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Do temporal and spatial heterogeneity modulate biodiversity–functioning relationships in com-munities of methanotrophic bacteria?

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ABSTRACT

Positive relationships between biodiversity functioning have been found in communities of plants but also of soil microbes. The beneficial effects of diversity are thought to be driven by niche partitioning among community members, which leads to more complete or more efficient community-level resource use through various mechanisms. An intriguing related question is whether environmentally more heterogeneous habitats provide a larger total niche space and support stronger diversity—functioning relationships because they harbor more species or allow species to partition the available niche space more efficiently. Here, we tested this hypothesis by assembling communities of 1, 2 or 4 methanotrophic isolates and exposing them to temporally (constant or diurnal temperature cycling) and structurally (one or two aggregate size classes) more heterogeneous conditions. In total, we incubated 396 microcosms for 41 days and found that more biodiverse communities consumed more methane (CH_4) and tended to have a larger community size (higher *pmoA* copy numbers). Diurnal temperature cycling strongly reduced CH_4 oxidation and growth, whereas soil aggregate composition and diversity had no detectable effect. Biodiversity effects varied greatly with the identity of the community members that were combined. With respect to community level CH_4 consumption, strain interactions were positive or neutral but never negative, and could neither be explained by 14 structural and function traits we collected or by the observed competitive hierarchy among the strains. Overall, our results indicate that methanotrophic diversity promotes methanotrophic community functioning. The strains that performed best varied with environmental conditions, suggesting that a high biodiversity is important for maintaining methanotrophic functioning as environmental conditions fluctuate over time.

1. Introduction

Studies with different groups of organisms and ecosystem types (Cardinale et al., 2012; O'Connor et al., 2017) have demonstrated that biodiversity promotes essential ecosystem functions such as resource uptake, biomass production and nutrient cycling. While such biodiversity-ecosystem functioning (BEF) relationships appear near-universal, an important related question is how much biological diversity a particular ecosystem can harbor. Ecological theory predicts that the number of species found in a given environment increases with available niche space. One important factor related to niche space is environmental heterogeneity. More heterogeneous habitats can provide a broader range of resource types and are structurally more complex (Stein et al., 2014). More heterogeneous habitats could therefore support stronger BEF relationships because they harbor more species (Jessup et al., 2005), and also because the larger total niche space available allows for more distinct realized niches and therefore a higher niche complementarity among the organisms present in the system (Dimitrakopoulos and Schmid, 2004; Tylianakis et al., 2008; Griffin et al., 2009). Environmental heterogeneity can further provide refugia to organisms during disturbance, thereby promoting species persistence and stabilizing ecosystem functioning over time (Hector et al., 1999; Loreau and de Mazancourt, 2013; Stein et al., 2014). However, the relationship between environmental heterogeneity and BEF

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relationships is currently not well investigated (Cardinale et al., 2012).

Most BEF research to date has focused on organisms that are predominantly above ground, such as plants or larger animals, and relatively little is known about the extent to which these relationships apply to soil microbial systems. Microorganisms drive important biogeochemical processes, including the decomposition of organic matter and nutrient cycling. Soil microbes are also critical regulators of greenhouse gas fluxes and are therefore important in the context of climate change (Bodelier and Steenbergh, 2014). The overall diversity of microorganisms in soils is typically very high, with 5000 to 20,000 species per gram of soil (Torsvik et al., 1990; Sogin et al., 2006; Roesch et al., 2007), suggesting a high degree of functional redundancy in soil microbial communities. However, microbial functions are taxonomically very diversified and soils are environmentally very heterogeneous. The diversity of organisms performing a given function in a given spatial niche may therefore be relatively low. Studies in which microbial diversity has been directly manipulated only recently began to emerge, and their results are controversial. Some studies have shown that loss of diversity reduces ecosystem functioning (e.g. Bell et al., 2005; Replansky and Bell, 2009; Salles et al., 2012; Philippot et al., 2013; Delgado-Baquerizo et al., 2016; Schnyder et al., 2018), while others have found no relationship between microbial diversity and ecosystem functions (e.g. Griffiths et al., 2000, 2001; Seghers et al., 2003; Wertz et al., 2006, 2007). Other studies have even suggested that community-level functioning may decrease in more diverse microbial systems due to an increased frequency of antagonistic interactions between microbial strains (Jousset et al., 2011; Becker et al., 2012). Overall, this suggests that the mechanisms mediating BEF relationships in microbial communities differ across scales and taxa.

The additive partitioning methods by Loreau and Hector (2001) and extensions of this scheme (Fox, 2005) have been used to get a handle on the mechanisms that promote BEF relationships. These methods are based on relative yields (RY), which is the yield of a species in a mixture relative to the yield of its monoculture. By analyzing patterns of RYs in mixtures, it is possible to partition the extra performance of species mixtures relative to the average monoculture (the so-called net biodiversity effect) into complementarity and selection effects. Complementarity effects generally are associated with beneficial effects of growing in mixture that are spread over many species. In contrast, selection effects typically indicate that a single species (or a few) dominates community functioning at the expense of other species. These methods have been successfully applied to plant communities. However, the application of these methods to microbial communities is usually impossible because the variable of interest (biomass, activity) cannot be measured at the species level, or only with complicated and expensive experimental procedures that are also prone to large errors (e.g. stable isotope probing, sequencing). An alternative approach, which to our knowledge has not been used to study species interactions in microbial communities, is mechanistic diallel analysis. Diallel analysis is commonly used in crop breeding experiments and allows the contributions of parental lines to the performance of crosses to be quantified (Griffing, 1956). Given a suitable study design, biodiversity experiments can be analyzed as a mechanistic diallel in which the species combined in a mixed community correspond to the parental alleles. In this framework, monocultures are considered to be a special case in which both of the "parents" are contributing the same allele. An advantage of this approach over additive partitioning methods is that the performance of mixtures only needs to be measured at the community level rather than at the species level, i.e. it avoids the difficulties encountered when applying the additive partitioning method. In diallel analysis, net biodiversity effects are then decomposed into "general combining abilities" (GCA) and "specific combination abilities" (SCA) by fitting the model $y_{a,b,i} \sim GCA_a + GCA_b + SCA_{a,b} + \varepsilon_{a,b,i}$, where $y_{a,b,i}$ is the performance of a mixture of strains a and b (where a equals b for monocultures) in replicate i, GCA_n is the average contribution of strain n to mixture performance over the entire study, $SCA_{a,b}$ is the average additional contribution of the specific strain combination a-b, and $\varepsilon_{a,b,i}$ is the corresponding residual. GCAs, SCAs, and their dependence on additional factors can then be analyzed using standard linear models. SCAs quantify community-level benefits of combining two species on an absolute scale, after adjustments for interaction-independent overall effects of the species by the GCAs. SCAs can then be related to properties of these species, testing hypotheses such as whether effects of species interactions are more positive if these are more dissimilar in traits.

