 23 | Deep-sea cluster 3p, OPU3 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 5 |
| 25 | Deep-sea cluster 2r | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 8 |
| 35 | Deep-sea cluster 1d | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 12 |
| 49 | Deep-sea cluster 5w, OPU1 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 7 |
| 63 | Deep-sea cluster 2t | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 9 |
| 95 | Deep-sea cluster 5h | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 5 |
| 119 | Deep-sea cluster 2q | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 7 |
| 132 | Deep-sea cluster 2g | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 5 |
| 161 | Deep-sea cluster 5d | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 5 |
| 33 | Deep-sea cluster 3q | 88 | 13 | 0 | 0 | 0 | 0 | 0 | 8 |
| 14 | Lake cluster 1a | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 9 |
| 30 | Aquatic cluster 5a | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 5 |
| 39 | *Methyloglobulus* like 13, LP20 | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 6 |
| 64 | Aquatic cluster 4a | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 8 |
| 44 | *Methylococcaceae* 12d | 0 | 92 | 0 | 0 | 0 | 8 | 0 | 13 |
| 38 | Aquatic cluster 2b | 11 | 89 | 0 | 0 | 0 | 0 | 0 | 9 |

*(Continued)*

**TABLE 8 | Continued**

| OTU | Name of cluster | Marine | Aquatic | Upland soils | Rice | Wetland | Bog | Soil, landfill | Number of non-redundant sequences |
|---|---|---|---|---|---|---|---|---|---|
| 2 | USCα 4, RA14 | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 26 |
| 31 | JR3a | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 7 |
| 17 | USCα 16, JR1, Cluster 5 | 0 | 0 | 93 | 7 | 0 | 0 | 0 | 14 |
| 22 | USCα 8, MHP | 0 | 0 | 86 | 0 | 14 | 0 | 0 | 7 |
| 9 | USCγ 1 | 0 | 0 | 81 | 6 | 0 | 6 | 6 | 16 |
| 100 | USCγ 2 | 0 | 0 | 80 | 0 | 0 | 0 | 20 | 5 |
| 28 | Cluster 2a, TUSC | 0 | 7 | 79 | 0 | 7 | 7 | 0 | 15 |
| 41 | Cluster 1l, *Crenothrix* related | 0 | 0 | 75 | 13 | 13 | 0 | 0 | 8 |

*OTUs are defined as habitat-specific if at least 75% of the non-redundant reads were detected in one habitat. Common OTUs were detected in at least five different habitats. The group of upland soils includes hydromorphic soils, arctic-alpine soils, volcanic soils and polluted soils. Cultivated OTUs contain at least one sequence of a cultivated strain, but not necessarily a type strain. Color coding reflects relative detection frequency across habitats.*

## UNDERSTANDING THE INFLUENCE OF ENVIRONMENTAL FACTORS ON METHANOTROPHIC COMMUNITY COMPOSITION AND ACTIVITY

It is obvious that the occurrence and activity of methanotrophic bacteria in different ecosystems is largely influenced by abiotic and biotic environmental conditions. Important factors are methane and oxygen concentrations, nutrient availability, pH, temperature, salinity and water availability (Semrau et al., 2010). Additional factors will influence these bacteria indirectly such as soil moisture content, which affects gas diffusion and thus methane and oxygen supply, or plant cover, which alters the water and nutrient status in soil. Among these factors, methane concentration, nitrogen status and the role of copper have been studied in most detail and were identified as very important for shaping methanotrophic communities and for influencing their activity (Conrad, 2007; Semrau et al., 2010; Ho et al., 2013). Future research needs to address the question how the different factors act alone and in combination on the members of methanotrophic communities in different ecosystems. The present study evaluated only the presence or absence of methanotrophic bacteria in the different ecosystems, but this does not implement information about metabolic activity. In particular type IIa methanotrophs are capable of forming resting stages, which enable prolonged survival under unfavorable conditions (Whittenbury et al., 1970a). To link ecosystem function with community composition, activity in dependence on environmental parameters needs to be analyzed in more detail in future studies.

The present review provides a comprehensive overview about habitat preferences of methanotrophic taxa, considering the complete diversity as represented by *pmoA* as marker and including all major ecosystems in which these bacteria occur. However, habitat preferences do also exist within these ecosystems. The preferential occurrence of USCα in acidic and USCγ in pH neutral upland soils or the plant genotype specific colonization of rice by uncultivated groups of methanotrophs are just two examples (Knief et al., 2003; Lüke et al., 2011). In the latter case, differences can be seen as shifts in the methanotrophic community composition, but not based on pure presence absence data. Likewise, shifts have been observed in aquatic ecosystems, where methanotrophic communities differ in dependence on depth or type of sediment (Pester et al., 2004; Rahalkar and Schink, 2007; Biderre-Petit et al., 2011; Deutzmann et al., 2011). In contrast, almost nothing is known about niche differentiation and habitat preferences among all those OTUs that represent uncultivated genera and species of the marine deep-sea clusters. These methanotrophs appear to coexist in marine habitats, or differentiation occurs at a finer scale. In-depth studies within the different ecosystems are needed to obtain further knowledge about habitat preferences of the individual clusters of methanotrophic bacteria. Such studies need to implement meta-data describing the physicochemical and biological characteristics of the habitat or have to be done under controlled conditions whereby specific parameters are manipulated.

There is a clear need to study the impact of environmental factors at different taxonomic resolution in order to gain comprehensive understanding about mechanisms that lead to niche differentiation among methanotrophs. In initial studies, a simple differentiation between type I and type II methanotrophs was made (e.g., Graham et al., 1993; Amaral et al., 1995; Henckel et al., 2000b), which is certainly appropriate due to some major differences that exist between these groups, e.g., in terms of physiology. Hence, these studies provided valuable insight concerning the differential responses of the studied methanotrophs to high and low methane, oxygen and nitrogen concentrations (Conrad, 2007; Ho et al., 2013). However, the compilation of ecophysiological characteristics from type strains in this study has shown that responses of methanotrophic bacteria to specific environmental factors are often not closely

**TABLE 9 | Broadly distributed and habitat-specific OTUs$_4$.**

| OTU | Name of cluster | Marine | Aquatic | Soil, weak methane supply | Rice | Wetland | Bog | Soil, landfill | Number of non-redundant sequences |
|---|---|---|---|---|---|---|---|---|---|
| **COMMON, CULTIVATED** | | | | | | | | | |
| 1 | *Methylocystis echinoides* | 2 | 24 | 16 | 47 | 4 | 2 | 4 | 47 |
| 3 | *Methylocystis rosea, hirsuta* | 0 | 29 | 26 | 19 | 6 | 5 | 15 | 93 |
| 10 | *Methylocystis* sp. | 0 | 11 | 14 | 68 | 4 | 0 | 4 | 30 |
| 28 | *Methylosarcina lacus* | 9 | 27 | 9 | 45 | 0 | 0 | 9 | 12 |
| 40 | *Methylobacter tundripaludum* | 0 | 20 | 10 | 0 | 30 | 10 | 30 | 10 |
| 127 | *Methylocystis sp., pmoA2* | 0 | 50 | 17 | 8 | 0 | 8 | 17 | 12 |
| **COMMON, UNCULTIVATED** | | | | | | | | | |
| 25 | *Methylosarcina* | 0 | 0 | 33 | 33 | 8 | 0 | 25 | 12 |
| 32 | *Methylosoma* | 0 | 17 | 17 | 42 | 17 | 0 | 8 | 13 |
| 41 | RCL a | 0 | 8 | 33 | 8 | 8 | 0 | 42 | 12 |
| 59 | *Methylocystis* | 0 | 14 | 29 | 0 | 14 | 29 | 14 | 7 |
| 73 | *Methylocystis* | 0 | 10 | 10 | 30 | 10 | 30 | 10 | 10 |
| **SPECIFIC, CULTIVATED** | | | | | | | | | |
| 94 | *Methylobacter* sp. | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 8 |
| 137 | *Methylocystis* sp. | 0 | 86 | 0 | 14 | 0 | 0 | 0 | 7 |
| 43 | *Methyloparacoccus murrellii* | 11 | 78 | 0 | 0 | 11 | 0 | 0 | 9 |
| 376 | *"Methylacidiphilum fumarolicum, kamchatkense," pmoA2* | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 5 |
| 149 | *Methylocystis parvus* | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 7 |
| 202 | *Methylocystis* sp. | 0 | 0 | 14 | 0 | 0 | 0 | 86 | 8 |
| 23 | *Methylocaldum gracile* | 0 | 8 | 8 | 0 | 0 | 0 | 83 | 13 |
| **SPECIFIC, UNCULTIVATED** | | | | | | | | | |
| 37 | Deep-sea cluster 2r | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 6 |
| 19 | Deep-sea cluster 3p, OPU3 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 5 |
| 156 | Deep-sea cluster 3p, OPU3 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 5 |
| 42 | Deep-sea cluster 5w, OPU1 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 6 |
| 64 | Aquatic cluster 2b | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 5 |
| 61 | Aquatic cluster 4a | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 8 |
| 7 | Lake cluster 1a | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 9 |
| 350 | *Methylobacter psychrophilus* | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 5 |
| 229 | *Methyloglobulus* like 13, LP20 | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 6 |
| 197 | *Methylomonas* | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 5 |
| 52 | *Methyloparacoccus* | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 5 |
| 158 | *Methyloparacoccus* | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 7 |
| 332 | *Methylosoma* | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 6 |
| 50 | FWs 1a | 0 | 80 | 20 | 0 | 0 | 0 | 0 | 5 |
| 270 | *Methylocystis* | 0 | 80 | 0 | 0 | 0 | 20 | 0 | 5 |
| 2 | USCα 4, RA14 | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 20 |
| 58 | USCα 4, RA14 | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 7 |
| 140 | USCα 4, RA14 | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 5 |
| 90 | USCγ 1 | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 6 |
| 33 | USCα 16, JR1, Cluster 5 | 0 | 0 | 88 | 13 | 0 | 0 | 0 | 8 |
| 205 | USCγ 1 | 0 | 0 | 80 | 0 | 0 | 0 | 20 | 5 |
| 242 | *Methylococcaceae* | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 5 |
| 54 | *Methylocystaceae* 11 | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 6 |

*(Continued)*

**TABLE 9 | Continued**

| OTU | Name of cluster | Marine | Aquatic | Soil, weak methane supply | Rice | Wetland | Bog | Soil, landfill | Number of non-redundant sequences |
|---|---|---|---|---|---|---|---|---|---|
| 343 | *Methylocystis* | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 5 |
| 14 | *Methylomonas* | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 8 |
| 150 | *Methyloparacoccus* | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 5 |
| 65 | RPC 2a | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 10 |
| 97 | RPC1_3 like 10, RPC1 | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 7 |
| 95 | RPC 2a | 0 | 0 | 7 | 93 | 0 | 0 | 0 | 14 |
| 26 | *Methyloparacoccus* | 0 | 0 | 9 | 91 | 0 | 0 | 0 | 11 |
| 102 | *Methylocystis* | 0 | 0 | 14 | 86 | 0 | 0 | 0 | 7 |
| 9 | *Methylosarcina* | 0 | 0 | 14 | 82 | 0 | 0 | 5 | 23 |
| 130 | *Methyloparacoccus* | 0 | 0 | 9 | 82 | 0 | 0 | 9 | 12 |
| 48 | *Methylosarcina* | 0 | 11 | 11 | 78 | 0 | 0 | 0 | 9 |
| 387 | *Methylocystis* | 0 | 0 | 0 | 0 | 0 | 100 | 0 | 5 |

*OTUs are defined as habitat-specific if at least 75% of the non-redundant reads were detected in one habitat. Common OTUs were detected in at least five different habitats. The group of upland soils includes hydromorphic soils, arctic-alpine soils, volcanic soils, and polluted soils. Cultivated OTUs contain at least one sequence of a cultivated strain, but this is not necessarily a type strain. Color coding reflects relative detection frequency across habitats.*

linked to phylogeny, a finding that was recently also reported by Krause et al. (2014), so that other approaches may be necessary to categorize methanotrophs. A concept that has several times been applied considers type I methanotrophs as r-strategists and type II methanotrophs as k-strategists (Steenbergh et al., 2010; Siljanen et al., 2011). A recent proposition is based on a classification of methanotrophic bacteria into more specific ecological response groups based on specific functional traits: methanotrophic genera were classified based on their life strategies as competitors, stress tolerators or ruderals (Bodelier et al., 2012; Ho et al., 2013). The data compiled in this review clearly support the assumption that methanotrophic bacteria have developed different life strategies. Several groups of methanotrophs, among them many uncultivated lineages, appear to be specifically adapted to a certain habitat type and may thus represent good competitors in this specific environment. Some others have been found more widespread in different habitat types and may thus represent stress tolerators and/or ruderals.

## IMPORTANCE TO OBTAIN FURTHER ISOLATES OF METHANOTROPHIC BACTERIA

The evaluation of the representativeness of cultured model strains has revealed that they cover already a substantial fraction of the frequently detected methanotrophs in environmental samples. Several of them are common colonizers in diverse habitats. This encompasses in particular those taxa that are easily recovered in enrichment studies, while other isolated species and genera have not (yet) been frequently detected in nature. The fact that major clusters of uncultivated methanotrophs are detected in diverse ecosystems clearly shows the need for further isolation efforts to get hands on these organisms. This applies in particular to the frequently detected methanotrophs belonging to the diverse rice paddy clusters, the marine deep-sea clusters, the upland soil clusters or the different aquatic clusters. It is likely that these organisms are well adapted to their respective habitats, so that specific enrichment strategies may have to be applied, which better mimic the natural conditions of these methanotrophs to stimulate their growth. Several attempts were already made to enrich USCα methanotrophs, but until now, these resulted in the retrieval of well-known methanotrophic genera such as *Methylocystis* and *Methylosinus* rather than an enrichment of bacteria harboring USCα gene sequences (Dunfield et al., 1999; Knief and Dunfield, 2005; Kravchenko et al., 2010).

Only the combination of community analyses in natural environments, under controlled conditions in microcosms or mesocosms and of pure cultures or enrichment cultures will allow to understand the physiological and regulatory mechanisms at cellular level that ultimately control activity and affect dispersal of methanotrophs in nature. The fact that many gene functions and regulatory mechanisms in methanotrophic bacteria are until now only little understood, e.g., the role of *pxmA*, limits also the gain of knowledge from cultivation-independent studies when global analysis approaches such as metagenomics, -transcriptomics or -proteomics are applied. This underlines the need for studying pure cultures under laboratory conditions.

The analysis of dispersal patterns at high taxonomic resolution needs a sufficiently large data basis. Conclusions about habitat preferences can only be drawn for frequently detected genera and species, but not so easily for those methanotrophic genera that are currently represented by a single strain or a very small

number of sequences. Their less frequent recovery in cultivation-dependent and -independent approaches might point toward higher specialization. In order to draw further conclusions about habitat preferences for these smaller groups, the detection of similar sequences in cultivation-independent studies and/or the isolation of further representatives are necessary. The application of next generation sequencing techniques will facilitate the detection of such rare methanotrophs due to the higher sequencing depth that can be reached. However, currently the integration of NGS data from studies into existing sequence databases is time consuming, as tools for data mining are still largely lacking. At the moment, this limitation can most conveniently be overcome if authors deposit representative *pmoA* sequences in the NCBI nucleotide database or provide them as fasta files. NGS sequencing technology is more and more frequently applied to characterize methanotrophic communities and will lead to an enormous amount of data in the next years. If these data are supplemented with detailed information about the sampling sites and the experimental conditions, it may become a very valuable data resource, enabling more detailed meta-analyses, focusing on specific ecosystems, environmental factors, or taxonomic groups.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01346

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# Activity and abundance of methane-oxidizing bacteria in secondary forest and manioc plantations of Amazonian Dark Earth and their adjacent soils

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The oxidation of atmospheric $CH_4$ in upland soils is mostly mediated by uncultivated groups of microorganisms that have been identified solely by molecular markers, such as the sequence of the *pmoA* gene encoding the β-subunit of the particulate methane monooxygenase enzyme. The objective of this work was to compare the activity and diversity of methanotrophs in Amazonian Dark Earth soil (ADE, Hortic Anthrosol) and their adjacent non-anthropic soil. Secondly, the effect of land use in the form of manioc cultivation was examined by comparing secondary forest and plantation soils. $CH_4$ oxidation potentials were measured and the structure of the methanotroph communities assessed by quantitative PCR (qPCR) and amplicon pyrosequencing of *pmoA* genes. The oxidation potentials at low $CH_4$ concentrations (10 ppm of volume) were relatively high in all the secondary forest sites of both ADE and adjacent soils. $CH_4$ oxidation by the ADE soil only recently converted to a manioc plantation was also relatively high. In contrast, both the adjacent soils used for manioc cultivation and the ADE soil with a long history of agriculture displayed lower $CH_4$ uptake rates. Amplicon pyrosequencing of *pmoA* genes indicated that USCα, *Methylocystis* and the tropical upland soil cluster (TUSC) were the dominant groups depending on the site. By qPCR analysis it was found that USCα *pmoA* genes, which are believed to belong to atmospheric $CH_4$ oxidizers, were more abundant in ADE than adjacent soil. USCα *pmoA* genes were abundant in both forested and cultivated ADE soil, but were below the qPCR detection limit in manioc plantations of adjacent soil. The results indicate that ADE soils can harbor high abundances of atmospheric $CH_4$ oxidizers and are potential $CH_4$ sinks, but as in other upland soils this activity can be inhibited by the conversion of forest to agricultural plantations.

**Keywords: methane oxidation, Amazonian Dark Earth, terra preta de índio, methanotroph, *pmoA*, USC-α, *Methylocystis***

## INTRODUCTION

Most soils in the Amazon region have low fertility. Typically, Amazonian soils are acidic, have low P contents, low cation exchange capacity and high levels of Al at levels that can be toxic to crops (Cochrane and Sanchez, 1982). In contrast, Amazonian Dark Earth (ADE) soils, also known as terra preta de índio, are fertile soil patches found dispersed throughout the Amazon that were formed by the ancient Amazonian indigenous populations. It is believed that these soils were unintentionally or intentionally formed by long-term habitation with casual addition of domestic refuse and by long-lasting agricultural activity based on the clearing of vegetation and the incomplete combustion of organic material (Smith, 1980; Denevan, 1998; Glaser, 1999). Unlike their adjacent soils, ADE have high contents of P, Ca, Mg, Zn, Mn, and stable organic matter (Costa and Kern, 1999; Woods and McCann, 1999).

Differences in bacterial community structure and composition have been observed under different land use systems in Amazonian soils (Jesus et al., 2009; Navarrete et al., 2010; Taketani et al., 2013), which will in turn influence ecosystem processes such as the decomposition of organic matter and nutrient mineralization or its immobilization (Neher, 1999). In addition, the bacterial communities in ADE soils were shown by 16S rRNA tag sequence analysis to be distinct from their adjacent soils, particularly when compared at taxonomic levels lower than phylum (Taketani et al., 2013). One of the possible influences on the microbial communities of ADE soils is the presence of large amounts of biochar, which have prompted research into the effect of biochar application on microbial community structure and composition (Anderson et al., 2011; Khodadad et al., 2011). Replicating the high carbon and biochar contents of ADE in other soils has been suggested as a mechanism of $CO_2$ sequestration (Sombroek et al., 2003; Lehmann, 2007); however, the presence of these relatively large amounts of carbon in ADE then raises concern whether changes in climate and land use may result in increased emissions of $CO_2$ and $CH_4$. One possible mechanism of increased $CH_4$ emissions would be the decomposition of labile components of biochar to form substrates for methanogens (Knoblauch et al.,

2008). To our knowledge, $CH_4$ cycling in ADE soil has not been investigated and one important question is whether methane-oxidizing bacteria (methanotrophs) are present and active in ADE soils. If present, methanotrophs could consume atmospheric $CH_4$ or potentially mitigate the release to the atmosphere of $CH_4$ produced endogenously in the soil.

Upland soils, defined as those that are typically well-drained and oxic, have an important role in the global $CH_4$ cycle by acting as a sink for atmospheric $CH_4$ (King, 1992), which globally is estimated at more than 30 Tg $y^{-1}$ (Denman et al., 2007). Although this activity is found in a wide variety of upland soils, pristine forest soils have been identified as the most efficient biological sinks of atmospheric $CH_4$ (Dunfield, 2007; Dalal and Allen, 2008). Numerous studies have shown that the conversion of pristine land to agriculture lowers the oxidation capacity of the soil (Keller et al., 1990; Mosier et al., 1991; Hütsch et al., 1994; Jensen and Olsen, 1998; Priemé and Christensen, 1999; Knief et al., 2005; Levine et al., 2011). Various factors associated with agriculture have been shown to inhibit atmospheric $CH_4$ oxidation, including soil compaction, acidification and fertilization (Dunfield, 2007). Conversely, the abandonment of agriculture can also lead to at least partial recovery of methanotroph populations and atmospheric $CH_4$ uptake (Levine et al., 2011). ADE soils are commonly found on well-drained areas of the Amazon region (*terra firme*), and may also be sinks for atmospheric $CH_4$.

Methanotroph diversity and activity has been assessed in different upland soils exhibiting atmospheric $CH_4$ oxidation (Dunfield, 2007; Kolb, 2009). The diversity of atmospheric $CH_4$ oxidizers is typically assessed by the detection of the *pmoA* gene, which encodes the β-subunit of methane monooxygenase (pMMO) enzyme (McDonald et al., 2008; Semrau et al., 2010). For the most part, as yet uncultivated microorganisms mediate atmospheric $CH_4$ oxidation and are characterized by their *pmoA* gene sequences alone. In addition, phospholipid fatty acids have been used to identify atmospheric $CH_4$ oxidizers (Bodelier et al., 2009). The USCα *pmoA* clade is widely distributed in upland soils (Knief et al., 2003) and based on gene analyses are believed to belong to Alphaproteobacteria most closely related to *Methylocapsa* (Ricke et al., 2005). The USCγ *pmoA* clade is another associated with upland soils exhibiting atmospheric $CH_4$ uptake, and appear to favor neutral or somewhat alkaline soils (Knief et al., 2003). Another clade termed JR3, initially identified in grassland soil (Horz et al., 2005) was found to dominate in desert soils with atmospheric $CH_4$ oxidation capacity (Angel and Conrad, 2009). *Methylocystis*-related species have been shown to use $CH_4$ at relatively low concentrations (Knief and Dunfield, 2005; Knief et al., 2006; Baani and Liesack, 2008), but whether they are important consumers of atmospheric $CH_4$ in upland soils is not clear.

To our knowledge, no studies have previously examined $CH_4$ oxidation or the diversity of methanotrophs in ADE soils. The primary objective of this study was to determine the extent to which ADE soils are a potential sink for atmospheric $CH_4$ and secondly to determine how the methanotroph community structure and their $CH_4$ uptake potential compares between forested and agricultural sites.

## MATERIALS AND METHODS

### STUDY AREA, SOIL SAMPLING, AND SOIL ANALYSIS

Soil samples were collected from two different areas, Caldeirão and Barro Branco. The Caldeirão experimental research station from Embrapa Amazônia Ocidental is located in Iranduba County in the Brazilian Central Amazon (03°26′00″ S, 60°23′00″ W). The other sampling area near the Barro Branco community is located in the Manacapuru County in the Brazilian Central Amazon (03°18′12″ S, 60°31′45″ W). ADE soils and their adjacent soils were collected from both areas. In both cases, the distance between the ADE soil zone and the adjacent soil zone was ∼2 km.

The soils were classified based on the World Reference Base for Soil Resources (FAO, 1998). ADE soils were classified as Hortic Anthrosol (i.e., reference horizon that results from prolonged habitation with casual additions of domestic organic refuse and cultural material). The adjacent soil from Caldeirão was classified as Haplic Acrisol (i.e., clay-rich soils with low fertility and toxic amounts of Al). The adjacent soil from Barro Branco was classified as Oxisol (i.e., red or yellowish soils with <10% weatherable minerals and low cation exchange capacity). At both areas, ADE soil and adjacent soil were sampled from secondary forest sites and agricultural sites cultivated with manioc (*Manihot esculenta*). The forested ADE and adjacent soil sites at Caldeirão were under ∼40-year-old secondary forest stands. At Barro Branco, the secondary forests were about 20 years-old. The agricultural sites in ADE and adjacent soils at Caldeirão had been used for manioc cultivation for at least 40 years, whereas the sites at Barro Branco had been deforested 5-years previously for conversion to plantations.

Soil samples were collected in February 2013. Three environmental replicates were collected from each sampling site. The sample plot (location) at each site was determined by choosing a random point, and from this reference point three sampling points (sublocations) 5 m apart were chosen for the collection of intact soil cores of 5 cm in diameter and 15 cm in length. Soil samples were collected in triplicate from each sublocation, which were subsequently homogenized to produce a composite soil sample for each sublocation. A total of 24 samples corresponding to the four sites (forested ADE, cultivated ADE, forested adjacent, and cultivated adjacent) from each of the two areas (Caldeirão and Barro Branco) were prepared. The samples for DNA extraction were transported from the field to the laboratory in an insulated box with dry ice. Approximately 1 kg of soil samples were collected from each of the 24 sublocations and sent to the department of Soil and Plant Nutrition of Embrapa Western Amazon. The frozen and unsieved soil samples were used for DNA extraction, whereas the 1 kg samples of fresh soil were sieved (2 mm mesh diameter) and used for the determination of soil chemical properties and $CH_4$ oxidation potentials. Soil pH ($H_2O$, 1:1), soil extractable Al, Ca, Fe, K, Mg, Mn, P, Zn, soil organic carbon (SOC), total C, total N, and cation exchange capacity were determined according to the methods described by Embrapa (1997).

### $CH_4$ OXIDATION

Potential $CH_4$ oxidation rates were measured using soil from each sampling point (sublocation). Ten grams of fresh sieved soil was

placed into a 120 ml serum vial in duplicate (Bull et al., 2000; Horz et al., 2002; Shrestha et al., 2012). The bottles were sealed with butyl rubber stoppers, and final mixing ratios of 10, 100, 1000, and 10 000 ppmv of $CH_4$ was injected into the gas headspace of the vials. The incubation of soil microcosms was performed at 25°C in the dark with shaking at 150 rpm for up to 19 days. $CH_4$ concentrations were measured on a daily basis by gas chromatography with a flame ionization detector using 0.5 ml gas samples from the bottle headspaces, as described previously (Shrestha et al., 2012). $CH_4$ oxidation rates were calculated by linear regression of $CH_4$ consumption versus time for the incubations with 10 ppm $CH_4$.

#### DNA EXTRACTION FROM SOIL SAMPLES

Soil DNA extractions were carried out in triplicate from 0.3 *g* wet weight subsamples of each soil sample. Extractions were performed using the Nucleospin soil DNA extraction kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. DNA was quantified using a Qubit dsDNA HS Assay (Molecular Probes, Invitrogen, USA). The triplicate DNA extracts of each sampling sublocation were pooled.

#### REAL-TIME QUANTITATIVE PCR ASSAYS

Real-time quantitative PCR (qPCR) with three technical replicates for each sublocation DNA sample was performed to determine the copy numbers of the *pmoA* genes. The qPCR assay using the primer set A189f-mb661r was used to target the conventional *pmoA* genes of *Methylocystaceae* and *Methylococcaceae* methanotrophs (Costello and Lidstrom, 1999; Kolb et al., 2003). The assay using primers A189f-Forest675r was used to target USCα *pmoA* genes (Kolb et al., 2003). The qPCRs were performed with the SYBR Green JumpStart Taq ReadyMix System (Sigma, Taufkirchen, Germany) on an iCycler instrument (Bio-Rad, Munich, Germany). The data were analyzed using Bio-Rad CFX Manager (version 3.0) software. PCR mixtures and thermal cycling conditions were performed as described previously by Kolb et al. (2003). Briefly, the A189f-Forest675r assay was performed in 25 μl reaction mixtures containing 12.5 μl of SYBR Green Jump-Start Taq Ready Mix (Sigma), 1 μM of each primer, 50 ng of BSA (Roche, Mannheim, Germany), and 4 mM $MgCl_2$ (Sigma). The assay for the abundance of conventional *pmoA* genes (A189f-mb661r) was performed in 25-μl reaction mixtures containing 12.5 μl of SYBR Green Jump-Start Taq Ready Mix (Sigma), 0.667 μM of each primer and 4 mM $MgCl_2$. Standards for qPCR were generated by serial dilution of stocks of a known number of plasmids containing a single cloned copy of a *Methylococcus pmoA* gene or a USCα *pmoA* gene, according to the assay. All samples from an experiment were run on a single plate.

#### HIGH-THROUGHPUT SEQUENCING AND ANALYSIS

PCR was performed using the primers A189f and A682r that amplify a broad range of *pmoA*, *amoA*, and related sequences (Holmes et al., 1995; Lüke and Frenzel, 2011). The PCR components and conditions were identical to that described previously (Angel and Conrad, 2009). Briefly, the 50 μl reaction contained 5 μl of 10x AccuPrime™ PCR Buffer II (Invitrogen, Karlsruhe, Germany), additional 1.5 mM $MgCl_2$ (to a final concentration of 3 mM), 0.5 mM of each primer (Sigma), 50 ng of BSA (Roche) and 1 μl of Taq DNA polymerase (Invitrogen). All ADE samples could be amplified directly with the barcoded primer sets; however, it was not possible to obtain amplicons of the expected size for the adjacent soil samples using these primers. Therefore, a 2-step PCR procedure in which conventional primers (i.e., without barcodes) was used in the first step followed by a successive low-cycle-number amplification using the barcoded primers, as described by Berry et al. (2011). This approach successfully produced PCR amplicons of the expected size. To allow comparisons, the same 2-step PCR approach was used for all samples. Five replicate PCR reactions were performed for each sample. After amplification, PCR reactions were pooled and loaded on 1% agarose gel stained in GelRed™ (Biotium Inc., Hayward, CA, USA). The DNA fragment of the correct size was excised from the agarose gel and eluted in 30 μl $H_2O$ using the QIAquick gel extraction kit (Qiagen, Hilden, Germany). The purified PCR products from all samples were mixed in a 1:1 ratio and sequenced at the Max Planck-Genome-Centre Cologne (Cologne, Germany) using a Roche 454 Genome Sequencer FLX System.

A detailed description of the procedures used for sequence analysis was described previously (Dumont et al., 2014). In this study, only sequences with read lengths longer than 300 bp were used for further analysis. The sorting of sequences according to barcodes, trimming and quality filtering were processed using mothur version 1.29.2 (Schloss et al., 2009). Chimeric sequences were identified and removed using uchime (Edgar et al., 2011) implemented in mothur. Classification of *pmoA* sequences was performed using standalone TBLASTN version 2.2.26+ against a curated database of *pmoA* sequences and the lowest common ancestor (LCA) algorithm in MEGAN version 4.70.4 (Huson et al., 2011), as described previously (Dumont et al., 2014). A total of 110,437 sequences were obtained. 42,213 reads (a range from 9022 to 2977 reads per library) remained after basic quality filtering. The amplification of non-target sequences is common with these primers (Bourne et al., 2001) and these contaminants were identified by an absence of similarity to the reference database and removed from further analysis. The contaminants corresponded to an average of 57% from ADE samples and 87% from adjacent soil samples. A total of 13,595 reads remained after removing these contaminant sequences, corresponding to an average of 2802 reads from ADE and 597 from adjacent soil samples.

Representative sequences from each *pmoA* clade identified during the sequence analysis were selected for further analysis. These reads were translated into amino acid sequences and added to a reference *pmoA/amoA* phylogenetic tree using parsimony in ARB (Ludwig et al., 2004).

Sequences are available through the Metagenomics Rapid Annotation (MG-RAST) server[1] with accession numbers 4577576.3 (TPISFBB2), 4577577.3 (TPISFBB3), 4577578.3 (TPISFBB4), 4577570.3 (TPIMBB2), 4577571.3 (TPIMBB3), 4577572.3 (TPIMBB5), 4577565.3 (ADJSFBB2), 4577566.3 (ADJSFBB3), 4577560.3 (ADJMBB2), 4577561.3 (ADJMBB3), 4577562.3 (ADJMBB4), 4577579.3 (TPISFC3), 4577580.3

[1] http://metagenomics.anl.gov/

(TPISFC4), 4577581.3 (TPISFC5), 4577573.3 (TPIMC2), 4577574.3 (TPIMC3), 4577575.3 (TPIMC4), 4577567.3 (ADJSFC2), 4577568.3 (ADJSFC4), 4577569.3 (ADJSFC5), 4577563.3 (ADJMC3), 4577564.3 (ADJMC4).

### STATISTICS

Differences in soil chemical properties were tested by one-way analysis of variance. Two-way analysis of variance model was used to assess differences in *pmoA* gene abundances between land uses and soil types. Test of proportions was used to observe significance of proportion difference in *pmoA* gene relative abundance generated by amplicon pyrosequencing between ADE and adjacent soils using prop.test in the R Stats Package[2]. Significance level of $p < 0.05$ was applied for all statistical analyses and performed using R version 3.03 (R Foundation for Statistical Computing).

## RESULTS

### SOIL CHEMICAL PROPERTIES

The soil chemical properties are presented in **Table 1**. As previously reported, the measured soil chemical properties at the Caldeirão Experimental Station showed a clear distinction between ADE and adjacent soil samples (Taketani et al., 2013; Brossi et al., 2014). ADE soils from Barro Branco had similar properties to those at Caldeirão, with relatively high pH, Ca, CEC, K, Mg, Mn, P, SOC, and Zn compared to their adjacent soils. These characteristics indicate the potential for high agricultural productivity. In contrast, the adjacent soils (i.e., Haplic Acrisol and Oxisol) had lower pH and higher Al and Fe.

### SOIL $CH_4$ OXIDATION POTENTIALS

$CH_4$ oxidation was immediate at concentrations of 10 and 100 ppmv, but a lag phase of 6–10 days was observed for concentrations of 1000 and 10,000 ppmv (results not shown). Relatively high rates of high-affinity $CH_4$ oxidation (10 ppm $CH_4$) were observed in all soils from the forested sites and the ADE soil used for manioc cultivation at the Barro Branco area (**Table 2**). In contrast, the $CH_4$ oxidation rates were more than one-order of magnitude lower in both plantations in adjacent soil and the ADE plantation soil at Caldeirão. The precise history of these soils is not available, but members of the local communities indicated that manioc has been cultivated in ADE soil at the Caldeirão site for living memory (>40 years), whereas the Barro Branco ADE soil was only recently (5 years) converted from forest to agriculture by slash-and-burn.

### ABUNDANCE OF METHANOTROPHS

Quantitative real-time PCR assays were used to determine the copy numbers of *pmoA* genes in ADE and adjacent soils from both secondary forest and the manioc cultivation sites (**Figure 1**). The *pmoA* qPCR assay with primers A189f-mb661r targets methanotrophs belonging to the *Methylococcaceae* and *Methylocystaceae* families and generally has poor specificity for the genes from other families of methanotrophs. The abundance of genes detected with this assay (**Figure 1A**) was not significantly affected by soil type or land use. Based on the diversity of *pmoA* genes detected in the soils (**Figure 2**), these results correspond to *Methylocystis pmoA* genes. Another qPCR assay was used to specifically enumerate USCα *pmoA*, which are a common uncultivated group associated with atmospheric $CH_4$ oxidation. In ADE soils, the abundances of USCα *pmoA* (**Figure 1B**) were more than two-orders of magnitude higher than *Methylocystis pmoA* genes (**Figure 1A**). USCα were below the detection limit ($1 \times 10^4$ copies $g^{-1}$ dry weight soil) in the plantations of adjacent soils. Taking the data from Barro Branco and Caldeirão sites together, the abundance of USCα *pmoA* was significantly higher in ADE than adjacent soil (ANOVA, $p < 0.0001$), but the difference in abundance based on land use (forested versus cultivated) was not significant (ANOVA, $p = 0.77$).

### COMPOSITION OF METHANOTROPH COMMUNITIES

The methanotroph communities in the soils were analyzed by *pmoA* gene pyrosequencing. PCR using the A189f-A682r primer combination retrieves diverse *pmoA*-related genes, including the proteobacterial *pmoA* genes and those from uncultivated methanotrophs believed to be responsible for atmospheric $CH_4$ uptake in upland soils (McDonald et al., 2008). A known problem with these primers is a tendency to co-amplify non-specific sequences, which can make clone libraries useless (Bourne et al., 2001). Non-specific amplification with these primers was also observed in our pyrosequencing data, with an average of 87% of reads from adjacent soils corresponding to non-target reads. The advantage of relatively high number of reads obtainable by pyrosequencing compared with clone libraries meant that sufficient numbers of genuine *pmoA* sequences were still available to allow for comparisons in *pmoA* diversity between the samples.

Almost all sequences passing the quality-filtering steps were assigned to seven clades, which were defined and described previously (Lüke and Frenzel, 2011). Representative sequences from each of these clades were added to a database of *pmoA* and *amoA* sequences and are shown in a simplified phylogenetic tree (**Figure 2**). The most abundant clades identified were USCα, tropical upland soil cluster (TUSC) and *Methylocystis*. The other less abundant clades were RA21, M84-P105, AOB-rel, and the AOB-like group. AOB-rel is also referred to in the literature as Cluster 1 (Kolb et al., 2005).

The relative abundance of the clades from each of the sites is shown in **Figure 3**. A test of proportions indicated that, with the exception of AOB-like sequences, the relative abundances of these clades were significantly different ($p < 0.05$) between the ADE and the adjacent soils (Table S1).

### COMPARISON OF RELATIVE *pmoA* GENE ABUNDANCES OBTAINED BY qPCR AND PYROSEQUENCING

Data from the *pmoA* qPCR assays and amplicon pyrosequencing approaches provided independent numbers to compare the relative abundance of *pmoA* clades in the soils. Based on the diversity of *pmoA* detected by pyrosequencing, *Methylocystis* was the only group present that was a target for the A189f-mb661r *pmoA* qPCR assay. Therefore, the abundance of *pmoA* detected with this qPCR assay was taken as the abundance of *Methylocystis pmoA* genes. Calculating the relative abundance of *Methylocystis* and USCα from the qPCR assays (**Figure 4A**) and the pyrosequencing dataset (**Figure 4B**) showed relatively good agreement.

[2]http://www.r-project.org

**Table 1 | Soil chemical properties of Amazonian Dark Earth (ADE) and their adjacent (ADJ) soils under secondary forest and manioc cultivation.**

| Soil properties | Amazonian Dark Earth | | | | Adjacent soil | | | | Statistics |
|---|---|---|---|---|---|---|---|---|---|
| | Secondary forest | | Manioc plantation | | Secondary forest | | Manioc plantation | | ADE vs. ADJ |
| | Barro Branco | Caldeirão | Barro Branco | Caldeirão | Barro Branco | Caldeirão | Barro Branco | Caldeirão | |
| Al[a] | 0.03 ± 0.03[b] | 0.01 ± 0 | 0.09 ± 0.08 | 0.16 ± 0.13 | 2.01 ± 0.26 | 1.80 ± 0.08 | 2.51 ± 0.35 | 1.37 ± 0.08 | ***[c] |
| Ca | 5.19 ± 1.35 | 2.79 ± 0.50 | 3.45 ± 0.41 | 3.36 ± 0.11 | 0.17 ± 0.06 | 0.09 ± 0.04 | 0.23 ± 0.17 | 0.32 ± 0.06 | *** |
| CEC[†] | 6.64 ± 1.24 | 3.49 ± 0.51 | 4.50 ± 0.47 | 5.64 ± 0.29 | 2.32 ± 0.23 | 2.06 ± 0.11 | 2.90 ± 0.17 | 1.89 ± 0.03 | *** |
| Fe | 5.00 ± 1.00 | 26.33 ± 1.53 | 13.00 ± 3.46 | 51.33 ± 3.51 | 112.33 ± 19.30 | 313.00 ± 53.26 | 75.33 ± 31.21 | 259.67 ± 26.84 | ** |
| K | 21.00 ± 1.00 | 44.67 ± 5.03 | 20.33 ± 3.79 | 20.33 ± 6.50 | 15.33 ± 1.15 | 19.67 ± 5.69 | 16.33 ± 0.58 | 17.67 ± 3.51 | * |
| Mg | 1.36 ± 0.09 | 1.33 ± 0.26 | 0.90 ± 0.16 | 1.08 ± 0.31 | 0.09 ± 0.01 | 0.08 ± 0.03 | 0.09 ± 0.03 | 0.15 ± 0.01 | *** |
| Mn | 48.65 ± 9.40 | 52.1 ± 12.04 | 31.63 ± 3.24 | 22.02 ± 2.85 | 3.9 ± 0.04 | 1.88 ± 0.75 | 4.93 ± 0.33 | 1.84 ± 0.29 | ** |
| P | 26.00 ± 9.54 | 56.33 ± 12.66 | 51.33 ± 13.86 | 73.33 ± 4.50 | 6.00 ± 0.64 | 5.67 ± 1.53 | 5.33 ± 2.08 | 1.33 ± 0.58 | *** |
| $pH_{water}$ | 5.63 ± 0.13 | 5.85 ± 0.27 | 5.53 ± 0.13 | 5.30 ± 0.29 | 4.33 ± 0.06 | 3.84 ± 0.09 | 4.38 ± 0.08 | 4.33 ± 0.13 | ** |
| SOC | 29.90 ± 1.39 | 32.31 ± 3.36 | 24.7 ± 2.50 | 13.15 ± 1.34 | 19.23 ± 0.97 | 11.72 ± 1.88 | 21.2 ± 1.11 | 10.84 ± 0.25 | ** |
| Total C | 3.23 ± 0.45 | 2.99 ± 0.32 | 3.07 ± 0.33 | 2.14 ± 0.05 | 2.71 ± 0.43 | 2.12 ± 0.36 | 2.33 ± 0.55 | 1.81 ± 0.26 | * |
| Total N | 0.24 ± 0.08 | 0.25 ± 0.04 | 0.24 ± 0.02 | 0.16 ± 0.01 | 0.19 ± 0.02 | 0.17 ± 0.02 | 0.16 ± 0.02 | 0.14 ± 0.02 | ns |
| Zn | 10.74 ± 1.96 | 6.76 ± 0.49 | 4.40 ± 0.69 | 2.32 ± 0.28 | 5.63 ± 1.15 | 0.32 ± 0.03 | 5.63 ± 1.15 | 0.51 ± 0.16 | ** |

[†] *Cation exchange capacity.*

[a] *Al, Ca, CEC, and Mg are expressed in centimoles per cubic decimeter; Fe, K, Mn, P, and Zn are expressed in milligram per cubic decimeter; soil organic C (SOC) is expressed in gram per kilogram; Total C and Total N in percentage.*

[b] *Values are means (n = 3) followed by the standard deviation.*

[c] *ANOVA, n = 12, *p < 0.05, **p < 0.01, ***p < 0.001, ns indicates p ≥ 0.05.*

**Table 2 | $CH_4$ oxidation rates in Amazonian Dark Earth and their adjacent soils under secondary forest and manioc cultivation.**

| Soil type and site | Land use | $CH_4$ oxidation rate[a] [pmol of $CH_4$ (*g* dw)$^{-1}$ $h^{-1}$] |
|---|---|---|
| Amazonian Dark Earth | | |
| Barro Branco | Secondary forest | 33.5 ± 1.6 |
| Caldeirão | Secondary forest | 48.1 ± 4.5 |
| Barro Branco | Manioc plantation | 50.0 ± 1.4 |
| Caldeirão | Manioc plantation | 6.0 ± 1.1 |
| Adjacent soil | | |
| Barro Branco | Secondary forest | 31.0 ± 1.9 |
| Caldeirão | Secondary forest | 21.0 ± 1.7 |
| Barro Branco | Manioc plantation | 8.0 ± 1.9 |
| Caldeirão | Manioc plantation | 6.1 ± 2.2 |

[a] *Errors are standard deviation (n = 3).*

The major difference between these data was that USCα in the cultivated adjacent soils was below the detection limit of the qPCR assay (**Figure 1B**) and therefore its relative abundance was calculated as 0 (**Figure 4A**); however, USCα sequences were detected of ∼20% of *Methylocystis* in the pyrosequencing dataset from these samples (**Figure 4B**).

## DISCUSSION

Many processes, such as $CH_4$ oxidation, are crucial for soil ecosystem functioning and have an impact on global biogeochemistry. Forest soils in particular have been identified as an efficient sink for atmospheric $CH_4$ and are highly sensitive to land use change (Dunfield, 2007). Here, we have characterized methanotrophs in ADE and their adjacent soils (Haplic Acrisol and Oxisol) under two different land uses (i.e., secondary forest and manioc cultivation). These approaches showed two major outcomes with respect to ADE soils: (1) high $CH_4$ oxidation rates were observed in three of four ADE soils examined, and (2) high relative and absolute abundances of methanotrophs belonging to the USCα *pmoA* cluster associated with atmospheric $CH_4$ oxidation in upland soils were observed in all ADE soil samples, independent of land use.

### $CH_4$ OXIDATION POTENTIALS

The $CH_4$ oxidation rates were relatively high in forested sites. This is in agreement with other studies of tropical forests soils (Verchot et al., 2000; Veldkamp et al., 2008; Zhang et al., 2008; Dörr et al., 2010). Surprisingly, the ADE soil at the Barro Branco site under manioc cultivation showed a $CH_4$ oxidation rate similar to that of the forested sites. Many studies have shown that conversion of forest to agriculture diminishes $CH_4$ uptake. For example, after 2 years of agriculture a Norwegian soil showed a fivefold decrease in $CH_4$ oxidation rate (Jensen and Olsen, 1998). At the time of sampling, the ADE soil at Barro Branco had been used for manioc cultivation for ∼5 years, suggesting that it too should have shown a decreased $CH_4$ oxidation potential. The ADE soil at the manioc planation at the Caldeirão area, which has a longer history of cultivation, showed a decreased $CH_4$ oxidation potential. The cultivated ADE site at Barro Branco had been burned to clear the land, which may have also influenced in $CH_4$ oxidation capacity as in some cases fire has been shown stimulate atmospheric $CH_4$ oxidation (Jaatinen et al., 2004).

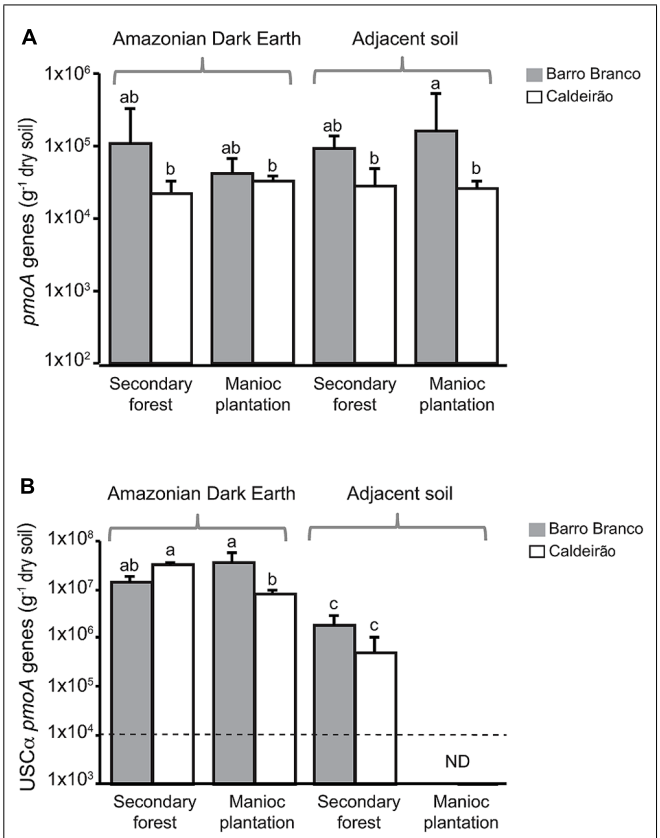


**FIGURE 1 | Methanotroph *pmoA* abundances in Amazonian Dark Earth and adjacent soils under secondary forest and manioc cultivation at Barro Branco and Caldeirão sites. (A)** Abundance of *pmoA* genes using the A189f-mb661r qPCR assay targeting conventional *pmoA* genes (i.e., mostly *Methylocystis* in these soils). **(B)** Abundance of USCα *pmoA* genes determined with the A189f-Forest675r qPCR assay. Abundances denoted with different letters above the bars are significantly different from each other (Tukey's HSD, $p < 0.05$). ND indicates that the target gene was below the detection limit of the qPCR assay, which is indicated by the dashed line.

### ABUNDANCE AND COMMUNITY COMPOSITION OF METHANOTROPHS

Differences in the methanotroph communities were found between ADE and adjacent soils under secondary forest and manioc cultivation, indicating that the methanotrophic community is altered depending on soil type and land use. USCα were the predominant methanotrophs in all ADE soils and the forested adjacent soils. This group is as yet uncultivated, but is believed to be responsible for atmospheric $CH_4$ consumption in many forest soils (Dunfield, 2007; Kolb, 2009; Nazaries et al., 2013). The abundance of USCα *pmoA* genes was $\sim 1 \times 10^7$ per gram dry weight in the ADE soils, which was one-order of magnitude higher than in the forested sites of the adjacent soils. In comparison, the same assay used to quantify USCα in a German forest soil detected $\sim 1 \times 10^6$ gene copies per gram dry weight of soil (Kolb

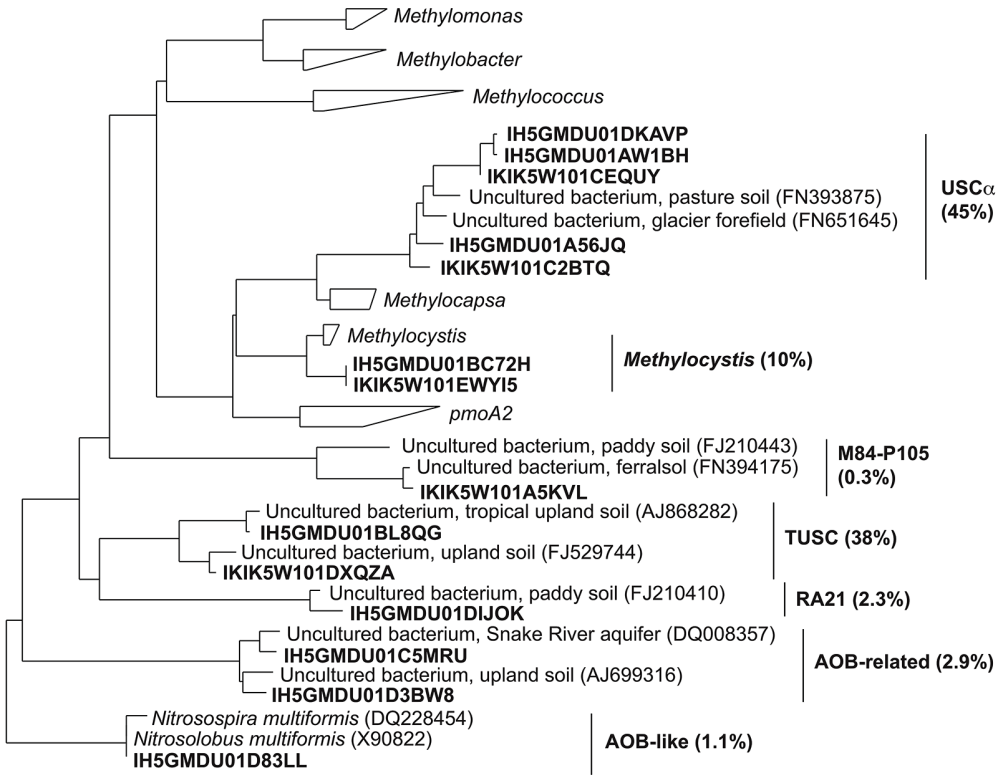


**FIGURE 2 | Phylogenetic tree of representative *pmoA* pyrosequences (bold type) obtained in this study.** The percentages of sequences corresponding to each clade are shown in parentheses.

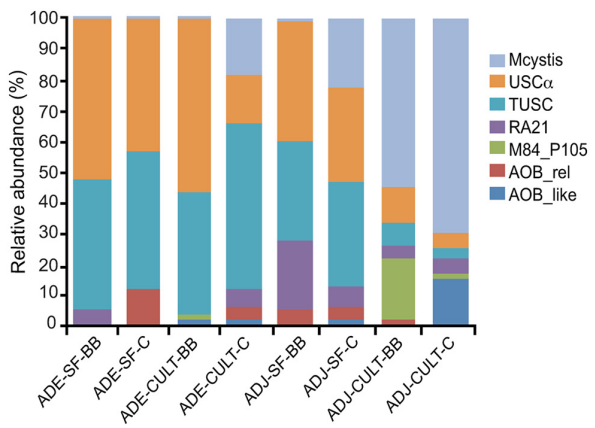


**FIGURE 3 | Relative abundance of detected *pmoA*-related clades obtained by amplicon pyrosequencing of Amazonian Dark Earth (ADE) and adjacent soils (ADJ) under secondary forest (SF) and manioc cultivation (CULT) at Barro Branco (BB) and Caldeirão (C) sites.** The sequence clades are described in the text. "*Mcystis*" indicates *Methylocystis*.

et al., 2005), suggesting that their abundance in ADE was relatively high.

