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MINI-REVIEW

Methylotrophs in natural habitats: current insights through metagenomics

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Abstract The focus of this review is on the recent data from the omics approaches, measuring the presence of methylotrophs in natural environments. Both Bacteria and Archaea are considered. The data are discussed in the context of the current knowledge on the biochemistry of methylotrophy and the physiology of cultivated methylotrophs. One major issue discussed is the recent metagenomic data pointing toward the activity of "aerobic" methanotrophs, such as *Methylobacter*, in microoxic or hypoxic conditions. A related issue of the metabolic distinction between aerobic and "anaerobic" methylotrophy is addressed in the light of the genomic and metagenomic data for respective organisms. The role of communities, as opposed to single-organism activities in environmental cycling of single-carbon compounds, such as methane, is also discussed. In addition, the emerging issue of the role of non-traditional methylotrophs in global metabolism of single-carbon compounds and the role of methylotrophy pathways in non-methylotrophs is briefly mentioned.

Keywords Methylotrophy · Methane oxidation · Metagenomics · *Methylobacter* · *Methylophilaceae* · Environmental detection

Introduction

Methylotrophy, the ability to utilize reduced carbon substrates containing no carbon-carbon bonds (C1 substrates) as their sources of carbon and energy is an example of a specialized mode of microbial metabolism (Anthony 1982; Chistoserdova and Lidstrom 2013). One of the important functions carried out by methylotrophs is methane oxidation. Methanotrophs act as a natural and very efficient filter capturing the majority (up to over 95 %) of methane produced via methanogenesis or non-biogenic methane (Kuivila et al. 1988; Le Mer and Roger 2011). This methanotroph-enabled filter also works, to a degree, in mitigating human-initiated disasters such as oil spills (Kessler et al. 2011; Crespo-Medina et al. 2014). The non-methanotrophic methylotrophs constitute important barriers for accumulation of other environmentally important C1 compounds, such as methanol, naturally generated by plants (Galbally and Kirstine 2002; Jacob et al. 2005), methylated amines generated in aquatic environments (Naqvi et al. 2005; Lidbury et al. 2015), and halogenated methanes, which occur naturally as well as through human activities (Muller et al. 2011). Methylotrophs are also active in transformation of methylated sulfur species, some of which, such as dimethylsulfide, are considered to have significant climate impact (Sunda et al 2002; Vallina and Simo 2007). The biotic impact on this process, however, has been recently debated (Quinn and Bates 2011). Methylotrophs are also involved in the global nitrogen cycle, different species being capable of nitrogen fixation, denitrification, or both (Chistoserdova and Lidstrom 2013). These are the well-recognized roles of methylotrophs in the environment. However, evidence is mounting that our understanding of the role of the methylotrophs may be somewhat simplistic and methylotrophy may be much more widespread in the bacterial world than previously assumed (Chistoserdova 2011a).

As a metabolic framework, methylotrophy has been known since the early twentieth century, when bacteria capable of growth on methane were first characterized (Kaserer 1906; Söhngen 1906). Since then, methylotrophic species have been

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characterized belonging to *Alpha-*, *Beta-*, and *Gammaproteobacteria*, *Actinobacteria*, *Firmicutes*, *Verrucomicrobia*, and a *Candidatus phylum* NC10 (Chistoserdova and Lidstrom 2013). Specific lineages within the Archaea (the ANME types) have also been shown to be active in methane oxidation (Knittel and Boetius 2009; Haroon et al. 2013; Offre et al. 2013). However, none of the anaerobic methylotrophs, bacterial or archaeal, have yet been obtained in pure cultures. So far, they are being studied as enrichment cultures.

As a field, methylotrophy has been formed and further defined by a number of landmark studies devoted to the biochemistry of specific enzymes and pathways involved in oxidation and assimilation of C1 substrates, using a handful of model organisms. The three enzymes for primary oxidation of C1 substrates that became the hallmarks of methylotrophy are the methane monooxygenase (MMO) of which two unrelated forms are known (particulate, pMMO and soluble, sMMO; Hakemian and Rosenzweig 2007), the two-subunit methanol dehydrogenase (MDH) encoded by *mxaFI* genes (Anthony 2004; Williams et al. 2005), and methylamine dehydrogenase (MADH), encoded by *mau* genes (Davidson 2003, 2004). These enzymes demanded years of research, culminating in a plethora of crystal structures and catalytic models. Thus, the methylotrophy capability in the environment has often been identified through the presence of these enzymes (Dumont and Murrell 2005; Neufeld et al. 2007; Lau et al. 2013). For C1 assimilation, three different pathways have been characterized, two specific to methylotrophy, the serine cycle and the ribulose monophosphate cycle, and the classic Calvin-Benson-Bassham (CBB) cycle for CO_2 assimilation (Anthony 1982; Chistoserdova and Lidstrom 2013).

Some of the details of the pathways responsible for methylotrophy have only recently been revealed, for example, the question of regeneration of glyoxylate from acetyl-CoA, the transformation required for the operation of the serine cycle in organisms lacking isocitrate lyase, required over 50 years of research (Anthony 2011), being finally resolved in 2007, by defining the reactions of the novel pathway, the ethylmalonyl-CoA (EMC) pathway (Erb et al. 2007; Chistoserdova et al. 2009). Likewise, the alternative pathway for methylamine oxidation, involving N-methylglutamate (NMG) as an intermediate, while biochemically identified decades ago, has only been described in genetic terms recently (Latypova et al. 2010; Chen et al. 2010).

A major metabolic challenge for methylotrophs is dealing with formaldehyde, the extremely toxic intermediate of many of the C1 oxidative pathways. One of the main and the most widespread pathways for formaldehyde oxidation was only discovered in 1998 (Chistoserdova et al. 1998), and this pathway, involving tetrahydromethanopterin (H_4MPT) and methanofuran as cofactors, caused an unexpected and a surprising turn of events, connecting the two opposing metabolic processes, methanogenesis and methylotrophy (Chistoserdova et al. 1998, 2003, 2004). Today, this connection appears less surprising, considering that in both processes, C1 units get transferred between different oxidation states. Moreover, close relatives of the methane-generating Archaea turned out to be the species that can carry out anaerobic methane oxidation (Knittel and Boetius 2009). Anaerobic methane oxidation has also been discovered in freshwater environments and has been attributed to a new phylum of bacteria, NC10, their metabolism also employing H_4MPT and methanofuran as cofactors for formaldehyde oxidation (Ettwig et al. 2010). These findings further reinforced the significance of H_4MPT/methanofuran as mediators of C1 transfers in omnipresent and environmentally important microbes, both Bacteria and Archaea. The role of these cofactors in taxa not known for methylotrophy or methanogenesis (Chistoserdova 2013) awaits further investigation.

Obviously, reliance on pure cultures has been limiting our understanding of C1 metabolism on a global scale. Comparisons of signature gene sequences from cultivated methylotrophs with sequences retrieved from environmental samples strongly suggested that the diversity of methylotrophs has been barely sampled by pure cultures, including the potential existence of major novel taxa capable of methylotrophy (Kalyuzhnaya et al. 2005a). Recent revelations about the environmental prevalence of the primary C1 oxidation enzymes/pathways are also changing the outlook on methylotrophy. While the MxaFI-type MDH is one of the most well-studied enzymes in the methylotrophy field (Anthony 2004), it now appears not to be the most widespread of the MDH enzymes. Instead, the enzyme that mystified the field for nearly two decades, designated as XoxF, has recently emerged as a more widespread MDH (Chistoserdova 2011a; Beck et al. 2013). The long-standing question of the activity of this enzyme (Chistoserdova and Lidstrom 1997) has been resolved, by identifying its reliance on lanthanides, as part of its catalytic centre (Hibi et al. 2011; Fitriyanto et al. 2011; Nakagawa et al. 2012; Pol et al. 2014). Likewise, the much-celebrated MADH (Davidson 2003) appears to be much less environmentally widespread, compared to the alternative NMG pathway (Latypova et al. 2010; Chen et al., 2010; Beck et al. 2014, 2015; Wischer et al. 2015). These recent findings set a stage for revisiting methylotrophy as likely a more encompassing and more widespread metabolic feature in the bacterial world than previously thought.

