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Living apart together – Bacterial volatiles influence methanotrophic growth and activity.

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Volatile organic compounds play an important role in microbial interactions. However, little is known about how volatile-mediated interactions modulate biogeochemical processes. In this study, we show the effect of volatile-mediated interaction on growth and functioning of aerobic methane-oxidizing bacteria, grown in co-culture with five different heterotrophs. Both growth and methane oxidation of *Methylobacter luteus* were stimulated by interaction with specific heterotrophs. In *Methylocystis parvus* we observed significant growth promotion, while methane oxidation was inhibited. Volatolomics of the interaction of each of the methanotrophs with *Pseudomonas mandelii*, revealed presence of a complex blend of volatiles, including dimethylsulfide, dimethyldisulfide and bicyclic sesquiterpenes. Although the ecological role of the detected compounds remains to be elucidated, our results provide unprecedented insights into interspecific relations and associated volatiles for stimulating methanotroph functioning, which is of substantial environmental and biotechnological significance.

Methane oxidation by methanotrophic bacteria and archaea is the only known biological sink for the greenhouse gas methane (Conrad 2009). Besides performing an important ecosystem service, aerobic methanotrophs also have industrial potential. They can be applied in methane removal,

bioremediation (Jiang et al 2010), and production of biofuels and other added-value chemicals (Lee et al 2016). Despite decades of research on controls of methane oxidation and methanotroph physiology, links between methanotrophs and other microbes remain to be elucidated (Ho et al 2016). In laboratory settings, methanotrophs benefit from the presence of non-methanotrophic heterotrophs, but the mechanisms driving the interaction remain unknown (Ho et al 2014). Methanotrophs and heterotrophs may be mutually co-dependent. For example, heterotrophs may provide them with essential nutrients (Iguchi et al 2011, Stock et al 2013), or alleviate toxic effects of methane-oxidation metabolites such as methanol (Krause et al 2017), while exuded methanotrophic metabolites serve as a carbon source to the heterotrophs (Ho et al 2016, Stock et al 2013).

Moreover, microbial interaction can occur across physical barriers. Thus far, little is known about the influence of volatile secondary metabolites on growth and function of methanotrophs. Given their dependence on gaseous substrates, we hypothesize methanotrophs to be especially receptive to volatile organic compounds (hereafter: volatiles), which rapidly diffuse through water- and air filled pores. Volatiles play an important role in the long-distance interaction between soil microorganisms (Schmidt et al 2015). However, despite recent increased research interest and technological advances, the ecological role of volatile secondary metabolites remains unclear (Tyc et al 2016). Moreover, volatile effects on important biogeochemical processes are virtually unknown. Here, we measured growth and functioning of two strains of methane-oxidizing bacteria (*Methylobacter luteus 53v* and *Methylocystis parvus* OBBP), cultured in the presence of – but not in physical contact with – five different strains of heterotrophic bacteria (*Bacillus pumilus* isolate YXY-10, *Bacillus simplex* strain DUCC3713, *Exiguobacterium undae* strain B111, *Pseudomonas mandelii* JR-1 and Stenotrophomonas maltophilia strain ATCC 13637), isolated from a methanotrophic enrichment culture. To this end, we spread 50 µl of one of the heterotroph strains on one half of a two-compartment Petri dish, containing 0.1x-TSB agar (see inset of Fig. 1 and supplementary methods), and after two days applied seven 4 µl droplets of methanotroph-culture (OD_{600nm} = 0.5) on the other half, containing NMS agar. Plates with only methanotrophs, only heterotrophs and methanotrophs with added CO_2 served as controls, with

five replicates per treatment. After incubation at 20% CH_4 until growth developed (5-7 days), we quantified the cell biomass and methane oxidation rates.

In four out of five *Methylobacter luteus*-heterotroph interactions, heterotroph presence promoted growth, and all these interactions stimulated CH_4-oxidation relative to the controls (Mann-Whitney U test, $P<0.05$). CO_2 did not stimulate growth of *M. luteus* (Fig. S1), but growth of *Methylocystis parvus* was promoted by heterotroph presence, and CO_2, as may be expected from its carbon assimilation pathway (Jiang et al 2010) . Only in interaction with *Pseudomonas mandelii* growth exceeded the CO_2 control. Total CH_4 consumption per plate of *M. parvus* was lower than both controls in the presence of most heterotrophs.