It was surprising that USCα abundances were equally high in the cultivated and forested ADE soils (**Figure 1B**). This pattern was different for the adjacent soils where they were below the qPCR detection limit in the cultivated soils, indicating abundances at least two-orders of magnitude lower than the forested sites. In comparison, the manioc plantation in ADE soil at Caldeirão has a long history of agriculture use, yet the USCα abundance was only threefold lower than in the corresponding forested soil. To the best of our knowledge, this is the first study to detect a high absolute and relative abundance of USCα in agricultural soils. Priemé et al. (1997) showed that $CH_4$ oxidation rates took more than 100 years to reach pre-cultivation levels and that the highest rates were in the oldest (200 years) woodlands. The apparent resilience of USCα populations in ADE soil compared with other upland soils, possibly from a protective property of ADE, suggests that recovery of $CH_4$ oxidation capacity after agricultural abandonment might be faster in ADE than other types of upland soil.

Also of note in this study was that the $CH_4$ uptakes rates were relatively low in cultivated ADE soil at Caldeirão, but USCα abundance in this soil was relatively high. One possible explanation for this lack of correlation is that USCα methanotrophs can incorporate acetate and possibly other organic carbon substrates (Pratscher et al., 2011), suggesting that $CH_4$ oxidation is a facultative trait in these organisms and $CH_4$ is oxidized only under certain conditions. Evidence that USCα are not obligate methanotrophs include reported failures to sufficiently label their nucleic acids with $^{13}CH_4$ for stable isotope probing (Bengtson

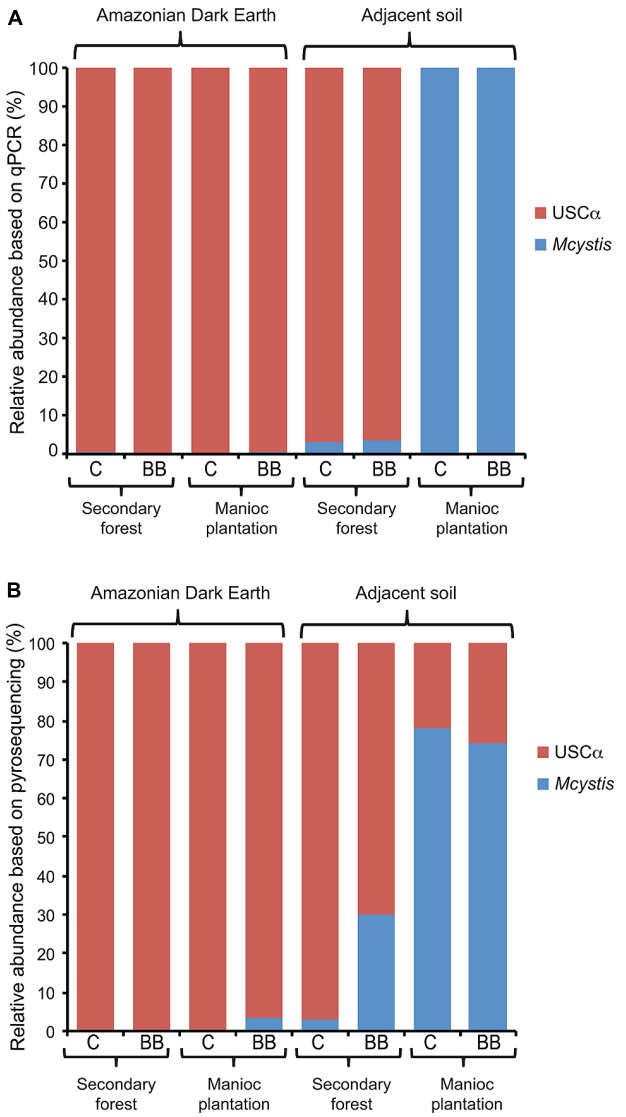


**FIGURE 4 | Relative abundance of *Methylocystis* (*Mcystis*) and USCα *pmoA* genes in Amazonian Dark Earth and their adjacent soils under secondary forest and manioc cultivation at Barro Branco (BB) and Caldeirão (C) sites.** Abundances calculated based on **(A)** qPCR or **(B)** pyrosequencing data.

et al., 2009; Pratscher et al., 2011), and an ability of many of their closest cultivated relatives to grow using multicarbon compounds (Tamas et al., 2014). Another possibility is that the USCα methanotrophs in this ADE soil at Caldeirão have been able to remain dormant, or possibly that DNA from dead cells is relatively stable in ADE soil.

The diversity of methanotrophs observed in this study was similar to the observations of Dörr et al. (2010), who observed in Brazilian ferralsols a prevalence of USCα in natural and afforested sites and higher relative abundances of *Methylocystis* and *Methylococcus* spp. in agricultural soil under conventional farming. Among the cultivated methanotrophs, we only detected *Methylocystis pmoA* and no conventional *pmoA* genes from *Methylococcaceae* methanotrophs; however, the unconventional M84-P105 *pxmA* sequences, which have been shown to belong to members of the *Methylococceae* (Tavormina et al., 2011), were detected in cultivated adjacent soils suggesting a low abundance of these methanotrophs in some soils (**Figure 3**). Although the relative abundance of *Methylocystis* was high in the adjacent soils from manioc plantation sites (**Figure 3**), no difference in their absolute abundance between ADE and adjacent soil, or between forested and cultivated sites was observed at this sampling time (**Figure 1**). *Methylocystis* have been shown to be important consumers of $CH_4$ in hydromorphic soils under dry conditions when $CH_4$ concentrations are relatively low (Knief et al., 2005). These *Methylocystis* possess an unconventional pMMO gene, termed pMMO2 (Ricke et al., 2004), which is expressed under low $CH_4$ (Baani and Liesack, 2008). We only detected two *pmoA2* gene sequences in our pyrosequencing dataset (data not shown), suggesting that conditions in these Amazonian soils at the time of this analysis were not favorable for pMMO2-possessing oligotrophic *Methylocystis* species.

Other *pmoA*-related gene sequences were detected, such as TUSC, AOB-rel and AOB-like groups. The AOB-like sequences correspond to the *amoA* genes of *Nitrosospira* and *Nitrosomonas* (**Figure 2**). In ADE soils, these *amoA* sequences were only detected in plantation soil, which is likely a consequence of enrichment by ammonium fertilizer applied to the soil for manioc cultivation. The TUSC and AOB-rel groups have not been linked to cultivated organisms and the function of the enzyme encoded by these genes is not known (Lüke and Frenzel, 2011). TUSC or "tropical upland soil cluster" is also termed "Cluster 2" elsewhere (Knief et al., 2005). As the name implies, they were found to be abundant in some tropical upland soils (Knief et al., 2005), but have also been detected in temperate forest soil (Knief et al., 2003). It is noteworthy that the relative abundance of TUSC tended to mirror USCα in these Amazonian soils. One possibility to explain this correlation is that TUSC sequences are a divergent *pmoA* gene found in USCα methanotrophs, such as the case with M84-P105 *pxmA* in *Methylomonas* and *pmoA2* in *Methylocystis*; however, other studies have not observed a correlation between USCα and TUSC relative abundances (Kolb, 2009; Dörr et al., 2010).

## CONCLUSION

This study has shown that ADE soils are a potential sink for atmospheric $CH_4$. The relatively high rate of "high-affinity" $CH_4$ uptake by the ADE soil with a 5-year history of agriculture contradicts many studies showing the process to be sensitive to land use change. All the ADE soils examined had a high abundance of USCα methanotrophs ($\sim 10^7$ *pmoA* genes $g^{-1}$ soil), which was particularly surprising for the ADE soil at the Caldeirão site that had a long history of manioc cultivation. In comparison, the abundance of USCα methanotrophs was up to 1000-fold lower in adjacent than ADE soil, and both the adjacent soils used for agriculture displayed relatively low $CH_4$ uptake rates. This raises the question if USCα methanotrophs are indeed more resistant to disturbance in ADE than in other upland soils and whether this apparent resilience of ADE extends to the protection of other groups of vulnerable microorganisms and their associated functions.

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## SUPPLEMENTARY MATERIAL

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# Land-use influences the distribution and activity of high affinity CO-oxidizing bacteria associated to type I-*coxL* genotype in soil

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Soil carboxydovore bacteria are the biological sink of atmospheric carbon monoxide (CO). The initial oxidation of CO is catalyzed by a CO-dehydrogenase (CODH), and the gene *coxL* encodes the large subunit of the enzyme. Only a few carboxydovore isolates were shown to oxidize atmospheric CO and little is known about the potential impact of global change on the ecophysiology of this functional group. The main objective of this study was to assess the impact of land-use and soil properties on *coxL* gene diversity and identify molecular indicators for the soil uptake of atmospheric CO. Soil samples were collected in three neighboring sites encompassing different land-use types, namely deciduous forest, larch plantation and maize field. CO uptake activity was related to total carbon and nitrogen content in soil, with the highest activity observed in deciduous forest. An extensive *coxL* database was assembled to optimize a PCR detection assay targeting sequences belonging to functional type I-CODH and hypothetical type II-CODH. Fully replicated *coxL* gene libraries unveiled a unique molecular signature in deciduous forest soil, with enrichment of type I sequences. Genetic profiles of larch and maize monocultures were not statistically different and showed higher level of *coxL* gene richness than deciduous forest. Soil water content and CO uptake activity explained 38% of the variation of *coxL* gene profiles in a canonical ordination analysis, leading to the identification of sequences belonging to the $\delta$-Proteobacteria cluster as indicator for high affinity CO uptake activity. Enrichment of type I and $\delta$-Proteobacteria *coxL* sequences in deciduous forest were confirmed by qPCR in an independent soil survey. CO uptake activity in model carboxydovore bacteria suggested that a significant fraction of detected putative high affinity CO oxidizers were active in soil. Land-use was a driving force separating *coxL* diversity in deciduous forest from monocultures.

**Keywords: trace gas, soil uptake, atmosphere, global change, gas exchanges**

## INTRODUCTION

Carbon monoxide (CO) is present at the trace level in the atmosphere, with typical background levels ranging from 1 ppmv in urban areas to 0.1 ppmv in remote locations (Novelli et al., 1998; Chan et al., 2002). A combination of modeling approaches attributed biogenic hydrocarbons and methane oxidation, biomass burning and fossil fuel utilization as the main sources of CO in the atmosphere, representing 2500 Tg CO $year^{-1}$ global emissions (Holloway and Levy, 2000). This trace gas has a relatively short atmospheric lifetime of 0.4–2 months, owing to its strong reactivity toward hydroxyl radicals (OH·), the cleansing molecules in the atmosphere. Because of this OH·-mediated reaction, CO indirectly influences the distribution of methane, and thus is considered as an indirect greenhouse gas (Daniel and Solomon, 1998). The uptake of atmospheric CO, catalyzed by specialized microorganisms thriving in oxic soil, is the most uncertain term of CO budget, representing about 15% of the global loss of this trace gas in the atmosphere. Despite the fact that industrialization has increased CO global emissions in the last century (Assonov et al., 2007), current CO concentrations are relatively stable in the atmosphere. Long-term time series analysis unveiled slight but significant decreasing trends of CO concentration in response to reduced industrial and motor vehicle emissions, disturbed by episodic CO pulses originating from forest fires (Novelli et al., 2003; Chevalier et al., 2008). This delicate balance in the atmospheric burden of CO largely relies on microbiological and chemical processes responsible for the abatement of global emissions. Resilience, resistance, or vulnerability of the biological sink of CO to global change, including changes in land-use and climate, must be assessed to predict the fate of atmospheric CO budget. Identification and characterization of soil CO-oxidizing bacteria are mandatory to achieve this challenging task.

In general, CO is a highly toxic gas due to its high affinity to metal-containing enzymes involved in respiratory chains. Sophisticated CO-insensitive metabolisms have evolved in bacteria adapted to exploit this trace gas distributed ubiquitously in the environment. CO-oxidizing bacteria possess a CO-dehydrogenase

(CODH) catalyzing the reaction: $CO + H_2O \rightarrow CO_2 + 2H^+ + 2e^-$. The enzyme is a member of the molybdenum-containing hydroxylases comprising aldehyde oxidoreductase and xanthine dehydrogenase participating in purine nucleotide metabolism (Hille, 2005). In aerobic CO-oxidizing bacteria, CODH is a dimer of heterotrimers consisting of the small (CoxS), medium (CoxM), and large (CoxL) subunits (Dobbek et al., 2002). The active site embedded in the large subunit comprises a dinuclear molybdenum and copper heterometal cluster unique to CODH. Two physiological groups of aerobic CO-oxidizing bacteria have been identified. Carboxydotrophic bacteria are generally facultative chemolithoautotrophs, using CO as a sole carbon and energy source if organic substrates are growth limiting (Mörsdorf et al., 1992). These bacteria have a low affinity toward CO and are incapable of scavenging atmospheric CO (Conrad et al., 1981). In contrast, carboxydovore bacteria cannot grow in presence of elevated level of CO. These bacteria exhibit a versatile mixotrophic metabolism, allowing them to grow on mixtures of inorganic and organic substrates (King and Weber, 2007). In soil, carboxydovore bacteria scavenge atmospheric CO and take advantage of CO diffusing in soil from nitrogen-fixing nodules as well as chemical degradation of organic matter.

A few high affinity carboxydovore bacteria have been isolated so far and very little is known about the environmental control on their distribution and activity (King and Weber, 2007). Phylogenetic analysis of *coxL* gene sequences revealed the occurrence of two different groups of CODH, namely the functional type I-CODH and the hypothetical type II-CODH. Type I-CODH are the most extensively studied and are found in the model carboxydotrophic bacterium *Oligotropha carboxidovorans* as well as carboxidovores such as *Stappia* and *Mycobacterium* isolates demonstrating the ability to scavenge atmospheric CO (King, 2003a,b; Weber and King, 2007). Comparatively less is known about the second group, since the occurrence of a functional type II-CODH never has been reported. Distribution of type I- and type II-*coxL* sequence has been investigated in the environment. Although both phylogenetic groups are ubiquitous in soil, the environmental control on their distribution remains to be elucidated. The objective of this study is to assess the impact of land-use on carboxydovore activity and diversity. We tested the hypothesis that adjacent sites encompassing different land-use types harbor distinct CO-oxidizing bacterial community structure and density, resulting in a site-specific CO uptake activity and *coxL* diversity profile. Soil physicochemical parameters, known to vary within the three ecosystems, such as carbon content and pH, were expected to explain the spatial distribution of this functional group in soil due to the importance of these factors in shaping soil microbial communities structure (Vasileiadis et al., 2013).

## MATERIALS AND METHODS

### SITES AND SAMPLING

Soil samples were collected at the Verchères Arboretum in St. Amable, (QC, Canada), located about 40 km from Montreal on the south shore of the St. Lawrence River (45°67′N; 73°32′W). The landscape of the sampling site is a mosaic encompassing a broad range of ecosystem types arranged over a relatively small area ($<1\,km^2$). Among these ecosystems are tree nurseries (e.g., spruce, larch, pine) established by the *ministère des ressources naturelles-Québec* (MRNQ) for seed production to support reforestation programs. Fifteen years ago, the MRNQ converted part of the original agricultural area to tree plantations, leaving some parcels for agronomic production as well as unseeded lands that led to the emergence of a natural deciduous forest (MRNQ, pers. Commun.) For the purpose of this study, three adjacent areas with contrasting land-use types were sampled: maize monoculture area (stations A1, A2, A3), hybrid larch (*Larix marschlinsii Coaz*) plantation established by the MRNQ (stations M1, M2, M3) and the natural deciduous forest (stations F1, F2, F3). Three stations were identified in each ecosystem to collect composite soil samples (3 land-use types × 3 stations = 9 composite samples). Each composite soil sample consisted of six subsamples collected along a 2-m radius traced from a central point. The A-horizon was collected, from the soil surface to a depth of 10 cm as this zone comprises the highest CO uptake activity (King, 1999b). Surface litter in the forests and debris from the previous crop in the maize sites were removed before sampling. Samples were placed in plastic bags and transferred at 4°C within 2–4 h following their collection in the field. All samples were stored less than 3 months before laboratory analyses. A first soil survey was undertaken in April 2012. Soil of the maize monoculture was bare and unplowed, with a few crop residues remaining on the surface. Samples were used for CO uptake measurements, physicochemical analyses and *coxL* clone libraries. Sampling sites were visited for a second soil survey in November 2013. In contrast to the first survey, crop residues (i.e., senescent maize) resulting from plowing, were present at the maize monoculture sampling sites. Soil samples collected in 2013 were used for CO uptake and *coxL* qPCR assays.

### SOIL PHYSICOCHEMICAL PROPERTIES

Soil texture was determined with the hydrometer method and particle size distribution (**Table 1**) assigned soil samples to the silt loam textural class (Elghamry and Elashkar, 1962). Soil pH was analyzed in soil:water suspensions (1:2) and soil water content was measured using standard gravimetric method. Soil nutrients were analyzed in external service laboratories (INRS-Centre Eau, Terre et Environnement, QC, Canada). Phosphorus and potassium were analyzed by inductively coupled plasma optical emission spectrometry (ICP-AES) after acid extraction, while total carbon and total nitrogen soil content were determined using an elemental combustion system.

### MICROORGANISMS

*Mycobacterium smegmatis* (DSMZ 43756) and *Stappia kahanamokuae* (DSMZ 18969) were purchased at the Leibniz Institute DSMZ—German Collection of Microorganisms and Cell Cultures, while *Burkholderia xenovorans* LB400 was kindly provided by the laboratory of Dr. Michel Sylvestre (INRS-Institut Armand-Frappier). *M. smegmatis* and *B. xenovorans* were grown in PYE broth (Kimble et al., 1995) and Bacto Marine Broth (Difco 2216) was used for the growth of *S. kahanamokuae*. Cultures were incubated at 30°C under 200 rpm agitation. Triplicate cultures dedicated to CO uptake measurements (20 ml) were incubated in gastight 500 ml Wheaton® glass bottles equipped with a rubber

septum cap. Defined volume of CO gas mixture (508 ± 10 ppmv CO, GST-Welco, PA, USA) was injected to get ∼3 ppmv in the static headspace after inoculation. Headspace samples were collected during the incubation period to measure CO oxidation activity (see below). Independent triplicate cultures were also prepared in parallel to monitor bacterial growth by optical density readings. The biomass of stationary phase cultures was quantified by agar plate enumeration using PYE and Bacto Marine agar media.

### CO UPTAKE ACTIVITY

CO uptake activity was measured using either soil samples [∼50 $g_{(drybasis)}$] or bacterial cultures. Soil samples were placed into 500 ml gastight Wheaton® glass bottles with rubber septum caps. Diffusion limitation of the activity was negligible since preliminary experiments showed proportional CO uptake activity as a function of the amount of soil in the assay using 25, 50, 75, and 100 g soil samples (data not shown). Bottles containing soil samples were tightly closed and CO gas mixture (508 ± 10 ppmv CO GST-Welco, PA, USA) was injected to get ∼1 ppmv initial concentration in the static headspace. Decrease of the CO mixing ratio was monitored as a function of time by analyzing aliquots (10 ml) of the headspace air in a Trace Analytical Reduced Gas Analyzer (ta3000R, Ametek Process Instruments®, DE, USA) as previously described (King, 1999a). Apparent first order CO uptake rate constants were obtained by integrating the logarithmic decrease of headspace CO mixing ratio. Measurements were performed using biologically independent triplicates and at least five CO concentration points were used for data integration. Experiments involving soil samples were accomplished over 20–60 min, depending on microbial activity. CO uptake activity in bacterial cultures was measured over a 7-day period. Cell-specific CO oxidation rate (pmol $cfu^{-1}$ $h^{-1}$) was calculated by normalizing the activity to biomass as determined by agar plate enumeration. Reproducibility of the CO analyses was assessed before each set of measurements by repeated analysis of certified CO standard gas (2.05 ± 0.10 ppmv, GST-Welco, PA, USA), and standard deviations were lower than 5%. No significant CO uptake was observed for blank experiments involving sterile media or empty glass bottles. Considering the occurrence of simultaneous CO production and consumption activities in nature and their dependence on temperature, moisture and solar radiations, rates of CO oxidation in soil presented in this study must be considered as potential CO uptake activities.

### *coxL* PHYLOGENETIC ANALYSIS AND PCR DETECTION ASSAYS

Sequences similar to *coxL* in *Mycobacterium smegmatis* (type I-CODH) and *Burkholderia xenovorans* (type I- and type II-CODH) were retrieved from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) using the protein Basic Local Alignment Search Tool (Altschul et al., 1990). Nucleic acid sequences were imported in the software Mega (Tamura et al., 2007), translated *in silico*, and the amino acid sequences were aligned using Muscle (Edgar, 2004). Alignments were manually refined and functional amino acid sequence motifs of the active site distinguishing *coxL* sequences belonging to type I-CODH (AYXCSFR) from

**Table 1 | Physicochemical properties, potential CO uptake activity, and *coxL* richness in soil.**

| Land-use type | Texture (sand/silt/clay) | C (%) | N (%) | P (mg $kg^{-1}$) | K (mg $kg^{-1}$) | pH | $H_2O$ (%) | CO (pmol $g^{-1}_{(dw)}$ $h^{-1}$) | Simpson (*D*) | Shannon (*H'*) | Number of *coxL* clones |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Maize | 0.05/0.84/0.11 | 1.6 ± 0.2 | 0.09 ± 0.04 | 616 ± 24 | 471 ± 186 | 5.4 ± 0.4 | 17 ± 6 | 389 ± 52 | 0.01 | 4.0 | 24 ± 3 |
| Larch | 0.03/0.88/0.09 | 2.3 ± 0.2 | 0.14 ± 0.01 | 332 ± 17 | 382 ± 115 | 4.7 ± 0.2 | 38 ± 10 | 227 ± 221 | 0.01 | 4.0 | 31 ± 7 |
| Deciduous | 0.14/0.84/0.02 | 11 ± 4 | 0.59 ± 0.23 | 290 ± 95 | 174 ± 78 | 4.0 ± 0.3 | 59 ± 4 | 1962 ± 1117 | 0.03 | 3.4 | 37 ± 8 |

*Average values (three sampling stations per land-use type) are represented with standard deviations.*

type II-CODH (AYRGAGR) were examined in order to validate specificity of the retrieved sequences (King and Weber, 2007). Phylogenetic tree of amino acid sequences translated from *coxL* gene sequences was constructed with maximum-likelihood algorithm. The alignment was used to identify consensus regions to design *coxL*-specific oligonucleotides. Three sets of degenerated primers were developed to detect and quantify presumptive CO-oxidizers in soil, so-called universal-*coxL*, type I-*coxL*, and $\delta$-Proteobacteria-coxL assays (**Table 2**).

16S rRNA gene sequences were retrieved from the genome of 102 presumptive CO-oxidizing bacteria identified in the *coxL* database (Table S1). 16S sequences were classified into two databases: type I-*coxL* and type II-*coxL* groups, as a function of *coxL* gene harbored by the bacteria, and then aligned. Pairwise difference ($D$) matrices were computed to obtain the similarity scores $S$ ($S = 1 - D$) of all possible combinations of 16S rRNA and *coxL* gene sequences for both databases. Comparisons between the percentages of similarity of all *coxL* pairs and the sequence similarities of the 16S rRNA genes of the same bacteria were performed by regression analysis ($n = 820$ and $n = 1830$ for type I and type II databases, respectively). This pairwise similarity score analysis has recently been utilized to establish a similarity score threshold value for nitrate/nitrous oxide reductases (Palmer et al., 2009), particulate methane monooxygenase (Degelmann et al., 2010), and hydrogenase (Constant et al., 2011b) gene sequences at the species level.

### DNA EXTRACTION AND UNIVERSAL-*coxL* PCR

Soil DNA was extracted from an exact amount of soil (∼500 mg) using the FastDNA Spin Kit (MP Biomedicals®, OH, USA) for soil according to the manufacturers protocol. DNA was eluted in 50 μL nuclease-free water. DNA samples were diluted (1:10, 1:100, and 1:500) before the PCR due to residual humic acids inhibition. All PCR mixtures consisted of 1× reaction buffer (15 mM $MgCl_2$), 0.2 mM deoxynucleotide triphosphates, 10 μM of each primer (**Table 2**), 0.8 mg $ml^{-1}$ bovine serum albumin, 1.25 U Fast-Taq polymerase (Feldan®, QC, Canada), 2 μl diluted DNA and nuclease-free water to obtain a final volume of 50 μL. A touchdown PCR protocol was used for the universal-*coxL* assay as follow: 95°C for 5 min, 16 cycles of "touchdown steps" denaturing at 95°C for 20 s, annealing temperature starting at 65°C decreasing 0.5°C in every cycle to reach a temperature of 55° (40 s at each cycle), and a elongation step of 72°C for 45 s, completed with a final set of 19 regular PCR cycles of 95°C for 20 s, 55°C for 40 s and 72°C during 45 s with a final extension of 72°C for 5 min.

**Table 2 | List of the primers designed in this study.**

| Assays (*coxL*) | Primers | PCR-amplified fragment size (bp) |
|---|---|---|
| Universal (PCR) | uni820-forward: 5′-GGBGGBGGYTTYGGCWMSAA-3′<br>uni1611-reverse: 5′-GTBKCRTGNCCCTGNCC-3′ | 800 |
| Type I (qPCR) | type I-1288-forward: 5′-TSKKYACSGGCWSSTA-3′<br>type I-1540-reverse: 5′-TAYGAYWSSGGYRAYTA-3′ | 252 |
| $\delta$-Proteobacteria (qPCR) | D967-forward: 5′-TTCTWCKCYGAYGCVCARCC-3′<br>D1285-reverse: 5′-CBGAGTCGGDSASGAADCC-3′ | 291 |

### *coxL* GENE LIBRARIES

One *coxL* gene library was derived from each sampling station, resulting in nine fully replicated libraries. Partial *coxL* gene sequences were PCR-amplified using the universal-*coxL* assay and cloned in pGEM-T® Easy Vector cloning Kit (Promega, WI, USA). Recombinant colonies were selected, plasmid DNA extracted following standard procedure (Sambrook and Russell, 2001) and *coxL* inserts were PCR-amplified and sequenced using the Sanger's Method (Génome Québec Innovation Centre, McGill University, QC, Canada). In total, 279 clones were obtained. Clone sequences were aligned and *in silico* translated to verify the canonical signature of the active site characterizing type I and type II *coxL* sequences. The OTU representative sequences (0.90 similarity cut-off) obtained using the universal-*coxL* assay were deposited in the GenBank database with accession numbers KJ395119 to KJ395310. UniFrac distance matrix, reflecting the pairwise phylogenetic distance between the sequences retrieved from each sampling site was calculated to verify if land-use types have significantly different microbial communities (Lozupone and Knight, 2005).

### TYPE I- AND $\delta$-PROTEOBACTERIA-*coxL* qPCR ASSAYS

Type I- and $\delta$-Proteobacteria-*coxL* genes were PCR-amplified using the universal-*coxL* assay and plasmid DNA of clone 55M3 (accession number KJ395179) and genomic DNA of *Haliangium ochraceum* DSM 14365 as matrices, respectively. PCR products were concentrated and purified with standard commercial kits (E.Z.N.A. Cycle Pure Kit, Omega Bio-Tek®, GA, USA). Purified DNA was quantified with fluorescent DNA-binding dye (Quantifluor dsDNA, Promega, WI, USA). Standard curves for type I- and $\delta$-Proteobacteria-*coxL* qPCR assays were obtained using serial dilutions of quantified DNA ($10^1$–$10^9$ copies $\mu l^{-1}$). Reactions contained 1× Perfecta SYBR Green Fast Mix reaction buffer (Quanta Biosciences®, MD, USA), 15 μM of each primer (**Table 2**), 0.3 mg $ml^{-1}$ bovine serum albumin, 5 μL diluted DNA (1:500) and nuclease-free water to obtain a final volume of 20 μL. Preliminary experiments with internal standard DNA spiked in soil DNA extracts (Deer et al., 2010; Decoste et al., 2011) were conducted and showed undistinguishable qPCR-signal recovery between the samples using 1:500 DNA dilutions. Furthermore, qPCR results from 1:500 to 1:1000 dilutions were undistinguishable, providing no significant incidence of PCR inhibitors on *coxL* abundance data (data not shown). Reactions were performed in the Rotor Gene 6000 (Corbett Life Science®, NSW, Australia) with the following conditions: 94°C for 5 min, 35 cycles of 94°C for 30 s, 51°C (Type I-*coxL*) or 56°C ($\delta$-Proteobacteria-*coxL*) for 30 s, 68°C for 20 s (Type I-*coxL*) or 15 s ($\delta$-Proteobacteria-*coxL*) with fluorescence acquisition following each 68°C step and a

melting cycle with a ramp from 75 to 99°C, rising 0.2°C every 5 s. Replicate calibration curves were performed to verify the accuracy of the qPCR resulting in an efficiency of 0.70 ($R^2 = 0.98$) and 0.73 ($R^2 = 0.96$) for type I- and δ-Proteobacteria-*coxL* assays, respectively. Type I- and δ-Proteobacteria-*coxL* gene libraries were also performed to confirm the specificity of the assays. The resulting type I- and δ-Proteobacteria-*coxL* sequences with more than 200 pb length have been deposited in the GenBank database with accession numbers KJ567007 to KJ567022 and KJ567023 to KJ567040, respectively.

### STATISTICAL ANALYSIS

Gene libraries were normalized to the sequencing effort of the smallest *coxL* library to avoid biases in comparative analyses introduced by the sampling depth. Using the software Mothur (Schloss et al., 2009), 24 *coxL* sequences were randomly selected from the nine libraries. The resulting sequences were grouped into operational taxonomic units (OTU) defined by a similarity level of 0.90. These files were used for diversity index calculation and statistical analysis. Redundancy analysis (RDA) was computed using the Vegan package (Dixon, 2003) implemented in R (R Development Core Team, 2008) according to the comprehensive procedure described by Borcard et al. (2011). RDA is a constrained analysis, used to extract structures of an observational dataset related to explanatory variables. In this study, RDA was considered to identify environmental variables influencing the structure of *coxL* gene profile in soil, in addition to highlight *coxL* sequences whose presence is related to elevated CO soil uptake activity. This test was preferred from canonical analysis due to the occurrence of several null values in the *coxL* data matrix. Soil variables (e.g., pH, carbon, nitrogen, water content, CO uptake activity) were standardized by subtracting individual values by the average and dividing them by the standard deviation. This transformation procedure resulted in centered data or *z*-scores, generating variables characterized by an average of zero and a standard deviation of 1. The Hellinger transformation was applied to *coxL* OTU frequency distribution before computing the distance matrix to avoid unduly relationships between explanatory variables and *coxL* composition supported by the high weight of rare species (Legendre and Gallagher, 2001). The most parsimonious constrained model to explain *coxL* composition was obtained by forward selection of the environmental variables (Blanchet et al., 2008) and permutation tests ($n = 1000$) were performed to assess the significance of the RDA. Pearson correlation analyses were conducted to identify environmental variables related to soil CO uptake activity. Analysis of variance with Bonferroni *post-hoc* statistical test was performed to compare CO uptake activity and abundance of *coxL* genes between the three land-use types (SigmaPlot 12®, Systat Software Inc., CA, USA).

## RESULTS

### SOIL PROPERTIES AND CO UPTAKE ACTIVITY

Triplicate composite soil samples (A-horizon) were collected in April 2012 to relate CO uptake activity to soil physicochemical properties and *coxL* diversity profiles. The highest carbon and nitrogen contents were detected in deciduous forest soil, while maize monoculture showed the maximum levels of potassium and phosphorus (**Table 1**). Distribution of the measured variables showed some level of co-linearity. Indeed, soil water content was positively related to K, P, and pH (Pearson correlation, $P < 0.05$) and inversely related to total carbon and nitrogen content (Pearson correlation, $P < 0.01$). Variations in soil physicochemical properties resulted in a broad range of CO uptake activities, from 45 pmol $g^{-1}_{(dw)}$ $h^{-1}$ in larch plantation (station M2) to 3243 pmol $g^{-1}_{(dw)}$ $h^{-1}$ in deciduous forest (station F3). The activity was positively correlated to carbon and nitrogen content in soil (Pearson correlation, $P < 0.01$), whereas no significant relationship was observed with the other variables. In accordance with total carbon and nitrogen profiles, CO uptake activity observed in deciduous forest soil was greater than in maize and larch plantations (ANOVA, $P < 0.05$), while soil samples collected from these two sites could not be distinguished based on their CO uptake activity. CO compensation concentration, reached when CO production and consumption rates are equivalent, was at the detection limit of the gas chromatographic system for the three ecosystem types (i.e., <25 ppbv), impairing estimation of the gross production and consumption rates of CO (Conrad, 1994).

### DETECTION OF *coxL* GENOTYPES

An extensive phylogenetic analysis of *coxL* gene sequences was essential to get fundamental information regarding the evolution of functional type I-CODH and hypothetical type II-CODH, to optimize the universal-*coxL* PCR detection assay as well as to interpret gene libraries data. Putative *coxL* gene sequences were obtained from genome sequencing projects and CO-oxidizing bacteria exhibiting high affinity CO uptake activity (**Figure 1**). A parsimonious phylogenetic reconstruction of the type I-CODH group was obtained, while type II-*coxL* sequences were distributed in several clusters for which topology was poorly supported by bootstrap analysis (**Figure 1A**). Inspection of the conserved amino acid signature of the active site unveiled the occurrence of atypical motif in *Saccharomonospora viridis* and *Streptospotangium roseum* (**Figure 1A**). The PYRGAGR signature observed in these bacteria diverged from the canonical AYRGAGR motif of type II sequences. Pairwise sequence similarity scores of 16S rRNA and *coxL* genes were calculated to test whether standardization of the classification of *coxL* sequences is possible under "species-level" OTU and to assign environmental *coxL* sequences to taxonomic groups in phylogenetic analyses. The pairwise sequence similarity scores were correlated in bacteria possessing type I-*coxL* sequence, where the linear regression model ($n = 820$, $P < 0.001$) predicts a species-level similarity score threshold of 0.89 ± 0.04 (**Figure 2A**). For type II sequences, the regression model ($n = 1830$, $P < 0.001$) was associated to a species-level similarity score threshold of 1 ± 0.07 (**Figure 2B**), providing indication for different evolution histories for both types of CODH. Evidence of lateral transfer was noticed for type II-CODH. For instance, type II-*coxL* sequence detected in the aerobic hyperthermophilic Crenarchaeota *Aeropyrum pernix* was affiliated with that of a member of the Chloroflexi phylum (*Sphaerobacter thermophilus*), supporting potential lateral gene transfer event in the Archaea (**Figure 1A**). The extensive *coxL* database was

utilized to optimize previous universal *coxL* PCR detection assay (Table S2).

Genomic DNA was extracted from nine composite soil samples and *coxL* genes were PCR-amplified, cloned and sequenced. In total, 279 clones were derived from the maize (73), larch (93), and deciduous forest (113) samples. Sequences were classified into 192 different OTU using an arbitrary cut-off of 10% difference to accommodate both type I- and type II-*coxL* sequences.

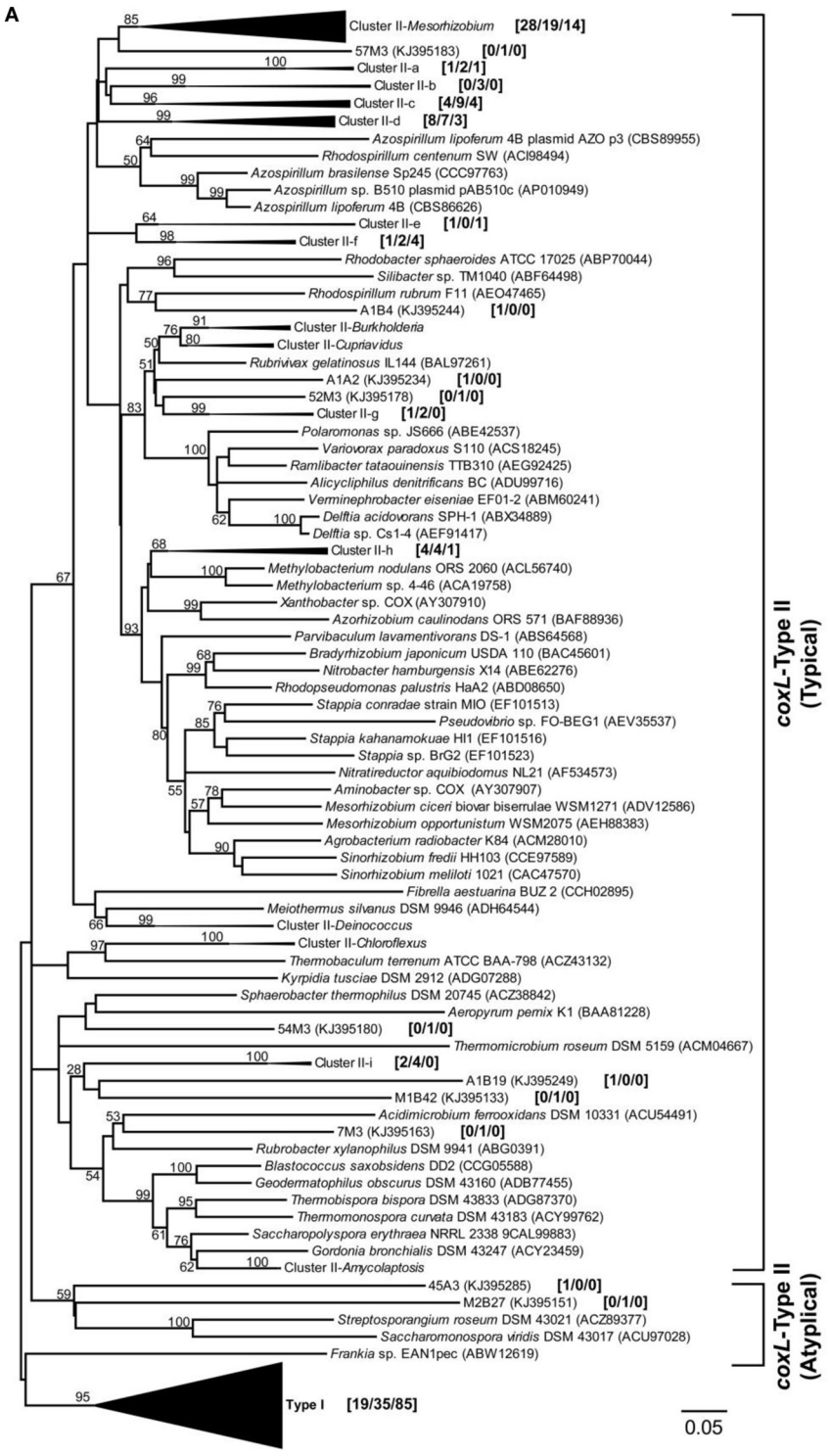


**FIGURE 1 |** ***(Continued)***

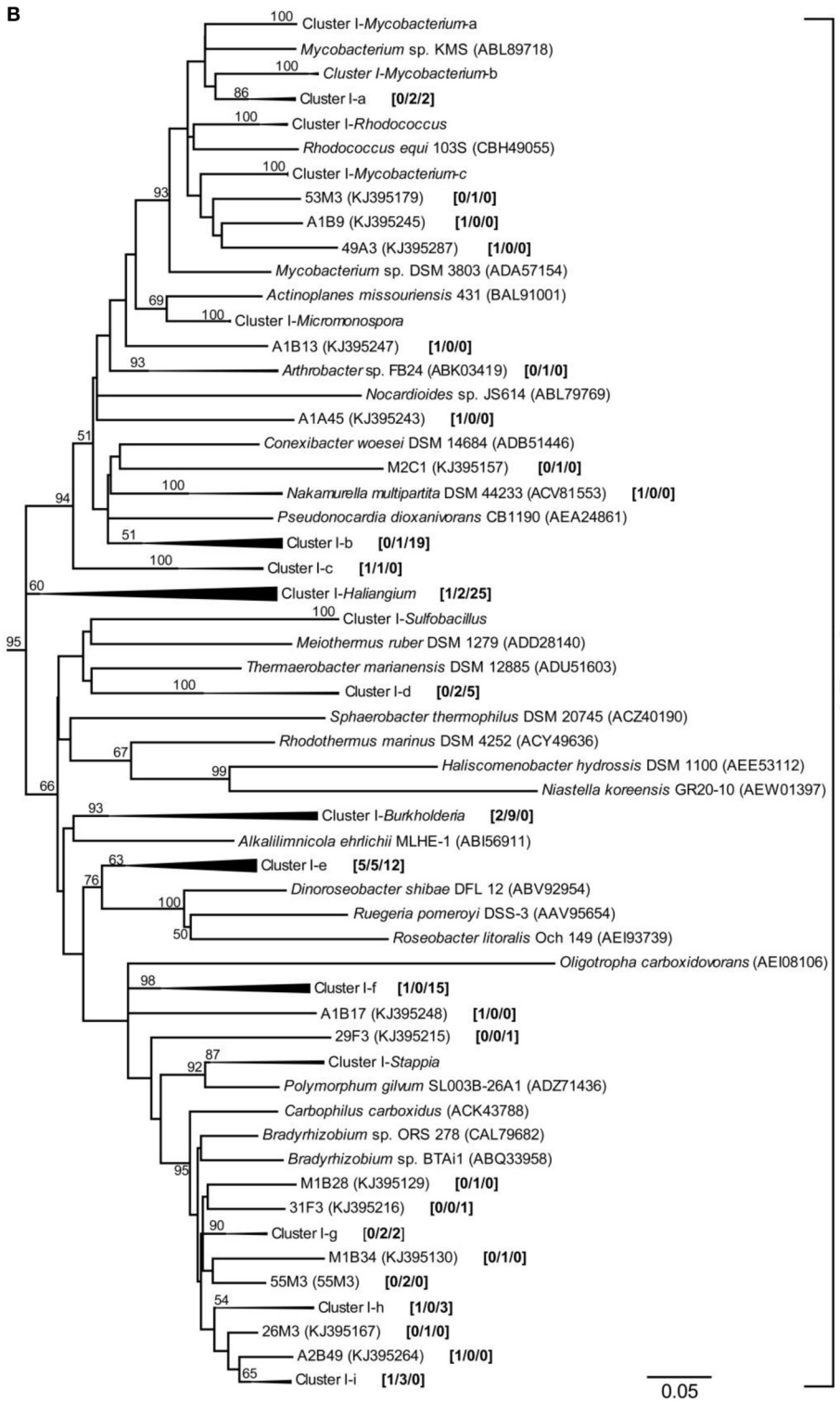


**FIGURE 1 | Phylogenetic analysis of *coxL*-inferred amino acid sequences (313 residues) by the maximum-likelihood algorithm (model WAG+G).** Global analysis including both type I- and type II-*coxL* sequences is shown **(A)** with a detailed view of *coxL*-type I phylogenetic group **(B)**. The analysis included sequences retrieved from public database along with the 192 OTUs identified in this study. The numbers in brackets show the number of *coxL* sequences from the nine clone libraries belonging to individual OTUs and clusters [maize/larch/deciduous]. The percentage of replicated trees in which the associated CoxL sequences clustered together in the bootstrap test (1000 replicates) are shown for nodes supported by ≥50% of the replicates. Prefixes of OTUs encompassing type I- and type II-*coxL* indicate land-use type as follow: A, maize monoculture; M, larch monoculture; and F, deciduous forest. Scale = number of substitutions per site.

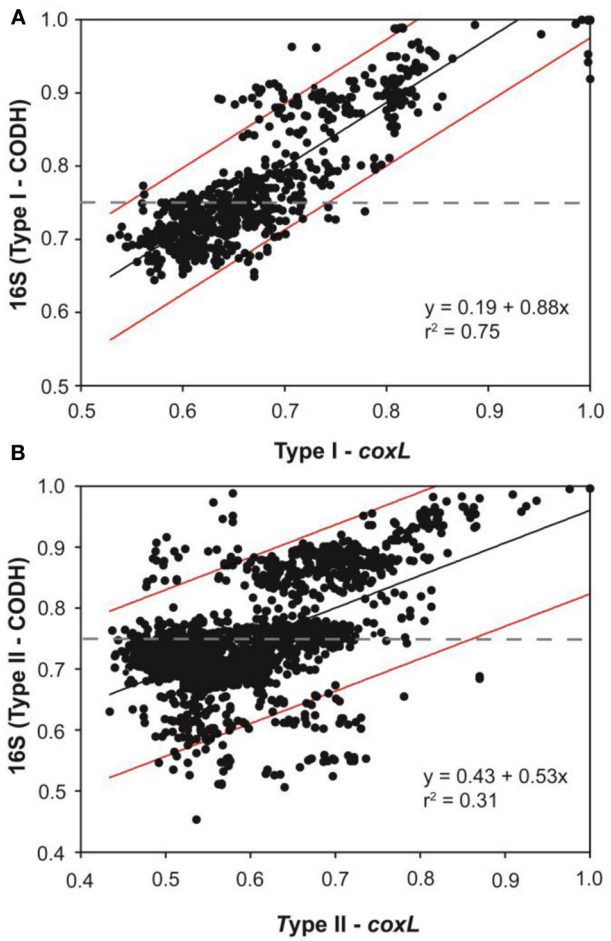


**FIGURE 2 | Correlation between the pairwise sequence similarity scores of *coxL* and the 16S rRNA gene in presumptive CO-oxidizing bacteria belonging to (A) type I-CODH and (B) type II-CODH.** The black line represents the linear regression and red lines delineate the prediction interval of the model for the 95% confidence level.

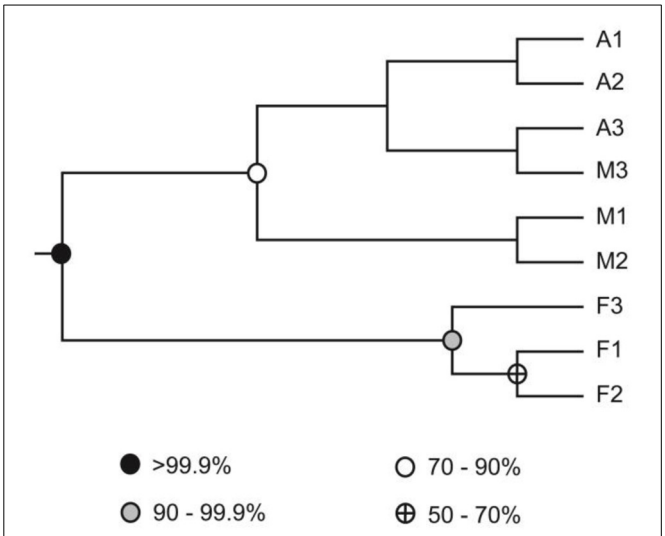


**FIGURE 3 | UPGMA dendrogram of soil samples collected in deciduous forest (stations F1, F2, F3), maize monoculture (stations A1, A2, A3), and larch plantation (stations M1, M2, M3).** The UPGMA was derived from the UniFrac distance matrix, reflecting the pairwise phylogenetic distance between the sequences retrieved from each sampling station. Nodes are filled as a function of the frequency at which they were found in Jackknife procedure keeping 75% sequences for the analysis.

According to a rarefaction analysis, sampling effort was insufficient to cover the whole diversity of presumptive CO-oxidizing bacteria communities (data not shown). Comparison of the gene libraries thus are representative of the dominant members of this functional group in soil. Diversity metrics indicate lower richness of *coxL* sequences sampled in deciduous forest soil than the monocultures (**Table 1**). The lower value of the Simpson index in deciduous forest reflects dominance of the sampled community by a small number of OTU. UniFrac analysis of the nine *coxL* gene libraries was in accordance with the diversity metrics. Composition of deciduous forest *coxL* gene libraries differed significantly from maize and larch monocultures, while conversion of the agricultural field to larch monoculture 15 years ago did not influence the composition of dominant presumptive CO-oxidizing bacteria (**Figure 3**). The relative abundance of clones belonging to types I and II varied as a function of land-use type (**Figure 4A**). Type I-*coxL* sequences dominated deciduous forest soil, while maize and larch plantations displayed higher proportion of type II. Clone sequences belonging to type II were phylogenetically-distant from cultured representative bacteria. With the exception of the OTU A2B46, affiliated to *coxL* sequence from *Mesorhizobium loti* (73% similarity score, implying both sequences are derived from bacteria that could belong to two different phyla; **Figure 2B**), no type II sequence related to clusters comprising known CO-oxidizing bacteria was detected (**Figure 1A**). Phylotypes affiliated to the atypical *coxL* sequence of *S. viridis* and *S. roseum* were detected in the three ecosystem types, representing 0.7% of the analyzed clones. Most of the clones encompassing type I-*coxL* were comprised in *Actinobacteria* (16%), $\alpha$-Proteobacteria (14%), and $\delta$-Proteobacteria (10%) clusters. The proportion of clone sequences related to these phyla varied as a function of land-use type. For instance, 27% type I sequences detected in deciduous forest encompassed the $\delta$-Proteobacteria cluster, while this group represented 6 and 10% in maize and larch monocultures (**Figure 4B**).

## RELATIONSHIP BETWEEN *coxL* GENE SEQUENCES AND ENVIRONMENTAL VARIABLES

A RDA was performed to infer the relationship of *coxL* gene sequences with environmental variables (**Figure 5**). The most parsimonious model to explain variation of *coxL* sequences included soil CO uptake activity and water content. The other variables being redundant to CO uptake and soil moisture, their addition in the analysis increased the variance inflation factor unduly and they were therefore ignored in the analysis. The first two canonical axes explained 38% of the total variance of *coxL* OTU frequency distribution. Significance of the RDA was confirmed with 1000 permutations of *coxL* data matrix ($P = 0.003$). Soil water content played an important role for the dispersion

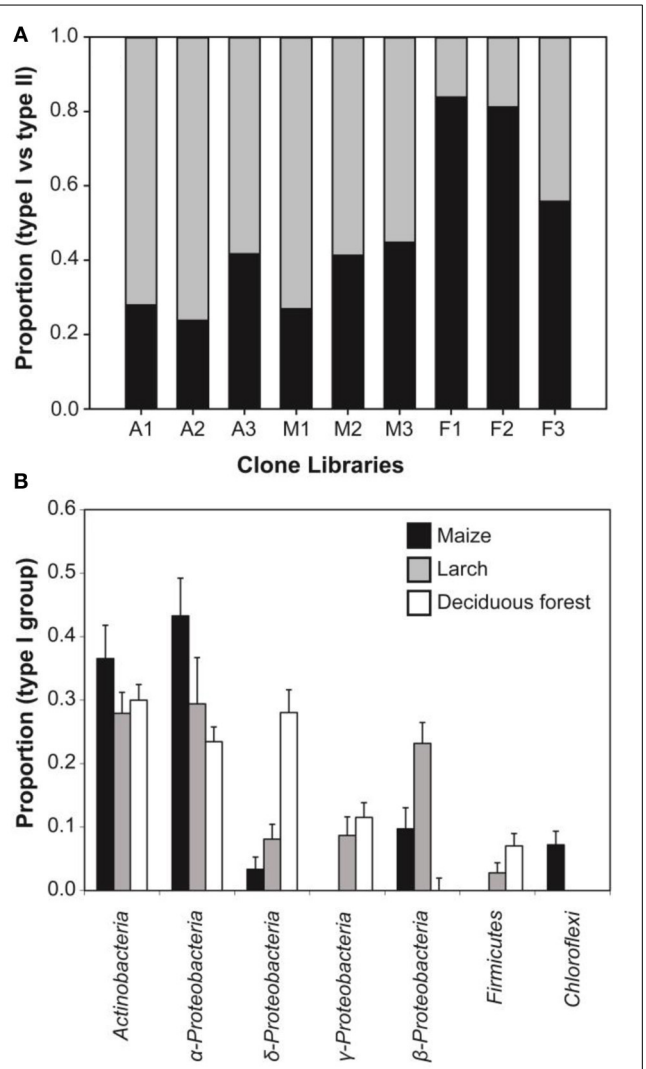


**FIGURE 4 | (A)** Relative abundance of *coxL* clone sequences belonging to type I- (black) and type II-CODH (gray) groups in the nine clone libraries. **(B)** Percent composition (average ± standard error of three replicated clone libraries) of bacterial phyla represented by type I-*coxL* sequences detected in the three different ecosystems.

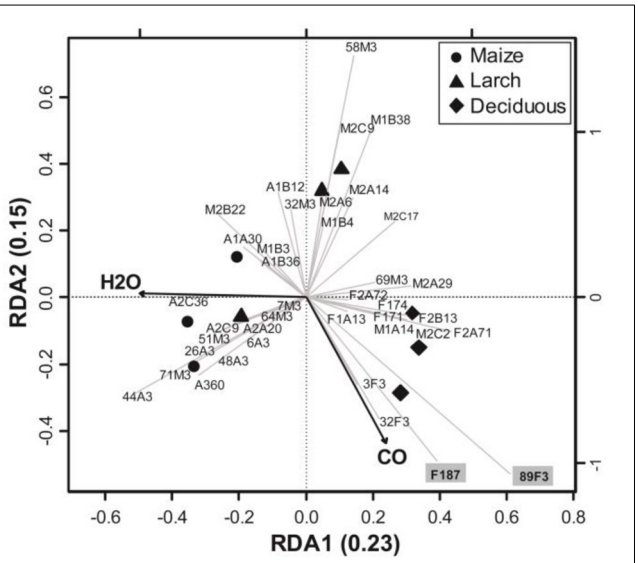


**FIGURE 5 | Parsimonious RDA triplot of Hellinger-transformed *coxL* OTU frequency matrix explained by soil water content and CO uptake activity.** One OTU representative was kept for the analysis in the case of collinearly in *coxL* OTU profiles.

of the samples along the first axis, while CO uptake activity discriminated the samples along the second. According to UniFrac analysis, axes clearly separated samples collected in deciduous forest from those originating from both monocultures (**Figure 5**). The occurrence of 12 OTU was related to higher CO uptake activities. Among them, 10 encompassed type I phylogenetic group (89F3, F187, 3F3 F2A71, M2C2, M1A14, F1A13, F2B13, F171, F2A72), while 2 belonged to type II (32F3, F174). Combined with the higher relative abundance of type I sequences detected in deciduous forest, this observation led us to consider that type I-*coxL* might be a better indicator of CO uptake activity in soil than type II sequences. OTUs 89F3 and F187 were related to deciduous forest samples characterized with the highest CO uptake activity (**Figure 5**). These sequences encompass the δ-Proteobacteria cluster, suggesting the relevance of this type I-*coxL* subgroup to predict CO oxidation activity in the soil samples. The obligate halophile myxobacterium *Haliangium ochraceum* isolated from coastal seaweed (Fudou et al., 2002) was the sole cultivated representative of the δ-Proteobacteria cluster, with no report on its CO uptake activity. We tested the CO uptake activity of *H. ochraceum* and confirmed its capability to scavenge atmospheric CO (Figure S1). This is the first demonstration of CO oxidation activity in the δ-Proteobacteria class.