Methylotrophy is modular in its nature (Chistoserdova 2011a). This means that, to enable methylotrophy, a minimum set of the critical metabolic modules need to be present, as follows: (1) a primary oxidation module that generates energy from a C1 substrate; (2) a module for oxidation of the product of primary oxidation, which may be either formaldehyde or a formaldehyde adduct; and (3) a C1 assimilation module. Many species are known that contain multiple modules in

each category. The steadily growing databases of methylotroph genomes now provide strong support for the concept of modularity, by demonstrating that the number of configurations of essential methylotrophy metabolic modules is likely unlimited (Chistoserdova 2011a; Beck et al. 2014, 2015). This notion provides a solid framework for detecting methylotrophy through in silico (meta)genome analysis. Such predictions are important not only for organisms whose environmental functions remain unknown but also for organism with well-defined environmental functions that may also rely on or benefit from the methylotrophy capability.

Methylotrophs are ubiquitous in nature and found essentially in every environment that was tested, including extreme environments (Chistoserdova and Lidstrom 2013). However, in most environments, they are typically present at low relative abundances (Dedysh et al. 2001; Sauter et al. 2012; Beck et al. 2013). Thus, the knowledge on the types inhabiting specific habitats has relied for a long time on the cultivated representatives (Anthony 1982) or on detection of specific molecular markers, such as polymerase chain reaction (PCR)-amplified gene fragments (Kalyuzhnaya et al. 2004, 2005b; Dumont and Murrell 2005; Neufeld et al. 2007; Lau et al. 2013). However, both types of methods are prone to biases as not all species are readily cultivable, and not all species possess the markers that are targeted.

Metagenomics, also referred to as environmental genomics or ecogenomics, is a modern method for analyzing microbial communities in their natural habitats, omitting cultivation. While the term metagenomics typically refers to analysis of DNA molecules, other omics approaches target, respectively, the composition or environmental RNA molecules (metatranscriptomics), proteins (metaproteomics) or metabolites (metametabolomics) (Chistoserdova 2009, 2010; Kalyuzhnaya et al 2015a). The state of the art in the field now is application of a combination of different omics analyses, in order to increase robustness of the data. Accessing multiple omics datasets, at multiple sampling points, through the use of special computational pipelines forms the basis for the systems biology paradigm (see Fig. 1 for overview).

Omics and system approaches are now applied to biological systems at all levels of complexity, some of the most complex systems being microbial communities in soils and sediments (Castelle et al. 2013; Howe et al. 2014; Zhou et al. 2015). As, despite their well-documented role in biogeochemical cycles, methylotrophs in many environmental niches constitute only minor fractions of total populations, special metagenomics approaches have been traditionally applied to studying methylotrophs in semi-in situ conditions, such as "functional" metagenomics combining stable isotope probing (SIP) with high-throughput sequencing. This approach involves feeding the natural populations a substrate of interest, labeled by a heavy isotope (e.g., ^{13}C), followed by characterization of the heavy fraction of communal DNA that is enriched in DNA (DNA-SIP). RNA (RNA-SIP) and proteins (protein-SIP) of microbes that actively metabolize the labeled substrate could also be labeled. The SIP technique has been effective in identifying methylotrophs in a variety of environmental niches. Labeled DNA (RNA) can either be analyzed for the presence of specific phylogenetic (16S rRNA gene) or functional gene markers, or total DNA (RNA) could be sequenced (Kalyuzhnaya et al. 2015). The latter method is referred to as whole genome shotgun (WGS) sequencing. Below, results from both types of metagenomics and metatranscriptomics approaches will be mentioned. First, studies of methylotroph communities in Lake Washington, a long-term model system, will be summarized, with a focus on genomic and metagenomic approaches. Next, results will be highlighted from application of the omics approaches for detection of methylotrophs in other major types of natural habitats.

Methylotrophs in lake sediments

Pre-genomic studies

Freshwater lakes are environments characterized by dynamic cycling of methane, serving both as its major sources and major sinks. Lake Washington in Seattle, USA, has served as a model for studying methylotrophy for a number of years (Chistoserdova 2011b; Chistoserdova et al. 2013). Methane oxidation takes place in the top layer of the sediment, in which methane and oxygen form steep counter gradients (Kuivila et al 1988; Auman et al. 2000). Early cultivation-based experiments readily produced pure cultures of methanotrophs, and these were separated into two groups, Gammaproteobacteria of the family *Methylococcaceae* (type I methanotrophs) and Alphaproteobacteria of the family *Methylocystaceae* (type II methanotrophs; Auman et al. 2000). PCR amplification of 16S rRNA genes and of functional genes involved in methane oxidation (*pmoA*, *mmoX*) and nitrogen fixation (*nifH*) from the same environment confirmed their presence, identifying the *Methylomonas* species as the dominant methanotroph type (Auman et al. 2001; Auman and Lidstrom 2002).

Later application of environmental tools for a broader detection of methylotrophy potential in the same study site, specifically PCR amplification of genes for the H_4MPT-linked formaldehyde oxidation functions, has uncovered the presence not only of the two types of methanotrophs but also of a variety of species without an obvious role in methane oxidation such as *Methylophilaceae*, *Methylobacterium*, *Xanthobacter*, and *Hyphomicrobium* (Kalyuzhnaya et al. 2004, 2005b). These findings were further supported by the analysis of rRNA and mRNA molecules isolated directly from the sediment and by SIP with a variety of C1 substrates, followed by PCR amplification and sequencing of select

