The ISME Journal (2014) 8, 1945–1948
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www.nature.com/ismej

SHORT COMMUNICATION

The more, the merrier: heterotroph richness stimulates methanotrophic activity

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Although microorganisms coexist in the same environment, it is still unclear how their interaction regulates ecosystem functioning. Using a methanotroph as a model microorganism, we determined how methane oxidation responds to heterotroph diversity. Artificial communities comprising of a methanotroph and increasing heterotroph richness, while holding equal starting cell numbers were assembled. We considered methane oxidation rate as a functional response variable. Our results showed a significant increase of methane oxidation with increasing heterotroph richness, suggesting a complex interaction in the cocultures leading to a stimulation of methanotrophic activity. Therefore, not only is the methanotroph diversity directly correlated to methanotrophic activity for some methanotroph groups as shown before, but also the richness of heterotroph interacting partners is relevant to enhance methane oxidation too. In this unprecedented study, we provide direct evidence showing how heterotroph richness exerts a response in methanotroph–heterotroph interaction, resulting in increased methanotrophic activity. Our study has broad implications in how methanotroph and heterotroph interact to regulate methane oxidation, and is particularly relevant in methane-driven ecosystems.

The ISME Journal (2014) **8,** 1945–1948; doi:10.1038/ismej.2014.74; published online 2 May 2014
Subject Category: Microbial population and community ecology
Keywords: autotroph–heterotroph interaction; microbial networking; methanotroph; methane oxidation; ecosystem functioning

Biodiversity is claimed to be essential for sustainable ecosystem functioning (Tilman *et al.*, 1997; Bell *et al.*, 2005; Cardinale *et al.*, 2006). Prokaryotes, however, exist in vast abundance with an enormous uncultured diversity, and have been assumed to be highly redundant (Yachi and Loreau, 1999). Nevertheless, microbes are sensitive to environmental perturbation (Allison and Martiny, 2008; Wittebolle *et al.*, 2009; Philippot *et al.*, 2013), but functioning could be compensated despite diversity loss even among minority microbial guilds catalyzing well-defined processes (Wertz *et al.*, 2007; Ho *et al.*, 2011). Owing to their vast diversity and versatile metabolic capability, microorganisms form complex communities interacting at multi-trophic levels (Naeem and Li, 1997; Naeem *et al.*, 2000). Depending on their metabolic capabilities, microorganisms can be broadly grouped into autotrophs (primary producer) and heterotrophs (decomposers), with the autotrophs forming the base of food webs. Similarly, in methane-driven ecosystems, the methanotroph can be considered as a primary producer and interacts to form close association with the heterotrophs (Hutchens *et al.*, 2004; Iguchi *et al.*, 2011; van Duinen *et al.*, 2013; Agasild *et al.*, 2014). Possibly, the methanotroph and heterotrophs are mutually codependent. Hence, methanotroph diversity alone may be insufficient to account for effects caused by diversity loss. Therefore, not only is methanotroph diversity important (Levine *et al.*, 2011), but also the variation and richness of heterotrophic microorganisms coexisting in the same environment is highly relevant in studies addressing how methanotroph–heterotroph interaction and diversity exert a response in ecosystem

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Received 27 November 2013; revised 31 March 2014; accepted 31 March 2014; published online 2 May 2014

functioning. Yet, studies on methanotroph diversity–ecosystem function relationship with regard to this biotic component are still scarce.

We used *Methylomonas methanica* NCIMB 11130^T as a model methanotroph and considered the methane oxidation rate as the functional response variable. Methanotrophs are able to oxidize methane, a potent greenhouse gas, for growth and reproduction. Hence, methanotrophs have an important function in the global carbon cycle. *Methylomonas* spp., in particular, along with some other gammaproteobacterial methanotrophs form a minority of the total methanotroph population, but appear to be key players in aerobic methane oxidation in many important environments with high methane emission (Bodelier *et al.*, 2013; Ho *et al.*, 2013). Although some methanotrophic communities are more resilient than others (Horz *et al.*, 2005; Ho *et al.*, 2011; Levine *et al.*, 2011; Ho and Frenzel, 2012), diversity loss and/or shifts in composition of other microorganisms cohabiting the same environment may have ecological implications, particularly in a methane-driven ecosystem. Here, we aim to determine how heterotroph richness exerts a response in methanotrophic activity in an environment where the methanotroph is the primary producer.