Here, we use experimental communities of methanotrophic bacteria to investigate effects of their diversity on their growth and activity. Methanotrophic bacteria are obligate aerobic bacteria that use methane (CH_4) as their primary carbon and energy source. All known aerobic methanotrophic bacteria belong to either the Gammaproteobacteria (type I), Alphaproteobacteria (type II), Verrucomicrobia (Type III; McDonald et al., 2008; Knief, 2015), or a novel type of methanotrophs recently discovered in Actinobacteria (van Spanning et al., 2022). Methanotrophs live in a wide variety of environments, including wetlands, lakes, oceans, soils and landfills (Conrad, 2007). They are one of the major sinks for atmospheric CH_4 and play an important role in regulating CH_4 emission from soils and wetlands by oxidizing CH_4 produced in the anoxic layers of these environments before it enters the atmosphere. They therefore play a key role in the CH_4 cycle and the associated greenhouse effect (Canadell et al., 2021). In the present study, we used eight isolates of methanotrophic bacteria that we systematically combined in a microcosm experiment, creating communities with one, two or four strains. We included two factorial experimental treatments in the study to vary spatio-temporal heterogeneity in environmental conditions, hypothesizing that BEF relationships would be stronger in systems that offer more colonizable niche space. The first treatment addressed effects of spatial heterogeneity. An important determinant of soil heterogeneity is aggregate structure. Methanotrophs are typically active on aggregate surfaces (Stiehl-Braun et al., 2011; Rime and Niklaus, 2017) where they have access to CH_4. On the other hand, they may be better protected from predation or desiccation if they are further inside aggregates. Furthermore, methanotrophs have been found to differ in their kinetics of CH_4 oxidation, suggesting that they may occupy different spatial niches along gas diffusion gradients. In our study, we manipulated soil aggregate structure by creating treatments with different numbers of aggregate size classes (an "aggregate diversity treatment"). The second treatment consisted of incubation temperatures that were either constant or cycled diurnally. The methanotrophic strains we used differed in their optimum growth temperatures, and we reasoned that a temperature cycle would provide growth opportunities for different strains at different times, i.e. temporal complementarity. The experimental microcosms were incubated for several weeks and CH_4 consumption was measured regularly. We determined the community size by copy number of a characteristic functional gene (*pmoA*, encoding a subunit of particulate methane mono-oxygenase) and community composition by next generation sequencing of the *pmoA* gene. The overall objectives of our study were to test whether (1) methanotroph diversity promotes CH_4 consumption and methanotroph growth, (2) environmental heterogeneity (in space and time) enhances these ecosystem functions, and (3) to analyze species interactions underlying the observed BEF relationship.

2. Materials and methods

2.1. Experimental setup

We set up a replicated microcosm experiment factorially combining a methanotroph species richness treatment, a soil aggregate diversity treatment, and a temperature treatment. The goals of the aggregate diversity and temperature treatments were to create a greater diversity (heterogeneity) in environmental conditions (colonizable niche space), either spatially or temporally.

The experiment was conducted in microcosms made from 50 mL

centrifuge tubes (Sarstedt, Nümbrecht, Germany), which were sealed with gas-permeable cotton plugs to minimize the risk of cross-contamination. These microcosms contained a matrix of 4 g γ-sterilized soil collected from a calcareous meadow (Nenzlingen, Switzerland; 47° 33′ N, 7° 34' E, 520 m a.s.l.; silty clay loam soil, 41% clay, 52% silt 3.9% C, 0.33% N, pH ≈ 7,6; Niklaus et al., 2003), which has a very low abundance of methanotrophs. The relative abundance of the *pmoA* gene measured by qPCR in the Nenzlingen soil matrix was <0.01% of that measured in inoculated microcosms, indicating that potentially remaining background DNA in the soil matrix did not interfere with the analysis of active methanotrophic communities.

The methanotroph species richness treatment consisted of communities assembled from two pools of four pure strains of methanotrophic bacteria each (Table 1). Each pool contained methanotrophs that were phylogenetically and functionally distinct, at least relative to the variation present in the total set of strains. Prior to the experiment, all strains were grown in liquid batch cultures in nitrate mineral salt medium (NMS medium; Dedysh et al., 1998). The liquid batch cultures consisted of 100 mL serum flasks with 20 mL NMS medium and 20 mL CH_4 in the headspace. These cultures were maintained at 25 °C (except for *M. capsulatus* at 36 °C) until the cultures reached the stationary phase of growth, after which they were stored at 4 °C. We measured the cell density of all pure cultures (CASY TTC cell counter, Roche Innovatis AG, Germany) and diluted them with NMS medium to approximately 10×10^6 cells mL^{-1}. These cultures of standardized cell density were then combined within each pool to create communities of three different diversity levels: four strain monocultures, six possible two-strain mixtures, and the mixture of all four strains. The amounts of pure culture we mixed were inversely proportional to the target strain richness. We added a total of 0.9 mL of these culture solutions (approx. 9×10^6 methanotroph cells) along with 0.5 mL NMS medium to each microcosm.