## LINKING CO UPTAKE ACTIVITY TO THE ABUNDANCE OF *coxL* SEQUENCES AND THEORETICAL POPULATIONS OF CARBOXYDOVORES BACTERIA IN SOIL

Gene libraries suggested that distribution of *coxL* sequences belonging to type I or the δ-Proteobacteria cluster reflect CO soil uptake activity. The analysis was however limited by insufficient sampling effort to cover the whole diversity of *coxL* sequences in soil as well as PCR and cloning bias. In order to challenge the results of clone libraries, the three sampling sites were visited for a second soil survey in 2013. CO uptake activity was measured and showed the same trend than the 2012 soil survey, with higher oxidation rates in deciduous forest than in monocultures, and total DNA was extracted for qPCR analyses. Degenerated oligonucleotides were designed to quantify *coxL* sequences belonging to type I group and δ-Proteobacteria subgroup. Optimization of a broad assay, specific type I-*coxL* sequences derived from public database and clone sequences obtained in this study was unsuccessful due to no or unspecific amplification signals induced by consensus degenerated primers (data not shown). As an alternative, oligonucleotides were designed based on the clone sequences only. Specificity of the assays was confirmed by *coxL* gene libraries (23 clones per

assay) with 13 and 0% unspecific sequences for type I and δ-Proteobacteria, respectively. The abundance of type I-*coxL* varied between $10^9$ and $10^{10}$ genes $g^{-1}_{(dw)}$ in maize and larch monocultures and $10^{10}$–$10^{11}$ genes $g^{-1}_{(dw)}$ in deciduous forest (**Figure 6A**). A similar trend was observed for the δ-Proteobacteria subgroup with an average of $10^9$ and $10^{11}$ genes $g^{-1}_{(dw)}$ for monocultures and deciduous forest, respectively, (**Figure 6A**). According to *coxL* gene libraries, type I-*coxL* sequences were more abundant in deciduous forest soil than both monocultures ($P = 0.004$). The abundance of δ-Proteobacteria *coxL* sequences and the relative proportion of this group was significantly higher in deciduous forest than maize monoculture ($P = 0.01$), while maize and larch monocultures pair as well as larch plantation and deciduous forest pair could not be distinguished based on the abundance of δ-Proteobacteria *coxL* sequences. Linear regression analyses showed that abundance of both *coxL* subgroups, as estimated by qPCR, was proportional to CO oxidation activity in soil ($P < 0.003$), but the relationships were largely driven by the contrasting properties of deciduous forest samples (**Figure 7**).

### THEORETICAL POPULATIONS OF CARBOXYDOVORE BACTERIA IN SOIL

Although differences in the efficiency of the qPCR reaction between standard and environmental DNA templates are expected due to the utilization of degenerated primers, the absolute quantification method remains a standard choice in environmental microbiology (Brankatschk et al., 2012). In order to verify the reliability of the qPCR estimates, three carboxydovore bacteria known to oxidize atmospheric CO and available in public microorganism culture collections were selected and characterized in term of cell-specific CO uptake activity. CO oxidation activity was detected at the onset of the stationary phase of the strains demonstrating a broad range in specific activities, from 29 to 2171 zmol $cfu^{-1}$ $h^{-1}$ (**Figure 6B**). This range in specific activities was used to calculate theoretical populations [*N*; cell $g^{-1}_{(dw)}$] of metabolically active carboxydovore bacteria necessary to explain the CO uptake activity measured in the second soil survey (Equation 1):

$$N = \frac{CO_{Soil}}{CO_{Bacteria}} \quad (1)$$

where $CO_{Soil}$ is the CO uptake activity measured in soil [pmol $g^{-1}_{(dw)}$ $h^{-1}$] and $CO_{Bacteria}$ is the CO oxidation rate in carboxydovore bacteria (pmol $cfu^{-1}$ $h^{-1}$; cfu = colony forming unit). The lowest and highest cell-specific CO oxidation activities ($CO_{Bacteria}$) measured in *B. xenovorans* and *M. smegmatis* (**Figure 6B**) were utilized to calculate the upper and lower limits of *N*, respectively (**Figure 6A**, shaded areas). For this calculation, it is assumed that each bacterium harbors a single *coxL* operon and that one cfu corresponds to a unique viable cell. Considering the facts that some bacteria possess two *coxL* operons, the potential impact of cultivation conditions on CO uptake activity and the possibility that bacterial colonies arise from cell aggregates, this calculation provided a rough estimate of theoretical carboxydovore populations in soil. Nevertheless, with the exception of the abundance of δ-Proteobacteria *coxL* sequences in maize monoculture where qPCR data were below theoretical estimates, there was an agreement between theoretical populations and qPCR data (**Figure 6A**).

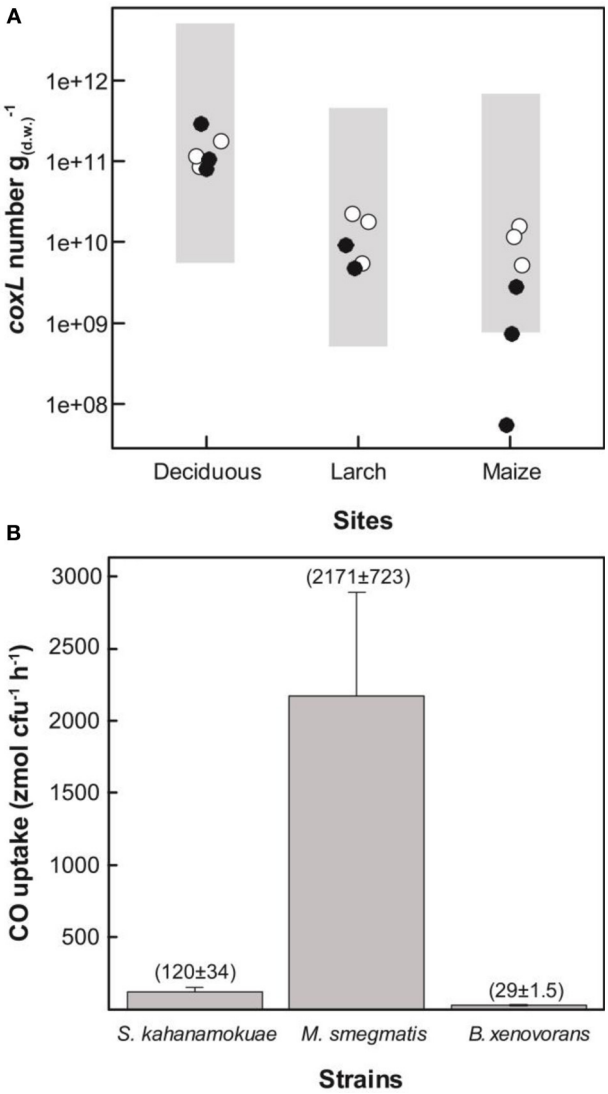


**FIGURE 6 | (A)** Abundance of type I-*coxL* sequences (○) and δ-Proteobacteria-*coxL* sequences (●) in soil. Shaded areas represent the lower and upper limit of theoretical populations of CO-oxidizing bacteria. Average CO uptake rates measured in soil samples collected in 2013 (1.5, 1.0, and 11 nmol $g^{-1}_{(dw)}$ $h^{-1}$ for maize, larch and deciduous forest, respectively), and cell-specific activity of *B. xenovorans* (upper limit of the population) and *M. smegmatis* (lower limit of the population) were used for the calculations (see panel **B**). **(B)** Specific CO uptake activity of selected CO-oxidizing bacteria.

## DISCUSSION

Land-use change exerts strong impact on biogeochemical cycles and soil microbial communities. These environmental pressures are especially marked for biogeochemical processes involving specific metabolisms, restricted to specialist microbes. For instance, afforestation of bog and grassland altered methanotrophic bacteria communities structure in soil, which was directly linked to an

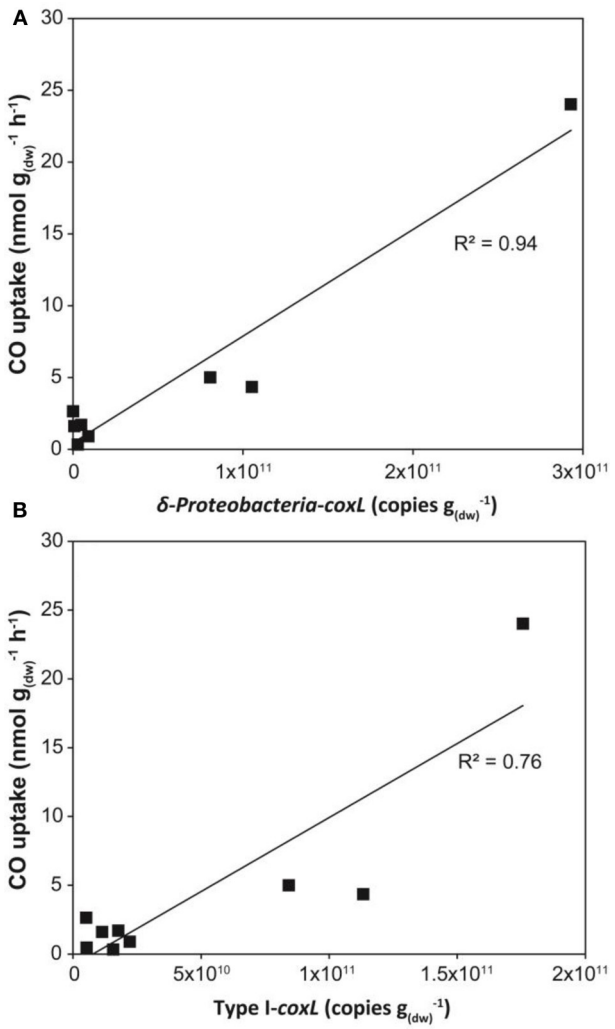


**FIGURE 7 | Linear regression modeling dependence of CO uptake activity on the abundance of (A) δ-Proteobacteria-*coxL* and (B) type I-*coxL* gene number in soil assessed by qPCR.**

enhanced atmospheric methane soil uptake activity in land management experimental stations (Nazaries et al., 2013). Similarly, composition of $N_2$-fixing and denitrifying microbial communities responded to soil physicochemical properties and land-use change, resulting in alteration of nitrous oxide fluxes following afforestation of pastures (Singh et al., 2011). The impact of land-use change on CO soil-to-air exchanges has been investigated in tropical and temperate climates. Exchanges measured in the field being influenced by temperature and soil water content, these investigations resulted in conflicting observations where agricultural areas represented either more important (King, 2000; King and Hungria, 2002; Pendall et al., 2010) or less important (Moxley and Smith, 1998) sinks for atmospheric CO than native forests. An unanswered question is whether variance of CO uptake rates observed in soil was due to change in CO-oxidizing bacteria populations in term of density, specific activity or diversity. Very few field studies combined CO uptake rate measurements with molecular survey of CO-oxidizing bacteria, impairing a clear assessment of the environmental control on their distribution and activity. One notable exception is an extensive survey of CO-oxidizing bacteria accomplished along volcanic deposits, demonstrating a gradient in community structure parallels to CO uptake activity (Weber and King, 2010a). Here, we seek to examine the occurrence of such microbial succession in three neighboring land-use types. The sampling strategy as well as replication of *coxL* gene libraries and physicochemical analyses were essential to assess the spatial distribution of CO-oxidizing bacteria in the surveyed ecosystems with confidence, in addition to identify the environmental factors best explaining their distribution (Prosser, 2010).

Although conversion of the maize plantation to larch monoculture 15 years ago resulted in significant changes in soil physicochemical properties (**Table 1**), it exerted no significant impact on CO uptake activity and *coxL* diversity. However, higher activity and distinct CO-oxidizing bacteria community structure were observed in deciduous forest soil, which emerged from fallow land without human intervention. CO uptake rates reported in **Table 1** were in the same magnitude than the 0.3–50 nmol $g_{(dw)}^{-1}$ $h^{-1}$ observed in temperate forest soil samples exposed to atmospheric CO (King, 1999a; Hardy and King, 2001). Soil carbon and nitrogen content were the best variables to explain variations of CO uptake activity. Even though such relationships have been observed in previous investigations, the mechanistic aspects of the simulation of CO uptake activity by soil nutrients have received little attention. In the case of nitrogen, correlation does not imply causation since previous investigations excluded nitrogen limitation of the activity. Indeed, ammonium soil amendments caused no influence on CO uptake rate, while nitrite addition resulted in transient inhibition of the activity (King, 1999a; Chan and Steudler, 2006). On the other hand, two main mechanisms have been proposed to explain how soil carbon content enhances CO uptake activity. Considering the fact that soil carbon content determines microbial biomass and soil respiration activity, it was first proposed that higher soil carbon content supported more abundant communities of CO-oxidizing bacteria (Inman et al., 1971; Moxley and Smith, 1998; King, 1999a). Secondly, an increase of the relative importance of *coxL* OTU to 16S rRNA gene OTU ratio as a function of soil organic carbon has been noticed, suggesting that soil carbon enhances diversity of CO-oxidizing bacteria relative to the whole microbial population in soil, resulting in an alteration in CO uptake activity (Weber and King, 2010a). Variation of the abundance and community structure of CO-oxidizing bacteria in response to carbon content in soil are likely induced by the occurrence of a larger pool of CO in organic rich soils, due to abiotic CO production reactions resulting from thermal- and UV irradiation-mediated soil organic matter decomposition (Conrad and Seiler, 1985; Sanhueza et al., 1998; Derendorp et al., 2011). Therefore, considering that low pH and high carbon content are known to promote CO production in soil (Moxley and Smith, 1998; King, 1999a), sampled deciduous forest may represent a more favorable niche for CO-oxidizing bacteria relative to maize and larch monocultures, resulting in the distribution of *coxL* sequences and CO uptake activities measured in this study.

The occurrence of *coxL* gene sequences belonging to type I and type II groups has been documented in forest, agricultural soils, and volcanic deposits. Investigations undertaken in volcanic deposits showed that type I-*coxL* diversity was correlated to soil respiration and CO uptake activity, while no significant correlation was found for type II-*coxL* sequences (Dunfield and King, 2005). This observation suggested that microbes belonging to type I and type II groups responded differently to environmental factors. Our analysis extends this proposal and unveils that type I-*coxL* sequences are better proxy for soil CO uptake activity than those encompassing the type II clade. The relative proportion of type I sequences was higher in deciduous forest soil demonstrating the highest CO uptake activity (**Figure 4A**), while maize and larch monocultures comprised higher proportion of *coxL* sequences belonging to type II-CODH. This was further supported by the qPCR assay, showing a direct link between type I-*coxL* gene number and CO uptake activity (**Figure 7B**). These observations, combined with experimental evidence obtained in previous investigations, question the physiological role of the hypothetical type II-CODH in bacteria and the relevance of this genetic marker for CO uptake activity. In contrast to type I-CODH, functional type II-CODH remains to be experimentally demonstrated. The best characterized type I-CODH is the enzyme from *Oligotropha carboxidovorans*, a carboxydotrophic bacterium unable to oxidize atmospheric CO due to its low affinity for this substrate (Conrad et al., 1981). Nevertheless, this classical CODH model unveiled critical features on genetic regulation and architecture of the active site (Santiago et al., 1999; Dobbek et al., 2002). Functional type II-CODH was proposed following the PCR-detection of type II *coxL* (and no detection of type I-*coxL*) in *Aminobacter* sp. COX, demonstrating high affinity CO-uptake activity (King, 2003a). The involvement of type II-CODH in CO oxidation reaction is however puzzling since the canonical cysteine residue accommodating the copper atom directly involved in the CO oxidation catalysis (Dobbek et al., 2002) is replaced by a glycine residue in these hypothetical enzymes. Furthermore, characterization of CO oxidation activity in marine *Roseobacter* spp. revealed that strains harboring type II-*coxL* only were not active (Cunliffe, 2010). Genetic investigations are mandatory to assess the physiological role of type II-CODH, but *coxL* sequences belonging to functional type I-CODH phylogenetic group appear more relevant to predict CO uptake activity in soil.

Previous soil survey for CO-oxidizing bacteria realized along volcanic deposits, agricultural areas and forests unveiled dominance of type I-*coxL* sequences belonging to $\alpha$-, $\beta$-Proteobacteria, *Actinobacteria*, and *Chloroflexi*, with each group represented by strains for which CO uptake activity has been demonstrated (King and Weber, 2007). The $\alpha$-Proteobacteria cluster comprises the model CO-oxidizing bacterium *Oligotropha carboxidovorans* able to grow using CO as only carbon source, as well as *Bradyrhizobium*, *Roseobacter*, *Ruegeria*, and *Stappia* representatives. Among these, *Stappia* isolates displayed a high affinity CO oxidation activity and thus, the ability to oxidize ambient and sub-ambient levels of CO (Weber and King, 2007). *Bradyrhizobium*, *Roseobacter*, and *Ruegeria* representatives were also shown to oxidize CO, but their affinity for CO has not been reported (King, 2003a; Tolli et al., 2006; Cunliffe, 2010). Oxidation of atmospheric CO in $\beta$-Proteobacteria mainly has been examined in *Burkholderia*. Metabolism of CO was unevenly distributed in this genus, with more prevalence in strains thriving in the rhizosphere, and CO oxidation rates were shown to be higher when heterotrophic growth substrates were limiting (King, 2003a; Weber and King, 2012). *Actinobacteria* were also shown to oxidize atmospheric CO, with mycobacterium as the most extensively studied group (King, 2003b; Song et al., 2010; Kim and Park, 2012). Finally, recent investigations demonstrated that capacity for CO uptake is a common trait among the *Ktedonobacteria*, in agreement with the detection of *coxL* sequence affiliated to this taxonomic group in cinder volcanic deposits (Weber and King, 2010a; King and King, 2014). These observations suggest that carboxydovore bacteria responsible for the measured CO uptake activity harbored the type I-*coxL* sequences detected in this study. Comparison of our analysis with previous investigations combining type I-*coxL* and CO uptake activity analysis suggests that environmental conditions select different groups of carboxydovores in soil. Indeed, analysis of a vegetation chronosequence in Hawaii highlighted an increase in $\beta$-Proteobacteria-*coxL* sequences in sites characterized by higher CO uptake activity, suggesting the importance of this taxonomic group for CO uptake activity (Weber and King, 2010a,b; King and King, 2014). It was proposed that CO-oxidizing *Burkholderia* spp. were favored with plant development, benefiting of root exudates for growth and elevated CO levels as energy source in the rhizosphere (King and Crosby, 2002; Weber and King, 2012). In this study, assignation of type I-*coxL* clone sequences to taxonomic groups showed a higher relative abundance of $\delta$-Proteobacteria-*coxL* sequences in deciduous forest showing the maximal CO uptake activity (**Figure 4B**). Rare sequences affiliated to this cluster were detected in bare soil of volcanic deposits, with *H. ochraceum* as the closest cultivated relative (Weber and King, 2010a). We confirmed the ability of *H. ochraceum* to oxidize atmospheric CO, but the origin of detected $\delta$-Proteobacteria-*coxL* sequences remains unknown as they share less than 75% similarity score with *H. ochraceum*. Although this carboxydovore is halophile, myxobacteria related to this genus are diverse and were detected in recent soil metagenomic surveys (Luo et al., 2014; Zhou et al., 2014). Myxobacteria are ubiquitous in soil and are characterized by the formation of fruiting bodies enclosing stress-resistant myxospores structures as well as their ability to metabolize recalcitrant carbon macromolecules and feed on prey microorganisms through exoenzyme secretion (Reichenbach, 1999; Dawid, 2000). As no other genome sequence of $\delta$-Proteobacteria was shown to harbor *coxL* gene sequence in our genome data mining, isolation of more representatives within this taxonomic group deserves peculiar attention to investigate their contribution in the biogeochemical cycle of CO.

This article provides the first absolute abundance of type I-*coxL* sequences in soil. The abundance of type I sequences determined in this study was higher than the $10^8$ genes $g^{-1}$ reported in volcanic deposits using a qPCR assay specific to *Burkholderia* (Weber and King, 2010b). Analysis of cell-specific CO oxidation activity in three selected carboxydovores was undertaken to assess reliability of the qPCR assays. There was a general agreement

with the *coxL* gene numbers and theoretical populations of carboxydovores bacteria necessary to explain the CO uptake activity measured in soil. The broad range in theoretical population predictions was explained by variance in specific CO oxidation activities, varying from 29 to 2171 zmol cfu$^{-1}$ h$^{-1}$ among the tested isolates. Even though potential variability induced by the formation of cfu from cell aggregates cannot be excluded, similar variations were observed in previous comparison of CO uptake activity in carboxydovore bacteria. Indeed, activity measured in axenic cultures of *Stappia* sp. and *Stenotrophomonas* sp. varied between 6 and 100 μg CO mg$^{-1}_{(protein)}$ h$^{-1}$ (King, 2003a). Substrate affinity, cell physiology and metabolic activity are potential explanations for such variability in specific activity estimates (Knief and Dunfield, 2005) and will need more attention in future investigations to address how CO shapes microbial communities in the environment. Gene libraries suggested a more pronounced enrichment of carboxydovores belonging to δ-Proteobacteria in deciduous forest relative to both monocultures, but a qPCR assay targeting this specific subpopulation contradicted this observation. Incongruence of theoretical populations of carboxydovores and the abundance of δ-Proteobacteria-*coxL* sequences estimated by qPCR in maize plantation highlights the fact that carboxydovores belonging to this class cannot be used as an universal proxy for CO uptake activity in soil, due to the response of CO-oxidizer to their environment resulting in the dominance of different taxonomic groups of carboxydovores in contrasting ecosystems (Dunfield and King, 2005; King et al., 2008; Weber and King, 2010a). Considering this observation, we recommend the broader qPCR assay we developed, targeting the whole type I-*coxL* cluster, to test the relevance of this molecular marker in predicting CO uptake activity in soil for future investigations. These additional efforts, including samples displaying a broad range of CO uptake activity, are necessary because the regression analysis reported in **Figure 7B** was largely supported by the high CO uptake activity and *coxL* abundance in deciduous forest.

In conclusion, this work demonstrates the non-random distribution of CO-oxidizing bacteria in contrasting ecosystems, with land-use as a driver of diversification for this functional group. We showed that composition and abundance of CO-oxidizing bacteria community structure reflected CO uptake activity in soil. The combination of two complementary methodological approaches applied to independent soil surveys provides strong support and confidence to these observations. In contrast to the functional type I-CODH, the physiological role of type II-CODH remains to be defined as their distribution does not appear directly linked to CO uptake activity in soil and CO-oxidizing bacteria. Although this study was limited to three ecosystems, the soil survey resulted in the development of a reliable qPCR assay targeting presumptive CO-oxidizing bacteria in soil. A more extensive survey, including more ecosystem types is however necessary to challenge this quantitative indicator to predict CO oxidation rate in the environment. Finally, in addition to describe diversity of carboxydovore bacteria, this work suggests this functional group represents a significant proportion of soil microbiota. For instance, density of high affinity $H_2$-oxidizing bacteria responsible for 80% of the global loss of atmospheric $H_2$ is typically between $10^6$ and $10^8$ cells g$^{-1}_{(soil-dw)}$, as estimated by qPCR targeting the gene *hhyL* specifying the large subunit of their high affinity hydrogenase (Constant et al., 2011b). These microorganisms compensate their low abundance by a much higher cell specific activity than carboxydovores, oxidizing $H_2$ at a rate of 2–3 amol cfu$^{-1}$ h$^{-1}$ in some streptomycetes (Constant et al., 2011a). Because carboxydovores are abundant and taxonomically diverse, they should exert a significant impact on soil microbiota and biological processes. Future work thus should focus on the interactions of CO-oxidizing bacteria with microorganisms involved in other globally important biogeochemical functions. In addition to alter global budget of atmospheric CO, alteration of the distribution and activity of this functional group may have significant impacts on ecosystem services.

## AUTHOR CONTRIBUTIONS

Liliana Quiza and Isabelle Lalonde performed the experiments. Liliana Quiza, Isabelle Lalonde, and Claude Guertin participated to manuscript redaction. Claude Guertin introduced Philippe Constant to the sampling site. Philippe Constant designed the research and wrote the article.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00271/abstract

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# Anaerobic carboxydotrophic bacteria in geothermal springs identified using stable isotope probing

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Carbon monoxide (CO) is a potential energy and carbon source for thermophilic bacteria in geothermal environments. Geothermal sites ranging in temperature from 45 to 65°C were investigated for the presence and activity of anaerobic CO-oxidizing bacteria. Anaerobic CO oxidation potentials were measured at up to 48.9 μmoles CO $g^{-1}$ (wet weight) $day^{-1}$ within five selected sites. Active anaerobic carboxydotrophic bacteria were identified using $^{13}CO$ DNA stable isotope probing (SIP) combined with pyrosequencing of 16S rRNA genes amplified from labeled DNA. Bacterial communities identified in heavy DNA fractions were predominated by *Firmicutes*, which comprised up to 95% of all sequences in $^{13}CO$ incubations. The predominant bacteria that assimilated $^{13}C$ derived from CO were closely related (>98% 16S rRNA gene sequence identity) to genera of known carboxydotrophs including *Thermincola*, *Desulfotomaculum*, *Thermolithobacter,* and *Carboxydocella,* although a few species with lower similarity to known bacteria were also found that may represent previously unconfirmed CO-oxidizers. While the distribution was variable, many of the same OTUs were identified across sample sites from different temperature regimes. These results show that bacteria capable of using CO as a carbon source are common in geothermal springs, and that thermophilic carboxydotrophs are probably already quite well known from cultivation studies.

**Keywords: carboxydotrophs, stable isotope probing, geothermal, carbon monoxide (CO), thermophile**

## Introduction

Carbon monoxide (CO) is an odorless gas toxic to many animals due to its competitive binding to hemoglobin (Haab, 1990). It has been estimated that about $3.3 \times 10^9$ metric tons of CO are released annually to the atmosphere (Conrad, 1996). There are numerous natural biogenic and abiogenic sources of CO. Thermal decomposition and photochemical degradation of organic compounds are important sources of abiotic CO (Sipma et al., 2006). CO is also a component of volcanic emissions, which may contain as much as 1–2% of CO per volume of total gas (Giggenbach, 1980; Svetlichny et al., 1991a; Sokolova et al., 2009 and references therein). Biogenic CO may also be produced in microbial ecosystems, and net CO production has been reported for marine algae (Conrad, 1988) and hypersaline cyanobacterial mats (Hoehler et al., 2001) during photosynthesis. Sulfate-reducing bacteria (SRB) have also been shown to produce CO during fermentation (Voordouw, 2002). Some microbes growing in high temperature environments are likely capable of growth at low concentrations of CO (Sokolova et al., 2009). It has also been suggested that CO-oxidizing microbes

may occupy micro-niches in which biogenic CO locally accumulates to high levels (Techtmann et al., 2009).

Microorganisms that have the ability to oxidize CO are termed "carboxydotrophs" (King and Weber, 2007). A number of aerobic and anaerobic bacteria as well as some anaerobic archaea (e.g., methanogens) are capable of using CO as a source of energy and/or carbon (e.g., Mörsdorf et al., 1992; Oelgeschläger and Rother, 2008; Sokolova et al., 2009). Carboxydotrophic energy generation employs the enzyme CO dehydrogenase (CODH) that oxidizes CO to $CO_2$, generating electrons. The aerobic and anaerobic versions of this enzyme differ. Anaerobic CODH in bacteria is encoded by *cooS* genes (Techtmann et al., 2011) and contains nickel in the active site, while aerobic CODH is encoded by *cox* genes and contains molybdenum (e.g., Dobbek et al., 2001; King and Weber, 2007). Within the Domain *Bacteria*, anaerobic carboxydotrophs are typically found within the phylum *Firmicutes* and some in the class *Alphaproteobacteria* of the *Proteobacteria* (see Techtmann et al., 2009). Purple non-sulfur bacteria (i.e., phototrophic *Alphaproteobacteria*) exist among the known anaerobic CO oxidizers and were among the first discovered (Uffen, 1981; Kerby et al., 1995). However, an increasing number have been identified that are strictly anaerobic thermophiles belonging to the phylum *Firmicutes* (e.g., Svetlichny et al., 1991b; Sokolova et al., 2002). Hydrothermal systems have been proposed as early ecosystems supporting chemolithotrophic life, including thermophilic anaerobic bacteria and archaea using CO as an energy and carbon source (e.g., Cavicchioli, 2002; Wächtershäuser, 2006; King and Weber, 2007). Examples of thermophilic archaea that use CO include *Thermococcus* sp. NA1, capable of both heterotrophic and carboxydotrophic growth (Lee et al., 2008) and *Archaeoglobus fulgidus* capable of using CO as an autotrophic growth substrate (Henstra et al., 2007a).

While the anaerobic oxidation of CO may be coupled to a variety of respiratory processes such as sulfate reduction and acetogenesis (Oelgeschläger and Rother, 2008), hydrogenogenic carboxydotrophs make up the majority of thermophilic CO oxidizing microbes that have been identified in geothermal environments (e.g., Svetlichny et al., 1991a,b; Sokolova et al., 2004, 2005; Slepova et al., 2006). These bacteria oxidize CO via the water-gas-shift reaction (Uffen, 1981; Sipma et al., 2006):

$$CO + H_2O \rightarrow CO_2 + H_2 \quad (\Delta G^0 = -20\text{kJ}) \qquad (1)$$

Thermophilic bacteria and archaea with the capacity for hydrogenogenic carboxydotrophy have been isolated from various locations around the world including the Kunashir Island, Russia (Svetlichny et al., 1991a), Kamchatka (Sokolova et al., 2002; Slepova et al., 2006), Yellowstone National Park (Sokolova et al., 2004), and Iceland (Novikov et al., 2011). The isolates share similar ranges of optimal pH (ca. 6.8–7.0) and temperature (ca. 55–83°C) (see Henstra et al., 2007b; Techtmann et al., 2009) but are phylogenetically divergent (Techtmann et al., 2009). Unlike mesophilic carboxydotrophs, the thermophilic hydrogenogenic species isolated so far do not show growth inhibition by high levels of CO. In fact, most grow under atmospheres of 100% CO, far above natural CO concentrations in geothermal systems (e.g., Svetlichny et al., 1991a,b).

CO oxidizing thermophiles are of potential biotechnological interest for the anaerobic fermentation of synthesis gas ("syngas"). Syngas is a product comprised mostly of $H_2$, CO, and $CO_2$ resulting from the high temperature gasification of waste biomass, into higher-value bioalcohol fuel (Henstra et al., 2007b). As syngas is produced at high temperatures, bacteria from geothermal sites are of particular interest due to the expected high rates of substrate conversion at high temperatures. Characterization of anaerobic CO-oxidizing bacteria in geothermal systems therefore could provide fundamental information about the natural diversity of thermophilic carboxydotrophs available for these biotechnological applications. DNA-Stable Isotope Probing (SIP) is a valuable tool in assessment of functional bacterial groups. It has been used in a variety of environments to identify active consumers of substrates such as methane (He et al., 2012; Sharp et al., 2012, 2014b). There are however some difficulties in applying SIP to identify carboxydotrophs. Firstly, while some carboxydotrophic bacteria directly incorporate CO-carbon into the carboxyl group of acetate via acetyl-CoA synthases (ACSs) using the Wood-Ljungdahl pathway (e.g., Henstra et al., 2007b), others incorporate the $CO_2$ produced from CO oxidation as the direct source of cellular carbon, using reductive $CO_2$ pathways such as the Calvin-Benson-Bassham (CBB) Cycle, or the reverse tricarboxylic acid (TCA) cycle (Uffen, 1981; Ragsdale, 1991, 2004; Berg, 2011). As such, $CO_2$ present in the atmosphere may be incorporated rather than the $CO_2$ produced directly from the oxidation of CO diluting the labeling effect. Another issue with CO-SIP is that the products of CO oxidation, i.e., $H_2$ and $CO_2$, may result in labeling of other autotrophs. Cross-feeding is a caveat in any SIP experiment, but the severity of the cross-feeding, especially via $CO_2$, can often be assessed with controls such as the addition of exogenous $^{12}CO_2$ (e.g., Sharp et al., 2012). CO-SIP will also not detect carboxydoheterotrophs that may oxidize CO but use a different carbon source. Therefore, in this study we assessed the value of CO-SIP to identify anaerobic thermophilic carboxydoautotrophic bacteria in some geothermal springs.

## Materials and Methods

### Geothermal Spring Sample Collection

Five geothermal springs were selected for CO-SIP investigations from among geothermal sites in Western Canada (Grasby et al., 2000; Sharp et al., 2012). Soil or sediment or biomat samples were collected at various times of the year between fall 2010 and fall 2012 into sterile screw-cap tubes (**Table 1**). Samples were kept cold as soon as possible to minimize changes in the microbial community during transport. Collected material was sub-sampled within approximately 5 days of sampling for DNA extraction, and the remainder was stored at 4°C for 1–2 d prior to incubation studies.

### Soil Microcosms and CO Oxidation

Approximately 2–5 g of sample material (wet weight) was added to 120-ml serum bottles and crimp-sealed with sterile blue butyl rubber stoppers. An anoxic environment was created by repeated (3×) evacuation and refilling with $N_2$ gas. CO was

**TABLE 1 | Name of geothermal spring, measured *in situ* pH and temperature, and anaerobic CO-oxidation potentials for sites included in the current study.**

| Geothermal site | Sample ID | Description | Environmental temperature (°C) | Incubation temperature (°C) | pH | CO oxidation potentials (μmol CO $g^{-1}$ $d^{-1}$) |
|---|---|---|---|---|---|---|
| Dewar Creek | DCm2010 | biomat | 54.9 | 55 | 8.30 | 20.4 ± 1.8 |
| | DCmN11 | biomat | 45.0 | 45 | 8.30 | 24.6 ± 1.1 |
| | DCs9 | sediment | 64.7 | 65 | 7.94 | 48.9 ± 5.4 |
| Lakelse | L3 | organic rich sediment | 45.1 | 55 | 8.27 | 13.6 ± 2.7 |
| Grayling River | GR1 | sediment | 56.1 | 55 | 7.02 | 18.6 ± 4.2 |
| Liard | Liard2 | biomat | 53.5 | 55 | 6.76 | 30.2 ± 1.0 |
| Portage Brûlé | PB1 | biomat | 45.9 | 45 | 6.34 | 35.7 ± 3.7 |

*Oxidation potentials represent duplicate incubations and are listed in μmol CO $g^{-1}$ (wet weight) $d^{-1}$ with one s.d.*

added to final mixing ratios of 5–10% in the headspace to assess oxidation potential and act as a $^{12}C$ incubation for stable isotope probing (SIP) experiments. For SIP experiments, labeled gases $^{13}CO$ (99 atom % $^{13}C$, Sigma Aldrich) and $^{13}CO_2$ (99 atom % $^{13}C$, Sigma Aldrich) were used at mixing ratios of 10% v/v, both separately and in combination with non-labeled CO and $CO_2$ gases in different SIP trials. "Control" is used to refer to the un-incubated (i.e., no CO or $CO_2$ added) environmental samples. Microcosms were incubated at close to environmental temperatures (**Table 1**). Headspace CO was monitored at ca. 1-d intervals using a Varian 450-Gas Chromatograph equipped with a 0.5-m Hayseep N and a 1.2-m Mol Sieve 16X column in series coupled to a Thermal Conductivity Detector (GC/TCD). Potential production of methane was monitored using a GC-Flame Ionization Detector (FID). Incubations proceeded until approximately 95–100% of the added CO had been consumed, typically within a week. Samples were then harvested and frozen immediately at −85°C. CO consumption rates were based on duplicates of any sample ($^{12}CO$ and $^{13}CO$ only incubations), while duplicate SIP experiments were performed only in some cases.

## DNA Extraction and Density Fractionation

DNA was extracted from approximately 500 mg of sample using the FastDNA Spin Kit (MP Biomedicals) with the addition of washing steps using guanidine thiocyanate (Knief et al., 2003). Quantification was performed using the Quant-iT™ dsDNA HS Assay Kit (Invitrogen) and extracted DNA was stored at −20°C prior to ultracentrifugation separation. Heavy and light DNA were separated by density gradient ultracentrifugation using cesium chloride (CsCl) as described by Neufeld et al. (2007), with minor modifications. Five hundred nanograms to one microgram of total DNA was typically used for each SIP assay. To account for any variability in DNA distribution patterns that may arise due to using inconsistent amounts of DNA in CsCl gradients, within an individual sample set (i.e., all trials from the same sample site) similar total DNA amounts were used. Centrifugation and gradient fractionation were performed as described by Sharp et al. (2014b). DNA was precipitated from each fraction using polyethylene glycol (PEG) and glycogen as in Neufeld et al. (2007). DNA present in each density gradient was quantified using the Quant-iT™ dsDNA HS Assay Kit (Invitrogen). As the focus of this paper is assessing bacterial rather than archaeal carboxydotrophy, 16S rRNA gene PCR assays for each fraction were set-up using a QIAgility (v. 4.13.5) with bacterial specific primers 519f and 907r (Stubner, 2002). 16S rRNA gene copies were quantified on a Rotor-Gene Q (Qiagen) as in Sharp et al. (2012).

## Microbial Community Analysis

The density gradients of DNA extracted from incubations with $^{13}C$-labeled vs. $^{12}C$-labeled substrates were compared. Heavy SIP fractions with increases in the relative amounts of DNA and/or 16S rRNA gene copies were selected for pyrosequencing analysis of the 16S rRNA gene. "Light" fractions (density ca. 1.690 g $ml^{-1}$) from $^{13}CO$ incubated samples were also analyzed in some cases for comparison to heavy fractions (Table S1). Where possible, DNA was also amplified from the corresponding heavy fractions from untreated controls (**Figure 2**, Table S1). However, in some cases the amount of DNA present in the fractions was below detection, or too low to obtain enough for pyrosequencing (e.g., DCm2010) (Supplementary Figure S1). Samples were prepared for sequencing analysis as described previously (Grasby et al., 2013; Sharp et al., 2014a) using FLX Titanium amplicon primers 454T_RA_X and 454T_F, which contain 16S rRNA gene targeted primers 926fw (5′-aaactYaaaKgaattgRcgg-3′) and 1392r (5′-acgggcggtgtgtRc-3′) designed to target both bacteria and archaea (Ramos-Padrón et al., 2011). PCR reactions and purification were performed as described in Sharp et al. (2014a). Purified PCR products (ca. 150 ng total DNA) were analyzed at the Genome Quebec and McGill University Innovation Centre, Montreal, Quebec on a 454 Life Sciences Genome Sequencer FLX (Roche) machine running the Titanium chemistry.

## Sequence Data Processing

Quantitative Insights Into Microbial Ecology (QIIME) pipeline version 1.8 (Caporaso et al., 2010) was used to process raw sequence data as in Sharp et al. (2014a). A minimum quality score of 25 was used and sequences were screened using ChimeraSlayer (Haas et al., 2011). Taxonomic identification of a representative sequence (most common) for each phylotype (clustered at 97% similarity) was determined using nucleotide

Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) against the Silva 111 reference database (Pruesse et al., 2007). Eukaryotic and chloroplast sequences were removed from further analysis. Final numbers ranged from 2195 to 21118 sequences per sample (Table S1). A phylogenetic tree was constructed using the parsimony-add function in ARB (Ludwig et al., 2004).

16S rRNA gene sequences obtained from this study have been deposited in the SRA database under accession numbers SRP028305 and SRP059036. Representative sequences of identified OTUs present at 25 fold enrichment are provided in the Supplementary Material.

## Results

### Biodegradation of Carbon Monoxide

Anaerobic oxidation of carbon monoxide was detected via monitoring of the CO mixing ratio in serum bottle headspaces after adding CO. Communities that showed evidence for CO consumption were selected for further investigation using stable isotope probing (SIP). The average values for anaerobic CO oxidation potentials for each sample is listed in **Table 1**. Rates ranged from 13.6 to 48.9 μmol CO $g^{-1}$ $d^{-1}$. The fastest rates were observed in samples from the Dewar Creek hot spring. Despite the anaerobic conditions, no methane production was observed in sample sites GraylingRiver1, Liard2, and PortageBrûlé1 during any incubation and only minor amounts were observed in other Dewar Creek and Lakelse samples, with estimated $CH_4$ production rates of 0.013–11.4 nmol mol $day^{-1}$ $g^{-1}$. The amount of $CH_4$ produced corresponds to ca. <0.1% of the added CO being converted to methane. One exception was a Lakelse sample that produced more total $CH_4$ (∼ 18 μmol) than CO added, indicating other substrates were present for methanogenesis.

### Identification of Active CO Consuming Bacteria Using SIP Combined with 16S rRNA Gene Sequencing

Density profiles of DNA extracted from samples after incubation with $^{13}CO$ generally ranged from 1.660 to 1.800 g $ml^{-1}$. Shifts in DNA density compared to un-amended control samples were often subtle (Supplementary Figure S1). Therefore, quantitative real-time PCR (qPCR) counts of total bacterial 16S rRNA gene abundance were used to identify density fractions that had an increase in the relative number of gene copies (**Figure 1**, Supplementary Figure S2).

Representative profiles of 16S rRNA gene copies vs. DNA density for GraylingRiver1 (GR1) and PortageBrûlé1 (PB1) SIP experiments are shown in **Figure 1**. The number of 16S rRNA gene copies in the control environmental communities peaked in the density range of 1.690–1.700 g $ml^{-1}$. Incubations using $^{12}CO$ showed the same peaks, demonstrating that incubation with CO itself had little effect on the overall DNA density profile of the community. However, incubation of GR1 with $^{13}CO$ resulted in a marked shift in 16S rRNA gene copies toward heavier fractions, indicating assimilation of the $^{13}C$ from $^{13}CO$ into DNA. In contrast, no significant shift in DNA density or 16S rRNA gene copies was observed over multiple incubations of the PB1 sample under $^{13}CO$. Although this sample also oxidized CO, no assimilation of $^{13}C$ was evident. These two samples were representative of the two major patterns observed. Other samples analyzed are shown in Supplementary Figures S1, S2.

A fundamental issue with interpreting SIP results is cross-labeling of other bacteria via metabolic products, especially $CO_2$. The severity of this problem can be estimated via several controls. In GR1 (**Figure 1A**), as well as in several other samples tested (Supplementary Figures S1, S2) incubations with only $^{13}CO_2$ and no added CO always showed very minor density shifts in DNA and 16S rRNA gene counts. This indicated that other autotrophs

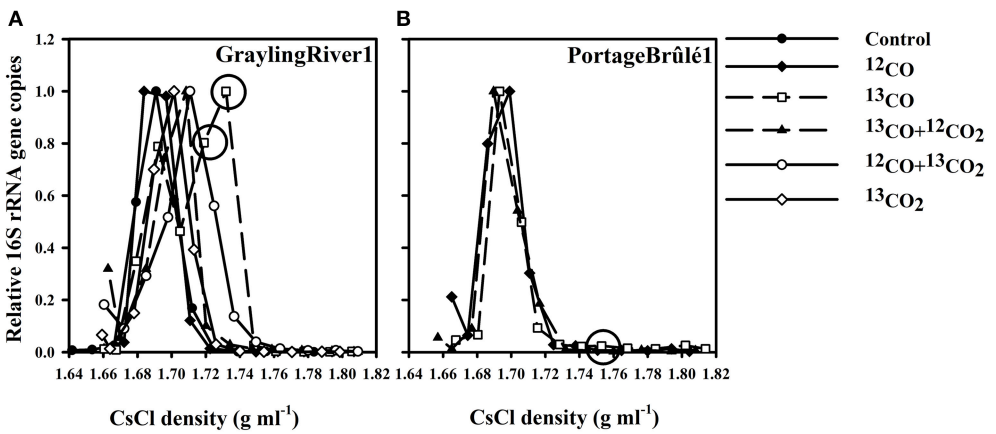


**FIGURE 1 | (A)** Representative 16S rRNA gene counts vs. DNA density for SIP experiments using GraylingRiver1 sediment, illustrating the shifts in relative 16S rRNA gene copies associated with different incubations. The incubations using only $^{13}CO$ as the sole carbon and energy source showed the greatest increase in the relative number of 16S rRNA gene copies in heavy fractions at densities of approximately 1.730 g $ml^{-1}$. DNA from these heavy fractions was used for 16S rRNA gene pyrosequencing. **(B)** Relative 16S rRNA gene copies from PB1 sediment showing no density shift with $^{13}CO$ incubations despite active CO oxidation. Circles indicate $^{13}CO$ incubation fractions used for 16S rRNA gene pyrosequencing analyses. "Control" represents the un-incubated (no CO or $CO_2$ added) environmental sample.

growing on substrates such as sulfur and ammonia were of minor importance. CO was therefore the primary energy source in the incubations, and the food webs detected were ultimately based on CO oxidation.

In samples like GR1, microcosms containing $^{13}CO$ as the sole carbon and energy source (with no $CO_2$ addition) displayed the greatest shift in density (**Figure 1**). Less of a shift in density was observed in incubation with $^{13}CO + {}^{12}CO_2$, probably because assimilation of $^{12}C$ from the added $CO_2$ diluted the labeling effect. Incubation with $^{12}CO + {}^{13}CO_2$ showed some increase in 16S rRNA gene copies compared to the un-incubated control, albeit to a lesser extent than in $^{13}CO$. The apparent assimilation of C preferably from $^{13}CO$ but also from $^{13}CO_2$ indicates that $CO_2$ was probably the primary C source of the CO oxidizers, but that there may be a diffusion effect whereby the $^{13}CO_2$ produced directly by a carboxydotroph from $^{13}CO$ is preferentially assimilated compared to exogenous $^{12}CO_2$ supplied in the atmosphere.

In the majority of cases, the "heavy" fractions that showed the greatest increase in 16S rRNA gene copies were at a density of ca. 1.730 g $ml^{-1}$ (**Figure 1**, Supplementary Figure S2). Heavy fractions that contained a large increase in DNA amounts and/or 16S rRNA gene copies as compared to an un-incubated, non-labeled control were selected for 16S rRNA gene sequencing. In some cases (e.g., DCm2010), the amount of DNA present in the corresponding high-density fractions of the unlabeled controls was too low to obtain enough for successful pyrotag sequencing despite inputs of similar total amounts of DNA (see Supplementary Figure S1).

Most predominant operational taxonomic units (OTUs) in the heavy fractions of all samples (i.e., most putative carboxydotrophs) belonged to the phylum *Firmicutes*, in particular to the class *Clostridia*. In heavy fractions recovered from $^{13}CO$ incubations that showed observable shifts in 16S rRNA gene copies, members of the phylum *Firmicutes* accounted for 31–95% of the reads (Table S1). One of the lowest % of *Firmicutes* was from GR1 which had a very high proportion of *Crenarchaeota* in both the original community and in all heavy fractions. However, of bacterial sequences only, *Firmicutes* accounted for 81.5% in the heavy fraction of GR1_$^{13}CO$. The proportions of top phyla (>1% of sequences) of both the un-amended environmental samples and the heavy fractions for two sites that showed strong DNA labeling are shown in **Figure 2**.

The taxonomic identifications of OTUs recovered from$^{13}CO$ microcosm heavy fractions that were present at 25 fold enrichment compared to the original environmental sample are shown in **Table 2**. A 16S rRNA gene phylogenetic tree was constructed using the top OTUs from each $^{13}CO$ heavy fraction showing an observable shift compared to reference sequences (**Figure 3**). Taxonomic identifications of OTUs present at >1% of all sequences are presented in Supplementary Table S2. Most of the putative carboxydotrophs identified belonged with >98% sequence identity to genera that include known CO-oxidizers, such as *Thermincola*, *Desulfotomaculum*, *Carboxydocella,* and *Thermolithobacter* (Sokolova et al., 2002, 2005, 2007; Parshina et al., 2005a,b). For example, the most abundant OTU (OTU_17948) recovered from $^{13}CO$ heavy fractions from Dewar Creek (DCm2010 and DCmN11) and Lakelse springs showed 99% sequence identity to both *Thermincola potens* and to *Thermincola carboxydiphila,* known CO-oxidizing bacteria (Sokolova et al., 2005; Byrne-Bailey et al., 2010) (**Table 2**). Members of the genus *Carboxydocella* were

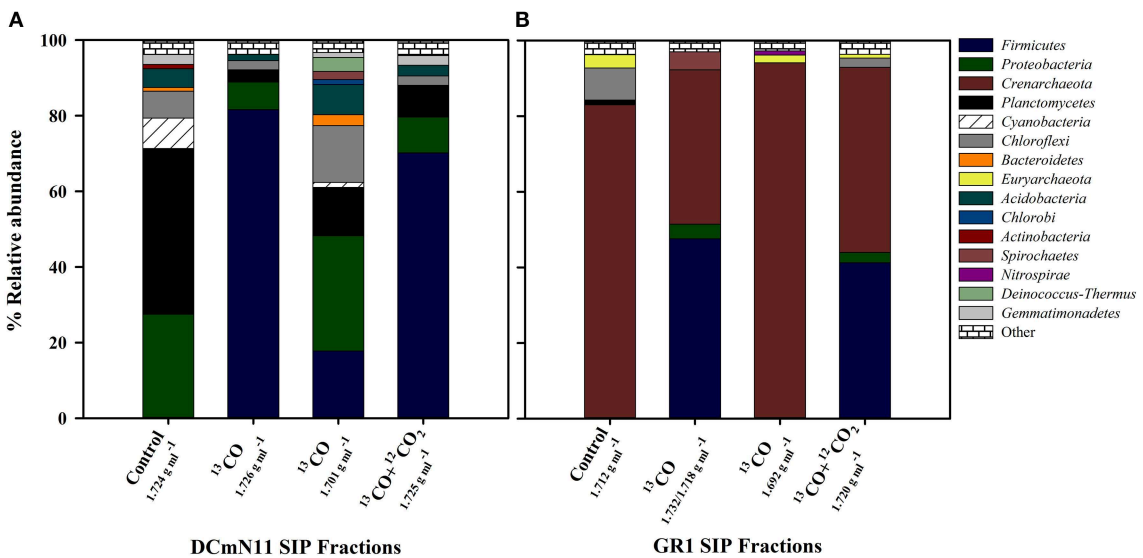


**FIGURE 2 | Relative abundance (%) of different phyla for SIP incubations showing results from heavy (density ca. 1.730 g $ml^{-1}$) fractions and light fractions (density ca. 1.700 g $ml^{-1}$) of samples incubated with $^{13}CO$ or $^{13}CO + {}^{12}CO_2$ compared to the community detected in heavy fractions of un-incubated Control samples (i.e., no CO or $CO_2$ added). (A)** DCmN11 showing significant increase in the proportion of *Firmicutes* in heavy fractions and **(B)** GR1 showing an increase in *Firmicutes* but also the large proportion of *Crenarchaeota* present in both the un-incubated environmental control sample and SIP fractions. 16S rRNA gene sequences were clustered at 97% similarity and classified using QIIME. "Other" includes phyla present at <1%.

**TABLE 2 | The percent of total sequences associated with top OTUs identified in heavy fractions of $^{13}$CO incubations.**

| OTU | Phylum | BLAST identification | Identity (%) | DCm2010 1.733 g ml$^{-1}$ | DCmN11 1.726 g ml$^{-1}$ | DCs9 1.740 g ml$^{-1}$ | L3 1.721 g ml$^{-1}$ | GR1 1.726 g ml$^{-1}$ | Liard2 1.734 g ml$^{-1}$ | PB1 0.753 g ml$^{-1}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| 3148 | *Firmicutes* | *Desulfotomaculum kuznetsovii/D. luciae* | 99 | | | 30.0 (n.d.) | | | | |
| 3442 | *Firmicutes* | *Thermolithobacter carboxydivorans* | 100 | | | 8.9 (n.d.) | | | | |
| 7600 | *Firmicutes* | *Carboxydocella thermautrotrophica* | 98 | 7.6 (n.d.) | 2.9 (n.d.) | 17.9 (0.0) | | 17.0 (n.d.) | 28.0 (n.d.) | |
| 9076 | *Firmicutes* | *Sporomusa sphaeroides* | 94 | | | | | | | 15. 7 (n.d.) |
| 12486 | *Firmicutes* | *Caloramator australicus* | 99 | | | | 7.3 (n.d.) | | | |
| 14221 | *Firmicutes* | *Desulfurispora thermophila* | 97 | | 1.6 (n.d.) | | 7.5 (0.0) | | | |
| 17597 | *Firmicutes* | *Streptococcus thermophilus* | 100 | | | | | | | 26.7 (n.d.) |
| 17948 | *Firmicutes* | *Thermincola potens/ T. carboxydiphila* | 99 | 68.2 (n.d.) | 65.5 (n.d.) | | 48.4 (n.d.) | 3.9 (n.d.) | | |
| 17986 | *Firmicutes* | *Thermincola potens/ T. carboxydiphila* | 90 | | | 8.1 (n.d.) | | | | |
| 18478 | *Deinococcus-Thermus* | *Thermus scotoductus* | 100 | | | 5.4 (0.2) | | | | |
| 20883 | *Firmicutes* | *Candidatus* Desulforudis audaxviator | 93 | | | | | 15.1 (n.d.) | | |
| 21098 | *Proteobacteria* | *Azonexus caeni* | 98 | | | | | | | 19.3 (0.2) |

*The density of the heavy fraction analyzed is reported in g ml$^{-1}$. OTUs reported are those with a 25 fold enrichment compared to the original community in at least one sample site. The percent of total sequences for each OTU is reported with BLAST identification and percent sequence identity to the top cultured BLAST hit. Numbers in brackets represent the proportion (%) of sequences present in the same OTU in the original environmental sample. n.d., not detected.*

also detected in the majority of heavy fractions across all sites. This genus was represented by multiple OTUs, however the top OTU_7600 identified in most samples corresponded to *Carboxydocella thermautotrophica* (98% similarity). Members of the genus *Desulfotomaculum* were most predominant in DCs9, a sediment sample collected from Dewar Creek. In DCs9_$^{13}$CO incubations, 31.9% of sequences were attributed to the genus *Desulfotomaculum.* 30.0% of sequences were in OTU_3148, which showed 99% sequence identity to *D. kuznetsovii* and *D. luciae*.