We directly manipulated the initial heterotroph richness in artificially assembled communities consisting of a single methanotroph, and increased heterotroph richness by selecting up to 10 heterotrophic species (Table 1) from 2 phyla (*Firmicutes* and *Proteobacteria*) and 3 proteobacterial classes (detailed methodology in Supplementary Information). Methanotroph and heterotrophs were enumerated using the flow cytometer (Accuri C6, BD Biosciences, Erembodegem, Belgium), and assembled at equal starting cell numbers (10^7 cells ml^{-1} each), hence, the total cell numbers were held constant in the cocultures regardless of heterotroph richness. The heterotrophs were selected on the basis of their growth-promoting or neutral effect on the methanotroph in methanotroph–heterotroph cocultures (Stock *et al.*, 2013) and comparable growth conditions (for example, pH, temperature; Table 1). Moreover, some heterotrophs were randomly selected from the BCCM/LMG Bacteria Collection (Ghent University, Ghent, Belgium) to represent a diverse group of microorganisms. Batch incubations containing the cocultures in 10 ml nitrate medium salts (NMS) were performed in 120 ml opaque bottles. Headspace methane was adjusted to ~20 vol% and incubation was carried out on a shaker (120 r.p.m.) at 28 °C in the dark. Methane depletion in the headspace was followed over ~3 days to determine the methane oxidation rate by linear regression. The experiment was set up using quasi-optimal design methods (Goos and Jones, 2011). As the primary objective was to allow assessment of heterotroph richness effect on methane oxidation, while accounting for batch effects (discrepancy between independent batch incubations) and eliminating the risk of confounding effects of the heterotroph composition, the optimality criterion was set to the average correlation between the design matrix column of the richness and the design space spanned by the design matrix columns of the absence/presence indicator columns of the 10 heterotrophs. This criterion is minimized by using a modified Federov exchange algorithm under the additional constraint that the total number of incubations is 80, equally distributed over the two batches. Moreover, incubations with the methanotroph in pure culture served as a reference.

High heterotroph richness stimulated methane oxidation; in the absence of heterotrophs, methane oxidation was still detected, but significantly increased ($P<0.0001$) in cocultures with high heterotroph richness despite comparable total cell numbers after incubation (Figure 1 and Supplementary Figure S1). A discrepancy in the activity measured between batch incubations was observed (Supplementary Figure S2), but the design

Table 1 Heterotroph species used in this study, and the growth-promoting or neutral effects of some heterotrophs on *Methylomonas* spp.

Phylum/Class	*Species*	*Code*[a]	*Strain number*	*Growth conditions*[b]	*Growth effects*[c]
Alphaproteobacteria					
	Paracoccus denitrificans	H1	LMG 4049	pH 7.4, 26 °C	NA
	Rhizobium radiobacter	H2	LMG 287	pH 7.3, 28 °C	Growth-promoting
	Ochrobactrum anthropi	H3	LMG 2134	pH 7.4, 25 °C	Growth-promoting
Betaproteobacteria					
	Cupriavidus metallidurans	H4	LMG 1195^T	pH 7.3, 28 °C	NA
	Comamonas terrigena	H5	LMG 1249	pH 7.4, 30 °C	NA
	Acidovorax delafieldii	H6	LMG 1792	pH 7.4, 28 °C	NA
	Achromobacter denitrificans	H7	LMG 1231^T	pH 7.4, 28 °C	NA
Gammaproteobacteria					
	Pseudomonas putida	H8	LMG 24210	pH 7.4, 28 °C	Growth promoting
	Escherichia coli	H9	LMG 2092^T	pH 7.4, 28 °C	Growth promoting
Firmicutes					
	Bacillus azotoformans	H10	LMG 9581^T	pH 7.4, 30 °C	Neutral

Abbreviation: NA, data not available.
[a]Referred code of heterotroph species in this article.
[b]Growth conditions for each species: pH, optimum temperature.
[c]Growth effects on *Methylomonas* spp. based on optical density measurements as described in Stock *et al.* (2013).

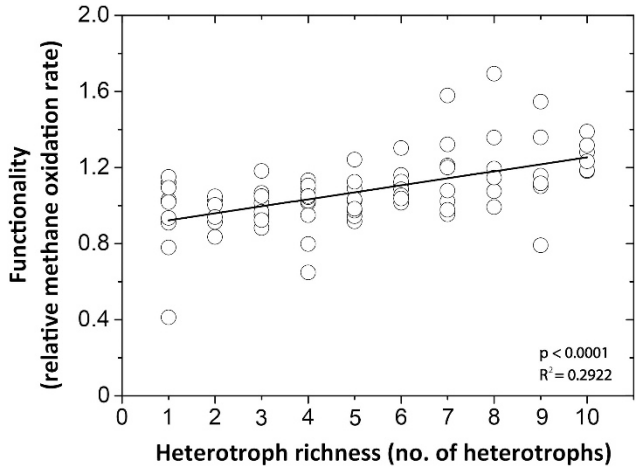

Figure 1 Stimulation of methane oxidation with increasing heterotroph richness, corrected for batch effects (normalized relative to batch 1). Increased methane oxidation was significantly correlated to heterotroph diversity ($P<0.0001$). Functionality given as the ratio of methane oxidation rate in a coculture compared with the methane oxidation rate in a pure methanotroph culture ($n=86$). Values >1 represent a stimulation of methane oxidation in the cocultures. The methane oxidation rates are given in Supplementary Figure S2, and further supported by two independent batch incubations (Supplementary Figure S1). During incubation, pH fluctuated within a narrow range (7.0–7.2).

