Functional stabilisation and partner selection during repeated co-culivation in a methanotrophic interactome

Frederiek - Maarten Kerckhof, Charlotte De Rudder, Varvara Tsilia, Ruben Props, Adrian Ho, Kim Heylen, Nico Boon

Background

Biological oxidation of methane (CH_4) is an essential ecosystem function. Accumulating evidence indicated that this function is mediated by associations of methanotrophic bacteria (MOB) with non-methanotrophic partners; together referred to as a methanotrophic *interactome*. Given the potency of CH4 as a greenhouse gas, a thorough understanding of how these *interactomes* exert an effect on methane oxidation is of special interest. Furthermore, MOB - non-MOB associations could be exploited for sustainable biotechnological applications in light of the renewed interest in MOB as natural and cost-efficient biocatalysts. The selectivity of MOB for non-MOB partners, as well as the stimulation of MOB activity (CH_4 oxidation rate, MOR) with increasing non-MOB richness have both been recently described for a single batch incubation period. Therefore, we hypothesized that during repeated co-cultivation of MOB with non-MOB, ecological sorting would guide the methanotrophic *interactome* towards its optimal composition, which could additionally boost functionality (MOR).

Methods

Co-cultures of 8 non-MOB partners with a single alpha- or a single gammaproteobacterial MOB were repeatedly sub-cultivated. In every cycle, the headspace CH_4 concentration was measured to over time to determine the MOR, while headspace CO2 concentrations and total protein in the culture were determined to track the fate of CH4-derived carbon (catabolism and assimilation respectively). Finally, the relative abundance of each co-culture partner was assessed using a 16S rRNA gene-targeted denaturing gradient gel electrophoresis (DGGE).

Results and Discussion

While no significant improvement of functionality was observed, the biological variability of MOR was stabilized by co-cultivation with non-MOB partners. Overall, higher biomass yields were obtained when MOB were co-cultivated with non-MOB partners and the alphaproteobacterial MOB appeared to be able to support more non-MOB biomass than the gammaproteobacterial MOB, which could be linked to the proposed life-strategies of these clades. A clear partner selection was observed as only 4 out of 8 initial partners were

found to persist during repeated cycles of co-cultivation. While 2 of the persisting partners could coexist with either MOB type, the other two were more restricted to a specific MOB. Differential metabolic potential of non-MOB was resolved by genome mining publicly available genomes; our attempt to find clues for the partner selectivity did not reveal a clear link with the potential for C1-compound metabolism. However, genes for sugar metabolism (fructose, mannose, sucrose) were restricted to the persisting partners while genes encoding an ATP-dependent vitamin B12 importer were restricted to the non-persisting partners, underlining the importance of metabolic exchange in the methanotrophic *interactome*.

FUNCTIONAL STABILISATION AND PARTNER SELECTION DURING REPEATED CO-CULTIVATION IN A METHANOTROPHIC INTERACTOME

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ABSTRACT

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KEYWORDS

Aerobic methanotroph, synthetic ecology, microbial co-cultivation, microbial ecology, microbiology, functional stability

DEFINITIONS

A microbial ***interactome*** is referred here as an entire community interacting in concert to contribute to a specific microbial process. These interactions encompass (but are not limited to) (syn)trophic interactions where interacting partners exchange molecular building blocks and

63 energy while feeding on a defined substrate. Others include antagonistic interactions such as
64 selective predation.

INTRODUCTION

The biological oxidation of methane (CH_4), a greenhouse gas which contributes greatly to radiative forcing (Ciais et al. 2014; Nisbet et al. 2014), is an essential ecosystem service which both contributes to atmospheric CH_4 removal (Kirschke et al. 2013), as well as to attenuation of terrestrial and marine methane emissions (Conrad 2009; Hinrichs & Boetius 2002). Biological methane oxidation is mainly facilitated by aerobic methane-oxidizing bacteria (MOB, methanotrophs) which possess the unique ability to use CH_4 as a sole carbon and energy source (Hanson & Hanson 1996). Due to this trait, they can act as primary producers of organic carbon in methane-fueled ecosystems (Petersen & Dubilier 2009; Ruff et al. 2013). Accumulating evidence shows that MOB are generally present in so-called methanotrophic *interactomes,* consisting of MOB and non-MOB partners, and these *interactomes* as a whole are likely the main contributors to enhanced biological methane oxidation (Ho et al. 2014; Iguchi et al. 2015; Oshkin et al. 2014; Stock et al. 2013). Furthermore, apart from cycling of CH_4, interactions within the methanotrophic *interactome* or its interactions with other non-methanotrophic *interactomes* may influence biogeochemical cycling of other building blocks of life on earth (e.g. N or S) (Costa et al. 2006; Hanke et al. 2014; Ho et al. 2014; Joye 2012; Koch et al. 2015; Kraft et al. 2014; Luesken et al. 2011; Oshkin et al. 2015). This is evidenced, for instance, by the reports on the interactions between MOB and ammonia-oxidizing Archaea and Bacteria (AOA/AOB) (Daebeler et al. 2014; Zheng et al. 2014). Additionally, the importance of accompanying bacteria for MOB-driven biotechnological processes has been illustrated in the case of single-cell protein production (SCP) (Bothe et al. 2002), CH_4-driven denitrification (Amaral et al. 1995; Modin et al. 2007; Zhu et al. 2016), biodegradation of organic pollutants (Hrsak & Begonja 2000), and the methane-driven production of biopolymers (Helm et al. 2006).