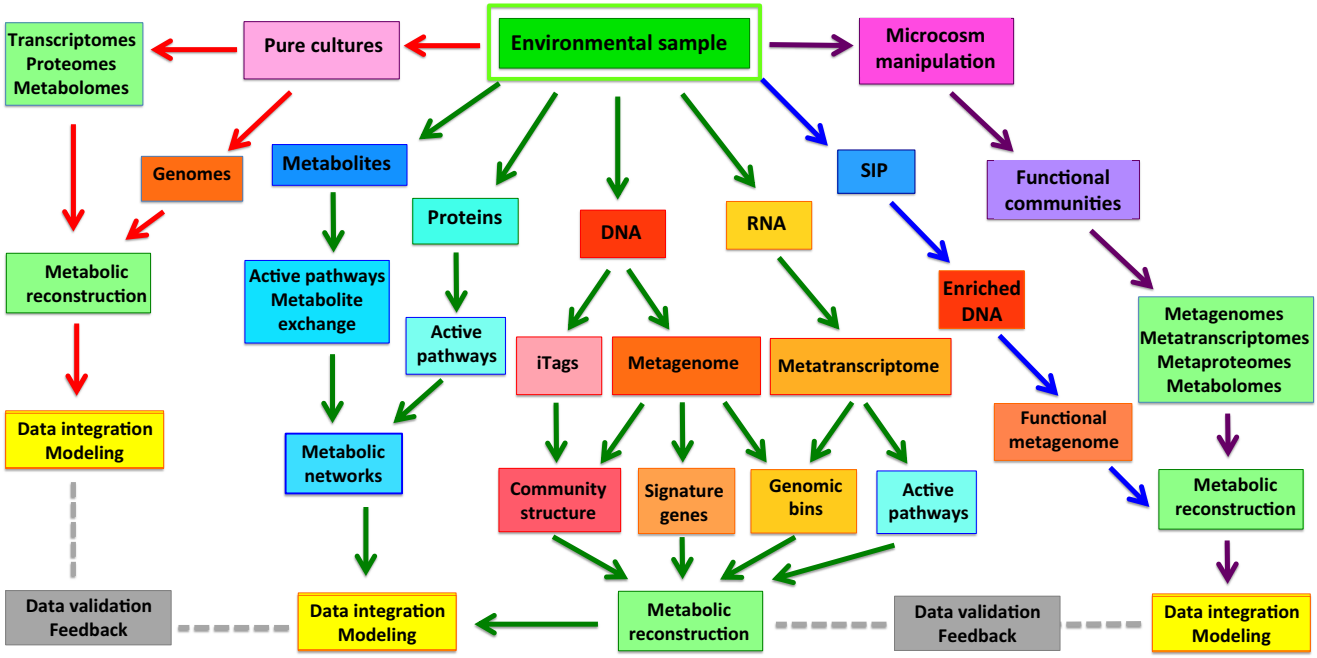

Fig. 1 A schematic overview of omics approaches to studying methylotrophy in natural environments. Due to low abundance of methylotrophs in most environments, data from direct metagenomic sequencing, proteomics, or metabolomics (*green arrows*) may be fragmented. Thus, omics experiments with relevant pure cultures (*orange arrows*), sequencing of functionally enriched molecules (*blue arrows*), or manipulation of microcosms (*purple arrows*) are helpful in generating the accurate systems-level knowledge (*dashed gray lines*)

genes (Nercessian et al. 2005). The data on the presence and activity of a variety of known methylotrophs, most prominently members of the *Methylophilaceae* family, suggested that either C1 substrates other than methane were supporting methylotroph communities, or that methane oxidation to CO_2, instead of being carried out by a single type of bacteria (methanotrophs), may be carried out by bacterial consortia. However, this phase of the community analysis has been limited to detection of single signature genes, as few lake cultures were in hand, and no genomic data were available for any methylotrophs.

Functional metagenomics of C1 metabolism

The first complete genomic sequence of a methanotroph became available in 2004 (Ward et al. 2004), coinciding with the onset of WGS sequencing of environmental DNA (Tyson et al. 2004; Venter et al. 2004). This was the approach utilized for obtaining further insights into the methylotroph communities in Lake Washington sediment. In order to generate high-resolution genomic data for key methylotrophs, the power of metagenomics was combined with functional enrichment by the way of SIP (Kalyuzhnaya et al. 2008a; also see Kalyuzhnaya et al 2015a for method details). Sediment samples from Lake Washington were exposed separately to ^{13}C-labeled methane, methanol, methylamine, formaldehyde, and formate, to target populations actively utilizing each of these representative C1 compounds. Total DNA was extracted from each microcosm, and the ^{13}C-labeled fractions were separated from unlabeled DNA by isopycnic centrifugation. Shotgun libraries were generated from these DNA samples, and these were sequenced. Analysis of taxonomic and functional gene signatures in the respective metagenomes revealed shifts toward specific functional guilds in each functional community, highlighting roles of specific guilds in utilization of specific single-carbon compounds (Kalyuzhnaya et al. 2008a). From these analyses, the methylamine microcosm was one of the least complex in terms of species richness, dominated by a group of closely related strains identified as representatives of the genus *Methylotenera*, at that time a novel and poorly studied guild of bacteria. High coverage of DNA representing these species allowed extraction of a composite (i.e., representing a few closely related strains) genome of *Methylotenera* from the metagenomic sequence, followed by detailed reconstruction of their metabolism, including genome-wide comparisons with close relatives. Somewhat surprisingly, the *Methylotenera* species were also highly enriched in microcosms fed methane, while not having any genes for methane oxidation (Kalyuzhnaya et al. 2008a).

The other highly-covered genome we were able to retrieve from the metagenomic dataset was the (composite) genome of a *Methylobacter* species closely related to a described species *Methylobacter tundripaludum* (Wartiainen et al. 2006), mostly from the methane microcosm dataset (Kalyuzhnaya et al. 2008a). This suggested that uncultivated *Methylobacter*-type methanotrophs and not the previously

cultivated methanotroph species (Auman et al. 2000) were the main active methanotroph species. While less covered than the *Methylotenera* composite genome, the *Methylobacter* genomic sequence bin still revealed detailed information for a high-level metabolic reconstruction (Kalyuzhnaya et al. 2008a).

While, through the metagenomic approach, two major species have been identified in the methane microcosm, *Methylobacter* and *Methylotenera*, their respective roles remained uncertain. The *Methylobacter* species were obviously equipped for methane utilization. However, the *Methylotenera* species lacked genes for methane oxidation (Kalyuzhnaya et al. 2008a; Lapidus et al. 2011). Thus, the role of *Methylotenera* in methane metabolism remained enigmatic.

Functional metagenomics were further applied in order to test for which organisms were active in methane metabolism in hypoxic conditions and whether methane oxidation could be linked to denitrification. Lake Washington sediment samples were incubated with ^{13}C-labeled methane in the presence of ambient air atmosphere or in hypoxic conditions, and nitrate was added to select samples (Beck et al. 2013). DNA sequencing revealed that, again, the most active species were the *Methylococcaceae* with the major type being *Methylobacter* species, and the *Methylophilaceae* with the major type being *Methylotenera* species. The dominance of these species in the metagenomes and their coordinated responses to both methane and nitrate stimuli suggested that the *Methylococcaceae* and the *Methylophilaceae* species might engage in cooperative behavior (Beck et al 2013).

Recent insights from cultivation approaches

As culture-independent approaches hinted at the unsuspected diversity of known methylotrophs in lake sediments, activities on pure culture isolation were renewed, expanding the range of substrates and conditions and focusing on the most active groups, *Methylobacter* and *Methylophilaceae*. The *Methylophilaceae* types were successfully enriched on either methanol or methylamine, and multiple pure cultures were isolated (Beck et al. 2014; McTaggart et al. 2015a). While highly enriched cultures of *Methylobacter* were easy to establish at semi-in situ temperatures (Oshkin et al. 2015), pure cultures were extremely difficult to obtain. Most of the cultures remained mixed even after months of incubation and after multiple transfers with dilutions. Analysis of the satellite types in select microcosms revealed the narrow and overlapping range of bacteria, suggesting that these types were non-random, and likely they were selected for under specific environmental pressures. The most persistent satellite taxa included *Methylotenera*, *Flavobacterium*, *Pseudomonas*, *Janthinobacterium*, and *Achromobacter*. *Methylophilus* sequences were also detected in some of the microcosms. Notably, in these microcosms, very few sequences belonged to other *Methylococcaceae* or to *Methylocystaceae*, including the previously cultivated species (Oshkin et al. 2015). So far, only two pure *Methylobacter* cultures resulted from these experiments (Kalyuzhnaya et al. 2015b). The two cultures have identical 16S rRNA genes that are also highly similar to the sequences uncovered through metagenomics (Kalyuzhnaya et al. 2008a; Beck et al. 2013).

A variety of other methylotroph types were cultivated on methanol or methylamine, and these were classified within *Alphaproteobacteria* and within *Actinobacteria* and *Firmicutes* (Beck et al. 2015; McTaggart et al. 2015b). Genomes of more than 50 representative methylotrophs from Lake Washington have been now sequenced (Lapidus et al. 2011; Kittichotirat et al. 2011; Beck et al. 2014, 2015; Kalyuzhnaya et al. 2015b; McTaggart et al. 2015a, b). However, of these, only the *Methylococcaceae* and the *Methylophilaceae* genomes have been matched to the metagenomic sequences or detected in microcosm communities (Oshkin et al 2015; Hernandez et al 2015; see below).