and statistical analysis accounted for batch effects. After batch correction, activity was positively correlated to heterotroph richness (Figure 1 and Supplementary Figure S2). The linear relationship suggests that heterotrophs' contribution to methane oxidation was additive, that is, each heterotroph contributed equally to stimulate methane oxidation. Further increase in heterotroph richness may induce a decelerating curve where some level of functional or metabolic complementarity may render further heterotroph addition redundant (Bell *et al.*, 2005). To determine whether increased activity was a result of higher net nutrient/metabolite availability from lysed heterotroph cells, the methanotroph was incubated in heterotroph-spent medium where activity decreased (Supplementary Figure S3). The cause of the inhibitory effect on methane oxidation remains to be elucidated. However, the inhibition of methanotrophic activity in heterotroph-spent medium emphasized the importance of the heterotroph as the inhibition was apparently alleviated in the methanotroph–heterotroph cocultures, and the methanotrophic activity was stimulated. Considering that residual Luria Bertani (LB) medium from heterotroph cultures may have been unintentionally added into the artificial communities during assembly (detailed methodology in Supplementary Information), the methanotroph was incubated in undiluted LB or LB diluted with NMS medium ($\times 0.1$, $\times 0.01$ and $\times 0.001$) to determine the potential adverse effects of the growth medium on methane oxidation (Hanson and Hanson, 1996). Methane oxidation was inhibited only in incubation with undiluted LB (Supplementary Figure S4), excluding any adverse effect of residual LB on methane oxidation in our incubations. Therefore, higher nutrient/metabolite availability potentially derived from lysed cells does not appear to be a cause for the increased methanotrophic activity, and further suggests that activity was stimulated by a more direct mechanism.

In a mutualistic interaction, methanotrophic symbionts serve as a carbon source (for example, CO_2) for *Sphagnum* and brown mosses, and microalgae in return for molecular oxygen (Raghoebarsing *et al.*, 2005; Liebner *et al.*, 2011; van der Ha *et al.*, 2011). Moreover, methanotrophs may benefit from interactions with heterotrophs; specific heterotrophs are thought to provide methanotrophs with essential metabolites (cobalamin; Iguchi *et al.*, 2011; Stock *et al.*, 2013). Conversely, the methanotrophs are known to sustain whole communities in methane-driven ecosystems (Hutchens *et al.*, 2004). They provide an accessible carbon source for the heterotrophs through different mechanisms (for example, exudates via methane-based fermentation under oxygen-limited conditions; Kalyuzhnaya *et al.*, 2013). Besides, methanotrophs interact with their biotic components in a predator–prey relationship (selective grazing; Murase and Frenzel, 2008). These studies demonstrate a tight association between methanotrophs and their biotic environment. In contrast to these findings, we show that methanotroph-interacting partners are not necessarily exclusive. Whereas single heterotrophs had no appreciable effect (Supplementary Figure S1), an increasing heterotroph richness, regardless of the heterotroph combination and corrected for batch effects, on average results in significantly increasing methane oxidation (Figure 1; $P<0.0001$). The experiment was designed to assess the effect of richness by eliminating the risk of confounding effects of the heterotroph composition. The confounding effect was insignificant ($P=0.8389$), unequivocally showing that heterotroph richness alone induced a higher methane oxidation rate. Nevertheless, it is still unclear how heterotroph richness stimulated methanotrophic activity. However, a high heterotroph richness may possess versatility in metabolic capacity to relieve accumulated inhibitory compounds (for example, methanol, formaldehyde; Hanson and Hanson, 1996) that could not have been achieved in single heterotroph cocultures, thus, relieving potentially adverse effects on the methanotrophs. Taken together, we provide direct evidence showing the relevance of a diverse heterotroph community to enhance methane oxidation. However, further studies are needed to determine whether the effect of heterotroph richness is species specific and can be extrapolated to other methanotrophs. Similarly, future studies elucidating the underlying mechanisms causing the stimulatory effect warrant attention.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

We extend our gratitude to Professor Peter Frenzel (Max-Planck Institute, Marburg) for fruitful discussions. This work was supported by the Flemish Fund for Scientific research (FWO Vlaanderen) grant no. G.0700.10N (to KDR) and FWO11/PDO/084 (to KH), a research grant from the Geconcentreerde Onderzoeksactie (GOA) of Ghent University (BOF09/GOA/005) and the Inter-University Attraction Poles (IUAP) 'μ-manager' and 'StUDyS' funded by the Belgian Science Policy (BELSPO, P7/25 and P7/06). OT and JDN acknowledge the N2N Multidisciplinary Research Partnership project of Ghent University. This publication is Publication no. 5602 of the Netherlands Institute of Ecology.

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All authors have seen and approved the final version submitted.

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Supplementary Information accompanies this paper on The ISME Journal website (http://www.nature.com/ismej)