We limit our discussion of the methanotrophic *interactome* here to associations between MOB and bacterial (rather than eukaryotic) non-MOB partners (Hanson & Hanson 1996; Hrsak & Begonja 2000; Iguchi et al. 2011; Jeong et al. 2014; Stock et al. 2013). While the benefit of associating with the MOB is clear for non-MOB, i.e. acquisition of organic carbon from CH_4 through MOB (Modin et al. 2010; Murase & Frenzel 2007)), it is less straightforward what benefit the MOB acquire from their non-MOB partners (Iguchi et al. 2015). Non-MOB partners could potentially aid MOB in a multitude of ways such as the removal of inhibitory intermediates from CH_4 metabolism, such as methanol (as evidenced by the frequent association of methylotrophic non-MOB with MOB (Beck et al. 2013; He et al. 2012; Takeuchi et al. 2014)), the detoxification of toxic intermediates of MOB-driven degradation of organic (micro)pollutants (Benner et al. 2014; Hesselsoe et al. 2005; Hrsak & Begonja 2000) or non-MOB could supply growth factors (such as vitamins) to the MOB, as evidenced by rhizobial strains which stimulated gammaproteobacterial growth and CH_4 oxidation by excreting cobalamin (vitamin B12) (Iguchi et al. 2011). Although some experiments have shown that partnerships between the MOB and non-MOB partners are highly specific (Hernandez et al. 2015; Stock et al. 2013; van der Ha et al. 2013), an increase in community diversity (richness, evenness), rather than specific partnerships, influenced ecosystems functionality (Ho et al. 2014; Wittebolle et al. 2009).

The detailed study of these complex microbial interactions is hampered by technical limitations. Even by means of an established top-down method such as stable isotope probing (SIP) (Dumont & Murrell 2005; Neufeld et al. 2007) causal evidence for cooperation and/or evidence for a bidirectional flux between partners in natural ecosystems is difficult to establish. Hence recently, synthetic ecology experiments have been considered as a bottom-up approach in the toolkit of the environmental microbiologist (Bai et al. 2015; De Roy et al. 2014; Faith et al. 2014;

Großkopf & Soyer 2014; Jousset et al. 2011; Mee & Wang 2012; Stenuit & Agathos 2015). Although (re)assembling an ecosystem by means of isolates is not a new concept (Namsaraev & Zavarzin 1972; Wilkinson et al. 1974), the recent rise in interest for these type of experiments illustrates the need for a greater understanding as to how biotic interactions influence biogeochemical cycling.

In this study, we hypothesized that MOB select for specific non-MOB partners based on their ability to stimulate the functionality of the MOB (i.e. methane oxidation rate; MOR). We tested our hypothesis by repeated co-cultivation of a methanotrophic *interactome* as recently employed by Ho et al. (2014) in the context of richness-functionality relationships. Additionally, we included both a representative alpha- and gammaproteobacterial MOB, as these clades harbor the majority of the currently cultivated MOB (Knief 2015; Semrau et al. 2010), and both clades are hypothesized to have distinct life strategies (Ho et al. 2013). Although these life strategies (e.g. competitiveness) could potentially impact biotic interactions with non-MOB in a methane-driven ecosystem, it is not yet known if and how they influence the methanotrophic *interactome*. If MOB show preference for specific partners, prolonged co-cultivation will select for these preferred partners. Conversely, the incompatible non-MOB partners will recede with successive cycles of co-cultivation. Hence, in this experiment, repeated co-cultivation will result in an unsupervised self-selection of the most optimal methanotrophic *interactome* from the initial partner combination. In support, the difference in the genetic make-up of the preferred partners was mined for cues on the potential mechanism of selectivity. Additionally, the influence of these preferred partnerships on the stability of ecosystems functionality (i.e. variability in MOR) was determined.

MATERIALS & METHODS

1. Strains and growth conditions

Methylomonas methanica NCIMB 11130^T (Gammaproteobacteria; type I) and *Methylosinus* sp. LMG 26262 (Alphaproteobacteria, type II) were chosen as methanotrophs. Non-methanotrophic partner cultures were selected and cultivated as described previously (Ho et al. 2014) (Table 1). After resuscitation from a -80 °C stock, the bacteria were inoculated on nutrient agar (Sigma-Aldrich, 70148) for 14 h at 28° C, after which they were transferred to liquid LB broth (Lennox, Sigma L3022) and placed on an orbital shaker (120 rpm) at 28° C for 30 h. MOB (triplicate) and non-MOB partner co-cultures (quadruplicate), as well as a partner-only control (unreplicated) were grown on 20 mL NMS medium (with copper) (Whittenbury et al. 1970) in 120 mL opaque serum bottles closed air-tight with grey butyl rubber stoppers. The bottles were incubated on a rotary shaker (150 rpm) at 28 °C. At the start of the batch incubation, the headspace of the serum bottles contained of 20% (v/v) of CH_4 (N45, Air Liquide, Belgium) in the headspace. The synthetic communities were assembled at equal starting numbers for each strain based on cell count using flow cytometry (Van Nevel et al. 2013). The initial MOB-non-MOB co-culture inoculum contained 10^8 cells mL^{-1}. Both axenic cultures of MOB and partners contained the same number of cells as was present in the synthetic ecosystem per organism type. Serial co-cultivation was performed by transferring 10% (v/v) to fresh NMS medium after approximately 72 hours per cycle. An overview of the experimental design and sampling amounts is given in Fig. S1.

2. Analytical methods

Headspace gas composition was sampled concurrently at 0, 4, 6, 24, 48 and 72 hours during cycle 1, 3, 4 and 5. The headspace was sampled and injected in a Compact GC® (Global Analyser Solutions, the Netherlands) equipped with a PoraBOND Q pre-column (Agilent, USA), a Molsieve 5A column, one channel connected to a flame ionization detector and two channels connected to a thermal conductivity detector. The system was controlled by EZChrom Elite software (Agilent, USA).