To explore which compounds are responsible for the observed effects on methanotroph growth and functioning, we trapped volatiles (Tyc et al 2015), and compared profiles of plates containing methanotrophs only, heterotrophs only, or their interaction, with un-inoculated plates serving as controls (four replicates each, see supplementary methods). *Pseudomonas mandelii* was selected as a model heterotroph in this comparison, due to its varying impact on the methanotrophs. For both methanotrophs each treatment had a distinct volatile profile, with interacting bacteria showing different volatile composition than the monocultures (PLS-DA, Fig. S2), albeit mostly resembling the volatile profile of the heterotroph (Fig. 2). We identified compounds that differed in abundance between the *methanotroph * P. mandelii* interaction and their monocultures (Table S3). *Pseudomonas mandelii* monocultures and their interaction with each of the methanotrophs produced dimethylsulfide (DMS), dimethyldisulfide (DMDS) and low concentrations of dimethyltrisulfide (DMTS, Fig. 2). These small sulphur compounds are well known and ubiquitous bacterial volatiles (Effmert et al 2012, Lemfack et al 2014). DMTS can affect microbial growth and colony morphology, which may be related to quorum-sensing inhibition (Chernin et al 2011, Garbeva et al 2014, Tyc et al 2015). Indeed, *Pseudomonas* strains have been observed to produce DMTS and DMS in interaction with other bacteria (Yang et al 2015). Moreover, DMS has been found to stimulate methane oxidation in landfill-soil

biofilters, and alter methanotroph community structure, with no evidence of co-metabolization of DMS by the methanotrophs (Kim et al 2013). We tested effects of low concentrations (0.05-5 pM) of DMS, DMDS and their combination on methanotroph growth and activity (SI 1.4), and found no significant effect on growth of *M. parvus* at these low concentrations, whereas methane oxidation tended to decrease with DMS concentration (SI 4, Fig S4). At higher concentrations (100 µM), both compounds and their mixture were inhibitive to *M. luteus* and tended to inhibit *M. parvus* (Fig. S5-6).

Interestingly, two bicyclic sesquiterpenes were observed in the *M. luteus* * *P. mandelii* interaction: cadinene and alpha-muurolene (Fig. S3). Their (trace) presence in *M. luteus* cultures, but not in *P. mandelii* indicates potential production by *M. luteus*. This is supported by the presence of terpene-synthesis gene clusters in the *M. luteus* genome, which lacks in the genome of *P. mandelii* (Table S4 (Weber et al 2015)). Terpenes are generally considered plant secondary metabolites, but recent chemical analyses and sequencing of microbial genomes shows that terpenes and their cyclases are widespread in bacteria as well (Yamada et al 2015). However, no study to our knowledge has shown terpene production by methanotrophs, highlighting a promising avenue of further research. Although terpenes can have antimicrobial properties, and indeed monoterpenes have been found to inhibit methane oxidation (Maurer et al 2008), the occurrence of sesquiterpenes in interaction with potentially beneficial heterotrophs, also hints at a potential role as an infochemical.

In conclusion, volatile organic compounds produced when methanotrophs grow in the presence of heterotrophs can affect methanotroph growth and activity. Although the underlying mechanisms of these effects, as well as the blend of compounds involved remain to be elucidated, our findings provide a first insight into the growth-promoting effects of volatile organic compounds produced in heterotroph-methanotroph interactions.

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Conflict of Interest

The authors declare no conflict of interest.