The microcosms contained matrix material of different structure. We separated soil aggregates from the γ-sterilized soil by sieving into two size fractions: 0.5–2 mm (S: small) and 2–4 mm (L: large). Some microcosms contained only one type of aggregate (one size fraction: S or L), while other microcosms contained a mixture of two aggregates types (two size fractions: S and L). We incubated the microcosms in two separate incubators, both of which had an average temperature of 23 °C. However, in the first incubator the temperature was kept constant, while in the second incubator the temperature was ramped up from 18 to 28 °C in 12 h, and then down to 18 °C in another 12 h. In total, we incubated 132 different community composition × treatment combinations (2 pools × 11 strain compositions × 3 aggregate compositions × 2 temperature treatments). For each of these combinations, there were three replicate microcosms (resulting in a total of 396 microcosms), plus an additional replicate that was frozen at −80 °C immediately at the start of the experiment (total of 66 communities; no temperature treatment applied). Every two weeks, one replicate was removed and frozen for later DNA extraction. This resulted in samples for days 0, 13, 28, and 41 from the start. The study also included controls with sterile soil only (negative control), two for each temperature treatment, which were harvested with the other samples on days 0 and 41.

Within the incubators, microcosms were maintained in airtight boxes connected to a custom-built system that measured and controlled CH_4 concentrations. Headspace gas was pumped through a tube filled with silica gel beads to dry the sample before CH_4 concentrations were determined in a cell equipped with a CH_4 detector (TGS 2611, Figaro Inc., Arlington Heights, IL). A mixture of 5% CH_4 and 10% O_2 in N_2 was added through a solenoid valve when CH_4 concentrations fell below a threshold. The readings from the semiconductor gas sensor were not very accurate; therefore, we used the same sensor and electronics to control the CH_4 concentration in both incubators, periodically switching between incubators with a solenoid valve to ensure that CH_4 concentration did not differ systematically between temperature treatments. CH_4 concentrations were additionally monitored by taking headspace samples from the boxes with a syringe 1–2 times per day and measuring the concentration by gas chromatography. CH_4 concentrations averaged 6200 μmol CH_4 mol^{-1} throughout the experiment (gas chromatographic analysis).

2.2. CH_4 consumption and CO_2 production

The CH_4 consumption and CO_2 production of each individual microcosm was determined by removing them from the incubators and placing them in an airtight 3 L jar with 1% CH_4 in the headspace. Over a

Table 1
Methanotrophic strains used to assemble the artificial communities and their traits. Four strains each were organised in a pool within which all possible community compositions consisting of 1, 2 or 4 strains were realized. The trait were taken from Table 1 in Schnyder et al. (2018) and Oshkin et al. (2020; genomic information for M. hirsuta and M. sporium). Code is the three-letter abbreviation used in the Figures; sMMO: soluble methane mono-oxigenase; PLFA_PCoA: ordination axis values in principle coordinate analayis of PLFA profiles.

Trait	Pool 1				Pool 2			
	Methylobacter luteus ATCC 49878 T	*Methylocystis echinoides* IMET 10491	*Methylocystis hirsuta* CSC1	*Methylosinus sporium* NCIMB 11126	*Methylococcus capsulatus* Bath	*Methylocystis parvus* OBBP	*Methylocystis rosea* SV97	*Methylosinus trichosporium* OB3b
Code	mbl	mce	mch	mss	mcc	mcp	mcr	mst
Type	I	II	II	II	I	II	II	II
Motility	No	no	no	yes	no	no	no	yes
Fixes N_2	No	yes	yes	yes	yes	yes	yes	yes
Cell length (μm)	2.1	1.25	0.85	2.05	1.25	1	1.75	2.5
Cell width (μm)	1.15	0.6	0.45	0.8	0.9	0.4	0.8	1
Has sMMO	no	no	yes	yes	yes	no	yes	yes
Forms aggregates, chains, or rosettes	Yes	no	no	yes	yes	yes	yes	yes
pH optimum	6.6	7.0	7.0	6.6	–	7.0	5.5	6.6
Temperature optimum	30	27	30	30	42	28.5	23	28.5
Genome size (Mbp)	5.1	–	4.2	3.8	3.3	4.5	3.9	4.9
G + C mol%	50	62	62.4	63	63.6	63.4	63	66
Initial growth rate (d^{-1})	0.182	0.503	0.0	0.172	0.042	0.233	0.076	0.247
PLFA_PCoA1	−37.06	31.25	36.01	−27.04	−31.21	24.41	−27.84	37.63
PLFA_PCoA2	−26.48	−4.09	−6.78	39.83	−33.47	1.53	56.78	−11.43

period of 24 h, three 25 mL headspace samples were collected and analyzed for CH_4 and CO_2 concentrations by gas chromatography (Agilent 7890N gas chromatograph; CH_4 was detected with a flame ionization detector; 12' Porapak Q column; isothermal at 80 °C; He carrier gas; CO_2 was determined on the same detector after reduction of CO_2 with H_2 on a Ni-catalyst; Agilent Technologies Inc., Santa Clara, CA). The microcosms were then returned to the incubators. Gas exchange rates were calculated by linear regression of headspace concentrations against sampling time, with consumption rates converted to μmol microcosm^{-1} using the ideal gas law.

2.3. DNA extraction

DNA was extracted from replicates collected at days 0, 28 and 41 using the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA) according to the manufacturer's instructions. The concentration of extracted DNA was measured fluorometrically using the Qubit dsDNA BR Assay Kit (ThermoFischer, Waltham, MA) on the Spark 10M Multimode Microplate Reader (Tecan, Männedorf ZH, Switzerland) using a standard curve (0–100 ng/uL DNA). Samples were then diluted to 1 ng DNA μL^{-1}.

2.4. qPCR of pmoA gene

Methanotrophic community size was quantified as *pmoA* gene copy number ((subunit A of the particulate methane mono-oxygenase gene; primers A189F and mb661; Holmes et al., 1995; Costello and Lidstrom, 1999) using quantitative PCR (StepOne real-time PCR system Applied Biosystems, Foster City, CA). A serial dilution of purified DNA from *Methylococcus capsulatus* (quantified with the Qubit Fluorometer, Invitrogen, Carlsbad, CA) was included in duplicate in each run to determine the calibration curve. In addition, we included reference samples of DNA from *Methylococcus capsulatus* on all plates to standardize between plates using the geometric mean (Ruijter et al., 2015).