Total cellular protein was determined using the Bio-Rad DC Protein Assay, which is a modified version of the Lowry protein quantification as per the manufacturer's instructions. Lyophilized bovine serum albumin was used as a protein standard. Previously, MOB growth was shown to have a linear correlation with total protein measurements (Hoefman et al. 2013).

Total heterotrophic plate counts were performed at the end of the fifth co-cultivation cycle by decimal serial dilution plating (using sterile physiological solution) on nutrient agar (Sigma-Aldrich, 70148). Plates were counted after 48h of incubation at 28°C. Only plates with more than 30 or less than 300 cells were counted, and counts were assumed to be Poisson distributed.

3. PCR-DGGE

The 16S rRNA gene region was amplified by PCR using 338F and 518R primers targeting the V3 region (Muyzer et al. 1993; Ovreas et al. 1997). A GC clamp of 40 bp (Muyzer et al. 1993; Ovreas et al. 1997) was added to the forward primer. The PCR program consisted of 10 min 95°C; 35 cycles of 1 min. 94°C, 1 min. of 53°C, 2 min. of 72°C; and a final elongation for 10 min. at 72°C. Amplification products were analysed by electrophoresis in 1.5% (wt/vol) agarose gels stained with ethidium bromide. DGGE (Denaturing Gradient Gel Electrophoresis) based on the protocol of Muyzer *et al*. (Muyzer et al. 1993) was performed using the INGENYphorU

System (Ingeny International BV, The Netherlands). PCR fragments were loaded onto 8% (w/v) polyacrylamide gels in 1 × TAE buffer (20 mM Tris, 10 mM acetate, 0.5 mM EDTA pH 7.4). To process and compare the different gels, a homemade marker of different PCR fragments was loaded on each gel (Boon et al. 2002). The polyacrylamide gels were made with denaturing gradients ranging from 40% to 60% (where 100% denaturant contains 7 M urea and 40% formamide). The electrophoresis was run for 16 hours at 60°C and 120V. Staining and analysis of the gels was performed as described previously (Boon et al. 2000). The normalization and analysis of DGGE gel patterns was done with the BioNumerics software 5.10 (Applied Maths, Sint-Martens-Latem, Belgium), which was also used to assign band classes. Distinctive band classes for each *interactome* partner were selected based upon patterns from individual axenic cultures that were loaded on the DGGE.

4. Statistical data analysis

All statistical analyses and data visualizations were performed using R 3.2.3 (http://r-project.org). Multiple comparisons were performed as follows: after checking normality of residuals (both by means of a Q-Q normal plot as well as the Shapiro-Wilks' normality test) and homogeneity of variances (both by means of inspecting boxplots as well as robust Levene-type testing or Brown-Forsythe testing if normality could not be assumed) the following general and post hoc tests were performed: if the normality and homoscedasticity hypothesis could be retained an ANOVA was performed with Tukey HSD Post-Hoc testing. If normality was retained but homoscedasticity rejected a weighted-least squares ANOVA was run with a Games-Howell post-hoc test. If normality could not be retained nonparametric multiple contrast effects were employed with Tukey contrasts. To model the methane removal generalized additive models were employed in R. Variance-to-mean ratios (VMR) were used as relative dispersion

metrics and were calculated by dividing the cycle means and variances of normalized methane oxidation rates.

5. Comparative genomics

Publically available genomes were acquired from their respective sources (Table 1) and annotated by the rapid annotations using subsystems technology (RAST) server using default settings ("classic RAST" annotation scheme, RAST as a gene caller, FIGfam release 70, Genetic code 11, automatically fix errors and backfill gaps) with additional fixing of frame shifts and construction of a metabolic model (Aziz et al. 2008; Overbeek et al. 2014). After annotation, a genbank file was downloaded from RAST and pathway-genome databases were built using the PathoLogic tool in PathwayTools (Karp et al. 2010) v. 19.5 with automatic build and all automated options in the consistency checker. Subsequently, comparative analyses were run using the web interface of the PathwayTools web server.

RESULTS AND DISCUSSION

1. MOB partner selection

The specificity of MOB interaction with non-MOB partners in the co-cultures could lead to the specific selection of partners by the MOB after repeated co-cultivation. Previously, we reported on 1:1 co-cultivation experiments of 25 non-MOB with 9 MOB partners and showed that specific combinations of MOB and non-MOB differentially stimulated maximal growth and growth rates of the resulting 1:1 *interactomes* during a single co-cultivation cycle (Stock et al. 2013). Now, we incubated 8 partners at equal concentrations of $3.17*10^7 \pm 1.75*10^7$ cells mL^{-1} with $3.23*10^7 \pm 2.31*10^7$ cells mL^{-1} of either *Methylomonas methanica* NCIMB 11130^T or *Methylosinus* sp. LMG 26262. By adding an initial excess of non-MOB as compared to MOB only the non-MOB best adapted for interaction with the MOB will be able to persist in the *interactome* after repeated cycles of co-cultivation. Hence, this unsupervised selection for specific partners by the MOB will be mediated by competition among the non-MOB partners for specific CH_4-derived carbon sources supplied by the MOB. Alternatively, the negative and neutral interactions we observed earlier (Stock et al. 2013), could indicate partner incompatibility with MOB-derived carbon, leading to immediate washout upon repeated sub-cultivation. The presence/absence (and relative abundance) of each individual constituent partner of the methanotrophic co-culture was determined at the end of each co-cultivation cycle using denaturing gradient gel electrophoresis (DGGE). As expected, the MOB were observed in each cycle and each treatment condition (Fig. 1 and Fig. S2). Additionally, of the eight initial non-MOB partners, only the same four strains (*R. radiobacter* LMG 287, *C. metallidurans* LMG 1195^T, *A. denitrificans* LMG 1231^T, *P. putida* LMG 24210) could be observed at the end of the first co-cultivation cycle with each of the MOB. These partners could hence be considered