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Figure legends

Figure 1. Biomass (A-B), and population methane oxidation (C-D) of the methanotrophs *Methylobacter luteus* and *Methylocystis parvus* grown on two-compartment agar plates under 20% CH_4 headspace, incubated alone (Control), alone with 5% CO_2 (CO_2 control) or in the presence of a heterotroph: *Bacillus pumilus* isolate YXY-10, *Bacillus simplex* strain DUCC3713, *Stenotrophomonas maltophilia* strain ATCC 13637, *Exiguobacterium undae* strain B111, *Pseudomonas mandelii* JR-1. Growth of *M. luteus* CO_2 control was performed in a separate experiment (SI 2). Boxes represent median, first and third quartiles. Whiskers indicate the 5^{th} and 95^{th} percentile. Inset shows two-compartment Petri dish with methanotroph droplets. Grey areas mark difference between control and CO_2 control means. Asterisks indicate significant difference from controls, diamonds indicate significant difference from CO_2 controls (pairwise comparisons against controls, Mann-Whitney U test, *=≤0.05, **≤0.01). Abbreviations; b.d, below detection: n.a, not applicable.

Figure 2. Euclidian distance based clustering of samples based on volatile presence. Each column is a sample, each row represents a compound. Left: Interaction between *Methylobacter luteus* and *Pseudomonas mandellii.* Right: Interaction between *Methylocystis parvus* and *Pseudomonas mandellii.* * Indicates tentative annotation, 'Bs' denotes bad spectrum. 'Unknown' indicates no match was found in the most recent NIST library or NIOO-KNAW library, based on mass spectra, retention time and retention index (SI 1).