Some OTUs identified may reflect bacteria with as yet unknown or unconfirmed CO-oxidizing capabilities. One such cluster, OTU_20883, present in both the GR1_$^{13}$CO and GR1_$^{13}$CO + $^{12}CO_2$ heavy fraction at 15.1 and 5.3% of sequences respectively, is 93% similar to *Candidatus* "Desulforudis audaxviator." Neither this OTU nor any others that showed any similarity to this bacterium were detected in the control environmental sample from this site. BLAST results for OTU_17986 representing 8.1% of the total for DCs9_$^{13}$CO returned equal results for *Thermincola potens* and *Thermincola carboxydiphila*. However, the sequence identity to both was only 90%, and it branches distantly from *Thermincola* in the phylogenetic tree (**Figure 3**). This may represent another related CO-oxidizing genus, the exact nature of which requires further study.

While PB1 and Liard2 sediments did oxidize CO, incubations with $^{13}$CO did not show any observable shift in 16S rRNA gene density profiles. Nevertheless, we did for comparison analyze the heavy fractions from these incubations. The heavy fraction of PB1 showed a slight increase in *Firmicutes* as compared to the proportion present in the original community (Table S1), however no OTUs with sequences >1% were associated with known CO-oxidizing bacteria (Table S2). In comparison, the heavy fraction from Liard2_$^{13}$CO had a number of OTUs associated with CO-oxidizing bacteria. Only one OTU was present at 25 fold enrichment and represented the top OTU of this fraction (**Table 2**). OTU_7600 had a 98% BLAST identity to *Carboxydocella thermoautotrophica.* It represented 28.0% of sequences and was the same *Carboxydocella* OTU found in other sites at relatively high abundance. Therefore, growth of carboxydotrophs was probably occurring in these samples as well, albeit at slow rates.

High G + C content may be responsible for the observation of some organisms in heavy fractions, including uncultured *Crenarchaeota* present in heavy fractions recovered from GR1_$^{13}$CO (**Table 2**). The G + C content of previously identified CO-oxidizing thermophiles ranges from ca. 40–48% (Svetlichny et al., 1991b; Sokolova et al., 2002, 2004, 2005), corresponding to a density range of 1.698–1.705 g ml$^{-1}$. Increased G + C content results in a higher buoyant density that may initially suggest

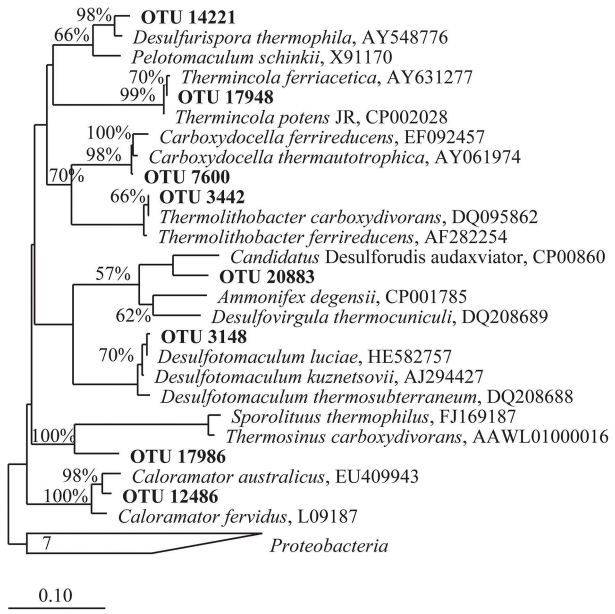


**FIGURE 3 | Phylogenetic tree of partial 16S rRNA gene sequences belonging to OTUs identified in heavy fractions from $^{13}$CO SIP incubations present at a minimum of 25 fold enrichment.** OTUs presented are from those incubations that showed an observable shift in density and are hypothesized to represent microbes that oxidized CO. A skeleton tree was constructed from near complete 16S rRNA gene sequences (>1400 bp) via Neighbor-joining with a Jukes-Cantor correction and 10,000 bootstraps. Shorter sequences produced via 454 pyrosequencing obtained in this study were added by parsimony using ARB (in bold). The scale bar represents 0.1 change per nucleotide position. Bootstrap support values greater than 55% for the major nodes are given. The tree was rooted using 7 *Proteobacteria* 16S rRNA gene sequences.

$^{13}$C incorporation (Schildkraut et al., 1962). However, the high proportions in un-amended and $^{12}$C controls of *Crenarchaeota* in the case of GR1 suggest that these microbes were not carboxydotrophs but rather are naturally present at that density due to relatively high G + C content.

## Discussion

In this study, bacteria potentially involved in the anaerobic oxidation of carbon monoxide were identified from hot spring environments using DNA-SIP. Five geographically diverse geological settings were identified in which potential anaerobic CO-oxidation was detectable. These five locations are widely dispersed geographically over an area of approximately 1 million km$^2$. The measured CO-oxidation potentials were variable between geothermal springs. Comparative rates from other environments are rarely reported, however CO-oxidation rates of 120 μmol l$^{-1}$ of sediment d$^{-1}$ were estimated in slurries from Uzon Caldera, Kamchatka (Kochetkova et al., 2011), and a $^{14}$CO tracer was used to estimate a rate of 40.75 nmol CO cm$^{-3}$ sediment d$^{-1}$ for another anaerobic hot spring community in Kamchatka (Slepova et al., 2007). While obtained following different methodology, these *in vitro* rate estimates are 2–4 orders of magnitude lower than those measured in the present study, and show that potential rates of CO oxidation may vary greatly between environments. The observation of little to no methane production in most samples was consistent with the negligible proportions of *Euryarchaeota* detected in the original environmental communities and the lack of archaeal sequences detected in heavy SIP fractions (e.g., **Figure 2A**).

Distinct differences were noted between the original microbial communities and the communities detected in $^{13}$C-labeled heavy DNA fractions for each sample. In most cases, *Firmicutes* were present at <1% in the original communities but increased in abundance in $^{13}$CO incubated heavy fractions, reaching up to ca. 95% of all 16S rRNA gene reads (Table S1). These results indicate that CO-metabolizing bacteria make up a relatively minor component of the overall population within these geothermal systems but are still present and may become active if CO is provided. Most of the bacteria identified in heavy fractions showed high identities (>98%) to known CO-oxidizing bacteria described from other geothermal springs, particularly *Carboxydocella* and *Thermincola* species (**Table 2**). This finding suggests not only that the CO-SIP procedure was successful in identifying primary carboxydotrophs without cross-feeding artifacts, but also that the predominant carboxydotrophic bacteria in geothermal environments may in fact already be well described from cultivation studies. This is a rather unusual finding for SIP experiments (e.g., Redmond et al., 2010). Geographically, it also indicates that anaerobic thermophilic carboxydotrophic bacteria are highly cosmopolitan (at least at the species/genus level), since most described isolates have been obtained from Russian geothermal sites. Aerobic carobyxdotrophy is taxonomically diverse (e.g., King and Weber, 2007). And while the presence of CO in geothermal spring emissions may suggest the potential for wide-spread CO metabolism, the current study supports the notion that the capacity for anaerobic carboxydotrophy among thermophiles is more limited.

While the most predominant bacteria identified via SIP were similar to carboxydotrophs isolated from other geothermal springs, there were a few exceptions. Among the predominant OTUs detected in heavy DNA fractions (**Table 2**), three OTUs showed <95% 16S rRNA gene sequence identity to any described species. For example, an OTU making up 15% of the heavy DNA fraction in sample GR1 had only a moderate similarity to the proposed genus "Desulforudis." *Candidatus* "Desulforudis audaxviator" was identified in the fracture water of a South African gold mine. This isolate has components of the Wood-Ljungdahl pathway and may be capable of CO oxidation and assimilation (Chivian et al., 2008). However, the low identity (93%) of our OTU indicates a genus-level divergence to Ca. "D. audaxviator."

While the use of 16S rRNA gene qPCR greatly improved the detection of shifts in density within the CsCl gradients, in general the observed shifts were subtle. Approximately 0.47 mmol of total $^{13}$C was added to each of the $^{13}$CO SIP incubations. However, previous studies show that relatively little of the CO oxidized microbially in geothermal habitats is incorporated into

biomass. Using radioisotope tracers to examine a hot spring community from Kamchatka, it was estimated that 85% of the $^{14}CO$ was oxidized to $CO_2$ while only 0.5% was used for cell biomass production. The remainder was distributed between dissolved organic matter and minor (0.001%) amounts of methane (Slepova et al., 2007). At 0.5% incorporation, a maximum of ca. 2.35 μmol of $^{13}C$ would have been incorporated into the bacteria identified in the current study and may explain why shifts in the density of labeled DNA were minor compared to SIP experiments using substrates such as $^{13}CH_4$ where more C is incorporated into the cells over a short incubation period (Dumont et al., 2011). The use of qPCR and the examination of the 16S rRNA gene copy profiles provided a means by which to identify subtle shifts in DNA density (Lueders et al., 2004; Sharp et al., 2012). The lack of observable shifts in 16S rRNA gene copies in one of our samples ($^{13}CO$-incubated PortageBrûlé) indicated that some geothermal communities may contain bacteria that are using CO as an energy source to maintain the population but are perhaps growing slowly, maybe due to other nutrient limitations, or more likely are incorporating other, possibly organic, C sources into biomass (**Figure 1B**). This does indicate limitations in the $^{13}CO$-SIP technique. While it appeared to be effective in identifying some carboxydotrophs, it cannot identify all of these metabolically diverse microorganisms including potential carboxydoheterotrophs.

The $^{13}CO$-SIP technique is also challenging because carboxydotrophs may incorporate $CO_2$ rather than CO directly, and because the initial products of CO-oxidation, $H_2$ and $CO_2$, may lead to labeling of other autotrophs. Our experiments included controls suggesting that these problems were minor. The greatest shifts in density were observed when $^{13}CO$ was provided as the sole carbon source (i.e., no extra $CO_2$ was added), but incubations with $^{13}CO$ + $^{12}CO_2$ showed smaller shifts in DNA density compared to $^{13}CO$ alone. Most likely, an initial oxidation of $^{13}CO$ to $^{13}CO_2$ via the gas-water shift reaction is followed by assimilation of the produced $^{13}CO_2$. Incubations with $^{13}CO_2$ alone (and no CO added) showed little or no apparent shifts in DNA density, indicating that labeling of autotrophs growing on substrates already present in the samples, such as sulfur or ammonia, was not an issue, and that CO was ultimately the primary energy source in the incubations. However, there is the distinct possibility of hydrogenotrophic organisms using the $H_2$ and $^{13}CO_2$ produced via the gas-water shift reaction. This particular form of cross-feeding cannot be eliminated from the current results, but lines of evidence suggest that it was minor: (1) Cross-feeding with $H_2$ may occur only in addition to primary CO oxidation- i.e., it can only be a secondary process given that CO was the major energy source available; (2) almost all of the detected bacteria in the present study were closely related to known carboxydotrophs; and (3) potentially hydrogenotrophic but non-carboxydotrophic bacteria such as the members of the phylum *Deinococcus-Thermus* were detected in some heavy DNA fractions, but were always of minor importance compared to known carboxydotrophs (e.g., *Thermus scotoductus* in DCs9, **Table 2**; Table S2). Complete genomes of both *T. scotoductus* and *T. antranikianii* (Table S2) lack genes related to CO metabolism (http://img.jgi.doe.gov/).

Many bacterial species that possess *cooS* genes have a primary metabolism that does not focus on CO, however their presence may imply a potential underlying or backup CO-dependent physiology should conditions vary and become more optimal for CO-oxidation (Techtmann et al., 2011). While challenging to measure *in situ*, localized accumulations of CO may create microniches within geothermal systems in which low abundance carboxydotrophic population members may thrive. For example, the species composition of the heavy fractions was similar for two biomat samples DCm2010 and DCmN11 with a dominance of *Thermincola potens* in both cases. The presence of CO oxidizing bacteria in these samples is perhaps not surprising given the observation of net CO production within microbial mats. Saline and intertidal sand flat photosynthetic microbial mats exhibited a net production of CO (3.1–5.4 μmol $m^{-2}$ $d^{-1}$) during daylight hours and were also observed to have a net production of $H_2$ (Hoehler et al., 2001). As many of the Dewar Creek samples that showed evidence of CO-oxidation were comprised of biomats with relatively high proportions of cyanobacteria (**Figure 2B**, Table S1), these results support CO as a potentially important carbon and energy source in microenvironments within geothermal systems. Variation in CO metabolizing bacteria was observed between samples collected from the same geothermal system but under different temperature regimes. OTU_3148 was 99% similar to both *Desulfotomaculum kuznetsovii* and *Desulfotomaculum luciae*, detected in $^{13}CO$ incubations of DCs9, another site from the Dewar Creek hot spring. *Desulfotomaculum kuznetsovii* is an obligate anaerobe and is capable of growth with CO as the sole carbon and energy source (Parshina et al., 2005a). *Thermolithobacter carboxydivorans* was also detected in this particular sample site but was not detected in any other microcosm. The optimum growth temperature for *T. carboxydivorans* of 70°C may also explain the presence of this bacterium in DCs9 with an environmental and incubation temperature of 65°C as opposed to other Dewar Creek samples with lower *in situ* temperatures incubated at 55°C (Sokolova et al., 2005).

The dominance of *Firmicutes* in heavy-density DNA fractions of geothermal samples incubated under $^{13}CO$ confirms that representatives of this phylum may play a predominant role relative to other phyla in anaerobic oxidation of CO in geothermal environments. In particular, they may reflect minor populations within geothermal microenvironments where localized CO concentrations may be high. While the detection of such microenvironments *in situ* is challenging, the hypothesized presence of these localized CO-rich niches (e.g., Techtmann et al., 2009) suggests a mechanism by which these carboxydotrophs may exist. Despite comprising a relatively small proportion of *in situ* communities, the CO oxidizing bacteria are active and show some variation across geothermal environments. Oxidation potentials are higher than the few previously reported rates for mixed geothermal communities. Despite geographical differences, thermophilic bacteria associated with anaerobic CO-oxidation are widely distributed geographically and the predominant species are well-described from cultivation studies. The presence of a few OTUs that do not show high degrees of similarity to any known cultured representatives indicates that a

few new lithoautotrophs that have not been previously identified as CO-oxidizers may also be present in the geothermal springs tested and require further study.

The detection of sequences associated with known CO-oxidizing bacteria in high abundance supports the applicability of the CO-SIP technique. SIP can be applied to target autotrophs by adding an energy substrate along with $^{13}CO_2$. This works as long as the added substrate is the primary energy source for the autotrophic community. We have previously used this approach to identify autotrophic methanotrophs (Sharp et al., 2012). We therefore conclude that the CO-SIP technique, which works in a similar way to identify autotrophic carboxydotrophs, does have some value, although of course the results still need to be interpreted with caution. Controls are necessary to demonstrate a low rate of assimilation of $^{13}CO_2$ by other autotrophs. It should also be stressed that heterotrophic carboxydotrophs will be missed- this was a possible explanation for the failure of some of the samples assayed in this study. Cross feeding via $H_2$ + $CO_2$ produced via the gas-water shift reaction is also a potential issue, although this appeared to be minor in this particular study. This study appeared to work because the bacteria identified were primarily known carboxydotrophs, however identification of a new potential carboxydotroph should only be taken as initial evidence that requires verification with other methods including sequencing of potential *cooS* genes.

## Author Contributions

AB, CS and SG collected samples; AB performed SIP incubations with input from PD; AB with aid from CS extracted DNA, prepared samples for sequencing and carried out data processing. AB and PD wrote the initial draft of the paper; all authors designed the study, discussed the results and commented on the manuscript.

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## Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00897

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# Metagenomic evidence for metabolism of trace atmospheric gases by high-elevation desert Actinobacteria

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Previous surveys of very dry Atacama Desert mineral soils have consistently revealed sparse communities of non-photosynthetic microbes. The functional nature of these microorganisms remains debatable given the harshness of the environment and low levels of biomass and diversity. The aim of this study was to gain an understanding of the phylogenetic community structure and metabolic potential of a low-diversity mineral soil metagenome that was collected from a high-elevation Atacama Desert volcano debris field. We pooled DNA extractions from over 15 g of volcanic material, and using whole genome shotgun sequencing, observed only 75–78 total 16S rRNA gene $OTUs_{3\%}$. The phylogenetic structure of this community is significantly under dispersed, with actinobacterial lineages making up 97.9–98.6% of the 16S rRNA genes, suggesting a high degree of environmental selection. Due to this low diversity and uneven community composition, we assembled and analyzed the metabolic pathways of the most abundant genome, a *Pseudonocardia* sp. (56–72% of total 16S genes). Our assembly and binning efforts yielded almost 4.9 Mb of *Pseudonocardia* sp. contigs, which accounts for an estimated 99.3% of its non-repetitive genomic content. This genome contains a limited array of carbohydrate catabolic pathways, but encodes for $CO_2$ fixation via the Calvin cycle. The genome also encodes complete pathways for the catabolism of various trace gases ($H_2$, CO and several organic C1 compounds) and the assimilation of ammonia and nitrate. We compared genomic content among related *Pseudonocardia* spp. and estimated rates of non-synonymous and synonymous nucleic acid substitutions between protein coding homologs. Collectively, these comparative analyses suggest that the community structure and various functional genes have undergone strong selection in the nutrient poor desert mineral soils and high-elevation atmospheric conditions.

**Keywords: aerobiology, Atacama Desert, methylotrophy, *Pseudonocardia*, trace gas oxidation**

## INTRODUCTION

The Atacama Desert is the driest and perhaps oldest desert on Earth, where an estimated 150 My of sustained aridity and 3–4 My of hyperaridity across the central plateau have shaped the landscape (Hartley et al., 2005). The Atacama region is bounded by the Andes to the east and by the coastal mountain range and the cold water Pacific Humboldt current to the west (Gómez-Silva et al., 2008). These barriers restrict the flow of atmospheric moisture, which in turn results in some of the most inhospitable proto-mineral soils on the planet that contain nearly undetectable organic carbon stocks and microbial biomass pools (Navarro-González et al., 2003). The eastern boundary of the region hosts large volcanoes that are situated in the leeward rain-shadow of the Andes. The upper plant-free reaches of these peaks are distinct from other more well studied Atacama geographic zones in that the higher elevation increases rates of precipitation, yet also increases rates of evaporation, sublimation, solar incidence and freeze-thaw cycling (Schmidt et al., 2009). Despite these additional stressors, the barren high volcanic deposits are a habitat still principally limited by water availability (Costello et al., 2009). Photo-atmospheric processes (e.g., lightning derived nitrate deposition, Michalski et al., 2004), likely play defining roles in these gravel-like mineral soils where biotic geochemical cycling is constrained to nearly undetectable levels.

Although meteorological data from the high-elevation reaches of the Atacama volcanoes are sparse (Richter and Schmidt, 2002), the restrictiveness of the conditions to biological activity is manifest in the biomass levels of the mineral soils, which are barely above detection limits, as well as microbial diversity estimates that rival the lowest ever sampled for exposed terrestrial systems (Costello et al., 2009; Lynch et al., 2012). The physical conditions that exclude nearly all microbial life seem to have been overcome by a limited spectrum of bacterial and fungal lineages that may have evolved the capacity for *in situ* activity. The most abundant of these organisms are Chloroflexi and certain Actinobacteria, mainly of the Actinomycetales, Acidimicrobiales

and Rubrobacterales orders (Costello et al., 2009; Lynch et al., 2012).

Based on our initial molecular survey of these volcanic samples (Costello et al., 2009; Lynch et al., 2012), and work carried out in other areas of the Atacama where plant and microbial phototrophs are absent (Neilson et al., 2012), we hypothesized that chemoautotrophic microbes may be supplying organic carbon to simple and low-energy flux communities. Previous studies elsewhere have demonstrated the biological uptake of trace gases (CO and $H_2$, but not $CH_4$) in 26 year old plant-free and carbon limited Hawaiian volcanic deposits (King, 2003a), implying trace gases may be important energy sources where organic carbon accumulations are limited. The present metagenomic study was undertaken to develop a more comprehensive understanding of the potential metabolic traits, particularly focused on energy and nutrient acquisition, which the few community members found at the Llullaillaco Volcano study sites possess. The functional hypotheses developed through this study will be considered in light of the known environmental conditions present at these sites, and support the ongoing development of realistic growth conditions for culture based experiments.

Here we present a shotgun metagenomic study of a low-diversity and phylogenetically under-dispersed community, composed almost exclusively of Actinobacteria (>98% of all bacteria) found in the high-elevation (>6000 m elevation) Atacama Desert volcanic deposits. By leveraging the natural low diversity of these samples with deep coverage from long-read whole metagenome shotgun sequencing, we were able to characterize the genomic makeup of the community members at a high level of detail through reference database classification of raw sequence reads. Our high sequencing depth and coverage also enabled *de novo* assembly based analyses of selection through estimation of non-synonymous and synonymous mutation rates for protein coding genes of the most abundant community member's genome.

## MATERIALS AND METHODS

### SAMPLE COLLECTION AND PRESERVATION

Two snow free mineral soil samples located approximately 5 m apart were collected from the Llullaillaco Volcano (−24.718, −68.529) at an elevation of 6034 m above sea level (m.a.s.l.) during the austral summer in mid-February 2009. The top 4 cm of surface material, excluding rocks larger than 2 cm in diameter, were aseptically collected and frozen the same day in the field using blue ice packs. By the evening of the day the samples were collected, they were transferred to a −20°C freezer at the army barracks (on the Chile-Argentina border) near the field site. The next day they were driven (on ice in a cooler) to Salta, Argentina where they were again placed in a −20°C freezer until they were hand carried to Colorado in a thick-walled cooler on blue ice packs. They arrived in Boulder, Colorado within 24 h of being taken out of the freezer in Salta and were still frozen upon arrival (i.e., the ice packs hadn't melted). The samples have since been continuously stored at −20°C. Further details regarding these and other samples collected from the Llullaillaco Volcano can be found in Lynch et al. (2012).

### DNA EXTRACTION AND SEQUENCING AND QUALITY CONTROL

We utilized a modified serial silica filter binding protocol (Fierer et al., 2012) to overcome the low DNA yields of these low biomass samples and to avoid the potential biases introduced from random genomic amplification techniques. DNA extractions were quantified using PicoGreen dsDNA fluorometry (Thermo Fisher Scientific Inc.). We recovered 1 μg of gDNA from each of the samples, which required 10.4 g of volcanic debris from sample 1 and 4.8 g from sample 3 (**Table 1**). Negative extraction controls were run with the same batch of extraction reagents, but no soils were added. These negative control extractions were excluded from the sequencing libraries due to insufficient quantities of dsDNA. Samples were shipped to the Duke University Genome Sequencing and Analysis Core Resource where the long-read 454 GS FLX+ platform was used to sequence randomly fragmented bulk nucleic acid extractions.

Library parsing and removal of the 454 MIDs was achieved with the sfffiles package (454 Life Sciences) and manually confirmed using the Geneious (6.1.3) viewer. Reads were trimmed so they contained no more than five bases with quality scores of 15 or lower (Cox et al., 2010). Sequence length was required to be within two standard deviations of the mean length, and no more than five ambiguous bases per read were permitted. We found very low rates of artificial read duplication (Gomez-Alvarez et al., 2009, 0.31 and 0.13% for the sites 1 and 3 libraries respectively), which was tested using CD HIT (Fu et al., 2012), with settings 1 1 3 that require 100% sequence identity and length.

We used a 15-mer spectrum analysis (Supplementary Figure 2, Marçais and Kingsford, 2011) to visualize how sequencing depth relates to the total metagenomic complexity of the samples. Additional desert and non-desert metagenomes were downloaded from the MG RAST server (Meyer et al., 2008), ID 4446153.3 and all datasets from Fierer et al. (2012).

#### rDNAs

A closed reference operational taxonomic unit (OTU) picking method (pick_closed_reference_otus.py, Caporaso et al., 2010) was applied to a UCLUST (Edgar, 2010) identified set of

**Table 1 | Summary of sample characteristics for volcano metagenomes.**

| Sample | Mass for DNA extraction | 16S RNAs | Observed OTUs | Shannon | Simpson | NRI (*p*-value) | pH | TOC |
|---|---|---|---|---|---|---|---|---|
| 1 | 10.4 g | 363 | 78 | 5.146 | 0.954 | 4.99 (<0.001) | 4.13 | 0.027 |
| 3 | 4.8 g | 334 | 75 | 4.862 | 0.938 | 2.37 (0.01) | 4.23 | 0.016 |

*The significant phylogenetic clustering of the microbial community is summarized by the positive Net Relatedness Index (NRI) of these low diversity low biomass communities. TOC, percent total organic carbon. pH and TOC values are from data and methods reported in Lynch et al. (2012).*

candidate 16S RNAs genes. This method overcomes the issue of sequencing different regions for the 16S rRNA gene with the shotgun technique. A 97% similarity was required for each candidate sequence alignment to the most current Green Genes reference dataset available (Release 13_5, McDonald et al., 2012). For the analysis of phylogenetic dispersion, near full length 16S rRNA gene sequences that have been previously published (JX098304—JX098810) were used to construct a maximum likelihood tree (Price et al., 2009) with the Green Genes reference dataset (13_5) clustered into 5088 $OTUs_{85\%}$. Phylocom 4.2 (Webb et al., 2008) was used to calculate a net relatedness index (NRI) value and associated one-tail *P*-values with 999 randomization iterations and the null hypothesis setting 2 (sample OTUs are drawn at random from the total species pool without replacement). This null hypothesis is intended to model the homogenizing effects of long distance atmospheric transport and deposition of bacterial cells from diverse sources, with a total absence of selection.

Fine scale phylogenetic trees were constructed with $OTUs_{1\%}$ of the full length 16S sequences determined by the QIIME pick_de_novo_otus.py workflow. SINA alignments (Pruesse et al., 2012) were built with Silva (115) reference database representatives (Quast et al., 2013) and maximum likelihood phylogenies were inferred with PhyML 3.0 (Guindon et al., 2010) using a GTR model of nucleic acid evolution.

#### GENETIC INVENTORY

The SEED database (Overbeek et al., 2005) uses a hierarchical classification system where the broadest level (level 1) includes many anabolic and catabolic pathways and their associated single enzyme catalyzed intermediaries. Pairwise *t*-tests were used to calculate significance of gene category count differences (level 1) between the Llullaillaco Volcano libraries and a collection of desert and non-desert metagenomes, using the pooled SD option and a Bonferroni correction for multiple comparisons ($\alpha = 0.05/(28 \times 2) = 0.0009$) in R (http://www.r-project.org/). Gene calls were made based on minimum ID of 60% and a maximum *e*-value of $1\ e^{-5}$ for all BLAT alignments that were generated from MG RAST, and the SEED database.

#### ASSEMBLY

*De novo* assembly was attempted on each of the two separate Llullaillaco site metagenomes with the MIRA V3.4.0 (Chevreux et al., 1999) signal trace assembly platform using the following settings: --job=denovo,genome,accurate,454 --highlyrepetitive --noclipping --notraceinfo --fasta -project=RL1All -SK:not=46 -AS:sep=yes 454_SETTINGS -ED:ace=yes -AL:mo=40:ms=30 -CL:bsqc=yes -LR:lsd=yes:ft=fastq. These settings require that each fragment addition to a contig have at least 40 high quality scoring bases of overlap and minimum quality scores of 30. They also restrict the variance of coverage levels across each contig to reflect the expectation that random shotgun sampling of each community member's genome should result in a unique coverage level that reflects its natural relative abundance in the community of genomes. This assembly approach assumes a theoretical copy number of one per unique genomic element leading to exclusion of repetitive elements, and also assumes that the main community members have significantly different relative abundances.

#### ASSEMBLY EVALUATION AND ANNOTATION

Tetramer based emergent self-organizing maps (ESOMs) http://databionic-esom.sourceforge.net/ were used to help evaluate contig binning (Dick et al., 2009) in conjunction with analysis coverage levels. Descriptions of the databionic ESOM settings and the Perl scripts used to calculate tetramer frequencies can be found at https://github.com/tetramerfreqs/binning. Consensus sequences from contigs were called with a majority rule to filter out all but the most abundant strains and low coverage ends were trimmed.

Bins of contigs that represent draft genomes and associated metadata were uploaded to the JGI IMG/ER database (Markowitz et al., 2012) for initial annotation. The phylogenetic origins of the JGI protein annotations were inspected and annotations for select coding DNA sequences (CDS) were checked manually. Completeness of the metagenome assembles was assessed by comparing protein family database (Punta et al., 2012) annotations to the list of conserved single copy genes (CSCGs, Rinke et al., 2013). Putative genes involved in major metabolic pathways were manual curated by evaluating blastx alignments and through literature-based refinement of functional annotations.

#### COMPARATIVE GENOMICS AND ANALYSIS OF SELECTION

Clusters of orthologs genes (COGs, Tatusov, 1997) for the three publically available *Pseudonocardia* sp. genomes were downloaded from the IMG/ER database. COG count data were subjected to hierarchal centroid clustering with Cluster 3.0 http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm and visualized with heatmaps drawn in TreeView (Saldanha, 2004).

Even when genes share clear homologous relationships they may perform divergent functions. One way to detect the signature of divergent selection between orthologous genes is through the comparison of rates of non-synonymous ($K_a$) to synonymous ($K_s$) mutations. When selection is weak or absent $K_a$:$K_s$ratios should be close to one since genetic drift should have an equal chance of causing either non-synonymous or synonymous mutations. However, when divergent selection drives altered amino acid coding potential, rates of non-synonymous mutations should be elevated relative to synonymous mutations (Yang, 1998). A Perl pipeline was used to link the following steps together for an iterative $K_a$:$K_s$ analysis. Pairs of candidate CDS orthologs between our best volcano *Pseudonocardia* sp. draft genome and the *Pseudonocardia asaccharolytica* (IMG ID 13496) draft genome were identified as reciprocal blastn hits (with $\geq$70% identity for 100 bp). Protein guided DNA alignments were generated for each CDS pair through the TranslatorX approach (Abascal et al., 2010), which relies on Muscle (Edgar, 2004) to align predicted amino acid sequences. Codeml (PAML 4.7, Yang, 2007) was then used to estimate rates of non-synonymous ($K_a$) and synonymous ($K_s$) nucleic acid substitutions for each ortholog pair alignment, using the WAG model of amino acid evolution. Ortholog pairs found with signatures of positive selection for amino acids substitutions ($K_a$:$K_s$ ratios of $\geq$ 1) were checked manually and annotated with a database of genes from the *P. asaccharolytica* draft genome using blastx.

#### HYDROGENASE PHYLOGENETICS

To place the [NiFe]-hydrogenase genes from our volcano *Pseudonocardia* sp. assembly into a broader phylogenetic context,

we constructed a phylogeny using available sequence data from other studies. A broad sampling of [NiFe]-hydrogenase large subunit amino acid sequences was obtained from the list of sequences provided by Vignais and Billoud (2007), along with their subgroup annotations. Sequences for a fifth subgroup were obtained through blastn searches using our assembled sequence, as well as from Constant et al. (2010). Incomplete sequences were not included in our analysis. All amino acid sequences were aligned using ClustalW2 (Larkin et al., 2007) using default parameters, and a phylogeny was made using the neighbor-joining algorithm implemented in MEGA 6 (Tamura et al., 2013) using the Poisson model with 1000 bootstrap replications.

## RESULTS

### SEQUENCING AND rDNA DIVERSITY

After trimming we were left with 3.85 million reads that total 1.3 Gb of DNA sequence data for downstream analysis. Each of the two site libraries contained nearly identical distributions of bacterial (99.2%), eukaryotic (0.5%) and archaeal (0.3%) reads, based on all MG RAST annotation databases. We found a low diversity community populated mostly by Actinobacteria (**Table 1**), which make up 98.6 and 97.9% of the 16S rRNA genes from the site 1 and 3 libraries, respectively. This highly uneven community structure is significantly under dispersed ($P < 0.001$ and 0.01 for the phylogenetic randomization tests on the two samples), indicating a likely non-random assemblage of bacterial lineages. All lineages shown in **Figure 1** belong within the Actinomycetales, other than an $OTU_{3\%}$ belonging to the Acidimicrobiales order (Supplementary Figure 1) that makes up 15.6% of the site 3 library, but only 1.9% of the site 1 library. The *Pseudonocardia* are by far the most abundant lineages (72.2% of site 1 and 56.3% of site 3 total 16S reads) and the *Saccharopolyspora* (Pseudonocardiaceae) also make up 8.8% and 12.6% of total 16S rRNA gene reads from sites 1 and 3, respectively.

### GENETIC INVENTORY

The Llullaillaco metagenomes show a pronounced reduction in genes associated with carbohydrate metabolism compared with other desert and non-desert metagenomes (**Figure 2**). By contrast we found significant enrichment of pathways categorized as membrane transport, nucleotide metabolism, regulation and cell signaling, nitrogen metabolism and virulence and defense. Examining the presence and absence of metabolic pathways within the total metagenome, we found no evidence for complete photosynthetic pathways, yet found complete gene sets for the oxidation of CO and $H_2$, and for $CO_2$ fixation with the Calvin cycle. Methylotrophic pathways also suggest a role for other C1 compound oxidation and assimilation including: methanol, formaldehyde, formate and perhaps methane. No nitrogen ($N_2$) fixation or ammonia monooxygenase genes were identified, but genes for nitrate ($NO_3^-$) reduction (nitrate reductase) and

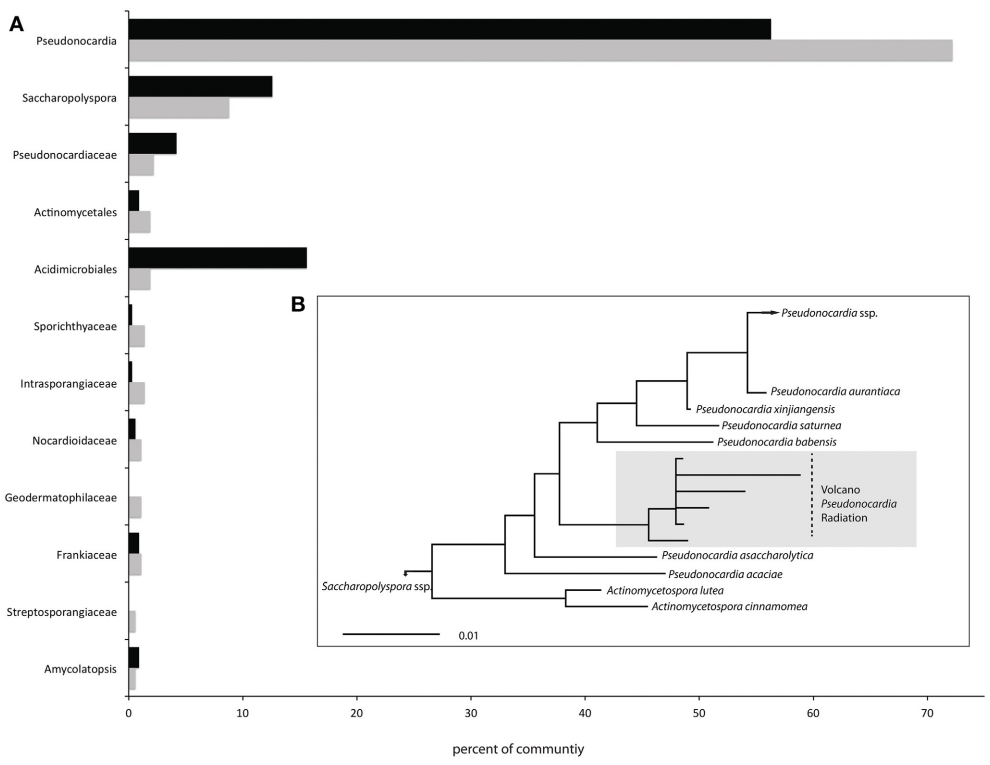


**FIGURE 1 | Community profile (A) All $OTU_{3\%}$ taxonomic assignments from each site that represent at least 1% of the total metagenome 16S gene reads.** These 12 OTUs constitute 94.3 and 92.6% of the total 16S gene reads from sites 1 (gray bars) and 3 (black bars) respectively and are all members of Actinomycetales other than the single Acidimicrobiales $OTU_{3\%}$. **(B)** Maximum likelihood phylogeny of the most abundant Pseudonocardia $OTU_{3\%}$, split into sub $OTUs_{1\%}$. The scale bar represents 1% divergence between nucleic acid sequences.

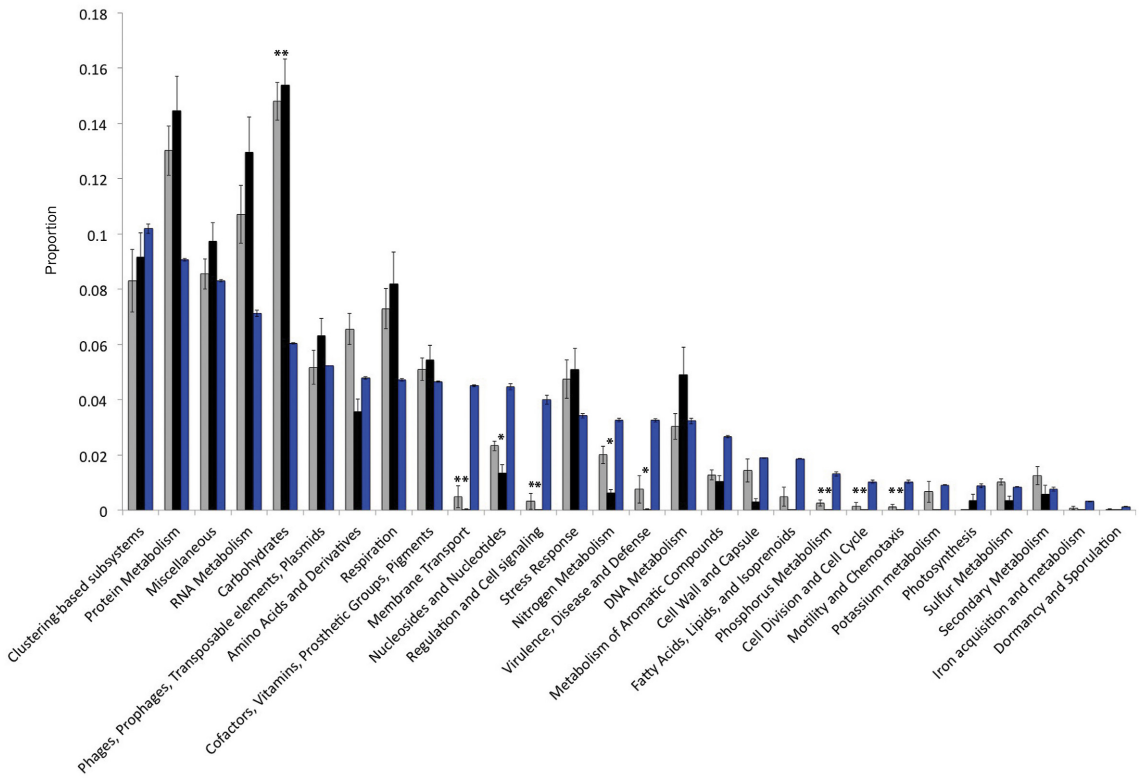


**FIGURE 2 | Inventories of gene functional categories, comparing non-desert (gray), desert (black) biomes to the high-elevation volcano metagenomes (blue).** Asterisks indicate Bonferroni corrected significant differences ($P < 0.0009$) between the volcano data and desert or non-desert data (desert to non-desert comparisons not shown) for all pairwise *T*-tests.

ammonia ($NH_3$) assimilation (glutamine synthetase) were found in high abundance.

## GENOME ASSEMBLY RESULTS

We were able to assemble and bin contigs (Supplementary Figures 3, 4) that represent composite genomes of the most abundant *Pseudonocardia* sp. (**Table 2**), as well as the other lower abundance community members, such as a member of the Acidimicrobiales (Supplementary Figures 1, 3). The best *Pseudonocardia* assembly appears to represent a nearly complete set of non-repetitive genomic elements since it contains 138/139 CSCGs (missing a DNA uptake competence gene, PF03772). None of the CSCGs were present in more than one copy in the metagenome assemblies, suggesting we did not greatly over-assemble this genome. The CSCGs are 139 protein coding genes that were found to occur only once in at least 90% of the 1515 finished bacterial genomes available in the IMG/ER database (Rinke et al., 2013). Within each of the new *Pseudonocardia* sp. assemblies, 2–3 single nucleotide polymorphisms (SNPs) were present in many of the CDS regions, which are likely indicative of strain and population level variation.

## COG COMPARISONS

COG counts from our highest quality *Pseudonocardia* sp. assembly (68–115 × coverage bin from site 1) and the three other publicly available genomes for named *Pseudonocardia* spp. (Supplementary Table 1, **Figure 3**) highlight some of the specific differences in genome content. We found certain COGs like those needed for CO oxidation are conserved at high copy numbers across all the *Pseudonocardia* spp., and that COGs such as those required for assimilatory nitrate reduction and carbon fixation (RuBisCO) show relatively higher counts in both our metagenome assembly and *P. asaccharolytica.* Other highly abundant gene clusters within our metagenome assembly bear resemblance to the more phylogenetically distant *Pseudonocardia* spp. These clusters include the antibiotic producing non-ribosomal peptide synthesis pathway (NRPS), various ABC peptide importers, cytochrome P450 monooxygenase, and several recombinases.

## SIGNATURES OF SELECTION ANALYSIS

Of the 5024 annotated CDS from the draft *P. asaccharolytica* genome we were able to initially align 1722 orthologous coding sequences from our best metagenome *Pseudonocardia* sp. assembly with at least 70% nucleotide identity. Of these, manual inspection filtered out 462 gene pairs that were poorly aligned or were not true homologs across the entire sequence. There were 59 remaining ortholog pairs (4.7%) with estimated $K_a$:$K_s$ ratios $\geq 1$, which reflects elevated rates of non-synonymous mutations brought about through strong divergent selection acting upon the amino acid sequences (**Figure 4**, Supplementary Table 2).

**Table 2 | Summary of metagenome *Pseudonocardia* sp. assemblies and nearest phylogenetic reference genome, *P. asaccharolytica* (JGI IMG id 13496).**

| Genome | Ave. coverage | Size | Max. contig | Contigs | CDS | CSCGs | GC % | rRNA operons |
|---|---|---|---|---|---|---|---|---|
| Site 1 volcano *Pseudonocardia* sp. | 68–115 | 4.87 Mb | 92,460 bp | 336 | 5434 | 138/139 | 70.7 | 3 |
| Site 3 volcano *Pseudonocardia* sp. | 45–68 | 4.63 Mb | 117,339 bp | 332 | 5055 | 129/139 | 70.8 | 3 |
| *Pseudonocardia asaccharolytica* | Na | 5.05 Mb | 441,096 bp | 72 | 5024 | Na | 71.7 | 3 |

*Conserved single copy genes (CSCGs) ratio estimates the completeness of the non-repetitive components of the metagenome assembles.*

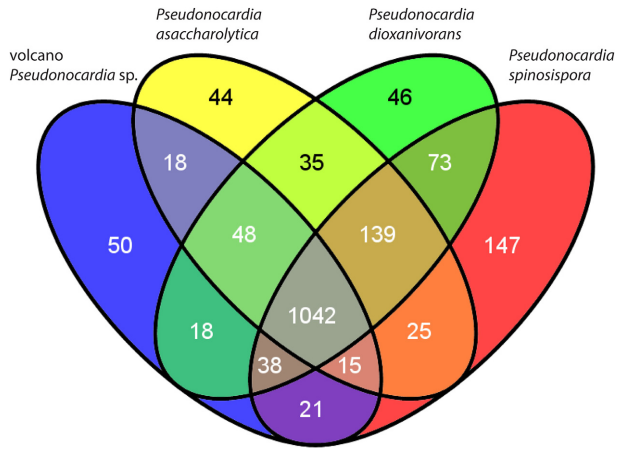


**FIGURE 3 | Venn diagram of the shared and unique genes (COGs) among named *Pseudonocardia* spp. with complete genomes and the volcano *Pseudonocardia* sp. genome assembly.** Although most of the 50 COGs unique to the volcano *Pseudonocardia* sp. (Supplementary Table 1) are classified as "function unknown" or "general function prediction only," the six additional defense mechanism related COGs and the nine fewer carbohydrate transport and metabolism COGs in the volcano *Pseudonocardia* sp. stand out as potentially relevant functional differences with other *Pseudonocardia* spp.

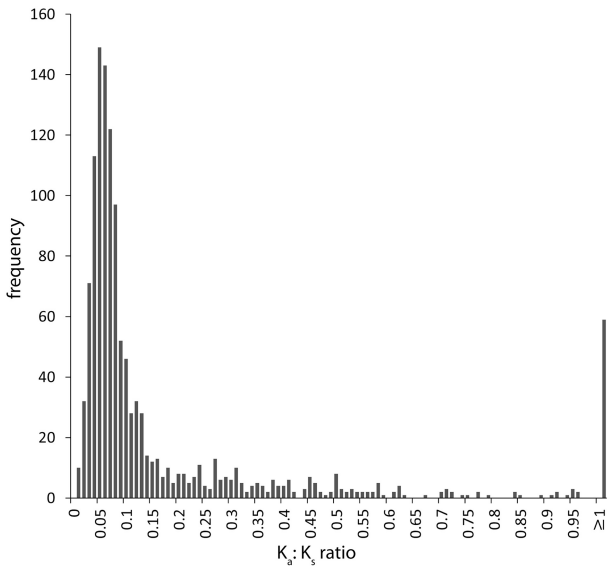


**FIGURE 4 | Distribution of $K_a$:$K_s$ ratios for 1260 pairwise orthologous protein coding sequences between the best volcano *Pseudonocardia* sp. assembly and its closest fully-sequenced relative, *Pseudonocardia asaccharolytica*, showing the majority of genes (95.3%) to be under purifying or relaxed selection regimes, where synonymous substitutions that do not alter the amino acid coding potential dominate the gene.** However, some outliers (4.7%) display higher levels of non-synonymous mutations (≥1 $K_a$:$K_s$) likely driven by divergent selection from the harsh high-elevation desert conditions. This analysis was limited to 23% of total volcano *Pseudonocardia* sp. genes due to the high degree of overall genomic divergence between these two species.

### CHARACTERISTICS OF THE VOLCANO *PSEUDONOCARDIA* SP. GENOME

The volcano *Pseudonocardia* sp. genome is at least 4.9 Mb (**Table 2**) and contains many of the pathways that define the total community metabolic potential (e.g., aerobic heterotrophic metabolism, $NO_3^-$ and $NH_3$ utilization, $H_2$ and CO oxidation, $CO_2$ fixation and methylotrophic pathways, **Figure 5**). Many genes (33%) were found with multiple copies in the genome, suggesting a possible role for gene duplication events during the divergence of this genome. Potential carbohydrate oxidation pathways are quite limited, with genes present only for the utilization of glucose, mannose, ribose, gluconate, maltose, trehalose, lactose, and galactose that feed into the Embden-Meyerhof-Parnas pathway or the pentose phosphate pathway. Carbohydrate uptake potential is apparently even more restricted as only a single annotated maltose ABC importer was identified. A complete list of putative gene annotations can be found in the IMG/ER database (id 45716).

### HYDROGENASE PHYLOGENY RESULTS

Our phylogenetic analysis of [NiFe]-hydrogenase sequences confirmed that the volcano *Pseudonocardia* sp. assembly includes a group 5 [NiFe]-hydrogenase gene (**Figure 6**). Our phylogeny resolved a monophyletic clade for hydrogenase group 5, which includes the group 5 hydrogenase sequences from Constant et al. (2010) as well as several other Actinobacterial phylogypes. [NiFe]-hydrogenase protein sequences that are most closely related to the volcano *Pseudonocardia* sp. came from *P. asaccharolytica*, *Pseudonocardia spinosispora*, and *Actinomycetospora chiangmaiensis*.

## DISCUSSION

The conditions present in the most extreme Atacama Desert soils exclude most life and leaves open the questions of if and how microbes may survive there. Previous studies of Atacama Desert soil microbiota have used either 16S gene based culture-independent approaches (Navarro-González et al., 2003; Costello et al., 2009; Lynch et al., 2012; Neilson et al., 2012), or to a limited extent culture-dependent methods (Lester et al., 2007;

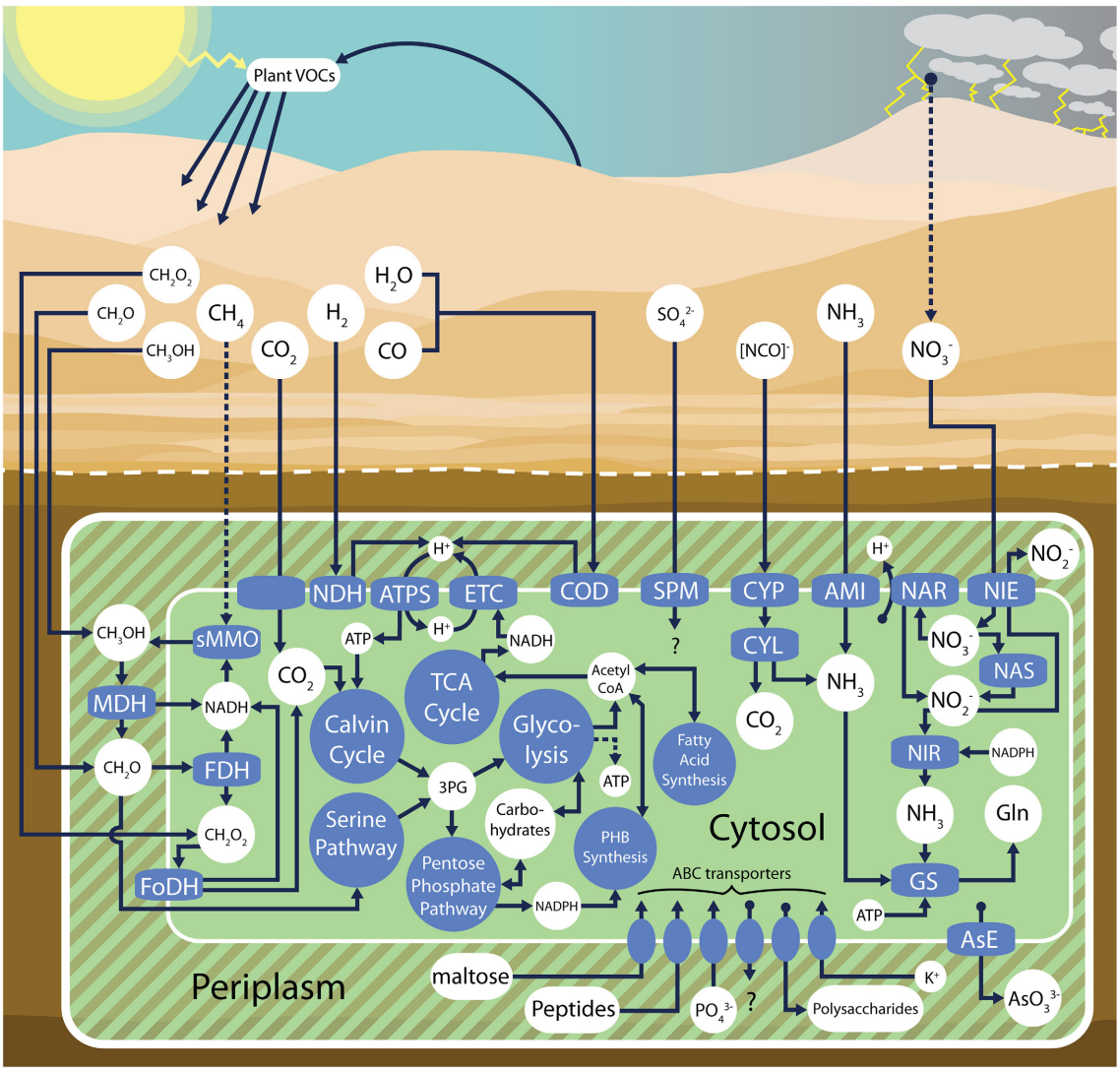


**FIGURE 5 | Ecophysiological overview of the volcano *Pseudonocardia* sp. metabolic pathways as inferred from assembled metagenomic data.** sMMO, soluble methane monooxygenase; MDH, (PQQ)-dependent methanol dehydrogenase; FDH, formaldehyde dehydrogenase; FoDH formate dehydrogenase-O; NDH, group 5 high-affinity NiFe hydrogenase, ATPS, ATP synthase; ETC electron transport chain; COD, form I carbon monoxide dehydrogenase; AsE, arsenite efflux; CYP, cyanate permease; CYL cyanate lyase; AMI, ammonium importer; NAS, assimilatory nitrate reductase; NAR, respiratory nitrate reductase; NIE, nitrite extrusion protein; NIR, nitrite reductase; GS, glutamine synthetase; SPM, sulfate permease; 3PG 3-phosphoglyceric acid; PHB, polyhydroxybutyrate; Gln, glutamine.