persisting partners. *R. radiobacter* LMG 287 and *C. metallidurans* LMG 1195^T were present in all cycles for both MOB and were therefore viewed as "promiscuous" partners (Fig. 1). *A. denitrificans* LMG 1231^T and *P. putida* LMG 24210 were more specific partners for *Methylosinus* sp. LMG 26262 and *M. methanica* NCIMB 11130^T, respectively (Fig. 1). Of the partners which could not be detected in the first cycle (the non-persisting partners), only *P. denitrificans* LMG 4049 re-appeared in cycle 5 of co-cultivation with LMG 26262. This suggests that during previous cycles its biomass did not wash out but rather remained present at levels below DGGE detection limits. We have previously observed this behavior when *Methylosinus* sp. LMG 26262 was combined with a GFP-tagged *Pseudomonas putida* (obtained from prof. Søren Molin, Sternberg et al. (1999)): low initial inoculum concentrations and a 5/95 non-MOB/MOB ratio were found to be required for optimal adaptation and development of a mutualistic interaction (F.M. Kerckhof & Charlotte De Rudder, unpublished data). To our knowledge, this is the first report of a repeated co-cultivation experiment which tracks the presence/absence of both MOB and non-MOB partners and further shows specific partner selection depending upon the MOB type for a defined set of non-MOB partners.

2. From CH_4 to interactome biomass: CH_4 oxidation and CH_4-C distribution

To investigate the significance of the observed association between the two MOB and the eight different non-MOB partners belonging to 3 phyla and 8 genera, CH_4-oxidizing activity and the fate of CH_4-derived carbon were determined during the repeated co-cultivation experiments. Unexpectedly, no significant differences in methane oxidation rates (MOR, mmol CH_4 oxidized L^{-1} h^{-1}) were observed between axenic MOB and MOB with partners ($p>0.05$) in cycle 1 (whereas they were observed byHo et al. (2014)) and subsequent cycles of repeated co-cultivation. Additionally, fitting of generalized additive (mixed) models did not show a

significant effect of non-MOB partners on the methane removal profiles (Fig. S3) at the 5% significance level. However, the type of MOB always had a significant effect on the methane removal profiles ($p<0.05$, data not shown) which was also supported by the overall higher average MOR for *M. methanica* NCIMB 11130^T (0.60±0.33 mmol CH_4 oxidized L^{-1} h^{-1}, averaged over all cycles) as compared to *Methylosinus* sp. LMG 26262 (0.24±0.25 mmol CH_4 oxidized L^{-1} h^{-1}). This could reflect the different traits, and possibly, life strategies adopted by the MOB (Ho et al. 2013): gammaproteobacterial MOB are believed to thrive at high CH_4 availability and were classified as competitors/competitors-ruderals in a competitor-stress tolerator-ruderals (C-S-R) framework (Grime 1977). This implies that gammaproteobacterial MOB are considered to be highly competitive for nutrients, trace elements and CH_4 and oxygen (C), while simultaneously being robust to disturbances (such as grazing, heat/cold stress, desiccation/rewetting (Ho et al. 2016): C-R). Conversely alphaproteobacterial MOB are believed to be stress tolerators/stress tolerators-ruderals which implies that they thrive with increasing stress (such as low levels of CH_4, O_2, nutrients but also physicochemical stress: S).

In contrast to our previous findings (Ho et al. 2014), this experiment showed no significant activity differences between incubation of *M. methanica* NCIMB 11130^T (and *Methylosinus* sp. LMG 26262, specific to this experiment) with or without non-MOB partners. This could be attributed to differences in the experimental setup, e.g. we used a subset of the partners of our previous work and added these partners in higher initial amounts than before. Nevertheless, our results showed that addition of non-MOB partners reduced the overall variability of the MOR, hence "stabilizing" the biological variability of methane removal by both axenic MOB strains, regardless of the co-cultivation cycle. The 'stabilizing' effect appears to become stronger with repeated sub-cultivation as compared to Cycle 1 (Fig. 2, Fig. S4). This stabilization of MOR in

the presence of non-MOB partners may be attributed to the removal of inhibitory compounds (Hanson & Hanson 1996; Wilkinson et al. 1974), and corroborates with earlier findings that community diversity enhances functional stability (Wittebolle et al. 2009). Process stabilization of the production of single cell protein (SCP, BioProtein, Danmark) by *Methylococcus capsulatus* Bath was shown for an *interactome* with *Ralstonia* and Bacillales (Bothe et al. 2002). These non-MOB are deliberately introduced to the *M. capsulatus* culture to enhance growth and process stability. Predictable and reliable functionality (in this study represented by methane removal and biomass growth) are essential for biotechnological application of synthetic ecosystems (Pandhal & Noirel 2014).