Figure 1

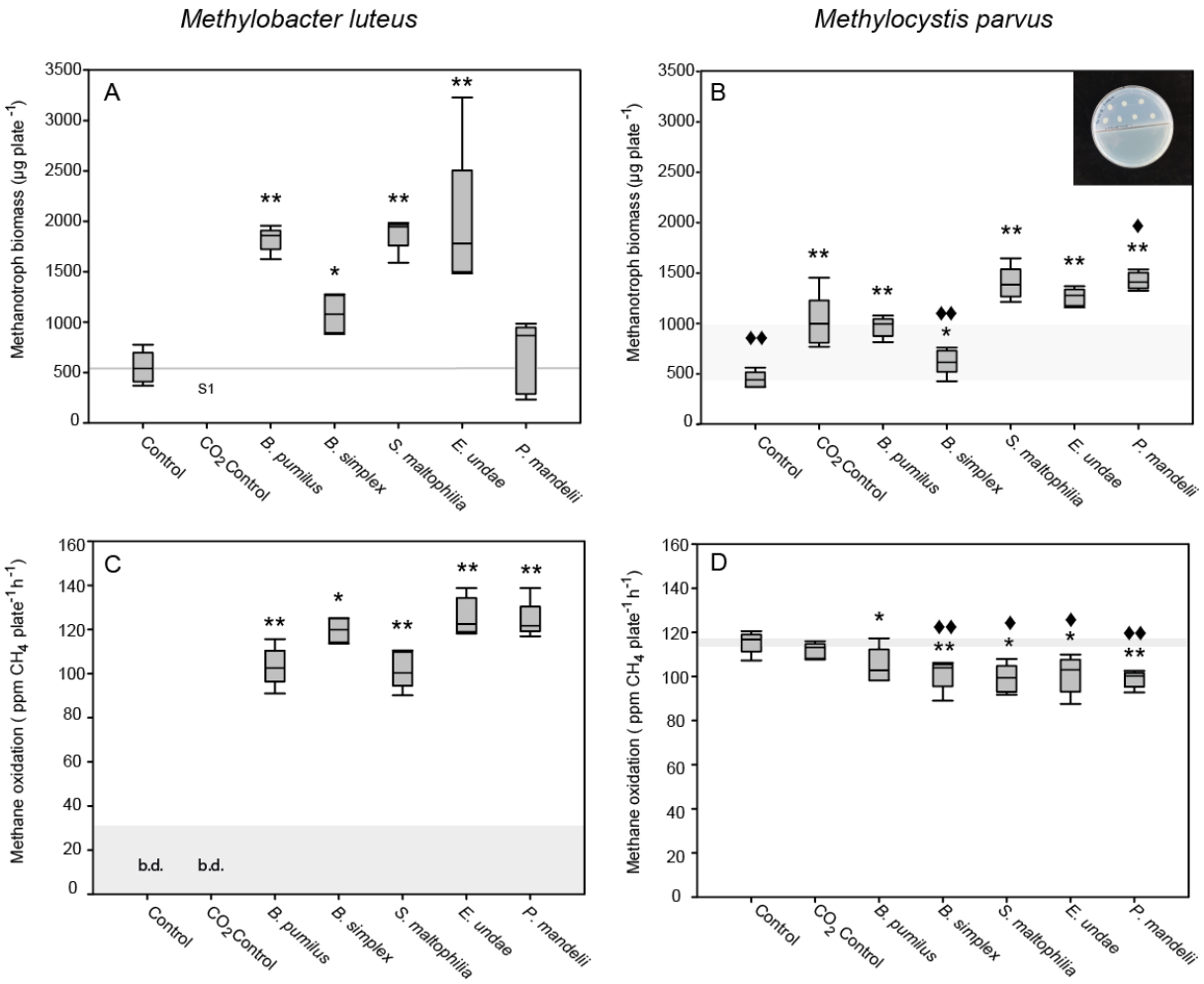
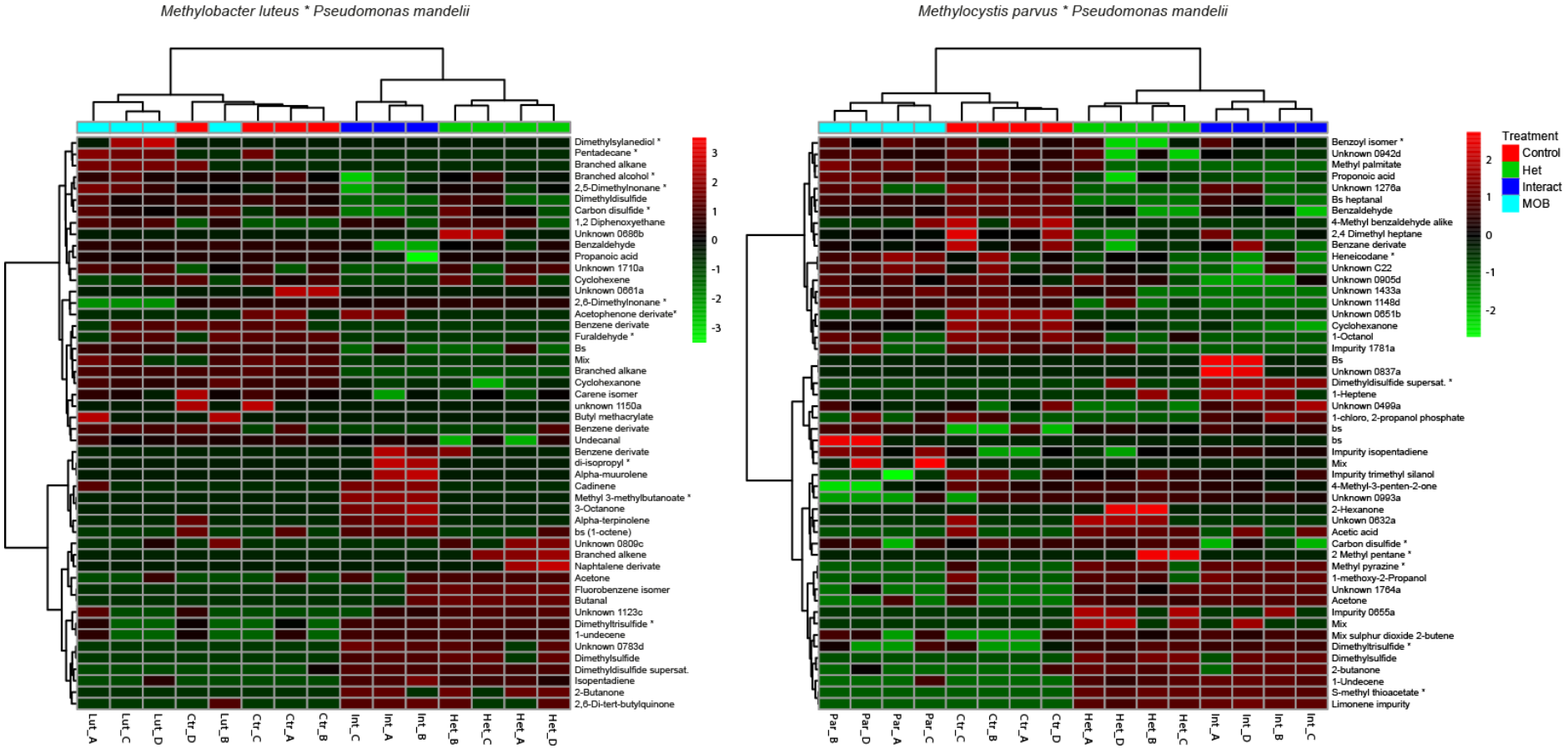