2.5. Sequencing of pmoA gene

To assess changes in methanotrophic community composition, we amplified the *pmoA* gene (using primers A189F/mb661 with universal sequences CS1 and CS2 for later extension with sequencing adapters and indices). PCR products were purified (GeneJET PCR purification kit, Thermo Scientific, Waltham, MA) and quantified fluorometrically (Qubit dsDNA BR assay kit, ThermoFischer, Waltham, MA) using a standard curve (0–100 ng/uL DNA). Samples were barcoded using the Fluidigm Access Array technology and paired-end sequenced using the Illumina MiSeq v3 platform at the Genome Quebec Innovation Center, Montreal, Canada.

Analysis of the *pmoA* gene sequences was performed according to a published pipeline (Frey et al., 2016), using algorithms implemented in USEAECH v9 (Edgar, 2010), unless otherwise stated. Forward and reverse read pairs were merged using the fastq_mergepairs algorithm (Edgar and Flyvbjerg 2015, minimum length = 500 bp, minimum overlap = 50 bp and max. Number of mismatches = 20), and primer sequences were trimmed using Cutadapt (Martin, 2011). The trimmed sequences were then quality filtered using the fastq_filter function (Edgar and Flyvbjerg, 2015 maximum expected error = 1). Since we knew the strains present, we did not perform OTU clustering and taxonomic assignment but instead mapped all quality-filtered reads against the known *pmoA* sequences of the methanotroph strains used in this experiment (sequences were retrieved from GenBank but verified by Sanger-sequencing the pure methanotroph cultures) using the usearch_global algorithm (Edgar, 2010; options id = 93, maxrejects = 0, maxaccepts = 0, and top hit only). We counted the number of matched sequences of each strain in each sample and calculated realized strain richness (i.e. the number of strains found in the sample, which may differ from the original richness of the inoculum). For this calculation, we only counted strains with a relative abundance greater than 2%. We also calculated the Shannon diversity index (H') and from this the effective strain richness ($e^{H'}$), which gives a lower weight to the low abundant strains.

2.6. Statistical analysis

We used analysis of variance based on general linear models (R 4.2; http://r-project.org) and linear mixed models (ASReml-R 4.1, VSNI, Hemel Hempstead, UK) to test for effects of the experimental treatments strain diversity, soil aggregate diversity, temperature regime, and their interactions on the dependent variables CH_4 consumption and community size. All strain richness metrics were *log*-transformed, which places the diversity treatments (richness of 1, 2 and 4) at regular distances from each other and is compatible with the positive decelerating shape of BEF-effects that is often found. We included pool in the model (two-level factor), similar to how blocks are fitted in linear models. Strain composition was fitted as a random term, as it is the unit of replication for testing diversity effects (Schmid et al., 2017).

PCR amplification is an exponential process, which leads to a corresponding error distribution. To counteract this effect, one could *log*-transform the data. However, this would put all values on a relative scale and cause problems by giving undue weight to differences between small numbers; for example, the difference between 1 and 2 would have the same weight than the difference between 1000 and 2000, even though the second is clearly much larger. To balance these two effects, we therefore analyzed *pmoA* copy numbers after square-root transformation.

Mechanistic diallel models were fitted separately for each combination of strain pool, temperature, and soil aggregate composition, except for the 4-strain mixtures that we excluded from this analysis. These models have the form $y_{ab} = GCA_a + GCA_b + SCA_{ab}$, where GCA and SCA are the general and specific combining abilities of the mixture of strains "a" and "b". Strain monocultures are treated as a special case in which the two components of the mixture are identical, i.e. as $y_{aa} = 2\,GCA_a + SCA_{aa}$. Note that this model has no overall intercept. The term GCA occupies 4 columns in the model matrix (one for each strain contained in the respective pool) and coefficients of 2 for monocultures and two coefficients of 1 for the 2-strain mixtures. Specific combining abilities were estimated as deviations of observations from predictions based on GCA, i.e. as residuals. Because the soil aggregate composition treatment had no significant effect, we used these three replicates to calculate means and standard errors of GCA and SCA.

To analyze the relationship of GCA to the traits of the methanotrophic strains, we first replaced cell length and width by cell volume (assuming an ellipsoid shape) and a shape parameter (length/width). We then created Gower distance matrices for traits, after standardizing the traits to a uniform range and replacing missing values by means (function "vegdist" from the "vegan" library). We then tested for an association of trait distances with GCA distances using Mantel tests. We proceeded similarly for SCAs, testing for an association of the matrix containing the SCAs with the trait distance matrix.

3. Results

3.1. Community compositions

Of the total 19×10^6 reads obtained through sequencing, 7×10^6 could be mapped to one of the eight strains used to create the communities. This corresponds to $(2.1 \pm 0.5) \times 10^4$ valid *pmoA* sequences per microcosm. Importantly, we found no evidence that strains other than the ones inoculated had grown in the microcosms; specifically, >98% of the quality-filtered sequences mapped to the eight target strain sequences, and sterile control microcosms, which we had included as negative controls, did not show any measurable methane consumption.

The strains that we recovered on day 0 showed that we had achieved

the intended community composition. The experimental gradient in methanotroph diversity was maintained throughout the incubation (Pearson's product moment correlation r = 0.67 and 0.53, P < 0.001 for realized and effective strain richness, respectively; Fig. 1). Very few strains became extinct during the course of the study; at the end of the study, *Methylobacter luteus* was lost from two two-strain and two four-strain mixtures, and *Methylosinus sporium* was lost from one four-strain and one two-strain mixture. Realized strain richness exceeded one in some monocultures, probably due to cross-contamination during DNA extraction and analysis in the laboratory.