To track how CH_4-derived carbon was distributed towards CO_2 (catabolism) and biomass (anabolism), the total cellular protein, as well as the headspace CO_2 were measured during all cycles. Overall, more CH_4-C was converted to biomass, and thus less to CO_2, when MOB were incubated with partners (Fig. 3). This suggests a higher yield of total protein produced per mg of CH_4 consumed when partners were co-cultivated with the MOB and, to a lesser extent, to a decrease of CO_2 produced per mg of CH_4 consumed (Table S1). More protein per mg of CH_4 was synthesized during co-cultivation by *M. methanica* NCIMB 11130^T than *Methylosinus* sp. LMG 26262. Interestingly, the total heterotrophic plate counts were lower for NCIMB 111130^T ($1.30*10^8 \pm 1.14*10^4$ CFU mL^{-1}) than LMG 26262 ($1.68*10^9 \pm 4.10*10^4$ CFU mL^{-1}) suggesting that the anabolic stimulation of partners might be MOB-type specific and could be related to their proposed life-strategies. For instance, the competitor *M. methanica* NCIMB 11130^T likely assimilated more of the CH_4-C than the stress-tolerator *Methylosinus* sp. LMG 26262, leaving less C available to the non-MOB partners. Here too, we confirmed this observation by an experiment in which both MOB were combined in same amounts with a GFP-tagged

305 *Pseudomonas putida* (obtained from prof. Søren Molin, Sternberg et al. (1999)), which resulted
306 in a higher GFP event count (determined by flow cytometry) for co-cultivation with
307 *Methylosinus* sp. LMG 26262 than with *M. methanica* NCIMB 11130^T after 6 repeated cycles of
308 72 h (F.M. Kerckhof & C. De Rudder, unpublished data). An additional differentiating feature
309 between both MOB was the increased CO_2 production during each cycle for *Methylosinus* sp.
310 LMG26262, which could not be observed for *M. methanica* NCIMB 11130^T. When partners
311 were added, the ratio of CO_2-C to protein was generally lower for each respective cycle in the
312 case of *Methylosinus* sp. LMG 26262 (Fig. 3), mainly due to an increase in the amount of total
313 protein (biomass) synthesized. This effect could not be observed for *M. methanica* NCIMB
314 11130^T. Negative controls with axenic non-MOB partners confirmed absence of growth and lack
315 of CO_2 production indicating carbon was primarily derived from CH_4 in the presence of MOB.
316 Moreover, protein was below the detection limit in all cycles and time points in these negative
317 controls, which suggest a lack of growth without the MOB and CH_4 (data not shown).
318 Consequently, the increased amount of biomass-protein synthesized without an increase in MOR
319 indicates that non-MOB growth is sustained by more efficient CH_4-C turnover. This would
320 require metabolic fine-tuning of MOB and non-MOB partners to optimally distribute and
321 assimilate the CH_4-C. Overall, reports on co-cultivation influencing MOR (Ho et al. 2014; Jeong
322 et al. 2014) are more scarce than reports on increased growth (biomass yield) of the
323 methanotrophic *interactome* as compared to a pure culture (Bothe et al. 2002; Hrsak & Begonja
324 2000; Jeong et al. 2014; Stock et al. 2013; Wilkinson et al. 1974), which could indicate that
325 although MOR and biomass increase are highly correlated for axenic MOB cultures (Hoefman et
326 al. 2014a), a decoupling of these processes may occur within the methanotrophic *interactome*.
327 Hence the use of an *interactome* rather than axenic MOB may not be advantageous if CH_4

328 removal is the desired application (e.g. CH_4 bio filtration), though it may be advantageous in
329 cases where overall biomass production is the goal (e.g. SCP or PHB production from CH_4).

330 **3. On the mechanism of partner selection: clues from genome mining**

331 Although the underlying mechanisms governing the specificity of non-MOB partner selection
332 remained inconclusive, we found several clues on the ecological significance of the observed
333 partner selectivity through comparative genomics. This was greatly facilitated by the synthetic
334 ecology approach in this work which allowed the use of strains with full genome availability.

335 A thorough comparison of genes encoding for different methylotrophy modules (compiled from
336 Chistoserdova (2011) and RAST scenario's/subsystems) showed limited distinctive genes in any
337 module investigated (Dataset S1). In primary oxidation modules, pyrroloquinoline quinone
338 (PQQ) synthase, a cofactor of (methanol) dehydrogenases, was found to be primarily restricted
339 to persisting partners (except for *R. radiobacter* LMG 287). No corresponding genes for
340 methanol oxidation were found in any non-MOB partner except *P. denitrificans* LMG 4049. The
341 promiscuous partners exclusively encoded for presumed oxidation modules for methylated sulfur
342 species (methylsulfonates, dimethylsulfide and dimethylsulfoniopropionate). Finally, both *P.*
343 *denitrificans* LMG 4049 and *A. denitrificans* LMG 1231^T were the only non-MOB encoding for
344 a primary oxidation module for methylamine. No further discrimination between persisting and
345 non-persisting partners could be made based on genes for C1-metabolism (Dataset S1).
346 However, other differences in carbon metabolism could be observed: D-threo-aldose-1-
347 dehydrogenase (E.C. 1.1.1.122), an enzyme involved in fructose and mannose metabolism, was
348 only encoded by persisting partners. That was also the case for a gene encoding glucoamylase
349 (E.C. 3.2.1.3), involved in starch and sucrose metabolism. These genes may be involved in

metabolism of sugars derived from soluble extracellular polymeric substances (EPS) synthesized by the MOB (van der Ha 2013; Wei et al. 2015; Wilshusen et al. 2004); extremophile MOB have even been reported to directly produce sucrose (But et al. 2015; Khmelenina et al. 2015; Medvedkova et al. 2007). Several genes involved in β-alanine biosynthesis and degradation were also found to be restricted to persisting partners.