The multiple *Methylophilaceae* isolates were sorted into specific ecotypes (a total of nine), based on a combination of the information on their sequences and their phenotypes (Beck et al 2014; McTaggart et al 2015a). Not only some of these ecotypes displayed significant genome-genome divergence, but they also differed in terms of core methylotrophy functions, such as the presence of genes for the traditional methanol dehydrogenase (*mxaFI*) or the presence of genes for methylamine utilization. The organisms classified into different ecotypes also differed in terms of the denitrification potential, from encoding no respiratory nitrate reduction functions to encoding partial or complete respiratory nitrate reduction pathways (Beck et al. 2014; McTaggart et al. 2015a). Overall, such divergence in terms of the methylotropy and nitrogen metabolism metabolic modules suggests that specific metabolic schemes may correspond to specific micro-niches within the sediment.

Insights from microcosm metagenomics

To gain further insights into the populations active in methane metabolism, a microcosm approach has been implemented. Dynamics of bacterial populations were followed under the selective pressure of methane and under varying conditions of oxygen availability (Oshkin et al. 2015). In the microcosms fed methane as a single source of carbon, with weekly transfers with dilutions, a very rapid loss of complexity was observed over the course of a few weeks. In these microcosms, again, the previously observed species were detected, the *Methylobacter* and the *Methylophilaceae* being the most relatively abundant operational taxonomic units (OTUs). Of the OTUs representing non-methylotrophic heterotrophs, the most relatively abundant belonged to *Flavobacterium* species. In these experiments, a clear separation was observed in the

types of *Methylophilaceae* prevailing under "low" oxygen versus "high" oxygen conditions (Oshkin et al. 2015), hinting again at specific niche adaptations for different *Methylophilaceae* ecotypes.

Metagenomic sequencing has been carried out for select microcosms, in order to evaluate the metabolic potential of the dominant species, through matching metagenomic sequence reads to the proxy genomes of the closest cultivated relatives. Through these analyses, methylotrophy blueprints for the dominant *Methylobacter* species were reconstructed, and these matched closely to the ones of the cultivated *Methylobacter* species (Kalyuzhnaya et al. 2015b). For the OTUs representing *Methylophilaceae* ecotypes, the *Methylophilus* types in high oxygen microcosms were metabolically close to the cultivated *Methylophilus* ecotype White (Beck et al. 2014), while two dominant *Methylotenera* types in low oxygen microcosms were most closely related to, respectively, ecotypes 301 and 13 (Beck et al. 2014). The metabolic distinctions between these two ecotypes, as revealed through metagenomics, were the absence of the *mxaFI* genes in the former and the presence of the respiratory denitrification genes in the latter.

The microcosm approach was further expanded by employing a gradient of oxygen tensions, mimicking the situation in the native lake sediment (Hernandez et al. 2015). By analyzing the composition of the communities established after a number of weeks under methane, through sequencing rRNA gene amplicons, it was demonstrated that, in support of prior observations, the methane-consuming communities were dominated by two major types, the *Methylococcaceae* and the *Methylophilaceae*. However, different species persisted under different oxygen tensions. At high initial oxygen tensions (corresponding to the upper part of the oxygen gradient in the sediment), the major players were, respectively, species of the genera *Methylosarcina* and *Methylophilus*, while at low initial oxygen tensions (lower part of the oxygen gradient), the major players were *Methylobacter* and *Methylotenera*. These data suggest that oxygen availability is at least one major factor determining specific partnerships in methane oxidation. The data also suggest that speciation within *Methylococcaceae* and *Methylophilaceae* may be driven by niche adaptation tailored toward specific placements within the oxygen gradient.

Comparative analysis of the genomes of cultivated representatives most closely related to the native populations has revealed conspicuous differences in the nitrogen metabolism potential. The *Methylosarcina* genome only encoded functions for nitrate conversion into ammonium (assimilatory denitrification; Kalyuzhnaya et al. 2015b). On the contrary, the *Methylobacter* genomes encoded, in addition, respiratory nitrate and nitrite reductases. The *Methylobacter* genomes also contained genes predicted to encode functions essential to dinitrogen fixation, including the subunits of the Rnf complex (Kalyuzhnaya et al. 2015b). While at this moment, the potential role of dinitrogen fixation in the fitness of *Methylobacter* is not obvious, its ability to denitrify presents a mechanism by which it may be able to outcompete *Methylosarcina* during hypoxia. Methane oxidation coupled to nitrate reduction has been recently demonstrated during hypoxia in a related methanotroph, *Methylomonas denitrificans* (Kits et al. 2015).

Likewise, while the *Methylophilus* genomes only encoded assimilatory denitrification reactions, the *Methylotenera* genomes varied in terms of their denitrification potential, from assimilatory to partial respiratory to complete respiratory (Beck et al. 2014; McTaggart et al. 2015a). The denitrification capability has been experimentally demonstrated so far in one *Methylotenera* species (Mustakhimov et al. 2013).

It is tempting to speculate that nitrogen metabolism functions, and especially the denitrification capability, confer competitive advantage at low oxygen to both *Methylobacter* and *Methylotenera*. It is also possible that these organisms may exchange nitrogen species such as nitrite, nitric oxide, and/or nitrous oxide. However, as yet we do not have information regarding how a methanotroph can provide carbon to a community of non-methanotrophs and as to what advantage the methanotroph may be gaining from the satellite community. The question whether nitrogen metabolism plays a role in shaping methane-oxidizing communities also awaits further investigation.

Overall, data form the experiments described above question the "strictly" aerobic nature of the gammaproteobacterial methanotrophs. Their propensity to cope well with hypoxic situations has been previously reported (Roslev and King 1994, 1995). While the ability to denitrify presents one mechanism for coping with hypoxia (Kits et al. 2015), active *Methylococcaceae* have also been detected in anoxic environments devoid of nitrate. In this case, methane oxidation was proposed to be linked to oxygenic photosynthesis (Milucka et al. 2015).

"Anaerobic" methane oxidation in freshwater environments

Some freshwater environments are characterized by high concentrations of nitrogen species such as nitrate, mostly as a result of agricultural runoff. It has been proposed that in anoxic sediments in such environments, methane oxidation could be linked to denitrification, akin to sulfate reduction-linked methane oxidation by ANME-type Archaea. Indeed, enrichment cultures active in both methane oxidation and nitrate reduction could be established (Raghoebarsing et al. 2006). Initially, it was thought that methane oxidation was carried out by the ANME-type Archaea, while the concomitant nitrate reduction was carried out by a novel type of bacteria belonging to NC10 candidate phylum (Raghoebarsing et al. 2006). Later experiments with enrichment cultures

revealed that NC10 bacteria alone could carry out both nitrate reduction and methane oxidation, as in these enrichments, the population of the ANME Archaea was significantly diminished (Ettwig et al. 2010). Important insights into the metabolic make up of the NC10 methane-oxidizing bacteria, named *Methylomirabilis oxifera*, were gained via metagenomic sequencing of the highly enriched microcosm community (*M. oxifera* constituting approximately 80 % of total population). A single-scaffold sequence was generated for the genome of *M. oxifera*, revealing the presence of the complete set of genes for methane oxidation, very similar to the set utilized by aerobic methanotrophs. Carbon assimilation was predicted to be autotrophic, using the traditional CBB cycle, akin to verrucomicrobial aerobic methanotrophs (Rasigraf et al. 2014). In terms of nitrate reduction, a nearly complete pathway has been uncovered, with the predicted product being nitrous oxide, as is typical of many denitrifying microbes (Orellana et al. 2014). Interestingly, the original methane oxidizer suspect of the ANME-type Archaea was also later identified as a bona fide nitrate-dependent methane oxidizer (Haroon et al. 2013). Insights into the biochemistry of this process were also obtained via metagenomics of highly enriched cultures. In this case, the pathway for methane oxidation was the reversed methanogenesis, as is typical of Archaea. However, the encoded nitrate reductase was of a bacterial type, and the respective genes were proposed to be a result of horizontal transfer (Haroon et al. 2013).