Okoro et al., 2009). Taken together, the pioneering work done on Atacama soils indicates that low diversity microbial communities are present at many sites, though few details have emerged regarding the origins and functional nature of these microorganisms. In this study, we used a deep metagenomic sequencing strategy to examine the structure and functional potential of the Llullaillaco Volcano microbial community (Lynch et al., 2012). Difficulty with extracting DNA from very low biomass mineral soils required us to pool roughly the equivalent of 60 standard 0.25 g soil DNA extractions to achieve the quantity of genomic DNA necessary for shotgun metagenomic sequencing. As a result, this dataset is less spatially expansive than our previous amplicon based analysis (Lynch et al., 2012), yet still demonstrates the low-diversity community structure extends throughout a relatively large volume of soil. Despite the limitations of this study, the approach allowed for a more thorough description of the Llullaillaco Volcano microbial community structure, and provides an initial insight into the protein coding potential of the metagenome as well as the most abundant community member's genome.

Through this approach we found an extremely low-diversity community of organisms (**Figure 1**, **Table 1**) that host an unusual inventory of functional genes (**Figure 2**), including an absence of phototrophic pathways and limited capacity for heterotrophic carbohydrate metabolism. The low diversity community lacks many of the clades previously recovered from high-elevation air

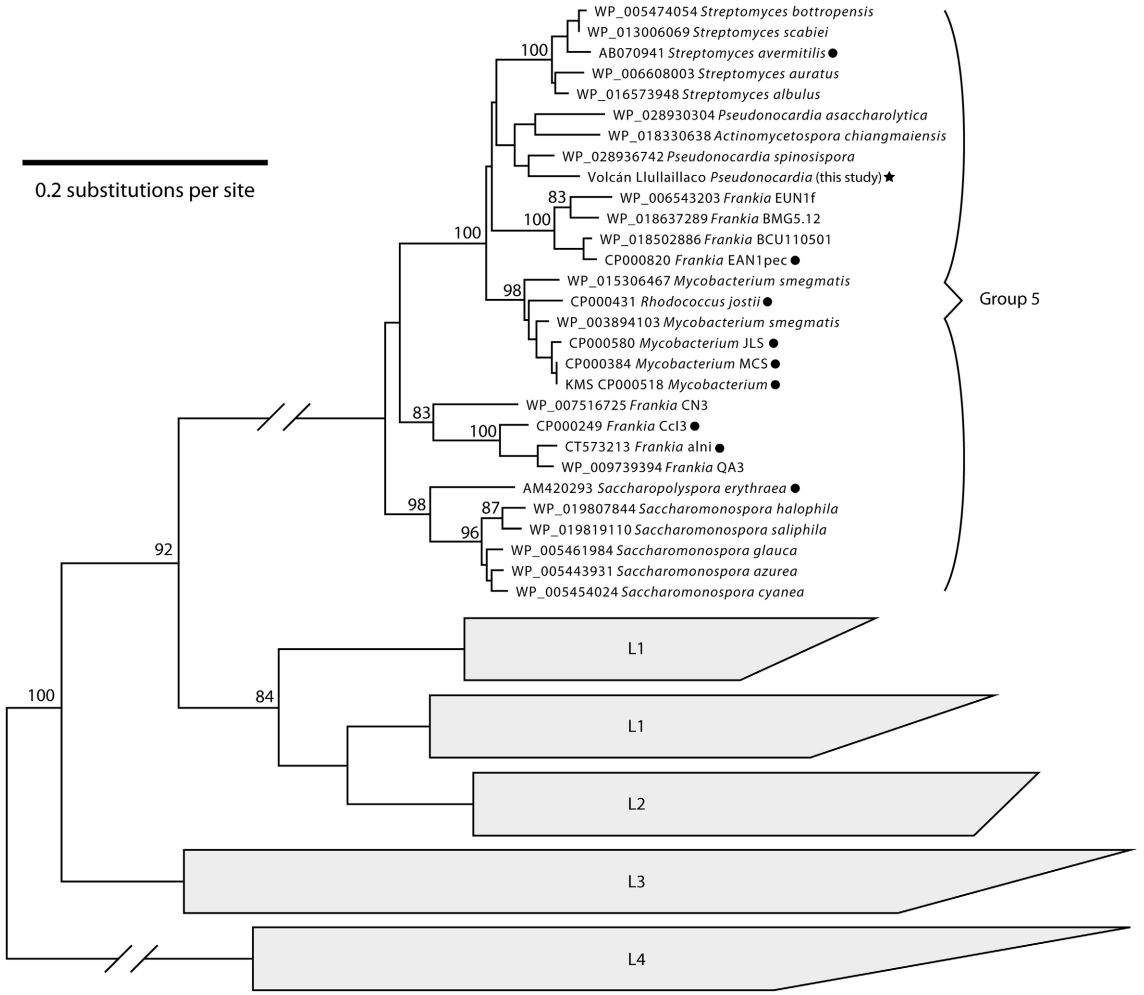


**FIGURE 6 | Neighbor-joinging phylogenetic tree of [NiFe]-hydrogenase amino acid sequences.** The phylotype from our *Pseudonocardia* sp. assembly (star) falls into the same clade as sequences shown in Constant et al. (2010), which are marked with circles. Sequences from other [NiFe]-hydrogenase large subunit subclades (L1–L4, Vignais and Billoud, 2007) are shown as the outgroup. Bootstrap support values are shown for nodes present in over 80% of bootstrapped trees. The scale bar represents 20% divergence between amino acid sequences.

(Bowers et al., 2012) and dust (Stres et al., 2013) microbiome studies, suggesting a high degree of environmental selection that could occur during atmospheric transport to these Atacama sites, or during active or dormant residence in the mineral soils.

The most abundant 16S gene OTU (*Pseudonocardia* sp.) recovered from the two sites used in this study (and from the third "low site" from Lynch et al., 2012), shares a relationship with *Pseudonocardia* sp. detected in other high elevation samples from Himalayan and Antarctic mineral soils (Rhodes et al., 2013), as well as with isolates from Icelandic volcanic deposits (Cockell et al., 2013) leaving open the possibilities it may be native to these sites or that it could be present at the Llullaillaco Volcano sites as a consequence of atmospheric transport (Stres et al., 2013). It is noteworthy that the Acidimicrobiales $OTUs_{3\%}$ (**Figure 1**) found in this environment (15.6% of the site 3 library, and 1.9% of the site 1 library) is related to known inhabitants of fumaroles (Supplementary Figure 1, Benson et al., 2011; Itoh et al., 2011), so it is likely that at least some of the organisms present at our research sites are the result of regional wind transport from active fumaroles on nearby Socompa Volcano (Costello et al., 2009), or from as yet undiscovered fumarolic activity on Llullaillaco Volcano. Indeed, we found Acidimicrobiales 16S gene sequences identical to those from the Llullaillaco Volcano in warm fumaroles of Socompa Volcano (Costello et al., 2009). It is also possible that the presence of known fumarole inhabitants indicates that our research sites are located on soils that were originally fumarolic and that the organisms found there are relics that have survived as dormant spores. This would explain the presence of genes for the utilization of gases that are found in fumarolic emissions (e.g., CO and $H_2$), rather than the idea that they serve to metabolize the exceedingly low concentrations of atmospheric gases found at elevations above 6000 m.a.s.l.

## ENERGETICS

Detailed examination of the most abundant community member's genome assembly reveals unique genetic content (**Figure 3**),

evidence for divergent natural selection acting on certain homologs (**Figure 4**, Supplementary Table 2) and complete metabolic pathways related to trace atmospheric substance metabolism (**Figure 5**). Unidentified soil oligotrophs have long been suspected of oxidizing ubiquitous trace gases like $H_2$, CO, and $CH_4$ based on evidence from bulk soil process studies (Conrad, 1996; Constant et al., 2011). Although unequivocal demonstrations of bacterial growth and cell division from trace gas metabolism have been elusive, several actinobacterial isolates have been shown to oxidize ambient $H_2$ and CO at atmospheric concentrations (Constant et al., 2008; King, 2003b). In certain actinobacteria, ambient $H_2$ oxidation has now been conclusively tied to the activity of high-affinity group 5 [NiFe] hydrogenases (Greening et al., 2014).

[NiFe] hydrogenases are membrane-bound enzymes that catalyze the splitting of periplasmic $H_2$, facilitating the production of a proton gradient for ATP synthesis (**Figure 5**, "NDH"). A novel group 5 [NiFe] hydrogenase gene set is present in our genome assembly of the most abundant volcano *Pseudonocardia* sp. (**Figure 6**), indicating that the dominant organism at this site likely has the ability to utilize atmospheric concentrations of $H_2$ (0.53 ppmv, at sea level, but about 0.24 ppmv at 6000 m.a.s.l.) for energy production. Greening et al. (2014) also found that *Mycobacterium smegmatis* group 5 [NiFe] hydrogenase expression levels increased under carbon starvation conditions, implicating the oxidation of $H_2$ as a source of electrons during low metabolic states. Given the low levels of organic carbon measured at the volcano sites (**Table 1**), and the phylogenetic affiliation between the group 5 volcano *Pseudonocardia* sp. [NiFe] hydrogenase and the *M. smegmatis* group 5 [NiFe] hydrogenase (sharing 80% amino acid identity) studied by Greening et al. (2014), oxidation of trace $H_2$ seems to be a plausible energy source for the new *Pseuodnocardia* sp. However, [NiFe] hydrogenase genes are not the only genes we observed that could be used to metabolize atmospheric substrates.

Previous studies have correlated a widespread occurrence of carbon monoxide dehydrogenase genes with soil CO uptake (King, 2003a; Weber and King, 2010; Quiza et al., 2014), and various soil bacterial isolates have been confirmed to oxidize CO at atmospheric concentrations (<400 ppbv at sea level, Hardy and King, 2001; King, 2003b). Carbon monoxide dehydrogenase functions similarly to [NiFe] hydrogenase, in that it is a membrane-bound enzyme that facilitates the generation of a proton gradient. In this case, the enzyme oxidizes CO and reduces $H_2O$, forming $CO_2$ and two periplasmic protons (**Figure 5**, "COD"). *M. smegmatis* has been shown to be capable of trace CO uptake, and hosts canonical type I carbon monoxide dehydrogenase genes (Quiza et al., 2014), similar to the CO dehydrogenase genes present in the volcano *Pseudonocardia* sp. assembly. However, it is not yet clear how this activity affects cellular physiology. It is likely that tropospheric CO oxidation is often a supplemental energy source, contributing to a mixotrophic metabolism (King and Weber, 2007). Thus, physiological work focused on high-affinity CO oxidizing bacteria must carefully consider the possible requirements and roles of organic carbon sources, in addition to tracking low-concentration CO uptake (King and King, 2014).

The volcano *Pseudonocardia* sp. genome encodes complete pathways for the oxidation and assimilation of methanol, formaldehyde, and formate (**Figure 5**). The atmosphere contains very low concentrations of these gases mainly due to plant volatile emission and photochemical reactions (Hu et al., 2011; Stavrakou et al., 2011; Luecken et al., 2012). The study of bacterial metabolism of atmospheric concentrations of these C1 compounds is limited, although efforts are underway to develop an understanding of the distributions of methylotrophs and how they influence the global methanol cycle (Kolb and Stacheter, 2013). Furthermore, some evidence suggests that various Actinobacteria (e.g., *Streptococcus* and *Rhodococcus* spp., Yoshida et al., 2007) are capable of "$CO_2$ dependent oligotrophic growth" under laboratory carbon starvation conditions by oxidizing ambient methanol and formaldehyde (Yoshida et al., 2011), suggesting these C1 gases can be atmospheric sources of energy and carbon for some bacteria.

Methane is the most abundant of the trace gases at 1.79 ppmv (or 0.80 ppmv at 6000 m.a.s.l.), so would seem to be a likely target for trace gas oxidizers. However, the Llullaillaco Volcano metagenome lacks any identifiable particulate methane monooxygenase (pMMO) genes, which have been previously identified as likely coding for the high-affinity methane oxidation enzymes in various soils (Bull et al., 2000; Kolb, 2009). Likewise the study of early-successional Kilauea Volcano soils by King (2003a) detected CO and $H_2$ uptake, but not $CH_4$. Yet the volcano *Pseudonocardia* sp. does encode all genes required for a putative iron-dependent soluble methane monooxygenase (sMMO) enzyme that could function to oxidize methane to methanol, which would then be fed into the abovementioned methylotrophic pathways. sMMOs are notoriously non-specific enzymes (Green and Dalton, 1989), and atmospheric concentrations of methane have not yet been reported to support bacterial growth (Theisen and Murrell, 2005; Conrad, 2009). Nevertheless, the evidence for widespread ambient methane oxidation (McDonald et al., 2008) and experimental confirmation of methane oxidation by members of the phylum Verrucomicrobia (Dunfield et al., 2007) illustrates the continued need to explore the phylogenetic and geographic distributions of methane oxidizers.

Given the presence of these various gas utilization pathways in the volcano *Pseudonocardia* sp. genome (**Figure 5**), and the constant availability of these substrates at low concentrations in the atmosphere, the high-elevation volcanic deposit community may rely on a mixture of diffuse atmospheric substrates in the absence of direct photosynthetic inputs to at least maintain redox balance, or perhaps even to drive carbon fixation. However, it is important to note the volcano *Pseudonocardia* sp. shares nearly all of these aforementioned trace gas oxidation pathways (**Figures 5**, **6**) with *P. asaccharolytica,* its nearest phylogenetic relative (**Figure 1**). *P. asaccharolytica* does lack a (PQQ)-dependent methanol dehydrogenase gene, but these were present in other *Pseudonocardia* spp. (**Figure 3**). While no studies to date have tested *P. asaccharolytica* for trace gas metabolism either *in situ* or in culture (Reichert et al., 1998), the trace gas metabolism related genes common to the *P. asaccharolytica* and the volcano *Pseudonocardia* sp. genomes have been shown to confer trace gas metabolism

capacity in other bacteria (**Figure 6**), making it a plausible trait shared by various members of this genus. Consequently, the relevance of trace gas utilization as a potential metabolic strategy in the harsh Atacama Desert mineral soils of this study is difficult to interpret, since trace gas metabolism genes are not exclusive to *Pseudonocardia* sp. recovered from desert environments.

Atmospheric gas metabolism is not mutually exclusive with other trophic strategies. The volcano *Pseudonocardia* sp. hosts fully encoded aerobic heterotrophic and autotrophic carbon acquisition pathways, and several energy storage pathways (**Figure 5**). The large and small RuBisCO subunit genes of the volcano *Pseudonocardia* sp. both cluster within the form IC clade, which contains other known bacterial facultative autotrophs (Yuan et al., 2012) including various Actinobacteria such as *P. asaccharolytica*, further suggesting a flexibility in carbon and energy acquisition physiology. It is certainly possible this organism is opportunistic, capable of survival at low metabolic rates through the utilization of a variety of low-concentration and constantly replenished atmospheric gases, but perhaps is also capable of capitalizing on pulses of other multi-carbon nutrients and water when they become available, such as after a snow melt event. Further understanding of the environmental conditions and how they vary through annual cycles at these difficult to access field sites combined with direct experimental growth assays will be required to test if and how this bacterium, or other members of the community, may grow under and respond to, variable and stressful conditions.

#### STRESS TOLERANCE AND OTHER TRAITS

Metabolism of various trace atmospheric substrates may be important adaptations to survival in the harsh and nutrient limited desert volcano environment, but the reduced and underdispersed phylogenetic diversity of the microbial community (**Figure 1**, **Table 1**) suggests that other traits must be important for fitness, given that $H_2$ and CO oxidizing genes are present in many species of several bacterial phyla. Actinobacteria have a seemingly ubiquitous distribution across varied terrestrial and aquatic environments (Dinsdale et al., 2008), but are relatively most abundant in cold-desert soil environments (Fierer et al., 2012). Some obvious traits of the actinobacteria are likely linked to desert fitness, such as gram positive cell wall architecture, which is perhaps an original adaptation to ancient terrestrial colonization (Battistuzzi and Hedges, 2009; Rinke et al., 2013), and the ability of many lineages to sporulate. However, given the metabolic diversity and rapid genomic evolution found within this phylum (Zaneveld et al., 2010), the full scope of desert actinobacteria traits remains largely uncharacterized.

The volcano *Pseudonocardia* sp. assembly contains COGs with relatively high copy number compared to other species of the genus that could possibly underlie stress tolerance adaptations including: DNA replication and repair machinery, transcriptional regulators, response regulators, cytochrome P450, arabinose efflux permeases, ABC-type multidrug transport systems and non-ribosomal peptide synthesis pathways (NRPS, Supplementary Table 1). It is not possible to determine the exact functional roles these genes play without experimental confirmation, but it is conceivable they could be linked to adaptations to the stresses of wet-dry or freeze-thaw cycling or UV exposure. The multiple copies ($\geq$18) of the NRPS genes are notable because they share sequence homology most similar to the antibiotic gramicidin D gene set (Kessler et al., 2004). Considering the known importance of extrapolymeric substance production as a xerotolerace trait for many microorganisms (Lennon et al., 2012), and the presence of arabinose and polysaccharide export genes in the volcano *Pseudonocardia* sp. genome, it is not surprising that investment in antibiotic defense mechanisms that may ward off scavengers of these vulnerable carbon sources (e.g., fungi, Schmidt et al., 2012) may also be necessary.

We compared all well aligned homologs between the volcano *Pseudonocardia* sp. to *P. asaccharolytica* in order to identity how selection may have affected the amino acid sequences (and functions) of certain genes. *P. asaccharolytica* was isolated from a dimethyl sulfide and tree-bark biofilter enrichment experiment (Reichert et al., 1998), but little else is known about its ecology or physiology other than the lack of ability to oxidize any of the single carbohydrates tested in the original report, and that it can be grown at moderate rates on TSA media at mesophillic temperatures. Our analysis identified 59 volcano *Pseudonocardia* sp. genes (4.7% of all analyzed homolog pairs, Supplementary Table 2) that have higher rates of non-synonymous mutations when compared to their homolog in *Pseudonocardia assaccharolytica* ($K_a$:$K_s \geq 1$) because they evolved under a strong divergent selection regime (**Figure 4**). These genes fall into categories of protein translation (four tRNA methyltransferase modification enzymes and a ribosomal modulation protein), respiration (succinate dehydrogenase), energy storage (acyl CoA dehydrogenase) and membrane transport (polysaccharide, multidrug, potassium, phosphate and cyanate). Other annotations of genes found with a $\geq 1$ $K_a$:$K_s$ratio are more difficult to interpret such as 13 uncharacterized conserved proteins and three transposases, but underscore the potential for discovery of novel microbial traits from understudied environments and taxa. Although this analysis cannot determine the particulars of how these genes differ in terms of the reaction kinetics or substrate specificities of the enzymes they code for, functions like membrane transport and energy storage could plausibly underlie important survival traits for conditions in the nutrient limited high-elevation volcanic deposits of this study.

Another interesting aspect of the $K_a$:$K_s$ ratio analysis is that only 23% of total volcano *Pseudonocardia* sp. protein coding genes could be unambiguously aligned to homologs from *P. asaccharolytica*. The remaining 77% of genes are too divergent to analyze with this method. This limits the power of the analysis somewhat, but highlights the genetic novelty of each of these organisms, and suggests that further genomic and culture work on the *Pseudonocardia* spp. is warranted.

We find the most abundant genome in the community is intermediately sized (4.9 Mb, not including highly repetitive content, **Table 2**), and codes for diverse metabolic potential. This size is not unexpected though, as work by Konstantinidis and Tiedje (2004) shows evidence that heterogeneous, variable, and low nutrient niches in soils select for larger genomes, which often contain enhanced regulatory and secondary metabolite synthesis pathways. Barberán et al. (2014) recently expanded this concept by showing that, to some extent, genome size is a reflection

of the complexity and variability of terrestrial bacterial niches. Thus, even though utilization of low concentration atmospheric substrates may be important traits for the volcano *Pseudonocardia* sp., we did not expect to find signatures of genome streamlining, as have been documented in oceanic bacteria that specialize in low concentration nutrient uptake (Giovannoni et al., 2014). Given the variability of a high mountain top environment (Lynch et al., 2012) that experiences frequent wet-dry and freeze-thaw cycling stresses (Stres et al., 2010), we are not surprised to find significantly higher numbers of genes classified in the regulation and cell signaling categories in the total metagenome (**Figure 2**), as well as specific examples of transcription and response regulator genes with high copy numbers (Supplementary Table 1), and with high $K_a$:$K_s$ ratios (Supplementary Table 2) in the genome of the most abundant community member.

## CONCLUSIONS

The functional inferences drawn from this culture-independent study can now serve as testable hypotheses for ongoing culture-based experiments. Although a modest collection of bacteria and fungi have been cultured and isolated from these volcano samples using a variety of selection techniques (unpublished), the most abundant lineages observed from culture-independent approaches have thus far resisted isolation. Nevertheless, the results we present here can inform future culture-based physiological analyses by providing information on potential electron donors and growth conditions.

The atmosphere interfaces with diverse terrestrial and aquatic environments, so it is possible that the pathways and signatures of selection we have detected result from activity and replication elsewhere. Selective dispersal and dormancy processes cannot be ruled out either; perhaps we have recovered genomic material from the most well-dispersing or longest surviving spores. Although there is little evidence to suggest that the most abundant organism from the Llullaillaco Volcano study sites is native to another environment, or is an exceptional spore producer, these are possibilities that cannot yet be rejected, especially considering the evidence for wind borne transport of other lower abundance lineages of the community (Supplementary Figure 1).

Overall, our initial analyses of these metagenomes indicates that despite, or perhaps because of, the intense solar radiation this sparsely populated high-elevation microbial community lacks endogenous photosynthesizing primary producers, but possesses the genetic potential for utilization of various low molecular weight atmospheric substrates and $CO_2$ fixation. This seems to support our hypothesis that chemoautotrophic, rather than photoautotrophic, microbes may be supplying organic carbon to simple and low-energy flux communities at these sites, but does not allow us to determine the relative roles that heterotrophic or mixotrophic metabolism may play. Bacterial growth on trace gases and aerosols is difficult to study and can likely support only low rates of metabolism. Answering whether or not the intriguing combination of metabolic pathways found in the volcano *Pseudonocardia* sp. genome indicates an actual dependency for growth on one or more atmospheric substrates requires direct physiological experimentation at relevant gas concentrations. These pathways could also be supplemental to more standard heterotrophic metabolism, and may not by themselves support growth and cell division. Future studies of these high-elevation actinobacteria and their relatives (Cockell et al., 2013; Rhodes et al., 2013) should consider the possibility that a mixture of atmospheric, precipitation and soil derived substrates may be required for growth, or that these organisms are but remnants of extinct ecosystems or windblown transients.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00698/abstract

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# Probing the diversity of chloromethane-degrading bacteria by comparative genomics and isotopic fractionation

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Chloromethane ($CH_3Cl$) is produced on earth by a variety of abiotic and biological processes. It is the most important halogenated trace gas in the atmosphere, where it contributes to ozone destruction. Current estimates of the global $CH_3Cl$ budget are uncertain and suggest that microorganisms might play a more important role in degrading atmospheric $CH_3Cl$ than previously thought. Its degradation by bacteria has been demonstrated in marine, terrestrial, and phyllospheric environments. Improving our knowledge of these degradation processes and their magnitude is thus highly relevant for a better understanding of the global budget of $CH_3Cl$. The *cmu* pathway, for *c*hloro*m*ethane *u*tilisation, is the only microbial pathway for $CH_3Cl$ degradation elucidated so far, and was characterized in detail in aerobic methylotrophic Alphaproteobacteria. Here, we reveal the potential of using a two-pronged approach involving a combination of comparative genomics and isotopic fractionation during $CH_3Cl$ degradation to newly address the question of the diversity of chloromethane-degrading bacteria in the environment. Analysis of available bacterial genome sequences reveals that several bacteria not yet known to degrade $CH_3Cl$ contain part or all of the complement of *cmu* genes required for $CH_3Cl$ degradation. These organisms, unlike bacteria shown to grow with $CH_3Cl$ using the *cmu* pathway, are obligate anaerobes. On the other hand, analysis of the complete genome of the chloromethane-degrading bacterium *Leisingera methylohalidivorans* MB2 showed that this bacterium does not contain *cmu* genes. Isotope fractionation experiments with *L. methylohalidivorans* MB2 suggest that the unknown pathway used by this bacterium for growth with $CH_3Cl$ can be differentiated from the *cmu* pathway. This result opens the prospect that contributions from bacteria with the *cmu* and *Leisingera*-type pathways to the atmospheric $CH_3Cl$ budget may be teased apart in the future.

**Keywords: bacteria, chloromethane, comparative genomics, isotope fractionation, diversity**

## INTRODUCTION

Halocarbons such as chloromethane ($CH_3Cl$) and bromomethane are known for their ozone depletion potential (Harper, 2000). $CH_3Cl$, the most abundant volatile halocarbon in the atmosphere (~600 ppt), is responsible for approximately 15% of halogen-dependent ozone destruction in the stratosphere (Harper, 2000; Montzka and Reimann, 2011). The largest sources of $CH_3Cl$ emissions to the atmosphere include terrestrial vegetation (Hamilton et al., 2003; Yoshida et al., 2004; Keppler et al., 2005) and in particular the phyllosphere (i.e., aboveground parts of vegetation, Saito and Yokouchi, 2008), biomass burning, and the oceans (Montzka and Reimann, 2011). Conversely, the dominant sink for $CH_3Cl$ is via reaction with hydroxyl radicals in the troposphere and represents 84% of the total, estimated at 4.1 Tg Cl $yr^{-1}$ (Yoshida et al., 2004). However, certain methylotrophic bacteria capable of using $CH_3Cl$ as their sole source of carbon and energy for growth may also participate in this process, but the magnitude of their contribution remains to be characterized. Chloromethane-degrading bacteria are quite widespread, with representatives affiliated to the genera *Aminobacter*, *Hyphomicrobium*, *Leisingera*, *Methylobacterium*, *Roseovarius* (Alpha-Proteobacteria), *Pseudomonas* (Gamma-Proteobacteria) and *Acetobacterium* (*Actinobacteria*), isolated from diverse environments such as soils (Doronina et al., 1996; Miller et al., 1997; Coulter et al., 1999; McAnulla et al., 2001), activated sludge (Hartmans et al., 1986; Traunecker et al., 1991; Freedman et al., 2004), freshwaters (McAnulla et al., 2001), and seawater (Schäfer et al., 2005).

The only pathway for $CH_3Cl$ degradation known so far is corrinoid- and tetrahydrofolate-dependent, and was characterized in detail for the aerobic facultative methylotrophic strain *Methylobacterium extorquens* CM4 (Vannelli et al., 1999). This pathway, termed *cmu* (abbreviation for chloromethane utilization), involves a set of genes that were subsequently detected in several other chloromethane-degrading strains (reviewed in Schäfer et al., 2007; also see Nadalig et al., 2011). The first step of the *cmu* pathway involves the methyltransferase/corrinoid-binding CmuA protein, which transfers the $CH_3Cl$ methyl group to a corrinoid cofactor, and CmuB, another methyltransferase which catalyzes the transfer of the methyl group from the methylated

corrinoid to tetrahydrofolate ($H_4F$). Methyl-$H_4F$ is then oxidized to methylene-$H_4F$ and further to $CO_2$ via formate to conserve energy, or exploited for biomass production. However, other yet to be characterized metabolic pathways may be involved in the degradation of $CH_3Cl$ in the environment. For example, *Leisingera methylohalidivorans* MB2 grows with methyl halides but was reported not to contain close homologs of *cmu* genes (Schäfer et al., 2007).

Evidence for a given metabolic pathway may be obtained through the use of stable isotope techniques, and this has been used to distinguish different sources and sinks for $CH_3Cl$ (Harper et al., 2001, 2003; Czapiewski et al., 2002; Keppler et al., 2004, 2005; Saito and Yokouchi, 2008; Greule et al., 2012; Redeker and Kalin, 2012). Degradation of $CH_3Cl$ by cell suspensions of strains with the *cmu* pathway is also associated with specific carbon fractionation (Miller et al., 2001) but also with hydrogen isotope fractionation (Nadalig et al., 2013). Thus, isotopic approaches combined with genomic approaches may prove decisive in constraining the bacterial contribution to the global $CH_3Cl$ budget.

In the present study, we review available bacterial genome sequences for the presence of *cmu* genes, thereby uncovering several bacteria that have not been described to degrade $CH_3Cl$. In parallel and as a proof of concept for the potential of isotope methods to characterize yet unknown pathways for $CH_3Cl$ degradation, we determined hydrogen and carbon isotopic fractionation patterns of $CH_3Cl$ during growth of the chloromethane-degrading strain *L. methylohalidivorans* MB2 lacking *cmu* genes, as compared to that observed for *cmu* pathway strains *M. extorquens* CM4 and *Hyphomicrobium* sp. MC1.

## MATERIALS AND METHODS

### BIOINFORMATIC ANALYSIS

Comparative genome analysis was performed with the software tools available on the Microscope platform at Genoscope (Vallenet et al., 2009), using the assembled sequences of *M. extorquens* CM4 (GenBank accession numbers CP001298, CP001299, CP001300), *Hyphomicrobium* sp. MC1 (FQ859181), *Desulfomonile tiedjei* (CP003360, CP003361), *Thermosediminibacter oceani* (CP002131), *Thermincola potens* (CP002028), and *L. methylohalidivorans* MB2 (CP006773, CP006774, CP006775), and the draft sequences for *Desulfotomaculum alcoholivorax* (GenBank AUMW00000000; 66 contigs), *Desulfurispora thermophila* (GenBank AQWN00000000; 19 contigs; **Table 1**).

### BACTERIAL STRAINS AND GROWTH CONDITIONS

Strains *M. extorquens* CM4 and *Hyphomicrobium* sp. MC1 were laboratory stocks and cultivated in a mineral medium for methylotrophic bacteria (M3; Roselli et al., 2013) containing ($L^{-1}$ of distilled water) $KH_2PO_4$ (6.8 g), $(NH_4)_2SO_4$ (0.2 g), NaOH (5 M) (5.85 mL), yielding a final pH of 7.2. After autoclaving, 1 mL $L^{-1}$ medium each of calcium nitrate solution (25 g $L^{-1}$) and of trace elements solution containing (mg $L^{-1}$) $FeSO_4$ $7H_2O$ (100), $MnSO_4$ $H_2O$ (100), $ZnSO_4$ (29.5), $Co(NO_3)_2$ $6H_2O$ (25), $CuCl_2$ $H_2O$ (25), $Na_2MoO_4$ $2H_2O$ (25), $NH_4VO_3$ (14.4), $NiSO_4$ $6H_2O$ (10), $H_3BO_3$ (10), and 0.5 mL $L^{-1}$of $H_2SO_4$ (95%) were added. Strain *L. methylohalidivorans* MB2 (DSM 14336) was obtained from DSMZ (Braunschweig, Germany) and cultivated in a mineral medium (MAMS) containing ($L^{-1}$ of distilled water) NaCl (16 g), $(NH_4)_2SO_4$ (1 g), $MgSO_4$ $7H_2O$ (1 g), $CaCl_2$ $2H_2O$ (0.2 g), $KH_2PO_4$ (0.36 g), and $K_2HPO_4$ (2.34 g) as described (Schaefer et al., 2002). After autoclaving, 1 mL $L^{-1}$ medium of trace elements solution was added. Strains CM4, MC1, and MB2 were grown with $CH_3Cl$ gas [10 mL (Fluka), effectively yielding approximately 10 mM final concentration], in 300 mL Erlenmeyer vials fitted with sealed mininert valve caps (Sigma) and containing 50 mL of medium. Cultures were incubated at 30°C on a rotary shaker (100 rpm). Abiotic controls (no bacteria added) were prepared and incubated in the same way. Growth was followed by absorbance measurement at 600 nm.

The headspace of cultures was sampled regularly (0.1 mL) for determination of $CH_3Cl$ concentration by gas chromatography, and 1 mL headspace samples were also taken at each point and conserved in 12 ml Exetainers® (Labco Limited, Lampeter, UK) for subsequent isotopic measurements. Concentration of chloride was measured in supernatants of cultures using the spectrophotometric method of Jörg and Bertau (2004), except for *L. methylohalidivorans* MB2 because of the high chloride content of MAMS medium.

### ANALYSIS OF CONCENTRATIONS AND STABLE ISOTOPE VALUES OF CHLOROMETHANE

Concentration and stable carbon and hydrogen isotope values for $CH_3Cl$ were performed by gas chromatography coupled with flame ionization detector (GC-FID) and isotope ratio mass spectrometry (IRMS), respectively, as described previously (Nadalig et al., 2013), except that helium flow entering the gas chromatograph in isotopic analysis was increased to 1.8 ml $min^{-1}$.

The conventional "delta" notation, which expresses the isotopic composition of a material relative to that of a standard on a per mil (‰) deviation basis, was used. Values of $\delta^2H$ (‰) are relative to that for V-SMOW (Vienna Standard Mean Ocean Water), and values of $\delta^{13}C$ (‰) are relative to that for V-PDB (Vienna Pee Dee Belemnite). Carbon and hydrogen isotope fractionations associated with $CH_3Cl$ degradation by *L. methylohalidivorans* MB2, *M. extorquens* CM4 and *H.* sp. MC1 were determined from the slopes ($b_C$ *and* $b_H$) of the linear regression of isotope variation ($^{13}C$ and $\delta^2H$) of $CH_3Cl$ on the logarithm of the remaining $CH_3Cl$ concentration (ln f):

$$b_C = \delta^{13}C/lnf \text{ and } b_H = \delta^2H/lnf.$$

Fractionation factors $\alpha_C$ and $\alpha_H$ were calculated as $\alpha = 1{,}000/(b{+}1{,}000)$, and also expressed as isotope enrichment factors ($\varepsilon_C$ and $\varepsilon_H$), calculated as $\varepsilon = (\alpha{-}1)10^3$. Errors represent 95% confidence intervals calculated on the least-squares regression.

## RESULTS

Several chloromethane-degrading bacteria with the *cmu* pathway have been characterized (Schäfer et al., 2007), and a complete and assembled genome sequence is available for two of them, i.e., *M. extorquens* CM4 (Marx et al., 2012) and *Hyphomicrobium* sp. MC1 (Vuilleumier et al., 2011). Two types of organization of *cmu* genes were identified (Nadalig et al., 2011). The usual gene organization involves a putative *cmuBCA* operon and was found in all

**Table 1 | Characteristics of the bacterial strains discussed in this study.**

| **Strains** | ***Methylobacterium extorquens* CM4** | ***Hyphomicrobium* sp. MC1** | ***Desulfotomaculum alcoholivorax* DSM 16058** | ***Desulfurispora thermophila* DSM 16022** | ***Desulfomonile tiedjei* DSM 6799** | ***Thermincola potens* JR** | ***Thermosedimi-nibacter oceani* DSM 16646** | ***Leisingera methylohalidivorans* MB2 DSM 14336** |
|---|---|---|---|---|---|---|---|---|
| Affiliation | Alphaproteobacteria | Alphaproteobacteria | Deltaproteobacteria | Deltaproteobacteria | Deltaproteobacteria | Clostridia | Clostridia | Alphaproteobacteria |
| Origin | Industrial soil | Sewage sludge | Fluidized-bed reactor | Fluidized-bed reactor | Sewage sludge | Anodic biofilm | Deep-sea sediment | Sea water |
| Genome size (MB) | 6.18 | 4.75 | 3.47 | 2.82 | 6.53 | 3.16 | 2.28 | 4.65 |
| GC (%) | 68 | 59 | 47 | 55 | 50 | 46 | 47 | 62 |
| Total CDS | 6035 | 4955 | 3588 | 2815 | 5664 | 3343 | 2460 | 4608 |
| Plasmids | 2 | 0 | Unknown | Unknown | 1 | 0 | 0 | 2 |
| Sequence status | Assembled, finished | Assembled, finished | 66 contigs Permanent draft | 19 contigs Permanent draft | Assembled, finished | Assembled, finished | Assembled, finished | Assembled, finished |
| Genbank accession number | CP001298 CP001299 CP001300 | FQ859181 | AUMW00000000 | AQWN00000000 | CP003360 CP003361 | CP002028 | CP002131 | CP006773 CP006774 CP006775 |
| Reference | Marx et al. (2012) | Vuilleumier et al. (2011) | Kaksonen et al. (2008) | Kaksonen et al. (2007) | DeWeerd et al. (1990) | Byrne-Bailey et al. (2010) | Pitluck et al. (2010) | Buddruhs et al. (2013) |

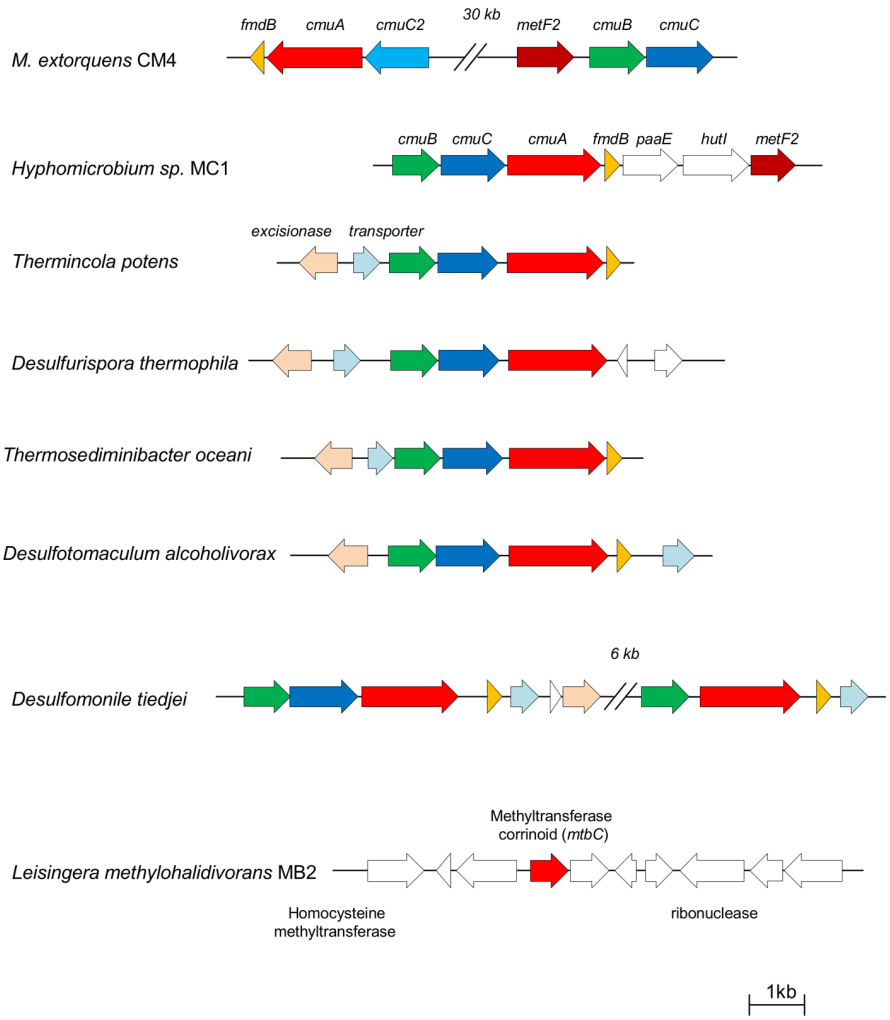


**FIGURE 1 | Comparison of *cmu* gene organization in sequenced genomes of chloromethane-degrading bacteria.** Arrows represent protein-coding genes, and homologous genes are shown in the same color. Gene clusters are drawn to scale.

experimentally characterized chloromethane-degrading bacteria with the *cmu* pathway except the reference chloromethane-degrading strain *M. extorquens* CM4, which harbors *cmu* genes in two clusters (**Figure 1**). The chloromethane-degrading strain *L. methylohalidivorans* MB2, in contrast, was known to lack *cmu* genes (Schäfer et al., 2007), so the recent report of its assembled genome sequence (Buddruhs et al., 2013) was of particular interest.

## COMPARATIVE GENOMICS

An exhaustive survey of the presence of *cmu* genes in available sequenced bacterial genomes was carried out, yielding several novel insights (**Table 2**). First, all strains with *cmu* homologs contained all three genes *cmuA*, *cmuB,* and *cmuC* essential for growth with $CH_3Cl$ using the *cmu* pathway. Second, these three genes were detected as a *cmuBCA* gene cluster (**Figure 1**) in the genomes of five bacterial strains that had not been reported to possess *cmu* genes or $CH_3Cl$ degradation activity. Strikingly and in contrast to all strains growing with $CH_3Cl$ using the *cmu* pathway described so far, all these strains are anaerobes. Three of them are Gram-negative bacteria from the class Deltaproteobacteria, i.e., *Desulfotomaculum alcoholivorax* (Kaksonen et al., 2008), *Desulfurispora thermophila* (Kaksonen et al., 2007) and *Desulfomonile tiedjei* (DeWeerd et al., 1990), and two belong to the class Clostridia, i.e., the Gram-positive *Thermincola potens* (Byrne-Bailey et al., 2010) and the Gram-negative *Thermosediminibacter oceani* (Pitluck et al., 2010). Notably, *Desulfomonile tiedjei* has a second *cmu* cluster containing only *cmuB* and *cmuA* (**Figure 1**) 6 kb away from a *cmuBCA* gene cluster. Levels of identity with homologs of the CM4 strain at the protein level are high, and range between 64 and 84%, 60 and 64%, and 34 and 39% for *cmuA*, *cmuB* and *cmuC* gene products, respectively (**Table 2**). Pairwise identity comparisons of the proteins encoded by *cmu* genes show that homologs of strains *Desulfotomaculum alcoholivorax*, *Desulfurispora thermophila*, *Thermincola potens* and *Thermosediminibacter oceani* are most related to each other, with identities at the protein level between 84–93%, 82–92%, and 66–87% for *cmuA*, *cmuB,* and *cmuC* gene products, respectively, and that

**Table 2 | Key CDS related to the *cmu* pathway in investigated genomes[a].**

| Protein | *Methylobacterium extorquens* CM4[b] | *Hyphomicrobium* sp. MC1 | *Desulfotomaculum alcoholivorax* DSM 16058 | *Desulfurispora thermophila* DSM 16022 | *Desulfomonile tiedjei* DSM 6799 | *Thermincola potens* JR | *Thermosediminibacter oceani* DSM 16646 | *Leisingera methylohalidivorans* MB2 DSM 14336 |
|---|---|---|---|---|---|---|---|---|
| CmuA | Mchl_5697 | HYPMCv2_2273 (84%) | DESALv160093 (69%) | AQWNv1_40006 (67%) | Desti_5447 (67%) Desti_5437 (64%) | TherJR_0143 (68%) | Toce_1533 (68%) | Leime_2531[c] (32%) |
| CmuB | Mchl_5727 | HYPMCv2_2275 (64%) | DESALv160091 (62%) | AQWNv1_40005 (62%) | Desti_5449 (60%) Desti_5438 (60%) | TherJR_0145 (61%) | Toce_1535 (62%) | n.d.[d] |
| CmuC | Mchl_5728 | HYPMCv2_2274 (39/35%)[e] | DESALv160092 (36/38%) | AQWNv1_40004 (35/34%) | Desti_5448 (37/36%) | TherJR_0144 (37/37%) | Toce_1534 (35/35%) | n.d. |
| CmuC2 | Mchl_5698 | | | | | | | |
| FmdB | Mchl_5696 | HYPMCv2_2271 (45%) | DESALv160094 (38%) | n.d. | Desti_5446 (42%) Desti_5436 (43%) | TherJR_0142 (42%) | Toce_1532 (37%) | n.d. |
| FolD | Mchl_5700 | HYPMCv2_2266 (47%) | DESALv110219 (40%) | AQWNv1_30228 (42%) | Desti_2379 (46%) | TherJR_1709 (41%) TherJR_1706 (39%) | Toce_0805 (40%) | Leime_3180 (52%) Leime_4077 (52%) |
| HutI | Mchl_5694 | HYPMCv2_2269 (53%) | DESALv150151 (33%) | AQWNv1_70146 (34%) | n.d. | n.d. | Toce_1473 (33%) | Leime_0109 (39%) |

*(Continued)*

**Table 2 | Continued**

| Protein | *Methylobacterium extorquens* CM4[b] | *Hyphomicrobium* sp. MC1 | *Desulfotomaculum alcoholivorax* DSM 16058 | *Desulfurispora thermophila* DSM 16022 | *Desulfomonile tiedjei* DSM 6799 | *Thermincola potens* JR | *Thermosediminibacter oceani* DSM 16646 | *Leisingera methylohalidivorans* MB2 DSM 14336 |
|---|---|---|---|---|---|---|---|---|
| MetF2 | Mchl_5726 | HYPMCv2_2268 (31%) | n.d. | n.d. | n.d. | n.d. | n.d. | Leime_2796 (35%) |
| MetF | Mchl_1881 | HYPMCv2_2119 (67%) | n.d. | n.d. | n.d. | n.d. | n.d. | Leime_1763 (46%) |
| PurU | Mchl_5699 | HYPMCv2_2267 (62%) | DESALv110045 (34%) | AQWNv1_20249 (32%) | n.d. | TherJR_0829 (33%) | Toce_1499 (33%) | Leime_2536 (36%) |
| Serine pathway (10 reactions) | Complete | Complete | 4 | 4 | 5 | 3 | 5 | 9 |
| Ethylmalonyl-CoA pathway (14 reactions) | Complete | Complete | 10 | 9 | 10 | 2 | 6 | Complete |
| $H_4$F pathway (3 reactions) | Complete | Complete | Complete | Complete | Complete | Complete | Complete | Complete |
| $H_4$MPT pathway (7 reactions) | Complete | Complete | 0 | 0 | 0 | 0 | 0 | 0 |

[a] *Sequence identity (>30%) at the protein level to* M. extorquens *CM4 CDS in brackets. All CDS homologs of CM4 genes are chromosomally encoded except hutI in* L. methylohalidivorans *MB2 which is plasmid-encoded.*
[b] *All CM4 CDS are plasmid-encoded except* metF.
[c] *Over the full length (211 aa) of the homolog.*
[d] *n.d., not detected.*
[e] *Sequence identity at the protein level to CmuC/CmuC2 of* M. extorquens *CM4 in brackets.*

CM4 homologs represent outliers for all three genes. It is interesting to note that the gene products of two copies of c*muA* and *cmuB* of *Desulfomonile tiedjei* (78 and 77% protein identity, respectively) are not each others' closest homologs. In addition, no evidence for substantial identity at the DNA level was detected between the two *cmu* gene clusters of this strain (data not shown). Further, CmuA encoded by the *cmuBCA* cluster of *Desulfomonile tiedjei* is closer to homologs from other strains (>80% identity at the protein level) than that encoded by the partial *cmu* cluster *cmuBA* (<75% identity).

Analysis of the *L. methylohalidivorans* MB2 genome (Buddruhs et al., 2013) confirmed the original report of Schäfer et al. (2007) that this $CH_3Cl$ strain did not contain *bona fide cmu* genes. As mentioned in the genome report, the closest homolog to *cmuA* is a gene coding a short (232 residues) corrinoid methyltransferase protein (MtbC) with only 32% identity to the *C*-terminal domain of CmuA (**Table 2**). However, no full-length homologs to *cmuB* and *cmuC* were detected in the genome sequence (**Table 2**). Taken together, these data confirm that the metabolic pathway used by *L. methylohalidivorans* MB2 to grow with $CH_3Cl$ is different to that of other known chloromethane-degrading strains with the *cmu* pathway.

The presence of downstream genes in the *cmu* pathway in strains containing *cmuABC* genes was also evaluated (**Table 2**). Genes potentially involved in the tetrahydrofolate ($H_4F$) dependent pathway for oxidation of methyl-$H_4F$ to formate via methylene-$H_4F$ are present in all genomes (**Table 2**), but close homologs of *metF* encoding methylene-$H_4F$ reductase were not detected except in strain MC1. Notably, only Alphaproteobacterial strains CM4, MC1, and *L. methylohalidivorans* MB2 possess the genes involved in the serine and ethylmalonyl-CoA pathways involved in growth of strains CM4 and MC1 with C1 compounds. Moreover, the tetrahydromethanopterin ($H_4MPT$) pathway crucial for growth of *Methylobacterium* with methanol (Marx et al., 2005), but thought to be dispensable for growth with $CH_3Cl$ (Studer et al., 2002), is only present in *M. extorquens* CM4 and *Hyphomicrobium* sp. MC1 which also grow with methanol, but absent in *L. methylohalidivorans* MB2, which is unable to grow with methanol, as well as in all other strains containing *cmu* genes investigated here. Finally, a search for genes common to chloromethane-degrading strains (including or excluding *L. methylohalidivorans* MB2) failed to reveal genes other than essential housekeeping genes (data not shown). This suggests that identification of the genes involved in $CH_3Cl$ degradation or in adaptation to $CH_3Cl$ metabolism is not possible by comparative genomics analysis alone.

## GROWTH OF STRAINS WITH CHLOROMETHANE AS SOLE CARBON AND ENERGY SOURCE

*Methylobacterium extorquens* CM4, *Hyphomicrobium* sp. MC1, and *L. methylohalidivorans* MB2 were cultivated with 10 mM $CH_3Cl$ as sole carbon and energy source in the recommended medium allowing fastest growth, i.e., minimal mineral medium for strains CM4 and MC1, and high-salt mineral medium for strain MB2 (**Figure 2A**). Chloromethane consumption during growth was measured in the gaseous phase by gas chromatography (**Figure 2B**). In cultures of *M. extorquens* CM4 and *H.*

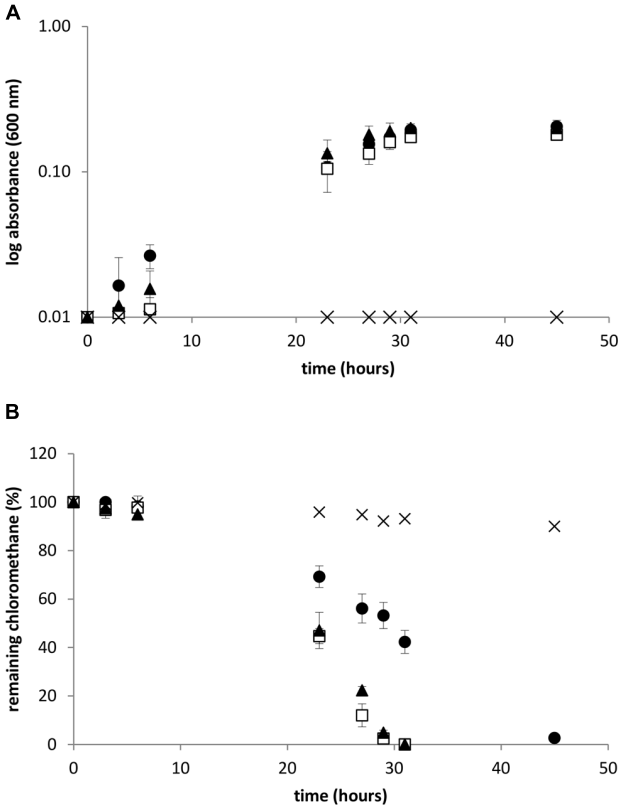


**FIGURE 2 | Growth and chloromethane degradation during bacterial cultivation**. Absorbance at 600 nm **(A)** and consumption of chloromethane **(B).** *Leisingera methylohalidivorans* MB2 (•), *M. extorquens* CM4 (□) *Hyphomicrobium* sp. MC1 (▲) and abiotic control (×). Error bars indicate the standard deviation of the mean of three biological replicates. Chloride concentration at the end of cultivation were 9.2 ± 0.2 and 9.2 ± 0.3 mol $L^{-1}$ for strains CM4 and MC1, respectively, but could not be measured in the high-salt medium used for growth of strains MB2.

sp. MC1, $CH_3Cl$ was completely degraded after 30 h under the chosen growth conditions. In contrast, consumption of $CH_3Cl$ by the *L. methylohalidivorans* MB2 culture required a longer time (∼45 h) to proceed to completion, although its growth behavior was similar to that of the other two strains.

## CARBON AND HYDROGEN ISOTOPE FRACTIONATION OF CHLOROMETHANE DURING GROWTH

During degradation of $CH_3Cl$, $\delta^{13}C$ values of residual chloromethane increased from approximately −32‰ (initial value) to 55, 9, and 33‰ for strains CM4, MC1, and MB2 respectively (**Figure 3A**). No carbon or hydrogen fractionation was observed in abiotic controls with media M3 and MAMS (data not shown). Derived values of isotope fractionation factor ($\alpha_C$) and of the corresponding enrichment factor were very similar for *cmu* pathway strains CM4 and MC1, and substantially larger for *L. methylohalidivorans* MB2 (**Table 3**).