Nearly all non-persisting partners (except *O. anthropi* LMG 2134) encoded for an ATP-dependent cobalamin importer (Vitamin B12 ABC transporter, ATPase component BtuD) in their genome, while all the persisting partners were lacking this gene. This may indicate selection against partners competing with the MOB for cobalamin. Interestingly, only the genome of *M. methanica* NCIMB 11130^T was found to encode for this gene, while it was not observed in *Methylosinus* sp. LMG 26262. Cobalamin has previously been shown to stimulate gammaproteobacterial methanotrophs (Iguchi et al. 2011), although there was only a weak effect for *Methylomonas methanica* S1. A gene encoding cobalamin synthase (E.C. 2.7.8.26) was encoded by all non-MOB partners (except for *A. denitrificans* LMG 1231^T), but not by the MOB. Regardless of the gene inventory, measurement of cobalamin synthesis under the current growth conditions should be performed to assess gene expression (and cobalamin export). However, the differential presence of a cobalamin transporter in persisting versus non-persisting partners adds to the increasing evidence that cobalamin is of importance for biological methane oxidation (Hoefman et al. 2014b; Iguchi et al. 2011; Iguchi et al. 2015; Lamb & Garver 1980) and its role requires further in-depth investigation.

Conclusion & Perspectives

We have shown that co-cultivation of non-MOB partners reduced the variability in MOR that was observed with axenically-grown MOB. Furthermore, the co-cultivation with the

alphaproteobacterial methanotroph *Methylosinus* sp. LMG 26262 had a higher biomass yield (measured as total protein) as compared to a pure culture, however this effect was not observed for the gammaproteobacterial MOB *Methylomonas methanica* NCIMB 11130^T. Of the 8 non-MOB partners that were initially added to both MOB, only 4 could be detected after the first sub-cultivation, showing selectivity of the MOB towards their non-MOB partners. The underlying mechanisms for partner selection still need to be resolved. Therefore, we searched the genomes of the non-MOB for clues on possible driving mechanisms of MOB- non-MOB interactions. Indeed, the availability of the full genome of each constituent strain (as in this experiment) is advantageous for other 'omics' applications to further unravel how gene-expression (metatranscriptomics) and translation to proteins (metaproteomics) is influenced by repeated co-cultivation and possible adaptation of MOB and their partners in a methanotrophic *interactome*. Ultimately, these findings suggested some interesting approaches for future synthetic ecology experiments which could be used to generate an appropriate model of the methanotrophic *interactome* (Jagmann & Philipp 2014; Larsen et al. 2012; Widder et al. 2016). This modeling could supply the tools for adequate microbial resource management (Read et al. 2011; Verstraete et al. 2007) for increased *recovery* of the carbon and energy harnessed in CH_4 rather than merely converting CH_4 to CO_2 (Verstraete 2015).

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AUTHOR'S CONTRIBUTIONS

F.-M. Kerckhof & C. De Rudder designed and performed the experiment, analyzed the data and wrote the manuscript. R. Props performed statistical data analysis. A. Ho, K. Heylen & N. Boon designed the experiment and performed a critical review of the manuscript.

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Figure 1 (on next page)

Community structure heat map by 16S rRNA DGGE.

Representative band classes were assigned to the MOB and each non-MOB partner. Each cell represents the average relative band intensity over all biological replicates (n=3 for pure MOB and n=4 with non-MOB partners, n=1 if cycle="N"). The color intensity of the cells is proportional to the relative abundance. Cycles 1 through 5 are indicated with their corresponding number, and "N" indicates amplicon of an axenic culture was loaded in the lane. "+8 HET" indicates co-cultivation incubations with 8 initial non-MOB partners (as described in the materials & methods section).