3.2. CH_4 consumption and CO_2 production

The analysis of CH_4 consumption and CO_2 emissions showed essentially the same patterns. Therefore, we present results for CH_4 only. We integrated the CH_4 fluxes over the entire experimental period, linearly interpolating between adjacent time points. Temperature cycling caused a large reduction in soil CH_4 oxidation compared to constant temperature ($F_{1,41} = 33$, $P < 10^{-6}$; Fig. 2), whereas neither soil composition ($F_{2,127} = 0.28$; $P = 0.8$) nor soil aggregate diversity ($F_{1,127} = 0.44$; $P = 0.5$) significantly affected CH_4 oxidation (Fig. 3). Also, the residual variances were much lower under cycling temperature. Therefore, we analyzed the two temperature treatments separately. Under constant temperature, CH_4 consumption was significantly higher in mixed-strain communities than in monocultures. Due to the large variation in CH_4 consumption between strain monocultures, this effect was not significant when tested using linear models with *log*-transformed strain richness (Fig. 2; $F_{1,19} = 2.8$, $P = 0.11$). However, when adjusting for the monoculture differences by analyzing the net biodiversity effects of the mixtures, the differences were statistically significant (Fig. 4; $t_{13} = 3.6$, $P = 0.003$). Under temperature cycling, no effect of strain richness was detected ($F_{1,19} = 0.003$, $P > 0.9$ and $t_{13} = -0.8$, $P = 0.46$).

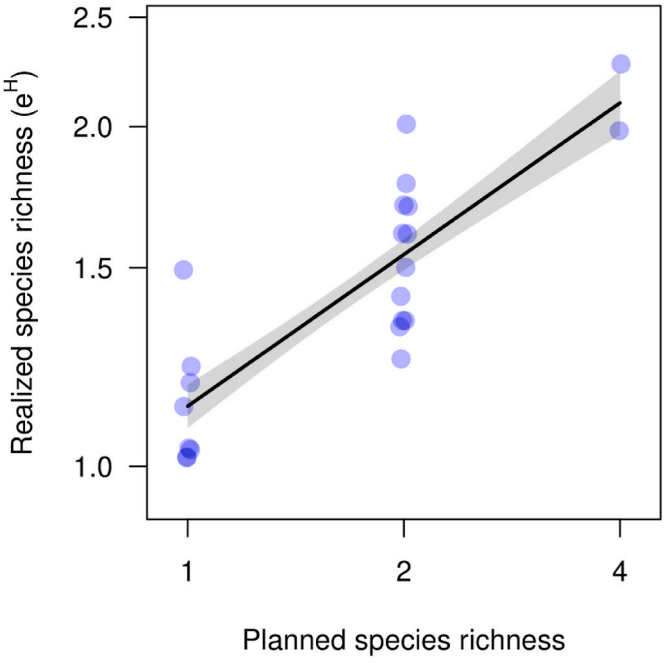

Fig. 1. Realized methanotrophic strain richness (exponential of Shannon diversity index determined based on *pmoA* sequence abundances) in dependence of strain richness according to the experimental design. Blue symbols: average per composition over DNA samplings at days 0, 28 and 41. Grey area: standard error or linear regression line.

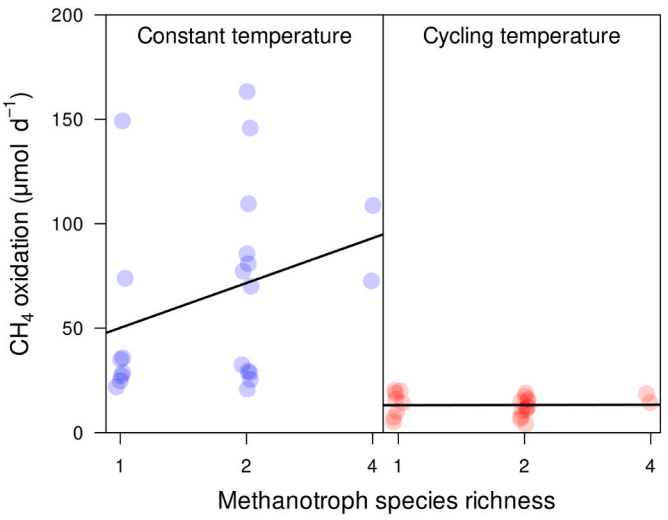

Fig. 2. Methane oxidation rate in dependence of methanotrophic strain richness treatment and temperature (constant or cycling). Symbols are average values per community composition.

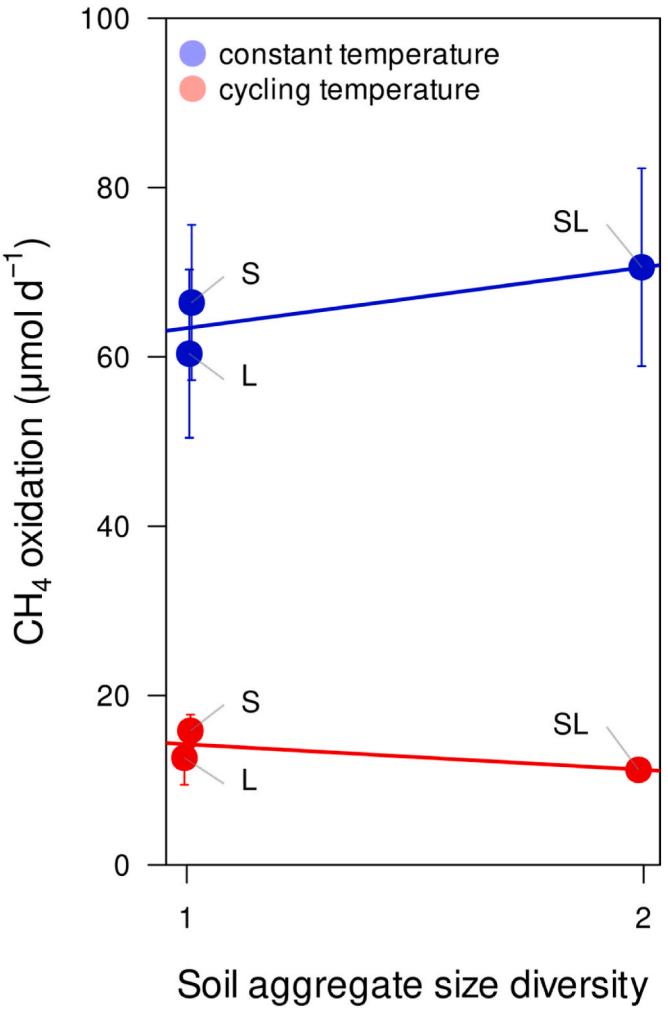

Fig. 3. Methane oxidation rate in dependence of soil aggregate composition treatment and temperature treatment. S: small aggregates; L: large aggregates; S + L mixture of small and large aggregates.