Identification of the novel guild of methanotrophs, the NC10 type, has prompted revisiting methane-oxidizing populations in some well-studied freshwater environments. In Lake Washington samples described above, NC10 types were detected at very low abundance (Beck et al. 2013). However, all these samples originated from oxic to semi-oxic sediment layers. In Lake Constance, a stratified lake with high input of nitrate, the populations of NC10 bacteria were recently measured via culture-independent approaches, concluding that, while in shallow water sediments aerobic methane oxidation was prevalent, in the anoxic profundal sediments, NC10 type bacteria were abundant, and their presence was co-located with active methane and nitrate consumption. However, in these anoxic niches, along with NC10 bacteria, the traditional "aerobic" methanotrophs were also detected (Deutzmann et al. 2014).

Methylotrophs in freshwater columns

Few metagenomic studies addressed pelagic freshwater methylotrophs. However, recently, Salcher et al. (2015) enumerated pelagic *Methylophilaceae* in Lake Zurich using flow cytometry and CARD-FISH, targeting two previously detected specific groups, LD28 and PRD01a001B. High-resolution sampling across water column and over multiple years has revealed high abundance of the LD28 group (up to 4 % of total cell counts), with pronounced peaks in spring and autumn-winter, coinciding with peaks in primary productivity. The LD28 cells were especially abundant in the cold hypolimnion samples. The PRD01a001B group was generally less abundant, showing a single peak over 4 years (Salcher et al. 2015). The genomes of species representing these groups of *Methylophilaceae* were sequenced by the same group. Remarkably, these were unusually small genomes, similarly to the genomes of the marine pelagic *Methylophilaceae* (Giovanoni et al. 2008; Huggett et al. 2012; see below), rather than to the genomes of the *Methylophilaceae* from lake sediments (Beck et al. 2014; McTaggart et al. 2015a). Like marine *Methylophilaceae*, the freshwater pelagic *Methylophilaceae* only encoded XoxF enzymes of the recognized primary oxidation methylotrophy modules. This study further concludes on the common evolutionary origin of the freshwater and marine pelagic methylotrophs, their genomes being shaped by significant genome reduction through gene loss and novel gene (such as rhodopsin genes) acquisition through horizontal transfers (Salcher et al. 2015). The abundance of the pelagic *Methylophilaceae* in this study negatively correlated with nitrate concentrations, further suggesting metabolic differences between pelagic and sedimental types.

Methanotrophs are also found in lake water columns, through a variety of techniques, including PCR amplification of the marker genes. In a subtropical water reservoir, both type I and type II proteobacterial methanotrophs were identified, along with representatives of the NC10 phylum. The methanotroph sequences were generally more abundant in the oxygen-depleted zone, compared to the oxygenated zone (Kojima et al. 2014). In a deep south-Alpine Lake Lugano, sequences of *Methylobacter*-type methanotrophs were detected in the anoxic but not oxic zones, coinciding with the peak in methane-oxidizing potential and suggesting that aerobic methanotrophs are most abundant and most active in the water column below the oxic-anoxic interface (Blees et al. 2014). In the well-studied lake Pavin, *Methylobacter*-type methanotrophs were also detected in anoxic layers (Biderre-Petit et al. 2011). These data strongly point toward aerobic methanotrophs, especially *Methylobacter* species, being active in hypoxic conditions, further challenging the long-term dogma of "strictly" aerobic nature of these organisms (Kalyuzhnaya et al 2013).

What is the metabolic distinction between aerobic and anaerobic methane toxidation?

The data available from genomic and metagenomic datasets for methane-oxidizing Bacteria and Archaea, in conjunction with respective metabolic activities/phenotypes now allow for addressing the metabolic distinctions between the two processes. Of all the metabolic guilds capable of methane

oxidation, only proteobacterial and verrucomicrobial methanotrophs are available in pure cultures (Chistoserdova and Lidstrom 2013). Pure cultures of methanotrophs are known to thrive at high oxygen when cultivated in laboratory. However, this type of cultivation is very artificial, as in most natural environments methanotrophs rarely face high oxygen where methane is present. Thus, by nature, most methanotrophs must be adapted to life in oxygen-limited conditions. Obviously, methane-oxidizing performance, including doubling times seen in laboratory cultures, cannot be expected from methanotrophs inhabiting natural environments. The other major difference between natural communities and laboratory cultures is the cooperative nature of metabolism. While labeled dinitrogen is measured in NC10-dominated microcosms fed ^{15}N-nitrite, it is possible that not only NC10 bacteria but also other organisms in the enrichment culture contributed to this activity. On the contrary, in experiments with pure cultures of *M. denitrificans*, also lacking genes for nitrous oxide reduction, evolution of nitrous oxide could be seen, in agreement with predictions from the genomic sequence (Kits et al. 2015).

From the available genomic and metagenomic data, we can now delineate the encoded pathways for methane oxidation and denitrification in the known methane-oxidizing species. From these data, the metabolic make up of the archaeal methane oxidizers, whether nitrate-dependent or sulfate-dependent, is clearly distinct from the metabolic schemes of the bacterial methanotrophs. The former utilize the reverse methanogenesis pathway (Knittel and Boetius 2009, Offre et al. 2013; Haroon et al. 2013). However, both aerobic and anaerobic bacterial methanotrophs encode very similar pathways for methane oxidation as well as for nitrate reduction (Ettwig et al. 2010; Kits et al. 2015; Kalyuzhnaya et al. 2015b). This begs a question: are the two modes for bacterial methane oxidation really different? At the genomic/metagenomic level, we have to conclude that they must use similar strategies. At the physiological level, they also seem to be very similar, thriving in low oxygen environments. The main difference so far is the oxygen sensitivity of the NC10 type organisms (Luesken et al. 2012), compared to high oxygen tolerance of (cultivated) proteobacterial methanotrophs (Chistoserdova and Lidstrom 2013).

While a novel mechanism has been proposed for oxygen acquisition by the NC10 microbes in conditions of limited oxygen, by the means of a hypothetical NO dismutase, this enzyme has never been demonstrated experimentally. Meantime, Blees et al. (2014) presented a compelling evidence that methane oxidation must be operating in the aerobic mode even when oxygen concentrations are below detection limit of common sensing devices. In this case, nitrate or nitrite concentrations were deemed too low to account for the observed methane turnover (Blees et al. 2014). Overall, the evidence for the aerobic methane oxidizers activity in the anaerobic environments points toward a significant gap in our understanding of in vivo methane oxidation.

Methylotrophy in soils

Methylotrophs in arctic soils

Wetlands are known as significant emitters of methane (Conrad 2009). Of special concern is the predicted future thaw of the permafrost, potentially releasing large amounts of the trapped methane into the atmosphere (Stolaroff et al. 2012). Therefore, information on microbial communities involved in production and consumption of methane in these environments is crucial. $^{13}CH_4$-SIP experiments with High Arctic soils in Norway have identified *Methylobacter* species as the most active methane oxidizers in these environments (Graef et al. 2011). *M. tundripaludum* was previously isolated from the same sampling site (Wartiainen et al. 2006). Notably, in these experiments, communities from the anoxic and from the oxygen transition layers of soil displayed higher activity in methane oxidation, including oxidation of atmospheric methane, compared to the oxic layer (Graef et al. 2011). *M. tundripaludum* sequences were further identified as most abundant methanotroph sequences in active layers of Norway arctic soils, via metagenomic and metatranscriptomic approaches (Tveit et al. 2013, 2014). In these studies, *M. tundripaludum* sequences were once again detected in both oxic and anoxic soil layers. However, the abundance of transcripts assigned to *M. tundripaludum* was the highest in anoxic layers, and the most highly transcribed genes were the pMMO genes. While both alphaproteobacterial methanotrophs and methanotrophs of the NC10 phylum were detectable in these sites, they were observed at much lower relative abundances (Tveit et al. 2013, 2014). The *M. tundripaludum* species identified in arctic soils were remarkably similar to the sequences identified in Lake Washington samples, and the methylotrophy and nitrogen metabolism pathways reconstructed from respective genomes and metagenomes (Kalyuzhnaya et al. 2008a; Svenning et al. 2011; Oshkin et al. 2015; Kalyuzhnaya et al. 2015b) were essentially identical, pointing to similar physiological strategies for survival and activity in hypoxic conditions for this species.