Methylobacter luteus
Methylocystis parvus
A
B
C
D
Methanotroph biomass (µg plate -1)
Methane oxidation (ppm CH4 plate-1 h-1)
S1
b.d.
b.d.
Control
CO2 Control
B. pumilus
B. simplex
S. maltophilia
E. undae
P. mandelii

Figure 2


Methylobacter luteus * Pseudomonas mandelii
Dimethylsylanediol *
Pentadecane *
Branched alkane
Branched alcohol *
2,5-Dimethylnonane *
Dimethyldisulfide
Carbon disulfide *
1,2 Diphenoxyethane
Unknown 0686b
Benzaldehyde
Propanoic acid
Unknown 1710a
Cyclohexene
Unknown 0661a
2,6-Dimethylnonane *
Acetophenone derivate*
Benzene derivate
Furaldehyde *
Bs
Mix
Branched alkane
Cyclohexanone
Carene isomer
unknown 1150a
Butyl methacrylate
Benzene derivate
Undecanal
Benzene derivate
di-isopropyl *
Alpha-muurolene
Cadinene
Methyl 3-methylbutanoate *
3-Octanone
Alpha-terpinolene
bs (1-octene)
Unknown 0809c
Branched alkene
Naphtalene derivate
Acetone
Fluorobenzene isomer
Butanal
Unknown 1123c
Dimethyltrisulfide *
1-undecene
Unknown 0783d
Dimethylsulfide
Dimethyldisulfide supersat.
Isopentadiene
2-Butanone
2,6-Di-tert-butylquinone
3
2
1
0
-1
-2
-3
Lut_A
Lut_C
Lut_D
Ctr_D
Lut_B
Ctr_C
Ctr_A
Ctr_B
Int_C
Int_A
Int_B
Het_B
Het_C
Het_A
Het_D
Methylocystis parvus * Pseudomonas mandelii
Benzoyl isomer *
Unknown 0942d
Methyl palmitate
Proponoic acid
Unknown 1276a
Bs heptanal
Benzaldehyde
4-Methyl benzaldehyde alike
2,4 Dimethyl heptane
Benzane derivate
Heneicodane *
Unknown C22
Unknown 0905d
Unknown 1433a
Unknown 1148d
Unknown 0651b
Cyclohexanone
1-Octanol
Impurity 1781a
Bs
Unknown 0837a
Dimethyldisulfide supersat. *
1-Heptene
Unknown 0499a
1-chloro, 2-propanol phosphate
bs
bs
Impurity isopentadiene
Mix
Impurity trimethyl silanol
4-Methyl-3-penten-2-one
Unknown 0993a
2-Hexanone
Unkown 0832a
Acetic acid
Carbon disulfide *
2 Methyl pentane *
Methyl pyrazine *
1-methoxy-2-Propanol
Unknown 1764a
Acetone
Impurity 0655a
Mix
Mix sulphur dioxide 2-butene
Dimethyltrisulfide *
Dimethylsulfide
2-butanone
1-Undecene
S-methyl thioacetate *
Limonene impurity
2
1
0
-1
-2
Treatment
Control
Het
Interact
MOB
Par_B
Par_D
Par_A
Par_C
Ctr_C
Ctr_B
Ctr_A
Ctr_D
Het_A
Het_D
Het_B
Het_C
Int_A
Int_D
Int_B
Int_C