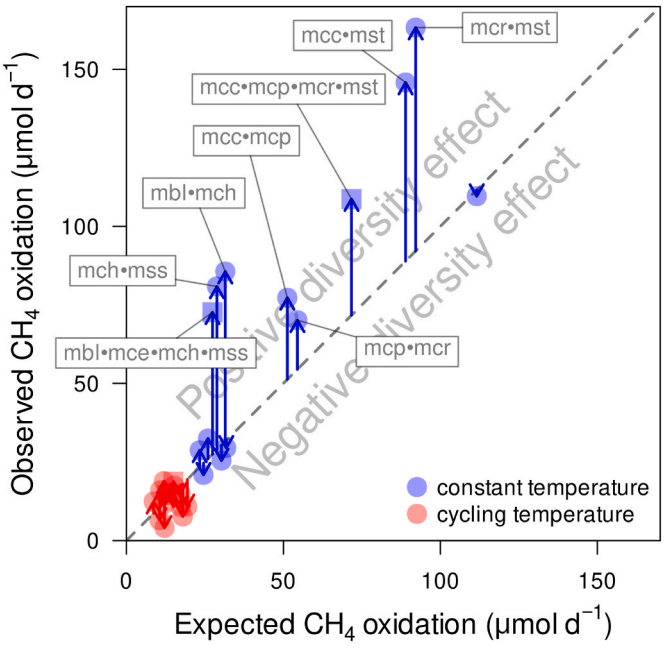

Fig. 4. Methane oxidation rate observed in strain mixtures in dependence of expected methane oxidation rate (average of strain monoculture rates). Arrows indicate net biodiversity effects. Community compositions are indicated for the strain mixtures that show pronounced non-additive mixting effects (codes see Table 1).

3.3. Methanotroph community size

Methanotroph communities grew over the course of the experiment (Fig. 5; day 0: 29 ± 5; day 28: 1024 ± 243; day 41: 4505 ± 906; data in 10^6 *pmoA* copies (g soil)$^{-1}$), and this growth was much greater in the constant temperature treatment than in the temperature cycling treatment ($t_{69} = 3.5$, $P = 0.0007$). Soil aggregate size composition ($F_{2,129} = 0.88$, $P = 0.42$) did not affect *pmoA* copy numbers. Because the copy numbers in the two temperature treatments were very different, we analyzed them separately. Broadly, the pattern followed that observed for CH_4 consumption. *pmoA* copy numbers increased with strain richness in the constant temperature treatment, but this effect was only statistically significant on day 28 ($t_{13} = 3.0$, $P = 0.005$).

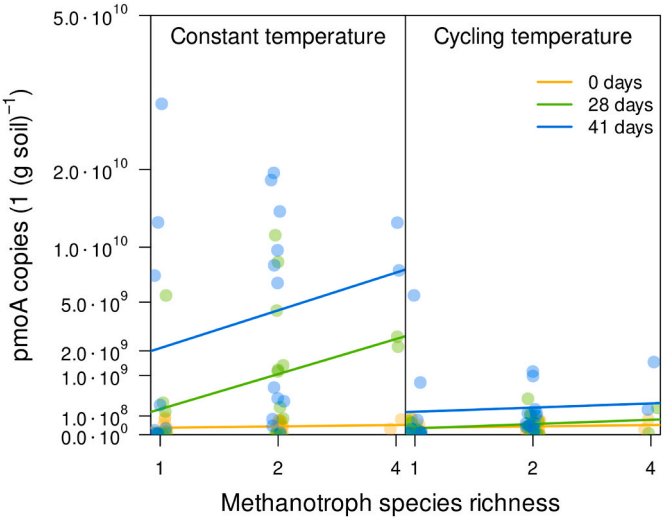

Fig. 5. Copy numbers of *pmoA* gene as determined by quantitative PCR, in dependence of strain richness treatment, temperature treatment, and sampling day. Copy numbers were square-root transformed prior to averaging (see Methods for reasons) and the Y axis is scale accordingly.

3.4. General and specific combining abilities

Decomposing CH_4 consumption (Fig. 2) into general (GCA) and specific (SCA) combining abilities using mechanistic diallel models, we found that GCAs were all positive and differed between strains ($F_{7,32} = 25$, $P < 10^{-6}$; Fig. 6), and that the effect of strain identity depended on temperature treatment ($F_{7,32} = 28$, $P < 10^{-6}$). Important drivers of this interaction were *Methylosinus trichsporium* and *Methylocystis parvus*, which had the highest GCAs at constant temperature but the lowest GCAs at cycling temperature.

SCAs (Fig. 7), which model the non-additive components of a mixture's CH_4 consumption, significantly depended on the particular strain combination ($F_{19,80} = 6.4$, $P < 10^{-6}$), and the effect of strain combination was temperature dependent ($F_{19,80} = 6.0$, $P < 10^{-6}$). Consistent with the diversity effect on CH_4 consumption, SCAs for strain monocultures were significantly lower than SCAs for two-strain mixtures ($t_{17} = 2.9$, $P = 0.01$).

3.5. Competitive hierarchy

To determine the competitive relationship of the methanotrophic strains in the constant temperature treatment, we determined the fraction of *pmoA* sequences of the component strains of all pairwise mixtures, and determined the change in this fraction from day 0 to day 41. Values > 1 (<1) indicate that a strain increased (decreased) in relative abundance over time. We found clear transitive competitive hierarchies in both pools (pool 1: Methylocystis echinoides < Methylocystis hirsuta < Methylobacter luteus < Methylosinus sporium; pool 2: Methylococcus capsulatus < Methylocystis rosea < Methylocystis parvus < Methylosinus trichosporium).