However, trends were markedly different for the three strains when considering the enrichment of $^2H$ in residual $CH_3Cl$ during cultivation. For strains CM4 and MC1, $\delta^2H$ values increased

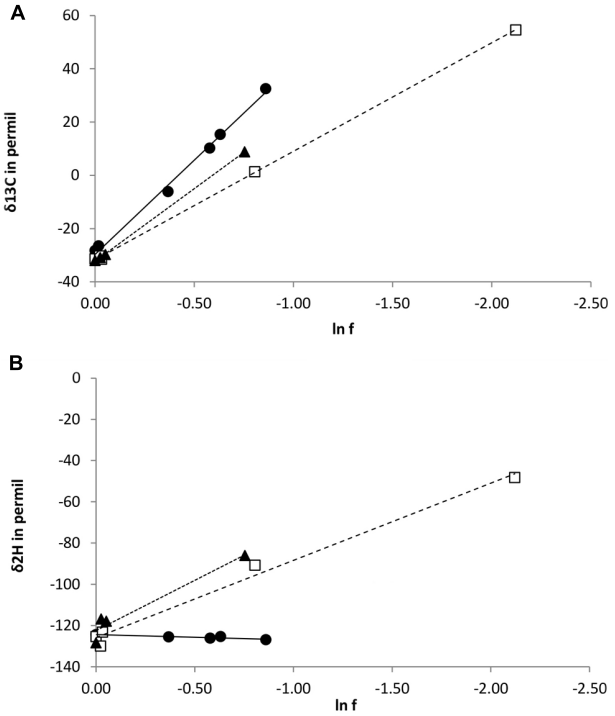


**FIGURE 3 | Isotope variations of chloromethane during bacterial cultivation with chloromethane. (A)** $\delta^{13}C$ and **(B)** $\delta^{2}H$ in relation to the fraction of remaining chloromethane (f). Lines represent best-fit linear regressions. *L. methylohalidivorans* MB2 (•), *M. extorquens* CM4 (□), and *Hyphomicrobium* sp. MC1 (▲).

from approximately −124‰ at the start of the experiment to 16‰ and −12‰ for strains CM4 and MC1, respectively (**Figure 3B**). In marked contrast, however, no substantial change of $\delta^{2}H$ was observed during degradation of $CH_3Cl$ by *L. methylohalidivorans* MB2 (**Figure 3B**). This resulted in large differences of hydrogen stable isotope fractionation factor ($\alpha_H$) and of the corresponding enrichment factor between strains CM4 and MC1 containing the *cmu* pathway for $CH_3Cl$ degradation on the one hand, and strain MB2 lacking the corresponding genes on the other hand (**Table 3**).

## DISCUSSION

The presence of *cmu* genes in recently completed genome sequences was somewhat expected, but their detection in exclusively anaerobic bacteria came as a surprise considering that they had so far only been found in aerobic chloromethane-degrading bacteria. Anaerobic chloromethane-degrading bacteria reported so far use a different, although in one case at least also corrinoid-dependent, pathway (Traunecker et al., 1991; Messmer et al., 1993; Freedman et al., 2004). Worthy of note, $CH_3Cl$ dehalogenation by the *cmu* pathway does not require aerobic conditions and is actually sensitive to oxygen (Studer et al., 2001). It is thus possible that anaerobic bacteria with *cmu* genes identified here (**Table 2**) are actually able to use $CH_3Cl$ as a carbon and energy source, although this remains to be tested experimentally.

The conserved *cmuBCA* gene organization (**Figure 1**) in $CH_3Cl$-degrading bacteria (Nadalig et al., 2011), and the high level of identity between the protein sequences encoded by *cmu* genes of *Thermincola potens*, *Desulfurispora thermophila*, *Thermosediminibacter oceani*, *Desulfotomaculum alcoholivorax,* and *Desulfomonile tiejdei* (>81, >74, and >58% for *cmuA*, *cmuB,* and *cmuC,* respectively), suggests a common evolutionary origin for these genes and their dissemination in the environment by horizontal gene transfer. The presence of an excisionase in the immediate environment of *cmu* genes in these five strains (**Figure 1**) further supports acquisition of *cmu* genes by horizontal transfer in these strains, as does the presence of two *cmu* gene clusters in *Desulfomonile tiedjei* whose sequences are not closer related to each other than to those of other chloromethane-degrading strains. To our knowledge, however, potential sources of $CH_3Cl$ that would support dissemination of *cmu* genes in anaerobic environments have not yet been reported.

Incidentally, our analysis also confirms the particular status in the *cmu* pathway of *cmuC*, a gene shown to be essential for growth of strain CM4 with $CH_3Cl$ (Studer et al., 2002; Roselli et al., 2013) but whose function remains elusive. Indeed, sequence conservation among the proteins encoded by genes *cmuA*, *cmuB,* and *cmuC* are lowest for the CmuC gene product (**Table 2**). Moreover, the probable loss of a *cmuC* homolog in one of the two *cmu* gene clusters of *Desulfomonile tiedjei* strain CM4 (**Figure 1**) also hints at its possibly lesser role in $CH_3Cl$ metabolism.

As to the chloromethane-degrading strain *L. methylohalidivorans* MB2, analysis of its genome (Buddruhs et al., 2013) confirms the initial report (Schäfer et al., 2007) that it lacks the *cmu* pathway. The best partial hit to CmuA (32% amino acid identity) is a 211-residue corrinoid protein, and *cmuB* or *cmuC* homologs were not detected in the *L. methylohalidivorans* MB2 genome (**Table 2**). However, downstream genes of the $H_4F$-dependent *cmu* pathway (*metF*, *folD*, *purU*) were all found, so an $H_4F$-dependent metabolic

**Table 3 | Isotopic enrichment ($\varepsilon$) and fractionation ($\alpha$) factors for carbon and hydrogen during growth with chloromethane.**

| | $\varepsilon_C$(‰) | $R^{2a}$ | $\alpha_C$ | $\varepsilon_H$ (‰) | $R^{2a}$ | $\alpha_H$ |
|---|---|---|---|---|---|---|
| *Methylobacterium extorquens* CM4 | 42 | 0.9997 | 1.042 | 39 | 0.9886 | 1.039 |
| *Hyphomicrobium* sp. MC1 | 54 | 0.9999 | 1.054 | 51 | 0.9418 | 1.051 |
| *Leisingera methylohalidivorans* MB2 | 76 | 0.9951 | 1.076 | 0 | 0.8230 | 1.000 |

[a] *Quality of fit to linear least-squares regression.*

pathway for growth of *L. methylohalidivorans* MB2 with $CH_3Cl$ remains a possibility.

In our experiments, we showed that *L. methylohalidivorans* MB2, previously grown with $CH_3Cl$ (0.37 mM; Schaefer et al., 2002), is capable of using this one-carbon compound as sole carbon and energy source at an initial concentration of 10 mM. A direct comparison of its growth behavior with that of strains CM4 and MC1 is prevented by the fact that the latter two strains do not grow in high-salt mineral medium, whereas *L. methylohalidivorans* MB2 does not grow in the standard low-salt mineral medium used for strains CM4 and MC1. Incidentally, this suggests that salt adaptation may be unrelated to adaptation to intracellular chloride production during dehalogenation, as observed recently for bacteria growing with dichloromethane (Michener et al., 2014).

The differences in $CH_3Cl$ metabolism of *L. methylohalidivorans* MB2 suggested by comparative genomics were experimentally supported by isotope analysis (**Figure 3**; **Table 3**). For *L. methylohalidivorans* MB2, isotopic enrichment factor for carbon during growth was substantially larger than for CM4 and MC1, indicating a larger primary isotope effect and providing further evidence for operation of another pathway for utilization of $CH_3Cl$ in this strain. In contrast, a previous study on carbon isotopic fractionation of $CH_3Cl$ by cell suspensions of three bacterial strains, including *L. methylohalidivorans* MB2, gave similar isotopic enrichment values (ranging between 42 and 47‰; Miller et al., 2001). In particular, the value obtained for *Aminobacter ciceronei* strain IMB1, the only strain so far shown to possess *cmuA* but not *cmuB* (Woodall et al., 2001), was similar to those of strains CM4 and MC1 (Miller et al., 2001). This suggests that the corrinoid dehalogenase protein CmuA drives carbon isotopic fractionation in chloromethane-degrading strains with the *cmu* pathway. Moreover and unlike for carbon, a larger isotope effect than in previous resting cell experiments (Nadalig et al., 2013) was observed for hydrogen during growth in strains CM4 and MC1. However, the most striking finding of the present study was the lack of substantial hydrogen isotope enrichment upon $CH_3Cl$ degradation by *L. methylohalidivorans* MB2. This suggests that unlike CmuAB chloromethane dehalogenase, the unknown dehalogenase of this strain does not cause hydrogen fractionation during degradation of the chloromethane methyl group. Nevertheless and as a common denominator to all three chloromethane-degrading strains investigated here (**Table 3**), carbon isotope fractionation (the primary isotope effect in cleavage of the carbon-halogen bond) was more pronounced than hydrogen isotope fractionation (a secondary isotope effect in $CH_3Cl$ dehalogenation), as expected (Elsner et al., 2005).

The observed differences in isotopic fractionation of $CH_3Cl$ carbon and hydrogen between the three strains CM4, MC1, and MB2 are best visualized in **Figure 4**, which shows the trends in enrichment of the heavier isotope of carbon and hydrogen for the different strains at different time points during growth. As proposed by Elsner et al. (2005), the slopes in these graphs constitute a clear indication that *L. methylohalidivorans* MB2 uses a different pathway for growth with $CH_3Cl$ than strains CM4 and MC1, which utilize the same pathway.

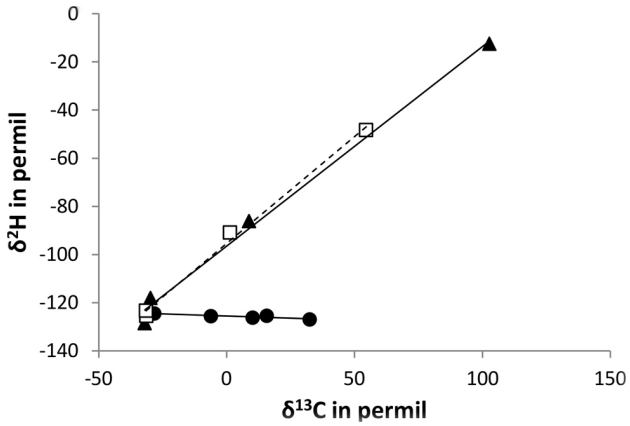


**FIGURE 4 | Changes in carbon and hydrogen isotope ratios for degradation of chloromethane by *L. methylohalidivorans* MB2 (•), *M. extorquens* CM4 (□), and *Hyphomicrobium* sp. MC1 (▲) at different time points during growth (increase in time from left to right of the graph).** Lines (slope = −0.03 ($R^2$: 0.8014) for strain MB2, slope = 0.89 ($R^2$: 0.9954) for strain CM4, and slope = 0.83 ($R^2$: 0.9939) for strain MC1, respectively) represent best fit regressions.

Measurements of isotopic fractionation for a given environmental compartment will include the overall contribution of the metabolic diversity of chloromethane-degrading bacteria and their relative occurrence in that environment. It is tempting to speculate that chloromethane degradation in the soil environment, for which an isotopic fractionation of 49‰ similar to that found here for strains CM4 and MC1 was obtained in a previous study (Miller et al., 2004), is predominantly performed by bacteria with the *cmu* pathway. Our results on microbially driven hydrogen and carbon isotope fractionation suggest that using in a two-dimensional isotope scheme might help to confirm this hypothesis. Thus, a combination of genomic studies with physiological and isotopic characterisation of chloromethane-degrading bacterial strains, as performed here, will remain a major objective for the near future in order to constrain the bacterial sink strength of the atmospheric budget of $CH_3Cl$.

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# Microbial acetone oxidation in coastal seawater

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Acetone is an important oxygenated volatile organic compound (OVOC) in the troposphere where it influences the oxidizing capacity of the atmosphere. However, the air-sea flux is not well quantified, in part due to a lack of knowledge regarding which processes control oceanic concentrations, and, specifically whether microbial oxidation to $CO_2$ represents a significant loss process. We demonstrate that $^{14}C$ labeled acetone can be used to determine microbial oxidation to $^{14}CO_2$. Linear microbial rates of acetone oxidation to $CO_2$ were observed for between 0.75-3.5 h at a seasonally eutrophic coastal station located in the western English Channel (L4). A kinetic experiment in summer at station L4 gave a $V_{max}$ of 4.1 pmol $L^{-1}$ $h^{-1}$, with a $K_m$ constant of 54 pM. We then used this technique to obtain microbial acetone loss rates ranging between 1.2 and 42 pmol $L^{-1}$ $h^{-1}$ (monthly averages) over an annual cycle at L4, with maximum rates observed during winter months. The biological turnover time of acetone (*in situ* concentration divided by microbial oxidation rate) in surface waters varied from ~3 days in February 2011, when *in situ* concentrations were 3 ± 1 nM, to >240 days in June 2011, when concentrations were more than twofold higher at 7.5 ± 0.7 nM. These relatively low marine microbial acetone oxidation rates, when normalized to *in situ* concentrations, suggest that marine microbes preferentially utilize other OVOCs such as methanol and acetaldehyde.

**Keywords: bacteria, kinetics, acetone oxidation, Western English Channel (L4), radioactive labeling, seasonality, acetone turnover**

## INTRODUCTION

Acetone is a ubiquitous oxygenated volatile organic compound (OVOC) in the troposphere [e.g., Singh et al., 1995, 2003; Lewis et al., 2005], and is thought to play an important role in the chemistry of the atmosphere by sequestering nitrogen oxides, and by providing $HO_x$ radicals through photolysis (Singh et al., 1995; Wennberg et al., 1998), thus influencing the oxidizing capacity and ozone formation (Singh et al., 2001). The composition of OVOCs in the troposphere and lower stratosphere is dominated by acetone, acetaldehyde, and methanol, e.g., Read et al. (2012). Total global sources of acetone range between 37 and 95 million tons per year (Singh et al., 2000, 2001, 2004; Jacob et al., 2002). Primary terrestrial, e.g., pasture and forest emissions and secondary anthropogenic sources (including biogenic propane oxidation) account for approximately half of known acetone sources (Singh et al., 2000). The oceans are thought to play a major role in controlling atmospheric acetone levels (Fischer et al., 2012), although whether the oceans currently act as a net source or sink to the atmosphere is not clear (Williams et al., 2004; Lewis et al., 2005; Marandino et al., 2005; Taddei et al., 2009; Fischer et al., 2012). However, recent data suggest that the North and South oligotrophic gyres of the Atlantic Ocean are a source of acetone to the atmosphere, whilst near air–sea equilibrium conditions dominates over equatorial waters, and temperate open ocean regions (high northern and southern latitudes) show a flux from the atmosphere to the oceans (Beale et al., 2013).

Acetone is thought to be produced photochemically in seawater from chromophoric dissolved organic matter (Mopper and Stahovec, 1986; Kieber et al., 1990; Mopper et al., 1991; de Bruyn et al., 2011; Dixon et al., 2013a), with strong diurnal variability (Zhou and Mopper, 1997). Acetone production due to photochemical processes was recently estimated at 48–100% of gross production for remote Atlantic Ocean surface waters (Dixon et al., 2013a). Biological production of substantial amounts of acetone (up to 8.7 mM) by cultured marine *Vibrio* species during degradation of leucine has also been reported (Nemecek-Marshall et al., 1995). Acetone is also an intermediate in the metabolism of propane, and is converted, via acetol to either acetaldehyde (+formaldehyde), acetic acid (+formaldehyde) or ultimately to pyruvic acid by a number of bacteria such as *Rhodococcus* and *Mycobacterium*. As both of these species are widespread in terrestrial and marine environments (Hartmans and de Bont, 1986; Ashraf et al., 1994), biological production of acetone is considered likely in agreement with recent marine incubation experiments (Dixon et al., 2013a).

Acetone losses in seawater are less well understood. Previous bacterial culture experiments have shown microbial uptake of acetone (Rathbun et al., 1982; Sluis and Ensign, 1997) with insignificant losses due to direct photolysis in fresh and riverine waters (Rathbun et al., 1982). Loss of acetone in seawater samples from a coastal station in the Pacific Ocean (33.6N, 118W) have recently suggested a short half-life of 5.8 ± 2.4 h with significant diurnal and seasonal variability (higher loss rates observed during winter and earlier in the day, de Bruyn et al., 2013). However, this contrasts with estimates from surface open ocean Atlantic waters where a comparison of *in situ* acetone concentrations with microbial oxidation rates from incubation experiments suggest much longer biological lifetimes ranging between 3 and 82 days (Beale

et al., 2013; Dixon et al., 2013a). Acetone oxidation rates have been shown to linearly positively correlate with bacterial production (Dixon et al., 2013a), and an inverse linear relationship has also been observed between acetone seawater concentrations and bacterial production (Beale et al., 2013). Thus, despite relatively low microbial acetone oxidation rates (compared to other OVOCs like methanol and acetaldehyde, Dixon et al., 2011a,b, 2013a; Dixon and Nightingale, 2012) these relationships suggest that as bacterial production increases, so does the rate of microbial acetone oxidation, leading to a reduction in the *in situ* concentration of acetone.

The aim of this study was to make a comprehensive assessment of the range and significance of microbial acetone oxidation rates over an annual cycle at a coastal observatory situated in the western English Channel.

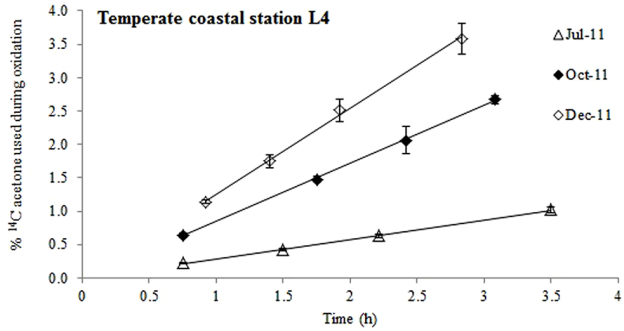


**FIGURE 1 | Time course experiments showing the % of added $^{14}$C-labeled acetone that was used during oxidation at station L4 during different months of the year.** The error bars represent ±1 standard deviation based on three replicates.

## MATERIALS AND METHODS

We have used a radiochemical technique with pico-molar additions of $^{14}$C labeled acetone ($^{14}CH_3CO^{14}CH_3$) to seawater to determine the microbial transformation (oxidation) of acetone to carbon dioxide, in a similar approach to that of Dixon et al. (2011a) for $^{14}$C labeled methanol.

### SAMPLE COLLECTION

Surface water samples (≤10 m) were collected from a long term monitoring station, situated approximately 10 nautical miles south-west of Plymouth, called L4 (50.3N, 04.22W, water depth ∼55 m, Smyth et al., 2010). Samples were pumped directly into acid-washed quartz Duran bottles and stored in the dark for the 2–3 h transit back to the laboratory. Labeled $^{14}$C acetone was purchased from American Radiolabeled Chemicals, Inc with a specific activity of 30 Ci mmol$^{-1}$ (ARC0469, neat liquid in sealed ampoule). Primary stocks were made by diluting 1 mCi into 40 mls of 18 MΩ Milli Q water (0.025 mCi mL$^{-1}$) and were stored in gas-tight amber vials in the dark at 4°C. Stability and storage trials suggested a loss in activity of <5% over 12 months. Addition volumes of $^{14}$C acetone to seawater samples were always <1% of the sample volume and typically ≤5% of the label was used during incubations ≤3.5 h.

### TIME COURSE EXPERIMENTS

Time course experiments were initially carried out to determine the period of linear incorporation of the $^{14}$C label. Labeled acetone ($^{14}$C) was added to seawater samples to yield final concentrations of 40–90 pM (2700–6100 disintegrations per minute mL$^{-1}$) depending on the experiment (**Figure 1**). Samples were incubated in acid washed polycarbonate bottles in the dark for between <1-6.5 h at *in situ* sea surface temperature. At selected times, triplicate sub-samples were taken to assess microbial oxidation to $^{14}CO_2$. Oxidation of $^{14}$C labeled acetone to $^{14}CO_2$was determined by pipetting 1 ml samples into 2 ml micro centrifuge tubes and adding 0.5 ml of $SrCl_2.6H_2O$ (1 M), to precipitate the $^{14}CO_2$ as $Sr^{14}CO_3$, 20 μl of NaOH (1 M), to neutralize the HCl produced, and 100 μl of $Na_2CO_3$(1 M), to ensure adequate pellet formation (Connell et al., 1997; Goodwin et al., 1998). After centrifugation the supernatant was aspirated, the pellet washed twice with ethanol (80%), resuspended in 1 ml of concentrated NaOH solution (∼ 10 nM) that had been adjusted to a pH of 11.7, before addition of Optiphase HiSafe III to create a slurry. The samples were vortex mixed and stored in the dark for >24 h before being analyzed on a scintillation counter (Tricarb 3100 or 2910, Perkin Elmer). This period ensures that any chemiluminescence arising from interactions between NaOH and Optiphase scintillant subside (Kiene and Hoffmann Williams, 1998).

### KINETIC DETERMINATIONS

The kinetics of microbial acetone oxidation were investigated at L4 during February and June 2011 using 1.0 ml surface seawater samples. Surface samples received an addition of $^{14}$C-labeled acetone, and a series of tubes for microbial oxidation were treated to yield a range of $^{14}$C concentrations between 2 and 47 nM (∼2.5% of added $^{14}$C acetone was oxidized) during February and between 6 and 1006 pM (1.4–5.5% of added $^{14}$C acetone was oxidized) during June 2011. Samples were incubated in screw topped, O-ring sealed micro tubes in the dark at *in situ* temperature. Three replicates from each acetone concentration were processed, as detailed above, after approximately 1 h incubation period.

### ACETONE OXIDATION RATES

Triplicate seawater samples (1 ml) were amended with $^{14}$C labeled acetone as detailed previously. Microbial acetone oxidation rates (pmol L$^{-1}$ h$^{-1}$) were calculated by multiplying the sample counts (nCi mL$^{-1}$ h$^{-1}$, where 1 Ci = 3.7 × 10$^{10}$ Bq) by the specific activity of $^{14}$C acetone (30 Ci mmol$^{-1}$). All rates were corrected by subtracting killed sample counts (Trichloroacetic acid, TCA, 5% final concentration) to correct for non-biological processes. TCA is regularly used for killed controls, e.g., when measuring bacterial production indirectly via $^{3}$H-leucine incorporation (Smith and Azam, 1992), and does not lyse cells.

### SEAWATER ACETONE CONCENTRATIONS

Surface seawater was collected in Niskin bottles, and transferred into brown glass sample bottles with gas-tight stoppers using Tygon$^{TM}$ tubing. Acetone concentrations were determined using a

membrane inlet system coupled to a proton transfer reaction mass spectrometer (Beale et al., 2011).

### BACTERIAL PRODUCTION, CHLOROPHYLL A CONCENTRATION, AND COMMUNITY COMPOSITION

Rates of bacterial protein production (BP) and the numbers of heterotrophic bacteria, *Synechococcus* spp and picoeukaryotes were also determined to investigate any trends. BP was determined by measuring the incorporation of $^{3}$H-leucine (20 nM final concentration) into bacterial protein on 1.7 ml seawater samples following the method of Smith and Azam (1992). The numbers of bacterioplankton cells were determined by flow cytometry on SYBR Green I DNA-stained cells from 1.8 ml seawater samples fixed in paraformaldehyde (0.5–1%, final concentration), flash frozen in liquid nitrogen immediately after fixation, and stored frozen at −80°C (Marie et al., 1997). Numbers of *Synechococcus* spp and picoeukaryotes were analyzed on unstained samples by flow cytometry (Zubkov et al., 2000). Chlorophyll a samples were determined by fluorometric analysis of acetone-extracted pigments (Holm-Hansen et al., 1965).

## RESULTS

### LINEAR TIME COURSE EXPERIMENTS

When pico-molar concentrations of $^{14}$C labeled acetone were added to surface waters from station L4, radioactive carbon was expired to $^{14}CO_2$(**Figure 1**) suggesting that acetone was used as a microbial energy source. At this coastal station, acetone oxidation was linear for up to ∼3.5 h, after which between 1 and 3.6% of the added label had been oxidized to $^{14}CO_2$. Microbial acetone oxidation rates were highest in December 2011 (9.5 pCi mL$^{-1}$ h$^{-1}$, $R^2 = 0.997$, $n = 4$) and lowest during July 2011 (2.5 pCi mL$^{-1}$ h$^{-1}$, $R^2 = 0.999$, $n = 4$).

### UPTAKE KINETICS

The microbial oxidation of $^{14}$C labeled acetone displayed non-saturation type kinetics for nano-molar additions of acetone between 2 and 47 nmol L$^{-1}$ during February 2011 (**Figure 2A**), which, when plotted as a modified Lineweaver-Burke plot (**Figure 2C**, △), showed a constant fraction of added label ($f = 0.025 \pm 0.001$) had been oxidized to $CO_2$, irrespective of the initial addition concentration. Pico-molar $^{14}$C-acetone additions (6-1006 pmol L$^{-1}$) were made in the following June which resulted in saturation kinetics (**Figure 2B**), where the fraction of acetone oxidized reduced from 5.5 to 1.4% with increasing addition concentrations (**Figure 2C**; ▲). Saturation kinetics displayed during June 2011 allowed the first estimates of $V_{max}$ and $K_m$ to be determined from an Eadie-Hofstee plot (**Figure 2D**) of 4.1 pmol L$^{-1}$ h$^{-1}$ and 54 pmol L$^{-1}$, respectively, for surface coastal waters of station L4.

### SURFACE SEASONAL TRENDS IN MICROBIAL ACETONE OXIDATION

The average monthly rates of microbial oxidation of acetone in surface waters at station L4 varied between 1.2 and 42 pmol L$^{-1}$ h$^{-1}$

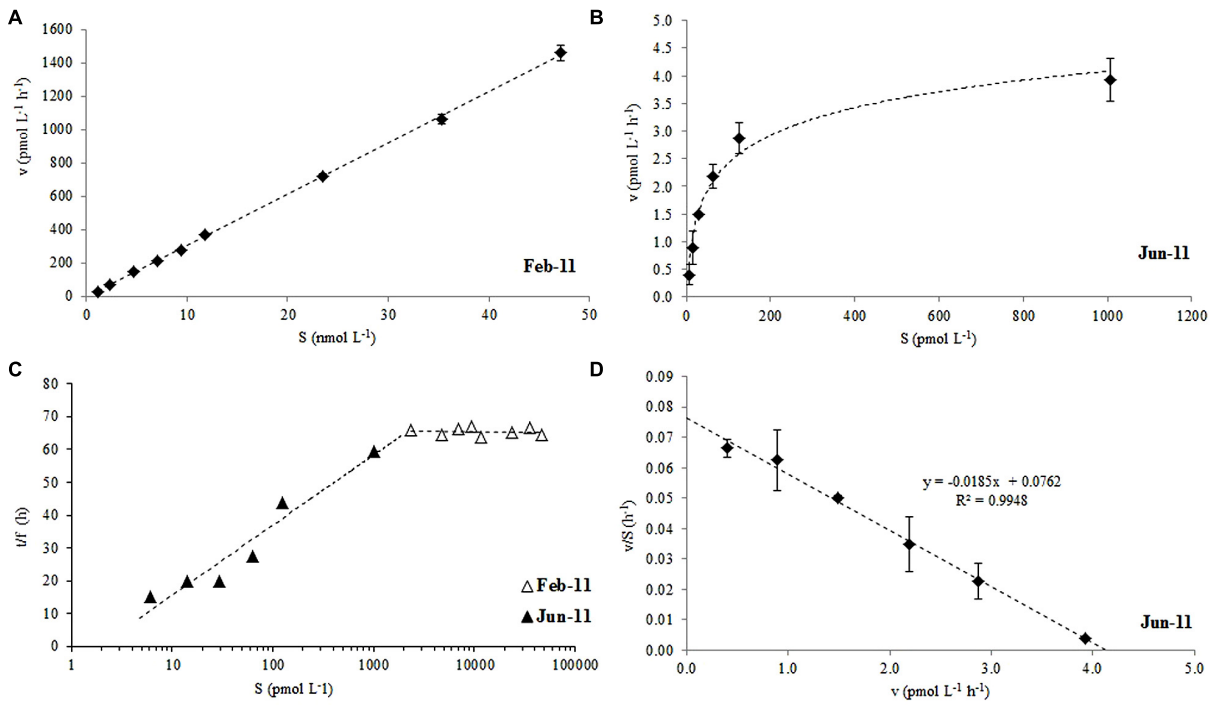


**FIGURE 2 | Rate of acetone oxidation (v) against added substrate concentration ($^{14}$C-labeled acetone, S) at (A) nano-molar additions in February, (B) pico-molar additions in June, (C) modified Lineweaver-Burke plot of combined Feb and June 2011 data and, (D) Eadie-Hofstee plot of June data used to derive $V_{max}$ and $K_m$ for surface waters of station L4.** For **(C)** the time of incubation (t) divided by the fraction of label oxidized to $CO_2$ (f) is plotted against S. The error bars represent ±1 standard deviation based on three replicates.

(**Figure 3B**) and showed significant changes with season. Oxidation rates were highest during winter (January and February 2011) at 36.2 ± 8.7 pmol $L^{-1}$ $h^{-1}$ and were 15-fold lower during the summer (June, July, and August 2011) at 2.4 ± 1.7 pmol $L^{-1}$ $h^{-1}$, with intermediate spring (March, April, May) and autumn (September, October, November) rates averaging 7.5 ± 4.0 and 4.5 ± 0.4 pmol $L^{-1}$ $h^{-1}$, respectively. When *in situ* seawater acetone concentrations are divided by microbial oxidation rates, biological turnover times are estimated, ranging between just over 3 days in February to ∼243 days in June during 2011 (**Figure 3C**). This suggests a clear seasonal trend of longer microbial turnover times in spring and summer months compared to autumn and winter. Corresponding monthly averaged changes in low nucleic acid containing bacteria are also shown in **Figure 3C** ranging between 0.44 and 3.9 × $10^5$ cells $mL^{-1}$, which show an opposite trend to microbial acetone turnover times ($r = -0.589$, $n = 16$, $P < 0.02$). Sea surface temperature at station L4 varied between 8.5 and 16.4°C, with typical low chlorophyll a values of ∼0.4 μg $L^{-1}$ during winter months rising fourfold to 1.6 μg $L^{-1}$ in July 2011 (**Figure 3A**). Additionally, average monthly numbers of high nucleic acid containing bacteria (1.3–5.8 × $10^5$cells $mL^{-1}$), *Synechococcus* sp. (0.7–36 × $10^3$cells $mL^{-1}$), pico- (0.6–16 × $10^3$cells $mL^{-1}$), and nano- (0.2–1.5 × $10^3$cells $mL^{-1}$), phytoplankton cell, and bacterial leucine incorporation rates (8–96 pmol leucine $L^{-1}$ $h^{-1}$), are summarized in **Table 1**.

#### DEPTH VARIABILITY IN MICROBIAL ACETONE OXIDATION

The variability of microbial acetone oxidation rates with depth at the relatively shallow (∼55 m) coastal station L4 was investigated during June 2011, when surface rates were at their lowest, but the water column was seasonally stratified (see **Figure 4**). Microbial acetone oxidation rates were lowest (0.78 ± 0.02 pmol $L^{-1}$ $h^{-1}$) in the shallow surface layer (<10 m), which showed enhanced surface warming and relatively lower salinity. Rates were on average, more than 30% higher at greater depths (average of 1.07 ± 0.04 pmol $L^{-1}$ $h^{-1}$).

## DISCUSSION

This study shows that $^{14}C$ labeled acetone can be used successfully to determine microbial oxidation rates (to $^{14}CO_2$) in seawater samples. We report the first estimates of $V_{max}$ (4.1 pmol $L^{-1}$ $h^{-1}$) and $K_m$ (54 pmol $L^{-1}$) for surface coastal waters during summer, when *in situ* surface oxidation rates were at their lowest (1.2 ± 0.39 pmol $L^{-1}$ $h^{-1}$, **Figure 3B**), despite relatively high average *in situ* acetone concentrations of 7.5 ± 0.7 nmol $L^{-1}$. When nano-molar (2–47 nM) $^{14}C$ acetone additions were made during winter months, first order kinetics were observed, but **Figure 2C** shows that a constant fraction of added label was oxidized to $CO_2$, suggesting that any microbial enzyme systems involved in the conversion of acetone to $CO_2$ were saturated. Pico-molar additions made during the summer, when acetone concentrations had more than doubled, showed first order reaction kinetics for approximately <100 pM acetone additions (**Figure 2B**). Both sets of data combined in a modified Lineweaver-Burke plot (**Figure 2C**, which assumes that if pico-molar additions had been made during winter, similar first order kinetics to summer would be observed) suggest *in situ* enzyme system saturation of 1–2 nM of mixed natural communities. Although the microbial composition of surface waters at L4 are highly likely to be different between the two seasons (e.g., Gilbert et al., 2009, 2012), it is unknown which microbes actively respire acetone to $CO_2$. However, it is noteworthy that seasonal changes in bacterial structure have been linked to change in day length (Gilbert et al., 2012) and other environmental variables (e.g., temperature, Gilbert et al., 2009) rather than trophic interactions.

The microbial acetone oxidation kinetics observed during February for nano-molar additions does not show rate limitation with increasing substrate concentration, and thus does not comply with Michaelis–Menten kinetics (Wright and Hobbie, 1966), which could indicate no active microbial enzyme transport systems for acetone oxidation. These authors also showed that the slope of such a linear relationship between uptake rates and added substrate concentration (as in **Figure 2A**) was identical to the kinetics of simple diffusion. In addition, when samples were killed with TCA (5% final concentration), acetone oxidation did not increase over time, suggesting that, despite a possible lack of active transport systems, the uptake was nevertheless due to microbial metabolic activity. Wright and Hobbie (1966) suggested that at very low concentrations of added substrate, most glucose was incorporated using active bacterial transport systems, while at higher concentrations diffusion across algal cells dominated. Our results suggest that when pico-molar additions are made (June 2011) active transport systems dominated with a resultant mixed community $V_{max}$ of 4.1 pmol $L^{-1}$ $h^{-1}$ and a $K_m$ of 54 pmol $L^{-1}$. However when nano-molar additions are made (February 2011) non saturation kinetics were observed, with possible diffusion across cell walls dominating (*cf.* methanol Dixon et al., 2011a).

Acetone oxidation by natural marine microbial communities could also be due to mixotrophic and heterotrophic phytoplankton in addition to heterotrophic bacteria. For rates of microbial acetone oxidation during February, which increased linearly with substrate concentration ($y = 0.031x - 0.003$, $n = 9$, $R^2 = 0.999$ for 1.7 h incubation period, **Figure 2A**) a diffusion constant ($K_d$) can be calculated from the slope of the linear relationship (Wright and Hobbie, 1965). This constant assumes that organisms oxidize the acetone as rapidly as it diffuses in (Wright and Hobbie, 1965). A $K_d$ of 0.003 $h^{-1}$ is equivalent to a turnover time of ∼1.4 days (Wright and Hobbie, 1965) which is comparable to the average estimate of 3.2 days for February 2011 determined in **Figure 3C**. This also compares well with the turnover of other organic compounds like DMS (e.g., 0.3–2.1 days, Simó et al., 2000) and methanol (e.g., 7 days in productive shelf waters, Dixon et al., 2011a). Despite the faster (i.e., hours) estimated acetone turnover times of de Bruyn et al. (2013), they also reported higher loss rates during the winter compared to other times of the year. However, the acetone turnover times reported by de Bruyn et al. (2013) originate from riverine and very near-shore costal environments (average salinity of 25.8 ± 2.1), that experience much less seasonal variability (average surface temperature of 17.5 ± 1.2°C) and higher average *in situ* acetone concentrations (59 ± 56 nM) compared to L4 waters (average salinity of 35.2 ± 0.1, average surface temperature of 12.5 ± 2.8°C,

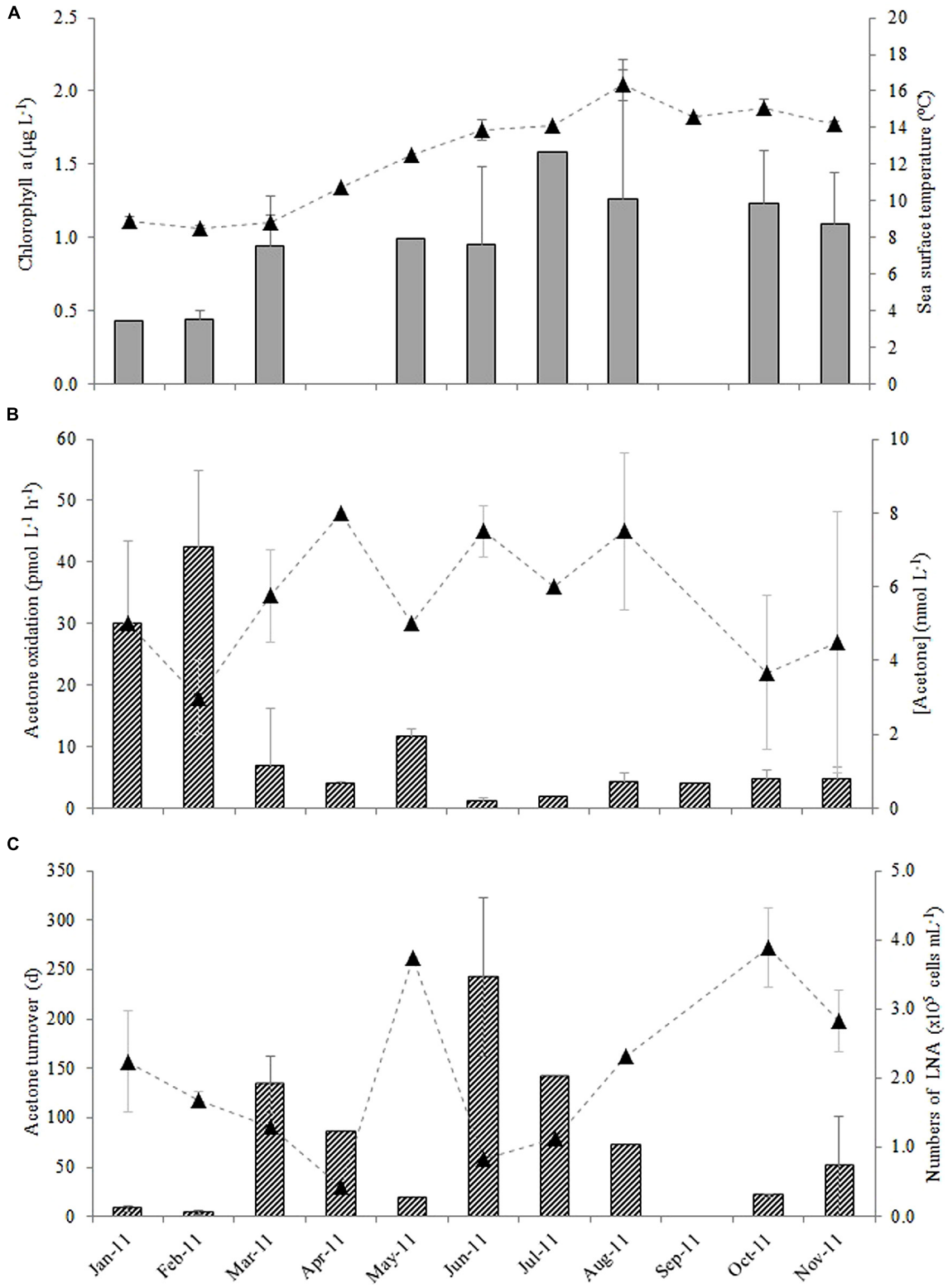


**FIGURE 3 | Monthly variability in surface waters at station L4 for (A) chlorophyll a (bars) and sea surface temperature (▲), (B) acetone oxidation rates (bars) and *in situ* seawater acetone concentrations (▲) and (C) resulting microbial turnover times (bars) with corresponding changes in the numbers of low nucleic acid containing bacteria (▲, LNA where there is a significant linear correlation between the microbial turnover time of acetone and the numbers of low nucleic acid containing bacteria, $r = -0.589$, $n = 16$, $P < 0.02$).** The error bars represent ±1 standard deviation based on three replicates.

average surface acetone concentrations of 5.6 ± 2.3 nM). Furthermore, de Bruyn et al. (2013) report higher acetone loss rates after rain events, which could suggest faster microbial removal associated with less saline waters, although this is not reflected in **Figure 4**.

Acetone production in seawater is largely thought to be a photochemical process (Kieber et al., 1990; Zhou and Mopper, 1997; de Bruyn et al., 2011; Dixon et al., 2013a), possibly related to UV breakdown of chromophoric dissolved organic matter (CDOM) originating from eukaryotic cells (Dixon et al., 2013a). Given the relatively high microbial acetone oxidation rates found during January/February 2011 (in this study and in de Bruyn et al., 2013), with turnover times estimated at 1.4–3.2 days, it is not presently understood what process maintains acetone levels during winter months, when average acetone concentrations are 3.4 ± 1.1 nM. Typically, during winter at L4, UV levels and phytoplankton biomass are relatively low (e.g., Smyth et al., 2010). However, the water column is fully mixed and more influenced by riverine waters, i.e., maximum river flows and re-suspension events of bottom sediments (Groom et al., 2009). Thus during these periods it is probable that the dissolved organic matter is dominated by terrestrial sources and re-suspended sediments rather than phytoplankton.

Relationships between microbial oxidation and turnover of acetone with other biogeochemical variables (see **Table 1**) have been explored, and reveal statistically significant negative linear relationships between acetone oxidation rates and both sea surface

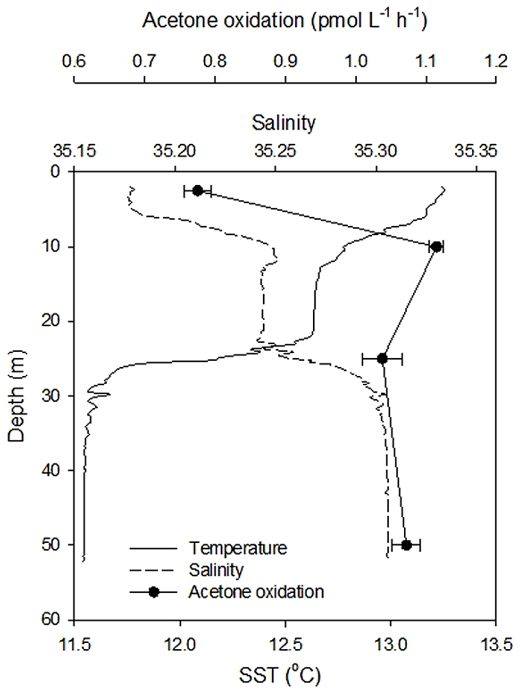


**FIGURE 4 | Variability in acetone oxidation rates at coastal station L4 with depth during June 2011.** The error bars represent ± 1 standard deviation based on three replicates.

**Table 1 | Summary of sampling at coastal station L4.**

| Month 2011 | SST[a] (°C) | Chl a[b] (μg L$^{-1}$) | LNA bacteria[c] (×10$^5$ cells mL$^{-1}$) | HNA bacteria[d] (×10$^5$ cells mL$^{-1}$) | Syns[e] (×10$^3$ cells mL$^{-1}$) | Peuks[f] (×10$^3$ cells mL$^{-1}$) | Nano[g] (×10$^3$ cells mL$^{-1}$) | BP[h] (pmol Leu L$^{-1}$ h$^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| **Winter** | | | | | | | | |
| January | 8.9 ± 0.3 | 0.43 | 2.2 ± 0.73 | 2.2 ± 0.70 | 9.3 ± 0.14 | 5.8 ± 0.77 | 0.41 ± 0.08 | – |
| February | 8.5 ± 0.2 | 0.44 ± 0.06 | 1.7 ± 0.1 | 1.7 ± 0.40 | 12.5 ± 2.4 | 8.9 ± 2.5 | 0.22 ± 0.04 | – |
| **Spring** | | | | | | | | |
| March | 8.9 ± 0.4 | 0.94 ± 0.34 | 1.3 ± 0.1 | 1.3 ± 0.02 | 5.0 ± 2.3 | 7.9 ± 0.31 | 1.0 ± 0.46 | – |
| April | 10.8 | – | 0.44 | 1.8 | 0.74 | 3.0 | 1.3 | 28.6 |
| May | 12.5 | 0.99 | 3.7 | 5.8 | 2.8 | 26.7 | 3.9 | 61.4 |
| **Summer** | | | | | | | | |
| June | 13.8 ± 0.6 | 0.95 ± 0.54 | 0.83 ± 0.05 | 4.4 ± 0.4 | 2.7 ± 2.3 | 9.7 ± 1.9 | 1.5 ± 0.44 | 49.9 ± 30.3 |
| July | 14.1 | 1.59 | 1.1 | 2.3 | 3.5 | 3.7 | 0.97 | 96.2 |
| August | 16.4 ± 0.8 | 1.26 ± 0.96 | 2.3 | 4.4 | 27.0 | 0.63 | 0.60 | 52.0 ± 23.5 |
| **Autumn** | | | | | | | | |
| September | 14.6 ± 0.1 | – | – | – | – | – | – | – |
| October | 15.1 ± 0.4 | 1.24 ± 0.36 | 3.9 ± 0.6 | 3.4 ± 0.72 | 35.9 ± 9.8 | 15.8 ± 1.9 | 1.1 ± 0.09 | 8.0 ± 0.02 |
| November | 14.2 ± 0.1 | 1.1 ± 0.3 | 2.8 ± 0.5 | 2.6 ± 0.09 | 13.1 ± 5.3 | 7.2 ± 0.86 | 0.72 ± 0.06 | 10.2 ± 6.2 |

*All samples were collected from the surface (≤10 m). [a]Sea surface temperature, [b]Surface concentration of chlorophyll a, [c]Number of low nucleic acid containing bacteria, [d]Number of high nucleic acid containing bacteria, [e]Synechococcus sp., [f]Picophytolankton (<2 μm), [g]Nanophytolankton (~2-12 μm), [h]Bacterial production, When there is >1 sampling date contributing to the monthly average, ± 1 SD is quoted. All parameters except BP were obtained from the L4 database, which is provided by the Plymouth Marine Laboratory, Western Channel Observatory.*

**Table 2 | Surface microbial oxidation rates normalized to in situ concentration ($h^{-1}$) and resulting turnover times, as a function of season for coastal station, L4.**

| Season[a] | Acetone | | Acetaldehyde | | Methanol | |
|---|---|---|---|---|---|---|
| | $h^{-1}$ | Days | $h^{-1}$ | Hours | $h^{-1}$ | Day |
| Winter | 0.012 ± 0.007 (5) | 3.5 | 0.86 ± 0.55 (4) | 1.2 | n/a | n/a |
| Spring | 0.001 ± 0.001 (6) | 42 | 0.87 ± 0.38 (6) | 1.1 | 0.03 ± 0.03 (4) | 1.3 |
| Summer | 0.0004 ± 0.0002 (5) | 104 | 0.95 ± 0.50 (4) | 1.1 | 0.06 ± 0.06 (4) | 0.73 |
| Autumn | 0.002 ± 0.001 (5) | 21 | 2.4 ± 2.5 (5) | 0.4 | 0.16 ± 0.04 (5) | 0.25 |

*Where the numbers in brackets denote number sampling dates.* [a]*Winter is defined a December, January, February; Spring as March, April, May; Summer as June, July and August; Autumn as September, October, November during 2011. n/a data not available.*

temperature and concentration of chlorophyll *a* ($r = -0.604$ and $-0.543$, respectively for $n = 21$, $P \leq 0.02$). This is largely because the highest acetone oxidation rates, were found during winter when sea surface temperatures and phytoplankton biomass were at their minima.

A statistically significant inverse relationship was also found between biological acetone turnover times and the numbers of low nucleic acid bacteria (LNA, $r = -0.589$, $n = 16$, $P < 0.02$). As previously noted, we do not know which marine microbes are capable of utilizing acetone, or the enzyme system(s) involved in the conversion of acetone to $CO_2$, but this relationship indicates that low nucleic acid containing bacteria could be responsible for marine acetone consumption in surface coastal waters. SAR11 *Alphaproteobacteria*, are often significant components of the LNA (Mary et al., 2006) and are the most abundant heterotrophs in the oceans. SAR11 cells are believed to play a major role in mineralizing dissolved organic carbon (Sun et al., 2011) being efficient competitors for resources (Morris et al., 2002). Whilst in culture, Sun et al. (2011) found that *Candidatus Pelagibacter ubique* (a subgroup of SAR11) have the genome encoded pathways for the oxidation of a variety of one-carbon compounds, including the OVOC compound methanol. We found that the SAR11 clade were the second most numerically dominant bacterial order of surface bacterial populations found at station L4 during the annual sampling period 2011–2012, and contributed between 16 and 46% during winter months (Sargeant, 2014). *Alphaproteobacteria* were also the most abundant bacterial Class found at station L4 over a 6 year study (Gilbert et al., 2012). This study further reported that members of the *Rickettsiales* (SAR11) and *Rhodobacteriales* were the most frequently recorded operational taxonomic units, with the abundance of *Rickettsiale*s reaching a maxima in winter (Gilbert et al., 2012), coincident with relatively fast acetone turnover times of ∼3 days, found in this study.

The acetone biological turnover times determined here should be considered as conservative, because it is possible that some heterotrophic bacteria also assimilate acetone carbon into particulate carbon biomass *cf.* methanol, Dixon et al. (2013b). Furthermore, microbial acetone uptake that gets transformed and excreted as more refractory DOC compounds (as in the microbial carbon pump, e.g., Ogawa et al., 2001; Jiao and Azam, 2011), possibly via some overflow metabolism strategies as previously suggested for methanol (Dixon et al., 2013a) will also not be revealed via the experimental approach of this study.

Coastal surface water microbial acetone oxidation rates have been normalized to *in situ* concentration as a function of season, and are compared to other biologically utilized OVOC compounds (acetaldehyde and methanol, e.g., Dixon et al., 2013a) in **Table 2**. Acetone is a less preferred organic compound for marine microbes compared to methanol and acetaldehyde, although acetone oxidation rates shows a much more pronounced seasonality. In addition, the one depth profile undertaken during summer suggests near-surface reduction in microbial acetone oxidation rates associated with a less saline, warmer tongue of water in the top 10 m.

The kinetic characteristics of microbial acetone oxidation can be compared to those of other substrates commonly used by bacteria, so that the ecological significance of acetone to marine microbial metabolism can be evaluated. Both $V_{max}$ and $K_m$ are more than 2 orders of magnitude smaller for acetone oxidation compared to methanol oxidation (Dixon et al., 2011a), which if compared further with proteins and carbohydrates gives the following order; proteins >>carbohydrates ≈ methanol>>acetone (refer to Dixon et al., 2011a for protein, carbohydrate, and methanol $V_{max}$ and $K_m$ data).

This research offers the first comprehensive seasonally resolved study combining microbial acetone oxidation rates with *in situ* concentrations in order to derive biological turnover times that ranged between ∼3 days in winter to >240 days in summer. We have experimentally derived the first $V_{max}$ and $K_m$ estimates of microbial acetone oxidation. We have also highlighted that there must be an unrecognized production mechanism for acetone during winter in coastal regions, possibly relating in some way, to enhanced dissolved organic matter from terrestrial sources. Further research should investigate possible winter acetone production mechanisms, identify which microbial species are utilizing acetone in marine environments, and characterize what enzyme systems are involved in the oxidation process.

## ACKNOWLEDGMENTS

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# Microbial monoterpene transformations—a review

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Isoprene and monoterpenes constitute a significant fraction of new plant biomass. Emission rates into the atmosphere alone are estimated to be over 500 Tg per year. These natural hydrocarbons are mineralized annually in similar quantities. In the atmosphere, abiotic photochemical processes cause lifetimes of minutes to hours. Microorganisms encounter isoprene, monoterpenes, and other volatiles of plant origin while living in and on plants, in the soil and in aquatic habitats. Below toxic concentrations, the compounds can serve as carbon and energy source for aerobic and anaerobic microorganisms. Besides these catabolic reactions, transformations may occur as part of detoxification processes. Initial transformations of monoterpenes involve the introduction of functional groups, oxidation reactions, and molecular rearrangements catalyzed by various enzymes. *Pseudomonas* and *Rhodococcus* strains and members of the genera *Castellaniella* and *Thauera* have become model organisms for the elucidation of biochemical pathways. We review here the enzymes and their genes together with microorganisms known for a monoterpene metabolism, with a strong focus on microorganisms that are taxonomically validly described and currently available from culture collections. Metagenomes of microbiomes with a monoterpene-rich diet confirmed the ecological relevance of monoterpene metabolism and raised concerns on the quality of our insights based on the limited biochemical knowledge.