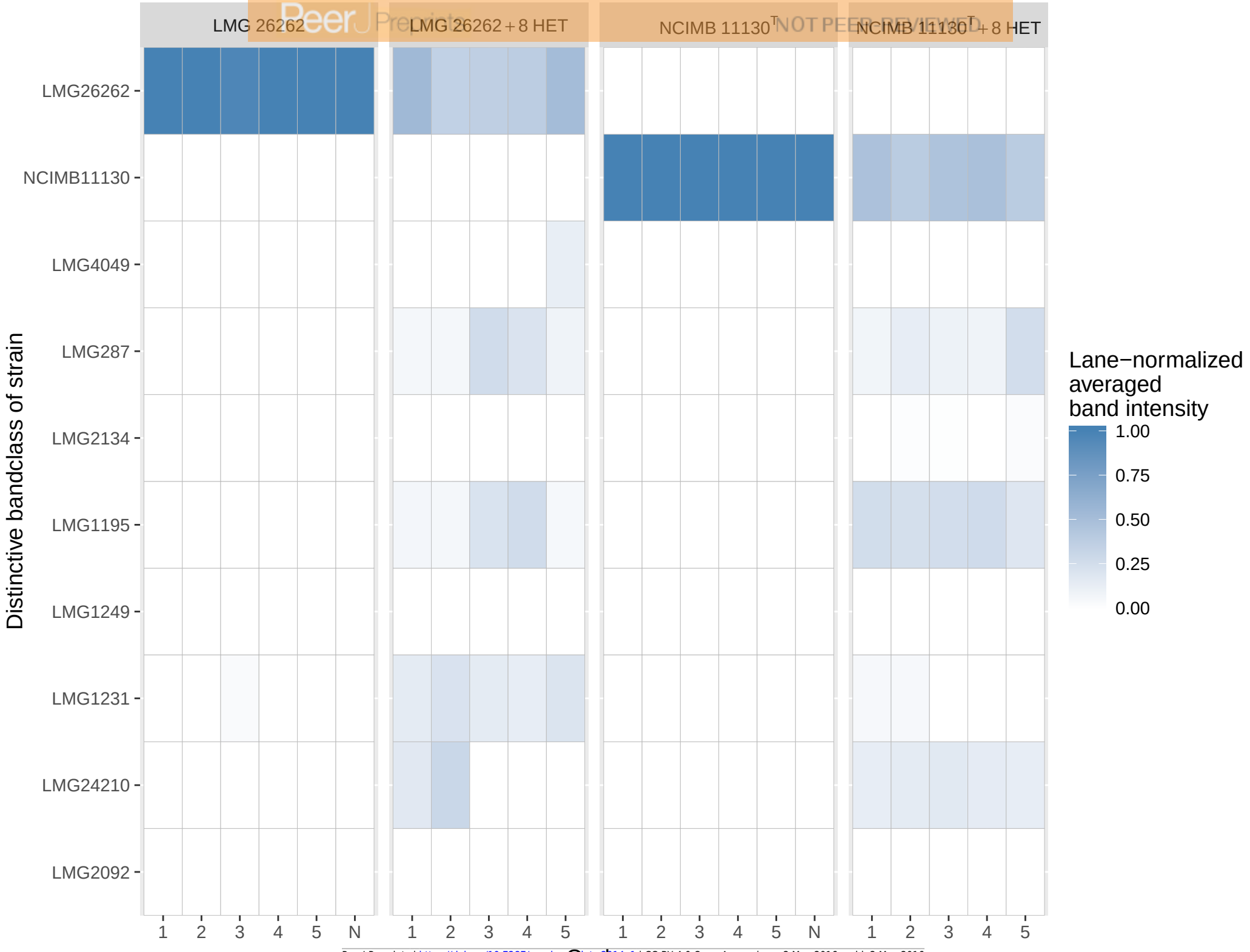

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Figure 2(on next page)

Stabilization of methane oxidation rates (MOR) by co-cultivation.

The stabilization of MOR is visualized by the ratio of Variance-to-Mean ratios (VMR) of the MOR from the co-cultivation of *Methylosinus* sp. LMG 26262 or *M. methanica* NCIMB 11130^T with eight heterotrophic partners over the MOR from the axenic MOB incubations. A dashed line represents a ratio of 1 in which case the relative MOR variance with partners is as high as without partners. Values below the dashed line indicate a lower VMR when the MOB is accompanied by the partners.

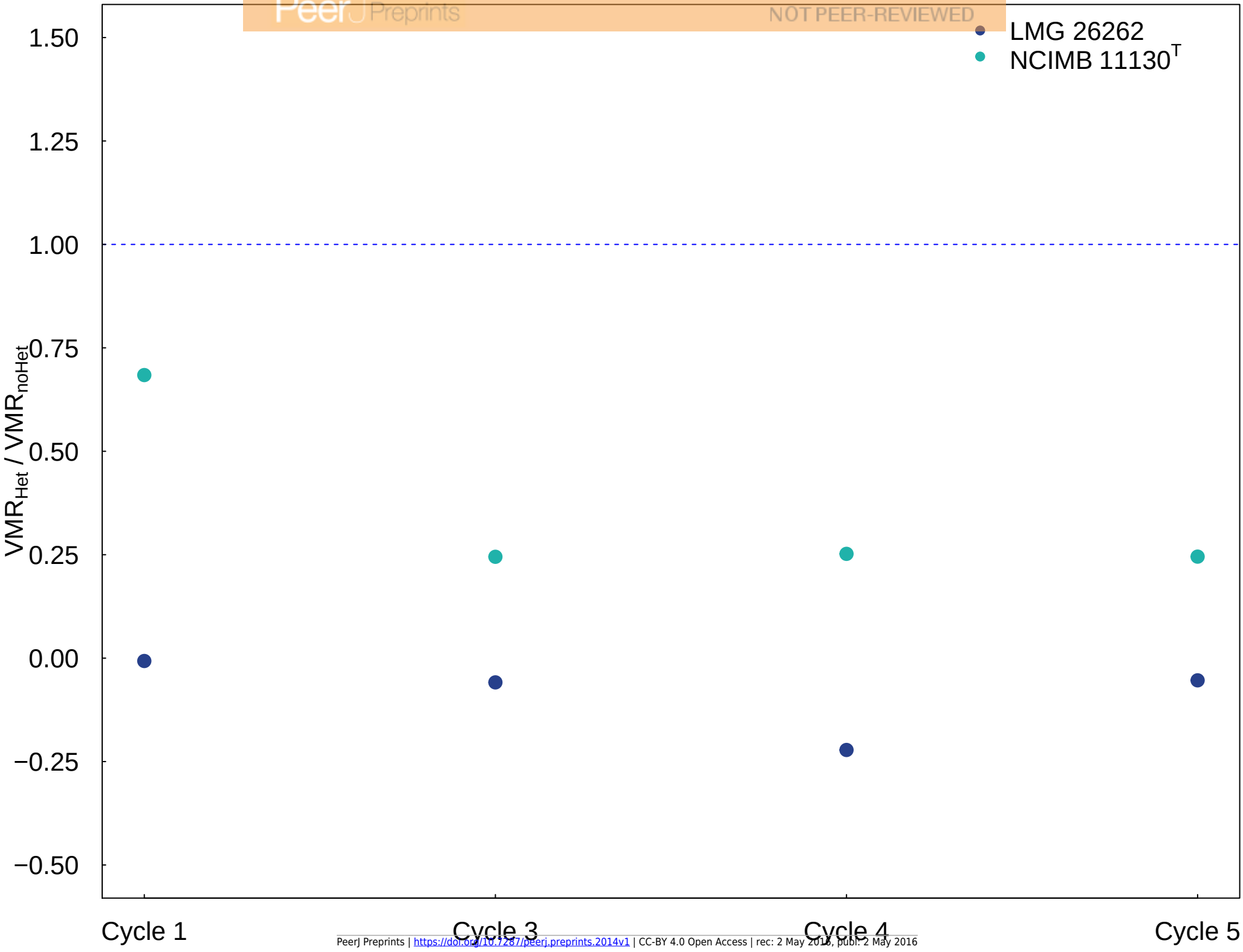

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Figure 3(on next page)

Box- and whisker plots of carbon ratio of CO_2-C formed per mL of culture over mg total protein per mL of culture at the end of each cycle.