3.6. Traits

Gower trait distances calculated using the full set of 14 traits (Table 1) were neither associated with pairwise differences in strain GCAs nor with SCAs (Mantel tests). Effects of relevant traits may be masked by the inclusion of unimportant traits in the distance calculation. Therefore, in an exploratory procedure, we repeated all Mantel tests with trait matrices computed using all possible combinations of

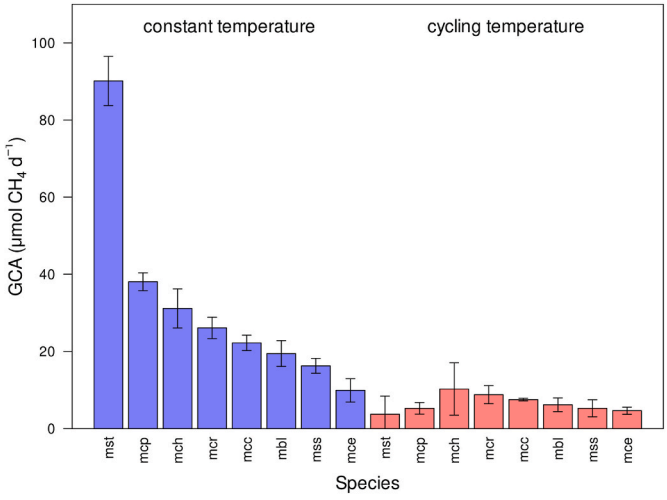

Fig. 6. General combining abilities (GCAs) of methanotrophic strains, obtained using mechanistic diallel analyses. GCAs indicate the additive contributions of the component strains to the methane oxidation of the individual mixtures. See code in Table 1 for strain designators. Error bars indicate standard errors calculated using the three aggregate size combinations as replicates.

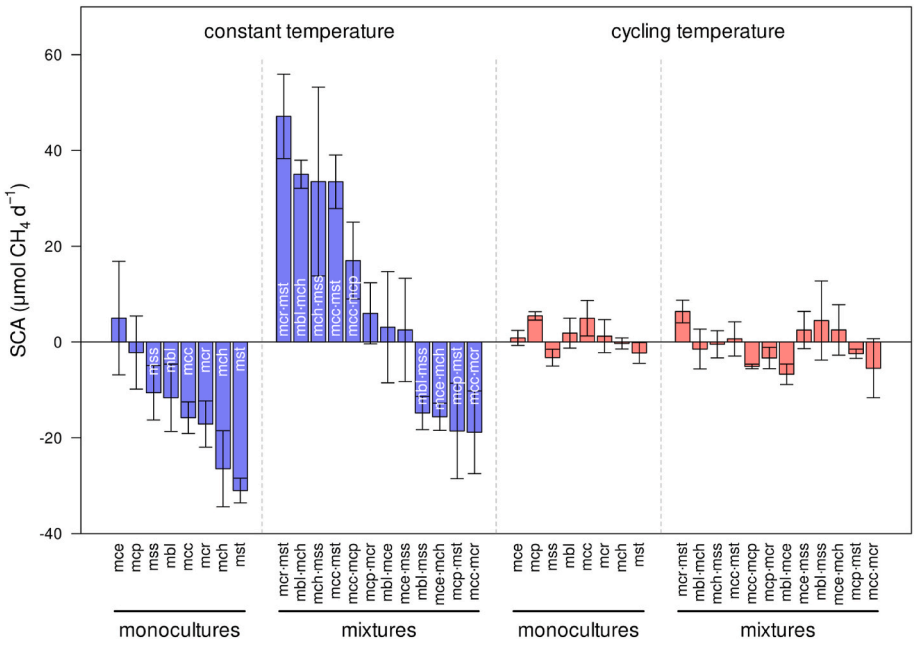

Fig. 7. Specific combining abilities (SCAs) of methanotrophic strains, obtained using mechanistic diallel analyses. SCAs indicate the non-additive contributions of strain combinations, i.e. the deviation of observed methane oxidation from predictions based on GCAs (see Fig. 6 and Methods). See code in Table 1 for strain designators. Error bars indicate standard errors calculated using the three aggregate size combinations as replicates.

traits. We found 99 (2) trait combinations that produced P-values<0.1 ($P < 0.05$) in the Mantel tests for GCA differences, and 297 (55) trait combinations for SCA. Of course, given the very large number of trait combinations and associated tests, these P-values do not reflect proper type I error probabilities. Nevertheless, the tests may provide an indication of which functional trait differences may be important. For GCA differences, the traits "motility" (does the strain have flagella?) and "PLFA_PCoA1" (first component value of principle coordinate ordination of PLFA profiles first ordination axis value of PLFA profiles) were the traits most frequently present in the combinations with $P < 0.1$. For SCAs, "motility" and "sMMO" (does the strain produces a soluble methane monooxygenase enzyme?) were the most common traits.

4. Discussion

We investigated the effect of habitat heterogeneity on the biodiversity–ecosystem functioning (BEF) relationship in a biogeochemically relevant group of bacteria, the methanotrophs. This was achieved by combining experimental gradients in methanotroph diversity with different levels of spatial (aggregate structure) and temporal (temperature cycle) heterogeneity. We found that CH_4 consumption indeed increased with the number of methanotrophic strains in the community. Contrary to our expectations, this relationship broke down under temperature heterogeneity and did not depend on soil structural heterogeneity. Strain mixtures differed strongly in diversity effects, but we did not identify traits or trait sets that predict which strain mixtures have particularly high diversity effects.

Few studies to date have examined BEF-relationships in microbial communities, and even fewer were concerned with soil CH_4 fluxes. These studies can be grouped into three broad categories. In the first, existing environmental gradients along which methanotrophic diversity varies are investigated. For example, Siljanen et al. (2011) reported a positive correlation between methanotrophic CH_4 consumption and diversity along a 16 m moisture gradient in a natural wetland. The problem with such correlational evidence is that the diversity gradient studied is the result of a pre-existing environmental gradient, and it therefore remains unclear whether changes in methanotrophic activity are the result of the diversity change or a diversity-independent consequence of the environmental differences. In the second type of study, diversity is manipulated by serial dilutions along which rare taxa are lost. Using this technique, we demonstrated that CH_4 consumption decreased as methanotrophic diversity was lost from a landfill cover soil (Schnyder et al., 2020). When using such dilution-to-extinction techniques, the taxonomic units that are lost first are either those with low abundance or those that fail to recover, for example due to low growth rates. While this may be a realistic scenario, the effects of diversity per se cannot be separated from effects driven by the specific traits of the removed organisms. In natural communities, a further complication is that the diversity of non-target groups is also reduced, which may also modify methanotrophic activity (Ho et al., 2014). In the third type of study, communities are systematically assembled de novo, as we did here. To our knowledge, the present study, together with a previous simpler one (Schnyder et al., 2018), are the first to use such systematic experimental designs in methanotrophic communities, and both demonstrate positive effects of methanotrophic diversity on CH_4 consumption. While causality can be clearly inferred from such designs, a drawback is that the resulting community compositions may be rather artificial. All these experimental approaches (observational gradients, diversity loss by dilution, assembled communities) are complementary and taken together strongly support the notion that methanotrophic diversity promotes CH_4 consumption.

