

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

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

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

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

34 Moreover, microbial interaction can occur across physical barriers. Thus far, little is known
35 about the influence of volatile secondary metabolites on growth and function of methanotrophs. Given
36 their dependence on gaseous substrates, we hypothesize methanotrophs to be especially receptive to
37 volatile organic compounds (hereafter: volatiles), which rapidly diffuse through water- and air filled
38 pores. Volatiles play an important role in the long-distance interaction between soil microorganisms
39 (Schmidt et al 2015). However, despite recent increased research interest and technological advances,
40 the ecological role of volatile secondary metabolites remains unclear (Tyc et al 2016). Moreover,
41 volatile effects on important biogeochemical processes are virtually unknown. Here, we measured
42 growth and functioning of two strains of methane-oxidizing bacteria (*Methylobacter luteus* 53v and
43 *Methylocystis parvus* OBBP), cultured in the presence of – but not in physical contact with – five
44 different strains of heterotrophic bacteria (*Bacillus pumilus* isolate YXY-10, *Bacillus simplex* strain
45 DUCC3713, *Exiguobacterium undae* strain B111, *Pseudomonas mandelii* JR-1 and *Stenotrophomonas*
46 *maltophilia* strain ATCC 13637), isolated from a methanotrophic enrichment culture. To this end, we
47 spread 50 μ l of one of the heterotroph strains on one half of a two-compartment Petri dish, containing
48 0.1x-TSB agar (see inset of Fig. 1 and supplementary methods), and after two days applied seven 4 μ l
49 droplets of methanotroph-culture ($OD_{600nm} = 0.5$) on the other half, containing NMS agar. Plates with
50 only methanotrophs, only heterotrophs and methanotrophs with added CO₂ served as controls, with

51 five replicates per treatment. After incubation at 20% CH₄ until growth developed (5-7 days), we
52 quantified the cell biomass and methane oxidation rates.

53 In four out of five *Methylobacter luteus*-heterotroph interactions, heterotroph presence
54 promoted growth, and all these interactions stimulated CH₄-oxidation relative to the controls (Mann-
55 Whitney U test, P<0.05). CO₂ did not stimulate growth of *M. luteus* (Fig. S1), but growth of
56 *Methylocystis parvus* was promoted by heterotroph presence, and CO₂, as may be expected from its
57 carbon assimilation pathway (Jiang et al 2010) . Only in interaction with *Pseudomonas mandelii* growth
58 exceeded the CO₂ control. Total CH₄ consumption per plate of *M. parvus* was lower than both controls
59 in the presence of most heterotrophs.

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

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

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

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

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104 GC-QTOF\2016\BODELIER\Bodelier](https://nioo0039/Lab-Shares-3/GC-QTOF-2_RAW-DATA_TD-GC-QTOF/2016/BODELIER/Bodelier). This is NIOO publication 6451.

105 **Conflict of Interest**

106 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% CH₄ headspace, incubated alone (Control), alone with 5% CO₂ (CO₂ 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* CO₂ control was performed in a separate experiment (SI 2). Boxes represent median, first and third quartiles. Whiskers indicate the 5th and 95th percentile. Inset shows two-compartment Petri dish with methanotroph droplets. Grey areas mark difference between control and CO₂ control means. Asterisks indicate significant difference from controls, diamonds indicate significant difference from CO₂ controls (pairwise comparisons against controls, Mann-Whitney U test, * \leq 0.05, ** \leq 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 mandelii*. Right: Interaction between *Methylocystis parvus* and *Pseudomonas mandelii*. * 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

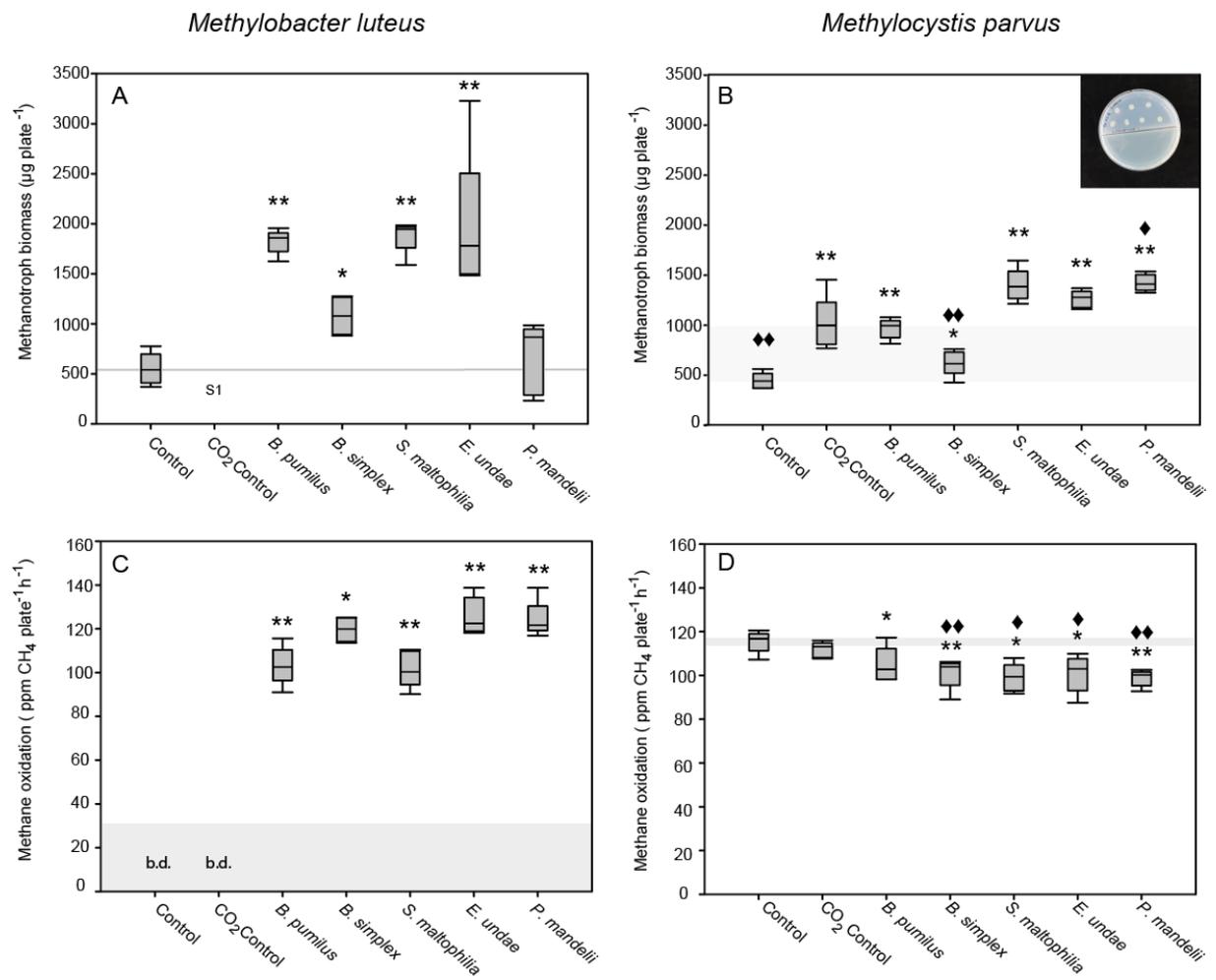


Figure 2