**Keywords: isoprenoids, acyclic monoterpene utilization, camphor, pinene, limonene, linalool, myrcene, eucalyptol**

## INTRODUCTION

Annually about 120 Pg of carbon dioxide are assimilated by plants. A part is transformed into chemically complex molecules and released into the environment by emission or excretion (Ghirardo et al., 2011). Volatile organic compounds (VOCs) comprise a large number of molecules, including various hydrocarbons, single carbon compounds (e.g. methane), isoprene and terpenes (e.g. mono- and sesquiterpenes). The atmosphere is loaded with an estimated VOC emission rate of about 1150 Tg C $yr^{-1}$ (Stotzky and Schenck, 1976; Guenther et al., 1995; Atkinson and Arey, 2003). These estimates included only non-methane VOCs of biogenic origin (BVOCs); a second source are anthropogenic VOCs. Among the BVOCs, isoprene and monoterpenes dominate with estimated emission rates of about 500 Tg C $yr^{-1}$ and 127 Tg C $yr^{-1}$, respectively (Guenther et al., 1995). Monoterpenes ($C_{10}H_{16}$) consist of two linked isoprene ($C_5H_8$) units and include in the strict sense only hydrocarbons. Often the term monoterpene is applied including monoterpenoids which are characterized by oxygen-containing functional groups. Structural isomers—acyclic, mono-, and bicyclic monoterpenes—, stereoisomers as well as a variety of substitutions result in a large diversity of molecules. Today, more than 55,000 different isoprenoids are known (Ajikumar et al., 2008). Monoterpenes are not only emitted as cooling substances (Sharkey et al., 2008), but can also be stored intracellularly serving mainly as deterrent or infochemical (Dudareva et al., 2013). Wood plants mainly accumulate pinene and other pure hydrocarbon monoterpenes as constituents of their resins, whereas citrus plants are the major source of limonene. Flowers, however, produce and emit a variety of oxygenated monoterpenes (e.g. linalool) (Kesselmeier and Staudt, 1999 and references therein, Sharkey and Yeh, 2001; Bicas et al., 2009).

In the atmosphere, monoterpenes are transformed in purely chemical reactions within hours. Photolysis and reactions with molecular oxygen, ozone, hydroxyl radicals, $NO_x$ species, and chlorine atoms result in carbonyls, alcohols, esters, halogenated hydrocarbons, and peroxynitrates. These products condense and lead to the formation of secondary aerosols. Rain or precipitation transports them to soils (Atkinson and Arey, 2003; Fu et al., 2009; Ziemann and Atkinson, 2012). Monoterpenes reach the surface layers of soils by leaf fall and excreted resins. Also roots emit monoterpenes into the rhizosphere (Wilt et al., 1993; Kainulainen and Holopainen, 2002). Deeper soil layers do contain significant less monoterpenes than the surface soil layer. Emission into the atmosphere and biotransformations in the surface layer mainly by microorganisms are the major sinks. An alternative, abiotic photoreactions like in the atmosphere, is limited by light availability in soil (Kainulainen and Holopainen, 2002; Insam and Seewald, 2010).

Bacteria encountering monoterpenes have to deal with their toxic effects (reviewed by Bakkali et al., 2008). In order to prevent the accumulation of monoterpenes in the cell and cytoplasmatic membrane, bacteria modify their membrane lipids, transform monoterpenes and use active transport by efflux pumps (Papadopoulos et al., 2008; Martinez et al., 2009). Below toxic concentrations monoterpenes are used by microorganisms

as sole carbon and energy source. The mineralization of the hydrocarbons requires the introduction of functional groups to access beta-oxidation like fragmentation reactions yielding central metabolites, e.g. acetyl-CoA. In many aerobic microorganisms molecular oxygen serves as reactive agent to functionalize the monoterpenes (**Figure 1**). Strains of *Pseudomonas* and *Rhodococcus* have become model organisms for the elucidation of pathways in aerobic bacteria. Nearly 40 years after the first reports on aerobic mineralization (Seubert, 1960; Seubert and Fass, 1964; Dhavalikar and Bhattacharyya, 1966; Dhavalikar et al., 1966), the mineralization of monoterpenes in denitrifying bacteria and methanogenic communities was discovered (Harder and Probian, 1995; Harder and Foss, 1999). Betaproteobacterial strains of the genera *Castellaniella* and *Thauera* are the study objects for the elucidation of anaerobic pathways. All these bacteria were obtained in single-fed batch enrichments with high substrate concentrations ($mmol*L^{-1}$), in contrast to low concentrations in nature ($\mu mol*L^{-1}$). Consequently, in batch enrichments isolated strains exhibit often a solvent tolerance; they grow in the presence of a pure monoterpene phase. Cultivation was rarely attempted by physical separation followed by single-fed batch cultivations. Such dilution-to-extinction series performed in replicates—also known as most-probable-number (MPN) method—revealed a frequent presence of the degradative capacities in natural populations: denitrifying communities in sewage sludge and forest soil yielded $10^6$–$10^7$ monoterpene-utilizing cells $ml^{-1}$, representing 0.7–100% of the total cultivable nitrate-reducing microorganisms (Harder et al., 2000). MPN cultivations for aerobic bacteria have not been reported so far, and for both cases the highly abundant bacteria with the capacity to grow on monoterpenes have not been identified.

Over the last 50 years, many monoterpene transformations have been reported for microbial cultures, but the biochemical pathways were rarely disclosed. More important for the maintenance of our knowledge, only a small portion of the investigated strains were deposited in culture collections. Without detailed knowledge of genes or the availability of strains, the observations of biotransformation experiments are of limited value for future studies. Therefore, this review on the transformation of monoterpenes focusses on enzymes for which the gene and protein sequences are available in public databases as well as on microorganisms that at least have been deposited in a public culture collection and ideally are validly described (**Table 1**). A broad overview on microbial biotransformations is also provided by a number of older review articles (Trudgill, 1990, 1994; van der Werf et al., 1997; Hylemon and Harder, 1998; Duetz et al., 2003; Ishida, 2005; Li et al., 2006; Bicas et al., 2009; Li and Lan, 2011; Schewe et al., 2011; Tong, 2013). KEGG and MetaCyc, two widely used reference datasets of metabolic pathways (reviewed by Altman et al., 2013), include degradation pathways of limonene, pinene, geraniol, and citronellol. Single reactions of *p*-cymene and *p*-cumate degradation are covered. MetaCyc additionally covers the metabolism of myrcene, camphor, eucalyptol, and carveol.

## BICYCLIC MONOTERPENES

(+)-Camphor [**1**, **Figure 2**] ($C_{10}H_{16}O$) is the substrate of one of the first and best described monoterpene transforming enzymes, a specific cytochrome P450 monooxygenase (*camABC*, P450cam, EC 1.14.15.1) from *Pseudomonas putida* (ATCC 17453). Initially, (+)-camphor is hydroxylated. The resulting 5-*exo*-hydroxycamphor [**2**] is oxidized by a NAD-reducing dehydrogenase (EC 1.1.1.327) which gene *camD* is part of the operon *camDCAB*. The diketone is oxidized in a Baeyer–Villiger like oxidation to a lactone, either by a 2,5-diketocamphane 1,2-monooxygenase or a 3,6-diketocamphane 1,6-monooxygenase

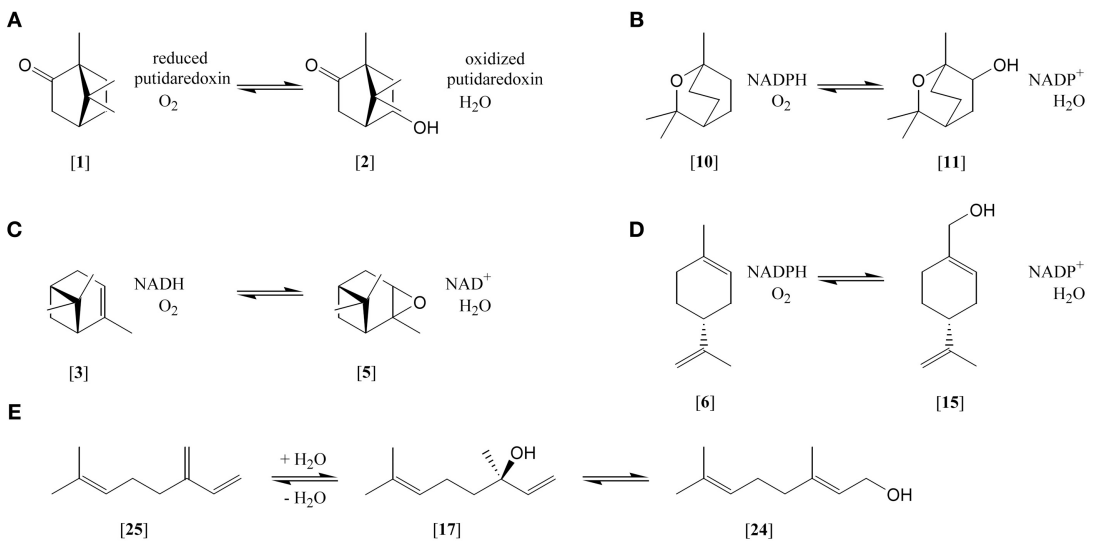


**FIGURE 1 | Selected monoterpene transformations. (A)** (+)-camphor [1] hydroxlation to 5-hydroxycamphor [2]; **(B)** 1,8-cineole [10] hydroxylation to hydroxy-1,8-cineole [11]; **(C)** α-pinene [3] epoxidation to α-pinene oxide [5]; **(D)** (*R*)-limonene [6] hydroxylation to perillyl alcohol [15]; **(E)** myrcene [25] hydration to (*S*)-(+)-linalool [17] and isomerization to geraniol [24].

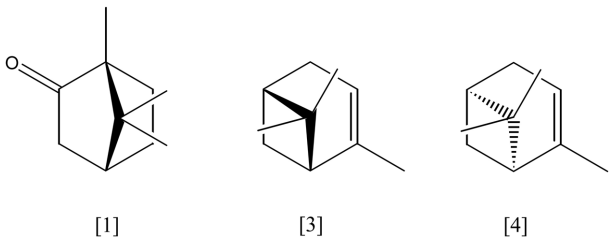


**FIGURE 2 | (+)-camphor [1]; α-pinene [3]; β-pinene [4].**

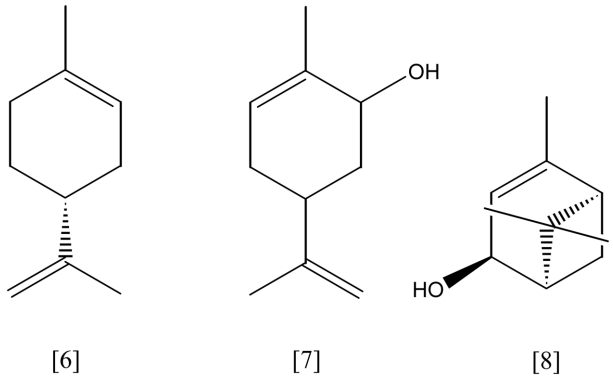


**FIGURE 3 | (*R*)-limonene [6]; carveol [7]; *trans*-verbenol [8].**

($camE_{25-1}E_{25-2}$ or $camE_{36}$, EC 1.14.13.162). The lactone spontaneously hydrolyses to 2-oxo-$\Delta^3$-4,5,5-trimethylcyclopentenyl-acetic acid which is activated as coenzyme A thioester by a specific synthase ($camF_{1,2}$, EC 6.2.1.38). This CoA-ester serves as substrate for another specific monooxygenase (*camG*, EC 1.14.13.160), which initiates the cleavage of the second ring by formation of a lactone. After hydrolysis of the lactone, the linear product is oxidized to isobutanoyl-CoA and three acetyl-CoA. All corresponding genes (*camABCDEFG*) have been identified on a linear plasmid (Ougham et al., 1983; Taylor and Trudgill, 1986; Aramaki et al., 1993; Kadow et al., 2012; Leisch et al., 2012; Iwaki et al., 2013).

The most abundant bicyclic monoterpene is pinene with the isomers α-pinene [**3**] and β-pinene [**4**] ($C_{10}H_{16}$), a main constituent of wood resins (e.g. conifers). *Pseudomonas rhodesiae* (CIP 107491) and *P. fluorescens* (NCIMB 11671) grew on α-pinene as sole carbon source. α-pinene is oxidized to α-pinene oxide [**5**] by a NADH-dependent α-pinene oxygenase (EC 1.14.12.155) and undergoes ring cleavage by action of a specific α-pinene oxide lyase (EC 5.5.1.10), forming apparently isonovalal as first product which is isomerized to novalal (Best et al., 1987; Bicas et al., 2008; Linares et al., 2009). The cleavage reaction of α-pinene oxide was also described for a *Nocardia sp.* strain P18.3 (Griffiths et al., 1987; Trudgill, 1990, 1994).

An alternative route for pinene degradation via a monocyclic *p*-menthene derivate has been described for *Pseudomonas* sp. strain PIN (Yoo and Day, 2002). *Bacillus pallidus* BR425 degrades α- and β-pinene apparently via limonene [**6**] and pinocarveol. While α-pinene is transformed into limonene and pinocarveol, β-pinene yields pinocarveol only. Both intermediates may be further transformed into carveol [**7**] and carvone. The activity of a specific monooxygenases has been suggested, but experimental evidence is lacking (Savithiry et al., 1998). *Serratia marcescens* uses α-pinene as sole carbon source. *Trans*-verbenol [**8**] was a detectable metabolite. In glucose and nitrogen supplemented medium, this strain formed α-terpineol [**9**]. The two oxidation products were considered to be dead-end products as they accumulated in cultures (Wright et al., 1986). A general precaution has to be mentioned here for many biotransformation studies: monoterpenes contain often impurities and oxidation products which may be utilized as substrates resulting in traces of monoterpene and monoterpenoid transformation products that are not further metabolized. Stoichiometric experiments have to show that the amount of metabolite is larger than the amount of impurity in the substrate. Only such careful stoichiometric experiments, mutants in functional genes or the characterization of enzymes *in vitro* can provide a proof of the presence of a biotransformation.

Eucalyptol, the bicyclic monoterpene 1,8-cineole [**10**] ($C_{10}H_{18}O$), is transformed in several pathways. *Novosphingobium subterranea* converts 1,8-cineole initially into 2-*endo*-hydroxycineole, 2,2-oxo-cineole, and 2-*exo*-hydroxycineole. Acidic products from ring cleavages have been identified *in situ* (Rasmussen et al., 2005). Hydroxy-cineole formation occurred in 1,8-cineole-grown cultures of *Pseudomonas flava* (Carman et al., 1986). A cytochrome P450 monooxygenase from *Bacillus cereus* UI-1477 catalyzes the hydroxylation of 1,8-cineole, yielding either 2*R*-*endo*- or 2*R*-*exo*-hydroxy-1,8-cineole [**11**] (Liu and Rosazza, 1990, 1993). Another 1,8-cineole-specific P450 monooxygenase (EC 1.14.13.156) has been purified and characterized from *Citrobacter braakii*, which yielded 2-*endo*-hydroxy-1,8-cineole only. Further oxidation and lactonization were followed by a spontaneous lactone ring hydrolysis (Hawkes et al., 2002). Biotransformation in *Rhodococcus* sp. C1 involves an initial hydroxylation to 6-*endo*-hydroxycineol [**12**] and further oxidation to 6-oxocineole by a 6-*endo*-hydroxycineol dehydrogenase (EC 1.1.1.241). A 6-oxocineole monooxygenase (EC 1.14.13.51) converts the ketone into an unstable lactone. Spontaneous decomposition results in (*R*)-5,5-dimethyl-4-(3′-oxobutyl)-4,5-dihydrofuran-2(3H)-one. An initial monooxygenase activity has not been detected in cell-free systems, while the dehydrogenase and oxygenase activities have been measured in crude cell extracts (Williams et al., 1989).

## MONOCYCLIC MONOTERPENES

Limonene [**6**, **Figure 3**] ($C_{10}H_{16}$) is the most abundant monocyclic monoterpene, besides toluene the second most abundant VOC indoors (Brown et al., 1994). It represents the main component of essential oils from citrus plants, e.g. lemon and orange. *Rhodococcus erythropolis* DCL14 transforms (*R*/*S*)-limonene via limonene-1,2-epoxide into limonene-1,2-diol [**13**, **Figure 5**], applying a limonene-1,2 monooxygenase (EC 1.14.13.107) and a limonene-1,2-epoxide hydrolase (EC 3.3.2.8),

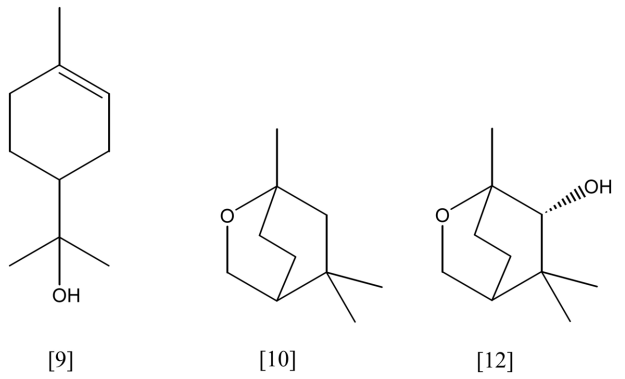


**FIGURE 4 | α-terpineol [9]; 1,8-cineole [10]; 6-hydroxycineol [12].**

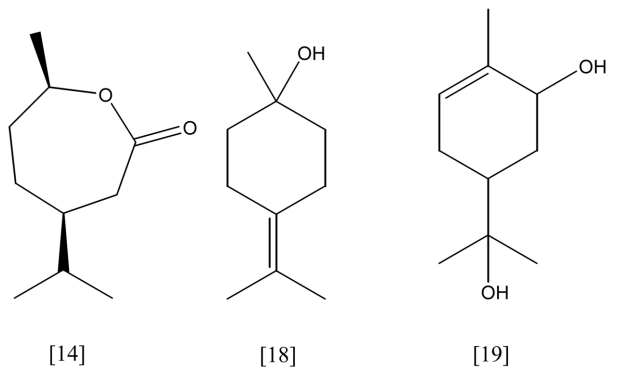


**FIGURE 6 | isopropenyl-7-methyl-2-oxo-oxepanone [14]; γ-terpineol [18]; *p*-menth-1-ene-6,8-diol [19].**

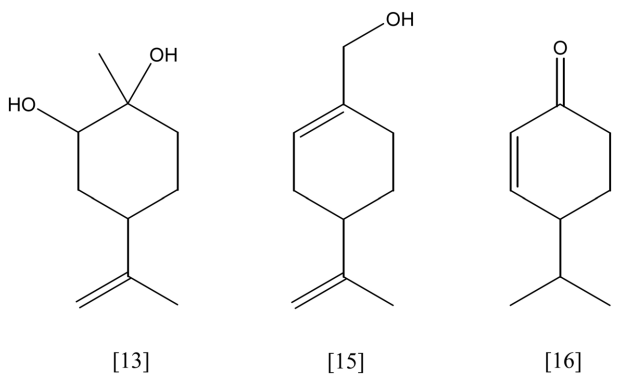


**FIGURE 5 | limonene-1,2-diol [13]; perillyl alcohol [15]; cryptone [16].**

respectively. A specific dehydrogenase (EC 1.1.1.297) forms the ketone, 1-hydroxy-2-oxolimonene, which is oxidized to a lactone by a 1-hydroxy-2-oxolimonene 1,2-monooxygenase (EC 1.14.13.105). Enzyme activities were only detected in limonene-induced cells, suggesting a tight regulation of the limonene degradation. *R. erythropolis* DCL14 harbors a second pathway for limonene degradation. Initially, (*R*)-limonene is hydroxylated by a NADPH-dependent limonene 6-monooxygenase (EC 1.14.13.48) to *trans*-carveol [**7**]. Subsequently, *trans*-carveol is oxidized to carvone and dihydrocarvone by a carveol dehydrogenase (EC 1.1.1.243) and carvone reductase (EC 1.3.99.25), respectively. A monocyclic monoterpene ketone monooxygenase (EC 1.14.13.105) inserts an oxygen atom, forming isopropenyl-7-methyl-2-oxo-oxepanone [14, **Figure 6**]. This lactone is cleaved by a specific ε-lactone hydrolase (EC 3.1.1.83) yielding hydroxyl-3-isopropenyl-heptanoate. Oxidation and activation as coenzyme A thioester enable a further degradation in accordance to the beta-oxidation (van der Werf et al., 1999b; van der Werf and Boot, 2000). *R. opacus* PWD4 uses (*R*)-limonene on the same pathway. Biomass from a glucose-toluene chemostat culture transformed limonene into *trans*-carveol, which was further oxidized to carvone by a *trans*-carveol dehydrogenase (EC 1.1.1.275) (Duetz et al., 2001).

Studies on the limonene metabolism in *P. gladioli* identified α-terpineol [**9**, **Figure 4**] and perillyl alcohol [**15**] as major metabolites. However, none of the involved enzymes has been purified or further characterized (Cadwallader et al., 1989). A α-terpineol dehydratase from *P. gladioli* was isolated and partially purified. The hydration reaction to the isopropenyl double bond of (4*R*)-(+)-limonene resulted in (4*R*)-(+)-α-terpineol as only product (Cadwallader et al., 1992). *Geobacillus stearothermophilus* (ex *Bacillus*) showed growth on limonene as sole carbon source. The main limonene transformation product was perillyl alcohol, while α-terpineol and perillyl aldehyde were found in minor concentrations. After heterologous expression of a putative limonene degradation pathway in *E. coli*, α-terpineol was identified as major product of the biotransformation. Other studies reported a limonene hydroxylation on the methyl group yielding perillyl alcohol, which underwent further oxidation to perillic acid (Chang and Oriel, 1994; Chang et al., 1995). Additional studies on the recombinant limonene hydroxylase confirmed the production of perillyl alcohol from limonene but revealed in addition the formation of carveol. The limonene hydroxylase showed dependency on molecular oxygen and NADH as cofactors and was suggested to belong to the (*S*)-limonene 7-monooxygenase family (EC 1.14.13.49) (Cheong and Oriel, 2000).

*Enterobacter agglomerans* 6L and *Kosakonia cowanii* 6L (ex *Enterobacter cowanii*) transformed (*R*)-limonene [**6**]. The main metabolites detected in ether extracts of *E. agglomerans* 6L cultures were γ-valerolactone and cryptone [**16**]. In assays using four recombinant expressed limonene-transforming enzymes from *K. cowanii* 6L, linalool [**17**, **Figure 8**] was identified as main product besides smaller amounts of dihydrolinalool. It was proposed that the potential limonene hydroxylase converts limonene into linalool, perillyl alcohol, α-terpineol and γ-terpineol [**18**] (Park et al., 2003; Yang et al., 2007).

*Pseudomonas putida* (MTCC 1072) converts limonene to *p*-menth-1-ene-6,8-diol [**19**] and perillyl alcohol (Chatterjee and Bhattacharyya, 2001). No sequence information was found in public databases. Two other strains of *Pseudomonas putida* (F1 and GS1) have been found to convert (+)-limonene to perillic

acid in co-substrate fed-batch cultures (Speelmans et al., 1998). Experimental results indicated the participation of the *p*-cymene pathway (CYM) (Mars et al., 2001). *Castellaniella defragrans* grows anaerobically on cyclic monoterpenes as sole carbon and energy source under denitrifying conditions (Foss et al., 1998). Recent experiments suggested an oxygen-independent hydroxylation on the methyl group of limonene to perillyl alcohol as the initial activation step, followed by subsequent oxidation to perillic acid (Petasch et al., 2014).

*P*-cymene [**20**, **Figure 7**] ($C_{10}H_{14}$) is an aromatic monoterpene (*p*-isopropyl-toluene). *Pseudomonas putida* F1 (ATCC 700007) degrades *p*-cymene to *p*-cumate [**21**] via the CYM-pathway (*cymBCAaAbDE*). A two-component *p*-cymene monooxygenase (*cymAaAb*, EC 1.14.13.-) introduces a hydroxyl group on the methyl group of *p*-cymene. The resulting *p*-cumic alcohol is oxidized to the corresponding carboxylic acid by an alcohol and an aldehyde dehydrogenase (*cymB* and *cymC*, EC 1.1.1.- and EC 1.2.1.-). The genes *cymD* and *cymE* encode for a putative outer membrane protein and an acetyl coenzyme A synthetase, respectively. However, their role in the pathway remains unclear (Eaton, 1997). Upstream of the *cym*-operon, the genes for the further degradation of *p*-cumate are located. They are organized in another operon and comprise eight genes (*cmtABCDEFGH*). *P. putida* F1 has been shown to use *p*-cumate as sole carbon source. It is hydroxylated by a ferredoxin dependent *p*-cumate 2,3-dioxygenase. The genes *cmtAaAd* encode a ferredoxin reductase and a ferredoxin, and *cmtAbAc* encode the large and the small subunits of the dioxygenase (EC 1.14.12.-). The resulting *cis*-2,3-dihydroxy-2,3-dihydro-*p*-cumate is oxidized and ring cleavage occurs by introduction of another oxygen molecule. The responsible enzymes are a specific dehydrogenase (*cmtB*, EC 1.3.1.58) and a 2,3-dihydroxy-*p*-cumate dioxygenase (*cmtC*, EC 1.13.11.-), respectively. Further degradation is accomplished by a decarboxylation and elimination of an isobutyrate molecule, catalyzed by a 2-hydroxy-3-carboxy-6-oxo-7-methylocta-2,4-dienoate decarboxylase (*cmtD*, EC 4.1.1.-) and a 2-hydroxy-6-oxo-7-methylocta-2,4-dienoate hydrolase (*cmtE*, EC 3.7.1.-). The product, 2-hydroxypenta-2,4-dienoate, undergoes a water addition by a specific hydratase (*cmtF*, EC 4.2.1.80). Then, a carbon-carbon lyase reaction yields pyruvate and acetaldehyde, catalyzed by 2-oxo-4-hydroxyvalerate aldolase (*cmtG*, EC 4.1.3.39). Acetaldehyde is oxidized and enters as acetyl-CoA the citrate cycle (Eaton, 1996).

*Thauera terpenica* 21 Mol utilizes menthol [**22**] as sole carbon source. The proposed degradation mechanism involves two initial oxidation reactions leading to menth-2-enone, followed by a hydration and an additional oxidation step. Finally, ring cleavage may occur and the molecule is attached to coenzyme A to yield 3,7-dimethyl-5-oxo-octyl-CoA (Foss and Harder, 1998; Hylemon and Harder, 1998).

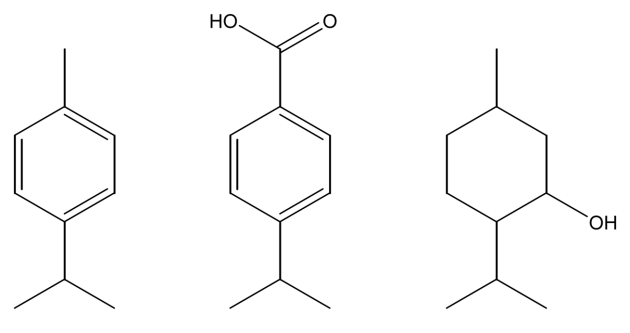


**FIGURE 7 | *p*-cymene [20]; *p*-cumate [21]; menthol [22].**

## ACYCLIC MONOTERPENES

First studies on acyclic monoterpenoids in the early sixties by Seubert and colleagues described the degradation of citronellol [**23**], geraniol [**24**], and nerol via an oxidation of the alcohol to an acid, followed by the formation of a CoA-thioester and subsequent beta-oxidation in *Pseudomonas citronellolis* (ATCC 13674) (Seubert, 1960; Seubert and Remberger, 1963; Seubert et al., 1963; Seubert and Fass, 1964). This knowledge has been extended toward other *Pseudomonas* strains (Cantwell et al., 1978). The complete degradation pathway has been classified as the acyclic terpene utilization and leucine utilization (ATU/LIU) pathway involving the genes *atuABCDEFGH* and

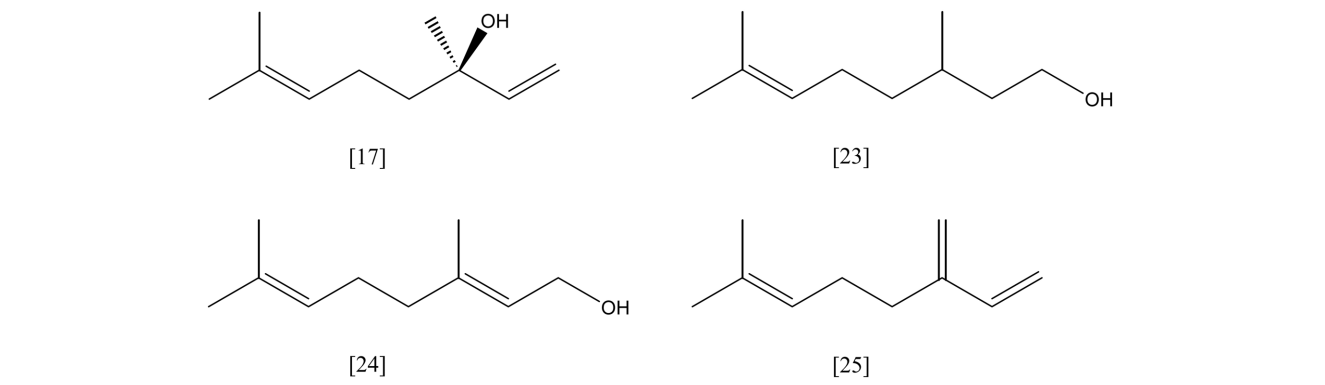


**FIGURE 8 | (*S*)-(+)-linalool [17]; citronellol [23]; geraniol [24]; β-myrcene [25].**

*liuRABCDE*. After the initial formation of *cis*-geranyl-CoA, a geranyl-coenzyme-A carboxylase (*atuCF*, EC 6.4.1.5) elongates the methylgroup. A hydroxyl group is introduced by an isohexenyl-glutaconyl-CoA hydratase (*atuE*, EC 4.2.1.57), followed by a water addition and elimination of an acetate molecule catalyzed by a 3-hydroxy-3-isohexenylglutaryl-CoA lyase (*liu*E, EC 4.1.3.26). The resulting 7-methyl-3-oxooct-6-enoyl-CoA is further degraded via two beta-oxidation like reactions to yield 3-methylcrotonyl-CoA, which enters the leucine degradation pathway (*liuRABCDE*) (Höschle et al., 2005; Aguilar et al., 2006; Förster-Fromme et al., 2006; Chávez-Avilés et al., 2010; Förster-Fromme and Jendrossek, 2010). Citronellol degradation is reported for many *Pseudomonas* strains, including *P. aeruginosa* PAO1 (ATCC 15692), *P. mendocina* (ATCC 25411), and *P. delhiensis* (DSM 18900) (Cantwell et al., 1978; Prakash et al., 2007; Förster-Fromme and Jendrossek, 2010). Among the few reactions described in detail is a molybdenum dependent dehydrogenase responsible for the geranial oxidation to geranylate in *P. aeruginosa* PAO1 (Höschle and Jendrossek, 2005).

The acyclic monoterpene β-myrcene [**25**] ($C_{10}H_{16}$) is transformed by *Pseudomonas aeruginosa* (PTCC 1074) into dihydrolinalool, 2,6-dimethyloctane and α-terpineol. Limonene has been proposed as possible intermediate in α-terpineol formation but was not detected in the culture broth (Esmaeili and Hashemi, 2011). *Pseudomonas* sp. M1 accomplishes degradation by hydroxylation on the C8 position to myrcene-8-ol, which is further oxidized, linked to coenzyme A and metabolized in a beta-oxidation like manner (Iurescia et al., 1999). The formation of geraniol from β-myrcene has been observed with resting cells of *Rhodococcus erythropolis* MLT1, regardless of the presence of a cytochrome P450 inhibitor. The reaction was dependent on aerobic conditions, however it remains unclear if a monooxygenase or lyase system is involved (Thompson et al., 2010).

The tertiary alcohol linalool is also transformed at the C8 position. A linalool monooxygenase (EC 1.14.13.151) has been described in *P. putida* PpG777 and *Novosphingobium aromaticivorans* (ATCC 700278D-5) (Ullah et al., 1990; Bell et al., 2010). In the absence of molecular oxygen, *Castellaniella defragrans* 65Phen has an unique enzyme for the linalool transformation, the linalool dehydratase-isomerase (Brodkorb et al., 2010). *Castellaniella* and *Thauera* strains were the first anaerobic microorganisms shown to anaerobically degrade and mineralize monoterpenes (Harder and Probian, 1995; Harder et al., 2000). The linalool dehydratase-isomerase (EC 4.2.1.127 and 5.4.4.4) of *C. defragrans* 65Phen catalyzes a regio- and stereo-specific hydration of β-myrcene yielding the tertiary alcohol (*S*)-(+)-linalool [**17**] and the isomerization to the primary alcohol geraniol (Brodkorb et al., 2010; Lueddeke and Harder, 2011). Geraniol and geranial dehydrogenases formed geranic acid (Heyen and Harder, 2000; Lueddeke et al., 2012). *T. linaloolentis* 47Lol grows on linalool as sole carbon and energy source. A similar isomerization of linalool to geraniol with subsequent oxidation of geraniol to geranial has been observed in cultures (Foss and Harder, 1997).

## MONOTERPENE TRANSFORMATION BY FUNGI

Fungi excrete laccases which are copper-containing oxidases. Utilizing molecular oxygen as a cosubstrate, an unspecific oxidation of organic molecules is initiated by these enzymes. Additionally, fungi express a variety of cytochrome P450 mono- and di-oxygenases. Thus, several fungi were described to transform monoterpenes during growth in rich medium (reviewed by Farooq et al., 2004). Species with a reported capacity to transform monoterpenes are *Aspergillus niger, Botrytis cinerea, Diplodia gossypina, Mucor circinelloides, Penicillium italicum, Penicillium digitatum, Corynespora cassiicola,* and *Glomerella cingulata*. For a long time, no species have been described to use monoterpenes as sole carbon and energy source for growth (Trudgill, 1994 and references therein). Recently, *Grosmannia clavigera*, a bark beetle-associated fungal pathogen of pine trees, was shown to grow on a mono- and diterpene mixture, containing α/β-pinene and 3-carene (Diguistini et al., 2011). ABC efflux transporter and cytochrome P450 enzymes confer a monoterpene resistance to the blue-stain fungi (Lah et al., 2013; Wang et al., 2013).

## MONOTERPENES IN THE CARBON CYCLE

Habitats with a dense vegetation of wood and flowers are expected to contain larger populations of monoterpene transforming microorganisms. Whereas coniferous forests emit up to 6.7 g carbon*$m^{-2}$*$yr^{-1}$, broadleaf evergreen forest and grassland emit only 3.5 and 2.5 g carbon*$m^{-2}$*$yr^{-1}$, respectively (Tanaka et al., 2012). Monoterpene emission rates between 0.3 and 7 g carbon*$m^{-2}$*$yr^{-1}$ for the United States—mainly α- and β-pinene, limonene and β-myrcene (Geron et al., 2000)—can support the aerobic growth of 0.15–3.5 g bacteria*$m^{-2}$*$yr^{-1}$, assuming 50% of carbon incorporated into biomass. This is a significant potential, considering the presence of around 10 g microbial biomass in the top centimeter of soil per square meter.

In marine systems, isoprene and monoterpenes (mainly α-pinene) are produced by phytoplankton and algae and partially emitted into the atmosphere (reviewed by Yassaa et al., 2008; Shaw et al., 2010). Isoprene emission was estimated to 0.2–1.2 Tg carbon*$yr^{-1}$ (Palmer and Shaw, 2005; Gantt et al., 2009; Shaw et al., 2010). For the ocean surface area this results in an emission rate of 0.0025 g carbon*$m^{-2}$*$yr^{-1}$. Current uncertainties in the size of emission based on shipborne measurements in comparison to satellite data (Luo and Yu, 2010) may be resolved by incorporating an export from the continental atmosphere to the oceanic atmosphere (Hu et al., 2013). Isoprene-amended samples from marine habitats were enriched in bacteria affiliating with *Actinobacteria*, *Alphaproteobacteria,* and *Bacteroidetes* and first strains were shown to degrade isoprene and aliphatic hydrocarbons (Acuña Alvarez et al., 2009).

In summary, these findings indicate a higher abundance of monoterpene transforming and mineralizing bacteria in soils than in the ocean. Indeed, most monoterpene transforming bacteria have been enriched or isolated from soil and freshwater samples in habitats with monoterpene emitting vegetation.

**Table 1 | Summary table of monoterpene transforming enzymes in validly described species of *Bacteria*.**

| EC number | Enzyme name | Organism | Substrate | Co-substrate | Product | Co-product | References |
|---|---|---|---|---|---|---|---|
| 1.14.13.155 | α-pinene monooxygenase | *Pseudomonas fluorescens* NCIMB 11671 | α-pinene | Oxygen, NADH | α-pinene oxide | Water, $NAD^+$ | Best et al., 1987 |
| 5.5.1.10 | α-pinene oxide lyase | *Pseudomonas fluorescens* NCIMB 11671 | α-pinene oxide | | (*E*)-2,6-dimethyl-5-methylidene-hept-2-enal (iso-novalal) | | Best et al., 1987 |
| | | *Pseudomonas rhodesiae* PF1 (CIP 107491) | | | | | Fontanille et al., 2002 |
| 1.14.13.156 | 1,8-cineole 2-endo-monooxygenase | *Citrobacter braakii* | 1,8-cineole | Oxygen, NADPH | 2-*endo*-hydroxy-1,8-cineole | Water, $NADP^+$ | Hawkes et al., 2002 |
| 1.14.13.105 | Monocyclic monoterpene ketone monooxygenase | *Rhodococcus erythropolis* DCL 14 | (−)-menthone | Oxygen, NADPH | (4*R*,7*S*)-4-methyl-7-(propan-2-yl) oxepan-2-one | Water, $NADP^+$ | van der Werf et al., 1999b |
| 1.1.1.297 | Limonene 1,2-diol dehydrogenase | *Rhodococcus erythropolis* DCL 14 | Limonene 1,2-diol | $NAD^+$ | 1-hydroxy-*p*-menth-8-en-2-one | NADH | van der Werf et al., 1999b |
| 1.14.13.107 | Limonene 1,2-monooxygenase | *Rhodococcus erythropolis* DCL 14 | (*R*)-limonene | Oxygen, NAD(P)H | 1,2-epoxy-menth-8-ene | Water, $NAD(P)^+$ | van der Werf et al., 1999b |
| 1.14.13.48 | (*S*)-limonene 6-monooxygenase | *Rhodococcus erythropolis* DCL 14 | (*S*)-limonene | Oxygen, NADPH | (−)-*trans*-carveol | Water, $NADP^+$ | van der Werf et al., 1999b |
| 1.1.1.243 | Carveol dehydrogenase | *Rhodococcus erythropolis* DCL 14 | (−)-*trans*-carveol | $NADP^+$ | (−)-carvone | NADPH | van der Werf et al., 1999b |
| 1.3.99.25 | Carvone reductase | *Rhodococcus erythropolis* DCL 14 | (+)-dihydrocarvone | Oxidized electron acceptor | (−)-carvone | Reduced electron acceptor | van der Werf et al., 1999b |
| 1.1.1.275 | *Trans*-carveol dehydrogenase | *Rhodococcus opacus* PWD4 (DSM 44313) | (+)-*trans*-carveol | $NAD^+$ | (+)-carvone | NADH | Duetz et al., 2001 |
| 3.1.1.83 | Monoterpene ε-lactone hydrolase | *Rhodococcus erythropolis* DCL 14 | (4*S*,7*R*)-7-methyl-4-prop-1-en-2-yl-oxepan-2-one | Water | 6-hydroxy-3-prop-1-en-2-yl-heptanoate | | van der Werf et al., 1999a |
| 3.3.2.8 | (4*R*)-limonene-1,2-epoxide hydrolase | *Rhodococcus erythropolis* DCL 14 | 1,2-epoxy-*p*-menth-8-ene | Water | Menth-8-ene-1,2-diol | | van der Werf et al., 1999a |
| 1.1.1.297 | (1*S*,2*S*,4*R*)-limonene-1,2-diol dehydrogenase | *Rhodococcus erythropolis* DCL 14 | Menth-8-ene-1,2-diol | $NAD^+$ | 1-hydroxy-*p*-menth-8-en-2-one | NADH | van der Werf et al., 1999a |
| 1.14.13.49 | (*S*)-limonene 7-monooxygenase | *Geobacillus stearothermophilus* (ex *Bacillus* strain BR388) | (*S*)-limonene | Oxygen, NADPH | (−)-perillyl alcohol | Water, $NADP^+$ | Cheong and Oriel, 2000 |
| 1.14.13.151 | Linalool 8-monooxgenase | *Novosphingobium aromaticivorans* ATCC 700278D-5 | Linalool | 2 oxygen, 2 NADH | (6*E*)-8-oxolinalool | 3 Water, 2 $NAD^+$ | Bell et al., 2010 |
| | | *Pseudomonas putida* PpG777 | | | | | Ullah et al., 1990 |

*(Continued)*

**Table 1 | Continued**

| EC number | Enzyme name | Organism | Substrate | Co-substrate | Product | Co-product | References |
|---|---|---|---|---|---|---|---|
| 4.2.1.127 | Linalool dehydratase (-isomerase) | *Castellaniella defragrans* 65Phen (DSM 12143) | β-myrcene | Water | (*S*)-(+)-linalool | | Brodkorb et al., 2010 |
| 5.4.4.4 | Linalool (dehydratase)-isomerase | *Castellaniella defragrans* 65Phen (DSM 12143) | (*S*)-(+)-linalool | | Geraniol | | Brodkorb et al., 2010 |
| 1.1.1.347 | Geraniol dehydrogenase | *Castellaniella defragrans* 65Phen (DSM 12143) | Geraniol | $NAD^+$ | Geranial | NADH | Lueddeke et al., 2012 |
| 1.2.1.86 | Geranial dehydrogenase | *Castellaniella defragrans* 65Phen (DSM 12143) | Geranial | Water, $NAD^+$ | Geranic acid | NADH | Lueddeke et al., 2012 |
| **Cym PATHWAY** | | | | | | | |
| 1.14.13.- | *p*-cymene monooxygenase, hydroxylase subunit (CymAa) | *Pseudomonas putida* F1 (ATCC 700007) | *p*-cymene | Oxygen, NADH | *p*-cumic alcohol | Water, $NAD^+$ | Eaton, 1997 |
| 1.14.13.- | *p*-cymene monooxygenase, reductase subunit (CymAb) | *Pseudomonas putida* F1 (ATCC 700007) | *p*-cymene | Oxygen, NADH | *p*-cumic alcohol | Water, $NAD^+$ | Eaton, 1997 |
| 1.1.1.- | *p*-cumic alcohol dehydrogenase (CymB) | *Pseudomonas putida* F1 (ATCC 700007) | *p*-cumic alcohol | $NAD^+$ | *p*-cumic aldehyde | NADH | Eaton, 1996 |
| 1.2.1.- | *p*-cumic aldehyde dehydrogenase (CymC) | *Pseudomonas putida* F1 (ATCC 700007) | *p*-cumic aldehyde | Water, $NAD^+$ | *p*-cumic acid | NADH | Eaton, 1996 |
| -.-.-.- | Putative outer membrane protein, unknown function (CymD) | *Pseudomonas putida* F1 (ATCC 700007) | | | | | Eaton, 1996 |
| 6.2.1.1 | Acetyl-CoA synthetase (CymE) | *Pseudomonas putida* F1 (ATCC 700007) | Acetate | CoA, ATP | Acetyl-CoA | Diphosphate, AMP | Eaton, 1996 |
| **Cmt PATHWAY** | | | | | | | |
| 1.14.12.- | *p*-cumate 2,3-dioxygenase (CmtAaAbAcAd) | *Pseudomonas putida* F1 (ATCC 700007) | *p*-cumate | Oxygen, NADH | *Cis*-2,3-dihydroxy-2,3-dihydro-*p*-cumate | $NAD^+$ | Eaton, 1996 |
| 1.3.1.58 | 2,3-dihydroxy-2,3-dihydro-*p*-cumate dehydrogenase (CmtB) | *Pseudomonas putida* F1 (ATCC 700007) | *Cis*-2,3-dihydroxy-2,3-dihydro-*p*-cumate | $NAD^+$ | 2,3-dihydroxy-*p*-cumate | NADH | Eaton, 1996 |
| 1.13.11.- | 2,3-dihydroxy-*p*-cumate dioxygenase (CmtC) | *Pseudomonas putida* F1 (ATCC 700007) | 2,3-dihydroxy-*p*-cumate | Oxygen | 2-hydroxy-3-carboxy-6-oxo-7-methylocta-2,4-dienoate | | Eaton, 1996 |
| 4.1.1.- | 2-hydroxy-3-carboxy-6-oxo-7-methylocta-2,4-dienoate decarboxylase (CmtD) | *Pseudomonas putida* F1 (ATCC 700007) | 2-hydroxy-3-carboxy-6-oxo-7-methylocta-2,4-dienoate | | 2-hydroxy-6-oxo-7-methylocta-2,4-dienoate | Carbon dioxide | Eaton, 1996 |

*(Continued)*

**Table 1 | Continued**

| EC number | Enzyme name | Organism | Substrate | Co-substrate | Product | Co-product | References |
|---|---|---|---|---|---|---|---|
| 3.7.1.- | 2-hydroxy-6-oxo-7-methylocta-2,4-dienoate hydrolase (CmtE) | *Pseudomonas putida* F1 (ATCC 700007) | 2-hydroxy-6-oxo-7-methylocta-2,4-dienoate | Water | 2-hydroxypenta-2,4-dienoate | Isobutyrate | Eaton, 1996 |
| 4.2.1.80 | 2-hydroxypenta-2,4-dienoate hydratase (CmtF) | *Pseudomonas putida* MT-2 (ATCC 33015) | 2-hydroxy-penta-2,4-dienoate | Water | 2-oxo-4-hydroxy-pentanoate | | Harayama et al., 1989 |
| 4.1.3.39 | 2-oxo-4-hydroxyvalerate aldolase (CmtG) | *Pseudomonas putida* PG (DSM 8368) | 2-oxo-4-hydroxy-pentanoate | | Acetaldehyde | Pyruvate | Platt et al., 1995 |
| 1.2.1.10 | Acetaldehyde dehydrogenase (CmtH) | *Pseudomonas putida* PG (DSM 8368) | Acetaldehyde | $NAD^+$, CoA | Acetyl-CoA | NADH | Platt et al., 1995 |
| **Atu PATHWAY** | | | | | | | |
| 1.1.99.-/1.2.99.- | Citronellol/citronellal dehydrogenase (AtuB; AtuG) | *Pseudomonas citronellolis* (ATCC 13674) | Citronellol/citronellal | Water, oxidized electron acceptor | Citronellal/citronellate | Reduced electron acceptor | Förster-Fromme et al., 2006 |
| 6.2.1.- | Putative citronellyl-CoA synthetase (AtuH) | *Pseudomonas citronellolis* (ATCC 13674) | Citronellate | CoA, ATP | Citronellyl-CoA | Diphosphate, AMP | Förster-Fromme et al., 2006 |
| 1.3.99.- | Putative citronellyl-CoA desaturase (AtuD) | *Pseudomonas citronellolis* (ATCC 13674) | Citronellyl-CoA | Oxidized electron acceptor | *Cis*-geranyl-CoA | Reduced electron acceptor | Förster-Fromme et al., 2006 |
| 6.4.1.5 | Geranyl-CoA carboxylase, carboxylase alpha-subunit (AtuF) | *Pseudomonas citronellolis* (ATCC 13674) | *Cis*-geranyl-CoA | Bicarbonate, ATP | Isohexenyl-glutaconyl-CoA | ADP, phosphate | Förster-Fromme et al., 2006 |
| 6.4.1.5 | Geranyl-CoA carboxylase, carboxylase beta-subunit (AtuC) | *Pseudomonas citronellolis* (ATCC 13674) | *Cis*-geranyl-CoA | Bicarbonate, ATP | Isohexenyl-glutaconyl-CoA | ADP, phosphate | Fall and Hector, 1977; Förster-Fromme et al., 2006 |
| | AtuC, AtuF | *Pseudomonas mendocina* (ATCC 25411) | | | | | Cantwell et al., 1978 |
| | AtuC, AtuF | *Pseudomonas aeruginosa* PAO1 (ATCC 15692) | | | | | Díaz-Pérez et al., 2004; Höschle et al., 2005 |
| 4.2.1.57 | Isohexenyl-glutaconyl-CoA hydratase (AtuE) | *Pseudomonas citronellolis* (ATCC 13674) | Isohexenyl-glutaconyl-CoA | Water | 3-hydroxy-3-isohexenyl-glutaryl-CoA | | Förster-Fromme et al., 2006 |
| | AtuE | *Pseudomonas aeruginosa* PAO1 (ATCC 15692) | | | | | Díaz-Pérez et al., 2004; Höschle et al., 2005 |
| | AtuE | *Pseudomonas mendocina* (ATCC 25411) | | | | | Cantwell et al., 1978 |
| 4.1.3.26 | 3-hydroxy-3-iso-hexenyl-glutaryl-CoA:acetate lyase (LiuE) | *Pseudomonas citronellolis* (ATCC 13674) | 3-hydroxy-3-isohexenyl-glutaryl-CoA | | 7-methyl-3-oxo-6-octenoyl-CoA | Acetate | Förster-Fromme et al., 2006; Chávez-Avilés et al., 2010 |

*(Continued)*

**Table 1 | Continued**

| EC number | Enzyme name | Organism | Substrate | Co-substrate | Product | Co-product | References |
|---|---|---|---|---|---|---|---|
| | LiuE | *Pseudomonas aerugenosa* PAO1 (ATCC 15692) | | | | | Chávez-Avilés et al., 2010 |
| **Cam PATHWAY** | | | | | | | |
| | | | | | | | (Iwaki et al., 2013, and references therein) |
| 1.14.15.1 | Camphor 5-monooxygenase (CamABC) | *Pseudomonas putida* (ATCC 29607) | (+)(-)-camphor | Oxygen, reduced putidaredoxin | 5-oxo-hydroxy-camphor | Water, oxidized putidaredoxin | Poulos et al., 1985 |
| | | *Novosphingobium aromaticivorans* (ATCC 700278D-5) | | | | | Bell et al., 2010 |
| 1.1.1.327 | 5-exo-hydroxycamphor dehydrogenase (CamD) | *Pseudomonas putida* (ATCC 17453) | 5-oxo-hydroxy-camphor | $NAD^+$ | 2,5-diketocamphane/ 3,6-diketocamphane | NADH | Aramaki et al., 1993 |
| 1.14.13.162 | 2,5-diketocamphane 1,2-monooxygenase ($CamE_{25-1}$, $CamE_{25-2}$, $CamE_{36}$) | *Pseudomonas putida* (ATCC 17453) | 2,5-diketocamphane | Oxygen, NADH | (+)-5-oxo-1,2-campholide | Water, $NAD^+$ | Taylor and Trudgill, 1986 |
| 1.14.13.162 | 3,6-diketocamphane 1,6-monooxygenase ($CamE_{36}$) | *Pseudomonas putida* (ATCC 17453) | 3,6-diketocamphane | Oxygen, NADH | (−)-5-oxo-1,2-campholide | Water, $NAD^+$ | Taylor and Trudgill, 1986 |
| 6.2.1.38 | (2,2,3-trimethyl-5-oxocyclopent-3-enyl) acetyl-CoA synthase (CamF1, CamF2) | *Pseudomonas putida* (ATCC 17453) | [(1*R*)-2,2,3-trimethyl-5-oxocyclopent-3-enyl] acetate | ATP, CoA | [(1*R*)-2,2,3-trimethyl-5-oxocyclopent-3-enyl] acetyl-CoA | Diphosphate, AMP | Ougham et al., 1983 |
| 1.14.13.160 | 2-oxo-$\Delta^3$-4,5,5-trimethyl cyclopentenyl acetyl-CoA 1,2-monooxygenase (CamG) | *Pseudomonas putida* (ATCC 17453) | [(1*R*)-2,2,3-trimethyl-5-oxocyclopent-3-enyl] acetyl-CoA | Oxygen, NADPH | [(2*R*)-3,3,4-trimethyl-6-oxo-3,6-dihydro-1H-pyran-2-yl] acetyl-CoA | Water, $NADP^+$ | Ougham et al., 1983; Leisch et al., 2012 |

## DATABASES FOR PATHWAY ANALYSIS AND A LOOK AT METAGENOMES

Databases are nowadays available for the analysis of enzymatic reactions and metabolic pathways in metagenomic and genomic sequence datasets. The most relevant are the Kyoto Encyclopedia of Genes and Genomes (KEGG), MetaCyc and the Biocatalysis/Biodegradation database of the University of Minnesota.

First studies used KEGG to identify monoterpene-related genes in metagenomes of microbiomes in insects and nematodes feeding on a monoterpene-rich diet. Pine beetles encounter the high terpenoid concentrations of conifers and may take advantage of detoxification processes catalyzed by their symbionts/microbiomes (Adams et al., 2013). The KEGG pathway for limonene and pinene degradation (ko00903) was used to identify genes encoding enzymes putatively involved in monoterpene degradation. Five enzymes were present and more abundant in the metagenomes than in a combined metagenomic set of plant biomass-degrading communities. These enzymes were an aldehyde dehydrogenase, an oxidoreductase, an enoyl-CoA hydratase and two hydratases/epimerases. Whether these genes are truly involved in monoterpene metabolism or the degradation of cyclic

compounds, e.g. related aromatic lignin monomers, is an open question. Taxonomically, these genes affiliated with the genera *Pseudomonas*, *Rahnella*, *Serratia*, and *Stenotrophomonas*.