Environmental heterogeneity is thought to provide additional niche space and thus facilitate the differentiation of organisms within a community, an effect that is a prerequisite for positive BEF relationships. Indeed, such positive effects of additional spatial niches have been observed in communities of plants (Dimitrakopoulos and Schmid, 2004) and mollusks (Griffin et al., 2009), and similar positive effects have been reported for both spatial and temporal niches in methane-oxidizing bacteria in natural environments (Bodelier et al., 2013). In contrast, a homogeneous environment should tend to favor the dominance of a single species. In our study, a higher diversity of aggregates sizes (i.e. a structurally more complex environment) did not enhance the BEF effects we found. The organization of microbial communities within soil aggregate structure is currently not well understood. Isotope labeling, combined with microautoradiographic analysis of soil sections, has shown that the active methanotrophs occupy the surface of soil

aggregates (Stiehl-Braun et al., 2011; Karbin et al., 2017; Rime and Niklaus, 2017). This likely reflects trade-offs between access to substrates and protection from adverse environmental effects. Important similar gradients can also be found with soil depth, with typical vertically profiles of CH_4 concentration, water availability, temperature, soil nutrient concentration, acidity, and organic matter content, and experiments have demonstrated functional complementarity of methanotrophs with depth, where methanotrophs near the soil surface consume atmospheric CH_4 under benign conditions and preempt this resource. Under adverse conditions of drought or fertilizer applications, the activity of these methanotrophs is inhibited, and CH_4 is consumed at greater depths by methanotrophs protected from these effects (Hartmann et al., 2011; Stiehl-Braun et al., 2011; Rime and Niklaus, 2017). Thus, one possible interpretation of our results is that the different aggregate sizes we used did not provide microhabitats that were sufficiently different to create a greater diversity of functional niches, and that stronger effects may have occurred with more vertical space. Alternatively, the aggregates were not preserved in their original form in the microcosms.

Temporal heterogeneity in the form of a diurnal temperature cycle strongly reduced methanotrophic activity and essentially eliminated the BEF-relationship. Thus, despite the different temperature optima of the strains, it appears that temporal variation in temperature did not provide additional growth opportunities, but rather acted as a perturbation. This result was surprising given that identical temperature regimes had no discernible effect on methanotrophic activities and BEF relationships in an experiment with a diversity gradient created by serial dilutions of a landfill cover soil community (Schnyder et al., 2020). A possible explanation may be that the communities in the latter experiment were adapted to diurnal temperature fluctuations, whereas the strains we used here were not. Conversely, the strains we used were likely isolated under constant and relatively high laboratory temperatures and thus did not perform well in the variable temperature incubation.

Biodiversity effects are ultimately mediated by functional differences between organisms. In our study, the CH_4 consumption rates of monocultures varied widely, supporting the idea that the strains differed in traits relevant for their growth rate. Also, the individual strain mixtures differed greatly in their net biodiversity effect, with some showing large benefits ("overyielding"). Interestingly, none of the mixtures showed a clearly negative mixing effect. The traits we compiled were carefully selected based on functional considerations; for example, we used traits related to cell size and aggregate formation because we reasoned that these were important for surface to volume ratios and thus growth rates; similarly, different temperature optima might have indicated different thermal niches. We also included more aggregate metrics such PLFA profiles; their fatty acids are biomarkers that inform about the physiology of the respective organisms. However, these traits did not explain the observed diversity benefits.

The mechanistic diallel analysis aimed to identify the contributions of individual strains, and strain combinations to the functioning of the mixtures. One mechanism that may lead to higher levels of functioning in more diverse communities is the selection probability effect (Aarssen, 1997). Essentially, if a few strains dominate the functioning of communities, then the more diverse mixtures will have a higher statistical probability of containing these strains, and the functioning of these mixtures will then be driven primarily by these dominant strains. In Pool 1, *Methylocystis hirsuta* had the highest GCA of all strains, indicating that its presence contributed most to CH_4 consumption. However, *pmoA* sequencing data indicated that *M. hirsuta* ranked only 3rd in the competitive hierarchy, and only two binary mixtures containing *M. hirsuta* showed positive net biodiversity effects (the mixture with *Methylocystis echinoides* did not). Conversely, *Methylosinus sporium* dominated the competitive hierarchy, but had a low GCA and was only present in one of the binary mixtures that had a pronounced positive diversity effect (namely the mixture with *M. hirsuta*). In Pool 2, *Methylosinus trichosporium* had the largest GCA, and dominated the competitive hierarchy revealed by *pmoA* sequencing. However, only two of the three binary mixtures containing *M. trichosporium* showed clear positive diversity effects on CH_4 consumption (the mixture with *Methylocystis parvus* did not). These results indicate that the diversity effects we found are not dominated by simple selection probability effects; they further suggest that total CH_4 consumption over the incubation period is decoupled from growth, or that pmoA sequence fractions, although standardized day 0 conditions, do not even qualitatively reflect biomass fractions in methanotroph communities.

Overall, our study provides evidence that soil methanotrophic diversity is an important promoter of CH_4 consumption. Specifically, we found evidence for positive but not for negative interactions among strains. Some strains performed extraordinarily well under certain environmental conditions (e.g. *M. trichosporium* at constant temperature), but failed once these conditions change (e.g. under cycling temperature). Hence, a high diversity is important for the maintenance of ecosystem functioning, in particular when environmental conditions change through time.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Pascal Niklaus reports financial support was provided by Swiss National Science Foundation.

Data availability

Data will be made available on request.

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