No headspace or protein measurements were performed for cycle 2. The heading of each group of boxplots describes the treatment. Strain numbers of the MOB alone represent axenic culture conditions. “+8 HET” designates co-culture with 8 non-MOB partners (as described in the materials & methods section).

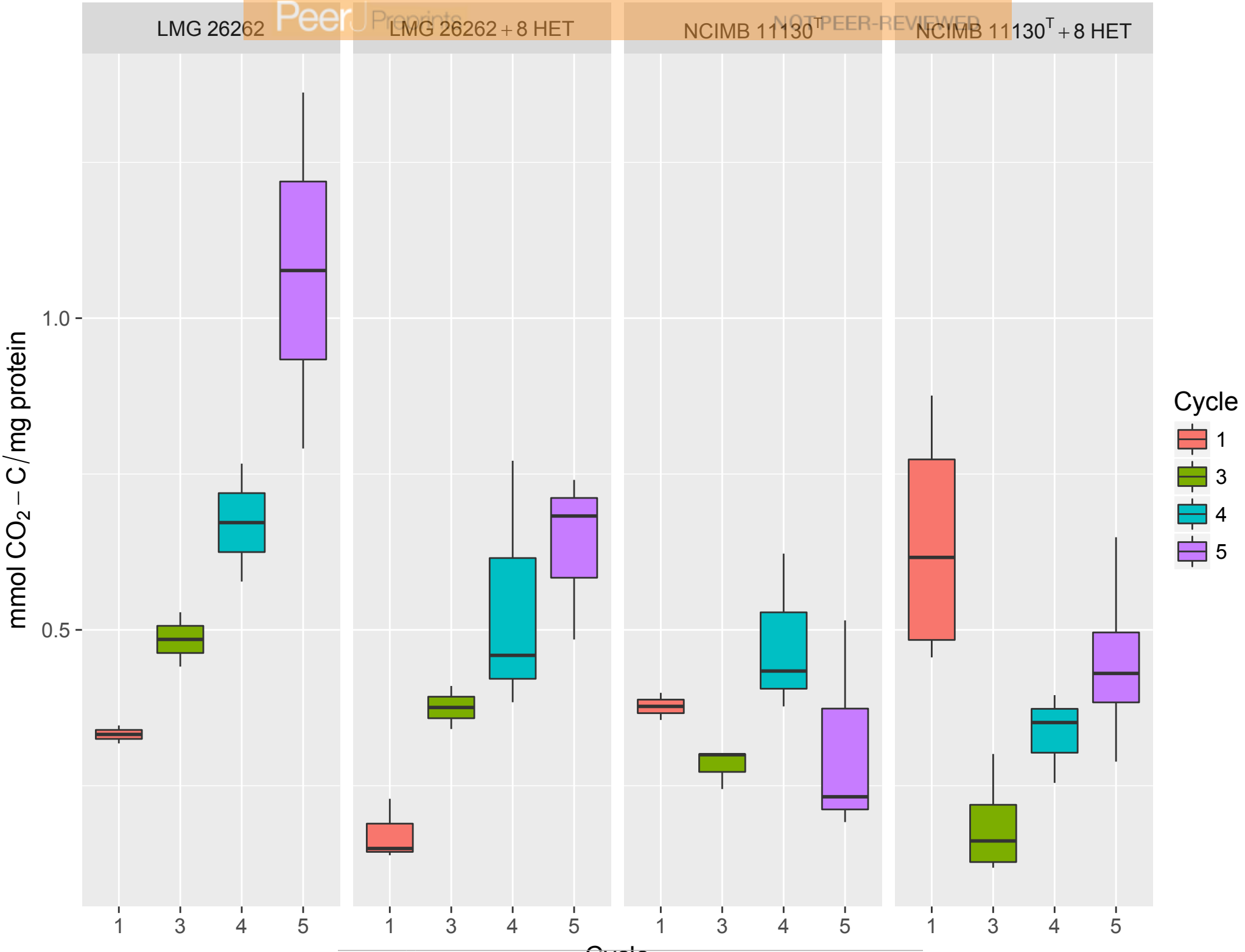

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Table 1(on next page)

Primary consumer strains used in the co-cultivation experiment (Ho et al., 2014).

([a]) “yes”: restricted facultative (obligate) or facultative methylotrophy is described within the species; “yes for strain”: genomic evidence shows possibility of methylotrophy (Chistoserdova 2011), within confirmed methylotrophic species; “unclear”: contradictory information was found in the literature, the genome, or both; “no”: no evidence for methylotrophy was found in literature or in the genome

1

Strain	Strain number	Methylotrophic ([a])	Genome source
Paracoccus denitrificans	LMG 4049	Yes for strain	IMG/ER: tax ID 2597490357
Rhizobium radiobacter	LMG 287	No	GOLD project: Gp0000707
Ochrobactrum anthropi	LMG 2134	No	GOLD project: Gp0000090
Cupriavidus metalluridans	LMG 1195^T	No	GOLD project: Gp0000357
Comamonas terrigena	LMG 1249	No	GOLD project: Gp0023602
Achromobacter denitrificans	LMG 1231^T	unclear	GOLD project: Gp0033444
Pseudomonas putida	LMG 24210	No	GOLD project: Gp0000136
Escherichia coli	LMG 2092^T	No	GOLD project: Gp0110161

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