The pinewood nematode *Bursaphlenchus xylophilus* transcribes cytochrome P450 genes as main metabolic pathway for xenobiotics detoxification, but not all enzymes needed for terpenoid metabolism were detected by transcriptomic analysis. Metagenomic data of nematode bacterial symbionts included the complete α-pinene degradation pathway (Cheng et al., 2013). Annotation based on KEGG revealed that the degradation pathways for limonene and pinene (map00903) and for geraniol (map00281) accounted for 2.5% of mapped metagenes. The majority of these genes affiliated to *Pseudomonas*, *Achromobacter*, and *Agrobacterium*. Strains isolated from the nematode and capable of growth on α-pinene affiliated to *Pseudomonas*, *Achromobacter*, *Agrobacterium*, *Cytophaga*, *Herbaspirillum,* and *Stenotrophomonas*.

## CONCLUSION

The synthesis and transformation of BVOCs, especially terpenoids, by plants is well studied (Kesselmeier and Staudt, 1999). Corresponding pathways have been elucidated and a variety of corresponding enzymes have been isolated and characterized (Mahmoud and Croteau, 2002; Yu and Utsumi, 2009). In contrast, the exploration of the microbial transformation and mineralization of monoterpenes has accumulated a small coverage of the field. Simply, over the last 50 years, research on bacterial monoterpene metabolism had only found the interest of very few principal investigators. Now, large sequence datasets of organisms and biological communities provide an unprecedented insight into the diversity of pathways and provide us with challenging hypotheses. However, the basis for the annotation is the biochemical characterization of enzymes which is only available for few monoterpenes. Only three pathways are completely known on the genetic and enzymatic level: the ones for camphor (CAM), *p*-cymene (CYM/CMT), and citronellol/geraniol (ATU/LIU). For pinene, the gene for a key enzyme, the α-pinene oxide lyase (EC 5.5.1.10), is still unknown. The lack of such a key enzyme sequence for a KEGG pathway (map00903) illustrates our uncertainty in the interpretation of metagenomic and genomic datasets. Progress in proteomic and metabolomic analyses in the last years support now biochemical and genetic experiments which will swiftly reveal the desired identification of key enzymes in the monoterpene metabolism.

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REVIEW
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# Pivotal roles of phyllosphere microorganisms at the interface between plant functioning and atmospheric trace gas dynamics

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The phyllosphere, which *lato sensu* consists of the aerial parts of plants, and therefore primarily, of the set of photosynthetic leaves, is one of the most prevalent microbial habitats on earth. Phyllosphere microbiota are related to original and specific processes at the interface between plants, microorganisms and the atmosphere. Recent –omics studies have opened fascinating opportunities for characterizing the spatio-temporal structure of phyllosphere microbial communities in relation with structural, functional, and ecological properties of host plants, and with physico-chemical properties of the environment, such as climate dynamics and trace gas composition of the surrounding atmosphere. This review will analyze recent advances, especially those resulting from environmental genomics, and how this novel knowledge has revealed the extent of the ecosystemic impact of the phyllosphere at the interface between plants and atmosphere.

## Highlights

- The phyllosphere is one of the most prevalent microbial habitats on earth.
- Phyllosphere microbiota colonize extreme, stressful, and changing environments.
- Plants, phyllosphere microbiota and the atmosphere present a dynamic continuum.
- Phyllosphere microbiota interact with the dynamics of volatile organic compounds and atmospheric trace gasses.

**Keywords: plant–microorganism interactions, aerial plant organs, environmental genomics, volatile organic compounds, phyllosphere–atmosphere interface, global change**

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## Introduction

Microbial communities on or around plants play a major role in plant functioning and vigor. Rhizospheric microbial communities, associated with root systems, have been extensively studied and best characterized, as they have been shown to be directly involved in crop productivity through their roles in bioaccessibility of mineral nutrients, protection against pathogens and release of phytohormones to stimulate plant growth. However, the phyllosphere, which *lato sensu* consists of the aerial parts of plants, and therefore primarily, of the set of photosynthetic leaves, is one of the most prevalent microbial habitats on earth.

The total extent of lower and upper surfaces of leaves is thought to represent $10^9$ $km^2$ that could harbor $10^{26}$ bacterial cells (Vorholt, 2012) and is a major potential entrance for phytopathogenic organisms, whose colonization of the plant must not only overcome plant defenses, but also confront competition from existing microorganisms. Although their numbers are much lower than those of bacteria, phyllosphere-associated fungi are potentially involved in major ecophysiological functions, such as interactions with pathogenic fungi, C/N dynamics or the initial steps of leaf litter degradation (Voříšková and Baldrian, 2013). As for archaea, the first studies that have investigated their occurrence suggest that they are a rather minor component of phyllospheric communities and are more present in the rhizosphere (Knief et al., 2012). Plant microbiota, and especially phyllosphere microbiota, are thus an important field of study for understanding community assemblage processes and the mechanisms of community maintenance *in natura*.

Knowledge on phyllosphere microbiota can reveal the mechanisms that govern processes at the interface between plants, microorganisms and the atmosphere, either in pristine environments, or in agricultural or anthropogenic environments. In the case of epiphytic microorganisms, which live on the surface of plant tissues, the phyllosphere is an extreme and unstable habitat, with characteristics of oligotrophy such as limitation in carbon and nitrogen nutrients, and of multiple and highly fluctuating physicochemical constraints (high light, ultraviolet radiation, temperature, dessiccation). Recent high-throughput –omics technologies have lifted a range of analytical bottlenecks, thus raising fascinating opportunities for characterizing in an exhaustive way the spatio-temporal structure of phyllosphere microbial communities in relation with the structural, functional, and ecological properties of host plants (genotype, anatomy, developmental, nutritional and physiological status, biogeography). The recent advances that have been brought to the field of microbial life in the phyllosphere, especially through the development of environmental genomics and metagenomics, have considerably expanded our understanding of the roles of phyllosphere microbial communities in plant–environment interactions and of the ecosystemic impact of the phyllosphere. Thus, major progress is expected in order to understand the impacts on the physicochemical properties of the environment, such as climate dynamics, the dynamics of numerous gaseous compounds [levels of volatile organic compounds (VOCs), gaseous plant hormones, and volatile pollutants] and the trace gas composition of the surrounding atmosphere.

## Confrontation of Microorganisms with the Extreme and Stressful Physicochemical Conditions of the Phyllosphere

Among the different above-ground portions of plants found in the phyllosphere such as the caulosphere (stems), the anthosphere (flowers) and the carposphere (fruits), the phyllophane (surface of leaves; **Figure 1**) presents many peculiar features for microbial life (Kowalchuk et al., 2010; Vorholt, 2012; Rastogi et al., 2013; Turner et al., 2013; Müller and Ruppel, 2014). Leaf surfaces are by themselves a complex architecture of microenvironments showing bidimensionally and tridimensionally heterogeneous structures. The characteristics of upper or lower phylloplane (Eglinton and Hamilton, 1967; Schreiber et al., 2004; Reisberg et al., 2013) affect the interactions between epiphytic microorganisms, which live on plant surfaces, in particular by modulating the access to nutrients from leaf tissues (Ruinen, 1961; Bulgarelli et al., 2013), by providing more or less protection from incoming sunlight (Atamna-Ismaeel et al., 2012a), or by presenting gateways for penetration within the plant endosphere (Hirano and Upper, 2000; Schreiber et al., 2004). Epiphytic microorganisms must adjust to multiple fluctuations involving the season cycle, the day/night cycle, and the developmental, morphological and anatomical dynamics of the plant, from the bud to the senescing leaf, or from the flower to the fruit. Plant photoassimilates like sucrose, fructose, and glucose are present on leaf surfaces (Trouvelot et al., 2014), but day/night fluctuations result in important modifications of the plant metabolite profile, and therefore of nutrient availability for the growth of epiphytic microorganisms. Moreover, plant metabolic status, especially carbohydrate status, is highly responsive to conditions of abiotic or biotic stresses (Couée et al., 2006; Trouvelot et al., 2014). Plant metabolites, such as soluble sugars, polyols, amino acids, amines, VOCs such as isoprenoids, halogenated compounds or alcohols, as well as plant water and salts, are not freely and directly available for epiphytic microorganisms. Plant leaf surfaces are generally protected by lipidic and waxy cuticles that greatly limit water and metabolite fluxes, and biochemical exchanges therefore depend on multiple pathways including excretion, exudation, guttation, wounding, leaching, or infiltration (**Figure 1**). All of these characteristics result in an oligotrophic habitat with limitations in carbon and nitrogen resources.

Phyllospheric microorganisms are subjected to multiple physicochemical stresses that can very rapidly vary through leaching, temperature changes, variations of sunlight-exposure, fluctuations of reactive oxygen species production and therefore of oxidative stress intensity. Trees adapted to desertic conditions can secrete soluble compounds that result in alkalization and salinization of leaf surfaces, thus generating saline or alkaline stress in phyllosphere microbes (Finkel et al., 2012). PhyR, which is a general stress response regulator necessary for plant colonization by several alpha-proteobacteria, is enhanced during growth on the phyllosphere compared to growth in liquid media in the laboratory (Gourion et al., 2006; Iguchi et al., 2013). A Δ*phyR* deletion mutant of the methanotroph *Methylosinus* sp. B4S, that colonizes *Arabidopsis* leaf surfaces, was demonstrated to be more sensitive to heat shock and ultraviolet light than the wild-type strain (Iguchi et al., 2013), thus emphasizing the importance of general stress responses for microorganisms living in the phyllosphere.

Adaptation to the phyllospheric lifestyle appears to rely on a variety of mechanisms related to a diversity of physico-chemical and biotic constraints. Epiphytic microorganisms can develop

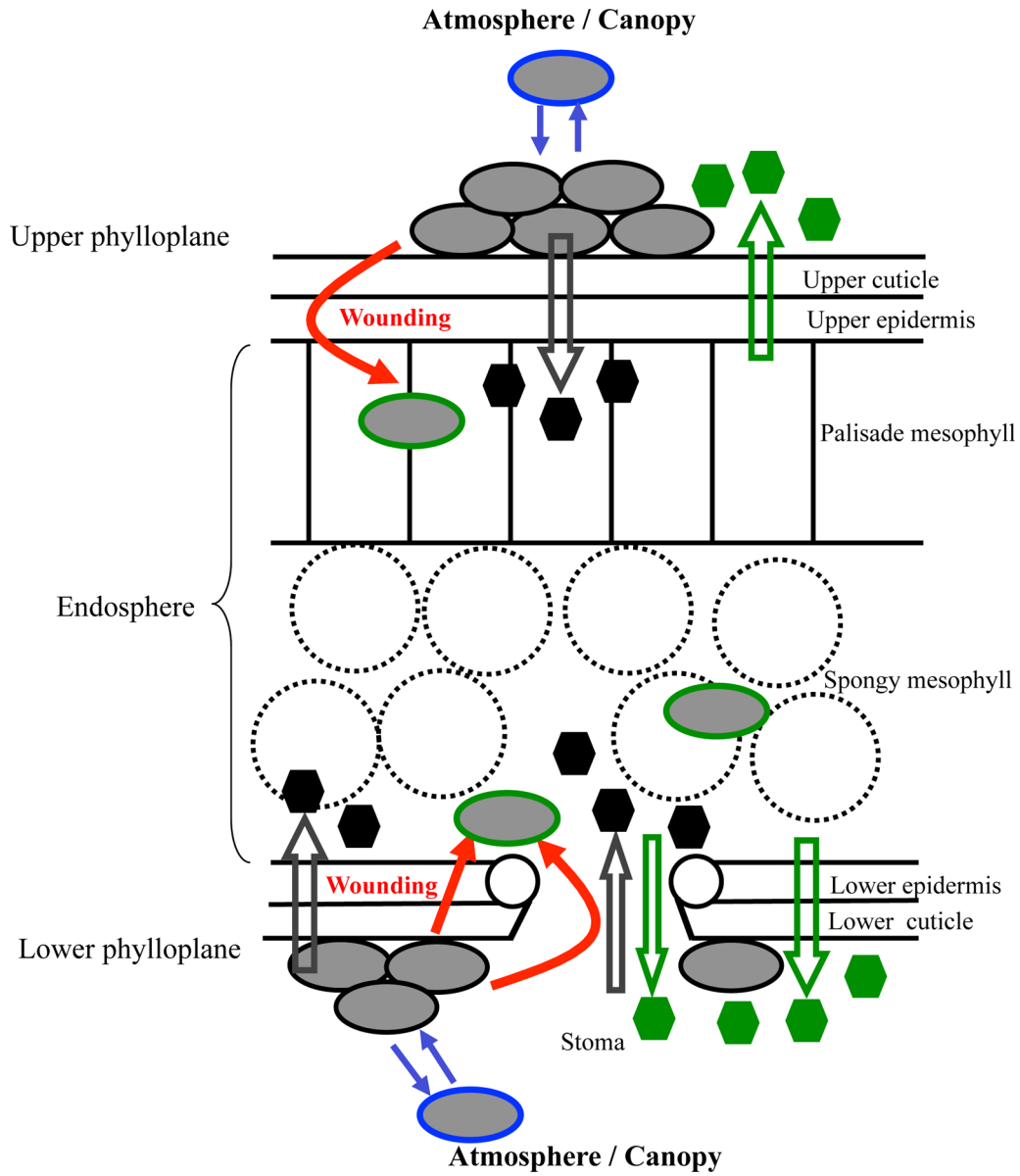


**FIGURE 1 | General histological features and biochemical exchanges of the phylloplane.** The schematic leaf cross section shows interactive exchanges (solid arrows) involving epiphytic microorganisms (gray ellipsoids with black edge) or endophytic microorganisms (gray ellipsoids with green edge) and leaf structures. It also shows the potential fluxes of plant (open green arrows) and microbial (open gray arrows) chemicals (green or black hexagons) that can occur through excretion, exudation, guttation, wounding, leaching, or infiltration. Blue arrows indicate the dynamics of the epiphyte communities with sweeping of bacteria from plant leaves and colonization from airborne microorganisms (gray ellipsoids with blue edge).

tolerance and resistance mechanisms against the antimicrobial and immunity compounds produced by plant tissues or against competing microorganisms (Trouvelot et al., 2014). Numerous studies have focused on the interactions between bacterial quorum-sensing signals and plant roots (Mathesius et al., 2003; Patel et al., 2013), while few studies have dealt with plant leaf microbial communities. Epiphytic microorganisms that display enzymes degrading *N*-acylhomoserine lactone (AHL) quorum-sensing signals have been reported in the tobacco phyllosphere (Ma et al., 2013), thus suggesting that signaling circuits may be involved in shaping complex epiphyllic microbial communities. Epiphytic microorganisms can also develop mechanisms of aggregate formation or of exopolysaccharide synthesis- in order to improve adhesion or protection from dessication (Yu et al., 1999; Monier and Lindow, 2003). Finally, they can also synthesize and secrete phytohormonal compounds, such as indole-3-acetic acid, which facilitates nutrient exudation from plant tissues as a result of plant cell wall relaxation (see details in Vorholt, 2012). However, the complete understanding of these adaptive mechanisms remains incomplete.

## Structure and Diversity of Phyllosphere Microbiota

The structural analysis of phyllosphere microbial communities (Ruinen, 1961; Hirano and Upper, 2000; Schreiber et al., 2004) has been deeply renewed by the development of culture-independent mass sequencing in a growing number of plant species and cultivars of agricultural or ecological interest (**Figure 2**). Supplementary Table S1 gives a list of phyllosphere microbiota that have been characterized by high-throughput molecular analysis and summarizes the main findings of these studies. It must be noted that up to now most of these studies are based mainly on sequencing of PCR-amplified DNA-level conserved taxonomic markers (16S rRNA for bacterial taxonomy; 18S rRNA and Internal Transcribed Spacer ribosomal regions for yeasts and fungi) and, less frequently, on markers of biological functions, such as key genes related to a given metabolism, to a regulatory process or to an adaptive mechanism (**Figure 2**; Supplementary Table S1). A potential bias resulting from primer design and PCR reaction conditions has been described (Mao et al., 2012). Most 16S rRNA universal primers also amplify chloroplast and mitochondrial sequences that result in less rRNA sequences of interest matched to bacteria (Santhanam et al., 2014; Jo et al., 2015). To minimize the amplification of host plant

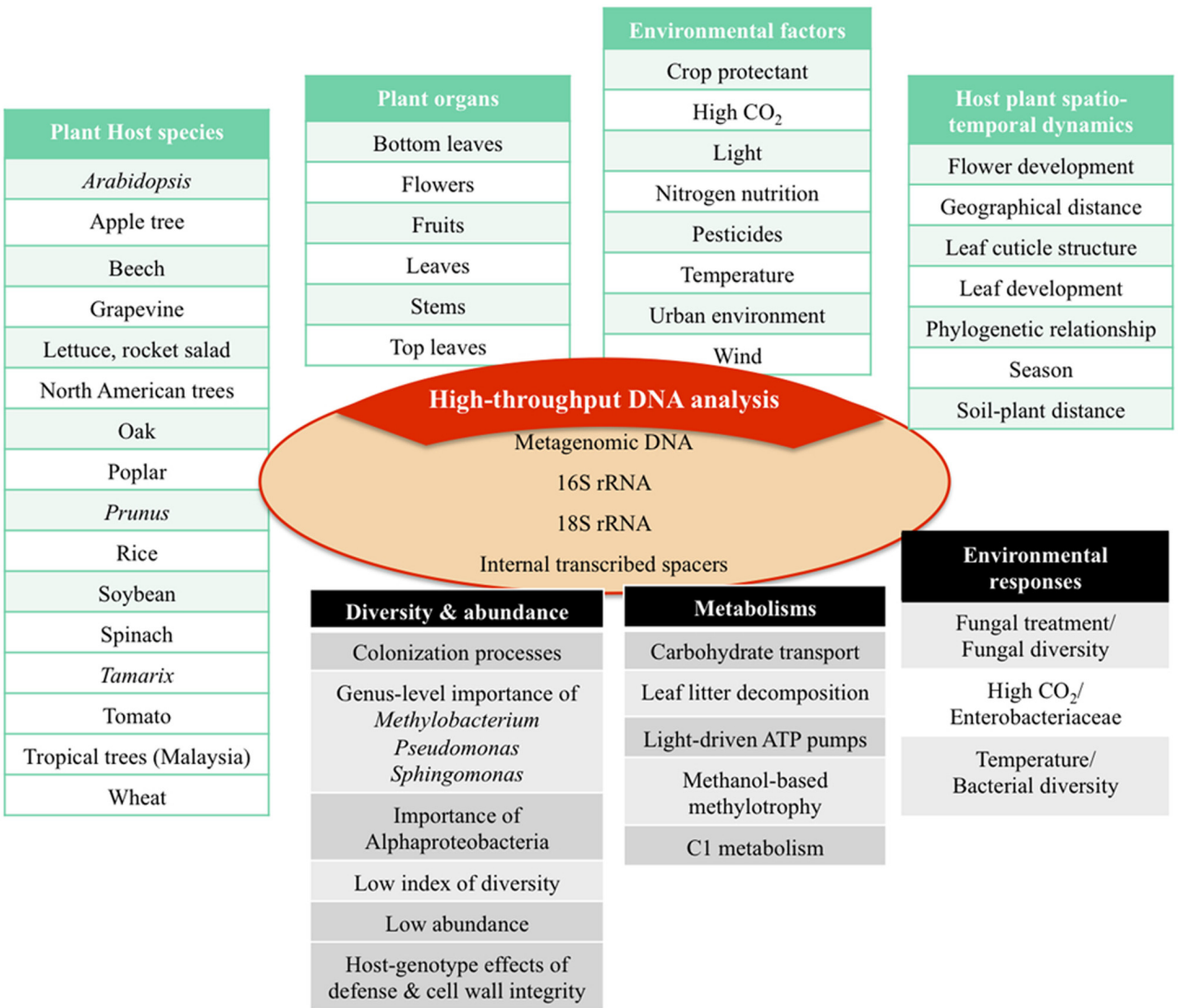


**FIGURE 2 | High-throughput molecular analysis of microbial communities of the phyllosphere: from direct DNA sequencing to sequencing of prokaryotic and eukaryotic taxonomical markers.** The present range of published studies covers a significant number of plant host species in various developmental and spatio-temporal contexts and under the influence of different environmental factors. The articles presented in Supplementary Table S1 represent a selection of studies that were performed in the last years using NGS technologies. Further studies can be found in tables of other recent reviews (Rastogi et al., 2013; Knief, 2014; Müller and Ruppel, 2014). Taken together, these studies give a dynamic view of phyllosphere microbiota biodiversity, metabolisms, and environmental plasticity.

DNA, primer 799F was designed to exclude chloroplast DNA, and the mtDNA sequences can be separated from the PCR-amplified bacterial sequences by size fractionation (Chelius and Triplett, 2001). Primer 799F has become a "standard" forward primer in recent phyllosphere microbiota analysis (Redford et al., 2010; Bodenhausen et al., 2013, 2014; Horton et al., 2014; Kembel et al., 2014; Maignien et al., 2014; Perazzolli et al., 2014; Santhanam et al., 2014; Williams and Marco, 2014; Copeland et al., 2015). Nevertheless, using primer 799F leads to systematic non-detection or underestimation of a few taxa such as cyanobacteria (Chelius and Triplett, 2001). Such bias can typically be avoided by direct mass sequencing and analysis of metagenomic DNA, which has been used so far only in a few studies of phyllosphere microbiota studies (Supplementary Table S1), but which is in constant increase as a result of ever lower sequencing costs and of ever improving bioinformatics tools.

The analysis of metagenomic data from phyllosphere microbial communities (**Figure 2**; Supplementary Table S1) essentially aims to correlate taxonomic composition (Which species is present? «Who is there?») and community structure (How abundant is each taxon? «How many are there?») with intrinsic features of the host plant (genotype, anatomy, metabolism, life history), with environmental features (geography, climate, season, pollutant exposure, phytosanitary treatments), or even with the evolutionary history of the plant species or of the plant population (domestication; relocalization). Specific studies have already shown that the assemblage of microbial communities in the phyllosphere is more similar in genetically related plants than in very divergent plant species (Redford et al., 2010; Kim et al., 2012; Bálint et al., 2013; Dees et al., 2015). Nevertheless, the spatial proximity between plants can also contribute to the composition of phyllospheric microbial communities (Finkel et al., 2012; Rastogi et al., 2012). Climatic factors such as temperature, seasons, occasional exposure to sand storms (Cordier et al., 2012; Rastogi et al., 2012; Bálint et al., 2013), or anthropogenic factors such as the use of pesticides (Shade et al., 2013; Karlsson et al., 2014; Ottesen et al., 2014; Glenn et al., 2015), play an important role in community structuration. Finally, anatomical location, whether on top leaves, bottom leaves nearer to the soil, flowers, fruits, or stems, strongly influence the structure of associated microbial communities (Ottesen et al., 2013; Shade et al., 2013).

The identification of generalist communities, usually present in phyllospheres of a given plant taxon, and specialized communities, adapted to a particular type of phyllospheric environment, is essential to achieve better understanding of the phyllosphere ecosystem and of functional interactions between plants, microbiota, and environmental features. Combinations of metagenomic and metaproteomic data have thus contributed to define the first catalogs of phyllosphere-associated generalist bacterial phyla present in different plant species, thus highlighting the involvement of Bacteroidetes, Actinobacteria, and Proteobacteria (Delmotte et al., 2009; Redford et al., 2010; Lopez-Velasco et al., 2011; Kim et al., 2012; Rastogi et al., 2012; Supplementary Table S1). In the case of yeasts, the prevalence of the phylum *Ascomycota* has been associated with microbiota from oak leaves in Europe (>90% of sequenced amplicon markers) and in Northern America, with however, significant differences of assemblages at the species level (Jumpponen and Jones, 2010; Voříšková and Baldrian, 2013).

Finally, it must be highlighted that these catalogs of phyllosphere microbial communities are magnifications and snapshots of spatially structured and highly dynamic communities. Complementary approaches such as *fluorescence in situ hybridization* (FISH) are therefore required to understand the structure and diversity of phyllospheric communities in the context of microscale spatio-temporal distributions (Remus-Emsermann et al., 2014).

## Processes of Recognition, Adhesion, and Colonization in the Phyllosphere

The cuticle is the exogenous wax layer of aerial plant surfaces which is the habitat of epiphytic bacteria and a barrier for invasive microorganisms. It is composed of long-chain fatty acids with additional pentacyclic triterpenoids and sterols and represents up to 15% of leaf dry weight (Eglinton and Hamilton, 1967). The composition of this highly lipophilic micro-structured wax layer shapes the associated phyllosphere bacterial communities as recently demonstrated using amplicon sequencing of the bacterial communities of a set of *Arabidopsis thaliana cer* mutants with different mutations in the cuticular wax biosynthesis pathways (Reisberg et al., 2013). "Plant-line-specific" bacterial communities, either positively or negatively affected by the wax phenotype, represented less than one third of the total sequence counts. "Permanent" residents, corresponding to bacterial communities that were not influenced by the wax phenotype, were affiliated with *Flavobacteriaceae*, *Flexibacteriaceae*, *Methylobacteriaceae*, *Rhizobiaceae*, *Sphingomonadaceae*, *Enterobacteriaceae*, and *Pseudomonadaceae*. Following outdoor growth, the resident bacterial community acquired as many as 2–7 bacterial clades for the wax mutant variant, unlike the wild-type plant, which was specifically enriched by only a single clade (Reisberg et al., 2013). The use of a gnotobiotic microbial community in relation with *A. thaliana* mutants has also revealed that genetic determinants of cuticle formation affected the dynamics of phyllosphere microbiota (Bodenhausen et al., 2014). All of this strongly suggests that the cuticular wax properties shape niches for specific adapted bacterial communities.

Among specific communities that are found in the phyllosphere of some plant species, the example of the *Massilia* genus is noteworthy. It is associated with lettuce leaves (Rastogi et al., 2012) and represents 7% of total bacterial population in the microbiome of spinach leaves (Lopez-Velasco et al., 2011). However, it has also been identified as a major contaminant of an aerosol with applications in agriculture, thus suggesting, as emphasized by Rastogi et al. (2012), that phyllosphere-associated *Massilia* bacteria stem from agricultural practices.

The endophytic microorganisms of the phyllosphere may be thought to be leaf epiphytic bacteria that cross the cuticle

and superficial tissue layers (**Figure 1**) or endophytic bacteria that migrate from the roots. This question was addressed by sequencing 16S RNA amplicons of the epiphytic and endophytic bacterial communities associated to roots and leaves of *A. thaliana* (Bodenhausen et al., 2013). In the epiphytic communities of the phyllosphere, bacterial richness was found to be lower compared to that of endophytic communities. The richness of bacterial endophytes in both the phyllosphere and the roots was similar, with higher abundance of *Burkholderiales*, *Actinomycetales,* and *Actinoplanes* than found in the leaf epiphytic communities. These observations suggest that leaf microbial endophytes would more likely result from migration of root endophytic microorganisms within the plant than from colonization of bacteria initially present on the surface of the leaf. Nevertheless, this does not preclude foliar entrance of endophytic microorganisms, as has been shown experimentally in other cases of host–endophyte systems (Hartley et al., 2015).

## Metabolic Dynamics of Phyllosphere Microbiota

Flavobacteria are found in high abundance in the rhizosphere and phyllosphere of terrestrial plants such as *A. thaliana* where it is one of the most dominant genera of the leaf microbiota (10%; Bodenhausen et al., 2013). Flavobacteria might be highly adapted to plant carbohydrate metabolism as recently deduced from genome comparison of Flavobacteria isolated from aquatic environments and from plants. Only the genomes of Flavobacteria from terrestrial plant communities and not from aquatic communities harbored genes encoding glycoside hydrolase families GH78 and GH106, that are responsible for utilization of rhamnogalacturonan, which is exclusively associated with terrestrial plant hemicelluloses (Kolton et al., 2013).

Phyllosphere-associated microorganisms live in a sunlight-exposed habitat. Photochemical conversion of this light resource into carbon and energy that may complement carbon resources from the host plant could be a major advantage for growth in a nutrient-limited environment. Analysis of metagenomic data has revealed the presence of bacterial rhodopsin genes in phyllospheric communities (Atamna-Ismaeel et al., 2012a). It thus seems that some epiphytic microorganisms possess retinal-dependent rhodopsin proton pumps that can be light-activated by radiations covering a span of wavelengths that are distinct from the absorption spectrum of chlorophylls and carotenoids, which drive plant photosynthetic processes and thus production of the plant carbon resources that are eventually available to epiphytic microorganisms (Atamna-Ismaeel et al., 2012a; Stiefel et al., 2013).

Given the roles of carbon and nitrogen resources in nutrient signaling and nutrient regulation affecting light-dependent processes (Tolonen et al., 2006; Moran and Miller, 2007), it is likely that several metabolic pathways of epiphytic bacteria can be influenced by the carbohydrate and nitrogen status of the host plant, and thus *in fine* by the fluctuations of plant–light interactions and photoassimilate production in the host plant (Athanasiou et al., 2010; Sulmon et al., 2011). Manching et al. (2014) have recently described global links between plant nitrogen balance and leaf epiphytic bacterial species richness in maize. In a free air $CO_2$ enrichment experiment, Ren et al. (2014) have also demonstrated important changes of phyllosphere bacterial communities in rice subjected to various combinations of elevated $CO_2$ and different levels of nitrogen fertilization. Conversely, enzymatic activities of phyllospheric microorganisms appear to act on important plant metabolites (Huang et al., 2014), thus raising the possibility of complex feedback metabolic loops between plant tissues and phyllospheric microorganisms.

A parallel study investigated the potential presence of gene markers associated with aerobic anoxygenic phototrophic bacteria (Atamna-Ismaeel et al., 2012b). Homologs of *bchY*, which encodes the Y subunit of chlorophyllide reductase, and of *pufM*, which encodes the M subunit of the photosynthetic reaction center, have been detected in five different metagenomes from phyllosphere microbiota (rice, soybean, tobacco, tamarix, clover). Epifluorescence microscopy was used to detect the presence of specific pigments associated with aerobic anoxygenic phototrophic bacteria. It was thus found that these bacteria accounted for 1–7% of the total community of epiphytic bacteria, with the presence of the genus *Methylobacterium*, and more surprisingly, with the presence of an unknown group of bacteria, that seem to be specific to the phyllosphere (Atamna-Ismaeel et al., 2012b).

Rarefaction curves using ribosomal genes indicates that bacterial diversity in the phyllosphere is similar in several different plant species and would be in the range of human microbiome diversity. However, phyllosphere bacterial diversity seems to be much lower than those of the rhizosphere, soil or marine ecosystems (Delmotte et al., 2009; Knief et al., 2012). Sequencing depth is a significant limitation for the detection of phyllosphere-specific bacterial communities, especially in the case of low-abundance species. Thus, markers of aerobic anoxygenic phototrophic bacteria communities, which show low-abundance (<0.4%), were not detected in metagenomic data of tamarix leaf microbiota (Atamna-Ismaeel et al., 2012a), whereas direct microscopic observation revealed their presence in a number of plant species (Atamna-Ismaeel et al., 2012b). It is therefore clear that complementary approaches ought to be developed in order to detect low-abundance bacterial species in the phyllosphere, especially through microscope observation methods, such as FISH or *fluidic force microscope* approaches (FluidFM; Stiefel et al., 2013). Complementary approaches, especially through combined meta-analysis of proteomics, transcriptomics, and metabolomics data (Delmotte et al., 2009; Knief et al., 2011, 2012), are also necessary to address the nutritional and functional mechanisms of microbial adaptation to life in the phyllosphere, such as the potential involvement of auxotrophic relationships and the potential dependence of phyllospheric microbial community structure on light availability and therefore foliage and canopy stratification.

## Impact of Phyllospheric Microorganisms on Plant–Plant, Plant–Insect Herbivory, and Plant-Atmosphere-Chemical Exchanges

Plants emit a great variety of VOCs that can promote or inhibit specific species and thus contribute to numerous biotic interactions and to the shaping of microbial communities. On the other hand, microbes can intercept or alter scent emissions by plants and subsequently plant signaling with other plants or animals (Shiojiri et al., 2006). Knowledge on plant surface microbiota can reveal the mechanisms that govern processes at the interface between plants, microorganisms and plant-interacting organisms, or between plants, microorganisms, and the atmosphere (**Figures 3** and **4**), either in pristine environments, or in agricultural or anthropogenic environments.

Major molecular regulations of plant responses to abiotic and biotic challenges rely on a diverse array of phytohormones, such as the gaseous hormone ethylene, the oxylipin hormone jasmonate and its volatile derivative, methyl jasmonate, that are induced by many herbivores, and the phenolic hormone salicylate and its volatile derivative, methyl salicylate, that are induced by many bacterial pathogens. Genetic analysis of *A. thaliana* mutants has revealed a link between the community composition of phyllosphere microbiota and plant ethylene signaling (Bodenhausen et al., 2014). Using a collection of 196 recombinant inbred lines of field-grown *A. thaliana*, genome-wide association study of associated leaf microbial community revealed that plant loci involved in defense such as reproduction of viruses and cell wall integrity, trichome branching, and morphogenesis shape microbial species richness of leaf microbiota (Horton et al., 2014). In another study focusing on tobacco plants, deficiency in the phytohormone jasmonic acid biosynthesis had no detectable effect on structuring the bacterial communities (Santhanam et al., 2014). Besides phytohormones, a myriad of plant defense and signaling chemicals, whether volatile or non-volatile, are involved in plant biotic interactions (Mason et al., 2014). Plant foliage-associated bacteria have been shown to degrade plant defense chemicals, thus resulting in reduced defense against insect defoliators (Mason et al., 2014). Bacterial symbionts of the genera *Stenotrophomonas*, *Pseudomonas*, and *Enterobacter*, when secreted by the Colorado potato beetle larvae on plant surfaces, suppress the anti-herbivore defenses of tomato by enhancing the microbial defense response and thus favor larval growth (Chung et al., 2013). In a recent study of interactions between a specialist chewing insect herbivore and its sole plant host, *Cardamine cordifolia*, experimental bacterial infections of the phyllosphere showed that individual *Pseudomonas* spp. strains promoted host choice by herbivores, and that bacterial strains exhibited variation in the way they ecologically impacted insect herbivores (Humphrey et al., 2014). As described above, pesticides have strong effects on community composition in the phyllosphere (Karlsson et al., 2014; Ottesen et al., 2014; Glenn et al., 2015), thus suggesting that pesticide treatments could interfere with natural interactions between phyllosphere microbiota and plant defenses. Better understanding of defense mechanisms involving multiple biotic interactions and phyllospheric bacteria may therefore result in novel pesticide usage in the context of sustainable agriculture.

Epiphytic microorganisms present the metabolic potential for degrading compounds that are toxic to plants, to humans or to the environment. Such detoxification potential could be recruited to carry out phyllosphere-based depollution processes. This phylloremediation can target organic compounds that are already known to be metabolized by epiphytic microorganisms, such as nicotin (Sguros, 1955), phenol (Sandhu et al., 2007), polycyclic aromatic hydrocarbons (acenaphthylene, acenaphthene, fluorene, phenanthrene) which are produced by car exhausts (Yutthammo et al., 2010), or chloromethane and isoprene, which are mainly emitted by plants, and are likely to

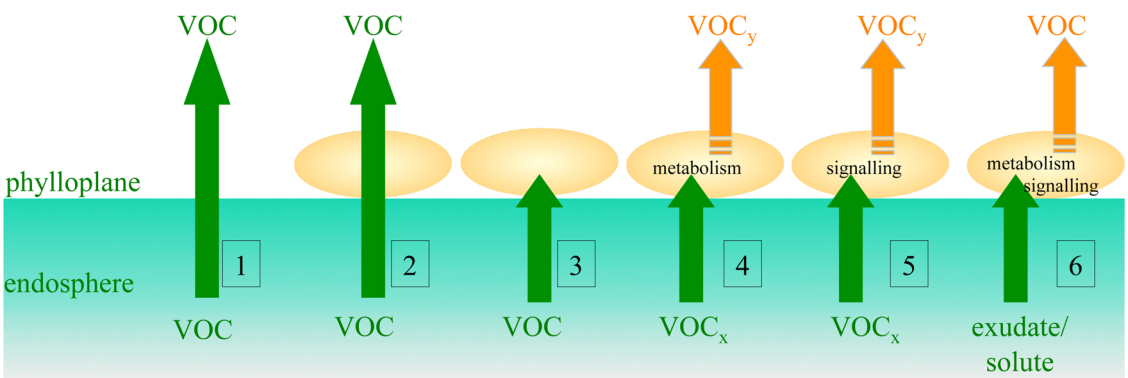


**FIGURE 3 | Theoretical scenarios of the fate of phyllosphere-emitted volatile organic compounds (VOCs).** Green and orange arrows respectively represent plant organic compound fluxes and VOCs fluxes generated from microbial epiphytes. (1) Free transfer through cuticle; (2) free transfer through cuticle and epiphytes; (3) interception by epiphytes via abiotic or metabolic processes with no VOC release; (4) biotransformation of plant VOCx by phyllospheric microbial metabolism resulting in emission of a microbial VOCy; (5) signaling by plant VOCs triggers the phyllosphere microbiota to produce a VOCy; (6) phyllosphere microbiota emit VOCs after exposure to plant non-volatile compounds. Similar scenarios involving endophytic microorganisms could also be envisaged.

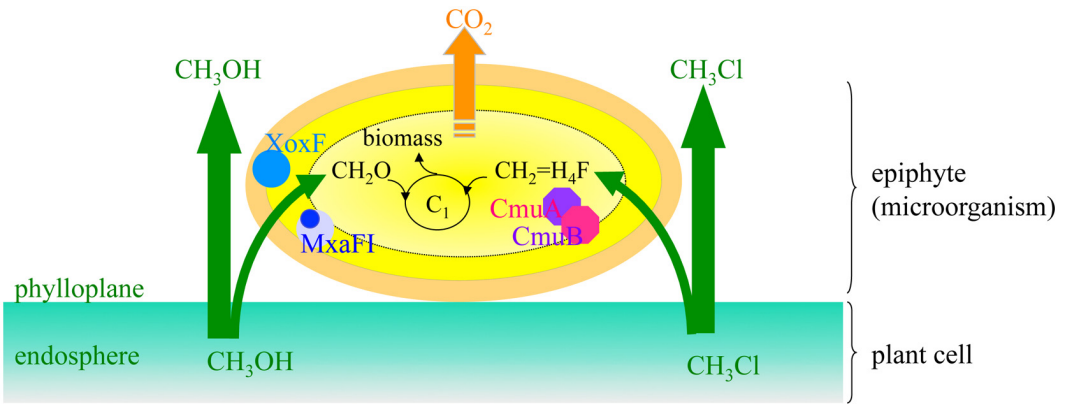


**FIGURE 4 | Biocapture of plant C1 VOCs by methylotrophs of the phyllosphere.** Key enzymes in methanol oxidation imply the MxaFI methanol dehydrogenase subunits (Chistoserdova et al., 2009), and a methanol-dehydrogenase-like protein XoxF whose mutation renders the bacteria less competitive than the wild-type *Methylobacterium extorquens* AM1 during colonization of the phyllosphere of *Arabidopsis thaliana* (Schmidt et al., 2010). Unlike methanol, chloromethane oxidation by the chloromethane utilization *cmu* pathway enters the C1 central metabolism as methylene-tetrahydrofolate ($CH_2 = H_4F$) and not as $CH_2O$ (Vannelli et al., 1999). Bacterial isolates of surface leaves of *A. thaliana* harbor the *cmu* genes (Nadalig et al., 2011).

affect ozone abundance in the atmosphere (Nakamiya et al., 2009; Nadalig et al., 2014).

It has thus been shown that, in the phylum of Actinobacteria, the *Arthrobacter* genus, which is able to degrade numerous organic compounds, can grow and remain in the phyllosphere (Scheublin and Leveau, 2013). Various species of *Arthrobacter* degrade aromatic hydrocarbons (phenol, chlorophenol, BTEX, phenanthrene), *s*-triazines (atrazine, cyanazine), and various other pesticides (phenylurea herbicides, glyphosate, malathion; Scheublin and Leveau, 2013).

Using custom-made microarrays of the Gram-positive *Arthrobacter* with species members commonly found in epiphytic bacterial communities (Rastogi et al., 2012), comparative transcriptome profiling with bacteria recovered from leaves of the common bean (*Phaseolus vulgaris*) or from growth on agar surfaces, demonstrated that several *cph* genes involved in 4-chlorophenol degradation had phyllosphere-induced expression (Scheublin et al., 2014), most likely resulting from the presence of natural plant-excreted phenolic compounds. The utilization of plants harboring adequate microbial communities that degrade a given set of organic compounds can be envisaged for processes of atmospheric depollution in urban or industrial environments, and for the depollution of atmospheric drifts of phytosanitary products in agricultural environments. Finally, it can also be envisaged that epiphytic microorganisms that have beneficial effects on plants could be used as probiotic agents (Berlec, 2012).

## Impact of Phyllospheric Microorganisms on Plant-Atmosphere-Climate Interactions

Plants emit a number of VOCs or VOC precursors that are transferred through the phyllosphere and that probably play a role in climate regulation (Otte et al., 2004; Peñuelas and Staudt, 2009; Schäfer et al., 2010). In the biosphere, plants are the main source of VOC emissions amounting to more than 1,000 Tg $year^{-1}$, with components as diverse as terpenes, monoterpenes and $C_1$ compounds, including methanol, methane, and halogenated methane. What is known of how plant emissions of VOCs interact at their surface with bacterial epiphytes has recently been reviewed (Junker and Tholl, 2013). It remains largely unknown how and to what extent VOCs emitted by plants could be biocaptured, intercepted or consumed through bacterial metabolism by epiphytes present directly on the surface of plants (**Figure 3**), or by transiently occurring airborne bacteria, and how the effects of climate change will impact the abundance, diversity, and ability of microbial metabolism in filtering of plant-emitted VOCs.

Methylotrophic microorganisms are able to utilize some of the plant organic compounds containing a single carbon atom or lacking C–C bonds such as methanol ($CH_3OH$), formaldehyde ($CH_2O$), and chloromethane ($CH_3Cl$). Methylotrophic microorganisms are ubiquitous and can be found in roots and leaves of plants (Delmotte et al., 2009; Knief et al., 2012; Jo et al., 2015), and in the air (DeLeon-Rodriguez et al., 2013). A prominent $C_1$ source for epiphyte microbiota (**Figure 4**) is methanol that has been proven to confer an advantage *in situ* to methylotrophic epiphytes such as the Alphaproteobacteria *Methylobacterium extorquens* and the methylotrophic yeast *Candida boidinii* (Sy et al., 2005; Kawaguchi et al., 2011). Seedlings of *Nicotiana* emitted methanol at 0.005 to 0.01 ppbv in the presence of *M. extorquens*, while plants not colonized by these bacteria showed much higher emissions (0.4–0.7 ppbv; Ababda-Nkpwatt et al., 2006).

Methane ($CH_4$) is the most abundant organic trace gas in the atmosphere (with a mixing ratio of ~1.8 ppm) and an important greenhouse gas. Both intact plants and detached leaves emit methane at an initial estimated source strength of 62–236 Tg/year for living plants and 1–7 Tg/year for plant litter (Keppler et al., 2006). Plants internally transport methane to

the atmosphere through the roots, stems, and leaves from the rhizosphere, where plant exudates provide the nutrients for growth of methanogenic bacteria. Pathways of direct methane production by plant tissues also exist (Althoff et al., 2014; Lenhart et al., 2015). Methane emissions rates depend on plant species and on abiotic factors such as the water regime and temperature (Bhullar et al., 2013). Moreover, directly at the phylloplane level, plant methane emissions would result from wax degradation, in addition to the previously suggested pectin degradation, in the presence of UV radiation and oxygen (Bruhn et al., 2014). Methanotrophic bacteria that utilize methane as a source of carbon and energy have been found in the phyllosphere of plants (Iguchi et al., 2012).

Isoprene (2-methyl-1,3-butadiene) is emitted by leaves of many plant species and emission was shown in some cases to increase with higher temperatures (Monson et al., 1992). The magnitude of global isoprene emissions to the atmosphere is similar to that of methane, and isoprene is an important precursor for photochemical ozone production when oxides of nitrogen levels are high (Arneth et al., 2008). A bacterial degradation pathway has been genetically characterized in a marine isolate, *Rhodococcus* sp. strain AD45 (van Hylckama Vlieg et al., 2000), but has not so far been demonstrated in plant isolates.

Chloromethane ($CH_3Cl$; methyl chloride) is the most abundant chlorinated organic compound in the atmosphere (currently ~550 ppt) and is considered to be responsible for over 16% of the halogen-catalyzed depletion of stratospheric ozone (World Meteorological Organization, 2014). In *A. thaliana*, chloromethane is the product of *S*-adenosylmethionine-dependent methylation of chloride, which is catalyzed by a protein encoded by the *HOL* (*HARMLESS TO OZONE LAYER*) gene, although a physiological *in planta* role for enzyme-produced chloromethane remains to be demonstrated (Nagatoshi and Nakamura, 2009). To assess if vegetation is the main contributor to global emissions of chloromethane to the atmosphere, a fluorescence-based bacterial bioreporter for chloromethane detection has been developed and validated in the model chloromethane-producing plant *A. thaliana* (Farhan Ul Haque et al., 2013). Bacterial adaptation to growth on chloromethane as the sole source of carbon and energy by the *chloromethane utilization* (*cmu*) pathway has been characterized in *M. extorquens* CM4 (Roselli et al., 2013). So far, the few cultivable chloromethane-degrading strains isolated from plants, which were affiliated to the genus *Hyphomicrobium* (Nadalig et al., 2011), were also degrading methanol, thus being able to filter several C1 VOCs emitted on plant leaf surfaces (**Figure 4**).

Volatile dimethyl sulphide (DMS) is considered to be an important global-climate regulator (Charlson et al., 1987; Schäfer et al., 2010; Nevitt, 2011). Fluxes and dynamics of DMS are strongly associated with oceanic sulfur cycles and with phytoplanktonic production of the DMS precursor dimethylsulphoniopropionate (DMSP; Charlson et al., 1987; Schäfer et al., 2010; Nevitt, 2011). However, some plant species (Otte et al., 2004), small in numbers, but ecologically significant, such as salt marsh grasses of the genus *Spartina* and sugar canes (*Saccharum* sp.), are efficient producers of DMSP, which can be metabolized to acrylate and DMS by plant-associated microbes possessing DMSP lyase (Ansede et al., 2001). Phyllosphere microbiota could therefore act on plant-related DMS dynamics both through DMSP-DMS transformation and through DMS metabolization. It is therefore highly likely that phyllosphere microbiota play major roles in carbon and sulfur biogeochemical cycles, in ecosystemic signaling and in climate regulation through their action on plant-related volatile compounds, thus requiring that understanding of the functional ecology of phyllospheric microbes, especially in species of the *Spartina* and *Saccharum* genera, is improved.

Pioneering studies on microbiota in clouds have shown the presence of prevalent bacteria that are common with phyllosphere microbiota, thus suggesting that at least some epiphytic microorganisms are adapted to the conditions of the troposphere (DeLeon-Rodriguez et al., 2013; Šantl-Temkiv et al., 2013). Tropospheric microorganisms are likely to act as water condensation or nucleation centers during cloud formation and to be involved in global carbon cycles through metabolization of the organic compounds that are present in clouds

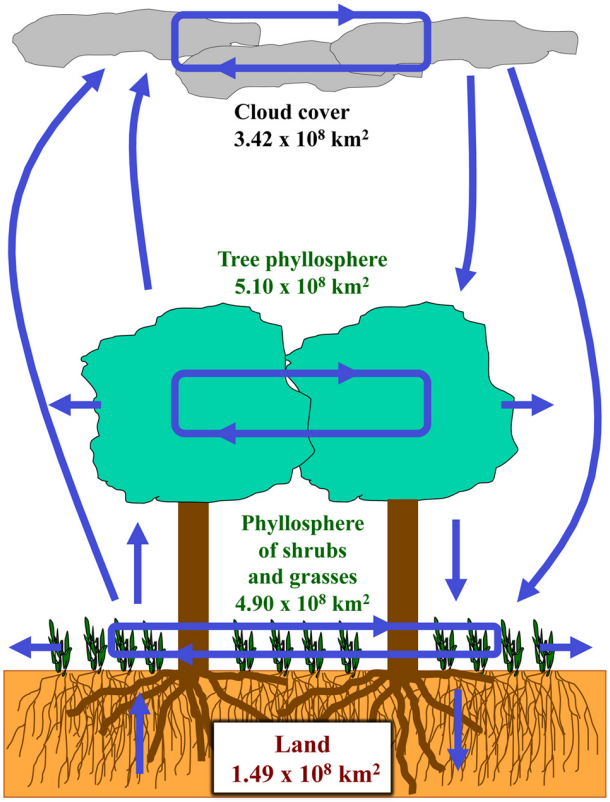


**FIGURE 5 | Dynamics of interactions of plant canopies and the atmosphere.** Land, plant strata, and cloud strata are represented, with estimates of global earth-wide surfaces, which were derived from recent studies (Friend, 2010; Probst et al., 2012; Vorholt, 2012). Interactions involving fluxes of microbial populations are symbolized by blue arrows (Lindemann et al., 1982; Finkel et al., 2012; Rastogi et al., 2012; DeLeon-Rodriguez et al., 2013; Šantl-Temkiv et al., 2013; Vaïtilingom et al., 2013; Hill et al., 2014).

(Vaïtilingom et al., 2013). Moreover, epiphytic microorganisms may constitute the major source of airborne bacteria, including ice nucleation-active (INA) bacteria. These bacteria mainly belong to the order of Gammaproteobacteria and possess common INA proteins encoded by *ina* genes that were qPCR quantified and estimated to reach up to $10^8$ *ina* genes per g of fresh weight in the foliage of cereals (Lindemann et al., 1982; Hill et al., 2014). The presence of INA proteins may also contribute to bacteria dissemination processes via deposition on cloud droplets. There may thus be strong links between phyllosphere microbiota and cloud microbiota (**Figure 5**) with important implications for climate regulation.

The combined activities of microbial communities in the phyllosphere, through the emission of VOCs and interactions with plant VOCs (**Figure 3**), through complex phyllosphere–atmosphere exchanges (**Figure 5**), and through ice-nucleation processes, are therefore potential mechanisms of major global impact on the biosphere. As described above, the composition of phyllosphere microbiota have an impact not only on plant growth and plant health, but also on plant-derived greenhouse and ozone-depleting gasses. Conversely, global change parameters such as elevated $CO_2$ and limited nitrogen have an impact on the composition of phyllosphere microbiota, at least through indirect effects on plant growth and metabolism. Such a network of see-saw relationships may thus be the basis for complex positive or negative feedback loops that could enhance or refrain global change processes.

## Novel Perspectives for Molecular, Physiological, and Ecological Studies of Phyllospheric Plant-Microorganism-Atmosphere Interactions

Current research is at the start of the characterization of terrestrial phyllosphere microbiota, and is likely to open new perspectives in microbial ecology, in plant ecophysiology, and in environmental sciences, that will go beyond the agronomical framework which, up to now, has been mostly taken into consideration. As is the case for numerous studies of microbial communities (Vandenkoornhuyse et al., 2010), phyllosphere metagenomics is greatly expanding this field of microbial ecology which covers a vast terrestrial compartment that was rather neglected.

Major issues of functional ecology related to phyllospheric microbial communities have already been identified and their potential importance requires a major effort of future research: (i) To what extent are epiphytic microorganisms involved in the chemical composition of the atmosphere through their potential action on gaseous molecules synthesized and emitted by plants? (ii) To what extent are epiphytic microorganisms able to act on toxic volatile products of anthropogenic origin? (iii) To what extent are phyllosphere microbiota interacting with microbe-associated molecular patterns triggering innate immunity in plants (Newman et al., 2013) and thus involved in the health and protection of plants? (iv) What is the impact of phyllospheric bacteria on gut microbial composition of herbivorous animals (Charrier et al., 2006; Hehemann et al., 2010; Thomas et al., 2011; Priya et al., 2012)?

Finally, in the context of land use and climate global changes (Peñuelas and Staudt, 2009), the complex dynamics between plants, phyllosphere microbiota, trace gasses, atmosphere microbiota and climate processes (**Figures 3–5**) urgently needs further investigation and, in particular, modeling analysis of potential regulatory feedback loops.

The dynamics of phyllosphere communities is also well-adapted as a model for studies in theoretical ecology concerning the origin of biodiversity, biotic interactions and community assemblage mechanisms (Finkel et al., 2012; Meyer and Leveau, 2012). It is thus bound to raise novel issues in evolutionary ecology, such as the co-evolutionary links between phyllosphere microbiota and host plants, and the possibility of symbiotic interactions in the phyllosphere, and especially on the phylloplane. However, the ongoing microbial colonization of plant surfaces and the ongoing sweeping of bacteria from plant surfaces (**Figure 5**) is likely to result in complex kinetics of plant–microorganisms interactions in the phyllosphere. Moreover, these interactions are likely to cover a wide range of affinities from loose associations to intimate symbioses. The complexity and the fluctuations of these interactions therefore entail that direct application of the holobiont concept (Zilber-Rosenberg and Rosenberg, 2008) to any kind of plant-phyllosphere system must be taken with caution, and that plant-phyllosphere systems should be better described by a fuzzy holobiont concept.

All of these novel and recent results and issues are the topics of active discussions and commentaries in the field of environmental microbiology. It is an effervescent field where further studies of phyllosphere microbiota are actively encouraged among environmental microbiologists by funding schemes at an international level. However, the importance of phyllosphere microbiota for plant functioning at physiological and ecological levels, the idiosyncracies of plant molecular mechanisms, and the complex regulatory loops between plants, microorganisms and the atmosphere advocate for the intensification of collaborations between plant scientists, biogeochemists and microbiologists.

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## Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00486/abstract

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