In another study, a metagenomic approach has been applied to test for the response of native permafrost microbial communities to thaw (Mackelprang et al. 2011). Communities of permafrost samples from the Alaska region were investigated before and after thaw in the laboratory, noting shifts in microbial communities in response. The methane trapped in the frozen soil was quickly consumed by aerobic methanotrophs. Remarkably, incubations in this study were carried out under helium headspace. Sequences of both gammaproteobacterial (type I) and alphaproteobacterial (type II) methanotrophs were

identified in the metagenomes. However, this study concluded that the latter (*Methylocystaceae*), rather than the former (*Methylococcaceae*) were active in response to thaw (Mackelprang et al. 2011). These results differ from the results obtained for the Norway permafrost samples described above and from earlier studies in which Canadian permafrost samples were investigated. Yergeau et al. (2010), using metagenomics, PCR, and microarrays, have compared communities in a permanently frozen layer (dating to 5000 years ago) to the communities in the active layer, finding them similar and finding the methanotroph population to be dominated by type I methanotrophs. The same group carried out methane-SIP experiments with the active layer sample, again finding type I methanotrophs (*Methylobacter* and *Methylosarcina* types) to be active in incorporating the label (Martineau et al. 2010). Moreover, among the labeled species, representatives of *Methylophilaceae* were also identified in this study.

Permafrost thawing leads to the formation of thermokarst shallow ponds that become stratified during summer months. A number of such ponds have been recently examined in northern Québec, Canada, for the active communities, using 16S rRNA transcript profiling (Crevecoeur et al. 2015). This study found type I methanotrophs, predominantly *Methylobacter*, in all pond samples, both in the oxygenated surface waters and in hypoxic bottom waters, their relative abundance reaching up to 25 % of total sequences (the highest abundance was measured in the sample with the lowest dissolved oxygen). *Methylotenera* sequences were found across all samples in this study. Interestingly, sequences belonging to *Methylacidifilales*, the verrucomicrobial methanotrophs, typically found in geothermal and acidic environments (Sharp et al. 2014), were also identified in some pond samples (Crevecoeur et al. 2015). The discrepancies on the abundances and the activity of type I, type II, and other types of methanotrophs in the permafrost soils may reflect the differences in environmental conditions in specific locations and obviate the need for a broader sampling.

Methylotrophs in the rhizosphere

Rice paddies generate large amounts of methane. However, much of it is oxidized by the methanotrophs within the same environmental niche (Conrad 2009). Typically, both type I and type II methanotrophs are detected via PCR. However, transcript analysis, along with methane-oxidizing potential measurements, indicated that type I methanotrophs are likely responsible for much of the activity, at least in some of the sampled sites (Reim et al. 2012; Ma et al. 2013). A metaproteogenomic approach has also been applied to rice rhizosphere environments (Knief et al. 2012). High complexity was uncovered for the communities inhabiting these environments. While most of the proteins identified were assigned to methanogenic Archaea, some of the relatively abundant proteins were of bacterial origin. Of the most relatively abundant bacterial proteins, proteins for aerobic methane oxidation were identified, including both types of MMO enzymes, soluble and particular. These were assigned to both type I and type II methanotrophs (Knief et al. 2012). In addition to the methanotroph sequences and proteins, sequences and proteins of *Methylobacterium* were detected in rice rizhosphere samples (Knief et al. 2012), and these were also relatively abundant in the rhizosphere of *Arabidopsis*, along with members of *Hyphomicrobiaceae* (Lundberg et al. 2012). Other methylotrophs detected in the rhizosphere through metaproteogenomics were *Methylotenera* species and members of *Burkholderiales*, expressing either the MxaFI-type or the XoxF-type MDH (Knief et al. 2012). These results further point toward the presence and the methylotrophic activity of these groups in environments experiencing hypoxic conditions.

Methylotrophs in the phyllosphere

Metaproteogenomics have been also applied to investigate methylotroph presence and abundance in the phyllospere of various plants (Delmotte et al. 2009; Knief et al. 2012). A general trend was identified, for less complex communities to inhabit the phyllosphere, compared to the rhizosphere, consistent with limited range of nutrients available in this environmental niche. However, some organisms were found to be thriving in the phyllosphere, most prominently the *Methylobacterium* species (up to 20 % of total phyllosphere community; Delmotte et al. 2009). While well equipped for multicarbon metabolism (Marx et al. 2012), the *Methylobacterium* species were shown to express proteins specific to the methylotrophic mode of life, including both types of the MDH enzymes, MxaFI-type and XoxF-type, the enzymes involved in the H_4MPT-linked formaldehyde oxidation, and the serine cycle enzymes (Delmotte et al. 2009). In addition, methylotrophy enzymes from Gram-positive organisms (*Amycolatopsis*) were detected, at somewhat lower abundance (Knief et al. 2012). Metaproteogenomics data argued against the methanotroph species being part of the microbial community potentially involved in atmospheric methane oxidation in the phyllosphere, as few DNA sequences or proteins for those organisms were detected in natural samples (Knief et al. 2013).

Methylotrophy in marine environments

Anaerobic oxidation of methane by ANME-type archaea

Anaerobic methane-oxidizing Archaea (ANME) play an important role in methane cycling in anoxic marine environments, and in these, they are considered to be the major filter preventing escape of methane to the atmosphere (Knittel and Boetius 2009). In specific environments, such as methane

seeps or mud volcanoes, their abundance can be very high, and they can form massive microbial mats (Michaelis et al. 2002; Lösekann et al. 2007) or be parts of gigantic carbonate chimneys (Brazelton et al. 2006). Typically, ANME organisms are found in association with sulfate-reducing bacteria, and in these cases, methane oxidation is likely coupled to sulfate reduction, through a yet unknown mechanism (Knittel and Boetius 2009). A recent study compared microbial community structures among 23 globally dispersed methane seeps, through pyrotag library analysis, concluding that all of these sites are characterized by the presence of key functional types, of which ANME methanotrophs and sulfate-reducing bacteria are major functional types, along with the aerobic methanotrophs (Ruff et al. 2015). The authors also conclude that few cosmopolitan microbial taxa mediate the bulk of methane oxidation. However, in each niche, specific, highly endemic populations appear to be present, likely selected for by local environmental factors (Ruff et al. 2015). Alternative electron acceptors for anaerobic methane oxidation have also been proposed. These include iron and manganese (Beal et al. 2009). Moreover, a mechanism has been recently proposed by which ANME organisms can couple methane oxidation to abiotic sulfate reduction (Milucka et al. 2012).

ANME organisms have not been isolated in culture, and their environmental detection and enumeration depend on a variety of culture-independent approaches (Knittel and Boetius 2009; Offre et al. 2013), while insights into their metabolism are only possible through metagenomic analysis. So far, all of the anaerobic archaeal methane oxidizers are classified within *Methanosarcinales* (*Euryarchaeota*), and these are separated into three distinct lineages, ANME-1, ANME-2, and ANME-3 (Offre et al. 2013). Metabolic reconstruction from assembled metagenomic data points toward reverse methanogenesis being the pathway for methane oxidation (Hallam et al. 2004). This conclusion is further supported by the relatively high abundance of proteins identified as subunits of methyl-CoM reductase (MCR) in a microbial mat dominated by ANME Archaea, supporting a role for this enzyme in methane oxidation (Krüger et al. 2003). Moreover, reverse reaction activity for MCR from a methanogen has been experimentally demonstrated (Scheller et al. 2010). A MCR homolog has been purified and crystallized from ANME-1-enriched microbial mat, revealing striking structural similarities between the MCR enzymes involved in methane oxidation and in methane generation (Shima et al. 2011). Overall, the data available point toward high abundance but relatively low diversity of archaeal methane oxidizers in anoxic marine environments and at their major role in methane cycling.

Methylotrophic bacteria in marine environments

In ocean waters, methanotrophs are typically present at very low abundances, constituting minor fractions of total microbial communities. Nevertheless, they are detectable via PCR amplification (Sauter et al. 2011). However, methanotroph populations may experience blooms in response to increased substrate supply, such as the unfortunate events of natural gas spills exemplified by the Deepwater Horizon disaster of 2010 (Rivers et al. 2013; Crespo-medina et al. 2014). The microbial blooms in this case were dominated by *Gammaproteobacteria*, with a significant proportion of *Methylococcaceae*, and their abundances were positively correlated with concentrations of hydrocarbons and negatively with the dissolved oxygen concentrations (Rivers et al. 2013). A significant number of transcripts, including the ones of pMMO, were assigned to *Methylobacter* and to other *Methylococcaceae* (Rivers et al. 2013). As part of these methane-induced blooms, non-methanotrophic methylotroph populations have also been identified, represented by *Methylopaga* and *Methylophilaceae* species (Kessler et al. 2011; Rivers et al. 2013; Crespo-Medina et al. 2014). *Flavobacteriaceae* DNA and transcripts were also abundant in these samples, such a response bearing striking similarities to the responses seen with lake sediment methane-utilizing communities, as described above.

Under natural conditions, the *Methylophaga* species are found at very low abundances. However, they are detectable through methanol-SIP (Neufeld et al. 2007, 2008). The metabolic potential of *Methylophaga* has been also connected to the degradation of high molecular weight dissolved organic carbon (DOC) in marine waters, through microcosm incubations followed by community transcriptomics (McCarren et al. 2010). From these experiments, it appears that *Methylophaga* species may be involved in the utilization of DOC as part of a community effort, active in consumption of methanol or formaldehyde (McCarren et al. 2010).

Microbial mats surrounding methane seeps present dramatically different habitats for aerobic methanotrophs. In the aerated and microoxic parts of the mats, aerobic methanotrophs (the *Methylobacter* type) can make up over 50 % of total microbial population and reach densities of over 1×10^9 cells cm^{-3} (Lösekann et al. 2007; Ruff et al. 2015). The two other most abundant types in such niches are *Thiotrichales* (including *Methylophaga*) and *Bacteroidetes* species (including *Flavobacteriaceae*), again bearing similarities to the responses characterized in lake sediments. Aerobic methanotrophs of the *Methylobacter* type have also been identified in anoxic *Beggiatoa* mats in such habitats. However, they were present at lower numbers, these mats being dominated by anaerobic ANME-type Archaea (Lösekann et al. 2007).

One type of a methylotroph that is ubiquitously found in surface marine waters is the OM43 clade *Betaproteobacteria* of the family *Methylophilaceae* (Rappé and Giovannoni 2003). The OM43 bacteria are similar, phylogenetically and metabolically, to the pelagic freshwater *Methylophilaceae*,

likely sharing a common ancestor (Salcher et al. 2015). They also possess significantly reduced genomes (Giovannoni et al. 2008; Hagget et al. 2012). Like in freshwater columns, their abundances are typically connected to phytoplankton blooms (Morris et al. 2006). However, it is not entirely clear what is their preferred substrate. Metaproteomic analysis of natural populations has identified MDH proteins (XoxF type) as some of the most abundant proteins in the samples (Sowell et al., 2011; Georges et al. 2014). Other C1 proteins were also identifiable (Georges et al. 2014), suggesting their involvement in metabolism of methanol. However, laboratory incubations of representative strain HTCC2181 demonstrated stimulation with other C1 compounds, such as methyl chloride, trimethylamine N-oxide (TMAO), or dimethylsulfoniopropyonate (DMSP; Halsey et al. 2012). A synergistic mode of metabolism has been proposed to explain this phenomenon, in which carbon from methanol is directed to assimilation, while other C1 substrates are used purely for energy generation. However, genome analysis did not reveal the presence of any known enzymatic systems that would allow for such metabolism (Halsey et al. 2012). Thus, such synergistic effect remains poorly understood and requires further investigation.

Methylotrophy in exotic environments

Methylotrophy potential has been tested through SIP-based metagenomics in some exotic environments. Sediment of a crater formed by meteor impact circa 52,000 years ago was subjected to SIP with methane, methanol, and methylamine, to test for the major active methylotroph species (Antony et al. 2010). These analyses uncovered the presence of, respectively, *Gammaproteobacteria* of the genus *Methylomicrobium*, known for their propensity to persist in high salt and high pH environments (Kalyuzhnaya et al 2008b) and *Methylophaga* and *Bacillus* species, all well-recognized methylotroph guilds (Chistoserdova and Lidstrom 2013).

Communities in another exotic environment, the underground cave ecosystem sealed from the outside world for millions of years and sustained by non-phototrophic carbon fixation, were assessed via methylamine-SIP (Wischer et al. 2015). *Methylotenera* species were determined as the primary methylamine utilizers, closely related to the species from Lake Washington. *Catellibacterium* species, closely related to the known methylotrophs within *Alphaproteobacteria*, were the other prominent methylamine utilizer type. Overall, these data suggest that the nature and the diversity of the "traditional" methylotrophs is limited to a number of previously described well-recognized taxa, and the data suggest that these taxa can adjust to specific environmental conditions in terms of temperature ranges, salinity, light availability, etc., while maintaining their overall metabolic strategies. The summary of major types of methylotrophs detected via metagenomics in a variety of environmental niches, with special mention of oxygen availability in these niches, is given in Table 1.

Non-traditional methylotrophs

Marine bacteria of the SAR11 clade are the most abundant aerobic heterotrophic bacteria in the ocean surface and one of the most successful organisms on the planet (Rappé and Giovannoni 2003). Like pelagic *Methylophilaceae*, they are characterized by possessing extremely small genomes (Giovannoni et al. 2005). Remarkably, these genomes encode a variety of functions for oxidation/demethylation of C1 compounds (Sun et al. 2011). These pathways were predicted to produce energy from C1 oxidation, while no pathways were encoded for C1 assimilation. Sun et al. (2011) demonstrated that pure cultures of *Pelagibacter ubique*, indeed, could convert into CO_2 substrates such as methanol, formaldehyde, methylamine, glycine betaine, trimethylamine (TMA), TMAO, and DMSP. Elevated levels of ATP were recorded in these cultures compared to controls. Moreover, natural communities of the Sargasso Sea microbial plankton revealed similar activities (Sun et al. 2011). These findings suggest that energy production from C1 compounds, rather than traditional methylotrophy, take place. A term has been coined, "methylovory," to refer to such type of metabolism (Sun et al. 2011). In a separate study, proteins implicated in C1 pathways of SAR11 were detected in coastal water samples through metaproteomics, suggesting high expression for the relevant genes (Williams et al. 2012).

Similar type of metabolism was demonstrated for another representative of abundant marine heterotrophs belonging to the Marine *Roseobacter* Clade. Pure cultures of *Ruegeria pomeroyi* were demonstrated to utilize TMA and TMAO as supplementary energy sources when grown on glucose. In this case as well, catabolism of TMA and TMAO resulted in the production of intracellular ATP and enhanced growth rate and growth yields, as well as enhanced cell survival during starvation (Lidbury et al. 2015).

The genes encoding TMA monooxygenase (*tmm*) and TMAO demethylase (*tdm*) have been characterized only recently (Chen et al. 2011; Lidbury et al. 2014). BLAST tests with the Global Ocean Sampling dataset (Rusch et al. 2007) have revealed that these genes were present in approximately 20 % of bacteria inhabiting surface ocean waters, most prominently in SAR11 types (Chen et al. 2011; Lidbury et al. 2014). Moreover, through re-examining published metaproteomic and metatranscriptomic datasets, these genes were found to be highly expressed (Lidbury et al. 2014). Thus, C1 metabolism by pelagic bacteria appears to be of significant environmental importance, potentially affecting both nutrient

Table 1 Major methylotroph types detected in different environmental niches

Study site	Method of detection	Major species	Oxygen level	References
Lake Washington sediment	Cultivation	Variety of strains belonging to 19 named genera and to novel taxa	Oxic/anoxic	Beck et al. 2014, 2015; McTaggart et al. 2015a, b
	SIP-MG	*Methylobacter*, *Methylotenera*	Oxic/anoxic	Kalyuzhnaya et al. 2008a; Beck et al. 2013
	Microcosm MG	*Methylosarcina*, *Methylophilus*	Oxic	Hernandez et al. 2015
		Methylobacter, *Methylotenera*	Oxic/anoxic	Hernandez et al. 2015; Oshkin et al. 2015
Lake Constance sediment littoral	qPCR	Aerobic methanotrophs	Oxic	Deutzmann et al. 2014
Lake Constance sediment profundal	qPCR	NC10, aerobic methanotrophs	Anoxic	Deutzmann et al. 2014
Subtropical water reservoir Taiwan	PCR	Type I, Type II methanotrophs	Oxic	Kojima et al. 2014
		NC10, Type I, Type II methanotrophs	Anoxic	
Lake Lugano	PCR	*Methylobacter*	Anoxic	Blees et al. 2014
Lake Pavin	PCR	*Methylobacter*	Anoxic	Biderre-Petit et al. 2011
Lake Zurich	CARD-FISH	*Methylophilaceae*	Oxic/anoxic	Salcher et al. 2015
High Arctic soil Norway	SIP-MG	*Methylobacter*	Oxic/anoxic	Graef et al. 2011
	MG, MT	*Methylobacter*	Oxic/anoxic	Tveit et al. 2013, 2014
Permafrost Alaska	MG	*Methylocystaceae*, *Methylococcaceae*	Anoxic	Mackelprang et al. 2011
Permafrost Canada	MG, PCR, microarray, SIP-MG	*Methylobacter*, *Methylosacrina*, *Methylophilaceae*	Oxic/anoxic	Yergeau et al. 2010; Martineau et al. 2010
Thermocarst ponds Canada	MT	*Methylobacter*, *Methylotenera*, *Methylacidifilales*	Oxic/anoxic	Crevecoeur et al. 2015
Rhizosphere rice	MP	Type I, Type II methanotrophs, *Methylobacterium*, *Methylotenera*	Oxic/anoxic	Knief et al. 2012
Rhizosphere *Arabidopsis*	MG	*Methylobacterium*, *Hyphomicrobiaceae*	Oxic/anoxic	Lundberg et al. 2012
Phyllosphere various plants	MP	*Methylobacterium*, *Amycolatopsis*	Oxic	Delmotte et al. 2009; Knief et al. 2012
Methane seeps 23 locations	MG	ANME Archaea, *Methylococcaceae*	Anoxic	Ruff et al. 2015
Microbial mat near methane sip Barentz Sea	MG	*Methylobacter*, *Methylophaga*	Oxic	Lösekann et al. 2007
Beggiatoa mat Barentz Sea	MG	ANME Archaea, *Methylobacter*	Anoxic	Lösekann et al. 2007
Natural gas spill Gulf of Mexico	MG, MT	*Methylobacter*, *Methylophaga*, *Methylophilaceae*	Oxic/anoxic	Kessler et al. 2011; Rivers et al. 2013; Crespo-Medina et al. 2014
Water Atlantic Ocean	SIP-MG	*Methylophaga*	Oxic	Neufeld et al. 2007, 2008
Water Pacific Ocean	Microcosm MT	*Methylophaga*	Oxic	McCarren et al. 2010
Water Saanich Inlet	Microcosm MG	*Methylomicrobium*, *Methylophaga*	Oxic/anoxic	Sauter et al. 2011
Surface water coastal Pacific Ocean	MP	*Methylophilaceae*	Oxic	Sowell et al. 2011
Water coastal Atlantic Ocean	MP	*Methylophilaceae*	Oxic/anoxic	Georges et al. 2014
Meteor crater	SIP-MG	*Methylomicrobium*, *Methylophaga*, *Bacillus*	Oxic	Antony et al. 2010
Underground cave	SIP-MG	*Methylotenera*, *Catellibacterium*	Oxic	Wisher et al. 2015

Oxic and anoxic refer, respectively, to high and low oxygen concentrations in each environmental niche. Oxic/anoxic indicates that a transition zone is present. See text for more details

MG metagenomics, *MT* metatranscriptomics, *MP* metaproteomics, *pPCR* quantitative PCR, *CARD-FISH*catalyzed reporter deposition fluorescence in situ hybridization

flow within marine surface waters and the flux of C1 compounds into the atmosphere.

A number of bacteria not formally characterized as methylotrophs encode not only modules for C1 oxidation but also modules for C1 assimilation, which makes them potentially true methylotrophs, even if conditions for methylotrophic growth have not been established in laboratory. One example is *R. pomeroyi*. In addition to the TMA and TMAO oxidation enzymes discussed above and the multiple DMSP degradation pathways (Reisch et al. 2011), it encodes a XoxF-type MDH, a glutathione-linked formaldehyde dehydrogenase, the H_4MPT-linked and the tetrahydrofolate-linked C1 transfer reactions, the serine cycle, the EMC cycle, and the CBB cycle (Chistoserdova 2011a).

Methylotrophy may also be overlooked in terrestrial bacteria. The recent discovery of lanthanide requirement for the XoxF-type MDH activity (Hibi et al. 2011; Fitriyanto et al. 2011; Nakagawa et al. 2012; Pol et al. 2014) poses questions about methylotrophy in abundant and broadly distributed guilds of bacteria such as diverse *Burkholderiales* or *Rhizobia* (Chistoserdova 2011a). Confirming these guilds as methylotrophs or methylovores may significantly shift understanding of their role in global carbon cycling.

Conclusions

While methylotrophy has been known, as a physiological phenomenon, for over 100 years, most of the knowledge on their biochemistry, physiology, and biogeochemical activities has relied on the cultivated representatives. The availability of the molecular tools for environmental detection has greatly expanded the means by which methylotrophs could be assessed in natural environments. However, it is the current omics technologies that provide the means for detecting, enumerating, and measuring the activities of methylotrophs in their natural habitats with most precision. The analysis of the recently published data presented in this manuscript highlights a few newly emerging issues. First, it questions the "strictly aerobic" nature of proteobacterial methanotrophs. The data presented here strongly point toward the activity of these organisms, especially the *Methylobacter* types, in microoxic and hypoxic conditions. The second and related issue is of the nature of anaerobic methane oxidation by Bacteria. Is it really different from aerobic? The similarity of the schemes for carbon and nitrogen metabolism uncovered through (meta)genomics argues against such differentiation. The third emerging issue is the role of communities in environmental C1 cycling. So far, community function in methane oxidation is well supported through both SIP experiments and through analysis of stable microcosm communities. Similar patterns may exist in utilization of other C1 compounds. The final emerging issue is the role of non-traditional methylotrophs in global C1 metabolism and the role of methylotrophy pathways in non-methylotrophs. The forthcoming omics data will either further support or challenge these new paradigms in methylotrophy. At the same time, the stage is set for further experimental tests toward the new state-of-the-art in methylotrophy.

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